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Supporting Information

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for

A New Protein Engineering Approach Combining Chemistry and Biology, Part II: Site-Specific Functionalization of Proteins by Organopalladium Reactions

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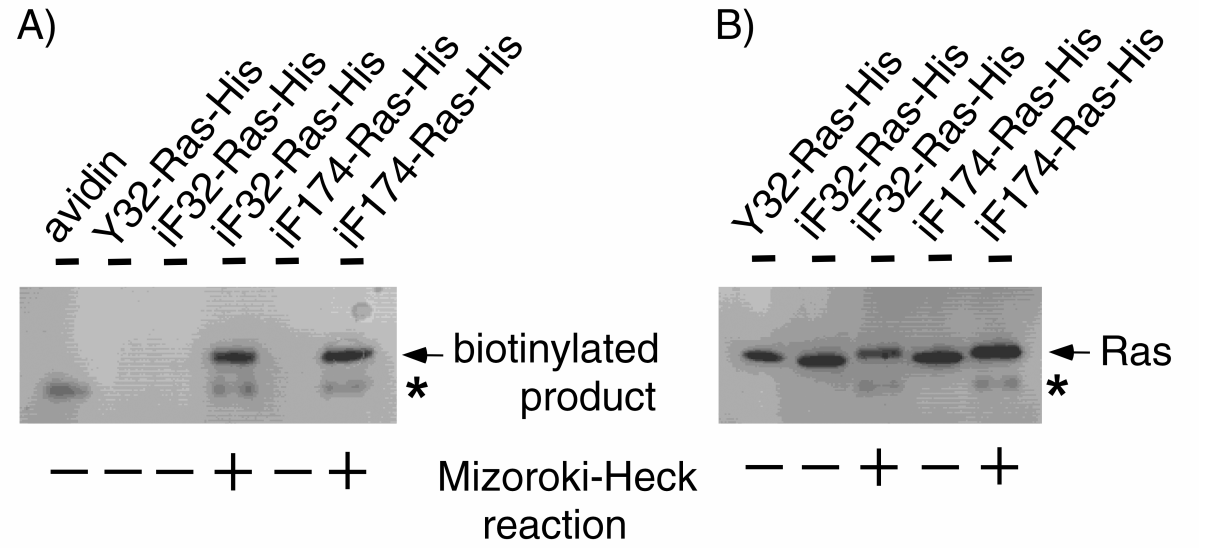


Figure S1. Detection of the biotinylated Ras protein using A) Streptavidin-HRP and B) an anti-Ras antibody. The iF32- and iF174-Ras-His proteins were subjected to the Mizoroki-Heck reaction, purified using monomeric avidin beads, and analyzed by Western blot. The Y32-Ras-His protein was used as a control. An asterisk indicates a cross reaction between avidin and Streptavidin-HRP (or an anti-Ras antibody).

