Direct inhibition of oncogenic KRAS by hydrocarbon-stapled SOS1 helices

Elizaveta S. Leshchiner, Andrey Parkhitko, Gregory H. Bird, James Luccarelli, Joseph A. Bellairs, Silvia Escudero, Kwadwo Opoku-Nsiah, Marina Godes, Norbert Perrimon, and Loren D. Walensky

Activating mutations in the Kirsten rat sarcoma viral oncogene homolog (KRAS) underlie the pathogenesis and chemoresistance of ∼30% of all human tumors, yet the development of high-affinity inhibitors that target the broad range of KRAS mutants remains a formidable challenge. Here, we report the development and validation of stabilized alpha helices of son of sevenless 1 (SAH-SOS1) as prototype therapeutics that directly inhibit wild-type and mutant forms of KRAS. SAH-SOS1 peptides bound in a sequence-specific manner to KRAS and its mutants, and dose-responsively blocked nucleotide association. Importantly, this functional binding activity correlated with SAH-SOS1 cytotoxicity in cancer cells expressing wild-type or mutant forms of KRAS. The mechanism of action of SAH-SOS1 peptides was demonstrated by sequence-specific down-regulation of the ERK-MAP kinase phosphosignaling cascade in KRAS-driven cancer cells and in a Drosophila melanogaster model of Ras85D→V12 activation. These studies provide evidence for the potential utility of SAH-SOS1 peptides in neutralizing oncogenic KRAS in human cancer.

Significance

KRAS is one of the most prevalent and vicious oncogenic proteins, yet no drugs are available to inhibit its pathologic activity in patients. We report that KRAS-targeting stapled peptides, modeled after the native son of sevenless 1 (SOS1) helical domain, engage wild-type and clinically relevant KRAS mutant proteins with nanomolar affinity. To our knowledge, these compounds represent the highest affinity and broadest spectrum binders of KRAS mutants reported to date. The stapled peptides disrupt the SOS1/KRAS protein interaction and directly inhibit nucleotide association to wild-type and mutant KRAS proteins. We correlate functional binding activity with SAH-SOS1 cytotoxicity across a 13-member panel of KRAS-driven cancer cells and demonstrate sequence- and dose-dependent inhibition of the ERK-MAP kinase phosphosignaling cascade downstream of KRAS in vitro and in vivo.
described small molecule hits engaged the RAS–SOS1–RAS ternary complex at a unique hydrophobic pocket, activated nucleotide exchange, and perturbed phosphosignaling downstream of RAS (13). Finally, a combinatorial screening approach identified several cyclic peptides with submicromolar RAS-binding activity, but no cellular activity was observed (14). Despite the recent progress in developing small molecules and synthetic peptides to directly target RAS or RAS-SOS1, high-affinity binders with promising cellular activity across the broad spectrum of wild-type and mutant KRAS-driven cancers have remained out of reach. Thus, there is a pressing need for next-generation agents to target and disarm KRAS in human cancer cells.

We have previously generated “stapled peptides” modeled after key α-helical interaction domains to disrupt oncogenic protein interactions of the BCL-2 family, p53, β-catenin, and EZH2 pathways (15–18). By sampling alternative staple positions, interrogating cellular uptake, and correlating biochemical function with anti-tumor activity and mechanism of action, we have generated lead compounds that form the basis for therapeutic development (16, 17, 19, 20). Here, we report the application of all-hydrocarbon stapling to recapitulate the native primary sequence and secondary structure of the RAS-interacting α-helix of SOS1. Our goal was to develop a direct inhibitor that binds the diversity of KRAS mutant forms, impairs nucleotide exchange and, importantly, broadly kills KRAS-mutant cancer cells in a sequence-specific manner by deactivating its downstream phosphosignaling cascade.

