Electronic Supplementary Information for

Site-Specific Chemical Fatty-acylation for Gain-of-Function Analysis of Protein *S*-Palmitoylation in Live Cells

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Supplementary Figures

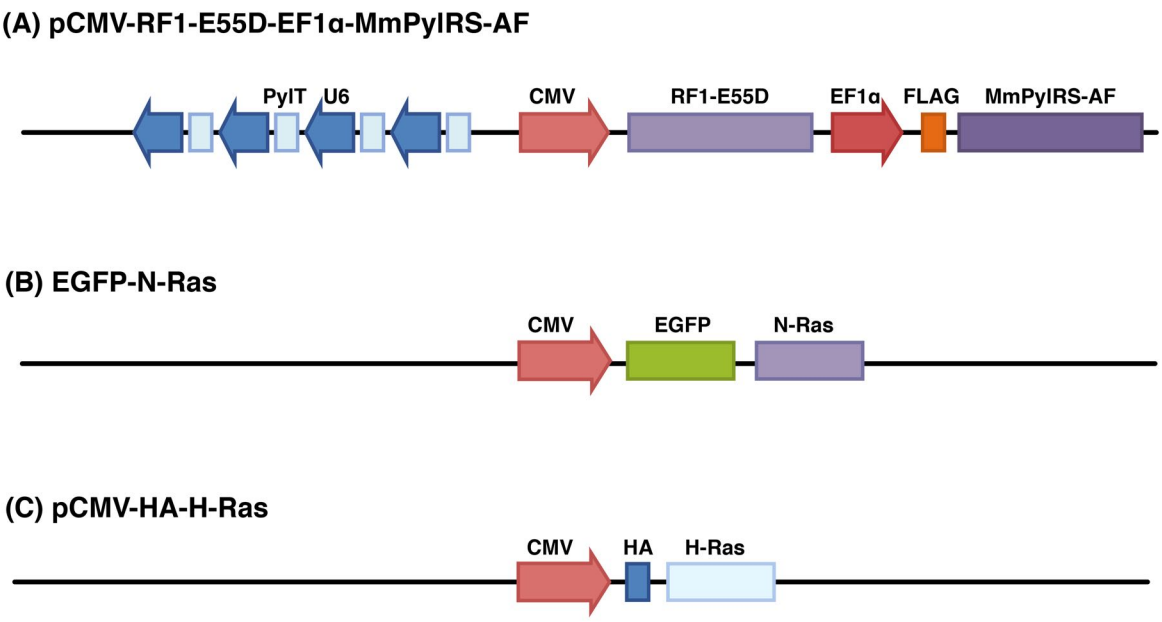


Figure S1. Plasmids used in this study. (A) pCMV-RF1-E55D-EF1α-MmPylRS-AF plasmid modified from pEF1α-MmPylRS-AF.[1] (B) and (C) N-Ras and H-Ras plasmids generated from pEGFP and pCMV-HA vectors.

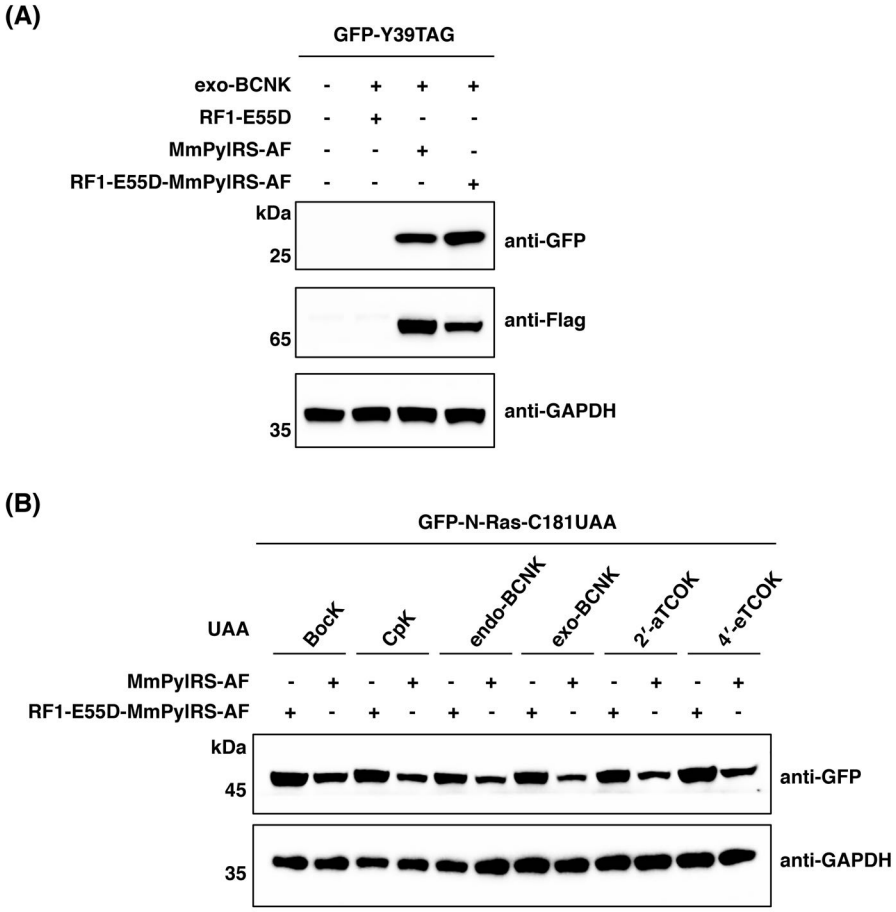


Figure S2. Insertion of RF1-E55D into the pEF1α-MmPylRS-AF plasmid enhances UAA incorporation efficiency. (A) Co-expression of RF1-E55D improves the incorporation of exo-BCNK into GFP-Y39TAG. HEK293T cells were co-transfected with plasmids of GFP-Y39TAG and RF1-E55D, FLAG-Mm-PylRS-AF, or RF1-E55D-FLAG-Mm-PylRS-AF in the presence of exo-BCNK (50 μM) for 24 h and lysed for western blot analysis. (B) Co-expression of RF1-E55D improves the incorporation of UAAs into GFP-N-Ras-C181TAG. HEK293T cells were co-transfected with plasmids of GFP-N-Ras-C181TAG and Mm-PylRS-AF or RF1-E55D-Mm-PylRS-AF in the presence of indicated UAA (50 μM) for 24 h and lysed for western blot analysis.

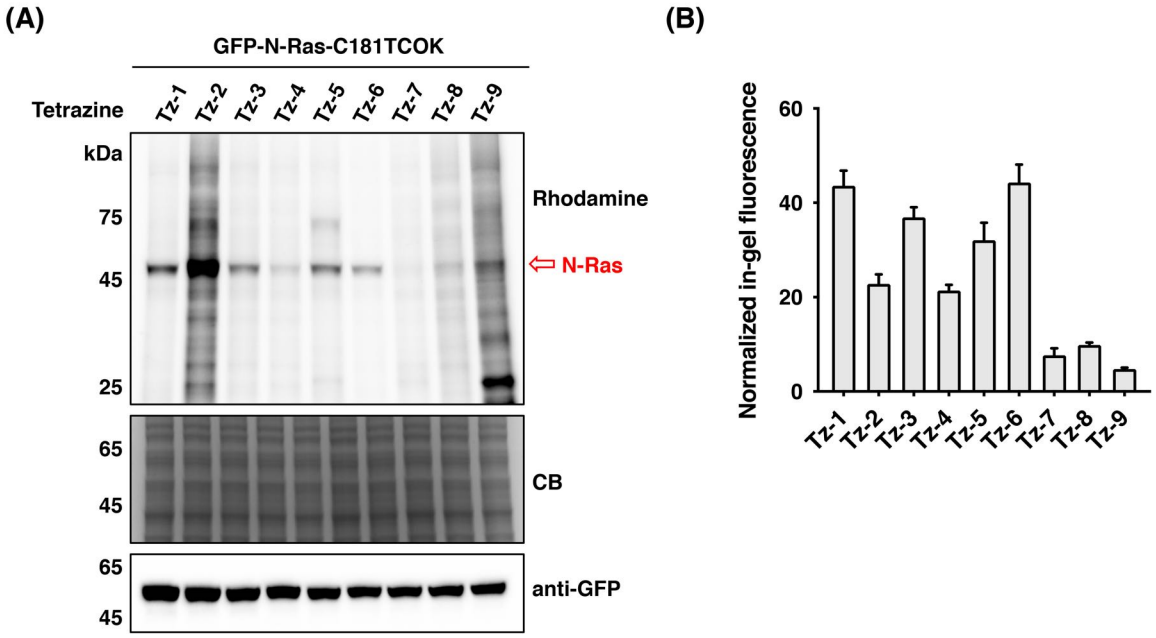


Figure S3. Screening of fatty-acyl tetrazines with GFP-N-Ras-C181TCOK. (A) HEK293T cells expressing GFP-N-Ras-C181TCOK (50 μM 2'-aTCOK) were incubated with fatty-acyl tetrazines (25 μM) and clicked with azido-rhodamine for in-gel fluorescence analysis. (B) Quantification of relative N-Ras fluorescence in (A). Fluorescence intensities of N-Ras bands were quantified and normalized to the overall lane intensities. Data are represented as mean ± s.d., n = 3.

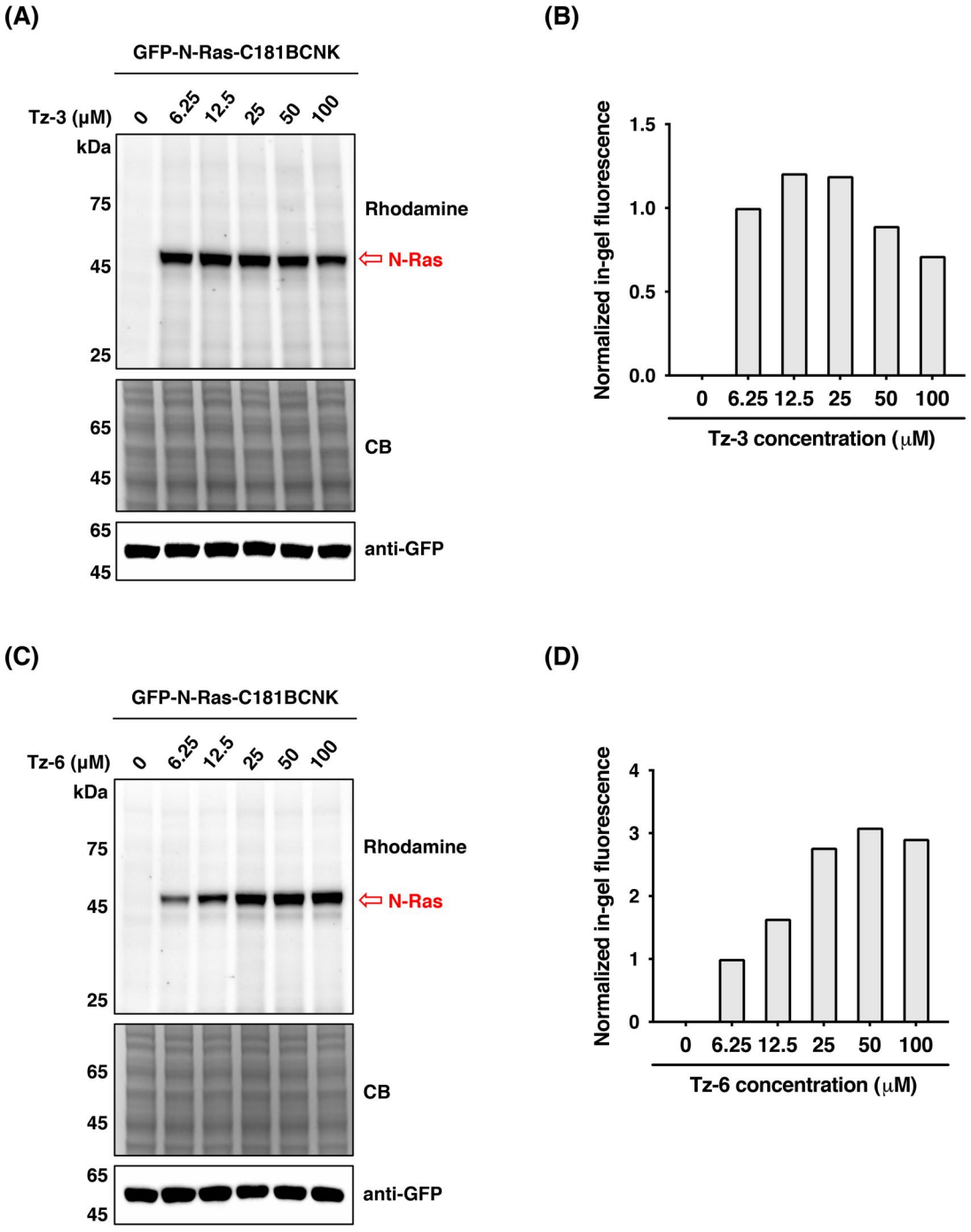


Figure S4. Optimization of fatty-acyl tetrazine concentrations for chemical fatty-acylation. (A) Optimization of Tz-3 concentration. HEK293T cells expressing GFP-N-Ras-C181BCNK (50 μM exo-BCNK) were incubated with Tz-3 of varying concentrations and clicked with azido-rhodamine for in-gel fluorescence analysis. (B) Quantification of N-Ras fluorescence bands in (A). (C) Optimization of Tz-6 concentration. HEK293T cells expressing GFP-N-Ras-C181BCNK (50 μM exo-BCNK) were incubated with Tz-6 of varying concentrations and clicked with azido-rhodamine for in-gel fluorescence analysis. (D) Quantification of N-Ras fluorescence bands in (C). Fluorescence intensity of every N-Ras band was quantified and normalized to corresponding anti-GFP level.

