Published in final edited form as:

J Proteome Res. 2018 May 04; 17(5): 1907–1922. doi:10.1021/acs.jproteome.8b00002.

Selective enrichment and direct analysis of protein S-palmitoylation sites

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Abstract

S-fatty-acylation is the covalent attachment of long chain fatty acids, predominately palmitate (C16:0, S-palmitoylation), to cysteine (Cys) residues via a thioester linkage on proteins. This post-translational and reversible lipid modification regulates protein function and localization in eukaryotes and is important in mammalian physiology and human diseases. While chemical labeling methods have improved the detection and enrichment of S-fatty-acylated proteins, mapping sites of modification and characterizing the endogenously attached fatty acids are still challenging. Here, we describe the integration and optimization of fatty acid chemical reporter labeling with hydroxylamine-mediated enrichment of S-fatty-acylated proteins, and direct tagging of modified Cys residues to selectively map lipid modification sites. This afforded improved enrichment and direct identification of many protein S-fatty-acylation sites compared to previously described methods. Notably, we directly identified the S-fatty-acylation sites of IFITM3, an important interferon-stimulated inhibitor of virus entry, and further demonstrated that the highly conserved Cys residues are primarily modified by palmitic acid. The methods described here should facilitate the direct analysis of protein S-fatty-acylation sites and their endogenously attached fatty acids in diverse cell types and activation states important for mammalian physiology and diseases.

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AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

SUPPORTING INFORMATION:

The following supporting information is available free of charge at ACS website https://urldefense.proofpoint.com/v2/url?u=http-3A__pubs.acs.org&d=DwlFaQ&c=JeTkUgVztGMmhKYjxsy2rfoWYibK1YmxXez1G3oNStg&r=VDqNBrd9MgHl2qXAUQVKBc7KRR9ZD25VTEzG2yNMhLQ&m=7Rf_qRBOH9hO7iVR-Y3fJyd5Zbkl3Ry2hKR8bS8RDmE&s=VUphYOJrgm2ajoHZlnu7m6xhBDgaoJXBnzc78WzxXvk&e=

Proteomics mass spectrometry datasets have been deposited with the ProteomeXchange Consortium via the PRIDE partner repository, with the dataset identifiers PXD003652, PXD008398, PXD008399, PXD008400, PXD008401.

Keywords

S-palmitoylation; fatty acylation; posttranslational modification; site identification; affinity enrichment; mass spectrometry-based proteomics

INTRODUCTION

The covalent addition of long-chain fatty acids to proteins, also referred to as protein fatty-acylation, is an important lipid modification that regulates the trafficking and function of membrane-associated proteins in eukaryotes.^{1–3} The main types of protein fatty-acylation are S-fatty-acylation, N-myristoylation, O-fatty acylation and NeLys-fatty-acylation. They differ in the fatty acid chain length and the modified amino acid. Many studies have revealed a greater diversity and number of fatty-acylated protein, and highlighted new potential functions and regulatory mechanisms for these lipid-modified proteins in physiology and disease.^{2–5} Nonetheless, the precise mapping of S-fatty-acylation sites on cysteine (Cys) residues of proteins and the characterization of endogenously attached lipids remains challenging.

Protein S-fatty-acylation, predominately the addition of palmitate (C16:0) to Cys (S-palmitoylation), is dynamically regulated and found in eukaryotes and plants.¹ The transfer of palmitate to Cys from palmitoyl-CoA to substrate proteins is catalyzed by a family of 23 palmitoyl transferases bearing a Cys-rich domain with a characteristic Asp-His-His-Cys (DHHC) motif. Cleavage of the palmitoyl group is accomplished by serine hydrolases (APT1/2)⁶ as well as α/β -hydrolase domain 17 (ABHD17).^{7,8} The main function of S-palmitoylation is to promote protein association to membranes.¹ Proteins can comprise a single palmitoyl moiety or can be dually modified with more palmitoyl groups or with other lipids, such as a myristoyl or prenyl moiety.⁹ S-palmitoylated proteins can be ordered in four different groups: i) soluble proteins modified with several palmitoyl groups (SNAP25, PSD-95),⁹ ii) membrane proteins modified with one or more palmitoyl group(s) adjacent to a transmembrane domain (Calnexin, IFITM3),^{10,11} iii) proteins dually modified with one of more palmitoyl group and a myristoyl group at the protein N-terminus (G α subunits, Src family kinases)⁹, iv) proteins dually modified with one of more palmitoyl group and a prenyl group proximal to the protein C-terminus (Ras proteins).⁹

The analysis of S-palmitoylated proteins remains challenging to study due to substoichiometric levels of lipid modification as well as hydrophobic and potentially labile thioester linkage of the S-fatty-acylated peptides.^{3,5} Several methods have been developed to visualize and capture S-fatty-acylated proteins (Figure 1). One approach employs alkyne-tagged palmitic acid chemical reporter, alk-16, for metabolic labeling followed by bioorthogonal tagging of cell lysates with azide-fluorophores for detection or affinity tags for enrichment (Figure 1A).² This method has been widely used to characterize S-fatty-acylated proteins in a wide range of cell lines and organisms.^{12–15} However, alk-16 can be metabolized in cells and could also be incorporated into other protein modifications such as N/O-fatty-acylated proteins, including O-palmitoylated¹⁶ and GPI-anchored proteins.¹⁷ To discriminate S-fatty-acylated proteins from N/O-fatty-acylated proteins, several studies have

employed hydroxylamine (NH₂OH) to selectively hydrolyze thioester bonds of alk-16 labeled proteins (Figure 1A).^{12,18}

Another approach, acyl-biotin exchange (ABE), exploits the NH₂OH sensitivity of thioester bonds to selectively label and enrich S-acylated proteins. Following cell lysis, free Cys residues are capped with N-ethyl maleimide (NEM), thioester bonds are hydrolyzed with NH₂OH, the newly liberated thiols on proteins are labeled with HPDP-biotin, captured with streptavidin beads and eluted for western blotting or proteomic analysis.¹⁹ Alternatively, NH₂OH-mediated acyl resin-assisted capture (acyl-RAC) has also been developed to selectively capture S-acylated proteins (Figure 1B).²⁰ Following capping and NH₂OH treatment, free thiols are captured on thiopropyl sepharose 6B resin via the formation of disulfide bonds, which can be reduced to elute captured proteins. Between the two methods, acyl-RAC circumvents biotinylation and affords a more direct protocol to capture S-acylated proteins and peptides on chemically-functionalized resins. Of note, these two NH₂OH-based methods do not distinguish between different fatty acid structures (chain length, unsaturation) and also capture other S-acylated proteins, such as those involved in ubiquitin conjugation in eukaryotes.^{20,21}

While several proteomic studies of S-fatty-acylated proteins have been reported using these methods,^{20,22–28} they are often limited by a high number of false-positive hits. For example, some proteomic studies report several hundred candidate S-fatty-acylated proteins from a specific cell line, but only ~100 S-palmitoylated proteins have been independently validated and annotated in Uniprot or SwissPalm²⁶. The combination of alk-16 labeling and NH₂OH-sensitive enrichment methods (acyl-RAC or ABE) should provide a more accurate method to identify S-fatty-acylated proteins. Indeed, the initial integration of these methods has afforded more robust S-fatty-acylated proteomic datasets in mammalian cells¹³ as well as *Plasmodium Falciparum*²¹. Nonetheless, the direct sites of S-fatty-acylation and lipid composition for many proteins remains to be determined. To overcome these limitations in site-specific proteomic analysis of protein S-fatty-acylation, we report the comparative analysis, integration and optimization of alk-16 metabolic labeling, NH₂OH-sensitivity and site-specific thiol labeling to selectively enrich S-fatty-acylated proteins and directly map their sites of lipid modification. Using our optimized conditions, we experimentally validate S-fatty-acylation of ~100 proteins in mammalian cells and identify more than 40 S-fatty-acylated peptides, including specific sites of modification for small type IV membrane proteins such as IFITM3. S-palmitoylation of IFITM3 was further validated by large-scale purification from mammalian cells and direct analysis of covalently attached fatty acids, which revealed palmitic acid as the major fatty acid modification on conserved Cys residues. Our optimized methods should significantly improve S-fatty-acylation proteomic studies and facilitate the direct analysis of lipid modification sites and composition on these hydrophobic membrane proteins.

MATERIALS AND METHODS

EDTA-free protease inhibitor was purchased from Roche Applied Science and Benzonase from EMD Millipore. Pre-stained protein ladders (Precision plus protein all blue standard and pre-cast polyacrylamide gels (Tris-HCl Criterion TGX stain-free gels, gradient 4–20 %)

were purchased from Bio-Rad Laboratories. Anti-HA magnetic beads, Invitrosol, High-capacity neutravidin beads were obtained from Thermo Scientific Pierce. Mass spectrometry grade trypsin and chymotrypsin were purchased from Promega. Rapigest was purchased from Waters. Protein concentration was measured with the BCA protein assay (Thermo Scientific Pierce). For proteomics, LC-MS grade H₂O and solvents were used. In-gel fluorescence scanning was performed using a BioRad ChemiDoc MP Imaging System. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA), azido-rhodamine and alk-16 were previously prepared in our lab as previously described.⁷ pCMV-HA-IFITM3 plasmid has previously been reported.³

Cell culture and transfection

RAW264.7, HeLa, HEK293T cells were obtained from ATCC. Cells were cultured in Dulbecco's modified Eagle media (DMEM, Gibco) supplemented with 10 % FBS (GE Healthcare *HyClone*TM) maintained in a humidified 37 °C incubator with 5% CO₂. HEK293T cells were transfected using Lipofectamine 3000 (Life Technologies) with a 3:1 ratio of transfection reagent/DNA according to the manufacturer's protocol in cell growth media at 80–90% confluence. RAW264.7 were stimulated with LPS (500 ng /mL, Enzo Life Sciences, *E. coli*, Serotype O111:B4, ALX-581–012-L001), and IFN- γ (100 U/mL IFN- γ , Thermo). HeLa were stimulated with Human Interferon- α 1 (8927SC) from Cell Signaling Technology.

