Supporting Information

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SI Materials and Methods

Materials. SW480 colorectal cancer cells were purchased from ATCC. The Pa02C, Pa08C, and Pa16C pancreatic cancer cell lines were derived as described (1). Colorectal tumors and cyst fluids were obtained from surgical resection specimens at the Johns Hopkins Hospital. Tissues and cyst fluids were flash frozen within 30 min of excision and stored at −80 °C. All samples were obtained in accordance with the Health Insurance Portability and Accountability Act and had Institutional Review Board approval.

A rabbit monoclonal (EP1125Y) antibody reactive with all three Ras isoforms [c-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-Ras), neuroblastoma rat sarcoma viral oncogene homolog (N-Ras), and Harvey rat sarcoma viral oncogene homolog (H-Ras)] (catalogue no. ab52939) was purchased from Abcam. A mouse monoclonal antibody specific to K-Ras (catalogue no. SC-30) was purchased from Santa Cruz Biotechnology. All other reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Preparation of Solutions. ***Antibody conjugation reaction buffer.*** Antibody conjugation reaction buffer (ACRB) [0.2 M triethanolamine (pH 8.2), 20 nM dimethyl pimelimidate dihydrochloride] was prepared fresh before each use.

Lysis buffer. Lysis buffer (10 mL) contained 6.87 mL RIPA buffer (68.7 μL Nonidet P-40, 687 μL 10% wt/vol sodium deoxycholate, 68.7 μL 10% wt/vol SDS) (Invitrogen), 206.1 μL 5 M NaCl, 68.7 μL 1 M sodium phosphate (pH 7.2), 1 mL water, one Complete EDTA-free Protease Inhibitor Mixture tablet (Roche), 1,000 μL 0.5 M NaF, 10 μL 80 mM b-glycerophosphate, 1,000 μL 20 mM Na pyrophosphate, 10 μL 300 mM Na orthovanadate, 10 μL 1 M DTT, and 100 μL 100 mM PMSF.

Modified RIPA buffer. Modified RIPA buffer (10 mL) contained 300 μL 5 M NaCl, 500 μL 1 M Tris (pH 7.4), 100 μL Nonidet P-40, 250 μL 10% (wt/vol) sodium deoxycholate, 20 μL 0.5 M EDTA, and 8.83 mL water.

Mass spectrometry solvents. Solvent A contained 3% (vol/vol) acetonitrile, 0.1% formic acid; Solvent B contained 90% (vol/vol) acetonitrile, 0.1% formic acid.

Immobilization of Antibody on Magnetic Beads. Conjugation of antibodies to beads was performed using slight modifications of the methods described by Whiteaker et al. (2). The rabbit monoclonal antibody to Ras (100 μL) was added directly to 500 μL Protein G Dynal Magnetic Beads (as obtained from Invitrogen, without further washing), and the antibody was bound to the beads on a rotator at room temperature for 1 h. The antibody-bound beads then were washed by incubation in 1 mL ACRB and collected on a magnet. The antibody was cross-linked to the protein G on the beads by incubation with 1 mL ACRB on a rotator at room temperature for 30 min. The beads then were washed twice with 1 mL 50 mM Tris-HCl (pH 7.5), resuspended in 1 mL 50 mM Tris-HCl (pH 7.5), and rotated at room temperature for 15 min. The incubation with Tris-HCl stopped the cross-linking reaction. The beads were resuspended in 300 μL 50 mM Tris-HCl (pH 7.5) and 200 μL glycerol and were stored at −20 °C.

Cell Lysis and Protein Quantification. Cultured cells were lysed by incubation in lysis buffer for 30 min on ice, with vortexing every 10 min. Tissues were lysed by placing 50 mg into a Covaris tube, which then was frozen in liquid nitrogen, and were pulverized twice using a Covaris CP02 instrument. The frozen tissue powder was transferred to Covaris glass vials, 1 mL of lysis buffer was added, and the suspension was sonicated once every 15 min for a total of four times using a Covaris S2 instrument with the following settings:

Step 1: Duty cycle 5%, Intensity 3%, Cycles/Burst 100, 5 s
Step 2: Duty cycle 20%, Intensity 8%, Cycles/Burst 100, 30 s
Step 3: Duty cycle 5%, Intensity 3%, Cycles/Burst 100, 5 s
Step 4: Duty cycle 5%, Intensity 3%, Cycles/Burst 100, 5 s
(Step 4 is a repeat of Step 3.)

