Supplementary data for:

Semisynthesis reveals Apoptin as a tumour-selective protein prodrug that causes cytoskeletal collapse

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Supplementary Figures S1-13

Figure S1: Synthesis of Apoptin C-terminal peptides 2
Figure S2: Production of Apoptin-thioester 3
Figure S3: Significant hydrolysis of thioester during NCL 4
Figure S4: Production of Apoptin-WT 5
Figure S5: Circular dichroism spectra of renatured Apoptin variants 6
Figure S6: Efficient transduction of Apoptin-WT using the TAT system. 7
Figure S7: Production of Apoptin-Mut 8
Figure S8: Apoptotic activity of Apoptin variants analysed via FACS 9
Figure S9: Synthesis of biotinylated Apoptin peptides 10
Figure S10: Apoptin interaction to known targets 11
Figure S11: Thr108 contributes to intracellular localisation of Apoptin. 12
Figure S12: Apoptin does not interfere with cytoskeleton of healthy cells 13
Figure S13: Lethal doses of Apoptin disrupt the cytoskeleton and perturb cell size. 14

Figure S1

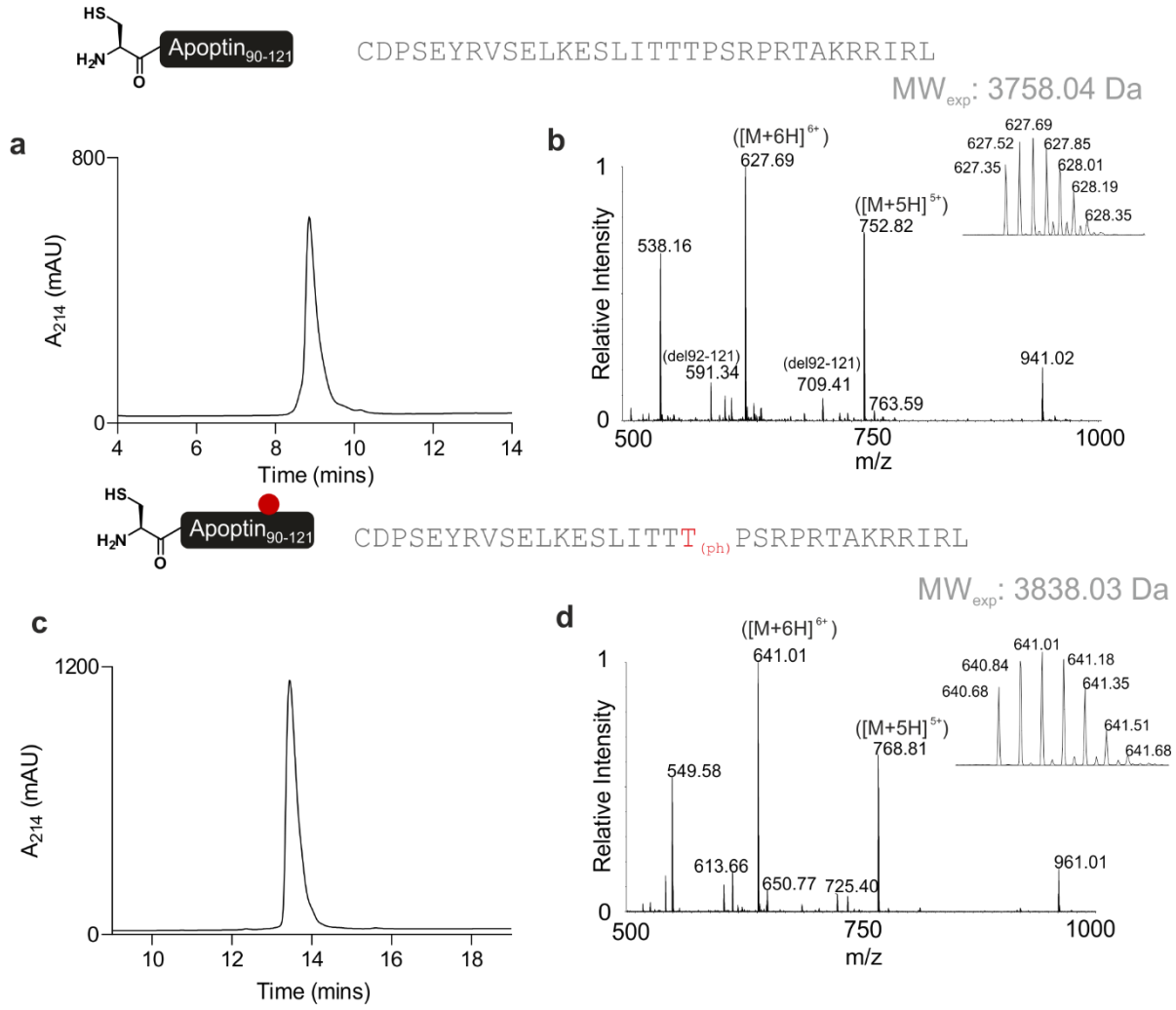

Figure S1: Synthesis of Apoptin C-terminal peptides

Peptides (Apoptin$_{90\text{-}121}$ and Apoptin$_{90\text{-}121}$-T108ph) were synthesis via Fmoc-SPPS and purified with RP-HPLC as described in the methods. a) RP-HPLC data and (b) MS data for the unmodified peptide (Expected mass 3758.04 Da, Observed mass 3758.06 Da). c) RP-HPLC data and (d) MS data for the T108ph peptide (Expected mass 3838.00 Da, Observed mass 3838.03 Da).

