The glutamate/aspartate transporter EAAT1 is crucial for T-cell acute lymphoblastic leukemia proliferation and survival


Received: May 3, 2023.
Accepted: May 20, 2024.


Publisher’s Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication.
E-publishing of this PDF file has been approved by the authors.
After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors’ final approval; the final version of the manuscript will then appear in a regular issue of the journal.
All legal disclaimers that apply to the journal also pertain to this production process.
The glutamate/aspartate transporter EAAT1 is crucial for T-cell acute lymphoblastic leukemia proliferation and survival

Vesna S. Stanulović¹, Shorog Al Omair¹, Michelle A.C. Reed¹, Jennie Roberts¹, Sandeep Potluri¹, Taylor Fulton-Ward², Nancy Gudgeon³, Emma L. Bishop², Juliette Roels³, Tracey A. Perry¹, Sovan Sarkar¹, Guy Pratt¹,⁴, Tom Taghon³, Sarah Dimeloe², Ulrich L. Günther¹, Christian Ludwig⁵ and Maarten Hoogenkamp¹, *

¹ Institute of Cancer and Genomic Sciences, University of Birmingham, Birmingham, United Kingdom
² Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom
³ Department of Diagnostic Sciences, Ghent University, Ghent, Belgium
⁴ Centre for Clinical Haematology, Queen Elizabeth Hospital Birmingham, Birmingham, United Kingdom
⁵ Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, United Kingdom

* Correspondence:
Maarten Hoogenkamp - m.hoogenkamp@bham.ac.uk

Running title: EAAT1 supports glutamine-driven T-ALL survival

Authorship Contributions


Authors’ disclosures

The authors declare no conflicts of interests.

Data sharing

T-ALL RNAseq data is available at NCBI-GEO GSE101566
Acknowledgments
We would like to thank Dr. A.W. Langerak, (Erasmus Medical Centre, Rotterdam, NL) for the provision of ARR and DU.528 cell lines and Prof P.N. Cockerill (University of Birmingham, UK) for HSB2 and CCRF-CEM. We would like to thank Dr. M. McGrew (University of Edinburgh, UK) for the PB_tet-on_Apple_shGFP plasmid and Prof. L. Bunch (University of Copenhagen, Denmark) for pcDNA3-EAAT1 plasmid. We would like to acknowledge the University of Birmingham FACS facility for cell sorting and the Biomolecular NMR Facility at the Henry Wellcome Building for Nuclear Magnetic Resonance (HWB-NMR), University of Birmingham. We gratefully acknowledge the contribution to this study made by the University of Birmingham’s Human Biomaterials Resource Centre, which was set up with support from the Birmingham Science City - Experimental Medicine Network of Excellence project.

Funding
This work was supported by Blood Cancer UK, through a Bennett Fellowship to M.H. [11002].
Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is a cancer of the immune system. Approximately 20% of paediatric and 50% of adult T-ALL patients have refractory disease or relapse and die from the disease. To improve patient outcome new therapeutics are needed. With the aim to identify new therapeutic targets, we combined the analysis of T-ALL gene expression and metabolism to identify the metabolic adaptations that T-ALL cells exhibit. We found that glutamine uptake is essential for T-ALL proliferation. Isotope tracing experiments showed that glutamine fuels aspartate synthesis through the TCA cycle and that glutamine and glutamine-derived aspartate together supply three nitrogen atoms in purines and all but one atom in pyrimidine rings. We show that the glutamate-aspartate transporter EAAT1 (SLC1A3), which is normally expressed in the central nervous system, is crucial for glutamine conversion to aspartate and nucleotides and that T-ALL cell proliferation depends on EAAT1 function. Through this work, we identify EAAT1 as a novel therapeutic target for T-ALL treatment.

Key words:

T-cell Acute Lymphoblastic Leukemia; glutamine; de novo nucleotide synthesis; EAAT1, SLC1A3; isotope tracing
Introduction

T-cell acute lymphoblastic leukemia (T-ALL) are hematological malignancies of the T-cell lineage. Subtypes are grouped based on immunophenotype, or more recently, gene expression profiling that correlates with the underlying genomic aberrations\textsuperscript{1,2}. T-ALL occurs both in adults and children and, despite improved treatment outcome in the last decades, up to 20% of pediatric and 50% of adult T-ALL patients have refractory disease or relapse\textsuperscript{3-6}. Alternative treatment options are not available for these patients and, therefore, novel therapeutic targets are needed.

For a cancer to proliferate, cells need to increase their size and replicate their DNA, which requires large quantities of proteins, lipids and nucleotides, as well as energy. Cell proliferation and survival require metabolic adaptations, involving glycolysis, glutaminolysis and the PI3K-AKT-mTOR pathway\textsuperscript{7-12}. Differences in the reliance on glutamine and glutaminolysis between B-ALL and T-ALL have been observed\textsuperscript{13,14}. Metabolic adaptations and mutations, such as Notch1 mutations, have been correlated with glutamine dependency in T-ALL\textsuperscript{15-17}. The compilation of metabolic changes results in distinct metabolic phenotypes and determination of the rate-limiting steps that support the oncogenic metabolic adaptations can lead to the identification of novel therapeutic targets.

With the aim to identify common oncogenic adaptations, we investigated T-ALL metabolism, using cell lines, representing different stages of T-cell development, and primary patient samples. We found that T-ALL cells uptake glutamine and use it to generate aspartate. Normally, glutamine and aspartate are used as substrates for de novo nucleotide production but in T-ALL, glutamine alone provides all of the nitrogen atoms and all but one carbon atom in the pyrimidine ring, and three of the four nitrogens in purines. Furthermore, we determined that T-ALL cells express the glutamate-aspartate antiporter EAAT1 (encoded by \textit{SLC1A3}), which is present in 95% of T-ALL patients\textsuperscript{2}. In the healthy adult body, EAAT1 is only present in the neurons and glia of the central nervous system where it removes cytotoxic glutamate from the glutamatergic synapses in exchange for aspartate\textsuperscript{18,19}. In T-ALL, EAAT1 is required for glutamate import into mitochondria in exchange for glutamine-derived aspartate which is then used as a substrate for nucleotide production in the cytoplasm. We show that T-ALL survival depends on glutamine uptake and EAAT1 function, which validates EAAT1 as a therapeutic target for treating T-ALL.

Methods

Detailed information is available in the Supplementary data.

\textit{Cell Culture}
T-ALL cell lines were grown in RPMI-1640 medium. For labelling experiments, glutamine-free RPMI was supplemented with 2mM [1,5-\textsuperscript{15}N]-L-Glutamine or [3,\textsuperscript{13}C]-L-Glutamine (Merck).

*In vitro* differentiation of human T-cell progenitors was performed using mononuclear cells isolated from cord blood; HBRC approval 15/NW/0079. CD34\(^+\) cells were co-cultured on OP9-DL4 cells in αMEM medium with hFLT3L and hIL7. CD4\(^+\)/CD8\(^+\) T-cells were isolated from leukocyte cones (UoB STEM Ethics ERN_17-1743) and left unstimulated or stimulated for 48h.

*Expression analysis*

RNA isolation and cDNA synthesis was performed as previously described\textsuperscript{20}. RNASeq libraries were prepared and indexed using the TruSeq Stranded mRNA Sample Preparation Kit LH. Libraries were pooled and sequenced as 100nt paired-end on Illumina HiSeq2500 sequencer.

*Western blot analysis*

Cell extracts were prepared in RIPA buffer. Proteins were separated on acrylamide gels, transferred to nitrocellulose membranes and stained with PonceauS to confirm equal loading, prior to o/n incubation with primary antibody. Secondary antibodies were visualized using an Odyssey CLx Imager.

*Immunofluorescent Staining*

Cells were fixed with formaldehyde, washed with PBS/0.05%Tween/2%FCS, followed by o/n incubation with primary antibody. Cells were washed and incubated with secondary antibody conjugated to Alexa dyes before being deposited onto glass slides using a Cytospin III centrifuge. MitoTracker Red CMXRos was used for mitochondria staining.

