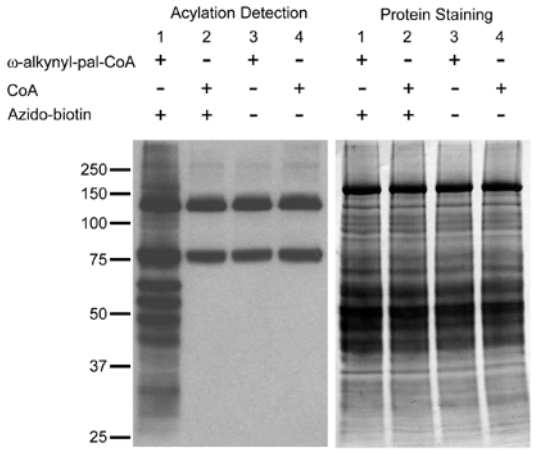
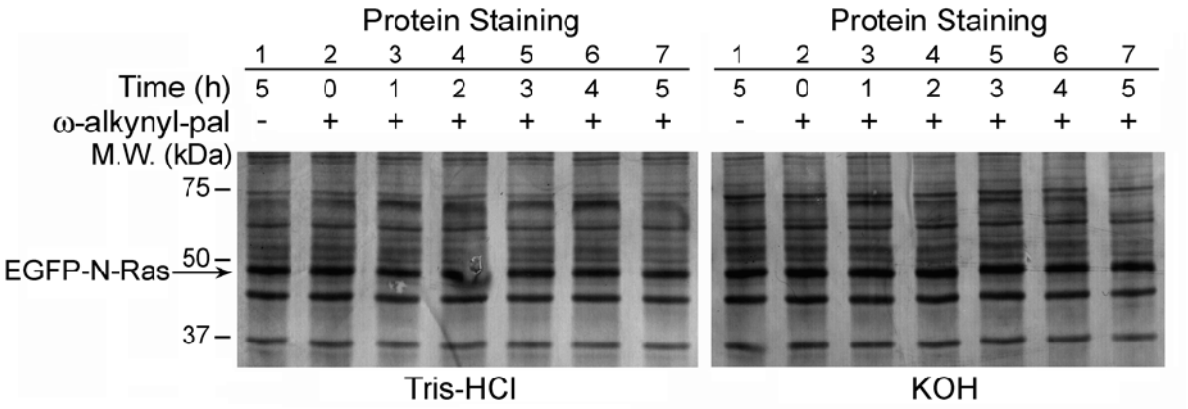
SUPPLEMENTARY MATERIALS:

Supplementary Figures:



Supplementary Figure S1. ω–Alkynyl-palmitate is readily imported into mitochondria resulting in palmitoylation of numerous proteins. P10 mitochondria enriched fractions from primary rat hepatocytes were incubated with 100 μM of ω–alkynyl-palmitoyl-CoA (lane 1) or CoA (lane 2) followed by click chemistry with 100 μM azido-biotin (lanes 1 and 2) or without (lanes 3 and 4). Following SDS-PAGE and Western blotting, PVDF membranes were developed with NeutrAvidin™-HRP/ECL (left panel) and corresponding Coomassie membrane staining (10 μg of protein per lane) is shown on the right panel.



Supplementary Figure S2. Optimization of incorporation of ω-alkynyl-palmitate into proteins in COS-7 cells transiently expressing various EGFP-Ras chimeric proteins or EGFP at various label concentrations and time points. Coomassie staining of membrane from figure 4C illustrates the typical equal loading of samples used throughout this study.

SUPPLEMENTARY TABLES:

Table 1. C-terminal sequences of mature farnesylated and carboxymethylated H-, N- and K-Ras

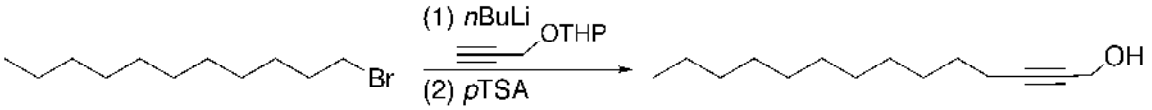
Ras Isoform	Amino Acid Sequence	Prenylation	Second Signal
	181 184 186 . . .		
H-RasWT	ESGPG**C**MS**C**K**C-OMe**	farnesyl	palmitates@cys181,184
	181 186 . .		
N-RasWT	DGTQG**C**MGLP**C-OMe**	farnesyl	palmitate@cys181
	185 + + + + + + + + .		
K-RasWT	**KKKKKK**S**K**T**KC-OMe**	farnesyl	polybasic region

Table 2. Expected and observed m/z ratio of the S-acylated tryptic peptides containing cysteine residue 305 of HMG-CoA synthase.

