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## **Cystine Uptake Inhibition Potentiates Front-Line Therapies In Acute Myeloid Leukemia**

Bryann Pardieu, Justine Pasanisi, Frank Ling, Reinaldo Dal Bello, Justine Penneroux, Angela Su, Romane Joudinaud, Laureen Chat, Hsin Chieh Wu, Matthieu Duchmann, et al.

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1 **CYSTINE UPTAKE INHIBITION POTENTIATES FRONT-LINE THERAPIES IN**  
2 **ACUTE MYELOID LEUKEMIA**

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27

28

1 **ABSTRACT**

2 By querying metabolic pathways associated with leukemic stemness and survival in  
3 multiple AML datasets, we nominated *SLC7A11* encoding the xCT cystine importer as  
4 a putative AML dependency. Genetic and chemical inhibition of *SLC7A11* impaired the  
5 viability and clonogenic capacity of AML cell lines in a cysteine-dependent manner.  
6 Sulfasalazine, a broadly available drug with xCT inhibitory activity, had anti-leukemic  
7 activity against primary AML samples in *ex vivo* cultures. Multiple metabolic pathways  
8 were impacted upon xCT inhibition, resulting in depletion of glutathione pools in  
9 leukemic cells and oxidative stress-dependent cell death, only in part through  
10 ferroptosis.

11 Higher expression of cysteine metabolism genes and greater cystine dependency was  
12 noted in *NPM1*-mutated AMLs. Among eight anti-leukemic drugs, the anthracycline  
13 daunorubicin was identified as the top synergistic agent in combination with  
14 sulfasalazine *in vitro*. Addition of sulfasalazine at a clinically relevant concentration  
15 significantly augmented the anti-leukemic activity of a daunorubicin-cytarabine  
16 combination in a panel of 45 primary samples enriched in *NPM1*-mutated AML. These  
17 results were confirmed *in vivo* in a patient-derived xenograft model. Collectively, our  
18 results nominate cystine import as a druggable target in AML and raise the possibility  
19 to repurpose sulfasalazine for the treatment of AML, notably in combination with  
20 chemotherapy.

21

## 1 INTRODUCTION

2 Acute Myeloid Leukemias (AML) encompass a genetically heterogenous group of  
3 neoplasms with poor outcome.<sup>1</sup> Long-term survival remains limited with standard of  
4 care chemotherapies combining anthracyclines with cytarabine.<sup>2</sup> New therapies are  
5 needed to improve chemotherapy efficacy in AML. Resistance to chemotherapy  
6 frequently emerges from the expression of stemness-associated transcriptional  
7 programs.<sup>3-5</sup> Metabolic rewiring plays a prominent role in the drug resistance of  
8 leukemic cells.<sup>6, 7</sup> Targeting the metabolic vulnerabilities associated with stemness  
9 programs is thus an appealing approach in AML.<sup>8, 9</sup> Repurposing of clinically approved  
10 medicines is a promising approach in oncology, including in leukemias.<sup>10, 11</sup> We  
11 therefore sought to identify targetable metabolic vulnerabilities in AML correlated with  
12 stemness programs, focusing on the druggable genome.

13 By querying correlations between expression of stemness signatures and metabolic  
14 pathways in AML datasets, we identify expression of *SLC7A11* as a poor prognostic  
15 factor and a therapeutic target in AML. *SLC7A11* encodes the antiporter xCT that  
16 imports cystine, the oxidized dimer and main source of intracellular cysteine, and  
17 exports glutamate. Targeting xCT induced a ROS-dependent death caused by cystine  
18 depletion which was at least partially attributable to ferroptosis. Finally, we show that  
19 clinically relevant concentrations of sulfasalazine (SSZ), a drug approved for  
20 inflammatory diseases but known to competitively inhibit xCT activity,<sup>12</sup> can  
21 recapitulate this effect *in vitro* and *in vivo* and improve the activity of anthracycline-  
22 based chemotherapies in AML.

23

24

## 1 **METHODS**

2 Detailed methods are provided in the *Supplementary Appendix* available online.

3

### 4 *Patient Derived Xenotransplants (PDX)*

5 The French National committee on animal care reviewed and approved all mouse  
6 experiments described in this study. Sample sizes were chosen in light of the fact that  
7 these *in vivo* models were historically highly penetrant and consistent. Animals were  
8 excluded from the study if any signs of distress were observed without clinical signs of  
9 leukemia: absence of leukemic blasts in bone marrow, spleen, and blood. Blinded  
10 observers visually inspected mice for obvious signs of distress, such as loss of  
11 appetite, hunched posture, and lethargy. In a first experiment,  $1 \times 10^6$  *CEBPA*, *RUNX1*,  
12 *ASXL1*, *EZH2*, *TET2* and *JAK2* mutated primary AML cells were tail vein injected into  
13 6 to 8 week-old sub-lethally irradiated recipient NSG-S (NSG-SGM3) males purchased  
14 from the Jackson Laboratory. This PDX model was chosen for its fast engraftment  
15 kinetics. Sixty days after injection, engraftment was confirmed by measuring circulating  
16 hCD45-positive blasts in blood (mean  $0.8\% \pm 0.3\%$ ), and mice were randomized to  
17 receive SSZ at 400 mg/kg/12h IP for 30 days or vehicle (10% DMSO + 90% HBSS),  
18 according to previously published treatment regimens.<sup>12, 13</sup> Mice were euthanized after  
19 30 days of treatment to evaluate leukemia burden in vehicle versus SSZ-treated  
20 groups. In a second experiment,  $2.5 \times 10^6$  *FLT3*-ITD, *NPM1c*, DNMT3A<sup>R882H</sup>, and  
21 IDH1<sup>R132H</sup> AML primary cells (chosen for the presence of an *NPM1c* mutation) were  
22 tail vein injected into 10 to 12 week-old sub-lethally irradiated recipient NOD.Cg-  
23 Prkdcscid IL2rgtm1Sug Tg(SV40/HTLV-IL3, CSF2) 10-7Jic/Jic Tac (hu NOG-EXL)  
24 males purchased from Taconic. This mouse strain provided optimal engraftment for

1 this primary sample. Fifty days after injection, engraftment was confirmed as *supra*  
2 (mean hCD45<sup>+</sup> blasts in blood 2.4%±1.3%). Following randomization, mice were  
3 treated once daily with chemotherapy (tail vein injection of 1mg/kg doxorubicin and  
4 50mg/kg cytarabine for 3 days and intraperitoneal injection of 50mg/kg cytarabine for  
5 2 additional days), twice daily with 150mg/kg SSZ (diluted in 10% DMSO + 90% HBSS)  
6 for 12 days, or both chemotherapy and SSZ. The '5+3' doxorubicin-based  
7 chemotherapy regimen was previously reported to mimic standard AML induction  
8 therapy.<sup>14</sup> Anthracycline dosing was reduced below the maximally tolerated dose and  
9 SSZ regimen lowered to allow concomitant administration. Bone marrow biopsies were  
10 performed on anesthetized animals at indicated time points. Sample were lysed in Red  
11 blood cell lysing buffer (Sigma-R7757), washed twice with PBS and resuspended in  
12 PBS 0.5%BSA (Sigma – A7906), 2mM EDTA prior to staining with APC-conjugated  
13 anti-human CD45 (BioLegend, 368512). Samples were washed 3 times before flow  
14 cytometry analysis. Mice were further followed-up for survival. No animals were  
15 excluded based on signs of distress in this experiment.

16

## 17 **RESULTS**

18 *The cysteine biosynthesis pathway gene SLC7A11 is a poor prognostic factor in AML.*

19 We first sought to determine through single-sample GSEA (ssGSEA) the metabolic  
20 pathways whose expression is positively correlated with stemness programs in two  
21 publicly available AML gene expression datasets (TCGA, GSE14468).<sup>15, 16</sup> In both  
22 cohorts, the cysteine/methionine biosynthesis pathway stood as the top metabolic  
23 pathway positively associated with expression of stemness programs, along with the  
24 branched-chain amino acid (valine, leucine and isoleucine) biosynthesis and lysine

1 degradation pathways (**Figure 1A-B** and **Supplementary Figure 1**). We next queried  
2 the metabolic dependencies of 12 AML cell lines with various genetic backgrounds,  
3 compared to 505 other cancer cell lines using the AVANA CRISPR library. This screen  
4 highlighted the specific functional dependency of AML cell lines on cysteine  
5 metabolism (**Figure 1C**). We therefore focused on cysteine metabolism pathway  
6 genes to identify those with prognostic relevance in AML patients. Our analysis  
7 uncovered a poor prognostic value for two genes, *CDO1* and *SLC7A11*, in both the  
8 TCGA and GSE14468 cohorts (**Figure 1D**). The poorer prognosis of AML patients  
9 expressing higher levels of *SLC7A11* which encodes the cystine/glutamate antiporter  
10 xCT was confirmed in a third cohort of 91 patients (GSE10358, **Figure 1E**).

11

#### 12 *Genetic and chemical inhibition of SLC7A11 has anti-leukemic activity*

13 To address the potential relationship between the poorer clinical outcome observed in  
14 patients with high levels of *SLC7A11* and AML cell dependence on this target, we  
15 transduced three AML cell lines, IMS-M2, OCI-AML3, and MOLM-14 with multiple  
16 hairpins whose expression reduced *SLC7A11* protein levels (**Figure 2A**). We observed  
17 a marked impairment in cell viability over a 6-day time course (**Figure 2B**). This effect  
18 was associated with a significant decrease in colony number of *SLC7A11*-depleted  
19 cells compared to those transduced with an empty vector control (**Figure 2C**). Of note,  
20 knockdown of *CDO1*, a gene downstream of *SLC7A11* in the cysteine metabolism  
21 pathway with an adverse prognostic value similar to *SLC7A11* (**Figure 1D**), did not  
22 decrease AML cell line viability (**Supplementary Figure 2**).