Metabolic labeling

Cells were plated 24 h before treatment. Cells were metabolically labeled with alk-16 (50 μ M, 4 h, unless otherwise stated) in DMEM supplemented with 10 % charcoal filtered FBS. Stimulated RAW264.7 were metabolically labeled with chemical reporters 16 h following stimulation with LPS/IFN- γ , unless otherwise stated. Cells were harvested, washed twice with cold PBS, and flash-frozen and stored at –80°C.

CuAAC click labeling and in-gel fluorescence

Frozen protein pellet was lysed in 4% SDS, 150 mM NaCl, 50 mM HEPES pH 7.4, benzonase, EDTA-free protease inhibitor (Roche). Lysates (100 μ g) were diluted with 150 mM NaCl, 50 mM HEPES pH 7.4 to 90 μ L and 10 μ L of freshly prepared CuAAC reactant solution [azido-rhodamine (1 μ L, 10 mM stock solution in DMSO), CuSO₄ (2 μ L, 50 mM in H₂O), TCEP (2 μ L, 50 mM in H₂O)), TBTA (5 μ L, 2 mM stock solution in DMSO/t-butanol)] were added and vortexed for 1 h at room temperature (protein concentration: 1 mg/mL, SDS concentration = 0.25%). EDTA (2 μ L of a 0.5 M solution in H₂O) was added to stop the CuAAC reaction. Samples were briefly vortexed and proteins were precipitated by CHCl₃/MeOH precipitation (4 volumes (400 μ L) MeOH, 1 volume (100 μ L) CHCl₃, 3 volumes (300 μ L) H₂O). The protein pellets were allowed to air-dry for 5–10 min, resuspended in 37.5 μ L of 4% SDS, 150 mM NaCl, 50 mM HEPES pH 7.4, diluted with 12.5 μ L 4 $\times$ sample loading buffer (4xLaemmli:BME 1:0.1), boiled for 5 min and separated by SDS-PAGE. The gel was washed twice with deionized water before in-gel fluorescence imaging and staining with Coomassie (InstantBlue, Expedeon).

NH₂OH treatment

Following capture with azido-rhodamine, EDTA addition and CHCl₃/MeOH precipitation, lysates were resuspended in 25 µL 4% SDS, 150 mM NaCl, 1 mM EDTA, 50 mM HEPES pH 7.4. 75 µL of 1.33 M NH₂OH pH7.4 in H₂O were added and the solution vortexed for 1 h at room temperature (RT), unless otherwise stated. Following CHCl₃/MeOH precipitation, lysates were resuspended in 25 µL 4% SDS, 150 mM NaCl, 50 mM HEPES, 1 mM EDTA. 75 µL of 0.67 M NH₂OH pH7.4 in H₂O were added and the solution boiled for 10 min. The “-NH₂OH” samples or “DMSO” samples were subjected to the same steps, replacing NH₂OH solution by 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.4.

Western Blot analysis

All primary antibodies were used at a 1:500 dilution and secondary antibody at 1:5000, unless otherwise stated. Primary antibodies were anti-calnexin (ab22595; Abcam, 1/2000), anti-pan Ras (Ras10, Millipore), anti-IFITM3 (10088–604, Proteintech), anti-VCP (ab11433, Abcam), anti-tubulin-β (ab6046, Abcam) and anti-p62 (18420–1-AP, Proteintech). Secondary antibodies were HRP-conjugated goat anti-rabbit (DC03L, Calbiochem), and HRP-conjugated goat anti-mouse IgG (115–035-003, Jackson ImmunoResearch).

Proteins were transferred on nitrocellulose membranes (0.2 µM, BioTrace™ NT Nitrocellulose Transfer Membranes, Pall Laboratory) using Towbin buffer and semi-dry transfer. Membranes were blocked for 1 h in 5% milk, incubated with primary antibody in 5% milk for 1 h, washed 4x with TBS-T (Tris-buffered saline, 0.1% Tween 20), incubated with secondary antibody and washed with 4 x TBS-T. Protein detection was performed with ECL detection reagent (GE Healthcare) on a Bio-Rad ChemiDoc MP Imaging System.

Acyl-Biotin Exchange (ABE), and Acyl-PEG-exchange (APE).

These experiments were performed as previously reported.^{4,5}

Acyl-Resin Assisted Capture (acyl-RAC)⁶

Frozen protein pellet was lysed in 4% SDS, 150 mM NaCl, 50 mM triethanolamine (TEA) pH 7.4, 1 mM EDTA, benzonase, EDTA-free protease inhibitor. Lysates (200 µg) were diluted with 150 mM NaCl, 50 mM TEA pH 7.4 to 92.5 µL. 5 µL of 200 mM neutralized TCEP (final concentration 10 mM) were added and incubated at RT for 30 min with end-over-end rotation. 2.5 µL N-ethylmaleimide (NEM, 1 M stock in ethanol, freshly prepared, 25 mM final concentration) were added and the solution was incubated for 2 h at RT. Proteins were precipitated by CHCl₃/MeOH precipitation. The protein pellets were allowed to air-dry for 5–10 min, resuspended in 92.5 µL of 4% SDS, 50 mM TEA pH 7.4, 150 mM NaCl, 1 mM EDTA. The reduction/alkylation was repeated and proteins were precipitated with CHCl₃/MeOH (3x) and resuspended in 50 µL 4% SDS, 50 mM TEA pH 7.4, 150 mM NaCl, 1 mM EDTA. Samples were split into two 25 µL aliquots - or + NH₂OH (100 µg/condition). 75 µL NH₂OH 1 M, 0.2% Triton X-100, pH 7.3 in H₂O were added to the “+NH₂OH”. For the negative control “-NH₂OH”, 75 µL 50 mM TEA, 0.2% Triton X-100, 150 mM NaCl pH 7.3 were added. Thiopropyl-Sephacrose 6B (T838, Sigma) were soaked in 1 mL H₂O for 30 min. Beads were washed three times with 0.5 mL of 50 mM TEA 150 mM

NaCl, 0.2% Triton X-100, pH 7.3. Each sample (100 μ L of $-/+$ NH₂OH) was added to the thiol-sepharose beads (1.5 mg beads / 100 μ g of proteins, unless otherwise stated) and incubated at RT for 3 h with end-over-end rotation. The supernatants from the beads were decanted and boiled with 4x Laemmli sample buffer (LSB:BME:supernatant 0.9:0.1:3) for 5 min. The beads were washed with 1% SDS in PBS (3 $\times$ 2 min), 4 M Urea in PBS (3x), PBS (3x). Beads were boiled at 95 °C for 10 min with 1x Laemmli sample buffer (LSB:BME:4% SDS 50 mM TEA, 150 mM NaCl pH 7.3, 0.9:0.1:3), separated by SDS-PAGE and analyzed by western blot.

S-palmitoylated IFITM3 peptide synthesis

Peptides ASTAKCLNIW and MNPCCLGF were prepared on Wang resin by the proteomics resource center at the Rockefeller University. N-terminal amino acid was Boc-protected while Cys were Mmt-protected.

Mmt-groups were deprotected with 1% TFA, 5% TIS in DCM for 1 h. The resin was washed with 3 x DCM and 3 x DMF. A mixture of NHS-palmitate (2 eq, typically 70 mmol for 7 mg of resin), TCEP (1 eq) in 50 μ L H₂O and TEA (1.5 eq) in 1.5 mL DMF was prepared and added to the resin. The mixture was vortexed for 1.5 h and the coupling repeated once overnight. The resin was washed with DMF (3x) and free Cys were reduce/alkylated with 10 mM TCEP in H₂O:DMF 1:1 for 30 min followed by the addition of 20 mM iodoacetamide for 30 min in the dark. The resin was washed with DMF (3 x) and DCM (3 x) and side chains were deprotected for 1 h with 95 % TFA, 2.5 % TIS and 2.5 % H₂O. Peptides were dried under an argon flow and in the desiccator overnight. Peptides were used without further purification.

Proteomics - Chemical reporter and acyl-RAC

-Sample preparation - Chemical reporter—Lysate were prepared as described above. For affinity purification of alkyne-modified proteins, 500 μ g of cell lysates / sample were used. CuAAC reactions and NH₂OH treatments were scaled up and performed as described above except azido-biotin was substituted for azido-rhodamine. Following the last CHCl₃/MeOH protein precipitation, protein pellets were air-dried for 10 min and resuspended in 500 μ L 0.4% SDS, 50 mM HEPES pH 7.4, 150 mM NaCl and added to 15 μ L of high-capacity Neutravidin beads (pre-washed three times with 0.4 % SDS, 50 mM HEPES pH 7.4, 150 mM NaCl). Samples were incubated at RT with end-over-end rotation for 90 min. The beads were washed with 1 mL 1 % SDS in PBS (3 $\times$ 5 min), 1 mL 4 M urea in PBS (2 $\times$ 5 min), 1 mL AMBIC (ammonium bicarbonate) (5 $\times$ 2 min). Samples were then reduced with TCEP (10 mM final, stock solution 100 mM TCEP pH 8 in AMBIC) for 30 min at RT. The supernatant was removed, and the beads were washed once with 1 mL AMBIC. Samples were alkylated with iodoacetamide (10 mM final, stock solution 100 mM iodoacetamide pH 8 in AMBIC) in the dark for 30 min. The supernatant was removed, and the beads were washed twice with 1 mL AMBIC. The supernatant was removed. 50 μ L AMBIC containing trypsin (typically 0.1 μ g Trypsin / 500 μ g of lysate) were added and samples were digested at 37 °C overnight. The samples were centrifuged, and the supernatant was transferred into clean tubes. The beads were washed with 50 μ L 1 % FA, 15 % acetonitrile in H₂O and 50 μ L 1 % FA in H₂O. These washes were combined with the

supernatant and peptides were cleaned-up using a C18 stage tip as previously described.⁷ Peptide pellets were resuspended in 5% acetonitrile/1% formic acid (FA) in H₂O for LC-MS analysis.

