

1 **TITLE**

2 The carnitine shuttle links mitochondrial metabolism to histone acetylation and lipogenesis

3

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25

26 **ABSTRACT**

27 Acetyl-CoA is a central metabolite used for lipid synthesis in the cytosol and histone acetylation  
28 in the nucleus, among other pathways. The two major precursors to acetyl-CoA in the nuclear-  
29 cytoplasmic compartment are citrate and acetate, which are processed to acetyl-CoA by ATP-  
30 citrate lyase (ACLY) and acyl-CoA synthetase short-chain 2 (ACSS2), respectively. While some  
31 evidence has suggested the existence of additional routes to nuclear-cytosolic acetyl-CoA, such  
32 pathways remain poorly defined. To investigate this, we generated cancer cell lines lacking both  
33 ACLY and ACSS2. Unexpectedly, and in contrast to observations in fibroblasts, ACLY and  
34 ACSS2 double knockout (DKO) cancer cells remain viable and proliferate, maintain pools of  
35 cytosolic acetyl-CoA, and are competent to acetylate proteins in both cytosolic and nuclear  
36 compartments. Using stable isotope tracing, we show that both glucose and fatty acids feed  
37 acetyl-CoA pools and histone acetylation in DKO cells. Moreover, we provide evidence for the  
38 carnitine shuttle and carnitine acetyltransferase (CrAT) as a substantial pathway to transfer two-  
39 carbon units from mitochondria to cytosol independent of ACLY. Indeed, in the absence of ACLY,  
40 glucose can feed fatty acid synthesis in a carnitine responsive and CrAT-dependent manner. This  
41 work defines a carnitine-facilitated route to produce nuclear-cytosolic acetyl-CoA, shedding light  
42 on the intricate regulation and compartmentalization of acetyl-CoA metabolism

43 **INTRODUCTION**

44 Acetyl-CoA is a central metabolic intermediate that is generated during nutrient  
45 catabolism, used as a building block for lipid synthesis, and serves as the substrate for protein  
46 and metabolite acetylation. In mitochondria, acetyl-CoA is generated from the breakdown of  
47 nutrients including carbohydrates, fatty acids, and amino acids. Mitochondrial acetyl-CoA enters  
48 the tricarboxylic acid cycle through a condensation reaction with oxaloacetate to generate citrate.  
49 The mitochondrial pool of acetyl-CoA is spatially distinct from acetyl-CoA found in the nucleus  
50 and cytosol due to the inability of acetyl-CoA to cross the inner mitochondrial membrane. Due to  
51 this compartmentalization, acetyl-CoA must be generated separately within the nucleus or cytosol  
52 for its use in these compartments. This is accomplished by ATP-citrate lyase (ACLY), which  
53 cleaves citrate exported from mitochondria into acetyl-CoA and oxaloacetate, and acyl-CoA short  
54 chain fatty acid synthase 2 (ACSS2), which condenses acetate with free coenzyme A. Acetyl-CoA  
55 generated by these enzymes is used for *de novo* lipogenesis, as well as for acetylation in the  
56 nucleus and cytosol<sup>1-3</sup>.

57 We previously demonstrated that in the absence of ACLY, ACSS2 is upregulated and  
58 acetate feeds acetyl-CoA pools for lipogenesis and histone acetylation<sup>4</sup>. Furthermore, *Acly*<sup>-/-</sup>  
59 mouse embryonic fibroblasts (MEFs) are dependent on exogenous acetate for viability,  
60 suggesting that acetyl-CoA synthesis by ACSS2 is the primary compensatory mechanism in the  
61 absence of ACLY<sup>4</sup>. Such compensation from acetate via ACSS2 is also observed *in vivo* upon  
62 deletion of *Acly* from adipose tissue or liver<sup>4-6</sup>. Yet, several clues suggested that additional acetyl-  
63 CoA generating mechanisms within the nuclear-cytosolic compartment must exist. First, in the  
64 absence of ACLY, about 20-40% of the acetyl-CoA pool in whole cells and in the cytosol remains  
65 unlabeled from exogenous acetate<sup>4,7</sup>. Secondly, although the downstream mevalonate pathway  
66 intermediate HMG-CoA is rapidly depleted in the absence of ACLY and acetate, acetyl-CoA levels  
67 decrease more moderately upon acute acetate withdrawal<sup>8</sup>. Third, histone acetylation is  
68 suppressed in the absence of ACLY at physiological acetate concentrations but does not appear  
69 to decline further when acetate is withdrawn<sup>4</sup>. Finally, though glucose use for fatty acid synthesis  
70 is strongly suppressed in the absence of ACLY, it is not fully blocked *in vitro* or *in vivo*<sup>4,6</sup>. Based  
71 on these clues, we hypothesized that two broad mechanisms could potentially account for these  
72 findings: 1) intracellular acetate production from other nutrients; and/or 2) another acetyl-CoA  
73 producer other than ACLY and ACSS2 (Figure 1A).

74 Both types of mechanisms have been reported, but the significance of such pathways  
75 remains poorly understood. In terms of endogenous sources, acetate production can occur  
76 directly from pyruvate non-enzymatically or via the pyruvate dehydrogenase complex (PDC)<sup>9,10</sup>.  
77 Additionally, acetate can be released from histone deacetylation<sup>11</sup> and acetylated metabolite  
78 hydrolysis<sup>12-14</sup>. Furthermore, non-canonical routes to acetyl-CoA production outside of  
79 mitochondria have also been proposed, with several studies reporting a moonlighting function of  
80 the pyruvate dehydrogenase complex (PDC), which can translocate to the nucleus under specific  
81 conditions to generate a local source of acetyl-CoA from pyruvate for histone acetylation<sup>15-18</sup>.  
82 Beyond pyruvate to acetate conversion and nuclear PDC, less well understood routes of nuclear-  
83 cytosolic acetyl-CoA metabolism have been suggested, including peroxisomal production and  
84 export of acetyl-CoA and acetylcarnitine shuttling out of the mitochondria<sup>19-21</sup>. The functional  
85 significance of such pathways relative to the canonical pathways via ACLY and ACSS2 remains  
86 poorly understood.

87 To evaluate potential alternative acetyl-CoA producing pathways, we generated cancer  
88 cell lines in which ACLY and ACSS2 are genetically deleted individually or in combination.  
89 Intriguingly, we show that cancer cells lacking both ACLY and ACSS2 (DKO cells) are viable,  
90 proliferate, contain a nuclear-cytosolic pool of acetyl-CoA, and sustain protein acetylation. Using  
91 carbon-13 tracing experiments, we demonstrate that fatty acids and glucose are prominent  
92 sources of acetyl-CoA that can feed acetyl-CoA pools and histone acetylation in the absence of  
93 ACLY and ACSS2. The data indicate that this is mediated at least in part via the carnitine shuttle  
94 and carnitine acetyltransferase (CrAT). Carnitine and CrAT function to transport acetyl-units out  
95 of the mitochondria and enable glucose dependent acetyl-CoA synthesis and *de novo* lipogenesis  
96 in an ACLY-independent manner. Overall, the data demonstrate that ACLY and ACSS2 are not  
97 the sole sources of acetyl-CoA in the nuclear-cytosolic compartment, and that the carnitine shuttle  
98 participates in the transit of acetyl-CoA from mitochondria to cytosol to support lipogenesis and  
99 histone acetylation.

100

## 101 **RESULTS**

### 102 **Cancer cells maintain viability and proliferation in the absence of ACLY and exogenous 103 acetate**

104 Since cancer cells are adept at engaging available metabolic flexibility mechanisms, we  
105 hypothesized that non-canonical acetyl-CoA production mechanisms could be revealed by  
106 developing ACLY KO cancer cell models that are not dependent on acetate for viability. We used  
107 murine *Acly*<sup>fl/fl</sup> hepatocellular carcinoma (HCC) cell lines and generated isogenic cell lines  
108 lacking ACLY or ACSS2 via adenoviral Cre treatment or CRISPR/Cas9 gene editing, respectively  
109 (Supplemental Figure 1A). ACLY KO cells had a dramatically decreased proliferation rate in cell  
110 growth and soft agar colony formation assays (Figure 1B, C). ACSS2 KO cells had no impairment  
111 of 2D cell growth but showed a modest defect in the numbers and size of soft agar colonies  
112 formed compared to parental controls (Figure 1B, C; Supplemental Figure 1B). As expected, loss  
113 of ACLY potently suppressed uniformly labeled <sup>13</sup>C<sub>6</sub>-glucose (<sup>13</sup>C-Glc) and increased <sup>13</sup>C<sub>2</sub>-acetate  
114 (<sup>13</sup>C-Ace) incorporation into acetyl-CoA and the downstream metabolite HMG-CoA (Figure 1D).  
115 ACSS2 KO cells used glucose similarly to their WT counterparts and labeling from acetate was  
116 completely ablated, showing that ACSS2 is the primary acyl-CoA synthetase that mediates  
117 synthesis of acetyl-CoA from acetate in these cells (Figure 1D). These data functionally validate  
118 our KO cell models.

119 We next tested if ACLY loss rendered these cancer cells reliant on acetate for proliferation  
120 and acetyl-CoA synthesis. While proliferation slows in ACLY KO HCC cells in the absence of  
121 acetate, the cells retain viability and continue to proliferate in the absence of exogenous acetate,  
122 in contrast to ACLY KO MEFs (Figure 1E; Supplemental Figure 1C)<sup>4</sup>. Similar observations were  
123 made in another cancer cell line, derived from *Kras*<sup>G12D</sup>-driven pancreatic cancer in mice with *Acly*  
124 deletion in the pancreas<sup>22</sup>, which also could proliferate in the absence of ACLY and exogenous  
125 acetate (Supplemental Figure 1E). Interestingly, these ACLY KO pancreatic cancer cells showed  
126 little to no reliance on exogenous acetate for proliferation, which is similar to that previously  
127 observed in ACLY null glioblastoma cells<sup>4</sup>. In the HCC ACLY KO cells, we quantified acetyl-CoA  
128 in the presence or absence of acetate, finding that acetyl-CoA abundance is elevated in ACLY  
129 KO cells in the presence of acetate and decreases back to that in WT cells in its absence (Figure  
130 1F). In contrast, HMG-CoA is almost entirely depleted upon acetate withdrawal, consistent with

131 that reported previously in ACLY KO cells<sup>8</sup>. Unexpectedly, malonyl-CoA accumulated under these  
132 conditions (Figure 1F). Together, these findings indicate that while exogenous acetate feeds  
133 acetyl-CoA pools in the absence of ACLY, additional mechanisms must also be available to cells  
134 to support proliferation.

135

### 136 **Cancer cells proliferate and maintain a pool of cytosolic acetyl-CoA in the absence of both 137 ACLY and ACSS2**

138 One possible explanation for these data is an endogenous source of acetate. To test this,  
139 we asked if ACSS2 is required for viability in ACLY KO cells, using CRISPR/Cas9 gene editing to  
140 delete *Acss2* in the ACLY KO HCC and pancreatic ACLY KO cells. Single cell clonal selection  
141 revealed that cells lacking both ACLY and ACSS2 (DKO) are viable and proliferate, albeit slower  
142 (Figure 2A, B; Supplemental Figure 2A, B, C).

143 We next examined acyl-CoA abundance, finding that acetyl-CoA is modestly reduced,  
144 HMG-CoA is dramatically reduced, and malonyl-CoA tends to be elevated in DKO cells, similar  
145 to the phenotype seen in ACLY KO cells in the absence of acetate (Figure 2C; Supplemental  
146 Figure 2D). To determine whether a substantial acetyl-CoA pool is present in the cytosol of DKO  
147 cells, we applied SILEC-SF, a recently developed technique for rigorous quantification of acyl-  
148 CoAs in subcellular compartments<sup>8</sup>. Subcellular measurements demonstrated distinct acyl-CoA  
149 profiles in mitochondria versus cytosol, including a clear cytosolic acetyl-CoA pool in the DKO  
150 cells (Figure 2D). The data indicate that cells are able to maintain nuclear-cytosolic acetyl-CoA  
151 pools independent of ACLY and ACSS2.

152

### 153 **Cells lacking ACLY and ACSS2 are dependent on exogenous fatty acids**

154 Having established the existence of an extramitochondrial acetyl-CoA pool in the DKO  
155 cells, we carried out RNA-sequencing to identify potential compensatory pathways. PCA analysis  
156 revealed distinct separation between ACLY deficient (ACLY KO and DKO1) and ACLY proficient  
157 genotypes (WT and ACSS2 KO) (Supplemental Figure 3A). Both ACLY KO and DKO cells  
158 showed marked transcriptional changes compared to WT with substantial overlap. However, there  
159 was also a distinct subset of genes specifically regulated in the DKO cells. (Figure 3A;  
160 Supplemental Figure 3B, C; Supplemental Table 1). Thus, we performed gene set enrichment  
161 analysis to identify functional groups of genes up- or down-regulated in the DKO cells (Figure 3B).  
162 Cell cycle related genes were among those suppressed, while fatty acid metabolism was notably  
163 enriched in the DKO cells, including fatty acid oxidation-related genes (Figure 3B, C;  
164 Supplemental Figure 3D). These included both mitochondrial and peroxisomal fatty acid oxidation  
165 genes (Figure 3C). These data suggest that lipid metabolism may be perturbed in the DKO cells  
166 and that fatty acid oxidation pathways might be upregulated as part of a compensatory  
167 mechanism.

168 To begin to investigate functional changes in lipid metabolism, we first tested if loss of  
169 ACLY and ACSS2 resulted in dependence on exogenous lipids. For this, DKO cells were cultured  
170 in media supplemented with serum treated with charcoal-dextran (CDT), a process that removes  
171 lipophilic compounds. DKO cells cultured in these lipid-depleted conditions failed to proliferate  
172 and began to undergo apoptosis (Figure 3D, E, F), while their ACLY KO counterparts proliferated  
173 with only a slight defect (Figure 3D). Interestingly, DKO cells also failed to proliferate in dialyzed  
174 serum (dFBS), which removes small molecules using a 10,000 MW cutoff membrane

175 (Supplemental Figure 3G). Acetate increased proliferation in ACLY KO cells but had no effect on  
176 DKO cells (Figure 3D; Supplemental Figure 3E). Addition of BSA-conjugated palmitic and oleic  
177 acids (PA/OA) fully rescued proliferation of DKO cells, and either PA or OA alone was also  
178 sufficient (Figure 3D, E; Supplemental Figure 3E, F, G). Supplementation of mevalonate,  
179 mevalonate-phosphate, or the medium chain fatty acid octanoate failed to rescue DKO cell  
180 proliferation (Figure 3D; Supplemental Figure 3E). Thus, cells lacking ACLY and ACSS2 are  
181 dependent on exogenous fatty acids for viability and proliferation.

