SUPPLEMENTARY MATERIAL:

Synthesis of 14-Azidotetradecanoic Acid:

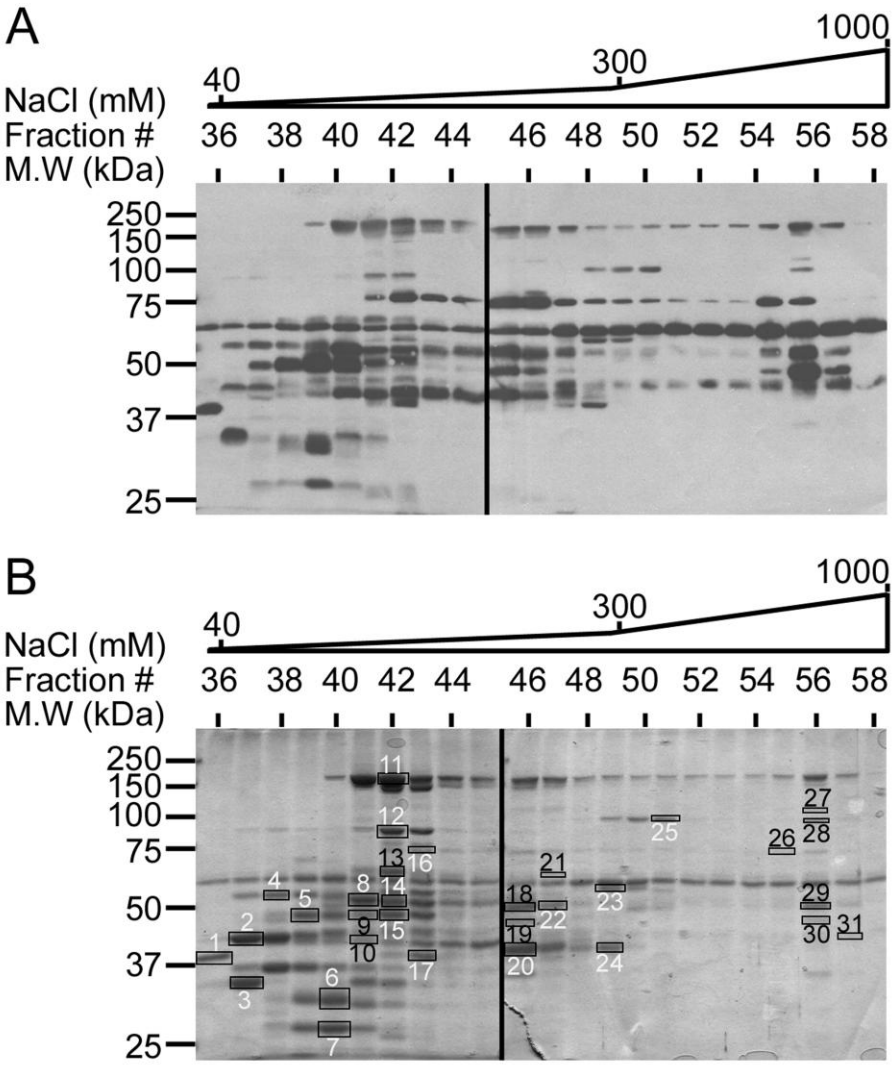
Methyl 14-Hydroxytetradecanoate: A stream of ozone was passed through a -78°C solution of methyl 13(*E*)-docosenoate (350 mg, 0.819 mmol; NuChek Prep, Inc) in CH_2Cl_2 (8 mL) until TLC analysis indicated complete consumption of the olefin (~30 min). Excess Ph_3P (214 mg, 1.24 mmol) was added in one portion and the mixture was allowed to stir at room temperature for 12 h, then all volatiles were removed in vacuum. The residue was dissolved in MeOH (4 mL), cooled to 0°C and $NaBH_4$ (15.5 mg, 0.62 mmol) was added in portions. After 1 h, the reaction mixture was concentrated in vacuo, the residue was diluted with sat. aq. NH_4Cl (10 mL), and extracted with EtOAc (3 × 20 mL). The combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified via SiO_2 column chromatography eluting with 30% EtOAc/hexanes to give methyl 14-hydroxytetradecanoate (182 mg, 78%) as colorless oil. NMR (300 MHz, $CDCl_3$) δ 1.23-1.39 (m, 8H), 1.52-1.68 (m, 4H), 2.30 (t, J = 7.2 Hz, 2 H), 3.64 (t, J = 6.9 Hz, 2 H), 3.66 (s, 3 H).

