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Supplemental Information

Carnitine Palmitoyltransferase 1A

Has a Lysine Succinyltransferase Activity

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Supplemental Figures

Figure S1

A

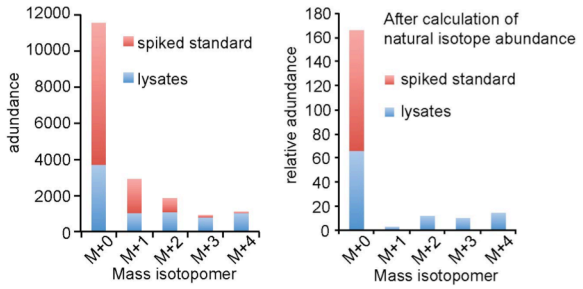
Swiss-Prot search for acyltransferase with coenzyme A binding (33 hits)

Exclude acyltransferases identified in yeast

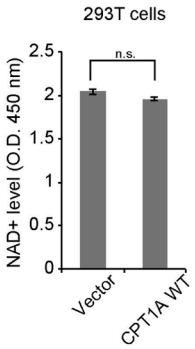
Potential lysine succinyltransferases (9 hits)

Entry	Protein names	Gene names
Q12983	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	BNIP3 NIP3
Q14032	Bile acid-CoA:amino acid N-acyltransferase (BACAT) (BAT)	BAAT
Q8TCG5	Carnitine O-palmitoyltransferase 1, brain isoform (CPT1-C)	CPT1C CATL1
P50416	Carnitine O-palmitoyltransferase 1, liver isoform (CPT1-L)	CPT1A CPT1
Q92523	Carnitine O-palmitoyltransferase 1, muscle isoform (CPT1-M)	CPT1B KIAA1670
P23786	Carnitine O-palmitoyltransferase 2, mitochondrial	CPT2
Q9Y232	Chromodomain Y-like protein (CDY-like)	CDYL CDYL1
Q6IB77	Glycine N-acyltransferase (EC 2.3.1.13)	GLYAT ACGNAT CAT GAT
Q9UKG9	Peroxisomal carnitine O-octanoyltransferase (COT)	CROT COT

B



C



D

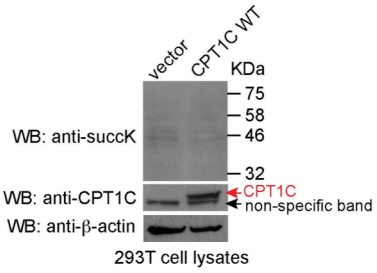


Figure S1, Screening of potential lysine succinyltranferases, Related to Figure 1.
(A) Screening for potential lysine succinyltransferases using SWISS-PROT database. (B) *left,* The isotopic distributions of succinate (m/z 289-293; M+0-4) extracted from cell lysates with or without spiking with unlabeled 20 nmoles of succinyl-CoA standard. The cells were incubate with [U-13C]glucose for 12 h before harvest to label endogenous succinyl-CoA. Succniyl-CoA was extracted and purified from the lysates first, and hydrolyzed and detected as succinate by GC/MS as described in Supplemental Experimental Procedures. *right,* The isotopic distributions of the extracted succinate after calculation of natural isotope abundance using IsoPat[2] software with pure succinate standard. Assuming a 200 µL volume for the 293T cell pellet, these figures correlate to intracellular concentrations of 100 µM for succinyl-CoA. (C) The results of NAD+ assay using 293T cells expressing vector control and CPT1A WT. (D) Western blotting results of lysates from 293T cells expressing vector control and CPT1C WT. P values were determined by a two-tailed Student's t test. n.s.: not significant.

Figure S2

A

1. PGK1: K131
2. GAPDH: K61,K194,K215,K263
3. ATB5B: K426
4. ENO1: K5,K64,K71,K80,K81,K89, K228,K233,K330,K335
5. PYGL: K29
6. GPI: K454
7. HSP70: K191,K362,K407
8. HDPD1: K218
9. HSPA: K523
10. ACADM: K217
11. CKB: K242,K307,K313
12. PGAM1: K100
13. TCP1: K400
14. NME2: K124
15. EEF1G: K434
16. CANX: K170
17. STIP: K325
18. CCT6A: K74,K78,K91
19. HADH: K644
20. ACAA2: K234
21. CCT5: K170
22. FACN: K1704
23. PSMB3: K77
24. ACADVL: K278
25. CCT8: K318,K326
26. HSD17B4: K424
27. ACLY: K978
28. HSPE1: K56
29. 14-3-3 zeta: K9,K11,K50
30. RPL1: K67
31. PPIA: K28
32. CLTC: K619
33. PRDX1: K35
34. PTGES3: K33
35. ARAD3B: K364
36. TFG: K103
37. ACO2: K736
38. CACYBP:K8
39. GSTK1: K71
40. MRPL15: K228