182 We next asked if this requirement for exogenous fatty acids reflected a limited ability to  
183 synthesize fatty acids *de novo*. Since the pathways and carbon sources supplying acetyl-CoA in  
184 the DKO cells were unknown, DKO cells were cultured in the presence of deuterated water and  
185 deuterium incorporation into palmitate was used to examine total *de novo* lipogenesis. Compared  
186 to WT and ACSS2 KO cells, DKO cells exhibited very low palmitate labeling (Figure 3G,  
187 Supplemental Figure 3H). This limited *de novo* lipogenesis suggests that DKO cells rely on  
188 exogenous fatty acids for proliferation due to reduced ability to adequately synthesize their own  
189 fat despite maintaining higher or similar concentrations of malonyl-CoA (Figure 2C).  
190

## 191 **Fatty acids regulate histone acetylation in the absence of ACLY and ACSS2**

192 To further understand how acetyl-CoA is used in the DKO cells, we assessed levels of  
193 histone acetylation, another major nutrient-sensitive acetyl-CoA-dependent process, across the  
194 4 genotypes. We quantified acetylation at sites on histone H3 by mass spectrometry, focusing on  
195 two high abundance acetylation sites that have been proposed as acetate reservoirs, H3K23ac  
196 and H3K14ac<sup>23</sup>. Consistent with prior studies<sup>4,24</sup>, ACLY KO cells maintain lower levels of histone  
197 acetylation at H3K23 and H3K14 than their WT and ACSS2 KO counterparts, and DKO cells  
198 exhibit similar levels of acetylation as single ACLY KO cells (Figure 4A, B). To investigate if the  
199 capacity of DKO cells to acetylate is intact, we blocked histone deacetylation with the broad HDAC  
200 inhibitor trichostatin A (TSA). Here we analyzed the putative reservoir site H3K23ac, the  
201 regulatory site H3K27ac, and pan acetyl-H4. TSA treatment causes an increase in global histone  
202 acetylation in all marks analyzed over time in ACLY KO cells, and this effect is comparable in  
203 DKO cells (Figure 4C). This suggests that acetyl-CoA is readily available for use for histone  
204 acetylation in cells lacking both ACLY and ACSS2. Similarly, tubulin acetylation and total lysine  
205 acetylation dynamics, as assessed by a pan K-ac antibody, were comparable between genotypes  
206 (Figure 4D; Supplemental Figure 4A), indicating that acetyl-CoA is available in both the nucleus  
207 and cytosol in these cells.

208 We hypothesized that serum lipids might play a role in sustaining histone acetylation  
209 independent of ACLY and ACSS2, because fatty acids oxidation produces acetyl-CoA and genes  
210 involved in oxidation are upregulated in DKO cells. To test this, DKO cells were incubated in lipid-  
211 depleted culture conditions. Lipid depletion led to a dramatic depletion of histone acetylation within  
212 24 hours, and this was rescued by addition of PA/OA (Figure 4E, F; Supplemental Figure 4B).  
213 Acetyl-CoA abundance increased modestly with PA/OA supplementation, with some variability  
214 between lines (Supplemental Figure 4C). Malonyl-CoA, on the other hand, was potently  
215 suppressed by fatty acid supplementation (Supplemental Figure 4D). This is consistent with the  
216 known role of fatty acyl-CoAs in allosteric inhibition of the acetyl-CoA consuming enzyme ACC  
217<sup>25,26</sup>, which may divert acetyl-CoA towards histone acetylation, as previously reported<sup>27-29</sup>.  
218 Consistently, ACC inhibition (ND630) also modestly increased histone acetylation and

219 proliferation in the DKO cells while suppressing malonyl-CoA levels (Supplemental Figure 4D, E,  
220 F), indicating that fatty acids may act to increase histone acetylation in part through acetyl-CoA  
221 sparing from ACC consumption. We also tested octanoate, which has been previously shown to  
222 promote histone acetylation<sup>30</sup> and found that octanoate also increased histone acetylation and  
223 acetyl-CoA abundance in the DKO cells, though did not rescue proliferation (Supplemental Figure  
224 4G, H; Supplemental Figure 3A). Together, these data show that exogenous fatty acids can  
225 regulate histone acetylation levels independently of ACLY and ACSS2.

226

### 227 **Fatty acids and glucose can feed acetyl-CoA pools and histone acetylation independent 228 of ACLY and ACSS2**

229 To determine if fatty acid oxidation contributes substantially to the acetyl-CoA pool in DKO  
230 cells, we used stable isotope tracing of uniformly labeled <sup>13</sup>C palmitate (<sup>13</sup>C<sub>16</sub>-PA) into whole cell  
231 acetyl-CoA pools (Figure 5A). A time-course experiment showed maximal labeling of acetyl-CoA  
232 in DKO cells within 2 hours, reaching up to 30% (Supplemental Figure 5A). These findings  
233 suggest rapid and substantial breakdown of palmitate into acetyl-CoA. We also examined labeling  
234 from <sup>13</sup>C-Glc, unexpectedly finding that although glucose was a minor contributor to acetyl-CoA  
235 pools in ACLY KO cells, it labeled a much greater fraction, similar to that of palmitate, in DKO  
236 cells (Figure 5B). Under these conditions, whole cell acetyl-CoA abundance was similar between  
237 genotypes (Supplemental Figure 5B). Additionally, in the absence of fatty acids, glucose labeled  
238 nearly 60% of the acetyl-CoA pool in DKO cells, though the pool size was reduced (Figure 5C;  
239 Supplemental Figure 5C). Malonyl-CoA accumulates in the absence of fatty acids in these cells  
240 in an ACC-dependent manner (Supplemental Figure 4D), and labeling paralleled that of acetyl-  
241 CoA, suggesting that glucose-derived carbon may feed into an extramitochondrial acetyl-CoA  
242 pool in these cells (Figure 5C). Acetate did not detectably contribute to acetyl-CoA pools in DKO  
243 cells (Figure 5C). Thus, both glucose and fatty acids are major carbon sources feeding acetyl-  
244 CoA pools in the absence of ACLY and ACSS2.

245 In order to determine if acetyl-CoA generated from glucose and palmitate is used for  
246 acetylation in the nucleus, we performed LC-MS analysis of histone acetylation in cells incubated  
247 with isotope labeled glucose or palmitate. WT cells used glucose derived carbons for histone  
248 acetylation, and this was blunted by ACLY KO as expected (Figure 5D). ACSS2 KO slightly  
249 blunted glucose carbon incorporation into histone acetylation, possibly reflecting the recycling of  
250 acetyl-groups over the 24-hour time<sup>11</sup>. DKO cells had greater histone acetylation labeling from  
251 glucose than ACLY KO, in line with acetyl-CoA labeling. Additionally, palmitate-derived carbon  
252 was used prominently by ACSS2 KO and DKO cells. The cumulative fractional labeling from  
253 palmitate and glucose in DKO cell histone acetylation matches that by WT and ACSS2 KO cells,  
254 while ACLY KO cells have much lower cumulative labeling from these two sources, consistent  
255 with unlabeled acetate feeding the acetyl-CoA pool only in those cells (Supplemental Figure 5D).  
256 Together, these data indicate that both glucose and fatty acids can supply nuclear-cytosolic  
257 acetyl-CoA through a pathway that does not require ACLY or ACSS2 and that can be used for  
258 histone acetylation.

259

### 260 **ACLY KO cells have altered glucose usage in the TCA cycle**

261 We next sought to define the mechanism(s) of ACLY- and ACSS2-independent production  
262 of nuclear-cytosolic acetyl-CoA. Since both glucose and fatty acids could contribute to histone

263 acetylation, we reasoned that this could occur either by two distinct substrate-specific  
264 mechanisms or a mechanism in which these substrates can feed into a common precursor acetyl-  
265 CoA pool (e.g., in mitochondria) that is then transported to the nuclear-cytosolic compartment  
266 (Supplemental Figure 5E).

267 Given multiple publications documenting a nuclear PDC<sup>15-18</sup>, we investigated the  
268 localization of PDH in the HCC cells. Using immunofluorescence and confocal microscopy, we  
269 observed prominent mitochondrial but minimal nuclear localization of catalytic subunit of the  
270 pyruvate dehydrogenase complex (PDHe1 $\alpha$ ) (Supplemental Figure 6A). Additionally, PDHe1 $\alpha$   
271 protein levels are unchanged in standard culture conditions and are unaffected by fatty acid  
272 availability (Supplemental Figure 6B, C). While not formally ruling out a role for a nuclear PDC,  
273 the data suggest that a different mechanism likely sustains the DKO cells.

274 We next asked if a mitochondrial route might be involved since both glucose and fatty  
275 acids can feed mitochondrial acetyl-CoA pools (Figure 5A; Supplemental Figure 5E). <sup>13</sup>C-glucose  
276 labeling into TCA cycle intermediates was elevated in ACLY KO and DKO cells despite modest  
277 to no change in abundance of these metabolites (Figure 5E, Supplemental Figure 5F). Palmitate  
278 was poorly used as a carbon source in the TCA cycle in all genotypes, suggesting that fatty acid  
279 oxidation in the mitochondria is a more minor source of mitochondrial acetyl-CoA (Figure 5E).  
280 Inhibition of mitochondrial (etomoxir) and peroxisomal (thioridazine) fatty acid oxidation revealed  
281 that each organelle contributes to approximately half of acetyl-CoA produced from palmitate in  
282 the DKO cells (Supplemental Figure 5G). Together, the data indicate that glucose derived carbon  
283 feeds the mitochondrial metabolite pool and suggest the mitochondria as a possible intermediate  
284 location for glucose carbons before supplying nuclear-cytosolic acetyl-CoA in DKO cells.  
285

## 286 **The carnitine shuttle facilitates glucose-dependent lipogenesis independent of ACLY**

287 Acetyl-CoA cannot directly cross organelle membranes, and thus a transport mechanism  
288 out of mitochondria and/or peroxisomes is needed to explain the data. We noticed that *Crat*,  
289 encoding the carnitine acetyltransferase (CrAT) is upregulated in DKO cells (Fig. 3C). CrAT is  
290 present in mitochondria and peroxisomes<sup>31,32</sup> and transfers the acetyl moiety from acetyl-CoA  
291 onto carnitine to generate acetylcarnitine for organelle export via the carnitine-acylcarnitine  
292 translocase (CACT; SLC25A20). CrAT is thought to play a buffering function to prevent high levels  
293 of mitochondrial acetyl-CoA, which can suppress pyruvate oxidation and cause non-enzymatic  
294 acetylation<sup>33-35</sup>. CrAT is a reversible enzyme, and thus if CrAT is also present within the cytosol  
295 or nucleus, it could enable the regeneration of acetyl-CoA in the cytosol or nucleus from  
296 acetylcarnitine. Some evidence suggests it may be present and functional in the cytosol and  
297 nucleus, although such a role remains poorly understood<sup>20,36-38</sup>.

298 To determine if acetylcarnitine could serve as a metabolic intermediate in the generation  
299 of acetyl-CoA in the nuclear-cytosolic compartment in DKO cells, we traced palmitate and glucose  
300 into acetylcarnitine. Acetylcarnitine is highly labeled from glucose in ACLY KO and DKO (Figure  
301 6A), similar to TCA cycle labeling, suggesting that acetylcarnitine reflects and may be in  
302 equilibrium with the mitochondrial acetyl-CoA pool. Palmitate labels the acetylcarnitine pool, but  
303 to a lesser extent than glucose (Figure 6A). These findings prompted us to ask if CrAT provides  
304 a mechanism for shuttling of acetyl-units, especially those derived from glucose, into the nuclear-  
305 cytosolic acetyl-CoA pool.

306 If CrAT can generate acetyl-CoA for histone acetylation, we anticipated that the  
307 supplementation of acetylcarnitine to the culture media should boost histone acetylation and  
308 proliferation in lipid-depleted DKO cells, and this was indeed the case (Figure 6B, C). Notably,  
309 supplementation of DKO cells with L-carnitine alone was also able to increase both histone  
310 acetylation and cell proliferation in lipid depleted conditions (Figure 6B, C), suggesting that it may  
311 promote acetylcarnitine shuttling out of mitochondria.

312 To further probe this possibility, we tested if L-carnitine supplementation increased the  
313 ability of DKO cells to carry out *de novo* lipogenesis. Using deuterated water labeling, we found  
314 that L-carnitine significantly increased synthesis of palmitate and stearate in DKO cells  
315 (Supplemental Figure 7A). Additionally, supplementation of L-carnitine enhanced glucose  
316 dependent *de novo* lipogenesis in the absence of ACLY and ACSS2, implicating the carnitine  
317 shuttle in transport of acetyl-units out of mitochondria (Figure 6D; Supplemental Figure 6B).

318 To understand if carnitine can also promote glucose dependent *de novo* lipogenesis  
319 outside of the DKO context, we tested the impact of carnitine supplementation on WT and ACLY  
320 KO cells. WT cell *de novo* lipogenesis from glucose was unaffected by carnitine supplementation  
321 (Figure 6E; Supplemental Figure 7C, D, E, F). ACLY KO cells, however, showed a significant  
322 increase in glucose derived *de novo* lipogenesis when supplemented with carnitine (Figure 6E;  
323 Supplemental Figure 7C, D, E, F). Additionally, supplementation of acetylcarnitine further reduced  
324 glucose-dependent *de novo* lipogenesis in ACLY KO cells, suggesting that the acetyl-units  
325 provided by acetylcarnitine supplementation dilute the glucose-derived acetyl-CoA pools (Figure  
326 6E; Supplemental Figure 7C, D, E). Supporting this model, glucose incorporation into whole cell  
327 acetyl-CoA was increased in ACLY KO and DKO cells with carnitine supplementation, and  
328 acetylcarnitine suppressed glucose-dependent labeling, particularly in ACLY KO cells (Figure 6F).  
329 Together, these findings show that acetyl-units from the mitochondria can be exported and used  
330 in the cytosol independent of ACLY in a manner facilitated by carnitine.

331 To test if CrAT is necessary for the carnitine-mediated shuttling of acetyl-units out of the  
332 mitochondria, we generated ACLY KO/CrAT KO cells (Supplemental Figure 7G, H). CrAT  
333 deficiency suppressed the residual glucose dependent *de novo* lipogenesis in ACLY KO cells and  
334 abrogated the effect of carnitine supplementation in promoting *de novo* lipogenesis (Figure 6G,  
335 Supplemental Figure 7I). Additionally, CrAT KO suppressed acetyl-carnitine labeling from glucose  
336 (Figure 7H). Next, we tested if the mitochondrial entry of pyruvate was necessary for carnitine  
337 driven glucose labeling of fatty acids in ACLY KO cells by inhibiting the mitochondrial pyruvate  
338 carrier (MPC). Inhibition of MPC by UK5099 causes a dramatic decrease in glucose contribution  
339 to *de novo* lipogenesis (Figure 6I, Supplemental Figure 7J). Notably, UK5099 in combination with  
340 carnitine supplementation appeared to cause toxicity (Supplemental Figure 7K). These data  
341 indicate that the mitochondrial carnitine shuttle and CrAT facilitate an alternative pathway for two  
342 carbon units to leave mitochondria for use in nuclear-cytosolic processes.

343

## 344 DISCUSSION

345 The two well established routes to nuclear-cytosolic acetyl-CoA pools used for lipid  
346 synthesis and histone acetylation are via mitochondrial citrate export and cleavage by ACLY and  
347 acetate activation by ACSS2. In this study we demonstrate that the carnitine shuttle and CrAT  
348 can provide an alternative route for two carbon transport from mitochondria to cytosol. In cancer  
349 cell lines lacking both ACLY and ACSS2, we show that a pool of cytosolic acetyl-CoA is

350 maintained, that histone acetylation is active, and that both glucose and fatty acids can supply  
351 acetyl-CoA. We further show that fatty acids boost histone acetylation both by serving as a carbon  
352 source and via acetyl-CoA sparing by ACC inhibition. In the absence of ACLY, we show that  
353 carnitine supplementation increases *de novo* lipogenesis from glucose in a CrAT-dependent  
354 manner, thus demonstrating that the carnitine-CrAT pathway can be used as an ACLY-  
355 independent means of transporting two carbon units to the cytosol (Figure 7A). Of note, our tracing  
356 data also suggest that a peroxisomal route is likely important for at least a portion of fatty acid-  
357 dependent acetyl-CoA production. This is confirmed and studied in depth in a complementary  
358 study reported in a manuscript co-submitted with this one (Kumar et al, co-submitted). Overall,  
359 this work broadens the understanding of nuclear-cytosolic acetyl-CoA metabolism and opens new  
360 avenues for investigation into the regulation of *de novo* lipogenesis and histone acetylation.