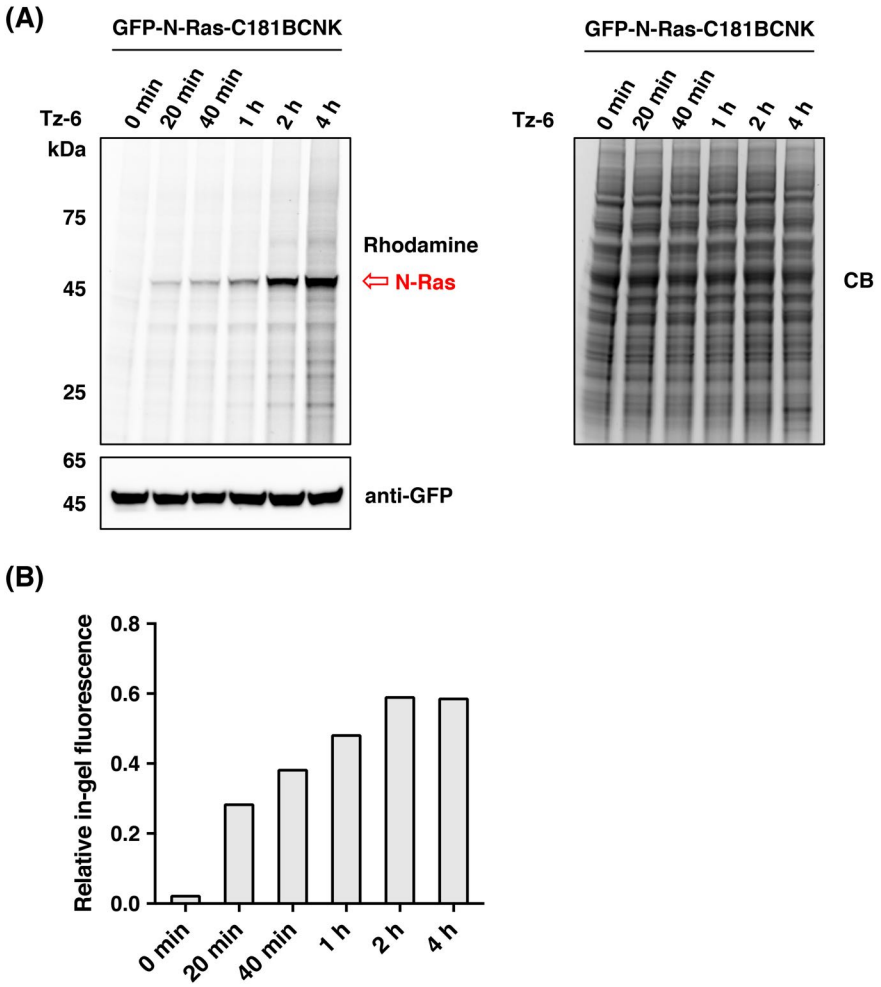


Figure S5. Investigation of fatty-acyl tetrazine labeling time for chemical fatty-acylation. (A) Investigation of Tz-6 labeling time. HEK293T cells expressing GFP-N-Ras-C181BCNK (50 μM exo-BCNK) were incubated with Tz-6 (50 μM) for varying time periods and clicked with azido-rhodamine for in-gel fluorescence analysis. (B) Quantification of N-Ras fluorescence bands relative to the whole fluorescence intensity of the corresponding lane in (A).

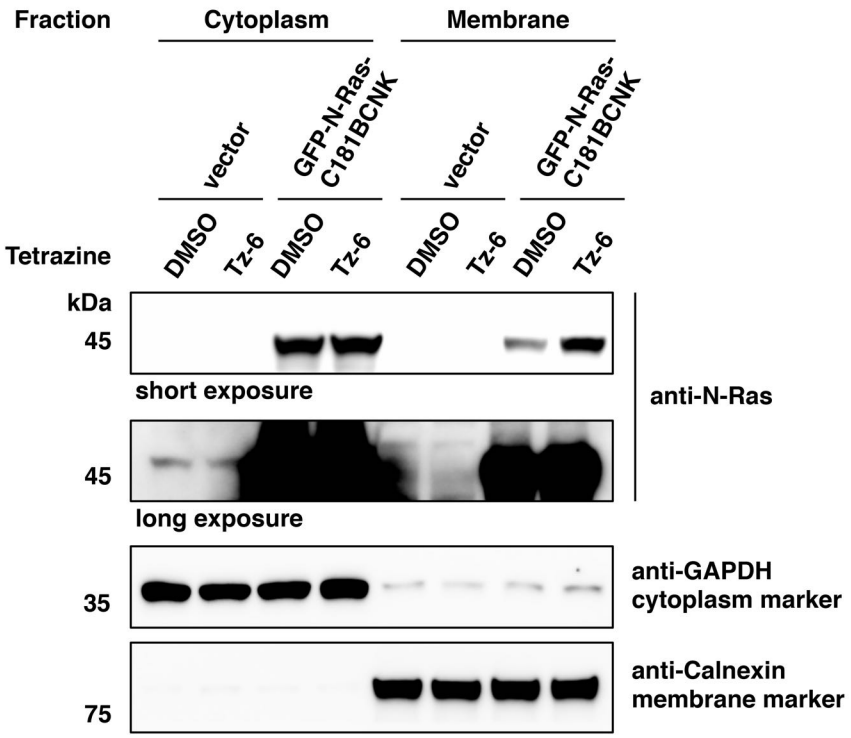


Figure S6. Detection of N-Ras membrane affinity upon chemical fatty-acylation. HEK293T cells were mock-transfected or transfected with GFP-N-Ras-C181BCNK, incubated with Tz-6 (50 μM), and lysed to separate membrane and cytoplasm fractions. GAPDH and calnexin were probed as cytoplasm and membrane controls, respectively. N-Ras was probed with an N-Ras antibody. Note that the endogenous N-Ras level is very low.

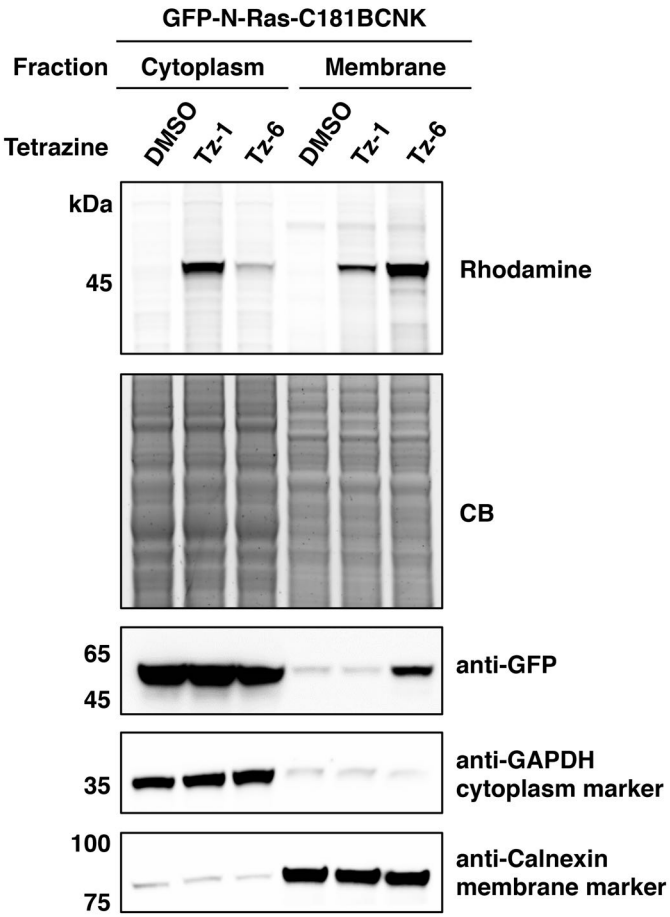


Figure S7. In-gel fluorescence analysis of chemically fatty-acylated N-Ras after fractionation. HEK293T cells were transfected with GFP-N-Ras-C181BCNK, incubated with Tz-1 or Tz-6 (50 μM), and lysed to separate membrane and cytoplasm fractions. The fractions were then clicked with azido-rhodamine and analyzed by in-gel fluorescence and western blot. GAPDH and calnexin were probed as cytoplasm and membrane controls, respectively.

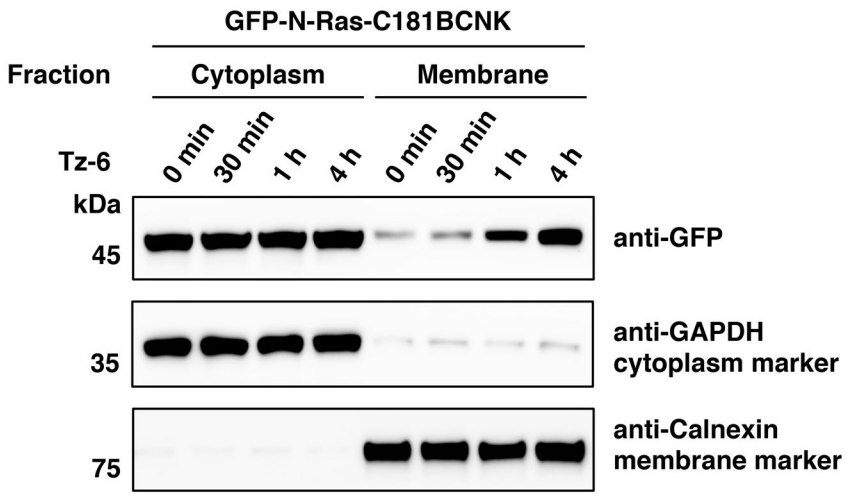


Figure S8. Investigation of Tz-6 labeling time for chemical fatty-acylation by fractionation. HEK293T cells expressing GFP-N-Ras-C181BCNK (50 μM exo-BCNK) were incubated with Tz-6 (50 μM) for varying time periods and lysed to separate membrane and cytoplasm fractions. GAPDH and calnexin were probed as cytoplasm and membrane controls, respectively.

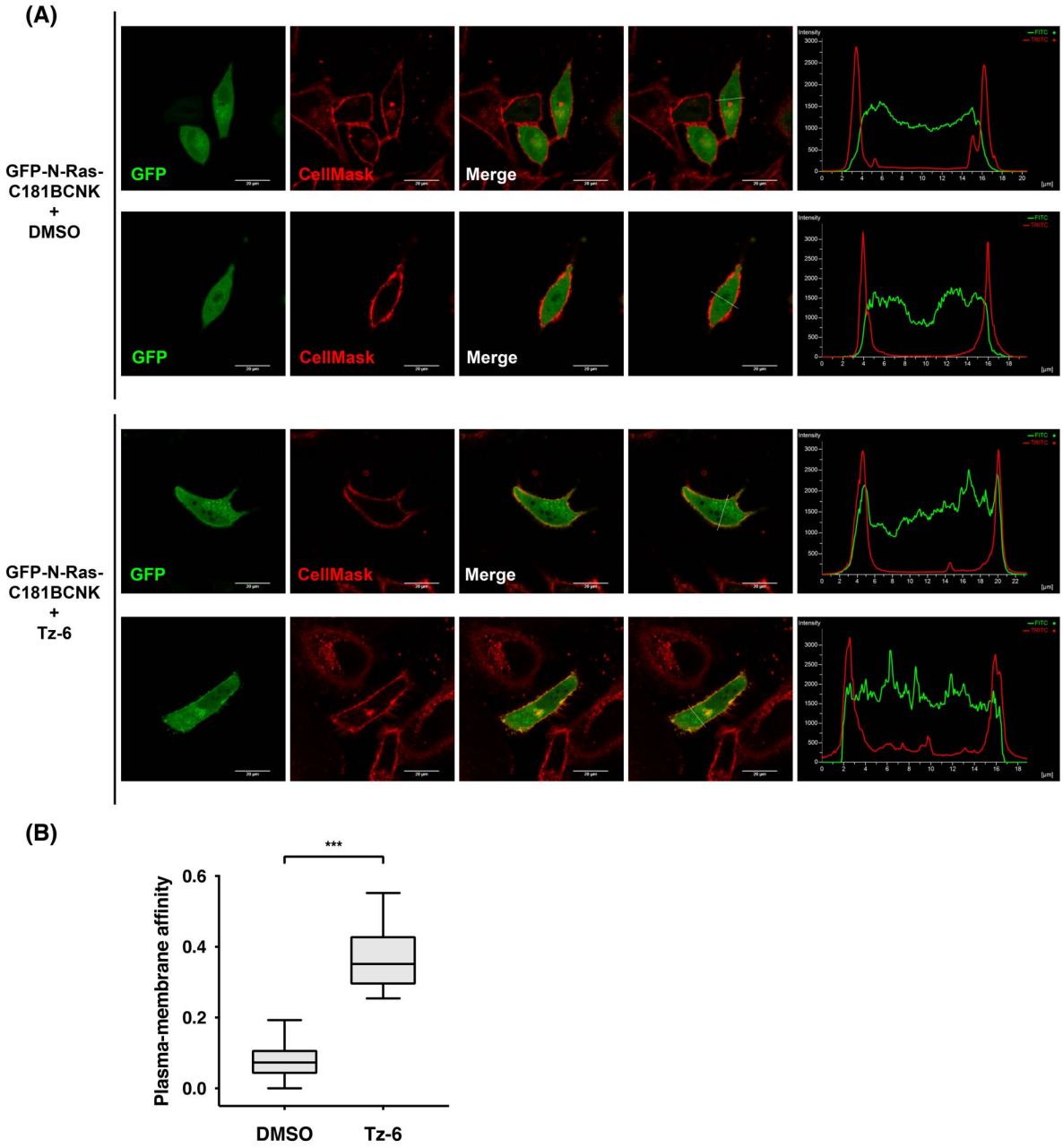


Figure S9. Confocal fluorescence microscopy of chemical fatty-acylated GFP-N-Ras. HeLa cells expressing GFP-N-Ras-C181BCNK (50 μM exo-BCNK) were incubated with DMSO or Tz-6 (50 μM), stained with CellMask Orange Plasma Membrane Stain, and imaged with confocal fluorescence microscopy. (A) Representative confocal fluorescence images of GFP-N-Ras-C181BCNK unlabeled or labeled with Tz-6. The fluorescence intensity profiles show colocalization of the GFP and CellMask signals. (B) Calculated plasma-membrane affinity of GFP-N-Ras-C181BCNK. Plasma-membrane affinity was calculated as previously reported[2] for at least 15 cells in each condition. Data are represented as mean ± s.d., and *** indicates a p-value <0.001, calculated by unpaired t test.