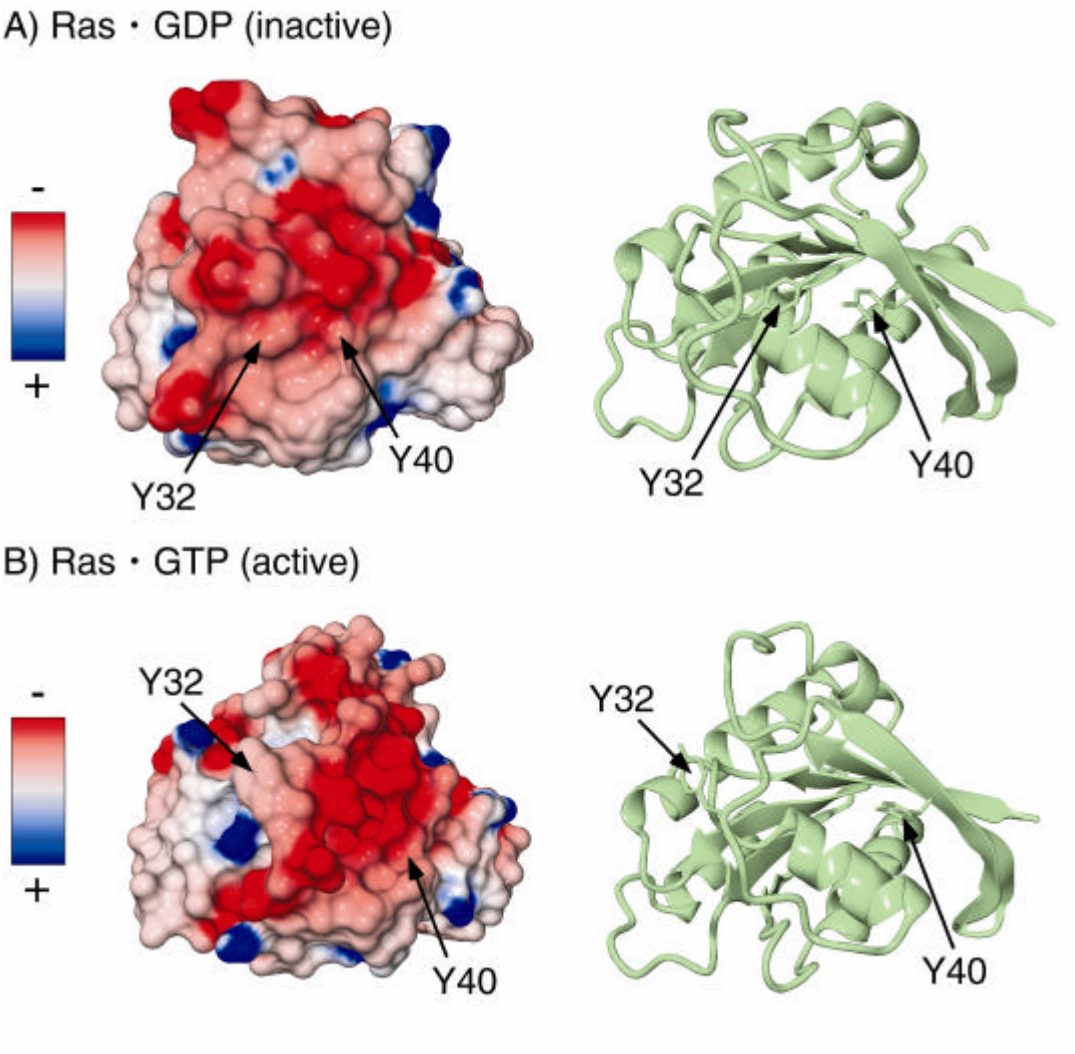


Figure S2. Electrostatic surface potentials (Left) and ribbon models (Right) of A) the GDP-bound form of the wild-type Ras protein (PDB: 4Q21)[1] and B) the GTP-bound form of the wild-type Ras protein (PDB ID: 5P21).[2] An arrow indicates tyrosine residues at position 32 and 40 of the wild-type Ras protein.

