Therapeutic targeting of ACLY in T-ALL *in vivo*

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Abstract

T-cell Acute Lymphoblastic Leukemia (T-ALL) is a hematological malignancy in need of novel therapeutic approaches. Here, we identify the ATP-citrate lyase ACLY as overexpressed and as a novel therapeutic target in T-ALL. To test the effects of ACLY in leukemia progression, we developed an isogenic model of NOTCH1-induced *Acly* conditional knockout leukemia. Importantly, we observed intrinsic antileukemic effects upon loss of ACLY, which further synergized with NOTCH1 inhibition *in vivo.* Metabolomic profiling upon ACLY loss revealed a metabolic crisis with reduced acetyl-CoA levels, as well as a decreased oxygen consumption rate. Gene expression profiling analyses showed that the transcriptional signature of ACLY loss very significantly correlates with the signature of MYC loss *in vivo*. Mechanistically, the decrease in acetyl-CoA led to reduced H3K27ac levels in *Myc*, resulting in transcriptional downregulation of *Myc* and drastically reduced MYC protein levels. Interestingly, our analyses also revealed a reciprocal relationship whereby *ACLY* itself is a direct transcriptional target of MYC, thus establishing a feedforward loop that is important for leukemia progression. Overall, our results identified a relevant ACLY-MYC axis and unveiled ACLY as a novel promising target for T-ALL treatment.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological disease of immature T-cell progenitor cells. Although most patients nowadays get cured thanks to intensive chemotherapy regimens, 20-50% of patients still relapse and have dismal prognosis[1]. Targeting different metabolic routes in patients has resulted in notable antileukemic effects, as exemplified by the clinical use of methotrexate, 6-mercaptopurine or L-asparaginase[2]. Moreover, we recently identified the antileukemic effects of either serine hydroxymethyltransferase inhibition[3] or mitochondrial uncoupling[4], highlighting the value of further exploring metabolic vulnerabilities in leukemia. We previously demonstrated that the metabolic consequences of NOTCH1 inhibition in T-ALL are critical for its antileukemic effects[5]. Since NOTCH1 activating mutations occur in over 60% of patients[6], we hypothesized that thorough analyses of the metabolic enzymes being downregulated upon NOTCH1 inhibition might yield novel therapeutic targets for T-ALL treatment and identified the ATP citrate-lyase *ACLY*, a critical metabolic and epigenetic regulator[7], as significantly downregulated upon NOTCH1 inhibition, suggesting a relevant role for ACLY in NOTCH1-driven leukemia. However, nothing is known about the role of ACLY in T-cell development or T-ALL.

Methods

Mice

Generation of NOTCH1-driven conditional inducible knockout leukemias was done as previously described[5]. All animal housing, handling, and procedures involving mice were

105 approved by the Rutgers Institutional Animal Care and Use Committee, in accordance
106 with all relevant ethical regulations.

107 **Profiling experiments**

108 Transcriptional, epigenetic, and metabolomic profiling experiments were done as
109 previously described[8].

110 **Molecular biology experiments**

111 Western Blot and luciferase reporter assays were done following standard procedures,
112 as previously described[8].

113

114 Detailed methods are described in Supplemental Methods.

115

116 Results and discussion

117 **ACLY is overexpressed in T-ALL**

118 We previously demonstrated the critical effects of the metabolic consequences of
119 NOTCH1 inhibition *in vivo* for its antileukemic effects[5]. Interestingly, we found the ATP-
120 citrate lyase *ACLY* among the metabolic genes downregulated by NOTCH1 inhibition in
121 T-ALL *in vivo* (**Figure 1A**). ACLY has been previously suggested as an important
122 therapeutic target in solid tumors such as pancreatic cancer[9], and recent studies have
123 described certain roles for ACLY in the normal physiological function of CD4 and CD8 T-
124 cells[10,11]. However, its putative role in the normal development of T-cells and other
125 hematological lineages, as well as in leukemic transformation and progression, remains
126 largely unknown. ACLY controls both metabolic and epigenetic processes, given its
127 critical role as the rate-limiting enzyme in fatty acid synthesis, as well as by its generation

128 of acetyl-CoA (critical for histone acetylation) as a product of its catalytic reaction[7]. Since
129 both metabolic and epigenetic mechanisms of resistance to NOTCH1 inhibition in T-ALL
130 have been previously described[5,8,12], our findings suggested a potential role for ACLY in
131 the response to NOTCH1 inhibition and, more broadly, as a relevant player in T-ALL.
132 Next, we analyzed available gene expression data from T-ALL patients[13] and observed a
133 broad upregulation of *ACLY* as compared to normal T-cell subsets (**Figure 1B**). In line
134 with this, *ACLY* levels were similar across all different T-ALL clinical subgroups[14].
135 However, samples with NOTCH1 or FBXW7 mutations showed a trend towards increased
136 *ACLY* levels, which reached statistical significance in the cases with simultaneous
137 NOTCH1/FBXW7 mutations (**Supplemental Figure 1A-B**). ACLY overexpression in T-
138 ALL was confirmed at the protein level, with human T-ALL cell lines showing higher
139 expression than normal human thymus or peripheral blood cells (**Figure 1C**). Taken
140 together, these results suggest a previously unknown but important role for ACLY in T-
141 ALL.
142

143 **ACLY is critical for T-ALL progression but dispensable for T-cell development**

144 Next, we hypothesized that targeting ACLY in T-ALL might confer therapeutic effects. To
145 test this hypothesis, we generated a model of NOTCH1-induced *Acly* conditional
146 knockout leukemia using a well-established protocol[5] of retroviral transduction of an
147 oncogenic form of NOTCH1 in bone marrow progenitor cells from *Acly* conditional
148 knockout mice, followed by transplantation into lethally irradiated recipients (**Figure 1D**).
149 In time, these mice developed NOTCH1-induced leukemias, which were subsequently
150 transplanted into a secondary cohort of mice and treated with vehicle control or tamoxifen,