Results
SAH-SOS1 Peptides Bind to Wild-Type and Mutant KRAS with Nanomolar Affinity. The crystal structure of the complex between the SOS1 catalytic domain and KRAS (PDB ID code: 1NVU) (21) demonstrated direct contact between a SOS1 α-helix (cyan) and KRAS, implicating this interface in catalyzing nucleotide exchange (Fig. L4). Using the primary sequence of this SOS1 α-helix (amino acids 929–944), we generated a series of stabilized alpha helices of SOS1 (SAH-SOS1) peptides spanning amino acids 929–944 (Fig. S1A). The resultant SAH-SOS1 a–d peptides were then screened by fluorescence polarization (FP) assay for binding activity to recombinant hexahistidine-tagged (His6)KRASt proteins, including wild-type and G12D, G12V, G12C, G12S, and G61H mutant forms (Fig. S1B). Whereas SAH-SOS1 peptides a, c, and d demonstrated nanomolar binding activity in the 60–160 nM range, SAH-SOS1b showed little to no interaction, consistent with the disruptive location of its staple at the KRAS binding interface (Fig. L4 and Fig. S1).

We selected SAH-SOS1 for further development based on its superior binding activity and solubility profile, and also advanced its stability (Fig. S1C). We generated SAH-SOS1b as an ideal negative control for biochemical and cellular studies. To optimize these constructs for cellular work, we further appended two Arg residues at the N termini to adjust the overall charge of the peptides from −1 to +1, based on our longstanding observation across multiple stapled peptide templates that cellular uptake is enhanced when overall charge is ≥0 (22, 23). We confirmed the cellular penetrance of these revised constructs (Table S1 and Fig. S2), hereafter referred to as SAH-SOS1b and SAH-SOS1γ (using capital letter subscripts), by confocal microscopy (Fig. S3A), fluorescence scan of electrophoresed lysates from treated cells (Fig. S3B), and high-content microscopic imaging analysis (Fig. S4). We then repeated the FP binding analyses by using our positive and negative control SAH-SOS1 constructs, documenting a 100- to 175-nM binding range for SAH-SOS1γ across the His6-KRAS proteins, with no binding activity observed for SAH-SOS1b (Fig. 1). As an additional measure of relative binding activity, we demonstrated that SAH-SOS1γ, but not SAH-SOS1b, disrupted the protein interaction between recombinant GST-KRAS and His6-SOS1 proteins in vitro (Fig. S5A). We confirmed that SAH-SOS1γ was capable of dissociating the protein complex owing to its capacity to directly target KRAS in solution, as demonstrated by streptavidin pull-down of biotinylated SAH-SOS1γ and anti-KRAS Western blot analysis (Fig. S5B). Thus, we find that insertion of an all-hydrocarbon staple into the native SOS1 sequence (amino acids 929–944) at the noninteracting helical face yields peptidic ligands that bind to KRAS in the nanomolar range, representing at least a two orders of magnitude improvement over the recently published peptide and small molecule inhibitors of the KRASSOS1 interface.

SAH-SOS1γ Engages both GDP-KRAS and GTP-KRAS. Because KRAS exists in two distinct conformations depending on its interaction with GDP vs. GTP (24), we next tested whether SAH-SOS1γ engages one or both forms of KRAS. FP analysis using GDP- and GTP-loaded, recombinant, wild-type KRAS revealed equivalent SAH-SOS1γ binding affinities (Fig. S6A), consistent with the capacity of SAH-SOS1γ to engage both forms of KRAS. As an additional measure of direct binding activity, we performed differential scanning fluorimetry using by GDP- and GTP-loaded forms of KRAS in the presence or absence of SAH-SOS1γ or the negative control peptide SAH-SOS1b. GTP-loaded KRAS demonstrated a lower melting temperature than GDP-loaded KRAS (Fig. S6B), reflecting the more rigid structure of the GDP-loaded form (and relative inaccessibility of the SYPRO dye until the GDP-loaded form unfolds at the higher temperature). Upon addition of SAH-SOS1γ, both forms of KRAS demonstrate a shift in the melting curve, consistent with a small increase in conformational flexibility from ligand binding. Indeed, the SOS1 interaction is believed to favor the KRAS “open” conformation (21), and allosteric release of nucleotide can also decrease the structural
stability of the KRAS protein (25). Importantly, the observed changes in KRAS are peptide sequence-specific, as the negative control stapled peptide, SAH-SOS1\(_{18}\), had no such effect (Fig. S6D).

