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The STAT3-MYC Axis Promotes Survival of Leukemia Stem Cells by Regulating SLC1A5 and Oxidative Phosphorylation

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Abstract:
AML is characterized by the presence of leukemia stem cells (LSCs), and failure to fully eradicate this population contributes to disease persistence/relapse. Prior studies have characterized metabolic vulnerabilities of LSCs, which demonstrate preferential reliance on oxidative phosphorylation (OXPHOS) for energy metabolism and survival. In the present study, using both genetic and pharmacologic strategies in primary human AML specimens, we show that signal transducer and activator of transcription 3 (STAT3) mediates OXPHOS in LSCs. STAT3 regulates AML-specific expression of MYC, which in turn controls transcription of the neutral amino acid transporter SLC1A5. We show that genetic inhibition of MYC or SLC1A5 acts to phenocopy the impairment of OXPHOS observed with STAT3 inhibition, thereby establishing this axis as a regulatory mechanism linking STAT3 to energy metabolism. Inhibition of SLC1A5 reduces intracellular levels of glutamine, glutathione and multiple TCA metabolites, leading to reduced TCA cycle activity and inhibition of OXPHOS. Based on these findings, we used a novel small molecule STAT3 inhibitor, that binds STAT3 and disrupts STAT3-DNA, to evaluate the biological role of STAT3. We show that STAT3 inhibition selectively leads to cell death in AML stem and progenitor cells derived from newly diagnosed and relapsed patients, while sparing normal hematopoietic cells. Together, these findings establish a STAT3-mediated mechanism that controls energy metabolism and survival in primitive AML cells.

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Running Title: STAT3 regulates OXPHOS in Leukemia Stem Cells

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- STAT3 regulates amino acid influx and glutaminolysis in leukemia stem cells by promoting expression of MYC and SLC1A5.
- Depletion of glutamine and its downstream metabolites leads to a decrease in OXPHOS activity in LSCs resulting in cell death.

Abstract

AML is characterized by the presence of leukemia stem cells (LSCs), and failure to fully eradicate this population contributes to disease persistence/relapse. Prior studies have characterized metabolic vulnerabilities of LSCs, which demonstrate preferential reliance on oxidative phosphorylation (OXPHOS) for energy metabolism and survival. In the present study, using both genetic and pharmacologic strategies in primary human AML specimens, we show that signal transducer and activator of transcription 3 (STAT3) mediates OXPHOS in LSCs. STAT3 regulates AML-specific expression of MYC, which in turn controls transcription of the neutral amino acid transporter SLC1A5. We show that genetic inhibition of MYC or SLC1A5 acts to phenocopy the impairment of OXPHOS observed with STAT3 inhibition, thereby establishing this axis as a regulatory mechanism linking STAT3 to energy metabolism. Inhibition of SLC1A5 reduces intracellular levels of glutamine, glutathione and multiple TCA metabolites, leading to reduced TCA cycle activity and inhibition of OXPHOS. Based on these findings, we used a novel small molecule STAT3 inhibitor, that binds STAT3 and disrupts STAT3-DNA, to evaluate the biological role of STAT3. We show that STAT3 inhibition selectively leads to cell death in AML stem and progenitor cells derived from newly diagnosed and relapsed patients, while sparing normal hematopoietic cells. Together, these findings establish a STAT3-mediated mechanism that controls energy metabolism and survival in primitive AML cells.

Introduction

AML is an aggressive disease with a dismal prognosis and high relapse rates.[1,2] AML is thought to arise from a subpopulation of leukemia stem cells (LSCs), which display unique molecular properties and negatively correlate with survival.[3,4] LSCs are often found in greater abundance at the time of relapse, suggesting they are more resistant to chemotherapy.[3-6] Therefore, identifying approaches to selectively target the LSC population is an important component of disease management. One unique vulnerability of LSCs is their preferential reliance on mitochondrial activity and oxidative phosphorylation (OXPHOS) for energy production.[6-8] This differs from normal hematopoietic cells, which are more flexible in their ability to utilize several pathways for energy production.[9-11] Strategies aimed at inhibiting OXPHOS are therefore of considerable interest and have been described in several reports.[7,12,13] While we have previously described the metabolic rewiring of LSCs towards increased reliance on OXPHOS,[7,13] limited mechanistic evidence has been generated to explain the etiological contributors to such events. In the present study, we identify the signal transducer and activator of transcription 3 (STAT3) as a modulator of OXPHOS and potential target for therapy. Prior studies have shown that STAT3 is important for leukemogenesis and is overexpressed in many AML patient samples.[14-16] STAT3 is activated by several signals including IL-6,[15,17] which induce phosphorylation of two residues on STAT3, Y705 and S727. Phosphorylation at Y705 leads to dimerization of STAT3 and translocation to the nucleus,[18] where STAT3 has a role as a nuclear transcription factor that mediates self-renewal and proliferation.[19] STAT3 also has a less-well characterized role regulating various functions of the mitochondria.[20] Its localization and function in the mitochondria is thought to be mediated by the phosphorylation of residue S727,[20,21] and some studies have suggested mitochondrial STAT3 regulates mitochondrial genes,[22] while others have demonstrated it can also act to enhance the activity of the electron transport chain (ETC).[21,23] Based on previous studies, and the known role of OXPHOS in supporting LSCs, we hypothesized that targeting STAT3 could facilitate eradication of primitive AML cell types. Here we show, similar to prior studies, that STAT3 is highly expressed in AML,[14-16,24,25] and by utilizing primary human AML specimens we show STAT3 serves as an important factor in regulating OXPHOS.

Methods

Cell culture

Base media of MEM and 5.5 mM of glucose was used, supplemented with physiologic levels of amino acids as previously reported[12] and 10nM human cytokines SCF, IL3, and FLT3.

Patient samples and leukemia stem cell enrichment

Primary human AML samples were obtained from apheresis products or bone marrow of patients with AML who gave written consent for sample procurement at the University of Colorado, according to the Colorado IRB Protocol #12-0173. For analysis of LSC enriched fractions, specimens were processed as previously described.[12,26,27] and detailed methods can be found in the supplemental materials.

Seahorse Cell Mito Stress Test

The extracellular flux assay XF96 kit was used to measure oxygen consumption rate (OCR). Drug-treated or siRNA-treated primary AML cells or LSCs were plated in Cell-Tak–coated XF96 cell culture microplates at 200K cells/well in five replicates and measured according to the manufacturer's protocol. OCR was measured at basal and after injection of 5 μg/mL oligomycin, 2 μmol/L FCCP, 5 μmol/L Antimycin A, and 5 μmol/L Rotenone.

SF25 synthesis and characterization

The chemical synthesis of SF25 (STAT3i) is described in the supplemental methods. Cell-based ELISA was used to determine the inhibition of cellular STAT3, STAT1α, STAT5A and STAT5B from binding to an immobilized DNA consensus sequence. Hela cells (1 million cells/100mm dish) that expressed constitutively active STAT3 were treated with STAT3i for 24 hours. Nuclear extracts were prepared using an extraction kit (Signosis) and diluted in complete lysis buffer. Evaluation of STAT3 from the nuclear extract binding to an immobilized DNA consensus sequence was determined using the TransAM STAT3 ELISA kit (ActiveMotif).[28]

RNA sequencing

LSCs were treated with either vehicle control or 5μM of STAT3i for 4 hours. mRNA isolation, quality check, library construction, and sequencing were performed according to a previously used protocol.[12] Single-end reads of 100 nt were generated for each sample on the Illumina HiSeq2500 platform. Methods for RNA sequencing analysis can be found in supplemental materials.

ETC complex activity assay

Complex I and Complex V activity was measured by a calorimetric assay (Abcam ab109721 and ab109714), according to the manufacturer's protocol. Complex II/III activity was measured utilizing Agilent's XF Plasma Membrane Permeabilizer (PMP) according to the manufacturer's protocol. Cells were treated with 2uM Rotenone, and succinate was added as a substrate at

10nM and baseline OCR was read in an XF96 Analyzer. 2uM of Antimycin A was then utilized to inhibit the complex.

Metabolic tracing experiments

Technical replicates of 250,000 LSCs were sorted and first incubated with vehicle control (DMSO) versus 5 μM of STAT3i for 3 hours. Cells were then incubated with $^{13}C_5$,$^{15}N_2$-L-Glutamine for 1, 8 or 16 hr. Heavy-labeled L-Glutamine was added at a physiologically relevant concentration (650μM). Extraction and mass spectrometry were performed as previously described,[29,30,31] and detailed methods can be found in supplemental materials.

CFU assay

Primary AML or normal cord blood mononuclear cells were first treated with drug versus vehicle control overnight in 37°C, and they were subsequently seeded in human methylcellulose media at 25K cells/mL. These were grown in 37°C for up to 14 days before counting on stereomicroscope.

Mouse studies

Xenograft studies were performed as previously described[33] and are detailed in the supplemental methods. In brief, human primary AML cells were treated with either vehicle control or 5μM of STAT3i overnight. On the second day, 8-9 busulfan-conditioned NSG-S mice per group were injected AML cells. Mice were sacrificed after 8-10 weeks and tested for engraftment using mouse and human specific CD45 antibodies. For in vivo mouse studies, mice were first engrafted with a human primary AML sample to approximately 15% human blast cells. They were then treated intraperitoneally with either 30mg/kg of STAT3i or vehicle control for 6 consecutive days.

