Supplementary Materials.

Profiling targets of the irreversible palmitoylation inhibitor 2-bromopalmitate

Dahvid Davda[1], Mahmoud El Azzouny[2], Christopher T.M.B. Tom[1], Jeannie L. Hernandez[2], Jaimeen D. Majmudar[2], Robert T. Kennedy[2,3], and Brent R. Martin[1,2]*

[1]Program in Chemical Biology, [2]Department of Chemistry, and [3]Department of Pharmacology, University of Michigan, 930 N. University Ave., Ann Arbor, MI 48109, USA.

16-Azidohexadecanoic acid (1). To a solution of 16-bromohexadecanoic acid (250 mg, 0.75 mmol) in DMF (2 mL) was added sodium azide (100 mg, 1.5 mmol) and the reaction was heated to 85 °C and stirred for 12 h. It was then cooled to room temperature, diluted with CH_2Cl_2 and quenched with 0.1 *N* HCl. The organic layer was dried over $MgSO_4$, filtered and concentrated to afford **1** as a white solid (183 mg, 82%) and was used without further purification. R_f 0.51 (30% EtOAc:hexanes). IR (thin film on NaCl): cm^{-1} = 2098 ($-N_3$). ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.18 (t, *J* = 7.0 Hz, 2H), 2.27 (t, *J* = 7.5 Hz, 2H), 1.58 – 1.49 (m, 4H), 1.30 – 1.19 (m, 22H). HRMS: *m/z* calculated for $C_{16}H_{30}N_3O_2$: 296.2338, found 296.2340 [*M* - H]$^-$.

16-Azido-2-bromohexadecanoic acid, 2BPN$_3$ (2). 1 (2.0 g, 6.7 mmol) was dissolved in thionyl chloride (40 mL) and stirred at reflux for 1 h. Then, bromine (450 µL, 8.7 mmol) was added dropwise over 3 h and the reaction was further refluxed for 3 h. The mixture was then cooled to room temperature, concentrated, dissolved in H_2O and stirred at room temperature for 12 h. The aqueous solution was extracted with diethyl ether (3x), and the organic layers were combined, washed with 0.1 *N* HCl, dried over $MgSO_4$, filtered and concentrated to afford an orange solid. Purification of this solid by flash chromatography (30% EtOAc:hexanes) yielded **2** (1.0 g, 40%) as a white solid. R_f 0.13 (30% EtOAc:hexanes). ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 4.20 (t, *J* = 7.4 Hz, 1H), 3.21 (t, *J* = 7.0 Hz, 2H), 2.05 – 1.92 (m, 2H), 1.59 – 1.52 (m, 2H), 1.47 – 1.17 (m, 22H). HRMS: *m/z* calculated for $C_{16}H_{29}BrN_3O_2$: 374.1443, found 374.1439 [*M* - H]$^-$.

***N*-(2-Bromohexadecanoyl)succinimide (3)**. The succinimidyl ester was prepared as previously described[1]. 2-bromopalmitic acid (500 mg, 1.49 mmol, Sigma) was added to a solution of *N*-hydroxysuccinimide (172 mg, 1.49 mol) in dry ethyl acetate (5 mL). *N,N'*-diisopropylcarbodiimide (257 μL, 1.64 mol) was added at room temperature, and the mixture was allowed to react overnight. Precipitated diisopropylurea was removed by filtration through a glass wool plug. The solvent was evaporated yielding a white powder, which was then recrystallized from absolute ethanol, to afford **3** (457 mg, 70.8%) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 4.43 (dd, *J* = 7.8, 6.9 Hz, 1H), 2.84 (s, 4H), 2.22 – 2.05 (m, 2H), 1.58 - 1.38 (m, 2H) 1.39 – 1.14 (m, 22H), 0.86 (t, *J* = 7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 168.44, 165.64, 40.90, 34.79, 31.90, 29.66, 29.65, 29.63, 29.61, 29.54, 29.42, 29.34, 29.21, 28.70, 26.93, 25.58, 22.67, 14.11.

2-Bromohexadecanoyl coenzyme A, 2BP-CoA (4). The acyl CoA was synthesized using a modified literature procedure[2]. **3** (26 mg, 61 μmol) was dissolved in freshly distilled THF (0.6 mL) and mixed with a solution of coenzyme A trilithiate dihydrate (5 mg, 6.1 μmol, Fisher) dissolved in deionized water (0.3 mL). Triethylamine (8.5 μL, 61 μmol) was added to the reaction and the homogeneous mixture was stirred on a rotisserie rotator for 3 hours at room temperature. The crude product was precipitated by adding 5% perchloric acid (1.2 mL) and excess THF was removed by rotary evaporation. The precipitate was collected by centrifugation (5 min, 3220x g, 4 ˚C) and the supernatant was discarded. The precipitate was washed, sonicated and centrifuged sequentially with 0.8% perchloric acid (2.5 mL), acetone (4 X 1.5 mL) and diethyl ether (3 X 1 mL) to remove excess starting materials. The white residue was extracted with 2-(*N*-morpholino)ethanesulfonic acid buffer (0.4 mL, 50 mM, pH 5) and insoluble side-products were removed by centrifuging the mixture. The product was precipitated again with 5% perchloric acid (0.6 mL). The precipitate was washed, sonicated and centrifuged sequentially with 0.8% perchloric acid (1 mL), acetone (3 x 0.5 mL), and dried under vacuum to