151 to induce isogenic loss of *Acly*. Mice were further subdivided into control-treated or treated
152 with the gamma-secretase inhibitor DBZ, to test the response to NOTCH1 inhibition *in*
153 *vivo* with or without ACLY expression (**Figure 1D**). Notably, our results demonstrated
154 intrinsic antileukemic effects for ACLY loss (**Figure 1E**), which further synergized with
155 NOTCH1 inhibition resulting in a 20% cure rate of leukemic mice (**Figure 1F**).
156 Interestingly, leukemias eventually developing in tamoxifen-treated mice were not genetic
157 escapers and did not show a concomitant feedback upregulation of ACSS2 (**Figure 1G**).
158 ACSS2 upregulation has been previously shown to mediate resistance to ACLY loss in
159 certain contexts[15], suggesting that T-ALL cells can still survive ACLY loss via alternative
160 mechanisms.
161 Many of the relevant therapeutic targets of T-ALL progression also show an effect in
162 normal T-cell development, and the potential effects of ACLY loss in normal healthy
163 hematopoietic cells would also be important to predict potential toxicities associated to
164 therapies aimed at inhibiting ACLY. To this end, we bred the *Acly* conditional knockout
165 mice with Vav-iCre expressing mice. Mice with hematopoietic-specific ACLY loss were
166 viable and showed no overt phenotype. Detailed immunophenotypic analyses of thymi
167 from these mice showed no differences in thymus weight or cellularity (**Supplemental**
168 **Figure 2A-B**), and we also failed to detect any significant difference in the numbers or
169 proportions of different thymocyte subsets (**Supplemental Figure 2C-F**). Overall, our
170 data suggest that ACLY is dispensable for normal T-cell development but critical for T-
171 ALL progression and, thus, may constitute a novel and attractive therapeutic target.
172
173 **ACLY loss leads to a metabolic crisis and reduced tumor burden**

174 To explore the mechanistic effects of ACLY loss, we next performed an acute deletion
175 experiment in which mice harboring *Acly* conditional knockout leukemias were first
176 allowed to become fully leukemic before treating them with vehicle (control) or tamoxifen
177 (to induce ACLY loss), followed by euthanasia 72h later (**Figure 2A**). In this setting, acute
178 ACLY loss translated into reduced *Acly* levels (**Figure 2B**) and led to reduced tumor
179 burden (**Figure 2C**). Mice harboring *Acly*-deleted leukemias showed significantly reduced
180 infiltration across tissues, including the spleen or the bone marrow and, most notably,
181 also showed reduced central nervous system infiltration in the meninges (**Figure 2D**).
182 These results were mainly driven by cytotoxic effects, as revealed by increased AnnexinV
183 staining upon ACLY loss (**Figure 2E**).
184 Next, given the well-known metabolic role of ACLY, we performed global metabolomic
185 analyses upon acute ACLY loss *in vivo*. Interestingly, we observed an expected reduction
186 in acetyl-CoA levels (**Figure 2F**), as well as a strong accumulation of AICAR (**Figure**
187 **2G**), a purine intermediate and a well-described activator of AMPK[16]. In addition, we also
188 detected accumulation of the pyrimidine intermediate N-carbamoyl-L-aspartate with a
189 concomitant global reduction in pyrimidines (**Figure 2H**), overall suggesting a block in
190 nucleotide biosynthesis. Finally, we also detected an accumulation of glucose and
191 glycolytic intermediates (**Figure 2I**), as well as an increase in oxidized glutathione levels
192 (**Figure 2J**). Consistent with these metabolic effects, loss of ACLY in an *Acly* conditional
193 knockout T-ALL cell line generated from our primary leukemia led to impaired proliferation
194 (**Figure 2K-L**), together with a significant reduction in oxygen consumption rate (**Figure**
195 **2M**). Overall, our results suggest that ACLY loss leads to a metabolic crisis in T-ALL cells.
196

197 **An ACLY – MYC feedforward loop regulates T-ALL progression**

198 Next, gene expression profiling analyses revealed a strong transcriptional signature upon
199 acute ACLY loss (**Figure 3A** and **Supplemental Table 1**). Pathway analyses using
200 EnrichPlot revealed strong downregulation of pathways related to oxidative
201 phosphorylation and translation (**Figure 3B**), consistent with the metabolic changes
202 previously observed. To gain a deeper understanding of the transcription factor/s that
203 may be governing these transcriptional effects, we analyzed our gene expression profile
204 data using Enrichr[17] and Dorothea[18]. Intriguingly, both algorithms identified a
205 downregulation of *MYC* activity as potentially related to the changes observed (**Figure**
206 **3C** and **Supplemental Figure 3A**). Previous reports suggested a potential regulation of
207 *MYC* by ACLY in endothelial cells[19], as well as the reverse regulation of *ACLY* by MYC
208 in prostate cancer[20]. To unravel the directionality of the regulation in our T-ALL setting,
209 we performed gene set enrichment analyses (GSEA)[21] against the signature of MYC loss
210 in T-ALL upon deletion of the N-Me enhancer[22]. Our results showed a very strong
211 correlation between both signatures (**Figure 3D**), and western blot analyses confirmed a
212 drastic downregulation in MYC protein levels (**Figure 3E**), which is consistent with the
213 strong antileukemic effects observed. Given the well-described role of ACLY in epigenetic
214 regulation and our own findings of reduced acetyl-coA levels upon ACLY loss (**Figure**
215 **2F**), we next performed ChIP-seq epigenetic profiling experiments, which revealed a
216 global reduction in H3K27ac mark with a concomitant milder reduction in H3K4me3 levels
217 (**Figure 3F**). Interestingly, the effect in histone acetylation might be specific for H3K27ac,
218 as we did not observe major changes in H3K9ac (**Figure 3F**). Importantly, these
219 epigenetic changes were evident in the *Myc* promoter, which showed markedly reduced

