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

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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.¹ Long-term survival remains limited with standard of
4 care chemotherapies combining anthracyclines with cytarabine.² 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.³⁻⁵ Metabolic rewiring plays a prominent role in the drug resistance of
8 leukemic cells.^{6, 7} Targeting the metabolic vulnerabilities associated with stemness
9 programs is thus an appealing approach in AML.^{8, 9} Repurposing of clinically approved
10 medicines is a promising approach in oncology, including in leukemias.^{10, 11} 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,¹² 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, 1×10^6 *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.^{12, 13} Mice were euthanized after
19 30 days of treatment to evaluate leukemia burden in vehicle versus SSZ-treated
20 groups. In a second experiment, 2.5×10^6 *FLT3*-ITD, *NPM1c*, DNMT3A^{R882H}, and
21 IDH1^{R132H} 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⁺ 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.¹⁴ 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).^{15, 16} 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.¹⁷ 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.¹⁸ 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)¹⁷. 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.¹⁹ 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 (IC₅₀), 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 IC₅₀s 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.¹⁷ 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,¹² 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⁺ 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⁺ cells (176 $\mu\text{M} \pm 40 \mu\text{M}$ versus 2.94 mM ± 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.²⁰ 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.^{21, 22} 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.²³ 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.^{24, 25} 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,²⁶ 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]).^{10, 11, 27-29} 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.³⁰ Specifically, we explored the addition of a fixed,
16 low concentration of SSZ (4 μ M, in the range of trough plasma concentrations of SSZ)³¹
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.³²

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 $\sim 100 \mu\text{M}$,³¹ i.e. in the range of 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.^{21, 22} 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.³³
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.⁷ 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.^{24, 25} 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,²⁶ which has recently been studied in AML.³⁴ 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.^{35, 36} 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.³⁷

12 Conventional cell culture does not recapitulate human plasma concentrations of key
13 metabolites,^{38, 39} bone marrow oxygen tension,⁴⁰ or anti-oxidant defenses provided by
14 the leukemic stroma.^{33, 41} 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,³⁰ limiting culture duration to 3 days, in
17 keeping with other drug screening platforms for primary AML cells.^{42, 43}

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.¹⁸ The latter are within the ~100 μ 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.¹⁹ SSZ is active in inflammatory diseases,
3 possibly through inhibition of NFκB pathways.⁴⁴ 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.^{45, 46} 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,³¹ 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,¹ is exquisitely sensitive to
16 oxidative stress, although the molecular underpinnings for this ROS vulnerability
17 remain unclear.^{11, 47} 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.⁴⁸⁻⁵⁰ 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.^{51, 52} 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.¹⁴

7 A formal comparison between xCT inhibitors, which may affect cancer viability by
8 multiple mechanisms,⁵³ more specific ferroptosis inducers such as GpX4 inhibitors,⁵⁴
9 and other GSH-depleting agents with promising anti-leukemic activity such as APR-
10 246,³⁴ will be the focus of future studies. Our findings strengthen a growing interest in
11 targeting cysteine metabolism in cancer cells,^{55, 56} 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.^{57, 58} Until then, our findings support clinical investigation of xCT
14 inhibition in combination with anthracycline-based chemotherapy in AML.

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12

13

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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 0.5×10^3 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.** IC₅₀ 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 IC₅₀s 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 0.5×10^3 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.** IC₅₀ 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% O₂) in limiting dilution with 250
15 μ 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.¹⁵ **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 μ 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 μ 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