afford **4** (1.3 mg, 19%) as a white powder. HRMS: *m/z* calculated for $C_{37}H_{63}BrN_7O_{17}P_3S^{2-}$: 540.6204, found 540.6194 $[M - 2H]^-$.

***N*-(16-Azido-2-bromohexadecanoyl)succinimide (5).** Following the procedure used to prepare *N*-(2-bromohexadecanoyl)succinimide, **2** (23 mg, 0.061 mmol), *N*-hydroxysuccinimide (7 mg, 0.061 mol), *N,N′*-diisopropylcarbodiimide (11 μL, 0.067 mol) in ethyl acetate (1 mL) were used to yield **5** (7.2 mg, 25%) as white crystals.

16-Azido-2-bromohexadecanoyl coenzyme A, $2BPN_3$-CoA (6). Following the procedure used to synthesize 2-bromohexadecanoyl coenzyme A, **5** (7.2 mg, 15.7 μmol), coenzyme A trilithiate dihydrate (1.3 mg, 1.57 μmol), and triethylamine (2.2uL, 15.7 μmol) in THF (200 μL) and deionized water (100 μL) were employed to afford **6** (1.6 mg, 24%) as a white powder HRMS: *m/z* calculated for $C_{37}H_{62}BrN_{10}O_{17}P_3S^{2-}$: 561.1211 $[M – 2H]^-$, found 561.1240 $[M - 2H]^-$.

Characterization of acyl-CoA products. The purity of 2BP-CoA and $2BPN_3$-CoA were assessed by TLC[3] and the thioester content of the preparations were determined spectroscopically[4]. The products were eluted on C18-bonded silica reverse phase TLC plates using *n*-butanol/water/acetic acid (5:3:2) as the solvent system. 2BP-CoA and $2BPN_3$-CoA were visualized under UV light, each revealing a single spot with R_f-value of 0.55 and 0.57 respectively (palmitoyl-CoA authentic standard R_f = 0.53). In addition, these spots gave a positive nitroprusside test for free thiol groups only after treatment with methanolic NaOH. 2BP-CoA and $2BPN_3$-CoA were dissolved in 75% isopropanol, 1 mM acetic acid to make 1 g / L stocks for spectroscopic analyses. The UV spectrum of each product was measured against an authentic palmitoyl-CoA (Sigma), with each acyl-CoA featuring a peak at 232 and at 260 nm. Furthermore, the 232 nm absorbance disappeared after methanolic NaOH treatment, indicating hydrolysis of the thioester bond. The relative concentrations of thioester to adenine in each acyl-CoA preparation was determined by measuring the ratio of the 232 and 260 nm absorptions and

using experimentally calculated extinction coefficients at 232 and 260 nm for the authentic palmitoyl-CoA standard.

Biochemical and Analytical Methods

Cell Culture. HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (FBS, JR Scientific) and 1% (vol/vol) 10,000 units penicillin, 10,000 μg streptomycin, 29.2 mg L-glutamate solution (PSQ, Invitrogen). Cells grown to ~60% confluency were transfected with pcDNA3 vectors expressing FLAG-epitope tagged proteins using Fugene HD transfection reagent (Promega). Cells were cultured for 48 hours to allow for sufficient protein expression.

Mass Spectrometry of endogenous CoA conjugates. 2-bromopalmitate and palmitate were both dissolved in BSA free fatty acid (0.05%) to achieve 1:6 molar ratio of BSA:fatty acid. INS-1(832/3) cells were incubated with 50 μM 2BP or 50 μM palmitic acid for 30 minutes in Kreb's Ringer Buffer with 0 mM glucose at 37°C. Cells were quenched and extracted as previously described[5], and normalized to protein concentrations using the BCA protein assay kit (Pierce). Detection was performed on an Agilent Technologies LC/MSD TOF using a dual electrospray ionization (ESI) source in negative-ion mode and analyzed as previously described[5]. The concentration of 2BP was determined by standard addition method, where a known concentration of standard was added to the unknown sample (pooled extract of 2BP treated cell, n = 4). Data was acquired from 2 biological replicates of 4 technical replicates processed on separate days. INS-1(832/3) cells were kindly provided by Dr. Christopher Newgard (Sarah W. Stedman Nutrition and Metabolism Center, Duke University, Durham, NC).

