Supporting Information

Leshchiner et al. 10.1073/pnas.1413185112

SI Methods

Fluorescence Size Exclusion Chromatography. FSEC was performed on an Agilent 1260 HPLC equipped with a fluorescence detector. A 100-μL solution of 0.5 μM or 10-μL solution of 10 μM FITC-SAH-SOS1$_A$ was injected onto a 10/100 GL Superdex 75 gel permeation column and eluted in 50 mM Tris(hydroxymethyl) aminomethane hydrochloride (pH 8) containing 150 mM NaCl, using a flow rate of 0.5 mL/min.

Differential Scanning Fluorimetry. Differential scanning fluorimetry (DSF), or thermal shift, assay was performed on a Roche LightCycler 480 II with ramp speed of 4.8 °C per min and 12 acquisitions per °C. Solutions of KRAS/SAH-SOS1 peptide (10 μM) with added SYPRO dye (Life Technologies; 1:1,000 dilution) was dispensed into 384-well polypropylene PCR plates (10 μL per well). The plates were sealed and heated in the instrument across a temperature range of 25–95 °C.

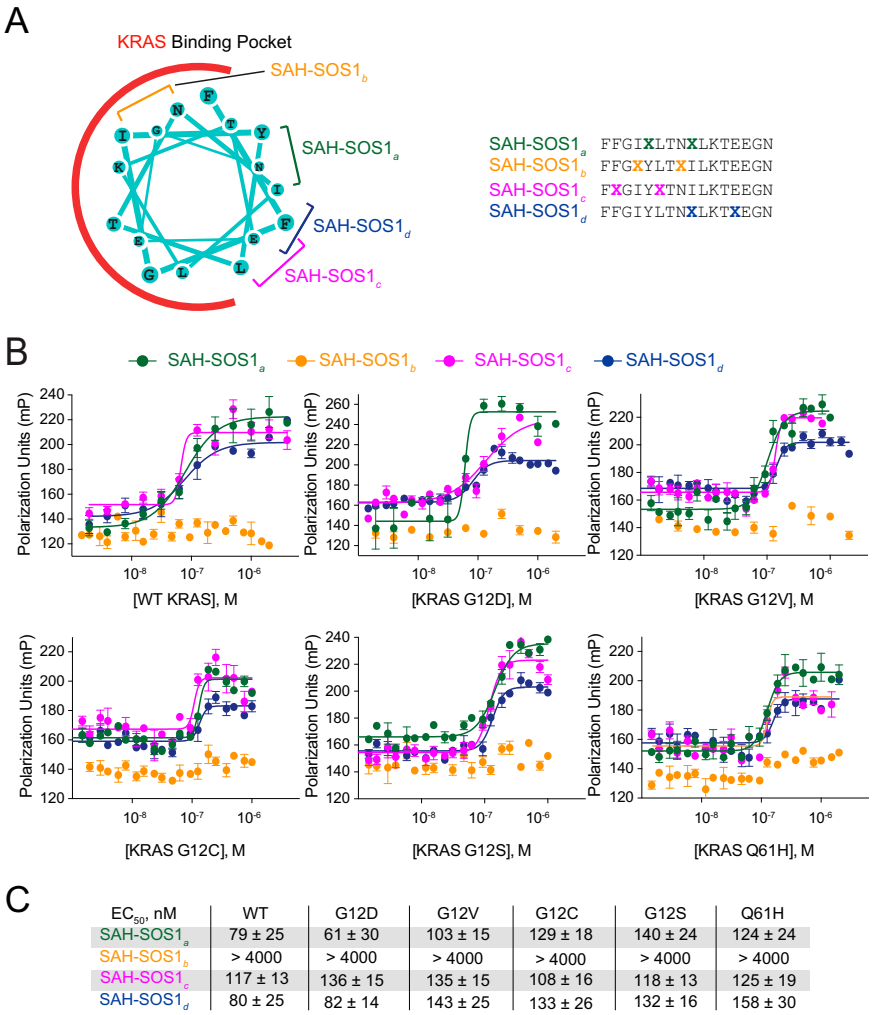
SOS1/KRAS Dissociation Assay. The catalytic domain of SOS1 protein (amino acids 564–1049) was cloned into the pET-28a vector and produced in *E. coli* BL21(DE3) as a His$_6$-tagged fusion protein. Protein expression was induced by 1 mM IPTG at 30 °C for 4 h, and His$_6$-SOS1 was purified using Ni-NTA affinity resin (Qiagen) and eluted with 150 mM imidazole in 50 mM Tris, 150 mM NaCl, pH 7.8, followed by size exclusion chromatography (GE Life Sciences) in 50 mM Tris, 150 mM NaCl, pH 7.8 buffer conditions. GST-KRAS (amino acids 1–188; Abnova) was subjected to buffer exchange (50 mM Tris, 150 mM NaCl, pH 7.8) using Amicon filter units (3 kDa molecular mass cutoff; Millipore). For in vitro immunoprecipitation studies, an equimolar mixture of recombinant GST-KRAS and His$_6$-SOS1 catalytic domain (amino acids 564–1049) (1 μM each, 300 μL of reaction volume) was incubated with vehicle, SAH-SOS1$_A$, or SAH-SOS1$_B$ peptides (5, 10 μM) at 25 °C for 40 min. The experimental solutions were transferred to preblocked [3% (wt/vol) BSA in PBS] glutathione resin beads (GE Healthcare Life Sciences) and incubated at 4 °C with constant mixing for 1 h, followed by three washes with 1% BSA in PBS, and resuspension of beads in LDS for SDS/PAGE and anti-KRAS (Cell Signaling) and anti-His$_6$ (Abcam) Western blot analysis.

