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 a novel therapeutic target in T-ALL. Our results show that ACLY is overexpressed in T-ALL, and its expression correlates with NOTCH1 activity. To test the effects of ACLY in leukemia progression and the response to NOTCH1 inhibition, 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.* Gene expression profiling analyses showed that the transcriptional signature of ACLY loss very significantly correlates with the signature of NOTCH1 inhibition *in vivo*, with significantly downregulated pathways related to oxidative phosphorylation, electron transport chain, ribosomal biogenesis and nucleosome biology. Consistently, metabolomic profiling upon ACLY loss revealed a metabolic crisis with accumulation of nucleotide intermediates and reduced levels of several amino acids. Overall, our results identify a link between NOTCH1 and ACLY and unveil ACLY as a novel promising target for T-ALL treatment.

75 T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological disease of
76 immature T-cell progenitor cells. Although most patients nowadays get cured thanks to
77 intensive multiagent chemotherapy regimens, 20-50% patients still relapse, and the
78 prognosis at this stage is dismal[1]. Thus, identifying novel targets that could translate into
79 improved therapeutic effects in T-ALL is an urgent medical need. Related to this, targeting
80 different metabolic routes in patients has resulted in notable antileukemic effects, as
81 exemplified by the clinical use of methotrexate, 6-mercaptopurine or L-asparaginase[2]. In
82 this context, we recently identified the antileukemic effects of either serine
83 hydroxymethyltransferase inhibition[3] or mitochondrial uncoupling[4], highlighting the value
84 of further exploring metabolic vulnerabilities in leukemia. Thus, since NOTCH1 activating
85 mutations occur in over 60% of patients[5], and we previously demonstrated that the
86 metabolic effects of NOTCH1 inhibition are also critical for its therapeutic effects[6], we
87 hypothesized that thorough analyses of the metabolic enzymes being downregulated
88 upon NOTCH1 inhibition might yield novel therapeutic targets for T-ALL treatment.
89 Indeed, we found that NOTCH1 inhibition in T-ALL *in vivo* led to downregulation of the
90 ATP-citrate lyase *ACLY* (**Fig. 1A**). ACLY controls both metabolic and epigenetic
91 processes, given its critical role in fatty acid synthesis as well as its generation of acetyl-
92 CoA (critical for histone acetylation) as a product of its catalytic reaction[7]. Since both
93 metabolic and epigenetic mechanisms of resistance to NOTCH1 inhibition in T-ALL have
94 been previously described[6,8], our findings suggested a potential role for ACLY in the
95 response to NOTCH1 inhibition and, more broadly, as a relevant player in T-ALL. Next,
96 we analyzed available gene expression data from T-ALL pediatric patients[9] and observed
97 a broad upregulation of *ACLY* as compared to normal T-cell subsets (**Fig. 1B**). In line with

98 this, *ACLY* levels were similar across different T-ALL clinical subgroups[10] (**Fig. 1C**).
99 However, samples with NOTCH1 or FBXW7 mutations showed a trend towards increased
100 *ACLY* levels, which reached statistical significance in the cases with simultaneous
101 NOTCH1/FBXW7 mutations (**Fig. 1D**). Finally, ACLY overexpression in T-ALL was
102 confirmed at the protein level, with human T-ALL cell lines showing higher expression
103 that normal human thymus or peripheral blood cells (**Fig. 1E**). Taken together, our results
104 suggest a previously unknown but important role for ACLY in T-ALL.
105 Next, we hypothesized that targeting ACLY in T-ALL might confer therapeutic effects. To
106 test this hypothesis, we generated a model of NOTCH1-induced *Acly* conditional
107 knockout leukemia using a well-established protocol[4,6,11,12] of retroviral transduction of an
108 oncogenic form of NOTCH1 in bone marrow progenitor cells from *Acly* conditional
109 knockout mice, followed by transplantation into lethally irradiated recipients (**Fig. 2A**). In
110 time, these mice developed NOTCH1-induced leukemias, which were subsequently
111 transplanted into a secondary cohort of mice and treated with vehicle control or tamoxifen,
112 to induce isogenic loss of *Acly*. Mice were further subdivided into control-treated or treated
113 with the gamma-secretase inhibitor DBZ, to test the response to NOTCH1 inhibition *in*
114 *vivo* with or without ACLY expression (**Fig. 2A**). Notably, our results demonstrated
115 intrinsic antileukemic effects for ACLY loss, which further synergized with NOTCH1
116 inhibition resulting in a 20% cure rate of leukemic mice (**Fig. 2B**). Interestingly, leukemias
117 eventually developing in tamoxifen-treated mice were not genetic escapers and did not
118 show a concomitant feedback upregulation of ACSS2 (observed in some other contexts
119 upon ACLY loss[7]) either (**Fig. 2C**), suggesting the T-ALL cells can still survive ACLY loss
120 via alternative mechanisms. To explore the mechanistic effects of ACLY loss, we next