23



1 The *SLC7A11* gene product heterodimerizes with the promiscuous solute carrier heavy  
2 subunit encoded by *SLC3A2* to form a cystine-glutamate antiporter known as the xCT  
3 system.<sup>17</sup> Though the Alanine/Serine/Cysteine/Threonine (ASCT) neutral amino acid  
4 transporter encoded by *SLC1A4* can import cysteine from the extra-cellular  
5 environment, cystine import is the main source of intracellular cysteine, owing to the  
6 very limited amounts of reduced cysteine in the plasma.<sup>18</sup> Three chemically unrelated  
7 compounds have been previously reported to inhibit the activity of xCT, including  
8 erastin, (S)-4-Carboxyphenylglycine (CpG), and sulfasalazine (SSZ)<sup>17</sup>. SSZ is a  
9 broadly available medicine with a well-known, low toxicity profile and would thus be  
10 ideally suited for drug repurposing in AML.<sup>19</sup> We therefore tested the activity of these  
11 three xCT inhibitors in a panel of 20 cell lines encompassing a broad range of AML  
12 genetic backgrounds. Though their potency, as estimated by half-maximal inhibitory  
13 concentrations (IC<sub>50</sub>), varied across cell lines, sub-millimolar activity was noted with  
14 both SSZ and CpG (**Figure 2D**) and to a lower extent with erastin (**Supplementary**  
15 **Figure 3**). To ascertain that the anti-leukemic activity of SSZ was due to xCT inhibition,  
16 we first compared dose-response curves of SSZ, CpG and erastin. Highly significant  
17 correlations were noted among the IC<sub>50</sub>s of all three drugs (**Figure 2E**), suggesting that  
18 their anti-leukemic activity is caused by their shared xCT targeting mechanism, despite  
19 differences in their chemical structures and xCT-independent activities.<sup>17</sup> Of note, there  
20 was no clear correlation between total *SLC7A11*, *SLC3A2* or ASCT expression by  
21 western blot and xCT dependence *in vitro* (**Supplementary Figure 4**). Neither of the  
22 two SSZ metabolites sulfapyridine and mesalazine, both of which lack xCT inhibitory  
23 activity,<sup>12</sup> inhibited the viability of the two cell lines most sensitive to SSZ  
24 (**Supplementary Figure 5**). Finally, given that the RPMI culture medium contains only  
25 cystine as an extracellular source of cysteine for cells, we sought to establish whether

1 supplementation with 1 mM cysteine of this cysteine-free culture medium counteracts  
2 the anti-leukemic effect of xCT inhibition by SSZ through ASCT-driven import of  
3 cysteine. Exogenous cysteine substantially alleviated response to SSZ of OCI-AML3  
4 cells, confirming that SSZ affects primarily AML cell viability through the import of  
5 cystine and that this response can be rescued by transport of extracellular cysteine  
6 and subsequent oxidation to cystine (**Figure 2F**).

7 To critically assess the pre-clinical potential of SSZ in AML, we next examined the  
8 response to SSZ of twelve primary AML samples exhibiting various genetic alterations  
9 (Supplementary Table 5) and four CD34<sup>+</sup> specimens derived from healthy donors  
10 (**Figure 2G**). Consistent with our previous observation with cell lines, the twelve  
11 leukemia samples showed an enhanced sensitivity to SSZ treatment compared to the  
12 healthy donor derived CD34<sup>+</sup> cells (176  $\mu\text{M} \pm 40 \mu\text{M}$  versus 2.94 mM  $\pm 4.21$  mM,  
13 respectively,  $p=0.0011$ ). *Ex vivo* treatment with SSZ of 6 primary AML samples  
14 impaired the long-term culture leukemic cell-initiating capacity, compared to vehicle  
15 (**Figure 2H**). Finally, *in vivo* administration of SSZ reduced the leukemic burden in a  
16 PDX model of AML (**Figure 2I**). Taken together, these studies indicate that targeting  
17 of cystine dependence either through SLC7A11 depletion or pharmacological xCT  
18 inhibition exhibits anti-leukemic activity in both human AML cell lines and primary  
19 patient specimens exposed to SSZ either in long-term culture or *in vivo*.

20

21 *SCL7A11 expression is BRD4-dependent in AML*

22 MYC-related transcriptional programs are important regulators of stem cell biology and  
23 regulate the self-renewal and survival of leukemic stem cells.<sup>20</sup> By querying various  
24 stemness-related transcriptional programs through ssGSEA, we found a significant

1 correlation between the activation of cysteine-methionine-related gene sets and  
2 multiple MYC-driven transcriptional signatures (**Figure 3A**). Bromo- and Extra-  
3 Terminal domain (BET) proteins, including BRD4, interact with acetylated histones in  
4 active regulatory domains (promoters and enhancers) and promote RNA Pol II activity.  
5 Despite the general nature of this mechanism, BET inhibitors such as OTX015, I-  
6 BET151, and JQ1, have been shown to have selective effects on gene expression  
7 through suppression of *MYC* and MYC-related transcriptional programs.<sup>21, 22</sup> Peaks  
8 corresponding to BRD4-binding regions and overlapping with the binding signal of the  
9 transcriptional activation histone mark H3K27Ac were present at the same promoter  
10 region of the human *SLC7A11* gene and at a putative super-enhancer in two  
11 independent ChIP-sequencing experiments performed in MOLM-14 and OCI-AML3  
12 cells (**Figure 3B**). Other activation histone marks including H3K4me1 and H3K9ac  
13 were present at this region (**Supplementary Figure 6**), and CRISPRi-mediated  
14 repression of this super-enhancer confirmed its role in *SLC7A11* expression (**Figure**  
15 **3C**). Consistent with its inhibitory effect on BET proteins, I-BET151 decreased the  
16 binding of BRD4 in these two regions, suggesting that BRD4 promotes the expression  
17 of *SLC7A11*. To test this hypothesis, we knocked down BRD4 in OCI-AML3 cells using  
18 two *BRD4*-directed shRNAs and observed a significant decrease in the expression of  
19 the canonical BRD4 transcriptional target *MYC* and *SLC7A11* both at RNA and protein  
20 levels (**Figures 3C and 3D**). Consistent with these results, the BET inhibitors, OTX015  
21 and JQ1 reduced both *SLC7A11* mRNA and protein levels in the three AML cell lines  
22 tested (**Figures 3E and 3F**). Taken together, our results suggest that *SLC7A11*  
23 expression is substantially regulated by the BET protein BRD4.

24

25 *xCT* inhibition induces global metabolic rewiring and ROS-mediated cell death in AML

1 Clinically active drugs in AML impair leukemic viability by inducing apoptosis, cell cycle  
2 arrest and/or differentiation.<sup>23</sup> However, treatment of three different xCT-dependent  
3 AML cell lines with SSZ or CpG did not induce apoptosis as assessed by flow  
4 cytometry, caspase-3 cleavage, cell cycle arrest or differentiation (**Supplementary**  
5 **Figure 7**). In addition, electron microscopy in two cell lines revealed that treatment with  
6 SSZ did not induce morphological features of apoptosis or autophagy (**Supplementary**  
7 **Figure 8**). To gain insights into the metabolic consequences of xCT inhibition, we used  
8 a mass spectrometry-based metabolism profiling approach on IMS-M2 cells treated  
9 with either CpG or SSZ. Among 117 annotated metabolites, steady state levels of 38  
10 (Supplementary Table 8) highly enriched in pathways directly coupled to  
11 cystine/cysteine metabolism, taurine and glutathione metabolism, as well as glycine,  
12 serine, and threonine metabolisms, or in pathways related to purine/pyrimidine,  
13 alanine/aspartate/glutamate, and glycerophospholipid metabolisms, were significantly  
14 altered upon xCT inhibition (**Figures 4A and 4B**).

15 Our analysis revealed a marked decrease in glutathione, one of the main products of  
16 the cystine/cysteine metabolism which exerts potent antioxidant activity by providing  
17 cellular protection against reactive oxygen species, ROS.<sup>24, 25</sup> Pronounced glutathione  
18 depletion upon xCT inhibition by CpG or SSZ was confirmed in IMS-M2 and OCI-AML3  
19 cells (**Figure 4C**). According to this observation, SSZ triggered an accumulation of  
20 ROS as reflected by increased H2DCFDA intracellular fluorescence (**Figure 4D**). This  
21 effect was abolished by the two ROS scavengers, N-acetyl-cysteine (NAC) and 2-  
22 mercaptoethanol (2-ME, **Figure 4D**). In addition, NAC and 2-ME supplementation  
23 substantially decreased the sensitivity to SSZ and CPG of IMS-M2 and OCI-AML3 cells  
24 (**Figures 4E and 4F**). Of note, genetic invalidation of the ROS sensor *PML* did not alter  
25 the sensitivity of OCI-AML3 cells to SSZ (**Supplementary Figure 9**). Finally, using a

1 C11: BODIPY staining approach, we showed that SSZ-induced ROS accumulation  
2 promotes lipid peroxidation in IMS-M2, and, to a lesser extent, OCI-AML3 cells, an  
3 effect that was abrogated by NAC and 2-ME supplementation (**Figure 4G**). A peculiar  
4 form of cell death, known as ferroptosis, has been described as resulting from the  
5 accumulation of lipid-based ROS, particularly lipid hydroperoxides. Given that this form of  
6 cell death is biochemically and morphologically distinct from other cell death  
7 modalities,<sup>26</sup> including apoptosis, necrosis, and necroptosis which are not induced by  
8 SSZ (**Supplementary Figure 7**), we investigated the effect of the ferroptosis inhibitor,  
9 ferrostatin-1, and showed this compound rescued partially the loss of viability induced  
10 by SSZ in IMS-M2 and OCI-AML3 cell lines (**Figure 4H**), as did the iron chelator  
11 deferoxamine, in contrast to inhibitors of apoptosis (QVD-OPH), autophagy  
12 (chloroquine) or necroptosis (necrostatin-1) (**Supplementary Figure 10**). Collectively  
13 these data show that xCT inhibition by SSZ in AML cells results in global metabolic  
14 rewiring and depletion of antioxidant defense systems including glutathione, resulting  
15 in ROS-dependent cell death.

16

### 17 *Sulfasalazine synergizes with anthracycline-based chemotherapies in NPM1c AML*

18 Among the panel of 20 AML cell lines tested for xCT dependency (**Figure 2D**), the two  
19 most sensitive to both SSZ and CpG were OCI-AML3 and IMS-M2, the only ones  
20 harboring *NPM1c* mutations. We therefore explored a possible specific dependency of  
21 *NPM1c* AMLs on xCT antiporter activity. Whereas *NPM1c* AMLs from both TCGA and  
22 GSE14468 exhibited comparable *SLC7A11* transcript levels (**Figure 5A**), they had  
23 higher expression of cysteine pathway genes (**Figure 5B**). In dose-response assays,  
24 OCI-AML3 and IMS-M2 required higher concentrations of cystine medium  
25 supplementation to rescue viability, compared to *NPM1* wildtype cell lines, suggesting

1 *that* NPM1c AML cell lines are more vulnerable to cystine import inhibition than their  
2 wild-type counterparts (**Figure 5C**). Of note, SSZ did not alter the cytoplasmic  
3 localization of the mutant NPM1c protein (**Supplementary Figure 11**).

4 Because of the trend towards greater xCT dependency in NPM1c AML, we performed  
5 dose-response viability assays in OCI-AML3 and IMS-M2 cells combining SSZ with a  
6 panel of 8 clinically available drugs all reported to have cytotoxic or differentiating  
7 activities in NPM1c AML (daunorubicin [DNR], cytarabine [AraC], actinomycinD  
8 [ActD], venetoclax [VEN]) or differentiating (arsenic trioxide [ATO], all-trans retinoic  
9 acid [ATRA], azacitidine [AZA] and selinexor [SEL]).<sup>10, 11, 27-29</sup> Synergism between SSZ  
10 and the anthracycline DNR was prominent in both cell lines (**Figure 5D**) and across all  
11 SSZ concentrations, including sub-micromolar (**Supplementary Figure 12**).

