An Orthosteric Inhibitor of the Ras-Sos Interaction

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**Supplementary Figure 3** | Circular dichroism spectra of the unconstrained peptides and HBS α-helices. Peptides were dissolved in 1 mM phosphate buffered saline (pH 7.4) containing 10% TFE.

![Circular dichroism spectra](image)

**Supplementary Figure 4** | Rates of nucleotide exchange from Ras in the presence or absence of Sos and αH mimetics. Exchange assays were performed with 1 µM Ras, 1 µM mant-GDP, 1 µM Sos, 100 µM GDP, and 25 µM HBS 3 or HBS 7.

![Rates of nucleotide exchange](image)
Supplementary Figure 5 | Determination of Flu-HBS 3 and Flu-HBS 7 affinities for Ras by a fluorescence polarization assay.

(a) Binding of HBS helices to nucleotide free Ras. EDTA was added to promote dissociation of nucleotide from Ras.

\[
K_d \text{ Flu-HBS 3–Ras} = 28 \pm 4.8 \mu \text{M}
\]

\[
K_d \text{ Flu-HBS 7–Ras} = 273 \pm 8.5 \mu \text{M}
\]

(b) Binding of Flu-HBS 3 to Ras•GDP

\[
K_d \text{ Flu-HBS 3–Ras•GDP} = 158 \pm 16 \mu \text{M}
\]
Supplementary Figure 6a | $^1$H-$^{15}$N HSQC overlaid spectrum of Ras (blue), Ras:HBS 3 (1:3, red) and Ras:HBS 3 (1:5, green).
Supplementary Figure 6b | Mean chemical shift difference ($\Delta \delta_{\text{NH}}$) plot depicting resonance shifts upon the addition of increasing amounts of HBS 3.
Supplementary Figure 6c | NMR studies implicate switch regions of Ras as the binding site for HBS 3. Ras residues that underwent significant shift (mean chemical shift difference > 0.02) are shown in orange. The color map was generated from the crystal structure of Ras in complex with Sos (PDB code 1nvw).
Supplementary Figure 7a | Cellular uptake of fluorescein (Flu) and fluorescein-labeled 3 (Flu-3), HBS 3 (Flu-HBS 3) and HBS 7 (Flu-HBS 7) into live HeLa cells. The structures of fluorescein-linked peptides are shown in Supplementary Figure 2. Cells were visualized by bright field and fluorescence microscopy after 12 hour incubation with the indicated peptides.
**Supplementary Figure 7b** | Effect of temperature on the cellular uptake of fluorescein-labeled HBS 3 (Flu-HBS 3). Cells were incubated with fluorescein (Flu) or Flu-HBS 3 at the indicated temperatures for 2.5 hours and fixed with paraformaldehyde. Nuclei were stained with DAPI. The images represent a single 0.25 µm Z-section through middle of the cell.
Supplementary Figure 8 | (a) HBS 3 attenuates EGF-induced Ras activation. HeLa cells were stimulated with EGF in the presence or the absence of the indicated peptides. GTP-bound Ras was isolated by the RBD pull-down assay (Supplementary Methods) and detected by immunoblotting (IB) with anti-Ras antibodies. To calculate fold activation, the levels of Ras-GTP were quantified by densitometry scanning and normalized to the levels of total Ras. Results are the mean ± SD of three independent experiments and are presented as the levels of active Ras relative to the level measured in the absence of EGF.

(b) HBS 3 downregulates Ras activation by directly interfering with the Ras-Sos complex. HeLa cells were transfected with HA-tagged SosCat-CAAX and treated with HBS 3 or vehicle. The expression of HA-SosCat-CAAX was detected by immunoblotting with anti-HA antibodies. GTP-bound Ras was isolated and quantified as described in panel (a). Results are presented as the level of active Ras in untreated cells relative to the level measured in treated cells.

(c) HBS 3 suppresses EGF-induced ERK activation. Serum-starved HeLa cells were treated with the indicated peptides or vehicle, and levels of ERK phosphorylation following EGF stimulation was monitored by immunoblotting with phospho-ERK-specific (pERK) antibodies. To calculate fold activation, the levels of pERK were quantified by densitometry scanning and normalized to the levels of total ERK2. Results are the mean ± SD of three independent experiments and are presented as the levels of pERK relative to the levels measured in the absence of EGF.