121 performed an acute deletion experiment in which mice harboring *Acly* conditional
122 knockout leukemias were first allowed to become fully leukemic before treating them with
123 vehicle-control or tamoxifen, followed by euthanasia 48h later. In this setting, acute ACLY
124 loss translated into reduced *Acly* levels (**Fig. 2D**) and led to reduced tumor burden (**Fig.**
125 **2E**), which was mainly driven by cytotoxic effects as revealed by increased AnnexinV
126 staining (**Fig. 2F**). Next, gene expression profiling analyses revealed a strong
127 transcriptional signature upon acute ACLY loss (**Fig. 2G**). Pathway analyses using the
128 STRING database[13] revealed strong downregulation of pathways related to TCA cycle,
129 electron transport chain and ribosomal biogenesis, together with a concomitant decrease
130 in nucleosome-biology related genes (**Fig. 2H** and **Supplemental Table 1**). Given the
131 correlation and effects on *ACLY* observed previously upon NOTCH1 inhibition (**Fig. 1**),
132 we next performed gene set enrichment analyses (GSEA)[14] against the signature
133 obtained upon NOTCH1 inhibition in mouse leukemias *in vivo*[6]. Of note, these analyses
134 revealed that the genes upregulated upon ACLY loss strongly correlate with the genes
135 upregulated by NOTCH1 inhibition and, conversely, genes downregulated upon ACLY
136 loss strongly correlate with the genes downregulated by NOTCH1 inhibition (**Fig. 2I**),
137 overall supporting a link between NOTCH1 and ACLY in T-ALL. Finally, given the strong
138 downregulation of transcriptional signatures linked to central metabolism, as well as the
139 well-known metabolic role of ACLY, we performed global metabolomic analyses upon
140 acute ACLY loss *in vivo*. Interestingly, while we did not observe significant changes in
141 pyruvate or acetyl CoA levels at this early time point, our results showed a strong
142 accumulation of AICAR (a purine intermediate and a well-described activator of AMPK[15])
143 together with concomitant accumulation of N-carbamoyl-L-aspartate (a pyrimidine

144 intermediate) and reduced levels of several amino acids, including aspartate, asparagine,
145 threonine and methionine (**Fig. 2J**), overall suggesting ACLY loss leads to a metabolic
146 crisis with compromised nucleotide biosynthesis in T-ALL cells.
147 ACLY has previously been suggested as an important therapeutic target in solid tumors
148 such as pancreatic cancer[16], and recent studies have described certain roles for ACLY in
149 the normal physiological function of CD4 and CD8 T-cells[17,18]. However, its putative role
150 in the normal development of T-cells and other hematological lineages, as well as in
151 leukemic transformation and progression, remains largely unknown. Our results
152 demonstrate that ACLY is upregulated in human T-ALL and, moreover, plays an important
153 role in leukemia progression *in vivo*. Our findings suggest a NOTCH1-ACLY link as
154 NOTCH1 inhibition leads to reduced *ACLY* levels, while NOTCH1 mutations in patients
155 correlate with higher levels of *ACLY*. In line with this, ACLY loss strongly synergized with
156 NOTCH1 inhibition *in vivo*, and the gene expression signature driven by ACLY loss very
157 significantly correlates with the signature obtained upon NOTCH1 inhibition. Intriguingly,
158 AKT is known to mediate NOTCH1-induced transformation[19], and AKT has also been
159 previously shown to directly phosphorylate and regulate ACLY[20]. Similarly, MYC is
160 another strong direct target of NOTCH1 in T-ALL[21], and recent studies support that MYC
161 might transcriptionally regulate ACLY[22], thus providing putative links between NOTCH1
162 and ACLY.
163 Taken together, our results demonstrate that targeting ACLY constitutes a novel and
164 attractive therapeutic option for leukemia treatment. While several ACLY inhibitors have
165 been described to date, these are either relatively weak and unspecific inhibitors, such as
166 bempedoic acid or BMS-303141[23,24], or potent inhibitors but not cell-permeable, such as