-Sample preparation – Acyl-RAC—250 µg of cell lysates / sample were used. Acyl-RAC protocol was scaled up and performed as described before with small modifications. Following enrichment of S-acylated proteins on Thiopropyl–Sephacrose 6B resin, beads were washed with 1 mL 2 % SDS in PBS (3 × 5 min), 1 mL 4 M urea in PBS (2 × 5 min), 1 mL PBS (2 x), 1 mL AMBIC (ammonium bicarbonate) (4x). 50 µL AMBIC containing trypsin (Promega, typically 0.05 µg Trypsin / 250 ug of lysate) were added and samples were digested at 37 °C overnight. The samples were centrifuged, and the supernatant was transferred into clean tubes. The beads were washed with 20 µL 50% acetonitrile in H₂O and 2 × 50 µL AMBIC. These washes were combined with the supernatant. Peptides were then reduced (10 mM final concentration, stock solution 100 mM TCEP pH 8 in AMBIC) for 30 min and alkylated with iodoacetamide (25 mM final, stock solution 100 mM iodoacetamide pH 8 in AMBIC) for 1 h in the dark. Peptide were cleaned-up using a C18 stage tip as previously described.⁷ Peptide pellets were resuspended in 5% acetonitrile/1% formic acid in H₂O for LC-MS analysis.

-Mass spectrometry analysis—Extracted tryptic peptides were desalted on a trap column prior to separation on a 12 cm/75 µm reversed phase C18 column (Nikkoy Technos Co., Ltd. Japan). A 120 min method increasing from 10% B to 45% B in 77 min (A: 0.1% Formic Acid, B: Acetonitrile/0.1% Formic Acid) were delivered at 200 nL/min. The liquid chromatography setup was coupled to an Orbitrap XL (Thermo, San Jose, CA, USA) mass spectrometer operated in top-8-CID-mode. Mass spectra were collected in a 300–1800 m/z mass range using 60,000 resolution.

-Maxquant search—The data were processed with MaxQuant version 1.5.0.25 or 1.5.3.8,⁸ and the peptides were identified from the MS/MS spectra searched against the database using the Andromeda search engine.⁹ Cysteine carbamidomethylation and Cysteine modified by NEM were used as variable modifications. For the identification, the false discovery rate was set to 0.01 for peptides, proteins and sites, the minimum peptide length allowed was seven amino acids, and the minimum number of unique peptides allowed was set to one. Other parameters were used as pre-set in the software. “Unique and razor peptides” mode was selected. Label free quantification experiments in MaxQuant was performed using the built-in label free quantification algorithm enabling the ‘Match between runs’ option. Data were elaborated using Microsoft Office Excel 2007 and Perseus version 1.5.0.9.

-Proteomics data processing – Chemical reporter—Label free intensities were logarithmized (base 2). The data were filtered to keep at least 3 valid values (out of 24) per protein. The data were filtered to remove reverse, contaminants, only identified by sites. The replicates were grouped together, and empty values were imputed with random numbers from a normal distribution (imputation criteria: width 0.3 and down shift 1.8). A two sample test (permutation-based, 250 permutations, FDR = 0.05 and S0 value = 1 for RAW264.7 and

FDR = 0.01 and S0 value = 1 for HeLa) was performed to compare the alk-16 with DMSO (fatty acylated proteins), alk-16 with alk-16+NH₂OH (S-fatty acylated proteins) and alk-16+NH₂OH with DMSO (N,O-linked alk-16).

Medium and high confidence protein hits were defined according to the following criteria for the analysis of alk-16 versus DMSO or alk-16+NH₂OH versus DMSO, based on the MS/MS counts (average from 4 replicates):

- **HC (High Confidence):** test significant and 5x MS/MS counts in the alk-16 sample compared to the DMSO sample or 5x MS/MS counts in the alk-16+NH₂OH compared to DMSO samples
- **MC (Medium Confidence):** test significant and 2.5x MS/MS counts
- **LC (Low Confidence):** significantly enriched over background but low MS/MS counts ratio

For the analysis of alk-16 versus alk-16+NH₂OH (S-fatty acylated proteins), proteins significantly enriched in the alk-16 samples over alk-16 + NH₂OH (i.e. significantly sensitive to the NH₂OH treatment) were attributed the same confidence as for the alk-16 versus DMSO analysis.

-Proteomics data processing – Acyl-RAC—Label free intensities were logarithmized (base 2). The data were filtered to keep at least 2 valid values (out of 16) per protein. The data were filtered to remove reverse, contaminants, only id by sites. The replicates were grouped together, and empty values were imputed with random numbers from a normal distribution (imputation criteria: width 0.3 and down shift 1.8). A two-sample test (permutation-based, 250 permutations, FDR = 0.01 and S0 value = 1 for RAW264.7 and FDR = 0.001 and S0 value = 1 for HeLa) was performed to compare the + NH₂OH sample (S-acylated proteins) with - NH₂OH (background).

Medium and high confidence protein hits were defined according to the following criteria, based on the MS/MS counts (average from 4 replicates):

- **HC (High Confidence):** test significant and 5x MS/MS counts in the + NH₂OH sample compared to the - NH₂OH sample.
- **MC (Medium Confidence):** test significant and 2.5x MS/MS counts in the + NH₂OH sample compared to the - NH₂OH sample.
- **LC (Low Confidence):** significantly enriched over background but >1x MS/MS counts in the + NH₂OH sample compared to the - NH₂OH sample.

Proteomics - Direct site ID

-Sample preparation

C18 stage-tipping/purification for direct site ID: The published protocol ⁷ was modified to recover hydrophobic peptides. 6 layers of C18 (3M Empore) were activated with 100 µL ACN 1% FA and equilibrated with 100 µL H₂O 1% FA. The sample was added, and the tip was centrifuged. Peptides were eluted with 100 µL 70% ACN 5% FA 25 % H₂O and 100 µL

90% isopropanol 5% FA 5 % H₂O. Eluates were combined, and peptides were dried in a speed-vac.

Cell lysis and CuAAC: To maximize alk-16 incorporation to S-palmitoylated proteins, HeLa were treated for 24 h with alk-16 adsorbed on BSA. Briefly, to 12 µL of 50 mM alk-16 in DMSO were added 60 µL of NaOH 0.01N. The mixture was warmed to 70 °C for 5 min until complete resuspension of alk-16 precipitate. 150 µL of a pre-warmed (37 °C) solution of 5% BSA in PBS were added and the mixture was vortexed. 10% FBS in DMEM was added up to 12 mL and the solution was filtered-sterilized (0.22 µm filter). To increase IFITM3 expression, cells were stimulated with IFN-α1 (Cell signaling) simultaneously to alk-16 treatment. Cell lysis and CuAAC reaction were performed as described above. For each sample, we typically used 2 mg of lysate. Following quenching of the CuAAC reaction with EDTA, samples were vortexed for 1 min and TCEP (final concentration 10 mM, stock solution 200 mM H₂O) was added. The solution was vortexed for 10 min before adding NEM (20 mM final concentration, stock solution 1M ethanol) for 1 h. Samples were subjected to 3x chloroform/methanol precipitation.

Peptide enrichment – digest before enrichment: Following the last protein precipitation, samples (typically 2 mg) were resuspended in 100 µL 1% Rapigest in 100 mM Tris, 10 mM CaCl₂. 900 µL 100 mM Tris, 10 mM CaCl₂ were added to dilute the sample to 0.1 % rapigest. Trypsin (20 µg) or Chymotrypsin (25 µg) were added and the sample was digested overnight (at 37 °C for trypsin or RT for chymotrypsin). Samples were boiled for 10 min to inactivate the enzyme and 20 µL of 10 % SDS were added (final concentration 0.2 % SDS for enrichment). Neutravidin beads were pre-washed with 0.2% SDS in 100 mM Tris pH8. Digested samples were enriched on Neutravidin beads (high capacity Pierce Neutravidin agarose beads, Thermo Fisher, typically 100 µL beads / 2 mg lysate) for 2 h. The supernatant was discarded, and beads were washed with 0.5 % SDS/PBS (3 × 1 mL), 4 M Urea/PBS (3 × 1 mL), PBS (5 × 1 mL), TEA buffer pH7.4 (2x). Thioester-linked alk-16 labeled peptides were hydrolyzed with 3 × 50 µL NH₂OH solution (25 µL 1% Rapigest, 2.5 µL 0.5 M EDTA, 1.25 µL TEA buffer pH 7.4 10 x, 21.25 µL 2M NH₂OH pH 7.4 (NH₂OH was dissolved in H₂O supplemented with 5 mM EDTA) for 30 min at 37 °C. After each elution, supernatant was transferred to an Eppendorf and 5 µL 250 mM IAA were added. Eluates were combined and vortexed for an additional 15 min in the dark. 2 µL FA were added and samples were subjected to a C18 purification (cf protocol above). Peptides were resuspended in 90 µL AMBIC, 10 µL ACN. 11 µL IAA (250 mM stock in H₂O) were added and the solution was vortexed for 1h30 in the dark. Rapigest was precipitated by adding 2 µL TFA and 5 µL FA, and incubating the samples at 37 °C for 30 min. Samples were spin down for 10 min at 8,000 xg and the supernatant was stage-tipped. The peptides were vacuum dried and kept at –80 °C prior to LC-MS/MS analysis.

