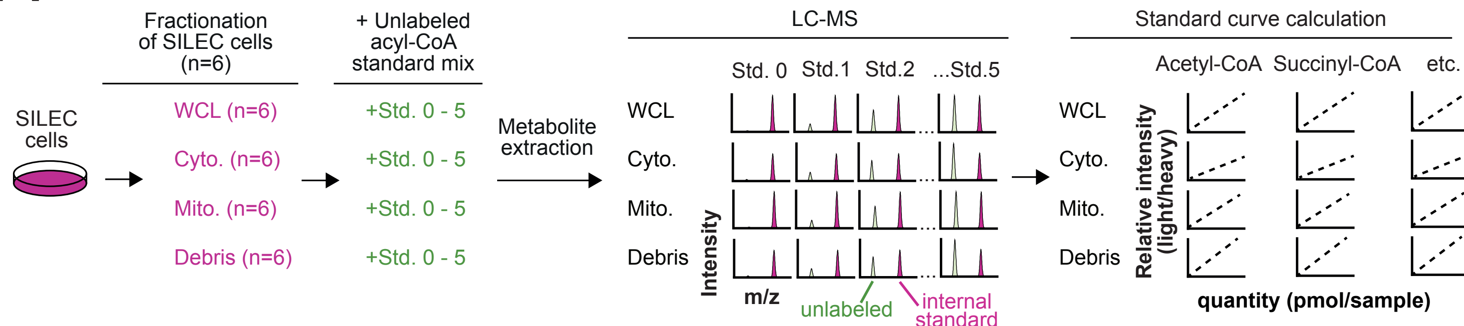
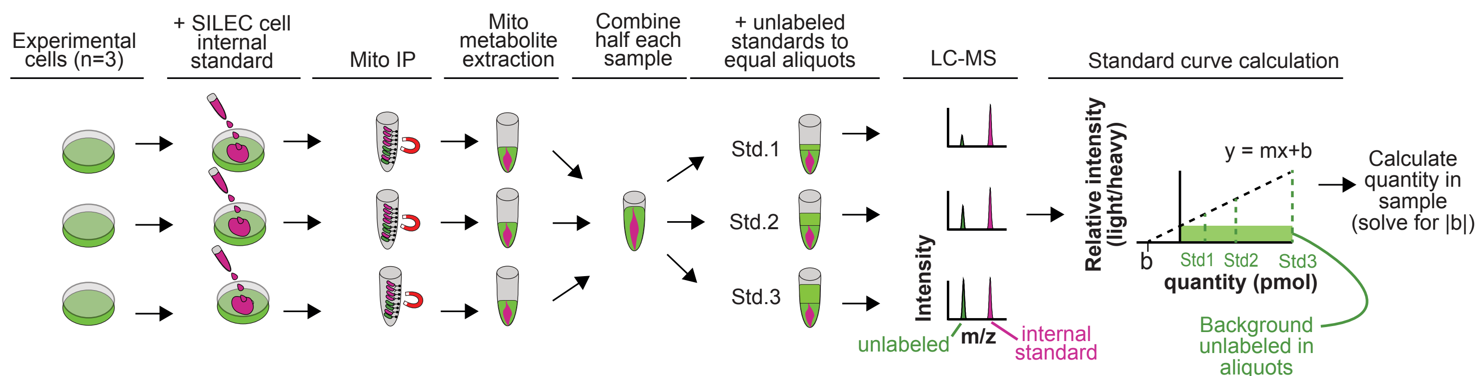




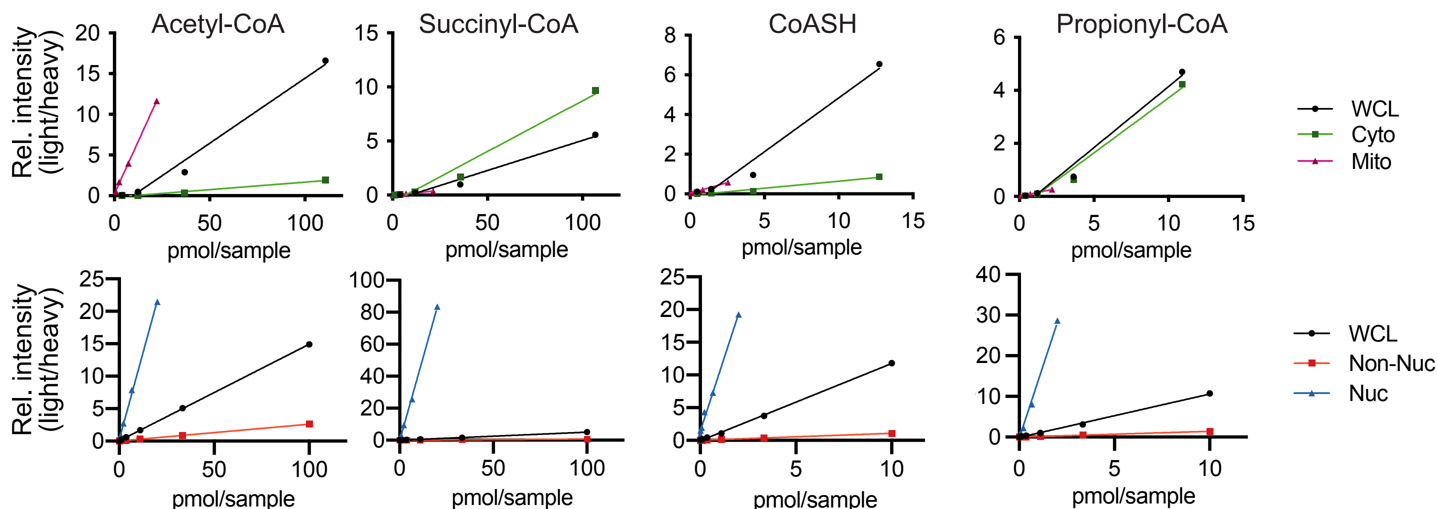


Figure S1: Standard curve generation, related to Figure 1 and STAR Methods
A) Schematic representation of standard curve generation in differential centrifugation protocols. Sub-cellular fractions were generated from equal aliquots of SILEC internal standard cells processed in parallel to the experimental + SILEC cell samples. After fractionation, known quantities of unlabeled standards were added to build standard curves. **B)** Schematic representation of standard curve generation by standard addition in Mito-IP protocol **C)** Representative standard curves generated in different subcellular fractions across several acyl-CoA species in HepG2 cells using differential centrifugation for mitochondrial/cytosol (upper panel) or nuclear (lower panel) isolation. WCL = whole cell lysate.

