A bioorthogonal chemical reporter for fatty acid synthase-dependent protein acylation

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Summary

Cells acquire fatty acids from dietary sources or via *de novo* palmitate production by fatty acid synthase (FASN). Although most cells express FASN at low levels, it is upregulated in cancers and during replication of many viruses. The precise role of FASN in disease pathogenesis is poorly understood, and whether *de novo* fatty acid synthesis contributes to host or viral protein acylation has been traditionally difficult to study. We describe a cell permeable, click-chemistry compatible alkynyl-acetate analog (Alk-4) that functions as a reporter of FASN-dependent protein acylation. In a FASN-dependent manner, Alk-4 selectively labeled the cellular protein interferon-induced transmembrane protein 3 (IFITM3) at its palmitoylation sites, and the HIV-1 matrix protein at its myristoylation site. Alk-4 metabolic labeling also enabled biotin-based purification and identification of more than 200 FASN-dependent acylated cellular proteins. Thus, Alk-4 is a useful bioorthogonal tool to selectively probe FASN-mediated protein acylation in normal and diseased states.

Keywords: fatty acid synthase, click chemistry, S-palmitoylation, N-myristoylation, HIV-1, Influenza, fatty acylation, IFITM3, HIV-1 Gag, CD9.

Introduction

Long chain fatty acids (FA) are essential components of lipid bilayers, are used to store energy liberated by β-oxidation, and are covalently attached to proteins to increase hydrophobicity and regulate subcellular localization.[1] Long chain fatty acids can be obtained exogenously through dietary sources, or endogenously via *de novo* fatty acid biosynthesis.[2] Mammalian fatty acid synthase (FASN) is a 272 kDa cytosolic enzyme that catalyzes the complete *de novo*

synthesis of palmitate from acetyl-CoA and malonyl-CoA. The final product, palmitic acid (16:0) is then released from FASN, where it can be metabolized by β-oxidation into myristic acid (14:0), or other long chain FA.[3] FASN expression is highly regulated in cells and its expression can change dramatically in response to stresses such as starvation, lactation or pathological states.[3] Increased *de novo* FA biosynthesis and FASN up-regulation have been observed in breast cancer, melanoma, and hepatocellular carcinoma.[4] Studies of enveloped viruses including hepatitis B virus,[5] Dengue virus,[6] Epstein-Barr virus,[7] hepatitis C virus,[8] HIV-1,[9] Chikungunya virus,[10,11] and West Nile virus[12,13] indicate that many viruses both upregulate and require host FASN activity for effective replication. The contributions of *de novo* synthesized FA to post-translational modifications of viral and host proteins remains understudied.

Identification of protein acylation has been challenging due to the lack of antibodies against lipid modifications, and inefficiencies of standard mass spectrometry techniques to identify acylated proteins.[14] While protein myristoylation site prediction is facilitated by a consensus sequence motif on nearly all myristoylated proteins (Met-Gly-XXX-Ser/Thr),[1] protein palmitoylation site prediction remains challenging due to the lack of a consensus sequence.[15] To measure acyl-group synthesis mediated by FASN and the fate of the *de novo* synthesized fatty acids, one must use ^{14}C labeled acetate, which suffers from low detection sensitivity, general complications associated with radioisotope work,[16] and an inability to selectively enrich acylated proteins. Over the last decade, bioorthogonal labeling and detection of protein fatty acylation using click chemistry compatible analogs of palmitate and myristate have provided quick and sensitive methods for detection of protein acylations.[17,18] The copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions enable labeling of cells with alkynyl analogs of fatty acids that can be reacted with azides conjugated to suitable detection tags, such as fluorophores, or affinity tags, including biotin.[19,20] Although very useful, palmitate and myristate analogs only measure the acylation state of proteins modified by the exogenous chemical

reporters. Given the critical role of FASN-dependent *de novo* synthesized fatty acids in cancer, metabolic disorders, and viral replication, we posit that a bioorthogonal reporter of FASN-dependent protein acylation will facilitate a better understanding of the contributions of FASN-dependent protein fatty acylation to protein function, protein localization, and FASN-mediated pathogenesis. Here we demonstrate the utility of 5-hexynoic acid, or Alk-4, a cell permeable, click-chemistry compound that labels proteins acylated by products of FASN-mediated *de novo* fatty acid biosynthesis.

Results

Alk-4 labels palmitoylated proteins at known palmitoylation sites. Bioorthogonal reporters such as alkynyl palmitate ("Alk-16") and alkynyl myristate ("Alk-12") are substrates of palmitoyltransferase and myristoyltransferase activity that are often used to identify palmitoylated and myristoylated proteins.[21–23] Because these reporters mimic the end product of FASN activity (palmitate) or palmitate oxidation (myristate), Alk-12 and Alk-16 cannot be used to determine the source of the fatty acyl adduct (i.e. exogenous/imported or endogenous/*de novo* synthesized). We hypothesized that a cell permeable, bioorthogonal mimic of a putative FASN substrate, 5-hexynoate (termed Alk-4 here)[24] could be used to study the contributions of FASN-mediated *de novo* fatty acid synthesis to protein acylation (Figure 1a,b). To determine whether Alk-4 selectively labels palmitoylated proteins, we tested whether a known palmitoylated protein, IFITM3, was labeled upon a 24 hour treatment of cells with alkynyl acetate analogues of different carbon chain lengths, Alk-3 and Alk-4, in comparison with the well-established palmitoylation reporter Alk-16 (Figure 1a, b). Alk-16 robustly labeled IFITM3 as detected by click-chemistry tagging of immunoprecipitated IFITM3 with azido-rhodamine and fluorescence gel scanning. Alk-4 also successfully labeled IFITM3, while Alk-3