Data sharing statement: For original data, please contact maria.l.amaya@cuanschutz.edu. RNA sequencing data will be deposited in Gene Expression Omnibus prior to publication.

Results

STAT3 is highly expressed in primary AML cells and plays a role in oxidative phosphorylation

To investigate the role of STAT3 in human AML cells, we utilized multiple primary AML specimens donated by patients at University of Colorado (characteristics outlined in Table 1). We first measured expression of total and phosphorylated STAT3 at Y705, a marker of STAT3 activation, via Western Blot. As shown in Figure 1A, 14 of 18 AML patient samples showed significantly higher STAT3 expression compared to the nearly absent STAT3 expression in normal mononuclear cells isolated from umbilical cord blood (CB). Furthermore, 8 out of 18 AML samples tested showed phosphorylation of STAT3 at the residue Y705, suggesting constitutive activation. To determine whether STAT3 expression mediates mitochondrial function of AML cells, we first performed siRNA-mediated knock-down studies and evaluated the results on mitochondrial respiration. STAT3 knockdown (Figures 1B-C) leads to a significant decrease in the oxygen consumption rate (OCR) after 48 hours as demonstrated in a representative primary

AML sample in Figure 1D, and in three primary AML samples (Figure 1E). Notably, our siRNA-mediated knockdown is specific to STAT3 and does not affect STAT5 (Figure S1A). Reduction in STAT3 is followed by decreased viability in knockdown cells at 60 hours (Figure 1F). These findings suggest a role for STAT3 in mitochondrial function. These findings were extended using a pharmacological small molecule STAT3 inhibitor, SF25 (hereafter termed STAT3i). STAT3i is a salicylamide that, like the previously described STAT3 inhibitor niclosamide, interacts with the DNA-binding domain of STAT3 and inhibits STAT3-DNA interaction (Figure 1G-H).[28] STAT3i is a more potent inhibitor of STAT3-DNA binding and has improved cell penetration and retention properties over niclosamide. STAT3i also shows selectivity to STAT3 inhibition compared to other members of the STAT family (Figure 1H). To test STAT3i, we first performed a dose response analysis using 8 primary AML samples. As shown in Figure 1I, after 24 hours of culture, significant cell death was observed at doses as low as 625nM. To ensure that subsequent studies of mitochondrial respiration could be performed prior to the onset of overt cell death events, which could confound analyses of STAT3 activity, we titrated time of exposure and dose (Figure S1B). These studies demonstrated no evident death in the first 12 hours of culture at a 5μM concentration. Subsequent studies performed at a dose of 5μM showed a profound inhibition of maximal respiratory capacity after only 4 hours for 3 of 3 primary AML specimens (Figure 1J), in agreement with the findings observed upon STAT3 knockdown and confirming OXPHOS inhibition occurs prior to cell death (Figure S1B). We do note the effect seen with STAT3i is more pronounced than with STAT3 knockdown, and although this could be due to the transient effect of siRNA technology, additional off-target effects from this compound cannot be ruled out. Given our primary samples are predominantly derived from leukapheresis, we also corroborated these findings in three primary bone marrow samples, which show a significant decrease in OXPHOS activity upon treatment of STAT3i (Figure S1C). To determine if this effect was specific to STAT3, we also evaluated the expression of STAT5, which has also been shown to be upregulated in primary AML cells.[34,35] As shown in Figure S1D, 6 out of 8 patient samples showed increased expression of STAT5, but only 1 sample shows significant phosphorylation of this protein. Further, siRNA-mediated knock-down of STAT5, shows no significant effect on maximal respiration in 4 primary AML samples (Figure S1E-F), suggesting the regulation of OXPHOS is a specific characteristic of STAT3.

MYC is a downstream target of STAT3 and regulates OXPHOS in LSCs

Given that OXPHOS is important for LSC survival,[7,12,13] and the above data showed that STAT3 can regulate OXPHOS in bulk leukemia, we next sought to determine whether STAT3 activity also plays a role in LSC biology. We have previously shown that LSCs are characterized by the presence of low reactive oxygen species (termed ROS-low), a characteristic that can be used to isolate LSC-enriched subpopulations from primary AML specimens (Figure 2A; Figure S2A).[7,26] Upon pharmacological inhibition with 5μM STAT3i, a similar decrease in maximal respiratory capacity was seen in ROS-low LSCs (Figure 2B) as observed for bulk AML specimens. Of note, ROS-high cells isolated from bulk leukemia specimens (Figure S2B) are also impaired, suggesting all populations of AML are affected by STAT3i. To investigate how STAT3 regulates OXPHOS in LSCs, we performed RNA-sequencing using specimens treated with 5μM STAT3i versus vehicle control for 4 hours (n = 4 individual patient samples). As observed for bulk tumor cells (Figure S1B), 4-hour incubation with STAT3i shows no significant cell death (Figure S2C) in LSCs. To analyze our RNA-seq data, we first confirmed that known STAT3 targets were downregulated on samples treated with STAT3i[36,37] (Figure S2D). This was followed by Gene Set Enrichment Analysis (GSEA), which showed changes in multiple pathways (Figure S2E-F), including several mitochondrial pathways and transcription factor targets (Figure S2G-H).

Interestingly, one of the top pathways changed upon STAT3 inhibition was "MYC targets" (Figure 2C)**.** MYC is a transcription factor and oncogene that is frequently activated in AML and can induce leukemogenesis.[38,39] This finding is supported by analyses derived from the BloodSpot database (http://www.bloodspot.eu/)[40] showing MYC is consistently up-regulated in AML cells (Figure 2D). In addition, prior studies show STAT3 regulates transcriptional activity of the MYC gene,[41] a finding confirmed in the present study where we observe STAT3 binding to the MYC promoter region by ChIP analysis (Figure 2E). To confirm STAT3 regulates MYC, LSCs were treated with STAT3i for 4 hours and qPCR and Western Blots were performed, showing significant downregulation of the Myc RNA and protein (Figure 2F-G). To test whether MYC plays a role in the OXPHOS changes seen upon STAT3 inhibition, we used siRNA to knock down MYC in primary AML patient samples and analyzed mitochondrial respiration. A reduction in maximal OCR indicated that knockdown of MYC leads to a significant decrease in OXPHOS AML cells (Figure 2H-I).

STAT3 regulates glutamine influx in LSCs via MYC

To better understand the ramifications of STAT3 inhibition, we next examined global energy metabolism of LSCs. To this end, we sorted ROS-low LSCs from four primary AML samples, and cultured them with 5μM STAT3i versus vehicle control for 4-hours, and performed metabolomic studies as previously described.[42] Steady-state analysis of the metabolomics data identified significant changes in metabolites that are part of the glutaminolysis pathway (Figure S3A), while glycolytic metabolites were unchanged (Figure S3B). This suggests STAT3 signaling could control OXPHOS by promoting glutaminolysis in LSCs. Since glutamine and glutamate were decreased, we also interrogated other amino acids. Some amino acids such as cysteine were non-significantly decreased, whereas aspartate and proline were increased (Figure S3C), likely as a compensatory response to the decreased glutamine pathway. Interestingly, even in the presence of higher aspartate levels, which can contribute to glutamate production, we saw a reduction in glutamate that could be related to decreased expression of aspartate aminotransferase upon STAT3 inhibition (Figure S3D). To further characterize the impact of STAT3 inhibition in the glutaminolysis pathway, we performed a metabolic tracing experiment with stable isotope-labeled glutamine, which captures dynamic changes in glutamine levels and catabolism.[43] Heavy labeled glutamine was employed for a time course experiment to determine how carbons mobilize into different energetic pathways upon modulation of STAT3. 5μM STAT3i or vehicle control were added to ROS-low AML cells 4-hours prior to the first time-point. $^{13}C_{5,}$ $^{15}N_2$ L-Glutamine was then added at physiologically relevant concentrations, and heavy-labeled metabolites were measured at 1, 8, and 16 hours by mass spectrometry as depicted in Figure 3A. STAT3 inhibition decreased the levels and percentage of heavy carbon accumulation in intracellular glutamine and glutamate in as little as one hour, and within 8 hours reduced glutathione (GSH) was also evident (Figure 3B). Furthermore, glutamine carbon accumulation was also decreased in TCA cycle intermediates including alpha-ketoglutarate, succinate and citrate shown in Figure S3E-F. This finding suggests a decreased influx of glutamine into AML LSCs upon STAT3 inhibition, likely mediated by glutamine transporter(s)**,** resulting in decreased levels of GSH and TCA cycle intermediates as depicted in Figure 3C. To test this hypothesis, we interrogated our RNA-seq dataset, and found that the sodium-dependent neutral amino acid transporter, SLC1A5, was significantly downregulated upon STAT3 inhibition (Figure S3G), and expression analysis from the BloodSpot database shows substantial up-regulation of this transporter in AML cells (Figure 3D), suggesting a possible dependence. SLC1A5 is a cell surface solute-carrying transporter that mediates uptake of neutral amino acids, and is known to transport glutamine in rapidly proliferating tumor cells.[44] To validate this finding, we performed Western Blots on LSCs after 4 hours of culture with 5μM