Metabolic Labeling. Labeling media was prepared with DMEM, 10% dialyzed FBS (Gemini Bio-Products), and 1% PSQ. 16-Azido-2-bromopalmitic acid ($2BPN_3$) or 2-bromopalmitic acid (2BP, Sigma) was dissolved in DMSO to make a 50 mM (1000X) stock, then diluted in labeling

media and briefly sonicated, and then added to cells at ~100% confluency. Cells were labeled with 2BP or $2BPN_3$ at the concentrations described for the indicated time period. Cells were collected by centrifugation at 800x g, washed twice with PBS, and then stored at -80ºC as cell pellets.

Cell Lysate Preparation. Pellets were re-suspended in phosphate buffered saline (without magnesium and calcium, PBS, Invitrogen), and sonicated briefly at 4°C. Cell debris was removed by low-speed centrifugation at 800x g for 2 minutes, and supernatant was placed into a new conical tube. Lysates were separated into soluble and membrane particulate fractions by ultracentrifugation at 100,000x g for 45 minutes. The insoluble pellet was sonicated in PBS to homogenize the lysate. The protein concentration was determined using the BCA assay (Biorad) using a Tecan F500 plate reader. Membrane particulate fractions were diluted in PBS to 1 mg / mL of proteome, then reacted with the indicated probe at the concentrations described for one hour at room temperature.

Copper-Catalyzed Azide-Alkyne Cycloaddition. 1 mg / mL of proteome was combined with 20 μM Carboxytetramethylrhodamine-alkyne (TAMRA-Alkyne, Click Chemistry Tools), 1 mM Tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich), 100 μM Tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (TBTA) (Sigma-Aldrich), and 1 mM CuSO4 in PBS (25 μl) at room temperature (~22°C). After 1 hour, samples were mixed with SDS loading buffer and dentaured at 90°C for 10 minutes. 8 μg of protein was loaded into each lane of a 10% SDS-PAGE, separated over 180 V-hours and fluorescence scanned on an Amersham Bioscience Typhoon scanner.

Mass Spectrometry Analysis. Samples were prepared as previously described[6], but biotin-alkyne (Click Chemistry Tools) was substituted at 20 μM. Furthermore, samples were chloroform/methanol precipitated following the click reaction to remove unreacted biotin-alkyne. Higher concentrations of biotin-alkyne led to increased non-specific background. Mass

spectrometry analysis was performed as previously described, using an Agilent 1100 HPLC coupled to a Thermo LTQ mass spectrometer using a 5-step MuDPIT protocol. Data analysis was preformed using previously reported algorithms.

Western blotting. Proteins were transferred to 0.45 μm polyvinylidine difluoride membrane (Immobilon-P, Millipore) and blocked with 5% bovine serum albumin (BSA, Fisher) in Tris buffered saline-Tween 20 buffer (TBS-T, pH 7.4) for 1 hour at room temperature. After washing, the membrane was incubated with a primary mouse-anti-FLAG antibody (M2 monoclonal, Sigma, 1 μg / mL antibody, 5% BSA, 0.02% NaN_3, TBS-T) for 1 hour at room temperature, washed, and probed with a secondary Alexa Fluor 647 nm donkey-anti-mouse antibody conjugate (IgG H +L, Invitrogen, 2 μg / mL antibody, 0.06% NaN_3, TBS-T) for 1 hour at room temperature. For streptavidin-based detection, the membrane was probed with a Cy5-streptavidin conjugate (50 ng / mL conjugate, 2.5% BSA, 0.06% NaN_3, TBS-T) for 1 hour at room temperature, and the washed as before. Blots were fluorescence scanned on an Amersham bioscience typhoon scanner.