B

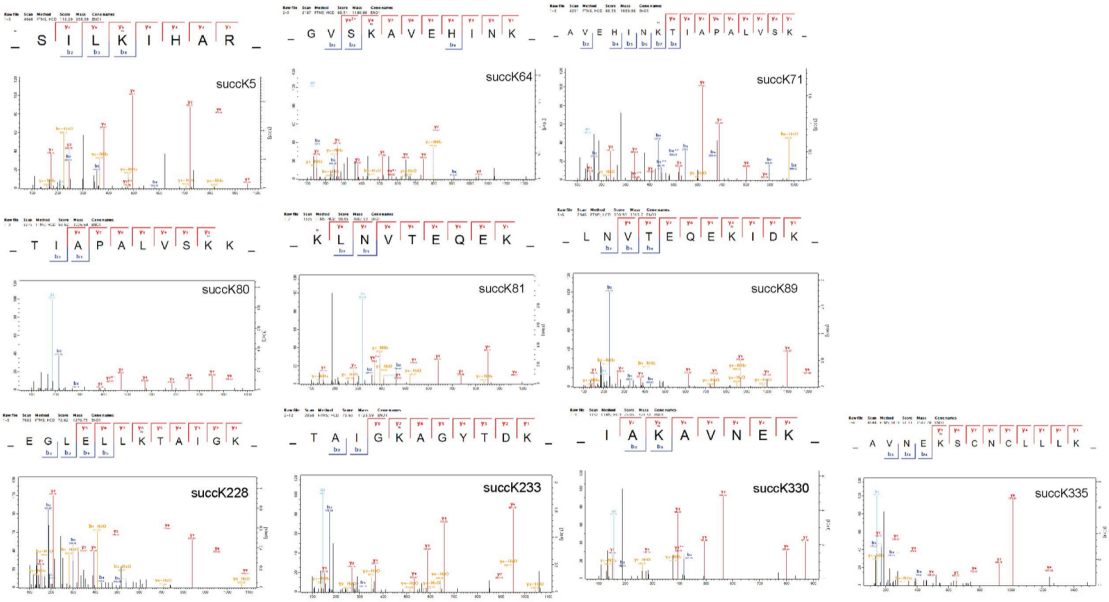


Figure S2, SILAC succinyl proteomic analysis of cells overexpressing CPT1A, Related to Figure 2. (A) A list of 40 proteins with succinylated lysine sites that are increased >2.0 fold by CPT1A WT expression in the SILAC-based quantitative lysine succinylation analysis. (B) MS/MS spectrum for the identification and quantification of succinylated enolase 1 peptides. Underlined nominal masses above and below the sequence denote the b and y ions that indicate peptide backbone fragment ions containing the N and C terminal, respectively.