14-Azidotetradecanoic Acid: The above methyl 14-hydroxytetradecanoate was converted to the corresponding mesylate (94%), azido methyl ester (92%), and finally to the known (1) azido free acid (94%) exactly as described above for its C_{12}-analogues. Chromatographic and spectral properties were also virtually identical except for the presence of two additional methylene units.

Synthesis of tagged triaryl-phosphine probes:

Phosphine-biotin and phosphine-fluorescein were synthesized as previously described (2-4).
Synthesis of myc peptide: The myc peptide with an N-terminal glycine linker (GGG-EQKLISEEDL) was prepared via standard solid phase peptide synthesis using fluorenylmethoxycarbonyl (Fmoc)-Leu-Wang resin (Advanced ChemTech, 0.6 mmol/g), N-alpha-Fmoc-protected amino acids, and 1,3-diisopropylcarbodiimide-mediated 1-hydroxybenzotriazole (HOBt) ester activation in 1-methyl-2-pyrrolidinone (NMP). All Fmoc groups were removed by treatment with piperidine in NMP (2 x 5 min). All coupling steps were performed by activating 5 equivalents of amino acid with 5 equivalents of both 1,3-diisopropylcarbodiimide and HOBt in NMP, adding the solution to the resin, and shaking the mixture for 30 min.

Synthesis of phosphine-myc: DMF (2 mL) was added to the reaction vessel containing the resin-bound, side chain-protected myc peptide (GGG-EQKLISEEDL) (250 mg, 150 umol), followed by a solution of phosphine-PFP (5) (238 mg, 449 umol) in DMF (2 mL) and diisopropylethylamine (157 uL, 936 umol). The reaction vessel was agitated under Ar for 4.5 h at room temperature. The reaction solution was drained, and the resin was washed with DMF followed by CH2Cl2. The washed and dried resin was treated with 95% trifluoroacetic acid (7 mL) for 3 h at room temperature. The peptide conjugate was precipitated in cold methyl tert-butyl ether (MTBE), rinsed with cold MTBE, and purified by reversed-phase HPLC. ESI-MS confirmed the identity of the product with *m/z* 861.0 (MHH++).



Supplementary Figure 1. Separation and labeling of mitochondrial protein from rat liver for the identification of palmitoylated proteins. Soluble mitochondrial proteins from rat liver were separated by ion exchange chromatography and aliquots from each fraction were labeled with azido-palmitoyl-CoA and phosphine-biotin. A) Acylated proteins detected by Western blot using neutravidin-HRP/ECL and B) Biosafe Coomassie blue stained duplicate gel. Rectangular boxes show the 31 bands corresponding to the labeled proteins that were cored from the 'Bio-safe' Coomassie stained gel. White numbering denotes the bands that were successfully identified and listed in tables 1-3 while the black numbering denotes the samples that we could not yet identified.

Identification of putative palmitoylated proteins by mass spectrometry:

In-gel digestion and peptide extraction

The sample preparation protocol followed closely the one published online for Coomassie-stained 1D gel bands by the University of the Missouri Proteomics Center (proteomics.missouri.edu).