The homogenate was kept on ice between sonications. The lysates from cells or tissues were clarified by centrifugation at 12,000 × *g* for 30 min at 4 °C. Lysates were stored at −80 °C, 2 mg of cellular protein per tube. A bicinchoninic acid (BCA) assay kit (Thermo-Scientific) was used to quantify protein concentrations.

Immunoprecipitation of Ras Proteins. Cell lysates containing 2 mg of total protein (generally ∼200 μL) were thawed on ice and diluted with four volumes of modified RIPA buffer. Antibody-conjugated Dynal beads (100 μL) were added, and the suspension incubated at 4 °C overnight (minimum of 12 h). The beads were collected on a magnet and were washed three times with freshly prepared modified RIPA buffer. The bound Ras proteins were eluted by vortexing the beads at 650 rpm in 100 μL 3% (vol/vol) acetic acid for 30 min at 37 °C on a Thermomixer (Eppendorf). The solution was neutralized by adding two volumes of 1 M ammonium bicarbonate.

Trypsin Digestion. Methanol (450 μL) was added to 300 μL of the neutralized Ras protein eluate, giving a final concentration of 60% (vol/vol) methanol. DTT was added to a final concentration of 1 mM, and the solution was incubated at 60 °C on a Thermomixer for 1 h at 650 rpm. The solution was cooled to room temperature. Iodoacetamide was added to a final concentration of 50 mM; then the solution was incubated at room temperature in the dark for 30 min. Distilled water (3.25 mL) was added to dilute the ammonium bicarbonate to 50 mM. The pH of the solution was ∼8.0. Sequencing grade trypsin (Promega) was added to a final concentration of 5 μg/mL, and the solution was incubated overnight at 37 °C. The peptide solution then was acidified by adding 1% trifluoroacetic acid (TFA) and was incubated at room temperature for 15 min. A Sep-Pak light C18 cartridge (Waters Corporation) was activated by loading 5 mL 100% (vol/vol) acetonitrile and was washed by 3.5 mL 0.1% TFA solution two times. Acidified digested peptide solution was centrifuged at 1,800 × *g* for 5 min, and the supernatant was loaded into the cartridge. To desalt the peptides bound to the cartridge, 1 mL, 3 mL, and 4 mL of 0.1% TFA were used sequentially. To elute the peptides from the cartridge, 2 mL of 40% (vol/vol) acetonitrile with 0.1% TFA was used, and this elution was repeated two more times (for a total of 6 mL of eluate). It was important to ensure that the cartridge had stopped dripping before each sequential wash and elution solution was applied. The eluted peptides were lyophilized overnight and redissolved in 40 μL of Solution A.

HPLC. Peptide samples were separated using a reversed-phase column (XBridge BEH130 C18 column, 5 μm, 2.1 × 250 mm) (Waters Corporation) on the liquid chromatography (LC) 1200 system (Agilent Technologies). After 40 μL of peptide sample was loaded into the column, the LC gradient was generated in 0.1% formic acid with increasing acetonitrile concentrations using solvent B: gradient 0–3% (vol/vol) for the first 6 min, then 3–10%

(vol/vol) for 4 min, and 10–40% (vol/vol) for the subsequent 20 min. The column was regenerated by continuing the gradient up to 100% (vol/vol) solvent B for the next 6 min, then reversing the gradient from 100–3% (vol/vol) solvent B over the next 2 min, and finally equilibrating in 3% (vol/vol) solvent B for 8 min. A sawtooth gradient consisting of alternating increases and decreases in solvent B concentration (0–100% vol/vol and 100–0% vol/vol for 10 min, repeated twice for a total of three times) was used to prevent carryover of the peptides. A blank sample (no protein) then was loaded into the LC and subjected to the gradient described above before the next experimental sample was loaded.