*SLC1A3 knockdown*

SLC1A3 was cloned into MigR1 in front of IRES-GFP\textsuperscript{21}. Short hairpin sequences targeting SLC1A3 were embedded into miR30 and cloned into pMSCVhygro\textsuperscript{22}. The mouse fibroblast cell line PlatE was transfected using TransIT-LT1 with MigRI-SLC1A3 and MSCVhyg\_shSLC1A3, MSCVhyg\_shGFP, or MSCVhyg\_shFF3\textsuperscript{23,24}. Functional shRNA was cloned into PiggyBac transposon doxycycline-inducible expression vector PB\_tet-on\_Apple\_shGFP\textsuperscript{25}. T-ALL cell lines were electroporated with pB\_shSLC1A3 and pCAGG-PBase\textsuperscript{26}, expanded and selected with puromycin prior to doxycycline induction.

*Patient Samples*
Patients' cells were from diagnostic samples from presentation cases, with ethical approval from the NHS National Research Ethics Committee (Reg:10/H1206/58). Blast cells were isolated from mononuclear cells using anti-human CD34 (T-ALL_1 and _2) or CD7 (T-ALL_3) MACS microbeads. For T-ALL_2, CD34⁺ cells were further sorted by FACS for CD7⁺, using anti-human CD7-FITC antibody.

NMR spectroscopy
For intracellular measurements, polar cell extracts were resuspended in NMR buffer and transferred to NMR tubes. Spectra were acquired using a Bruker 600MHz spectrometer. For uptake and release experiments, growth medium was collected and supplemented with 1mM TMSP, 10% D₂O. Spectra were acquired using a Bruker 500 MHz spectrometer. For live-cell real-time NMR spectroscopy, cells were resuspended at 10⁵/ml growth media containing 0.1% low melting agarose, 1mM TMSP, 10% D₂O. Spectra were acquired using a Bruker 500 MHz spectrometer. NMR spectroscopy data was processed in Topspin, MetaboLab in MATLAB²⁷ and MetaboLabPy (https://pypi.org/project/qtmetabolabpy).

Mouse studies
Animal experiments were performed at the University of Birmingham Biomedical Services Unit under an animal project license (PP8841933) in accordance with UK legislation. Female NOD.Cg-Prkdcscid Il2rgtm1Wjl/J (NSG) mice, aged 8-9 weeks at study commencement, were used. CCRF-CEM cells were transfected with pBshSLC1A3_2 or pBshGFP control vector and pCAGG-PBase. After selection, 3x10⁵ cells/mouse were injected into the tail vein. When approximately 1% engraftment was observed, next day all animals were transferred to a diet supplemented with 0.625g/kg doxycycline-hyclate.

Results
T-ALL metabolic phenotype
For our study, we chose four T-ALL cell lines that have different immunophenotypes, reflecting that the T-cells are blocked at different stages of development. Their immunophenotype and corresponding EGIL classification are indicated in Figure S1²⁸. To assess leukemic adaptation, we compared the gene expression of these cell lines to the gene expression of in vitro differentiated T-cell progenitors at the CD7⁺ CD5⁻ CD1a⁻, the CD7⁺ CD5⁺ CD1a⁻, and the CD7⁺ CD5⁺ CD1a⁺ CD3⁺ stage of differentiation. Analyses of the RNAseq data identified 2072 significantly differentially expressed genes, grouped by
hierarchical clustering into 2 clusters (Figure 1A, Table S1). Genes suppressed in the T-ALL cells were grouped in cluster 1 (C1, 1287 genes) while upregulated genes were in cluster 2 (C2, 786 genes). Gene functional annotation clustering analyses found 53 statistically significantly enriched functional clusters in C1 and 22 in C2 (Table S2, S3). Amongst the top 10 highest enriched clusters for C1 were: Glycyl-lysine isopeptide bond with SUMO2, RNA splicing, translation, DNA repair, DNA binding and localization to mitochondrion. For C2 genes encoding proteins containing BTB/POZ, LRR and PH protein domains, Phospholipase C-activating G-protein coupled receptor signaling, sequence-specific double stranded DNA-binding and extracellular matrix proteins were found (Figure 1B, Table S4). The largest cluster was composed of 123 genes whose product has lysine that is crosslinked through the isopeptide bond with C-terminal Glycine of SUMMO2 protein. Amongst the genes were transcription factors (FOXP1, ELK4, IKZF5, TCFL2, ZEB2) and chromatin modifiers (KMT2B, BRD7, CHD4, CHD9). The second largest (86 genes) was the group of mitochondrion proteins important for the mitochondrial respiratory chain complex I assembly (LYRM2, NDUFA2, NDUFB9 and TMEM186), regulation of mitochondrial membrane potential (PRELID1, SOD2 and ubiquitin B), ATP biosynthetic process (COX11, DGUOK and UQCC3) and TCA cycle (IDH3A, PDHB and SDHC).

We therefore investigated the metabolism of the TALL cell lines. Metabolite uptake was assessed by measuring metabolite levels in the media, 24h after medium change, by NMR spectroscopy and comparing it to the levels present in the media alone. We identified 22 metabolites with significantly changing levels in at least one of the T-ALL cell lines and found that all four cell lines utilized lysine, glucose, phenylalanine and glutamine and released lactate, pyruvate, glutamate and pyroglutamate (Figure 2A).

To assess how uptake influences intracellular metabolite levels we compared the abundance of intracellular metabolites at the time of growth-media change (0h) to that seen 8h and 24h later (Figure 2B). A similar response to media change in all four cell lines was found only for glutamate and myoinositol, whose intracellular concentration decreased upon media supplementation. Glutamate and myoinositol are important metabolites for every proliferating cell as they are used to fuel cell growth and proliferation. Myoinositol is used as a substrate for membrane synthesis while glutamate feeds into the TCA cycle hence, energy production. Observed glutamine uptake and glutamate utilization by T-ALL cell lines suggest that glutamine is used to support the cellular demand for glutamate (Figure 2A,B).

**Glutamine is Essential for T-ALL Proliferation and Survival**

Next, we wanted to determine the importance of glutamine for T-ALL. We therefore tested the effect of glutamine withdrawal on cellular proliferation. Omitting glutamine from the growth medium, while maintaining all other nutrients, including 10% fetal bovine serum,
reduced T-ALL cell numbers by 95% after 8 days of treatment, except for ARR where the proliferation decreased over the first four days, after which ARR resumed proliferation (Figure 2C). Another two cell lines, Molt-4 and Jurkat, were tested as well, which showed that Molt-4 was sensitive to glutamine withdrawal, whereas Jurkat showed growth kinetics similar to ARR (Figure S2A). Glutamine dependency do not correlate to oncogenic subtype, differentiation stage or Notch mutation status (Figure S2B)\textsuperscript{29}. Apoptosis assays of the cells grown in glutamine-free medium revealed that the T-ALL cell lines had an increased rate of apoptosis (Figure 2D), while cell cycle analysis showed a reduction of cells in S-phase (Figure S3), indicating that glutamine is important for T-ALL cell proliferation and survival.

**Glutamine Fuels Aspartate and Nucleotide Biosynthesis**

Glutamine has several significant roles in metabolic processes, such as fueling the TCA cycle through glutaminolysis, acting as a nitrogen donor in transamination and transamidation reactions, and de novo nucleotide synthesis. Aspartate, glycine, glutamate and alanine can be derived from glutamine, and glutamine, glycine and aspartate are used as substrates for nucleotide synthesis. To assess the glutamine contribution to T-ALL metabolic processes we performed tracer experiments using [2,5-\textsuperscript{15}N]Glutamine and [3-\textsuperscript{13}C]Glutamine. When cells were grown in the presence of [2,5-\textsuperscript{15}N]Glutamine, in addition to the expected \textsuperscript{15}N labelled glutamate (data not shown), we observed \textsuperscript{15}N incorporation into the amino group of aspartate and alanine within 8h of treatment for all four cell lines (Figure 3A,S4A). For aspartate, at 0h, a doublet was seen due to coupling to the H\textsubscript{α}. At later time points there was increased spectral complexity due to weak coupling of H\textsubscript{β}s to \textsuperscript{15}N. The resulting spectrum was a weighted average of a doublet of doublets (from \textsuperscript{15}N-labelled aspartate) and a simple doublet (unlabeled aspartate) (Figure 3A). This demonstrates that glutamine-derived \textsuperscript{15}N was incorporated into aspartate and alanine by transamination of oxaloacetate and pyruvate, respectively.