12 Anthracyclines are administered in combination with AraC in AML. We thus leveraged  
13 a previously reported *ex vivo* drug sensitivity screen performed in 45 primary AML  
14 samples (including 38 *NPM1c* AMLs) by multiparametric flow cytometry after a 72-hour  
15 treatment in niche-like conditions.<sup>30</sup> Specifically, we explored the addition of a fixed,  
16 low concentration of SSZ (4  $\mu$ M, in the range of trough plasma concentrations of SSZ)<sup>31</sup>  
17 to a 5-point 10-fold dilution of the DNR-AraC combination at a fixed 1:20 molar ratio  
18 mimicking conventional pharmacokinetics of these chemotherapeutic agents.<sup>32</sup>

19 Overall, low SSZ concentration resulted in higher activity of the DNR-AraC combination  
20 on both the total leukemic bulk ( $p < 0.0001$ ) and on GPR56+ leukemic stem cells  
21 ( $p = 0.0006$ , **Figure 5E**). To confirm the additive effect of SSZ on anthracycline-  
22 cytarabine chemotherapy combination *in vivo*, we transplanted primary leukemic blasts  
23 from a patient harboring NPM1c along with frequent NPM1c co-mutations (*DNMT3A*,  
24 *FLT3-ITD* and *IDH1*) into sub-lethally irradiated NOG-EXL recipient mice. Following  
25 engraftment, mice were randomly assigned to vehicle treatment, single-agent SSZ

1 (150 mg/kg twice daily) for two weeks, a maximally tolerated '5+3' regimen of  
2 doxorubicin (1 mg/kg/d for 3 days) and AraC (50 mg/kg/d for 5 days), or the  
3 combination of SSZ and Doxo-AraC chemotherapy (**Figure 5F**). Compared to vehicle-  
4 treated mice, single-agent SSZ significantly reduced leukemic burden in the bone  
5 marrow following treatment completion (**Figure 5G**). Importantly, the addition of SSZ  
6 to chemotherapy further reduced leukemic burden compared to chemotherapy alone,  
7 both at an early (**Figure 5G**) and a later time point following SSZ treatment completion  
8 (**Figure 5H**), and further expanded survival (**Figure 5I**).

9

#### 10 *Oxidative stress and anti-leukemic activity with clinical concentrations of sulfasalazine*

11 In patients, orally administered SSZ is cleaved in the gut into 5-aminosalicylic acid and  
12 sulfapyridine. The SSZ pro-drug endowed with xCT inhibitory activity has limited  
13 plasma bioavailability, with peak concentrations of ~100  $\mu\text{M}$ ,<sup>31</sup> i.e. in the range of  $\text{IC}_{50}$   
14 values of the most xCT-dependent cell lines (**Figure 2D**). We compassionately treated  
15 a patient with hyperleukocytic refractory AML with SSZ dosed in the lower range of  
16 regimens approved in adults (3-6 g/d). ROS induction was notable after 3 days SSZ  
17 exposure and increased at day 7 (**Figure 5J**), suggesting that SSZ-mediated SLC7A11  
18 inhibition is clinically achievable. Addition of SSZ to a stably dosed palliative regimen  
19 of the cytoreductive agent hydroxyurea (HY) also resulted in a prompt, though  
20 transient, drop in the peripheral blood leukemic burden, allowing discontinuation of HY  
21 (**Figure 5K**). Collectively, these data confirm *in vivo* the single-agent anti-leukemic  
22 activity of SSZ, but also provide rationale for further clinical investigation of SSZ  
23 combination with anthracycline-based chemotherapies in AML.

24

## 1 DISCUSSION

2 Here we report a framework for the systematic interrogation of metabolic vulnerabilities  
3 amenable to drug repurposing in AML. By inspecting correlations between expression  
4 of metabolic pathway and stemness signatures in multiple datasets, we nominated the  
5 *SLC7A11* gene as a poor prognostic factor in AML. Genetic and chemical inhibition of  
6 its gene product, encoding the xCT cystine importer, reduced the viability of multiple  
7 AML cell lines and primary patient samples with diverse genetic backgrounds.  
8 Conversely, the poor prognostic value of higher *CDO1* expression was not associated  
9 to a functional dependency in two cell lines.

10 The BET protein BRD4 regulates key gene expression programs involved in leukemic  
11 progression, including those executed by the MYC oncogene.<sup>21, 22</sup> We found  
12 expression of the cysteine metabolism pathway to be closely related to MYC signatures  
13 in primary AML datasets. ChIP-Seq analyses revealed BRD4 binding at the *SLC7A11*  
14 promoter and putative enhancers, while genetic and chemical repression of BRD4  
15 abrogated *SLC7A11* expression at both gene and protein levels. These findings are in  
16 keeping with the recent report of xCT downregulation upon BET protein degradation.<sup>33</sup>  
17 Thus, BRD4 appears to positively regulate the cysteine pathway through *SLC7A11*  
18 expression. Metabolic rewiring upon BET inhibition is increasingly recognized as an  
19 important mechanism of action and source of resistance to this drug class.<sup>7</sup> Further  
20 work is required to define the role of MYC itself in the regulation of *SLC7A11*.

21 In *SLC7A11*-dependent cell lines, chemical inhibition of xCT did not induce apoptosis,  
22 cell cycle arrest nor trigger differentiation. Unbiased metabolic profiling upon xCT  
23 inhibition revealed significant changes in multiple pathways, including in the anti-  
24 oxidant glutathione and taurine pathways.<sup>24, 25</sup> Glutathione levels were indeed reduced  
25 upon in vitro xCT inhibition, resulting in ROS induction. Addition of the ROS scavengers



1 NAC or 2-ME rescued viability upon xCT inhibition, demonstrating that xCT inhibition  
2 results in a form of ROS-dependent non-apoptotic cell death.

3 Impaired cystine import can trigger ferroptosis, a specific form of regulated cell death  
4 resulting from lipid peroxidation,<sup>26</sup> which has recently been studied in AML.<sup>34</sup> Though  
5 xCT inhibition in AML cell lines induced lipid peroxidation, the ferroptosis inhibitors  
6 ferrostatin-1 and deferoxamine only partly rescued cell viability upon xCT inhibition,  
7 suggesting that, beyond ferroptosis, other cell death mechanisms are triggered by  
8 ROS accumulation or glutathione depletion upon xCT inhibition in AML.<sup>35, 36</sup> Metabolic  
9 changes upon xCT inhibition were not limited to glutathione and taurine metabolism  
10 and included metabolites from the tricarboxylic acid cycle such as succinate or malate,  
11 and cystathionine, which is a precursor of cysteine but also of ketoglutaric acid.<sup>37</sup>

12 Conventional cell culture does not recapitulate human plasma concentrations of key  
13 metabolites,<sup>38, 39</sup> bone marrow oxygen tension,<sup>40</sup> or anti-oxidant defenses provided by  
14 the leukemic stroma.<sup>33, 41</sup> All of these limitations were taken in consideration in our  
15 niche-like culture conditions combining a stromal layer, physiological bone marrow  
16 oxygen tension (3%) and plasma-like medium,<sup>30</sup> limiting culture duration to 3 days, in  
17 keeping with other drug screening platforms for primary AML cells.<sup>42, 43</sup>

18 The ASCT cysteine transporter, which is expressed in AML cells, could provide  
19 resistance to xCT inhibition by allowing cysteine influx to compensate for cystine.  
20 However, concentrations of reduced cysteine are ~100 times lower than oxidized  
21 cystine in the human plasma.<sup>18</sup> The latter are within the ~100 $\mu$ M range, comparable to  
22 concentrations in conventional culture media, including our custom plasma-like  
23 medium (Supplementary Table 9).

1 Our study focused on SSZ as an xCT inhibitor because of its excellent safety profile  
2 and immediate availability for clinical trials.<sup>19</sup> SSZ is active in inflammatory diseases,  
3 possibly through inhibition of NFκB pathways.<sup>44</sup> Several lines of evidence nevertheless  
4 demonstrate that the anti-leukemic activity of SSZ results from xCT inhibition. The  
5 sensitivity profile of a panel of 20 AML cell lines to SSZ was highly correlated to that of  
6 other xCT inhibitors including CpG and erastin.<sup>45, 46</sup> This anti-leukemic activity was  
7 recapitulated by shRNA-mediated SLC7A11 repression and was rescued by cysteine.

8 The reduction in leukemic burden in 45 primary AML samples exposed to 4μM SSZ, a  
9 concentration within the range of SSZ trough concentrations in patients,<sup>31</sup> and the  
10 finding of ROS induction and cyto-reduction in a clinical setting, provide evidence that  
11 xCT inhibition can be achieved in patients by oral administration of conventional SSZ  
12 dosing regimens. The therapeutic window for xCT inhibitors in AML is further supported  
13 by >10-fold increased sensitivity of primary AML samples to SSZ compared to healthy  
14 CD34+ cells.

15 NPM1c AML, the most frequent AML genetic group,<sup>1</sup> is exquisitely sensitive to  
16 oxidative stress, although the molecular underpinnings for this ROS vulnerability  
17 remain unclear.<sup>11, 47</sup> Despite indications that NPM1c could be a biomarker of xCT  
18 dependence, dose-response assays to single-agent SSZ revealed no difference  
19 between *NPM1* mutated and wild type primary samples and chemosensitization by  
20 SSZ was noted regardless of *NPM1* status (*not shown*).

21 Repurposing of SSZ to inhibit xCT has been investigated alone or with standard of care  
22 therapies in non-hematological malignancies, so far with mitigated results.<sup>48-50</sup> Novel  
23 agents in AML often require combination therapy to fulfill their anti-leukemic potential.  
24 We uncovered robust synergism between SSZ and the anthracycline daunorubicin

1 (DNR), possibly through anthracycline mediated ROS induction.<sup>51, 52</sup> In patients,  
2 anthracyclines are administered in combination with cytarabine, which did not  
3 synergize with SSZ. The clinical relevance of this finding was however confirmed by *in*  
4 *vitro* and *in vivo* experiments combining anthracyclines and cytarabine with or without  
5 SSZ, though doxorubicin had to be substituted for daunorubicin for *in vivo*  
6 experiments.<sup>14</sup>

7 A formal comparison between xCT inhibitors, which may affect cancer viability by  
8 multiple mechanisms,<sup>53</sup> more specific ferroptosis inducers such as GpX4 inhibitors,<sup>54</sup>  
9 and other GSH-depleting agents with promising anti-leukemic activity such as APR-  
10 246,<sup>34</sup> will be the focus of future studies. Our findings strengthen a growing interest in  
11 targeting cysteine metabolism in cancer cells,<sup>55, 56</sup> and prompt further investigation of  
12 novel, more specific xCT inhibitors in AML, all of which are still at very early stages of  
13 drug development.<sup>57, 58</sup> Until then, our findings support clinical investigation of xCT  
14 inhibition in combination with anthracycline-based chemotherapy in AML.