(d) HBS 3 reduces the intensity and the duration of EGF-induced ERK activation. Serum-starved HeLa cells were treated with vehicle or HBS 3 for 12 hours and then stimulated with EGF for the indicated intervals. Levels of ERK activation were monitored as in (c). Results are
the mean ± SD of three independent experiments and are presented as the levels of pERK relative to the levels measured in the absence of EGF.

Supplementary Figure 8
**Supplementary Figure 9**  | Effect of HBS 3 on the levels and phosphorylation of EGFR. Serum-starved HeLa cells were treated with peptide and EGF as indicated. Cell lysates were fractionated on an SDS polyacrylamide gel and probed by Western blotting with the indicated antibodies.
Supplementary Figure 10 | Analytical HPLC traces of peptides 1, 2, 3, HBS 1, HBS 2, HBS 3, HBS 4, HBS 5, HBS 6, HBS 7, Flu-3, Flu-HBS 3, and Flu-HBS 7.
Supplementary Table 1 | Mass spectroscopic characterization of HBS helices and peptides.

| Compound     | Sequence<sup>a</sup>          | Mass Calculated [M+H]<sup>+</sup> | Mass Observed [M+H]<sup>+</sup> |
|--------------|--------------------------------|-----------------------------------|----------------------------------|
| wt (Sos<sup>929-944</sup>) | Ac-FFGIYLTNILKTEEGN-NH<sub>2</sub> | 1900.1                            | 1900.2                           |
| 1            | Ac-FEGIYRTDILRTEEGN-NH<sub>2</sub> | 1954.1                            | 1954.4                           |
| HBS 1        | XFE*GIYRTDILRTEEGN-NH<sub>2</sub> | 2006.2                            | 1003.9 ([M+H]<sup>+</sup>/2)    |
| 2            | Ac-FEGIYRTELLKAEEAN-NH<sub>2</sub> | 1924.1                            | 1924.1                           |
| HBS 2        | XFE*GIYRTELLKAEEAN-NH<sub>2</sub> | 1976.1                            | 1978.2                           |
| 3            | Ac-FEGIYRLELLKAEEAN-NH<sub>2</sub> | 1936.1                            | 968.7 ([M+H]<sup>+</sup>/2)     |
| HBS 3        | XFE*GIYRLELLKAEEAN-NH<sub>2</sub> | 1988.2                            | 1989.9                           |
| HBS 7        | XAE*GIYRLELLKAEAA-NH<sub>2</sub>  | 1811.0                            | 1813.3                           |
| Flu-3        | Ac-FE*GIYRLELLKAEEANK<sup>Flu</sup>-NH<sub>2</sub> | 2422.64                          | 2423.5                           |
| Flu-HBS 3    | XFE*GIYRLELLKAEEANK<sup>Flu</sup>-NH<sub>2</sub> | 2474.72                          | 2475.4                           |
| Flu-HBS 7    | XAE*GIYRLELLKAEAAAK<sup>Flu</sup>-NH<sub>2</sub> | 2297.56                          | 2298.5                           |
| HBS 4        | XFE*GIYRLELLK-NH<sub>2</sub> | 1473.7                            | 1474.9                           |
| HBS 5        | XFE*AIYRLELLKAEEAN-NH<sub>2</sub> | 1950.1                            | 1950.2                           |
| HBS 6        | XFE*GIYRLELLKAibEEAibN-NH<sub>2</sub> | 2016.2                            | 2017.1                           |

<sup>a</sup>X represents a 4-pentenoic acid residue; * = N-allyl residue (*G = N-allylglycine)
**Supplementary Table 2** | Rosetta computational alanine scanning results for two Ras-Sos crystal structures, PDB codes 1NVW and 1BKD.

| Residue | Δ∆G (kcal/mol) from 1NVW | Δ∆G (kcal/mol) from 1BKD |
|---------|---------------------------|---------------------------|
| F 929   | 1.64                      | 1.45                      |
| F 930   | 0.03                      | 0.05                      |
| G 931   | - -                       | - -                       |
| I 932   | 0.07                      | 0.05                      |
| Y 933   | 0.01                      | - -                       |
| L 934   | 0.65                      | 0.65                      |
| T 935   | 1.11                      | 1.59                      |
| R 936   | 0.28                      | 0.83                      |
| I 937   | - -                       | - -                       |
| L 938   | 0.63                      | 0.69                      |
| K 939   | 0.27                      | 0.44                      |
| T 940   | -0.13                     | 0.05                      |
| E 941   | - -                       | - -                       |
| E 942   | 1.10                      | 0.34                      |
| G 943   | - -                       | - -                       |
| N 944   | 2.35                      | 2.63                      |