To further confirm that SAH-SOS1\(_{14}\) peptide specifically targets KRAS at the SOS1 binding pocket, we performed NMR analysis of GDP-loaded \(^{15}\)N-KRAS upon incubation with SAH-SOS1\(_{14}\). We find that the overall fold of KRAS is preserved upon SAH-SOS1\(_{14}\) engagement, and discrete chemical shift changes occur in residues that colocalize at the SOS1-binding site (see below) and the very region of KRAS implicated in conformational opening during SOS1 protein engagement (e.g., H27, F28, V29, R149) (Fig. 2 A and B). A calculated model structure derived from docking analyses demonstrated the juxtaposition of SAH-SOS1\(_{14}\) residues with the majority of residues that undergo chemical shift change, such as L6, G15, L56, E63, Y64, R73, T74, and Q79 (Fig. 2C). Taken together, our biochemical data demonstrate that SAH-SOS1\(_{14}\) directly binds to both GDP- and GTP-loaded forms of KRAS, with the structural analysis implicating the SOS1-binding pocket as the functional site of SAH-SOS1\(_{14}\) interaction.

**SAH-SOS1\(_{14}\) Directly Inhibits Nucleotide Association to KRAS.** We next sought to determine whether the direct binding of SAH-SOS1\(_{14}\) could independently impact the capacity of KRAS to exchange nucleotide and, in particular, the activating nucleotide association process. The addition of the fluorescent GTP analog, mant-GTP (2′-5′-O-methylanthranilylguanosine-5′-triphosphate), associated with wild-type His\(_8\)-KRAS in a time-dependent fashion, as demonstrated by the increase in measured fluorescence (Fig. 3A). As a negative control, we incubated KRAS with mant-GTP and excess unlabeled GTP, which completely blocked mant-GTP association (Fig. 3A). We then coincubated KRAS with mant-GTP and either SAH-SOS1\(_{14}\) or SAH-SOS1\(_{18}\). Whereas SAH-SOS1\(_{14}\) dose-responsively inhibited mant-GTP association, SAH-SOS1\(_{18}\) had little to no effect (Fig. 3A).

To evaluate the functional impact of SAH-SOS1 peptides on the G12D mutant form of KRAS, mant-GTP association was examined because of the slower kinetics of mant-GTP binding in this experimental context. The incubation of His\(_8\)-KRAS G12D with mant-GDP resulted in time-responsive association, which was blocked upon coincubation with excess unlabeled GDP (Fig. 3B). As above, coincubation of KRAS G12D with mant-GDP and SAH-SOS1\(_{14}\) peptide dose-responsively blocked nucleotide association, whereas SAH-SOS1\(_{18}\), which does not bind to wild-type or mutant KRAS, had no effect. Thus, we find that engagement of wild-type or KRAS G12D by SAH-SOS1\(_{14}\) independently blocks the capacity of KRAS to engage nucleotides in both a dose-responsive and sequence-dependent fashion.

**SAH-SOS1\(_{14}\) Impairs the Viability of KRAS-Mutant Cancer Cells.** Whereas micromolar quantities of small molecules and peptides were shown to target the SOS1/KRAS binding region and disrupt nucleotide exchange activity (10–12), the effect of these agents on cancer cell viability was not explored. We therefore tested whether our lead SAH-SOS1\(_{14}\) construct, which binds to wild-type and mutant forms of KRAS in the 100- to 175-nM range, could inhibit the viability of pancreatic, colon, and lung cancer cells bearing distinct KRAS mutations. We found that SAH-SOS1\(_{14}\), but not the negative control peptide SAH-SOS1\(_{18}\), dose-responsively impaired the viability of cancer cells bearing KRAS G12D (Fig. 4A and Fig. S7A), G12C (Fig. S7B), G12V (Fig. S7C), G12S (Fig. S7D), G13D (Fig. S7E), and Q61H (Fig. S7F) mutations with IC\(_{50}\) values in the 5- to 15-µM range. Cancer cells expressing wild-type KRAS, such as HeLa and Colo320-HSR cells, were similarly affected (Fig. S8A). Importantly, we confirmed that at the doses that SAH-SOS1\(_{14}\) was cytotoxic to cells, the stapled peptide was soluble and monomeric (Fig. S9).