Briefly, excised protein bands in 1.5 mL polypropylene vials were minced to ~1 mm^3 pieces, washed 3 times with 1:1 v/v 100 mM NH_4HCO_3/acetonitrile, dehydrated with acetonitrile and air dried. Reduction was performed with 10 mM DTT in 100 mM NH_4HCO_3 sufficient to cover all gel pieces for 30 min. at 56° C. After cooling to room temperature, excess DTT solution was removed and discarded and samples were alkylated with sufficient 50 mM IAA in 100 mM NH_4HCO_3 for 30 min. at room temperature in the dark. Excess IAA solution was removed, gel pieces washed again once with 1:1 v/v 100 mM NH_4HCO_3/acetonitrile, dehydrated for 20 min. with acetonitrile, then excess acetonitrile removed. Gel pieces were rehydrated with sufficient 0.03 µg/µL bovine trypsin in 450/50/500 (V/V/V) 100 mM NH_4HCO_3/acetonitrile/dd. H_2O for 2 hours on ice. Excess trypsin solution was removed and sufficient 450/50/500 (V/V/V) 100 mM NH_4HCO_3/acetonitrile/dd. H_2O was added. Incubation was allowed to proceed for 16 hours at 37° C. After centrifugation, excess liquid was transferred into a new 1.5 mL vial, and gel pieces were extracted three times for each 10 minutes with 25-50 µL of 1% TFA in 60:40 acetonitrile: dd. H_2O. The combined extracts were then concentrated to approx. 20 µL volumes in a vacuum-centrifuge.

Mass spectrometric analysis of peptide extracts and database searching

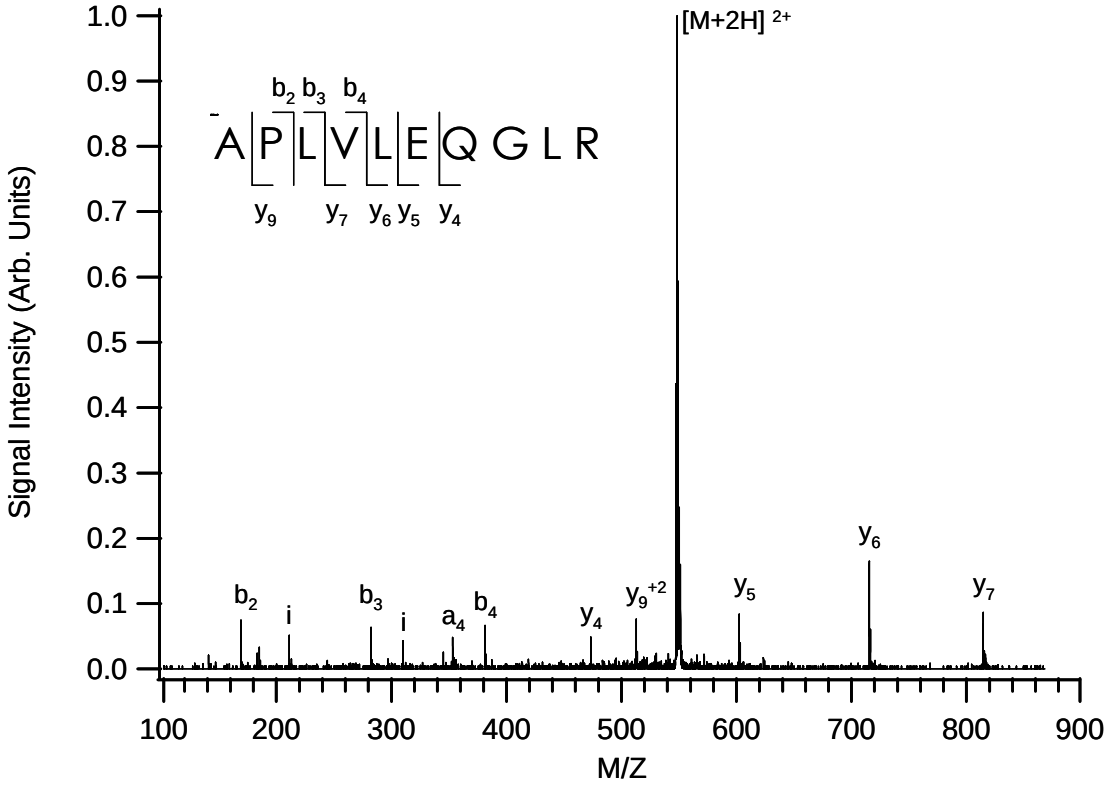
For peptide mapping with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS), 3 µL of the concentrated peptide extract were mixed 1:1 with a saturated 4-HCCA solution in 30:70 methanol:dd. H_2O. For sample spotting a two-layer method was employed as described by Dai et al. (6). 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. 2 washes with each 0.5 µL dd. H_2O were performed. For this, the water droplets were blown off with pressurized air 20 s after spotting. 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. Peptide masses were then tabulated and submitted to the Mascot peptide mapping program (www.matrixscience.com) for protein identification retrieval. Only probability scores, p, <0.05 were considered.