Figure S2

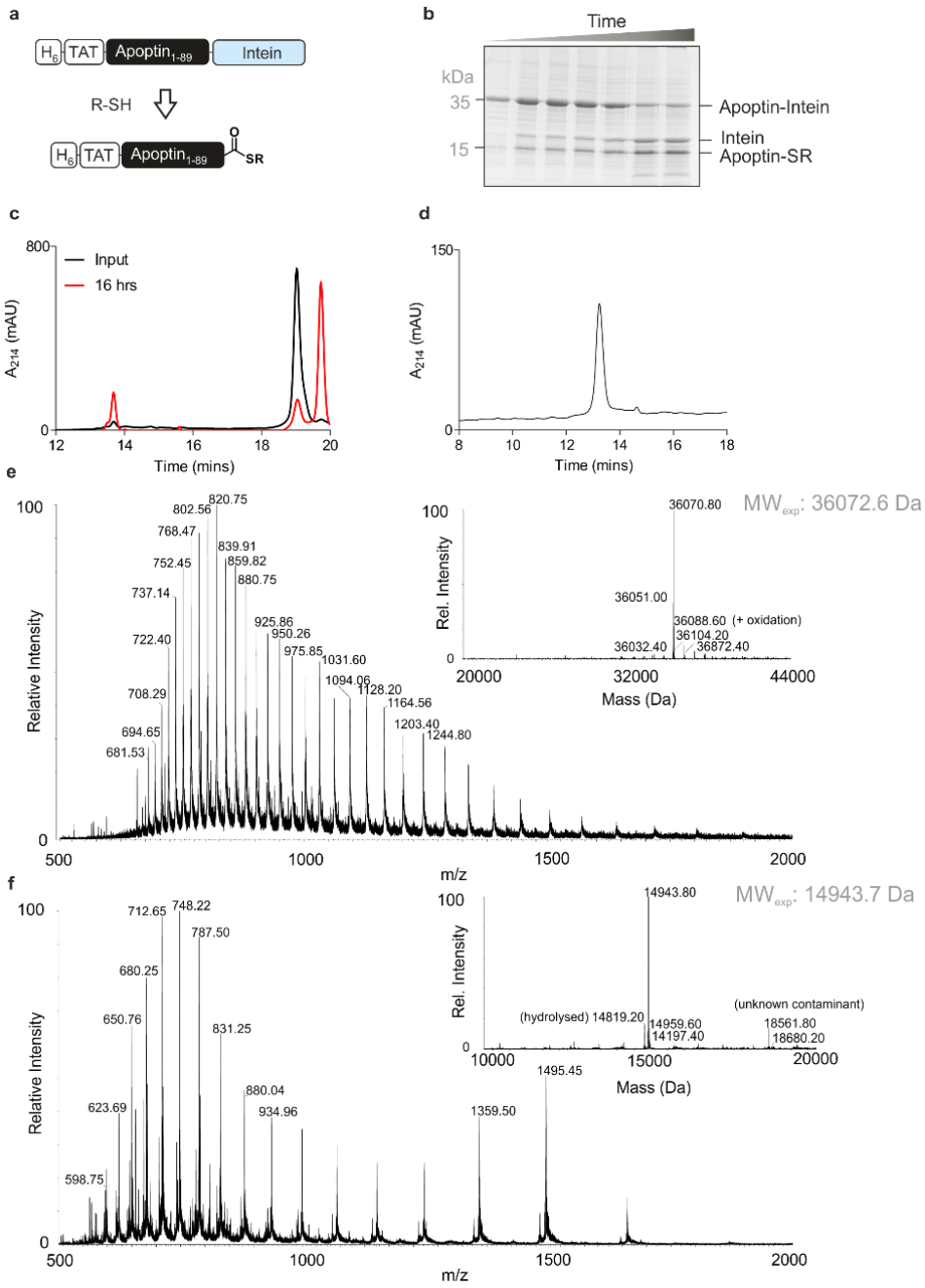

Figure S2: Production of Apoptin-thioester

a) Schematic for the thiolysis reaction. b) SDS-PAGE analysis of the thiolysis reaction over 16 hours. c) RP-HPLC analysis of the thiolysis reaction over 16 hours. d) RP-HPLC analysis of the isolated protein thioester. e) Mass spectrometry of the starting material corresponding to Apoptin-Intein (Expected mass 36072.6 Da, Observed mass 36070.8 Da). f) Mass spectrometry of the isolated protein thioester (Expected mass 14943.7 Da, Observed mass 14943.8 Da; hydrolysed expected mass 14819.2 Da).

Figure S3

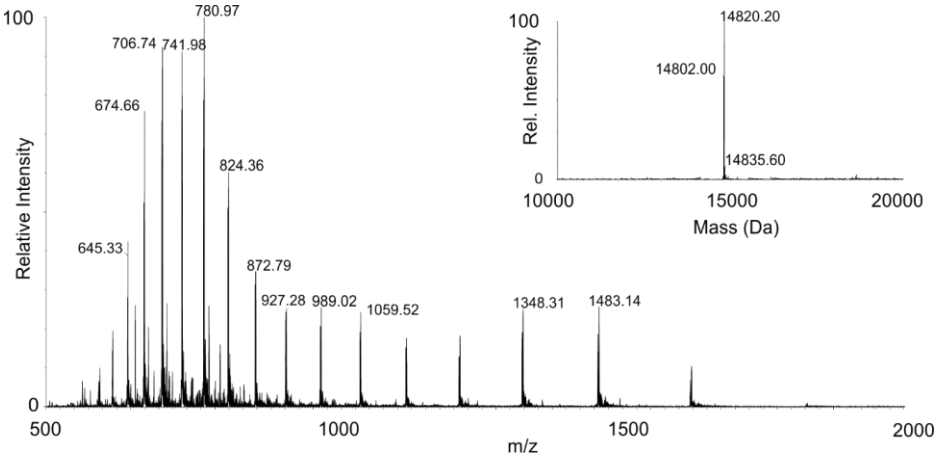

Figure S3: Significant hydrolysis of thioester during NCL

Mass spectrometry analysis of the thioester during the NCL reaction (Expected mass 14820.6 Da, observed mass 14820.2 Da).