KRAS Pull-Down Assay. Biotin-labeled SAH-SOS1$_A$ (10 μM) or vehicle (0.2% DMSO) was incubated with streptavidin-Sepharose in PBS at 4 °C for 1 h. The beads were then blocked with 2 mg/mL biotin (40 min) and 3% (wt/vol) BSA (40 min), and recombinant His$_6$-KRAS was added to the beads at a final concentration of 10 μM in 1% (wt/vol) BSA. The beads were then rotated at 4 °C for 1 h, washed three times with 1% BSA in PBS, and resuspended in LDS for SDS/PAGE and anti-KRAS (Cell Signaling) Western blot analysis.

Cellular Uptake Analysis. Panc 10.05 cells were plated at 4×10^5 cells per well in 35-mm poly-D-lysine–coated dishes (MatTek). The cells were treated with 0.5 μM FITC-SAH-SOS1 peptides for 2 h in serum-free DMEM and then stained with Hoechst dye and CellMask Orange (Invitrogen) for 10 min. The media was aspirated and the cells fixed with 4% (wt/vol) paraformaldehyde for 10 min at 4 °C, washed three times with PBS, and imaged using a Yokogawa CSU-X1 spinning disk confocal system (Andor Technology) attached to a Nikon Ti-E inverted microscope (Nikon Instruments; excitation with 405-, 488-, and 561-nm lasers). Images were acquired using a 100× Plan Apo objective lens with a Hamamatsu OrcaER camera (Hamamatsu Photonics). The data were analyzed by ImageJ software. At least six fields of view were collected for each experimental condition. As a second measure of peptide uptake, Panc 10.05 cells were plated in 12-well plates at 4×10^5 cells per well and then treated as above with the indicated doses of SAH-SOS1 peptides for 2 h, followed by aspiration of the media, PBS washes, and trypsinization. Cells were collected, washed three times with PBS, and lysed in buffer containing 1% (vol/vol) Nonidet P-40, 50 mM Tris, 150 mM NaCl, pH 7.4, supplemented with protease and phosphatase inhibitor tablets (Roche). The lysates were electrophoresed on 4–12% Bis-Tris gels (Invitrogen), which were then subjected to fluorescence imaging using a Typhoon 9400 (GE Healthcare).

High-content fluorescence microscopy analysis to evaluate intracellular FITC peptide was performed as described (1). The indicated cell lines were plated in black, clear bottom plates overnight at a density of 2×10^4 cells per well in DMEM supplemented with 10% (vol/vol) FBS, 1% penicillin/streptomycin, and 1% glutamine. The following day, cells were treated with 0.5 μM FITC-labeled SAH-SOS1 peptides or the equivalent amount of vehicle (0.1% DMSO) or FITC for 4 h in serum-free DMEM, and then stained with Hoechst dye and CellMask Deep Red (CMDR, Invitrogen) for 10 min. The media was aspirated, and cells were fixed with 4% (wt/vol) paraformaldehyde for 10 min, washed three times with PBS and imaged by ImageXpress Microscopy (high-throughput epifluorescence microscope; Molecular Devices). Data were collected for five sites per well, with each treatment performed in duplicate, 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. In addition, only FITC signal that exceeded an intensity threshold of 10 times above local background was incorporated into the analysis.

1. Moellering RE, et al. (2009) Direct inhibition of the NOTCH transcription factor complex. *Nature* 462(7270):182–188.



C

EC_{50}, nM	WT	G12D	G12V	G12C	G12S	Q61H
SAH-SOS1$_a$	79 ± 25	61 ± 30	103 ± 15	129 ± 18	140 ± 24	124 ± 24
SAH-SOS1$_b$	> 4000	> 4000	> 4000	> 4000	> 4000	> 4000
SAH-SOS1$_c$	117 ± 13	136 ± 15	135 ± 15	108 ± 16	118 ± 13	125 ± 19
SAH-SOS1$_d$	80 ± 25	82 ± 14	143 ± 25	133 ± 26	132 ± 16	158 ± 30

Fig. S1. Design and KRAS binding activity of SAH-SOS1 peptides. (*A*) SAH-SOS1 peptides for KRAS targeting were generated by inserting all-hydrocarbon staples at positions a (green), b (orange), c (pink), and d (blue) into a SOS1 peptide spanning amino acids 929–944. (*B*) Fluorescence polarization binding analysis of FITC-labeled SAH-SOS1 peptides and recombinant KRAS proteins, including wild-type and mutant variants. Data are mean ± SEM for experiments performed in technical triplicate and are representative of at least three biological replicates performed with independent preparations of recombinant KRAS proteins. (*C*) Table of EC_{50} values for the binding interactions between SAH-SOS1 peptides and the individual KRAS proteins.