MS. MS parameters were as follows: drying gas: 12 L/min, 300 °C; fragmentor: 130 V; dwell time: 10 ms; capillary voltage: 4,000 V; resolution of Q1 and Q3: unit mass; collision energy: optimized for each peptide (Table S1) with the Agilent MassHunter Peptide Optimizer. One picomole of synthetic peptide with $^{13}C/^{15}N$-labeled arginine or $^{13}C/^{15}N$-labeled lysine at its C terminus (Sigma) was used to optimize transition parameters. Selected reaction monitoring (SRM) analysis was carried out in positive mode using a 6460 Triple Quadrupole Mass Spectrometer (Agilent Technologies) equipped with capillary flow (100 μL/min) electrospray ionization connected to an Agilent 1200 series capillary pump.

SRM Data Analysis. A list of transitions was selected based on MassHunter Peptide Optimizer data for each heavy isotope-labeled peptide ($^{13}C_6{}^{15}N_2$-lysine and $^{13}C_6{}^{15}N_4$-arginine). The peaks of each y ion and b ion that could be generated from peptides with 2+ and 3+ charge states were optimized by altering the collision energy for each transition. The Skyline program (37) preloaded with WT and mutant Ras peptide sequences was used to analyze the data. The endogenous peptide-specific peaks were identified by comparison with the exogenously added $^{13}C/^{15}N$-labeled peptides, which were 8 Da and 10 Da heavier for lysine- and arginine-containing peptides, respectively. In addition, the retention times and transition profiles of the exogenous and endogenous peptides were inspected manually to ensure that they were internally consistent. Peptide abundance was calculated by integrating the areas representing the peaks of each detected exogenous and endogenous ion. Each analysis described in the text or listed in Table 1 or Table S2 was repeated at least once, and averages and SDs are reported.

Full-Scan LC-MS/MS and Data Analysis. The tryptic-digested peptides from immunoaffinity-enriched proteins were purified on a strong cation exchange-stage tip using binding and washing buffer [5 mM KH_2PO_4 (pH 2.7), 25% (vol/vol) acetonitrile] and an elution buffer containing 1% ammonium hydroxide in 25% (vol/vol) acetonitrile. LC-MS/MS analysis of dried peptides was carried out using a chip cube interfaced to an ultra–high-definition accurate-mass quadrupole-TOF LC/MS (Agilent Technologies). The chip LC system consisted of a 160-nL peptide enrichment column and a 150-mm analytical column packed with Zorbax 300SB-C18, 5-μm reversed-phase material. The peptides were separated by acetonitrile gradient (10–35% vol/vol) containing 0.1% formic acid. The MS/MS spectra were acquired in a data-dependent manner, targeting the four most abundant ions in each survey scan from 350–1,700 *m/z* and in each MS/MS scan from 100–1,700 *m/z* using a collision energy set-up of 3.0 V/100 Da, offset 2 V. Dynamic exclusion was enabled after acquisition of two spectra for 15 s. The data were searched using Spectrum Mill software against the human RefSeq database version 40 containing 31,789 protein sequences appended with different mutant Ras protein sequences. Carbamidomethylation was allowed as fixed modification, and oxidation of M and deamidation N and Q were permitted as variable modifications. One missed cleavage was allowed for searching tryptic peptides. Mass tolerances of 20 ppm and 50 ppm were allowed for MS and MS/MS spectra identification, respectively.

1. Jaffee EM, et al. (1998) Development and characterization of a cytokine-secreting pancreatic adenocarcinoma vaccine from primary tumors for use in clinical trials. *Cancer J Sci Am* 4:194–203.