Importantly, we feel very confident on our isolation/identification protocol since 19 of the 21 bands analyzed contained peptides or peptide masses originating from a single protein. Proteins for which identity was based on peptide mapping with 5 or fewer peptides, hypothetical proteins and proteins identified in gel bands with multiple proteins present (e.g. fraction 40, band 6), further confirmation of the proteins was performed with ESI-MS/MS analysis of selected tryptic peptides with protein-specific sequences (Supplementary Table 2). The instrument used was a microQTOF mass spectrometer (Waters/Micromass, Manchester, UK) equipped with a nanospray source. Protein digests were directly infused with a flow of ~250 nL/min in 20% acetonitrile in dd. H_2O (measured with a 10 µL syringe connected to the nanoLC outlet). Fragment masses were submitted to the Mascot sequence query program (www.matrixscience.com). All fragmented peptide spectra gave probability scores, p, <0.05. Supplementary Table 1 lists the identified putative palmitoylated rat liver mitochondrial protein. Supplementary Figure 2 shows an example of an MS/MS spectrum. Supplementary Table 2 shows the results from a few ESI-MS/MS analyses.

Supplementary table 1: Identification of putative palmitoylated proteins in various chromatographic anion exchanger fractions using in-gel digestion and MALDI-TOF-MS.

Fraction / Band #	Protein name	SwissProt# or NCBI ID	MW (kDa)	Matched peptides	Sequence coverage
36/1	Aspartate aminotransferase	P00507	47	11	25 %
37/2	3-ketoacyl-CoA thiolase	P13437	42	15	40 %
37/3	Malate dehydrogenase	P04636	36	23	59 %
38/4	Methylmalonate-semialdehyde dehydrogenase	Q02253	58	6	11 %
39/5	Hydroxymethylglutaryl-CoA synthase	P22791	56	5*)	8 %
40/6	(a) Electron transfer flavoprotein subunit alpha	P13803	35	8*)	32 %
	(b) Hydroxyacyl-coenzyme A dehydrogenase	Q9WVK7	34	5*)	13 %
40/7	(a) Electron transfer flavoprotein subunit beta	Q68FU3	28	11*)	47 %
	(b) Enoyl-CoA hydratase	P14604	32	7*)	24%
41/8	Aldehyde dehydrogenase family 7 member A1 (Antiquitin)	Q64057	56	5*)	11%
42/11	Carbamoyl-phosphate synthase [ammonia]	P07756	166	11	8 %
42/12	Dimethylglycine dehydrogenase	Q63342	96	13	15 %
42/14	Aldehyde dehydrogenase	P11884	57	8	15 %
42/15	Alanine--glyoxylate aminotransferase 2	Q64565	57	7	13 %
43/16	Heat shock protein 75 kDa	Q5XHZ0	80	7	12 %
43/17	Alpha-methylacyl-CoA racemase	P70473	42	9	23 %
46/20	Long-chain specific acyl-CoA dehydrogenase	P15650	48	18	46 %
48/22	Hypothetical protein LOC365699	gi|112984180	48	10*)	28 %
49/23	Sulfite oxidase	Q07116	54	9	18 %
49/24	Isovaleryl-CoA dehydrogenase	P12007	46	7	13 %
51/25	Sarcosine dehydrogenase	Q64380	101	16	13 %

*) These protein identities were further confirmed by ESI-MS/MS of a protein-specific peptide (see supplementary Table 2 and Figure 2).



Supplementary Figure 2. Example of ESI-MS/MS spectrum for the tryptic peptide APLVLEQGLR. The sequence is specific for hydroxymethylglutaryl-CoA synthase (HMGCS) in rats.