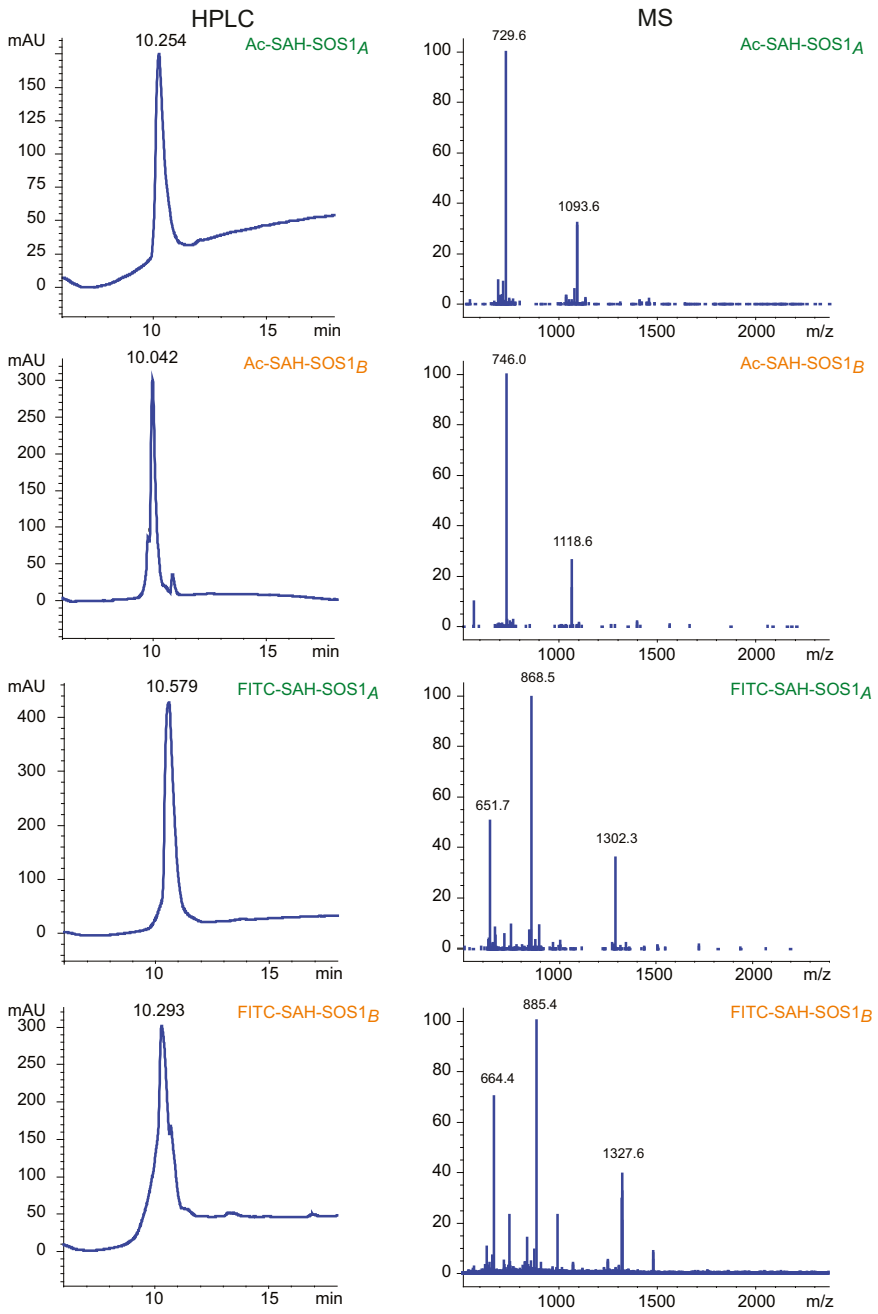


Fig. S2. Purification and mass spectrometry analysis of SAH-SOS1 peptides. HPLC (*Left*) and MS profiles (*Right*) of Ac-SAH-SOS1$_A$, Ac-SAH-SOS1$_B$, FITC-SAH-SOS1$_A$, and FITC-SAH-SOS1$_B$ peptides document compound purity (>90%) and mass identify, respectively.