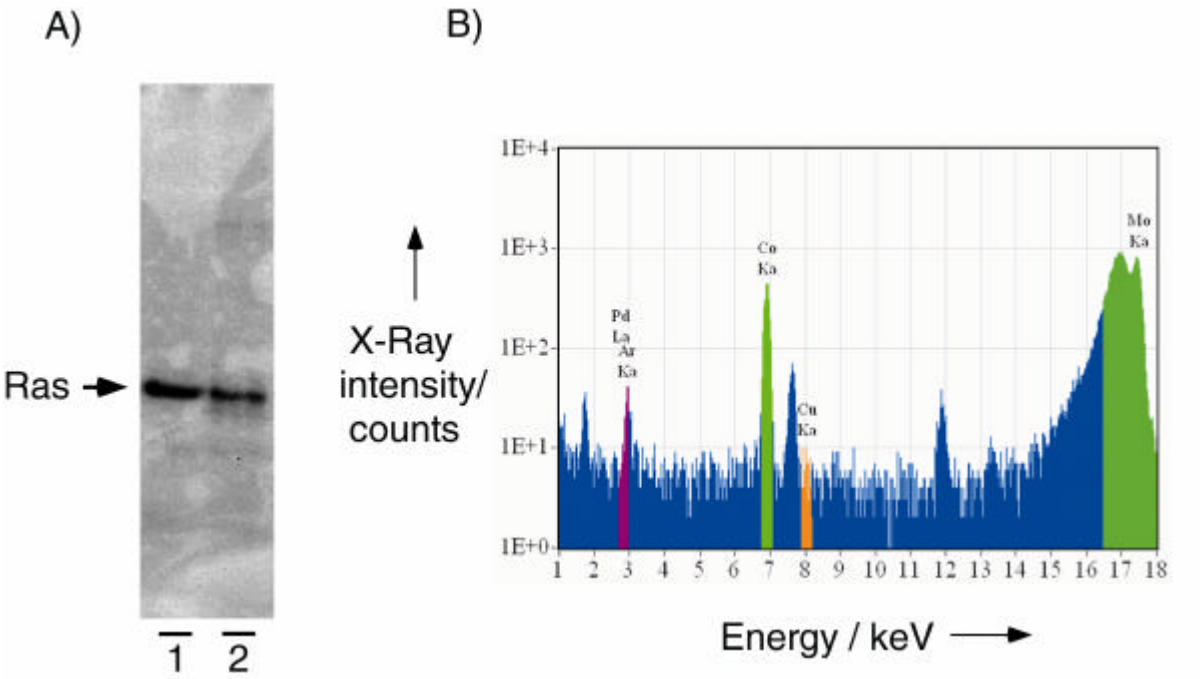
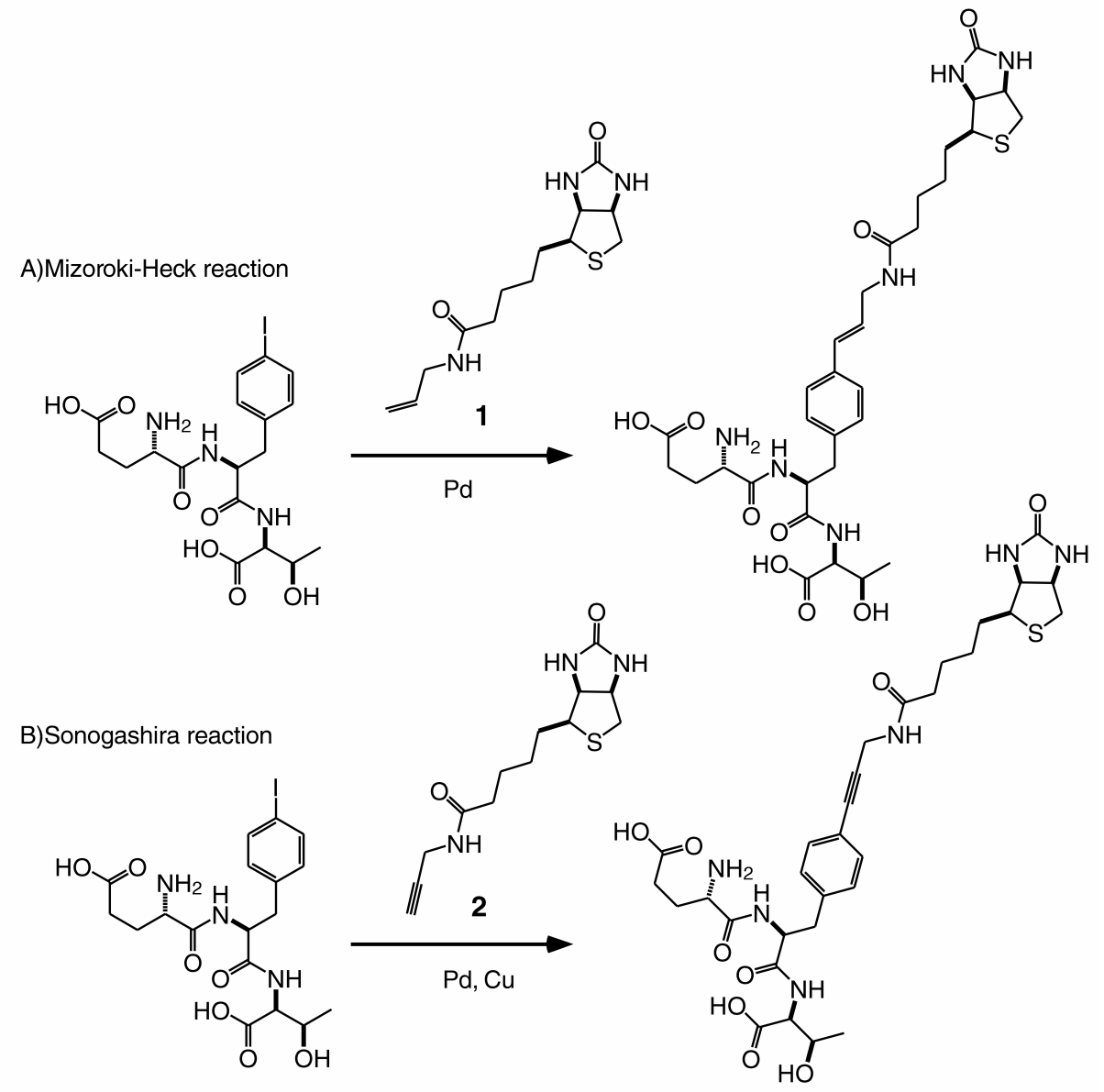


Figure S3. A) SDS-PAGE analysis of the wild-type Ras protein before (lane 1) and after (lane 2) being incubated under Sonogashira conditions. B) The X-Ray fluorescence (XRF) spectrum of the wild-type Ras protein (840 nM) after being incubated under Sonogashira conditions. The Pd-Lα peak (2.84 keV) was overlapped with the Ar-Kα peak (2.96 keV) derived from argon in air.



Scheme S1. Conjugation of the iF-peptide (**3**) with vinylated biotin (**1**) by A)Mizoroki-Heck reaction or propargylated biotin (**2**) by B)Sonogashira reaction.