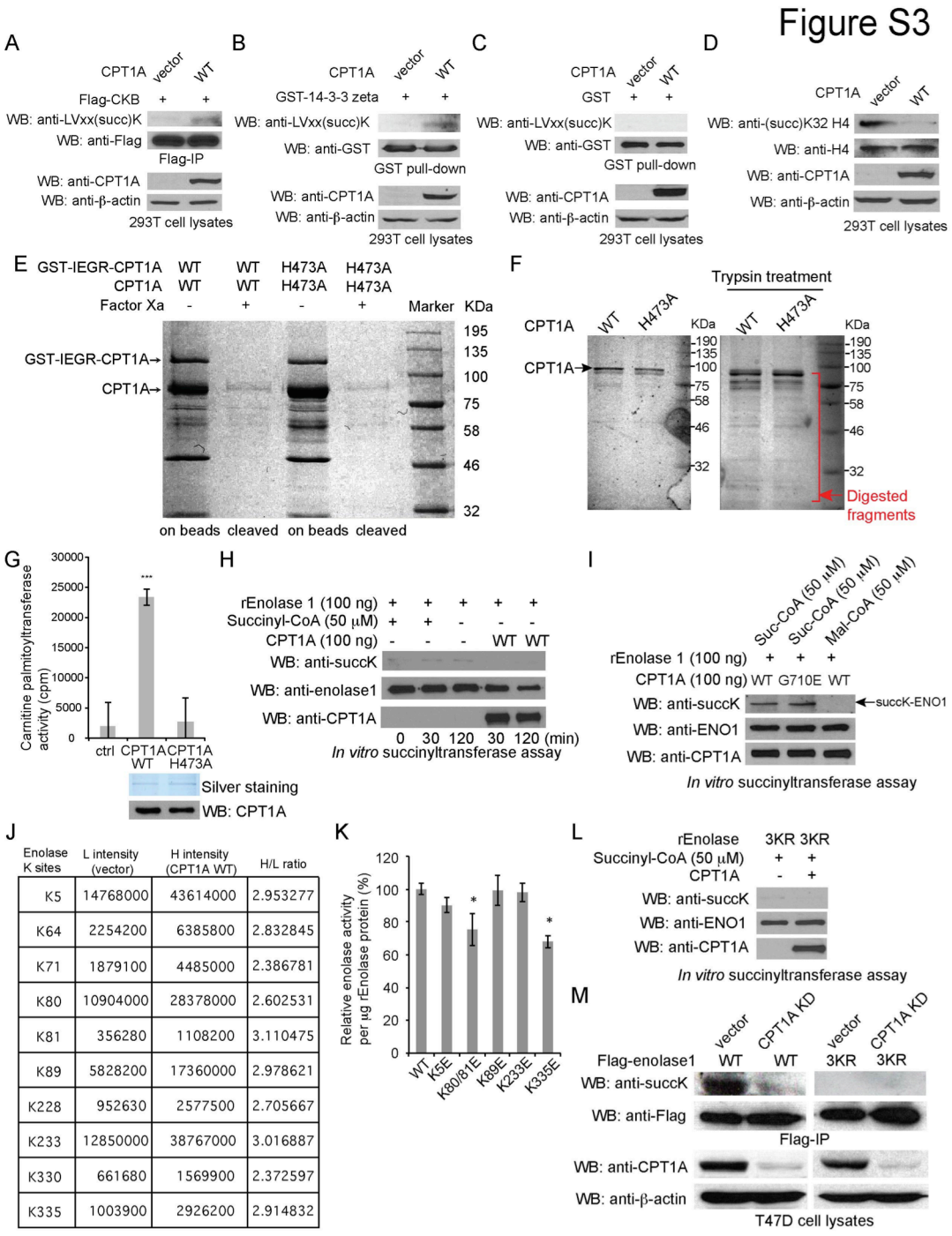


Figure S3, Validation of SILAC succinyl proteomics analysis, Related to Figure 3.
(A) Immunoprecipitated Flag-tagged CKB were Western blotted with anti-LVxx(succ)K succinylated lysine motif and anti-Flag antibodies. Lysates were Western blotted for CPT1A and β-actin. (B-C) Pulled down GST-tagged 14-3-3 zeta or GST alone were Western blotted with anti-LVxx(succ)K succinylated lysine motif and anti-GST antibodies. Lysates were Western blotted for CPT1A and β-actin. (D) Western blotting results of lysates from 293T cells stably expressing vector control and CPT1A WT with anti-(succ)K32 H4, anti-H4, anti-CPT1A, and anti-β-actin antibodies. (E) Coomassie blue staining of recombinant CPT1A WT and H473A proteins purified by Factor Xa cleavage. (F) Silver staining of

purified CPT1A WT and H473A proteins treated with or without sequence-grade trypsin (1 unit) for 15 min at room temperature. (G) Carnitine palmitoyltransferase activity assay results of purified CPT1A WT and H473A mutant. Silver staining results and Western blotting results of purified CPT1A proteins with anti-CPT1A antibody are also shown. (H) The *in vitro* succinyltransferase assay was performed using purified enolase 1 and succinyl-CoA without CPT1A protein or using purified CPT1A WT and enolase 1 without succinyl-CoA. The resulting samples were Western blotted with anti-pan-succinylated lysine, anti-CPT1A, and anti-enolase 1 antibodies. (I) The *in vitro* succinyltransferase assay was performed using purified CPT1A (WT or G710E) and enolase 1 with succinyl-CoA or malonyl-CoA. The resulting samples were Western blotted with anti-pan-succinylated lysine, anti-CPT1A, and anti-enolase 1 antibodies. (J) A list of the succinylated lysine sites of enolase 1 along with their peptide peak intensities detected by SILAC succinyl proteomics analysis of 293T cells expressing vector control and CPT1A WT. (K) Enolase activity assay results of purified enolase 1 WT and KE mutants. (L) The *in vitro* succinyltransferase assay was performed using purified CPT1A WT and enolase 1 3KR mutant with succinyl-CoA. The resulting samples were Western blotted with anti-pan-succinylated lysine, anti-CPT1A, and anti-enolase 1 antibodies. (M) Immunoprecipitated Flag-tagged enolase WT and 3KR mutant were Western blotted with anti-pan-succinylated lysine and anti-Flag antibodies. Lysates were Western blotted for CPT1A and β-actin.