showed minimal labeling (Figure 2a). Next, we tested whether Alk-4 labeling of IFITM3 occurred on its known palmitoylated cysteines.[22] A triple cysteine to alanine palmitoylation-deficient mutant of IFITM3 (termed PalmΔ), was not labeled by either Alk-16 or Alk-4 (Figure 2b). To further test the ability of Alk-4 to label palmitoylated proteins, we examined whether the tetraspanin CD9, which has 6 palmitoylated cysteines, was also labeled by Alk-4. Similar to IFITM3, CD9 was labeled by Alk-4, while a mutant CD9 in which its palmitoylated cysteines were mutated to alanine (termed CD9-PalmΔ), was not labeled (Figure 2c). These results indicate that Alk-4 is metabolized into a click-chemistry functionalized fatty acid adduct that is specifically incorporated onto protein palmitoylation sites.

Alk-4 metabolism provides a substrate used by DHHC palmitoyltransferases. Many proteins are reversibly palmitoylated at cysteine residues[25] by aspartate-histidine-histidine-cysteine (DHHC) palmitoyltransferases. DHHC palmitoyltransferases primarily use palmitoyl-CoA (C16:0) to modify cysteine residues on proteins, although DHHC's can tolerate substrates with carbon chain lengths as short as 14 and as long as 20.[26,27] Acyl chains with fewer than 14 carbons have not been detected on cysteines, indicating that DHHC enzymes disfavor short chain fatty acids as substrates.[28–31] Given the selectivity of the DHHC palmitoyltransferases for long chain fatty acids, we sought to determine whether labeling of IFITM3 by Alk-4 was affected by DHHC7 overexpression, which was previously shown to be among the enzymes that can catalyze IFITM3 palmitoylation.[32] In cells incubated with Alk-4, DHHC7 overexpression increased IFITM3 labeling, while overexpression of a dominant negative DHHC7 mutant decreased IFITM3 labeling (Figure 2d). These results indicate that Alk-4 is metabolized into a long chain fatty acid that can be used as a substrate by DHHC palmitoyltransferases for protein palmitoylation.

Labeling of IFITM3 by Alk-4 requires FASN. We have previously shown that IFITM3 palmitoylation is required for its antiviral activity against influenza virus infection.[22,33] To determine if FASN-mediated *de novo* fatty acid biosynthesis contributes to an IFNβ-regulated IFITM3-mediated antiviral response, we measured endogenous IFITM3 labeling by Alk-4 in wild-type (WT) and FASN knockout HAP1 cells. As expected, IFNβ induced endogenous IFITM3 expression, and IFITM3 upregulation was independent of FASN expression (Figure 2e). In WT cells, Alk-4 treatment resulted in robust endogenous IFITM3 labeling that was absent in FASN-deficient cells (Figure 2f). Thus, we show for the first time that FASN contributes to the palmitoylation of endogenous IFITM3. Owing to the observations that IFITM3 is required for an effective IFNβ-mediated anti-influenza response,[32] that palmitoylation of IFITM3 is required for its antiviral activity[32], and that FASN regulates Alk-4 mediated IFITM3 palmitoylation (Figure 2f), we sought to determine the effect of FASN expression on IFNβ-mediated inhibition of influenza virus infection. In the absence of IFNβ, FASN expression had no effect on influenza infection (Figure 2g). However, IFNβ-mediated inhibition of influenza virus infection was significantly decreased in the absence of FASN expression (Figure 2g), suggesting that FASN-dependent palmitate synthesis likely contributes to the palmitoylation-dependent antiviral activity of IFITM3.

Alk-4 labeling of myristoylated proteins is FASN dependent. Acetyl CoA is condensed with malonyl-CoA and elongated by FASN to generate palmitate for protein palmitoylation. To generate myristoyl CoA for myristoylation, palmitoyl CoA must be β-oxidized to myristoyl CoA before it is covalently attached to glycine residues by N-myristoyl transferases (Figure 4d). [34,1] To determine if Alk-4 is metabolized into a fatty acid analog that can selectively label myristoylated proteins, we tested whether a known myristoylated protein, HIV-1 matrix protein (MA), was labeled upon a 24-hour incubation with Alk-4. HEK293T cells were transfected with flag-tagged HIV-1 MA, or the myristoylation deficient matrix-G2A mutant (MA-G2A) and treated

with Alk-4 or Alk-12 (an established chemical reporter of myristoylation). Immunoprecipitation of flag-tagged matrix and subsequent click reaction with azido-rhodamine revealed labeling of HIV-1 matrix in cells incubated with Alk-4 or Alk-12 (Figure 3a). The G2A-MA Gag protein, which cannot be myristoylated, was not labeled by Alk-4, indicating myristoylation site-specific labeling of HIV-1 matrix protein by Alk-4. Treatment of cells with Fasnall,[9,35] a FASN inhibitor, abolished Alk-4 labeling of HIV-1 matrix protein. As a control, Fasnall treatment did not disrupt HIV-1 matrix protein labeling by Alk-12. To confirm the selective labeling of HIV-1 matrix protein that we observed with fluorescence-based click reactions, cell lysates were instead reacted with biotin azide. Biotin-conjugated proteins were precipitated with streptavidin agarose and bound proteins were released with sodium dithionite, which cleaves a diazo linker within the azido-biotin molecule, enabling selective elution of Alk-4 labeled proteins. Eluents were probed for the MA-Flag proteins, and, in the presence of Alk-4, HIV-1 matrix protein was recovered. HIV-1 matrix protein recovery was diminished both when a myristoylation deficient HIV-1 matrix protein variant was transfected (MA-G2A) (Figure 3b) and when FASN was inhibited by Fasnall. These results indicate that Alk-4 is metabolized into a fatty acid adduct that is specifically incorporated onto protein myristoylation sites in a FASN-dependent manner, and that can be detected by multiple labeling modalities.