Figure S4

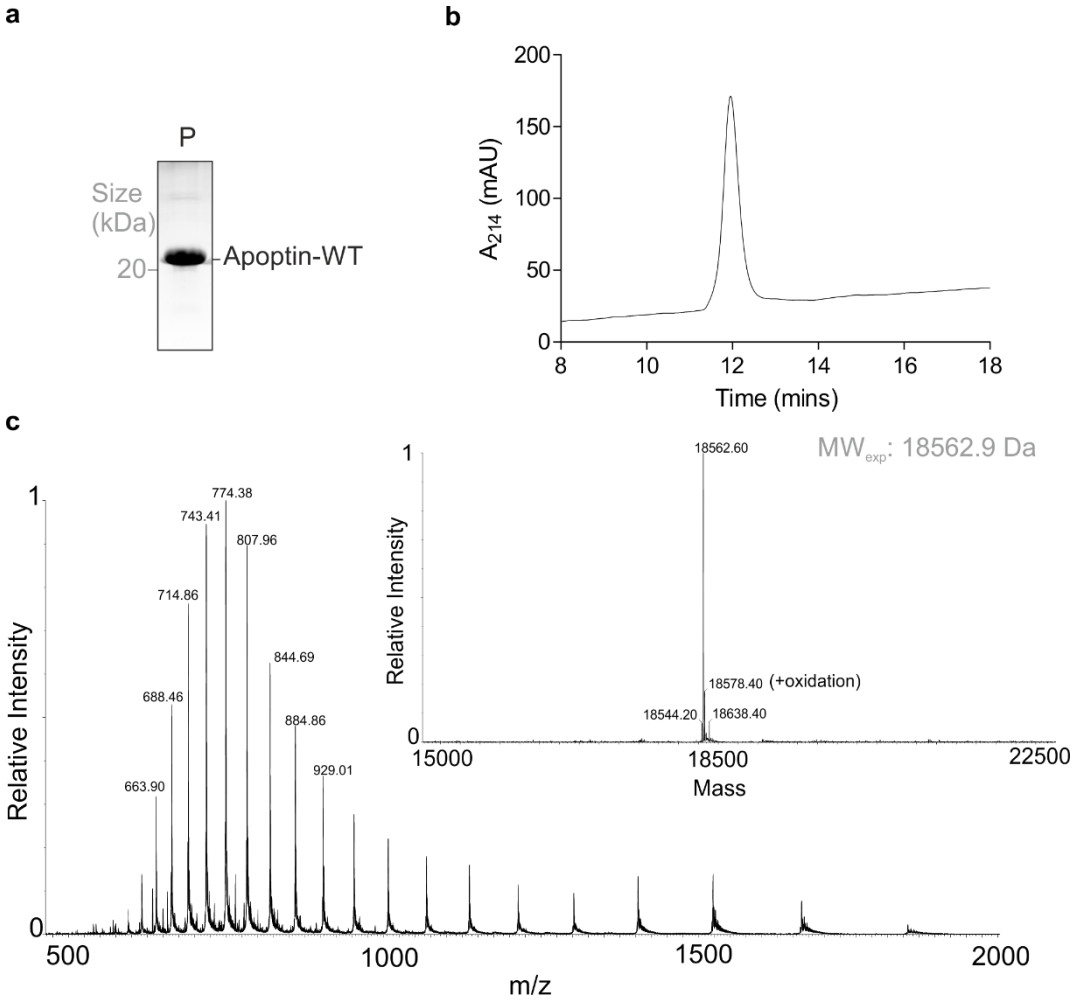

Figure S4: Production of Apoptin-WT

a) RP-HPLC and b) SDS-PAGE of purified and renatured Apoptin-WT. c) Mass spectrometry analysis of final product. Expected mass (-Met): 18562.9 Da, observed mass: 18562.6 Da.

Figure S5

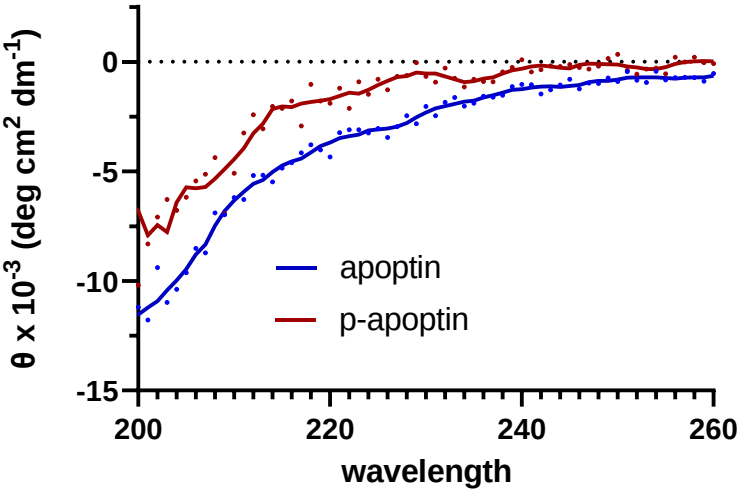

Figure S5: Circular dichroism spectra of renatured Apoptin variants.
Points represent an average from 3 circular dichroism wavelength scans of Apoptin-WT (blue) and Apoptin-T108ph (red). The lines represent smoothened spectra. Bestsel software was used to determine secondary structure content (Apoptin-WT: 27% Antiparallel β-Sheet, 20% Turn, 53% Others; Apoptin-T108ph: 28% Antiparallel β-Sheet, 24% Turn, 44% Others).[1]