of STAT3i. Significant downregulation of SLC1A5 was observed at the protein level (Figure 3E). Since previous studies in other systems have shown SLC1A5 can be regulated by MYC,[45,46] we tested whether this relationship was also evident in AML cells. Upon MYC inhibition by siRNA, we observed a decrease in SLC1A5 at the protein level (Figure 3F), suggesting SLC1A5 is regulated by MYC. On the other hand, although MYC has been shown to regulate glutaminase, which mediates the conversion from glutamine to glutamate, STAT3 inhibition does not appear to affect protein levels of glutaminase in LSCs (Figure S3H). These data suggest STAT3 regulates MYC, which in turn regulates SCL1A5 and the influx of glutamine into LSCs, ultimately leading to decreased glutamate, alpha-ketoglutarate and GSH levels. To further confirm this is the pathway by which STAT3 inhibition leads to a decrease in glutaminolysis, we knocked down SLC1A5 in primary AML cells, which resulted in a marked decrease in glutamine and its downstream metabolites (Figure 3G-H). As expected, the addition of excess glutamine to cell culture in the context of STAT3 inhibition did not rescue the OXPHOS activity as measured by Seahorse Assays (Figure 3I), since intake of this amino acid is reduced by less availability of its transporter. However, utilizing a cell permeabilizer agent to allow transporter-independent intake of glutamine did rescue the OXPHOS activity of LSCs, suggesting glutamine is an important driver of STAT3-mediated OXPHOS activity. To confirm the STAT3-MYC axis is involved in glutaminolysis leading to regulation of OXPHOS, we over-expressed Myc to determine whether we could rescue the OXPHOS phenotype. This was performed in the cell line MOLM-13 for feasibility. Even a modest increase in MYC expression (Figure S3I-J) leads to a partial rescue of OXPHOS activity (Figure S3K) and viability (Figure S3L) upon STAT3 inhibition.

STAT3-mediated TCA cycle changes lead to ETC dysfunction

We next sought to test whether STAT3-mediated TCA cycle changes affect ETC activity. Upon culturing 3 primary AML samples with 5μM STAT3i or vehicle control for 4 hours, we observed a decrease in ETC Complex I, II and III activity in STAT3 inhibited cells at 4 hours, but no decrease is seen in Complex V activity (Figure 4A). This is consistent with the metabolomics findings of decreased glutamate and decreased succinate that serve as substrates for complexes I and II/III respectively. Further, the addition of cell permeable glutamine and alpha-ketoglutarate rescue the effect of STAT3 in ETC Complex II/III (Figure 4B), suggesting this effect is TCA cycle mediated. Consistent with reduction of ETC activity upon STAT3i, mitochondrial reactive oxygen species is reduced upon STAT3 inhibition based on MitoSox stain as shown in Figure 4C. Given that STAT3 is a transcription factor, we questioned whether changes seen in the ETC activity could be related to expression changes of genes belonging to the ETC complexes, some of which are encoded in the nucleus and others in the mitochondria. We therefore probed our RNA-sequencing data, which showed no change in expression of the majority of ETC genes in LSCs treated with STAT3i (data not shown). This was validated by qPCR, which showed the majority of ETC complex genes are unchanged, with the exception of UQCC3 which belongs to Complex III (Figure 4D). Since STAT3 has also been shown to localize to the mitochondria and modulate expression of mitochondrial genes in other tissues,[22] we also tested whether these genes were changed upon STAT3 inhibition. Although we confirmed STAT3 is phosphorylated at S727 (Figure S4A) and it localizes to the mitochondria of primary AML cells (Figure S4B), gene expression analysis of our RNA-seq data shows no decrease in mitochondrial-encoded ETC genes upon STAT3 inhibition (Figure 4E). Similarly, we confirm no change at the protein level of two representative mitochondrial ETC genes, MT-CO1 and MT-ND2 (Figure 4F). We further confirmed this finding by utilizing siRNA-mediated knockdown of mitochondrial STAT3, which shows no change in mitochondrial protein expression of MT-CO1 (Figure S4C). These data suggest the decrease in ETC activity seen upon STAT3 inhibition is likely mediated by TCA cycle changes.

Inhibition of STAT3 leads to selective killing of LSCs compared to HSCs

We next investigated whether STAT3 activity influences the survival of AML stem and progenitor cells. As shown in Figure 5A, primary ROS-low AML specimens cultured with 5μM STAT3i for 24 hours showed a significant decrease in viability as assessed by flow cytometry, and there is no difference in the efficacy of STAT3i-mediated cytotoxicity in samples from newly diagnosed patients compared to specimens from relapsed patients after chemotherapy. Further viability of a paired sample shows its sensitivity to STAT3i is unchanged in the same patient at the time of diagnosis vs time of relapse (Figure S5A). This suggests STAT3i is equally efficacious in the chemo-resistant setting. Similar results are also seen in LSCs isolated from leukapheresis samples compared to LSCs isolated from bone marrow samples (Figure 5A and S5B, respectively), suggesting the proliferative state of the patient sample does not affect the sensitivity to STAT3 inhibition. Furthermore, culturing primary AML cells with 5μM STAT3i overnight, followed by progenitor analysis in colony-formation assays showed a significant reduction in CFUs (Figure 5B). Similarly, colony-formation was decreased by shRNA-mediated knock-down of STAT3 in primary human AML cells (Figure 5C-D), further supporting a direct role for STAT3 in maintaining the viability of primitive cells. This is in contrast to normal hematopoietic stem and progenitor cells (HSPCs), which show no significant cell death and no decrease in colony formation upon STAT3 inhibition (Figures 5E-F). These findings indicate STAT3 is a selective target for LSCs.

To directly test the impact of STAT3 inhibition on LSC function, we transplanted control and STAT3i-treated AML cells from three different patients into immunodeficient NSG-S mice and measured their engraftment ability.[3,47] At 8-10 weeks post-transplant, engraftment was measured as percentage of human blasts in total mononuclear cells in the bone marrow of experimental mice. These results showed a significant decrease of engraftment in the STAT3i-treated group (Figure 5G), suggesting loss of LSC potential as a consequence of STAT3 inhibition. Further, cells isolated from an engrafted mice (Figure 5G, bracket) were utilized for a secondary transplant, which showed complete eradication of blast cells. To show that STAT3i also has *in vivo* activity, we first engrafted mice with a primary AML sample, followed by intraperitoneal treatment of STAT3i at 30mg/kg or vehicle control for 6 days. As shown in Figure 5H, a short course of STAT3i treatment leads to significant death of blasts cells from AML engrafted mice.

Discussion

STAT3 is an important transcription factor that is involved in proliferation and transformation of cancer cells,[14,16,41,48] although its role in mitochondrial function is less-well understood. In this study, we confirm prior reports that STAT3 is highly expressed and activated in primary human AML samples, and identify a role for STAT3 in regulation of mitochondrial function. We show that blocking STAT3's transcriptional activity affects mitochondrial function via regulation of the TCA cycle and thereby regulation of the ETC. STAT3's effect on the TCA cycle is mediated by its regulation of MYC expression, which in turn, regulates the amino acid transporter SLC1A5.[46] This transporter is responsible for the influx of various amino acids into LSCs, and is particularly important for glutamine intake. Glutaminolysis has previously been shown to be important for cancer cells including AML,[12,30,31,49-51] as this process results in production of alpha-ketoglutarate which is crucial for TCA cycle activity. Other TCA cycle intermediates such as succinate are also decreased upon STAT3 inhibition, thereby affecting the ETC activity of complexes I, II and III. This hypothesized mechanism is depicted in **Figure 6**. GSH, which is essential for LSCs as previously shown,[13,52] is also decreased upon STAT3 inhibition. GSH is known to regulate

succinate dehydrogenase A by S-glutathionylation,[13] and a decrease in GSH could further contribute to a decrease ETC complex activity of LSCs. The decrease in OXPHOS as a result of STAT3 inhibition likely contributes to a decrease in LSC viability, as LSCs have a unique metabolic dependency on OXPHOS for energy production. Importantly, we have previously shown that depletion of glutamine alone is not sufficient to eradicate LSCs,[12] likely due to compensation from other amino acids such as glutamate, aspartate and cysteine. However, we propose that in the context of STAT3 inhibition cells are unable to fully compensate given their decreased expression of aspartate aminotransferase which is consistent with less formation of glutamate. These studies highlight the finding that although STAT3 is a transcription factor, it indirectly exerts regulatory mitochondrial functions that are critical to leukemia stem cells. Other potential roles of STAT3 in mitochondrial function as suggested by our RNA sequencing data or localization of STAT3 to the mitochondria remain to be investigated, and possible off-target effects of our STAT3i compound that could be contributing to the metabolic changes seen in LSCs have not been further explored.

In terms of clinical potential, targeting STAT3 leads to effective killing of both the bulk leukemia and the LSC population of primary AML samples while having no identifiable impact on the normal HSPC population. Although longer-term studies will be needed to further corroborate a lack of effect of STAT3 inhibition in normal HSPCS, our studies suggest inhibition of STAT3 is a potentially useful target for therapy. Further, STAT3's sensitivity does not appear to change between newly diagnosed AML or chemo-resistant cells, broadening its potential application to patients who have failed front-line chemotherapy regimens. In the future, it will be important to determine whether STAT3 inhibition can have longer-term added toxicities, and whether can be used in combination with current standard of therapy treatments.

In conclusion, we have shown a distinct role for STAT3 in regulating mitochondrial function of AML bulk and LSCs, which occurs via a MYC-SLC1A5 mediated pathway. This important STAT3 function could be used to target the LSC vulnerability of OXPHOS dependence, and thereby serve as a potential new therapy for AML patients.