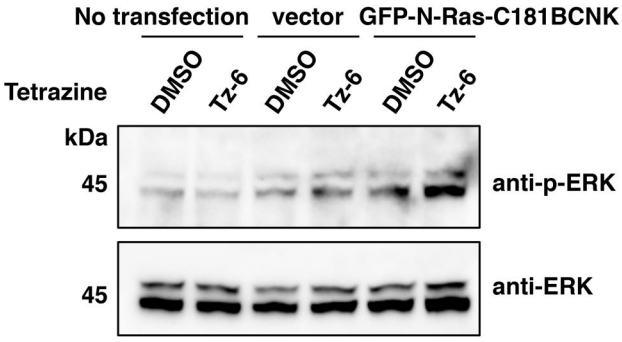


Figure S10. Control experiments for chemical fatty-acylation-induced ERK phosphorylation. HEK293T cells were not transfected, mock-transfected, or transfected with GFP-N-Ras-C181BCNK, incubated with Tz-6 (50 μM), and lysed for western blot analysis.

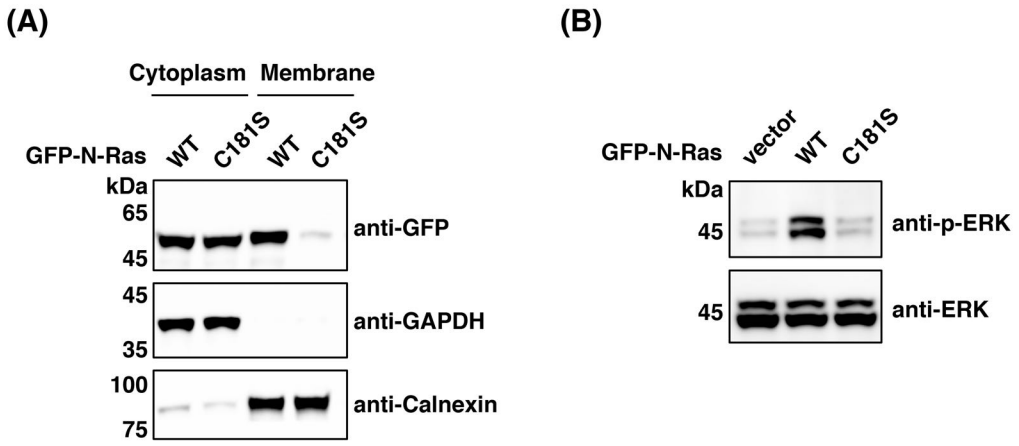


Figure S11. Effects of cysteine 181 mutation on N-Ras distribution and signaling. (A) HEK293T cells were transfected with GFP-N-Ras wild-type or C181S mutant and lysed to separate membrane and cytoplasm fractions. GAPDH and calnexin were probed as cytoplasm and membrane controls, respectively. (B) HEK293T cells were transfected with GFP-N-Ras wild-type or C181S mutant, lysed, and analyzed for phosphorylated ERK.

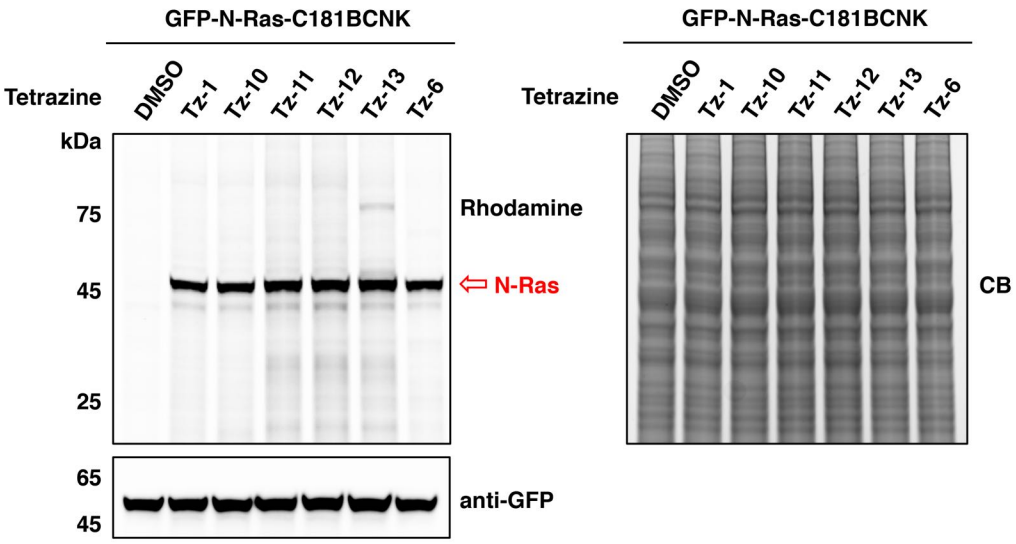


Figure S12. Modification of GFP-N-Ras-C181BCNK with fatty-acyl tetrazines of varying fatty-acyl chain lengths. HEK293T cells expressing GFP-N-Ras-C181BCNK (50 μM exo-BCNK) were incubated with fatty-acyl tetrazines of varying fatty-acyl chain lengths (50 μM) and clicked with azido-rhodamine for in-gel fluorescence analysis.

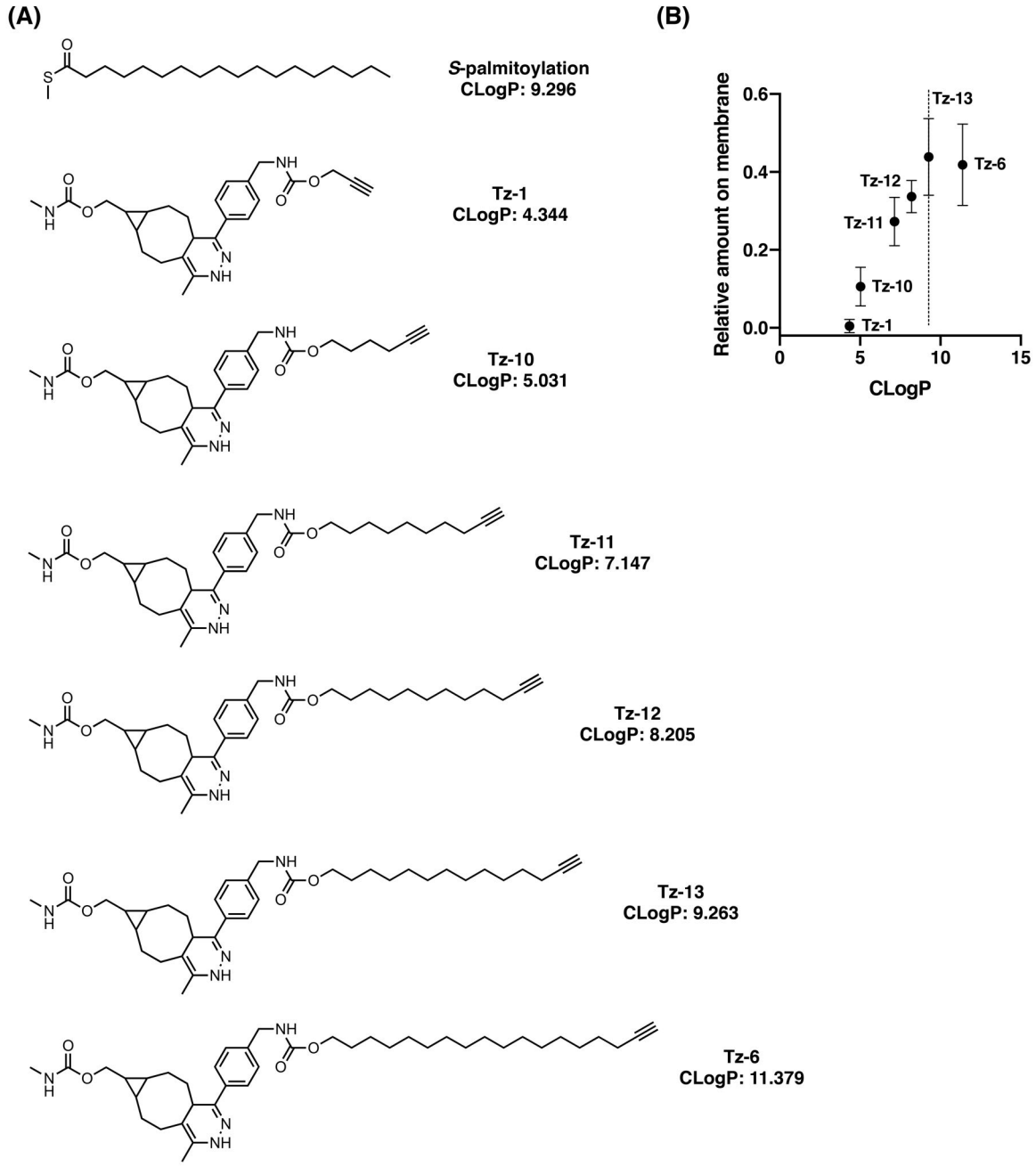


Figure S13. Correlation between CLogP of chemical fatty-acylation mimics and N-Ras membrane affinity. (A) Structures and CLogP values of *S*-palmitoylation and fatty-acylation mimics. CLogP values were calculated by ChemDraw 19. (B) Plot of CLogP values and N-Ras membrane affinities. N-Ras membrane affinities were obtained from Fig. 2B. The dashed line indicates CLogP value of *S*-palmitoylation.

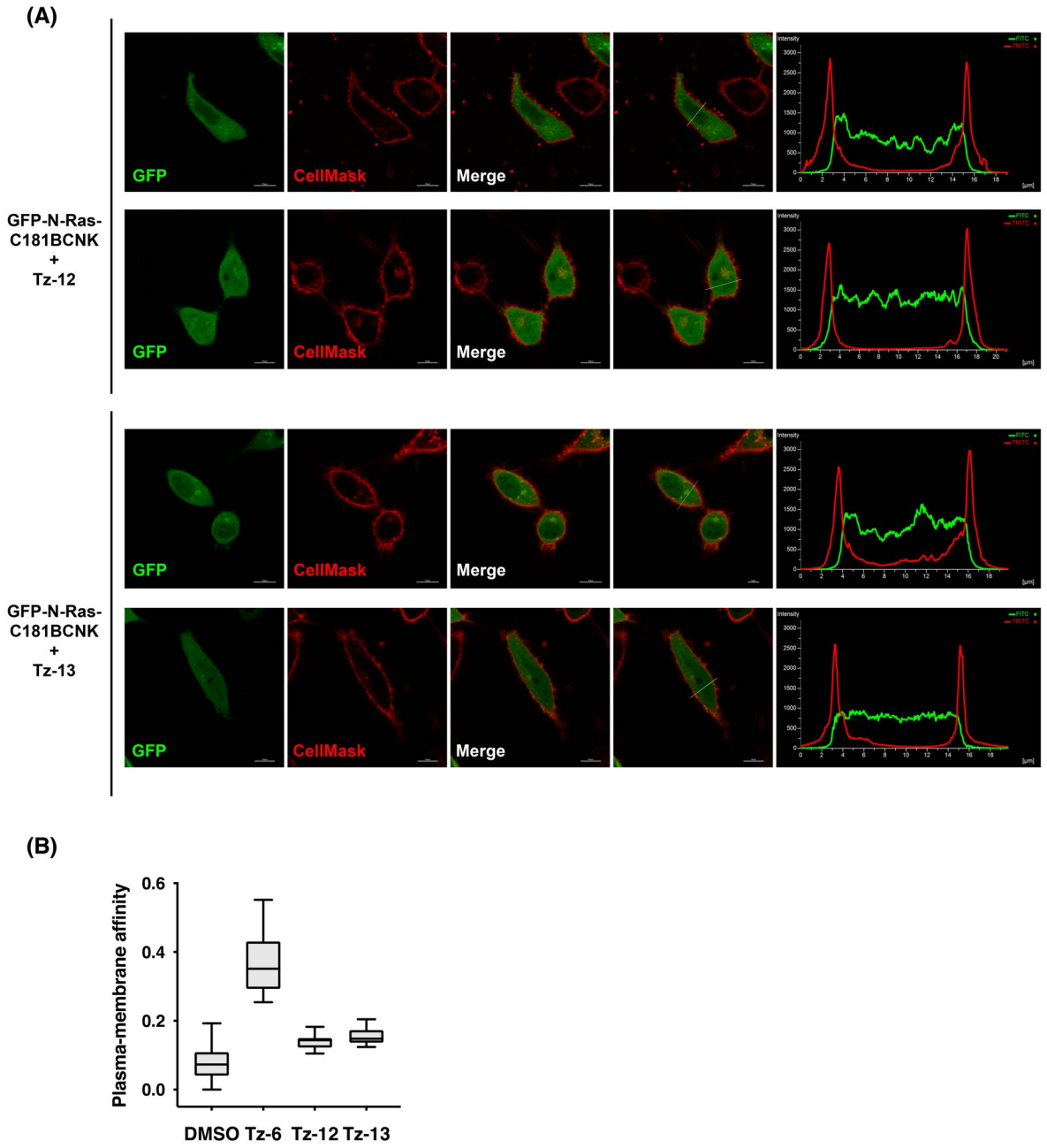


Figure S14. Confocal fluorescence microscopy of Tz-12- and Tz-13-modified GFP-N-Ras. HeLa cells expressing GFP-N-Ras-C181BCNK (50 μM exo-BCNK) were incubated with Tz-12 or Tz-13 (50 μM), stained with CellMask Orange Plasma Membrane Stain, and imaged with confocal fluorescence microscopy. (A) Representative confocal fluorescence images of GFP-N-Ras-C181BCNK labeled with Tz-12 or Tz-13. The fluorescence intensity profiles show colocalization of the GFP and CellMask signals. (B) Calculated plasma-membrane affinity of GFP-N-Ras-C181BCNK. Plasma-membrane affinity was calculated as previously reported[2] for at least 15 cells in each condition. Data are represented as mean ± s.d.. Data on DMSO and Tz-6 were from Figure S9.