Residues that contribute most strongly to binding are highlighted in bold.
Supplementary Table 3 | Effect of Sos αH derivatives on Sos-mediated guanine nucleotide exchange. Exchange assays were performed with 1 μM Ras, 1 μM mant-GDP, 1 μM Sos, 100 μM GDP, and 25 μM of the peptide.

| Compound | % Exchange Inhibition |
|----------|-----------------------|
| wt (Sos
\(^{929-944}\)) | < 10 |
| 1        | 13        |
| HBS 1    | 11        |
| 2        | 20        |
| HBS 2    | 40        |
| 3        | 37        |
| HBS 3    | 64        |
| HBS 7    | 15        |
| HBS 4    | <10       |
| HBS 5    | 50        |
| HBS 6    | 20        |

Supplementary Table 4 | Summary of circular dichroism data for unconstrained peptides and HBS α-helices.

| Compound | % helicity |
|----------|------------|
| wt (Sos
\(^{929-944}\)) | ** |
| 1        | 9          |
| HBS 1    | 19         |
| 2        | 16         |
| HBS 2    | 23         |
| 3        | 24         |
| HBS 3    | 56         |
| HBS 7    | 52         |
| HBS 4    | 22         |
| HBS 5    | ND         |
| HBS 6    | 23         |

**Peptide aggregation observed at 20-50 μM concentrations. ND = not determined**
Description of Peptide Design. Computational alanine scan\(^4\) of the Ras/Sos interface from two crystal structures suggested that while several \(\alpha\)H residues form weak contacts with Ras (Supplementary Table 2), residues F929, T935, E942 and N944 contribute most strongly to binding. Computational alanine scanning studies were performed using the Rosetta software as described.\(^4,5\) The predicted computational results are consistent with the experimental mutational data.\(^6\)

Peptide mimics of the wild-type Sos (929-944) proved to be only partly soluble in aqueous buffers at 25 \(\mu\)M and higher concentrations. We therefore optimized the native peptide sequence by incorporating charged residues at non-interfacial positions to enhance solubility. An arginine residue was placed at position 934 in place of a leucine residue to form a potential \(i, i+4\) ionic interaction with a glutamic acid at position 930; such salt-bridges are known to stabilize \(\alpha\)-helical conformations.\(^7,8\) Similarly, N936 was replaced with an aspartic acid residue to establish a potential ionic interaction with the positively charged residue at position 939. These mutations resulted in sequences with enhanced solubility (Supplementary Table 1). Peptide 1 and its HBS helix analog were partially soluble and weak inhibitors of nucleotide exchange; although the inhibition was dose dependent (Supplementary Fig. 11 and Supplementary Table 3).

Encouraged by these initial results, we sought to further improve the potency of HBS Sos helices by judicious replacement of \(\beta\)-branched amino acids, which have low helix-forming propensities,\(^9\) in an iterative manner to obtain sequences 2 and 3. T935 is a key hydrophobic residue at the interface and was replaced by a hydrophobic amino acid residue, leucine, that features high helix propensity, while T940 is a non-interfacial residue and was replaced with
alanine. D936 in peptide 1 was replaced with a glutamic acid residue as it has a higher helix propensity than aspartic acid. As expected, HBS 3 displayed higher helicity (56%) than HBS 2 (24%) (Supplementary Fig. 3 and Supplementary Table 4.).

Supplementary Figure 11 | Effect of Sos α-H mimics on the nucleotide exchange stimulation by Sos. Exchange assays were performed with 1 µM Ras, 1 µM mant-GDP, 1 µM Sos, 100 µM GDP, and 25 µM of the peptide, except as indicated for 1.

HBS 4 was designed as a truncated control that lacks two key C-terminal residues (E942 and N944). This compound was found to be inactive in the nucleotide exchange assay (Supplementary Table 3). HBS 5 and HBS 6 were designed to further improve on the activity on HBS 3 by incorporation of residues that improve helicity. In HBS 5, we substituted G931 with an alanine residue as to improve the helicity of the designed helices as glycine is known to be a helix breaker.9-11 HBS 6 features two aminoisobutyric acid (Aib) residues in place of two C-terminal alanine groups. Aib residues are known to support helical conformations,12 and are expected to resist proteolytic degradation. Both HBS 5 and HBS 6 performed poorly in the exchange assay as compared to HBS 3 (Supplementary Table 3).
Supplementary Methods

**General.** Commercial grade solvents and reagents were used without further purification. Fmoc amino acids and peptide synthesis reagents were purchased from Novabiochem. Hoveyda-Grubbs II catalyst was obtained from Sigma. Salts and buffers were obtained from Sigma (molecular biology grade). Cell culture media and reagents were purchased from Invitrogen, unless otherwise stated.