Figure S6

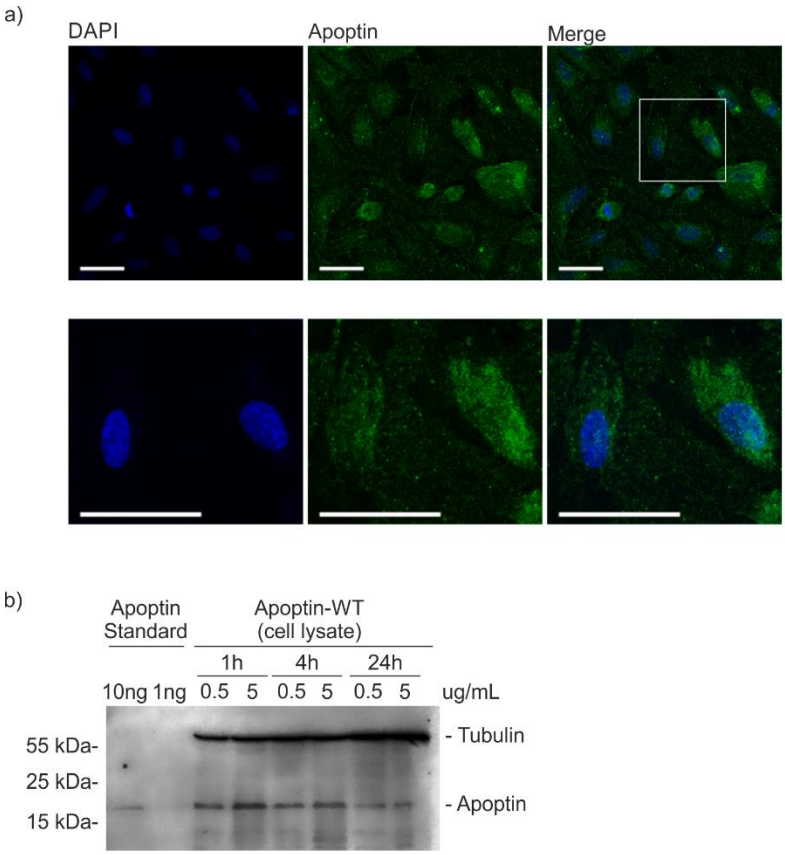

Figure S6: Efficient transduction of Apoptin-WT using the TAT system.
a) Immunofluorescence analysis of SAOS-2 cells delivered with Apoptin-WT. Cells were incubated with recombinant Apoptin-WT for 15 minutes, then fixed and stained with anti-HA antibodies. Cells were imaged at 60x magnification. The white box indicates the area that has been zoomed in as seen below. Scale bar=40 μm. b) Western blot analysis of Apoptin-WT delivery to HSC-3 cells. Cells were treated with 1 mL of 0.5 or 5 μg/mL Apoptin-WT for 15 minutes, washed and incubated for the indicated duration. Each lane contains lysate from approx. 200'000-300'000 cells of a total of $1.5*10^6$ (1h), $2.4*10^6$ (4h) and $3.4*10^6$ (24h) cells. 10 ng and 1 ng of purified Apoptin-WT were analysed as a control. Apoptin was visualised with an anti-HA antibody, Tubulin was used as a loading control.