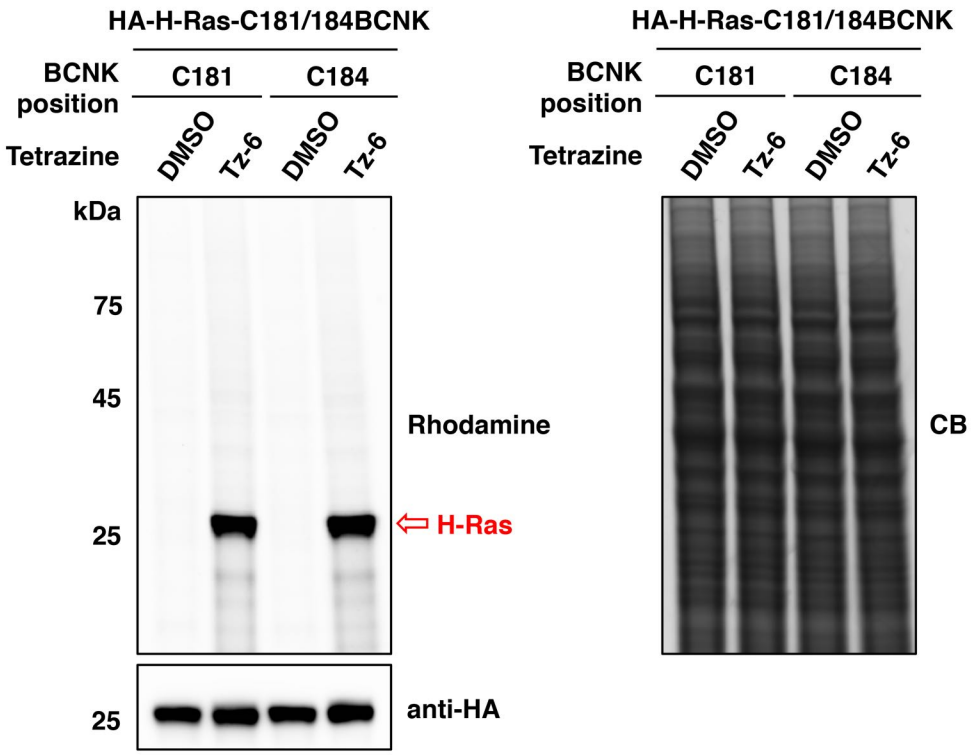


Figure S15. Chemical fatty-acylation of H-Ras with Tz-6. HEK293T cells expressing HA-H-Ras-C181BCNK or HA-H-Ras-C184BCNK were incubated with DMSO or Tz-6 (50 μM) and clicked with azido-rhodamine for in-gel fluorescence analysis.

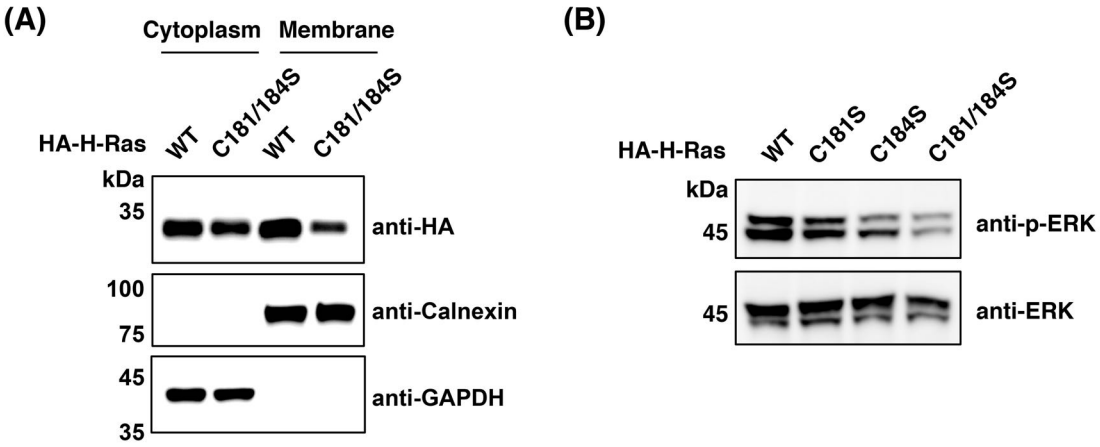
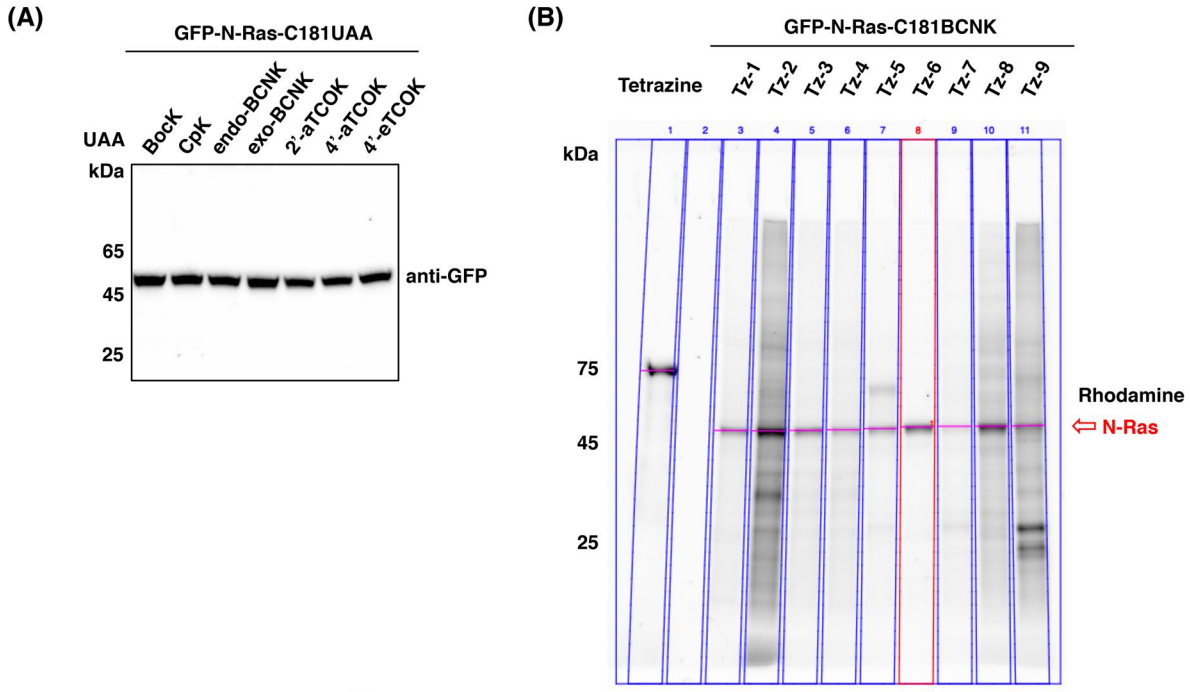


Figure S16. Effects of cysteine 181 and 184 mutation on H-Ras distribution and signaling. (A) HEK293T cells were transfected with HA-H-Ras wild-type or C181/184S mutant and lysed to separate membrane and cytoplasm fractions. GAPDH and calnexin were probed as cytoplasm and membrane controls, respectively. (B) HEK293T cells were transfected with HA-H-Ras wild-type, C181S, C184S, or C181/184S mutant, lysed, and analyzed for phosphorylated ERK.



(C)

	Lane	Relative Front	Adj. Volume (Int)	Volume (Int)	Lane %
Marker	1	0.424731	24724646	27467756	66.81353
Tz-1	3	0.534409	8553898	11719190	38.98777
Tz-2	4	0.535484	20392220	41233012	18.36586
Tz-3	5	0.534409	9891262	13611092	36.31163
Tz-4	6	0.533333	5993952	9264456	24.37134
Tz-5	7	0.531183	7225234	12031810	35.8201
Tz-6	8	0.527957	15201742	17427724	64.59682
Tz-7	9	0.526882	3215984	6955766	21.74832
Tz-8	10	0.526882	16972888	26201036	35.43122
Tz-9	11	0.525806	8634750	19081652	8.390316

Figure S17. Supporting figure for Fig. 2 in main text. (A) Whole western blot of anti-GFP shown in Fig. 2A. (B) Example for quantification of relative N-Ras fluorescence shown in Fig. 2C. The fluorescence intensities of every N-Ras band and corresponding lane were quantified in Image Lab (Biorad). The N-Ras band fluorescence relative to the whole lane was then calculated (Lane %). (C) Quantification data of the in-gel fluorescence shown in (B).

General methods and materials

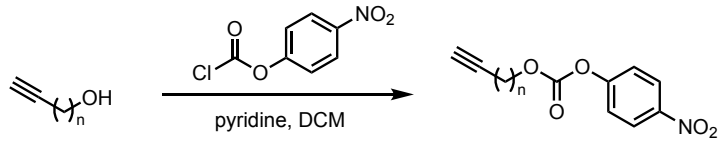
Unless otherwise noted, chemicals and solvents were obtained from Sigma-Aldrich, Acros, or TCI, and were used as received without further purification. Chemical reactions were performed in oven-dried flasks under a N_2 or Ar atmosphere when necessary. Anhydrous dichloromethane (DCM) and tetrahydrofuran (THF) were distilled from calcium hydride and sodium/benzophenone, respectively. TLC was conducted on silica gel plates from Qingdao Haiyang Chemical Co., Ltd, and spots were visualized by illumination with a 254 nm UV light and/or staining with basic $KMnO_4$. Silica gel (230-400 mesh, reagent grade) from Qingdao Haiyang Chemical Co., Ltd was used for flash column chromatography. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ or CD_3OD at room temperature on Bruker Avance NMR Spectrometers operating at 300 MHz, 400 MHz, or 500 MHz for ^1H. Chemical shifts are reported in δ ppm, and J values are reported in Hz. ^1H NMR chemical shifts were reported using tetramethylsilane (TMS, δ = 0.00 ppm) in $CDCl_3$ or CD_3OD (CD_3, δ = 3.31 ppm) as the internal standard. ^{13}C NMR chemical shifts were reported using $CDCl_3$ (δ = 77.00 ppm) or CD_3OD (δ = 49.00 ppm) as the internal standard. High resolution ESI mass spectra were recorded with an ABI QSTAR Elite mass spectrometer.

N$^{\varepsilon}$-Boc-L-Lysine (BocK) was purchased from Adamas. CpK, 2'-aTCOK, exo-BCNK, endo-BCNK, 4'-aTCOK, and 4'-eTCOK were synthesized in the laboratory according to previous reports.[3] The characterization data are in accordance with those in literature.[3] The unnatural amino acids (UAAs) were dissolved at 100 mM concentrations in 0.2 M NaOH with 20% DMSO to be the stock solutions, which were stored at -20 °C. The UAA stock solutions were diluted with complete cell growth media to 50 μM for incubation with cells.

Ampicillin and kanamycin were purchased from Sangon Biotech. Protease inhibitor cocktail was purchased from Roche (cOmplete ULTRA mini Tablets). Phosphatase inhibitor cocktail was purchased from Thermo Fisher Scientific. PfuUltra High-Fidelity DNA Polymerase was obtained from Agilent Technologies; restriction enzymes and dNTPs were obtained from New England Biolabs; oligonucleotide primers and gene fragments were synthesized by Genewiz. Plasmid DNA isolation was carried out with the Plasmid Mini Kit (Omega). Polyethylenimine (PEI) was purchased from Polysciences. Viafect transfection reagent was purchased from Promega. In-gel fluorescence and western blotting were recorded on a Chemidoc MP imaging system (Biorad). Confocal fluorescence imaging was performed with a Nikon A1R confocal fluorescence microscope.

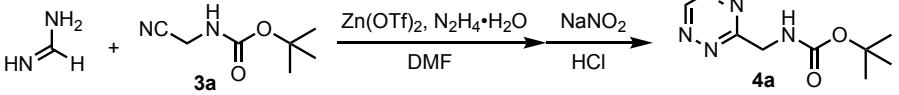
Synthesis of fatty-acyl tetrazines

Scheme S1. Synthesis of fatty-acyl tetrazines.