**Peptide Synthesis.** Peptides were synthesized on a CEM Liberty series microwave peptide synthesizer and purified by reversed-phase HPLC. The identity and the purity of the peptides were confirmed by LCMS.

**Synthesis of HBS α-helices.** HBS helices were synthesized as previously described. Peptide sequences up to the $i+3^{rd}$ residue of the putative helix (8 in Supplementary Fig.1) were synthesized on solid phase on a CEM Liberty Series microwave peptide synthesizer. Addition of $N$-allylglycine residue to 8 to obtain $N$-allylpeptide 9 was accomplished over two steps. Resin bound peptide 8 was treated with a solution of bromoacetic acid (20 eq), DIC (20 eq) and HOAt (10 eq) in DMF, and the mixture shaken for 2 h. Resin was washed sequentially with DMF (x3), DCM (x3) and DMF (x3), suspended in 1M allylamine (20 eq) in DMF and shaken for 20 min. Resin (containing 9) was washed with DMF (x 3), methanol (x 3) and DCM (x 3), and treated with the desired Fmoc amino acid (20 eq), DIC (20 eq) and HOAt (10 eq) in DMF under microwave irradiation for 30 min at 60 °C. Resin was washed with DMF (x 3), DCM (x 3), DMF (x 3), and coupled to the desired Fmoc amino acid residue (5 eq) and 4-pentenoic acid (5 eq) with HBTU (4.95 eq) and DIEA (10 eq) in NMP. Ring-closing metathesis of bis-olefin 11
was performed with Hoveyda-Grubbs II catalyst (20 mol%) in dichloroethane under microwave irradiation at 120 ºC for 10 min as described.\textsuperscript{2,3} Peptides were cleaved from the resin using TFA/TIS/water (95%: 2.5%: 2.5%), and purified by reversed-phase HPLC (C\textsubscript{18} column) (Supplementary Fig. 10).

**Synthesis of 5-carboxyfluorescein labeled peptides.** HBS helices and unconstrained peptides containing a C-terminal Mtt-protected Lys residues were synthesized as described above. The Mtt group was removed prior to peptide cleavage from resin, with TFA/TIS/DCM (1%: 2%: 97%), the resin was washed with DCM (x 3), MeOH (x 3) and DMF (x 3), and treated with 5-carboxyfluorescein-N-hydroxysuccinamide (5-FAMSE, 1 eq), DIEA (5 eq), and NMP for 2 h. The fluorescein-conjugates peptides were cleaved from resin and purified by reversed-phase HPLC (Supplementary Fig. 10).

**Circular Dichroism Studies.** CD spectra were recorded on an AVIV 202SF CD spectrometer equipped with a temperature controller using 1 mm length cells and a scan speed of 1.0 nm/min at 298 K. The spectra were averaged over 10 scans with the baseline subtracted from analogous conditions as those for the samples. The samples were prepared in 1 mM phosphate buffered saline, containing 10% trifluoroethanol, with the final peptide concentration 10-50 µM. The concentrations of unfolded peptides were determined by the UV absorption of tyrosine residues at 280 nm in 6.0 M guanidinium hydrochloride aqueous solution. The helix content of each peptide was determined from the mean residue ellipticity at 222 nm, as described.\textsuperscript{13} Percent helicity was calculated from the ratio $[\theta]_{222}/[\theta]_{\text{max}}$, where $[\theta]_{\text{max}} = (-44,000 + 250T)(1 - k/n)$; where $k = 4.0$ and $n = 16$ (number of amino acid residues in the peptide).\textsuperscript{13}
**His$_6$-Ras (1-166) and His$_6$-Sos-Cat (564-1049) Expression.** His$_6$-tagged HRas (residues 1-166) and His$_6$-tagged SosCat (residues 564-1049) both in pProEx HTb expression vectors, were expressed in *Escherichia coli* (BL21) by induction with 500 µM IPTG at a cell density corresponding to an absorbance of OD$_{600}$ = 1.0. Pellets were resuspended in buffer containing 20 mM Tris pH 7.6, 200 mM NaCl, 2.5 mM MgCl$_2$, 2 µM phenylmethlysulfonylfluride (PMSF), 1% aprotinin, 10 µg/ml leupeptin, 10 µM benzamidine, 10 µg/ml soybean trypsin inhibitor, and 10 µg/ml pepstatin, and sonicated using a Branson Cell Disrupter 200. Clarified lysates containing polyhistidine tagged proteins were incubated with charged nickel resin (Invitrogen) at 4 °C for 1 hour. The resin was washed five times in resuspension buffer containing 50 mM imidazole. The tagged proteins were eluted with buffer containing 200 mM imidazole in 20 mM Tris pH 7.6, 200 mM NaCl. Eluted proteins were dialyzed against buffer containing 20 mM Tris pH 7.6 and 200 mM NaCl for His-tagged SosCat, and 20 mM Tris pH 7.6, 200 mM NaCl and 1mM MgCl$_2$ for His-tagged Ras. The eluted proteins were concentrated with 5 kD molecular cut-off Amicon ultra centrifugal columns (Millipore). Purified proteins were snap frozen in liquid N$_2$ and stored at -80 °C until further use.