Table S1. The average mass calculated from the amino acid sequence of the Sonogashira reaction product (bF32-Ras-His).

product ion	$(M+H)^+$ [*m/z*]	$(M+2H)^{2+}$ [*m/z*]	product ion	$(M+H)^+$ [*m/z*]	$(M+2H)^{2+}$ [*m/z*]
b_1	88.09	44.55	y_{25}	3273.69	1637.35
b_2	159.17	80.09	y_{24}	3202.62	1601.81
b_3	272.32	136.67	y_{23}	3089.46	1545.23
b_4	373.43	187.22	y_{22}	2988.35	1494.68
b_5	486.59	243.80	y_{21}	2875.19	1438.10
b_6	614.72	307.86	y_{20}	2747.06	1374.03
b_7	727.88	364.44	y_{19}	2633.90	1317.45
b_8	841.04	421.02	y_{18}	2520.74	1260.87
b_9	969.17	485.09	y_{17}	2392.61	1196.81
b_{10}	1083.27	542.14	y_{16}	2278.51	1139.76
b_{11}	1220.41	610.71	y_{15}	2141.37	1071.19
b_{12}	1367.59[a]	684.30	y_{14}	1994.19[a]	997.60
b_{13}	1466.72[a]	733.87	y_{13}	1895.06[a]	948.03
b_{14}	1581.81[a]	791.41	y_{12}	1779.97[a]	890.50
b_{15}	1710.93[a]	855.97	y_{11}	1650.85[a]	825.93
b_{16}	2137.47	1069.24	y_{10}	1224.31[a]	612.66[a]
b_{17}	2252.56	1126.78	y_9	1109.22[a]	555.12
b_{18}	2349.67[a]	1175.34	y_8	1012.11	506.56
b_{19}	2450.78	1225.89	y_7	911.00	456.01
b_{20}	2563.94[a]	1282.47	y_6	797.84	399.43
b_{21}	2693.05	1347.03	y_5	668.73[a]	334.87
b_{22}	2808.14	1404.57	y_4	553.64[a]	277.32
b_{23}	2895.22[a]	1448.11	y_3	466.56	233.78
b_{24}	3058.40	1529.70	y_2	303.39	152.20
b_{25}	3214.58	1607.80	y_1	147.20	74.10

[a] The mass value that agreed with the experimental mass value in Figure 5.

Experimental Section

Materials: 9-Fluorenylmethoxycarbonyl (Fmoc) and side-chain protected amino acids, and Fmoc-CLEAR-Amide Resin were purchased from Peptide Institute (Minoo, Japan). $Pd(OAc)_2$ (99.9999% purity) was purchased from Sojitz Chemical (Tokyo, Japan). Copper(I) trifluoromethanesulfonate (CuOTf) benzene complex, sodium ascorbate, and decyl-β-D-glucopyranoside (DG) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) was obtained from Dojindo (Kumamoto, Japan). Bovine serum albumin (Ultrapure BSA) was purchased from Ambion (Austin, TX, USA). The expression plasmids pK7-Ras-His and pK7-Ras-His-32am were gifts from Dr. Takashi Ohtsuki, Okayama University. Ni-NTA agarose (Ni nitriloacetic acid-agarose beads) were purchased from QIAGEN GmbH (Hilden, Germany). 12% NuPAGE® Bis-Tris gel was purchased from Invitrogen (Carlsbad, CA, USA). The *Achromobacter* Protease I (Lys-C) was a gift from Prof. Takeharu Masaki, Ibaraki University. The anti-Ras antibody (H-Ras F235) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The streptavidin-horseradish peroxidase conjugate, anti-Mouse Ig (NA931V), ECL Plus Western blot detection reagents (RPN2132), Superdex 75 (HiLoad 16/60 pg), and prepacked disposable PD-10® Column, were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ).

Methods: An automated peptide synthesizer (PSSM-8) was a product of Shimadzu (Kyoto, Japan). The oxygen monitor (JKO-O2LD II) was a product of JIKCO (Tokyo, Japan). The oxygen absorber (A-500HS) was purchased from ISO (Yokohama, Japan). The preparative HPLC system consisting of a PU980 gradient pump and a UV980 UV-Vis detector was purchased from JASCO (Tokyo, Japan), and the Waters 600E multisolvent delivery system was purchased from Waters (Milford, MA, USA). Low- and high-resolution mass spectra were recorded on a JEOL JMS-MS-700P (Akishima, Japan) mass spectrometer under fast atom bombardment (FAB) conditions using glycerol (positive) or triethanolamine (negative) as a matrix. The LC-MS system was a combination of a model 1100 liquid chromatograph with a diode-array detector manufactured by Agilent technologies (Waldbronn, Germany), and a Thermoelectron LCQ ion-trap mass spectrometer (San José, CA, USA). Mightysil® C18, a reversed phase chromatographic medium, was purchased from Kanto Kagaku (Tokyo, Japan). The Ras or biotinylated Ras band was detected with a fluorescent imaging

analyzer FLA2000® or LAS-3000® (Fuji Photo Film, Tokyo, Japan). The structure of the Ras protein was displayed using the CueMol program (R. Ishitani, CueMol: Molecular Visualization Framework; cuemol.sourceforge.jp). The X-Ray fluorescence (XRF) analysis was performed using NANOHUNTER®, a tabletop TXRP Spectrometer manufactured by Rigaku (Tokyo, Japan).