Protein enrichment – digest after enrichment: Following CuAAC and protein precipitation, samples (typically 2 mg proteins / sample) were resuspended in 2 % SDS in PBS and diluted to 0.2 % SDS (1 mg/mL final protein concentration). Neutravidin beads were pre-washed with 0.2% SDS PBS. Samples were enriched on Neutravidin beads (high capacity Pierce Neutravidin agarose beads, Thermo Fisher, typically 100 µL beads / 2 mg

lysate) for 2 h. The supernatant was discarded, and beads were washed with 0.5 % SDS/PBS (3×1 mL), 4 M Urea/PBS (3×1 mL), PBS (5×1 mL).

Trypsin digest: Following enrichment and washes, beads were washed with TEA buffer pH7.4 (2x). Thioester-linked alk-16 labeled proteins were hydrolyzed with 3×50 μ L NH₂OH solution (25 μ L 1% Rapigest, 2.5 μ L 0.5 M EDTA, 1.25 μ L TEA buffer pH 7.4 10 x, 21.25 μ L 2M NH₂OH pH 7.4 (NH₂OH was dissolved in H₂O supplemented with 5 mM EDTA) for 30 min at 37 °C. After each elution, supernatant was transferred to an Eppendorf and 5 μ L 250 mM IAA were added. Eluates were combined and vortexed for an additional 15 min in the dark. Samples were desalted using a 7kDa Zeba column (Thermo Fisher), according to the supplier's protocol. Samples were eluted in TEA buffer pH8. IAA (10 mM final concentration) was added and samples were incubated in the dark for 1 h. Samples were desalted again and eluted in AMBIC. Samples were diluted with AMBIC up to 1 mL and 1 μ g Trypsin was added. Samples were incubated at 37 °C overnight. Samples were concentrated under vacuum until ~150 μ L remained. Rapigest was precipitated by adding 2 μ L TFA and 5 μ L FA, and samples were incubated at 37 °C for 30 min. Samples were spin down for 10 min at 8,000 xg and the supernatant was stage-tipped. The peptides were vacuum dried and kept at -80 °C prior to LC-MS/MS analysis.

Chymotrypsin digest: Following enrichment and washes, beads were washed with 2×100 μ L 2 M Urea/PBS. Protein were on-bead digested overnight at RT with 2 μ g chymotrypsin in 200 μ L 2M Urea/PBS, 1 mM CaCl₂.¹⁰ Beads were washed with PBS 4 x and TEA buffer pH8 2x. Thioester-linked alk-16 labeled peptides were hydrolyzed with 3×50 μ L NH₂OH solution (25 μ L 1% Rapigest, 2.5 μ L 0.5 M EDTA, 1.25 μ L TEA buffer pH 7.4 10 x, 21.25 μ L 2M NH₂OH pH 7.4 in H₂O supplemented with 5 mM EDTA) for 30 min at 37 °C. After each elution, supernatant was transferred to an Eppendorf and 5 μ L 250 mM IAA were added. Eluates were combined and vortexed for an additional 15 min in the dark. 2 μ L FA were added and samples were subjected to a C18 purification (cf protocol above). Peptides were resuspended in 90 μ L AMBIC, 10 μ L ACN. 11 μ L IAA (250 mM stock in H₂O) were added and the solution was vortexed for 1h30 in the dark. 20 μ L of 6M urea were added. Rapigest was precipitated by adding 2 μ L TFA and 5 μ L FA, and samples were incubated at 37 °C for 30 min. Samples were spin down for 10 min at 8,000 xg and the supernatant was stage-tipped. The peptides were vacuum dried and kept at -80 °C prior to LC-MS/MS analysis.

-Mass spectrometry for direct site ID—Samples were resuspended in 5 μ L 1:1:1 FA : 75% ACN : 1% TFA and then diluted by adding 25 μ L 1% TFA.

Peptides were analyzed by LC-MS/MS (Ultimate 3000 nano-HPLC system coupled to a Q-Exactive Plus mass spectrometer, Thermo Scientific). Peptides were separated on a C18 column (12 cm/75 μ m, 3 μ m beads, Nikkyo Technologies) at 200 nl/min with a gradient increasing from 1% Buffer B/99% buffer A to 95% buffer B/10% Buffer A in 120 min (buffer A, 0.1% formic acid; buffer B, 0.1% formic acid in acetonitrile; "gradient 1": 1% B for 10 min, 1% to 6 % B in 4 min, 6% to 50 % B in 77 min, 50 to 95 % in 1 min, remaining at 95% B for 17 min, 95% to 1% in 1 min and 1 % for 9 min) and were analyzed in a data-dependent acquisition manner. MS spectra were recorded at 17,500 resolution with m/z 100

as lowest mass. Normalized collision energy was set at 27, with AGC target and maximum injection time being 2e5, and 60 ms, respectively. Another gradient (“gradient 2”) was also tested: buffer A, 0.1% formic acid; buffer B, 0.1% formic acid in acetonitrile; 1% B for 10 min, 1% to 5 % B in 2 min, 5% to 90 % B in 55 min, remaining at 90% B for 17 min, 90% to 1% in 1 min and 1 % for 9 min).

-Maxquant search—The data were processed with MaxQuant version 1.5.3.8,⁸ and the peptides were identified from the MS/MS spectra searched against the database using the Andromeda search engine.⁹ Acetyl on protein N-terminus, oxidation of Met, Cysteine carbamidomethylation and Cysteine modified by NEM were used as variable modifications. For the identification, the false discovery rate was set to 0.01 for peptides, proteins and sites, the minimum peptide length allowed was five amino acids, and the minimum number of unique peptides allowed was set to one. Up to 10 miscleavages were allowed for chymotrypsin. Other parameters were used as pre-set in the software.

Direct analysis of S-fatty acylated peptides

-Sample preparation—Hek293T cells were transfected for 48 h with HA-hIFITM3 (typically 150 mm dish, transfected with 15 µg HA-IFITM3 using Lipofectamine 3000. Cells were harvested, washed twice with ice-cold PBS and cell pellets were resuspended in 1.5 mL lysis buffer (50 mM HEPES pH7.4, 150 mM NaCl, 1% Brij 97, Protease inhibitor cocktail 1x). Samples were kept on ice for 15 min, spun down at 500 xg for 10 min at 4 °C. The supernatant was added to 200 µL anti-HA magnetic beads (Thermo) and incubated at 4 °C for 2 h with end-over-end rotation. Beads were washed 6× 1 mL of chilled RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM triethanolamine pH 7.4), 6x PBS, 6 x H₂O. Beads aggregation was typically observed after washing with PBS and H₂O.

Elution and in-solution digest: The supernatant was removed. HA-IFITM3 was eluted with 1N NH₄OH (3 × 50 µL, 10 min each). Supernatants were combined in a Protein Lobind tube (Eppendorf). Samples were vacuum dried using a speed vacuum, and the pellet was resuspended in 25 µL 8M Urea in AMBIC (50 mM ammonium bicarbonate). 2 µL 200 mM TCEP pH8 were added and the sample was vortexed for 30 min at RT. 5 µL 100 mM IAA in AMBIC were added and the sample was vortexed for 30 min at RT. 66 µL of AMBIC, 1 µL CaCl₂, 1 µL Chymotrypsin (0.25 µg / µL in 1 mM HCl). The sample was incubated at RT for 6 h. 60 µL of ACN, 39 µL of AMBIC and 1 µL Chymotrypsin were added and the samples were incubated a further 12 h at RT. Samples were snap freezed, dried in a speed-vac and kept at –80 °C before analysis.

In-gel digest with Invitrosol: Beads were boiled in sample loading buffer (1:1:2 4 xLaemmli buffer: TCEP 200 mM : 4% SDS 50 mM triethanolamine pH7.4 150 mM NaCl) and samples loaded onto 4–20% Bio-Rad Criterion Tris-HCl gels for separation by SDS-PAGE. Lysosyme was also loaded on the gel to estimate the amount of IP-ed IFITM3. The gel was washed 3 x LC-MS grade H₂O, stained with Simply blue Safestain Coomassie (Thermo) for 1 h, washed with LC-MS grade H₂O for 1 h. The band of interest was excised, cut in small pieces (~1 mm x 1 mm) and transferred to a Protein Lobind tube (Eppendorf).