167 NDI-091143[25]. In this context, our unequivocal genetic results demonstrating a
168 therapeutic role for targeting ACLY in leukemia *in vivo* will hopefully spur additional efforts
169 to develop clinical-grade ACLY inhibitors that can be used in leukemia patients in the near
170 future.
171

172 **METHODS**

173 ***Cell lines and culture conditions***

174 Cells were cultured in standard conditions in a humidified atmosphere in 5 % CO_2 at 37C
175 in HyClone RPMI 1640 Media (Fisher Scientific, SH3002701) with 10 % FBS (Gemini Bio-
176 Products, 900-108) and 100 U/mL penicillin and 100 µg/mL streptomycin (VWR, 45000-
177 652). DND41 (ACC 525), HPB-ALL (ACC 483), and JURKAT (ACC 282) cells were
178 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).
179 CUTTL1 and KOPTK1 were gifts from Adolfo Ferrando.

180 ***In vivo models of NOTCH1-driven mouse T-ALLs***

181 Animals were maintained in ventilated caging in specific pathogen-free facilities at New
182 Brunswick RBHS Rutgers Campus. All animal housing, handling, and procedures
183 involving mice were approved by Rutgers Institutional Animal Care and Use Committee
184 (IACUC), in accordance with all relevant ethical regulations.

185 In order to generate *Acly* conditional inducible knockout leukemias, we used bone marrow
186 progenitor cells (fresh cells provided by K.E.W.) from *Acly*$^{flox/flox}$ mice (JAX #043555)
187 harboring a tamoxifen-inducible Cre recombinase from the ubiquitous UBC locus (JAX
188 #007001). Then, we performed retroviral transduction of lineage-negative enriched cells
189 from with retrovirus encoding a ΔE-NOTCH1-GFP oncogenic activated form of NOTCH1
190 with concomitant expression of GFP, as previously described[6]. Cells were then
191 transplanted via retro-orbital injection into lethally irradiated (7.5 Gy) recipient mice.

192 For leukemia-progression studies, already generated ΔE-NOTCH1-GFP-induced
193 *Acly*$^{flox/flox}$-Cre-ERT2/+ leukemias (1 x 10^6 leukemia cells) were transplanted from primary
194 recipients into sub-lethally irradiated (4.5 Gy) 6-8-week-old secondary recipient C57BL/6

195 mice (Taconic Farms) by retro-orbital injection. 48 hours after leukemic cell
196 transplantation, recipient mice were treated with vehicle only (corn oil; Sigma, C8267) or
197 tamoxifen (Sigma, T5648; 3 mg per mouse in corn oil), to induce isogenic loss of *Acly* in
198 established leukemias. Subsequently, 5 days post-transplantation, mice in each arm were
199 divided randomly into two different groups: control groups were subsequently treated with
200 vehicle only (2.3 % DMSO in 0.005% methylcellulose, 0.1 % Tween-80) or with DBZ (5
201 mg per Kg in vehicle solution; Syncom, 29762) on a 4-day-ON and 3-day-OFF schedule,
202 as previously described[6]. Investigators were not blinded to group allocation. Animals were
203 monitored for signs of distress or motor function at least twice daily until they were
204 terminally ill, whereupon they were euthanized.
205 For *Acly* acute deletion analyses in mouse primary leukemias, we transplanted
206 lymphoblasts from spleens of *Acly* conditional knockout NOTCH1-induced T-ALL-bearing
207 mice into a secondary cohort of recipient mice, as before. We monitored mice until they
208 presented clear leukemic signs with >60% GFP-positive leukemic cells in peripheral
209 blood; then, mice were treated with vehicle or tamoxifen and, 48 hours after treatment,
210 mice were euthanized and spleen samples were collected for further analyses.
211 ***Western blotting***
212 Human peripheral blood mononuclear cells (PBMCs) and peripheral blood CD4-positive
213 cells were purchased from Lonza (product numbers CC-2702 and 2W-200, respectively).
214 Samples from normal human thymus were kindly provided by Dr. Adolfo Ferrando
215 (Columbia University). Whole-cell extracts were prepared using standard procedures.
216 After protein transfer, membranes were incubated with the antibodies anti-ACLY (1:1000,
217 15421-1-AP, Proteintech), anti-ACSS2 (1:1000, 3658, Cell Signaling) and anti-β-actin-

218 HRP (1:50000; A3854, Sigma). Antibody binding was detected with a secondary antibody
219 coupled to horseradish peroxidase (Sigma, NA934 and NA931) using enhanced
220 chemiluminescence (Thermo Scientific, 34578).