FASN-dependent, Alk-4 mediated metabolic labeling of endogenous fatty acylated proteins. To test the utility of Alk-4 as a global indicator of FASN-dependent protein acylation, we incubated the human fibroblast-like cell line HAP1 or a FASN-deficient clone of the HAP1 cells with Alk-4 or a vehicle control (DMSO). Following metabolic labeling of HAP1 cells with Alk-4, cell lysates were reacted with azido-biotin and labeled proteins were precipitated as described in Figure 3b. Eluents were then probed for proteins known to be palmitoylated (Calnexin)[36] or myristoylated (Src).[37,38] In wild-type HAP1 cells, Alk-4 labeling recovered both Calnexin and Src, while in FASN-deficient cells, incubation with Alk-4 did not enable Calnexin

158 or Src recovery (Figure 4a). To determine the breadth of proteins recovered from cells
159 incubated with Alk-4, we next used mass spectrometry to identify biotinylated proteins from
160 HAP1 cells with or without Alk-4 and with or without FASN. FASN was only recovered from WT
161 HAP1 cells incubated with Alk-4, consistent with the acyl intermediates formed between FASN
162 and the elongating fatty acid chain (Figure 4d).[39] This experiment also recovered, in an Alk-4
163 dependent manner, several enzymes involved in the metabolism of acetate to palmitate and
164 myristate, including acetyl CoA synthetase (ACSA), acetyl CoA carboxylase 1 (ACACA), and
165 multiple enzymes involved in fatty acid beta-oxidation (Figure 4d, Supplementary table 2). In
166 total, Alk-4 labeling enabled recovery of 264 proteins in an Alk-4 and FASN-dependent manner
167 (Figure 4b). Of these, 77% (203) have previously been identified in at least one palmitoyl
168 proteome or they have been experimentally validated to be palmitoylated. These included well
169 characterized palmitoylation substrates, such as Guanine nucleotide-binding protein G
170 (GNAI1) and Catenin beta-1 (CTNB1) (Figure 4c, Supplementary table 1 & 3). Of the
171 remaining proteins that were purified, 17% were predicted to be palmitoylated (e.g. SAM
172 domain and HD domain-containing protein 1 [SAMHD1]), and 3% were predicted to be
173 myristoylated (e.g. ribosomal protein S6 kinase alpha [KS6A1])[40,41] (Figure 4c, Supplementary
174 Table 1 & 4).

175

176 **Discussion**

177 We describe a click-chemistry compatible FASN-substrate, Alk-4 (5-hexynoate), which
178 selectively labels both palmitoylated (*e.g.* IFITM3, CD9) and myristoylated (*e.g.* HIV-1 matrix)
179 proteins. Click chemistry compatible substrate analogs like Alk-4 overcome several inherent
180 disadvantages of radiolabeling, such as long sample processing and film exposure times with
181 low sensitivity.[16] Moreover, click chemistry reactions can be combined with several detection

methods that are compatible with high throughput applications including mass spectrometry-based proteomics, flow cytometry, fluorescence microscopy, and live cell imaging.[21] Beyond identification of FASN-dependent protein acylation, Alk-4 has more functionality than a radiolabeled FASN substrate because it can be reacted with azido-biotin to facilitate streptavidin-based purification of FASN-dependent acylated proteins. We are not the first to suggest the utility of Alk-4, which has previously been evaluated as a chemical tool to monitor protein acetylation at shorter timescales, although it was noted that some of the acetylation reporters were incorporated onto proteins by chemical acylation.[24] Nevertheless, our finding that FASN activity is required for Alk-4 labeling of multiple proteins such as IFITM3, HIV-1 matrix, Calnexin, and Src strongly supports the use of Alk-4 as a selective reporter of FASN-dependent protein acylation.

FASN activity is required for replication of several enveloped viruses, including Chikungunya,[10] HIV-1,[9] Influenza,[42] and SARS-CoV-2,[43] and many others.[6,7,13] FASN inhibitors, including Fasnall[9,35] and the TVB compounds[44,45] have therapeutic potential, and pharmacological inhibition of FASN has been shown to modulate fatty acylation of viral proteins, including Chikungunya virus nsP1 palmitoylation,[46] SARS-CoV-2 spike palmitoylation,[43] and HIV-1 Gag myristoylation (Figure 3). In other cases, modulation of FASN activity affects host proteins that regulate infection, including MYD88 palmitoylation,[47] and the results presented here that reveal that FASN activity is required for an effective IFNβ immune response against influenza virus, possibly by providing fatty acyl moieties for modification of IFITM3. Increased *de novo* FA biosynthesis and FASN up-regulation has also been observed in breast cancer, melanoma, and hepatocellular carcinoma[4], and *de novo* fatty acid biosynthesis and lipogenesis has been shown to be essential for protein palmitoylation of Ras, Wnt[48], Calnexin[49], and Src[50] in proliferating cells.[51,52] In tumor cells, FASN inhibition can have consequences beyond

inhibition of protein acylation[53]; *de novo* fatty acid biosynthesis has also been shown to be essential for membrane remodeling in tumor cells, where palmitate depletion via FASN inhibition led to disruption of lipid rafts and signaling pathways, ultimately resulting in apoptosis of tumor cells.[54] Thus, *de novo* fatty acid biosynthesis is a broadly utilized, fundamental metabolic pathway exploited during carcinogenesis and virus replication, and Alk-4 and its ability to measure flux through the *de novo* FASN pathway provides a new tool to better understand the role of FASN-dependent protein acylation during FASN-dependent pathologies.