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## 1 REFERENCES

- 2 1. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, *et al.* Genomic  
3 Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med* 2016 Jun 09; **374**(23):  
4 2209-2221.
- 5  
6 2. Dombret H, Gardin C. An update of current treatments for adult acute myeloid leukemia. *Blood*  
7 2016 Jan 07; **127**(1): 53-61.
- 8  
9 3. Eppert K, Takenaka K, Lechman ER, Waldron L, Nilsson B, van Galen P, *et al.* Stem cell gene  
10 expression programs influence clinical outcome in human leukemia. *Nat Med* 2011 Aug 28;  
11 **17**(9): 1086-1093.
- 12  
13 4. Shlush LI, Mitchell A, Heisler L, Abelson S, Ng SWK, Trotman-Grant A, *et al.* Tracing the origins  
14 of relapse in acute myeloid leukaemia to stem cells. *Nature* 2017 Jul 06; **547**(7661): 104-108.
- 15  
16 5. Ng SW, Mitchell A, Kennedy JA, Chen WC, McLeod J, Ibrahimova N, *et al.* A 17-gene stemness  
17 score for rapid determination of risk in acute leukaemia. *Nature* 2016 Dec 15; **540**(7633): 433-  
18 437.
- 19  
20 6. Farge T, Saland E, de Toni F, Aroua N, Hosseini M, Perry R, *et al.* Chemotherapy-Resistant  
21 Human Acute Myeloid Leukemia Cells Are Not Enriched for Leukemic Stem Cells but Require  
22 Oxidative Metabolism. *Cancer Discov* 2017 Jul; **7**(7): 716-735.
- 23  
24 7. Su A, Ling F, Vaganay C, Sodaro G, Benaksas C, Dal Bello R, *et al.* The Folate Cycle Enzyme  
25 MTHFR Is a Critical Regulator of Cell Response to MYC-Targeting Therapies. *Cancer Discov* 2020  
26 Dec; **10**(12): 1894-1911.
- 27  
28 8. Jones CL, Stevens BM, D'Alessandro A, Reisz JA, Culp-Hill R, Nemkov T, *et al.* Inhibition of Amino  
29 Acid Metabolism Selectively Targets Human Leukemia Stem Cells. *Cancer Cell* 2018 Nov 12;  
30 **34**(5): 724-740 e724.
- 31  
32 9. Pollyea DA, Stevens BM, Jones CL, Winters A, Pei S, Minhajuddin M, *et al.* Venetoclax with  
33 azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute  
34 myeloid leukemia. *Nat Med* 2018 Dec; **24**(12): 1859-1866.
- 35  
36 10. Falini B, Brunetti L, Martelli MP. Dactinomycin in NPM1-Mutated Acute Myeloid Leukemia. *N*  
37 *Engl J Med* 2015 Sep 17; **373**(12): 1180-1182.
- 38  
39 11. El Hajj H, Dassouki Z, Berthier C, Raffoux E, Ades L, Legrand O, *et al.* Retinoic acid and arsenic  
40 trioxide trigger degradation of mutated NPM1, resulting in apoptosis of AML cells. *Blood* 2015  
41 May 28; **125**(22): 3447-3454.

42

- 1 12. Gout PW, Buckley AR, Simms CR, Bruchovsky N. Sulfasalazine, a potent suppressor of  
2 lymphoma growth by inhibition of the x(c)- cystine transporter: a new action for an old drug.  
3 *Leukemia* 2001 Oct; **15**(10): 1633-1640.
- 4
- 5 13. Chung WJ, Lyons SA, Nelson GM, Hamza H, Gladson CL, Gillespie GY, *et al.* Inhibition of Cystine  
6 Uptake Disrupts the Growth of Primary Brain Tumors. *The Journal of Neuroscience* 2005;  
7 **25**(31): 7101-7110.
- 8
- 9 14. Wunderlich M, Mizukawa B, Chou FS, Sexton C, Shrestha M, Sauntharajah Y, *et al.* AML cells  
10 are differentially sensitive to chemotherapy treatment in a human xenograft model. *Blood*  
11 2013 Mar 21; **121**(12): e90-97.
- 12
- 13 15. TCGA TCGAC. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia.  
14 *N Engl J Med* 2013 May 30; **368**(22): 2059-2074.
- 15
- 16 16. Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double  
17 CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid  
18 leukemia with a distinctive gene expression profile that is uniquely associated with a favorable  
19 outcome. *Blood* 2009 Mar 26; **113**(13): 3088-3091.
- 20
- 21 17. Liu J, Xia X, Huang P. xCT: A Critical Molecule That Links Cancer Metabolism to Redox Signaling.  
22 *Mol Ther* 2020 Nov 4; **28**(11): 2358-2366.
- 23
- 24 18. Fu X, Cate SA, Dominguez M, Osborn W, Özpolat T, Konkle BA, *et al.* Cysteine Disulfides (Cys-  
25 ss-X) as Sensitive Plasma Biomarkers of Oxidative Stress. *Scientific Reports* 2019 2019/01/14;  
26 **9**(1): 115.
- 27
- 28 19. Chen J, Lin S, Liu C. Sulfasalazine for ankylosing spondylitis. *Cochrane Database Syst Rev* 2014  
29 Nov 27; (11): CD004800.
- 30
- 31 20. Yamashita M, Dellorusso PV, Olson OC, Passegue E. Dysregulated haematopoietic stem cell  
32 behaviour in myeloid leukaemogenesis. *Nat Rev Cancer* 2020 Jul; **20**(7): 365-382.
- 33
- 34 21. Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI, *et al.* Inhibition  
35 of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature*  
36 2011 Oct 27; **478**(7370): 529-533.
- 37
- 38 22. Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA, *et al.* RNAi screen identifies Brd4  
39 as a therapeutic target in acute myeloid leukaemia. *Nature* 2011 Oct 27; **478**(7370): 524-528.
- 40
- 41 23. Carter JL, Hege K, Yang J, Kalpage HA, Su Y, Edwards H, *et al.* Targeting multiple signaling  
42 pathways: the new approach to acute myeloid leukemia therapy. *Signal Transduct Target Ther*  
43 2020 Dec 18; **5**(1): 288.
- 44

- 1 24. Schaffer SW, Azuma J, Mozaffari M. Role of antioxidant activity of taurine in diabetes. *Can J*  
2 *Physiol Pharmacol* 2009 Feb; **87**(2): 91-99.
- 3
- 4 25. Muri J, Kopf M. Redox regulation of immunometabolism. *Nat Rev Immunol* 2020 Dec 18.
- 5
- 6 26. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, *et al.* Ferroptosis: A  
7 Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* 2017 Oct 5;  
8 **171**(2): 273-285.
- 9
- 10 27. Balsat M, Renneville A, Thomas X, de Botton S, Caillot D, Marceau A, *et al.* Postinduction  
11 Minimal Residual Disease Predicts Outcome and Benefit From Allogeneic Stem Cell  
12 Transplantation in Acute Myeloid Leukemia With NPM1 Mutation: A Study by the Acute  
13 Leukemia French Association Group. *J Clin Oncol* 2017 Jan 10; **35**(2): 185-193.
- 14
- 15 28. Brunetti L, Gundry MC, Sorcini D, Guzman AG, Huang YH, Ramabadran R, *et al.* Mutant NPM1  
16 Maintains the Leukemic State through HOX Expression. *Cancer Cell* 2018 Sep 10; **34**(3): 499-  
17 512 e499.
- 18
- 19 29. DiNardo CD, Tiong IS, Quaglieri A, MacRaid S, Loghavi S, Brown FC, *et al.* Molecular patterns  
20 of response and treatment failure after frontline venetoclax combinations in older patients  
21 with AML. *Blood* 2020 Mar 12; **135**(11): 791-803.
- 22
- 23 30. Figueiras RDB, Pasanisi J, Joudinaud R, Duchmann M, Sodaro G, Chauvel C, *et al.* Niche-like Ex  
24 Vivo High Throughput (NEXT) Drug Screening Platform in Acute Myeloid Leukemia. *Blood* 2020;  
25 **136**(Supplement 1): 12-13.
- 26
- 27 31. Yamasaki Y, Ieiri I, Kusuhara H, Sasaki T, Kimura M, Tabuchi H, *et al.* Pharmacogenetic  
28 characterization of sulfasalazine disposition based on NAT2 and ABCG2 (BCRP) gene  
29 polymorphisms in humans. *Clin Pharmacol Ther* 2008 Jul; **84**(1): 95-103.
- 30
- 31 32. Lim WS, Tardi PG, Dos Santos N, Xie X, Fan M, Liboiron BD, *et al.* Leukemia-selective uptake  
32 and cytotoxicity of CPX-351, a synergistic fixed-ratio cytarabine:daunorubicin formulation, in  
33 bone marrow xenografts. *Leuk Res* 2010 Sep; **34**(9): 1214-1223.
- 34
- 35 33. Piya S, Mu H, Bhattacharya S, Lorenzi PL, Davis RE, McQueen T, *et al.* BETP degradation  
36 simultaneously targets acute myelogenous leukemia stem cells and the microenvironment. *J*  
37 *Clin Invest* 2019 May 1; **129**(5): 1878-1894.
- 38
- 39 34. Birsén R, Larrue C, Decroocq J, Johnson N, Guiraud N, Gotanegre M, *et al.* APR-246 induces  
40 early cell death by ferroptosis in acute myeloid leukemia. *Haematologica* 2021 Jan 7.
- 41
- 42 35. Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. *Nature*  
43 *Chemical Biology* 2014 2014/01/01; **10**(1): 9-17.
- 44