Figure S7

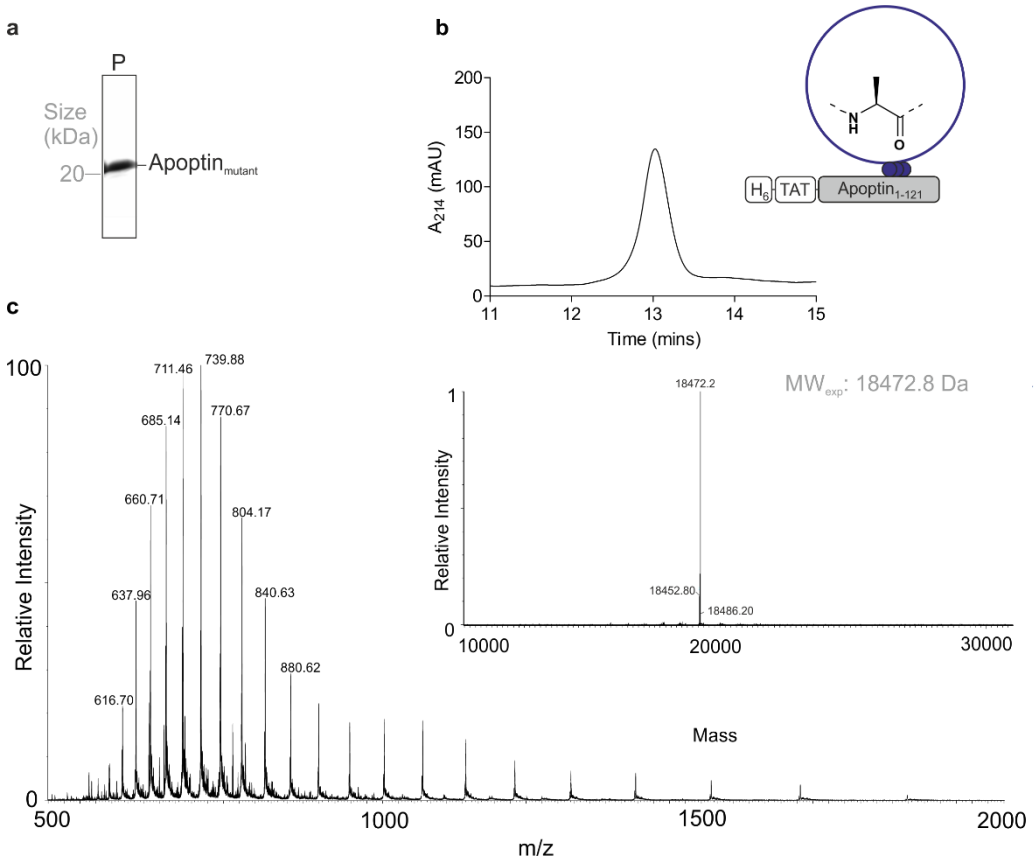

Figure S7: Production of Apoptin-Mut

a) RP-HPLC and b) SDS-PAGE of purified and renatured mutant Apoptin. c) Mass spectrometry analysis of final product (expected mass: 18472.8 Da, observed mass 18472.2 Da).

Figure S8

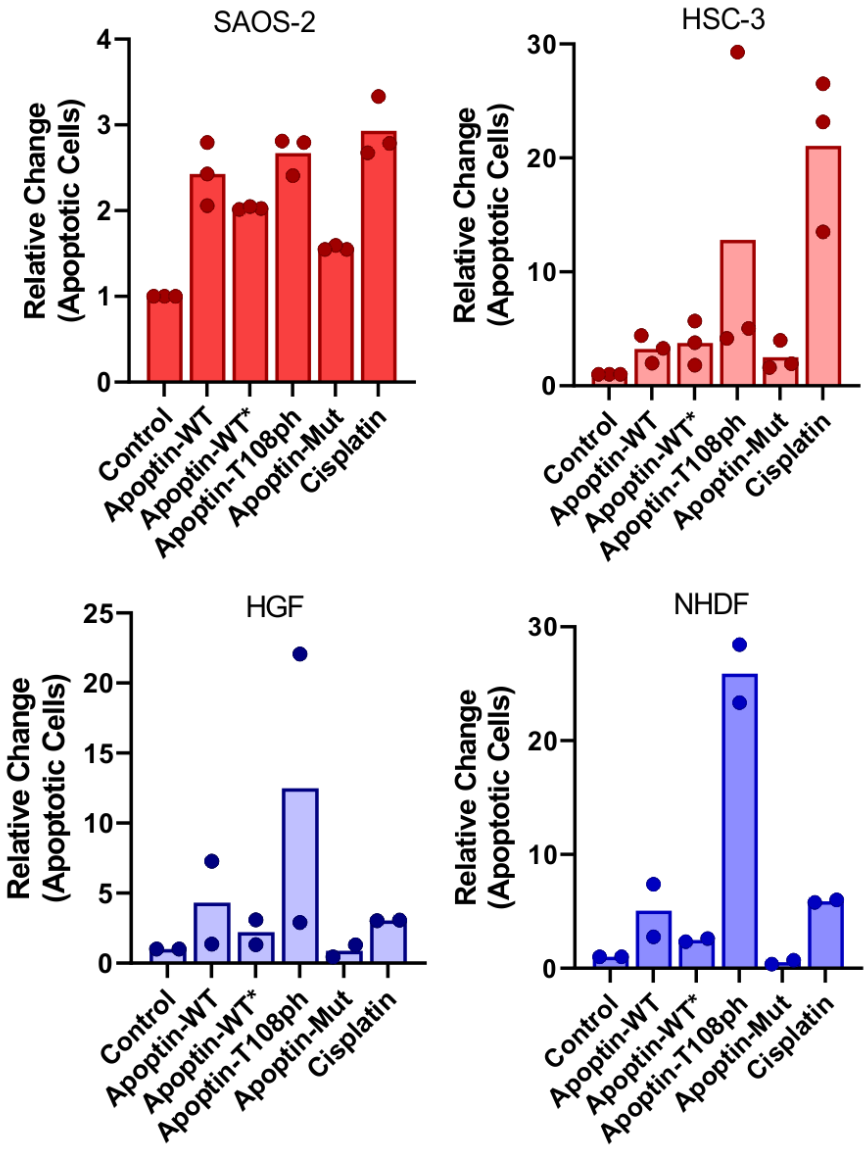

Figure S8: Apoptotic activity of Apoptin variants analysed via FACS.
Showing relative total Annexin V positive populations for cancer cell-lines (red, n=3) and healthy lines (blue, n=2).