METHODS

Reagents, transfections, and infections. Reagents, including 5-hexynoic acid (Alk-4), were purchased from Sigma (St. Louis, MO) or Thermo Fisher Scientific, unless stated otherwise. Alk-12 and Alk-16 were synthesized by the Hang lab according to published protocols.[21] Alk compounds were diluted in dimethyl sulfoxide (DMSO). HEK 293Ts were obtained from the American Type Culture Collection and were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Serum Source International, Charlotte, NC) and 1% Penicillin/Streptomycin. HAP1 wild-type and HAP-1 FASN knockout cells were obtained from Horizon Discovery (Lafayette, Colorado) and were maintained in IMDM supplemented with 10% heat-inactivated FBS and 1% Penicillin/Streptomycin. HIV-1 matrix plasmids pQCXIP-MA-FH (pMA-Flag) and pQCXIP-G2A-FH (pG2A-MA-Flag) were kindly provided by Dr. Stephen Goff, Columbia University.[55] HA-tagged IFITM3[22] and HA-tagged ZDHHC7[56] have been described. Cells were transfected using Genefect (Alkali Scientific, Fort Lauderdale, FL) or LipoJet transfection reagents (Signagen Laboratories, Rockville, MD), according to each manufacturer's protocol. Fasnall was synthesized as described. [35] Influenza virus H1N1 strain PR8 was propagated in 10-day-old embryonated chicken eggs (Charles River) for 48 hours at 37 °C as described previously.[57,58] IFNβ-treated HAP1 WT and FASN KO cells were treated

with IFN overnight or left untreated as described previously[32], and infected with H1N1 for 24 hours (MOI = 1). Cells were stained with anti-influenza virus nucleoprotein antibodies to measure percentage of infection by flow cytometry.

Metabolic labeling, immunoprecipitations, and CuAAC: Cells were incubated for 24 hours with the indicated concentrations of Alk-3, Alk-4, Alk-12, Alk-16, or 0.001% DMSO in media supplemented with 1% charcoal-stripped FBS (Serum Source International, Catalog number FB02- 500CS), and then collected and washed thrice in ice-cold 1x phosphate buffered saline (PBS). Cells were lysed for 10 minutes on ice in 100μl 1% Brij buffer (1% (w/v) Brij O10, 150mM NaCl, 50 mM triethanolamine with 1x EDTA-free complete protease inhibitor cocktail. Protein concentration was determined using the BCA assay. Flag precipitations used 500 μg of cell lysate mixed with Protein G coated agarose beads and incubated with Anti-Flag antibody (catalog number F3165) for 2 hours at 4^{o}C.Anti-HA IPs were performed using EZview Red Anti-HA Affinity Gel. Protein-conjugated beads were washed thrice with radioimmunoprecipitation assay (RIPA) buffer (50 mM triethanolamine, 150 mM NaCl, 1% sodium deoxycholate, 1% triton-X-100, 0.1 % SDS). Protein complexes bound to antibody coated beads were released by adding 4% SDS buffer (150 mM NaCl, 50 mM triethanolamine, 4% [w/v] SDS), and the click reaction was initiation by addition of 2.75μl of click chemistry master mix (0.5μl of 5mM azido-rhodamine or tetramethylrhodamine-5-carbonyl azide [Click Chemistry Tools, Scottsdale, AZ] in DMSO, 0.5μl of 50mM tris(2-carboxyethyl)phosphine [TCEP], 0.5μl of 50mM CuSO4, and 1.5μl of 2mM tris (1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (TBTA) in 1:4 [v/v] DMSO/butanol). Reactions were incubated for one hour at room temperature, and proteins were eluted from the beads by heating at 95^{o}C for 5 minutes in 4X SDS sample loading buffer (40% (v/v) glycerol, 240 mM Tris·Cl, pH 6.8, 8% (w/v) sodium dodecyl sulfate (SDS), 0.04% (w/v) bromophenol blue, 5% 2-mercaptoethanol). Eluted proteins

were resolved on 4-20% tris-glycine gels. To detect fluorescently labeled proteins, the gel was destained in 40% distilled water(v/v), 50%(v/v) methanol, 10%(v/v) acetic acid and visualized using on an Amersham Typhoon 9410 with 532-nm excitation and 580-nm detection filters. For biotin-based click reactions, Alk-4 incubated cells were lysed with 50μl 4% SDS buffer with 1x EDTA-free protease inhibitors supplemented with benzonase nuclease (Catalog number E1014). One mg of cell lysate was resuspended in 445μl 1x SDS buffer with 1x EDTA-free protease inhibitors and incubated for 1.5 hours with 55μl of click reaction master-mix consisting of 10μl of 5mM diazo biotin azide (Click Chemistry Tools), 10μl of 50mM TCEP, 25 ul of 2mM TBTA, 10μl of 50mM CuSO4. Proteins were precipitated using chloroform-methanol to remove unreacted biotin azide, and the precipitant was resuspended in 100μl 4% SDS buffer containing protease inhibitors supplemented and 2μl of 0.5M EDTA solution to chelate residual copper. Equivalent amount of protein in 100μl 4%SDS buffer and 200 μl 1% Brij buffer with EDTA-free protease inhibitors was incubated with 75ul streptavidin agarose (EMD Millipore) for two hours at room temperature. Protein-conjugated beads were washed once in PBS/0.2-1% SDS, and thrice in PBS. Labeled proteins were selectively eluted by two elutions with 50mM sodium dithionite, desalted using spin desalting columns, mixed with 4X SDS sample loading buffer, and resolved on 10-12% Tris-Glycine gel. Coomassie staining was done using standard techniques.[59]