References

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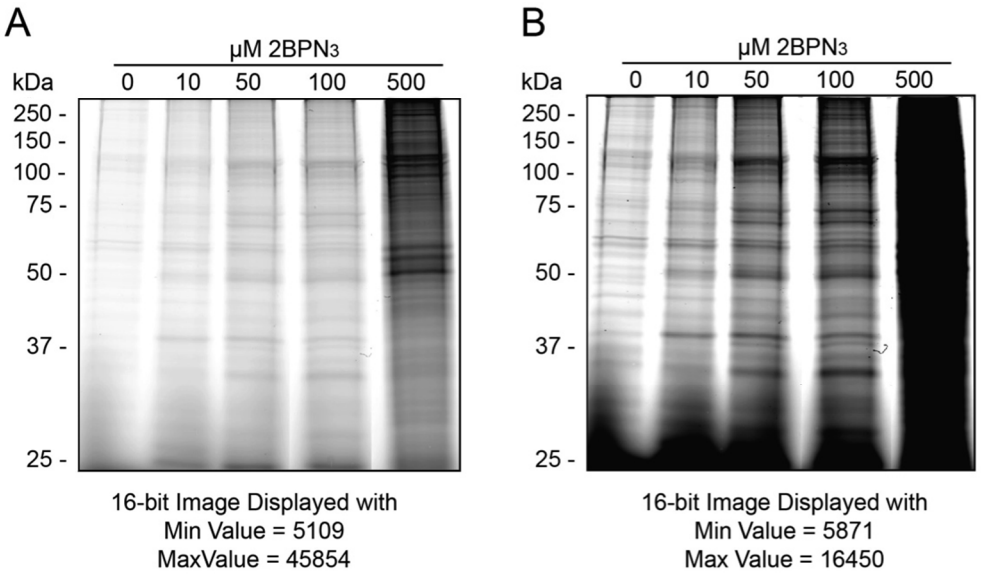
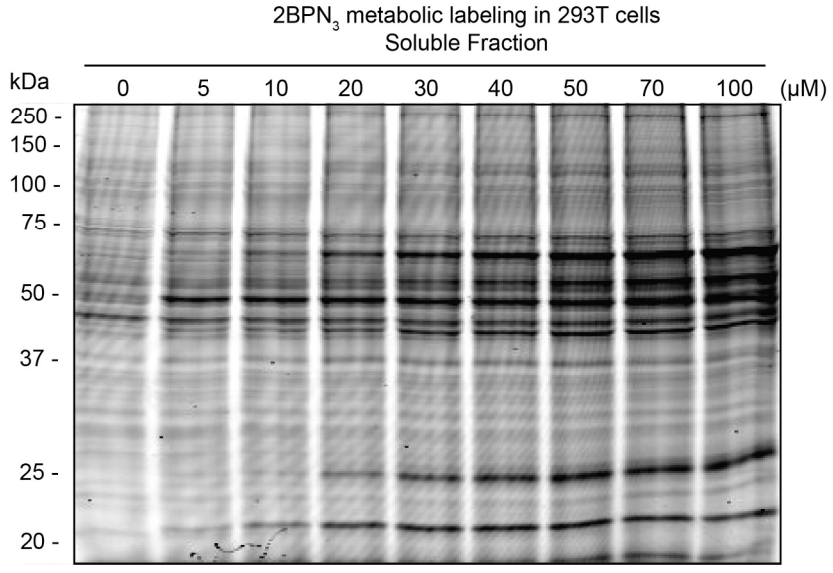
2. Al-Arif, A., and Blecher, M. (1969) Synthesis of fatty acyl CoA and other thiol esters using N-hydroxysuccinimide esters of fatty acids, *J Lipid Res 10*, 344-345.
3. Pullman, M. E. (1973) A convenient and versatile method for the purification of CoA thiol esters, *Anal Biochem 54*, 188-198.
4. Seubert, W. (1960) S-Palmitoyl CoA, *Biochem Prep 7*, 80-83.
5. Lorenz, M. A., El Azzouny, M. A., Kennedy, R. T., and Burant, C. F. (2013) Metabolome Response to Glucose in the beta-Cell Line INS-1 832/13, *J Biol Chem 288*, 10923-10935.
6. Martin, B. R., and Cravatt, B. F. (2009) Large-scale profiling of protein palmitoylation in mammalian cells, *Nat Methods 6*, 135-138.

Supplementary Figures

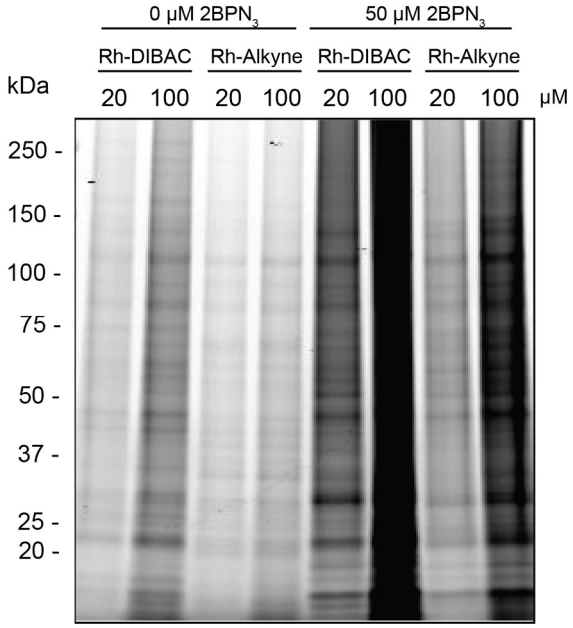
Supplementary Figure 1. $2BPN_3$ labeling is significantly increased at higher concentrations in 293T cells. Cells were labeled for 1 hour. (A) $2BPN_3$ labeling adjusted to maximize contrast at 10-fold higher than the standard labeling concentration. (B) $2BPN_3$ labeling adjusted to maximize contrast at 50 μM.