221 ***Flow cytometry analysis***

222 To analyze leukemic spleen samples, single cell suspensions were prepared by
223 disrupting spleens through a 70 μm filter. Red cells were removed by incubation with
224 ammonium-chloride-potassium lysing buffer (155 mM NH4Cl, 12 mM KHCO3 and 0.1 mM
225 EDTA) for 5 minutes in ice. Apoptotic cells in leukemic spleens were quantified with PE-
226 AnnexinV Apoptosis Detection Kit I (BD Pharmingen, 559763). All flow cytometry data
227 was collected on an Attune NxT Flow Cytometer (ThermoFisher Scientific) and analyzed
228 with FlowJo v10.6.2 software (BD).

229 ***Metabolite extraction and LC-MS-based metabolomics***

230 Leukemic spleen samples were processed and analyzed as previously described[4,11].

231 ***Quantitative RT-PCR***

232 Total RNA was extracted from cells using RNeasy Plus Mini Kit (Qiagen), and cDNA was
233 generated with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).
234 Quantitative PCR was performed on a QuantStudio 3 Real-Time PCR System (Applied
235 Biosystems) using SYBR Green PCR Master Mix (Takara), and transcript levels were
236 normalized to actin as an internal control. Primers used: *Acly* (Forward: 5′-
237 TTCGTCAAACAGCACTTCC-3′; Reverse: 5′- ATTTGGCTTCTTGGAGGTG-3′) and *Actin*
238 (5′-GGCTGTATTCCCCTCCATCG-3′; 5′-CCAGTTGGTAACAATGCCATGT-3′).

239 ***RNA-seq gene expression profiling***

240 *Acly* conditional knockout ΔE-NOTCH1-induced T-ALL-bearing mice were treated with
241 vehicle only (corn oil; Sigma, C8267) or tamoxifen (Sigma, T5648; 3 mg per mouse in
242 corn oil) to induce isogenic loss of *Acly* via intraperitoneal injection. 48 hours later, single-
243 cell suspensions of total leukemic splenocytes were prepared by pressing leukemic
244 spleens through a 70 μm filter. We removed red cells in spleen samples by incubation
245 with red blood cell lysis buffer (155 mmol/L NH4Cl, 12 mmol/L KHCO3, and 0.1 mmol/L
246 EDTA) for 5 minutes on ice. RNA was extracted using QIAshredder (QIAGEN, 79656)
247 and RNeasy Mini (QIAGEN, 74106) kits. RNA library preparations and next-generation
248 sequencing were performed using Illumina Next-Seq platform (Illumina). We estimated
249 gene-level raw counts and performed differential expression and/or GSEA analyses as
250 previously described[11].

251 ***RNA-seq analysis of Normal vs T-ALL***

252 *ACLY* expression was analyzed among T-ALL samples (n=57) and physiological
253 thymocyte subsets (n=21) from published literature[9]. Quantile normalization was
254 performed across samples. Differential expression was performed using Mann-Whitney
255 U-Test (P<0.001), and genes with FDR<0.05 using the Benjamini-Hochberg correction
256 were shortlisted as differentially expressed.

257 ***Statistical analysis***

258 Statistical analyses were performed with Prism 8.0 (GaphPad). Unless otherwise
259 indicated in figure legends, statistical significance between groups was calculated using
260 an unpaired two-tailed Student's *t*-test. Survival in mouse experiments was represented
261 with Kaplan–Meier curves, and significance was estimated with the log-rank test.

262 ***Data availability***

263 RNA-seq data from *Acly* conditional knockout leukemias was deposited in the NCBI Gene
264 Expression Omnibus repository under the following accession number: GSE227492.