To assess the contribution of glutamine to nucleotide biosynthesis, we first acquired 2D-\textsuperscript{1}H,\textsuperscript{15}N heteronuclear single quantum coherence (HSQC) NMR spectra of \textsuperscript{15}N uniformly labelled ATP, GTP and UTP standards, where we observed five \textsuperscript{1}H-\textsuperscript{15}N interactions for [U-\textsuperscript{15}N]ATP (a-d), three for [U-\textsuperscript{15}N]GTP (a-c) and three for [U-\textsuperscript{15}N]UTP (a-c) (Figure 3B). Spectra acquired from [2,5-\textsuperscript{15}N]Glutamine-labelled T-ALL cells showed \textsuperscript{15}N incorporation into nucleotides at the 1, 3 and 9 positions, the 3 and 9 positions and the 1 and 3 positions for adenine, guanine and uracil respectively (Figure 3B). As expected, transamidation using \textsuperscript{15}N-glutamine resulted in label incorporation at N-3 and N-9 in purines and the N-3 in pyrimidines. Additional label incorporation at N-1 in purines and N-3 in pyrimidines is sourced from glutamine-derived \textsuperscript{15}N-aspartate and shows that glutamine ultimately supplies all but one nitrogen in purine and both nitrogens in the pyrimidine ring.
We demonstrated that glutamine transamination of oxaloacetate gives rise to aspartate. It is possible that glutamine supplies oxaloacetate, through glutaminolysis, which would consequently mean that aspartate is completely derived from glutamine. We used [3-13C]Glutamine in labelling experiments to test this. Within the TCA cycle, [3-13C]Glutamine is converted to [2-13C]Fumarate, a symmetrical molecule which is hydrated equally to [2-13C]Malate and [3-13C]Malate, and further to [2-13C] or [3-13C]Oxaloacetate and aspartate (SI3). Acquired 2D-1H,13C HSQC NMR spectra revealed 13C incorporation in fumarate, malate, oxaloacetate and aspartate (Figure 4A, S4B). Resonances for 1H-13C moieties were derived from both [2-13C] and [3-13C] labelling, as shown for aspartate and malate (a and b respectively, Fig 4A,S2B). Quantification of the signals from the labelled samples, relative to the naturally occurring 13C in the control samples, showed that within 24h, 40-70% of aspartate was [2-13C] or [3-13C]Aspartate (Figure 4B). These findings confirm that two molecules of glutamine give rise to a single aspartate molecule; first glutamine supplies the backbone via the TCA cycle, while the second is used for transamination. These metabolic conversions occur in all four tested T-ALL cell lines even though we observed that ARR also uptake aspartate from the media (Figure 2A), suggesting that the aspartate uptake in ARR is not sufficient to support its demand.

A further implication of these findings was that carbons derived from glutamine would, via aspartate, get incorporated into pyrimidines, giving rise to [5-13C] or [6-13C]Uridine. This was indeed observed in 2D-1H,13C HSQC NMR spectra (peaks a and b, Figure 4A,B), showing that the carbon at position 4 also originates from glutamine via aspartate. Together, our results show that glutamine serves as a source for all but one of the atoms in the pyrimidine ring.

Additionally, examination of the 13C incorporation in other detectable metabolites found a significant label accumulation into proline, as would be expected since glutamate is used as a substrate for proline synthesis (Figure S4C).

**Patient-derived T-ALL and T-ALL cell lines have similar metabolic uptake**

To determine if glutamine is not only used by patient derived cell lines, but also by primary T-ALL patient isolates, we measured live-cell real-time metabolite uptake by NMR. T-ALL CD34+/CD7+ cells were isolated from three T-ALL patients at presentation. The flow cytometry profile revealed that samples had cytoplasmic CD3 expression but lacked CD3 surface expression. T-ALL_1 had normal karyotype and consisted of 60% CD34+ blasts, half of which were CD7+. For T-ALL_2, karyotyping showed two lines of roughly equal proportion; normal 46XY and 47XY,add(1)(q3),add(7)(p1)x2,add(16)(q2),+21 and immunophenotypically was predominantly CD7+, as well as CD5+, CD2+, CD38+ and CD4+. The T-ALL_3 karyotype was 46 XY,t(10;14) with a trisomy 21 subclone and cells were CD7+, CD13+, CD5−/−, CD2−/−,
CD117⁺, while CD1a⁻, CD4⁻ and CD8⁻. CD34⁺ (T-ALL_1) or CD7⁺ (T-ALL_2/3) cells were isolated and 10⁶ cells were resuspended in RPMI medium supplemented with GlutaMAX (L-glutamine/L-alanine dipeptide) and used for measuring metabolite uptake. GlutaMAX is a temperature-stable source of glutamine, which is hydrolyzed by peptidases located on the plasma membrane, into L-Glutamine and L-Alanine. Using live-cell NMR we observed for all the patient samples that GlutaMAX concentrations decreased without the equivalent increase in glutamine availability showing that patient-derived T-ALL utilize glutamine (Figure 5A).

**T-ALL cells express EAAT1 in the mitochondria**

Our finding that T-ALL rely on deriving their aspartate from glutamine highlights that this metabolic step is a T-ALL liability. Therefore, we focused on identifying potential therapeutic targets for T-ALL treatment within the metabolic processes supporting glutamine-derived aspartate synthesis. Inhibiting the glutamate-aspartate anti-port across the mitochondrial membrane would be a good strategy for targeting T-ALL as it is not directed towards the enzymatic conversions that would restrict aspartate availability for nucleotide synthesis in healthy cells. Aspartate export and glutamate import into mitochondria is known to be facilitated by 3 different antiporters SLC1A3, SLC25A12 and SLC25A13. While SLC25A12 and SLC25A13 are expressed in most tissues, the expression of SLC1A3 is mainly restricted to the CNS. Published gene expression data of 264 T-ALL patient samples revealed that SLC1A3 is expressed at various levels in 95% of patient samples (Figure 5B). Further analysis of the patient samples showed that higher SLC1A3 expression correlated with the LMO1/2 and TAL1/2 subgroups (Figure S5). There also seemed to be higher SLC1A3 expression associated with an ETP or near ETP status, as defined by Liu et al., although this was not statistically significant due to the low number of patients in these two groups. The presence of Notch mutations did not significantly correlate with SLC1A3 expression levels.

Analysis of our RNAseq data showed SLC1A3 expression in three of the T-ALL cell lines and an absence in T-cell progenitors, with subsequent qPCR and Western blotting confirming the expression of SLC1A3 mRNA and EAAT1 protein (encoded by SLC1A3), in all four T-ALL cell lines (Figure 5C,D). We observed that EAAT1 was detected in the mitochondria of the T-ALL cell lines but not in the acute myeloid leukemia (AML) cell line Kasumi-1, which does not express SLC1A3 (Figure 5C,D,E). SLC1A3 expression was not observed in mature CD4⁺ or CD8⁺ T-cells, regardless of whether cells were stimulated (Figure 5F). For a direct comparison of SLC1A3 expression between T-ALL patient samples and T-cell progenitors isolated from healthy thymus, we combined and analyzed published RNAseq data from ten different thymocyte populations and sixty T-ALL patients. These
data sets were generated using the same platform at similar sequencing depth and were analyzed together. The results show that significant SLC1A3 expression only occurs in T-ALL samples (Figure S6A). Higher expression was particularly found in the TAL oncogenic subgroup (Figure S6B) and less frequently in the immature, HoxA, and TLX3 subgroups, in line with the above findings (Figure S5).

Together, these results show that T-ALL cells aberrantly overexpress the high affinity glutamate-aspartate antiporter EAAT1.

**SLC1A3/EAAT1 is essential for oncogenic de novo nucleotide synthesis**

To assess the importance of EAAT1 for T-ALL survival we performed SLC1A3 knock-down using shRNA. Five shSLC1A3 were designed and tested for their capacity to suppress the expression of SLC1A3 cDNA. Mouse fibroblasts were co-transduced with retrovirus expressing SLC1A3-IRES-GFP and shRNAs. shSLC1A3 efficiency was measured relative to the negative control, shFF3, and the positive control shGFP. shSLC1A3_1 and shSLC1A3_2 had the capacity to suppress EAAT1 protein levels similar to shGFP, while the negative control shFF3 did not have any effect on EAAT1 protein levels (Figure 6A). shSLC1A3_1 and shSLC1A3_2 were cloned into a PiggyBac backbone that supports stable doxycycline-inducible shRNA expression. Induction of shSLC1A3 led to rapid cell death within 5 days from the start of the doxycycline treatment (Figure 6B, S7).