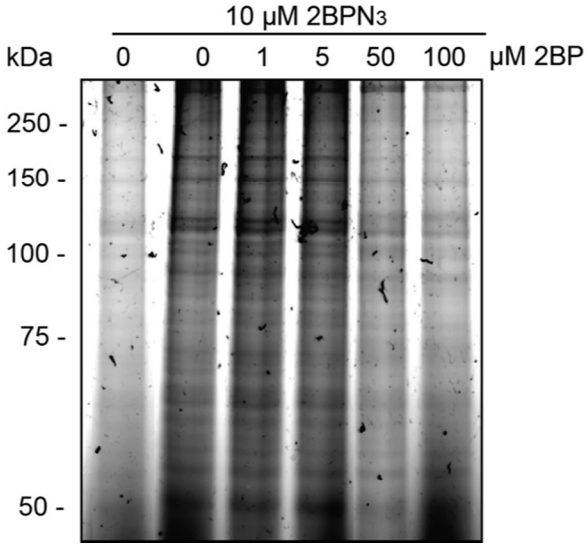
Supplementary Figure 2. Soluble cell fractions show significant labeling with rhodamine-alkyne in the absence of $2BPN_3$. 293T cells were labeled with 2BPN3 for 1 hour, then lysed by sonication and centrifuged at 100,000x g for 45 minutes. The soluble fraction was quantified using the BCA assay and mixed with 20 μM rhodamine-alkyne and other click chemistry reagents for 1 hour. Sample were separated using a 4-20% gradient SDS-PAGE gel.



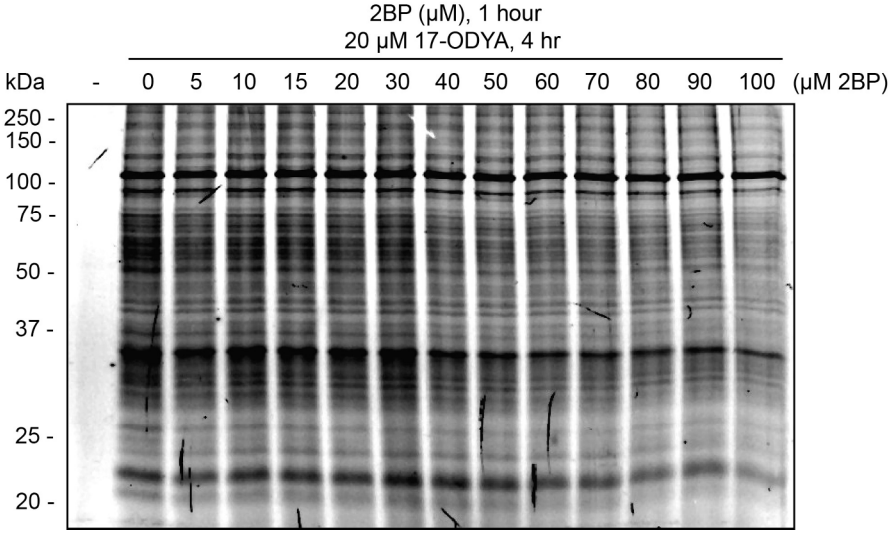
Supplementary Figure 3. Comparison of copper and copper-free click chemistry labeling of $2BPN_3$. Cells were labeled for 1 hour with $2BPN_3$, followed by incubation with either rhodamine-alkyne or rhodamine-aza-dibenzocyclooctyne (DIBAC). Samples were separated using a 4-20% gradient gel. DIBAC labeling is significantly more efficient, but has no clear increase in contrast over no-probe controls.



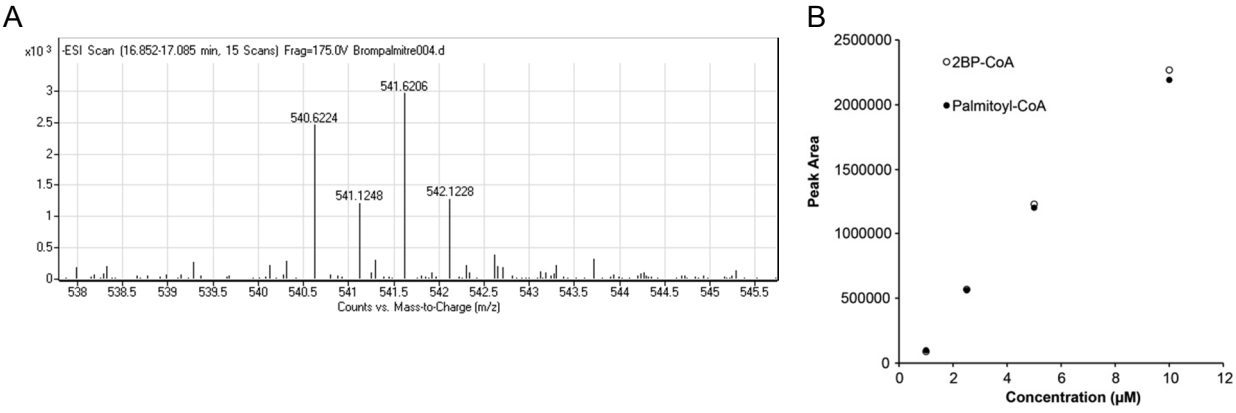
Supplementary Figure 4. Efficient *in vitro* competition of $2BPN_3$ labeling with 2BP. 293T cell lysates were labeled with increasing concentrations of 2BP for 1 hour, followed by labeling with $2BPN_3$ for 1 hour. Competition in vitro is by far more effective than live cell competition, as shown in **Figure 1D**.