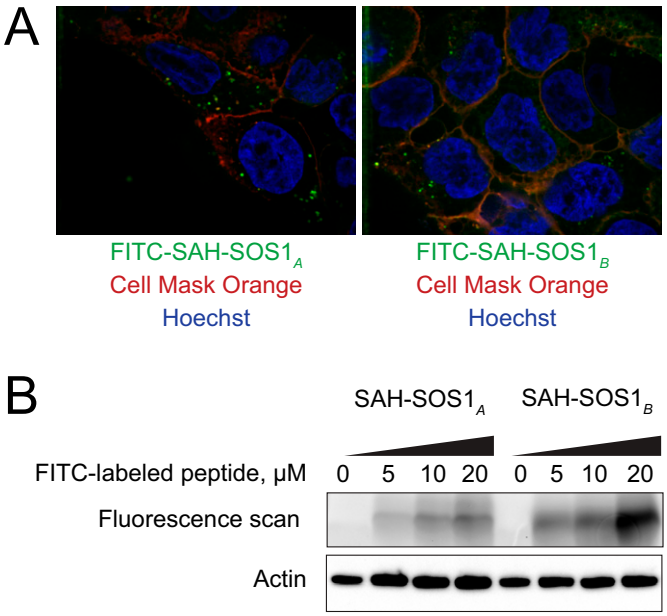


Fig. S3. Cellular uptake of SAH-SOS1$_A$ and SAH-SOS1$_B$ peptides. (*A*) FITC-derivatized analogs of SAH-SOS1$_A$ and SAH-SOS1$_B$ peptides bearing N-terminal Arg-Arg residues were applied to cultured Panc 10.05 cells, and cellular fluorescence was examined by confocal microscopy. Cell Mask Orange (plasma membrane), red; FITC, green; Hoechst (nucleus), blue. At least six fields of view were acquired per experimental condition; the data are representative of two biological replicates performed with independent cancer cell cultures. (*B*) FITC-SAH-SOS1 peptide uptake was also monitored by fluorescence scan of electrophoresed lysates from treated Panc 10.05 cells. Data are representative of at least three biological replicates performed with independent cancer cell cultures.