2. Whiteaker JR, et al. (2007) Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. *Anal Biochem* 362: 44–54.

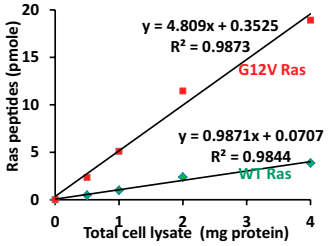

Fig. S1. Correlations between input amounts of lysate and WT and mutant Ras peptides detected by SRM. The endogenous WT peptides (green diamonds) and G12V-mutant Ras peptides (red squares) were quantified by comparison with the exogenously added heavy isotope-labeled synthetic peptides.

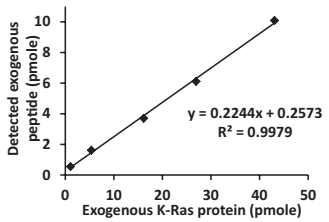

Fig. S2. Determination of peptide loss during the SRM procedure. K-Ras recombinant protein (50–2,000 ng, corresponding to 1–43 pmol of the GST-tagged recombinant K-Ras protein; molecular weight: 46.4 kDa) was spiked into SW480 cell lysates each containing 2 mg of total cellular protein, and SRM was performed. The *y* axis represents the calculated amount of peptide observed in the MS after subtraction of the 1.6 pmoles contributed by the endogenous WT Ras proteins present in SW480 cells. From the slope of the trend line, the recovery was determined to be 22.4%.

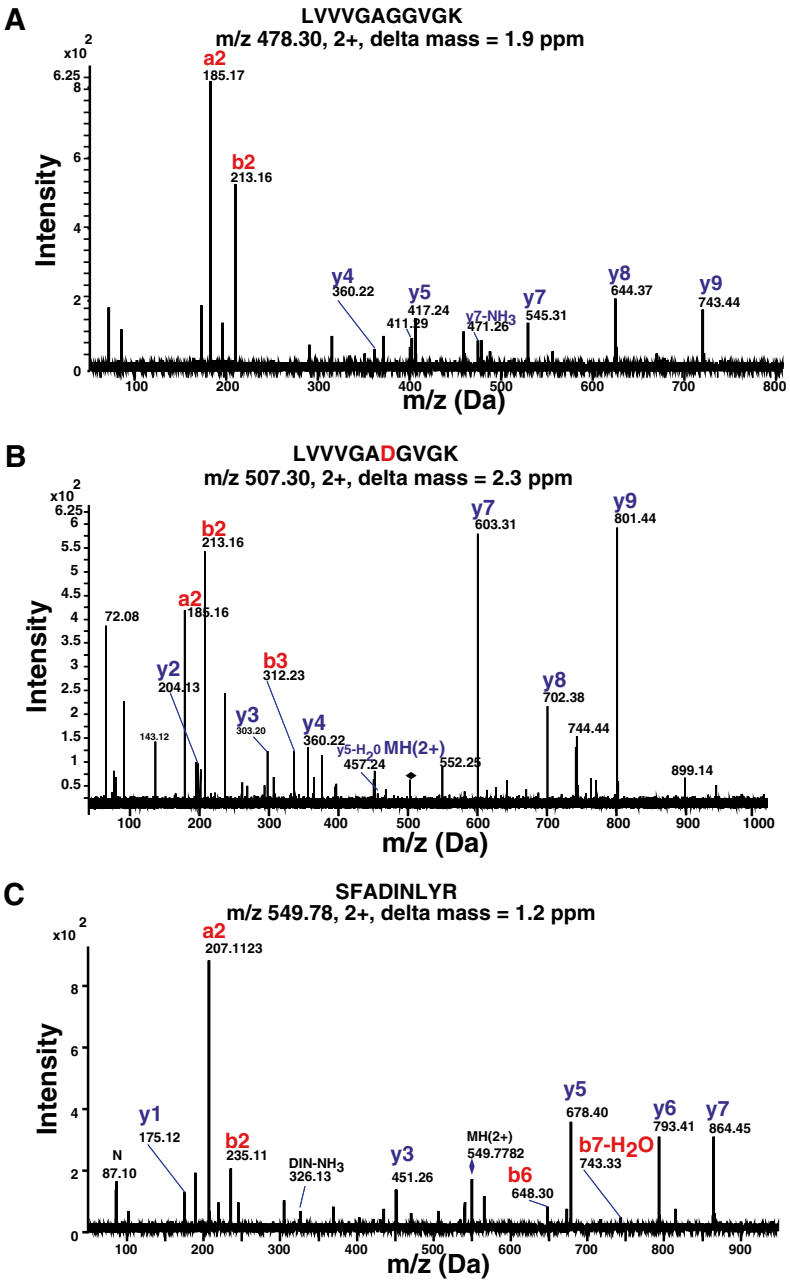

Fig. S3. (Continued)