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Authorship contributions

Contribution: M.L.A, C.T.J designed research; M.L.A, A.I, S.P, C.J, A.K, F.G, S.L.F, B.S, A.D performed and/or analyzed experiments; SF25 was designed and synthesized by P.R. and S.L.F. M.L.A prepared the figures; and M.L.A and C.T.J. wrote the manuscript with input from S.P, A.I, P.R., A.D, D.A.P, H.Y, M.M, and A.W.

Conflict of interest disclosures

Authors declare no competing interests.

References

1. Shah A, Andersson TM, Rachet B, Bjorkholm M, Lambert PC. Survival and cure of acute myeloid leukaemia in England, 1971-2006: a population-based study. *Br J Haematol*. 2013;162(4):509-516.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin*. 2019;69(1):7-34.
3. Eppert K, Takenaka K, Lechman ER, et al. Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat Med*. 2011;17(9):1086-1093.
4. Gentles AJ, Plevritis SK, Majeti R, Alizadeh AA. Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia. *JAMA*. 2010;304(24):2706-2715.
5. Ho TC, LaMere M, Stevens BM, et al. Evolution of acute myelogenous leukemia stem cell properties after treatment and progression. *Blood*. 2016;128(13):1671-1678.
6. Pollyea DA, Jordan CT. Therapeutic targeting of acute myeloid leukemia stem cells. *Blood*. 2017;129(12):1627-1635.
7. Lagadinou ED, Sach A, Callahan K, et al. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell*. 2013;12(3):329-341.
8. Liyanage SU, Hurren R, Voisin V, et al. Leveraging increased cytoplasmic nucleoside kinase activity to target mtDNA and oxidative phosphorylation in AML. *Blood*. 2017;129(19):2657-2666.
9. Simsek T, Kocabas F, Zheng J, et al. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell Stem Cell*. 2010;7(3):380-390.
10. Suda T, Takubo K, Semenza GL. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. *Cell Stem Cell*. 2011;9(4):298-310.
11. Takubo K, Nagamatsu G, Kobayashi CI, et al. Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. *Cell Stem Cell*. 2013;12(1):49-61.
12. Jones CL, Stevens BM, D'Alessandro A, et al. Inhibition of Amino Acid Metabolism Selectively Targets Human Leukemia Stem Cells. *Cancer Cell*. 2018;34(5):724-740 e724.
13. Pollyea DA, Stevens BM, Jones CL, et al. Venetoclax with azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute myeloid leukemia. *Nat Med*. 2018;24(12):1859-1866.
14. Redell MS, Ruiz MJ, Alonzo TA, Gerbing RB, Tweardy DJ. Stat3 signaling in acute myeloid leukemia: ligand-dependent and -independent activation and induction of apoptosis by a novel small-molecule Stat3 inhibitor. *Blood*. 2011;117(21):5701-5709.
15. Schuringa JJ, Wierenga AT, Kruijer W, Vellenga E. Constitutive Stat3, Tyr705, and Ser727 phosphorylation in acute myeloid leukemia cells caused by the autocrine secretion of interleukin-6. *Blood*. 2000;95(12):3765-3770.
16. Shastri A, Choudhary G, Teixeira M, et al. Antisense STAT3 inhibitor decreases viability of myelodysplastic and leukemic stem cells. *J Clin Invest*. 2018;128(12):5479-5488.
17. Zhou J, Wulfkuhle J, Zhang H, et al. Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. *Proc Natl Acad Sci U S A*. 2007;104(41):16158-16163.
18. Levy DE, Lee CK. What does Stat3 do? *J Clin Invest*. 2002;109(9):1143-1148.

19. Wingelhofer B, Neubauer HA, Valent P, et al. Implications of STAT3 and STAT5 signaling on gene regulation and chromatin remodeling in hematopoietic cancer. *Leukemia*. 2018;32(8):1713-1726.
20. Yang R, Rincon M. Mitochondrial Stat3, the Need for Design Thinking. *Int J Biol Sci*. 2016;12(5):532-544.
21. Zhang Q, Raje V, Yakovlev VA, et al. Mitochondrial localized Stat3 promotes breast cancer growth via phosphorylation of serine 727. *J Biol Chem*. 2013;288(43):31280-31288.
22. Macias E, Rao D, Carbajal S, Kiguchi K, DiGiovanni J. Stat3 binds to mtDNA and regulates mitochondrial gene expression in keratinocytes. *J Invest Dermatol*. 2014;134(7):1971-1980.
23. Wegrzyn J, Potla R, Chwae YJ, et al. Function of mitochondrial Stat3 in cellular respiration. *Science*. 2009;323(5915):793-797.
24. Benekli M, Xia Z, Donohue KA, et al. Constitutive activity of signal transducer and activator of transcription 3 protein in acute myeloid leukemia blasts is associated with short disease-free survival. *Blood*. 2002;99(1):252-257.
25. Habbel J, Arnold L, Chen Y, et al. Inflammation-driven activation of JAK/STAT signaling reversibly accelerates acute myeloid leukemia in vitro. *Blood Adv*. 2020;4(13):3000-3010.
26. Pei S, Minhajuddin M, Adane B, et al. AMPK/FIS1-Mediated Mitophagy Is Required for Self-Renewal of Human AML Stem Cells. *Cell Stem Cell*. 2018;23(1):86-100 e106.
27. Stevens BM, O'Brien C, Jordan CT, Jones CL. Enriching for human acute myeloid leukemia stem cells using reactive oxygen species-based cell sorting. *STAR Protoc*. 2021;2(1):100248.
28. Furtek SL, Matheson CJ, Backos DS, Reigan P. Evaluation of quantitative assays for the identification of direct signal transducer and activator of transcription 3 (STAT3) inhibitors. *Oncotarget*. 2016;7(47):77998-78008.
29. Nemkov T, Reisz JA, Gehrke S, Hansen KC, D'Alessandro A. High-Throughput Metabolomics: Isocratic and Gradient Mass Spectrometry-Based Methods. *Methods Mol Biol*. 2019;1978:13-26.
30. Gregory MA, Nemkov T, Park HJ, et al. Targeting Glutamine Metabolism and Redox State for Leukemia Therapy. *Clin Cancer Res*. 2019;25(13):4079-4090.
31. Gregory MA, Nemkov T, Reisz JA, et al. Glutaminase inhibition improves FLT3 inhibitor therapy for acute myeloid leukemia. *Exp Hematol*. 2018;58:52-58.
32. Ashton JM, Balys M, Neering SJ, et al. Gene sets identified with oncogene cooperativity analysis regulate in vivo growth and survival of leukemia stem cells. *Cell Stem Cell*. 2012;11(3):359-372.
33. Pei S, Pollyea DA, Gustafson A, et al. Monocytic Subclones Confer Resistance to Venetoclax-Based Therapy in Patients with Acute Myeloid Leukemia. *Cancer Discov*. 2020;10(4):536-551.
34. Baskiewicz-Masiuk M, Machalinski B. The role of the STAT5 proteins in the proliferation and apoptosis of the CML and AML cells. *Eur J Haematol*. 2004;72(6):420-429.
35. Wingelhofer B, Maurer B, Heyes EC, et al. Pharmacologic inhibition of STAT5 in acute myeloid leukemia. *Leukemia*. 2018;32(5):1135-1146.
36. Barre B, Vigneron A, Coqueret O. The STAT3 transcription factor is a target for the Myc and riboblastoma proteins on the Cdc25A promoter. *J Biol Chem*. 2005;280(16):15673-15681.

37. Snyder M, Huang XY, Zhang JJ. Identification of novel direct Stat3 target genes for control of growth and differentiation. *J Biol Chem*. 2008;283(7):3791-3798.
38. Hoffman B, Amanullah A, Shafarenko M, Liebermann DA. The proto-oncogene c-myc in hematopoietic development and leukemogenesis. *Oncogene*. 2002;21(21):3414-3421.
39. Renneville A, Roumier C, Biggio V, et al. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia*. 2008;22(5):915-931.
40. Bagger FO, Sasivarevic D, Sohi SH, et al. BloodSpot: a database of gene expression profiles and transcriptional programs for healthy and malignant haematopoiesis. *Nucleic Acids Res*. 2016;44(D1):D917-924.
41. Bowman T, Broome MA, Sinibaldi D, et al. Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proc Natl Acad Sci U S A*. 2001;98(13):7319-7324.
42. Nemkov T, D'Alessandro A, Hansen KC. Three-minute method for amino acid analysis by UHPLC and high-resolution quadrupole orbitrap mass spectrometry. *Amino Acids*. 2015;47(11):2345-2357.
43. Munger J, Bennett BD, Parikh A, et al. Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. *Nat Biotechnol*. 2008;26(10):1179-1186.
44. van Geldermalsen M, Wang Q, Nagarajah R, et al. ASCT2/SLC1A5 controls glutamine uptake and tumour growth in triple-negative basal-like breast cancer. *Oncogene*. 2016;35(24):3201-3208.
45. White MA, Lin C, Rajapakshe K, et al. Glutamine Transporters Are Targets of Multiple Oncogenic Signaling Pathways in Prostate Cancer. *Mol Cancer Res*. 2017;15(8):1017-1028.
46. Zhao X, Petrashen AP, Sanders JA, Peterson AL, Sedivy JM. SLC1A5 glutamine transporter is a target of MYC and mediates reduced mTORC1 signaling and increased fatty acid oxidation in long-lived Myc hypomorphic mice. *Aging Cell*. 2019;18(3):e12947.
47. Kreso A, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell*. 2014;14(3):275-291.
48. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. *Cell*. 1999;98(3):295-303.
49. Gallipoli P, Giotopoulos G, Tzelepis K, et al. Glutaminolysis is a metabolic dependency in FLT3(ITD) acute myeloid leukemia unmasked by FLT3 tyrosine kinase inhibition. *Blood*. 2018;131(15):1639-1653.
50. Jacque N, Ronchetti AM, Larrue C, et al. Targeting glutaminolysis has antileukemic activity in acute myeloid leukemia and synergizes with BCL-2 inhibition. *Blood*. 2015;126(11):1346-1356.
51. Wise DR, DeBerardinis RJ, Mancuso A, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci U S A*. 2008;105(48):18782-18787.
52. Pei S, Minhajuddin M, Callahan KP, et al. Targeting aberrant glutathione metabolism to eradicate human acute myelogenous leukemia cells. *J Biol Chem*. 2013;288(47):33542-33558.