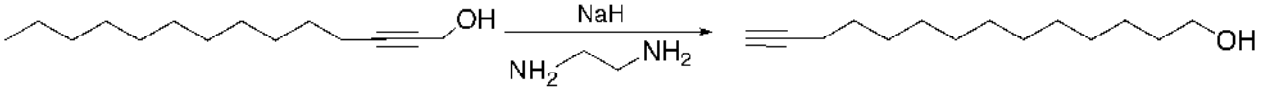
Compound	**Formula**	Calc. monoisot. m/z for$[M+H]^+$	Obs. monoisot. m/z for$[M+H]^+$
Palmitic acid	$C_{16}H_{32}O_2$	257.2	n/a
Alkyne analogue	$C_{16}H_{28}O_2$	253.2	n/a
Azido analogue	$C_{14}H_{27}N_3O_2$	270.2	n/a
Cys305 containing tryptic peptide (non S-acylated)	QAGSDRPFTLDDLQ YMIFHTPFCK	2830.3	Not observed
Cys305 containing tryptic peptide (S-acylated with Palmitic acid)	QAGSDRPFTLDDLQ YMIFHTPFCK + $C_{16}H_{32}O_2 - H_2O$	3068.5	3068.6
Cys305 containing tryptic peptide (S-acylated with Alkyne analogue)	QAGSDRPFTLDDLQ YMIFHTPFCK + $C_{16}H_{28}O_2 - H_2O$	3064.5	3064.5
Cys305 containing tryptic peptide (S-acylated with Azido analogue)	QAGSDRPFTLDDLQ YMIFHTPFCK + $C_{14}H_{27}N_3O_2 - H_2O$	3081.5	3081.6

CHEMICAL SYNTHESES:

Synthesis of Tetradec-13-ynoic acid (terminal acetylenic analogue of myristic acid)

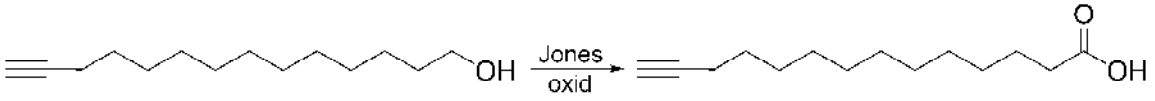


To a stirring, -78°C solution of 2-(prop-2-ynyloxy)tetrahydro-2*H*-pyran (1.25 g, 8.92 mmol) in THF:HMPA (10 mL, 5:1) was added *n*-BuLi (4 mL, 2.5 M solution in hexane) under an argon atmosphere. The reaction mixture was brought to 0°C over 30 min and stirred at that temperature for 1 h. To this was slowly added a solution of 1-bromoundecane (2.34 g, 9.99 mmol) in THF (5 mL). After 12 h at room temperature the reaction mixture was quenched by adding aq. NH_4Cl, extracted into EtOAc (2 × 100 mL). The combined organic phases were washed with water, brine and dried over Na_2SO_4, and all volatiles were removed in vacuo. The residue was dissolved in MeOH (10 mL) containing *p*-toluenesulfonic acid (10 mg). After 12 h at 40°C, the solvent was removed in vacuo and the residue was purified by SiO2 column chromatography to give tetradec-2-yn-1-ol (704 mg, 88% overall for two steps). TLC: 10% EtOAc/hexanes, R*f* ~ 0.33; 1H NMR ($CDCl_3$, 300 MHz) δ 4.26-4.22 (m, 2H), 2.22-2.16 (m, 2H), 1.52-1.43 (m, 3H), 1.35-1.15 (m, 16H), 0.87 (t, *J* = 6.6 Hz, 3H).