Figure S9

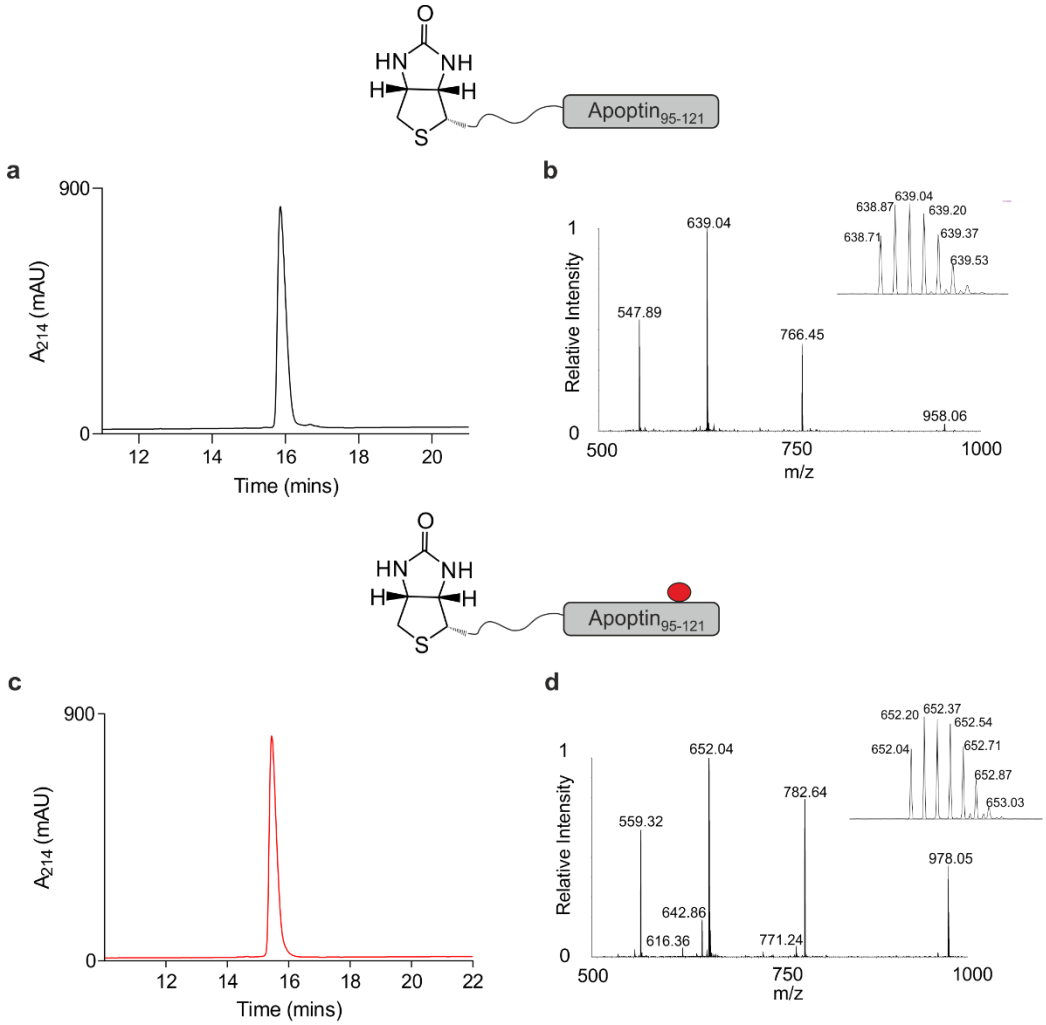

Figure S9: Synthesis of biotinylated Apoptin peptides

Apoptin-derived peptides were synthesis with an N-terminal biotin handle via Fmoc-SPPS and purified with RP-HPLC as described in the methods section. RP-HPLC and MS data of unmodified peptide (a, b; Expected mass: 3826.17 Da, Observed mass: 3826.26 Da) and Thr108ph peptide (c, d; Expected mass: 3906.14 Da, Observed mass: 3906.20 Da).