Preparation of iF-peptide: The tripeptide containing a 4-iodo-L-phenylalanine residue (iF-peptide, **3**) was prepared as described below. 4-Iodo-L-phenylalanine was derivatized with 9-Fluorenylmethyl chloroformate in the presence of *N*,*N*-diisoprpopylethylamine (DIEA) to afford Fmoc-4-iodo-L-phenylalanine. The iF-peptide was synthesized on Fmoc-CLEAR-Amide Resin (0.1 mmol) using Fmoc chemistry. Fmoc and side-chain protected amino acids were used in 4-fold molar excess over the N-termini. Fmoc amino acids were activated *in situ* by standard reagents such as 2-(1*H*-benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), and DIEA. The Fmoc group was removed with piperidine (20 % (*v*/*v*)) in *N*,*N*-dimethylformamide (DMF). The peptide was cleaved and deblocked by stirring them at RT for 6 h in trifluoroacetic acid (TFA; 8.25 mL), phenol (0.75 g), ethane dithiol (0.25 mL), thioanisole (0.5 mL), and water (0.5 mL). The yellow-orange crude peptide solution was filtered and the resin was washed with TFA (3 mL); the filtrate and wash were collected in a 50-mL Falcon tube. Ice-cold diethyl ether (40 mL) was added, and a white precipitate was immediately appeared. After centrifugation at 5000 rpm for 5 min the precipitate (the material) was washed once with ice-cold diethyl ether. The resulting crude peptide was dried in vacuo and purified via preparative ODS-HPLC (COSMOSIL® 5C-18 AR-II, 20x250 mm) using a linear gradient of 0-45% acetonitrile/0.1% TFA. The pure iF-peptide was lyophilized to give a colorless amorphous solid (10 mg).

Preparation of Y32-Ras-His protein: The human c-Ha-Ras protein (1-171) fused with hexahistidine (H_6) residues (Y32-Ras-His; MTEYKLVVVGAGGVGKSALTIQLIQ-NHFVDE-Y32-DPTIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFL CVFAINNTKSFEDIHQYREQIKRVKDSDDVPMVLVGNKCDLAARTVESRQAQDLARS YGIPYIETSAKTRQGVEDAFYTLVREIRQHKLRKL-GSLVPRGSHHHHHH) was expressed from pK7-Ras-His under control of the *lac* promoter in the *E. coli* DH5α strain. The Y32-Ras-His protein was purified using Ni-NTA agarose (Ni nitriloacetic acid-agarose beads) and a Superdex 75 column. The Y32-Ras-His protein (0.5 $mgmL^{-1}$)

was sealed in a laminated aluminum bag with an oxygen absorber, and stored at -20 °C in a solution containing Tris-HCl (50 mM, pH 7.6), NaCl (75 mM), $MgCl_2$ (1 mM), and glycerol (5.5 M).

Preparation of Y32-Ras protein: The wild-type Ras protein (1-171) without a hexa-histidine (H_6) tag (Y32-Ras; MTEYKLVVVGAGGVGKSALTIQLIQNHFVDE-Y32-DPTIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHQYREQIKRVKDSDDVPMVLVGNKCDLAARTVESRQAQDLARSYGIPYIETSAKTRQGVEDAFYTLVREIRQHKLRKL) was expressed from pRGH in the *E. coli* TG1 strain.[3] The Y32-Ras protein was purified using DEAE-Sephacel and a Superdex 75 column. The Y32-Ras protein (20 mg mL^{-1}) was sealed in a laminated aluminum bag with an oxygen absorber, and stored at –20 °C in a solution containing HEPES-KOH (40 mM, pH 8.0), NaCl (80 mM), $MgCl_2$ (0.8 mM), and glycerol (4.3 M).