To further probe the specificity of SAH-SOS1\(_{14}\) activity, we generated five mutant constructs that incorporated, for example, conversions of select hydrophobic residues to charged residues, and hydrophilic residues to hydrophobic residues (Fig. 4B). We then measured His\(_8\)-KRAS G12D binding activity and cell viability responses in the KRAS G12D-bearing Panc 10.05 cancer...
line. Strikingly, those SAH-SOS1Δ mutant peptides that retained KRAS G12D binding activity (mut1-2) inhibited cell viability, whereas those mutants that lost binding activity (mut3-5) had no effect on cancer cell viability (Fig. 4 C and D). Thus, we find that SAH-SOS1Δ manifests dose-responsive and sequence-specific antitumor activity across a broad spectrum of wild-type and mutant KRAS-expressing cancer cell lines.

SAH-SOS1Δ inhibits phosphosignaling downstream of KRAS in vitro and in vivo. To determine whether the observed inhibitory effect of SAH-SOS1Δ on cancer cell viability could be linked to negative modulation of KRAS signaling activity, we examined the dose-dependent effects of SAH-SOS1Δ treatment on the phosphorylation state of MEK, ERK, and AKT in Panc 10.05 cells exposed to EGF. Whereas SAH-SOS1Δ dose-responsively inhibited MEK1/2, ERK1/2, and AKT phosphorylation, SAH-SOS1Δ had no effect (Fig. 5A). An analogous SAH-SOS1Δ-specific effect was observed in HeLa cells bearing wild-type KRAS (Fig. S8B). Importantly, the dosing in which SAH-SOS1Δ down-regulated phosphorylation matched the concentrations required to impair cancer cell viability (Figs. 4A and 5A).

Finally, we evaluated whether the inhibitory effects of SAH-SOS1Δ on KRAS signaling observed in cancer cells could be extended to an in vivo context. For this work, we used an inducible system in Drosophila melanogaster for Ras1, the major Drosophila homolog of N-ras, H-ras, and K-ras genes (26). In this model, Actin Geneswitch (ActinGS) drives broad and high level tissue expression of a V12-mutant form of RAS85D upon treatment with RU486 (27). For our first study, we induced RAS85DV12 for 3 d by using 150 µM RU486, which was added directly to the fly food. We then injected vehicle or SAH-SOS1Δ directly into the fly abdomen, and 48 h later performed Western blotting for phosphorylated-ERK1/2 on the harvested tissue extracts. SAH-SOS1Δ treatment notably decreased the phosphorylation state of ERK1/2 (Fig. 5B). Next, we added SAH-SOS1Δ directly to the fly food, as for RU486, and after 3 d of RAS85DV12 induction, Western blotting for phospho-ERK1/2 and phospho-AKT was performed. We observed near complete suppression of ERK1/2 and AKT phosphorylation at the 100 µM SAH-SOS1Δ dosing level (Fig. 5C). To probe the specificity of the response, we repeated the experiment comparing responses to vehicle, SAH-SOS1Δ, and SAH-SOS1Δ after 3 and 5 d treatment with RU486 and SAH-SOS1Δ. In each case, we observed a decrease in ERK1/2 phosphorylation upon SAH-SOS1Δ treatment, whereas SAH-SOS1Δ had no effect (Fig. S10). Of note, SAH-SOS1Δ peptides had no independent effect on the levels of RAS or ERK protein, and no fly toxicity was observed from the treatments.

Discussion

KRAS is one of the most pervasive pathogenic factors in human cancer but no drugs are available for clinical use to directly bind and block this deadly protein. Over the last few years, there has been a flurry of new reports suggesting that small molecules and peptide prototypes may, in fact, be capable of engaging KRAS to block its GTP binding site, impair nucleotide exchange, tip the equilibrium in favor of the inactive state, and/or block interactions with effectors. Whereas the majority of approaches have achieved proof-of-concept for engagement of RAS or KRAS, and consequent biochemical modulation (albeit at mid- to high-micromolar concentrations), anticancer activity was either not yet explored, not observed, or required dosing levels that would preclude clinical translation. A notable exception are compounds that engage a novel binding pocket beneath the effector binding pocket of KRAS.
switch-II region of the G12C mutant form of KRAS. These new agents exhibited submicromolar antiproliferative activity specifically in KRAS G12C-driven cancer cells. Indeed, all of these new strategies hold promise that further iteration and optimization could lead to a breakthrough anti-KRAS agent. In the meantime, continued exploration of chemical space and extension of structural and biochemical studies into the cellular and in vivo realms is required.