Figure S10

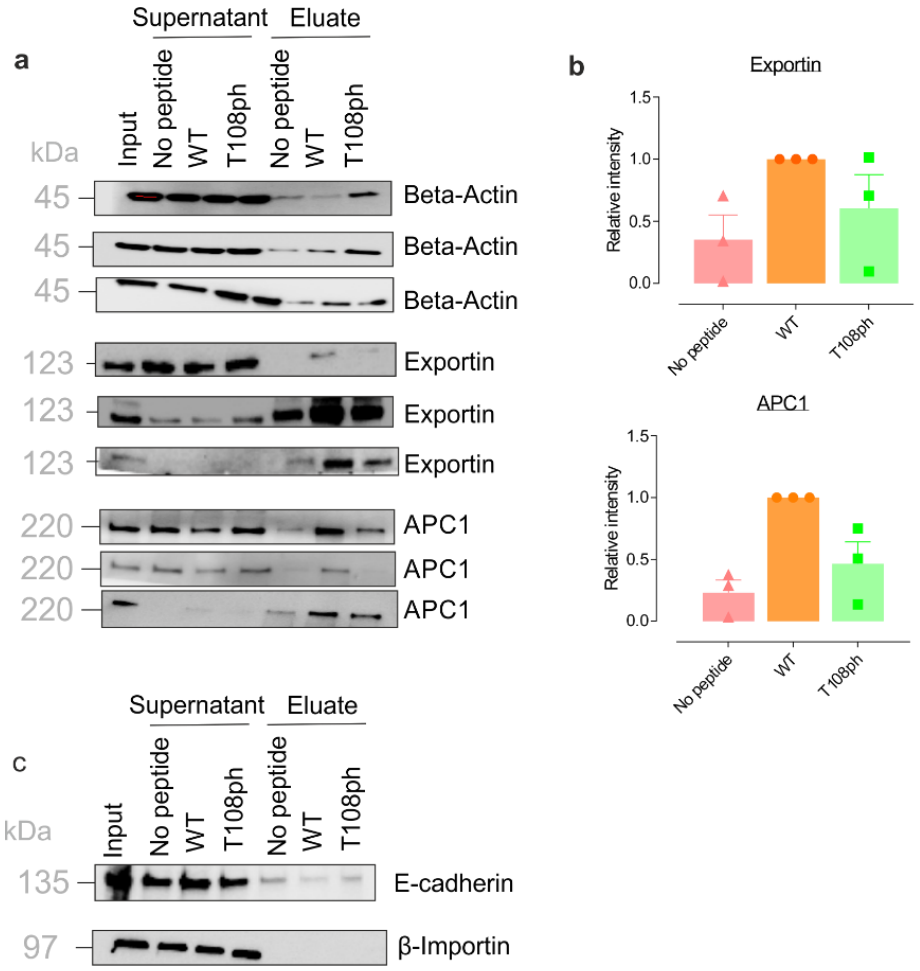

Figure S10: Apoptin interaction to known targets

a) Western blot analysis of beta-actin, exportin and APC1 pull-downs with wild-type and T108ph Apoptin peptides. Three independent replicates are shown. b) Quantification of Exportin (CRM-1) and APC1 pull-downs (n=3, ± SEM). c) Western blot analysis downs of E-cadherin and beta-importin pull-downs, showing that C-terminal Apoptin peptides does not interact with these targets.

Figure S11

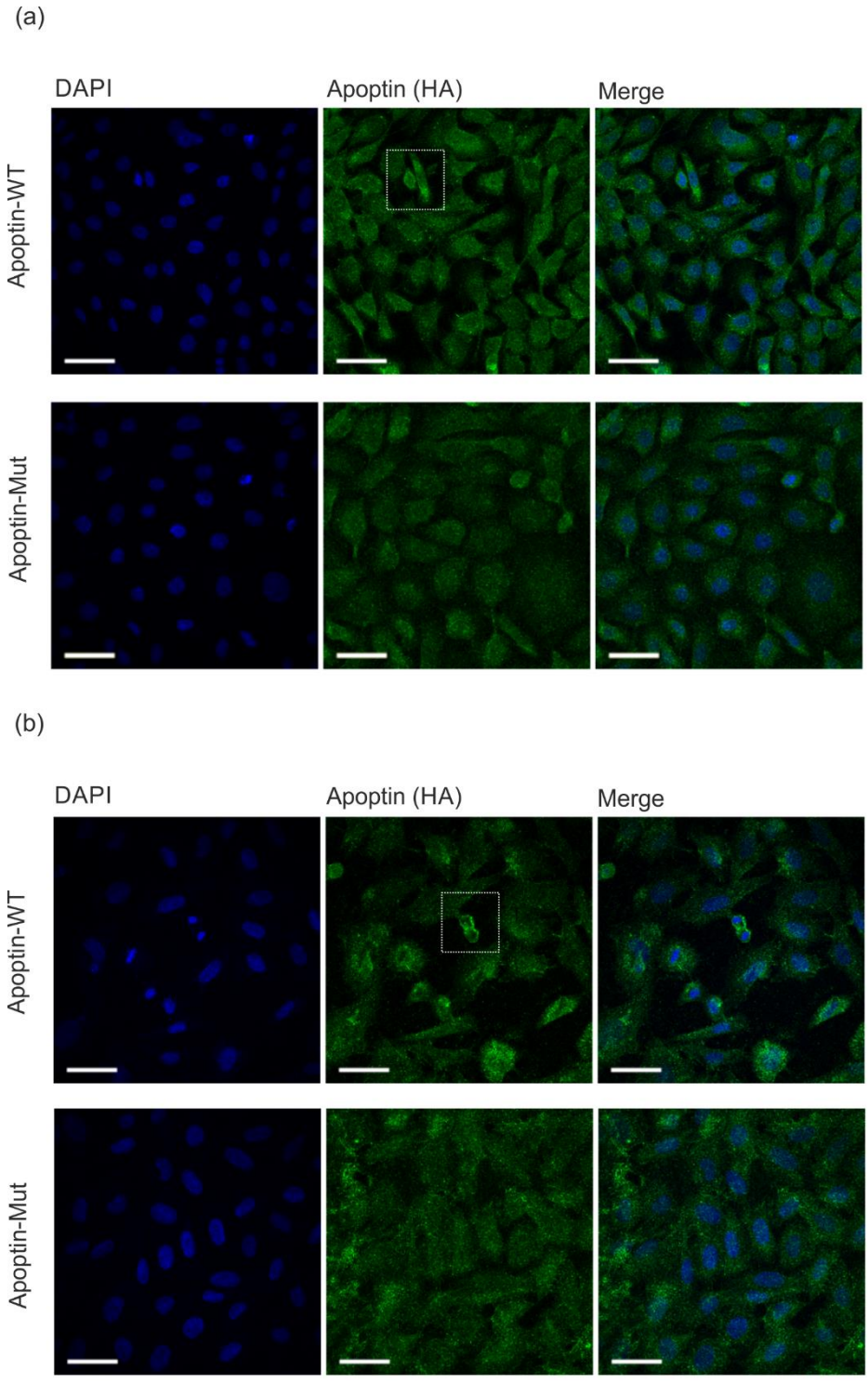

Figure S11: Thr108 contributes to intracellular localisation of Apoptin.
Immuno-fluorescence performed in SAOS-2 (a) and HSC-3 (b) cells after treatment with Apoptin-WT (top) and Apoptin-Mut (bottom) for 24h. Dotted boxes highlight regions of peri-nuclear accumulation in cells with condensed nuclei. 60x magnification. Scale bar= 40 μM.