Preparation of iF32-Ras protein: The H_6-fused iF32-Ras protein (iF32-Ras-His; MTEYKLVVVGAGGVGKSALTIQLIQNHFVDE-iF32-DPTIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHQYREQIKRVKDSDDVPMVLVGNKCDLAARTVESRQAQDLARSYGIPYIETSAKTRQGVEDAFYTLVREIRQHKLRKL-GSLVPRGSHHHHHH) was expressed using an *E. coli* cell-free translational system. The 4-iodo-L-phenylalanyl-$tRNA^{Phe}_{CUA}$ was prepared as described previously.[4] The pre-acylated tRNA was dissolved in the *E. coli* cell-free translation mixture[5] containing pK7-Ras-His-32am, encoding the H_6-fused Ras protein with a nonsense codon TGA at position 32. The cell-free system was incubated at 30 °C for 30 min. The resulting in vitro translation product was purified using Ni-NTA agarose (Ni nitriloacetic acid-agarose beads). The bound iF32-Ras-His was eluted with Tris-HCl (50 mM, pH 7.9), NaCl (0.3 M), imidazole-HCl (0.25 M, pH 7), and $Mg(OAc)_2$ (10 mM). The eluant was concentrated by ultrafiltration. Ascorbic acid (final concentration 4 mM) was mixed with the protein solution. It was stored at –80°C for the Sonogashira reaction. It was also buffer-exchanged into HEPES-KOH (40 mM, pH 8.0) containing glycerol (4.3 M, 40%(*v*/*v*)), NaCl (80 mM), and $MgCl_2$ (0.8 mM) for the Mizoroki-Heck reaction. The iF32-Ras-His stock solution (96% purity)[4] was sealed in a laminated aluminum bag with an oxygen absorber, and stored at -20°C.

Preparation of iF174-Ras protein: The H_6-fused Ras protein containing 4-iodo-L-phenylalanine at position 174 (iF174-Ras-His; MTEYKLVVVGAGGVGKSALTIQLIQ-NHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVF

AINNTKSFEDIHQYREQIKRVKDSDDVPMVLVGNKCDLAARTVESRQAQDLARSYGI PYIETSAKTRQGVEDAFYTLVREIRQHKLRKL-DP-iF174-SHHHHHHKL) was expressed from the PCR amplified template in the cell-free translational system. Purification and buffer exchange of the iF174-Ras-His protein was carried out in the same manner as for iF32-Ras-His.

Preparation of biotin analogues: Vinylated biotin (**1**) was synthesized through a condensation between D-(+)-biotin and 3-amino-1-propene by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) hydrochloride and DIEA. Propargylated biotin (**2**) was synthesized through a condensation between D-(+)-biotin and 3-amino-1-propyne in the same manner.

Preparation of palladium catalysts: The catalyst solution for the Mizoroki-Heck reaction was prepared under argon or nitrogen as described below. A stock solution of $Pd(OAc)_2$ (20 mM) in DMSO (12.8 M) and TPPTS (20 mM) in water was mixed in a molar ratio of 1:1, and this was incubated for 30 min at RT before use.

The catalyst solution for the Sonogashira reaction was prepared according to the procedure in the literature with some modifications.[6] $Pd(OAc)_2$ (10 mM) and TPPTS (50 mM) in water was mixed with CuOTf (20 mM) in DMSO in a volumetric ratio of 5:1. The catalyst was sealed in a laminated aluminum bag with the oxygen absorber, and then rotated at 4°C for five days.

Mizoroki-Heck reaction using a peptide substrate: The Mizoroki-Heck reaction was screened using the iF-peptide (Table 1). The prereaction mixtures containing various concentrations of iF-peptide, vinylated biotin (**1**), salts and bases were thoroughly dried on a vacuum centrifuge. They were freshly resuspended in a reaction mixture (9.5 µL) containing DMSO and BSA under nitrogen or argon in a glove box. The oxygen concentration was kept below 2%. The catalyst solution (0.5 µL) was finally added to start the Mizoroki-Heck reaction. The reaction mixtures were sealed in a laminated aluminum bag with an oxygen absorber and incubated under various conditions. The reaction mixtures were dried in vacuo and stored at –20 °C. These samples were dissolved in water (20 µL) and immediately analyzed on COSMOSIL® 3C-18 AR-II (4.6x 100 mm) at a flow rate of 1 mL/min using a linear gradient of 0-60% acetonitrile/0.1% TFA in 12 min. The molar absorption coefficient of the Mizoroki-Heck reaction product (**4**) was 1.1 X 10^4 $M^{-1}cm^{-1}$ at 215 nm, while that of the substrate (**3**, iF-peptide) was 1.5 x 10^4 $M^{-1}cm^{-1}$ at 215 nm.