220 levels of H3K27ac and a milder decrease in H3K4me3 (**Figure 3G**), consistent with the
221 changes in MYC activity previously observed.
222 Conversely, reverse GSEA analyses also showed that the signature of MYC loss upon
223 N-Me deletion is very similar to the one obtained upon ACLY loss (**Figure 3H**), suggesting
224 that MYC might also be upstream of *Acly*. Indeed, luciferase reporter assays testing this
225 regulation showed that MYC overexpression positively regulates the transcriptional
226 activity of the *ACLY* promoter, whereas treatment with the BRD4 inhibitor JQ1, which
227 prominently inhibits MYC activity[23], resulted in reduced transactivation of *ACLY* (**Figure**
228 **3I**). In line with this, epigenetic profiling of the *ACLY* promoter using publicly available
229 ChIP-seq data in human T-ALL cells uncovered the binding of multiple relevant T-cell-
230 specific transcription factors, including both MYC and NOTCH1 (**Supplemental Figure**
231 **3B**). Finally, we took advantage of our mouse N-Me conditional knockout primary T-ALL
232 cell line[22] to demonstrate that MYC loss in T-ALL resulted in significantly reduced ACLY
233 protein levels (**Figure 3J**), further supporting that MYC also regulates *Acly*. This is
234 consistent with the broad ACLY overexpression observed in T-ALL and with its
235 downregulation upon NOTCH1 inhibition, given the tight link between NOTCH1 and MYC,
236 and the critical role of MYC in T-ALL[22].
237
238 **Resistance to ACLY loss is associated with restored MYC levels**
239 Even if targeting ACLY shows strong intrinsic antileukemic effects *in vivo*, tamoxifen-
240 treated leukemic mice eventually relapse and succumb, and these leukemias are not
241 genetic escapers nor show compensatory upregulation of ACSS2 (**Figure 1**), supporting
242 alternative mechanisms of resistance. Interestingly, western blot analyses of relapsing

243 leukemias showed restored expression of MYC protein levels (**Supplemental Figure**
244 **3C**). Similarly, gene expression profiling analyses of relapsing leukemias confirmed the
245 restoration of MYC-driven transcriptional programs and additional pathways that were
246 significantly changing upon acute ACLY loss (**Supplemental Figure 3D-E**).
247 Collectively, our results identify a therapeutically relevant ACLY-MYC feedforward loop in
248 T-ALL (**Supplemental Figure 4**) in which ACLY provides acetyl-coA units needed for the
249 sustained transcriptional regulation of *MYC*, whereas MYC itself further reinforces this
250 loop by transcriptionally upregulating *ACLY* (**Supplemental Figure 4**). Importantly, while
251 our results identified a critical role for ACLY in T-ALL progression *in vivo*, ACLY is
252 dispensable for normal T-cell development, and mice lacking ACLY in the heme
253 compartment show no gross abnormalities, suggesting that targeting ACLY might have
254 limited toxicities for normal healthy cells. MYC and AKT are two of the most relevant
255 oncogenic factors downstream of NOTCH1 in T-ALL[5,22]. Intriguingly, AKT has also been
256 previously shown to directly phosphorylate and positively regulate ACLY[24]. Thus, the
257 transcriptional program controlled by NOTCH1 in T-ALL leads to increased ACLY activity
258 via multiple pathways, highlighting the key role of ACLY in leukemogenesis. Overall, our
259 results demonstrate that targeting ACLY constitutes a novel and attractive therapeutic
260 option for leukemia treatment. While several ACLY inhibitors have been described to
261 date, these are either relatively weak and unspecific inhibitors, such as bempedoic acid
262 or BMS-303141, or potent but not cell-permeable inhibitors, such as NDI-091143[25]. In this
263 context, our unequivocal genetic results demonstrating a therapeutic role for targeting
264 ACLY in leukemia *in vivo* will hopefully spur additional efforts to develop clinical-grade
265 ACLY inhibitors that can be used in leukemia patients in the near future.

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272 **Author contributions**

273 V.dS. performed most molecular biology experiments. A.S. and C.T. performed all
274 computational analyses. M.A., O.K., O.L., P.R.N., and H.A. assisted with several
275 experiments. X.S. supervised metabolomic analyses. K.E.W. contributed critical reagents
276 and gave critical input. D.H. designed the study, supervised the research, and wrote the
277 manuscript together with V.dS., with input from all authors.

278

279 **References**

280 1. Pagliaro L, Chen SJ, Herranz D, et al. Acute lymphoblastic leukaemia. *Nat Rev*
281 *Dis Primers*. 2024;10(1):41.

282 2. Rashkovan M, Ferrando A. Metabolic dependencies and vulnerabilities in
283 leukemia. *Genes Dev*. 2019;33(21-22):1460-1474.

284 3. Garcia-Canaveras JC, Lancho O, Ducker GS, et al. SHMT inhibition is effective
285 and synergizes with methotrexate in T-cell acute lymphoblastic leukemia. *Leukemia*.
286 2021;35(2):377-388.

287 4. da Silva-Diz V, Cao B, Lancho O, et al. A novel and highly effective mitochondrial
288 uncoupling drug in T-cell leukemia. *Blood*. 2021;138(15):1317-1330.