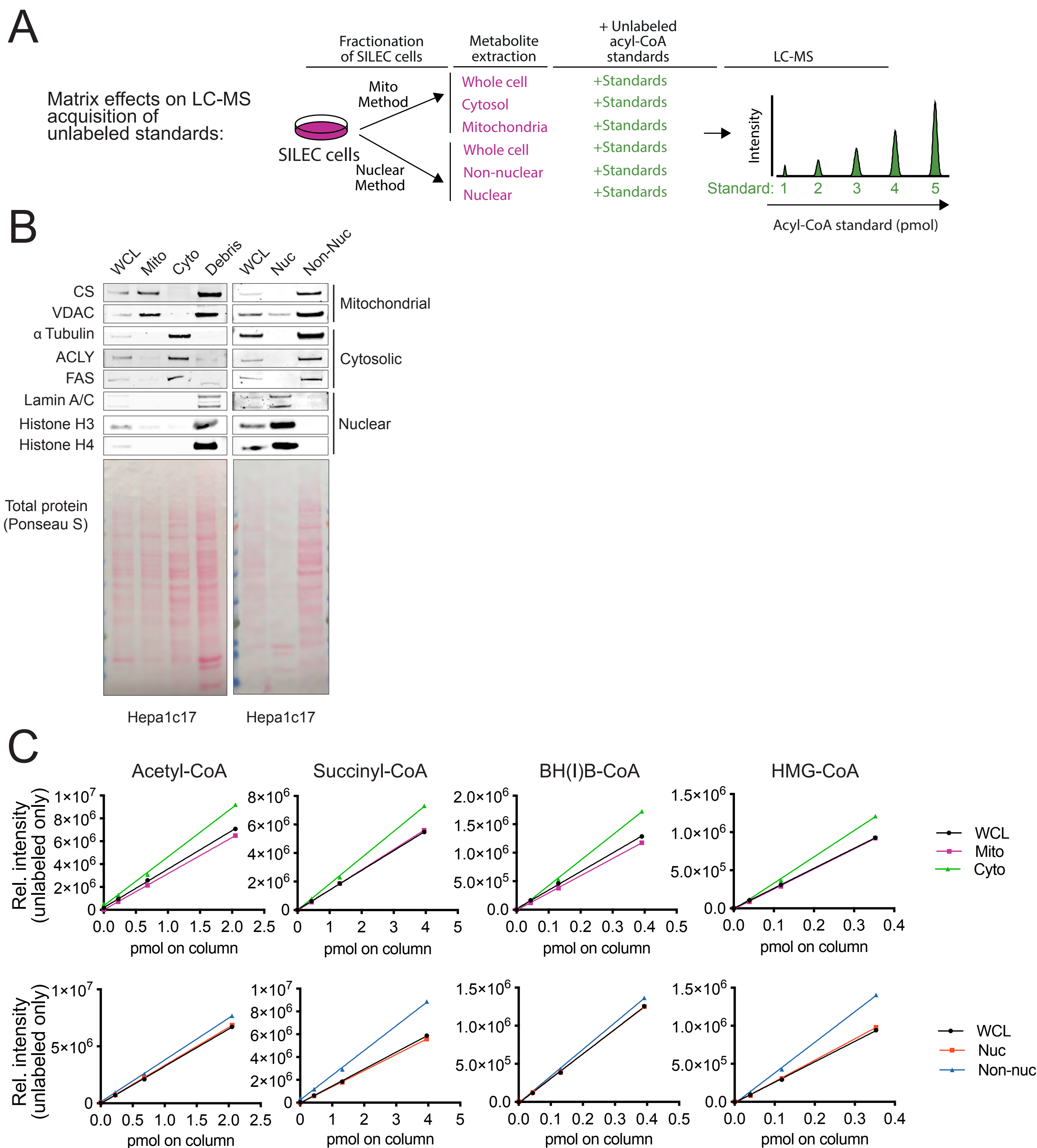


Figure S2: Sub-cellular matrix-specific effects on acyl-CoA acquisition, related to Figure 1
A) SILEC subcellular matrices were extracted from fully labeled Hepa1c17 SILEC cells. Following extraction, known quantities of unlabeled standards were added to equal aliquots of each SILEC matrix and analysed by LC-MS. **B)** Western blot analysis comparing protein enrichment for representative marker proteins for mitochondria, cytosol and nucleus. Equal protein quantity was loaded for each fraction. **C)** Raw signal intensity for unlabeled acyl-CoAs are displayed across a range of concentrations in the presence of different SILEC sub-cellular matrices. Abbreviations: CS (citrate synthase), VDAC (voltage dependent anion channel), FAS (fatty acid synthase), ACSS2 (Acyl-CoA synthetase short chain family member 2), ACLY (ATP-citrate lyase).

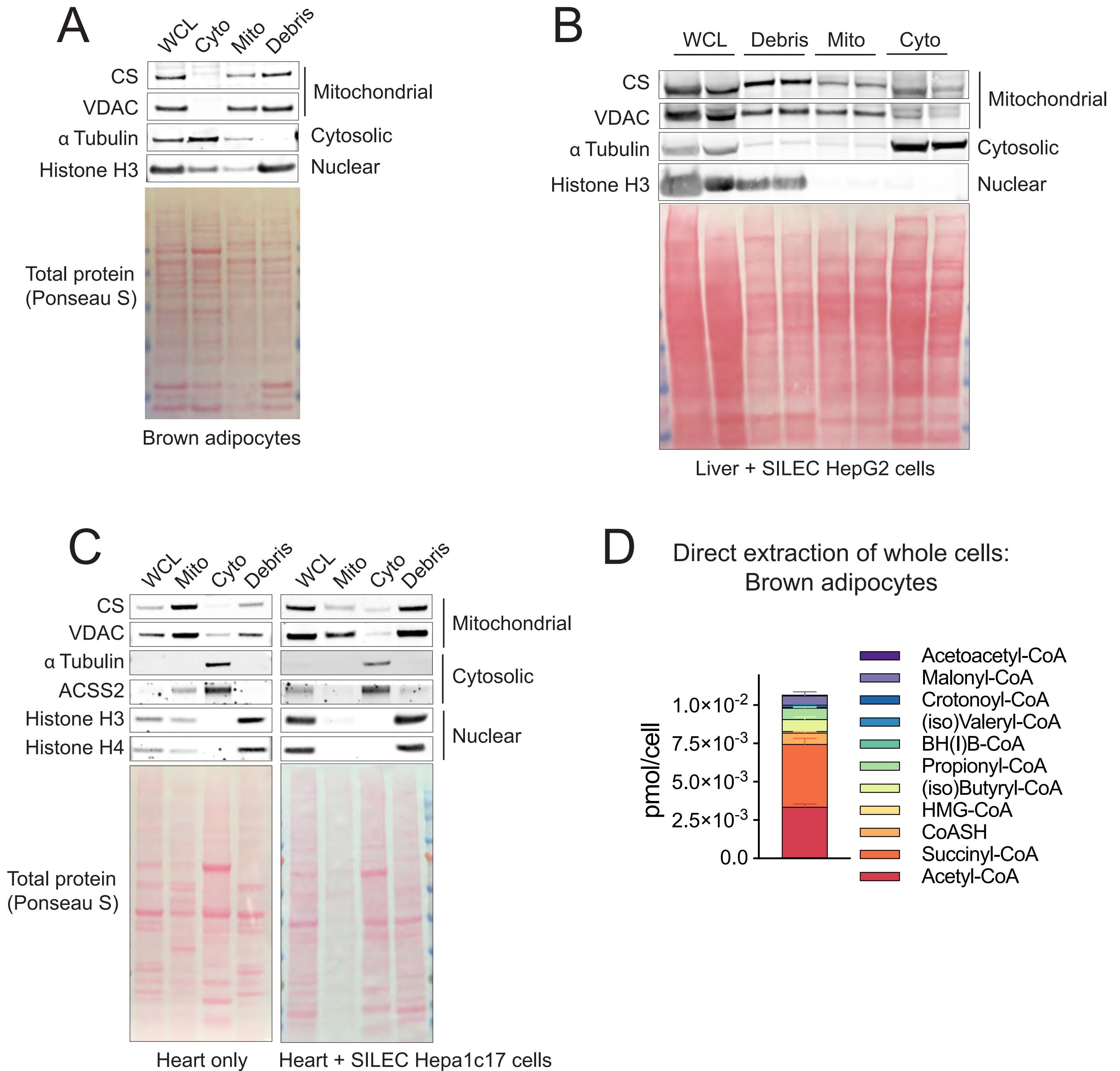


Figure S3: Western blots and acyl-CoA quantitation by direct extraction, related to Figure 2
A-C) Western blot analysis comparing protein enrichment for representative marker proteins for mitochondria, cytosol and nucleus. Equal protein quantity was loaded for each fraction. **A**) Brown adipocytes combined with SILEC brown adipocytes. **B**) Liver tissue combined with SILEC HepG2 cells. **C**) Human heart fractionation for heart only, and heart combined with SILEC Hepa1c17 cell internal standard. **D**) Acyl-CoA quantitation from direct extraction of whole brown adipocytes relating to **Figure 2B**. Abbreviations: CS (citrate synthase), VDAC (voltage dependent anion channel), ACSS2 (Acyl-CoA synthetase short chain family member 2).