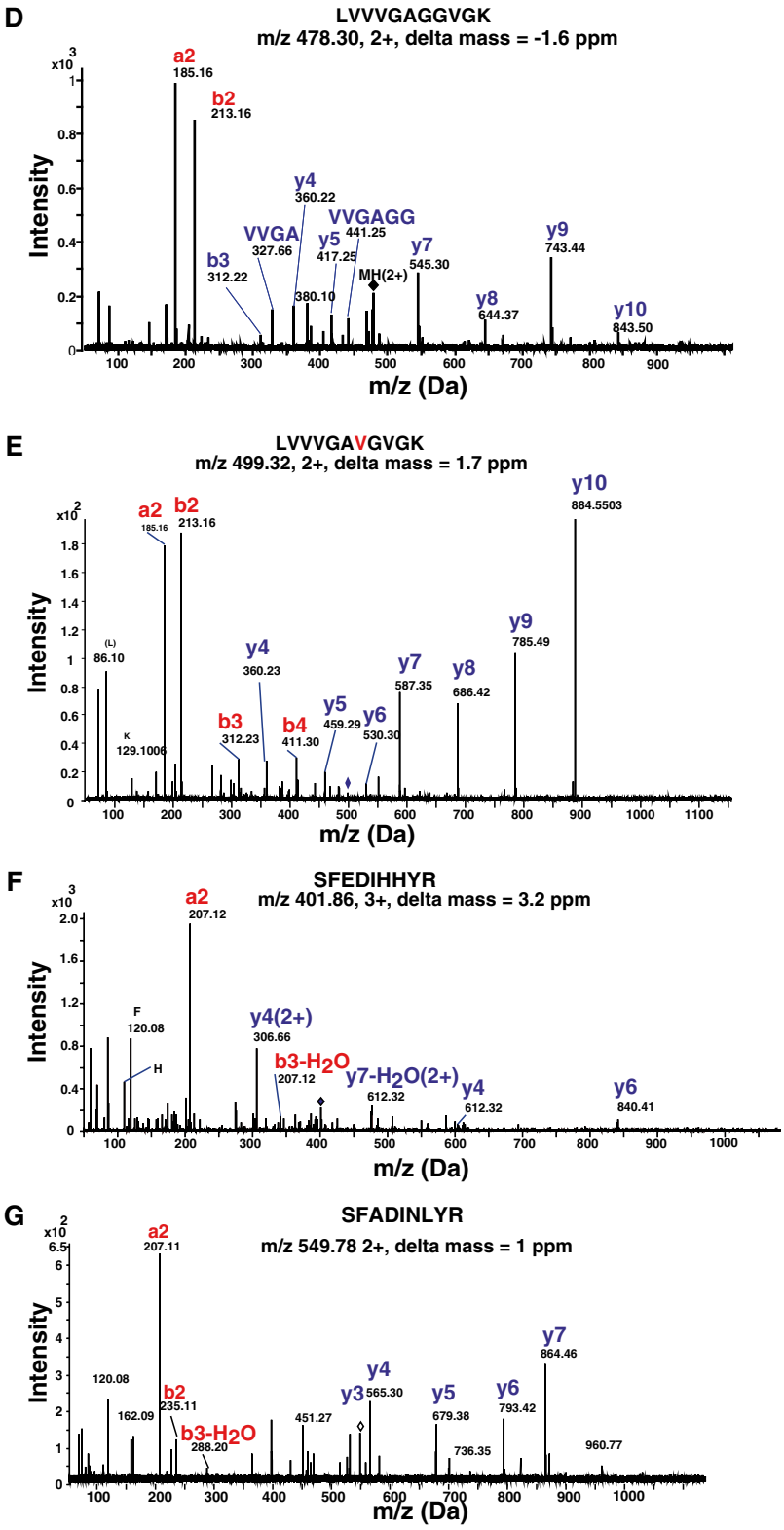

Fig. S3. Confirmation of peptides used for SRM-based quantification. (*A*–*C*) MS/MS spectra of the indicated peptides from WT Ras (*A*), mutant Ras (*B*), and N-Ras (*C*) proteins of Pa16c cells. (*D*–*G*) MS/MS spectra of the indicated peptides from WT Ras (*D*), mutant Ras (*E*), K-Ras (*F*), and N-Ras (*G*) proteins from SW480 cell line. The transitions of the indicated peptides are described in Table S1.

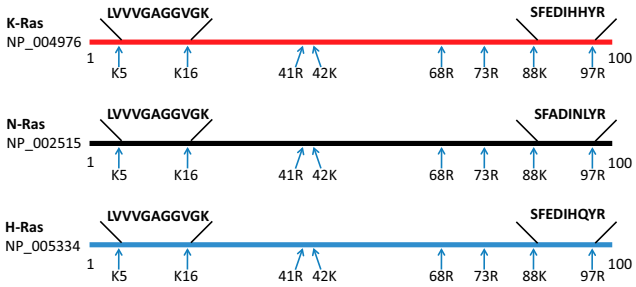

Fig. S4. Trypsin digestion maps of the first 100 residues of K-Ras, N-Ras, and H-Ras proteins.

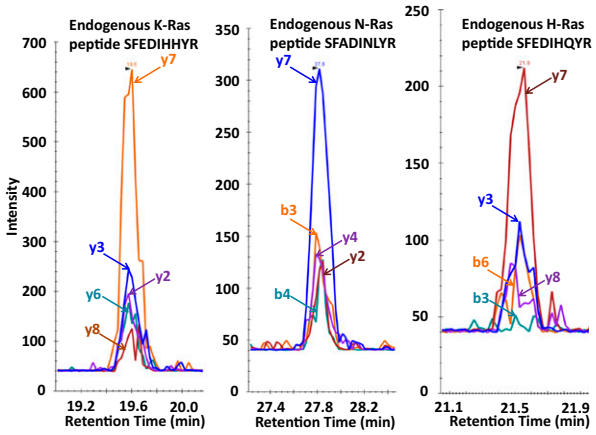

Fig. S5. Chromatograms of peptides derived from K-Ras, N-Ras, and H-Ras proteins derived from SW480 cells. The transitions of the indicated peptides are described in Table S1.

Table S1. SRM transition parameters

		Light*		Heavy*			
Peptide name	Peptide sequence	Q1	Q3	Q1	Q3	Collision energy	Fragment ion