Tables

AML	Diagnosis	Cytogenetics	Mutations	Age/Gender	Phereses or Bone Marrow
AML1	Relapse	Complex	IDH1, cKIT	47 yo M	Pheresis
AML2	De Novo	Complex	None	73 yo F	Pheresis
AML3	De Novo	Normal	NPM1, FLT3-TKD, PTPN11	74 yo F	Pheresis
AML4	Relapse	t(6;9)(p21;q34)	FLT3-ITD, FLT3-TKD, IDH2	79 yo M	Pheresis
AML5	Relapse	Normal	FLT3-ITD	49 yo F	Pheresis
AML6	De Novo	Normal	FLT3-ITD		Pheresis
AML7	No information	-	-	-	Pheresis
AML8	Unknown	Complex	CBL, SRSF2, TET2	80 yo F	Pheresis
AML9	No information	-	-	-	Pheresis
AML10	De Novo	Complex	FLT3-ITD, BCOR, NOTCH1	52 yo M	Pheresis
AML11	De Novo	Complex	ASXL1, DNMT3A NOTCH1, NRAS	52 yo M	Pheresis
AML12	Relapse	Normal	FLT3, NPM1, IDH1	65 yo F	Pheresis
AML13	De Novo	Complex	KRAS, PTPN11	60 yo F	Pheresis
AML14	De Novo	inv(16) (p13.1q22)	None	21 yo M	Pheresis
AML15	No Information	-	-	-	Bone Marrow
AML 16	No information	-	-	-	Bone Marrow
AML 17	Unknown	Normal	FLT3-ITD, MPN1	Unknown	Bone Marrow
AML 18	Unknown	Normal	MPN1	Unknown	Bone Marrow
AML19	No information	-	-	-	-

Table 1. Characteristics of primary AML samples used for these studies

Experiment	Samples Used
RNA sequencing	AML: 1, 4, 6, 14
ChIP Assay	AML: 1, 4, 6
Metabolomics - Global	AML: 6, 11, 14
Metabolomics - Flux	AML: 4, 5, 6
siRNA Experiments	AML: 1, 3, 4, 14
Complex Activity Assays	AML: 11, 14, 3
MitoSox Assay	AML: 5, 6,14
Viability Assays LSCs	AML: 5, 6, 14, 11, 15, 16, 17, 18
Colony Assays	AML: 1, 2, 4, 6, 11, 13
PDX Ex Vivo Assays	AML: 1, 6, 11
In Vivo Assay	AML 1

Table 2. Samples utilized for each experiment

Figure Legends

Figure 1. STAT3 is expressed in primary AML cells and it plays a role in OXPHOS. (A) Western Blot showing total and phosphorylated STAT3 levels in mononuclear cells derived from two cord blood (CB) and 18 human primary AML samples. (B) qPCR showing mRNA expression levels of STAT3 in 3 primary AML samples treated with scramble siRNA (siSCR) control or siRNA against STAT3 (siSTAT3) after 48 hours of culture. (C) Western blot showing STAT3 protein levels 48-hours post transfection of siSTAT3 compared to siSCR. (D) Seahorse Cell Mito Stress Test showing oxygen consumption rate (OCR) at the time of highest STAT3 knockdown (24-48 hours) of a representative primary AML sample. (E) Combined data showing changes in basal OCR, ATP Production and Maximal Respiratory Capacity in 4 primary AML samples, including sample 1D, upon STAT3 inhibition by siRNA. (F) Viability assay of STAT3-deficient cells compared to scramble control (siSCR) after 60 hours in culture (48h siRNA KD+ 12h viability). (G) Structure of the salicylamide STAT3 inhibitor SF25 (STAT3i). (H) Cell-based ELISA performed in HeLa cells showing cellular STAT1α, STAT3, STAT5A and STAT5B binding to an immobilized DNA consensus sequence in the presence or absence of STAT3i as well as Niclosamide. (I) Viability assay in eight primary AML samples treated with various doses of STAT3i for 24 hours compared to vehicle control. (J) Maximal respiratory capacity changes based on Seahorse Cell Mito Stress Test in 3 primary AML samples upon treatment with 5μM STAT3i for 4 hours compared to vehicle control. Statistical analyses were performed using a Student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 2. STAT3 regulates OXPHOS in LSCs via transcriptional regulation of MYC. (A) Cartoon depiction of sorting scheme for LSCs. (B) Maximal respiratory capacity changes based on Seahorse Cell Mito Stress Test in LSCs isolated form three primary AML samples after treatment with 5μM STAT3i for 4 hours compared to vehicle control. (C) RNA-sequencing showing expression changes in the MYC Targets pathway in LSCs isolated from 4 primary AML samples after treatment with 5μM STAT3i compared to vehicle control. (D) Expression data from BloodSpot showing differences in MYC expression in AML cells compared to hematopoietic stem cells (HSCs). CBF: Core-binding factor, Complex: Complex cytogenetics. (E) ChIP-PCR showing STAT3 binding to *MYC* promoter region. Data is shown as percent input, and normalized to negative IgG control. AcH3 is used as a positive control. n = 3 (F) qPCR comparing MYC expression of LSCs treated with 5μM STAT3i for 4 hours compared to vehicle control. (G) Western Blot comparing protein levels of Myc in LSCs treated with 5μM STAT3i for 4 hours compared to vehicle control. (H) Western blot showing Myc protein expression 48 hours after siRNA transfection. (I) Seahorse Cell Mitro Stress Test showing changes in maximal respiratory capacity of 4 primary AML samples 48 hours after transfection of either siRNA against MYC or scramble control. Statistical analyses were performed using a Student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 3. STAT3 promotes glutaminolysis in LSCs via MYC's regulation of the transporter SLC1A5. (A) Cartoon depiction of experimental design. LSCs are isolated from primary human AML cells, then treated with 5μM STAT3i versus vehicle control for 3 hours. Stable-isotope labeled $^{13}C_5$ $^{15}N_2$-L-Glutamine is then added to the media and cells are collected at 1, 8 and 16 hours and analyzed by mass spectrometry. (B) Tracing experiments showing changes in intracellular glutamine, glutamate and glutathione in LSCs after 4 hours of treatment with 5μM STAT3i compared to vehicle control, n = 4. (C) Cartoon depiction of hypothesized pathway by which STAT3 regulates glutaminolysis. (D) Data from BloodSpot showing SLC1A5 gene

expression in AML cells compared to HSCs. CBF: Core-binding factor, Complex: Complex cytogenetics. (E) Western Blot showing protein levels of SLC1A5 in LSCs after a 4-hour incubation period with 5μM STAT3i compared to vehicle control. (F) Western Blot showing protein levels of SLC1A5 after knocking down MYC in a human primary AML patient sample. (G) Western Blot showing SLC1A5 protein levels after 48 hours of siRNA knockdown compared to scramble control. (H) Heat map of steady-state metabolomics in primary AML cells treated with siSCR versus siSLC1A5. (I) Seahorse assay showing OCR after 40 minutes of 5μM STAT3i-treated LSCs in the presence or absence of media supplemented with 10X Glutamine. These conditions were then recapitulated in the presence of a plasma membrane permeabilizer. n = 4. Statistical analyses were performed using a Student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