289 5. Herranz D, Ambesi-Impiombato A, Sudderth J, et al. Metabolic reprogramming
290 induces resistance to anti-NOTCH1 therapies in T cell acute lymphoblastic leukemia. *Nat*
291 *Med*. 2015;21(10):1182-1189.
292 6. Weng PA, Ferrando A, Lee W, et al. Activating Mutations of NOTCH1 in Human T
293 Cell Acute Lymphoblastic Leukemia. *Science*. 2004;306:269-271.
294 7. Guertin DA, Wellen KE. Acetyl-CoA metabolism in cancer. *Nat Rev Cancer*.
295 2023;23(3):156-172.
296 8. Lancho O, Singh A, da Silva-Diz V, et al. A Therapeutically Targetable NOTCH1-
297 SIRT1-KAT7 Axis in T-cell Leukemia. *Blood Cancer Discov*. 2023;4(1):12-33.
298 9. Carrer A, Trefely S, Zhao S, et al. Acetyl-CoA Metabolism Supports Multistep
299 Pancreatic Tumorigenesis. *Cancer Discov*. 2019;9(3):416-435.
300 10. Chowdhury S, Kar A, Bhowmik D, et al. Intracellular Acetyl CoA Potentiates the
301 Therapeutic Efficacy of Antitumor CD8+ T Cells. *Cancer Res*. 2022;82(14):2640-2655.
302 11. Osinalde N, Mitxelena J, Sanchez-Quiles V, et al. Nuclear Phosphoproteomic
303 Screen Uncovers ACLY as Mediator of IL-2-induced Proliferation of CD4+ T lymphocytes.
304 *Mol Cell Proteomics*. 2016;15(6):2076-2092.
305 12. Knoechel B, Roderick JE, Williamson KE, et al. An epigenetic mechanism of
306 resistance to targeted therapy in T cell acute lymphoblastic leukemia. *Nat Genet*.
307 2014;46(4):364-370.
308 13. Van Vlierberghe P, Ambesi-Impiombato A, Perez-Garcia A, et al. ETV6 mutations
309 in early immature human T cell leukemias. *J Exp Med*. 2011;208(13):2571-2579.
310 14. Liu Y, Easton J, Shao Y, et al. The genomic landscape of pediatric and young adult
311 T-lineage acute lymphoblastic leukemia. *Nat Genet*. 2017;49(8):1211-1218.

312 15. Zhao S, Torres A, Henry RA, et al. ATP-Citrate Lyase Controls a Glucose-to-
313 Acetate Metabolic Switch. *Cell Rep*. 2016;17(4):1037-1052.
314 16. Vakana E, Altman JK, Glaser H, Donato NJ, Platanias LC. Antileukemic effects of
315 AMPK activators on BCR-ABL-expressing cells. *Blood*. 2011;118(24):6399-6402.
316 17. Kuleshov MV, Jones MR, Rouillard AD, et al. Enrichr: a comprehensive gene set
317 enrichment analysis web server 2016 update. *Nucleic Acids Res*. 2016;44(W1):W90-97.
318 18. Garcia-Alonso L, Holland CH, Ibrahim MM, Turei D, Saez-Rodriguez J. Benchmark
319 and integration of resources for the estimation of human transcription factor activities.
320 *Genome Res*. 2019;29(8):1363-1375.
321 19. Li R, Meng M, Chen Y, et al. ATP-citrate lyase controls endothelial gluco-lipogenic
322 metabolism and vascular inflammation in sepsis-associated organ injury. *Cell Death Dis*.
323 2023;14(7):401.
324 20. Singh KB, Hahm ER, Kim SH, Wendell SG, Singh SV. A novel metabolic function
325 of Myc in regulation of fatty acid synthesis in prostate cancer. *Oncogene*. 2021;40(3):592-
326 602.
327 21. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a
328 knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl*
329 *Acad Sci U S A*. 2005;102(43):15545-15550.
330 22. Herranz D, Ambesi-Impiombato A, Palomero T, et al. A NOTCH1-driven MYC
331 enhancer promotes T cell development, transformation and acute lymphoblastic
332 leukemia. *Nat Med*. 2014;20(10):1130-1137.
333 23. Zuber J, Shi J, Wang E, et al. RNAi screen identifies Brd4 as a therapeutic target
334 in acute myeloid leukaemia. *Nature*. 2011;478(7370):524-528.

335 24. Lee JV, Carrer A, Shah S, et al. Akt-dependent metabolic reprogramming
336 regulates tumor cell histone acetylation. *Cell Metab*. 2014;20(2):306-319.
337 25. Liang JJ, Zhou XF, Long H, et al. Recent advance of ATP citrate lyase inhibitors
338 for the treatment of cancer and related diseases. *Bioorg Chem*. 2024;142:106933.
339