Ras WT	LVVVGAGGVGK^{2+}	478.3	743.4	482.3	751.4	12	y9
			644.4		652.4	12	y8
			545.3		553.3	12	y7
			411.3		411.3	8	b4
			312.2		312.2	12	b3
			213.2		213.2	12	b2
Ras G12V	LVVVGAVGVGK^{2+}	499.3	686.4	503.3	694.4	13	y8
			587.4		595.4	13	y7
			360.2		368.2	21	y4
			204.1		212.1	21	y2
			312.2		312.2	13	b3
			213.2		213.2	13	b2
Ras G12D	LVVVGADGVGK^{2+}	507.3	702.4	511.3	710.4	13	y8
			603.3		611.3	13	y7
			360.2		368.2	21	y4
			411.3		411.3	9	b4
			312.2		312.2	13	b3
			213.2		213.2	13	b2
K-Ras–specific	SFEDIHHYR^{2+}	401.9	558.8	405.2	563.8	6	y8^{2+}
			485.2		490.2	6	y7^{2+}
			420.7		425.7	10	y6^{2+}
			475.2		485.2	14	y3
			338.2		348.2	18	y2
N-Ras–specific	SFADINLYR^{2+}	549.8	864.5	554.8	874.5	15	y7
			565.3		575.3	15	y4
			338.2		348.2	11	y2
			421.2		421.2	11	b4
			306.1		306.1	11	b3
H-Ras–specific	SFEDIHQYR^{3+}	398.9	554.3	402.2	559.3	6	y8^{2+}
			480.7		485.7	6	y7^{2+}
			416.2		421.2	10	y6^{2+}
			466.2		476.2	14	y3
			364.2		364.2	6	b3