Stapled peptides are a relatively new class of structured peptides, designed to mimic bioactive helices yet remedy the traditional shortcomings of peptide therapeutics, namely loss of shape, proteolytic liability, and difficulty accessing intracellular targets. When appropriately designed and optimized, stapled peptides can manifest remarkable structural stability, extracellular and intracellular protease resistance, and the capacity to gain entry to the cell via energy-dependent macropinocytosis (18, 23). Sampling a series of staple locations along the length of the peptide can yield high-affinity binders and, importantly, negative control compounds based on the replacement of key amino acid residues or steric interference of the staple at the binding interface. Here, we find that SAH-SOS1 can target the SOS1-binding pocket on KRAS with nanomolar affinity, directly and independently block nucleotide association, impair KRAS-driven cancer cell viability, and exert its effects by on-mechanism blockade of the phosphosignaling cascade downstream of KRAS. Importantly, all of these activities are dose- and sequence-dependent. Using a Drosophila model of induced mutant RAS expression, we find that the effects of SAH-SOS1, administered by injection or orally, extend to an in vivo context. Thus, a peptide-stapling approach to targeting the SOS1 regulatory groove on KRAS may be a viable strategy for therapeutic development.

One of the most promising features of our stapled peptide results is the broad spectrum, nanomolar targeting capacity of SAH-SOS1 across both wild-type and a diversity of KRAS mutant forms. Indeed, SAH-SOS1 peptides are among the highest affinity KRAS binders reported to date, and address the most clinically relevant forms of mutant KRAS, including the G12V, G12D, G12C, G12S, G12C, and Q61H variants. Importantly, the SAH-SOS1 approach can harness dual biochemical activities, in that it can both disrupt the activating SOS1/KRAS protein interaction, while also independently block nucleotide association as a direct result of its interaction with KRAS. It appears that the SOS1-binding interface provides a platform for SAH-SOS1 engagement of KRAS irrespective of mutant form, with peptide interaction effectively and uniformly blocking nucleotide association, as exemplified by our biochemical results using recombinant wild-type and G12D-mutant KRAS. Indeed, more than a dozen cancer lines expressing either wild-type or mutant KRAS protein were susceptible to SAH-SOS1 treatment.

A potential therapeutic window for this treatment strategy may derive from the relative dependency of such cancer cells on KRAS compared with normal cells. Our studies in Drosophila did not reveal toxicity from SAH-SOS1−induced down-regulation of RAS-driven phosphosignaling. It is also noteworthy that pancreatic cancer cells have been shown to up-regulate macropinocytosis as a unique means of nutrient loading (28). Because appropriately designed stapled peptides can achieve cellular penetration via the same macropinocytic pathway, preferential uptake of therapeutic peptide by KRAS-driven pancreatic cancer cells may provide a further measure of tissue selectivity. Future directions include further optimizing the potency of prototype SAH-SOS1 peptides by using structure-activity relationships, exploring the therapeutic window for KRAS modulation in vivo by building on the Drosophila results, and advancing lead constructs to preclinical testing in murine models of KRAS-driven cancers.

Methods

Peptide Synthesis and Characterization. Fmoc-based automated peptide synthesis, hydrocarbon stapling by olefin metathesis, and N-terminal derivatization with FITC-p-Ala, acetyl, or Biotin-p-Ala were performed according to our established methods (22). All peptides were purified by liquid chromatography/MS to >90% purity and quantified by amino acid analysis (Table S1 and Fig. S2).