Figure S12

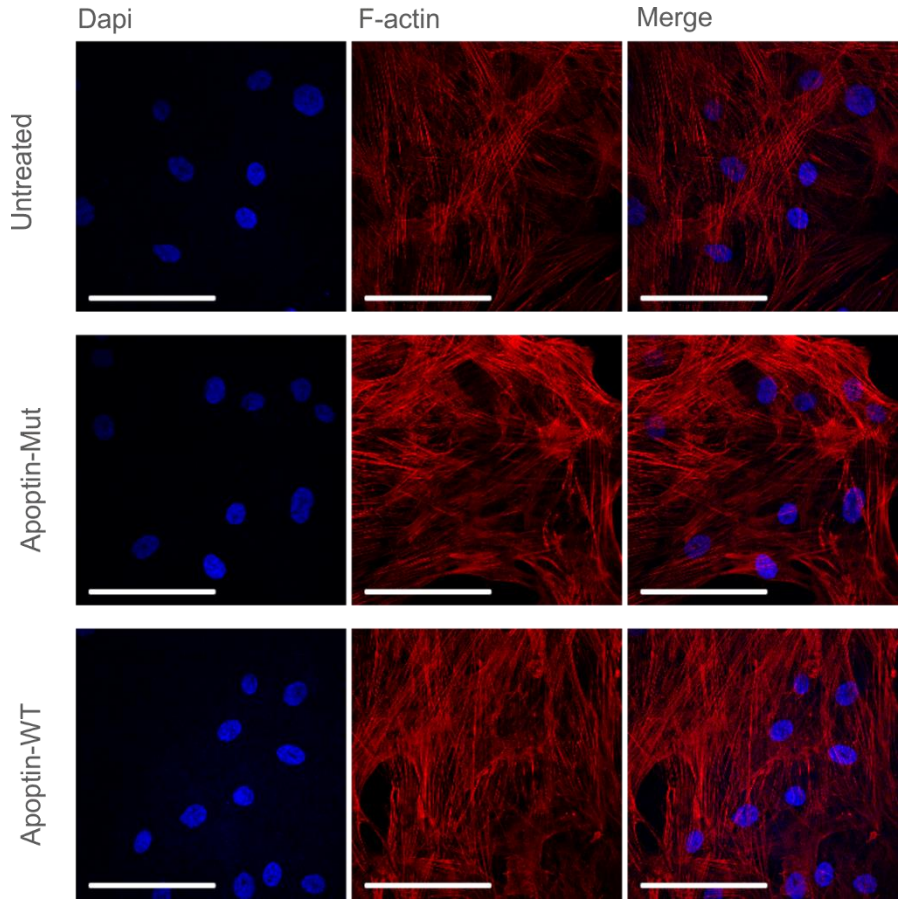

Figure S12: Apoptin does not interfere with cytoskeleton of healthy cells
Immuno-fluorescence images of HGF cells treated with sub-lethal doses (0.5 μg/mL) recombinant and mutant Apoptin and stained for F-Actin. Cells were imaged at 60x magnification, scale bar=40 μm.

Figure S13

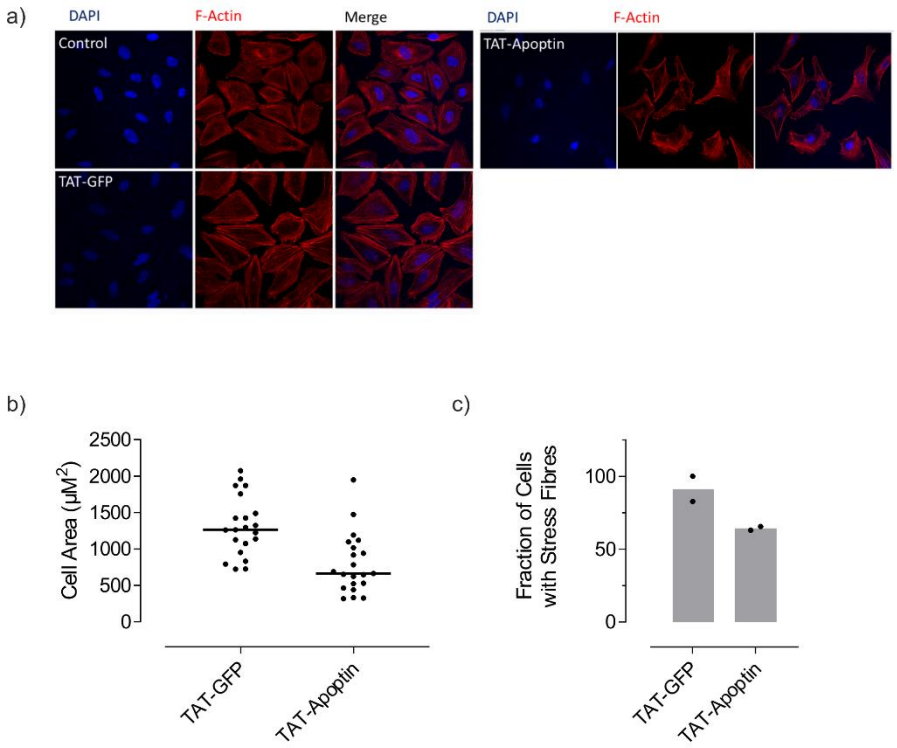

Figure S13: Lethal doses of Apoptin disrupt the cytoskeleton and perturb cell size.
a) Immuno-fluorescence of SAOS-2 cells treated with 5 μg/mL Apoptin for 15 min and incubated for 24h before fixing and staining with TRITC-phalloidin and DAPI. Cells were imaged at a 60x magnification. At least 20 cells from field of view were analysed for cell size (b) and scored for prominent stress fibre formation (c) (56 cells from 2 independent experiments).

Supplementary References

[1] A. Micsonai, F. Wien, L. Kernya, Y.-H. Lee, Y. Goto, M. Réfrégiers, J. Kardos, *PNAS* **2015**, *112*, E3095-E3103.