With the purpose to inhibit EAAT1 function in the CNS, two specific allosteric inhibitors, UCPH-101 and UCPH-102 were developed. Both drugs exhibited inhibitory effects on T-ALL proliferation (Figure 6C, S8). UCPH-101 also affected the growth and survival of the control AML cell line Kasumi-1 when cultured for more than two days, which can be explained by the presence of known toxophores. UCPH-102 had a specific anti-proliferative effect only on T-ALL cells. Next we performed [3-13C]Glutamine tracing experiments upon EAAT1 inhibition, which showed a marked decrease in the production of aspartate and UTP from glutamine (Figure 6D). Altogether, our results show that oncogenic nucleotide production depends on EAAT1 function and glutamine conversion to aspartate.

**EAAT1 is required for T-ALL xenograft development**

To test the importance of EAAT1 during disease progression in a mouse xenograft model, CCRF-CEM cells, carrying doxycycline-inducible shSLC1A3_2 or shGFP in conjunction with TdTomato, were injected into the tail vein of NSG mice. When engraftment was apparent at approximately 1% of total CD45+ blood mononuclear cells, the diet was supplemented with doxycycline to induce shRNA expression. After 6 days, significantly less human CD45+ cells were detected in mice with shSLC1A3-expressing CCRF-CEM cells, compared to mice that received shGFP-expressing cells (Figure 7A, S9). Doxycycline treatment resulted in a
survival advantage for the shSLC1A3 mice compared to the shGFP control mice (Figure 7B). While all human CD45+ shGFP-expressing cells were TdTomato-positive, this was the case for only 40% of human CD45+ cells in mice with shSLC1A3-expressing cells, indicating the outgrow of cells that did no longer express the shSLC1A3 construct (Figure 7C). These in vivo results show that EAAT1 gives a proliferative advantage to T-ALL and validates EAAT1 as therapeutic target.

Discussion

Understanding the metabolic pathways that support oncogenic proliferation can help to identify cancer-specific processes and rate-limiting steps that can be used for developing new therapies. We show that in T-ALL cells, glutamine is converted to glutamate that enters the mitochondria. Here, glutamate dehydrogenase (GLUD1,2) uses it to generate α-ketoglutarate that will enter the TCA cycle (Figure 8). Mitochondrial glutamate oxaloacetate transaminase (GOT2) uses glutamate as a donor for the amino group, which is transferred to the TCA cycle intermediate oxaloacetate, resulting in the generation of aspartate. Aspartate is then transported out of the mitochondria in exchange for a new glutamate molecule. Together, glutamine and aspartate are used as substrates for nucleotide synthesis (Figure 8). Similar processes are likely to occur in other cancers. Indeed, a recent study of genetic cancer dependencies has shown that T-ALL and several other cancer types are dependent on SLC1A3 expression40. Additionally, EAAT1 has been implicated in supporting proliferation in several solid cancer cell lines41,42. These studies showed a role for EAAT1 in the uptake of aspartate from the medium, especially under conditions of glutamine deprivation or asparaginase treatment. In both studies, removing EAAT1 under normal cell culture conditions showed little or no effect, in contrast to our work with T-ALL cells. This difference can be explained as the solid tumor cell lines were mainly dependent on EAAT1 for aspartate/glutamate uptake from the environment and the lack thereof resulted in a combinatorial effect on the TCA cycle, the electron transport chain, and de novo glutamine/glutamate and nucleotide synthesis. T-ALL cells on the other hand are dependent on the availability of glutamine. They rely on the function of EAAT1 at the mitochondria, where the glutamate/aspartate antiport is required for de novo nucleotide production. Our isotope tracing experiments show that the carbon and nitrogen atoms of glutamine are used for aspartate, purine and pyrimidine biosynthesis. Sun et al. also showed that asparaginase treatment resulted in a cellular depletion of glutamine/glutamate, possibly explaining why asparaginase treatment is often effective in treatment of T-ALL43.
EAAT1 is normally present on the plasma membrane of neurons and glia in the CNS, where it uptakes glutamate from the glutamatergic synapses\textsuperscript{18}. Based on the RNA expression and protein localization assessed with three different specific antibodies, the Human Protein Atlas Database reports that EAAT1 is not found at significant levels outside of the CNS (https://www.proteinatlas.org/ENSG00000079215-SLC1A3/tissue)\textsuperscript{44}. However, EAAT1 expression has been reported in neonatal cardiomyocytes where, similar to T-ALL, it is also localized at the mitochondria. Here it is present together with two other glutamate-aspartate antiporters, \textit{i.e.} ARALAR1 and CITRIN\textsuperscript{32}. ARALAR1 and CITRIN are calcium-binding mitochondrial carrier proteins that import a glutamate molecule together with a H\textsuperscript{+} into the mitochondria in exchange for the export of an aspartate anion\textsuperscript{45-47}. Their activity depends on the mitochondrial membrane potential that is maintained by cytoplasmic ATP\textsuperscript{48}. Both transporters are present in most cells and tissues (reviewed in \textsuperscript{49}). We found that SLC25A12 (encoding ARALAR1) is expressed in all four T-ALL cell lines, while SLC25A13 (encoding CITRIN) was found only in DU.528. T-ALL cells likely rely on EAAT1 because intracellular conditions, such as the pH, ATP availability and mitochondrial action potential, are restrictive for ARALAR1 and CITRIN, whereas EAAT1 is independent of these factors. It is known that T-ALL is dependent on AMPK activity, indicating low ATP availability\textsuperscript{50}. Consequently, EAAT1 might also be important for maintaining mitochondrial potential when ATP availability is reduced.

Altogether, we show that \textit{SLC1A3} is aberrantly expressed in T-ALL cells, where it supports \textit{de novo} nucleotide production which uses glutamine as the main substrate, and that EAAT1 is required for T-ALL proliferation, identifying it as a relevant therapeutic target. Unfortunately, the selective and potent EAAT1 inhibitor UCPH-102, which has been developed for the inhibition of EAAT1 in the CNS, is not suitable for \textit{in vivo} use and therefore new therapeutic agents need to be developed that can target the oncogenic function of EAAT1.
References


Figure Legends

**Figure 1. Differential gene expression and gene ontology analysis for T-ALL cell lines.**

A) Heat map showing hierarchical clustering of the significantly differentially expressed genes between the T-ALL cell lines (ARR, DU.528, HSB2 and CCRF-CEM) and the healthy CD34+ cells isolated from the umbilical cord blood and T-cell progenitors (CD7+ CD5- CD1a-, the CD7+ CD5+ CD1a-, and the CD7+ CD5+ CD1a+ CD3-) derived from the differentiation of the CD34+ progenitors on OP9-DL4 stromal cells. RNAseq gene expression data are gene-normalized counts clustered based on Pearson correlation with average linkage clustering. Two clusters are marked by blue triangles and numbered 1 and 2. Scale bar represents gene normalized counts from 0 to 50. B) Gene functional annotation clustering for clusters 1 and 2. Terms are ordered based on their Modified Fisher Extract P-value. Further details are available in Table S4.

**Figure 2. Metabolite levels in T-ALL cells are dynamic.**

A) Heat map showing hierarchical clustering of the growth medium metabolites after 24h. Data are the average of four independent experiments ± StDev. Scale bar represents log2 relative metabolite concentration. Metabolites with significantly different levels are shown. B) Abundance of intracellular metabolites at 8h or 24h, relative to the time of medium change (0h). Fold change was presented only for metabolites with significantly different relative levels with p<0.05. GPC, L-Alpha glycerylphosphorylcholine; GalNAc, Acetylgalactosamine, GlcNAc, N-Acetylglucosamine. C) Glutamine deprivation inhibits T-ALL cell proliferation. T-ALL cell lines were cultured in medium with 10% FCS, with or without 2mM GlutaMax. Four independent cell cultures were assayed per cell line and each point represents the mean ± StDev. Statistically significant differences were found when ARR was compared to any of the other cell lines at day 6 and 8 (p<0.05). D) Glutamine deprivation induces apoptosis in T-ALL cell lines. Scatter plots show flow-cytometry Annexin V and propidium iodide staining by flow-cytometry of cells grown in RPMI media supplemented with 10% FBS in the absence of glutamine. Gating was determined based on the staining of T-ALL cells grown in the RPMI media supplemented with 2mM glutamine. Cells were grown for the indicated number of days (D0-D8) prior to staining and analysis.