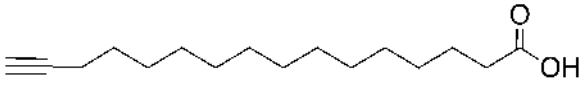
Freshly distilled ethylenediamine (10 g) was added dropwise to NaH (530 mg, Fluka 60% in mineral oil) in a 500 mL RBF under an argon atmosphere with cooling to 0°C. After complete addition, the reaction mixture was heated to 70°C. After 2 h, the reaction mixture was re-cooled to 0°C and a

solution of tetradec-2-yn-1-ol (580 mg) in toluene (3 mL) was added. After stirring at 70°C overnight, the reaction mixture was cooled to 0°C, acidified with aq. 6 N HCl, and extracted into EtOAc (3x50 mL), the combined organic phases were washed with water, brine and dried over Na_2SO_4. The solvent was removed and the resulting residue was purified by short column chromatography to afford tetradec-13-yn-1-ol (481 mg, 83%). 1H NMR ($CDCl_3$, 300 MHz) δ 3.60 (t, *J* = 5.1 Hz, 2H), 2.15 (dt, *J*= 5.1, 1.8 Hz, 2H), 1.91 (t, *J* = 2.1 Hz, 1H), 1.55-1.45 (m, 6H), 1.37-1.24 (m, 20H).



Jones' reagent (2 mL of a 10 N aqueous solution) was added to a stirring, 0°C solution of tetradec-13-yn-1-ol (280 mg, 1.33 mmol) in acetone (10 mL). After stirring at room temperature for 2 h, the reaction mixture was filtered through a pad of diatomaceous earth and the filtrate was concentrated *in vacuo*. The residue was dissolved in ether (150 mL), washed with water, brine and dried over Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified by SiO_2 column chromatography to furnish tetradec-13-ynoic acid (262 mg, 90%) as a colorless oil. 1H NMR ($CDCl_3$, 300 MHz) δ 2.34 (t, *J* = 7.2 Hz, 2H), 2.17 (dt, *J* = 6.9, 2.7 Hz, 2H), 1.93 (t, *J*= 2.7 Hz, 1H).

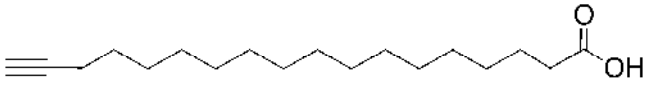
Synthesis of Hexadec-15-ynoic acid (terminal acetylenic analogue of palmitic acid)



Hexadec-15-ynoic acid was prepared in comparable yields by modification of the proceeding procedures using 1-bromotridecane and 2-(prop-2-ynyloxy)tetrahydro-2*H*-pyran. 1H NMR ($CDCl_3$,

300 MHz) δ 2.34 (t, *J* = 7.2 Hz, 2H), 2.17 (dt, *J* = 6.9, 2.7 Hz, 2H), 1.93 (t, *J* = 2.7 Hz, 1H), 1.75-1.10 (m, 22H).

Synthesis of Octadec-17-ynoic acid (terminal acetylenic analogue of stearic acid)

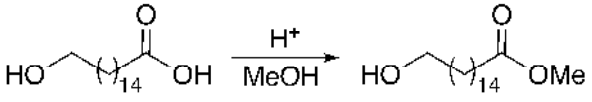


Octadec-17-ynoic acid was prepared in comparable yields by modification of the proceeding procedures using 1-bromopentadecane and 2-(prop-2-ynyloxy)tetrahydro-2*H*-pyran. 1H NMR ($CDCl_3$, 300 MHz) δ 2.34 (t, *J* = 7.2 Hz, 2H), 2.17 (dt, *J* = 6.9, 2.7 Hz, 2H), 1.93 (t, *J* = 2.7 Hz, 1H), 1.75-1.10 (m, 26H).

Synthesis of ω-azido-fatty acids.

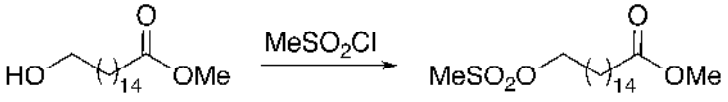
ω-azido-dodecanoic and ω-azido-tetradecanoic acids were synthesized as described in Martin *et al.* (2008) (1) and Kostiuk *et al.* (2008) (2).

Synthesis of and ω-azido-hexadecanoic acid

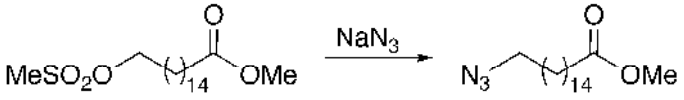


A solution of 16-hydroxyhexadecanoic acid (50 mg, 0.18 mmol) and conc. H_2SO_4 (2 mg) in anhydrous MeOH (20 mL) was maintained at room temperature for 12 h, then concentrated in vacuo. The residue was dissolved in EtOAc (25 mL), washed with saturated aqueous $NaHCO_3$ solution (2 × 10 mL), and dried over anhydrous Na_2SO_4. Evaporation of all volatiles and SiO_2 chromatographic

purification of the residue afforded methyl 16-hydroxyhexadecanoate (94%) as a colorless oil. TLC: 50% EtOAc/hexane, Rf ≈ 0.65; 1H NMR ($CDCl_3$, 300 MHz) d 1.19-1.41 (m, 20H), 1.52-1.69 (m, 6H), 2.18 (s, 1H), 2.29 (t, J = 7.2 Hz, 2H), 3.67 (t, J = 6.6 Hz, 2H), 3.68 (s, 3H).