Western blotting: Proteins were transferred onto PVDF membrane (Bio-Rad, Hercules, CA), blocked with 5% bovine serum albumin dissolved in 1x tris-buffered saline (TBS) containing 0.1% Tween-20. Anti-Flag (catalog number F3165) and Anti-FASN (catalog number SAB4300700) antibodies were purchased from Sigma and used at a final concentration of 1:1000; anti-Calnexin (catalog number 2679S) and anti-Src (catalog number 2109S) antibodies were purchased from Cell Signaling Technologies (Danvers, MA) and used at a final

concentration of 1:2000. Anti-rabbit secondary antibody (Thermo Fisher Scientific catalog number 31460) was used at a final concentration of 1: 5000, and anti-mouse secondary antibody (Cell Signaling Technology catalog number 7076S) was used at a final concentration of 1:2000.

Protein identification: Capillary-LC-MS/MS was performed using a Thermo Scientific orbitrap fusion mass spectrometer equipped with an EASY-Spray™ Sources operated in positive ion mode. Samples were separated on an easy spray nano column (Pepmap™ RSLC, C18 3μ 100A, 75μm X150mm Thermo Scientific) using a 2D RSLC HPLC system from Thermo Scientific. The full scan was performed at FT mode and the resolution was set at 120,000. EASY-IC was used for internal mass calibration. Mass spectra were searched using Mascot Daemon by Matrix Science version 2.3.2 (Boston, MA) and the database searched against Uniprot Human database (version 12032015). Data from two independent experiments were compiled on Scaffold Visualization software (Scaffold 4.9.0, Proteome Software Inc., Portland, OR). Proteins were identified based on total spectrum count with a 1% false discovery rate (FDR) and a minimum of two peptides. Proteins were considered high confidence hits if they had DMSO spectral count of zero, and a minimum spectral count of five in both replicates. Putative fatty acylation sites in high confidence proteins were identified in the SwissPalm protein S-palmitoylation database (Version 3, https://SwissPalm.org/) using Dataset 3 (proteins found in at least one palmitoyl-proteome or experimentally validated to be palmitoylated) and Dataset 1 (All datasets). Protein sequences were also searched against GPS-Lipid using high threshold settings (Version 1.0, http://lipid.biocuckoo.org/), as described in the supplementary methods section.

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Author Contributions

K.P.K., J.S.Y., and J.J.K. conceived the project and wrote the paper. K.P.K. and L.Z. performed all experiments. K.P.K., L.Z., J.S.Y., and J.J.K. performed data analysis. M.B., D.R.L., and T.A.J.H. provided experimental expertise. All authors edited and approved the manuscript.

Competing Interests

The authors declare no competing interests.