**Figure 3. T-ALL cells utilize glutamine-derived nitrogen for de novo nucleotide synthesis.** Metabolite tracing experiments using T-ALL cell lines that were grown in the presence of 2mM [2,5-15N]Glutamine. A) Overlay of 1D 1H-NMR spectra showing the Hβ-aspartate resonance after 0, 8 and 24h and schematic representations of [2,5-15N] Glutamine and the observed [2-15N] Aspartate. 15N are in red and shading indicates the
observed $^3$J scalar couplings between the aspartate Hβs and glutamine-derived $^{15}$N. The X-axis shows the chemical shift relative to TMSP in ppm and the Y-axis indicates TSA scaled intensity. **B)** Resonances observed in $^1$H-$^{15}$N-HSQC for [U-$^{15}$N]ATP, [U-$^{15}$N]GTP, [U-$^{15}$N]UTP standards and for T-ALL cells extracts grown for 24h in the presence of [2,5-$^{15}$N]Glutamine. Resonances are marked by letters a-e. Schematics show ATP, GTP and UTP with color-coded atoms based on the substrate of their origin (glutamine-purple, aspartate-orange, glycine-green, carbonate-black and $^{15}$N-red). Blue shaded lines indicate observed couplings annotated a-e.

**Figure 4. T-ALL cells utilize glutamine-derived carbon for de novo nucleotide synthesis.** **A)** Resonances observed in $^1$H-$^{13}$C-HSQC for T-ALL cells grown for 24h in the presence of [3-$^{13}$C]Glutamine. Resonances are marked by letters a-e. Schematics on the right show glutamine, aspartate and UTP with colour-coded atoms based on the substrate of their origin. Blue shaded lines indicate observed couplings annotated a-e. **B)** Quantification of glutamine-derived aspartate and UDP. Bar graphs show $^{13}$C signal intensity acquired from the cells grown in the presence of [3-$^{13}$C]Glutamine for 24h, relative to the signal acquired from the naturally occurring $^{13}$C observed in the extracts from cells grown without the label. Each bar represents one of the interactions shown in the schematics in (A). Data are the average of at least three independent experimental measurements ± StDev.

**Figure 5. EAAT1 is expressed in T-ALL and localized in mitochondria.** **A)** Patient derived T-ALL cells have similar metabolic uptake to T-ALL cell lines. Only metabolites with changing concentration are illustrated. **B)** Summary of the SLC1A3 expression levels as assessed by FPKM values from the RNA-seq of 265 patient samples. Data are grouped by the level of expression. **C)** SLC1A3 mRNA expression level in T-ALL cell lines and AML cell line Kasumi-1, relative to rRNA level assessed by qPCR. **D)** Western blot analysis of EAAT1 protein level using 150 µg total cell extract. PonceauS shows equal loading. **E)** SLC1A3 mRNA expression level in ARR and in unstimulated or stimulated CD4+ or CD8+ human T-cells, relative to rRNA expression. CD4+ and CD8+ thymocytes data represent the average ± StDev of six independent samples. **F)** Immuno-fluorescent imaging shows that EAAT1 (green) co-localizes with MitoTracker Red CMXRos (magenta) in T-ALL but not Kasumi-1.

**Figure 6. EAAT1 is essential for T-ALL proliferation and survival.** **A)** Protein level of EAAT1 and the reporter GFP protein upon the suppression of SLC1A3-IRES-GFP mRNA by shFF3 (negative control), shGFP (positive control) and five different shSLC1A3_1-5. Experiment was performed in duplicate. PonceauS staining illustrates equal loading. **B)** Knock-down of the SLC1A3 gene by shSLC1A3_1 and shSLC1A3_2 leads to ARR, DU.528
and CCRF_M cell death. C) To test the effect of EAAT1 inhibition, T-ALL and AML cells were cultured for six days in the presence of vehicle (DMSO), 25 µM UCPH-101, or 25 µM UCPH-102. Each data point is an average of three independent measurements ± StDev. D) EAAT1 inhibition leads to a loss of aspartate and UTP production from glutamine. T-ALL cells were grown for 24h in culture media containing [3-¹³C]Glutamine in the presence of 25µM UCPH-101 or vehicle (DMSO). Bar graphs show ¹³C signal intensity relative to the DMSO control. Each bar represents one of the interactions shown in the schematics in Figure 4A. Data are the average of three independent experiments ± StDev.

Figure 7. EAAT1 is required for T-ALL xenograft development. A) Mice were injected (iv) with 3 x10⁵ cells CCRF-CEM cells carrying doxycycline-inducible shSLC1A3_2 or shGFP. At day 16, the food was supplemented with doxycycline. The bar graph shows the expansion of human CD45⁺ cells in the two cohorts of mice (n=5) during 6 days of doxycycline treatment. Two-tailed t-test identified the difference between the two cohorts as significantly different (p<0.01). B) Kaplan Meier curve comparing the survival of mice injected with CCRF-CEM cells carrying doxycycline-inducible shSLC1A3 or shGFP. The induction of shRNA expression through addition of doxycycline in the food on day 16 is indicated. Log rank test (right-tailed) identified the difference between the two cohorts as significantly different (p<0.05). C) The number of TdTomato-positive cells relative to the total number of hCD45⁺ cells at day 6. Two-tailed t-test identified the difference between the two cohorts as significantly different (p<0.01).

Figure 8. Model illustrating the function of mitochondrial EAAT1.
Figure 1

A

B

<table>
<thead>
<tr>
<th>Enrichment Score</th>
<th>Term</th>
<th>PValue</th>
<th>Gene Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.04</td>
<td>Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in SUMO2)</td>
<td>5.6E-20</td>
<td>123</td>
</tr>
<tr>
<td>5.06</td>
<td>RRM (RNA-binding domain)</td>
<td>1.3E-07</td>
<td>30</td>
</tr>
<tr>
<td>4.72</td>
<td>Cytoplasmic translation</td>
<td>5.4E-07</td>
<td>17</td>
</tr>
<tr>
<td>4.54</td>
<td>RNA splicing</td>
<td>3.5E-07</td>
<td>27</td>
</tr>
<tr>
<td>3.99</td>
<td>DNA repair</td>
<td>3.9E-05</td>
<td>35</td>
</tr>
<tr>
<td>2.94</td>
<td>Chaperone</td>
<td>1.2E-04</td>
<td>25</td>
</tr>
<tr>
<td>2.78</td>
<td>Mitochondrion</td>
<td>1.6E-04</td>
<td>84</td>
</tr>
<tr>
<td>1.80</td>
<td>Cytosolic small ribosomal subunit</td>
<td>2.3E-03</td>
<td>8</td>
</tr>
<tr>
<td>1.78</td>
<td>Damaged DNA binding</td>
<td>6.8E-03</td>
<td>9</td>
</tr>
<tr>
<td>1.65</td>
<td>Protein biosynthesis</td>
<td>1.3E-02</td>
<td>15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enrichment Score</th>
<th>Term</th>
<th>PValue</th>
<th>Gene Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.74</td>
<td>Nuclear testis protein, N-terminal</td>
<td>6.2E-05</td>
<td>4</td>
</tr>
<tr>
<td>3.00</td>
<td>Proteins containing BTB domain</td>
<td>4.6E-04</td>
<td>9</td>
</tr>
<tr>
<td>1.95</td>
<td>LRR 5 (Leucine-rich repeat 5) domain</td>
<td>1.8E-03</td>
<td>10</td>
</tr>
<tr>
<td>1.93</td>
<td>Phospholipase C-activating G-protein coupled receptor signaling pathway</td>
<td>3.8E-04</td>
<td>7</td>
</tr>
<tr>
<td>1.22</td>
<td>FBG (Fibrinogen)</td>
<td>2.8E-02</td>
<td>3</td>
</tr>
<tr>
<td>1.19</td>
<td>Sequence-specific double-stranded DNA binding</td>
<td>4.7E-03</td>
<td>14</td>
</tr>
<tr>
<td>1.17</td>
<td>Melanogenesis</td>
<td>2.1E-02</td>
<td>5</td>
</tr>
<tr>
<td>0.95</td>
<td>Palmitate</td>
<td>3.5E-02</td>
<td>9</td>
</tr>
<tr>
<td>0.91</td>
<td>Extracellular matrix</td>
<td>1.4E-02</td>
<td>8</td>
</tr>
<tr>
<td>0.90</td>
<td>PH (Pleckstrin homology domain)</td>
<td>7.2E-02</td>
<td>7</td>
</tr>
</tbody>
</table>
**Figure 4**

### A

#### Asp

<table>
<thead>
<tr>
<th></th>
<th>ARR</th>
<th>DU.528</th>
<th>HSB2</th>
<th>CCRF_M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp [ppm]</td>
<td>3.9</td>
<td>3.9</td>
<td>3.9</td>
<td>3.9</td>
</tr>
<tr>
<td>55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>144</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>145</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### UTP