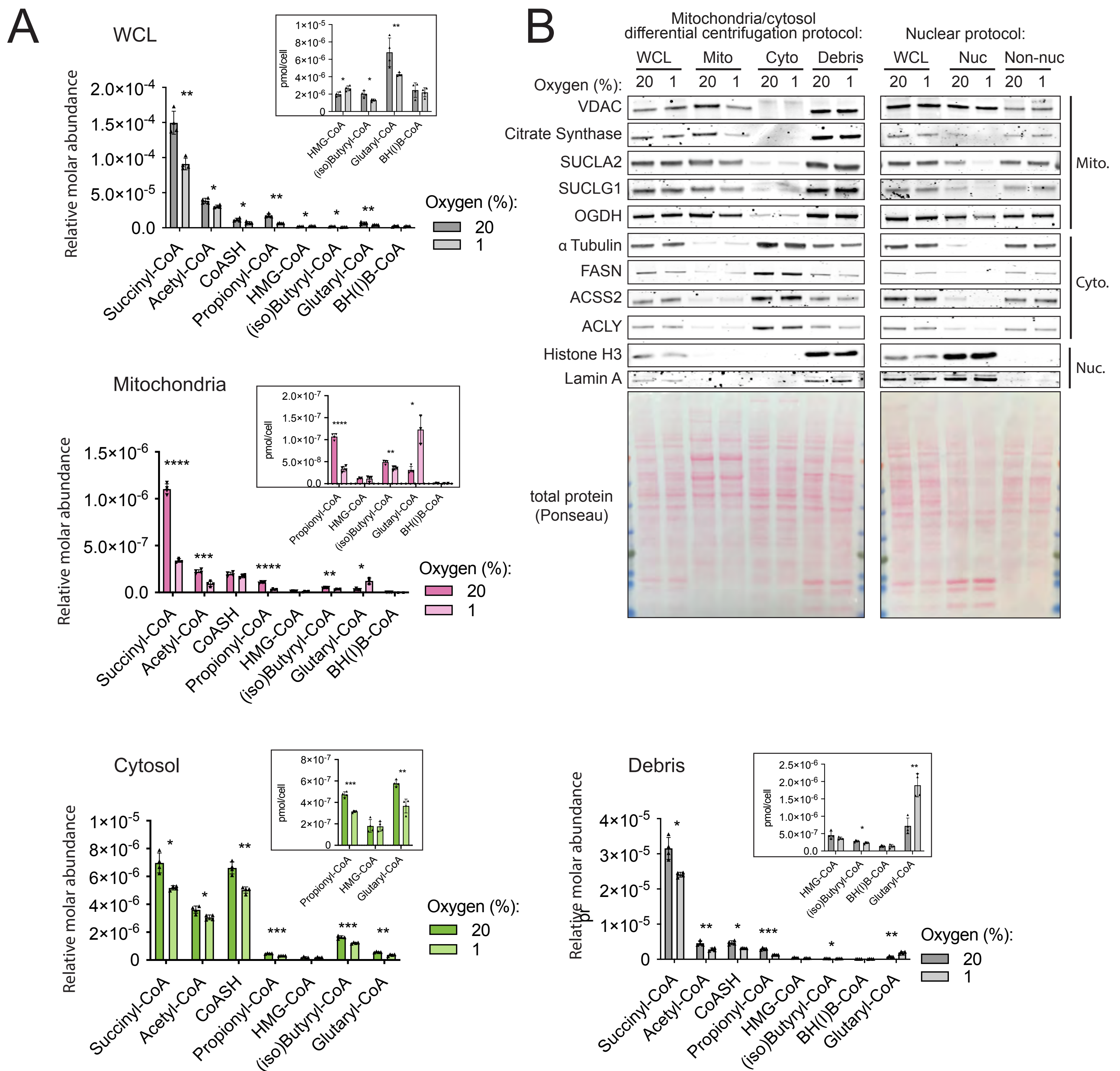
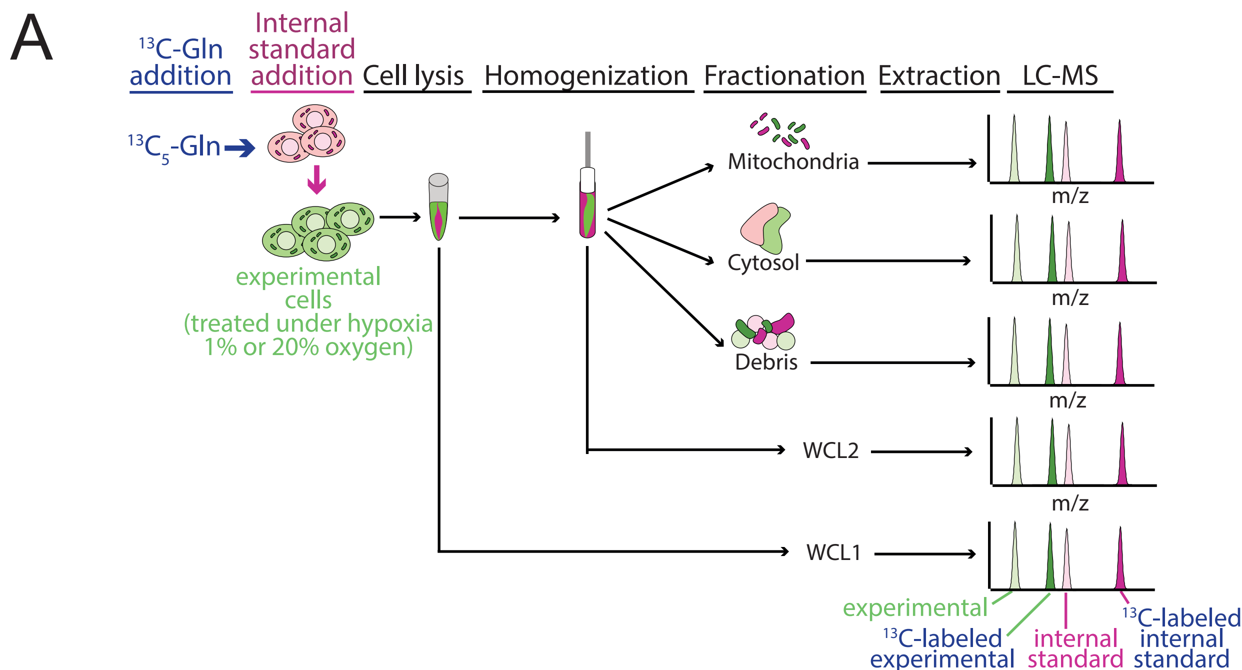
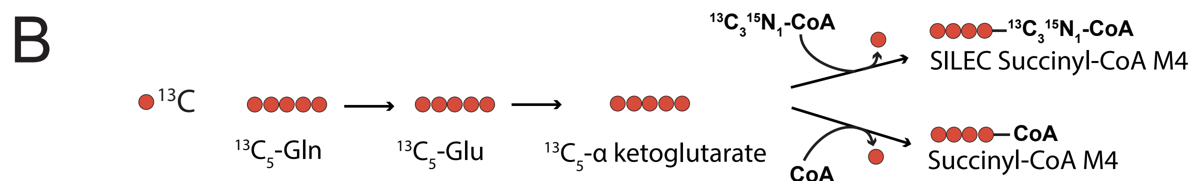


Figure S4: Subcellular acyl-CoA response to hypoxia and Western blots, related to Figure 3
HepG2 cells were incubated under 20% (normoxia) or 1% (hypoxia) oxygen for 24 h. **A)** Complete set of short chain acyl-CoA species quantified by SILEC-SF using mitochondrial/cytosolic differential centrifugation procedure from a representative experiment shown in **Figure 3D**. Symbols represent individual replicate dishes (n=4) and error bars represent standard deviation. Lower abundance metabolites are magnified in the upper right corner of each panel. **B)** Western blots comparing protein enrichment for representative marker proteins for mitochondria, cytosol and nucleus. HepG2 cells were subject to sub-cellular fractionation by both the mitochondrial/cytosol and nuclear/non-nuclear protocols. Equal protein quantity was loaded for each fraction. Abbreviations: VDAC (voltage dependent anion channel), SUCLA2 (Succinyl-CoA ligase [ADP-Forming] subunit beta), SUCLG1 (Succinyl-CoA ligase [GDP-forming] subunit alpha), OGDH (oxoglutarate dehydrogenase), FAS (fatty acid synthase), ACSS2 (Acyl-CoA synthetase short chain family member 2), ACLY (ATP-citrate lyase). For comparison between two groups, datasets were analyzed by two-tailed Student's t-test with Welch's correction and statistical significance defined as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).





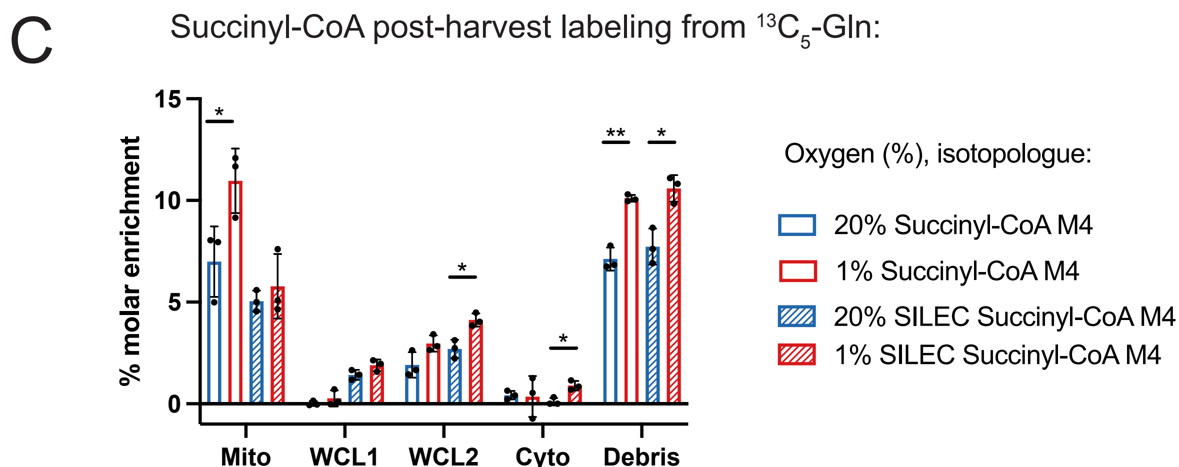
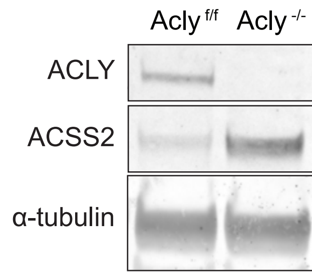


Figure S5: Post-harvest metabolism, related to Figure 3

Post-harvest labeling with 13C5-Gln was applied to SILEC-SF mitochondrial/cytosolic differential centrifugation method. HepG2 cells were incubated under 20% (normoxia) or 1% (hypoxia) oxygen for 24 h before harvest and 13C5-Gln was added in fractionation buffer at harvest. 13C incorporation into the acyl chain of acyl-CoAs derived from experimental cells and SILEC internal standard labeled cells can be differentiated by mass. **A)** Schemtic representation of experimental protocol. **B)** 13C5-Gln may be used as a substrate for SILEC labeled or unalbeled succinyl-CoA. **C)** Post-harvest 13C5-Gln incorporation into succinyl-CoA M4 was compared between experimental and SILEC internal standard succinyl-CoA across 2 experimental pre-treatment conditions (hypoxia and normoxia).



