Supporting information

MATERIALS AND METHODS

Materials. Rotenone, acetyl-CoA, succinyl-CoA, BHB-CoA, propionyl-CoA, HMG-CoA, reduced CoA (CoASH), trichloroacetic acid, 5-sulfosalicylic acid and [U-$^{13}C_6$]-glucose were purchased from Sigma-Aldrich (St. Louis, MO). [$^{13}C_3{}^{15}N_1$]-pantothenate was purchased from Isosciences (King of Prussia, PA). Pantothenate-free RPMI 1640 media was prepared by AthenaES, (Baltimore, MD). Charcoal-dextran stripped fetal bovine serum was purchased from Gemini Bio-products, (West Sacramento, CA). Oasis HLB 1cc (30 mg) SPE columns were purchased from Waters (Milford, MA). Methanol, acetonitrile and water were all purchased from Fisher Scientific (Optima grade).

Generation of Stable Isotope Labeled Internal Standards. Stable isotope labeled CoASH and CoA thioester internal standards were generated using [$^{13}C_3{}^{15}N_1$]-pantothenate as described in our recent reports.[18,19] Briefly, murine hepatocytes (Hepa 1c1c7) were cultured and expanded for three passages in pantothenate-free RPMI media supplemented with 10% charcoal-dextran stripped fetal bovine serum and 1 mg/L [$^{13}C_3{}^{15}N_1$]-pantothenate. After three passages, the media were replaced for 24 h with "ultra-labeling" media containing 3 mg/L labeled pantothenate and 3% charcoal-dextran stripped fetal bovine serum. Cells were washed, harvested by scraping and pulse sonicated in ice-cold 10% trichloroacetic acid (1 ml/plate). Protein and cellular debris were pelleted and the remaining acidified extract containing stable isotope labeled CoA thioesters with > 99% [$^{13}C_3{}^{15}N_1$]-pantothenate labeling were pooled, aliquoted, frozen, and stored at -80°C.

Cell Culture and Rotenone Treatments. HepG2 and H358 were maintained in RPMI 1640 media and SH-SY5Y cells were maintained in 1:1 F12:DMEM, each media supplemented with

10% fetal bovine serum, 2mM L-glutamine, penicillin, and streptomycin. Cells were incubated at 37 °C and 95% humidity with 5% CO_2. Rotenone solutions in DMSO were freshly prepared prior to treatment. Serial dilutions of rotenone were made for dosing and time course studies and treatments were performed when cells were approximately 80% confluent and added directly to the plate (DMSO < 1% volume). Control cells were treated with an equal amount of DMSO containing no rotenone.

Extraction and Quantification of CoASH and Thioesters using SILEC standards. Analyses of were performed using modifications of methodology developed previously by our group.[18,19] Briefly, after treatment, cells were harvested by scraping, centrifuged at 500*g*, and resuspended in 1 mL ice-cold 10% trichloroacetic acid containing biosynthetically generated stable coenzyme A thioesters (SILEC). Cells were then pulse-sonicated on ice followed by centrifugation to remove protein debris. The supernatant containing the short chain acyl-CoA species were purified by solid-phase extraction using Oasis HLB SPE columns. The eluant was dried down under nitrogen and resuspended in 50 µL 5% 5-sulfosalicylic acid. CoA standards were processed in a similar fashion.

LC-MS analysis of CoASH and Thioester derivatives. Samples were maintained at 4°C using a Leap CTC autosampler (CTC Analytics, Switzerland) and 10uL injections were used for analysis. Reversed-phase HPLC was used to separate analytes using a Luna C18 column (2.0 x 150 mm, pore size 3 µm, Phenomenex) with a guard column. Solvent A was water and solvent B was 95/5 acetonitrile/water (v/v), each containing 5 mM ammonium acetate. Solvent C was 80/20/0.1 (v/v/v) acetonitrile/water/formic acid. Gradient conditions 98% A/2% B for 1.5 min, increased to 75% A/25% B over 3.5 min, increased to 100% B over 0.5 min, maintained at 100%B for 8.5 min, washed with 100% C for 5 min, before equilibration for 5 min, with a flow

rate of 200 μL/min. Samples were analyzed using an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) in the positive ESI mode. A Valco valve was used to divert the column effluent to the mass spectrometer from 8 min to 13 min and to waste for the remainder of the run. The mass spectrometer operating conditions were as follows: ion spray voltage (5.0 kV), nitrogen as curtain gas (15 units), ion source gas 1 (8 units), gas 2 (15 units), and collision-induced dissociation (CID) gas (5 units). The ESI probe temperature was 450°C, declustering potential was 105 V, entrance potential was 10 V, collision energy was 45 eV, and the collision exit potential was 15 V. Transitions (*m/z*) for precursor → product ions for the LC-SRM/MS analyses were as follows: CoASH (768.1→261.1), [$^{13}C_3{}^{15}N_1$]-CoASH (772.1→265.1), acetyl-CoA (810.1→303.1), [$^{13}C_3{}^{15}N_1$]-acetyl-CoA (814.1→307.1), propionyl-CoA (824.1→317.1), [$^{13}C_3{}^{15}N_1$]-propionyl-CoA (828.1→321.1), malonyl-CoA and BHB-CoA (854.1→347.1), [$^{13}C_3{}^{15}N_1$]-malonyl-CoA and [$^{13}C_3{}^{15}N_1$]-BHB-CoA (858.1→351.1), succinyl-CoA (868.1→361.1), [$^{13}C_3{}^{15}N_1$]-succinyl-CoA (872→365.1), and HMG-CoA (912.1→405.1), [$^{13}C_3{}^{15}N_1$]-HMG-CoA (916.1→409.1). Data was analyzed with Analyst 1.4.1 software. Concentrations were determined from regression lines of standard curves and normalized for protein concentration.