<table>
<thead>
<tr>
<th></th>
<th>ARR</th>
<th>DU.528</th>
<th>HSB2</th>
<th>CCRF_M</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTP [ppm]</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>105</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>105.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
</tr>
<tr>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Myo-Inositol

<table>
<thead>
<tr>
<th></th>
<th>ARR</th>
<th>DU.528</th>
<th>HSB2</th>
<th>CCRF_M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo-Inositol [ppm]</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
</tbody>
</table>

### B

#### 

- **13C incorporation into Asp**

- **13C incorporation into UTP**
Figure 7

A

Human CD45^+ cells in blood

<table>
<thead>
<tr>
<th></th>
<th>shGFP</th>
<th>shSLC1A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold Increase D22/D15</td>
<td>**</td>
<td></td>
</tr>
</tbody>
</table>

B

Survival

+ Dox

C

TdTomato^+ / hCD45^+ cells in blood

<table>
<thead>
<tr>
<th></th>
<th>shGFP</th>
<th>shSLC1A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ARR (T I/ETP)</td>
<td>DU.528 (T II)</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>CD34</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CD1a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure S2**

A

![Cell Proliferation](image)

B

<table>
<thead>
<tr>
<th></th>
<th>Oncogenic subtype</th>
<th>Notch1 Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARR</td>
<td>ETP</td>
<td>-</td>
</tr>
<tr>
<td>DU.528</td>
<td>TAL</td>
<td>WT</td>
</tr>
<tr>
<td>HSB2</td>
<td>TAL</td>
<td>WT</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>TAL</td>
<td>Mut</td>
</tr>
<tr>
<td>Molt-4</td>
<td>TAL</td>
<td>Mut</td>
</tr>
<tr>
<td>Jurkat</td>
<td>TAL</td>
<td>Mut</td>
</tr>
</tbody>
</table>
Figure S3

A

<table>
<thead>
<tr>
<th></th>
<th>ARR</th>
<th>DU.528</th>
<th>HSB2</th>
<th>CCRF_M</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0</td>
<td>12</td>
<td>3.8</td>
<td>1.7</td>
<td>0.8</td>
</tr>
<tr>
<td>D2</td>
<td>23</td>
<td>13.5</td>
<td>3.2</td>
<td>5.0</td>
</tr>
<tr>
<td>D4</td>
<td>11</td>
<td>10.5</td>
<td>2.6</td>
<td>5.5</td>
</tr>
<tr>
<td>D8</td>
<td>10</td>
<td>0.8</td>
<td>5.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>-Gln</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARR</td>
<td>56% 31% 13%</td>
<td>60% 30% 10%</td>
</tr>
<tr>
<td>DU.528</td>
<td>60% 36% 7%</td>
<td>60% 16% 23%</td>
</tr>
<tr>
<td>HSB2</td>
<td>57% 33% 12%</td>
<td>72% 20% 8%</td>
</tr>
<tr>
<td>CCRF_M</td>
<td>52% 40% 8%</td>
<td>63% 25% 8%</td>
</tr>
</tbody>
</table>
Figure S4

A

Alanine

Intensity [AU]

0h

20

8h

24h

1.5

1.48

1H [ppm]

Black: ARR

Blue: DU.528

Green: HSB2

Red: CCRF_M

B

13C [ppm]

Fumarate

ARR

DU.528

HSB2

CCRF_M

138.2

6.60

6.52

6.60

6.52

6.60

6.52

6.60

6.52

1H [ppm]

13C [ppm]

Malate

ARR

DU.528

HSB2

CCRF_M

73.2

4.32

4.36

4.32

4.36

4.32

4.36

4.32

4.36

1H [ppm]

13C [ppm]

Oxaloacetate

ARR

DU.528

HSB2

CCRF_M

30.0

2.4

2.4

2.4

2.4

1H [ppm]

13C [ppm]

Proline

ARR

DU.528

HSB2

CCRF_M

31.8

4.2

4.15

4.2

4.15

4.2

4.15

4.2

4.15

1H [ppm]

0h

8h

24h
### SLC1A3 expression

<table>
<thead>
<tr>
<th>FPKM</th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
<th>FPKM</th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC1A3</td>
<td>3</td>
<td>50</td>
<td>32</td>
<td>14</td>
<td>9</td>
<td>133</td>
<td>85</td>
</tr>
</tbody>
</table>

### Maturation stage

<table>
<thead>
<tr>
<th></th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-cortical</td>
<td>10</td>
<td>50</td>
<td>35</td>
<td>5</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>cortical</td>
<td>1</td>
<td>53</td>
<td>29</td>
<td>17</td>
<td>2</td>
<td>83</td>
</tr>
<tr>
<td>post-cortical</td>
<td>8</td>
<td>40</td>
<td>44</td>
<td>8</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>N/A</td>
<td>3</td>
<td>46</td>
<td>31</td>
<td>21</td>
<td>1</td>
<td>18</td>
</tr>
</tbody>
</table>

### Oncogenic subtype

<table>
<thead>
<tr>
<th></th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HoxA</td>
<td>6</td>
<td>73</td>
<td>18</td>
<td>3</td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>LMO1/2</td>
<td>11</td>
<td>25</td>
<td>54</td>
<td>11</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>NKX2_1</td>
<td>0</td>
<td>86</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>TAL2/1</td>
<td>0</td>
<td>35</td>
<td>32</td>
<td>34</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>TLX1/3</td>
<td>4</td>
<td>55</td>
<td>38</td>
<td>3</td>
<td>3</td>
<td>43</td>
</tr>
<tr>
<td>unknown</td>
<td>5</td>
<td>62</td>
<td>29</td>
<td>5</td>
<td>1</td>
<td>13</td>
</tr>
</tbody>
</table>

### ETP status

<table>
<thead>
<tr>
<th></th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP</td>
<td>11</td>
<td>28</td>
<td>39</td>
<td>22</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>near ETP</td>
<td>4</td>
<td>38</td>
<td>25</td>
<td>33</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>not ETP</td>
<td>3</td>
<td>58</td>
<td>31</td>
<td>9</td>
<td>4</td>
<td>84</td>
</tr>
<tr>
<td>N/A</td>
<td>3</td>
<td>45</td>
<td>34</td>
<td>18</td>
<td>2</td>
<td>33</td>
</tr>
</tbody>
</table>

### Notch mutations

<table>
<thead>
<tr>
<th></th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
<th>low (&lt;0.1)</th>
<th>medium (0.1-1)</th>
<th>high (&gt;1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mutated</td>
<td>4</td>
<td>56</td>
<td>32</td>
<td>8</td>
<td>7</td>
<td>110</td>
</tr>
</tbody>
</table>
Figure S6

A

SLC1A3 Expression

Gene Expression (TPM)

Thymocytes  T-ALL

B

SLC1A3 Expression

Gene Expression (TPM)

CD34+CD1-  CD34+CD1+
CD34+CD4+  ISP CD28+  DP CD3+
SP CD4+  DP CD8+  SN CD1+
9d CD1-
Figure S7

**SLC1A3 mRNA level**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARR -dox</td>
<td></td>
</tr>
<tr>
<td>ARR +dox</td>
<td></td>
</tr>
<tr>
<td>DU528 -dox</td>
<td></td>
</tr>
<tr>
<td>DU528 +dox</td>
<td></td>
</tr>
<tr>
<td>Kasumi-1</td>
<td></td>
</tr>
</tbody>
</table>

Figure S8

**Cell number vs. Days**

- **ARR**
- **HUVEC**
- **Kasumi-1**
- **Molt4**
- **Jurkat**

Cell lines: Arr, HUVEC, Kasumi-1, Molt4, Jurkat.
Figure S9

Human CD45+ cells in blood

- shGFP
- shSLC1A3

D15
D22
Supplemental Figure Legends

**Figure S1.** Immunophenotypes of the T-ALL cell lines, with their EGIL classification, used within this study Sandberg et al. (2007).

**Figure S2.** A) Molt-4 and Jurkat cell lines were cultured in medium with 10% FCS, with or without 2mM GlutaMax. Four independent cell cultures were assayed per cell line and each point represents the mean ± StDev. B) Indication of oncogenic subgroup and Notch1 mutation status for the T-ALL cell lines used within this study (Etchin et al., 2013).