A

Figure S4

Protein names	Gene names	Protein ratio (WT/vector)
Very long-chain specific acyl-CoA dehydrogenase	ACADVL	0.64635
Acyl-CoA dehydrogenase family member 11	ACAD11	0.56261
Acyl-CoA dehydrogenase family member 9	ACAD9	0.80496
3-ketoacyl-CoA thiolase, mitochondrial	ACAA2	0.72823
3-hydroxyacyl-CoA dehydrogenase type-2	HSD17B10	0.81856

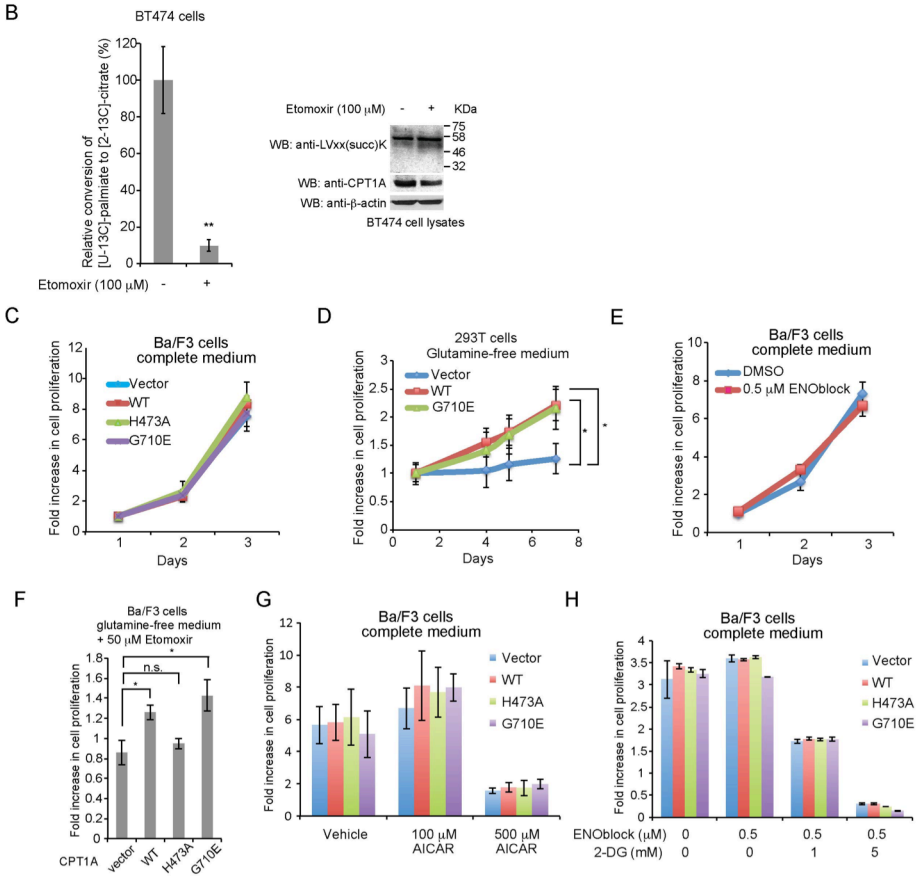


Figure S4, Functional consequences of CPT1A-dependent lysine succinylation in cells, Related to Figure 4. (A) A list of enzymes related to fatty acid oxidation pathway whose protein levels were decreased by expression of CPT1A WT in 293T cells. (B) *left,* Metabolic flux analysis of fatty acid oxidation activity in BT474 cells treated with or without etomoxir. *right,* Western blotting results of lysates from etomoxir- or vehicle-treated BT474 cells with anti-LVxx(succ)K, anti-CPT1A, and anti-β-actin antibodies. (C) Proliferation assay results of Ba/F3 cells expressing CPT1A WT and mutants as well as vector control in complete medium. (D) Proliferation assay results of 293T cells expressing CPT1A WT and G710E as well as vector control in glutamine-free medium. (E) Proliferation assay results of Ba/F3 cells treated with 0.5 μM ENOblock and vehicle control in complete medium. (F) Proliferation assay results of Ba/F3 cells expressing CPT1A WT and mutants as well as vector control treated with etomoxir in glutamine-free medium. Cell proliferation was measured at day 5 after the treatment. (G) Proliferation assay results of Ba/F3 cells expressing CPT1A WT and mutants as well as vector control treated with AICAR in complete medium. Cell proliferation was measured at day 2 after the treatment. (H) Proliferation assay results of Ba/F3 cells expressing CPT1A WT and mutants as well as vector control treated with ENOblock and 2-DG in complete medium. Cell proliferation was measured at day 2 after the treatment.