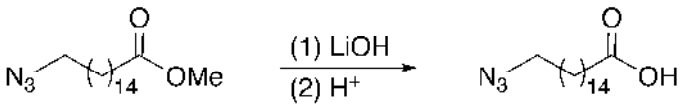


Methanesulfonyl chloride (32 mg, 0.28 mmol) was added slowly with stirring to a 0ºC solution of methyl 16-hydroxyhexadecanoate (40 mg, 0.14 mmol) and triethylamine (35 mg, 0.35 mmol) in anhydrous CH_2Cl_2 (5 mL). After 20 min, the reaction mixture was diluted with more CH_2Cl_2 (20 mL), washed with water (25 mL), saturated aqueous $NaHCO_3$ solution (2 × 10 mL), and dried over anhydrous Na_2SO_4. Evaporation of all volatiles and SiO_2 chromatographic purification of the residue afforded methyl 16-methansulfonyloxyhexadecanoate as a colorless oil which was used without further purification. TLC: 30% EtOAc/hexane, Rf ≈ 0.45; 1H NMR ($CDCl_3$, 300 MHz) d 1.22-1.49 (m, 22H), 1.51-1.69 (m, 4H), 2.31 (t, J = 7.2 Hz, 2H), 2.99 (s, 3H), 3.66 (s, 3H), 4.23 (t, J = 6.6 Hz, 2H).



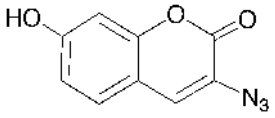
A solution of the above crude methyl 16-methansulfonyloxyhexadecanoate (45 mg, 0.12 mmol) and NaN_3 (24 mg, 0.37 mmol) in dry DMF (3 mL) was heated at 70ºC for 14 h, cooled to room temperature, and extracted with Et_2O (3 × 15 mL). The combined ethereal extracts were washed with water (2 × 15 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification of the residue via SiO_2 column chromatography gave methyl 16-azidohexadecanoate (36 mg, 90% for two

steps). TLC: 20% EtOAc/hexane, Rf ≈ 0.90; 1H NMR ($CDCl_3$, 300 MHz) d 1.21-1.42 (m, 22H), 1.55-1.69 (m, 4H), 2.30 (t, J = 7.5 Hz, 2H), 3.25 (t, J = 6.9 Hz, 2H), 3.66 (s, 3H).



LiOH (19.4 mg, 0.46 mmol) was added to a solution of methyl 16-azidohexadecanoate (36 mg, 0.115 mmol) in THF (2 mL) and H_2O (0.5 mL). After stirring at room temperature for 14 h, the reaction mixture was acidified to pH 4 using 1 M HCl, extracted with EtOAc (3 × 15 mL). The combined organic extracts were washed with brine (20 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification of the residue via SiO_2 column chromatography gave 16-azidohexadecanoic acid (33 mg, 96%). TLC: 20% EtOAc/hexane, Rf ≈ 0.20; 1H NMR ($CDCl_3$, 300 MHz) d 1.21-1.42 (m, 22H), 1.52-1.72 (m, 4H), 2.35 (t, J = 7.2 Hz, 2H), 3.25 (t, J = 6.9 Hz, 2H).

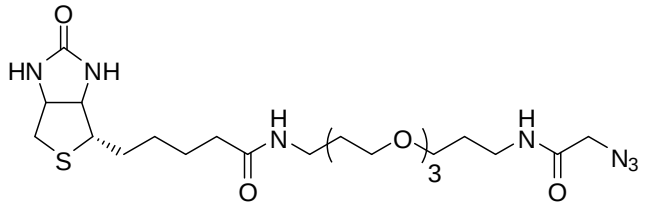
Synthesis of 3-Azido-7-hydroxycoumarin



A mixture of 2,4-dihydroxy benzaldehyde (1.38 g, 10 mmol), *N*-acetylglycine (1.17 g, 10 mmol), anhydrous sodium acetate (2.5 g, 30 mmol) in acetic anhydride (50 mL) was heated under reflux for 4 h, then cooled to room temperature, and poured onto ice water. The resultant yellow precipitate was collected by filtration and washed with a fresh portion of ice water. The product was dissolved in conc. HCl and ethanol (2:1, 30 mL) and heated under reflux for 1 hour and then diluted with ice water (40 mL). To this was added $NaNO_2$ (20 mmol) while maintained in an ice bath. After 10 min, NaN_3 (30