- 1 36. Jones CL, Stevens BM, D'Alessandro A, Culp-Hill R, Reisz JA, Pei S, *et al.* Cysteine depletion  
2 targets leukemia stem cells through inhibition of electron transport complex II. *Blood* 2019 Jul  
3 25; **134**(4): 389-394.
- 4  
5 37. Locasale JW. Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat Rev*  
6 *Cancer* 2013 Aug; **13**(8): 572-583.
- 7  
8 38. Cantor JR, Abu-Remaileh M, Kanarek N, Freinkman E, Gao X, Louissaint A, Jr., *et al.* Physiologic  
9 Medium Rewires Cellular Metabolism and Reveals Uric Acid as an Endogenous Inhibitor of  
10 UMP Synthase. *Cell* 2017 Apr 06; **169**(2): 258-272 e217.
- 11  
12 39. Vande Voorde J, Ackermann T, Pfetzer N, Sumpton D, Mackay G, Kalna G, *et al.* Improving the  
13 metabolic fidelity of cancer models with a physiological cell culture medium. *Sci Adv* 2019 Jan;  
14 **5**(1): eaau7314.
- 15  
16 40. Spencer JA, Ferraro F, Roussakis E, Klein A, Wu J, Runnels JM, *et al.* Direct measurement of  
17 local oxygen concentration in the bone marrow of live animals. *Nature* 2014 Apr 10; **508**(7495):  
18 269-273.
- 19  
20 41. Forte D, Garcia-Fernandez M, Sanchez-Aguilera A, Stavropoulou V, Fielding C, Martin-Perez D,  
21 *et al.* Bone Marrow Mesenchymal Stem Cells Support Acute Myeloid Leukemia Bioenergetics  
22 and Enhance Antioxidant Defense and Escape from Chemotherapy. *Cell Metab* 2020 Nov 3;  
23 **32**(5): 829-843 e829.
- 24  
25 42. Tyner JW, Tognon CE, Bottomly D, Wilmot B, Kurtz SE, Savage SL, *et al.* Functional genomic  
26 landscape of acute myeloid leukaemia. *Nature* 2018 Oct; **562**(7728): 526-531.
- 27  
28 43. Pemovska T, Kontro M, Yadav B, Edgren H, Eldfors S, Szwajda A, *et al.* Individualized systems  
29 medicine strategy to tailor treatments for patients with chemorefractory acute myeloid  
30 leukemia. *Cancer Discov* 2013 Dec; **3**(12): 1416-1429.
- 31  
32 44. Wahl C, Liptay S, Adler G, Schmid RM. Sulfasalazine: a potent and specific inhibitor of nuclear  
33 factor kappa B. *J Clin Invest* 1998 Mar 1; **101**(5): 1163-1174.
- 34  
35 45. Gasol E, Jiménez-Vidal M, Chillarón J, Zorzano A, Palacín M. Membrane topology of system xc-  
36 light subunit reveals a re-entrant loop with substrate-restricted accessibility. *The Journal of*  
37 *biological chemistry* 2004 Jul 23; **279**(30): 31228-31236.
- 38  
39 46. Dixon SJ, Patel DN, Welsch M, Skouta R, Lee ED, Hayano M, *et al.* Pharmacological inhibition  
40 of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis. *Elife*  
41 2014 May 20; **3**: e02523.
- 42  
43 47. Huang M, Thomas D, Li MX, Feng W, Chan SM, Majeti R, *et al.* Role of cysteine 288 in  
44 nucleophosmin cytoplasmic mutations: sensitization to toxicity induced by arsenic trioxide and  
45 bortezomib. *Leukemia* 2013 2013/10/01; **27**(10): 1970-1980.

- 1  
2 48. Robe PA, Martin DH, Nguyen-Khac MT, Artesi M, Deprez M, Albert A, *et al.* Early termination  
3 of ISRCTN45828668, a phase 1/2 prospective, randomized study of Sulfasalazine for the  
4 treatment of progressing malignant gliomas in adults. *BMC Cancer* 2009 2009/10/19; **9**(1): 372.
- 5  
6 49. Otsubo K, Nosaki K, Imamura CK, Ogata H, Fujita A, Sakata S, *et al.* Phase I study of  
7 salazosulfapyridine in combination with cisplatin and pemetrexed for advanced non-small-cell  
8 lung cancer. *Cancer Sci* 2017; **108**(9): 1843-1849.
- 9  
10 50. Shitara K, Doi T, Nagano O, Imamura CK, Ozeki T, Ishii Y, *et al.* Dose-escalation study for the  
11 targeting of CD44v+ cancer stem cells by sulfasalazine in patients with advanced gastric cancer  
12 (EPOC1205). *Gastric Cancer* 2017 2017/03/01; **20**(2): 341-349.
- 13  
14 51. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor  
15 effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol* 1999  
16 Apr 1; **57**(7): 727-741.
- 17  
18 52. Tadokoro T, Ikeda M, Ide T, Deguchi H, Ikeda S, Okabe K, *et al.* Mitochondria-dependent  
19 ferroptosis plays a pivotal role in doxorubicin cardiotoxicity. *JCI Insight* 2020 May 7; **5**(9).
- 20  
21 53. Soula M, Weber RA, Zilka O, Alwaseem H, La K, Yen F, *et al.* Metabolic determinants of cancer  
22 cell sensitivity to canonical ferroptosis inducers. *Nature Chemical Biology* 2020 2020/12/01;  
23 **16**(12): 1351-1360.
- 24  
25 54. Yusuf RZ, Saez B, Sharda A, van Gastel N, Yu VWC, Baryawno N, *et al.* Aldehyde dehydrogenase  
26 3a2 protects AML cells from oxidative death and the synthetic lethality of ferroptosis inducers.  
27 *Blood* 2020 Sep 10; **136**(11): 1303-1316.
- 28  
29 55. Badgley MA, Kremer DM, Maurer HC, DelGiorno KE, Lee HJ, Purohit V, *et al.* Cysteine depletion  
30 induces pancreatic tumor ferroptosis in mice. *Science* 2020 Apr 3; **368**(6486): 85-89.
- 31  
32 56. Hu K, Li K, Lv J, Feng J, Chen J, Wu H, *et al.* Suppression of the SLC7A11/glutathione axis causes  
33 synthetic lethality in KRAS-mutant lung adenocarcinoma. *The Journal of Clinical Investigation*  
34 2020 04/01/; **130**(4): 1752-1766.
- 35  
36 57. Lanzardo S, Conti L, Rooke R, Ruiu R, Accart N, Bolli E, *et al.* Immunotargeting of Antigen xCT  
37 Attenuates Stem-like Cell Behavior and Metastatic Progression in Breast Cancer. *Cancer Res*  
38 2016 Jan 1; **76**(1): 62-72.
- 39  
40 58. Zhang Y, Tan H, Daniels JD, Zandkarimi F, Liu H, Brown LM, *et al.* Imidazole Ketone Erastin  
41 Induces Ferroptosis and Slows Tumor Growth in a Mouse Lymphoma Model. *Cell Chemical*  
42 *Biology* 2019 2019/05/16/; **26**(5): 623-633.e629.
- 43  
44

## 1 **FIGURE LEGENDS**

2 **Figure 1. The cysteine biosynthesis pathway gene *SLC7A11* is a poor prognostic**  
3 **factor in AML. A-B.** Heatmaps of ssGSEA z-scores for selected stemness and  
4 metabolic pathway gene expression signatures (Supplementary Table 1) from (A.) the  
5 TCGA-LAML (n=179) and (B.) the GSE14468 (n=526) AML cohorts. C. Volcano plot  
6 of gene set enrichment analysis of 90 metabolic pathways (Supplementary Table 3)  
7 with CERES dependency as a metric in AML (n=12) vs non-AML (n=505) cancer cell  
8 lines from the AVANA 18Q4 CRISPR/Cas9 screen library. D. Log-transformed  
9 unadjusted p values of univariable Cox models inspecting the prognostic value of each  
10 gene from the cysteine/methionine pathway (Supplementary Table 2) as continuous  
11 variables in patients from the TCGA-LAML (n=149) and GSE14468 (n=522) AML  
12 cohorts with available overall survival data. E. Overall survival of patients according to  
13 *SLC7A11* expression higher or lower than median values in the TCGA-LAML (n=149),  
14 GSE14468 (n=522) and GSE10358 (n = 91) AML cohorts, p values from univariable  
15 analyses (log-rank tests).

16

17 **Figure 2. Genetic and chemical inhibition of *SLC7A11* has anti-leukemic activity**  
18 **A.** Western blot of *SLC7A11* and vinculin in IMS-M2, OCI-AML3 and MOLM14 cell  
19 lines transduced with *SLC7A11* targeting shRNAs or empty vector. **B.** Viability  
20 assessed by CellTiterGlo at different time points after doxycycline induction in IMS-M2,  
21 OCI-AML3 and MOLM14 cell lines transduced with *SLC7A11* targeting or empty vector  
22 control. Mean  $\pm$ SD of CellTiterGlo luminescence relative to day 0 (7 technical  
23 replicates). Statistical difference between control and each shRNA across all time  
24 points was inspected by two-way ANOVA. **C.** Number of colonies (relative to control)  
25 after plating of  $0.5 \times 10^3$  OCI-AML3 cells transduced with shRNAs targeting *SLC7A11*

1 or empty vector for 14 days in methylcellulose. Mean  $\pm$  SD of 3 technical replicates.  
2 Unpaired t tests with Welch correction. **D.** IC<sub>50</sub> of SSZ and CpG in a panel of 20 AML  
3 cell lines after 5 days of culture measured by the CellTiterGlo viability assay. **E.**  
4 Pairwise Pearson correlation coefficients  $r$  and resulting  $p$  values between the IC<sub>50</sub>s of  
5 the 3 xCT inhibitors across the 20 AML cell lines panel. **F.** Number of colonies (relative  
6 to untreated control) after plating of  $0.5 \times 10^3$  OCI-AML3 cells cultured for 14 days in  
7 methylcellulose in the presence of SSZ at indicated concentrations or DMSO vehicle  
8 with (red) or without (black) addition of cysteine (100mM). Mean  $\pm$ SD of 3 technical  
9 replicates. Unpaired t tests with Welch correction. **G.** IC<sub>50</sub> of SSZ in a panel of 12  
10 primary AML samples and CD34+ cells from 4 healthy donors after 3 days of culture  
11 measured by CellTiterGlo. The dashed line indicates the 0.25 mM concentration  
12 retained for the long-term culture limiting dilution assay. P value from a Mann-Whitney  
13 test. **H.** Long-term culture initiating cell frequency determined by a 3-week liquid culture  
14 in niche-like conditions (hTERT-MSC-GFP feeder, 3% O<sub>2</sub>) in limiting dilution with 250  
15  $\mu$ M SSZ or DMSO vehicle in 6 primary AML samples. Wilcoxon matched-pairs signed  
16 rank test. Characteristics of primary AML samples are in [Supplementary Table 5](#). **I.**  
17 Proportion of hCD45+ leukemic cells in the bone marrow of NSG-S mice engrafted  
18 with a PDX model of AML with *CEBPA*, *RUNX1*, *ASXL1*, *EZH2*, *TET2* and *JAK2*  
19 mutations euthanized after 30 days of treatment with SSZ (400 mg/kg/12h, IP) or  
20 vehicle. Mann-Whitney test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

21

22 **Figure 3. SLC7A11 expression is BRD4 dependent in AML.** **A.** Heatmap of ssGSEA  
23 z-scores for cysteine-methione pathway program as reference and multiple MYC  
24 expression signatures from MSigDB and KEGG databases in the GSE14468 AML  
25 cohort.<sup>15</sup> **B.** ChIP-Seq data at the *SLC7A11* locus for H3K27Ac in untreated OCI-AML3