Synthesis of biotinylated peptide 4 under optimized Mizoroki-Heck reaction conditions: Vinylated biotin (**1**; 10 mM, 2.5 mL), iF-peptide (**3**, 2 mg, 7 mM, 560 μL), and TAPS-NaOH (2M, 1.6 mL, pH 8.3), were mixed and thoroughly dried on a vacuum centrifuge. This mixture was freshly resuspended in DG (1.6 mM, 3.2 mL) under nitrogen in a glove box. The oxygen concentration was kept below 2%. The catalyst solution (0.8 mL) was finally added. The reaction mixture was sealed in a laminated aluminum bag with an oxygen absorber and incubated at RT for 14h. The mixture was dried on a vacuum centrifuge and stored at –20°C. It was purified by ODS-HPLC (COSMOSIL® 5C-18 AR-II, 10x250 mm) at a flow rate of 3 mL/min using a linear gradient of 0-40% acetonitrile/0.1% TFA in 25 min to give the bF-peptide **4** (0.8 mg, 25%): HRMS (FAB) calculated for $C_{31}H_{45}N_6O_9S$ $[(M+H)^+]$ 677.2969, found 677.3015.

Sonogashira reaction using a peptide substrate: The Sonogashira reaction was screened using the iF-peptide (Table 2). The prereaction mixtures containing various concentrations of iF-peptide, propargylated biotin (**2**) and salts were thoroughly dried on a vacuum centrifuge. They were freshly resuspended in a reaction mixture (9.5 μL) containing DMSO (and proteins) under nitrogen or argon gas. The oxygen concentration was kept below 2%. The catalyst solution (0.5 μL) was finally added. The reaction mixtures were sealed in a laminated aluminum bag with an oxygen absorber and incubated under various conditions. The reaction mixtures were dried on a vacuum centrifuge and stored at –20°C. These samples were dissolved in water (20 μL) and immediately analyzed on COSMOSIL® 5C-18 AR-II (4.6x250 mm) at a flow rate of 1 mL/min using a linear gradient of 0-100% acetonitrile/0.1% TFA in 30 min. The molar absorption coefficient of the Sonogashira reaction product (**5**) was 1.3×10^4 $M^{-1}cm^{-1}$ at 215 nm, while that of the substrate (**3**, iF-peptide) was 1.5×10^4 $M^{-1}cm^{-1}$ at 215 nm.

Synthesis of biotinylated peptide 5 under optimized Sonogashira reaction conditions: Propargylated biotin (**2**; 10 mM, 320 μL), iF-peptide (**3**, 2 mg, 10 mM, 0.4 mL), and TAPS-NaOH (2M, 0.4 mL, pH 8.3) were mixed and thoroughly dried on a vacuum centrifuge. Next, Triton® X100 (100% (*v*/*v*), 20 μL) was added and further dried on a vacuum centrifuge. This mixture was freshly resuspended in DMSO in water (2.4 M, 3040 μL) under nitrogen in a glove box. The oxygen concentration was kept below 2%. The catalyst solution (960 μL) was finally added. The reaction mixture was sealed in a laminated aluminum bag with an oxygen absorber and incubated at RT for 90 min. The reaction mixture was dried on a vacuum centrifuge and stored at –20 °C. This

mixture was purified by ODS-HPLC (COSMOSIL® 5C-18 AR-II, 10x250 mm) at a flow rate of 3 mL/min using a linear gradient of 0-40% acetonitrile/0.1% TFA in 25 min to give bF-peptide **5** (0.5 mg, 15%): HRMS (FAB) calculated for $C_{31}H_{41}N_6O_9S$ [$(M-H)^-$] 673.2656, found 673.2708.

Protein functionalization by the Mizoroki-Heck reaction: The prereaction mixture (100 μL) containing vinylated biotin (0.4 mM), NaOAc (0.1 M), $MgCl_2$ (8 mM), tetrabutylammonium chloride (5 mM), decyl-β-D-glucopyranoside (0.16 mM, 0.005% (*w*/*v*)), tyramine-HCl (1 mM), and TAPS-NaOH (10 mM, pH 8.3) was thoroughly dried on a vacuum centrifuge. It was freshly dissolved in a reaction mixture (9.5 μL) containing DMSO (1.3 M, 10%(*v*/*v*)) and iF32-Ras-His (2.85 μg) under nitrogen or argon in a glove box. The oxygen concentration was kept below 2%. The catalyst solution (0.5 μL) was finally added to start the Mizoroki-Heck reaction. The reaction mixture was sealed in a laminated aluminum bag with an oxygen absorber, and this was incubated at 5 °C for 50 h. The crude reaction product was mixed well with the extraction buffer (100 μL) containing sodium stearate (2 mM, 0.06% (*w*/*v*)), Triton® X100 (0.03% (*v*/*v*)), NaCl (100 mM), and Tris-HCl (22 mM, pH 7.6). The precipitate was removed by centrifugation at 4 °C. The supernatant was subjected to a GST-pull down assay.