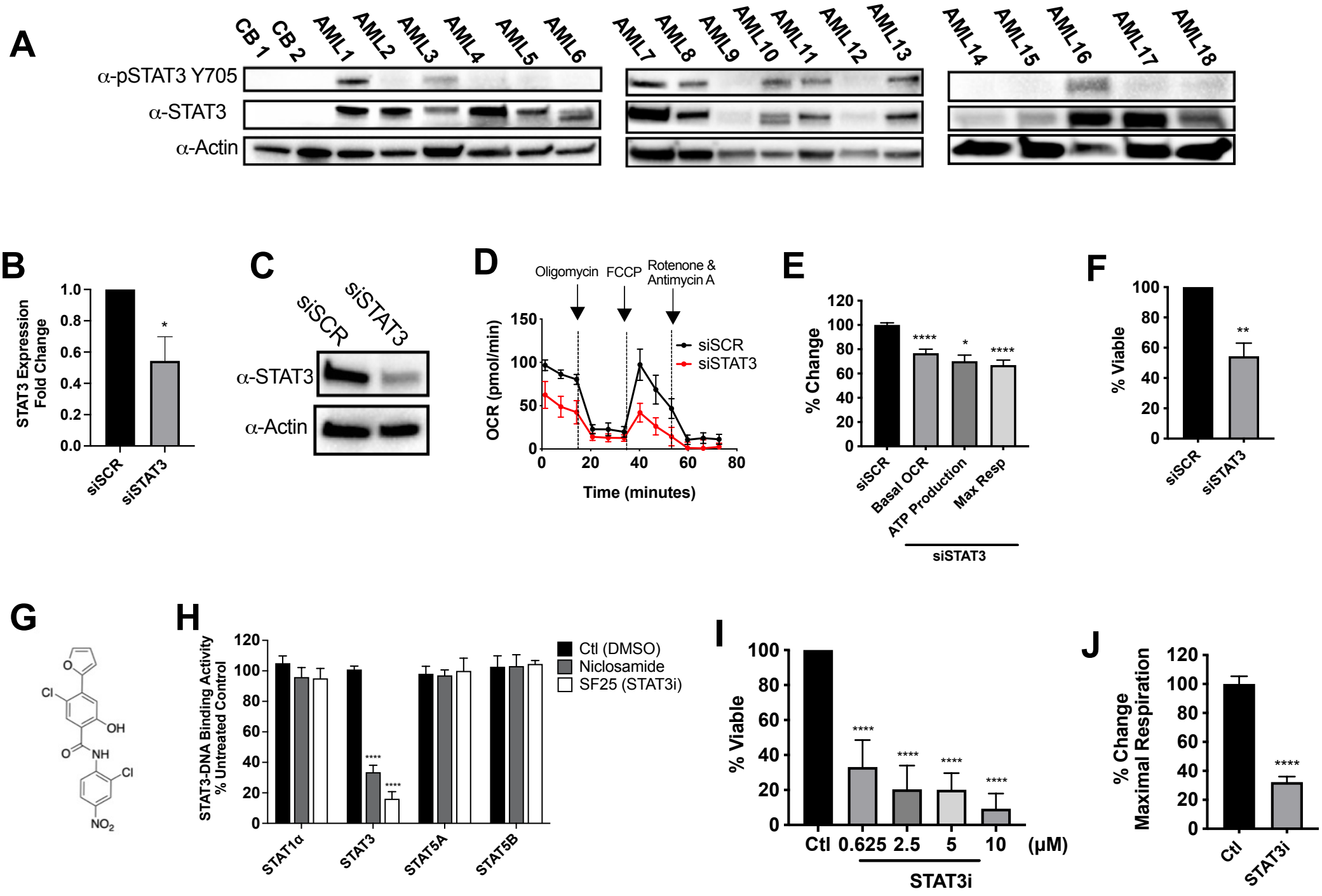
Figure 4. STAT3 regulates ETC activity of LSCs. (A) Bar graph representing complex activity of complexes I, II/III and V in three primary AML samples after treatment with 5μM STAT3i or vehicle control for 4 hours. (B) Complex II/II activity of 3 primary AML samples treated with 5μM STAT3i or vehicle control for 4 hours. Cells were incubated on metabolomics media or in the presence of 4mM glutamine and 4mM cell permeable α-KG. (C) Mitochondrial ROS as measured by flow cytometry in LSCs isolated from 3 primary AML samples and treated with 5μM STAT3i for 4 hours compared to vehicle control. 4μM of cell-permeable GSH was used as positive control. (D) qPCR showing gene expression of ETC complex genes in LSCs isolated from three primary AML samples and treated with 5μM STAT3i for 4 hours or vehicle control. (E) Gene expression from RNA-sequencing of mitochondrial genes in LSCs treated with 5μM STAT3i for 4 hours or vehicle control, n = 4. (F) Western blot showing protein levels of MT-CO1 and MT-ND2 in LSCs treated with or without 5μM STAT3i for 4 hours. Statistical analyses were performed using a Student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 5. Inhibition of STAT3 leads to selective eradication of LSCs compared to HSPCs. (A) Viability of LSCs isolated from six primary human AML samples, three at the time of diagnosis and three at the time of relapse, after 24-hour treatment with 5μM STAT3i compared to vehicle control. (B) Percent number of colonies from three primary AML samples treated with 5μM STAT3i or vehicle control for 24 hours and plated in human methylcellulose media for up to 14 days. (C) Western blot showing protein level of STAT3 after treatment with shRNA against STAT3 or scramble control (shSCR). (D) Percent number of colonies in 3 primary AML samples treated with either shSTAT3 or shSCR. (E) Viability of CD34+ cells (HSPCs) isolated from 3 human cord blood samples and treated with 5μM STAT3i vs vehicle control after 24 hours. NS = Not Significant. (F) Percent colonies of HSPCs isolated from 3 cord blood samples and treated with either 5μM STAT3i vs vehicle control for 24 hours and plated in human methylcellulose media for up to 14 days. NS = Not Significant. (G) Percent engraftment at 8-10 weeks of 3 primary AML samples pre-treated with either 5μM of STAT3i or vehicle control overnight and injected into Busulfan-treated NSG-S mice. The sample in bracket was utilized for secondary transplant, which shows complete eradication of LSCs. (H) % human blasts in mice engrafted with a primary AML samples and treated *in vivo* with 30mg/kg STAT3i or vehicle control intraperitoneally daily for 6 days. Statistical analyses were performed using a Student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 6. Hypothesized model. STAT3 is phosphorylated at Y705 which signals its localization to the nucleus to regulate the expression of several genes, including MYC. MYC, in turn,

promotes expression of the amino acid transporter SLC1A5, allowing influx of glutamine into the cell and ultimately abundance of TCA cycle intermediates and GSH. TCA cycle intermediates then promote oxidative phosphorylation by ETC activity. GSH is known to promote glutathionylation of the ETC Complex II, which is important for its activity. Upon inhibition of STAT3, glutaminolysis is compromised, decreasing levels of glutamate, GSH and TCA cycle intermediates thereby decreasing OXPHOS in LSCs.

Figure 1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AML16
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AML18
α-pSTAT3 Y705
α-STAT3
α-Actin
B
STAT3 Expression Fold Change
siSCR
siSTAT3
C
siSCR
siSTAT3
α-STAT3
α-Actin
D
Oligomycin
FCCP
Rotenone & Antimycin A
OCR (pmol/min)
siSCR
siSTAT3
Time (minutes)
E
% Change
siSCR
Basal OCR
ATP Production
Max Resp
siSTAT3
F
% Viable
siSCR
siSTAT3
G
Cl
OH
NH
Cl
NO2
H
STAT3-DNA Binding Activity % Untreated Control
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Niclosamide
SF25 (STAT3i)
STAT1α
STAT3
STAT5A
STAT5B
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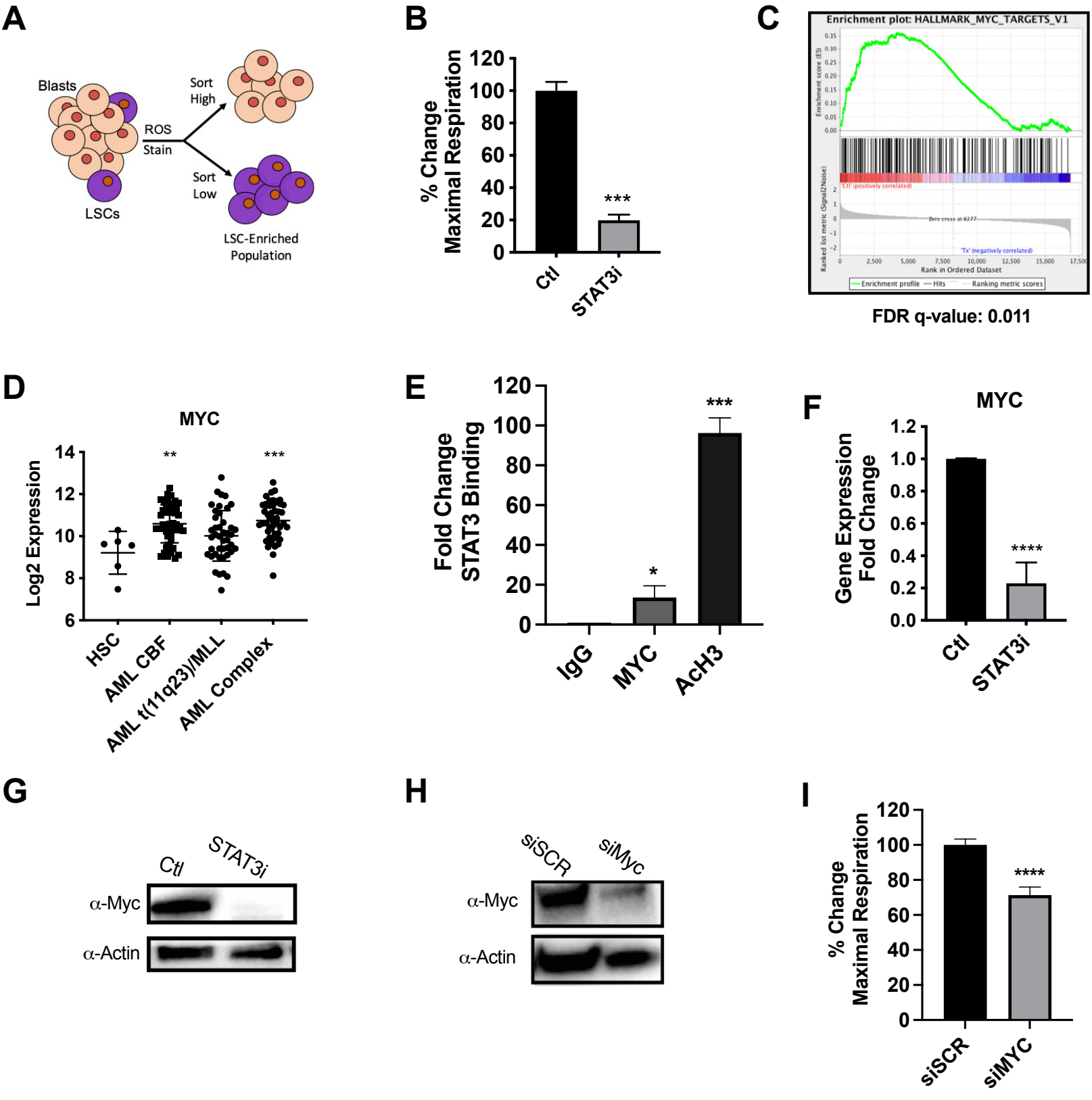
Ctl
STAT3i

