SLC25A1 reprograms mitochondrial and fatty acid metabolism to promote the progression of acute myeloid leukemia

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Abstract

Abnormal metabolic reprogramming is a hallmark of acute myeloid leukemia (AML), contributing to leukemia initiation, progression and drug resistance. The key mitochondrial citrate transporter SLC25A1 plays an essential role in regulating cellular energy metabolism and plays an important role in the regulation of lipid metabolism. However, the role of SLC25A1 in the pathogenesis and aberrant lipid metabolism in AML remain unexplored. In this study, our analysis of public datasets and patient samples revealed that SLC25A1 expression was markedly elevated in AML and was associated with poor prognosis. Knockdown or pharmacological inhibition of SLC25A1 significantly suppressed AML cell proliferation by inducing apoptosis, without affecting cell cycle progression or differentiation. Moreover, SLC25A1 proved vital for AML tumorigenesis *in vivo*. Mechanistically, we demonstrated that SLC25A1 inhibition disrupted citrate homeostasis, leading to mitochondrial dysfunction and reduced fatty acid metabolism. Notably, we developed a novel SLC25A1 inhibitor, CTPI3, which effectively inhibits the progression of AML *in vivo*, and synergizes with venetoclax to kill AML cells by mitochondrial and fatty acid metabolism regulation. In summary, our findings highlight that SLC25A1 plays a vital role in maintaining AML cell survival and regulating its drug sensitivity. Furthermore we developed a more effective novel drug targeting SLC25A1, providing additional therapeutic options for venetoclax-resistant patients and highlighting SLC25A1 as a promising biomarker and therapeutic target for AML.

Introduction

Acute myeloid leukemia (AML) is a group of hematopoietic malignancies, with characteristics of clonal proliferation and apoptosis or differentiation blockade.[1,2] Unfortunately, patients with AML generally have poor clinical outcomes and high mortality rates, as a result of frequent drug resistance and relapse.[3] Recent studies found that a shift in the metabolic pathway is driven by oncogene activation or loss of tumor-suppressors, as well as demands of an expanding biomass contributed to tumor initiation and progression.[4] In AML, both glycolysis and oxidative phosphorylation (OxPHOS) are dysregulated and usually enhanced compared with healthy counterparts,[5] and levels of glycolysis, OxPHOS, and fatty acid oxidation markedly affect proliferation and drug sensitivity of AML cells.[6-9] Understanding metabolic dependencies of cancer cells opens possibilities for targeted therapies. Such approaches include inhibiting key glycolytic enzymes, blocking nutrient transporters, or targeting altered lipid metabolism in cancer cells.

Solute carrier (SLC) transporters are mostly located in the cell membrane and include over 400 members organized into 66 families.[10] These transporters are critical for maintaining cellular homeostasis, mediating the uptake and efflux of various metabolites, ions, and other molecules, including glucose, amino acids, neurotransmitters, and drugs. Recently, accumulating data showed that several SLC family members play roles in both supporting can-

cer cell survival and mediating drug resistance, making them attractive targets for therapeutic intervention.[11] The mitochondrial citrate carrier SLC25A1, also known as CIC, is the transporter responsible for exporting citrate from mitochondria into cytoplasm.[12,13] This transporter is the only known protein responsible for facilitating the exchange of citrate or isocitrate from mitochondria into cytoplasm, against malate.[14] In addition to its pivotal role in tricarboxylic acid (TCA) cycle, SLC25A1 is crucial for *de novo* lipogenesis and protein acetylation by regulating the levels of cytoplasmic acetyl-CoA.[15,16] The citrate transporter SLC25A1 thus connects fatty acid metabolism with mitochondrial metabolism. SLC25A1 has been identified as an oncogene in some cancers[17-19] and dysregulation of SLC25A1 activity or expression has been associated with various diseases, where it promotes cancer cell growth and resistance to energy stress-induced apoptosis. However, the potential role of SLC25A1 in the initiation and progression of hematological malignancies through metabolic regulation, particularly AML, remains poorly understood.

In this study, we found SLC25A1 expression levels were significantly higher in AML patients. Furthermore, inhibiting SLC25A1 repressed AML cell proliferation both *in vitro* and *in vivo*, and led to mitochondrial damage and reduced fatty acid metabolism in AML cells. Overall, our findings suggest SLC25A1 could serve as a promising metabolic target for AML therapy.

Methods

Detailed descriptions of the experiments can be found in *Online Supplementary Methods*.

Ethics approval and consent to participate

The experimental protocol was approved by ethics committee of The First Hospital of China Medical University ([2022] number 174). Collection of tissue and clinicopathologic information was obtained with informed consent. Animal experiments were conducted in accordance with the China Medical University Animal Care and Use Committee guidelines and approved by the Institutional Review Board of the First Hospital of China Medical University.

Transplantation

AE9a (1×10^4) or MLL-AF9 cells (1×10^5) were injected into the tail vein of sublethally irradiated (4.75 Gy) C57BL/6N recipient mice. Survival curves, spleen weight, liver weight, and AML cell infiltration in bone marrow, spleen and peripheral blood were analyzed, respectively.

TEM

Treated Kasumi-1 and THP1 cells were harvested and fixed in TEM fixative at 4°C and pre-embedded in agarose solution. The cells were then post-fixed, dehydrated at room temperature, resin penetrated and embedded, polymerized, and stained. The grids were finally observed and photographed in a TEM.

Mitochondrial respiratory capacity

The mitochondrial respiratory capacity was determined using an XF Cell Mito Stress Test Kit (Agilent Technologies). Cells were seeded in an XF cell culture microplate and incubated for 24 hours (h) at 37°C, followed by addition of base medium containing 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM glucose for 1 h prior to assay. The OCR was measured using an XF^{e}96 extracellular flux analyzer (Agilent Technologies) with sequential injection of 1.5 μM oligomycin A, 0.5 μM FCCP, and 0.5 mM rotenone/antimycin A.

RNA sequencing

Kasumi-1 cells were infected with lentivirus expressing small hairpin RNA (shRNA) against *SLC25A1* (scrambled shRNA as control). Cellular RNA was then extracted with TRIzol reagent (Invitrogen). An RNA sequencing (RNA-seq) library was constructed using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) and sequenced with Illumina Novaseq platform. Hisat2 v2.0.5 was used to align reads to genome and featureCounts v1.5.0-p3 was used to count read numbers mapped to each gene. Gene ontology (GO) analyses were performed to analyze DEG.

Targeted metabolomics

Targeted metabolomics was performed by Novogene Co., Ltd. (Beijing, China) to quantify selected metabolites using liquid chromatography-mass spectrometry (LC-MS/MS) (SCIEX QTRAP® 6500+) in Multiple Reaction Monitoring (MRM) mode. Samples underwent targeted extraction based on metabolite properties. Method validation included assessment of linearity, precision, accuracy, and stability. Data were analyzed using standard curves generated from authentic reference compounds to ensure accurate and reproducible quantification.

Lipidomic

Kasumi-1 cells were infected with lentivirus expressing shRNA against *SLC25A1* (scrambled shRNA as control). The lipids were then extracted and subjected to ultra-performance liquid chromatography-mass spectrometry (UHPLC-MS/MS), as described above.

Statistical analysis

R language (version 3.5.2) and GraphPad Prism 8 were mainly used for statistical analysis and figure drawing. Kaplan-Meier survival analysis was used to indicate prognostic values. Pearson correlation analysis was performed to test correlation of two variants. Two-tailed *t* test was performed to calculate quantitative difference between two groups. Statistical significance was defined as $P<0.05$.