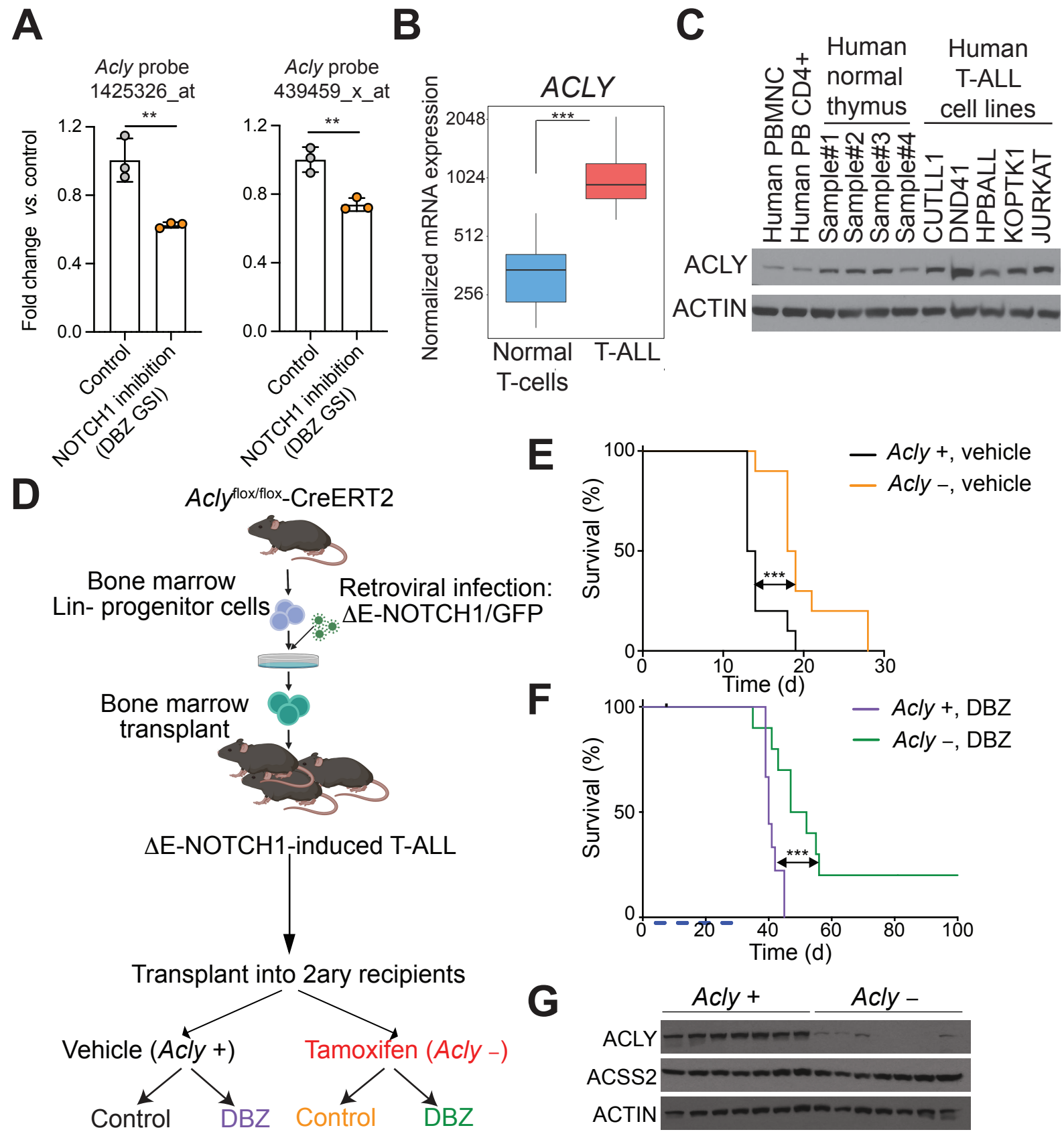
340 **Figure Legends**

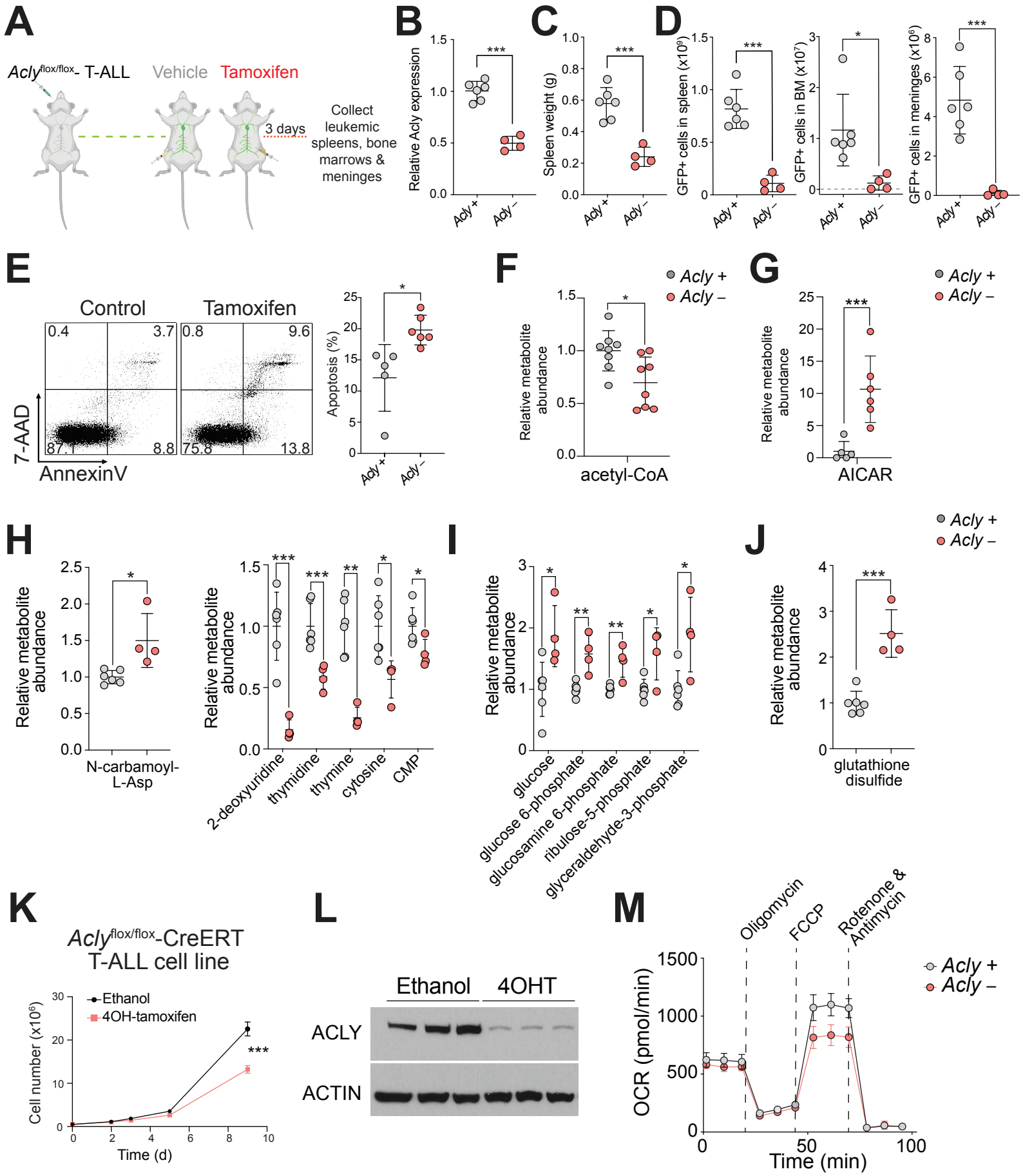
341 **Figure 1**. ACLY is a novel therapeutic target in T-ALL. (A) mRNA levels of *Acly* upon
342 NOTCH1 inhibition with the DBZ gamma-secretase inhibitor in mouse T-ALL *in vivo*[5]
343 (**P<0.01 calculated with 2-tailed Student's *t*-test). (B) Box-plot showing *ACLY*
344 expression among T-ALL samples (n=57) and physiological thymocyte subsets (n=21)[13].
345 Quantile normalization was performed across samples. Boxes represent first and third
346 quartiles and lines represent the median. Whiskers represent the upper and lower limits
347 (P<0.001 using Mann-Whitney U-Test; FDR<0.05 using Benjamini-Hochberg correction).
348 (C) Western blot analysis of ACLY and ACTIN expression in human peripheral blood
349 mononuclear cells (PBMNC), CD4+ T-cells, or healthy human thymocytes, as compared
350 to human T-ALL. (D) Schematic illustration of retroviral-transduction protocol for the
351 generation of NOTCH1-induced T-ALLs from inducible *Acly*-conditional knockout mice,
352 followed by transplant into secondary recipients treated with vehicle ($Acly^{+/+}$) or tamoxifen
353 ($Acly^{-/-}$), with or without DBZ. (E) Kaplan-Meier survival curves of mice harboring *Acly*-
354 positive and *Acly*-deleted isogenic leukemias (n = 10 per group). (F) Kaplan-Meier
355 survival curves of mice harboring *Acly*-positive and *Acly*-deleted isogenic leukemias
356 treated with 4 cycles DBZ (5 mg/kg) on a 4-days-ON (blue blocks at the bottom) and 3-
357 days-OFF schedule (n = 10 per group). (G) Western blot analysis of ACLY, ACSS2, and
358 ACTIN expression in leukemic spleens from terminally ill mice from the survival curve in
359 Figure 1E. ***P < 0.005 in panels B and C calculated with log-rank test; *P < 0.05; **P <
360 0.01; ***P < 0.005 in panels D-F calculated with log-rank test calculated with two-tailed
361 Student's *t*-test.