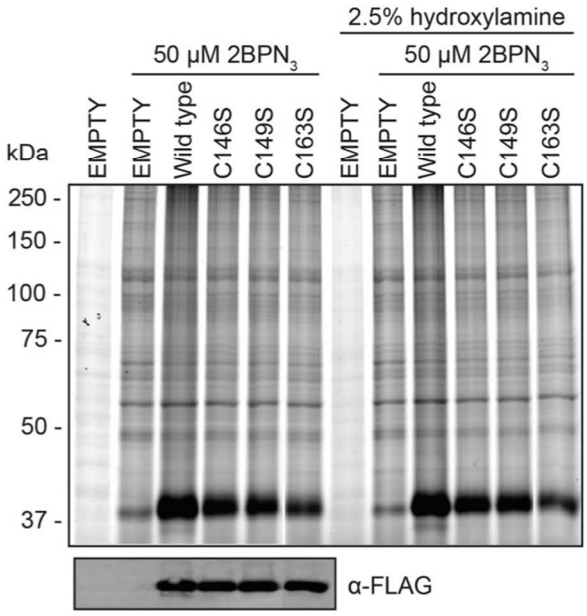
Supplementary Figure 5. 2BP reduces metabolic incorporation of the palmitate analogue 17-octadecynoic acid (17-ODYA) in cells. 293T cells were labeled for 1 hour with varying concentrations of 2BP, followed by addition of 17-ODYA for 4 additional hours. Palmitoylation levels are reduced most effectively at the highest concentrations of 2BP. Samples were separated using a 4-20% gradient SDS-PAGE gel.



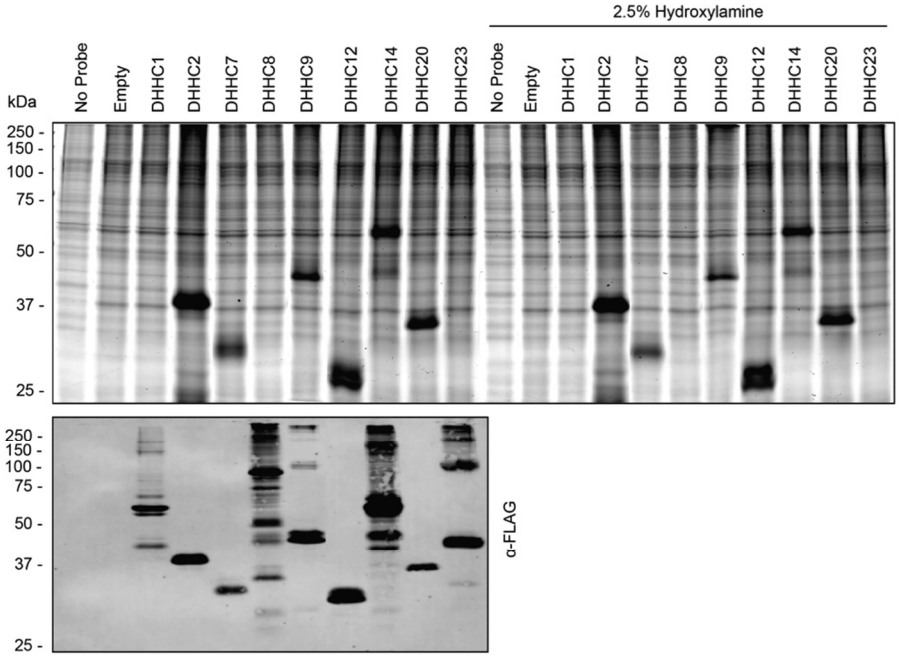
Supplementary Figure 6. Equal ionization efficiency of palmitoyl-CoA and 2BP-CoA in negative mode electrospray ionization mass spectrometry. (A) Synthetic standards of palmitoyl-CoA and 2BP-CoA ([M - 2H] shown) were doped into cell extracts and analyzed by LC-MS. The observed isotopic distribution correlates with the bromine adduct. (B) Equivalent intensities were measured across a 4-point dilution series. This data demonstrates equivalent ionization efficiency and allows direct comparison of ion intensities in profiling experiments.