361 One reason for undertaking this study to identify compensatory mechanisms of acetyl-  
362 CoA metabolism is that both ACLY and ACSS2 are of interest as therapeutic targets. The liver  
363 specific ACLY inhibitor bempedoic acid is FDA approved for LDL cholesterol lowering<sup>3,39</sup>, and the  
364 first ACSS2 inhibitor has entered oncology clinical trials (NCT04990739). At least in mice, ACLY  
365 deletion<sup>6</sup> or bempedoic acid<sup>40</sup> strongly suppresses glucose-dependent hepatic *de novo*  
366 lipogenesis, suggesting that ACLY is a dominant mechanism for glucose-dependent *de novo*  
367 lipogenesis in the liver, although acetate can sustain lipogenesis in the absence of ACLY<sup>6</sup>.  
368 However, looking to the future, ACLY is also of interest as a potential target in oncology, and  
369 given the metabolic plasticity of cancer cells and the ability to shift between nutrient sources<sup>41</sup>, a  
370 potential role of CrAT should be considered. Carnitine is synthesized in the liver, but also can be  
371 obtained through the diet. Carnitine is abundant in certain diets, such as those high in red meat,  
372 and the impact of dietary carnitine warrants exploration. Additionally, L-carnitine and acetyl-L-  
373 carnitine dietary supplements are widely available. Inhibiting carnitine metabolism would need to  
374 be evaluated with caution, as carnitine deficiency can cause severe phenotypes including muscle  
375 wasting, heart failure, liver damage, and cognitive delays<sup>42</sup>. In addition, it will be of interest to  
376 understand whether the carnitine shuttle plays a role in compensating for ACLY and ACSS2 under  
377 conditions such as high fat diet, in which both enzymes are suppressed in adipose tissue<sup>43</sup>;  
378 presumably acetyl-CoA would still be needed for histone acetylation and the mevalonate pathway  
379 even if fatty acid synthesis activity is low.

380 CrAT has mainly been studied for its role in mitochondrial metabolism, including fatty acid  
381 oxidation and acetyl-CoA buffering<sup>33-35,44</sup>; however there have been prior reports of CrAT activity  
382 outside of the mitochondria, as well as observations consistent with such activity. Early  
383 biochemical characterization of CrAT activity showed that it was at least partially localized on the  
384 cytosolic face of the ER membrane<sup>36</sup>. Yet, most reports suggest that CrAT is not a membrane  
385 bound protein and is localized within the mitochondrial lumen and the peroxisome, predominantly  
386 due to different CrAT isoform expression<sup>32</sup>. Tracing studies using labeled acetylcarnitine show  
387 that the acetyl-units from acetylcarnitine can indeed be used for lipid synthesis, and that their  
388 usage is increased with ACLY inhibition by hydroxycitrate<sup>37</sup>. Another study suggested that an  
389 isoform of CrAT is present in the nucleus and may promote histone acetylation in a manner  
390 dependent on the carnitine/acetylcarnitine translocase (CACT)<sup>20</sup>. Finally, acetyl-proteomics data in  
391 CrAT KO skeletal muscle showed that this perturbation increases mitochondrial acetylation,  
392 supporting the idea that CrAT can act as a buffer system for mitochondria acetyl-CoA, but also  
393 observed decreases in cytosolic protein acetylation<sup>33</sup>. In addition to acetyl-CoA, CrAT may also

394 be important for shuttling of other short-chain acyl-CoAs from mitochondria to the nuclear-  
395 cytosolic compartment. This is consistent with evidence that odd chain and branched chain fatty  
396 acid synthesis is CrAT dependent<sup>38</sup>, and that isoleucine catabolism, a mitochondrial process, can  
397 supply propionyl-CoA for histone propionylation and that this correlates with production of  
398 propionyl-carnitine<sup>8</sup>. Nevertheless, despite these reports in the literature, the significance of such  
399 a route has not been widely appreciated. Our findings, using genetic models and isotope tracing,  
400 are consistent with a model in which CrAT produces acetylcarnitine from acetyl-CoA in  
401 mitochondria and then following export to the cytosol, it converts acetylcarnitine back to acetyl-  
402 CoA as an alternative to ACLY for two carbon transfer from mitochondria (Figure 7A). However,  
403 while CrAT is required for this pathway in mitochondria, it remains possible that another unknown  
404 enzyme or a non-enzymatic process is responsible for nuclear-cytosolic conversion of  
405 acetylcarnitine into acetyl-CoA.

406 Further work will be needed to characterize the physiological contexts in which this  
407 pathway is employed versus the ACLY- or ACSS2-dependent routes to supply acetyl-CoA for lipid  
408 synthesis and chromatin modification. ACLY has been shown to participate in a non-canonical  
409 TCA cycle in a manner that influences cell fate<sup>45</sup>. This depends on oxaloacetate production by the  
410 ACLY reaction, which is returned to the TCA cycle after conversion to malate by MDH1. The  
411 carnitine shuttle offers a means to transport two carbon units to the cytosol without concomitant  
412 oxaloacetate production and thus one prediction is that this route might be important for  
413 supporting histone acetylation or lipogenesis in cell types that do not engage the non-canonical  
414 cycle.

415 An additional question emerging from this work is the function of histone acetate  
416 reservoirs. It has been proposed that histones provide a large reservoir of acetyl-units that can be  
417 mobilized either under metabolic stress or for a ready source of acetyl-CoA for site-specific  
418 histone acetylation and gene regulation<sup>23,46,47</sup>. Lipid deprivation in the DKO cells causes a rapid  
419 depletion in global histone acetylation in DKO cells. This model could uniquely enable studies of  
420 the consequences of histone acetylation reservoir depletion for gene regulation and chromatin  
421 structure.

422 Overall, this work provides evidence that the carnitine shuttle can contribute to both lipid  
423 synthesis and metabolites needed for chromatin modification. These data lay the groundwork for  
424 functional studies into the role of this pathway in physiological and disease contexts in regulation  
425 of cell state via histone acetylation and viability and growth potential via lipid synthesis.

426  
427

## 428 MATERIALS AND METHODS

429

### 430 Cell lines

431 Murine Aclyf/f hepatocellular carcinoma cell lines were generated from diethylnitrosamine  
432 induced tumors in Aclyf/f mice and have been previously described<sup>8</sup>. ACLY KO cells were  
433 generated by administration of adenoviral Cre recombinase, and single cell clonal populations  
434 were generated by limiting serial dilution. WT and ACLY KO cells were then transduced with a  
435 LentiCRISPR v2 vector with no guide RNA or containing a guide RNA targeting the first exon of  
436 ACSS2 or near the active site of CrAT:

437 ACSS2 KO, DKO1, DKO2 (and KPACs):

438 mACSS2sg2 - CGAGCTGCACCGGCGTTCTG

439 DKO3:

440 mACSS2sg6 - CTGCACCGGCGTTCTGTGG

441 ACLY KO CrAT KO1, ACLY KO CrAT KO2:

442 msgCRAT1F - CACCGTCCACAAGTGCAACTATGGG

443 Following transduction, cells were treated with puromycin until the entirety of an un-transduced  
444 cell population died. Following puromycin treatment single cell clonal populations were generated  
445 for ACSS2 KO, CrAT KO, ACLY/ACSS2 DKO, and ACLY/CrAT DKO cells by limiting serial  
446 dilution. The bulk cell of population of WT and ACLY KO cells transduced with the empty  
447 LentiCRISPR v2 were used as controls.

448 Murine pancreatic cancer cell lines were generated from pancreatic ductal  
449 adenocarcinoma tumors of Pdx1-Cre; LSL-KrasG12D; Tp53f/f; Aclyf/f mice<sup>22</sup>. A female mouse  
450 with palpable tumors in the peritoneal cavity was sacrificed at approximately 15 weeks of age.  
451 Pancreatic tumor was excised from the animal, minced in smaller pieces using sterile scissors  
452 and finally digested using a collagenase VI solution (2 mg/mL in DMEM/F12; Sigma #C9891) for  
453 20 minutes at 37°C. The solution was then filtered through a 70 µm mesh to obtain a single cell  
454 suspension. Cells were cultured in PDEC medium<sup>22</sup> and tested for mycoplasma contamination.  
455 Cells were passaged at confluence and ACLY deletion was confirmed by western blotting after 3  
456 passages. Pancreatic cancer ACLY KO cells were then transduced with a retroviral pOZ-N vector  
457 (addgene 3781) expressing ACLY or an empty pOZ-N vector to generate cells with reconstituted  
458 ACLY (ACLY KO plus ACLY cDNA) or an empty vector (ACLY KO). Cells were selected using IL-  
459 2R selection beads via pull down. To generate ACSS2 KO cells, cells were then transduced with  
460 a LentiCRISPR v2 empty vector or vector containing a guide RNA targeting the first exon of  
461 ACSS2. Following transduction, cells were treated with puromycin until the entirety of an un-  
462 transduced cell population died. Following puromycin treatment single cell clonal populations  
463 were generated for ACLY KO cells with ACLY cDNA and ACSS2 KO as well as ACLY KO cells  
464 with ACSS2 KO by limiting serial dilution.

465

#### 466 **Cell culture**

467 Murine HCC cells were cultured in DMEM/F12 (Gibco #11320033) supplemented with  
468 10% super calf serum (FS) (Gemini #100-510). Murine pancreatic cancer cells were cultured in  
469 DMEM (Gibco #11965084) supplemented with 10% super calf serum (FS) (Gemini #100-510).  
470 Cell growth experiments were performed by plating cells at the indicated density in DMEM/F12 +  
471 10% FS and cells were allowed to adhere overnight. Culture medium was changed the following  
472 day to the indicated conditions. Media for all experiments used high glucose DMEM (Gibco  
473 #11965084) or glucose and glutamine free DMEM (Gibco #A1443001) supplemented with 10 mM  
474 glucose and 4 mM glutamine unless otherwise indicated. Dialyzed fetal bovine serum (dFBS)  
475 (Gemini #100-108) and charcoal stripped FBS (charcoal dextran treated - CDT) (Corning #35-  
476 072-CV) or charcoal stripped FBS (CDT) (Sigma F6765) were used when indicated. All cell lines  
477 were routinely tested for mycoplasma contamination.

478 Metabolite addback and inhibitor treatment experiments were performed in the media  
479 conditions listed with supplementation of the listed metabolite or drug with equal volumes of  
480 vehicle control unless otherwise specified. Acetate, pyruvate, and mevalonate were  
481 supplemented after dissolving in water. Carnitine and acetylcarnitine were dissolved in media

482 used for each experiment. Fatty acid supplementation used palmitate and/or oleate conjugated  
483 to fatty acid free BSA (Bioworld 22070023) and fatty acid free BSA in an equivalent volume was  
484 supplemented as control.

485

#### 486 **Soft Agar Colony Formation Assay**

487 Cells were plated in 6 well plates at a density of  $2.5 \times 10^4$  cells per well. First, plates were  
488 coated with glucose and glutamine free DMEM media containing 10 mM glucose and 4 mM  
489 glutamine and supplemented with 10% FS and 0.6% Bacto Agar. Cells were plated on top of the  
490 0.6% Bacto agar layer in glucose and glutamine free DMEM media containing 10 mM glucose  
491 and 4 mM glutamine and supplemented with 10% FS and 0.3% Bacto Agar. Each cell line was  
492 plated in triplicate. Fresh media was added to the wells every 7 days for 3 weeks. Images were  
493 taken for analysis after 3 weeks. A total of 4 non-overlapping images were taken of each well  
494 totaling 12 images for analysis per cell line. Colonies were counted after blinding of images.

495

#### 496 **Acid Extraction of Histones**

497 Acid extraction on isolated nuclei was performed as previously described<sup>4</sup>. Cells were  
498 lysed with NIB-250 buffer (15 mM Tris-HCl (pH 7.5), 60 mM KCl, 15 mM NaCl, 5 mM MgCl<sub>2</sub>, 1  
499 mM CaCl<sub>2</sub>, 250 mM sucrose, 1 mM DTT, 10 mM sodium butyrate, protease inhibitors) with 0.1%  
500 NP-40 for 5 minutes on ice. Nuclei were pelleted from the cell lysate by centrifugation at 600g at  
501 4°C for 5 minutes. Extracted nuclei were washed with NIB-250 twice. Extracted nuclei were  
502 resuspending in 0.4N H<sub>2</sub>SO<sub>4</sub> and rotated for 4 hours or overnight at 4°C to extract histone proteins.  
503 The extracts were cleared by centrifugation at 11,000g at 4°C for 10 minutes. Clarified histone  
504 extracts were precipitated by adding 100% TCA to a final concentration of 20% TCA and the  
505 extracts were at 4°C overnight. Precipitated histones were centrifuged at 11,000g at 4°C for 10  
506 minutes. Histones were washed with 1 mL acetone + 0.1% 12 N HCl once and 1 mL acetone  
507 twice. The histone pellet was air dried at room temperature then resuspended in glass distilled  
508 H<sub>2</sub>O. Resuspended histones were used for western blotting.

509

#### 510 **Western blotting**

511 Cell for whole cell protein lysates were collected by trypsin mediated release from tissue  
512 culture plates. Cells were spun at 8000 rpm for 5 min and were kept on ice. Pellets were washed  
513 once with PBS. The cell pellet was resuspended in 50-100  $\mu$ L RIPA buffer [1% NP-40, 0.5%  
514 deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris plus protease inhibitor cocktail (Sigma-  
515 Aldrich, P8340) and phosSTOP if phosphorylated proteins were being investigated (Sigma-  
516 Aldrich, 04906845001)]. Cell lysis was allowed to occur on ice for 10 minutes. Cells were  
517 sonicated with a Branson Sonifier for 10 pulses at 20% amplitude. Cell lysate was clarified by  
518 centrifugation at 15,000xg for 10 minutes at 4°C and supernatant was transferred to a new tube.  
519 Samples were stored at -80C until analysis. All blots were developed using a LI-COR Odyssey  
520 CLx system. Antibodies used in this study were: ACLY (Proteintech #15421-1-AP), ACSS2 (CST  
521 #3658), Beta-actin (CST #3700), Alpha-tubulin (CST #2144), CrAT (Cloud-Clone Corp  
522 #PAC400Mu01), PDHe1 $\alpha$  (Santa Cruz sc-377092), Pan-acetyl-lysine (CST #9441), Acetyl-tubulin  
523 (CST #3971), Acetyl-H3K23 (CST #14932), Acetyl-H4 (Millipore 06-866), Acetyl-H3K27 (Abcam  
524 ab4729), Acetyl-H3K9 (Active Motif AB\_2793569), Acetyl-H4K5 (Millipore 07-327).

525

526 **RT-qPCR**

527 RNA was extracted from cells after trypsinization and pelleting by centrifugation at 8000xg  
528 for 5 minutes. Pellets were resuspended in 500  $\mu$ L Trizol (Life Technologies). RNA was extracted  
529 following the Trizol manufacturer protocol. Then, cDNA was prepared using high-capacity RNA-  
530 to-cDNA master mix (Applied Biosystems, 4368814) according to kit instructions. cDNA was  
531 diluted 1:20 and amplified with PowerUp SYBR Green Master Mix (Applied Biosystems, A25778)  
532 using a ViiA-7 Real-Time PCR system (Applied Biosystems). Fold change in expression was  
533 calculated by the  $\Delta\Delta C_t$  method using actin as a control. Primer sequences listed below.