B
Acly -/- MEFs

C
Acly -/- liver cancer cells

D
Acly f/f liver cancer cells

0 mM acetate
0.1 mM acetate
1 mM acetate

Figure S6: Whole cell acyl-CoA response to acetate availability, related to Figure 4
A) Western blot confirming ACLY deletion and ACSS2 upregulation in Acly-/- liver cancer cell line compared to control Aclyf/f liver cancer cell line. Equal protein was loaded. **B-D)** Data from **Figure 4B**, displayed as cellular acyl-CoA concentration. Cells incubated in DMEM supplemented with 10% dialyzed fetal calf serum with the addition of the indicated concentration of acetate for 4 h. Metabolites were directly extracted from whole cells. Each symbol represents an individual replicate sample (n=4) from representative experiments. Error bars show standard deviation and statistical comparisons between two groups, were made by two-tailed Student's t-test with Welch's correction and statistical significance defined as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

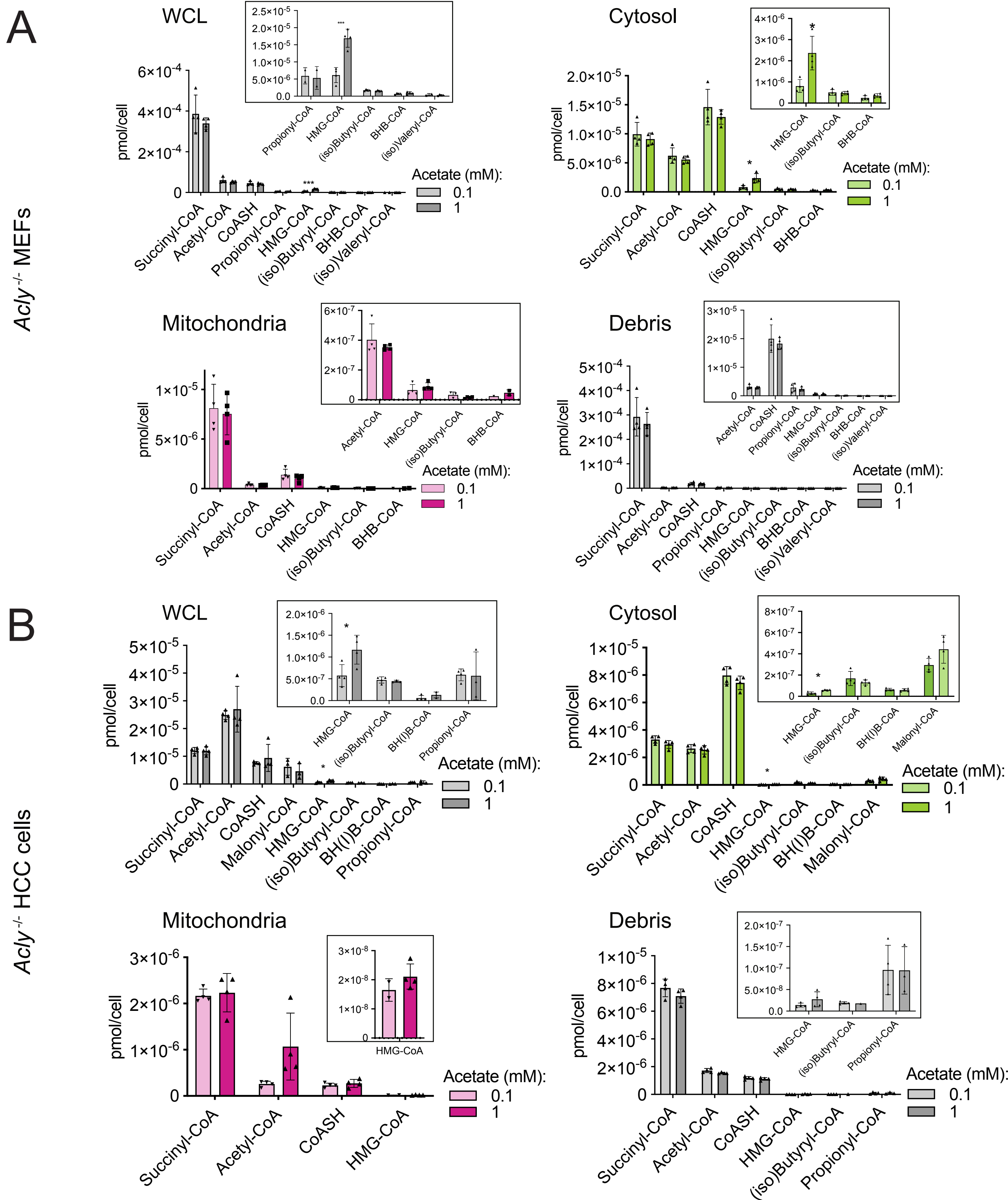


Figure S7: Sub-cellular acyl-CoA response to acetate availability, related to Figure 4

Complete set of short chain acyl-CoA species quantified by SILEC-SF using mitochondrial/cytosolic differential centrifugation procedure for **(A)** *Acly-/-* MEFs and **(B)** *Acly-/-* HCC cells. Acyl-CoA species that were not quantified showed insufficient signal intensity for the analyte, the internal standard or both. Low abundance metabolites are magnified in the upper right corner of each panel. Each symbol represents an individual replicate sample (n=4) from representative experiments. For all panels, error bars show standard deviation and statistical comparisons between two groups, were made by two-tailed Student's t-test with Welch's correction and statistical significance defined as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

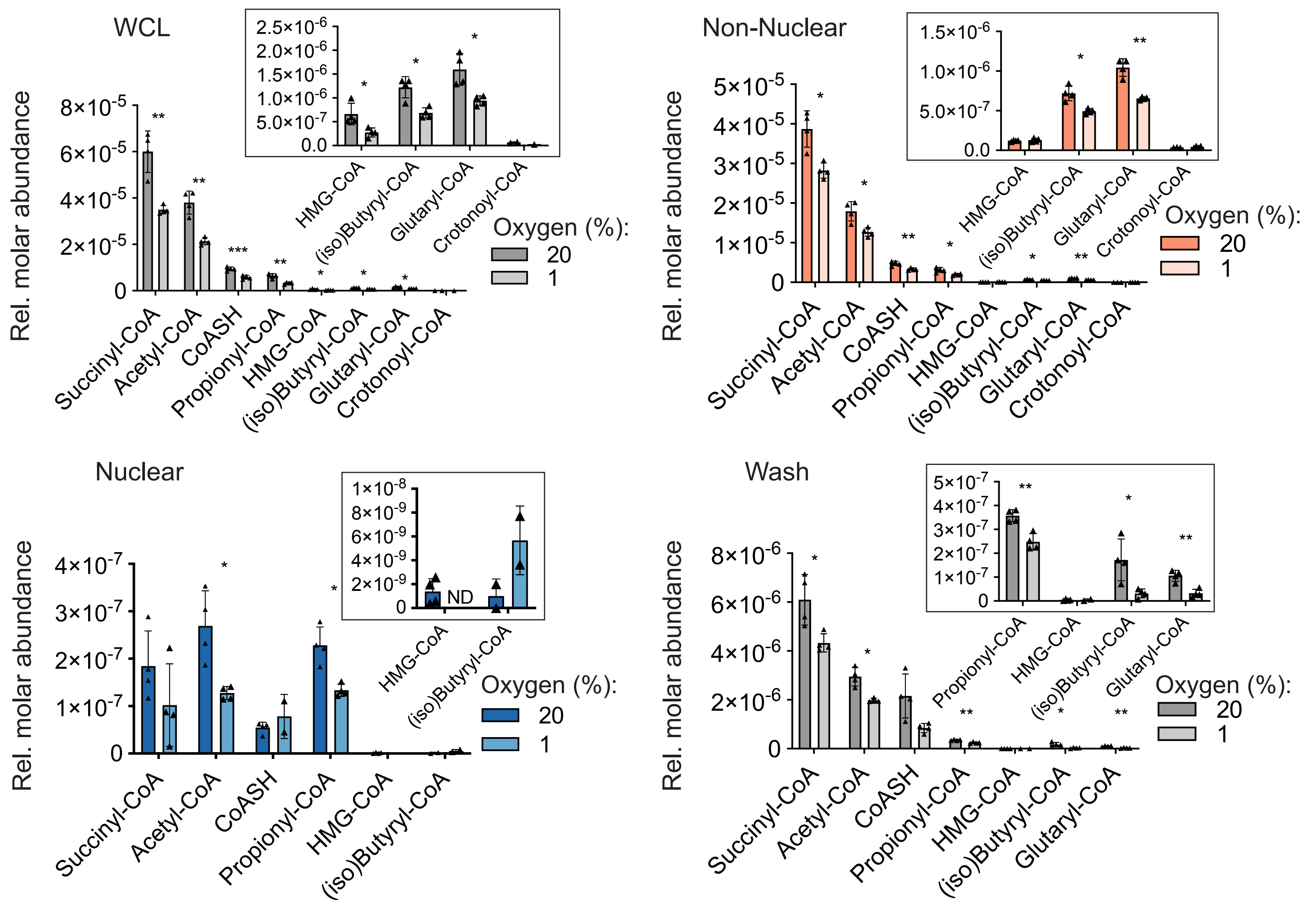


Figure S8: Nuclear acyl-CoA quantitation using SILEC-SF, related to Figure 5D
Complete set of short chain acyl-CoA species quantified by SILEC-SF using nuclear differential centrifugation procedure from a representative experiment. HepG2 cells were incubated under 20% (normoxia) or 1% (hypoxia). Lower abundance metabolites are magnified in the upper right corner of each panel. Error bars show standard deviation. Short chain acyl-CoA species that were not quantified showed insufficient signal intensity for the analyte, the internal standard or both. ND= not detected. For comparison between two groups, datasets were analyzed by two-tailed Student's t-test with Welch's correction and statistical significance defined as $p < 0.05$ (*), $p < 0.01$ (**).