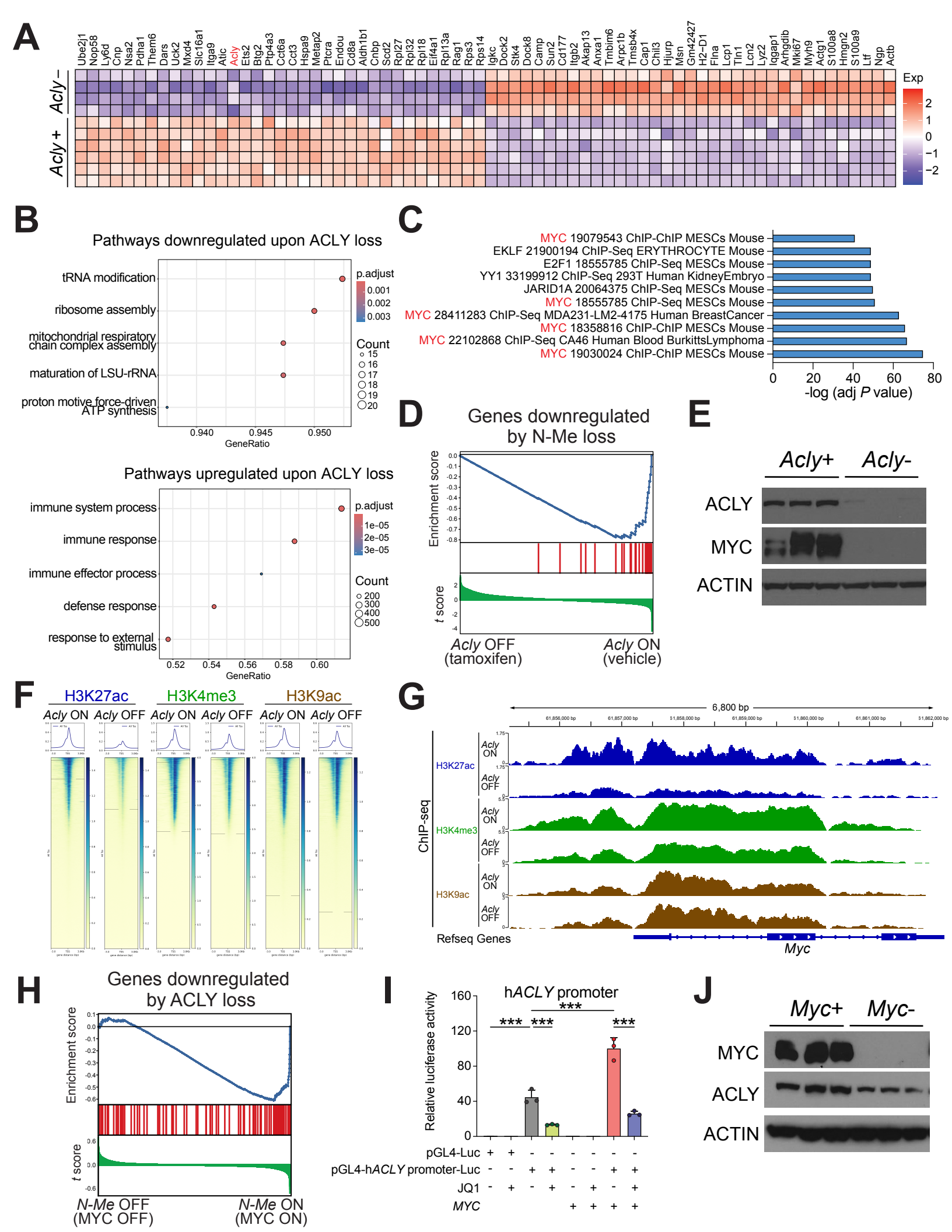
362 **Figure 2.** Metabolic consequences of ACLY loss in T-ALL. (A) Schematic of acute ACLY
363 deletion experiment in leukemic mice *in vivo*. (B) Quantitative RT-PCR analysis of *Acly*
364 mRNA expression in tumor cells isolated from ΔE-NOTCH1-induced *Acly* conditional
365 knockout leukemia–bearing mice 72 h after being treated with vehicle only ($Acly^{+/+}$) or
366 tamoxifen ($Acly^{-/-}$) *in vivo*. (C-D) Tumor burden in ΔE-NOTCH1-induced *Acly* conditional
367 knockout leukemia–bearing mice 72 h after being treated with vehicle only ($Acly^{+/+}$) or
368 tamoxifen ($Acly^{-/-}$) *in vivo* as revealed by total spleen weight (C) or by the infiltration of
369 GFP-positive leukemic cells in the spleen, bone marrow or meninges (D). (E)
370 Representative flow cytometry plots from of annexin V (apoptotic cells) and 7-AAD (dead
371 cells) staining (left) and quantification of apoptosis (right) in leukemic spleens from ΔE-
372 NOTCH1-induced *Acly* conditional knockout leukemia–bearing mice 48 h after being
373 treated with vehicle only ($Acly^{+/+}$) or tamoxifen ($Acly^{-/-}$) *in vivo*. (n = 4-5 per treatment in
374 panels B-E; $^{*}P < 0.05$ and $^{***}P < 0.005$ using two-tailed Student's *t*-test.) (F-G) Relative
375 Acetyl-CoA (F) and AICAR (G) abundance upon isogenic loss of *Acly* in leukemic spleens
376 48-72h after being treated with vehicle only ($Acly^{+/+}$) or tamoxifen ($Acly^{-/-}$) *in vivo*. (H)
377 Relative abundance of the indicated pyrimidine intermediates upon tamoxifen-induced
378 isogenic loss of *Acly* in leukemic spleens from mice treated as in A. (I) Relative abundance
379 of the indicated glycolytic intermediates upon tamoxifen-induced isogenic loss of *Acly* in
380 leukemic spleens from mice treated as in A. (J) Relative abundance of glutathione
381 disulfide upon tamoxifen-induced isogenic loss of *Acly* in leukemic spleens from mice
382 treated as in A. (K-L) Growth curve (K) and western blot analyses of ACLY and ACTIN
383 expression (L) in an *Acly* conditional knockout T-ALL cell line *in vitro* upon treatment with
384 ethanol (control) or 4-hydroxytamoxifen (4OHT) to induce ACLY loss. (M) Oxygen