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271 **Author contributions**

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

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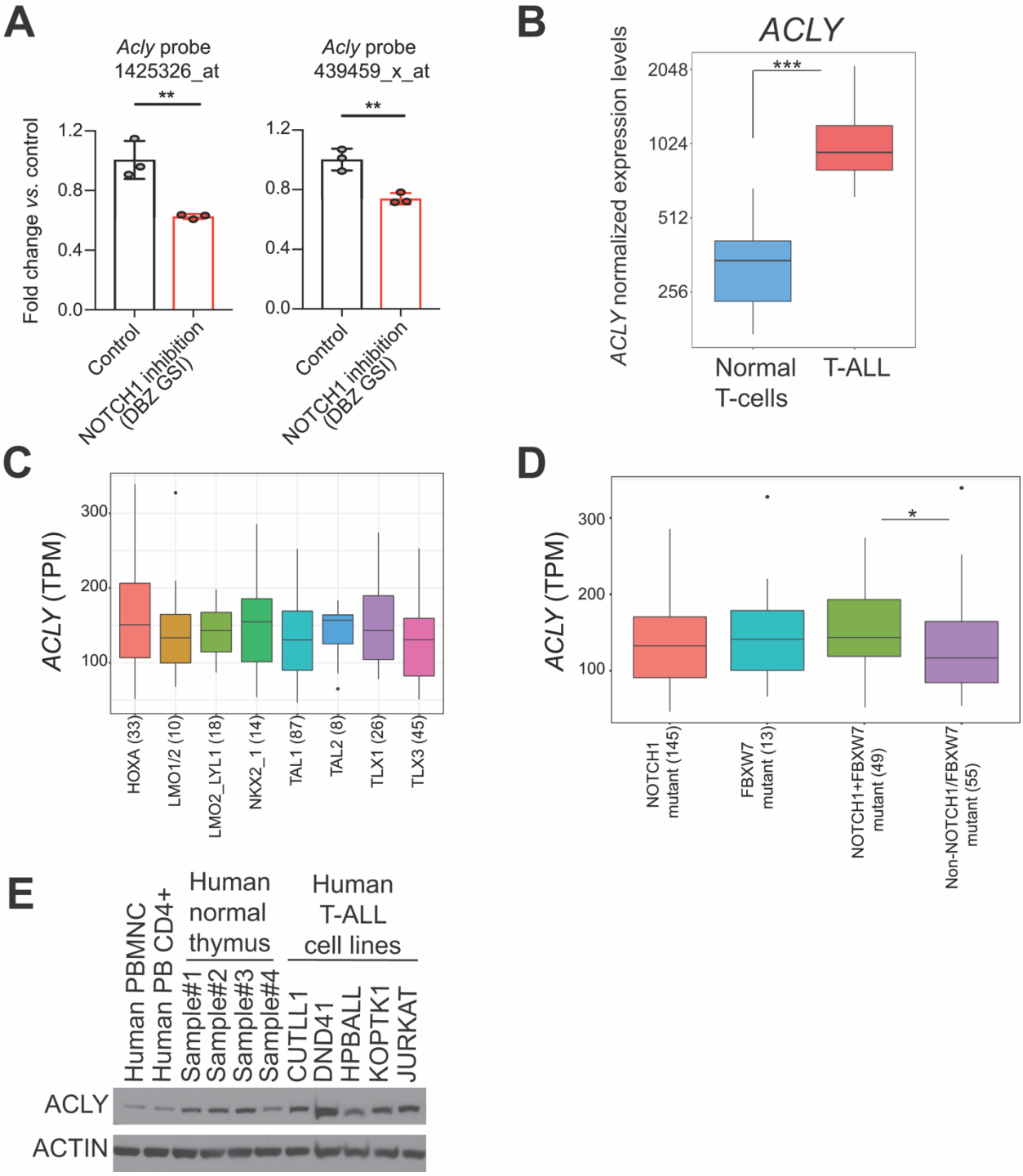
338