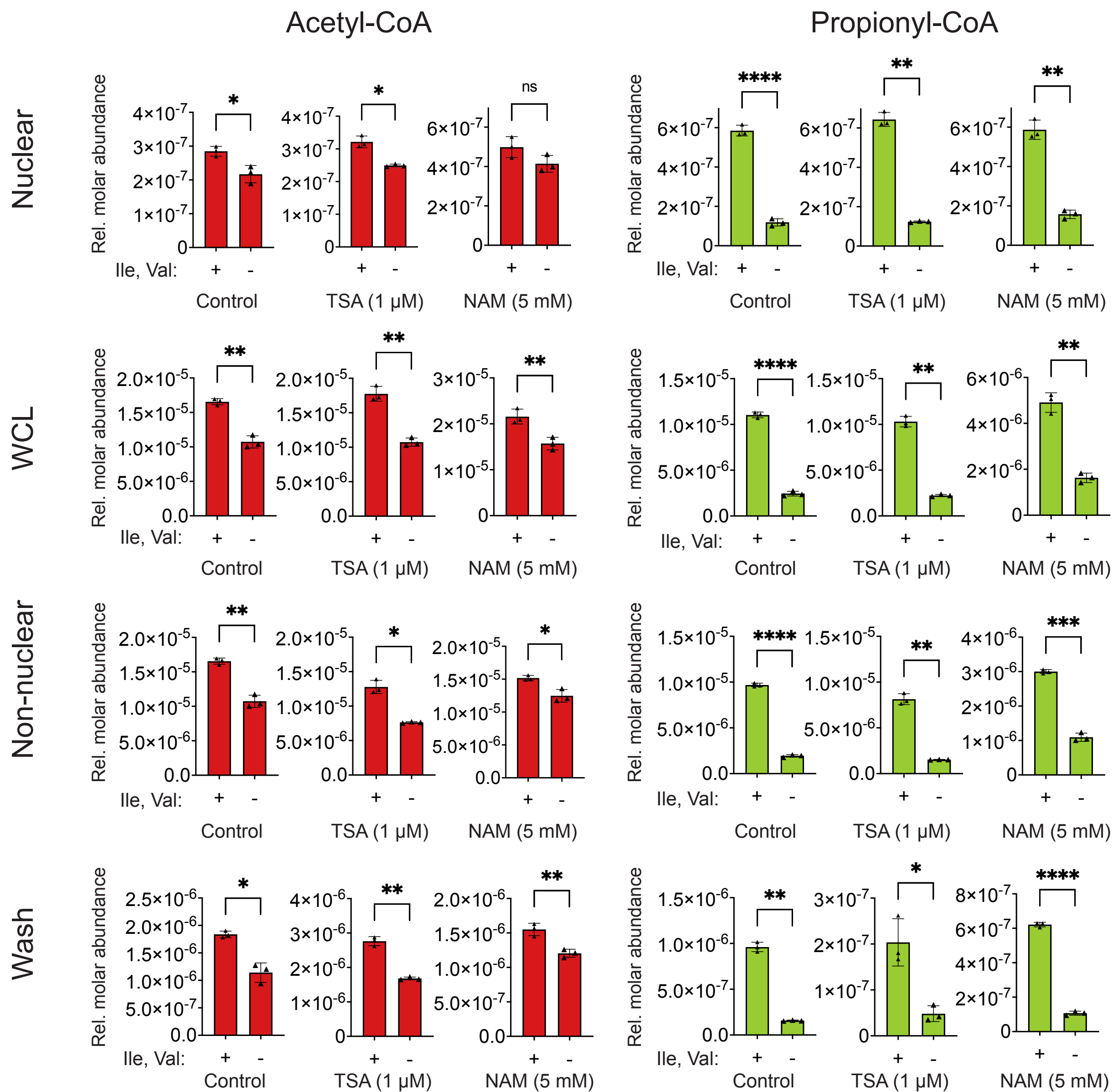


Figure S9: Nuclear acyl-CoA quantitation with deacetylase inhibition, related to Figure 7
HepG2 cells were incubated in DMEM with 10% dialyzed FBS for 24 h in the presence or absence of isoleucine and valine before harvest. To test potential for artifactual generation of propionyl-CoA or acetyl-CoA during SILEC-SF processing via deacylation, the class I and II HDAC inhibitor, trichostatin A (TSA) or the sirtuin inhibitor nicotinamide (NAM) were added to the fractionation buffer during SILEC-SF processing in separate experiments. Acetyl-CoA and propionyl-CoA quantitation after SILEC-SF analysis with nuclear procedure are displayed. Individual symbols represent replicate (n=3) samples. For all panels, symbols represent individual replicate samples and error bars represent standard deviation. For comparison between two groups, datasets were analyzed by two-tailed Student's t-test with Welch's correction and statistical significance defined as $p < 0.05$ (*), $p < 0.01$ (**).

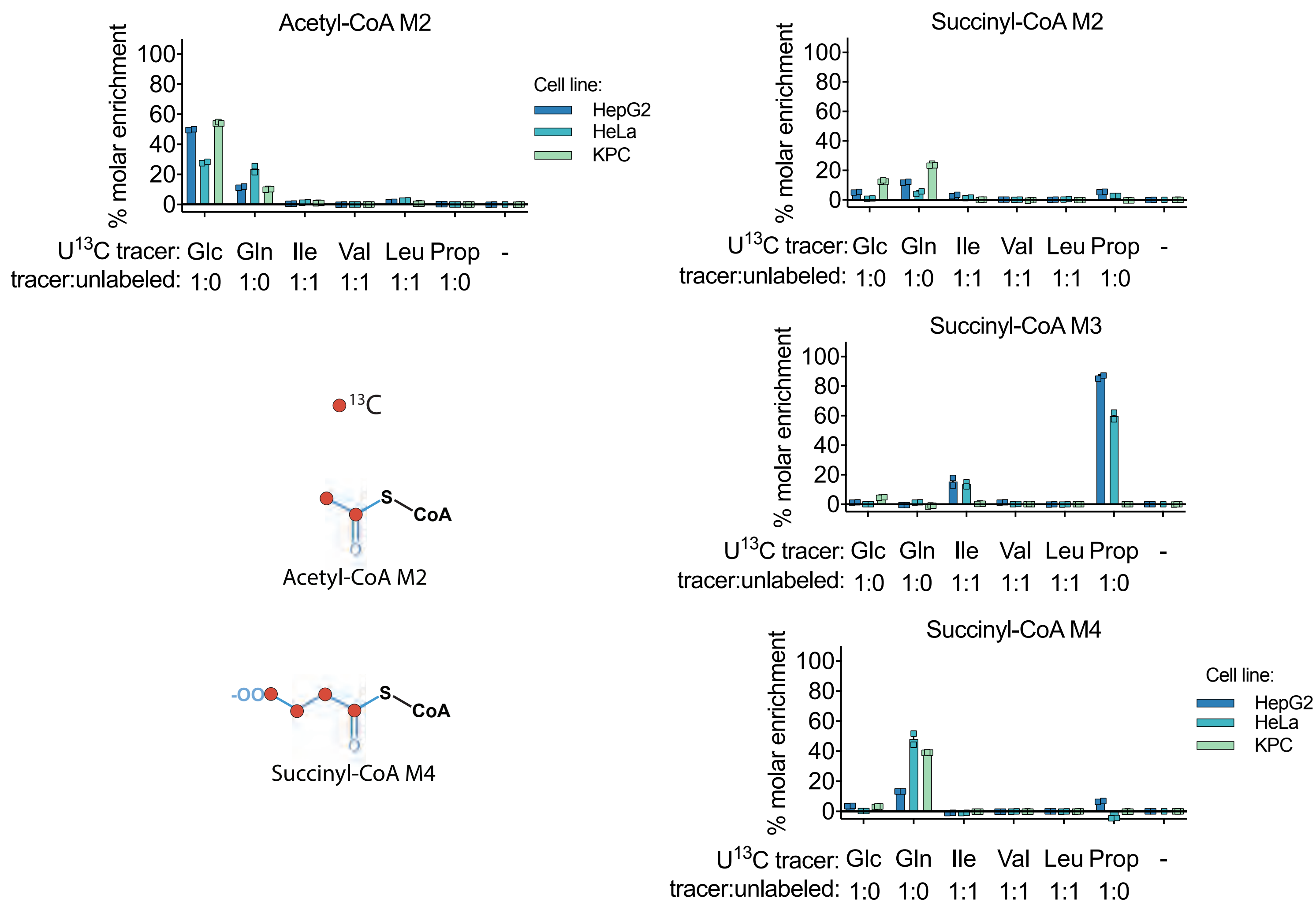
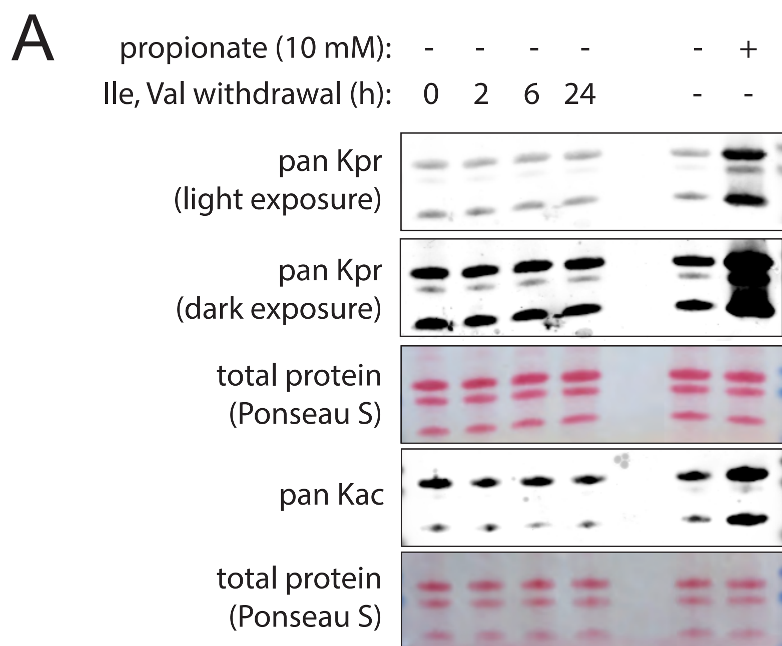