**Figure S3.** A) Glutamine deprivation induces apoptosis in T-ALL cell lines. Scatter plots show flow-cytometry Annexin V and propidium iodide staining by flow-cytometry of cells grown in RPMI media supplemented with 10% FBS in the absence of glutamine. Gating was determined based on the staining of T-ALL cells grown in the RPMI media supplemented with 2mM glutamine. Cells were grown for the indicated number of days (D0-D8) prior to staining and analysis. B) Cell cycle analysis after removal of glutamine. Cells were cultured for 4 days in the absence of glutamine from the medium and analysed by flow cytometry after the addition of DAPI to the samples. Live cells were gated and percentages indicate cells in the G0/G1, S, and G2 phase of the cell cycle respectively.

**Figure S4.** Metabolite tracing experiments using [2,5-15N]Glutamine and [3-13C]Glutamine. T-ALL cell lines were grown in the presence of 2mM [2,5-15N]Glutamine) (A) or [3-13C]Glutamine (B-D). A) Overlay of 1D 1H-NMR spectra showing the Hβ-alanine resonance after 0, 8 and 24h and schematic representations of [2,5-15N]Glutamine and the observed [2-15N]Alanine. 15N are in red and shading indicates the observed 3J scalar couplings between the alanine Hβs and glutamine-derived 15N. The X-axis shows the chemical shift relative to TMSP in ppm and the Y-axis indicates TSA scaled intensity. B,C) Resonances observed in 1H-13C-HSQC for T-ALL cells grown in the presence of [3-13C]Glutamine for 24h. Resonances are marked by letters a-b. Schematics on the right show fumarate, malate, oxaloacetate and proline with colour-coded atoms based on the substrate of their origin. Blue shaded lines indicate observed couplings.

**Figure S5.** Correlations between SLC1A3 expression levels and maturations stage, oncogenic subtype, or ETP status of 265 patient samples, as reported by Liu et al. (2017). Data are grouped by the level of expression. The left tables show the percentage of patients within each group, whereas the right panels show absolute counts. Significant differences between the total distribution pattern of expression levels and those of different subgroups
were determined using a Chi-square test and are indicated by shading and bold numbers. Additionally, percentages that changed more than a factor 1.5 compared to the percentages in the top panel (SLC1A3 expression), and were representative of more than 3 patient samples, are shown in green if raised and in red if reduced.

**Figure S6.** Comparison of SLC1A3 expression levels between healthy T-cell progenitors and T-ALL patient samples. Published RNAseq data for ten healthy thymocyte progenitor subsets (Roels et al., 2020) and sixty T-ALL patient samples (Verboom et al. 2018) were analyzed. (A) Overall comparison between the two data sets indicates a statistically significant difference (p<0.05). (B) The same data subdivided according to isolated T-cell progenitor stages and T-ALL oncogenic subtypes as determined in Verboom et al (2018). Purified thymocyte populations are: CD34+CD1- (CD34⁺CD1a⁻CD4⁻), CD34+CD1+ (CD34⁺CD1a⁺CD4⁻), CD34+CD4+ (CD34⁺CD1a⁺CD4⁺), ISP CD4+CD28+ (CD4⁺CD34⁺CD3⁺CD8⁻HLA-DR⁻CD28⁺), DP CD3– (CD4⁺CD8⁻CD3⁻), DP CD3+ (CD4⁺CD8⁺CD3⁺), SP CD4+ (CD4⁺CD8⁻CD3⁺), SP CD8+ (CD4⁻CD8⁺CD3⁺), gd CD1a+ (CD3⁺TCRγδ⁺CD1a⁺), gd CD1a– (CD3⁺TCRγδ⁻CD1a⁻). IMM; immature oncogenic subgroup of T-ALL. TPM; transcripts per million.

**Figure S7.** SLC1A3 mRNA expression level in T-ALL cell lines ARR and DU.528, in the presence or absence of shSCL1A3 knockdown, and the AML cell line Kasumi-1. Expression was measured by qPCR and shown relative to rRNA level. Data are the average ± StDev.

**Figure S8.** The effect of EAAT1 inhibition on individual cell lines, compared to the vehicle control (DMSO). Cell lines were cultured for six days in the presence of vehicle (DMSO), 25 μM UCPH-101, or 25 μM UCPH-102. Each data point is an average of three or four independent measurements ± StDev.

**Figure S9.** Mice were injected (iv) with 3 x10^5 cells CCRF-CEM cells carrying doxycycline-inducible shSLC1A3_2 or shGFP. At day 16, the food was supplemented with doxycycline. The bar diagram shows the frequency of human CD45⁺ cells in the two cohorts of mice (n=5) prior to (day 15) and after 6 days of doxycycline treatment (day 22).

**Table S1 provided as Excel file only.**
List of Differentially expressed genes.

**Table S2 provided as Excel file only.**
Gene functional annotation clusters identified for cluster C1 in Figure 1.
Table S3 provided as Excel file only.
Gene functional annotation clusters identified for cluster C2 in Figure 1.

Table S4 provided as Excel file only.
Gene lists of gene functional annotation clusters shown in Figure 1B.
Experimental Procedures

Cell Culture

T-ALL cell lines were grown in RPMI 1640 medium (Merck) supplemented with 10% FCS, Pen/Strep, 2mM Glutamine or GlutaMax (LifeTechnologies) and 0.075mM 1-Thyoglycerol (Merck) in a humidified incubator at 37°C and 5% CO₂. Where indicated, 25μM UCPH-101 or UCPH-102 (Abcam) was added. For labelling experiments, glutamine-free RPMI was supplemented with 2mM [1,5-15N]-L-Glutamine or [3-13C]-L-Glutamine (Merck).

For in vitro differentiation of human T-cell progenitors, mononuclear cells were isolated from cord blood using Lymphoprep™ (StemCell Technologies); HBRC Application 15-224 under generic approval 15/NW/0079. Red blood cells were lysed with 1x Lysing buffer (BD Biosciences) and CD34+ cells were isolated using a human CD34 MicroBead Kit (Miltenyi Biotec). CD34+ cells were co-cultured on 80% confluent OP9-DL4 cells in αMEM medium, supplemented with 5 ng/ml human FLT3L (Peprotech) and 5 ng/ml human IL7 (Peprotech). Cells were sorted at specific time points for RNA isolation and subsequent RNA sequencing; day 7 for CD7+,CD5-,CD1a- cells, day 14 for CD7+,CD5-,CD1a+ cells, and day 21 for CD7+,CD5- CD1a+,CD3- cells. Purification of cell populations was performed using a BD FACSARia Fusion (BD Biosciences) cell sorter.

CD4+ and CD8+ T cells were isolated using Miltenyi Microbeads (CD4 Cat#130-045-101, CD8 Cat#130-045-201) from mononuclear cells separated as above from leukocyte cones (NHS Blood and Transplant); University of Birmingham STEM Ethics ERN_17-1743. CD4+/CD8+ T cells were either left unstimulated or stimulated with Immunocult T cell activator (Stemcell Technologies Cat#10991) for 48 hours prior to snap freezing and RNA extraction.

Expression analysis

RNA isolation and cDNA synthesis was performed as previously described (Stanulovic et al 2017). Quantitative PCR (qPCR) was performed using an ABI 7500 Real-Time PCR System. Quantitation was carried out using a standard curve of serial dilutions and relative to rDNA. Primers used for quantification: SLC1A3F 5’AAGAGAAACAATGGCGTGGAC, SLC1A3R 5’ATTCCAGCTGCCCAATACT, 18SrRNAF 5’GGCCCGAAGCGTTTACTTTGA, 18SrRNAR 5’GAACCGCGGTCTCCTATCCATT.

RNaseq

RNaseq libraries were prepared and indexed using the TruSeq Stranded mRNA Sample Preparation Kit LH (Illumina) according to the manufacturer’s protocol. Libraries were pooled and sequenced as 100 nt paired-end on Illumina HiSeq 2500 sequencer at a depth of approximately 30 million reads per library.
Reads acquired from RNASeq were mapped as stranded libraries to the human genome (GRCh38), using HISAT2 at usegalaxy.org. Transcripts were assembled using StringTie and GENCODE gene annotation with quartile normalisation and effective length correction. Duplicate biological replicates were used. Differential gene expression was determined by DESeq2 between T-ALL samples and CD34+ hematopoietic progenitors and in vitro differentiated samples (CD7+ CD5- CD1α−, the CD7+ CD5+ CD1α−, and the CD7+ CD5+ CD1α+ CD3+). Normalised gene counts of the significantly differentially expressed genes were used to select the gene IDs that were significantly differentially expressed. Pearson average linkage hierarchical clustering and heat maps were computed by Multi Experiment Viewer software. Gene functional annotation clustering was performed using DAVID v2023q. Clusters with enriched terms with Modified Fisher Extract P-value <0.05 were considered significant, categories with redundant terms were filtered out. Data has been deposited at NCBI-GEO GSE101566.