The sample was reduced / alkylated / digested following a published protocol¹¹ with modifications. The gel pieces were washed 2x H₂O, dried with acetonitrile (ACN). The proteins were reduced by adding 200 µL 0.5x Invitrosol, 10 mM TCEP in AMBIC (50 mM ammonium bicarbonate). After 30 min at RT, the supernatant was removed and the gel pieces were dried with ACN. The proteins were alkylated by adding 200 µL 0.5x Invitrosol, 50 mM IAA in AMBIC (50 mM ammonium bicarbonate). Samples were incubated in the dark at RT for 20 min. The supernatant was removed and the gel pieces were dried with ACN. The gel pieces were destained 2 × 30 min with 500 µL ACN: AMBIC 1:1 and dried with ACN. Chymotrypsin was dissolved in 1 mM HCl (25 µg/25 µL). 1 µg of chymotrypsin was added to 4 µL Invitrosol 5x, 1 µL CaCl₂ 1M and 94 µL AMBIC. The solution was added to the gel pieces and the samples were kept on ice. After 15 min, 50 µL AMBIC were added and the samples were vortexed at RT for 16 h. The supernatant was removed and transferred to a Protein Lobe tube (Eppendorf). The gel pieces were washed (5 min at 37 °C for each wash) with 100 µL ACN:AMBIC 1:1, 100 µL isopropanol:AMBIC 1:1, 100 µL isopropanol:formic acid 80:20. The washes were combined to the supernatant, snap frozen and dried in a speed-vac. The pellet was kept at -80 °C before LC-MSMS analysis. Samples containing Invitrosol were run on high flow Orbitrap.

In-gel digest no Invitrosol: same protocol as above with modifications: The reduction was performed with 200 µL 10 mM TCEP in AMBIC and the alkylation with 200 µL 0.5x Invitrosol, 10 mM IAA in AMBIC. For the digest, 1 µg of chymotrypsin in 100 µL of 10 mM CaCl₂, 1M Urea, AMBIC was added to the gel pieces on ice. After 15 min, 50 µL 10 mM CaCl₂, 1M Urea, AMBIC were added and the samples were incubated at RT for 16 h.

-Mass spectrometry

Protocol low flow: Samples were solubilized in 10 µl formic acid, followed by the addition of 40 µl of 50% isopropanol/10% methanol/0.1% Formic Acid. Peptides were desalted on a trap column and separated on a 12 cm/75 µm reversed phase C18 column (Nikkoy Technos Co., Ltd. Japan). A 120 min method increasing from 5% B to 30% B in 70 min, and 30%B to 45%B in 7 min (A: 0.1% Formic Acid, B: Acetonitrile/0.1% Formic Acid) were delivered at 200 nL/min. The liquid chromatography setup (Dionex, Boston, MA, USA) was coupled to an Orbitrap XL (Thermo, San Jose, CA, USA) mass spectrometer operated in top-8-CID-mode. Mass spectra were collected in a 300–1800 m/z mass range using 60,000 resolution.

Protocol high flow: Samples were solubilized in 10 µl formic acid, followed by the addition of 40 µl of 50% isopropanol/10% methanol/0.1% Formic Acid. Peptides were separated on Thermo Acclaim 120 C18 (2.1×150mm) at 200 µl/min using the standard ACN/water gradient (A: 0.1% Formic Acid, B: Acetonitrile/0.1% Formic Acid) and a 30-minute method (0 % B for 6 min, increase from 0% B to 1% B in 1.65 min, increase to 40% B in 12.35 min, increase to 90 % B in 1 min, remaining at 90% B for 3 min, decrease to 0 % B in 1 min, remaining at 0% B for 5 min). The liquid chromatography setup (Dionex, Boston, MA, USA) was coupled to an Orbitrap XL (Thermo, San Jose, CA, USA) mass spectrometer operated in top-8-CID-mode. Mass spectra were collected in a 300–1800 m/z mass range using 60,000 resolution.

-Data analysis

Proteome Discoverer: The data were processed with Proteome Discoverer 1.14.1.14 software (Thermo Fisher Scientific) with minor modifications. Raw data files (.raw) were processed by using the Mascot node and searched against IFITM3, *E. Coli* database and common protein contaminants. The precursor mass tolerance was set to 5 ppm and fragment ion mass tolerance to 0.5 Da. Carbamidomethylation or palmitoylation on Cys, Acetyl on Protein N-terminus and oxidation of Met were used as variable modifications. Enzyme specificity was set as none; as chymotrypsin is non-specific, setting the enzyme specificity as none did not limit the digest specificity to F/W/Y/L/M and did not limit the number of miscleavages. Results included peptides identified with high, medium, and low confidence.

Maxquant: The data were processed as described above with the following modifications: Chymotrypsin was used as the digestion enzyme (F/W/Y/L/M cleavage sites), allowing 10 missed cleavage sites. Carbamidomethylation on Cys, Acetyl on protein N-terminus and oxidation of Met, addition of palmitate, myristate (C14; exact mass 210.1983656), oleate (C18:1; exact mass 264.2453156), stearate (C18; exact mass 266.2609657) and palmitoleate (C16:1; exact mass 236.2140155) were used as variable modifications (maximum 5 modifications per peptide).

Byonic search: Byonic (v2.10.21, Protein Metrics, Inc.) was used to search for unknown modification on human IFITM3 using the Wildcard Search function.

RESULTS AND DISCUSSION

alk-16 chemical reporter labeling and NH₂OH treatment optimization

To establish a more robust protocol for the proteomics analysis of S-fatty-acylated proteins, we first optimized Cu^I-azide-alkyne cycloaddition (CuAAC) and enrichment of alk-16 labeled proteins in RAW264.7 murine macrophages (SI Optimization of protocols and SI Figures S1-2). We focused on macrophages given our laboratory interests in innate and adaptive immunity to microbial pathogens. For these experiments, macrophages were labeled with alk-16 or treated with DMSO as a control. Following cell lysis, proteins were reacted with azido-rhodamine via CuAAC for in-gel fluorescence visualization of alk-16 labeled proteins or with azido-biotin for enrichment of alk-16 labeled proteins. Since Yang *et al.* reported that the buffer employed for the CuAAC reaction was important for labeling,²⁹ we further optimized our CuAAC reaction conditions and found HEPES buffer improved azido-rhodamine and azido-biotin ligation compared to other commonly used buffers.

To ensure maximum enrichment over background, we optimized the Neutravidin bead enrichment and NH₂OH thioester hydrolysis conditions to distinguish S-fatty-acylated proteins from N/O-fatty-acylated proteins (SI Optimization of protocols and SI Figures S3). CuAAC and NH₂OH thioester hydrolysis conditions have been reported in the literature,^{12,18} but required further investigation to avoid non-specific protein degradation. Quenching the CuAAC reaction with EDTA appeared essential to prevent protein degradation (SI Figure S3A-B),³⁰ and conditions of the NH₂OH treatment were further optimized to ensure known

S-fatty-acylated proteins, CANX,¹¹ Fyn,³¹ IFITM3¹⁰ labeled with alk-16 were sensitive to the NH₂OH treatment.

Comparative proteomic analysis of S-fatty-acylated proteins in RAW264.7 macrophages

We then employed alk-16 labeling +/- NH₂OH treatment, Neutravidin enrichment and on-bead tryptic digestion to identify S-fatty-acylated proteins in macrophages (Figure 1A). The recovered peptides from 4 biological replicates were analyzed on a nanoLC-MS/MS Orbitrap. The proteins were identified and quantified with the label-free MaxLFQ algorithm in Maxquant.³² The label-free quantification (LFQ) intensities were highly reproducible between 4 biological replicates (SI Figure S4A-D and Table S1). A statistical analysis (t-test, permutation-based, 250 permutations, FDR = 0.05 and s0 value = 1), was performed to identify proteins labeled with alk-16 over background (DMSO) (Figure 2A). Out of the >800 proteins identified in the alk-16 and DMSO samples, >400 proteins were significantly enriched in the alk-16 sample over the DMSO background (SI Table S1). This pool of alk-16-labeled proteins includes many known S-fatty-acylated proteins (CANX, Fyn, NRas), as well as a few N-myristoylated proteins (ARF1, LAMTOR1, NADH-cytochrome b5 reductase 3 (Cyb5r3)) and GPI-anchored proteins (Cd14). The data were further classified in high, medium and low confidence based on spectral counts (SI Table S1, SI experimental procedures) and provided a list of alk-16 labeled protein targets in naïve RAW264.7 macrophages (Figure 1A). A statistical analysis (t-test, permutation-based, 250 permutations, FDR = 0.05 and s0 value = 1), was then performed to identify only proteins labeled with alk-16 that are sensitive to the NH₂OH treatment (Figure 2B, SI Table S1 and SI Figure S4D). Out of the >400 alk-16 labeled proteins, less than 150 proteins were significantly sensitive to the NH₂OH treatment (Figure 2B), indicating that alk-16 was incorporated on a Cys residue via a thioester linkage, and that these proteins are S-fatty-acylated. The data were again further classified in high, medium and low confidence (SI Table S1, SI experimental procedures) and provided a list of 103 S-fatty-acylated proteins identified with high/medium confidence in naïve RAW264.7 macrophages (Figure 2C). This list of 103 high/medium proteins include many well-characterized S-fatty-acylated proteins, such as CANX, NRas, HRas, IFITM3 and SNAP23 (Table S1). Notably, most alk-16 labeled proteins were resistant to the NH₂OH treatment, as seen by their equal enrichment in both samples (alk16 and alk-16 + NH₂OH) and a t-test difference (log2) close to 0, confirming that our CuAAC and NH₂OH protocols did not non-specifically degrade proteins. As expected, GPI-anchored proteins and N-myristoylated proteins were not significantly sensitive to the NH₂OH treatment. The only N-myristoylated protein remaining in the list (Lamtor1) is also S-fatty-acylated (Figure S2B, SI Table S1).³³ Most known S-fatty-acylated proteins were NH₂OH sensitive (red dots, Figure 2B), confirming the efficiency of optimized NH₂OH treatment to hydrolyze thioesters. Only a small subset of known S-fatty acylated proteins (Fyn, Scimp, Phospholipid scramblase 1) were not enriched in the alk-16 sample over DMSO background or over the NH₂OH treated sample, due to their weak labeling with alk-16. Taken together, the data suggest that alk-16 labeling combined with optimized NH₂OH treatment provides a high confidence list of S-fatty-acylated proteins.