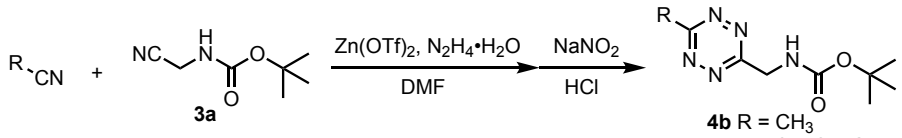
1a n = 1, **1b** n = 4
1c n = 8, **1d** n = 10
1e n = 12, **1f** n = 16

2a n = 1, **2b** n = 4
2c n = 8, **2d** n = 10
2e n = 12, **2f** n = 16



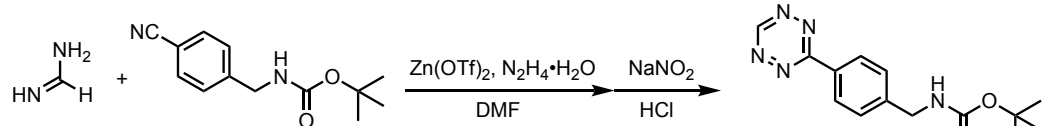
3a

4a



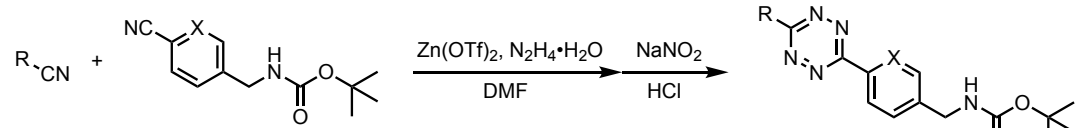
3a

4b R = CH_3
4c R = CH_2CH_2OH



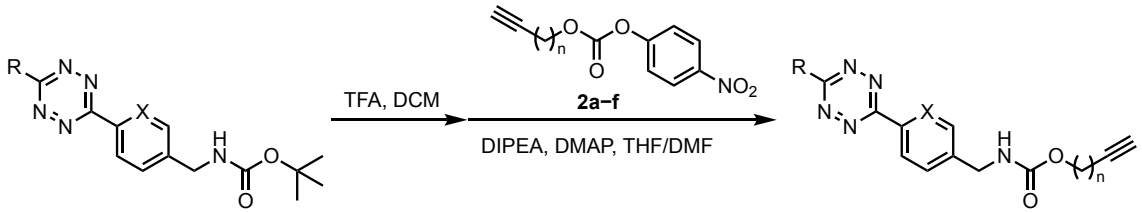
3b

4d



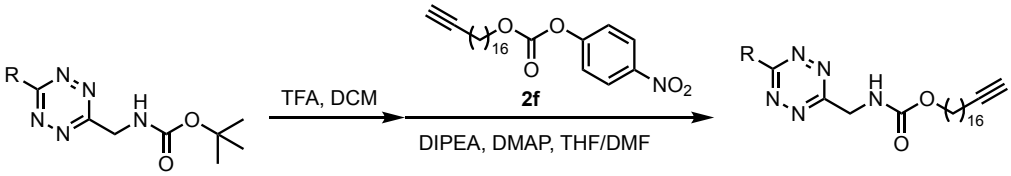
3b X = CH
3c X = N

4e R = CH_3, X = CH
4f R = $CHCH_2CH_2OH$, X = CH
4g R = CH_3, X = N
4h R = $CHCH_2CH_2OH$, X = N



4d–h

Tz-1, R = CH_3, X = CH, n = 1
Tz-5, R = H, X = CH, n = 16
Tz-6, R = CH_3, X = CH, n = 16
Tz-7, R = CH_2CH_2OH, X = CH, n = 16
Tz-8, R = CH_3, X = N, n = 16
Tz-9, R = CH_2CH_2OH, X = N, n = 16
Tz-10, R = CH_3, X = CH, n = 4
Tz-11, R = CH_3, X = CH, n = 8
Tz-12, R = CH_3, X = CH, n = 10
Tz-13, R = CH_3, X = CH, n = 12



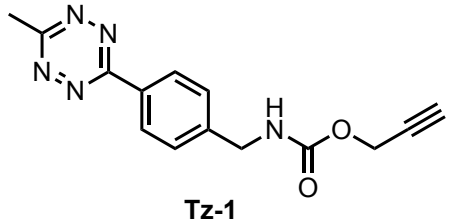
4a–c

Tz-2, R = H
Tz-3, R = CH_3
Tz-4, R = CH_2CH_2OH

Synthesis of 4-nitrophenyl carbonates 2a–f. Alkynyl alcohols **1a–d** were obtained from commercial sources. Tetradec-13-yn-1-ol **1e** and octadec-17-yn-1-ol **1f** were synthesized according to a previous report.[4] For synthesis of 4-nitrophenyl carbonates **2a–f**, to a stirred solution of the alkynyl alcohol **1a–f** (1.0 equiv.) in DCM was added pyridine (1.2 equiv.) and 4-nitrophenyl chloroformate (1.1 equiv.) successively at room temperature. The reaction mixture was stirred at room temperature for 10 h and quenched with 0.1 M HCl solution. The organic layer was separated and the aqueous layer was extracted with ethyl acetate. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting 4-nitrophenyl carbonates **2a–f** (70–88% yields) were used in the next steps without further purification.

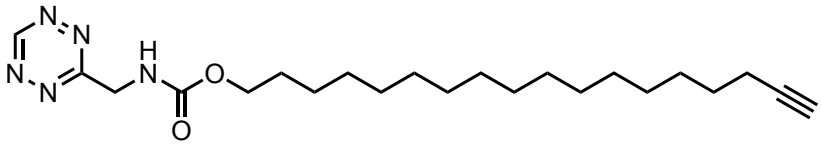
Synthesis of tetrazines 4a–h. The cyano compounds **3a–b** were obtained from Accela ChemBio. Compound **3c** was synthesized according to a previous report.[5] Tetrazines **4a–h** were synthesized according to previous reports.[6] Generally, to a sealed tube were added an equal amount of formamidine acetate (or nitrile) and the cyano compound **3a–c**, zinc triflate, DMF, and hydrazine hydrate. The mixture was heated at 70 °C for 3 h. Upon cooled to room temperature, the mixture was transferred into a beaker. Sodium nitrite in water was slowly added to the mixture, followed by slow addition of 1 M HCl until the pH was about 3. The resulting mixture was extracted with CH_2Cl_2 and the organic solution was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue purified by silica gel column chromatography to provide the tetrazines **4a–h**. The NMR spectra are consistent with those previously reported.

Synthesis of fatty-acyl tetrazines Tz-1–13. Generally, to a stirred solution of tetrazine **4a–h** (0.3 mmol) in DCM (2 mL) was added 1 mL of trifluoroacetic acid (TFA) at 0 °C. The reaction mixture was stirred at ambient temperature for 4 h. The solvent was then removed under reduced pressure and azeotropes with toluene three times to afford the Boc-deprotected amine as a red solid. The crude product was re-dissolved in THF (2 mL) and DMF (2 mL). To this solution was successively added diisopropylethylamine (DIPEA, 0.6 mmol), DMAP (0.06 mmol), and 4-nitrophenyl carbonate **2a–f** (0.36 mmol). The reaction mixture was stirred at room temperature for 10 h and quenched with 0.1 M HCl solution. The mixture was then extracted with ethyl acetate three times. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography to provide the fatty-acyl tetrazines **Tz-1–13**.



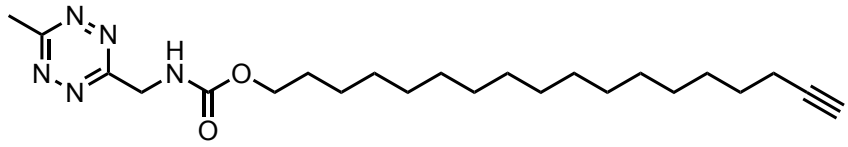
Tz-1

*Fatty-acyl tetrazine **Tz-1**.* Synthesized from compounds **4e** and **2a** according to the general procedure in 67% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.55 (d, *J* = 8.4 Hz, 2H), 7.51 (d, *J* = 8.4 Hz, 2H), 5.37 (s, 1H), 4.74 (d, *J* = 2.5 Hz, 2H), 4.51 (d, *J* = 6.1 Hz, 2H), 3.10 (s, 3H), 2.51 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.27, 163.80, 155.57, 142.95, 131.05, 128.26, 128.12, 78.04, 74.82, 52.79, 44.85, 21.15. HRMS calcd for $C_{14}H_{14}N_5O_2$ [M + H] 284.1147, found 284.1132.



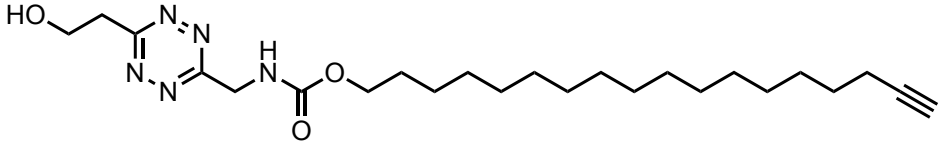
Tz-2

*Fatty-acyl tetrazine **Tz-2**.* Synthesized from compounds **4a** and **2f** according to the general procedure in 59% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.30 (s, 1H), 5.70 (s, 1H), 5.07 (d, *J* = 6.0 Hz, 2H), 4.10 (t, *J* = 6.7 Hz, 2H), 2.18 (td, *J* = 7.0, 2.7 Hz, 2H), 1.94 (t, *J* = 2.6 Hz, 1H), 1.70 – 1.58 (m, 2H), 1.56 – 1.47 (m, 2H), 1.32 – 1.22 (m, 24H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.16, 158.73, 129.87, 84.81, 68.00, 65.87, 65.38, 29.68, 29.64, 29.62, 29.59, 29.55, 29.51, 29.48, 29.26, 29.24, 29.09, 28.91, 28.75, 28.49, 25.80, 18.38. HRMS calcd for $C_{22}H_{38}N_5O_2$ [M + H] 404.3026, found 404.3024.



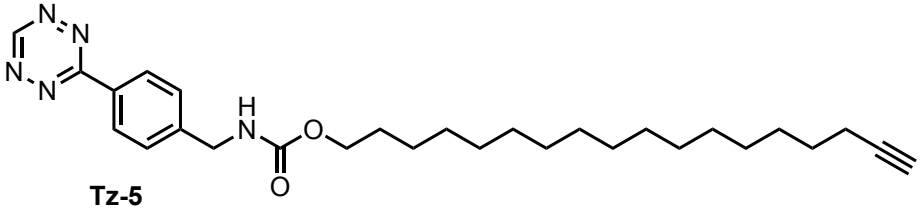
Tz-3

*Fatty-acyl tetrazine **Tz-3**.* Synthesized from compounds **4b** and **2f** according to the general procedure in 47% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.67 (s, 1H), 5.01 (d, *J* = 5.9 Hz, 2H), 4.10 (t, *J* = 6.7 Hz, 2H), 3.08 (s, 3H), 2.18 (td, *J* = 7.2, 2.7 Hz, 2H), 1.93 (t, *J* = 2.6 Hz, 1H), 1.68 – 1.56 (m, 2H), 1.56 – 1.49 (m, 2H), 1.41 – 1.25 (m, 24H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.45, 166.19, 156.75, 84.80, 68.00, 65.76, 43.66, 29.62, 29.58, 29.55, 29.50, 29.48, 29.24, 29.08, 28.91, 28.74, 28.47, 28.31, 25.78, 21.15, 18.37. HRMS calcd for $C_{23}H_{40}N_5O_2$ [M + H] 418.3182, found 418.3182.



Tz-4

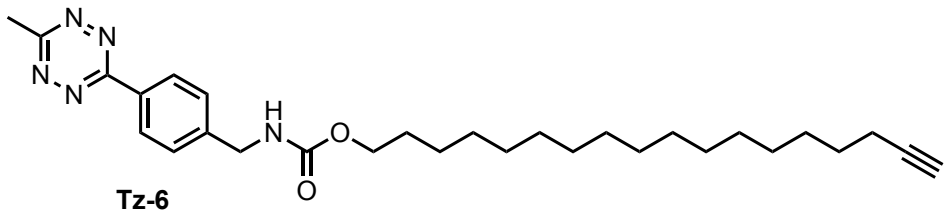
*Fatty-acyl tetrazine **Tz-4**.* Synthesized from compounds **4c** and **2f** according to the general procedure in 39% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.68 (s, 1H), 5.03 (d, *J* = 6.1 Hz, 2H), 4.31 – 4.24 (m, 2H), 4.10 (t, *J* = 6.7 Hz, 2H), 3.61 (t, *J* = 5.8 Hz, 2H), 2.38 (s, 1H), 2.18 (td, *J* = 7.1, 2.6 Hz, 2H), 1.94 (t, *J* = 2.7 Hz, 1H), 1.64 – 1.59 (m, 2H), 1.56 – 1.48 (m, 2H), 1.41 – 1.25 (m, 24H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.46, 166.77, 156.79, 84.83, 68.01, 65.83, 59.90, 43.69, 37.51, 29.67, 29.64, 29.59, 29.56, 29.51, 29.48, 29.34, 29.25, 29.09, 28.90, 28.87, 28.74, 28.47, 25.78, 18.37. HRMS calcd for $C_{24}H_{42}N_5O_3$ [M + H] 448.3288, found 448.3282.



Tz-5

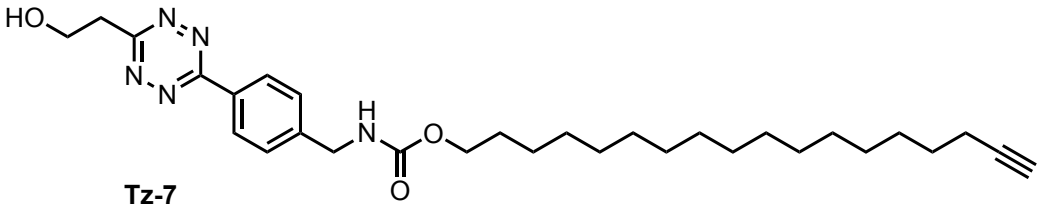
*Fatty-acyl tetrazine **Tz-5**.* Synthesized from compounds **4d** and **2f** according to the general procedure in 56% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.20 (s, 1H), 8.60 (d, *J* = 8.2 Hz, 2H),

7.51 (t, *J* = 18.4 Hz, 2H), 5.08 (s, 1H), 4.50 (d, *J* = 5.7 Hz, 2H), 4.12 (t, *J* = 6.7 Hz, 2H), 2.18 (td, *J* = 7.1, 2.5 Hz, 2H), 1.93 (t, *J* = 2.6 Hz, 1H), 1.60 – 1.66 (m, 2H), 1.54 – 1.48 (m, 2H), 1.40 – 1.24 (m, 24H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.32, 157.79, 156.86, 144.35, 130.81, 128.68, 128.22, 84.81, 68.00, 65.53, 44.79, 29.65, 29.63, 29.59, 29.57, 29.54, 29.49, 29.30, 29.28, 29.23, 29.10, 29.00, 28.77, 28.52, 25.87, 18.40. HRMS calcd for $C_{28}H_{42}N_5O_2$ [M + H] 480.3339, found 480.3356.