Protein functionalization by the Sonogashira reaction: The protein substrate (iF32 or iF174-Ras-His; 2 μL) was mixed with the prereaction mixture (30 μL) containing TAPS-NaOH (120 mM, pH 8.3), Triton® X100 (0.5% (*v*/*v*)), and propargylated biotin (20 mM) under nitrogen or argon in a glove bag. Next, the catalyst solution (8 μL) was added to start the Sonogashira reaction. The reaction mixture was incubated at 6 °C for 80 min.

LC-MS and LC-MS/MS analyses: LC-MS and LC-MS/MS analyses were performed as described previously[7,8] with some modifications. The crude reaction mixture (10 μL) was mixed with the SDS gel-loading buffer (20 μL) containing DTT (200 mM), SDS (140 mM, 4% (*w*/*v*)), glycerol (2.2 M, 20% (*v*/*v*)), bromophenol blue (0.1% (*w*/*v*)), and Tris-HCl (100 mM, pH 6.8), immediately after the Sonogashira reaction. The sample was incubated at 60 °C for 1 min and applied on SDS-PAGE (12% NuPAGE® Bis-Tris gel). The 21kDa protein band was excised from the gel, and it was incubated in the digestion buffer (50 μL) containing Lys-C (0.05 μg), decyl-β-D-glucopyranoside (1.6 mM, 0.05%(*w*/*v*)), and Tris-HCl (0.05 M, pH 9.0) at 37 °C for 12 h. The liberated peptides were separated on Mightysil® C18 (1 x 50 mm) at a flow rate of 30 μL/min using

a linear gradient of 2-60% solvent B in 40 min, where solvent A and B consisted of 0.09% (*v*/*v*) TFA in water, and 0.075% (*v*/*v*) TFA and 80% (*v*/*v*) acetonitrile in water, respectively. The eluant was analyzed with a LCQ ion-trap mass spectrometer with a house-made ESI probe.

GST pull-down assay: An in vitro binding assay was carried out as described below. A tenfold volume of the extraction buffer containing sodium stearate (2 mM, 0.06% (*w*/*v*)), Triton X100 (0.03% (*v*/*v*)), NaCl (20 mM), and Tris-HCl (22 mM, pH 7.6), was added the reaction mixture. The precipitate was removed by centrifugation at 4 °C. Next, the supernatant was mixed with *N,N'*-dimethylthiourea (50 mM), BSA (0.5 mg mL^{-1}) and a non-hydrolyzable GTP analogue (GTPγS; 0.1 mM) or GDP (0.1 mM). This was incubated at RT for 10 min, and mixed with $MgCl_2$ (10 mM), GST-Raf-1-RBD (1 μg) and glutathione-Sepharose® 4B beads suspended in water (20 μL), then was incubated on ice for 1h. The resin was washed with the washing buffer (1 mL) containing $MgCl_2$ (5 mM), NaCl (20 mM), Tris-HCl (20 mM, pH 7.6) and *N,N'*-dimethylthiourea (50 mM). The bound proteins were then separated on SDS-PAGE.

XRF analysis: The amount of transition-metal ions bound on proteins was estimated by XRF analysis as described below. The wild-type Ras protein (2 mg, 1 mL) was incubated at RT for 1h under nitrogen in the presence of $Pd(OAc)_2$ (2 mM), TPPTS (10 mM), CuOTf (0.8 mM), DMSO (2.3 M), TAPS-NaOH (200 mM, pH 8.3), Triton® X100 (0.4% (*v*/*v*)), HEPES-KOH (4 mM, pH 8.0), NaCl (8 mM), $MgCl_2$ (0.1 mM), and glycerol (0.4 M). *N,N'*-dimethylthiourea (1 M, 56 μL) was added, and this mixture was applied onto a PD-10 column that had been equilibrated with a desalting buffer containing Tris-HCl (10 mM, pH 7.6), NaCl (155 mM), $MgCl_2$ (2 mM), and MeOH (0.6 M). Furthermore, the desalting buffer (2 mL) was applied onto the PD-10 column and the sample (3.1 mL) was collected in a tube. The concentration of the Ras protein was determined by the Bradford method using BSA as a standard. The recovered Ras protein (82 μg, 3 mL) was lyophilized and dissolved in nitric acid (2 M, 5 mL). A known amount of cobalt ion (10 ppm) was mixed as an internal standard. This sample was spotted on a filter, dried under a heat lamp, and subjected to XRF analysis using $Mo_{K\alpha}$ radiation (17.5 keV). The amount of palladium and copper ions was estimated by measuring peak areas against the Co-Kα peak at 6.93 keV, assuming that the ratio of the Pd-Lα, Co-Kα, and Cu-Kα peak areas are 1:27.5:39 when they are at the same concentration. The lower detection limit is 0.004 ppm (60 nM; copper).

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