Figures

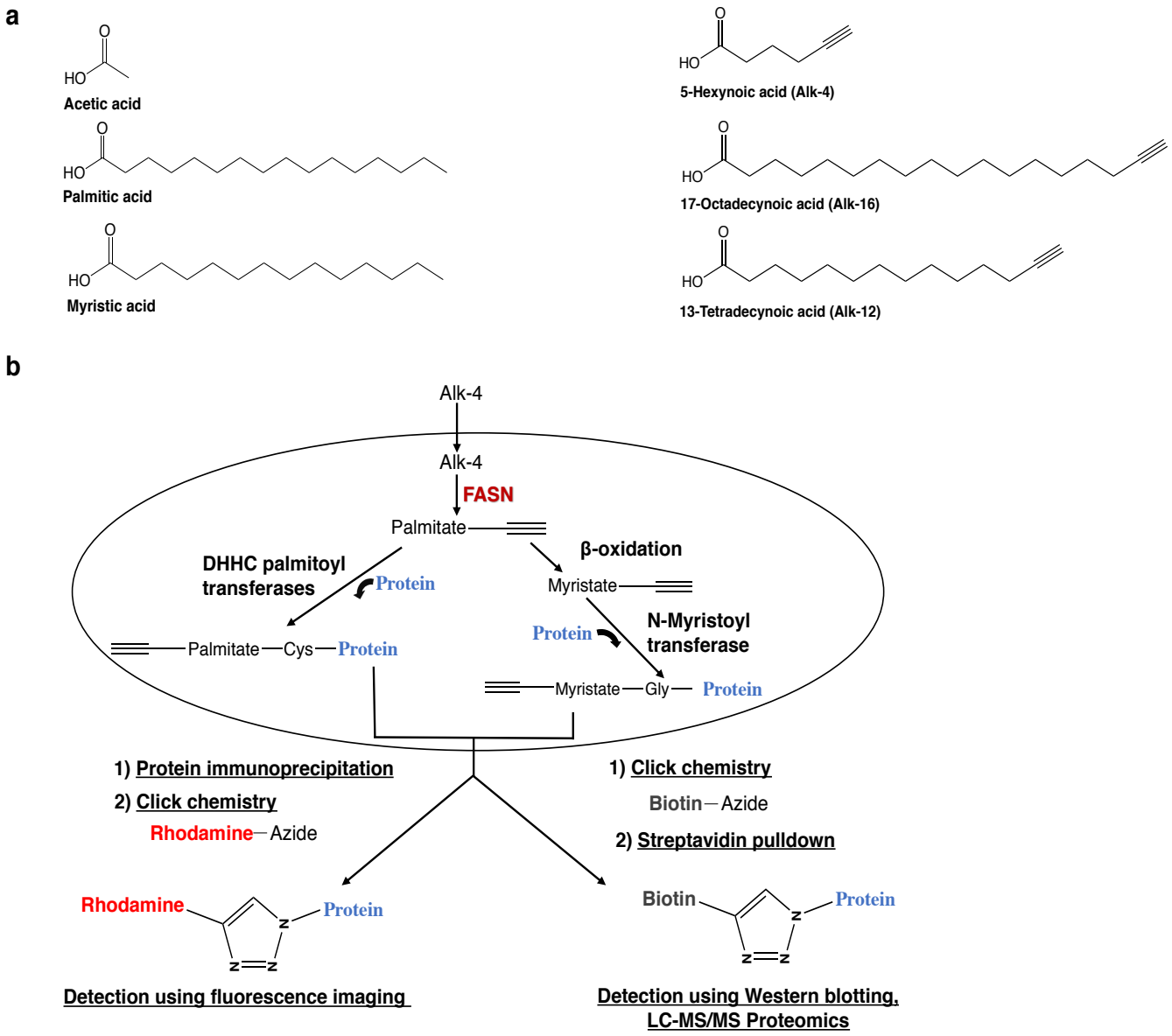

Figure 1: Analog structures and schema depicting Alk-4 metabolism and incorporation onto protein acylation sites. (a) Structures of acetate, palmitate, and myristate, followed by their click chemistry-compatible analogs Alk-4, Alk-16, and Alk-12. (**b)** 5-Hexynoic acid (Alk-4) can be metabolized through the endogenous FASN pathway to yield functionalized versions of fatty acyl groups that are transferred onto protein acylation sites by palmitoyl or N-myristoyl transferases. Copper-catalyzed azide-alkyne cycloaddition (CuAAC) click reaction of proteins containing the functionalized alkyne group to an azide-conjugated fluorophore such as rhodamine can be used for fluorescence imaging, while CuAAC click reaction of alkyne-containing proteins to an azide-conjugated biotin can be used for affinity purification and subsequent western blotting or proteomics.