Supplemental Experimental Procedures

Cell culture and Western blotting
293T cells stably transfected with CPT1A WT and mutants were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 100 μg/ml hygromycin. BT474 cells stably transfected with shRNAs against CPT1A were cultured in DMEM/F12 phenol red free medium with 10% FBS, 1% penicillin/streptomycin, and 1 μg/ml puromycin. Ba/F3 cells stably transfected with CPT1A WT and mutants were cultured in RPMI 1640 medium with 10% FBS, 1% penicillin/streptomycin, 10 ng/ml murine IL3, and 1 μg/ml puromycin. 293T and BT474 stable cells were directly lysed, sonicated, and heated at 95°C for 5 min in SDS-PAGE sample buffer containing 10 mM HEPES, pH 7.5, 2 mM sodium orthovanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 50 mM nicotinamide, 300 mM NaCl, 1% Nonidet P-40, 100 mM dithiothreitol, 5% glycerol, 1% SDS, and cOmplete™ EDTA-free protease inhibitor cocktail (Roche). Ba/F3 cells were lysed in 10 mM HEPES, pH 7.5, 2 mM sodium orthovanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 50 mM nicotinamide, 300 mM NaCl, 1% Nonidet P-40 and cOmplete™ EDTA-free protease inhibitor cocktail (Roche). Crude lysate was centrifuged at 12,000 x g for 10 min at 4˚C. Clarified lysate was mixed with 6x SDS sample buffer and heated at 95°C for 5 min for Western blotting. The following commercial antibodies were used: anti-pan-succinylated lysine antibody (PTM-401) and anti-succinylated H4 from PTM Bio, anti-β-actin (A1978) and anti-Flag antibodies from Sigma, anti-CPT1A antibody (15184-1-AP) and anti-H4 from Proteintech, and anti-enolase 1 antibody (3810S) from Cell Signaling Technology. Anti-LVxx(succ)K succinylated lysine motif antibody was developed by PTM Bio. All the peptide libraries used for dot blot assay were developed by PTM Bio. ENOblock was purchased from AdoQ bio.

Anti-Flag immunoprecipiation
Flag-tagged enolase 1 and CPT1A WT (or vector control) plasmids were transiently co-transfected in 293T cells. Cell lysates were obtained by homogenization in 20 mM HEPES, pH 7.5, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM nicotinamide, 5 mM dithiothreitol, and cOmplete™ EDTA-free protease inhibitor cocktail (Roche). Crude lysates were centrifuged at 12,000 x g for 10 min at 4˚C. Clarified lysate was diluted with equal volume of 10 mM HEPES, pH 7.5, 2 mM sodium orthovanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 50 mM nicotinamide, 300 mM NaCl, 1% Nonidet P-40 and then incubated with anti-Flag antibody conjugated beads (Sigma) for 60 min at 4˚C. After the incubation, the beads were washed twice with TBS and the Flag-tagged proteins were eluted with 3X Flag peptide (Sigma) in TBS.

Retroviral constructs and production of retroviruses.
Human CPT1A (NM_001876) cDNA was purchased from Genscript. Human enolase 1, 14-3-3, and CKB cDNA were purchased from GE Dharmacon. All cDNAs were amplified by PCR and subcloned into pLHCX-hygro- or pMSCV-puro-derived Gateway destination vector as described (Hitosugi et al., 2012). The pLHCX-hygro destination vectors were co-transfected with packaging plasmids into 293T cells, which were selected in 100 μg/ml hygromycin for 3 weeks. The pMSCV-puro destination vectors were co-transfected with packaging plasmids into 293T cells. Retrovirus was harvested 48 h after transfection, and 8 mg/ml of polybrene was added. Ba/F3 cells were infected with harvested retrovirus and selected in 1 μg/ml puromycin for 2 weeks.

shRNA constructs and production of lentivirus
The lentiviral human CPT1A shRNA construct (TRCN0000036279) in the pLKO.1-puro vector was purchased from Open Biosystems. shRNA plasmids were co-transfected into 293T cells along with lentiviral packaging plasmids as described (Hitosugi et al., 2012). Lentivirus was harvested 48 h after transfection, and 8 μg/ml of polybrene was added. Subconfluent BT474 cells were infected with harvested lentivirus and selected in 1 μg/ml puromycin for 2 weeks.

Site-directed mutagenesis

H473A and G710E mutations were introduced into CPT1A using a QuikChange-XL site-directed mutagenesis kit (Stratagene). All constructs were sequence verified using Sanger sequencing.