Figure 2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ROS
Stain
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High
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Low
LSCs
LSC-Enriched
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Ctl
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Enrichment plot: HALLMARK_MYC_TARGETS_V1
Enrichment score (ES)
Ranked list metric (Signal2Noise)
'Ctl' (positively correlated)
Zero cross at 8277
'Tx' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
FDR q-value: 0.011
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MYC
Log2 Expression
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AML CBF
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AML Complex
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Fold Change
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IgG
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STAT3i
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Ctl
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α-Myc
α-Actin
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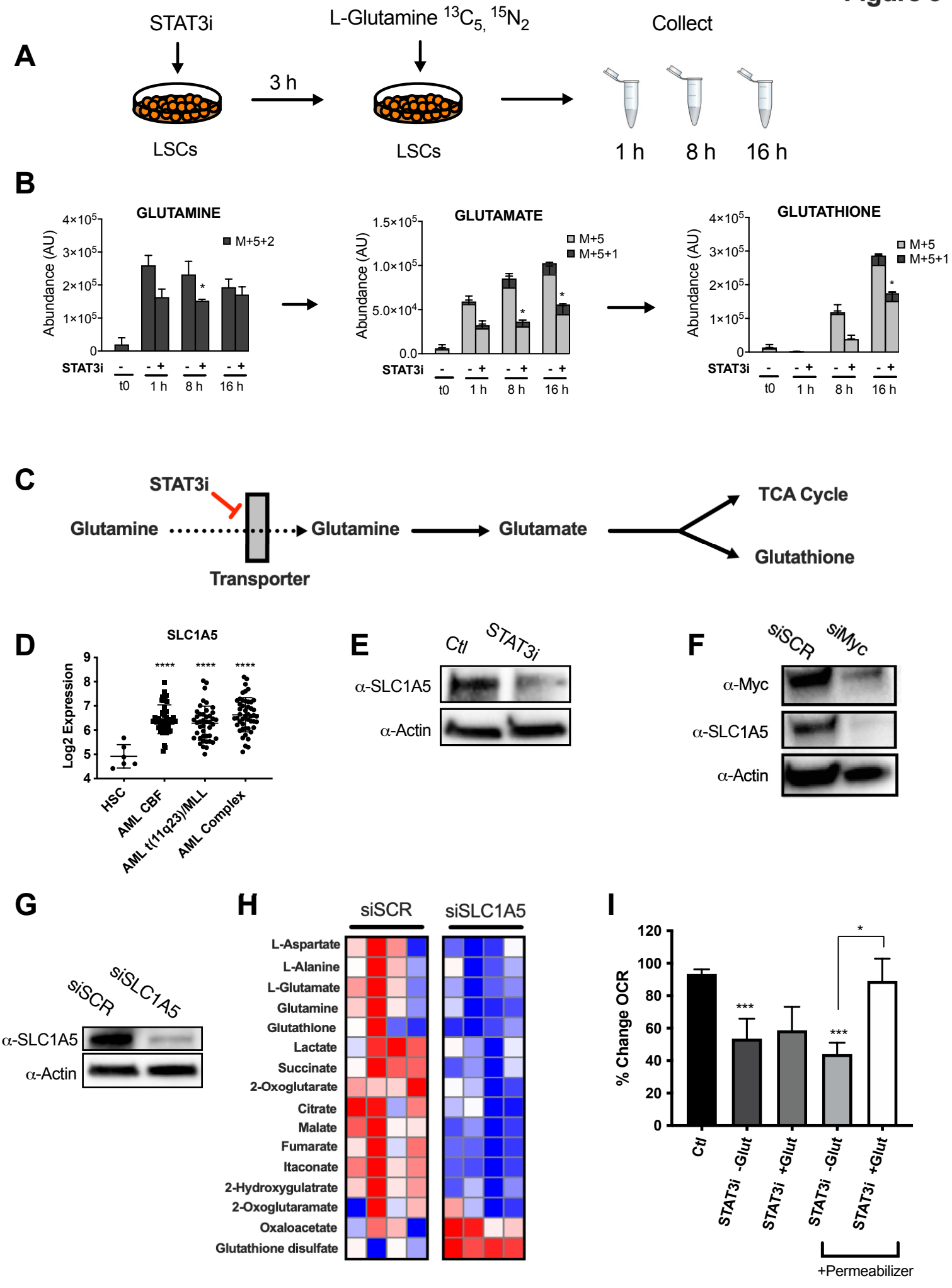
Figure 3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GLUTAMATE
GLUTATHIONE
Abundance (AU)
M+5+2
M+5
M+5+1
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STAT3i
Glutamine
Transporter
Glutamine
Glutamate
TCA Cycle
Glutathione
D
SLC1A5
Log2 Expression
HSC
AML CBF
AML t(11q23)/MLL
AML Complex
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Ctl
STAT3i
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α-Actin
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L-Aspartate
L-Alanine
L-Glutamate
Glutamine
Glutathione
Lactate
Succinate
2-Oxoglutarate
Citrate
Malate
Fumarate
Itaconate
2-Hydroxygulatrate
2-Oxoglutaramate
Oxaloacetate
Glutathione disulfate
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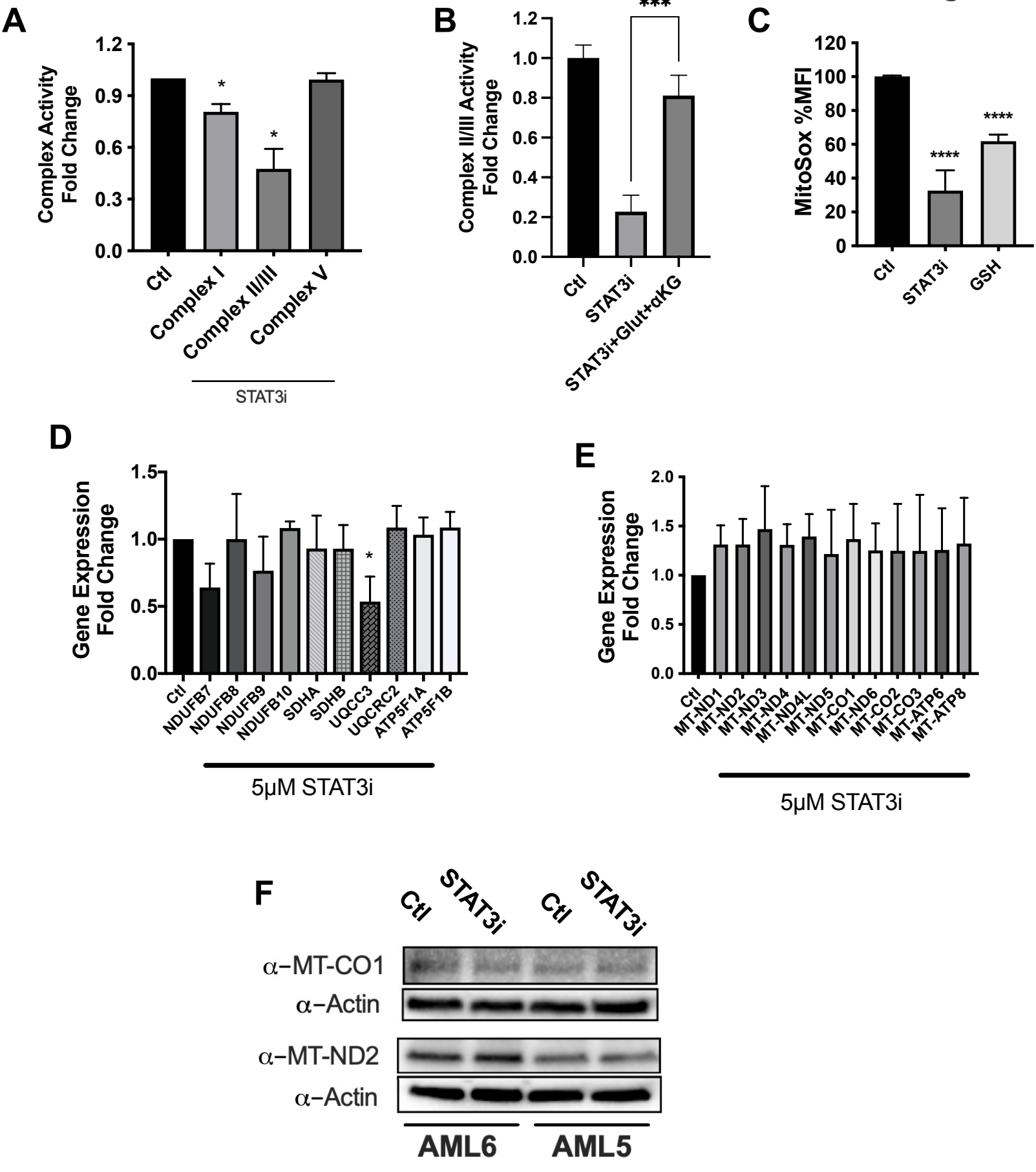
STAT3i +Glut
+Permeabilizer