1 and MOLM-14 cells, and for BRD4 after treatment with DMSO or with the I-BET151  
2 BET inhibitor. Blue boxes indicate the position of sgRNAs for CRISPRi inhibition of the  
3 Super-Enhancer region **C.** *SLC7A11* expression determined by RQ-PCR in MOLM14  
4 cells after CRISPRi inhibition of the Super-Enhancer region or control vector. Mean  
5  $\pm$ SD of 3 technical replicates. Unpaired t tests with Welch's correction. **D.** *BRD4*, *MYC*  
6 and *SLC7A11* expression levels determined by RQ-PCR in OCI-AML3, cells after 36-  
7 hour doxycycline induction of BRD4-targeting shRNAs or empty vector. Mean  $\pm$ SD of  
8 4 technical replicates. Unpaired t tests with Welch's correction. **E.** Western blot of  
9 *SLC7A11* and vinculin in OCI-AML3 cells after 36-hour doxycycline induction of BRD4-  
10 targeting shRNAs or empty vector. **F.** *MYC* and *SLC7A11* expression level determined  
11 by RQ-PCR in OCI-AML3, IMS-M2 and MOLM14 cells after 48-hour treatment with 1  
12  $\mu$ M JQ1, OTX15 or vehicle. Mean  $\pm$ SD of 4 technical replicates. Unpaired t tests with  
13 Welch's correction. **G.** Western blot of *SLC7A11* and vinculin (loading control) in  
14 protein extracts from OCI-AML3, IMS-M2 and MOLM14 cells after 48-hour treatment  
15 with 1  $\mu$ M JQ1, OTX15 or vehicle. P values are from unpaired t tests with Welch's  
16 correction of 4 technical replicates. \*\*\* $p < 0.001$ .

17

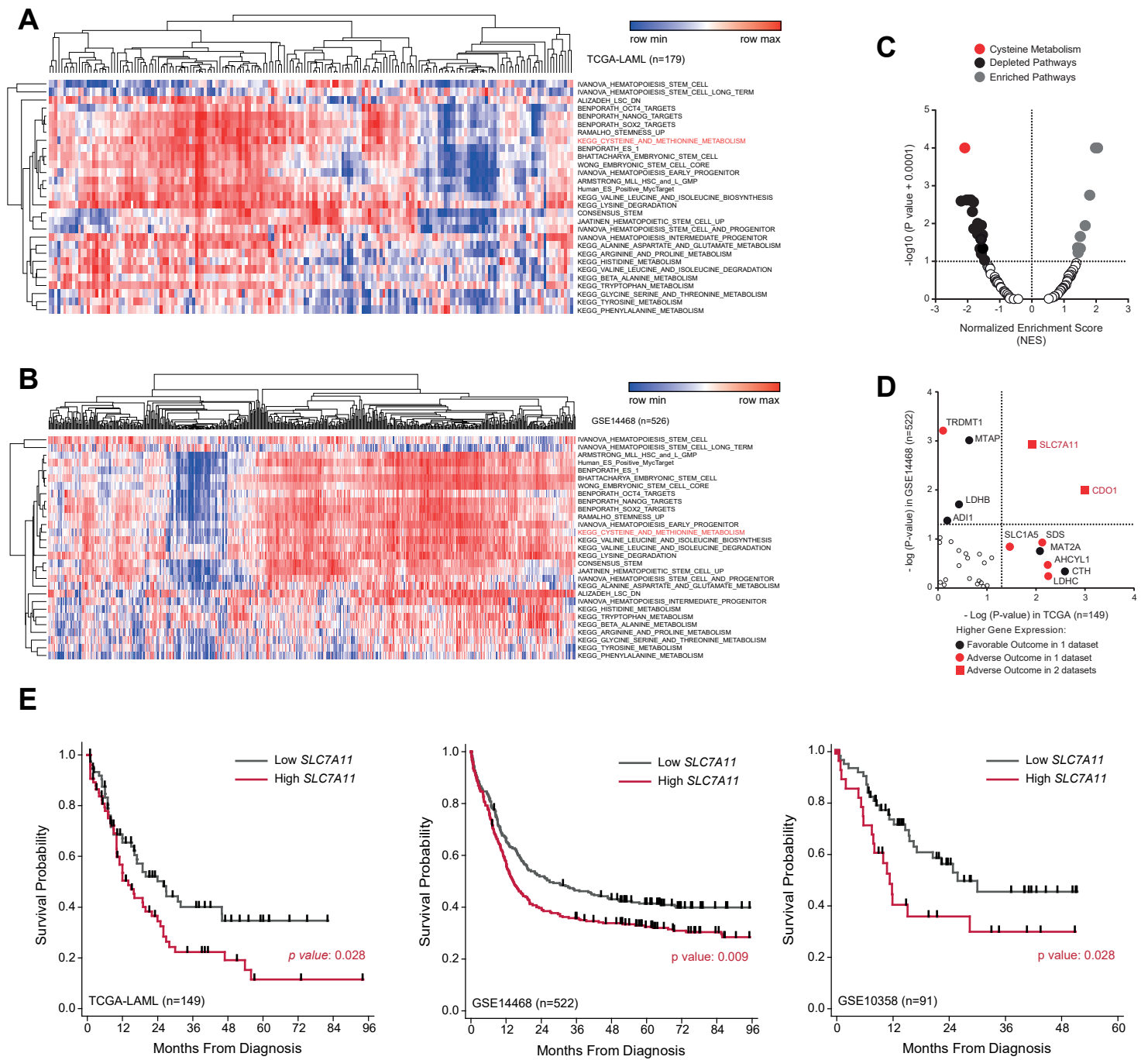
18 **Figure 4. xCT inhibition induces global metabolic rewiring and ROS-mediated**  
19 **cell death. A-B.** Heatmap (**A.**) and Pathway Impact by MetaboAnalyst (**B.**) of the top  
20 38 deregulated metabolites by LC-HRMS metabolomics in IMS-M2 cells treated for 72  
21 hours with SSZ or CpG at  $IC_{50}$  (115  $\mu$ M and 34.93  $\mu$ M respectively) or DMSO. Six  
22 technical replicates. Cut-off for this list of metabolites:  $abs[\log_2(FC)] = 1$ ,  $p\text{-val} < 0.05$   
23 **C.** Mean  $\pm$ SD of glutathione levels by colorimetric assay in IMS-M2 and OCI-AML3  
24 after a 72-hour treatment with half-maximal inhibitory concentrations of SSZ (115  $\mu$ M  
25 and 110  $\mu$ M respectively) or CpG (34.93  $\mu$ M and 28.22  $\mu$ M respectively) or DMSO.

1 Unpaired t tests with Welch's correction. **D.** Histogram plots of H2DCFDA staining of  
2 indicated cell lines after 72-hour treatment with DMSO vehicle, 250  $\mu$ M SSZ alone or  
3 combined with 55  $\mu$ M 2-mercaptoethanol (2-ME) or 2 mM N-acetylcysteine (NAC).  
4 Results are from one representative experiment of three independent replicates. **E-F.**  
5 Dose-response curves from CellTiter-Glo viability assays in increasing concentrations  
6 of **(E)** SSZ or **(F)** CpG in OCI-AML3 and IMS-M2 cells with or without 55  $\mu$ M 2-  
7 mercaptoethanol or 2 mM N-acetylcysteine. **G.** Histogram plots of C11-BODIPY  
8 staining of indicated cell lines after 72-hour treatment with DMSO vehicle, 250  $\mu$ M SSZ  
9 alone or combined with 55  $\mu$ M 2-mercaptoethanol, 2 mM N-acetylcysteine, or 10  $\mu$ M  
10 ferrostatin-1. Results from one representative of three independent replicates. **H.**  
11 Dose-response curves from CellTiter-Glo viability assays after 5-day culture with  
12 increasing concentrations of SSZ/CpG in OCI-AML3 and IMS-M2 cells, with or without  
13 10  $\mu$ M ferrostatin-1 (Fer-1).

14

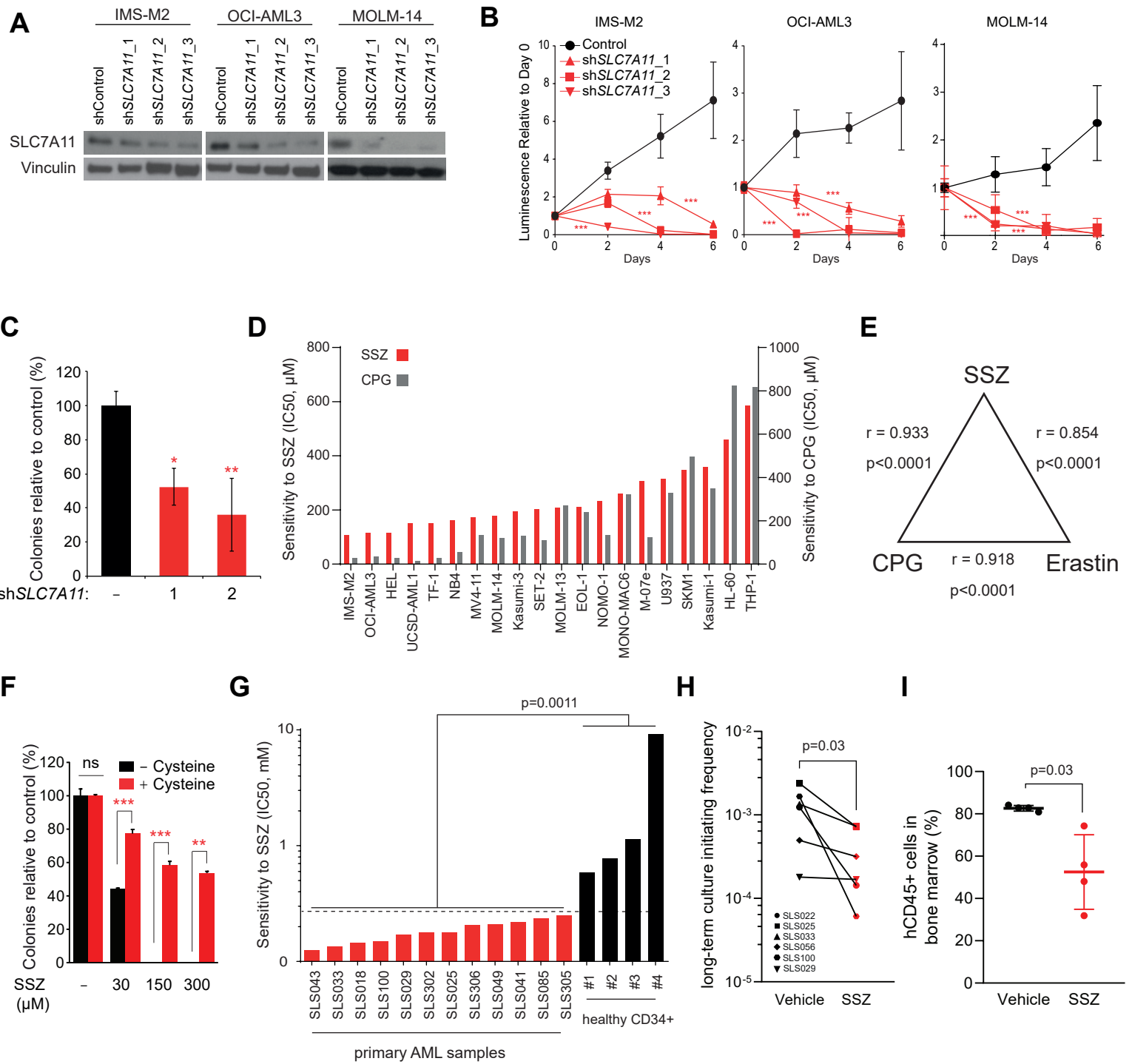
15 **Figure 5. Sulfasalazine synergizes with anthracycline-based chemotherapies in**  
16 **AML. A-B.** *SLC7A11* gene expression **(A.)** and ssGSEA z-scores of the Cysteine  
17 Metabolism Pathway **(B.)** in patients with mutant or wildtype *NPM1* from the TCGA-  
18 LAML and GSE14468 datasets (n=173 and n=525 with available *NPM1* status,  
19 respectively). P values from Mann-Whitney tests. **C.** Half maximal effective  
20 concentration ( $EC_{50}$ ) of cystine supplemented in dose-response assays (5-point 2-fold  
21 serial dilution) to cystine/cysteine-free RPM1 1640 medium. CellTiterGlo viability  
22 readout after 5 days culture in 10 AML cell lines, according to *NPM1c* status. P value  
23 from Mann Whitney test. **D.** Bliss synergy scores (mean  $\pm$  SE) across the full  
24 combination metrics of SSZ with indicated drugs (daunorubicin [DNR], cytarabine  
25 [AraC], actinomycinD [ActD], venetoclax [VEN], arsenic trioxide [ATO], all-trans retinoic