Figure S10: Stable isotope labeling of acetyl-CoA and succinyl-CoA, related to Figure 6B
Pancreatic adenocarcinoma (KPC) cells were incubated in media containing the indicated uniformly (U)13C-labeled substrates for 18 h. Whole cells were harvested by direct extraction. Total substrate concentrations were equal across all samples except for propionate, which was added only to the U13C3-propionate samples. U13C-labeled Val, Leu and Ile were diluted 1:1 with unlabeled substrate. A single experiment with n=3 replicate samples is displayed.



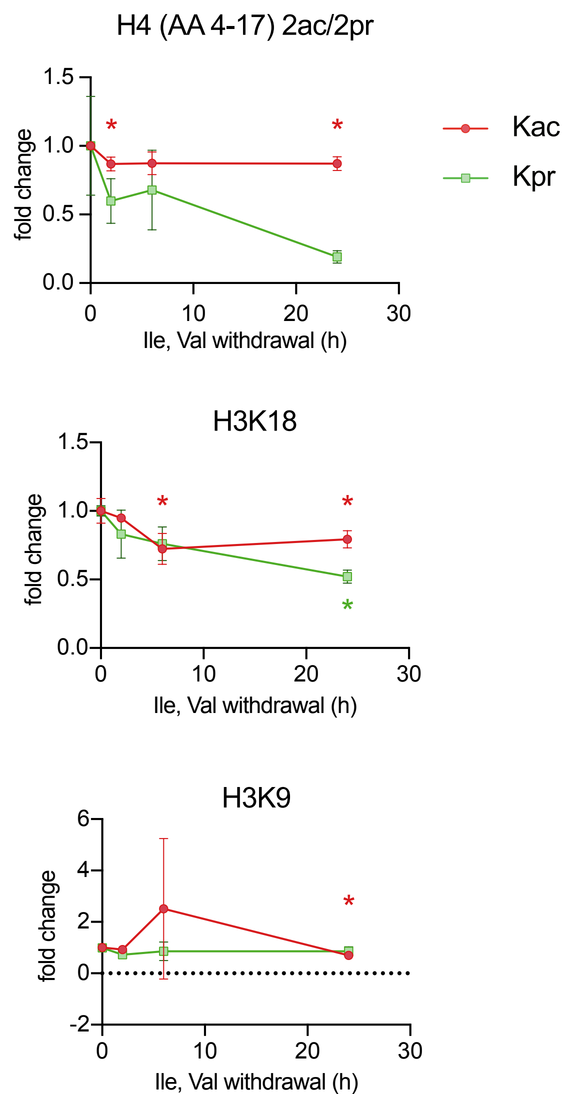
B All Kpr sites:

H3K14pr
H3K9pr
H4K59pr
H4K77pr
H4 (AA 4-17) 1pr
H4K79pr
H3K122pr
H4K31pr
H3K64pr
H3K4pr
H4K91pr
H3K18pr
H3K79pr
H3K23pr
H4 (AA 4-17) 2pr

relative intensity

Ile, Val withdrawal (h)

Figure S11: BCAAs support histone lysine propionylation, related to Figure 7
A) Western blot of acid-extracted histones from HepG2 cells incubated in DMEM with 10% dialyzed FBS. Media was changed on all timepoints 24 h before harvest and switched to dropout media at the indicated times before harvest. **B)** Relative intensity over time for all Kpr sites detected by acyl proteomic analysis (**Figure 7E & F**). For all panels, error bars represent standard deviation. Statistical significance in B was determined by comparison to t=0 control for each mark and is indicated above or below the specific time-point in the color matching the mark ($p < 0.05$ (*)).



B % intensity rankings for Kpr and Kac modified peptides:

	histone_peptide_modified site	% of peptide with this modification	significantly downregulated
1	H3_9_17 K14pr	4.9%	
2	H3_9_17 K9pr	2.3%	
3	H4_56_67 K59pr	1.4%	
4	H4_68_78 K77pr	1.1%	
5	H4_4_17 1pr	1.0%	*
6	H4_79_92 K79pr	0.64%	
7	H3_117_128 K122pr	0.61%	
8	H4_24_35 K31pr	0.59%	
9	H3_64_69 K64pr	0.49%	*
10	H3_3_8 K4pr	0.43%	
11	H4_79_92 K91pr	0.41%	
12	H3_18_26 K18pr	0.39%	*
13	H3_73_83 K79pr	0.27%	
14	H3_18_26 K23pr	0.15%	*
15	H4_4_17 2pr	0.12%	

	histone_peptide_modified site	% of peptide with this modification	significantly downregulated
1	H3_18_26 K23ac	34%	
2	H4_4_17 1ac	34%	
3	H3_73_83 K79ac	19%	
4	H3_9_17 K14ac	12%	
5	H4_4_17 2ac	7.2%	*
6	H4_41_45 K44ac	4.0%	
7	H3_18_26 K18ac	2.5%	*
8	H3_54_63 K56ac	2.5%	
9	H4_56_67 K59ac	0.98%	
10	H4_79_92 K79ac	0.61%	
11	H4_79_92 K91ac	0.53%	
12	H4_24_35 K31ac	0.51%	
13	H3_9_17 K9ac	0.44%	*
14	H3_117_128 K122ac	0.34%	
15	H3_64_69 K64ac	0.28%	
16	H4_68_78 K77ac	0.15%	
17	H3_27_40 K36ac	0.02%	
18	H3_3_8 K4ac	0.00%	
19	H3_27_40 K27ac	0.00%	

Figure S12: Histone lysine acetylation and propionylation, related to Figure 7
Acyl proteomics analysis from **Figure 7E & F**. **A)** Relative intensity over time for 3 Kac sites identified as significantly regulated and for corresponding Kpr marks. **B)** Ranked average intensity across all time-points of specific Kpr and Kac modified sites as percent total signal detected across all modifications for that peptide. Error bars represent standard deviation. Statistical significance in A was determined by comparison to t=0 control for each mark and is indicated above or below the specific timepoint in the color matching the mark ($p < 0.05$ (*)).