Figure 4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0.2
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STAT3i
STAT3i+Glut+αKG
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Gene Expression Fold Change
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MT-ND2
MT-ND3
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MT-ND5
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MT-CO2
MT-CO3
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5μM STAT3i
F
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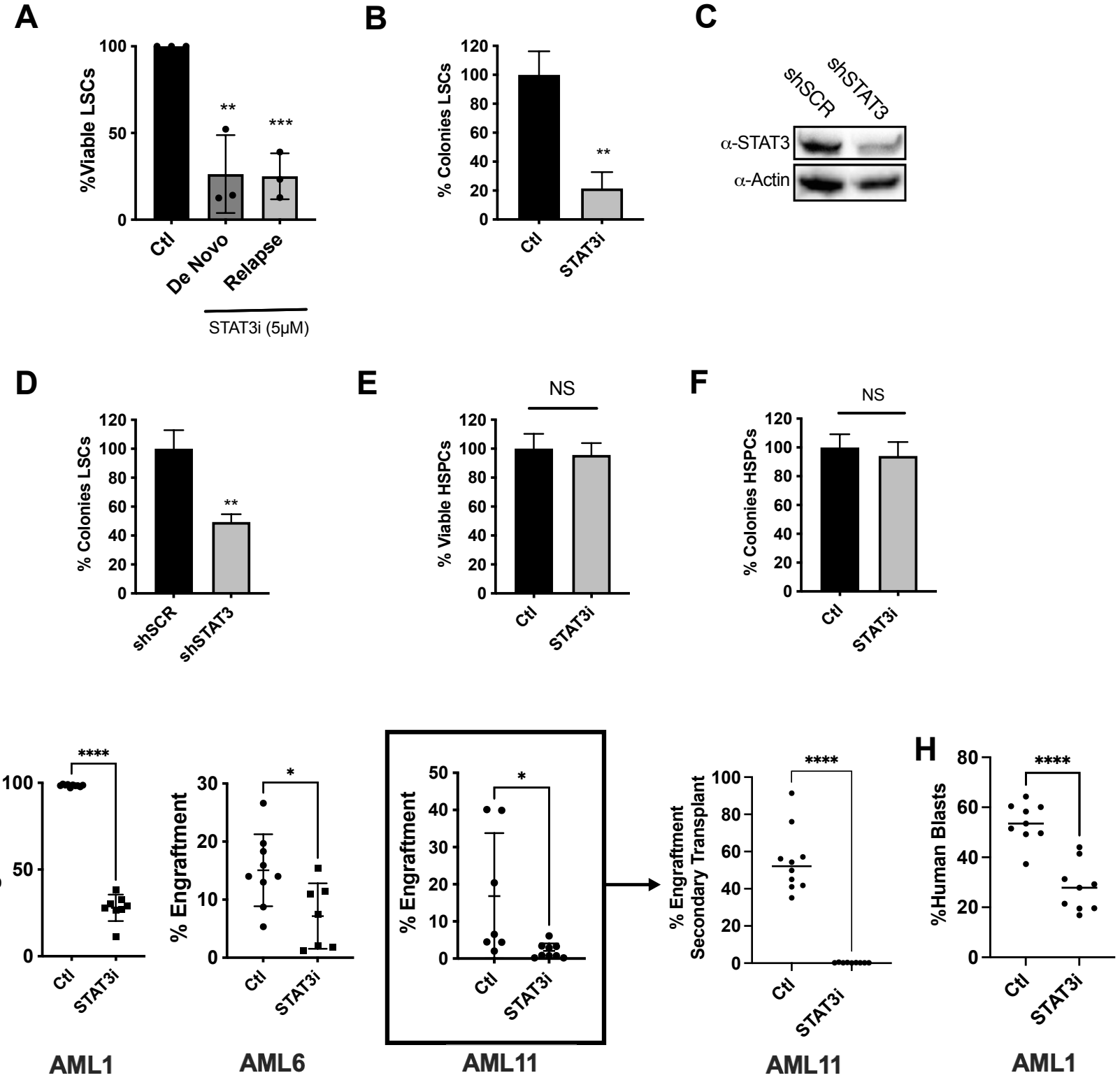
α−Actin
AML6
AML5

Figure 5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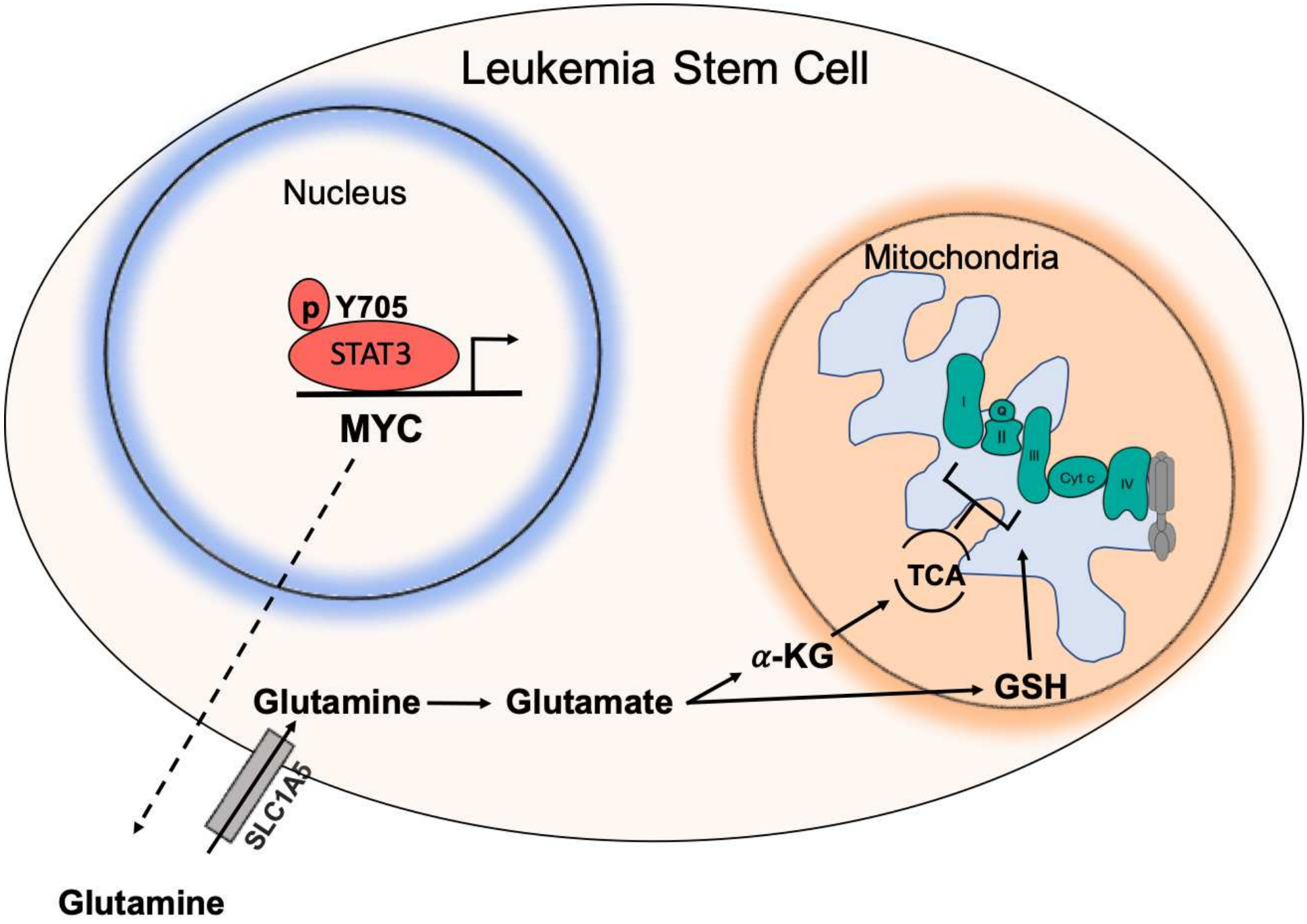
100
80
60
40
20
0
**
Ctl
STAT3i
C
shSCR
shSTAT3
α-STAT3
α-Actin
D
% Colonies LSCs
120
100
80
60
40
20
0
**
shSCR
shSTAT3
E
NS
% Viable HSPCs
120
100
80
60
40
20
0
Ctl
STAT3i
F
NS
% Colonies HSPCs
120
100
80
60
40
20
0
Ctl
STAT3i
G
% Engraftment
100
50
0
****
Ctl
STAT3i
AML1
% Engraftment
30
20
10
0
*
Ctl
STAT3i
AML6
% Engraftment
50
40
30
20
10
0
*
Ctl
STAT3i
AML11
% Engraftment Secondary Transplant
100
80
60
40
20
0
****
Ctl
STAT3i
AML11
H
%Human Blasts
80
60
40
20
0
****
Ctl
STAT3i
AML1

Figure 6


Leukemia Stem Cell
Nucleus
p Y705
STAT3
MYC
Mitochondria
I
Q
II
III
Cyt c
IV
TCA
α-KG
GSH
Glutamine
Glutamate
SLC1A5
Glutamine