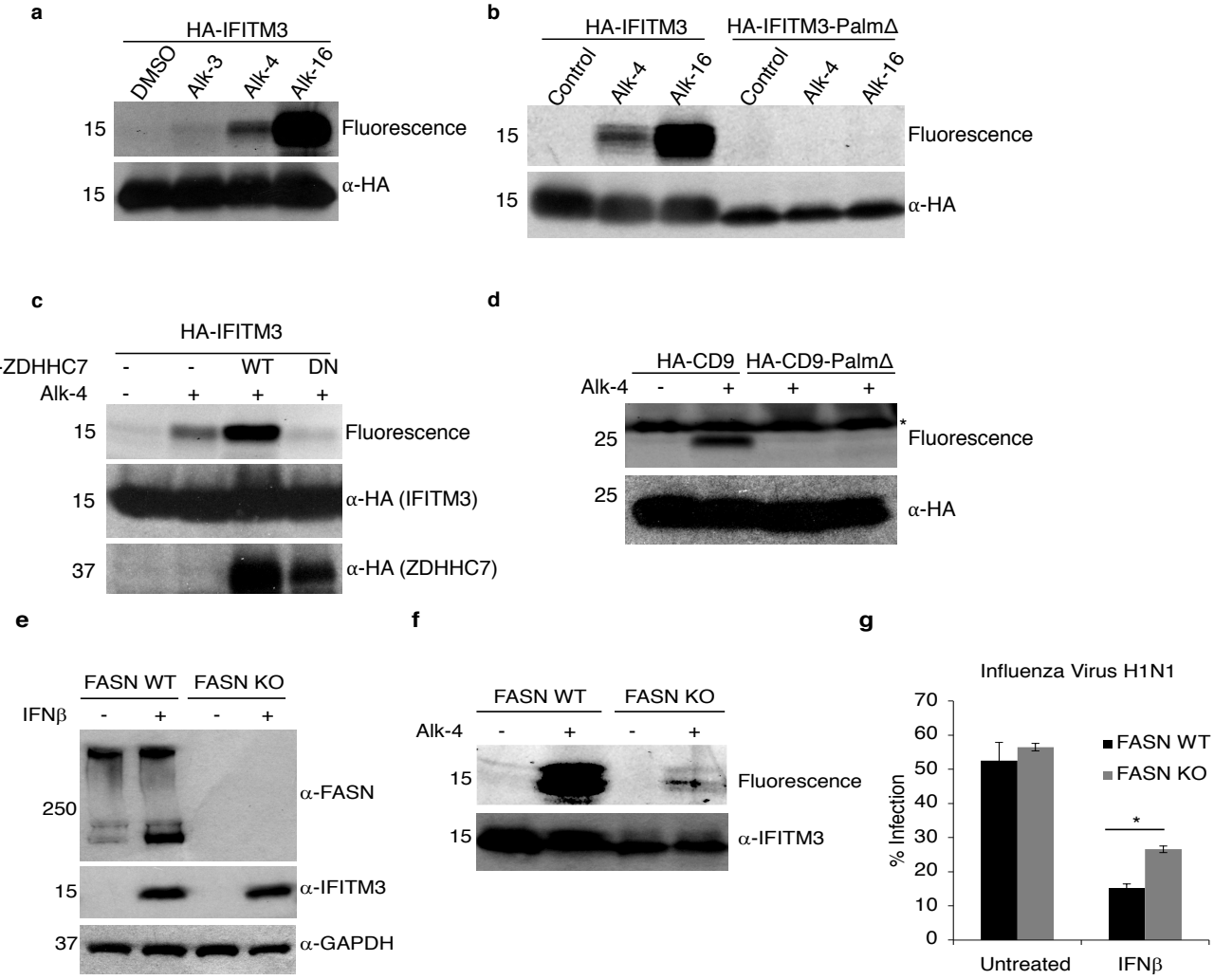

Figure 2: FASN-dependent incorporation of Alk-4 at known protein palmitoylation sites **(a)** Immunoprecipitated HA-IFITM3 from HA-IFITM3 transfected 293Ts followed by rhodamine azide click reaction revealed detectable labeling by Alk-16 and Alk-4 and minimal labeling by Alk-3. **(b)** A triple cysteine to alanine IFITM3 mutant (PalmΔ) was not labeled by Alk-4, suggesting that Alk-4 labeling of IFITM3 occurred on known palmitoylated cysteines. **(c)** Alk-4 labeled CD9, while a mutant where its six palmitoylated cysteines were mutated to alanine was not labeled, revealing that Alk-4 labels CD9 on known palmitoylated cysteines. **(d)** DHHC palmitoyltransferase overexpression increased Alk-4 labeling of IFITM3, while a dominant negative mutant partially decreased labeling, suggesting that Alk-4 is metabolized into a long chain fatty acid utilized by DHHC palmitoyltransferases (* indicates 25kDa fluorescent molecular weight standard bleed through). **(e)** To test the requirement of FASN for labeling of endogenous IFITM3 in cells treated with IFNβ, HAP1 WT and FASN KO cells were labeled with Alk-4. Western blotting to confirm FASN levels in WT and KO cells, and expression of endogenous IFITM3 on IFNβ treatment. **(f)** Alk-4 labeling of endogenous IFITM3 was only observed in WT cells and not detected in FASN KO cells, indicating that FASN contributes to palmitoylation of IFITM3. **(g)** IFNβ was significantly less effective at inhibiting Influenza virus strain H1N1 infection in FASN KO cells (*p = 0.0002), indicating that FASN is required for mounting of an effective IFNβ immune response against influenza virus, possibly through provision for fatty acyl groups for activation of IFITM3.

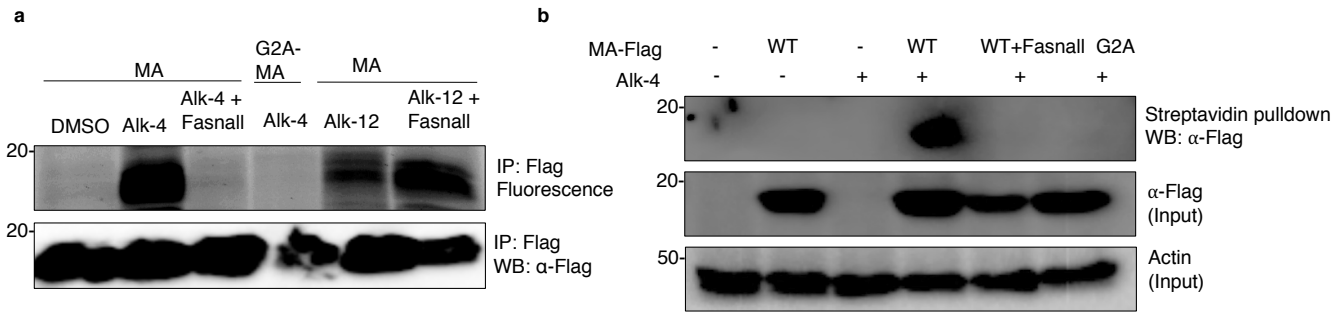

Figure 3: FASN-dependent incorporation of Alk-4 at known myristoylation sites. 293Ts were transfected overnight with plasmids encoding MA-Flag (MA), or the myristoylation deficient G2A-MA-Flag(G2A), and incubated for 24 hours either with Alk-4, or Alk-12. 10uM of the FASN inhibitor Fasnall was added one-hour post-transfection. **(a)** Immunoprecipitation of MA-Flag and click reaction with TAMRA azide revealed labeling of WT MA with Alk-4 and not G2A-MA, suggesting that Alk-4 labels MA at its known myristoylation site. FASN inhibition with Fasnall reduced labeling of MA by Alk-4, suggesting that FASN is required for labeling of MA by Alk-4. Alk-12 also labeled MA in both cells treated and untreated with Fasnall, indicating that Fasnall does not inhibit NMT function. **(b)** On click reaction with diazo azido biotin, streptavidin pulldown, and selective elution of Alk-4 labeled proteins, only WT-MA was labeled by Alk-4 and not G2A-MA, and Fasnall treatment also inhibited Alk-4 labeling of MA, corroborating our findings that FASN is required for labeling of MA by Alk-4 at its myristoylation site.