We next sought to evaluate the efficiency and selectivity of the NH₂OH-based enrichment methods, ABE and acyl-RAC methods to enrich S-fatty-acylated proteins. These methods

have been widely employed to identify S-fatty-acylated by proteomics but have not been compared to alk-16+/-NH₂OH-based proteomics.²⁶ ABE and acyl-RAC methods enrich any proteins modified via a thioester bond, including S-fatty-acylated proteins (Figure 1B). Using both ABE and acyl-RAC, three known S-fatty-acylated proteins, CANX, Ras, IFITM3 were specifically enriched in the presence of NH₂OH. Since acyl-RAC afforded a more efficient protocol (SI Figure S5A and Figure 1B), we focused on this method for comparative proteomic studies. To ensure maximum enrichment over background (-NH₂OH sample), we optimized the thiopropyl sepharose 6B bead enrichment (SI Figure S5B). Following on-bead tryptic digestion, the samples (n= 4 biological replicates) were analyzed by nanoLC-MS/MS and quantified by LFQ. This approach provided highly reproducible LFQ intensities of protein enrichment between replicates (SI Figure S6). A t-test (permutation-based, 250 permutations, FDR = 0.01 and s0 value = 1) was performed to identify only proteins enriched over background (-NH₂OH sample) (Figure 2C). The data was further classified in high, medium and low confidence based on the spectral counts (SI Table S2, SI experimental procedures). 347 proteins were identified with high or medium confidence as S-acylated, including known S-fatty-acylated proteins (CANX, IFITM3, NRas, SNAP23) and more than 286 proteins reported to be S-fatty-acylated in other proteomic studies (Figure 2C-E, SI Table S2).²⁶ Notably, we identified 69 proteins out of the 101 proteins previously identified as S-acylated in RAW264.7 macrophages.^{26,35}

The alk-16 fatty acid reporter labeling and acyl-RAC proteomic method gave overlapping but distinct protein substrates (Figure 2C-D, SI Table S1-3), which are consistent with previous proteomic datasets.²⁶ From the high/medium confidence hits, 322 and 347 proteins were identified by alk-16 and acyl-RAC proteomics, respectively, and 103 alk-16 labeled proteins were NH₂OH sensitive. 83 proteins were recovered by all three analyses (acyl-RAC, alk-16 versus DMSO, alk-16 versus alk-16 + NH₂OH), including 29 well-characterized S-fatty-acylated proteins such as CANX, IFITM3, NRas and SNAP23, and 78 proteins previously identified by S-fatty-acylation proteomic studies (Figure 2, Table S3). However, these methods can capture different populations of proteins from cell lysates. Acyl-RAC method recovers many S-acylated proteins that may not be directly fatty acid modified, including other thioester-modified proteins involved in ubiquitin conjugation in mammalian cells²⁰ (SI Table S2). Notably, 207 high/medium confidence hits, such as one of the top hits MYOF, are highly enriched by acyl-RAC but are not labeled with alk-16 fatty acid chemical reporter. These 207 proteins could nonetheless include some candidates S-fatty-acylated proteins poorly labeled by alk-16 due to their low abundance or slow S-fatty-acylation turnover. Alternatively, alk-16 preferentially labels more dynamically S-fatty-acylated proteins, N/O-fatty-acylated proteins^{2,5} and may also be metabolized in cells³⁶. The comparison of alk-16 labeled proteins +/- NH₂OH provides a more robust dataset of S-fatty-acylated proteins (alk-16 and NH₂OH-sensitive) and also highlights other candidate fatty-acylated proteins that may be modified on other amino acid residues besides Cys. Proteins recovery by alk-16 +/-NH₂OH may reflect low abundance proteins, such as the known S-fatty acylated protein HRas, which is labeled by alk-16 and sensitive to NH₂OH but not enriched by acyl-RAC. Proteins labeled with alk-16 but not NH₂OH sensitive, and also enriched by acyl-RAC (TUBB5, VDAC2, Figure 2C-D) might be false positive hits or could be insensitive to the NH₂OH treatment due to S- to N-transfer during sample preparation.³⁷

To explore protein S-fatty-acylation in host immunity, we employed both proteomic methods to quantify changes in fatty-acylation in naïve and LPS/IFN- γ stimulated RAW264.7 macrophages (Figure 2E-F, SI Figure S7-9, SI Table S1-3). The observed differences could originate from a change in fatty-acylation or change in protein expression. The list of S-fatty-acylated proteins more enriched in stimulated RAW264.7 macrophages includes IFN-effectors, such as inducible nitric oxide synthase (Nos2) and Irgm1. Other immune receptors (CD36, TLR2, TLR9), effectors (IFITM2, IFITM3,^{10,38} Tnf), regulators (CD74) and signaling factors (LAT2, HCK, Lyn, Fyn) were also recovered in naïve and stimulated macrophages (Figure 2D-E and SI Table 3). The data suggest that many proteins involved in host immunity may be regulated by protein fatty-acylation.

Comparative proteomic analysis of S-fatty-acylated proteins in HeLa epithelial cells.

To evaluate the generality of our proteomic methods, we employed the alk-16 +/- NH₂OH chemical reporter method and acyl-RAC to selectively identify S-fatty-acylated proteins in HeLa epithelial cells (Figure 3A-B, SI Figure S10 and Table S4). Similar numbers of proteins were identified in HeLa cells compared to RAW264.7 macrophages (Figure 3C) and 58 proteins were detected by both methods with medium to high confidence. Of note, this number might appear to be low and depends on the thresholds chosen for the two-sample tests. Here, severe thresholds have been applied to minimize the number of false positive hits. The specificity and ability of both acyl-RAC and chemical reporter methods to detect known and candidate S-fatty-acylated proteins were assessed by western blot analysis (Figure 3D-E, SI Figure S11). CANX, RAS (NRAS/KRAS) and IFITM3 are well-characterized S-fatty-acylated proteins that were detected by both methods with medium to high confidence. β -Tubulin³⁹ and VCP^{40,41} were previously suggested to be S-fatty-acylated using acyl-RAC or chemical reporter (alk-16/DMSO only). Sequestosome-1 (SQSTM1 gene), also known as p62, is a key protein in the autophagy pathway, that was highly enriched in the acyl-RAC experiment and appeared to be labelled with alk-16 but not NH₂OH sensitive. Enrichment of alk-16 labeled CANX, RAS (NRAS/KRAS) and IFITM3 is sensitive to NH₂OH treatment, suggesting alk-16 labels these proteins via a thioester linkage on Cys, as expected for S-fatty-acylated proteins. Of note, the small portion of enrichment persisting after NH₂OH treatment could be attributed to incomplete NH₂OH or S- to N-transfer during sample preparation.³⁷ Proteins circled in black (VCP, p62 and β -tubulin) are candidate S-fatty-acylated proteins in HeLa cells. While they are enriched by the acyl-RAC method, they are weakly labeled with alk-16 chemical reporter and are not sensitive to NH₂OH treatment. These results confirmed the proteomics analysis and suggest validation of candidate S-fatty-acylated proteins should be performed using both methods. Importantly, comparing alk-16 to alk-16 + NH₂OH appeared to be the most accurate method to identify S-fatty-acylated proteins and preferable for the direct identification of S-fatty-acylated sites. However, it is important to note that alk-16 labeling in cells is often short (4 h) and S-fatty-acylated proteins with a low turnover might be poorly labeled with alk-16. This could explain why some known S-fatty-acylated proteins are missing from the labeling alk-16 experiments.

Direct identification of protein S-fatty-acylation sites

Most methods for the indirect identification of S-fatty-acylation sites report up to hundreds of sites.^{20,22–25,27,28} However, these studies are not selective for S-fatty-acylation as they only enrich NH_2OH -sensitive S-acylated peptides. Additionally, most of the candidate S-acylation sites originate from soluble proteins and these methods often fail to identify S-fatty-acylation sites adjacent to transmembrane domains that often occur on integral membrane proteins. We thus developed a protocol for S-fatty-acylation site identification based on alk-16 enrichment and selective cleavage of thioester bonds with NH_2OH (Figure 4A). With this protocol, S-fatty-acylated proteins are metabolically labeled with alk-16, cells are lysed and alk-16 labeled proteins are biotinylated with azido-biotin via CuAAC reaction. Free Cys are capped with NEM, proteins are digested, and biotinylated proteins are enriched on Neutravidin beads. Thioesters are selectively hydrolyzed with NH_2OH and alkylated again with iodoacetamide to prevent subsequent oxidation, before being analyzed by LC-MS/MS. Any Cys modified by iodoacetamide will then be marked as a S-fatty-acylation site.

We initially optimize this protocol using three known S-fatty-acylated proteins, CANX, RAS and IFITM3 (SI Figure S12-14). After optimizing the NEM capping, biotinylation, Neutravidin enrichment and NH_2OH elution conditions (SI Figure 12-14), we found that inclusion of Rapigest, a MS-compatible detergent was essential for efficient thioester hydrolysis, solubility and final elution of transmembrane proteins (CANX and IFITM3) from the beads (SI figure S14A). However, elution of RAS from beads was not complete, suggesting that alk-16 could have been metabolized or incorporated to another residue than Cys, consistent with recent report of Ne-Lys fatty-acylation of Ras isoforms⁴². VCP was used as a control to confirm that the elution was thiol-specific (Figure 3). As expected, while VCP can be labeled by alk-16 (Figure 3D), it was not eluted following NH_2OH treatment.