385 consumption rate (OCR) in *Acly* conditional knockout T-ALL cells, under basal conditions
386 or after 4OHT-induced loss of ACLY, measured in real-time using a Seahorse XF24
387 instrument. Data are presented as mean ± SD of n = 4 wells. n = 6-8 per treatment in
388 panels F-G and n = 4-5 per treatment in panels H-J; *P < 0.05; **P < 0.01 and ***P <
389 0.005 using two-tailed Student's *t*-test.
390
391 **Figure 3**. ACLY-MYC axis in T-ALL (A) Heatmap representation of the top differentially
392 expressed genes between control (*Acly*$^{+/+}$) and tamoxifen-treated (*Acly*$^{-/-}$) *Acly*-
393 conditional knockout ΔE-NOTCH1–induced leukemias. Cutoffs used: top 40 up and
394 downregulated genes; *P*-adjusted value < 0.0001; log fold change >1. Scale bar shows
395 color-coded differential expression, with red indicating higher levels of expression and
396 blue indicating lower levels of expression. (B) Pathway analyses of significantly
397 downregulated or upregulated genes upon ACLY loss using EnrichPlot. (C) Enrichr
398 analyses of transcription factors controlling downregulated pathways upon ACLY loss.
399 (D) GSEA of genes downregulated by N-Me loss in control (vehicle only–treated; ACLY
400 ON) or *Acly*-deleted leukemias (tamoxifen–treated; ACLY OFF) *in vivo.* (E) Western blot
401 analyses of ACLY, MYC, and ACTIN expression 72 h after tamoxifen-induced deletion of
402 *Acly in vivo.* (F) Heatmap representation of genome-wide enrichment for H3K27ac (left),
403 H3K4me3 (middle), and H3K9ac (right) marks in control (vehicle only–treated; ACLY ON)
404 or *Acly*-deleted leukemias (tamoxifen–treated; ACLY OFF) *in vivo.* The color of the heat
405 map indicates the number of CPM-normalized reads within +/-3000bp of annotated
406 Transcription Start Site (TSS) for each gene. (G) Epigenetic profiling around the *Myc*
407 promoter showing ChIP-seq tracks for the indicated histones marks in mouse T-ALL

408 leukemic samples treated as in F. (H) GSEA of genes downregulated by ACLY loss in
409 control (vehicle only–treated; N-Me ON) or N-Me deleted leukemias (tamoxifen–treated;
410 N-Me OFF) *in vivo*[22]. (I) Luciferase reporter activity in 293T cells of a pGL4 promoter
411 empty construct (pGL4-Luc) or a pGL4 promoter plus the human *ACLY* promoter, in the
412 presence or absence of MYC overexpression or treatment with JQ1. Data from three
413 independent transfection replicates are shown. ***$P < 0.005$ using two-tailed Student's *t*-
414 test. (J) Western blot analyses of ACLY, MYC, and ACTIN expression 48 h after
415 tamoxifen-induced deletion *in vitro* of N-Me using an N-Me conditional knockout T-ALL
416 cell line[22].
417