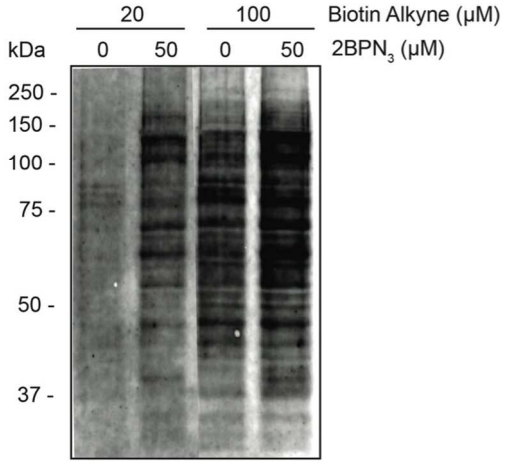
Supplementary Figure 7. Mutational analysis of select cysteines in the DHHC2 active site. 293T cells were transfected with FLAG epitope-tagged DHHC2 mutants and labeled with 50 μM $2BPN_3$ for 1 hour. Control lanes were labeled with 50 μM 2BP. Mutants showed reduced labeling, especially C163S. Labeling was not sensitive to hydroxylamine treatment, demonstrating stable alkylation of the enzyme.



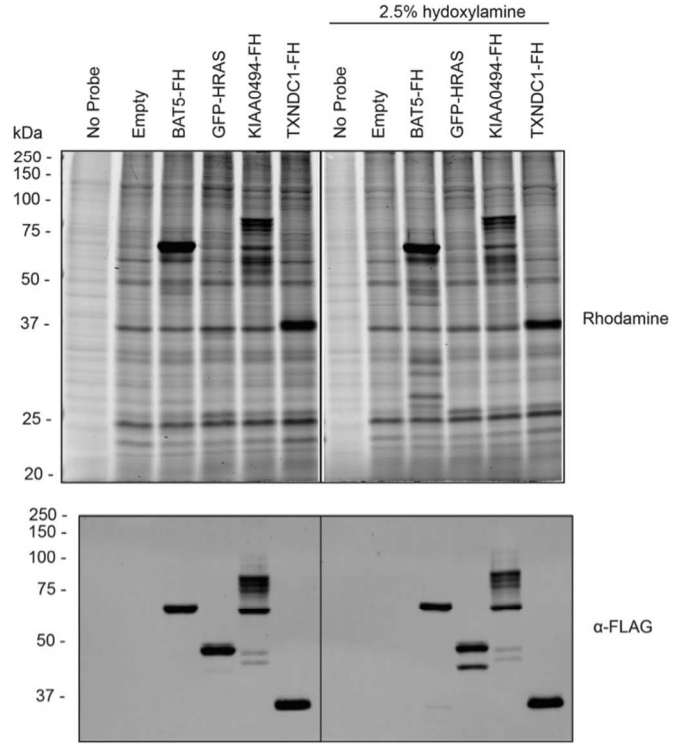
Supplementary Figure 8. Over-expressed DHHC PAT enzymes metabolically labeled with $2BPN_3$. Nine epitope-tagged DHHC PAT cDNAs were transfected into 293T cells for 48 hours, and labeled with $2BPN_3$ for 1 hour. Over-expressed DHHC PAT enzymes are clearly visible in transfected lysates as dominant bands, and confirmed by anti-FLAG western blotting.



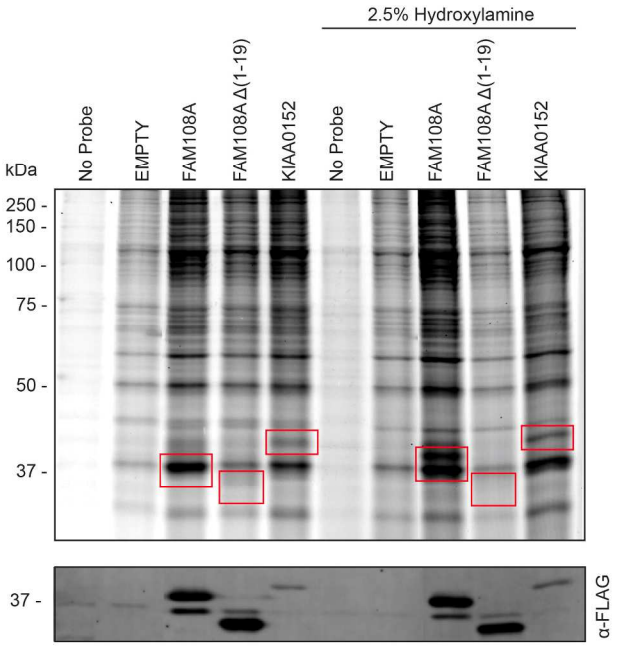
Supplementary Figure 9. Enhanced background at higher concentrations of biotin-alkyne in 2BPN$_3$-labeled lysates. Biotin-alkyne click chemistry was more selective at 20 μM than at 100 μM concentrations. 20 μM Biotin-alkyne was selected for further enrichment for mass spectrometry.