**Western blot analysis**

Cell extracts were prepared by lysing cells in RIPA buffer (50mM TRIS pH8, 150mM NaCl, 0.5% deoxycholic acid, 1% NP40, 0.1% SDS) on ice for 20 min. The insoluble fraction was precipitated by centrifuging at 20,000 g for 10min. Protease inhibitor and PhosSTOP (Roche) were used 1:100.

Proteins were separated on 4-12% gradient Bis-Tris Plus Bolt Mini Gels (LifeTechnologies), transferred to nitrocellulose membranes and stained with PonceauS to confirm equal loading prior to o/n incubation with antibodies. Primary antibodies raised against EAAT1 (D4166; Cell Signalling) and GFP (GF28R; eBiosciences) were used at a final concentration of 1µg/ml. Secondary antibodies, IRDye 680RD or 800RD (Li-Cor), were used at a 0.5µg/ml. Westerns were visualised using an Odyssey CLx Imager (Li-Cor).

**Immunofluorescent Staining**

Cells were fixed with 2% formaldehyde for 10min and washed twice in PBS/0.05%Tween/2%FCS, followed by o/n incubation with 1µg/ml primary antibody. Cells were washed and incubated with 1µg/ml secondary antibody conjugated to Alexa dyes (LifeTechnologies). Immuno-stained cells were deposited onto the glass slides using a Cytospin III centrifuge (Shandon). MitoTracker Red CMXRos (Invitrogen) was used for mitochondria staining as per manufacturer's instruction. Slides were dried, covered with Prolong Gold Anti-Fade DAPI reagent (LifeTechnologies) and imaged using a Zeiss LSM880 Confocal microscope.
**SLC1A3 knockdown**

SLC1A3 was amplified by PCR from pcDNA3-EAAT1. The PCR product was cloned into MigR1 in front of IRES-GFP. Short hairpin sequences targeting SLC1A3 were designed using [http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA](http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA). Designed shRNA (shSLC1A3_1 ACCATATCAACTGATTGCACAG, shSLC1A3_2 GGGTAACTCAGTGATTGAAGAG, shSLC1A3_3 GTGGCACACAATCCTATAAATG, shSLC1A3_4 AGGCCTCAGTGTCCTCATCTAT and shSLC1A3_5 CACTCCTCAACTGATGATAGAC) were embedded into mir30 and cloned into pMSCVhygro. The mouse fibroblast cell line PlatE was transfected using TransIT-LT1 (Mirusbio) with MigRI-SLC1A3 and MSCVhyg_shSLC1A3, MSCVhyg_shGFP, or MSCVhyg_shFF3. Functional shRNA was cloned into piggybac transposon inducible expression vector PB_tet-on_Apple_shGFP using HindIII and Kpn2L (ThermoFisher). T-ALL cell lines were electroporated with pB_shSLC1A3 and pCAGG-PBase, expanded and selected with puromycin (ThermoFisher). Expression of shRNA was induced with 0.1μg/ml doxycycline (Merck). Cells were counted every other day and the cell concentration was adjusted to 0.4x10^6/ml.

**Patient Samples**

The patients’ cells used in this study were from diagnostic samples from presentation cases before treatment. They were obtained from the Queen Elizabeth Hospital Birmingham, Birmingham, UK with ethical approval from the NHS National Research Ethics Committee (Reg:10/H1206/58). Cytogenetic abnormalities and sample immunophenotype were determined at the time of disease diagnosis at the West Midlands Regional Genetics Laboratory, Birmingham Women’s NHS Foundation Trust, Birmingham, UK.

Mononuclear cells were purified from peripheral blood by differential centrifugation using Lymphoprep (Axis-Sharp UK). Undifferentiated blast cells were isolated using anti-human CD34 (T-ALL_1 and _2) or CD7 (T-ALL_3) MACS microbeads (Miltenyi). For T-ALL_2, CD34 cells were further sorted by FACS for CD7 using anti-human CD7-FITC antibody (CD6-6B7; BioLegend).

**Intracellular NMR spectroscopy**

Methanol/chloroform extraction was used to prepare polar extracts from 5x10^7 cells. Following centrifugation, the polar phase was dried in a vacuum concentrator. Pellets were resuspended in 50μl NMR buffer (100 mM sodium phosphate pH7.0, 500μM Sodium 3-(trimethylsilyl)propionate (TMSP; Merck), 10% D_2O) and transferred to 1.7mm NMR tubes. NOESY 1D spectra with water pre-saturation were acquired using the standard Bruker pulse sequence noesygpr1d on a Bruker 600MHz spectrometer with a TCI 1.7mm z-PFG
CryoProbe™. The sample temperature was set to 300K. The $^1$H carrier was on the water frequency and the $^1$H 90° pulse was calibrated at a power of 0.326W. Key parameters were: spectral width 12.15ppm/7288.6Hz; complex data points, 16384; interscan relaxation delay, 4s; acquisition time, 2.25s; short NOE mixing time, 10ms; number of transients, 256; steady state scans, 4.

**Growth-media metabolite uptake and release**

Growth medium was collected and supplemented with 10% D$_2$O and 1mM TMSP. Samples were transferred to 5mm glass NMR tubes and spectra were acquired at 300K, using a Bruker 500 MHz spectrometer equipped with a TXI probe. The $^1$H carrier was on the water frequency and the $^1$H 90° pulse was calibrated at a power of 12.9W. Measurements were carried out after locking on deuterium frequency and shimming. The standard Bruker 1D NOESY pulse sequence (noesygpp1d) with water saturation was used. Spectra were acquired with 64 transients and 4 steady state scans.

**Live-cell real-time NMR spectroscopy**

Cells were resuspended at $10^6$/ml growth media containing 0.1% low melting agarose (Sigma), 1mM TMSP, 10% D$_2$O. Samples were loaded into 5mm NMR tubes and measurements were collected every 8.4 minutes, for a total of 100 time points. CPMG (Carr-Purcell-Meiboom-Gill) 1D spectra with water pre-saturation were acquired using the standard Bruker pulse sequence cpmgpr1d on a Bruker 500 MHz spectrometer with a TXI $^1$H/D-$^{13}$C/$^{15}$N probe at 310K. The $^1$H carrier was on the water frequency and the $^1$H 90° pulse was calibrated at a power of 12.9W. For the CPMG T$_2$ filter, a T$_2$ filter time of 68 ms arose from 100 loops over a 680 µs echo time between 180 pulses. Other key parameters were: spectral width 12.02 ppm/6009.6 Hz; complex data points, 16384; interscan relaxation delay, 4s; acquisition time, 2.73s; short NOE mixing time, 10ms; number of transients, 64; steady state scans, 4.

**NMR spectroscopy data processing and analysis**

NMR spectroscopy data was processed in Topspin (Bruker Ltd, UK), MetaboLab (version 20910688) in MATLAB (version R2015b)$^{13}$ and MetaboLabPy (https://pypi.org/project/qtmetabolabpy). All spectra were aligned to TMSP, a spline baseline correction was applied, the water and TMSP regions were excluded, and the total spectral area (TSA) of each spectrum was scaled to 1. To compare metabolite concentrations, a well-resolved peak was picked for each metabolite in the first spectrum and peaks were picked in the other spectra in an automated manner using in-house subroutines of MetaboLab (version 20910688). Spectral assignments were made using Chenomx software and acquired chemical standards.
Mouse studies

Animal experiments were performed at the University of Birmingham Biomedical Services Unit under an animal project licence (PP8841933) in accordance with UK legislation. Female NOD.Cg-Prkdscid Il2rgtm1Wjl/SzJ (NSG) mice aged 8-9 weeks at study commencement were used for the xenograft model. CCRF-CEM cells were transfected with pBshSLC1A3_2 or pBshGFP control vector and pCAGG-PBase as described above. After selection, 3x10^5 cells per mouse were injected *intra venous* using the tail vein. When approximately 1% engraftment was observed, the next day all animals were transferred to a diet supplemented with 0.625g/kg doxycycline hyclate.

References