**Fluorescence Polarization Assay.** The relative affinity of peptides for N-terminal His$_6$-tagged Ras was determined using fluorescence polarization based binding assay with fluorescein labeled Sos peptides **Flu-HBS 3** and **Flu-HBS 7**. The polarization experiments were performed with a DTX 880 Multimode Detector (Beckman) at 25° C, with excitation and emission wavelengths of 485 and 525 nm, respectively. Addition of an increasing concentration (0 nm to 750 µM) of His$_6$-Ras protein to a 15 nM solution of fluorescein labeled Sos peptide in 20 mM Tris pH 7.4, 300 mM NaCl, 1mM MgCl$_2$, and 0.1% pluronic F-68 (Sigma) in 96 well plates afforded the IC$_{50}$
value, which was fit into equation (1) to calculate the dissociation constant ($K_D$) for the Sos/ Ras complex. $^{14}$ 10 mM EDTA was added to the above solution to establish nucleotide-free Ras conditions (Supplementary Figure 5a). The binding affinity ($K_D$) reported for each peptide is the average of three individual experiments, and were determined by fitting the experimental data to a sigmoidal dose-response nonlinear regression model on GraphPad Prism 4.0.

$$K_D = \frac{(R_T \times (1 - F_{SB}) + L_{ST} \times F_{SB}^2)}{F_{SB} - L_{ST}} \quad (1)$$

where,

$R_T$ = Total concentration of Ras (1-166) protein

$L_{ST}$ = Total concentration of fluorescent peptide

$F_{SB}$ = Fraction of bound fluorescent peptide

**Nucleotide Exchange Assay.** Rate of Sos-catalyzed nucleotide exchange reaction was monitored as described previously. $^{17}$ Briefly, 1 μM Ras was loaded with the fluorescent nucleotide analog mant-GDP (Invitrogen) in buffer containing 20 mM Tris (pH 7.5), 50 mM NaCl and 4 mM EDTA for 5 min at room temperature. The mixture was supplemented with 14 mM MgCl$_2$ and incubated for at least 2 h on ice. Reactions were initiated by the addition of 100 μM unlabeled GDP, and the decrease in fluorescence (excitation: 355 nm emission: 470 nm) was monitored with a fluorescence spectrophotometer (Perkin Elmer, model LS55). For the inhibitory-peptide experiments, the reactions were supplemented with the indicated peptides (in 20 mM Tris pH 7.5, 50 mM NaCl and 14 mM MgCl$_2$) before the addition of 1 μM SosCat and unlabeled 100 μM GDP.
**1H-15N HSQC NMR Spectroscopy.** The purification procedure was essentially the same as described above with the following variation: the BL21 cells harbouring the His-tagged Ras construct were grown at 37 °C in M9 media supplemented with 15NH4Cl as the sole source of nitrogen. Protein production was induced with 500 µM IPTG at O.D. 1.0 for 16 hours at 16 °C. Protein purification and concentration were performed as described above. The His6-tag was removed by incubating the His6-tagged Ras with recombinant His6-tagged Tobacco Etch Virus (TEV) protease (Invitrogen) overnight at 4 °C following manufacturer’s protocol. The sample was loaded on a charged NiNTA agarose column and the tag-less protein collected in the flow through fraction. Uniformly 15N-labelled Ras was buffer exchanged against the NMR buffer (20 mM Na2HPO4-NaH2PO4, pH 5.5, 150 mM NaCl, 10 mM MgCl2) using Amicon Ultra centrifugal filter (Millipore) and supplemented with 10% D2O. Data was collected on a 900 MHz Bruker four channel NMR system equipped with cryoprobe at 30 °C and analyzed with the BioSpin software (Bruker).