Isotopomer analysis using [$^{13}C_6$]-glucose. SH-SY5Y cells were maintained in culture as described above. For isotopic tracer analysis, glucose-free DMEM media was prepared containing 10% charcoal-dextran stripped fetal bovine serum, penicillin, streptomycin, glutamine and supplemented with 1 mg/L of either unlabeled glucose or [U-$^{13}C_6$]-glucose. Cells were washed with DPBS and treated for 6 h with labeled media containing 100 nM rotenone or DMSO (control). After treatment, CoA species were extracted as previously described except without SILEC standards. LC-MS methods conducted in the same manner except that in

addition to the M0 CoA molecule, the M1, M2, M3 and M4 isotopomer of each metabolite was also quantified. Label-free cells were used to generate an isotopomer enrichment matrix for acetyl-CoA, succinyl-CoA, BHB-CoA and CoASH. An isotopomer array generated for each sample was multiplied by the inverse of the matrix generated from the label free cells to determine the absolute concentration of each isotopomer and presented as a percentage of the total isotopomers for each CoA derivative as described by Fernandez *et al.*[21]

Supplemental Table 1. Absolute levels of CoASH and several short chain acyl-CoA thioesters measured using SILEC methodology. Concentrations are presented as pmol/10^6 cells $\pm$ SEM (n=3 plates/group). When compared to DMSO controls, *p < 0.05 and **p < 0.005. Statistical analyses for all experiments were performed using an unpaired two tailed t test using GraphPad Prism software (La Jolla, CA).

		Acetyl-CoA	Succinyl-CoA	CoASH	BHB-CoA	Propionyl-CoA	HMG-CoA
SH-SY5Y	DMSO	8.68 ± 0.70	3.37 ± 0.10	2.42 ± 0.17	0.065 ± 0.002	0.047 ± 0.003	0.59 ± 0.01
	10 pM	7.05 ± 0.33	2.88 ± 0.30	3.12 ± 1.28	0.065 ± 0.002	0.036 ± 0.006	0.64 ± 0.02
	100 pM	8.99 ± 0.33	2.98 ± 0.07 *	2.97 ± 0.87	0.070 ± 0.001	0.042 ± 0.006	0.62 ± 0.07
	1 nM	9.53 ± 0.51	2.48 ± 0.10 **	3.52 ± 0.21 *	0.079 ± 0.008	0.049 ± 0.002	0.83 ± 0.12
	10 nM	8.21 ± 0.18	2.23 ± 0.09 **	2.13 ± 0.32	0.086 ± 0.002 **	0.041 ± 0.002	0.73 ± 0.06
	100 nM	9.54 ± 0.74	0.99 ± 0.02 **	1.93 ± 0.30	0.177 ± 0.008 **	0.019 ± 0.003 **	1.05 ± 0.16 *
	1 μM	10.25 ± 0.21	0.66 ± 0.04 **	2.19 ± 0.57	0.241 ± 0.027 **	0.010 ± 0.003 **	1.05 ± 0.21
HepG2	DMSO	11.46 ± 0.44	9.52 ± 0.81	10.76 ± 1.50	0.170 ± 0.008	0.704 ± 0.029	2.02 ± 0.21
	10 pM	8.78 ± 0.72 *	6.78 ± 0.35	6.73 ± 0.43	0.150 ± 0.003	0.601 ± 0.037	1.60 ± 0.04
	100 pM	11.49 ± 0.62	7.71 ± 0.45	8.77 ± 1.07	0.164 ± 0.002	0.740 ± 0.049	1.76 ± 0.10
	1 nM	9.35 ± 0.85	7.21 ± 0.69	6.09 ± 0.67 *	0.163 ± 0.019	0.586 ± 0.035	1.84 ± 0.20
	10 nM	10.13 ± 0.93	7.25 ± 0.13	6.78 ± 0.35	0.170 ± 0.011	0.783 ± 0.035	2.29 ± 0.08
	100 nM	10.51 ± 0.15	4.39 ± 0.23 **	5.36 ± 0.41 *	0.325 ± 0.012 **	0.735 ± 0.018	2.00 ± 0.08
	1 μM	12.05 ± 0.46	2.08 ± 0.06 **	5.42 ± 1.17 *	0.582 ± 0.003 **	0.719 ± 0.059	2.10 ± 0.16
H358	DMSO	14.76 ± 0.82	7.48 ± 0.60	5.82 ± 1.12	0.158 ± 0.006	0.157 ± 0.005	2.08 ± 0.19
	10 pM	12.32 ± 0.29 *	7.36 ± 0.20	4.86 ± 0.41	0.160 ± 0.015	0.157 ± 0.011	1.85 ± 0.04
	100 pM	12.20 ± 0.43	7.83 ± 0.40	4.44 ± 0.08	0.152 ± 0.006	0.146 ± 0.001	1.83 ± 0.23
	1 nM	10.94 ± 0.91 *	7.02 ± 0.12	5.13 ± 0.69	0.212 ± 0.010 *	0.179 ± 0.018	1.62 ± 0.07
	10 nM	11.44 ± 0.16 *	6.28 ± 0.20	5.19 ± 0.42	0.358 ± 0.002 **	0.222 ± 0.011 *	1.91 ± 0.19 *
	100 nM	9.05 ± 0.65 **	2.39 ± 0.13 **	3.45 ± 0.19	0.713 ± 0.006 **	0.174 ± 0.005	1.17 ± 0.11 *
	1 μM	10.44 ± 0.34 **	1.30 ± 0.14 **	3.87 ± 0.57	1.103 ± 0.101 **	0.150 ± 0.015	1.36 ± 0.07 *