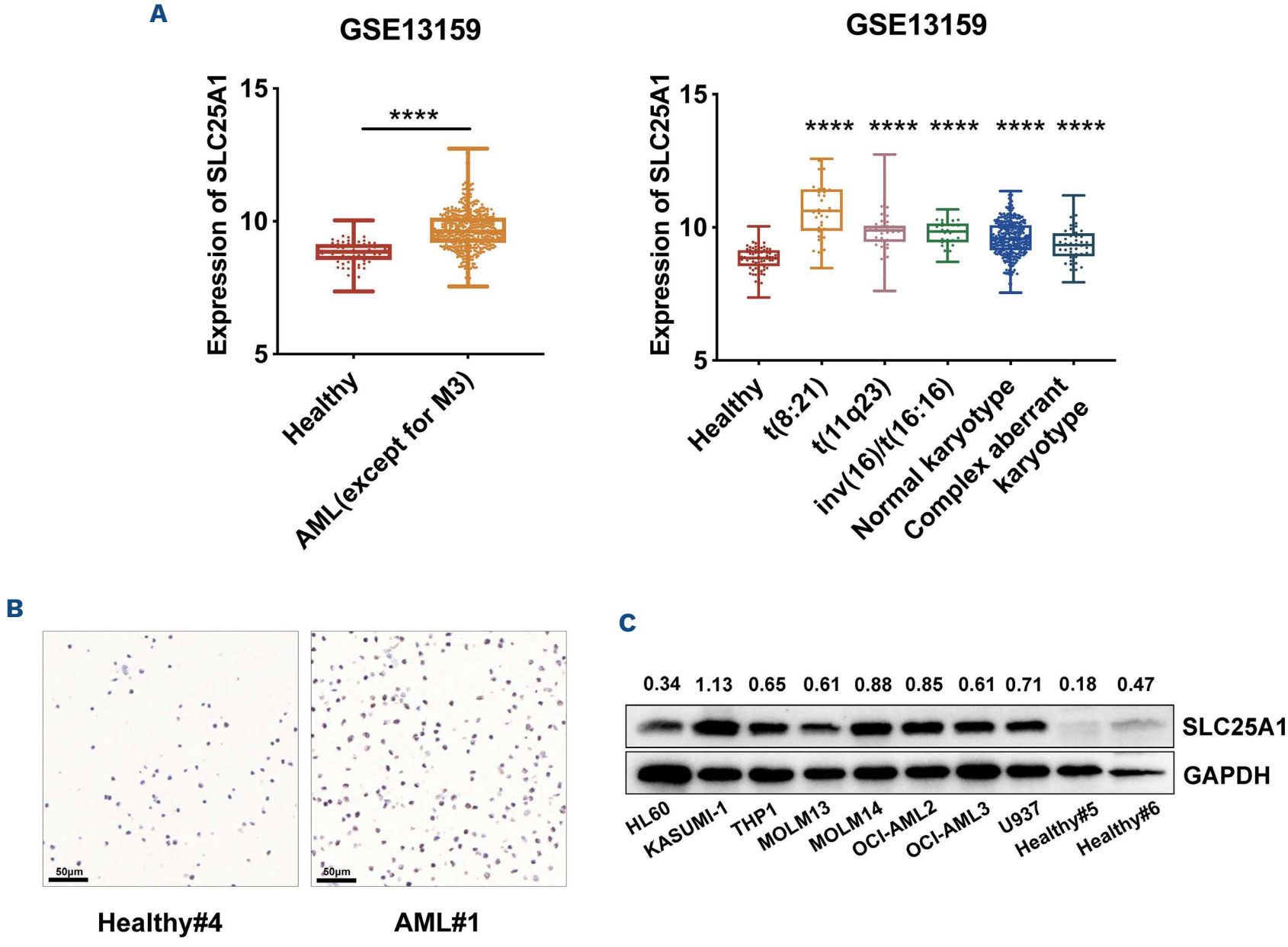
Results

SLC25A1 levels were elevated in acute myeloid leukemia

We investigated role of SLC25A1 in AML by analyzing CRISPR (DepMap 22Q2 Public+Score, Chronos) data for 26 AML cell lines. Chronos scores of SLC25A1 in most cell lines were <0, strongly suggesting that SLC25A1 was crucial in AML cells (*Online Supplementary Figure 1A*). Further analysis using GSE13159 AML database indicated a significant elevation of *SLC25A1* mRNA across different AML subtypes with specific genetic backgrounds, relative to healthy counterparts (Figure 1A). We confirmed this by using quantitative polymerase chain reaction (qPCR) analysis to analyze *SLC25A1* mRNA in AML samples from our hospital, which revealed notably higher expression in non-APL AML, compared to healthy controls (*Online Supplementary Figure S1B*). Protein levels of SLC25A1, validated by histochemistry and western blot, were also higher in AML patients (Figure 1B; *Online Supplementary Figure S1C*) and in most human AML cell lines (Figure 1C). Additionally, data from DepMap reveals that Kasumi-1 cells and THP1 cells exhibit comparatively high levels of SLC25A1 mRNA and protein expression among AML cell lines (*Online Supplementary Figure S1D, E*). To further explore roles of SLC25A1 in leukemia stem cells (LSC), we respectively analyzed two RNA sequencing databases of AML (GSE76009 and GSE230423) and found there was no significant difference in expression of SLC25A1 in LSC and blasts, indicating SLC25A1 was not a specific target in LSC (*Online Supplementary Figure S1F*). In distinct cohorts of AML patients, cases with higher *SLC25A1* expression were significantly associated with poorer outcomes (Figure 1D). Collectively, these findings underscore potential roles of SLC25A1 in the initiation and progression of AML.

SLC25A1 was crucial for the survival of acute myeloid leukemia cells

To explore the role of SLC25A1 in AML cell survival, we knocked down *SLC25A1* using two different shRNA (shRNA; shSLC25A1-1 and shSLC25A1-2) in AML cell lines that showed high levels of SLC25A1. Both shRNA effectively decreased expressions of SLC25A1 at both mRNA and protein levels (*Online Supplementary Figure S2A*). The knockdown (KD) of *SLC25A1* significantly inhibited proliferation and clonogenicity of Kasumi-1 and THP1 cells (Figure 2A, B; *Online Supplementary Figure 2B*). Furthermore, we extended our study to primary myeloid leukemia cells,



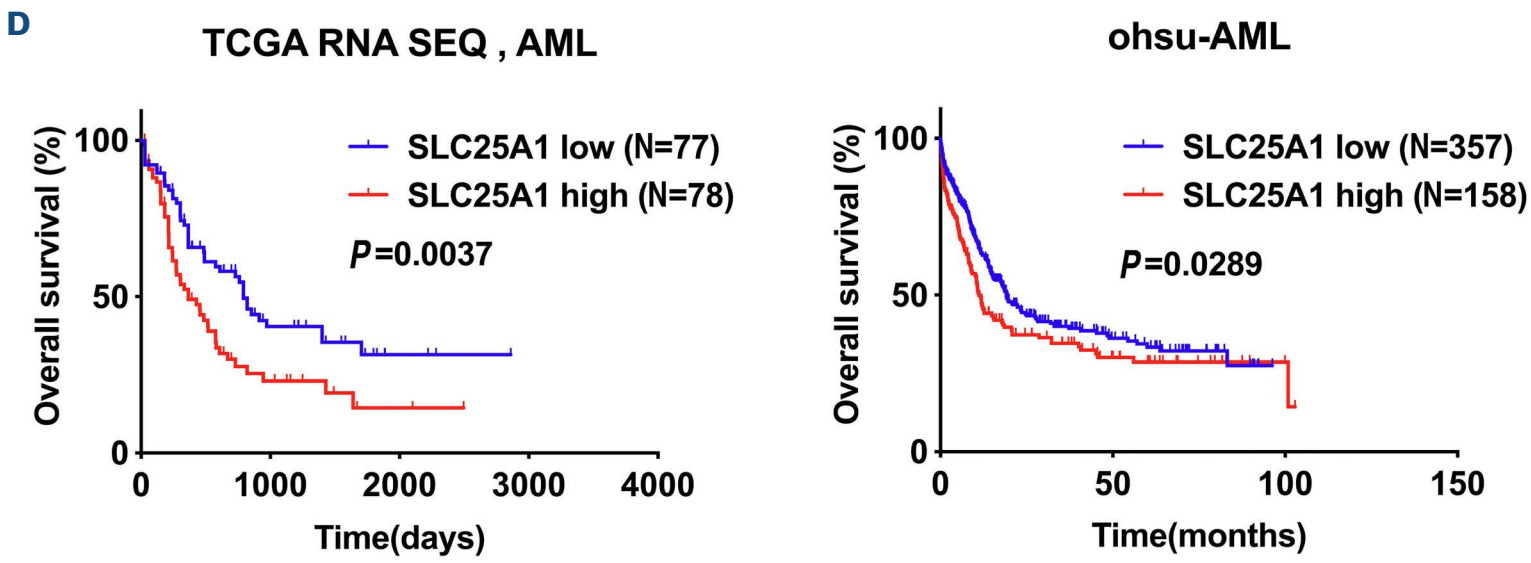


Figure 1. SLC25A1 is highly expressed in acute myeloid leukemia. (A) The mRNA expression of *SLC25A1* in healthy donors (N=74) and acute myeloid leukemia (AML) patients (N=542) in gene set enrichment (GSE)13159 database; mRNA expression of *SLC25A1* in healthy donors (N=74) and AML patients of various subtypes, including t(8:21) (N=40), t(11q23) (N=38), inv(16)/t(16:16) (N=28), Normal karyotype (N=351) and complex aberrant karyotype (N=48) in GSE13159 database. (B) The immunohistochemistry analysis of SLC25A1 protein expression in bone marrow mononuclear cells from healthy donors (N=3) and AML patients (N=3). (C) The western blotting analysis of SLC25A1 expression in healthy donors and various patient-derived AML cell lines. (D) The survival analysis of SLC25A1 in 2 AML databases (log-rank test).

where *SLC25A1* KD markedly reduced colony formation in AML#5 patient blasts (Figure 2B; *Online Supplementary Figure S2F*). To clarify mechanisms leading to the inhibition of cell proliferation, we assessed apoptosis, cell cycle, and cell differentiation in Kasumi-1 and THP1 cells, discovering that *SLC25A1* KD primarily increased apoptosis without impacting cell cycle or differentiation (Figure 2C; *Online Supplementary Figure S2C-E*). Additionally, inhibiting SLC25A1 expression also halted proliferation of MOLM14 and OCIAML2 cell lines (*Online Supplementary Figure S2G*). These findings indicate that SLC25A1 affects AML cell survival mainly by enhancing apoptosis.

HL60 cells displayed the lowest SLC25A1 levels among tested AML cell lines (Figure 1C). Notably, reducing SLC25A1 expression in HL60 cells had no impact on cell proliferation, clonogenicity, apoptosis, cell cycle, or differentiation (*Online Supplementary Figure S3A-E*). Conversely, overexpressing SLC25A1 in HL60 cells promoted cell proliferation and increased colony formation (*Online Supplementary Figure S3F, G*), yet it did not affect cell cycle or differentiation (*Online Supplementary Figure S3H, I*).

We treated Kasumi-1 and THP1 cells with the specific SLC25A1 inhibitor CTPI2, which reduced their proliferation and clonogenicity (Figure 2D; *Online Supplementary Figure S4A*). CTPI2 also inhibited growth of MOLM14 and OCIAML2 cells (*Online Supplementary Figure S4B*). CTPI2 treatment decreased viability of primary human AML cells (AML#1, AML#2, AML#4, AML#5) without affecting proliferation of healthy mononuclear cells (MNC) (Healthy#7, Healthy#8, Healthy#9, Healthy#10) at the same concentrations (Figure 2D; *Online Supplementary Figure 4C*). Furthermore, CTPI2 treatment enhanced apoptosis in Kasumi-1 and THP1 cells (*Online Supplementary Figure S4D*).

These results suggest that SLC25A1 plays a crucial role in survival of AML cells with high SLC25A1 expression but is not essential for survival of healthy MNC with low SLC25A1 expression.

SLC25A1 was required for the progression of acute myeloid leukemia *in vivo*

To further investigate necessity of SLC25A1 for AML maintenance and progression *in vivo*, we knocked down *SLC25A1* using shRNA (SLC25A1 KD) in murine AML cell lines, AE9a and MLL-AF9 (*Online Supplementary Figure S5A*). SLC25A1 KD significantly attenuated growth of these cell lines (Figure 3A). Moreover, CTPI2 markedly reduced survival of both AE9a and MLL-AF9 cells, at appropriate concentrations (*Online Supplementary Figure S5B*). Additionally, *SLC25A1* KD in AE9a leukemic stem cells (LSC) and MLL-AF9 leukemic granulocyte-macrophage progenitors (LG-MP) significantly decreased colony numbers, indicating a reduction of stemness (Figure 3B; *Online Supplementary Figure S5C, D*).

Following treatment, AE9a or MLL-AF9 cells were transplanted into sub-lethally irradiated recipient mice. The recipients of SLC25A1 KD cells exhibited extended survival (Figure 3C), smaller spleens and livers (Figure 3D; *Online Supplementary Figure S5E*), and lower percentages of leukemia blast cells (GFP^+ c-Kit^+ or GFP^+ $Mac\text{-}1^-$) in bone marrow, spleen, and peripheral blood (Figure 3E; *Online Supplementary Figure S5F*), compared to control mice. Histological examinations revealed fewer leukemia blasts in bone marrow and spleen of SLC25A1 KD recipients than in controls (*Online Supplementary Figure S5G*). In conclusion, these results underscore the critical role of SLC25A1 in supporting AML progression *in vivo*.