We then applied this optimized workflow to detect S-fatty-acylation sites by LC-MS/MS (SI Figure S15, SI Tables S5-6). Proteins in cell lysates were either digested before enrichment or after NH_2OH -elution with trypsin or chymotrypsin. We envisioned that the use of chymotrypsin would be advantageous to identify S-fatty-acylation sites of insoluble proteins where the modification is located in or in proximity to a transmembrane domain. This region of a protein might not contain any Arg and Lys, as required for trypsin specificity, yielding large peptides that might be difficult to identify by LC-MS/MS. Chymotrypsin cleaves proteins non-specifically after several hydrophobic amino acids, Phe, Tyr, Trp, Met, Leu. Score and delta score thresholds were applied for confident site identification and localization (70 and 50 respectively were applied for trypsin digest and 50 and 30 for chymotrypsin digest). This direct site identification protocol combined with trypsin digest provided a list of S-fatty-acylation sites for 156 proteins, 35 of which were identified in the alk-16 + NH_2OH experiment (SI table S4). These iodoacetamide marked sites, as expected, belong to soluble S-fatty-acylated proteins. The list includes some known S-fatty-acylated sites reported in Uniprot or recently published, including S-fatty-acylation sites of GNAS, GNAQ, GOLIM4,⁷ GNA13 and SNAP23 (Figure 4B, SI Figure S16, SI Table S5). Digestion with chymotrypsin yielded 137 modified peptides from 44 proteins, 19 of which were identified in the alk-16 + NH_2OH (SI Table S4). Some known S-fatty-acylation sites were identified, including sites for IFITM3,¹⁰ FLOT1,⁴³ Caveolin-1,⁴⁴ STOM,⁴⁵ and IFITM1⁴⁶

(SI Figure S17, SI Table S6). As expected, both enzymes produced distinct list of peptides due to different peptide length and solubility. Only a few sites, such as for caveolin-1 (Figure 4C) and FLOT1, were identified using both enzymes. Combining both dataset, S-fatty-acylation sites of 44 out of the 106 proteins (high, medium and low confidence) identified with the chemical reporter method were detected. For some proteins, all reported sites were identified such as for SNAP23²⁰ or caveolin-1⁴⁴ (SI Table S5-6) while for others, such as IFITM3, direct iodoacetamide modification of Cys105 was not observed and could be due to low levels of modification as previously suggested³⁰. As we suspected missing sites could also be lost during the LC-MS/MS analysis due to their high hydrophobicity, we compared two LC methods, one increasing to 50% acetonitrile in 91 min and the other increasing to 90 % acetonitrile in 67 min. While this modification of LC gradient decreased the retention time of peptides, it did not increase the number of identified sites. These results demonstrate our optimized protocol that integrates alk-16 labeling, NH₂OH treatment, enrichment and iodoacetamide tagging enables direct detection and robust analysis of S-fatty-acylation sites.

Direct analysis of IFITM3 S-fatty-acylation

Given our laboratory interest in IFITM3 S-fatty-acylation and antiviral activity,^{10,30,38,47} we sought to directly characterize fatty acids that are covalently attached to the Cys residues of IFITM3 (Figure 5A). IFITM3 comprises two S-fatty-acylation sites (C71 and C72) in proximity of a intramembrane domain, which were identified by our direct method (Figure 5B), and another site adjacent to a transmembrane domain (C105).^{10,48} Our recent analysis of IFITM3 S-fatty-acylation by acyl-PEG exchange (APE) and alk-16 labeling revealed that C72A was the major site of S-fatty-acylation.³⁰ Since trypsin digest of IFITM3 results in large hydrophobic peptides (>29 amino acids), it appeared advantageous to digest IFITM3 with chymotrypsin, yielding 8–10 amino acid long peptides (SI Figure 18). Due to the predicted hydrophobicity of the lipidated peptides, we first performed preliminary experiments to test whether synthetic S-palmitoylated peptides could be detected by LC-MS/MS. IFITM3 peptides MNPCCCLGF (C71/72 palmitoylation sites) and ASTAKCLNIW (C105 palmitoylation site) were synthesized by solid-phase peptide synthesis, reduced and alkylated for LC-MS analysis. Peptides were used without further purification and therefore comprised a mixture of unmodified and mono/doubly palmitoylated peptides (MNPCCCLGF, MNPCC(+palm)LGF, MNPC(+palm)CLGF and MNPC(+palm)C(+palm)LGF or ASTAKCLNIW and ASTAKC(+palm)LNIW), reflecting the potential mixture from biological samples (Figure S19). The S-palmitoylated peptides were highly hydrophobic and were not soluble in most solvents. Nonetheless, solubilization in formic acid (FA) was effective and the samples were then diluted with water and acetonitrile (ACN) to yield 50% ACN, 20% FA. Of Note, long incubation times in 100% FA or 20% FA resulted in formylation, oxidation or degradation of the peptides (SI Figure S20). LC-MS was then performed in the positive ion mode and MS/MS analysis were achieved using CID fragmentations. Thioester linkage appeared to be stable under low energy CID conditions since CID was successfully used to identify S-palmitoylated peptides.^{49,50} While CID was also shown to cause partially loss of S-palmitoylation, MNPCCCLGF (mono or doubly-palmitoylated) were detected as singly charged ions, while the doubly charged ions gave the strongest signal for ASTAKC(+palm)LNIW (SI Figure S21). As expected, the retention time of the peptides increased together with the S-palmitoylation status. All S-palmitoylated

peptides were detected by nanoflow in MS1 but MS2 was only detected for ASTAKC(+palm)LNIW. Other peptides did not fragment well in MS2. Nonetheless, these conditions could be applied to identify IFITM3 S-palmitoylated peptides in biological samples.

To characterize IFITM3 S-fatty-acylation from mammalian cells, human HA-IFITM3 was expressed in HEK293T cells and immunoprecipitated using anti-HA beads. Following washes, IFITM3 was either eluted from the beads followed by in-solution digestion or separated by SDS-PAGE and subjected to in-gel digestion before LC-MS/MS analysis (SI Table S7 and SI Figure S22). Of note, some of the identified peptides contained miscleavages, which is not surprising for chymotrypsin digest as this enzyme is known to be unspecific. The IFITM3 peptide ASTAKC(+palm)LNIW was identified from both in-gel and in-digestion samples (SI Figure S23 and 24 and SI Table S7). Peptide SLFNTLFMNPcc(+palm)LGFIAF was also identified with lower confidence in the in-solution digest sample. Peptide MNPc(+palm)c(+palm)LGF was only found with low confidence in the in-solution digest sample, probably due to poor MS/MS fragmentation or low abundance in the sample. Solubility of the S-palmitoylated peptides was suspected to be an issue during sample preparation and chymotrypsin digest, therefore, we tested whether adding a detergent could increase peptide identification by LC-MS/MS. Invitrosol is a proteomics compatible detergent which elutes in three peaks and well separated from the elution times of most peptides. It can be used to solubilize and denature hydrophobic proteins at concentrations that do not inhibit digestion enzymes such as chymotrypsin. Moreover, Invitrosol can improve peptide recovery and identification when used for the sample resuspension before LC-MS/MS analysis.⁵¹ IFITM3 was in-gel digested in the presence of Invitrosol and analyzed by high flow LC-MS/MS (SI Table S8). Many IFITM3 peptides containing miscleavages or additional modifications (Met oxidation) of peptide MNPCCCLGF (C71/72 of IFITM3) were identified (SI Figure S25, SI Table S8). Notably, peptides bearing palmitate on C72 were detected with a higher intensity in MS1 and higher confidence/score than peptides bearing palmitate on C71 (Figure 5C, SI Figure S25). These peptides are expected to have similar properties and should have similar solubility and fragmentation behaviors, which suggests that S-palmitoylation of IFITM3 at C72 is more prevalent than S-palmitoylation at C71, consistent with our previous indirect studies using alk-16 and APE³⁰. Peptide ASTAKC(+palm)LNIW containing C105 was also identified in the sample with lower confidence (SI Figure 26 and SI Table 8). As other lipids have been suggested to be incorporated onto Cys residues,^{5,52,53} we also searched the data to include other lipid modifications such as myristate (C14), oleate (C18:1), stearate (C18) and palmitoleate (C16:1). Interestingly, peptide MNPCCCLGFIAF modified by oleate or palmitoleate, most likely on C72, was also detected at low levels in the sample (SI Figure S26). These experiments demonstrate palmitate is the predominant fatty acid that is covalently attached to C71, C72 and C105 of IFITM3, with C72 being the major site of S-palmitoylation.

CONCLUSIONS

In this study, we optimized and compared two methods widely employed to identify S-fatty-acylated proteins by MS-based proteomics. While these methods identified an overlapping

list of fatty-acylated proteins, only alk-16 chemical reporter combined with NH_2OH specifically and robustly identified S-fatty-acylated proteins. Alk-16 chemical reporter labeling alone gave a list of S/N/O-fatty acylated proteins, while acyl-RAC provided a list of S-acylated proteins. Acyl-RAC identifies any proteins with a thioester modification and is often misused in the literature to validate S-fatty-acylated proteins. It would be preferable, when validating new S-fatty-acylated proteins, to use acyl-RAC and alk-16 (+/- NH_2OH) in combination.