A
Acly probe 1425326_at
Acly probe 439459_x_at
Fold change vs. control
1.2
0.8
0.4
0.0
**
Control
NOTCH1 inhibition (DBZ GSI)
B
ACLY
Normalized mRNA expression
2048
1024
512
256
***
Normal T-cells
T-ALL
C
Human PBMNC
Human PB CD4+
Human normal thymus
Sample#1
Sample#2
Sample#3
Sample#4
Human T-ALL cell lines
CUTLL1
DND41
HPBALL
KOPTK1
JURKAT
ACLY
ACTIN
D
Acly flox/flox-CreERT2
Bone marrow Lin- progenitor cells
Retroviral infection: ΔE-NOTCH1/GFP
Bone marrow transplant
ΔE-NOTCH1-induced T-ALL
Transplant into 2ary recipients
Vehicle (Acly +)
Tamoxifen (Acly –)
Control
DBZ
Control
DBZ
E
Survival (%)
100
50
0
0
10
20
30
Time (d)
***
Acly +, vehicle
Acly –, vehicle
F
Survival (%)
100
50
0
0
20
40
60
80
100
Time (d)
***
Acly +, DBZ
Acly –, DBZ
G
Acly +
Acly –
ACLY
ACSS2
ACTIN


A
Acly flox/flox- T-ALL
Vehicle
Tamoxifen
3 days
Collect leukemic spleens, bone marrows & meninges
B
Relative Acly expression
C
Spleen weight (g)
D
GFP+ cells in spleen (x10⁹)
GFP+ cells in BM (x10⁷)
GFP+ cells in meninges (x10⁶)
Acly +
Acly –
E
Control
Tamoxifen
7-AAD
AnnexinV
Apoptosis (%)
F
Relative metabolite abundance
acetyl-CoA
G
AICAR
H
N-carbamoyl-L-Asp
2-deoxyuridine
thymidine
thymine
cytosine
CMP
I
glucose
glucose 6-phosphate
glucosamine 6-phosphate
ribulose-5-phosphate
glyceraldehyde-3-phosphate
J
glutathione disulfide
K
Acly flox/flox-CreERT T-ALL cell line
Ethanol
4OH-tamoxifen
Cell number (x10⁶)
Time (d)
L
Ethanol
4OHT
ACLY
ACTIN
M
Oligomycin
FCCP
Rotenone & Antimycin
OCR (pmol/min)
Time (min)


A
Ube2j1 Nop58 Ly6d Cnp Nsa2 Pdha1 Therm6 Dars Uck2 Mxd4 Slc16a1 Itga9 Atic Acly Ets2 Btg2 Ptp4a3 Cct6a Cct3 Hspa9 Metap2 Ptcra Endou Cd8a Aldh1b1 Cnbp Scd2 Rpl27 Rpl32 Rpl18 Eif4a1 Rpl13a Rag1 Rps3 Rps14 Igkc Dock2 Stk4 Dock8 Camp Sun2 Cd177 Itgb2 Akap13 Anxa1 Tmbim6 Arpc1b Tmsb4x Cap1 Chil3 Hjurp Msn Gm42427 H2-D1 Flna Lcp1 Tln1 Lcn2 Lyz2 Iqgap1 Arhgdib Mki67 Myh9 Actg1 S100a8 Hmgn2 S100a9 Ltf Ngp Actb
Acly−
Acly+
Exp
2
1
0
−1
−2
B
Pathways downregulated upon ACLY loss
tRNA modification
ribosome assembly
mitochondrial respiratory chain complex assembly
maturation of LSU-rRNA
proton motive force-driven ATP synthesis
0.940
0.945
0.950
GeneRatio
p.adjust
0.001
0.002
0.003
Count
15
16
17
18
19
20
Pathways upregulated upon ACLY loss
immune system process
immune response
immune effector process
defense response
response to external stimulus
0.52
0.54
0.56
0.58
0.60
GeneRatio
p.adjust
1e-05
2e-05
3e-05
Count
200
300
400
500
C
MYC 19079543 ChIP-ChIP MESCs Mouse
EKLF 21900194 ChIP-Seq ERYTHROCYTE Mouse
E2F1 18555785 ChIP-Seq MESCs Mouse
YY1 33199912 ChIP-Seq 293T Human KidneyEmbryo
JARID1A 20064375 ChIP-Seq MESCs Mouse
MYC 18555785 ChIP-Seq MESCs Mouse
MYC 28411283 ChIP-Seq MDA231-LM2-4175 Human BreastCancer
MYC 18358816 ChIP-ChIP MESCs Mouse
MYC 22102868 ChIP-Seq CA46 Human Blood BurkittsLymphoma
MYC 19030024 ChIP-ChIP MESCs Mouse
0
20
40
60
80
-log (adj P value)
D
Genes downregulated by N-Me loss
Enrichment score
t score
Acly OFF (tamoxifen)
Acly ON (vehicle)
E
Acly+
Acly-
ACLY
MYC
ACTIN
F
H3K27ac
H3K4me3
H3K9ac
Acly ON
Acly OFF
G
6,800 bp
ChIP-seq
H3K27ac
H3K4me3
H3K9ac
Acly ON
Acly OFF
Refseq Genes
Myc
H
Genes downregulated by ACLY loss
Enrichment score
t score
N-Me OFF (MYC OFF)
N-Me ON (MYC ON)
I
hACLY promoter
Relative luciferase activity
160
120
80
40
0
***
pGL4-Luc
pGL4-hACLY promoter-Luc
JQ1
MYC
J
Myc+
Myc-
MYC
ACLY
ACTIN