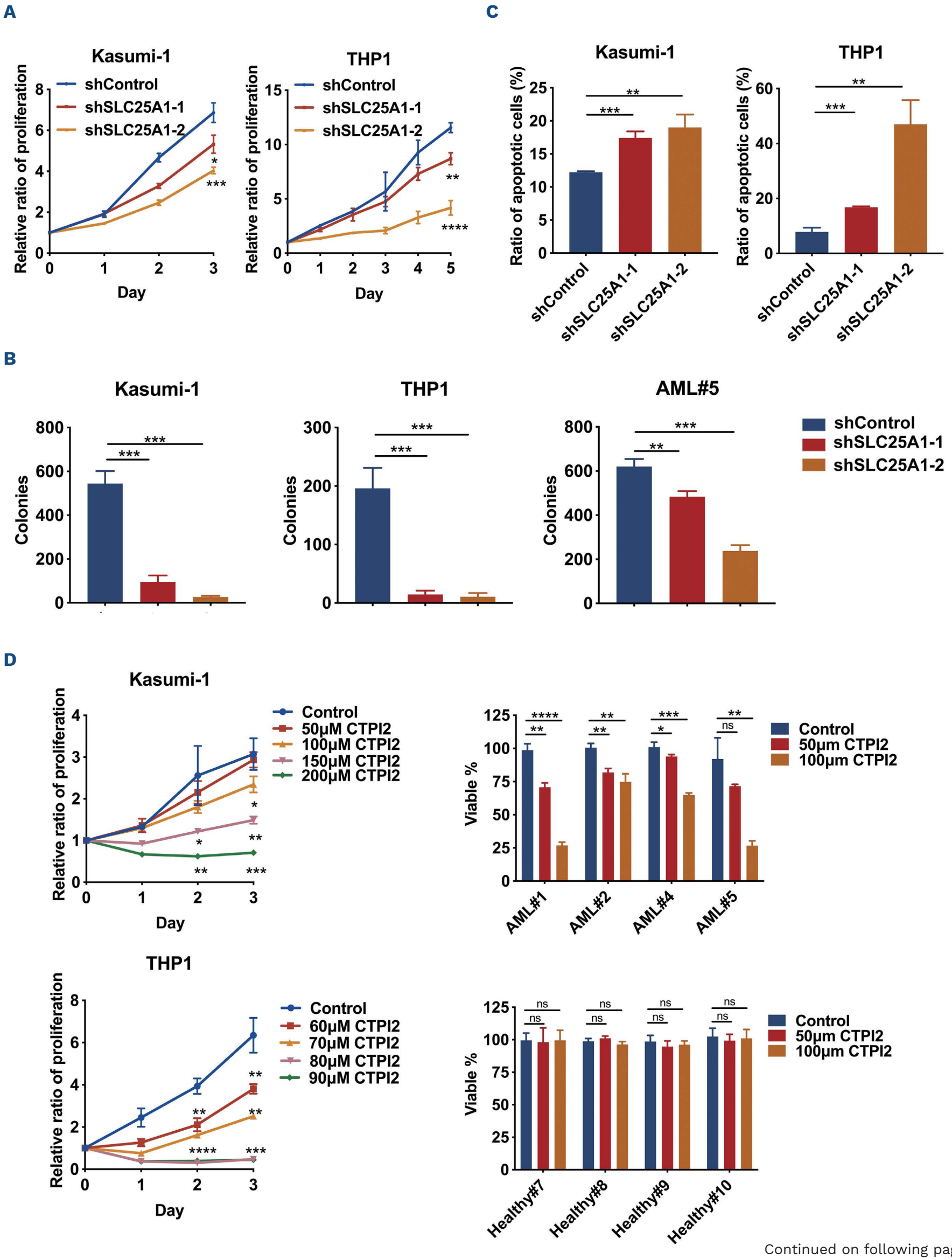


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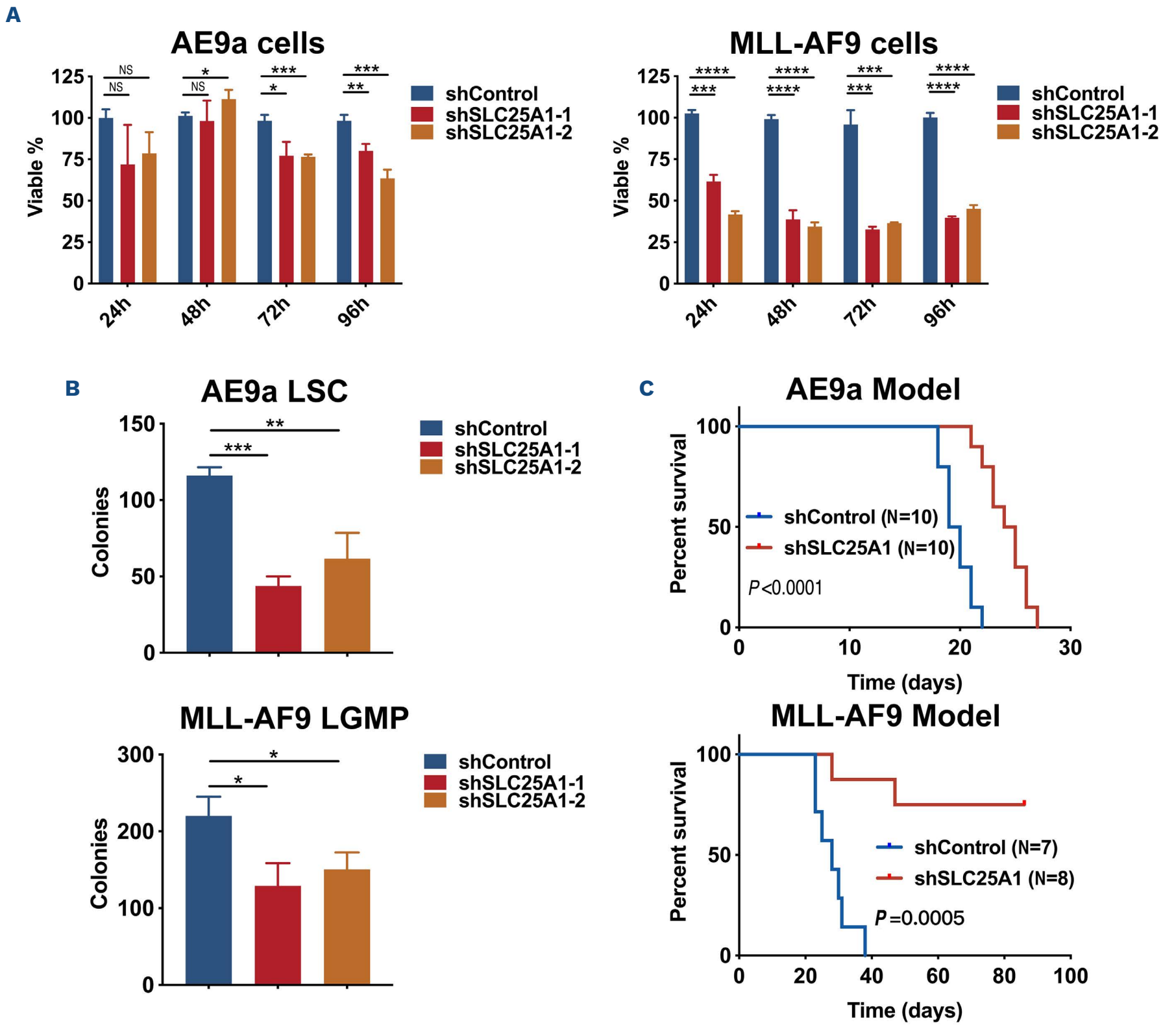
Figure 2. SLC25A1 is required for survival of acute myeloid leukemia cells *in vitro*. (A) The growth of Kasumi-1 and THP1 cells was inhibited after *SLC25A1* knockdown (KD) with small hairpin control (shControl), shSLC25A1-1 and shSLC25A1-2 lentiviruses. (B) The colony formation assay of Kasumi-1, THP1 cells and human primary acute myeloid leukemia (AML) cells (AML#5) after *SLC25A1* KD with shControl, shSLC25A1-1 and shSLC25A1-2 lentiviruses. (C) The percentage of apoptotic leukemia cells at day 2 after *SLC25A1* KD. (D) The growth of Kasumi-1 and THP1 cells was inhibited after SLC25A1 inhibitor (CTPI2) application. The growth of human primary AML cells (AML#1, #2, #4, #5) cells was inhibited after CTPI2 application, but the growth of mononuclear cells from healthy bone marrow (Healthy#7, #8, #9, #10) was not affected.

SLC25A1 deficiency compromised mitochondrial function and resulted in elevated reactive oxygen species level

Given that SLC25A1 is the citrate transporter on the mitochondrial membrane, we investigated its impact on mitochondrial function in AML cells. After *SLC25A1* KD or CTPI2 treatment, transmission electron microscopy (TEM) revealed a significant reduction in mitochondrial length, indicating structural damage (Figure 4A; *Online Supplementary Figure 6A*). Additionally, we assessed mitochondrial membrane potential using JC-1 probe via flow cytometry and fluorescence microscopy. The increased ratio of JC-1 monomers in cells with SLC25A1 KD or CTPI2 treatment



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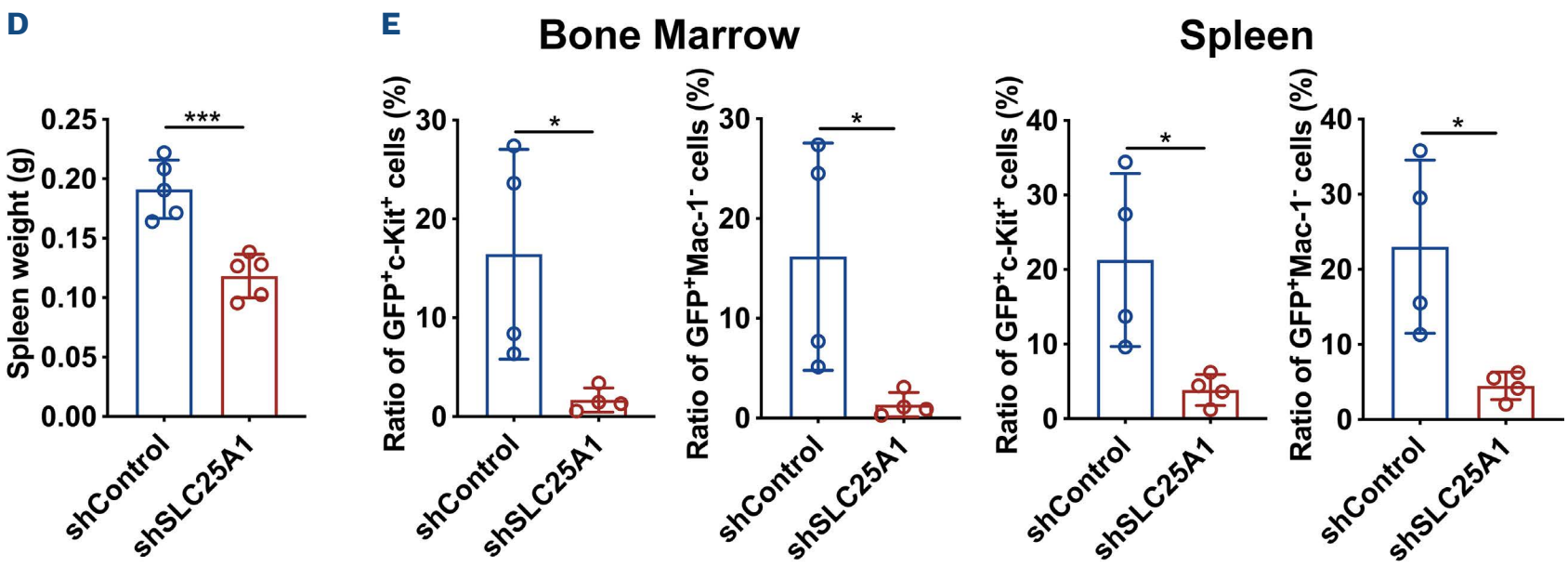