TABLE S1: Cell culture conditions, related to STAR methods

Cell line	Culture conditions
Acly-/- mouse embryonic fibroblasts (Clone: PC9)	DMEM, high glucose (Gibco #11965084) with 10% (v/v) calf serum (Gemini bio-products cat. #100-510, lot C93GOOH).
HepG2	DMEM, high glucose (Gibco #11965084) with 10% (v/v) fetal bovine serum (Cytiva cat. #SH30910.01). Used at <20 passages from ATCC stocks.
Murine pancreatic adenocarcinoma (KPC, Clone: 2838c3)	DMEM, high glucose (Thermo Fisher Scientific, Gibco #11965084) with 10% (v/v) calf serum (Gemini bio-products #100-510, lot C93GOOH).
Hepa1c7 cells	Alpha MEM (Gibco cat. #12561056) with 10% fetal bovine serum (Cytiva cat. #SH30910.01) and penicillin/streptomycin (Gibco cat. #10378016).
Brown adipocytes	Prepared from immortalized brown preadipocyte cells (Harms et al., 2014) through induction of differentiation as described previously (Huber et al., 2019).
Murine preadipocytes (Clone: 5A)	5A preadipocytes were passaged in DMEM/F12 (Gibco #11320033), 10% heat inactivated fetal bovine serum (Cytiva cat. #SH30910.01), 1% penicillin/streptomycin (Gibco cat. #10378016) and seeded in 12 well plates for experiments. Cells were cultured for 24-48 h post confluent before induction media was added for 3 days. Induction media; DMEM/F12 (Gibco cat. #11320033), 10% heat inactivated fetal bovine serum, 1% penicillin/streptomycin, 5ug/ml insulin, 0.05 mM IBMX, 10 µM Dexamethasone, 5µM Troglitazone.
Phoenix-AMPHO	DMEM, high glucose with 10% fetal bovine serum fetal bovine serum (Cytiva cat. #SH30910.01).
Murine liver cancer (Clone: D42 and D42C4)	DMEM, high glucose (Thermo Fisher Scientific, Gibco #11965084) with 10% calf serum (Gemini bio-products #100-510, lot C93GOOH).

Table S2: Acyl-CoA standard mixture, related to STAR Methods

Unlabeled standard	Concentration (pmol/100 µl)
Acetyl-CoA	5000
BHB-CoA	500
Butyryl-CoA	500
CoASH	500
Crotonoyl-CoA (2-butenoyl-CoA)	500
Glutaryl-CoA	500
HMG-CoA	500
Isovaleryl-CoA	500
Malonyl-CoA	500
Propionyl-CoA	500
Succinyl-CoA	5000

Table S3: Acyl-CoA Masses, related to STAR methods

Isotopologue	Precursor Formula [M]	Precursor Ion [M+H]+	Product Ion [M+H-507]+
Acetyl-CoA-M0	C23H38N7O17P3S	810.1331	303.1373
Acetyl-CoA ISTD	[13]C3C20H38[15]N1N6O17P3S	814.1402	307.1444
Acetyl-CoA-M1	[13]C1C22H38N7O17P3S	811.1364	304.1407
Acetyl-CoA-M2	[13]C2C21H38N7O17P3S	812.1398	305.1440
Acetyl-CoA-M3	[13]C3C20H38N7O17P3S	813.1431	306.1474
Acetyl-CoA-M4	[13]C4C19H38N7O17P3S	814.1465	307.1507
Acetyl-CoA-M5	[13]C5C18H38N7O17P3S	815.1498	308.1541
BH(I)B-CoA	C25H42N7O18P3S	854.1593	347.1635
BH(I)B-CoA ISTD	[13]C3C22H42[15]N1N6O18P3S	858.1664	351.1706
CoASH	C21H36N7O16P3S	768.1225	-
CoASH ISTD	[13]C3C18H36[15]N1N6O16P3S	772.1296	-
Crotonoyl-CoA	C25H40N7O17P3S	836.1487	329.1530
Crotonoyl-CoA ISTD	[13]C3C22H40[15]N1N6O17P3S	840.1558	333.1601
Glutaryl-CoA	C26H42N7O19P3S	882.1542	375.1584
Glutaryl-CoA ISTD	[13]C3C23H42[15]N1N6O19P3S	886.1613	379.1655
HMG-CoA	C27H44N7O20P3S	912.1647	405.1690
HMG-CoA ISTD	[13]C3C24H44[15]N1N6O20P3S	916.1718	409.1761
(iso)Butyryl-CoA	C25H42N7O17P3S	838.1644	331.1686
(iso)Butyryl-CoA ISTD	[13]C3C22H42[15]N1N6O17P3S	842.1715	335.1757
(iso)Valeryl-CoA	C26H44N7O17P3S	852.1800	345.1843
(iso)Valeryl-CoA ISTD	[13]C3C23H44[15]N1N6O17P3S	856.1871	349.1914
Propionyl-CoA-M0	C24H40N7O17P3S	824.1487	317.1530
Propionyl-CoA ISTD	[13]C3C21H40[15]N1N6O17P3S	828.1558	321.1601
Propionyl-CoA-M1	[13]C1C23H40N7O17P3S	825.1521	318.1563
Propionyl-CoA-M2	[13]C2C22H40N7O17P3S	826.1554	319.1488
Propionyl-CoA-M3	[13]C3C20H40N7O17P3S	827.1588	320.1521
Propionyl-CoA-M4	[13]C4C19H40N7O17P3S	828.1621	321.1555
Propionyl-CoA-M5	[13]C5C18H40N7O17P3S	829.1655	322.1564
Succinyl-CoA-M0	C25H40N7O19P3S	868.1385	361.1428
Succinyl-CoA-M1	[13]C1C24H40N7O19P3S	869.1419	362.1461
Succinyl-CoA-M2	[13]C2C23H40N7O19P3S	870.1452	363.1495
Succinyl-CoA-M3	[13]C3C22H40N7O19P3S	871.1486	364.1528
Succinyl-CoA-M4	[13]C4C21H40N7O19P3S	872.1520	365.1562
Succinyl-CoA-M5	[13]C5C20H40N7O19P3S	873.1553	366.1596
Succinyl-CoA-M6	[13]C6C19H40N7O19P3S	874.1587	367.1629
Succinyl-CoA-M7	[13]C7C18H40N7O19P3S	875.1620	368.1663
Succinyl-CoA ISTD	[13]C3C22H40[15]N1N6O19P3S	872.1456	365.1499
Succinyl-CoA ISTD M1	[13]C4C21H40[15]N1N6O19P3S	873.1490	366.1532
Succinyl-CoA ISTD M2	[13]C5C20H40[15]N1N6O19P3S	874.1523	367.1566
Succinyl-CoA ISTD M3	[13]C6C19H40[15]N1N6O19P3S	875.1557	368.1600
Succinyl-CoA ISTD M4	[13]C7C18H40[15]N1N6O19P3S	876.1591	369.1633
Succinyl-CoA ISTD M5	[13]C8C17H40[15]N1N6O19P3S	877.1624	370.1667
Succinyl-CoA ISTD M6	[13]C9C16H40[15]N1N6O19P3S	878.1658	371.1700
Succinyl-CoA ISTD M7	[13]C10C15H40[15]N1N6O19P3S	879.1691	372.1734

*3-hydroxymethylglutaryl-CoA (HMG-CoA)

*3-hydroxybutyrate-CoA/3-hydroxyisobutyrate-CoA (BH(I)B-CoA)

ISTD = internal standard

CoASH was quantified using MS1 Precursor ion

Data S1: Supplemental Experimental Procedures, related to STAR Methods

SILEC-SF

Mitochondrial/cytosolic differential centrifugation protocol

1. Media was poured from SILEC cell dishes into a waste container and cells were placed on an ice slope and residual media drained and aspirated.
2. Dishes were laid flat on ice and 1 ml ice-cold buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris–HCl (pH 7.5), 1 mM EDTA (pH 8), adjusted to pH 7.5) added to each dish.
3. Cells were detached by a cell scraper.
4. Cell clumps were broken up by pipetting the cell suspension against the culture dish with a P1000 pipette at least 6 times.
5. SILEC cell suspension from all dishes was combined in a 50 ml tube on ice.
6. The volume was adjusted to > n * 1 ml with buffer (1 ml required per experimental sample).
7. A homogenous SILEC cell suspension was made by mixing against the wall of the tube 6 times with a serological pipette immediately before addition to experimental dishes.
8. Experimental samples were combined with SILEC internal standard cells. For cell samples, media was removed from experimental cells in the same manner as for the SILEC cells, 1 ml of ice-cold homogenous SILEC cell suspension was added to each experimental dish/tissue sample on ice and cells were scraped into the SILEC suspension and transferred to a pre-chilled 1 ml Potter-Elvehjem Tissue Grinder (Corning cat. #7725T-1) in a beaker of ice and water. For tissue samples, weighted tissue pieces were added directly to a pre-chilled 1 ml Potter-Elvehjem Tissue Grinder in a beaker of ice and water containing 1 ml SILEC cell suspension. For standard curve samples, 1 ml SILEC cell suspension were transferred directly to pre-chilled 1 ml Potter-Elvehjem Tissue Grinder in a beaker of ice and water without experimental cells (6 standard curves samples were generated per experiment).
9. Cells were lysed by stroking with the pestle attached to an overhead stirrer (SOS20, Southwest Science) operated at 1,600 rpm. *Note: Optimal stroke number was determined for each cell line and tissue by assessing the purity and integrity of mitochondria and cytosol by Western blot and the intensity of acyl-CoA signal within each compartment across a range of up to 60 strokes.* Stroke numbers used in this study: Hepa1c17 cells (10 strokes), HepG2 and liver cancer cell lines (D42/D42C4) (15 strokes), heart tissue and brown adipocytes (20 strokes), MEF cells (30 strokes).
10. Cell homogenate was transferred to 1.5 ml tubes on ice.
11. For whole cell lysate (WCL) analysis, a 100 µl aliquot of homogenate (representing 10% of the total sample) was removed and quenched in 1 ml ice-cold 10% (w/v) Trichloroacetic in water.
12. Homogenate was centrifuged at 1,300 × *g* from 10 min at 4 °C and supernatant was transferred to a new pre-chilled 1.5 ml tube. *Note: Care was taken not to disturb the pellet. A small volume of supernatant was left above the pellet consistently across samples.*