Our new protocol for the identification of S-fatty-acylation sites, based on alk-16 labeling and enrichment combined with NH_2OH sensitivity and subsequent iodoacetamide tagging, is more specific than previously reported methods,^{20,22–28} which were mostly based on acyl-RAC. The use of different enzymes, trypsin and chymotrypsin, appeared advantageous to identify S-fatty-acylation sites of both soluble and insoluble proteins. In HeLa cells, sites for 44 proteins out of the 106 S-fatty-acylated proteins were identified. Dually lipidated peptides remain challenging to identify and modified peptides of some well-characterized S-fatty-acylated proteins such as CANX were not found. These hydrophobic modified peptides could be lost at different steps of the workflow. Some proteins might aggregate following chloroform/methanol precipitation,⁵⁴ alkylation with iodoacetamide following NH_2OH treatment could be difficult due to peptide precipitation, and highly hydrophobic peptides could be difficult to solubilize for LC-MS/MS analysis. Additionally, as chymotrypsin digest is non-specific, it could produce multiple modified peptides with various mis-cleavages that might be difficult to identify due to their low abundance. The list of modified peptides might still contain some false positive hits due to incomplete NEM capping. However, it could also include sites for S-fatty-acylated proteins which were not identified at the protein level using the alk-16 + NH_2OH method due to the different sensitivity and confidence thresholds applied. For instance, some modification sites of proteins known to be S-fatty-acylated (Uniprot), such as PLSRC1/3, were identified, while these proteins were not found at the protein level. Nonetheless, the methods we describe here still provides a robust protocol for identifying and profiling many protein S-fatty-acylation sites from mammalian cells.

To more thoroughly characterize IFITM3 S-fatty-acylation, we developed conditions to analyze fatty-acylation of Cys residues that should be generally applicable to integral membrane proteins. The use of S-palmitoylated peptide standards appeared essential to optimize the LC-MS/MS conditions and provided essential insight on peptide solubility and stability. Immunoprecipitation of HA-IFITM3 followed by chymotryptic digestion provided identification of mono-S-palmitoylated peptides, confirming that IFITM3 is indeed modified with palmitic acid C71, C72 and C105, and that C72 is the predominant site of modification.^{10,30} Additionally, our results confirm oleic acid can be added onto Cys residues, as reported in our recent proteomics study identifying substrates of alkyne-tagged analogues of oleic acid.⁵² IFITM3 is a good model to optimize detection of hydrophobic peptides, and applying similar sample preparation and LC-MS/MS conditions to other biological samples could allow the direct identification of S-palmitoylated peptides of other transmembrane proteins. High flow LC-MS/MS, combined with the use of Invitrosol as a detergent, provided the best results, although doubly S-palmitoylated peptide MNP(C(+palm)C(+palm)LGF was difficult to detect using this method. While the peptide was detected using our direct site ID method (Figure 5B), it was only found with low confidence (SI Table S7), probably due to its weak

fragmentation in the CID mode, low abundance in the biological sample or sample loss during sample preparation due to high hydrophobicity. Use of another MS/MS fragmentation method⁵⁵ or different LC conditions (C4 column or different buffer) might improve the detection of the dually lipidated peptides in the future.

S-fatty-acylation (S-palmitoylation) is important in physiology and disease, however, the diversity, abundance, and regulatory mechanisms of S-fatty-acylation *in vivo* are not fully understood. Notably, the specific sites and precise fatty acid composition of S-fatty-acylated/S-palmitoylated proteins are still challenging to characterize for individual proteins and proteome-wide. The improved methods described here should facilitate more precise analysis of S-fatty-acylation and should enable quantitative studies to explore the functions and regulation of this crucial fatty acid modification in eukaryotes and plants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We thank Hang lab members for helpful discussions and the Proteomics Resource Center of The Rockefeller University for mass spectrometry analysis and peptide synthesis.

Funding Sources

This work was supported by a Marie Skłodowska-Curie Individual Fellowship (“QuantPalm_immunity”) to E.T. H.C.H. acknowledges grant support from NIH-NIGMS R01GM087544.

ABBREVIATIONS

alk-16	alkyne-tagged analogue of palmitic acid
CuAAC	Cu(I)-catalyzed azide alkyne cycloaddition
GPI	Glycophosphatidylinositol
HPDP	(3aS,4S,6aR)-hexahydro-2-oxo-N-[6-[[1-oxo-3-(2-pyridinyldithio)propyl]amino]hexyl]-1H-thieno[3,4-d]imidazole-4-pentanamide
LPS/IFN-γ	lipopolysaccharides/interferon-gamma
NEM	N-ethylmaleimide
wt	wild-type

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Figure 1. Methods to enrich and identify S-fatty-acylated, alk-16 labeled and S-acylated proteins by proteomics.

(A) With the chemical reporter labeling, cells are treated with alk-16, an alkyne-tagged reporter of saturated fatty acids. Following cell lysis, modified proteins can be reacted with an azide-functionalized reagent via biorthogonal labeling and captured on Neutravidin™ beads. Lysate subjected to NH₂OH treatment or cells treated with DMSO are used as a negative control. Statistical comparison of samples enables the selective identification of candidate S-fatty-acylated, N/O-alk-16 labeled and alk-16 labeled proteins. (B) In the acyl-RAC method, following cell lysis, free Cys are capped with N-ethyl maleimide (NEM) in the presence of TCEP. Acylated Cys are hydrolyzed with hydroxylamine (NH₂OH) and reacted with the thiopropyl sepharose resin. Lysate not subjected to NH₂OH treatment is used as a negative control. Comparison of proteins enriched in the two different conditions as indicated by the two-headed arrows enabled the identification of candidate S-acylated proteins.

Figure 2. S-fatty-acylated proteins in naïve and LPS/IFN- γ stimulated RAW264.7 macrophages. (A-B) Volcano plot showing the results of the two-sample test (FDR = 0.05, s0 = 1) for the alk-16 chemical reporter strategy experiment (n = 4 replicates, LFQ quantification). (A) alk-16/DMSO and (B) alk-16/(alk-16+NH₂OH). Proteins reported to be S-fatty-acylated or GPI-anchored in Uniprot are shown in red and green, respectively. N-myristoylated proteins³⁴ are shown in blue. Dually lipidated proteins (N-myristoylated and S-fatty-acylated) are shown in red. Putative S-fatty-acylated proteins, identified with high, medium or low confidence are shown in dark grey (Supporting Information, experimental procedures). All other proteins are shown in light grey. (D) Venn diagram showing the number of proteins identified with medium and high confidence in experiments A, B, E. (D) Table showing a subset of proteins identified in experiments shown in A, B, E. The four first columns indicate if proteins have been reported to be S-palmitoylated (palm), N-myristoylated (myr), GPI-anchored (GPI) or were found in previous proteomics experiments

(prev prot)²⁶. For the chemical reporter or acyl-RAC experiments, the means of log₂ LFQ intensities and MS/MS counts, as well results of the two-sample tests (“+” = significant) are shown. “MS ratio” corresponds to the ratio of the mean MS/MS counts of the two samples being compared, and was used to further sort the data (“MS ratio” ≥ 5 was attributed to a high confidence hit (HC), $2.5 < \text{“MS ratio”} < 5$ to medium confidence and $1 < \text{“MS ratio”} < 2.5$ to low confidence). (E) Volcano plot showing the results of the two-sample test (FDR = 0.01, $s_0 = 1$, + NH₂OH versus – NH₂OH) for the acyl-RAC method (n= 4 replicates, LFQ quantification). Proteins shown in red are reported as S-fatty-acylated in Uniprot. Candidate S-fatty-acylated proteins, identified with high, medium or low confidence are shown in dark grey (Supporting Information, experimental procedures). All other proteins are shown in light grey. (F-G) Quantitative analysis of S-fatty-acylated proteome following LPS/IFN- γ stimulation. Volcano plot showing the statistical significance (FDR = 0.05, $s_0 = 1$) of changes in enrichment levels between stimulated and naïve cells as a function of the t-test difference for (F) the chemical reporter method or (G) acyl-RAC.

Figure 3. S-fatty-acylated proteins in HeLa cells.
(A) Volcano plot showing the statistical significance of enrichment level changes +/- NH2OH as a function of the t-test difference for (A) the chemical reporter method (p =0.01, s0=1) or (B) acyl-RAC (p =0.001, s0=1). (C) Venn diagram showing the number of proteins identified with medium and high confidence in experiments A-B. (D) Table showing a subset of proteins identified in experiments shown in A and B. The four first columns indicate if proteins have been reported to be S-palmitoylated (palm). For the chemical reporter or acyl-RAC experiments, the means of log2 LFQ intensities and MS/MS counts, as well results of the two-sample tests (“+” = significant) are shown. “MS ratio” corresponds to the ratio of the mean MS/MS counts of the two samples being compared, and was used to further sort the data (“MS ratio” > 5 was attributed to a high confidence hit (HC), 2.5 “MS ratio” < 5 to medium confidence and 1 < “MS ratio” < 2.5 to low confidence). (E-F) S-fatty-acylation of known and candidate S-fatty-acylation proteins were assessed by the chemical

reporter (D) and acyl-RAC (E) methods combined with western blot analysis. (D) alk-16 chemical reporter. Beads = 5x Input or Supernatant (Sup), except for VCP and p62: Beads = 10x Input or Sup. E) Acyl-RAC. alk-16 chemical reporter. Beads = 2x Input or Sup except for β -tubulin where Beads = 4x Input or Sup.

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