SILAC labeling and sample preparation for LC-MS/MS analysis

SILAC labeling was performed using SILAC Protein Quantification Kit (Pierce, Thermo) according to the supplier's instructions. The signal intensity of a lysine succinylated peptide with heavy (H) $^{13}C_6$ lysine labeling was divided by that with light (L) $^{12}C_6$ lysine labeling to calculate the lysine succinylation H/L ratio (Figure 1B). To accurately quantify CPT1A-dependent succinylation levels of each lysine site, the lysine succinylation H/L ratio on each peptide was normalized by the peptide H/L ratio, which was calculated by dividing the peptide counts of the corresponding peptide with heavy (H) $^{13}C_6$ lysine labeling by those with light (L) $^{12}C_6$ lysine labeling detected by the quantitative LC-MS analysis. 293T cells expressing CPT1A-WT were labeled with $_L$- ^{13}C-Lysine (heavy amino acid), while the control 293T cells expressing pLHCX vector were labeled with $_L$-^{12}C Lysine (light amino acid). The labelings were performed separately and the cells were passaged at least six times to ensure 97% labeling efficiency before harvest. The cells were expanded in SILAC media to obtain ~5 x 10^8 cells, collected and washed twice with ice-cold PBS supplemented with 3 μM trichostatin A (TSA) and 50 mM nicotinamide (NAM). The cell pellets were sonicated three times using high intensity ultrasonic processor (Scientz) in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0 and 0.5% NP-40) containing 5 mM DTT, 3 μM TSA, 1% protease inhibitor cocktail III, 2 mM EDTA and 50 mM NAM in addition to 8 M urea. Equal amounts of crude proteins from "heavy" and "light" labeled cells were mixed and precipitated with 15% trichloroacetic acid (TCA). The precipitated proteins were washed twice with -20˚C acetone, dissolved in 100 mM NH_4HCO_3, pH 8.0 and digested with trypsin at a trypsin-to-protein ratio of 1:50 (w/w) at 37˚C for 16 h. Following digestion, DTT was added to a final concentration of 5 mM. The samples were incubated at 50˚C for 30 min and alkylated using iodoacetamide (Sigma) at a final concentration of 15 mM at room temperature in the dark for 30 min. The alkylation reaction was quenched using 30 mM cysteine at room temperature for 30 min, and the samples were further digested by adding trypsin at a ratio of 1:100 (w/w) at 37˚C for 4 h. The digested peptide samples were separated into 18 fractions by using a C18 column (Agilent 300Extend C18 column, 250 x 4.6 mm, 5-μm particle size) using a gradient of 2% to 60% acetonitrile (ACN) in 10 mM ammonium bicarbonate, pH 10.0, over the period of 80 min. To enrich the lysine succinylated peptides, the tryptic peptides were first dissolved in NETN buffer and then incubated with anti-pan-succinylated lysine antibody-conjugated beads (PTM Bio) at 4˚C overnight with gentle shaking. The beads were washed four times with NETN buffer and twice with ddH_2O. The bound peptides were eluted from the beads by using 0.1% trifluoacetic acid (Sigma). The eluted fractions were combined, vacuum-dried and further cleaned with C18 ZipTips (Millipore) according to the supplier's instructions.

LC-MS/MS succinyl proteomics analyses and database searching

Enriched peptides were dissolved in 0.1% formic acid (Fluka) and directly loaded onto a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific) equipped with a pre-column (Acclaim PepMap 100). Peptides were separated with a constant flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system with a gradient composed of solvent B (0.1% formic acid in 98% ACN). The method was run from 6% to 22% solvent B for the first 26 min, 22% to 35% for 8 min and then to 80% in a 3 min duration and a final 3 min hold at 80%. The MS/MS analysis was performed on a Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific) using a nanospray ionization (NSI) source. For MS scans, the m/z scan range was from 350 to 1800 and the intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using NCE setting as 30; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 1E4 in the MS survey scan with 10.0 s dynamic exclusion. The electrospray voltage applied was 2.0 kV and automatic gain control (AGC) was used to prevent overfilling of the ion trap; 5E4 ions were accumulated for generation of MS/MS spectra. The resulting MS/MS data was processed using MaxQuant with integrated Andromeda search engine (v.1.4.1.2). Tandem mass spectra were searched against *SwissProt_Human* (20,274 sequences) database concatenated with reverse decoy database. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. False discovery rate (FDR) thresholds for protein, peptide and modification site were specified at 1% while the minimum peptide was set at 7. All the other parameters in the MaxQuant were set to default values. The site localization probability was set as >0.75. We normalized our whole proteomics data by the customary

calculation that divides all the signal intensity values by the average value, which is necessary to normalize the data based on the sample loading amount. While in succinyl proteomics, sample preparation with enrichment of succinylated peptides by immunoprecipitation should result in a difference in the total amount of succinylated peptides between the vector and CPT1A KD samples because CPT1A KD cells showed significant decrease in lysine succinylation in lysates as compared to vector control cells as shown in Figure 2A. This difference in the total amount of succinylated peptides makes the different averages of the signal intensities from succinylated peptides in vector control and CPT1A KD cells, and such different averages should represent biologically relevant difference in succinylation between vector control and CPT1A KD cells (the average of signal intensities from succinylated peptides in CPT1A KD cells divided by that in vector cells was 0.88 as shown in Table S3, suggesting that overall succinylation is decreased by CPT1A knockdown as expected from Western blotting results in Figure 2A) and should not be directly used for the customary calculation to normalize succinyl proteomics data. Instead, we performed the following two-step normalization for our succinyl proteomics data in Figure 2B as previously published (Hebert et al., 2013; Park et al., 2013): **1)** We normalized our succinyl proteomics data by the customary calculation using T47D whole proteomics data (all the numbers of the signal intensities from succinylated peptides in vector control and CPT1A KD cells were respectively divided by the average of the signal intensities from the "total peptides" in vector control and CPT1A KD cells, which enables to normalize the succinyl proteomics data based on the starting amount of the total peptides in vector control and CPT1A KD cells); **2)** We further divided the "H/L ratios" of succinylated peptides from step **1** by the "H/L ratios" of total peptides, which were taken from the same cell lysates as illustrated in Figure 1. This additional normalization makes sure that changes in H/L ratio are due to changes in lysine succinylation on each protein, not due to changes in its protein expression or experimental artifacts from incomplete incorporation of heavy amino acids into cells. We also normalized the succinyl proteomics data from 293T cells in Figure 1 in the exactly same way.