1 acid [ATRA], azacitidine [AZA] and selinexor [SEL]) in OCI-AML3 and IMS-M2 cells.  
2 Positive Bliss scores indicate synergism and negative scores antagonism. **E.**  
3 Difference in activity of DNR-AraC combination at a fixed 1:20 ratio on the in a 5-point  
4 dose-response assay in niche-like conditions with addition of a fixed 4  $\mu$ M  
5 concentration of SSZ compared to addition of SSZ vehicle (DMSO 0.1%). Activity is  
6 measured as the untruncated actual area over the curve of total leukemic bulk (viable  
7 CD19-/CD3-/CD45+ cells) and gated viable CD19-/CD3-/CD45+/GPR56+ leukemic  
8 stem cells (LSCs). P values from paired t tests. **F.** Scheme of the *in vivo* treatment of  
9 a PDX sample (harboring *NPM1c*, *FLT3<sup>ITD</sup>*, *DNMT3A<sup>R88H</sup>* and *IDH1<sup>R132H</sup>* mutations)  
10 transplanted into sub-lethally irradiated NOG-EXL recipient mice with vehicle (n=4),  
11 chemotherapy (n=4, doxorubicin 1 mg/kg/d d1-3 and cytarabine 50 mg/kg/d d1-5), SSZ  
12 (n=8, 150 mg/kg bid, d0-14) or chemotherapy + SSZ (n=8). **G-H.** Proportion of hCD45+  
13 leukemic cells in bone marrow aspirates performed at day 16 (**G.**) and day 29 (**H.**),  
14 hence 2 days and 15 days after the last SSZ or vehicle administration). Note that bone  
15 marrow aspiration was a technical failure at day 29 in one of 8 mice from the chemo  
16 only group and two of 8 mice from the chemo + SSZ group. P values from Mann-  
17 Whitney tests. **I.** Overall survival of the four mice groups since the first day of  
18 treatments. P values from log-rank tests. **J.** Histogram plot of H2DCFDA staining in  
19 PBMCs (>90% blasts) from patient SLS341 prior to (day 0), at day 3 (SSZ 1.5g thrice-  
20 daily) and 7 (SSZ 2g thrice-daily) of a compassionate SSZ treatment. **K.** Longitudinal  
21 monitoring of white blood cell (WBC) count (left axis) and dosing of compassionate  
22 SSZ and cytoreductive hydroxyurea (HY, both on right axis) in patient SLS341. Day 0  
23 refers to the start of SSZ therapy.

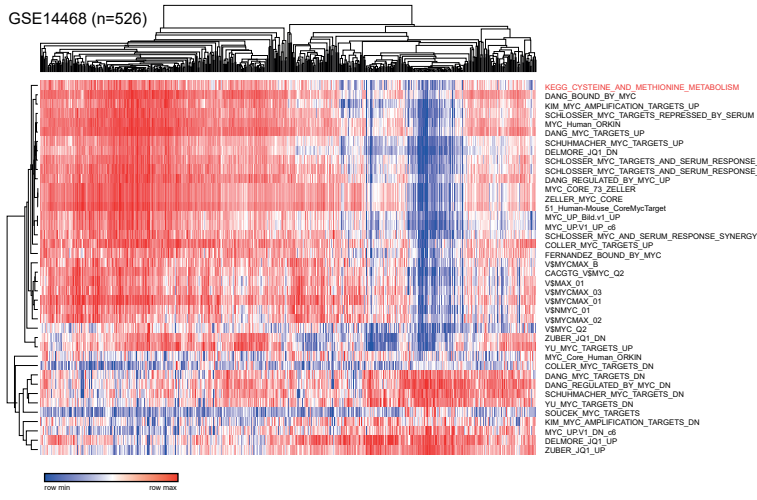
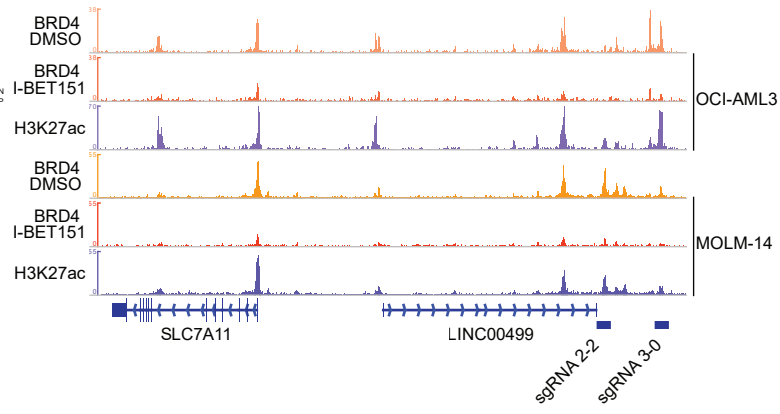
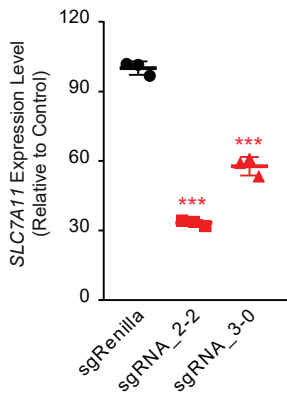
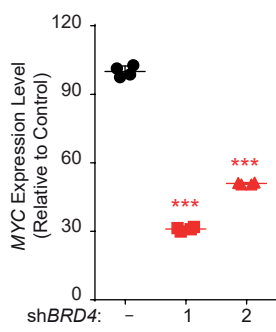
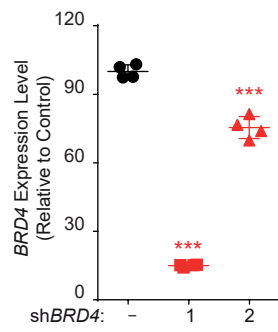
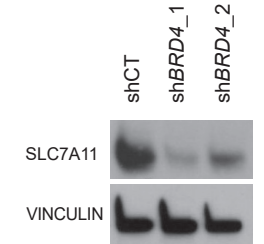
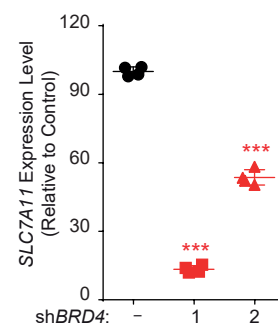
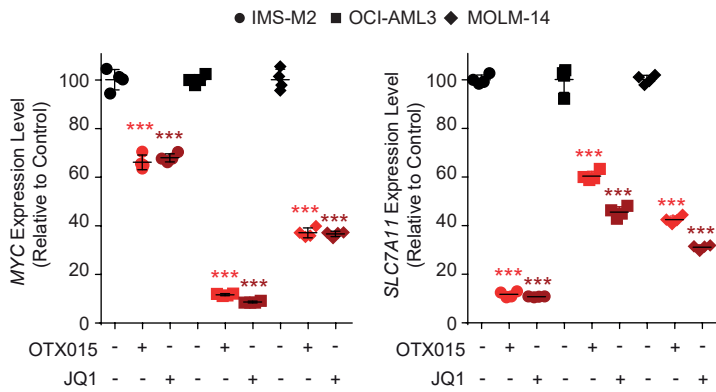
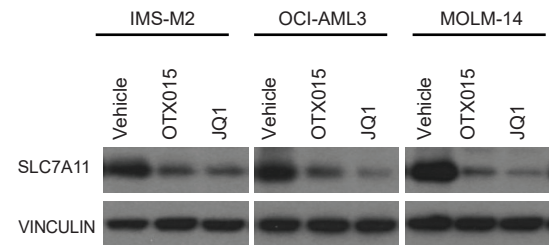