13. The high-density debris pellet was quenched by resuspension in 1 ml 10% trichloroacetic acid and the supernatant was centrifuged at 10,000 × *g* for 20 min at 4 °C to pellet mitochondria.
14. The supernatant (the cytosolic fraction) was quenched by transfer to a new 1.5 ml tube containing 0.25 ml of 50% (w/v) trichloroacetic acid in water to make a final concentration of 10% trichloroacetic acid. *Note: Care was taken not to disturb the pellet. A small volume of supernatant was left above the pellet consistently across samples.*
15. Residual cytosolic fraction was carefully removed from the mitochondrial pellet with P200 pipette and discarded, and the pellet was quenched by resuspension in 1 ml 10% (w/v) trichloroacetic acid in water.
16. Samples were stored at -80 °C before thawing on ice for acyl-CoA processing, or directly processed.

Nuclear isolation by differential centrifugation protocol

1. Media was poured from SILEC cell dishes into a waste container, and cells were placed on ice at a 45° angle and residual media drained and aspirated completely.
2. Dishes were laid flat on ice and 0.5 ml ice-cold lysis buffer (250 mM sucrose, 15 mM Tris–HCl (pH 7.5), 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$ adjusted to pH 7.4) added to each dish.
3. SILEC cells were scraped into the buffer, cell clumps were broken up by pipetting against the plate at least 4 times with a P1000.
4. Cells were combined in a 50 ml tube on ice.
5. The volume was made up to > 0.5*n ml (0.5 ml required per sample) with buffer and kept on ice. *Note: NP-40 addition was delayed to coordinate detergent lysis of SILEC cells with experimental cells.*
6. Immediately before addition to experimental dishes, NP-40 (1% v/v in lysis buffer) was added to achieve a final concentration of 0.1% (v/v).
7. Cell suspension was homogenized by careful mixing by laminar flow against the wall of the 15 ml tube 4 times with a serological pipette. *Note: Care was taken to avoid frothing.*
8. Media was removed from experimental cells in the same manner as for the SILEC cells and 0.5 ml of ice-cold homogenous SILEC cell suspension was added to each dish/sample.
9. Cells were scraped into the SILEC suspension
10. Cell suspension was mixed against the dish with a P1000 pipette at least 4 times to break up cell clumps. *Note: Care was taken to avoid frothing.*
11. Cell suspension was transferred to a 1.5 ml tube on ice.
12. For WCL analysis, 50 µl (representing 10% of the total sample) was removed after homogenization and quenched in 1 ml ice-cold 10% trichloroacetic acid in water.
13. Nuclei were pelleted by centrifugation at 600 × *g* for 5 min at 4 °C.
14. The supernatant ('non-nuclear' fraction) was quenched by transferal to a new 1.5 ml tube containing 0.125 ml of 50% (w/v) trichloroacetic acid in water to make a final

concentration of 10% trichloroacetic acid. *Note: Care was taken not to disturb the pellet. A small volume of supernatant was left above the pellet consistently across samples.*

15. The nuclear pellet was washed by the addition of 0.5 ml lysis buffer without NP-40 and re-centrifuged at 600 × *g* for 5 min at 4 °C.
16. The supernatant (the 'wash' fraction) was quenched by transferal to a new 1.5 ml tube containing 0.125 ml of 50% (w/v) trichloroacetic acid in water to make a final concentration of 10% trichloroacetic acid. *Note: Care was taken not to disturb the pellet. A small volume of supernatant was left above the pellet consistently across samples.*
17. Residual wash was carefully removed from the nuclear pellet with a P200 pipette and discarded.
18. The nuclear pellet was quenched by the resuspension in 1 ml 10% (w/v) trichloroacetic acid in water.
19. Samples were stored at -80 °C before thawing on ice for acyl-CoA processing, or directly processed.

Mito-IP protocol

1. Cells were harvested by addition of SILEC cells, and homogenized as described above for mitochondria and cytosol isolation by differential centrifugation except that the buffer used was KPBS (136 mM KCl and 10 mM KH2PO4, pH 7.25).
2. Homogenate was transferred to a 1.5 ml tube on ice and centrifuged at 1,000 x g for 2 min at 4 °C.
3. The post-nuclear supernatant was transferred to a 1.5 ml tube containing 200 ul of prewashed anti-HA magnetic beads and incubated by rotation for 3.5 min at 4 °C.
4. Beads were collected on a magnet and the overlying solution 'mitochondria depleted cytoplasm' was transferred to a 1.5 ml and quenched by addition of trichloroacetic acid to a final concentration of 10% (w/v).
5. Beads were washed twice with 1 ml KPBS before extraction of mitochondria by addition of trichloroacetic acid 10% (w/v).
6. Samples were incubated on ice for 10 min and vortexed vigorously to extract metabolites into solution.
7. Beads were collected on a magnet and the supernatant was collected in a fresh 1.5 ml and stored at -80 °C before thawing on ice for acyl-CoA processing, or directly processed.

Standard curve generation for differential centrifugation protocols

1. Equal aliquots of SILEC internal standard cells were fractionated in the absence of experimental unlabeled cells in parallel with each experiment and known quantities of unlabeled standards were added directly after isolation of each fraction.
2. 6-point standard curves (standard 0 to standard 5) were generated for each fraction by dilution from a stock mixture of unlabeled acyl-CoA standards.
3. The stock mixture was dissolved in 10% trichloroacetic acid in water according to **Table S2** and aliquots were frozen at -80 and thawed once at use.

4. Serial 3-fold dilutions were made from standard 5 stock in 10% trichloroacetic acid in water and each dilution was added in a volume of 100 µl to the appropriate samples containing SILEC internal standard immediately after fractionation protocol was complete (i.e. samples in 10% trichloroacetic acid).
5. The concentration of standard 5 (the highest standard) stock was adjusted to suit metabolite abundance in different samples. For most cell samples standard 5 stock was a 50-fold dilution of the original stock mixture (mitochondrial and nuclear fractions used a 250-fold dilution). For most tissue samples, standard 5 stock was a 20-fold dilution of stock mixture (mitochondrial and nuclear fractions used a 100-fold dilution).

8. After standard addition samples were stored at -80 °C before thawing on ice for acyl-CoA processing, or directly processed as indicated in **STAR Methods:** Acyl-CoA sample processing and analysis by LC-MS. *Note: Standard curve and experimental samples from the same experiment were stored, processed and analyzed as a batch.*

6. Following LC-MS analysis, standard curves were generated by plotting standard quantity against relative intensity (light/ SILEC internal standard) and linear curves were fitted (formula 1). This standard curve was used to determine the quantity recovered within each sample (formula 2). Adjustments were made to account for removal of 10% of sample for whole cell lysate aliquot and for cell number before fractionation with formula 3 for whole cell lysate and formula 4 for all other fractions.

   Where y = relative intensity, x_S = standard quantity, b = constant, x_Q = quantity recovered in sample, C = cell number before fractionation, Q = adjusted quantity in sample.

$$y = mx_S + b \qquad (1)$$

$$x_Q = (y - b)/ m \qquad (2)$$

$$Q = 10x_Q / C \qquad (3)$$

$$Q = 10x_Q / 9C \qquad (4)$$

Standard curve generation for Mito-IP protocol

1. Following sonication of Mito-IP extracts in 10% trichloroacetic acid during processing (**STAR Methods:** Acyl-CoA sample processing and analysis by LC-MS), half of each replicate sample was pooled together, vortex mixed and then aliquoted into 3 exact replicates (as indicated in **Figure S1B**).
2. Unlabeled acyl-CoA standard stock mixture (**Table S2**) was diluted in 10% trichloroacetic acid. For non-mitochondrial fractions the first dilution was 50-fold followed by 2 serial 3-fold dilutions, for mitochondria the first dilution was 500-fold followed by 2 serial 3-fold dilutions.

3. Each dilution was added in a volume of 100 µl to the appropriate fraction of a replicate sample and samples were processed and analyzed by LC-MS as described in **STAR Methods:** Acyl-CoA sample processing and analysis by LC-MS.
4. Following LC-MS analysis, standard curves were generated by plotting the known concentration of acyl-CoA standard stock against the relative signal intensity (unlabeled/SILEC internal standard) and fitting a curve by linear regression (**Figure S1B**). The linear equation ($y = mx + b$, where y = relative intensity and x = quantity) was adjusted to account for the presence of additional unlabeled analyte in the standard curve derived from the sample. Thus, the formula $y = mx$ was then used to calculate quantity within each of the replicate samples that did not contain standard. Values were doubled to account for half the sample being used in the standard curve.