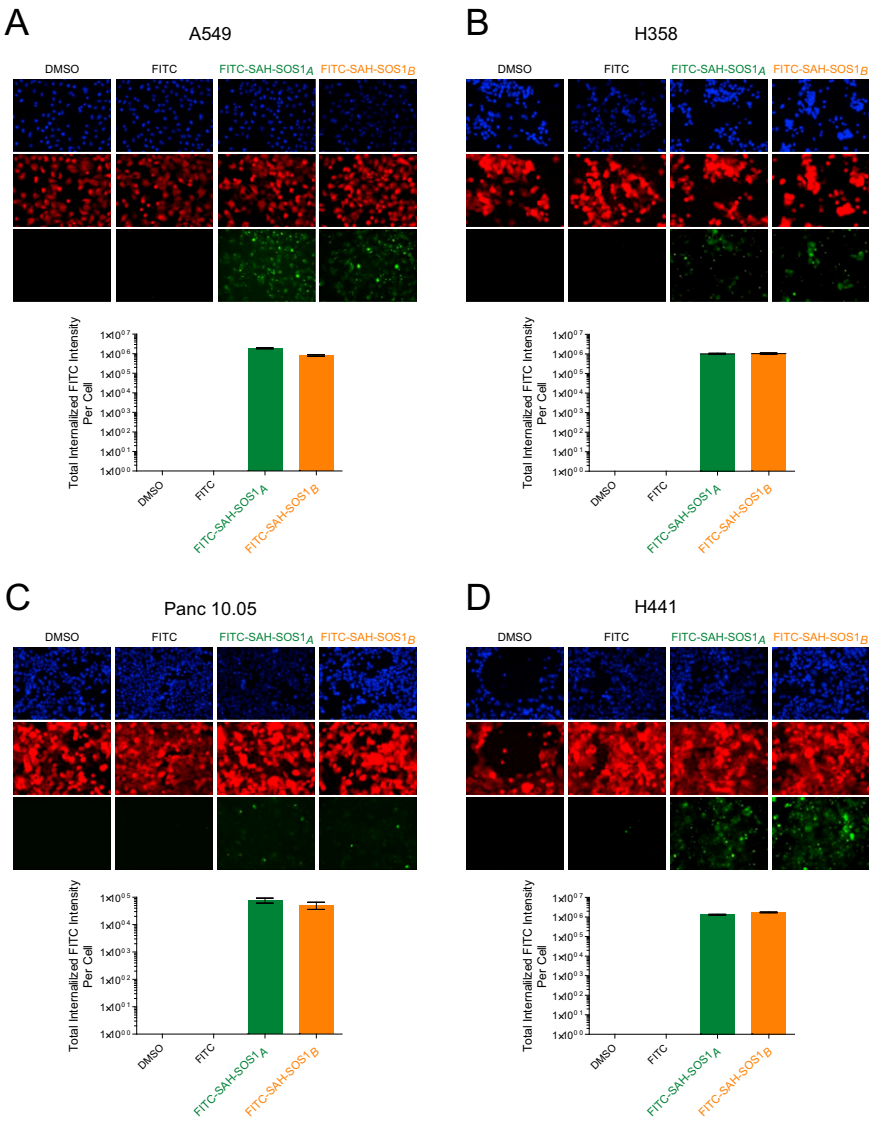


Fig. S4. High content microscopic imaging analysis of SAH-SOS1 peptide uptake by KRAS-driven cancer cell lines. High throughput epifluorescence microscopy and image analysis (MetaXpress) demonstrate fluorescence of A549 (*A*), H358 (*B*), Panc 10.05 (*C*), and H441 (*D*) cells treated with FITC-SAH-SOS1$_A$ or FITC-SAH-SOS1$_B$ peptides (0.5 μM), but not the vehicle (0.1% DMSO) or FITC-only controls. The nuclear (Hoechst 33342, blue) and plasma membrane (CellMask Deep Red, red) channels were used to create a mask, such that green intensity located inside the cellular boundary was measured on a per cell basis over five fields, averaged, and then plotted. Data are mean ± SEM for experiments performed in technical duplicate and then repeated with independent cancer cell cultures and treatment.

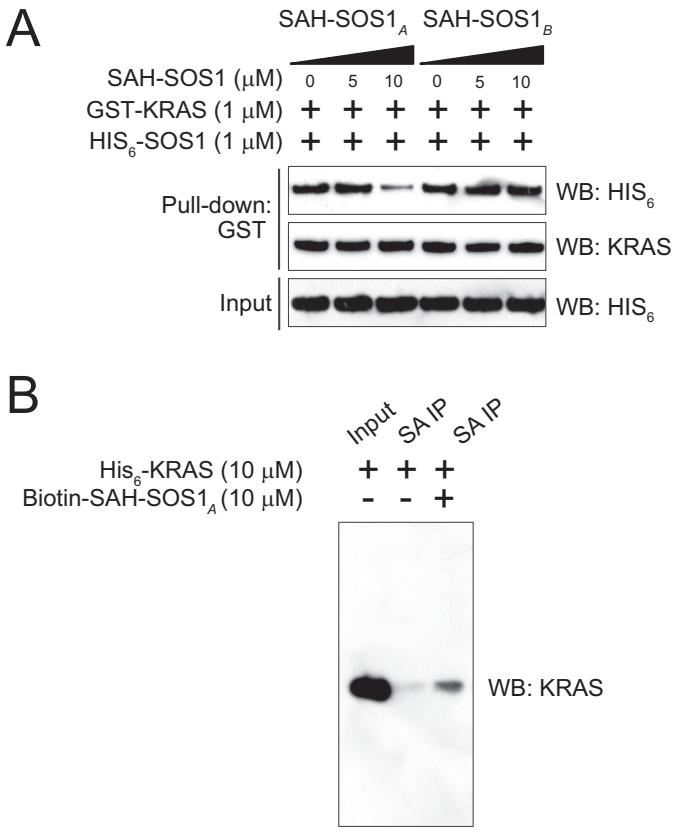


Fig. S5. Dissociation of the SOS1/KRAS protein interaction by SAH-SOS1$_A$. (*A*) SAH-SOS1$_A$, but not SAH-SOS1$_B$, disrupts the protein interaction between GST-KRAS (1 μM) and His$_6$-SOS1 (1 μM), as evidenced by GST pull-down and anti-KRAS and anti-His$_6$ Western blot analysis. (*B*) SAH-SOS1$_A$ directly targets recombinant KRAS in solution, as evidenced by streptavidin pull-down of the Biotin-SAH-SOS1$_A$/KRAS complex.