KRAS Protein Purification. Recombinant wild-type human KRAS protein (amino acids 1–156) and its G12D, G12V, G12C, G12C, and Q61H mutant variants were expressed in Escherichia coli BL21(DE3) as N-terminal hexahistidine-tagged (His) fusion proteins by using the pET28-MHL expression vector (Addgene plasmid 25153; a gift from C. Arrowsmith, University of Toronto, Toronto). Protein expression was induced with 1 mM IPTG for 4 h at 30 °C. Bacterial pellets were resuspended in 20 mM Tris, 250 mM NaCl, pH 7.8 lysed by microfluidization (Microfluidics M-110L), and centrifuged at 45,000 rpm for 1 h at 4 °C (Beckman L-90K). The cleared cellular lysates were subjected to Ni affinity resin (Qiagen) chromatography followed by elution with 150 mM imidazole in 50 mM Tris, 250 mM NaCl, pH 7.8. Concentrated eluates were subjected to size exclusion chromatography (GE Life Sciences) at 4 °C by using 50 mM Tris, 150 mM NaCl, pH 7.8 buffer conditions, and the corresponding monomeric peaks were collected. Protein concentration was determined by Bradford assay (Bio-Rad) and UV absorbance measurements, and the average value was used. His-tagged KRAS proteins were used in all biochemical experiments unless otherwise indicated.

Fluorescence Polarization Assay. FP assays were performed as described (18). Briefly, FITC-SAH-SOS1 peptides (15 nM) were incubated with the indicated serial dilution of KRAS wild-type or mutant protein in binding buffer (50 mM Tris, 1 mM NaCl, pH 8.0). Fluorescence polarization was measured using a SpectraMax MS microplate reader (Molecular Devices). Ecp values were calculated by nonlinear regression analysis of dose–response curves using Prism software (GraphPad).

Nucleotide Loading. Nucleotide loading for FP, differential scanning fluorimetry (DSF), and NMR spectroscopy studies were performed as described (21). Purified KRAS protein (200 μM) was incubated with 2 mM GDP, 6 μM GppNp in loading buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DTT) for 1.5 h on ice. Reactions were quenched with 12 mM MgCl2 and incubated for 30 min on ice. For DSF (SI Methods), the protein was used without further purification, whereas for NMR and FP, excess of the free nucleotides was removed and the buffer exchanged into PBS, 1 mM DTT, 2 mM MgCl2 using preequilibrated NAP-5 columns (GE Life Sciences).

NMR Spectroscopy. All spectra were collected by using an Agilent Inova 500 MHz system equipped with a triple resonance (H, C, N) 5-mm cold probe. Experiments were performed at 25 °C. KRAS was uniformly 15N isotopically labeled, charged with GDP as described above, and dissolved in phosphate buffer (95.5:4.5:O.D.O, PBS pH 7.4, 1.0 mM DTT, 2 mM MgCl2). The instrument was locked to the deuterium signal, and resonances are reported in parts per million relative to D2O (ν = 4.72 ppm). A 5 mM NMR tube was charged with a volume of sample (150 μM protein) and the 1H NMR spectrum in the absence or presence of ligand was obtained using 500 μM of each ligand. The change in chemical shift upon addition of ligand was plotted for each resonance. For titration experiments, up to 10 μM equivalents of ligand (in PBS) were added to the protein solution. Data were analyzed by using VNMR 3.2 software (Agilent Technologies). The change in chemical shift upon addition of ligand was plotted for each resonance. The presence of a probe, or a residue that is overlapped or not assigned.

Docking Calculations. The peptide sequence FFGIXLTNLXKTEEGN was built by using Maestro in an idealized α-helical form. The sequence was docked to the target protein by using the HADDOCK webserver default parameters (31, 32). Chain R of the 1NVU crystal structure (21) was used, with residues L6, G15, H27, F28, V29, L66, D57, E63, Y64, R73, T74, G77, and R149 defined as the active residues. Top-scoring clusters were analyzed visually with Pymol (Schrodinger, LLC).