mmol) was added in portions. After stirring for another 15 minutes, the resulting precipitate was collected by filtration, washed with water, and dried under reduced pressure to afford 3-azido-7-hydroxycoumarin (322 mg, ~25% overall yield). ^{1}H NMR (DMSO-d6 300 MHz) δ 6.74 (d, *J* = 2.2 Hz, 1 H), 6.79 (dd, *J* = 8.4, 2.2 Hz, 1 H), 7.47 (d, *J* = 8.5 Hz, 1 H), 7.56 (s, 1 H); ^{13}C NMR (DMSO-d6 75 MHz) δ 161.0, 158.0, 153.4, 128.7, 128.5, 121.8, 114.5, 112.0, 102.7.

Synthesis of Azido-biotin*



Azido-biotin conjugate was prepared as described in ***Hang *et al.* (3).

Synthesis of phosphine-biotin. Phosphine-biotin was synthesized as described previously in Kostiuk *et al.* (2)**.**

MASS SPECTROMETRY:

Enzymatic digestion, pre-fractionation

HMGCS-His_6 was labeled with 50 μM palmitoyl-CoA, ω-azido-tetradecanoyl-CoA, or ω-alkynyl-palmitoyl-CoA for 30 mins, followed by reduction, alkylation, and enzymatic digestion with bovine trypsin. Briefly, to a sample of ~20 μL with an approximate protein concentration of 1 μg/μL of palmitoylated protein sample, 1M NH_4HCO_3 was added to obtain an end concentration of ~0.1 M

NH_4HCO_3 and subsequently reduced and alkylated. To the sample solution, 5 µL of 90 mM DTT was added and the sample was incubated at 56°C for 30 mins. After this, the sample was cooled to room temperature and 5 µL of 200 mM IAA or NEM was added and the sample vials were left in the dark for 1 hour at room temperature. For enzymatic digestion, 1 µL of a 1µg/µL concentration of bovine trypsin was added and the sample incubated at room temperature over night. The next day, samples were purified with a stepwise fractionation procedure using µC18-Ziptips. After acidifying the samples with 1% TFA, the peptides were bound to the Ziptips, washed with 0.1 % TFA, and eluted sequentially with 4 µl portions of 20%, 45%, 65% and 85% acetonitrile/H_2O mixtures with a concentration of 0.1% TFA (palmitoylated peptides were detected in the 65% ACN fraction).

Mass spectrometric analysis of peptides

For peptide mapping with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS), each fraction was mixed 1:1 with a saturated 4-HCCA solution in 30:70 methanol:H_2O. For sample spotting a two-layer method was employed as previously described (4). Briefly, ~1µL of a 12 mg/mL 4-HCCA solution of 80:20 v/v acetone:methanol was spotted onto the MALDI stainless steel target to form a thin first layer. As a second layer 0.5 µL of the peptide extract matrix mixture was spotted onto the dried first layer and air dried. The MALDI targets were analyzed with a Voyager DE-STR MALDI TOF MS (Applied Biosystems, Framingham, MA), located at the UBC Proteome Core Facility. The instrument was operated in positive reflectron mode. Calibration was done first externally with known peptide standards, before data analysis internal calibration was performed with known matrix cluster signals and tryptic autolysis peptide signals, to achieve a mass accuracy of 100 ppm. About 100-200 single laser shot spectra were accumulated per sample.

SUPPLEMENTARY REFERENCES

1. Martin, D. D., Vilas, G. L., Prescher, J. A., Rajaiah, G., Falck, J. R., Bertozzi, C. R., and Berthiaume, L. G. (2008) *Faseb J* **22**, 797-806
2. Kostiuk, M. A., Corvi, M. M., Keller, B. O., Plummer, G., Prescher, J. A., Hangauer, M. J., Bertozzi, C. R., Rajaiah, G., Falck, J. R., and Berthiaume, L. G. (2008) *Faseb J* **22**, 721-732
3. Hang, H. C., Yu, C., Pratt, M. R., and Bertozzi, C. R. (2004) *J Am Chem Soc* **126**, 6-7
4. Dai, Y., Whittal, R. M., and Li, L. (1999) *Anal Chem* **71**, 1087-1091