534 CrAT-mF TGGTCATCTACTCCAGCCCC  
535 CrAT-mR AACTGGCAGCGTCTCATTGT  
536 Actin-mF TGGTGGGAATGGGTAGAA  
537 Actin-mR TCTCCATGTCGTCCCAGTTG

538

539 **D<sub>2</sub>O and glucose labeling of fatty acids and FAME GC-MS**

540 Cells were seeded at a density of  $7.5 \times 10^5$  cells per plate in DMEM/F12 containing 10%  
541 FS. For deuterium tracing, the following day the media was changed to DMEM media containing  
542 10% FS or 10% dFBS supplemented with 100  $\mu$ M acetate and 10% deuterium oxide (Sigma  
543 151882). For glucose tracing, the following day the media was changed to glucose and glutamine  
544 free DMEM media containing 10% dFBS or 10% CDT with 4 mM glutamine and 10 mM <sup>13</sup>C<sub>6</sub>  
545 glucose. After 24- or 48-hours cells were washed with ice cold DPBS and trypsinized. The trypsin  
546 reaction was stopped using cold 10% fatty acid free BSA in DPBS to remove any exogenous fatty  
547 acids. The cells were then washed twice with ice cold DPBS and the cell pellet was frozen at -  
548 80°C until extraction.

549 Lipids were extracted by resuspending the cell pellet in 2 ml ice cold methanol, followed  
550 by addition of 700  $\mu$ L ice cold glass distilled water. 10  $\mu$ L of 1 mM heptadecanoic acid in methanol  
551 was added to each sample as an internal standard. Cell suspensions were sonicated using a  
552 Branson Sonifier 250 at an output of 2.5 and a duty cycle of 20% for 15 pulses. Following  
553 sonication, 1 mL of ice-cold chloroform was added, and the suspension was mixed by vortexing.  
554 An additional 700  $\mu$ L of ice-cold chloroform and 700  $\mu$ L of ice-cold glass distilled water was added  
555 to the mixture and vortexed to mix. The suspension was centrifuged at 8000g for 10 minutes at  
556 4°C. The chloroform fraction was transferred to a new tube and the original suspension was re-  
557 extracted with 700  $\mu$ L ice cold chloroform and centrifuged at 8000g for 10 minutes at 4°C. The  
558 chloroform fraction from both extractions were pooled and 100  $\mu$ L ice cold water was added and  
559 the sample was vortexed to mix. The sample was centrifuged at 8000g for 10 minutes at 4°C and  
560 the chloroform fraction was transferred to a new tube and dried under nitrogen at 40°C.

561 Lipids were derivatized to methyl-esters by first resuspending the dried lipid extracts in 2  
562 mL methanol: toluene (80:20) containing butylated-hydroxy toluene (5 mg in 50 mL). Acetyl-  
563 chloride (2  $\mu$ L) was added, and the samples were heated to 95°C for 1 hour. Following heating, 5  
564 mL 6% potassium carbonate was added, and the samples were centrifuged at 8000 RFC for 10  
565 minutes at 4°C. The toluene layer was transferred to a new tube and centrifuged at 10000 RPM  
566 for 5 minutes at room temperature. The toluene layer was transferred to a glass GC/MS vial with  
567 a volume reducing insert. Fatty acid methyl esters were analyzed by GC/MS on an Agilent GC/MS  
568 7890A/5975A with a DB-5 column. Deuterium or carbon enrichment into palmitate was  
569 determined using Fluxfix<sup>48</sup>.

570

### 571 **Acyl-CoA analysis by LC-MS**

572 For extraction of acyl-CoAs, culture medium was completely aspirated from cells in 6cm  
573 plates before adding 1ml ice-cold 10% trichloroacetic acid to plates. For quantification  
574 experiments internal standard was added containing [ $^{13}\text{C}_3^{15}\text{N}_1$ ]-labeled acyl-CoAs generated in  
575 pan6-deficient yeast culture<sup>49</sup>. Plates were scraped to collect the cells. Samples were then  
576 sonicated for 10 × 0.5 s pulses to completely disrupt cellular membranes and incubated on ice to  
577 precipitate proteins. Protein was pelleted at 16,000g for 10 min at 4°C. Supernatant was collected  
578 and purified by solid-phase extraction using Oasis HLB 1cc (30 mg) SPE columns (Waters).  
579 Eluate was evaporated to dryness under nitrogen gas and re-suspended in 50  $\mu\text{L}$  of 5% 5-  
580 sulfosalicylic acid (w/v) for injection. Samples were analyzed by an Ultimate 3000 autosampler  
581 coupled to a Thermo Q-Exactive Plus Instrument in positive electrospray ionization (ESI) mode  
582 as previously described<sup>50</sup>. For quantitation, a calibration curve was generated using commercially  
583 available standards and internal standards containing [ $^{13}\text{C}_3^{15}\text{N}_1$ ]-labeled acyl-CoAs generated in  
584 pan6-deficient yeast culture<sup>49</sup> were added to each sample. For enrichment analysis, isotopically  
585 labeled glucose ( $^{13}\text{C}_6$  glucose), acetate ( $^{13}\text{C}_2$  acetate), or palmitate ( $^{13}\text{C}_{16}$  palmitate) was added  
586 to culture media and the enrichment into acyl-CoAs was determined using FluxFix based on  
587 samples treated with no isotope tracer<sup>48</sup>.

588

### 589 **Acyl-carnitine analysis by LC-MS**

590 For extraction of acyl-carnitines, cells were washed with 5ml ice-cold 0.9% NaCl to remove  
591 extracellular metabolites and scraped on ice in 1ml -80°C 80% HPLC-grade methanol/20% HPLC-  
592 grade water. Extracts were collected in 1.5ml tubes and 10ng of d3-propionyl-L-carnitine internal  
593 standard (Cayman 26579) in 50 $\mu\text{l}$  80% HPLC-grade methanol/20% HPLC-grade water was  
594 added to each sample. Samples were vortexed and incubated at -80°C for 30 min following  
595 centrifugation at 17,000g for 10 min at 4°C. Supernatants were transferred into a 96-well plate  
596 and dried under nitrogen gas at room temperature overnight. Dried metabolites were resuspended  
597 in 50 $\mu\text{l}$  95% HPLC-grade water/ 5% HPLC-grade methanol using TOMTEC QUADRA 4. For each  
598 sample, 1 $\mu\text{l}$  was injected and analyzed using a Vanquish Duo UHPLC system coupled to a  
599 Thermo Q-exactive Plus Orbitrap Instrument in positive electrospray ionization mode in full scan  
600 mode from 150-1000 m/z. The HPLC system used a hydrophilic interaction chromatography  
601 (HILIC) analytical column (Ascentis Express 2.1 mm x150 mm, 2.7 $\mu\text{m}$ ). The column was kept at  
602 30°C and the flow rate was 0.5ml/min. The mobile phase was solvent A (10 mM ammonium  
603 acetate and 0.2% formic acid in water) and solvent B (10 mM ammonium acetate and 0.2% formic  
604 acid in 95% HPLC-grade acetonitrile/5% HPLC-grade water). Elution gradients were run starting  
605 from 98% B to 86% B from 0-7 min; 86% B to 50% B from 7-7.3 min; 50% B to 10% B from 7.3-  
606 8.3; 10% B was held from 8.3-14.5 min, 10% B to 98% B from 14.5 to 14.510; 98% was held from  
607 14.510-15 min and then the column was equilibrated while eluting on the other identical column.  
608 For each analyte and the internal standard, the peak corresponding to the [M+H]<sup>+</sup> ion at 5ppm  
609 was integrated in Tracefinder 4.1 (Thermo Scientific). The enrichment of isotopically labelled  
610 glucose ( $^{13}\text{C}_6$  glucose) or palmitate ( $^{13}\text{C}_{16}$  palmitate) into acetyl-carnitine was determined using  
611 FluxFix based on samples treated with no isotope tracer<sup>48</sup>.

612

### 613 **TCA metabolite analysis by GC-MS**

614                   Polar metabolites were extracted from cells through addition of 1mL 80:20 methanol:water  
615 chilled to -80°C. Cells were collected by scraping after addition of norvaline as an internal  
616 standard, lysed by 3 rounds of freeze thawing, and insoluble material was removed through  
617 centrifugation at 12,000g at 4°C for 10 minutes. The pellet was used for protein quantification  
618 after resuspension in 2% SDS 0.1 mM Tris buffer. The supernatant containing polar metabolites  
619 was evaporated to dryness by SpeedVac. The dried pellet was stored at -80°C until derivatization.  
620 Samples were derivatized by addition of 30  $\mu$ L of 5 mg/mL methoxyamine in pyridine and heated  
621 for 15 minutes at 70°C. A total of 70  $\mu$ L MTBSTFA was then added, and the samples were heated  
622 at 70°C for 1 hour. After derivatization, samples were centrifuged at 12,000 rpm for 5 minutes.  
623 The supernatant was transferred to a glass GC/MS vial with a volume reducing insert. Samples  
624 were analyzed by GC/MS on an Agilent GC/MS 7890A/5975A with a DB-5 column. 13-C  
625 enrichment was determined using Fluxfix based on samples treated with no isotope tracer<sup>48</sup>.  
626 Relative quantification was performed by normalizing the sum of the AUC of all isotopologues for  
627 each metabolite to the sum of the AUC of all isotopologues of norvaline within each sample  
628 followed by normalization to the average protein quantification within each experimental group.  
629

### 630 **Histone acetylation UPLC-MS/MS analysis**

631                   Histones were extracted as described in “Acid Extraction of Histones”. Dry histone pellets  
632 were stored at -80°C until processing for LC/MS analysis. The unmodified lysines in dry histone  
633 were propionylated by propionic anhydride at pH 8 and 51°C for 1 hour followed by trypsin  
634 digestion at pH 8 and 37°C for overnight as in our previously published procedures<sup>51</sup>. A Waters  
635 Acquity H-class UPLC coupled with a Thermo TSQ Quantum Access triple quadrupole mass  
636 spectrometer was used to quantify the acetylated lysines on H3 tryptic peptides. The UPLC and  
637 MS/MS settings, solvent gradient and detailed mass transitions were reported previously<sup>52</sup>.  
638 Retention time and specific mass transitions were both used to identify individual acetylated and  
639 propionylated peaks. The <sup>13</sup>C-labeled acetyl-lysine peptide peaks were identified by considering  
640 the shift in mass by +2. The resolved peaks were integrated using Xcalibur software (version 2.1,  
641 Thermo). Relative quantitative analysis was used to determine the amount of modification on  
642 individual lysines.  
643

### 644 **Stable isotope labeling of essential nutrients in cell culture-subcellular fractionation 645 \*(SILEC-SF) acyl-CoA quantitation**

646                   SILEC-SF was performed as previously described<sup>8</sup>. In brief, WT HCC cells (D42) were  
647 used to generate SILEC internal standard by passaging of cells in <sup>15</sup>N<sup>13</sup>C<sub>3</sub>-pantothenate (Vitamin  
648 B5) for at least 9 passages as previously described<sup>8</sup>. SILEC WT cells were mixed with DKO1 cells  
649 before fractionation. Mitochondria and cytosolic fractions were separated through differential  
650 centrifugation. Extracted acyl-CoAs were subjected to analysis by LC-MS as described above.  
651

### 652 **RNA-Sequencing**

653                   RNA was extracted from HCC cells cultured in DMEM + 10% FS for 24 hours using a  
654 Qiagen RNeasy Plus Mini Kit (Qiagen #74134) with an additional on column DNA digestion using  
655 Qiagen TURBO DNase (Qiagen #AM2238) based on manufacturer protocols.

656                   RNA-seq libraries were prepared with the NEBNext poly(A) Magnetic Isolation Module  
657 (NEB #E7490L) followed by the NEBNext Ultra Directional RNA library preparation kit for Illumina

658 (NEB #E7420L) according to manufacturer's protocol. Library quality was assessed using an  
659 Agilent BioAnalyzer 2100 and libraries were quantified with the Library Quant Kit for Illumina (NEB  
660 #E7630L). Libraries were then diluted to 1.8pM and sequenced on the NextSeq500 platform using  
661 75-base-pair (bp) single-end reads. All RNA-seq read alignment was performed using Illumina  
662 RNA-seq alignment software (version 2.0.1). Briefly, reads were mapped to *Mus musculus*  
663 University of California Santa Cruz (UCSC) mouse GRCm38/mm10 reference genome with RNA  
664 STAR aligner under default settings (version 2.6.1a)<sup>53</sup>. Transcripts per million (TPM) generation  
665 and differential expression analysis was performed on aligned reads to *Mus musculus* UCSC  
666 GRCm38/mm10 reference genome using Illumina RNA-seq differential expression software  
667 (DESeq2, v1.0.1)<sup>54</sup>. Significance cut-offs are listed in figure legends for each analysis. GSEA<sup>55,56</sup>  
668 was performed by comparing DKO cells to all other genotypes combined (WT, ACLY KO, ACSS2  
669 KO).

670

## 671 Immunofluorescence confocal microscopy

672 Cells were plated on glass coverslips and allowed to adhere for 24 hours. Live cells were  
673 then incubated in DMEM + 10% FS with 1  $\mu$ M MitoTracker Deep Red FM (Thermo) for 30 minutes.  
674 Cells were washed with PBS 3 times then fixed with 4% PFA for 30 minutes. After fixation,  
675 coverslips were washed 3 times with TBST then blocked with 10% goat serum, 1% BSA, 0.1%  
676 gelatin, 22.52 mg/mL glycine, and 0.1% Triton X-100 in TBST for 30 minutes. Coverslips were  
677 washed with TBST 3 times then incubated with PDHe1 $\alpha$  antibody (Abcam ab110334) for 3 hours.  
678 Coverslips were washed with TBST 3 times then incubated with DAPI and donkey anti-rabbit  
679 Alexa Flour 488 (Invitrogen #a21206) for 1 hour. Coverslips were washed 3 times then mounted  
680 on slides for imaging. Slides were imaged on a Zeiss LSM 880. Z-stacks were compressed into  
681 a single plane for representation.

682

## 683 Statistical Analyses

684 All analyses were performed using GraphPad Prism or R (RNA sequencing analysis).  
685 Statistical test specifics are included in figure legends.

686

687

## 688 REFERENCES

1. Campbell, S. L. & Wollen, K. E. Metabolic Signaling to the Nucleus in Cancer. *Mol. Cell* **71**, 398–408 (2018).
2. Bose, S., Ramesh, V. & Locasale, J. W. Acetate Metabolism in Physiology, Cancer, and Beyond. *Trends Cell Biol.* (2019) doi:10.1016/j.tcb.2019.05.005.
3. Batchuluun, B., Pinkosky, S. L. & Steinberg, G. R. Lipogenesis inhibitors: therapeutic opportunities and challenges. *Nat. Rev. Drug Discov.* 2022 **21**, 283–305 (2022).
4. Zhao, S. *et al.* ATP-Citrate Lyase Controls a Glucose-to-Acetate Metabolic Switch. *Cell Rep.* **17**, 1037–1052 (2016).
5. Martinez Calejman, C. *et al.* mTORC2-AKT signaling to ATP-citrate lyase drives brown adipogenesis and de novo lipogenesis. *Nat. Commun.* **11**, 1–16 (2020).
6. Zhao, S. *et al.* Dietary fructose feeds hepatic lipogenesis via microbiota-derived acetate. *Nature* **579**, (2020).
7. Trefely, S. *et al.* Subcellular metabolic pathway kinetics are revealed by correcting for artifactual post harvest metabolism. *Mol. Metab.* **30**, 61–71 (2019).
8. Trefely, S. *et al.* Quantitative subcellular acyl-CoA analysis reveals distinct nuclear

704 metabolism and isoleucine-dependent histone propionylation. *Mol. Cell* **82**, 447–462.e6  
705 (2022).