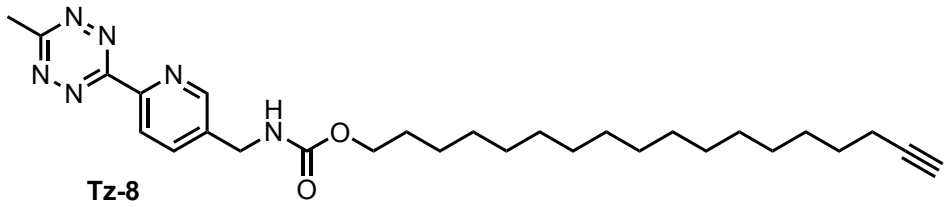
Tz-6

*Fatty-acyl tetrazine **Tz-6**.* Synthesized from compounds **4e** and **2f** according to the general procedure in 28% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 (d, *J* = 8.3 Hz, 2H), 7.51 (d, *J* = 8.0 Hz, 2H), 5.11 (s, 1H), 4.50 (d, *J* = 5.1 Hz, 2H), 4.11 (t, *J* = 6.7 Hz, 2H), 3.10 (s, 3H), 2.18 (td, *J* = 7.0, 2.7 Hz, 2H), 1.94 (t, *J* = 2.6 Hz, 1H), 1.71 – 1.54 (m, 2H), 1.54 – 1.43 (m, 2H), 1.39 – 1.25 (m, 24H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.24, 163.86, 156.85, 143.58, 130.89, 128.22, 128.10, 84.82, 68.01, 65.45, 44.70, 29.65, 29.63, 29.59, 29.56, 29.53, 29.49, 29.34, 29.27, 29.10, 29.00, 28.87, 28.75, 28.47, 25.84, 21.15, 18.38. HRMS calcd for $C_{29}H_{44}N_5O_2$ [M + H] 494.3495, found 494.3490.



Tz-7

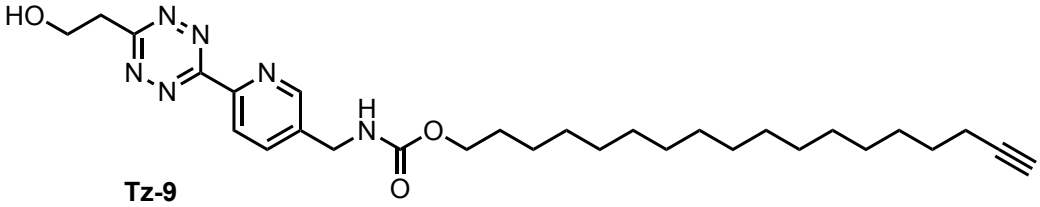
*Fatty-acyl tetrazine **Tz-7**.* Synthesized from compounds **4f** and **2f** according to the general procedure in 10% yield. ^{1}H NMR (500 MHz, d^6-DMSO) δ 8.44 (d, *J* = 8.1 Hz, 2H), 7.77 (t, *J* = 5.8 Hz, 1H), 7.53 (d, *J* = 8.2 Hz, 2H), 4.81 (t, *J* = 5.7 Hz, 1H), 4.30 (d, *J* = 5.8 Hz, 2H), 4.03 – 3.96 (m, 4H), 3.42 (t, *J* = 6.3 Hz, 2H), 2.71 (t, *J* = 2.4 Hz, 1H), 2.13 (td, *J* = 6.9, 2.5 Hz, 2H), 1.58 – 1.51 (m, 2H), 1.44 – 1.38 (m, 2H), 1.35 – 1.18 (m, 24H). ^{13}C NMR (126 MHz, d^6-DMSO) δ 168.65, 163.98, 157.22, 145.30, 130.15, 128.39, 127.98, 85.05, 71.54, 64.45, 59.81, 44.06, 38.64, 29.58, 29.52, 29.50, 29.48, 29.47, 29.41, 29.24, 29.20, 29.18, 29.07, 28.95, 28.60, 28.43, 25.86, 18.14. HRMS calcd for $C_{30}H_{46}N_5O_3$ [M + H] 524.3601, found 524.3594.



Tz-8

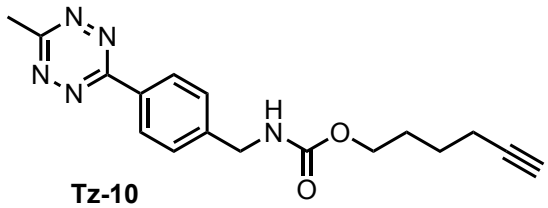
*Fatty-acyl tetrazine **Tz-8**.* Synthesized from compounds **4g** and **2f** according to the general procedure in 25% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.59 (d, *J* = 7.9 Hz, 1H), 7.94 (d, *J* = 7.3 Hz, 1H), 5.36 (s, 1H), 4.52 (d, *J* = 4.5 Hz, 2H), 4.08 (t, *J* = 6.8 Hz, 2H), 3.14 (s, 3H), 2.15 (dt, *J* = 7.1, 2.6 Hz, 2H), 1.91 (t, *J* = 2.6 Hz, 1H), 1.63 – 1.57 (m, 2H), 1.53 – 1.47 (m, 2H), 1.39 – 1.19 (m, 24H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.07, 163.34, 156.87, 149.87, 149.22, 137.71, 136.64, 123.72, 84.76, 67.98, 65.61, 42.32, 29.63, 29.60, 29.58, 29.54, 29.52,

29.49, 29.44, 29.22, 29.05, 28.95, 28.71, 28.45, 25.79, 21.28, 18.34. HRMS calcd for $C_{28}H_{43}N_6O_2$ [M + H] 495.3447, found 495.3441.



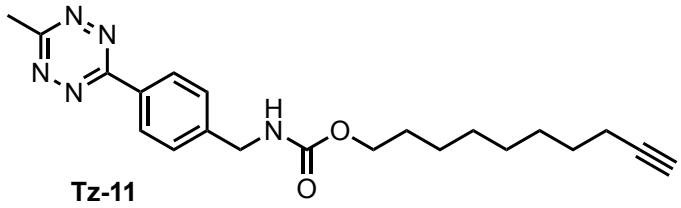
Tz-9

*Fatty-acyl tetrazine **Tz-9**.* Synthesized from compounds **4h** and **2f** according to the general procedure in 16% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.63 (d, *J* = 8.1 Hz, 1H), 7.97 (d, *J* = 7.2 Hz, 1H), 5.28 (s, 1H), 4.54 (d, *J* = 5.3 Hz, 2H), 4.31 (t, *J* = 5.8 Hz, 2H), 4.11 (t, *J* = 6.8 Hz, 2H), 3.68 (t, *J* = 5.7 Hz, 2H), 2.17 (dt, *J* = 7.1, 2.6 Hz, 2H), 2.03 (br, 1H), 1.93 (t, *J* = 2.6 Hz, 1H), 1.59 – 1.66 (m, 2H), 1.55 – 1.48 (m, 2H), 1.24 – 1.39 (m, 24H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.15, 163.72, 156.89, 149.87, 149.07, 137.89, 136.83, 123.93, 84.82, 68.01, 65.71, 60.01, 42.41, 37.68, 29.68, 29.65, 29.63, 29.59, 29.57, 29.53, 29.49, 29.34, 29.26, 29.09, 28.98, 28.75, 28.49, 25.83, 18.39. HRMS calcd for $C_{29}H_{45}N_6O_3$ [M + H] 525.3553, found 525.3550.



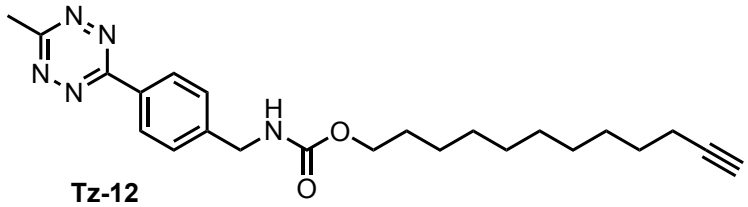
Tz-10

*Fatty-acyl tetrazine **Tz-10**.* Synthesized from compounds **4e** and **2b** according to the general procedure in 27% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.55 (d, *J* = 7.9 Hz, 2H), 7.50 (d, *J* = 8.0 Hz, 2H), 5.17 (s, 1H), 4.48 (d, *J* = 6.2 Hz, 2H), 4.14 (t, *J* = 6.4 Hz, 2H), 3.09 (s, 3H), 2.30 – 2.17 (m, 2H), 1.96 (s, 1H), 1.81 – 1.71 (m, 2H), 1.67 – 1.53 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.36, 163.96, 156.86, 143.62, 131.04, 128.35, 128.24, 84.03, 68.83, 64.83, 44.84, 28.17, 24.99, 21.28, 18.21. HRMS calcd for $C_{17}H_{20}N_5O_2$ [M + H] 326.1617, found 326.1609.



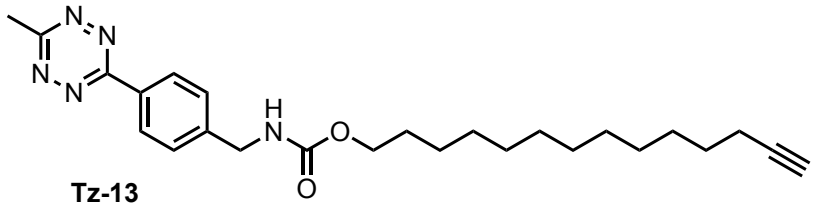
Tz-11

*Fatty-acyl tetrazine **Tz-11**.* Synthesized from compounds **4e** and **2c** according to the general procedure in 29% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.55 (d, *J* = 8.4 Hz, 2H), 7.51 (d, *J* = 8.1 Hz, 2H), 5.12 (s, 1H), 4.48 (d, *J* = 6.2 Hz, 2H), 4.11 (t, *J* = 6.7 Hz, 2H), 3.09 (s, 3H), 2.17 (td, *J* = 6.9, 2.7 Hz, 2H), 1.93 (t, *J* = 2.6 Hz, 1H), 1.65 – 1.61 (m, 2H), 1.56 – 1.46 (m, 2H), 1.41 – 1.27 (m, 8H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.37, 163.99, 156.98, 143.70, 131.03, 128.35, 128.24, 84.83, 68.23, 65.51, 44.84, 29.23, 29.22, 29.09, 28.74, 28.53, 25.91, 21.29, 18.48. HRMS calcd for $C_{21}H_{28}N_5O_2$ [M + H] 382.2243, found 382.2237.



Tz-12

*Fatty-acyl tetrazine **Tz-12**.* Synthesized from compounds **4e** and **2d** according to the general procedure in 26% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.52 (d, *J* = 8.4 Hz, 2H), 7.49 (d, *J* = 8.1 Hz, 2H), 5.21 (s, 1H), 4.47 (d, *J* = 6.2 Hz, 2H), 4.09 (t, *J* = 6.7 Hz, 2H), 3.08 (s, 3H), 2.16 (td, *J* = 7.0, 2.6 Hz, 2H), 1.92 (t, *J* = 2.6 Hz, 1H), 1.67 – 1.57 (m, 2H), 1.54 – 1.44 (m, 2H), 1.37 – 1.21 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.35, 163.96, 157.02, 143.75, 130.98, 128.31, 128.21, 84.90, 68.21, 65.53, 44.81, 29.57, 29.50, 29.36, 29.17, 29.13, 28.84, 28.57, 25.95, 21.27, 18.50. HRMS calcd for $C_{23}H_{31}N_5O_2Na$ [M + Na] 432.2375, found 432.2371.



Tz-13

*Fatty-acyl tetrazine **Tz-13**.* Synthesized from compounds **4e** and **2e** according to the general procedure in 19% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.55 (d, *J* = 8.2 Hz, 2H), 7.50 (d, *J* = 8.0 Hz, 2H), 5.14 (t, *J* = 6.3 Hz, 1H), 4.48 (d, *J* = 6.2 Hz, 2H), 4.10 (t, *J* = 6.7 Hz, 2H), 3.09 (s, 3H), 2.17 (td, *J* = 7.0, 2.7 Hz, 2H), 1.93 (t, *J* = 2.7 Hz, 1H), 1.66 – 1.59 (m, 2H), 1.55 – 1.46 (m, 2H), 1.39 – 1.23 (m, 16H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.36, 163.97, 156.99, 143.73, 131.01, 128.33, 128.23, 84.94, 68.17, 65.57, 44.83, 29.68, 29.65, 29.60, 29.40, 29.22, 29.21, 29.15, 28.87, 28.61, 25.97, 21.29, 18.51. HRMS calcd for $C_{25}H_{36}N_5O_2$ [M + H] 438.2869, found 438.2864.

Plasmids and cloning

The plasmid pCMV-RF1-E55D-EF1α-MmPylRS-AF was generated in the lab by inserting eRF1-E55D sequence under the CMV promoter into the plasmid pEF1α-MmPylRS-AF (Y306A and Y384F double mutant of wild-type PylRS).[1, 7] The plasmid pEGFP-N-Ras was generated by cloning N-Ras gene into pEGFP-C1 vector (Clontech) through restriction enzyme sites SalI and KpnI. The plasmid pCMV-HA-H-Ras was previously generated in the lab.[8] TAG codons were introduced into the pEGFP-N-Ras plasmid at C181 position and pCMV-HA-H-Ras plasmid at C181 or C184 position by site-directed mutagenesis. Site-directed mutagenesis was performed with QuikChange II Site-Directed Mutagenesis Kit (Agilent) with primers designed by Agilent Primer Design Program.

Cell culture and transfection

HEK293T and HeLa cells were grown in DMEM (Corning) supplemented with 10% FBS (Corning) at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. HEK293T cells were transfected using PEI (Polysciences) with a 2.5:1 ratio of transfection reagent/DNA in Opti-MEM media (Thermo Fisher) at 70% confluence. HeLa cells were transfected using Viafect (Promega) with a 3:1 ratio of transfection reagent/DNA according to the manufacturer's protocol in cell growth media at 70% confluence.