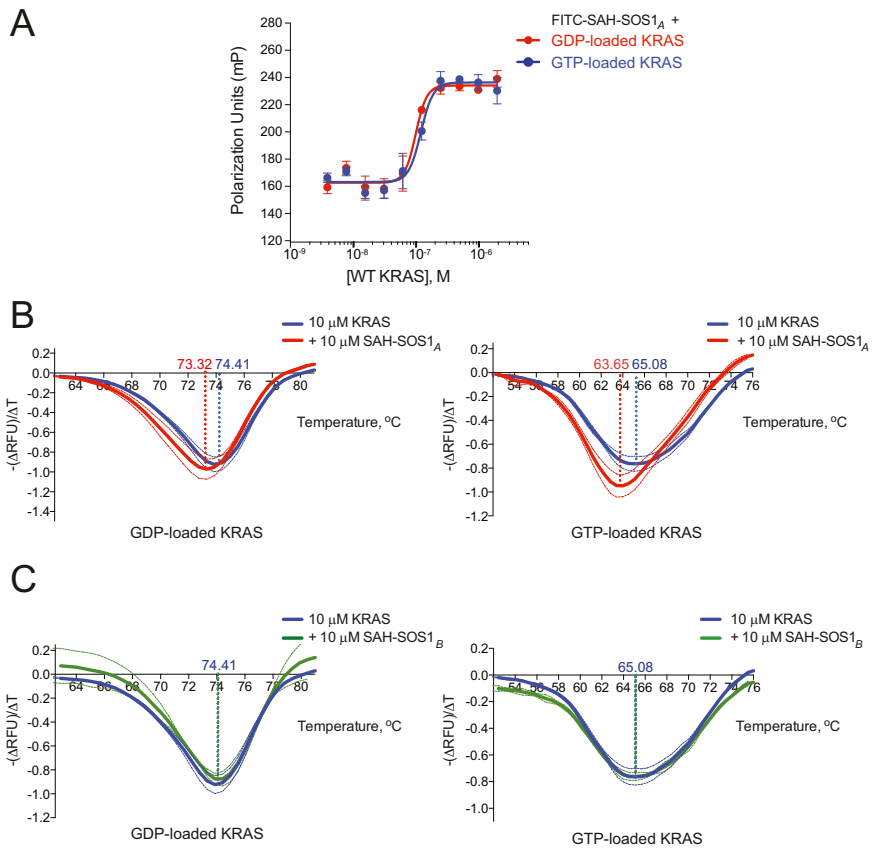


Fig. S6. SAH-SOS1$_A$ binds to GDP- and GTP-loaded forms of KRAS. (*A*) Fluorescence polarization binding analysis of FITC-SAH-SOS1$_A$ and recombinant wild-type KRAS protein, loaded with GDP (red) or GTP (blue). Data are mean ± SD for experiments performed in technical triplicate and representative of at least two biological replicates performed with independent preparations of recombinant KRAS proteins. (*B* and *C*) Differential scanning fluorimetry was performed on GDP- or GTP-loaded forms of recombinant wild-type KRAS (blue), in the presence or absence of SAH-SOS1$_A$ (red) (*B*) or the negative control SAH-SOS1$_B$ peptide (green) (*C*). Data are mean ± SD (dotted lines) for experiments performed in triplicate and representative of three biological replicates using independent KRAS preparations.