Nucleotide Association Assay. The association of mant-GTP with wild-type KRAS protein was monitored by fluorescence measurement over time on a Tecan X1000 fluorescence spectrometer (excitation 360 nm, emission 440 nm) (12). SAH-SOS1 peptides at the indicated amounts were incubated with 1 μM KRAS and 1 μM mant-GTP in buffer containing 25 mM Tris (pH 7.5), 50 mM NaCl, and 1 mM DTT at 25 °C. KRAS and mant-GTP alone established the positive control for association, and competition with 200-fold excess unlabeled GTP served as the negative control. The identical association experiment was performed with KRAS G12D except that mant-GDP was used instead, because
malt-GTP association was not detectable within the analogous experimental time frame.

Cell Viability Experiments. The indicated cell lines (American Type Culture Collection) were plated in 96-well plates at 10^4 cells per well by using high-glucose DMEM media supplemented with 10% (vol/vol) FBS, 1% penicillin/streptomycin and l-Glutamine (Invitrogen). The media was aspirated and glucose DMEM media supplemented with 10% (vol/vol) FBS, 1% penicillin/streptomycin, and L-Glutamine. After 24 h, cells were treated with the indicated concentrations in serum-free DMEM. After 4 h of peptide exposure, serum was replaced (10% (vol/vol) FBS final concentration), and cell viability was measured at 24 h by CellTiterGlo assay (Invitrogen).

Phosphosignaling Western Blot Analysis. The indicated cells were plated in 12-well plates at 10^5 cells per well by using high-glucose DMEM media supplemented with 10% (vol/vol) FBS, 1% penicillin/streptomycin, and l-Glutamine. After 24 h, cells were treated with the indicated amounts of SAH-SOS1 peptides for 4 h in serum-free DMEM, followed by addition of l-ethylendiamine (Egl) (Cell Signaling) at 10 nM/g for 15 min. Cells were lysed in buffer containing 0.5% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM MgCl2, Complete protease inhibitors and PhosphoOne phosphatase inhibitors (Roche), and the lysates were subjected to SDS/PAGE and Western blot analysis using antibodies to actin (Sigma) and phospho- and total ERK1/2, 56K, and MEK1/2 (Cell Signaling).

In Vivo Phosphosignaling Analysis. The Drosophila ActinGS-Gal4 line was a gift from J. Tower, University of Southern California, Los Angeles, and the UAS-RasBSTD12 line was obtained from the Bloomington Stock Center. Flies were reared and experiments were conducted at 25 °C on a 12:12h light-dark cycle at constant humidity by using standard sugar yeast medium, unless noted otherwise. Flies were reared on food (2 mL) containing high-glucose DMEM. After 4 h of peptide exposure, serum was replaced (10% (vol/vol) FBS) and the supernatants were analyzed by SDS/PAGE and Western blotting. For the time-course experiments, flies were reared on 2 mL of chemically defined food (Harlan) containing vehicle, RU486 alone (150 μg/mL), or the combination of RU486 with 20 μL of vehicle (DMSO) or 20 μL of SAH-SOS1 or SAH-SOS1 stock solutions to achieve final peptide concentrations of 1, 10, or 100 μM. Flies were collected after 4 d, processed, and lysates were analyzed as described above. For the time-course experiments, flies were reared on 2 mL of chemically defined food (Harlan) containing vehicle, RU486 alone (150 μg/mL), or the combination of RU486 with 20 μL of vehicle (DMSO) or 20 μL of 10 mM stock solutions of SAH-SOS1 or SAH-SOS1 peptides (final peptide concentration of 100 μM). After 3 or 5 d, the flies were collected, processed, and lysates analyzed as above. Antibodies for Western blot analysis included tubulin (Sigma), phospho- and total ERK1/2 (Cell Signaling), and Ras (gift of M. Therrien, University of Montreal, Montreal).

ACKNOWLEDGMENTS. We thank Eric D. Smith for figure preparation and graphics assistance, Marc Therrien for Ras antibodies, and John Tower for Actin-GS-Gal4 flies. This work was supported by NIH Grant 5R01GM090299 (to L.D.W.), NIH Grant P01CA120964 and Howard Hughes Medical Institute (to P.N.), an American Association of University Women International Fellowship (to E.S.L.), NIH Grant T32GM007753 (to J.L.), the Todd J. Schwartz Memorial Fund, and the Wolpoff Family Foundation.