706 9. Vysochan, A., Sengupta, A., Weljie, A. M., Alwine, J. C. & Yu, Y. ACSS2-mediated  
707 acetyl-CoA synthesis from acetate is necessary for human cytomegalovirus infection.  
708 *Proc. Natl. Acad. Sci. U. S. A.* **114**, E1528–E1535 (2017).

709 10. Liu, X. *et al.* Acetate Production from Glucose and Coupling to Mitochondrial Metabolism  
710 in Mammals. *Cell* **175**, 502–513.e13 (2018).

711 11. Bulusu, V. *et al.* Acetate Recapturing by Nuclear Acetyl-CoA Synthetase 2 Prevents Loss  
712 of Histone Acetylation during Oxygen and Serum Limitation. *Cell Rep.* **18**, 647–658  
713 (2017).

714 12. Lu, M. *et al.* ACOT12-Dependent Alteration of Acetyl-CoA Drives Hepatocellular  
715 Carcinoma Metastasis by Epigenetic Induction of Epithelial-Mesenchymal Transition. *Cell*  
716 *Metab.* **29**, 886–900.e5 (2019).

717 13. Prokesch, A. *et al.* N-acetylaspartate catabolism determines cytosolic acetyl-CoA levels  
718 and histone acetylation in brown adipocytes. *Sci. Reports* **2016** *6*, 1–12 (2016).

719 14. Huber, K. *et al.* N-acetylaspartate pathway is nutrient responsive and coordinates lipid  
720 and energy metabolism in brown adipocytes. *Biochim. Biophys. Acta - Mol. Cell Res.*  
721 **1866**, 337–348 (2019).

722 15. Sutendra, G. *et al.* A nuclear pyruvate dehydrogenase complex is important for the  
723 generation of Acetyl-CoA and histone acetylation. *Cell* **158**, 84–97 (2014).

724 16. Nagaraj, R. *et al.* Nuclear Localization of Mitochondrial TCA Cycle Enzymes as a Critical  
725 Step in Mammalian Zygotic Genome Activation. *Cell* **168**, 210–223.e11 (2017).

726 17. Chen, J. *et al.* Compartmentalized activities of the pyruvate dehydrogenase complex  
727 sustain lipogenesis in prostate cancer. *Nat. Genet.* **2018** *502* **50**, 219–228 (2018).

728 18. Zervopoulos, S. D. *et al.* MFN2-driven mitochondria-to-nucleus tethering allows a non-  
729 canonical nuclear entry pathway of the mitochondrial pyruvate dehydrogenase complex.  
730 *Mol. Cell* **82**, 1066–1077.e7 (2022).

731 19. He, A. *et al.* Acetyl-CoA Derived from Hepatic Peroxisomal  $\beta$ -Oxidation Inhibits  
732 Autophagy and Promotes Steatosis via mTORC1 Activation. *Mol. Cell* **79**, 30–42.e4  
733 (2020).

734 20. Madiraju, P., Pande, S. V., Prentki, M. & Madiraju, S. R. M. Mitochondrial acetyl carnitine  
735 provides acetyl groups for nuclear histone acetylation. *Epigenetics* **4**, 399–403 (2009).

736 21. Jo, C. *et al.* Histone acylation marks respond to metabolic perturbations and enable  
737 cellular adaptation. *Exp. Mol. Med.* **2020** *5212* **52**, 2005–2019 (2020).

738 22. Carrer, A. *et al.* Acetyl-CoA metabolism supports multistep pancreatic tumorigenesis.  
739 *Cancer Discov.* **9**, 416–435 (2019).

740 23. Mendoza, M. *et al.* Enzymatic transfer of acetate on histones from lysine reservoir sites to  
741 lysine activating sites. *Sci. Adv.* **8**, 5688 (2022).

742 24. Wellen, K. E. *et al.* ATP-citrate lyase links cellular metabolism to histone acetylation.  
743 *Science* **324**, 1076–80 (2009).

744 25. Ogiwara, H., Tanabe, T., Nikawa, J. -i & Numa, S. Inhibition of Rat-Liver Acetyl-  
745 Coenzyme-A Carboxylase by Palmitoyl-Coenzyme A. *Eur. J. Biochem.* **89**, 33–41 (1978).

746 26. Trumble, G. E., Smith, M. A. & Winder, W. W. Purification and Characterization of Rat  
747 Skeletal Muscle Acetyl-CoA Carboxylase. *Eur. J. Biochem.* **231**, 192–198 (1995).

748 27. Galdieri, L. & Vancura, A. Acetyl-CoA carboxylase regulates global histone acetylation. *J.*  
749 *Biol. Chem.* **287**, 23865–23876 (2012).

750 28. Galdieri, L., Gatla, H., Vancurova, I. & Vancura, A. Activation of AMP-activated protein  
751 kinase by metformin induces protein acetylation in prostate and ovarian cancer cells. *J.*  
752 *Biol. Chem.* **291**, 25154–25166 (2016).

753 29. Chow, J. D. Y. *et al.* Genetic inhibition of hepatic acetyl-CoA carboxylase activity  
754 increases liver fat and alters global protein acetylation. *Mol. Metab.* **3**, 419–431 (2014).

755 30. McDonnell, E. *et al.* Lipids Reprogram Metabolism to Become a Major Carbon Source for  
756 Histone Acetylation. *Cell Rep.* **17**, 1463–1472 (2016).

757 31. Houten, S. M., Wanders, R. J. A. & Ranea-Robles, P. Metabolic interactions between  
758 peroxisomes and mitochondria with a special focus on acylcarnitine metabolism. *Biochim.  
759 Biophys. Acta - Mol. Basis Dis.* **1866**, 165720 (2020).

760 32. Corti, O., DiDonato, S. & Finocchiaro, G. Divergent sequences in the 5' region of cDNA  
761 suggest alternative splicing as a mechanism for the generation of carnitine  
762 acetyltransferases with different subcellular localizations. *Biochem. J.* **303**, 37–41 (1994).

763 33. Davies, M. N. *et al.* The Acetyl Group Buffering Action of Carnitine Acetyltransferase  
764 Offsets Macronutrient-Induced Lysine Acetylation of Mitochondrial Proteins. *Cell Rep.* **14**,  
765 243–254 (2016).

766 34. Muoio, D. M. *et al.* Muscle-Specific Deletion of Carnitine Acetyltransferase Compromises  
767 Glucose Tolerance and Metabolic Flexibility. *Cell Metab.* **15**, 764–777 (2012).

768 35. Seiler, S. E. *et al.* Carnitine Acetyltransferase Mitigates Metabolic Inertia and Muscle  
769 Fatigue during Exercise. *Cell Metab.* **22**, 65–76 (2015).

770 36. Valkner, K. J. & Bieber, L. L. The sidedness of carnitine acetyltransferase and carnitine  
771 octanoyltransferase of rat liver endoplasmic reticulum. *Biochim. Biophys. Acta -  
772 Biomembr.* **689**, 73–79 (1982).

773 37. Lligona-Trulla, L. *et al.* Acetyl-L-carnitine flux to lipids in cells estimated using isotopomer  
774 spectral analysis. *J. Lipid Res.* **38**, 1454–1462 (1997).

775 38. Wallace, M. *et al.* Enzyme promiscuity drives branched-chain fatty acid synthesis in  
776 adipose tissues. *Nat. Chem. Biol.* **2018 1411** **14**, 1021–1031 (2018).

777 39. Goldberg, A. C. *et al.* Effect of Bempedoic Acid vs Placebo Added to Maximally Tolerated  
778 Statins on Low-Density Lipoprotein Cholesterol in Patients at High Risk for  
779 Cardiovascular Disease: The CLEAR Wisdom Randomized Clinical Trial. *JAMA* **322**,  
780 1780–1788 (2019).

781 40. Morrow, M. R. *et al.* Inhibition of ATP-citrate lyase improves NASH, liver fibrosis, and  
782 dyslipidemia. *Cell Metab.* **34**, 919–936.e8 (2022).

783 41. McGuirk, S., Audet-Delage, Y. & St-Pierre, J. Metabolic Fitness and Plasticity in Cancer  
784 Progression. *Trends in Cancer* **6**, 49–61 (2020).

785 42. Longo, N., Frigeni, M. & Pasquali, M. Carnitine transport and fatty acid oxidation.  
786 *Biochim. Biophys. Acta - Mol. Cell Res.* **1863**, 2422–2435 (2016).

787 43. Carrer, A. *et al.* Impact of a High-fat Diet on Tissue Acyl-CoA and Histone Acetylation  
788 Levels. *J. Biol. Chem.* **292**, 3312–3322 (2017).

789 44. Noland, R. C. *et al.* Carnitine insufficiency caused by aging and overnutrition  
790 compromises mitochondrial performance and metabolic control. *J. Biol. Chem.* **284**,  
791 22840–22852 (2009).

792 45. Arnold, P. K. *et al.* A non-canonical tricarboxylic acid cycle underlies cellular identity.  
793 *Nature* **603**, 477 (2022).

794 46. Ye, C. & Tu, B. P. Sink into the Epigenome: Histones as Repositories That Influence  
795 Cellular Metabolism. *Trends Endocrinol. Metab.* **29**, 626–637 (2018).

796 47. Boon, R., Silveira, G. G. & Mostoslavsky, R. Nuclear metabolism and the regulation of the  
797 epigenome. *Nat. Metab.* **2020 211** **2**, 1190–1203 (2020).

798 48. Trefely, S., Ashwell, P. & Snyder, N. W. FluxFix: Automatic isotopologue normalization for  
799 metabolic tracer analysis. *BMC Bioinformatics* **17**, 1–8 (2016).

800 49. Snyder, N. W. *et al.* Production of stable isotope-labeled acyl-coenzyme A thioesters by  
801 yeast stable isotope labeling by essential nutrients in cell culture HHS Public Access.  
802 *Anal. Biochem.* **474**, 59–65 (2015).

803 50. Frey, A. J. *et al.* LC-quadrupole/Orbitrap high-resolution mass spectrometry enables  
804 stable isotope-resolved simultaneous quantification and 13 C-isotopic labeling of acyl-  
805 coenzyme A thioesters. *Anal. Bioanal. Chem.* **408**, 3651–3658 (2016).

806 51. Kuo, Y. M., Henry, R. A. & Andrews, A. J. A quantitative multiplexed mass spectrometry  
807 assay for studying the kinetic of residue-specific histone acetylation. *Methods* **70**, 127–  
808 133 (2014).

809 52. Kuo, Y. M. & Andrews, A. J. Quantitating the Specificity and Selectivity of Gcn5-Mediated  
810 Acetylation of Histone H3. *PLoS One* **8**, e54896 (2013).

811 53. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21  
812 (2013).

813 54. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion  
814 for RNA-seq data with DESeq2. *Genome Biol.* **15**, 1–21 (2014).

815 55. Subramanian, A. *et al.* Gene set enrichment analysis: A knowledge-based approach for  
816 interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 15545–  
817 15550 (2005).

818 56. Mootha, V. K. *et al.* PGC-1 $\alpha$ -responsive genes involved in oxidative phosphorylation are  
819 coordinately downregulated in human diabetes. *Nat. Genet.* **2003** **34**, 267–273  
820 (2003).

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822

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834 Conceptualization: LI, KEW, NWS  
835 Methodology: LI, KEW, NWS  
836 Formal Analysis: LI, ST, CD, TM, NK, AF  
837 Investigation: LI, ST, CD, JD, TM, NK, AF, PN, LR, JS, HA, AC  
838 Visualization: LI  
839 Supervision: KEW, NWS, BC, AA  
840 Writing - original draft: LI, KEW  
841 Writing - review & editing: LI, KEW, NWS, ST, AC, BC, AA, CD, JD, TM, NK, AF, PN, LR, JS, HA

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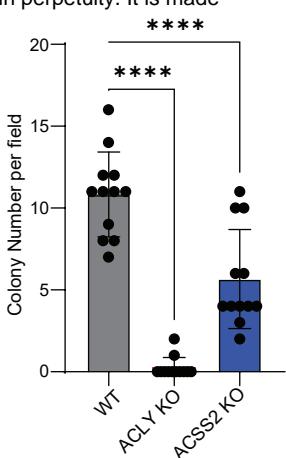
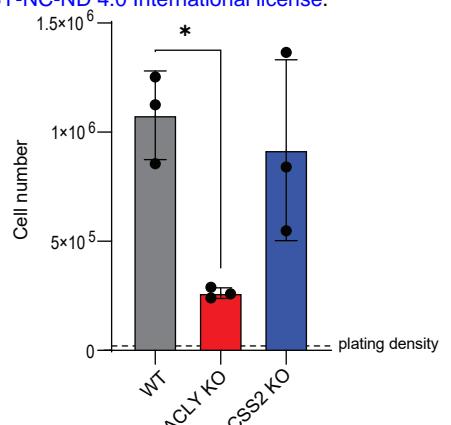
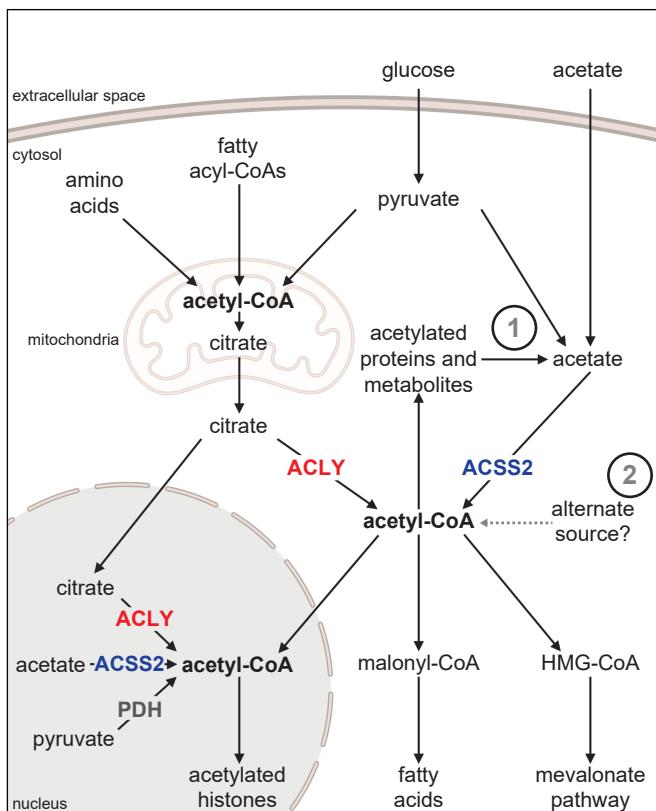
### 843 **Competing Interests:** No competing interests declared

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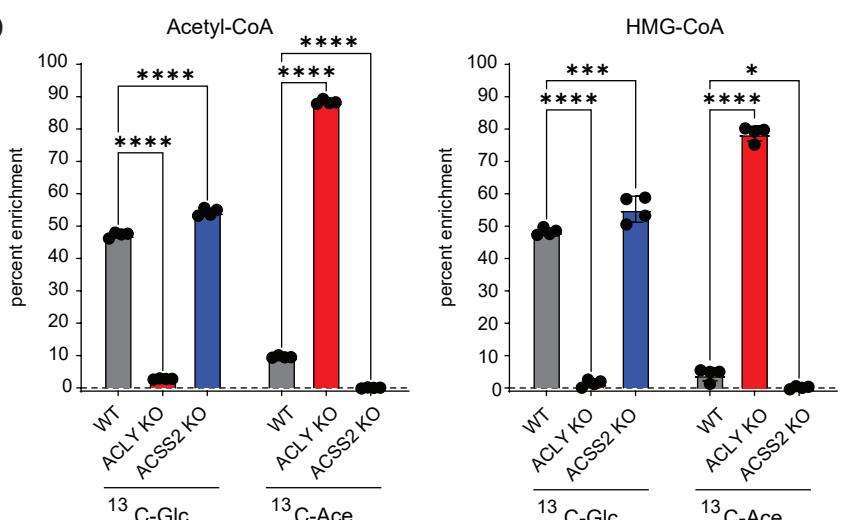
845 **Data and Material Availability:** All data needed to evaluate the conclusions in the paper are  
846 present in the paper and/or the Supplementary Materials. RNA sequencing data will be deposited  
847 in GEO.