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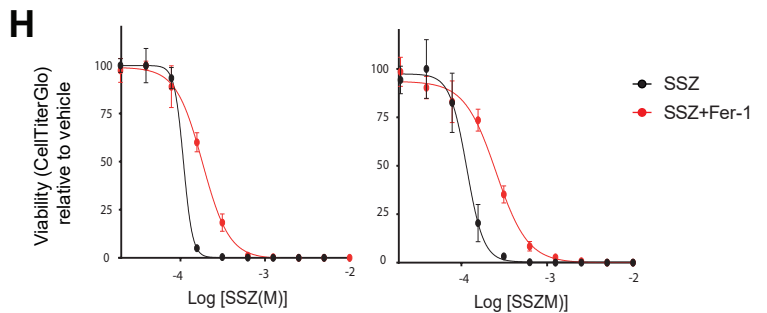
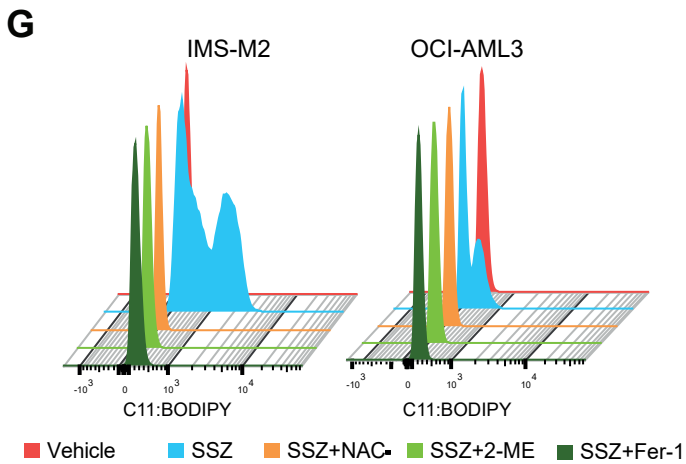
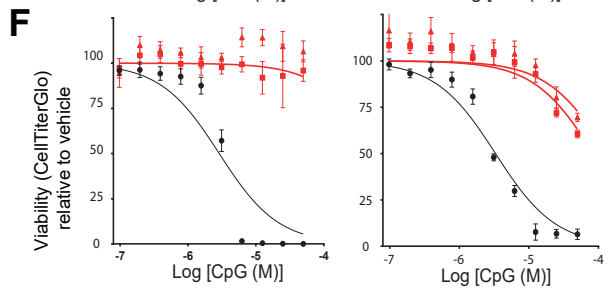
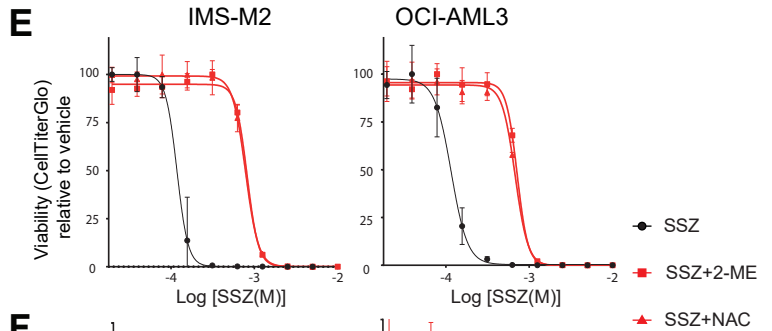
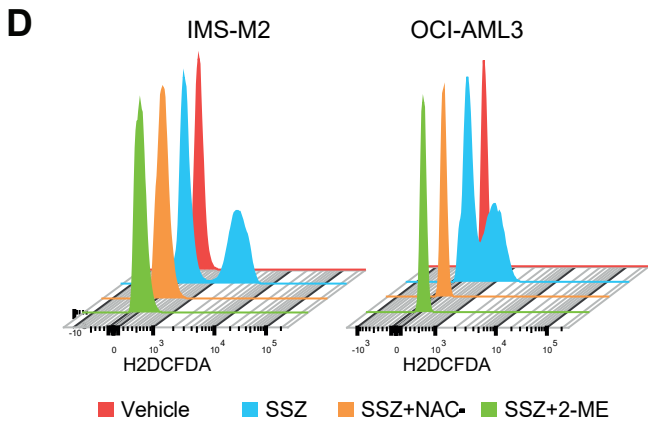
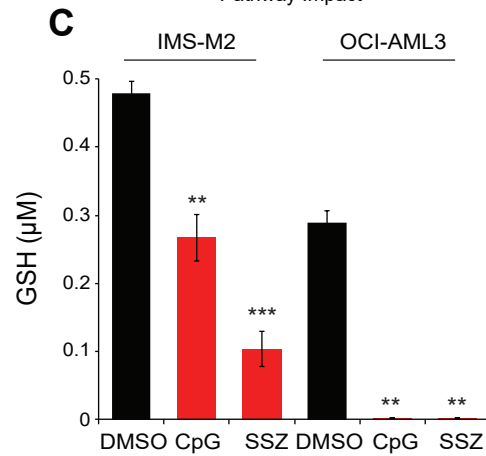
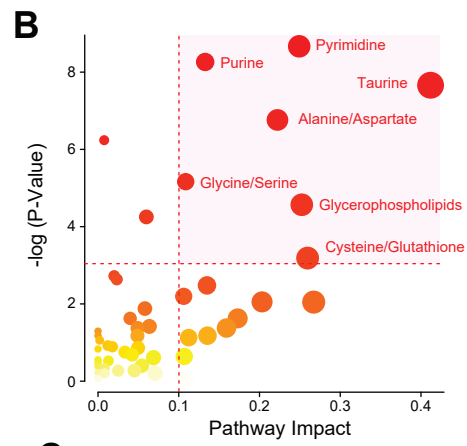
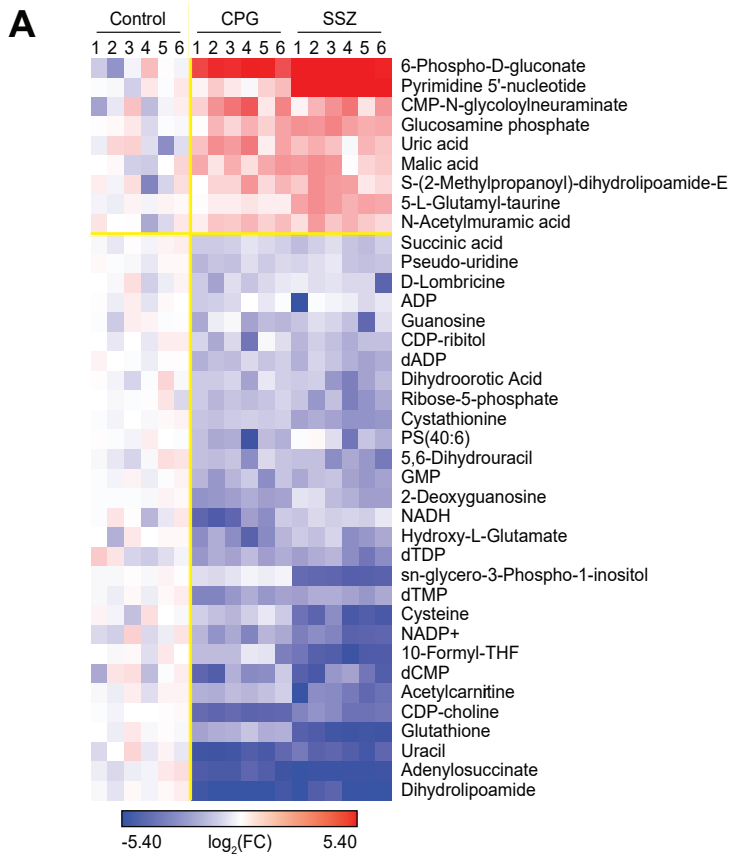




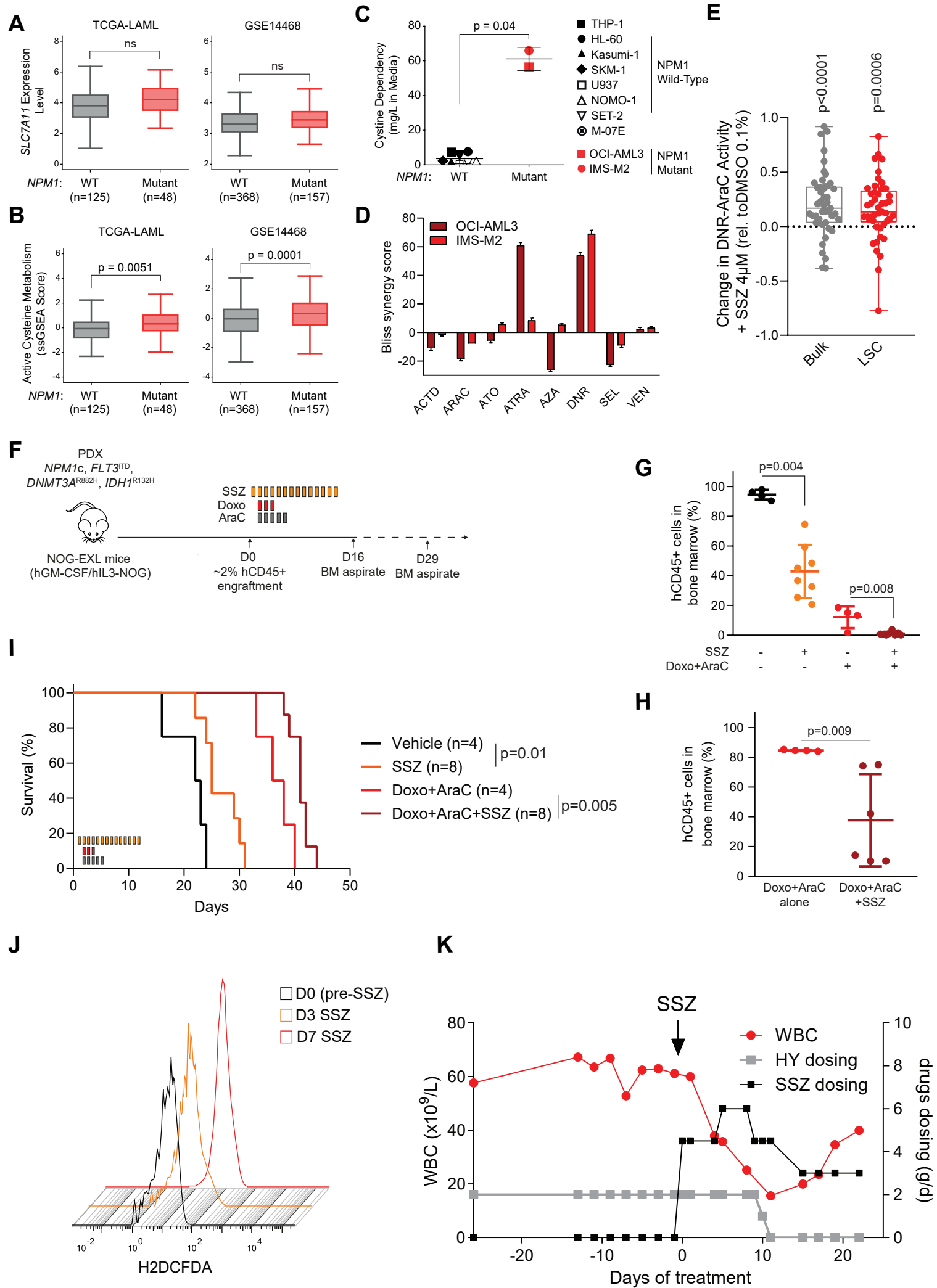
Pardieu et al. Figure 2

**A****B****B****C****D****E****F**

Pardieu et al. Figure 3



Pardieu et al. Figure 4



Pardieu et al. Figure 5