*"Heavy" and "Light" refer to the isotopic versions of the peptide. The heavy Ras WT, Ras G12V, and Ras G12D peptides have a heavy isotope-labeled lysine, making them 8 Da heavier than their light counterparts; the heavy Ras-specific peptides have a heavy isotope-labeled arginine, making them 10 Da heavier than their light counterparts.

Table S2. Relative levels of K-Ras, N-Ras, and H-Ras proteins

	K-Ras SFEDIHHYR	N-Ras SFADINLYR	H-Ras SFEDIHQYR	Total Ras	K-Ras/total Ras (%)	N-Ras/total Ras (%)	H-Ras/total Ras (%)
Cell lines							
SW480	4.3 ± 0.35	0.69 ± 0.21	0.96 ± 0.064	5.9 ± 0.71	72 ± 1.4	12 ± 2.1	17 ± 0.71
Pa08C	3.5 ± 0.50	0.94 ± 0.23	0.30 ± 0.028	4.8 ± 0.78	74 ± 2.1	20 ± 1.4	6.5 ± 0.71
Pa16C	1.8 ± 0.14	0.75 ± 0.14	0.40 ± 0.0071	2.9 ± 0.28	61 ± 0.71	26 ± 2.1	14 ± 0.71
Pa02C	0.92 ± 0.11	0.69 ± 0.10	0.92 ± 0.26	2.6 ± 0.50	37 ± 1.4	28 ± 0.71	35 ± 2.8
Normal tissues							
Spleen 10	1.9 ± 0.21	0.93 ± 0.014	0.47 ± 0.035	3.3 ± 0.21	57 ± 2.1	29 ± 2.1	14 ± 0.0
Spleen 12	2.6 ± 0.50	1.1 ± 0.17	0.43 ± 0.021	4.1 ± 0.71	63 ± 1.4	27 ± 0.0	11 ± 0.71
Colorectal mucosa 102	1.7 ± 0.14	0.53 ± 0.071	0.41 ± 0.18	2.6 ± 0.0071	65 ± 3.5	20 ± 2.8	20%, 11
Colorectal mucosa 104	1.8 ± 0.21	0.82 ± 0.028	0.36 ± 0.085	3.0 ± 0.071	64%, 56	26%, 29	16 ± 6.4
Tumor tissues							
CRC 2640	1.1 ± 0.23	0.35 ± 0.064	0.21 ± 0.042	1.8 ± 0.35	68 ± 0.71	21 ± 0.71	12 ± 0.0
CRC 2966	1.3 ± 0.071	0.37 ± 0.0071	0.25 ± 0.064	1.9 ± 0.071	67 ± 1.4	20 ± 1.4	13 ± 2.8
CRC 3106	1.9 ± 0.42	0.74 ± 0.19	0.29 ± 0.12	3.0 ± 0.14	66 ± 12	25 ± 7.1	10 ± 4.2
CRC 3107s	1.6 ± 0.50	0.61 ± 0.057	0.35 ± 0.23	2.5 ± 0.28	61 ± 11	25 ± 0.71	15 ± 11
CRC 3108	2.2 ± 0.85	0.83 ± 0.38	0.41 ± 0.25	3.5 ± 1.5	65 ± 2.8	24 ± 0.71	11 ± 2.8
Average	2.1	0.72	0.44	3.2	63	23	14
SD	0.071	0.11	0.071	0.28	10	5	7