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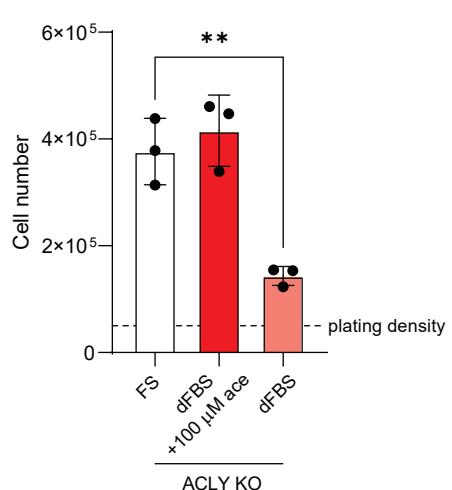
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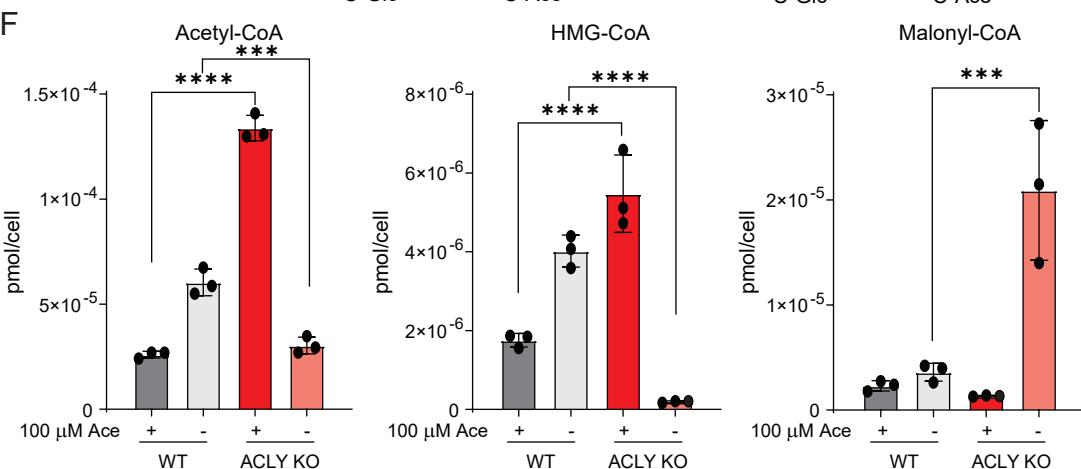
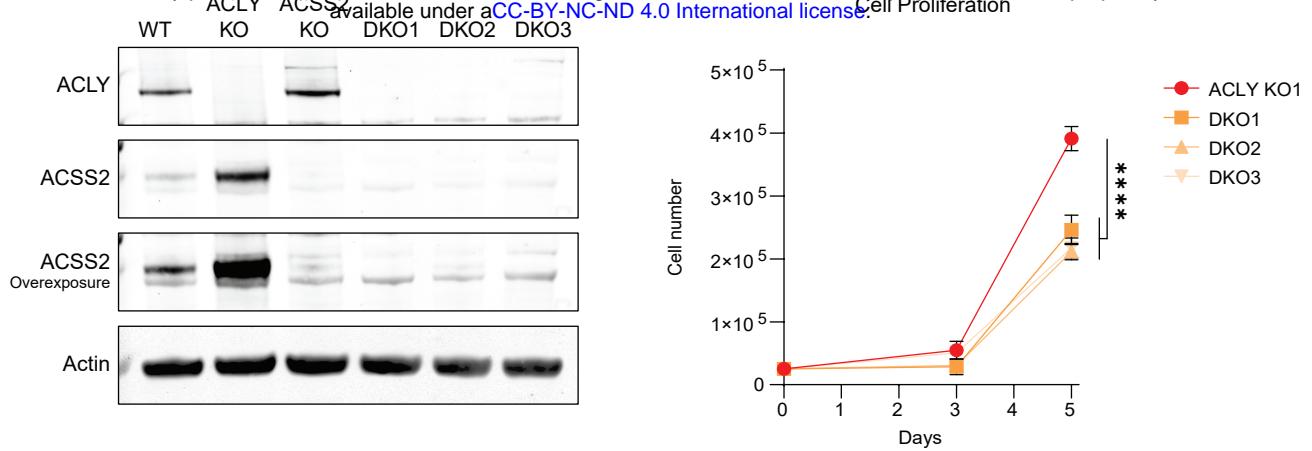
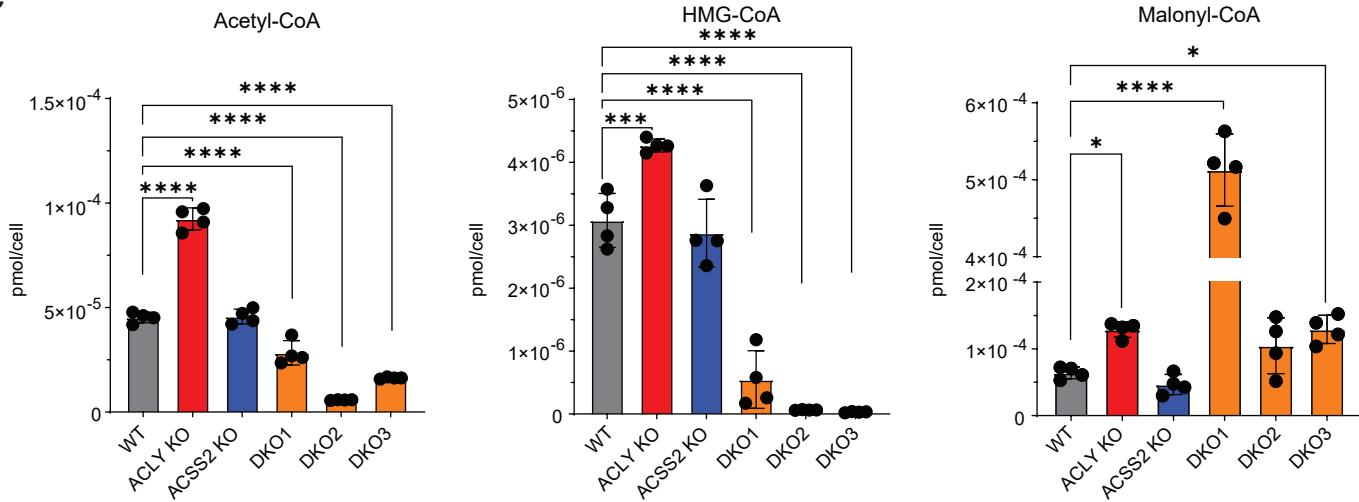


Figure 1: ACLY KO cancer cells proliferate in the absence of exogenous acetate

849 **Figure 1: ACLY KO cancer cells proliferate in the absence of exogenous acetate**  
850 A) Schematic diagram of acetyl-CoA metabolism. Arrows represent biochemical conversions.  
851 Numbers refer to potential ACLY- and exogenous acetate-independent acetyl-CoA generating  
852 pathways in the nuclear-cytosolic compartment. Created with BioRender.com.  
853 B) Proliferation of WT, ACLY KO, and ACSS2 KO HCC cell lines over 4 days in DMEM + 10%  
854 FS. Statistical significance was calculated by one-way ANOVA.  
855 C) Colony formation in soft agar of WT, ACLY KO, and ACSS2 KO HCC cells. Colonies were  
856 counted at 4x magnification. Data are from 3 replicate wells with 4 fields counted per well.  
857 Statistical significance was calculated by one-way ANOVA.  
858 D) Whole cell acetyl-CoA measurements and isotopologue distribution (labeled acetyl-CoA, m+2;  
859 labeled HMG-CoA sum of m+2, m+4, and m+6) of WT, ACLY KO, and ACSS2 KO HCC cells  
860 cultured in glucose and glutamine free DMEM + 10% dFBS + 10mM glucose + 100 $\mu$ M acetate for  
861 6 hours. Statistical significance was calculated by two-way ANOVA.  
862 E) ACLY KO proliferation in DMEM + 10% FS, DMEM + 10% dFBS, or DMEM + 10% dFBS +  
863 100 $\mu$ M acetate for 96 hours. Statistical significance was calculated by one-way ANOVA.  
864 F) Whole cell acyl-CoA measurements of WT and ACLY KO cells grown in DMEM + 10% dFBS  
865 or DMEM + 10% dFBS + 100 $\mu$ M acetate for 24 hours. Statistical significance was calculated by  
866 two-way ANOVA.  
867 FS, full serum (10% calf serum); dFBS, dialyzed FBS. Each point represents a biological replicate  
868 and error bars represent standard deviation. \*p $\leq$ 0.05; \*\*p $\leq$ 0.01; \*\*\*p $\leq$ 0.001; \*\*\*\*p $\leq$ 0.0001.  
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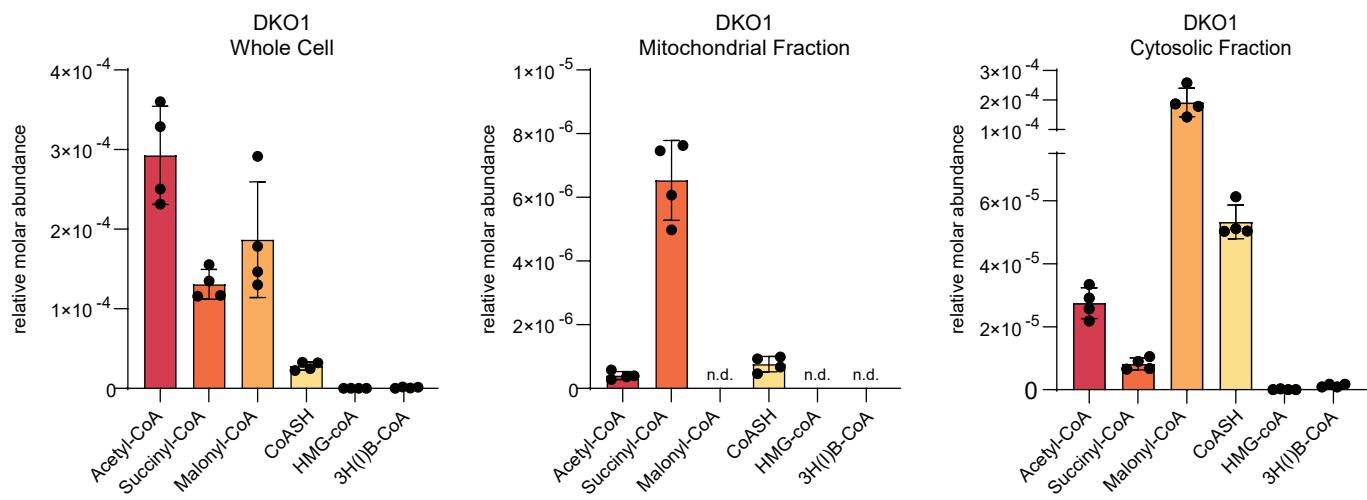
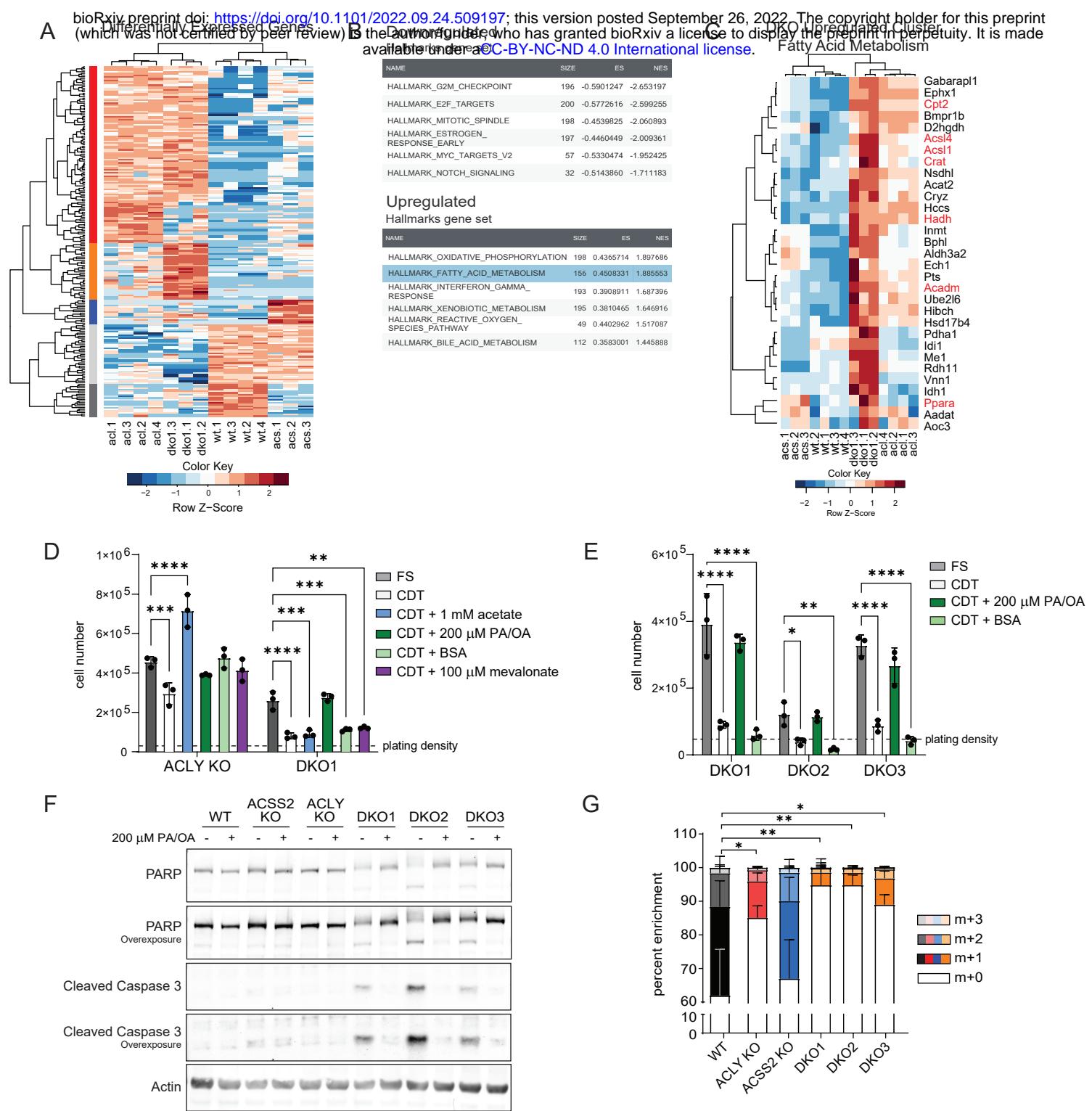