Figure 3. SLC25A1 is required for survival of acute myeloid leukemia cells *in vivo*. (A) The growth of AE9a cells and MLL-AF9 cells was inhibited after *SLC25A1* knockdown (KD) with small hairpin control (shControl), shSLC25A1-1 and shSLC25A1-2 lentiviruses. (B) The colony formation assay of AE9a leukemic stem cells (LSC) and MLL-AF9 leukemic granulocyte-macrophage progenitors (LGMP) after *SLC25A1* KD. (C) Kaplan-Meier survival curves of recipient mice transplanted with AE9a cells and MLL-AF9 cells after *SLC25A1* KD. A log-rank test was performed. (D) The weight changes of the spleen in recipient mice transplanted with AE9a cells after *SLC25A1* KD. (E) The frequencies of $GFP^+c\text{-}Kit^+$ or GFP^+Mac1^- leukemia blast cells in the bone marrow and the spleen of recipient mice transplanted with shControl AE9a cells and shSLC25A1 AE9a cells.

suggested a decrease in mitochondrial membrane potential (Figure 4B; *Online Supplementary Figure S6B*, C). Further examination of mitochondrial function through extracellular flux analysis showed suppressed oxygen consumption rates (OCR) in cells expressing shSLC25A1 or treated with CTPI2 (Figure 4C; *Online Supplementary Figure S5D*). These results suggest SLC25A1 deficiency impairs mitochondrial function in AML cells.

ROS are indicators of mitochondrial impairment. Consequently, we employed flow cytometry and fluorescence microscopy to measure ROS levels, confirming their increase following SLC25A1 suppression (Figure 4D; *Online Supplementary Figure S6E, F*). To assess whether ROS accumulation participated in AML cell mortality, we neutralized ROS in SLC25A1-inhibited cells using scavenger N-acetyl-L-cysteine (NAC). The restoration of cell viability by NAC in the context of SLC25A1 inhibition suggested SLC25A1 deficiency inhibited cell survival through the upregulation of ROS (Figure 4E; *Online Supplementary Figure 6G*).

Given this, we explored the interaction between SLC25A1 inhibition and BCL-2 inhibitor venetoclax, which suppresses cell growth partly through ROS induction.[20,21] Treatment of AML cells with venetoclax in SLC25A1 KD group resulted in heightened drug sensitivity compared to the control (*Online Supplementary Figure S7A*). Furthermore, addition of CTPI2 to treatments with venetoclax led to a synergistic suppression of AML cell growth (*Online Supplementary Figure S7B*). We also detected the effect on cell apoptosis by flow cytometry and found ratio of apoptotic cells was significantly increased in cells treated with CTPI2 plus venetoclax (*Online Supplementary Figure S7C*). An examination of ROS levels across control, single treatment, and combination treatment groups demonstrated the combination yielded the highest ROS levels (*Online Supplementary Figure 7D*). Collectively, these results indicate the potential of SLC25A1 inhibitor CTPI2 to enhance sensitivity of ROS-generating drugs like venetoclax by synergistically enhancing ROS-induced apoptosis.

Deletion of SLC25A1 disrupted fatty acid metabolism

To investigate deeper mechanism of SLC25A1 in AML, we analyzed *SLC25A1*-related genes (R>0.5) within The Cancer Genome Atlas (TCGA) and Vizome AML databases[22] using GO, revealing a substantial metabolic association in nearly half of identified GO terms (*Online Supplementary Figure S8A, B*). We also performed RNA-seq analysis of control and *SLC25A1* KD AML cells. GO analysis of differentially expressed genes (DEG) and GSEA showed SLC25A1 was closely correlated with ROS metabolic process, extrinsic apoptotic signaling pathway and oxidative phosphorylation (*Online Supplementary Figure S8C*). Besides the pathways stated above, we also found that metabolic pathways, including nicotinate metabolism and fatty acid metabolism, were enriched by analyzing DEG (*Online Supplementary Figure S8C*). We further analyzed differences in metabolites between the control and *SLC25A1* KD groups by metabolomics, finding that a series of fatty acid metabolism pathways were enriched, including α linolenic acid and linoleic acid metabolism, biosynthesis of unsaturated fatty acids, carnitine synthesis and transfer of acetyl groups into mitochondria (Figure 5A). Moreover, fatty acid homeostasis was enriched in SLC25A1-high group (*Online Supplementary Figure 8D*). Combining GO analysis of RNA-seq and metabolomics, we focused on changes in fatty acid metabolism (fatty acid