For the titration experiments, three and five molar equivalents of HBS 3 (in NMR buffer with 10% D2O) were added to Ras, and the data was collected as described above. Mean chemical shift difference (ΔδNH) observed for 1H and 15N nuclei of various resonances corresponding to residues in the switch and non-switch regions were calculated as described.

**Cellular Uptake of peptides.** HeLa cells were plated at sub-confluency in DMEM supplemented with 10% FBS in a 96 well plate with glass bottom. The following day, media was replaced with one supplemented with 8 µM fluorescein (5-FAM) only or fluorescein-tagged peptides (in DMEM supplemented with 10% FBS) as indicated. After 12 hours, the cells were washed twice with warm PBS and imaged directly with the Zeiss Axiovert 200M microscope.
For the temperature-dependence uptake studies, cells were incubated with 8 µM Flu-HBS 3 or fluorescein at 4 °C or 37 °C for 2.5 hours. Cells were washed three times with PBS, fixed with formaldehyde, and nuclei stained with DAPI. At least 12 serial Z-section of each cell were captured to identify intracellular fluorescence signals. The serial Z-sectioned images were deconvoluted by the nearest neighbor algorithm using the AxioVision 4.8 Software. The representative images in Supplementary Fig. 7b depict a single slice of the serial Z-section around the center of the cell.

**Ras Activation Assay.** The RBD-pull down assay was carried out as described earlier. Briefly, GST-Raf-RBD fusion proteins were expressed in E. coli by induction with 0.5 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 5 hours. The expressed fusion proteins were isolated from bacteria lysates by incubation with glutathione agarose beads for 1 hour at 4 °C. HeLa cells were grown to confluence, serum-starved for 4 hours, and incubated with 75 µM peptide (dissolved in DMEM) for an additional 12 hours. For experiments with SosCat-CAAX (SosCat with CAAX box of HRas), HeLa cells were transfected with HA-tagged SosCAAX twenty four hours prior to starvation. Cells were treated with the indicated peptides for 12 hours prior to stimulation. After stimulation with 10 ng/ml EGF for the indicated intervals at 37 °C, the cells were lysed in RBD lysis buffer containing 25 mM Tris-HCl (pH 7.4), 120 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 10% glycerol, 10 mg/ml pepstatin, 50 mM NaF, 1% aprotinin, 10 mg/ml leupeptin, 1 mM Na3VO4, 10 mM benzamidine, 10 mg/ml soybean trypsin inhibitor, 1% NP40, and 0.25% sodium deoxycholic acid. The lysates were then incubated with 20 µg of recombinant GST-Raf-RBD immobilized to agarose beads for 1.5 hours at 4 °C. The complexes
were collected by centrifugation and washed six times with the RBD lysis buffer. Bound proteins were eluted with SDS sample buffer, separated by SDS-12.5% PAGE and transferred to nitrocellulose membrane. The proteins were detected by blotting with anti-HA (12CA5; 1:10,000) for SosCatCAAX or anti-Ras10 (Millipore; 1:10,000) primary antibodies and Alexa Fluor 680 goat anti-mouse (Molecular Probes, 1:10,000) secondary antibody and visualized with the Odyssey Infrared Imaging System (LiCor).

**EGFR Activation Assay.** Cells were grown, treated and lysed as described above. EGFR and pEGFR levels were detected by blotting with anti-EGFR (Santa Cruz Biotech) and pEGFR pY1068 (Cell Signaling) antibodies.

**ERK Activation Assay.** Cells were treated and lysed as described above. Levels of total ERK2 and phosphorylated ERK were detected with anti-ERK2 (Upstate Biotechnology, 1:1,000) and phospho-ERK1/2 (Cell Signaling, 1:1,000) antibodies, respectively. ERK phosphorylation levels were quantified with the Odyssey software and normalized to total ERK expression.
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