339 **Figures and Figure Legends**

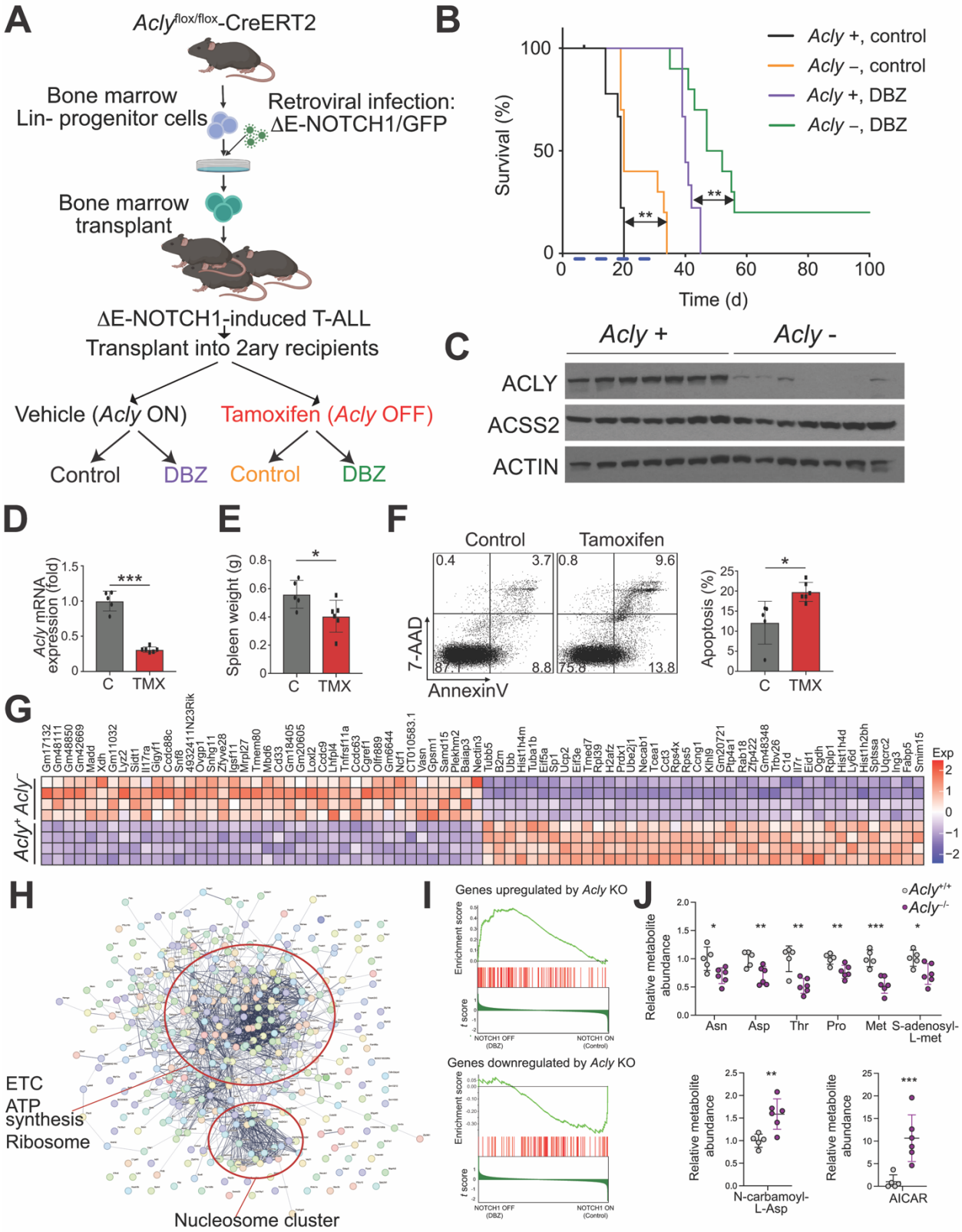


340

341 **Figure 1**. ACLY is overexpressed in human T-ALL. **A,** mRNA levels of *Acly* upon

342 NOTCH1 inhibition with the DBZ gamma-secretase inhibitor in mouse T-ALL *in vivo*[6]

343 (**P<0.01 calculated with 2-tailed Student's t-test). **B,** Box-plot showing *ACLY* expression
344 among T-ALL samples (n=57) and physiological thymocyte subsets (n=21)[9]. Quantile
345 normalization was performed across samples. Boxes represent first and third quartiles
346 and lines represent the median. Whiskers represent the upper and lower limits (P<0.001
347 using Mann-Whitney U-Test; FDR<0.05 using Benjamini-Hochberg correction). **C,** *ACLY*
348 expression levels in human primary T-ALL cases from different subclinical groups[10]. **D,**
349 *ACLY* expression levels in human primary T-ALL cases with/without *NOTCH1* and
350 *FBXW7* mutations[10] (Wilcoxon rank-sum test *P < 0.05). **E,** Western blot analysis of
351 ACLY, ACSS2 and ACTIN expression in human peripheral blood mononuclear cells
352 (PBMNC), CD4+ T-cells or healthy human thymocytes, as compared to human T-ALL.