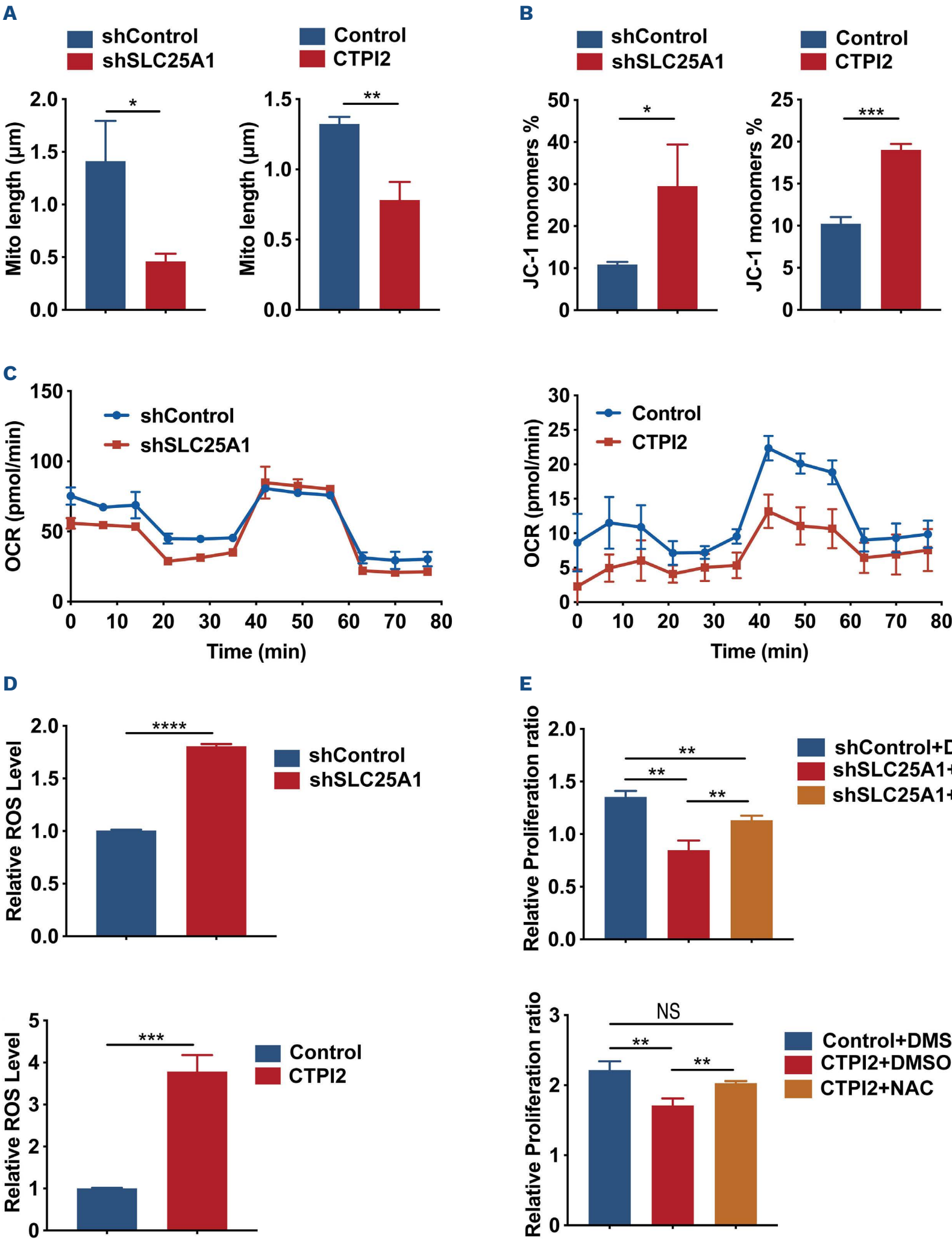
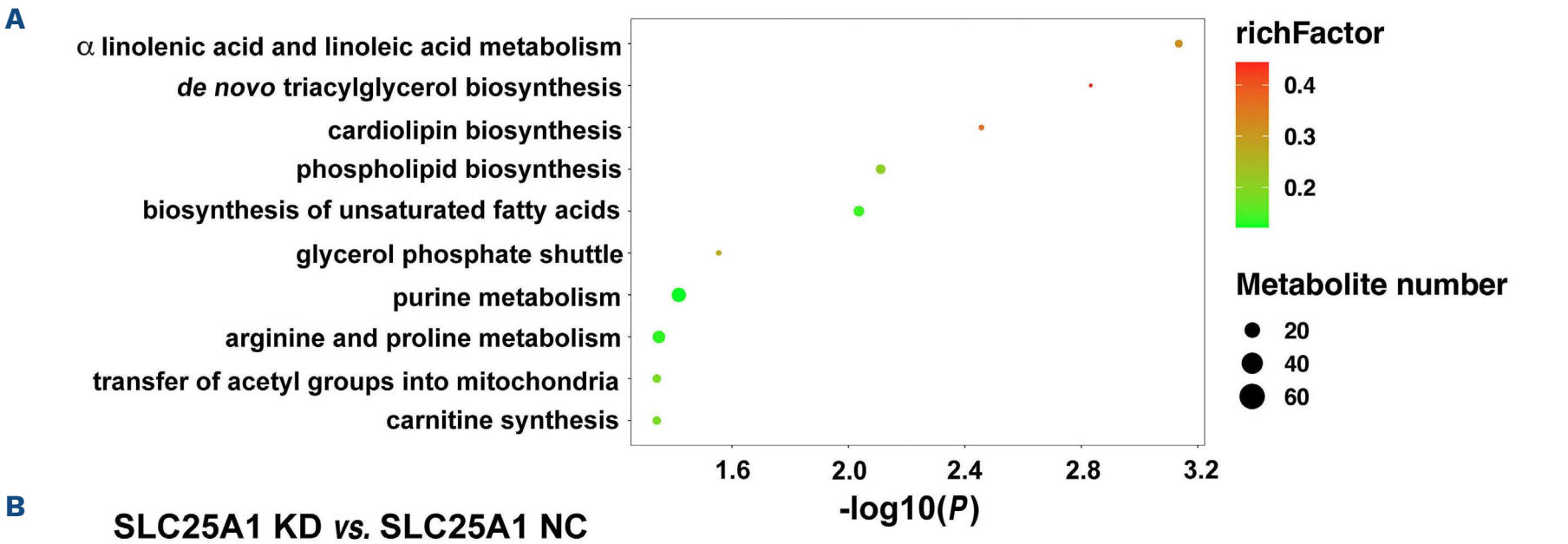
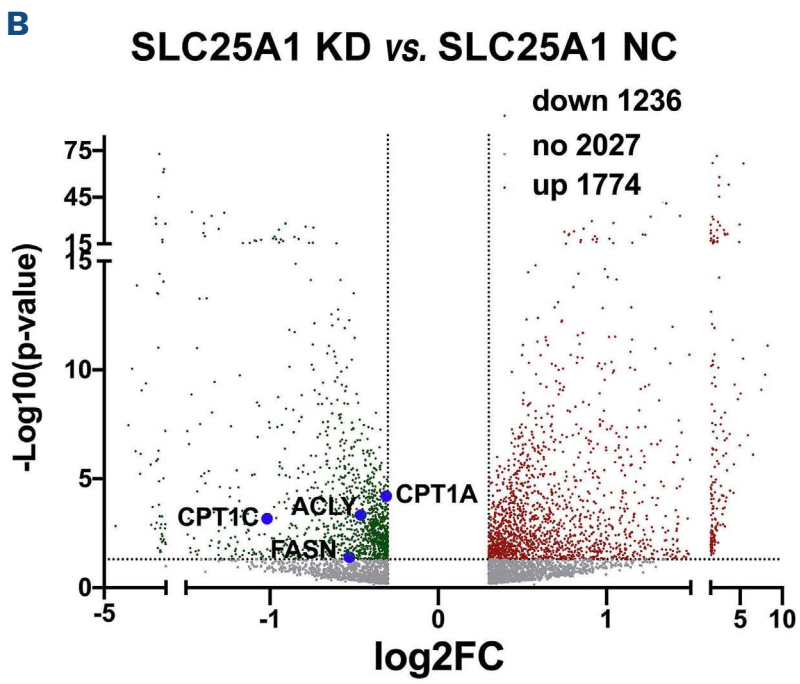
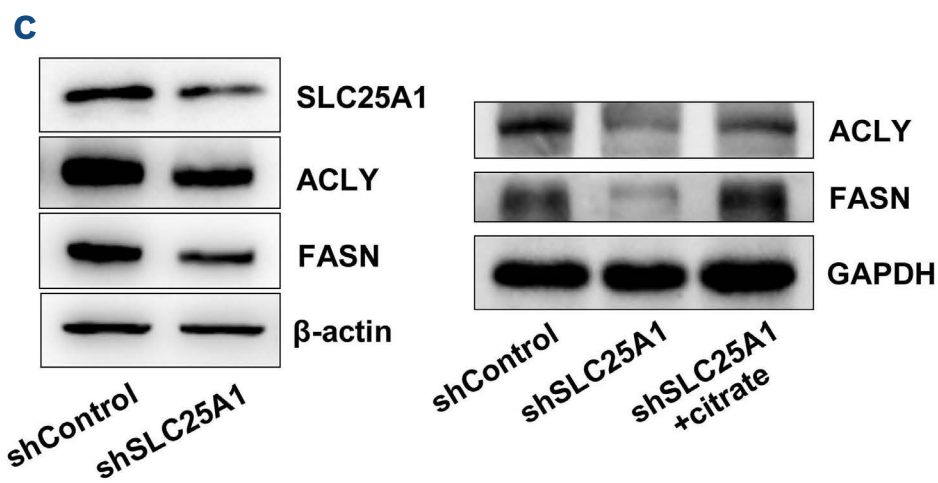
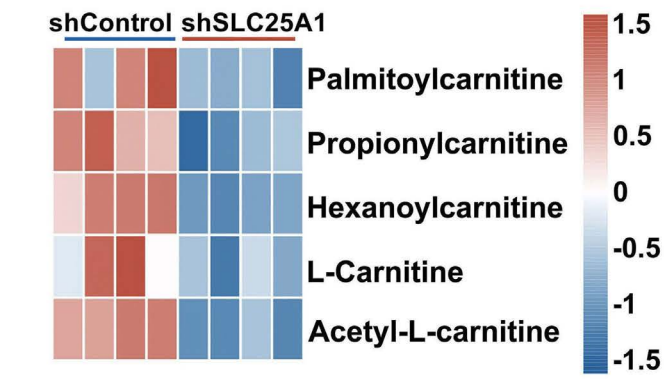


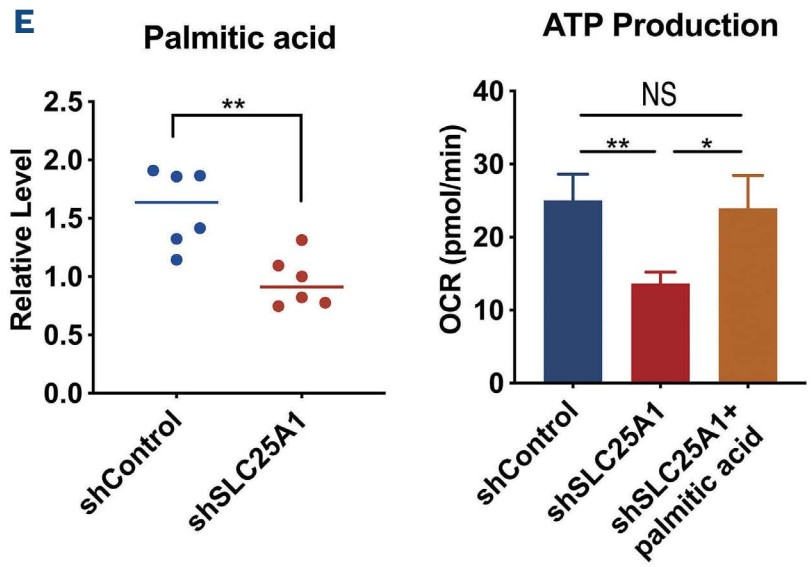
Figure 4. SLC25A1 affects the function of mitochondria and the cellular reactive oxygen species level. (A) The change of mitochondrial length under transmission electron microscope after *SLC25A1* knockdown (KD) and CTPI2 application in Kasumi-1. (B) The change of mitochondrial membrane potential through flow cytometry after *SLC25A1* KD and CTPI2 application in Kasumi-1. (C) The oxygen consumption rate (OCR) measured by seahorse extracellular flux analyzer experiment after *SLC25A1* KD and CTPI2 application in Kasumi-1. (D) The cellular reactive oxygen species (ROS) levels measured by flow cytometry after *SLC25A1* KD and CTPI2 application in Kasumi-1. (E) The growth of Kasumi-1 cells after *SLC25A1* KD and CTPI2 application is rescued by ROS remover (NAC).

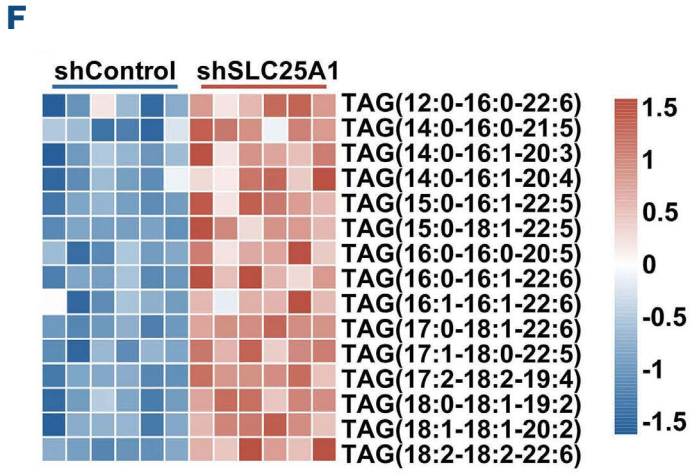












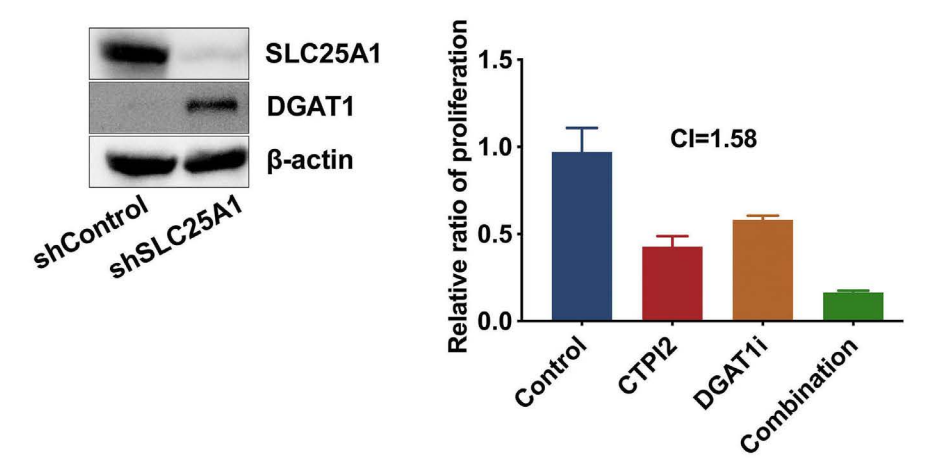


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Figure 5. Knockdown of *SLC25A1* inhibits fatty acid metabolism and initiates self-protection of acute myeloid leukemia cells. (A) The gene ontology (GO) enrichment analysis of differential metabolites performed by metabolomics on Kasumi-1 with *SLC25A1* knockdown (KD). (B) The volcano plot of differentially expressed genes (DEG) performed by RNA sequencing on Kasumi-1 with *SLC25A1* KD. (C) The western blotting (WB) validation of *SLC25A1* KD on the expression of ACLY and FASN in Kasumi-1 cells (left panel). The WB validation of ACLY and FASN expression following *SLC25A1* KD and citrate supplementation (right panel). (D) The heat map showing the change of carnitine related metabolites (palnitoylcarnitine, propionylcarnitine, hexanoylcarnitine, L (-)-carnitine and acetyl-L-carnitine) level detected by metabolomics after *SLC25A1* KD in Kasumi-1 cells. (E) The change of palmitic acid level detected by lipidomics after *SLC25A1* KD in Kasumi-1 cells (left panel). Palmitic acid supplementation partially rescues impaired mitochondrial function in *SLC25A1* KD cells (right panel). (F) The heat map showing the change of TAG level detected by lipidomics after *SLC25A1* KD in Kasumi-1 cells (left panel). The validation of DGAT1 expression by WB after *SLC25A1* KD in Kasumi-1 (middle panel). The synergistic effect of CTPI2 and DGAT1i (A922500) on inhibiting the growth of Kasumi-1 (right panel).