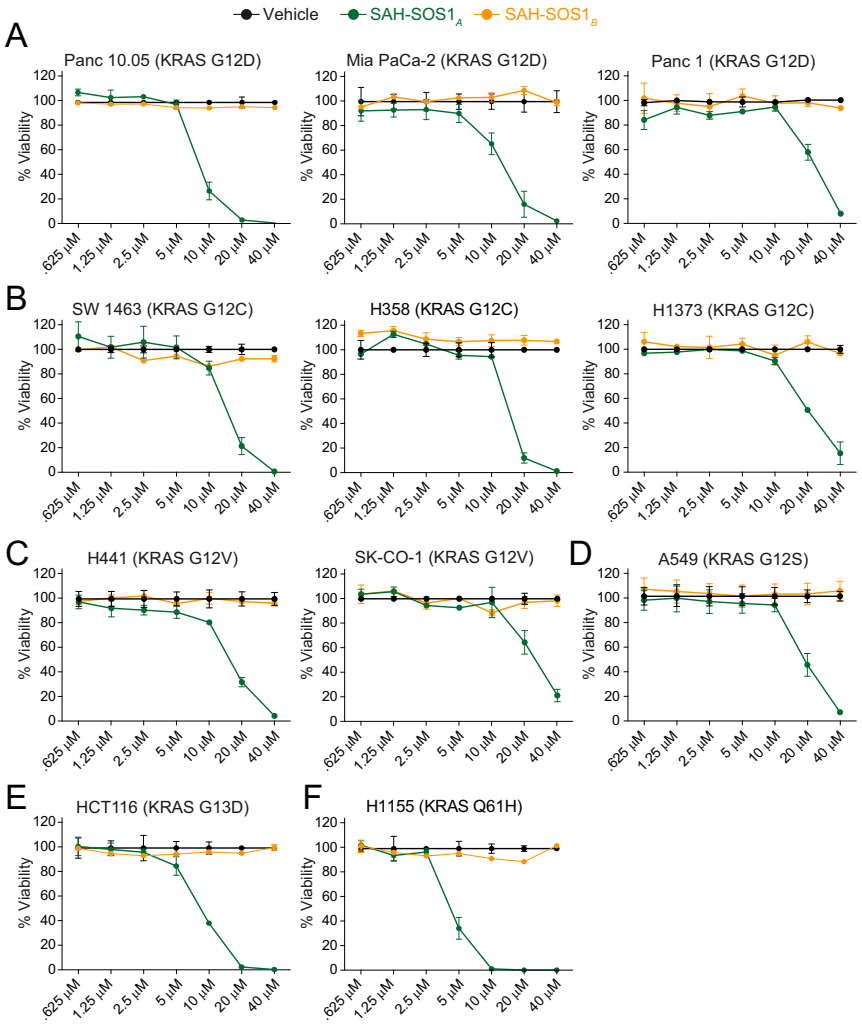


Fig. S7. SAH-SOS1$_A$ inhibits the viability of mutant KRAS-expressing cancer cells in a dose- and sequence-dependent manner. Cancer cells bearing KRAS G12D (*A*), KRAS G12C (*B*), KRAS G12V (*C*), KRAS G12S (*D*), KRAS G13D (*E*), and KRAS Q61H (*F*) were treated with vehicle, SAH-SOS1$_A$, or SAH-SOS1$_B$, and cell viability was measured at 24 h by CellTiterGlo assay. Data are mean ± SEM for experiments performed in at least duplicate and representative of at least two biological replicates performed with independent cancer cell cultures.

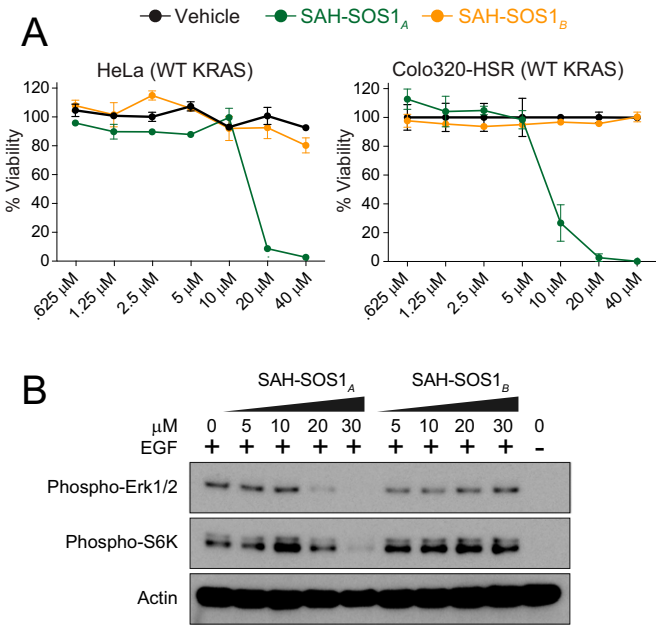


Fig. S8. SAH-SOS1$_A$ cytotoxicity correlates with inhibition of phosphosignaling downstream of KRAS. (*A*) HeLa and Colo320-HSR cells bearing wild-type KRAS were treated with vehicle, SAH-SOS1$_A$, or SAH-SOS1$_B$ peptides and cell viability measured at 24 h by CellTiterGlo assay. Data are mean ± SEM for experiments performed in at least technical duplicates and representative of at least two biological replicates performed with independent cancer cell cultures. (*B*) Panc 10.05 cells were incubated with vehicle, SAH-SOS1$_A$, or SAH-SOS1$_B$ at the indicated doses for 4 h, followed by 15 min of stimulation with EGF. Cellular lysates were then electrophoresed and subjected to Western blot analysis using antibodies to phospho- and total MEK1/2, ERK1/2, and Akt.

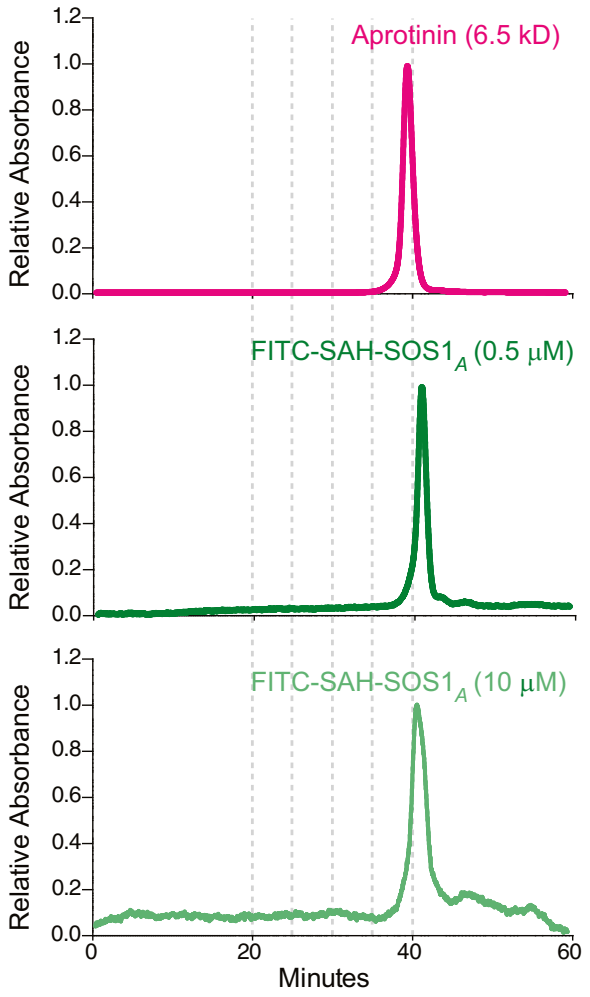


Fig. S9. Fluorescence size exclusion chromatography (FSEC) of SAH-SOS1$_A$ solutions. FSEC analysis of 0.5 μM and 10 μM solutions of FITC-SAH-SOS1$_A$ peptide demonstrate a monodispersed peak, reflecting a monomeric species eluting at ~3 kDa with respect to the aprotinin molecular mass standard (6.5 kDa).