A
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 ON)
Tamoxifen (Acly OFF)
Control
DBZ
Control
DBZ
B
Acly +, control
Acly –, control
Acly +, DBZ
Acly –, DBZ
Survival (%)
Time (d)
C
Acly +
Acly -
ACLY
ACSS2
ACTIN
D
Acly mRNA expression (fold)
C
TMX
E
Spleen weight (g)
C
TMX
F
Control
Tamoxifen
7-AAD
AnnexinV
Apoptosis (%)
C
TMX
G
Acly+ Acly−
Exp
H
ETC
ATP synthesis
Ribosome
Nucleosome cluster
I
Genes upregulated by Acly KO
Genes downregulated by Acly KO
Enrichment score
t score
NOTCH1 OFF (DBZ)
NOTCH1 ON (Control)
J
Acly+/+
Acly−/−
Relative metabolite abundance
Asn
Asp
Thr
Pro
Met
S-adenosyl-L-met
N-carbamoyl-L-Asp
AICAR

353

354 **Figure 2.** Targeting ACLY shows antileukemic and synergistic effects with NOTCH1
355 inhibition *in vivo*. **A,** Schematic of retroviral-transduction protocol for the generation of
356 NOTCH1-induced T-ALLs from inducible *Acly*-conditional knockout mice, followed by
357 transplant into secondary recipients treated with vehicle ($Acly^{+/+}$) or tamoxifen ($Acly^{-/-}$),
358 with or without DBZ. **B,** Kaplan-Meier survival curves of mice harboring *Acly*-positive and
359 *Acly*-deleted isogenic leukemias treated with 4 cycles of vehicle or DBZ (5 mg/kg) on a 4-
360 days-ON (blue blocks at the bottom) and 3-days-OFF schedule (log-rank test; $^{**}P < 0.01$;
361 n = 10 per group). **C,** Western blot analysis of ACLY, ACSS2 and ACTIN expression in
362 leukemic spleens from terminally ill mice from survival curve in B. **D**, Quantitative RT-
363 PCR analysis of *Acly* mRNA expression in tumor cells isolated from ΔE-NOTCH1-induced
364 *Acly* conditional knockout leukemia–bearing mice 48 h after being treated with vehicle
365 only ($Acly^{+/+}$) or tamoxifen ($Acly^{-/-}$) *in vivo*. **E,** Tumor burden in ΔE-NOTCH1-induced *Acly*
366 conditional knockout leukemia–bearing mice 48 h after being treated with vehicle only
367 ($Acly^{+/+}$) or tamoxifen ($Acly^{-/-}$) *in vivo* as revealed by total spleen weight. **F**,
368 Representative flow cytometry plots from of annexin V (apoptotic cells) and 7-AAD (dead
369 cells) staining (left) and quantification of apoptosis (right) in leukemic spleens from ΔE-
370 NOTCH1-induced *Acly* conditional knockout leukemia–bearing mice 48 h after being
371 treated with vehicle only ($Acly^{+/+}$) or tamoxifen ($Acly^{-/-}$) *in vivo*. (n = 5 per treatment; $^{*}P <$
372 0.05 and $^{***}P < 0.005$ using two-tailed Student's *t*-test. **G,** Heat map representation of the
373 top differentially expressed genes between control ($Acly^{+/+}$) and tamoxifen-treated
374 ($Acly^{-/-}$) *Acly*-conditional knockout ΔE-NOTCH1–induced leukemias. Cutoffs used: top 40
375 up and downregulated genes; *P*-adjusted value < 0.0001; log fold change >1. Scale bar
376 shows color-coded differential expression, with red indicating higher levels of expression

377 and blue indicating lower levels of expression **H,** Pathway analyses of significantly
378 downregulated genes upon ACLY loss (flod change > 1.7; $P < 0.001$) using the STRING
379 database[13]. **I,** GSEA of genes regulated by ACLY in control (vehicle only–treated;
380 NOTCH1 ON) compared with DBZ-treated (NOTCH1 OFF) ΔE-NOTCH1–induced mouse
381 leukemias *in vivo*[6]. **J,** Relative abundance of indicated metabolites upon tamoxifen-
382 induced isogenic loss of *Acly* in leukemic spleens from mice treated as in D (n = 5 per
383 treatment; *$P < 0.05$; **$P < 0.01$; *** $P < 0.005$ using two-tailed Student's *t*-test.).
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