Purification of recombinant CPT1A proteins

CPT1A proteins were purified using Factor Xa Cleavage Capture kit (Millipore) since neither of N-terminus nor C-terminus Flag-tagged CPT1A proteins showed any enzymatic activities (data not shown). The CPT1A variants with N-terminus Factor Xa cleavage sequence (IEGR) and the CPT1A variants without the IEGR sequence were respectively subcloned into pDEST27 (N-terminus GST-tag) destination vector and pcDNA 3.2 (no tag) Gateway destination vector (Life Technologies). GST-IEGR-CPT1A WT along with CPT1A WT (or GST-IEGR-CPT1A H473A mutant along with CPT1A H473A mutant) were co-transfected into 293T cells. Transfected 293T cells were lysed in buffer containing 10 mM HEPES, pH 7.5, 2 mM sodium orthovanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 300 mM NaCl, 1% Nonidet P-40, and protease inhibitor cocktail (Roche). GST-IEGR-CPT1A and CPT1A proteins were pulled down from the lysates by glutathione-Sepharose beads. The GST-IEGR-CPT1A and CPT1A protein bound beads were extensively washed with PBS and treated by Factor Xa enzyme in PBS to obtain cleaved CPT1A proteins. Factor Xa was removed by Factor Xa Cleavage Capture kit. The purification efficiency was examined by Coomassie blue staining.

Purification of recombinant enolase 1

Human enolase 1 (BC011130) cDNA (GE Dharmacon) was amplified by PCR and subcloned into pET53 Gateway destination vector, which appends an N-terminal His_6-tag, as described (Hitosugi et al., 2012). Transformed BL21(DE3)pLysS *E. coli* were grown to a density of 0.5 (OD600) at 37˚C and induced with 1 mM IPTG for 4 h at 37˚C. Bacterial cells were collected and sonicated in buffer containing 20 mM sodium phosphate, pH 8.0 0.5 M NaCl, and 20 mM imidazole in the presence of protease inhibitor and centrifuged at 4,800 x g for 15 min at 4˚C. Clarified lysate was loaded onto a Ni-NTA column and washed with 20 mM sodium phosphate, pH 8.0 and 0.5 M NaCl, and 20 mM imidazole. Bound proteins were eluted with 20 mM sodium phosphate, pH 8.0 and 0.5 M NaCl, and 250 mM imidazole. Proteins were desalted on a PD-10 column and purity of the preparation was assessed by Coomassie blue staining.

***In vitro* enolase activity assay**

Enolase activity was measured using an enzyme coupled assay. Enolase enzyme mix containing 100 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM ADP, 0.2 mM NADH, 0.5 unit/ml recombinant human pyruvate kinase M1 purified as described (Hitosugi et al., 2012), and 0.1 unit/ml rabbit muscle lactate dehydrogenase (EMD Millipore) was prepared. 2-phosphoglycerate (2-PG) was added last to initiate

the reaction at a final concentration of 2 mM. The decrease in autofluorescence (ex: 340 nm, em: 460 nm) due to NADH oxidation was measured as enolase activity. The reaction mixtures of *in vitro* succinyltransferase assay were directly used for the enolase activity assay. For the activity assay of enolase in Ba/F3 cells, cells were lysed in 10 mM HEPES, pH 7.5, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 300 mM NaCl, 1% Nonidet P-40 and protease inhibitor cocktails. Crude lysate was centrifuged at 12,000 x g for 10 min at 4˚C. Clarified lysate was used for enolase enzyme activity assay.