oxidation and fatty acid synthesis) following *SLC25A1* KD and identified four genes (*CPT1A*, *CPT1C*, *ACLY*, and *FASN*) downregulated in RNA-seq that are intimately connected to fatty acid metabolism (Figure 5B). Protein interaction network analysis via STRING and GeneMANIA online tools further implicated SLC25A1 in associations with enzymes of fatty acid synthesis (ACLY and FASN) (*Online Supplementary Figure S8E, F*). Additionally, positive correlation between SLC25A1 expression and levels of enzymes in AML databases was validated (*Online Supplementary Figure 8G, H*). Protein expression levels of CPT1A, CPT1C, ACLY, and FASN in Kasumi-1 and THP1 cells decreased after *SLC25A1* KD (Figure 5C; *Online Supplementary Figure S8I, J*). Based on changes in expression levels of key enzymes in fatty acid metabolism, we looked for related metabolite changes through metabolomics data. Citrate in mitochondria, the substrate of SLC25A1, was increased after *SLC25A1* KD, while coenzyme A, the product of ACLY from citrate and the substrate of fatty acid synthesis, decreased after *SLC25A1* KD (*Online Supplementary Figure S8K, L*), leading to TCA cycle overloading, elevated ROS levels, and changes in fatty acid metabolites. Meanwhile, supplementation with citrate in cytoplasm of SLC25A1 KD cells can restore expression of ACLY and FASN (Figure 5C), indicating direct regulation of SLC25A1 on ACLY and FASN through citrate. Considering the significance of fatty acid oxidation, where carnitine serves as a shuttle for fatty acids into mitochondria, we observed diminished levels of five carnitine-related intermediates (L-carnitine, palmitoylcarnitine, acetyl-carnitine, propionylcarnitine, hexanoylcarnitine) produced by carnitine acyl transferases CPT1A and CPT1C following *SLC25A1* KD (Figure 5D). Lipidomic assessment revealed a reduction in palmitic acid, a vital long-chain fatty acid synthesized by FASN, in *SLC25A1* KD group, and extra supplementation with palmitic acid in *SLC25A1* KD cells rescued the proliferation defect and restored the reduced oxygen consumption rate (OCR) caused by the KD (Figure 5E; *Online Supplementary Figure S8M*). Together, these results suggest SLC25A1 deletion reduces fatty acid oxidation and synthesis.

In-depth lipidomic analysis revealed most phosphoglycerides (PG) and triglycerides (TAG) were elevated after SLC25A1 KD (Figure 5F; *Online Supplementary Figure S8N*). The lipids play cytoprotective roles by forming lipid droplets,[23] suggesting that the effect might involve a self-protection mechanism after stress. DGAT1, a key enzyme catalyzing conversion of fatty acids to TAG,[24] was found to be upregulated in protein expression level after SLC25A1 KD (Figure 5F; *Online Supplementary Figure S8O*). When AML cells underwent treatment with a DGAT1 inhibitor, A922500[25] in combination with CTPI2, a notable synergistic effect was observed (Figure 5F; *Online Supplementary Figure S8P*). These results reveal SLC25A1 deletion initiates self-protection by increasing PG and TAG which can be reversed by inhibiting DGAT1.

A novel SLC25A1 inhibitor CTPI3 showed high effectiveness and safety, synergizing with venetoclax

Given the relatively high working concentration required for CTPI2 in AML cell lines, we sought to identify a more specific and potent inhibitor of SLC25A1. We screened Food and Drug Administration (FDA)-approved drug library based on “Fit Value” scores and selected compounds with Fit Value >1 for further structural optimization. Using Induced Fit Docking (IFD) module in the Schrödinger Suite, flexible molecular docking of candidate compounds was performed against active site of SLC25A1. This approach led to the identification of a new-generation inhibitor, CTPI3, with an improved predicted binding conformation (Figure 6A). CTPI3 significantly reduced the viability of primary AML cells, Kasumi-1, THP1, AE9a, and MLL-AF9, while showing less toxicity towards healthy MNC (Figure 6B). CTPI3 induced apoptosis in Kasumi-1 and THP1 cells (*Online Supplementary Figure S9A*). In both AE9a and MLL-AF9 murine leukemia models, CTPI3 treatment extended overall survival compared to vehicle controls (Figure 6C). *In vivo*, CTPI3 led to notable reductions in spleen size, less disruption of splenic architecture, and decreased GFP^+Mac-1^- cell populations in bone marrow, spleen, and peripheral blood (Figure 6D; *Online Supplementary Figure S9B*). The treatment group exhibited higher hemoglobin and platelet counts (*Online Supplementary Figure S9C*). Body weight remained stable during treatment, and hematoxilin and eosin staining as well as caspase 3 immunohistochemical staining of liver, kidney, and brain showed no significant toxicity (Figure 6E; *Online Supplementary Figure S9C*). These results indicate that the newly devoloped CTP13 potently inhibits AML cells growth *in vitro* and *in vivo*, with no obvious toxicity.

Following CTPI3 treatment, TCA metabolites (α-ketoglutarate, pyruvic acid, oxalic acid, fumaric acid, maleic acid,

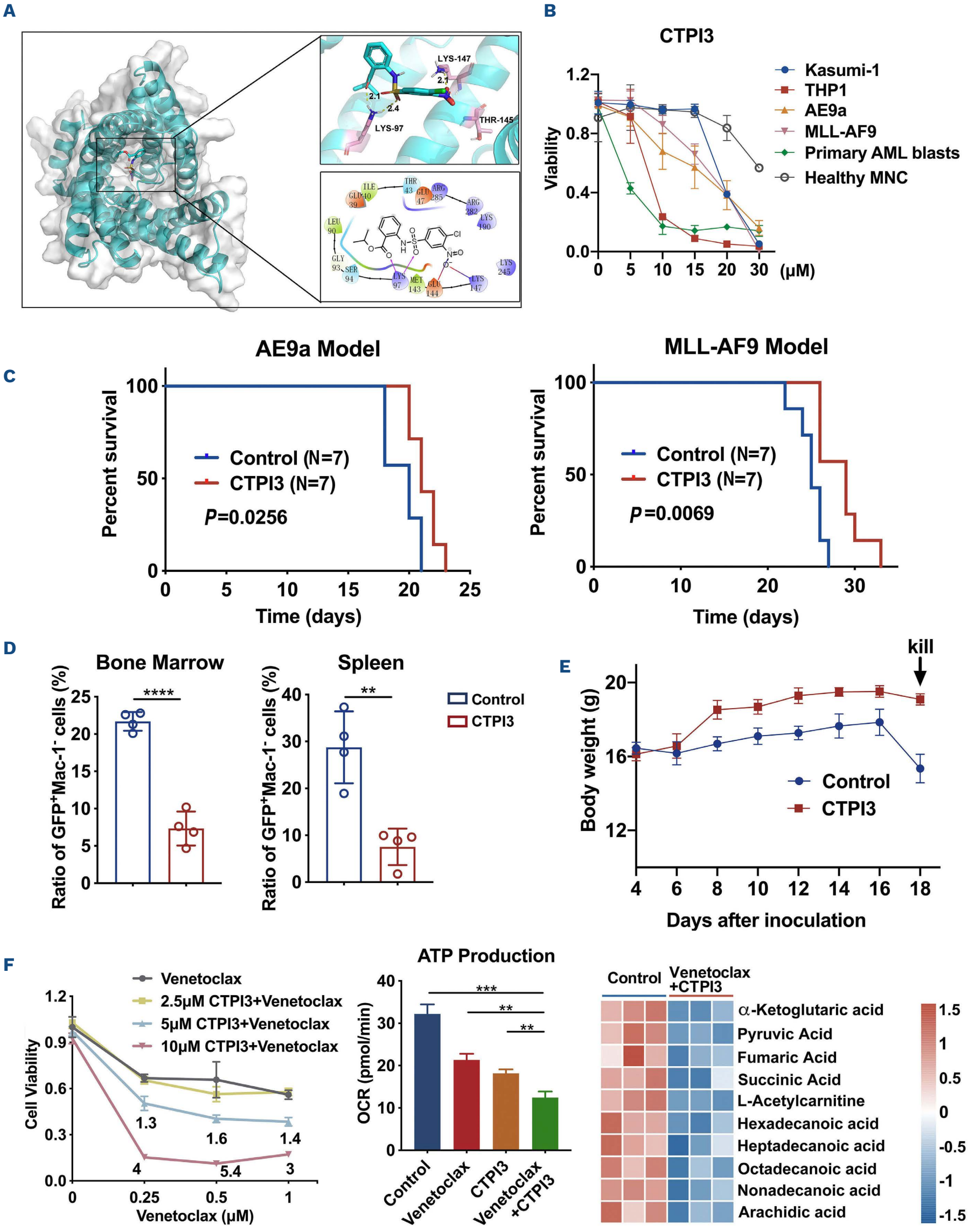

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Figure 6. CTPI3 inhibits acute myeloid leukemia cell viability, synergizes with venetoclax *in vitro*, and inhibits acute myeloid leukemia progression *in vivo*. (A) Predicted binding mode of CTPI3 to the active site of SLC25A1 based on molecular docking analysis. (B) Dose-response curves showing the inhibitory effects of CTPI3 on acute myeloid leukemia (AML) cell lines (Kasumi-1, THP1, AE9a, MLL-AF9), primary AML blasts, and healthy mononuclear cells (MNC). (C) Kaplan-Meier survival curves of mice bearing AE9a (left panel) or MLL-AF9 (right panel) AML models treated with CTPI3 (N=7 per group). (D) *In vivo* efficacy of CTPI3 in the AE9a model: reduction in $GFP^{+}Mac\text{-}1^{-}$ leukemia burden in bone marrow (BM), spleen (SP) compared to control mice. (E) Body weight monitoring post-CTPI3 treatment. (F) CTPI3 sensitizes AML cells to venetoclax. Left: cell viability in Kasumi-1 cells treated with venetoclax and CTPI3. Middle: mitochondrial ATP production is reduced upon CTPI3 or combination treatment. Right: heatmap showing altered levels of key TCA intermediates and fatty acids following combination treatment.

and succinic acid) were all reduced (*Online Supplementary Figure 10A*), which was in accord with results shown in Figure 4. Moreover, combining CTPI3 with venetoclax resulted in high synergistic anti-leukemic effects, including enhanced suppression of proliferation and ATP production (Figure 6F). Meanwhile, targeted metabolomics revealed a marked reduction in both TCA cycle intermediates and fatty acids following combined treatment, with significant reduction of CPT1A and CPT1C expression (Figure 6F; *Online Supplementary Figure S10B, C*). The outcomes reveal targeting SLC25A1 by CTPI3 effectively improves sensitivity of venetoclax through inhibiting fatty acid metabolism.