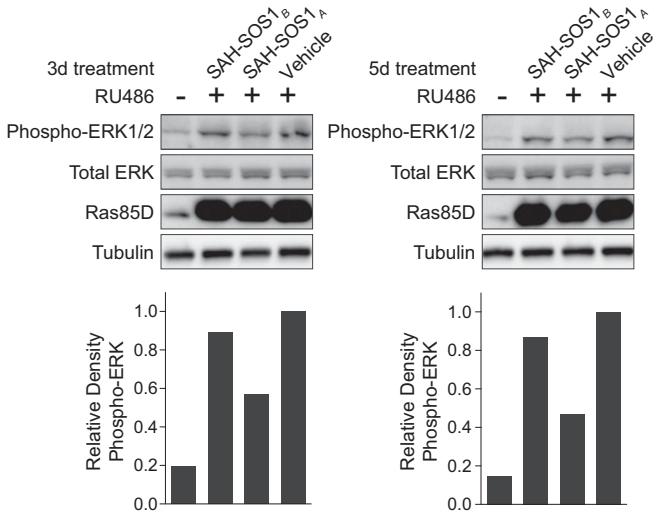


Fig. S10. SAH-SOS1$_A$ inhibits phosphosignaling downstream of KRAS in a *D. melanogaster* model of RAS activation. *D. melanogaster* Ras85D^{V12}/ActinGS (n = 20 per treatment arm) were exposed to chemically defined medium supplemented with vehicle or RU486 (150 μg/mL) for 2 d. RAS-induced flies were then treated with vehicle (DMSO), SAH-SOS1$_A$, or SAH-SOS1$_B$, added to a final concentration of 100 μM in 2 mL of fresh food containing RU486 (150 μg/mL). After 3 or 5 d of oral peptide treatment, the flies were collected and tissue lysates subjected to electrophoresis and Western blotting using RAS, and phospho- and total ERK1/2 antibodies. Western bands were quantified with ImageJ software.

Table S1. SAH-SOS1 peptide compositions

Peptide	Sequence	N terminus	MW	M+3/3	Fig.
SAH-SOS1$_a$	FFGIXLTNXLKTEEGN	FITC-βAla	2,292	765	S1
SAH-SOS1$_b$	FFGXYLTXILKTEEGN	FITC-βAla	2,341	781	S1
SAH-SOS1$_c$	FXGIYXTNILKTEEGN	FITC-βAla	2,308	770	S1
SAH-SOS1$_d$	FFGIYLTNXLKTXEGN	FITC-βAla	2,326	776	S1
SAH-SOS1$_A$	RRFFGIXLTNXLKTEEGN	FITC-βAla	2,604	869	1, S2–S4, S9
SAH-SOS1$_A$	RRFFGIXLTNXLKTEEGN	Ac	2,186	730	2–5, S2, S5–S8, S10
SAH-SOS1$_A$	RRFFGIXLTNXLKTEEGN	Biotin-βAla	2,441	815	S5
SAH-SOS1$_B$	RRFFGXYLTXILKTEEGN	FITC-βAla	2,654	886	1, S2–S4
SAH-SOS1$_B$	RRFFGXYLTXILKTEEGN	Ac	2,236	746	3–5,S2,S5–S8, S10
SAH-SOS1$_A$mut1	RRFFGKXLTNXLKTEEGN	FITC-βAla	2,619	874	4
SAH-SOS1$_A$mut1	RRFFGKXLTNXLKTEEGN	Ac	2,201	735	4
SAH-SOS1$_A$mut2	RRFFGIXLTRXLKTEEGN	FITC-βAla	2,647	883	4
SAH-SOS1$_A$mut2	RRFFGIXLTRXLKTEEGN	Ac	2,229	744	4
SAH-SOS1$_A$mut3	RRFFGIXDTNXLKTEEGN	FITC-βAla	2,606	870	4
SAH-SOS1$_A$mut3	RRFFGIXDTNXLKTEEGN	Ac	2,188	730	4
SAH-SOS1$_A$mut4	RRFFGIXKTNXLKTEEGN	FITC-βAla	2,619	874	4
SAH-SOS1$_A$mut4	RRFFGIXKTNXLKTEEGN	Ac	2,201	735	4
SAH-SOS1$_A$mut5	RRFEGIXRLEXLKAEEAN	FITC-βAla	2,640	881	4
SAH-SOS1$_A$mut5	RRFEGIXRLEXLKAEEAN	Ac	2,222	742	4