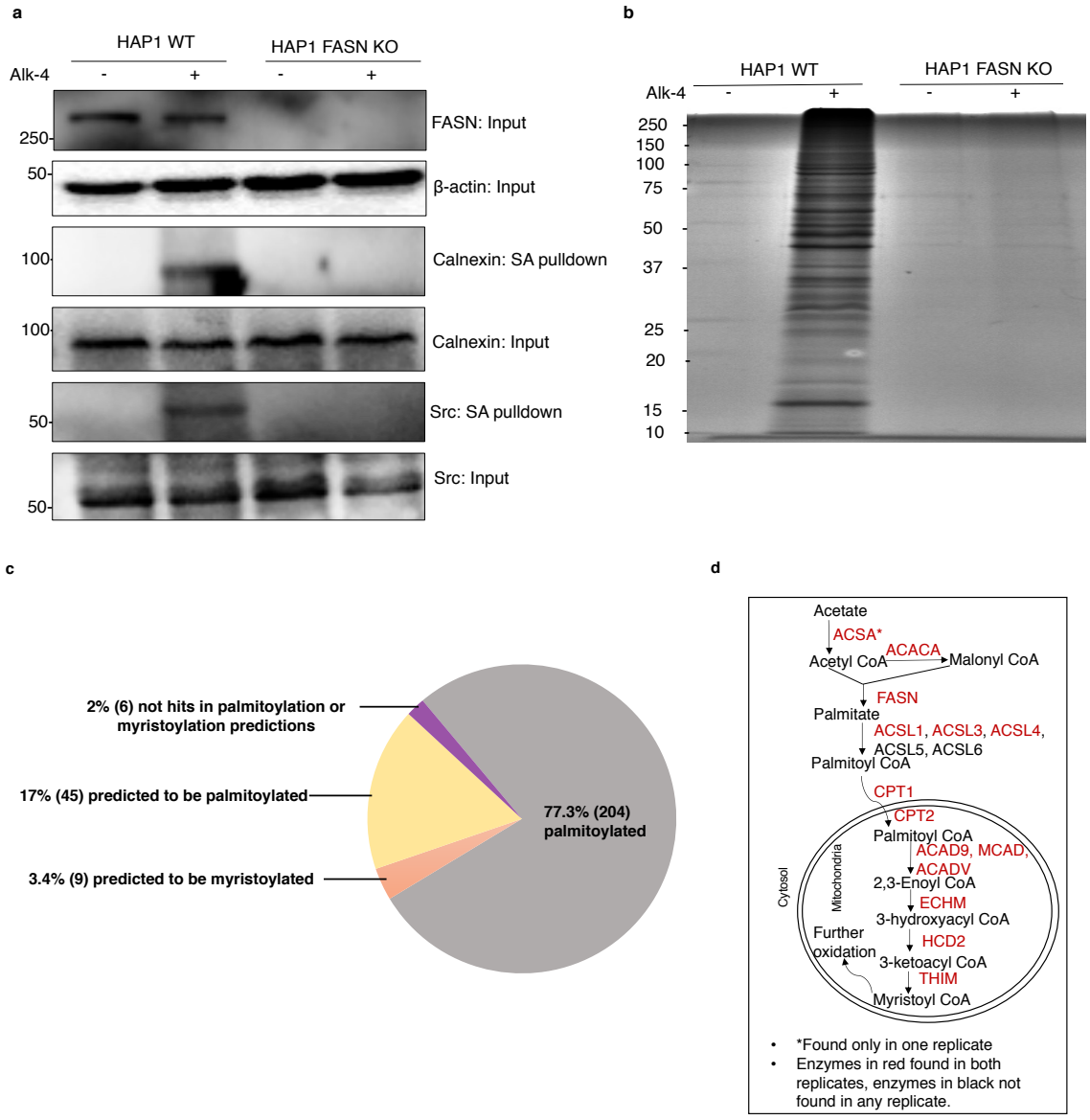

Figure 4: FASN-dependent purification of myristoylated and palmitoylated proteins from Alk-4 labeled cells. HAP1 wild-type (WT) and HAP1 FASN knockout (KO) cells were labeled with Alk-4, click reacted with diazo azido biotin, and labeled proteins were purified using streptavidin beads and eluted using sodium dithionite. **(a)** Western blotting indicates that proteins known to be palmitoylated (Calnexin) and myristoylated (Src) can be purified from cells in an Alk-4 and FASN dependent manner. **(b)** Coomassie staining of the streptavidin (SA) pulldown fraction revealed recovery of several proteins only in WT cells labeled with Alk-4. **(c)** Proteomics analysis of SA pulldown fraction revealed that 77% of proteins selectively recovered in the WT Alk-4 cells are found in at least one palmitoyl proteome or experimentally validated to be palmitoylated based on the SwissPalm database (Dataset 3). Additionally, 17% of proteins are predicted to be palmitoylated, based both on GPS-lipid analysis using high confidence settings, CSS-Palm, and SwissPalm (Dataset 1). GPS-Lipid analysis also revealed 3% of proteins are predicted to be myristoylated (consensus N-terminal Glycine and non-consensus sequence), while 2% of the proteins were not hits on prediction algorithms but had isoforms and cysteines as per SwissPalm analysis. **(d)** Proteomics analysis of SA pulldown fraction (n=2) revealed recovery of enzymes involved in elongation of short chain fatty acids, as well as enzymes involved in the mitochondrial beta-oxidation pathway for oxidation of long

chain fatty acids. Long chain fatty acid such as palmitic acid and its oxidized product myristic acid can be used for fatty acylation of proteins.

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