Discussion

The poor prognosis and resistance to existing pharmacotherapies for many AML patients leads to an urgent need for new therapeutic targets. Recently, accumulating data showed that dysregulated cellular metabolism is one of hallmarks of tumor and plays important roles in tumor growth, progression and drug resistance.[26-28] Citrate, an essential metabolite in both mitochondria and cytoplasm, regulates cellular energy homeostasis in tumors.[29] Here, we show citrate transporter SLC25A1, a clinical biomarker for AML patients, is required for survival of leukemic cells, both *in vitro* and *in vivo*. Depletion of SLC25A1 promotes ROS-induced apoptosis by impairing mitochondrial function through TCA cycle. Inhibition of SLC25A1 could enhance sensitivities of AML cells to venetoclax. Mechanistic studies show citrate homeostasis disturbance significantly suppresses growth of AML cells accompanied by affecting fatty acid metabolism. These findings suggest SLC25A1 plays a pivotal role in AML and represents a potential novel therapeutic target (Figure 7).

Previous studies revealed *SLC25A1* acted as an oncogene in the progression of solid tumors,[17-19,30,31] yet its molecular mechanism has not been fully elucidated and its role in AML remains unclear.[32] Our data shows SLC25A1 is a clinically significant biomarker highly expressed in patients with different subtypes of AML which is correlated with inferior prognosis in patients. Interestingly, suppressing SLC25A1 significantly inhibited growth of both AML cell lines and primary cells from AML patients, but had no effect on normal MNC. Moreover, KD of *SLC25A1* blocked the progression of AML *in vivo*. These findings collectively indicate SLC25A1 plays an important role in the promotion of AML and could serve as a therapeutic target for suppression of AML cell growth. We confirmed at cellular level that SLC25A1 inhibitor CTPI2 can effectively inhibit survival of AML cells, but the effective concentration of this inhibitor is relatively high, making it unsuitable for drug treatment exploration in mouse models. To improve clinical value of SLC25A1 targeting therapy, we synthesized new generation of SLC25A1 inhibitors based on structure of CTPI2. We named it as CTPI3. CTPI3 showed higher sensitivity in both AML cell lines and primary AML cells from patients, but had no toxicity in healthy MNC with the same concentration. More significantly, CTPI3 application *in vivo* expanded survival of AML mice and decreased infiltration of AML cells in the bone marrow, spleen and peripheral blood, without obvious toxicity in important organs. CTPI3 developed by our team might extremely promote SLC25A1 targeting therapy in future.

Metabolic reprogramming is an important tumor characteristic. In recent years, increasing evidence has shown besides glucose metabolism and oxidative phosphorylation, amino acid metabolism and fatty acid metabolism also play important roles in tumorigenesis and drug resistance.[33-36] Here we found inhibiting SLC25A1 damaged mitochondrial function and affected the growth of AML cells by promoting ROS-induced apoptosis, without affecting the cell cycle and differentiation. Mitochondria are the central hub for oxidative phosphorylation and fatty acid metabolism.[37] Indeed, we found SLC25A1 depletion disturbs fatty acid metabolism. Intriguingly, a series of important enzymes involved in fatty acid synthesis and oxidation are downregulated upon SLC25A1 blockage, including ACLY, FASN, CPT1A, and CPT1C. We verified the regulation of SLC25A1 on FA enzymes through citrate rescue experiment. We also confirmed the important role of palmitic acid in cell growth and oxidative phosphorylation by palmitic acid addition. Based on current evidence, we speculate that the downregulation of enzymes involved in fatty acid synthesis and oxidation is most likely an adaptive cellular response to the metabolic crisis caused by SLC25A1 deficiency, rather than a result of direct molecular interactions. However, whether SLC25A1 can directly interact with and regulate transcription factors such as MYC or SREBP1, which govern the expression of many enzymes involved in fatty acid

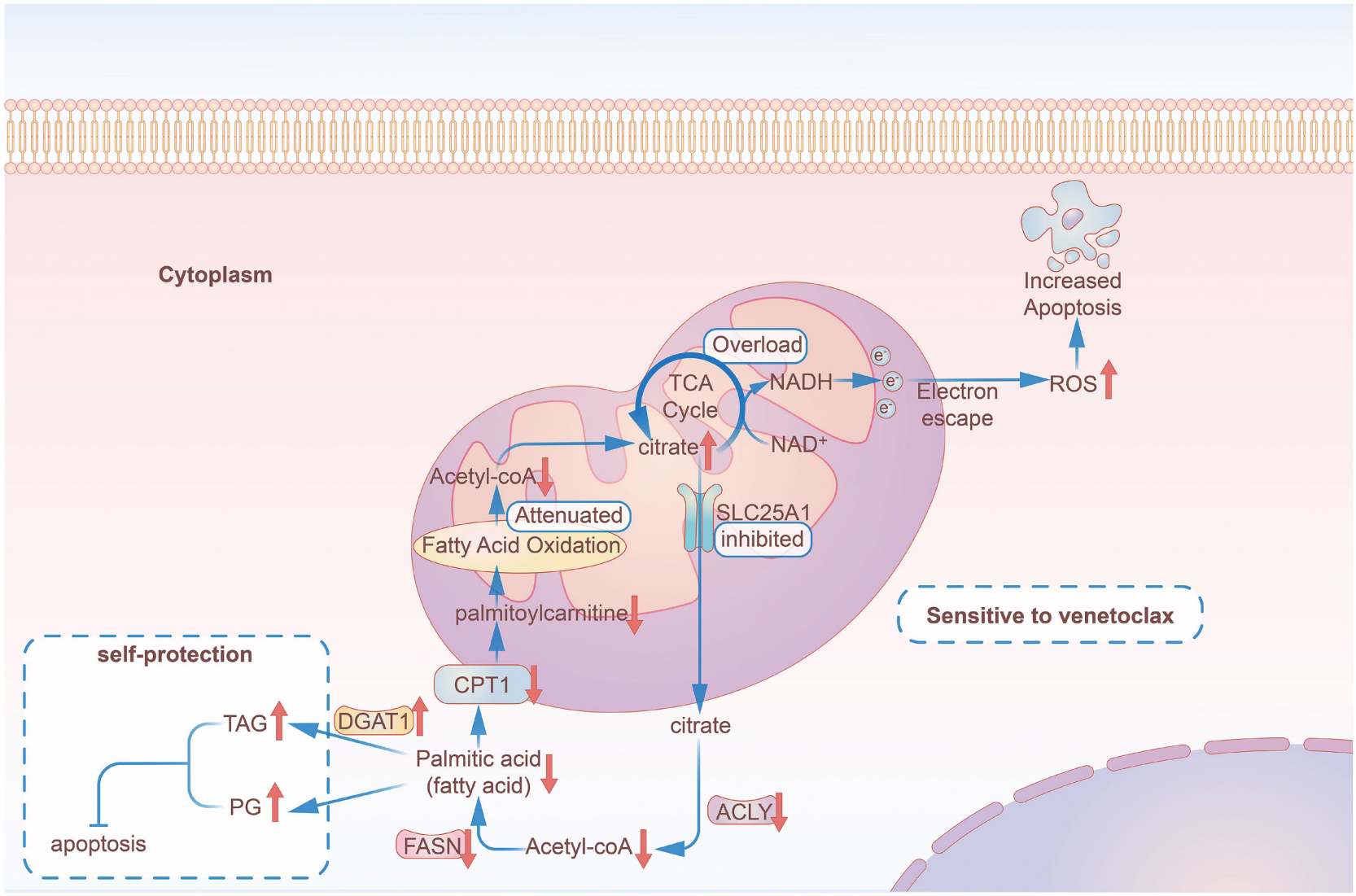

Figure 7. The work mechanism of SLC25A1 in acute myeloid leukemia cells.

metabolism, remains to be further explored. Mitochondrial metabolism and fatty acid metabolism are essential for cell energy supplementation, with important roles in the initiation and progression of AML.[26,38] Our results indicate that singly targeting SLC25A1 might effectively kill AML cells through simultaneously affecting multiple metabolic pathways including mitochondrial metabolism and fatty acid metabolism.

DGAT1, a key enzyme catalyzing conversion of fatty acids to TAG, is found to be upregulated. Lipid droplets formed by TAG and PG may protect cells from oxidative stress.[23,39] Cells initiate self-protective mechanisms by upregulating DGAT1 expression, producing more TAG and PG to provide alternative energy compensation and inhibit apoptosis, potentially contributing to cellular resistance mechanisms. Inhibiting DGAT1 can enhance AML cells' sensitivity to SLC25A1 inhibition, providing a new direction for improving the efficiency of SLC25A1-targeted therapies. SLC25A1 was previously shown to regulate metabolites in fatty acid metabolism in solid tumors[17,19,40] and our current study has extended these findings, contributing to more detailed molecular mechanism.

The targeted drug venetoclax is the first-line therapy in older AML, inducing apoptosis in AML cells by inhibiting BCL2.[41,42] However, besides classic mechanism, recent studies revealed venetoclax inhibited mitochondrial metabolism to induce cell death,[6,43] yet resistance and recurrence persist as significant challenges.[42,44-46] Up-regulation of FAO compensates the inhibition of amino acid metabolism by venetoclax, leading to venetoclax resistance.[47] But FAO direct inhibitor might have toxicity on other important organs, without clinical significance. Of note, a recent study identified SLC25A1 as a significant gene influencing venetoclax sensitivity through CRISPR screening,[1] which indicated targeting SLC25A1 might overcome the resistance of venetoclax. Our novel findings showed that SLC25A1 inhibitor, CTPI3 could synergistically target AML cells with venetoclax by inhibiting mitochondrial and fatty acid metabolism. As SLC25A1 was only high expressed in AML cells, targeting SLC25A1 might be safer than FAO inhibitor to improve sensitivity of venetoclax, which provides high clinical value.