Chemical fatty-acylation of proteins in cells

HEK293T or HeLa cells were seeded on 12-well plates (Corning) and cultured overnight. The next day cells were co-transfected with the plasmid of interest containing a TAG codon (0.75 μg per well) and the MmPylRS/Pyl-tRNA plasmid (0.25 μg per well) using PEI (2.5 μL per well) or Viafect (3 μL per well) in Opti-MEM media or complete cell growth media containing the corresponding UAA. After 16 h incubation, cell media was changed to fresh Opti-MEM media without UAA. After another 6 h culture at 37 °C/5% CO_2, cells were labeled with fatty-acyl tetrazines (25-50 μM) in DMEM supplemented with 10% charcoal/dextran treated FBS (Thermo Fisher) for 2 h at 37 °C.

CuAAC click labeling and in-gel fluorescence

Aforementioned HEK293T Cells were harvested, washed with cold PBS, and lysed in pre-chilled Brij lysis buffer (1% Brij 97, 150 mM NaCl, 50 mM triethanolamine pH 7.4, Roche EDTA-free protease inhibitor cocktail) with vigorous vortexing. The resulting cell lysate was centrifuged at 12,000 rcf for 20 min at 4 °C to remove cellular debris. Protein concentrations were determined by the BCA assay (Pierce). Cell lysates were normalized with Brij lysis buffer to a total of 50 μL with an equal protein concentration.

Cell lysates (100 μg) were diluted with SDS buffer (1% SDS, 150 mM NaCl, 50 mM HEPES pH 7.4) to 89 μL, and then reacted with 11 μL freshly prepared click chemistry reaction cocktail containing azido-rhodamine (2 μL, 10 mM stock solution in DMSO), tris-(2-carboxyethyl)phosphine hydrochloride (TCEP; 2 μL, 50 mM freshly prepared stock solution in deionized water), tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA; 5 μL, 10 mM stock solution in DMSO/t-butanol), and $CuSO_4 \cdot 5H_2O$ (2 μL, 50 mM freshly prepared stock solution in deionized water) for 2 h at room temperature. The click reactions were terminated by addition of ice-cold methanol (1 mL). The mixtures were placed at −20 °C overnight and then centrifuged at 20,000 rcf for 20 min at 4 °C to precipitate the proteins. The supernatants were discarded and protein pellets were air-dried for 2 h. The resulting protein pellets were resuspended with 70 μL of SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM triethanolamine pH 7.4), diluted with 25 μL 4X SDS-loading buffer (40% glycerol, 200 mM Tris-HCl pH 6.8, 8% SDS, 0.4% bromophenol blue) and 5 μL 2-mercaptoethanol. The resulting samples were heated for 5 min at 95 °C before loaded onto 4−20% Bis-Tris gels (Genscript) for SDS-PAGE separation. Generally, 20 μg of protein per gel lane is sufficient for in-gel fluorescence visualization. For in-gel fluorescence, gels were scanned on a Bio-Rad ChemiDoc MP Imager with the rhodamine filter. After in-gel fluorescence scanning, gels were stained with Coomassie staining reagent. Another gel was loaded with identical samples as in the fluorescence gel and subjected for Western blot analysis.

Subcellular fractionation

HEK293T cells were seeded on 60 mm dishes (Corning) and cultured overnight. The next day cells were co-transfected with the plasmid of interest containing a TAG codon and the MmPylRS/Pyl-tRNA plasmid using PEI in Opti-MEM media containing the corresponding UAA. After 16 h incubation, cell media was changed to fresh Opti-MEM media without UAA. After another 6 h culture at 37 °C/5% CO_2, cells were labeled with indicated fatty-acyl tetrazines (25-50 μM) in DMEM supplemented with 10% charcoal/dextran treated FBS

(Thermo Fisher) for 4 h at 37 °C. Cells were harvested, washed with cold PBS, and pelleted with centrifugation at 1,000 rcf for 3 min.

Cell pellets were permeabilized with 500 µL ice-cold digitonin extraction buffer (5 mM digitonin, 5 mM EDTA, 10 mM PIPES, pH 6.8, 1 mM PMSF), and incubated on ice with gentle agitation on a platform mixer for 10 min. The extraction mixture was centrifuged at 480 rcf and 4 °C for 10 min. The supernatant (i.e., the cytosolic fraction) was transferred to a clean microcentrifuge tube, and stored at -80 °C. The digitonin-insoluble pellets were resuspended with 500 µL ice-cold Triton X-100 extraction buffer (0.5% Triton X-100, 3 mM EDTA, 10 mM PIPES, pH 7.4, 1 mM PMSF), and incubated on ice with gentle agitation on a platform mixer for 30 min. The extraction mixture was centrifuged at 5,000 rcf and 4 °C for 10 min. The supernatant (i.e., the membrane/organelle fraction) was transferred to a clean microcentrifuge tube, and stored at -80 °C. The Triton-insoluble pellets were dissolved into 100 µL of SDS lysis buffer. The protein concentration of each fraction was determined by the BCA assay (Pierce). These subcellular fractions were normalized by protein concentrations, and diluted to a concentration of 0.7 µg/µL with 4X reducing SDS-loading buffer. The resulting samples were heated for 5 min at 95 °C before loaded onto 4−20% Bis-Tris gels (Genscript) for SDS-PAGE separation and Western blot analysis.

Protein phosphorylation

HEK293T cells were seeded on 12-well plates (Corning) and cultured overnight. The next day cells were transfected with plasmids of interesting in the presence of UAA for 16 h, and starved in Opti-MEM media without UAA. After another 6 h culture at 37 °C/5% CO_2, cells were labeled with indicated fatty-acyl tetrazines (25-50 µM) in DMEM containing 10% charcoal/dextran treated FBS (Thermo Fisher) for 4 h 37 °C. Cells were harvested and washed with PBS three times. Cell pellets were lysed in SDS lysis buffer (4% SDS, 150 mM NaCl, 50 mM triethanolamine pH 7.4, phosphatase inhibitor cocktail) with vigorous vortexing. The resulting cell lysates were centrifuged at 12,000 rcf for 20 min at room temperature to remove cellular debris. Protein concentrations were determined by the BCA assay (Pierce). Cell lysates were normalized by protein concentrations, and diluted to a concentration of 2 µg/µL with 4X reducing SDS-loading buffer. The resulting samples were heated for 5 min at 95 °C before loaded onto 4−20% Bis-Tris gels (Genscript) for SDS-PAGE separation and Western blot analysis.

Western blot

Gels were transferred to nitrocellulose membranes using Bio-Rad Trans-Blot Semi-Dry Cell (20 V, 40 min), which were blocked with PBST (0.05% Tween-20 in PBS) containing 5% nonfat milk for 1 h at room temperature. The membranes were then incubated with appropriate antibodies overnight at 4 °C. Membranes were washed with PBST three times, developed using Bio-Rad Clarity Western ECL substrate, and imaged with a Bio-Rad ChemiDoc MP Imager.

Anti-GFP-HRP (sc-9996-HRP, Santa Cruz, 1:1000 dilution), anti-HA-HRP (3F10, Roche, 1:1000 dilution), and anti-GAPDH-HRP (HRP-60004, Proteintech, 1:2500 dilution) were used for anti-GFP, anti-HA, and anti-GAPDH blots, respectively. Calnexin, H3, N-Ras, ERK, and p-ERK (phospho-p44/42 MAPK Thr202/Tyr204) protein levels were immunoblotted by incubating the blots with rabbit anti-calnexin (10427-2-AP, Proteintech, 1:1000 dilution), rabbit anti-H3 (17168-1-AP, Proteintech, 1:2000 dilution), rabbit anti-N-Ras (10724-1-AP,

Proteintech, 1:1000 dilution), rabbit anti-ERK (4695T, CST, 1:1000 dilution), and rabbit anti-p-ERK (4370S, CST, 1:2000 dilution) antibodies, respectively. Goat anti-rabbit-HRP secondary antibody (Jackson Immunoresearch Laboratories, 1:10000 dilution) was used for rabbit primary antibodies.

For quantification of Western blots, the intensity of protein band of interest relative to that of the corresponding loading control was calculated and quantified with ImageLab software (Bio-Rad).

Live cell imaging

HeLa cells were seeded on 12-well plates and cultured overnight. The next day cells were transfected with the plasmids of GFP-N-Ras-C181TAG (0.75 μg per well) and eRF1-E55D-MmPylRS-AF (0.25 μg per well) using 3 μL Viafect in complete cell growth media containing exo-BCNK. After 16 h incubation, cell media were changed into Opti-MEM media without UAA. After another 6 h culture at 37 °C/5% CO_2, cells were labeled with Tz-6 (50 μM) in DMEM containing 10% charcoal/dextran treated FBS (Thermo Fisher) for 4 h 37 °C and washed with PBS three times. Finally, cells were stained with CellMask Orange Plasma Membrane Stain (Thermo Fisher) for 5 min at 37 °C, and then immediately imaged in FluoroBrite DMEM (Thermo Fisher) with confocal microscopy. GFP was excited at 488 nm and CellMask at 543 nm. Emission was scanned at 500–550 nm and 580–650 nm for GFP and CellMask, respectively.

In order to quantitatively determine the plasma-membrane association of GFP-N-Ras-C181BCNK, a previously reported method[2] was used for image analysis. Briefly, a cytosolic localization model was constructed from the fluorescence intensity values in reference cells expressing cytosol localized GFP for cytoplasm signals and stained with CellMask stain as a plasma-membrane marker. The fluorescence distributions of Tz-6 labeled GFP-N-RasC181-BCNK are deconvoluted into the plasma-membrane and the cytoplasmic fractions as previously described[2] to evaluate the plasma-membrane partitioning and affinity of GFP-N-RasC181-BCNK. The overall plasma-membrane affinity was calculated in Fiji (NIH) using the “peripheral” macro. Computed data were square root transformed and tested by unpaired t test in GraphPad Prism.

Quantification and statistical analysis

Generally, data were presented as mean ± standard deviation determined from biological replicates. The method for determining error bars and significance is indicated in the corresponding figure legends with biological replicate number indicated. Statistical analysis was performed with GraphPad Prism.