Supplementary Table 2. Confirmation of the identification of selected proteins by ESI-MS/MS

FPLC-Fraction / Band #	Protein name	Swiss-Prot# or NCBI id	M_{avg}	Protein-specific sequence of fragmented peptide
39/5	Hydroxymethylglutaryl-CoA synthase	P22791	1095.3	APLVLEQGLR
40/6	(a) Electron transfer flavoprotein subunit alpha	P13803	1794.1	GLLPEELTPLILETQK
	(b) Hydroxyacyl-coenzyme A dehydrogenase	Q9WVK7	931.0	GIEESLKR
40/7	(a) Electron transfer flavoprotein subunit beta	Q68FU3	1958.2	EIIAVSCGPPQCQETIR
	(b) Enoyl-CoA hydratase	P14604	1425.6	NSSVGLIQLNRPK
41/8	Aldehyde dehydrogenase family 7 member A1 (Antiquitin)	Q64057	1938.2	STCTINYSTALPLAQGIK
48/22	Hypothetical protein LOC365699	gi| 112984180	2012.1	VTNEYNESLLYSPEEPK

Supplementary table 3: List of identified putative palmitoylated mitochondrial proteins and their function or involvement in metabolism

Fraction/Band	Identification	Comment
38/4	Methylmalonyl semialdehyde dehydrogenase (EC 1.2.1.2.7)	Valine catabolism
42/11	Carbamoyl phosphate synthetase (EC 6.3.4.16)	Urea cycle
37/3	Malate dehydrogenase (EC 1.1.1.37)	TCA cycle/malate aspartate shuttle
41/8	Aldehyde dehydrogenase family 7 member A1 (EC 1.2.1.3)	Lysine metabolism
42/14	Aldehyde dehydrogenase (EC 1.2.1.3)	Wide specificity
49/24	Isovaleryl-CoA dehydrogenase (EC 1.3.99.10)	Branch chain amino acid degradation
51/25	Sarcosine dehydrogenase (EC 1.5.99.1)	Gly, Ser and Thr metabolism
42/12	Dimethylglycine dehydrogenase (EC 1.5.99.2)	Gly, Ser and Thr metabolism
40/6b	Hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35)	β-Oxidation
46/20	Long-chain specific acyl-CoA dehydrogenase (EC 1.1.99.13)	β-Oxidation
37/2	3-ketoacyl-CoA thiolase (EC 2.3.1.16)	β-Oxidation
40/7b	Enoyl-CoA hydratase (EC 2.3.1.16)	β-Oxidation
40/6a	Electron transfer flavoprotein subunit α	Electron acceptor
40/7a	Electron transfer flavoprotein subunit β	Electron acceptor
42/15	Alanine--glyoxylate aminotransferase 2 (EC 2.6.1.44 and EC 2.6.1.40)	Amino acid transaminase Ala, Asp, Gly, Ser and Thr metabolism
36/1	Aspartate aminotransferase (EC 2.6.1.1)	Amino acid transaminase multiple pathways
39/5	Hydroxymethylglutaryl-CoA synthase (EC 2.3.3.10)	Ketone body synthesis
49/23	Sulfite oxidase (EC 1.8.3.1)	Sulfur metabolism
43/17	Alpha-methylacyl-CoA racemase (EC 5.1.99.4)	Bile acid synthesis and methyl-branched fatty acid degradation.
43/16	Heat shock protein 75 kDa	Chaperone with ATPase activity
48/22	Hypothetical protein LOC365699	Similarity to NAD, sugar and sphingosine kinases

Plasmids and primer design. C-terminal hexahistidine tagged versions of the mature cDNA for human mHMG-CoA synthase, was constructed in the bacterial expression vector pET 19b (Novagene) using a previously described HA tagged cDNA (7) in pcDNA3 (Initrogen) as the template for PCR reactions using the following primers: reverse, 5'CAGTTCTCGAGTTAATGATGATGATGATGATGGACGGGACGCCGGGCATACTTTCG (containing the c-terminal His_6 tag and a translational stop codon in frame followed by an Xho1 restriction enzyme recognition sequence), forward mature, 5'ATCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGACAGCCTCTGCTG TCCCCCTGGC (containing an Xba1 restriction enzyme recognition sequence, ribosome binding site, and a translational start codon). Following PCR the cDNAs were inserted into the pET 19b vector between the Xho1 and Xba1 sites.

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