Figure 2: ACLY/ACSS2 double knockout cells are viable and maintain a cytosolic pool of acetyl-CoA

870 **Figure 2: ACLY/ACSS2 double knockout cells are viable and maintain a cytosolic pool of**  
871 **acetyl-CoA**

872 A) Western blot for ACLY and ACSS2 in WT, ACLY KO, ACSS2 KO, and 3 DKO cell lines.  
873 B) Proliferation curve of ACLY KO and DKO HCC cell lines over 5 days in DMEM + 10% FS. Data  
874 are represented as mean of three replicates +/- standard deviation. Statistical significance was  
875 calculated by one-way ANOVA between samples at 5 days.  
876 C) Whole cell acyl-CoA measurements in WT, ACLY KO, ACSS2 KO, and DKO cell lines grown  
877 in DMEM + 10% dFBS + 100 $\mu$ M acetate for 24 hours. Statistical significance was calculated by  
878 one-way ANOVA.  
879 D) Acyl-CoA quantitation using SILEC-subcellular fractionation performed on DKO1 cells grown  
880 in DMEM + 10% dFBS + 100 $\mu$ M acetate for 24 hours. 3H(I)B-CoA, 3-HB-CoA and isobutyryl-CoA  
881 are not resolved and are represented together. Acyl-CoA species marked with n.d. were not  
882 detected in n=4 samples.  
883 Each point represents a biological replicate and error bars represent standard deviation. \*p $\leq$ 0.05;  
884 \*\*p $\leq$ 0.01; \*\*\*p $\leq$ 0.001; \*\*\*\*p $\leq$ 0.0001  
885



886 **Figure 3: Loss of ACLY and ACSS2 alters fatty acid metabolism and causes reliance on**  
887 **exogenous fatty acids**

888 A) Heatmap of all differentially expressed genes between all 4 genotypes,  $\log_2FC > 2$  and an  
889 adjusted  $p$ -value  $< 0.01$  expressed as row Z-score. DESeq counts were  $\log_2$  transformed before  
890 clustering. Row clusters (red, orange, blue, light gray, and dark gray) represent groups of genes  
891 commonly differentially regulated by sample cluster. See Table S1 for gene list by cluster.  
892 B) The top six upregulated and downregulated Hallmarks gene sets from GSEA analysis  
893 comparing DKO cells to all other genotypes.  
894 C) Genes from the hallmarks fatty acid metabolism gene set commonly upregulated across DKO1  
895 samples. Cluster expanded from supplemental figure 3D. DESeq counts were  $\log_2$  transformed  
896 before clustering.  
897 D) Cell proliferation after 96 hours. Cells were plated in DMEM/F12 media overnight then cultured  
898 in DMEM + 10% FS or CDT serum with or without the addition of metabolites. PA/OA is 100  $\mu$ M  
899 of each fatty acid conjugated to BSA (200  $\mu$ M total). Statistical significance was calculated by two-  
900 way ANOVA.  
901 E) Cell proliferation after 96 hours. Cells were plated in DMEM/F12 media overnight then cultured  
902 in DMEM + 10% FS or CDT serum with or without the addition of metabolites. PA/OA is 100  $\mu$ M  
903 of each fatty acid conjugated to BSA (200  $\mu$ M total). BSA condition is equal volume of fatty acid  
904 free BSA as added to PA/OA condition. Statistical significance was calculated by two-way  
905 ANOVA.  
906 F) Western blot analysis of cells cultured in DMEM + 10% CDT serum with or without PA/OA.  
907 PA/OA is 100  $\mu$ M of each fatty acid conjugated to BSA (200  $\mu$ M total). Without PA/OA conditions  
908 contain fatty acid free BSA.  
909 G) Isotopologue enrichment of palmitate measured by GC-MS. Cells were cultured in DMEM +  
910 10% D2O + 10% dFBS + 100  $\mu$ M acetate for 24 hours. Bars represent mean of 3 or 4 biological  
911 replicates for each cell line. Statistical analysis performed on total hydrogen enrichment.  
912 Statistical significance was calculated by one-way ANOVA.  
913 CDT, charcoal dextran treated. Each point represents a biological replicate and error bars  
914 represent standard deviation. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$

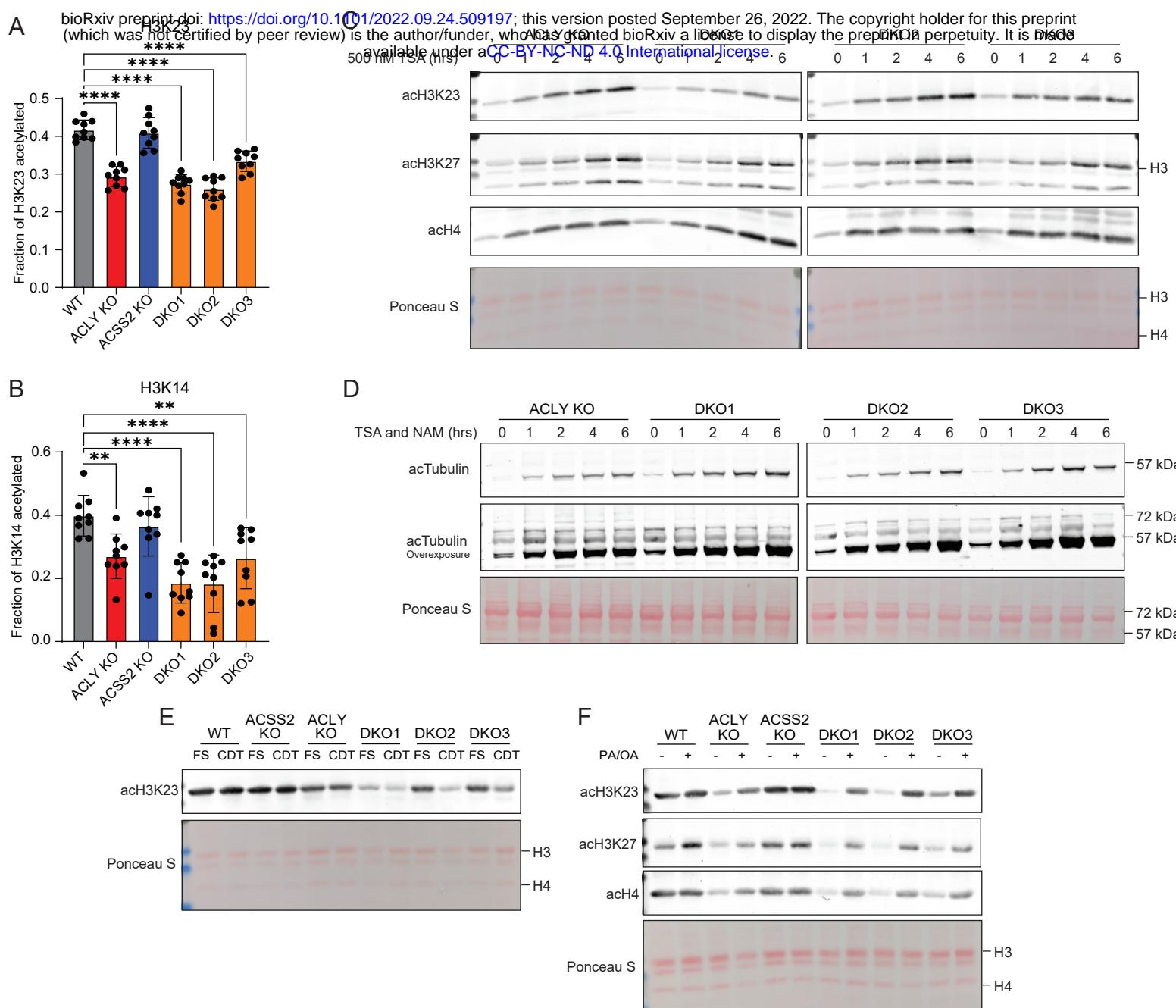


Figure 4: Fatty acid availability modulates histone acetylation independent of ACLY and ACSS2

916 **Figure 4: Fatty acid availability modulates histone acetylation independent of ACLY and**  
917 **ACSS2**

918 A) Fraction of the total quantified H3K23 residues acetylated by LC-MS. Statistical significance  
919 was calculated by one-way ANOVA.

920 B) Fraction of the total quantified H3K14 residues acetylated by LC-MS. Statistical significance  
921 was calculated by one-way ANOVA.

922 C) Acid extracted histone western blot from cells grown in DMEM + 10% FS and treated with 500  
923 nM trichostatin A (TSA) over a time course. Ponceau S stain for total protein in histone extracts  
924 used for western blot.

925 D) Whole cell protein extract western blot from cells grown in DMEM + 10% FS and treated with  
926 500 nM TSA and 500  $\mu$ M nicotinamide (NAM) over a time course. Ponceau S stain for total protein  
927 in histone extracts used for western blot.

928 E) Acid extracted histone western blot from cells cultured in DMEM + 10% FS or CDT for 24  
929 hours. Ponceau S stain for total protein in histone extracts used for western blot.

930 F) Acid extracted histone western blot from cells cultured for 24 hours in DMEM + 10% CDT and  
931 supplemented with PA/OA. PA/OA is 100  $\mu$ M of each fatty acid conjugated to BSA (200  $\mu$ M total).  
932 Ponceau S stain for total protein in histone extracts used for western blot.

933 Each point represents a biological replicate and error bars represent standard deviation. \* $p\leq 0.05$ ;  
934 \*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$ ; \*\*\*\* $p\leq 0.0001$

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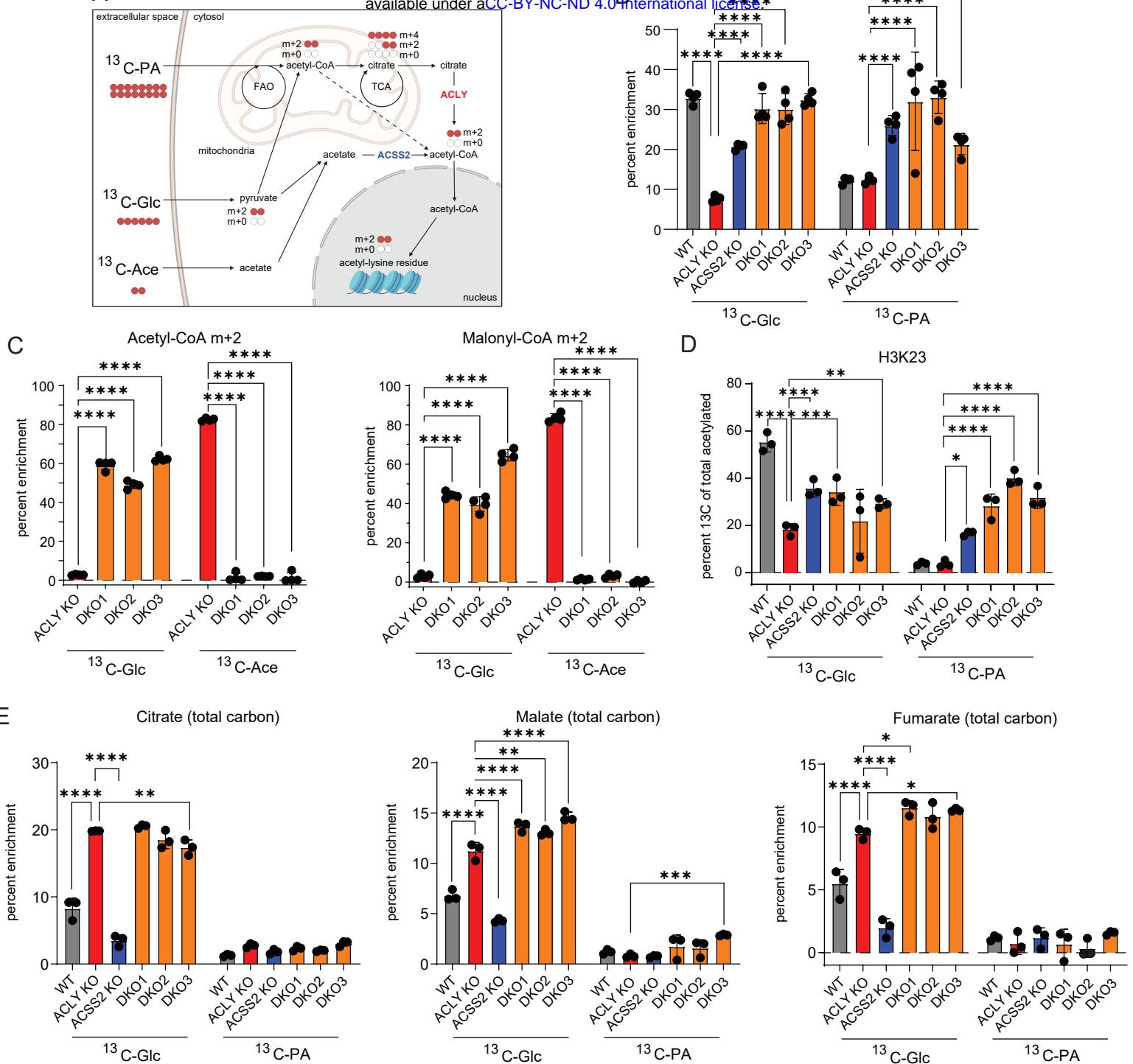


Figure 5: Fatty acids and glucose can supply acetyl-CoA for histone acetylation in a manner independent of ACLY and ACSS2

936 **Figure 5: Fatty acids and glucose can supply acetyl-CoA for histone acetylation in a**  
937 **manner independent of ACLY and ACSS2**

938 A) Schematic depicting glucose, palmitate, and acetate carbon tracing into acetyl-CoA and  
939 histone acetylation. Created with BioRender.com.

940 B)  $^{13}\text{C}_6$ -glucose and  $^{13}\text{C}_{16}$ -palmitate tracing into acetyl-CoA analyzed by LC-MS. Cells were  
941 cultured in glucose and glutamine free DMEM + 10% CDT supplemented with 4 mM glutamine  
942 and either 10 mM  $^{13}\text{C}_6$ -glucose and 100  $\mu\text{M}$  palmitate conjugated to BSA or 10 mM glucose and  
943 100  $\mu\text{M}$   $^{13}\text{C}_{16}$ -palmitate conjugated to BSA for 2 hours. Statistical significance was calculated by  
944 two-way ANOVA.

945 C) Acetyl-CoA and malonyl-CoA enrichment from  $^{13}\text{C}_6$ -glucose or  $^{13}\text{C}_2$ -acetate. Cells were  
946 cultured in glucose and glutamine free DMEM + 10% dFBS supplemented with 4 mM glutamine  
947 and either 10 mM  $^{13}\text{C}_6$ -glucose and 100  $\mu\text{M}$  acetate or 10 mM glucose and 100  $\mu\text{M}$   $^{13}\text{C}_2$ -acetate  
948 for 6 hours. Statistical significance was calculated by two-way ANOVA.