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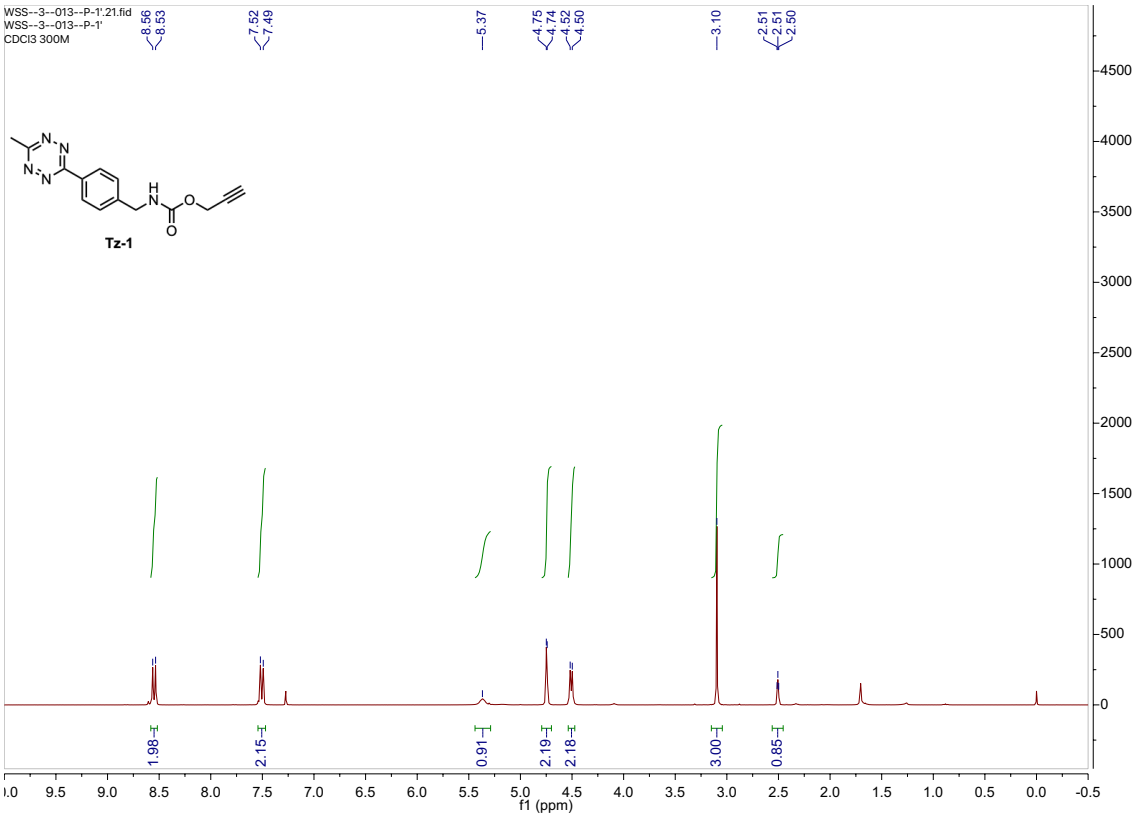
NMR spectra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4.50
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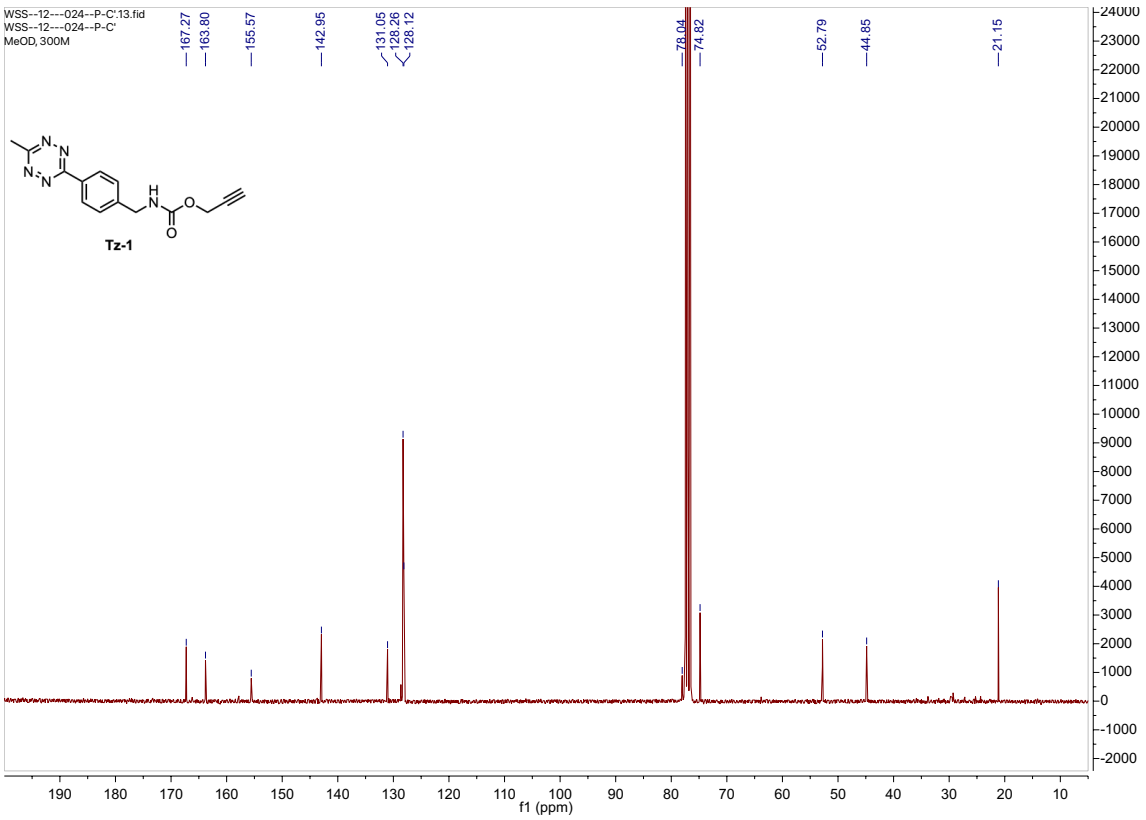
f1 (ppm)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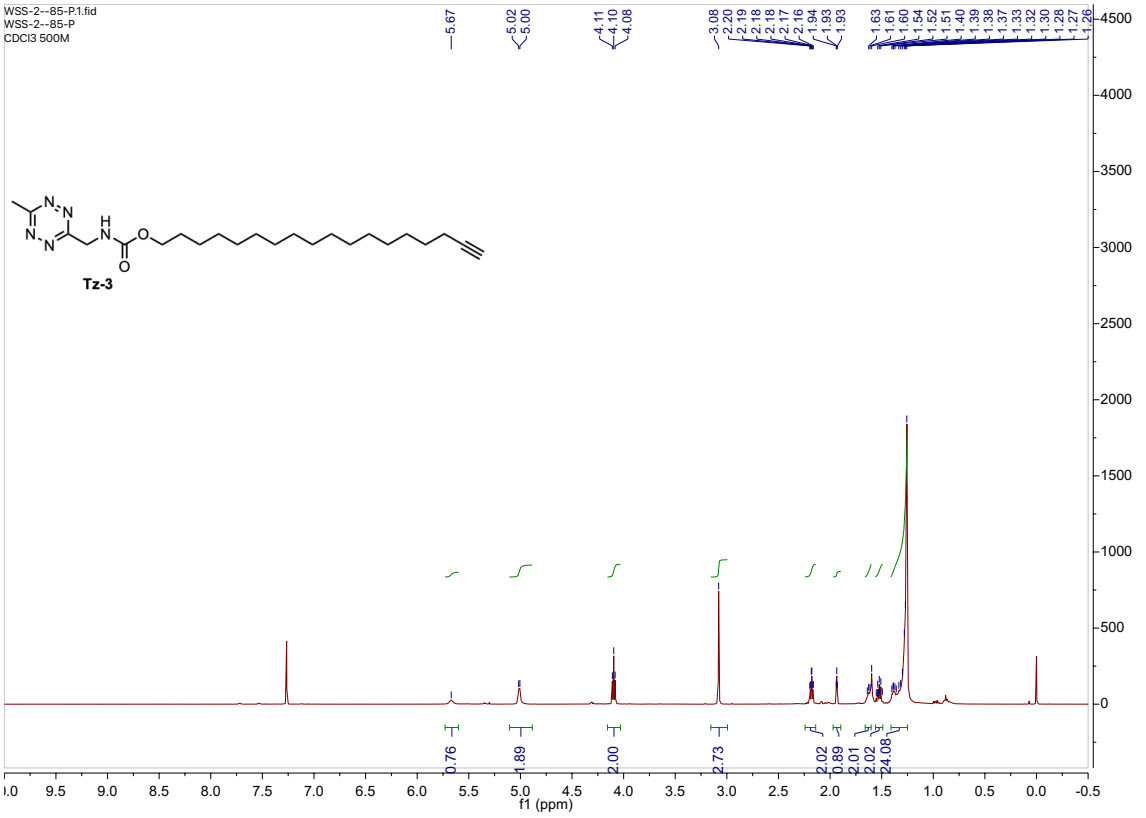
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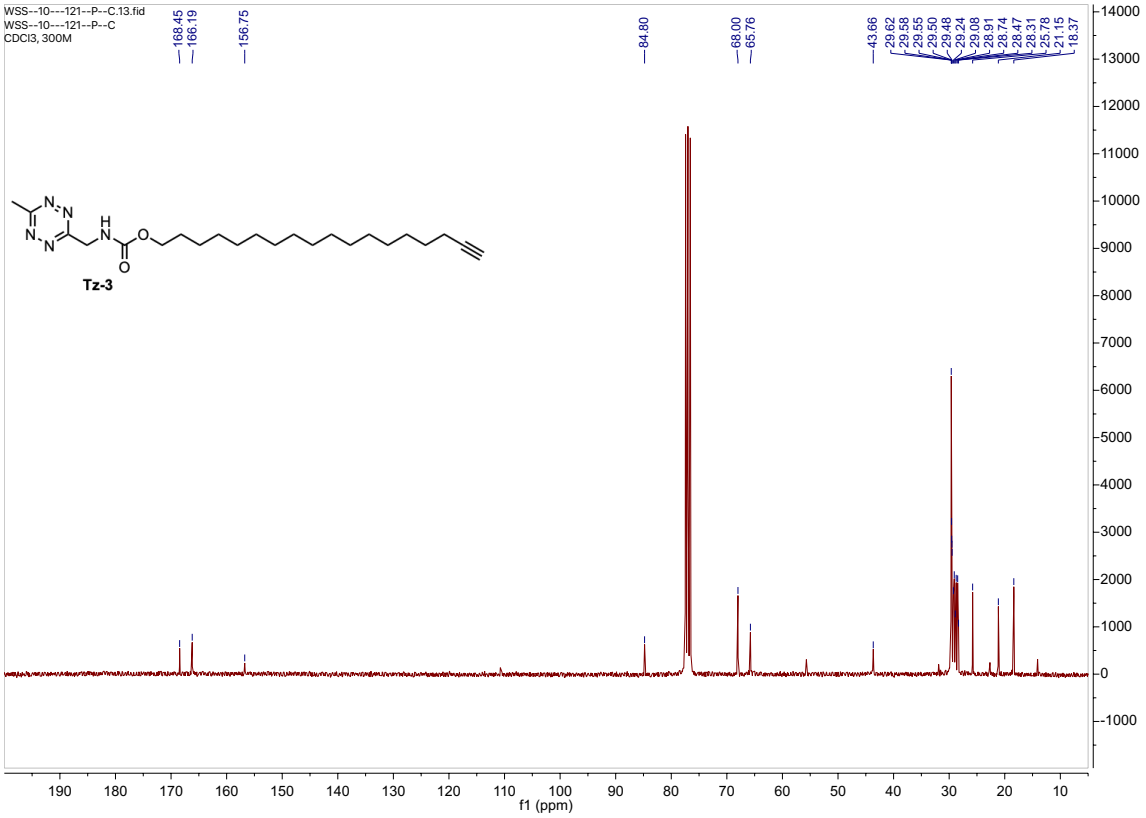
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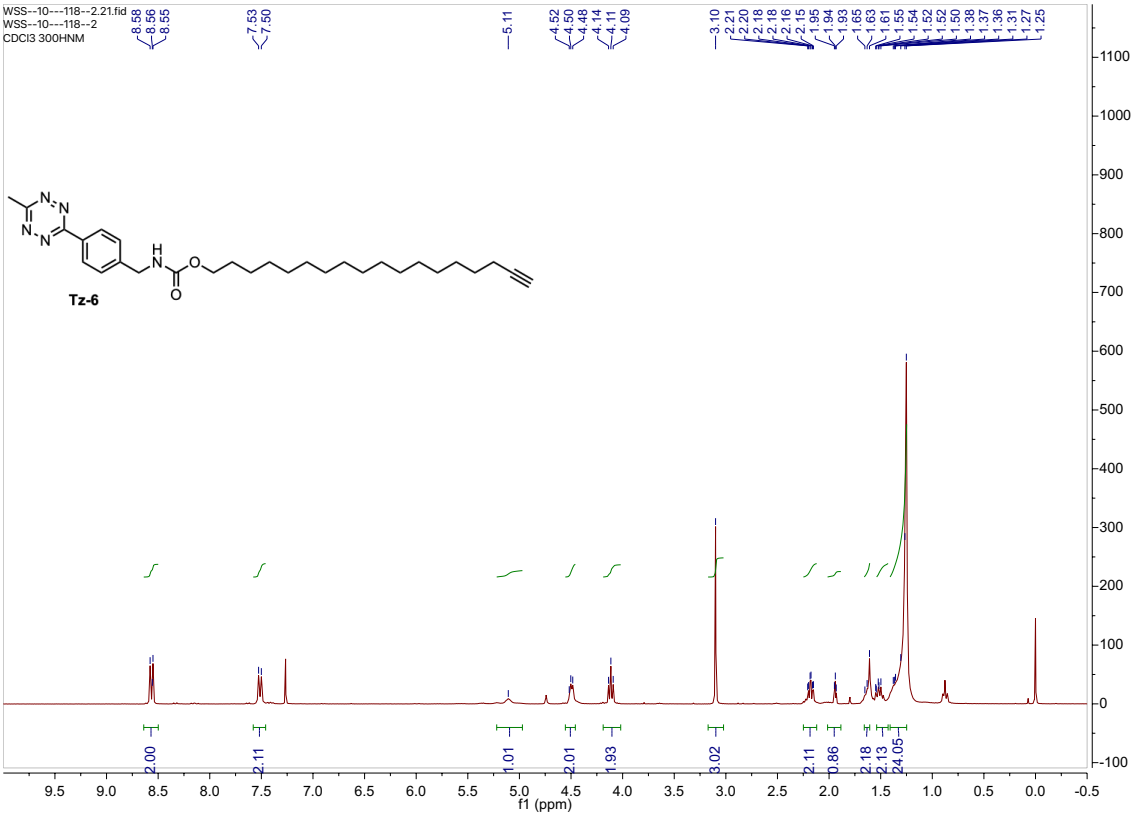
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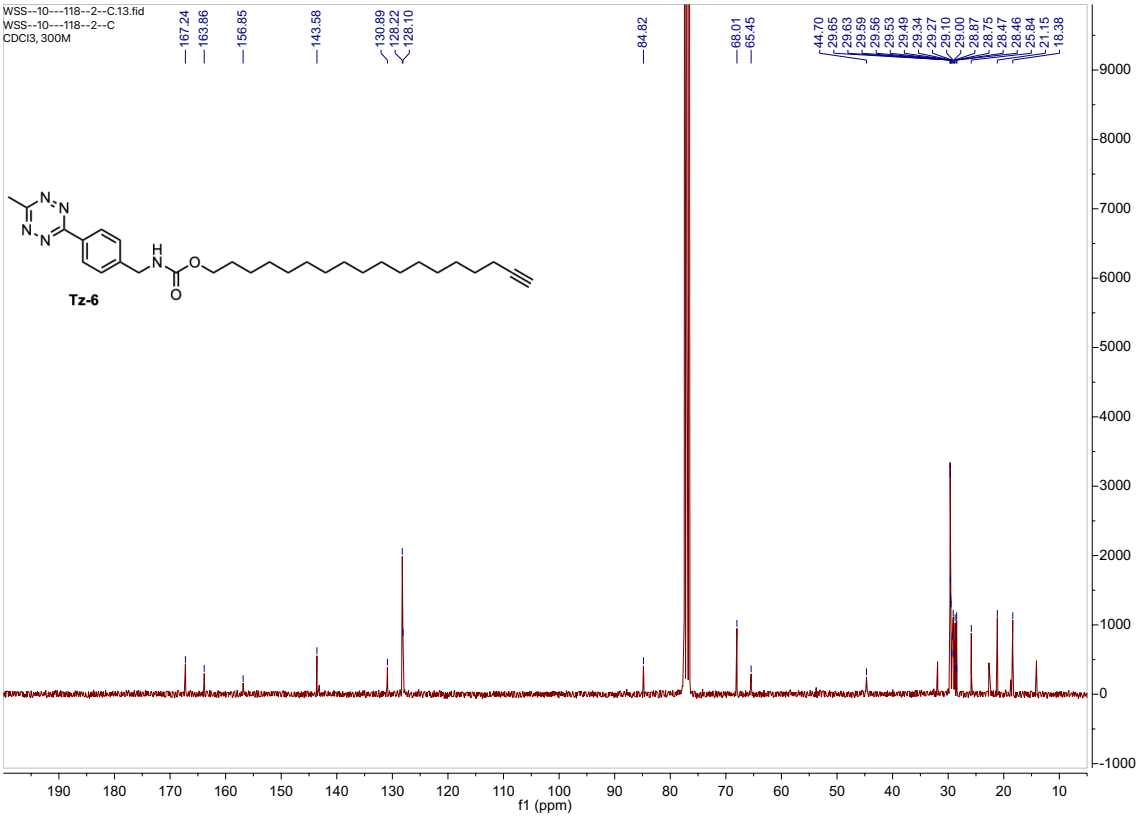
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