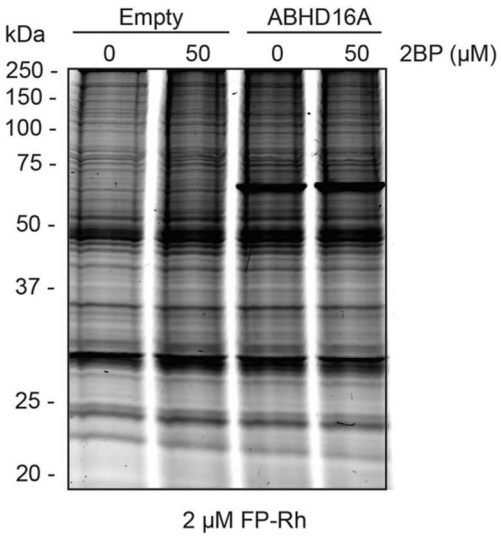
Supplementary Figure 10. Select palmitoylated proteins are metabolically labeled with 2BPN$_3$. Cells were transfected for 48 hours with epitope-tagged cDNAs for annotated palmitoylated proteins, and labeled for 1 hour with 2BPN$_3$. Labeling was resistant to hydroxylamine treatment, showing it is not due to a thioester linkage.



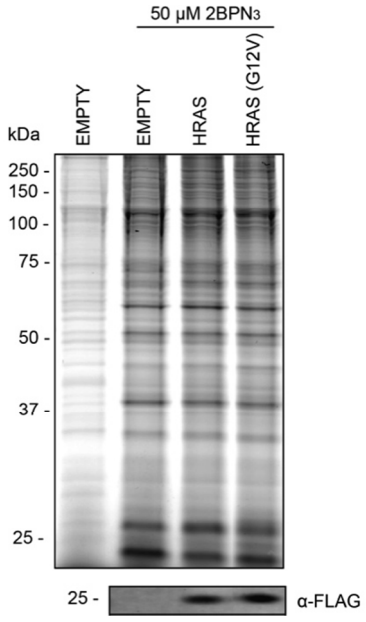
Supplementary Figure 11. 2BP competes with palmitoylation sites on FAM108A. FAM108A and FAM108(Δ1-19) were C-terminally tagged with the FLAG epitope and transfected in 293T cells. The palmitoylated protein KIAA0152 was also included. After 48 hours, cells were labeled with 50 μM 2BPN3 for 1 hour, conjugated to rhodamine-alkyne, and detected after separation by SDS-PAGE with or without incubation with 2.5% hydroxylamine. Red boxes denote recombinant protein bands.



Supplementary Figure 12. ABHD16A activity is unaffected by 2BP labeling. ABHD16A-transfected 293T cells were labeled with 50 μM 2BP for 1 hour, and then lysates were labeled with the serine hydrolase activity-based probe fluorophosphonate-rhodamine for 1 hour. There is no observed inhibition of ABHD16A by 2BP, suggesting non-catalytic residues, such as sites of palmitoylation, are labeled by 2BP in cells.



Supplementary Figure 13. H-Ras is not effectively labeled with $2BPN_3$. 293T cells were transfected with FLAG-H-Ras, FLAG-H-Ras (G12V), or empty vector and labeled with 2BPN3 for 1 hour. Labeling is equivalent across all experiments. Longer incubation times do lead to enhanced H-Ras labeling (not shown).



Supplementary Table 2. DHHC PAT enzymes identified by mass spectrometry profiling. Only DHHC5, 6, 7, 17, and 20 passed the filter thresholds, as described in the main text.

Protein	$2BPN_3$-1	$2BPN_3$-2	$2BPN_3$-3	2BP-1	2BP-2
ZDHHC3	0	4	1	0	0
ZDHHC4	0	2	0	0	0
ZDHHC5	7	9	6	1	0
ZDHHC6	3	13	10	0	0
ZDHHC7	6	3	2	0	0
ZDHHC9	1	1	2	0	0
ZDHHC12	1	2	0	0	0
ZDHHC13	0	3	0	0	0
ZDHHC14	0	1	0	0	0
ZDHHC17	0	6	1	0	0
ZDHHC18	1	2	2	0	0
ZDHHC20	7	5	5	0	0
ZDHHC21	1	0	0	0	0
ZDHHC24	1	3	0	0	0