Our study firstly demonstrates metabolic reprogramming induced by SLC25A1 in AML, which leads to the progression of AML as well as venetoclax resistance of patients. These findings improve our cognition in the roles of metabolic regulation in AML development and treatment, providing the basis of personalized therapy targeted on metabolism disorder. In conclusion, targeting mitochondrial metabolism and fatty acid metabolism using SLC25A1 inhibitor might be a potential novel strategy for enhancing AML therapy and overcoming drug resistance.

Disclosures
No conflicts of interest to disclose.

Contributions
MC designed the research, performed most of experiments and wrote the paper. WL performed parts of experiments and analyzed parts of the results. YT analyzed parts of the results. CH performed parts of the animal experiments. RG performed parts of drug experiments. SK participated in writing of the manuscript. PY participated in data analysis. CHM directed cell experiments. LW directed parts of the experiments and revised the article. XY directed the research design, the performance of experiments and revision of the article.

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Data-sharing statement
All data supporting the findings of this study are available within the article, its Online Supplementary Appendix or from the corresponding author upon reasonable request. For original data, please contact the corresponding author XY.

References

1. Chen X, Glytsou C, Zhou H, et al. Targeting mitochondrial structure sensitizes acute myeloid leukemia to venetoclax treatment. Cancer Discov. 2019;9(7):890-909.
2. MacPherson L, Anokye J, Yeung M, et al. HBO1 is required for the maintenance of leukaemia stem cells. Nature. 2020;577(7789):266-270.
3. Miller K, Nogueira L, Mariotto A, et al. Cancer treatment and survivorship statistics, 2019. CA Cancer J Clin. 2019;69(5):363-385.
4. Vander Heiden MG, DeBerardinis RJ. Understanding the intersections between metabolism and cancer biology. Cell. 2017;168(4):657-669.
5. DeBerardinis RJ, Chandel NS. We need to talk about the Warburg effect. Nat Metab. 2020;2(2):127-129.
6. Sharon D, Cathelin S, Mirali S, et al. Inhibition of mitochondrial translation overcomes venetoclax resistance in AML through activation of the integrated stress response. Sci Transl Med. 2019;11(516):eaax2863.
7. Skrtić M, Sriskanthadevan S, Jhas B, et al. Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. Cancer Cell. 2011;20(5):674-688.
8. Farge T, Saland E, de Toni F, et al. Chemotherapy-resistant human acute myeloid leukemia cells are not enriched for leukemic stem cells but require oxidative metabolism. Cancer Discov. 2017;7(7):716-735.
9. Samudio I, Harmancey R, Fiegl M, et al. Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. J Clin Invest. 2010;120(1):142-156.
10. Perland E, Fredriksson R. Classification systems of secondary active transporters. Trends Pharmacol Sci. 2017;38(3):305-315.
11. Elena P, Gert F, Mikko G. The role of solute carrier transporters in efficient anticancer drug delivery and therapy. Pharmaceutics. 2023;15(2):364.
12. Palmieri F, Monné M. Discoveries, metabolic roles and diseases of mitochondrial carriers: a review. Biochim Biophys Acta. 2016;1863(10):2362-2378.
13. Palmieri F. Mitochondrial transporters of the SLC25 family and associated diseases: a review. J Inherit Metab Dis. 2014;37(4):565-575.
14. Palmieri F. The mitochondrial transporter family SLC25: identification, properties and physiopathology. Mol Aspects Med. 2013;34(2-3):465-484.
15. Ma C, Gerhard E, Lu D, Yang J. Citrate chemistry and biology for biomaterials design. Biomaterials. 2018;178:383-400.
16. Zhang Z, Qiao Y, Sun Q, Peng L, Sun L. A novel SLC25A1 inhibitor, parthenolide, suppresses the growth and stemness of liver cancer stem cells with metabolic vulnerability. Cell Death Discov. 2023;9(1):350.
17. Fernandez HR, Gadre SM, Tan M, et al. The mitochondrial citrate carrier, SLC25A1, drives stemness and therapy resistance in non-small cell lung cancer. Cell Death Differ. 2018;25(7):1239-1258.
18. Ozkaya AB, Ak H, Atay S, Aydin HH. Targeting mitochondrial citrate transport in breast cancer cell lines. Anticancer Agents Med Chem. 2015;15(3):374-381.
19. Yang Y, He J, Zhang B, et al. SLC25A1 promotes tumor growth and survival by reprogramming energy metabolism in colorectal cancer. Cell Death Dis. 2021;12(12):1108.
20. Lee J, Khan D, Hurren R, et al. Venetoclax enhances T cell-mediated antileukemic activity by increasing ROS production. Blood. 2021;138(3):234-245.
21. Chiou J, Huang N, Huang C, et al. NOXA-mediated degradation of MCL1 and BCL2L1 causes apoptosis of daunorubicin-treated human acute myeloid leukemia cells. J Cell Physiol. 2021;236(11):7356-7375.
22. Tyner J, Tognon C, Bottomly D, et al. Functional genomic landscape of acute myeloid leukaemia. Nature. 2018;562(7728):526-531.
23. Olzmann JA, Carvalho P. Dynamics and functions of lipid droplets. Nat Rev Mol Cell Biol. 2019;20(3):137-155.
24. Dias S, Soares V, Ferreira A, et al. Lipid droplets fuel SARS-CoV-2 replication and production of inflammatory mediators. PLoS Pathog. 2020;16(12):e1009127.
25. Zhou J, Simon J, Liao C, et al. An oncogenic JMJD6-DGAT1 axis

tunes the epigenetic regulation of lipid droplet formation in clear cell renal cell carcinoma. Mol Cell. 2022;82(16):3030-3044.e8.
26. Mishra S, Millman S, Zhang L. Metabolism in acute myeloid leukemia: mechanistic insights and therapeutic targets. Blood. 2023;141(10):1119-1135.
27. Egan G, Schimmer A. Contribution of metabolic abnormalities to acute myeloid leukemia pathogenesis. Trends Cell Biol. 2023;33(6):455-462.
28. Hanahan D. Hallmarks of cancer: new dimensions. Cancer Discov. 2022;12(1):31-46.
29. Yangjing Z, Xia L, Fusheng S, et al. Citrate promotes excessive lipid biosynthesis and senescence in tumor cells for tumor therapy. Adv Sci (Weinh). 2021;9(1):e2101553.
30. Mosaoa R, Kasprzyk-Pawelec A, Fernandez H, Avantaggiati M. The mitochondrial citrate carrier SLC25A1/CIC and the fundamental role of citrate in cancer, inflammation and beyond. Biomolecules. 2021;11(2):141.
31. Hlouschek J, Hansel C, Jendrossek V, Matschke J. The mitochondrial citrate carrier (SLC25A1) sustains redox homeostasis and mitochondrial metabolism supporting radioresistance of cancer cells with tolerance to cycling severe hypoxia. Front Oncol. 2018;8:170.
32. Liu F, Deng S, Li Y, Du J, Zeng H. SLC25A1-associated prognostic signature predicts poor survival in acute myeloid leukemia patients. Front Genet. 2022;13:1081262.
33. Lee K, Giltnane J, Balko J, et al. MYC and MCL1 cooperatively promote chemotherapy-resistant breast cancer stem cells via regulation of mitochondrial oxidative phosphorylation. Cell Metab. 2017;26(4):633-647.e7.
34. Hattori A, Tsunoda M, Konuma T, et al. Cancer progression by reprogrammed BCAA metabolism in myeloid leukaemia. Nature. 2017;545(7655):500-504.
35. Ye H, Adane B, Khan N, et al. Leukemic stem cells evade chemotherapy by metabolic adaptation to an adipose tissue niche. Cell stem cell. 2016;19(1):23-37.
36. Martínez-Reyes I, Chandel N. Cancer metabolism: looking forward. Nat Rev Cancer. 2021;21(10):669-680.
37. Yashi M, Guoyuan Q, Francesca V, et al. Loss of fatty acid degradation by astrocytic mitochondria triggers neuroinflammation and neurodegeneration. Nat Metab. 2023;5(3):445-465.
38. Shao X, Xu A, Du W, et al. The palmitoyltransferase ZDHHC21 regulates oxidative phosphorylation to induce differentiation block and stemness in AML. Blood. 2023;142(4):365-381.
39. Lee S, Li J, Tai J, Ratliff T, Park K, Cheng J. Avasimibe encapsulated in human serum albumin blocks cholesterol esterification for selective cancer treatment. ACS Nano. 2015;9(3):2420-2432.
40. Zhang R, Peng X, Du J, et al. Oncogenic KRASG12D reprograms lipid metabolism by upregulating SLC25A1 to drive pancreatic tumorigenesis. Cancer Res. 2023;83(22):3739-3752.
41. Chan S, Thomas D, Corces-Zimmerman M, et al. Isocitrate dehydrogenase 1 and 2 mutations induce BCL-2 dependence in acute myeloid leukemia. Nat Med. 2015;21(2):178-184.
42. DiNardo C, Tiong I, Quaglieri A, et al. Molecular patterns of response and treatment failure after frontline venetoclax combinations in older patients with AML. Blood. 2020;135(11):791-803.
43. Jones C, Stevens B, D'Alessandro A, et al. Inhibition of amino acid metabolism selectively targets human leukemia stem cells. Cancer Cell. 2018;34(5):724-740.e4.
44. Thomalla D, Beckmann L, Grimm C, et al. Deregulation and epigenetic modification of BCL2-family genes cause resistance to venetoclax in hematologic malignancies. Blood. 2022;140(20):2113-2126.
45. Li X, Xu S, Tan Y, Chen J. The effects of idarubicin versus other anthracyclines for induction therapy of patients with newly diagnosed leukaemia. Cochrane Database Syst Rev. 2015;2015(6):CD010432.
46. Zhang X, Yang L, Liu X, et al. Regulatory role of RBM39 in acute myeloid leukemia: mediation through the PI3K/AKT pathway. Biochim Biophys Acta Mol Cell Res. 2024;1871(1):119607.
47. Stevens B, Jones C, Pollyea D, et al. Fatty acid metabolism underlies venetoclax resistance in acute myeloid leukemia stem cells. Nat Cancer. 2020;1(12):1176-1187.