Carnitine palmitoyltransferase activity assay

Carnitine palmitoyltransferase activity was measured as described (Dobrzyn et al., 2004). Cells were homogenized in buffer containing 20 mM HEPES, pH 7.5, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 μM PMSF, 10 mM EGTA, 2 mM sodium orthovanadate, 5 mM sodium fluoride, and 5 mM sodium pyrophosphate. The homogenates were centrifuged at 1,000 x g for 10 min at 4°C to remove nuclear material and cell debris. The supernatant was further centrifuged at 14,000 x g for 5 min at 4°C to obtain membrane proteins. 200 μg of the membrane proteins were added to the assay medium containing 20 mM HEPES, pH 7.5, 75 mM KCl, 1% BSA, 4 mM $MgCl_2$, 4 mM ATP, 250 μM GSH, 70 μM palmitoyl-CoA, 0.25 mM carnitine, 10 μCi L-[^{3}H]carnitine, with or without 100 μM malonyl-CoA. Samples were incubated at 37°C for 10 min. The reaction was stopped by the addition of 0.5 ml of ice-cold 1 M perchloric acid. The reaction mixtures were centrifuged and the supernatant was removed. The membrane pellets were resuspended in 0.5 ml of ddH_2O and extracted with 0.6 ml of 1-butanol. 0.4 ml of the 1-butanol phase was back-extracted with 60 μL of ddH_2O. Seventy microliters of the 1-butanol-phase was counted by liquid scintillation and malonyl-CoA-sensitive carnitine palmitoyltransferase activity was calculated.

GC/MS analysis of succinyl-CoA

Intracellular levels of succinyl-CoA in cell lines expressing CPT1A WT and mutants as well as vector control were measured with an isotope-ratio based approach using GC/MS as we have performed previously (Hitosugi et al., 2012). In brief, two dishes of cells were cultured in media supplemented with [U-13C]glucose for 12 hours to label endogenous succinyl-CoA first, one dish of cells were quickly rinsed in ice-cold PBS and homogenized in 10% TCA, and same number of cells in another dish were also quickly rinsed in ice-cold PBS and homogenized in 10% TCA spiked with 80 nmoles of pure succinyl-CoA standard (Sigma). The homogenates were centrifuged to remove debris and the resulting supernatants were applied to a solid-phase extraction column preconditioned with methanol followed by water. The columns were washed with water three times, and succinyl-CoA was eluted with methanol. The eluents were dried up and reconstituted in water. The succinyl-CoA solution was made alkaline with NaOH to hydrolyze succinyl-CoA at 40^{o}C for 1 hour and acidified with HCl. Succinate was extracted from the acidified solutions with ethylacetate, derivatized with MTBSTFA in DMF, and analyzed by GC/MS (Li et al., 2015). The detailed isotopic distributions of succinate extracted from cell lysates with or without spiking with unlabeled succinyl-CoA standard were presented in Figure S4A. The change in isotopic distribution of the extracted succinate (m/z 289-293) by spiking with unlabeled succinyl-CoA standard was measured to quantify intracellular endogenous succinyl-CoA levels. Natural isotope abundance was calculated with IsoPat^2 software using pure succinate (Gruber et al., 2007).

NAD+ quantification assay

Intracellular NAD+ levels were measured by a NAD+/NADH colorimetric kit (BioVision) as per manufacturer's instructions. Briefly, metabolites were extracted from 2 x 10^5 of 293T cells expressing CPT1A WT (or vector control) with freeze/thaw cycles in the extraction buffer. 1/4 of the extracted sample was used for total NAD+ and NADH detection and the rest samples were heated at 60 ^{o}C for 30 min to decompose all the NAD+. The samples were then mixed in the NAD cycling enzyme mix for 5 min at room temperature and incubated with NADH developer for 1-2 hours at room temperature. Total NAD+ and NADH as well as NADH were separately measured by OD_{450nm}. The concentration of NAD+ was then calculated by subtracting the NADH value from the total NAD+ and NADH.

GC/MS analysis of enolase activity by measuring the conversion rate of [3-13C]-2PG to [3-13C]-PEP in cells incubated with 13C-glucose

Enolase activity was measured by monitoring the conversion rate of [3-13C]-2PG to [3-13C]-PEP with GC/MS. In brief, the cells were incubated with [U-13C]-glucose for overnight. After the incubation, the

cells were quickly rinsed in ice-cold PBS and lysed with 50% methanol. The crude lysates were centrifuged to remove debris. The resulting supernatants were dried with nitrogen gas, derivatized with MSTFA, and then injected onto GC/MS as previously described (Hitosugi et al., 2012). The flux rate of [U-13C]-glucose to [3-13C]-2PG was determined by dividing the ion intensity of [3-13C]-2PG (m/z 462) by that of [12C]-2PG (m/z 459) and the flux rate of [U-13C]-glucose to [3-13C]-PEP was determined by dividing the ion intensity of [3-13C]-PEP (m/z 372) by that of [12C]-PEP (m/z 369). The enolase activity was calculated by dividing the [3-13C]-PEP flux rate by the [3-13C]-2PG flux rate.

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