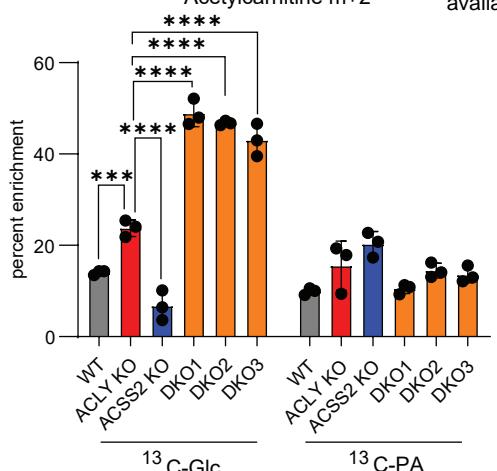
949 D)  $^{13}\text{C}_6$ -glucose and  $^{13}\text{C}_{16}$ -palmitate tracing into acetylation on extracted histones, analyzed by  
950 LC-MS. Cells were cultured in glucose and glutamine free DMEM + 10% CDT supplemented with 4 mM  
951 glutamine and either 10 mM  $^{13}\text{C}_6$ -glucose and 100  $\mu\text{M}$  palmitate conjugated to BSA or 10  
952 mM glucose and 100  $\mu\text{M}$   $^{13}\text{C}_{16}$ -palmitate conjugated to BSA for 24 hours. Statistical significance  
953 was calculated by two-way ANOVA.

954 E)  $^{13}\text{C}_6$ -glucose and  $^{13}\text{C}_{16}$ -palmitate tracing into TCA cycle intermediates, analyzed by GC-MS.  
955 Cells were cultured in glucose and glutamine free DMEM + 10% CDT supplemented with 4 mM  
956 glutamine and either 10 mM  $^{13}\text{C}_6$ -glucose and 100  $\mu\text{M}$  palmitate conjugated to BSA or 10 mM  
957 glucose and 100  $\mu\text{M}$   $^{13}\text{C}_{16}$ -palmitate conjugated to BSA for 6 hours. Statistical significance was  
958 calculated by two-way ANOVA.

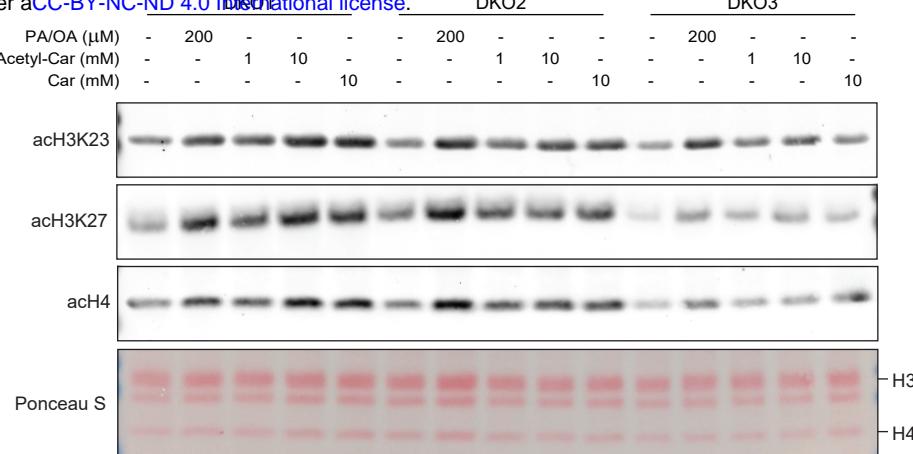
959 Each point represents a biological replicate and error bars represent standard deviation. All tests  
960 compared to ACLY KO as the control. \* $p\leq 0.05$ ; \*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$ ; \*\*\*\* $p\leq 0.0001$

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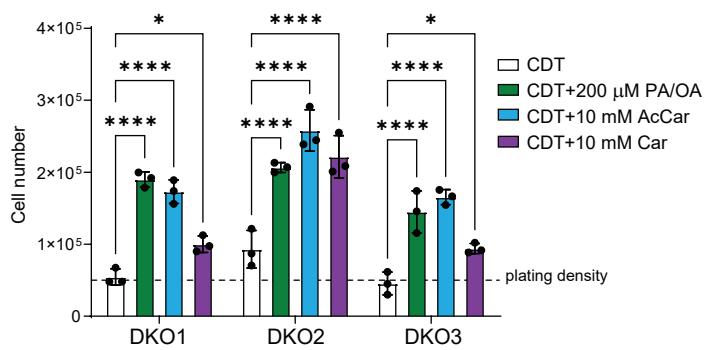
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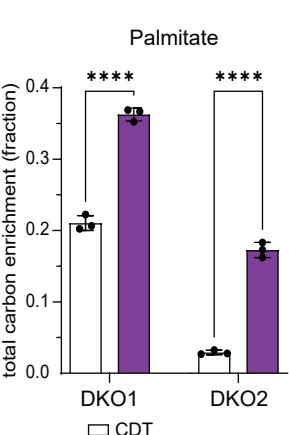
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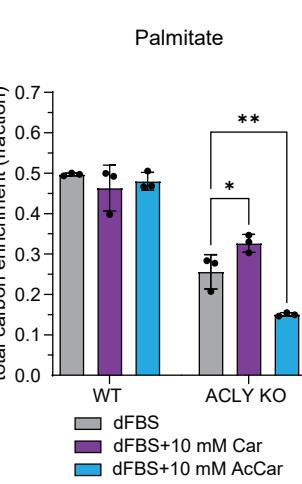
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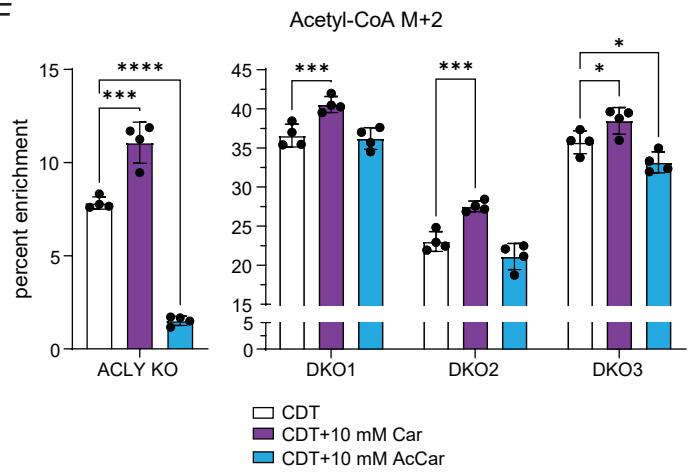
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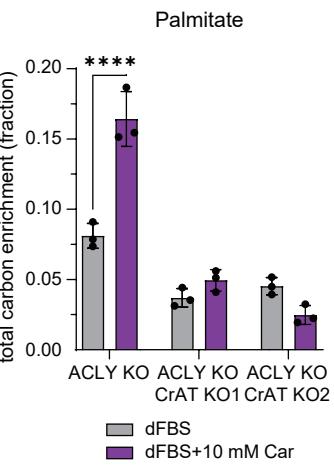
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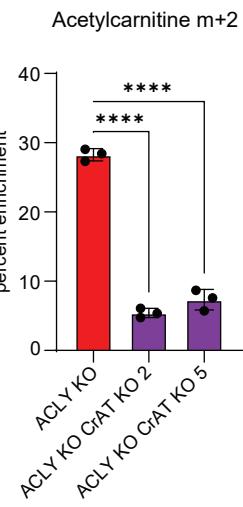
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G



H



I

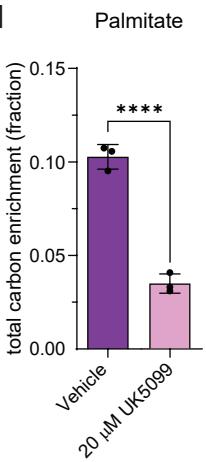


Figure 6: Carnitine facilitates histone acetylation and de novo lipogenesis from glucose derived carbon

962 **Figure 6: Carnitine facilitates histone acetylation and de novo lipogenesis from glucose**  
963 **derived carbon**

964 A)  $^{13}\text{C}_6$ -glucose and  $^{13}\text{C}_{16}$ -palmitate tracing into acetylcarnitine, analyzed by LC-MS. Cells were  
965 cultured in glucose and glutamine free DMEM + 10% CDT supplemented with 4 mM glutamine  
966 and either 10 mM  $^{13}\text{C}_6$ -glucose and 100  $\mu\text{M}$  palmitate conjugated to BSA or 10 mM glucose and  
967 100  $\mu\text{M}$   $^{13}\text{C}_{16}$ -palmitate conjugated to BSA for 6 hours. Statistical significance was calculated by  
968 two-way ANOVA.

969 B) Acid extracted histone western blot from cells cultured in DMEM + 10% CDT for 24 hours  
970 supplemented with PA/OA, acetylcarnitine, or carnitine. PA/OA is 100  $\mu\text{M}$  of each fatty acid  
971 conjugated to BSA (200  $\mu\text{M}$  total). Ponceau S stain for total protein in histone extracts used for  
972 western blot.

973 C) Cell proliferation after 96 hours. Cells were plated in DMEM/F12 media overnight then cultured  
974 in DMEM + 10% CDT serum with or without the addition of metabolites. PA/OA is 100  $\mu\text{M}$  of each  
975 fatty acid conjugated to BSA (200  $\mu\text{M}$  total). Statistical significance was calculated by two-way  
976 ANOVA.

977 D)  $^{13}\text{C}_6$ -glucose tracing into palmitate measured by GC-MS. Cells were cultured in glucose and  
978 glutamine free DMEM + 10% CDT supplemented with 4 mM glutamine and 10 mM  $^{13}\text{C}_6$ -glucose  
979 with or without 10 mM carnitine for 48 hours. Statistical significance was calculated by unpaired  
980 t-tests.

981 E)  $^{13}\text{C}_6$ -glucose tracing into palmitate measured by GC-MS. Cells were cultured in glucose and  
982 glutamine free DMEM + 10% dFBS supplemented with 4 mM glutamine and 10 mM  $^{13}\text{C}_6$ -glucose  
983 with or without 10 mM carnitine or 10 mM acetylcarnitine for 48 hours. Statistical significance was  
984 calculated by two-way ANOVA.

985 F)  $^{13}\text{C}_6$ -glucose tracing into acetyl-CoA, analyzed by LC-MS. Cells were cultured in glucose and  
986 glutamine free DMEM + 10% dFBS supplemented with 4 mM glutamine and 10 mM  $^{13}\text{C}_6$ -glucose  
987 supplemented with or without 10 mM carnitine or 10 mM acetylcarnitine for 6 hours. Statistical  
988 significance was calculated by two-way ANOVA.

989 G)  $^{13}\text{C}_6$ -glucose tracing into palmitate measured by GC-MS. Cells were cultured in glucose and  
990 glutamine free DMEM + 10% dFBS supplemented with 4 mM glutamine and 10 mM  $^{13}\text{C}_6$ -glucose  
991 with or without 10 mM carnitine for 48 hours. Statistical significance was calculated by unpaired  
992 t-tests.

993 H)  $^{13}\text{C}_6$ -glucose tracing into acetylcarnitine, analyzed by LC-MS. Cells were cultured in glucose  
994 and glutamine free DMEM + 10% dFBS supplemented with 4 mM glutamine and 10 mM  $^{13}\text{C}_6$ -  
995 glucose for 6 hours. Statistical significance was calculated by one-way ANOVA.I)  $^{13}\text{C}_6$ -glucose  
996 tracing into palmitate measured by GC-MS. Cells were cultured in glucose and glutamine free  
997 DMEM + 10% dFBS supplemented with 4 mM glutamine and 10 mM  $^{13}\text{C}_6$ -glucose with 10 mM  
998 carnitine with vehicle control or 20  $\mu\text{M}$  UK5099 for 48 hours. Statistical significance was calculated  
999 by unpaired t-tests.

1000 Each point represents a biological replicate and error bars represent standard deviation. \* $p\leq 0.05$ ;  
1001 \*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$ ; \*\*\*\* $p\leq 0.0001$

1002

A

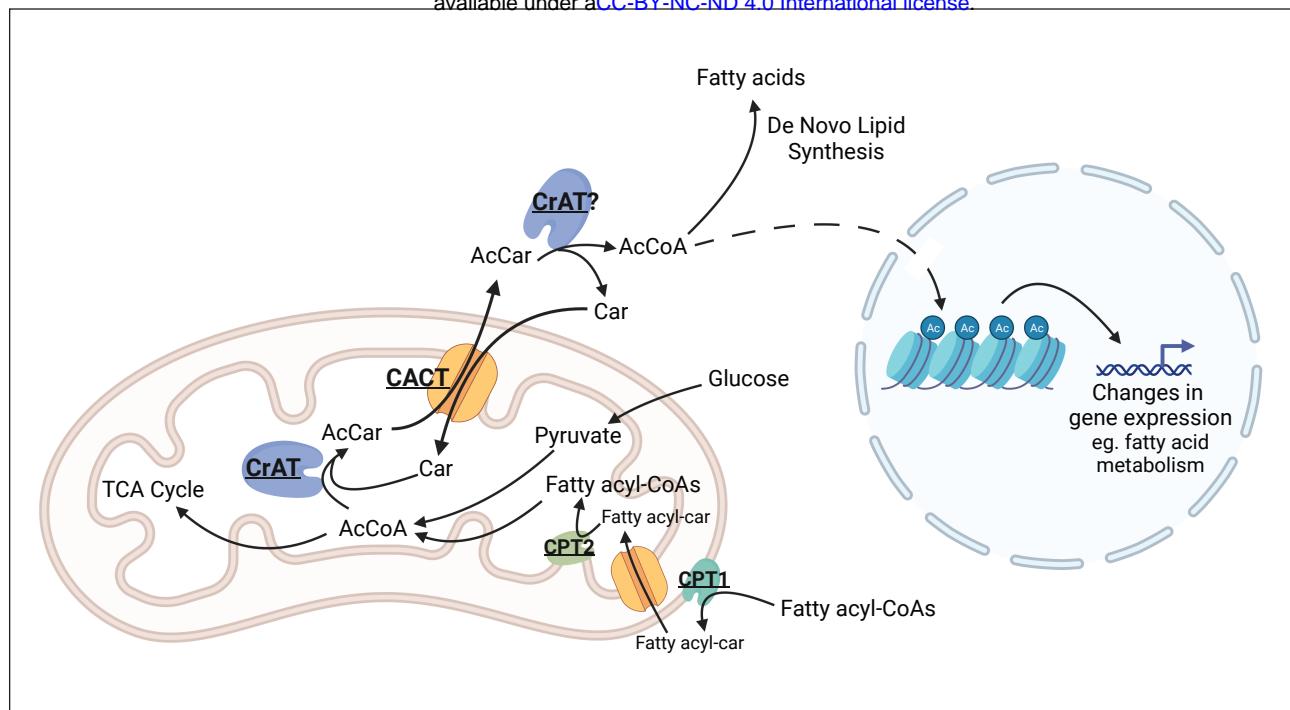


Figure 7: The carnitine shuttle provides acetyl-units to the nuclear cytosolic compartment

1003 **Figure 7: The carnitine shuttle provides acetyl-units to the nuclear cytosolic compartment**  
1004 A) Schematic depicting CrAT-dependent acetylcarnitine shuttling out of the mitochondria for  
1005 acetyl-CoA generation in the nuclear-cytosolic compartment. Arrows represent biochemical  
1006 conversions. Created with BioRender.com.  
1007