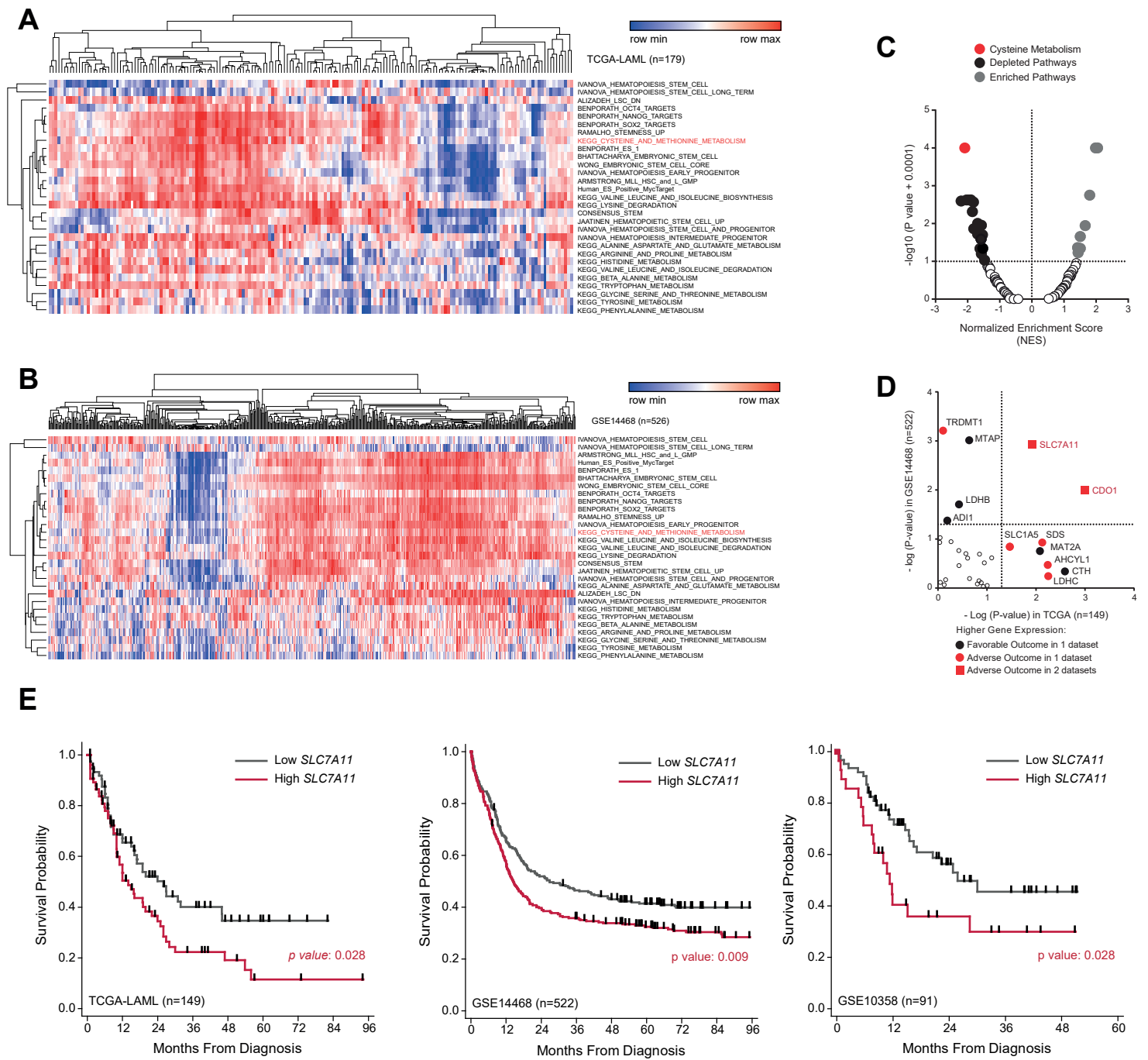
18 **Figure 4. xCT inhibition induces global metabolic rewiring and ROS-mediated**
19 **cell death. A-B.** Heatmap (**A.**) and Pathway Impact by MetaboAnalyst (**B.**) of the top
20 38 deregulated metabolites by LC-HRMS metabolomics in IMS-M2 cells treated for 72
21 hours with SSZ or CpG at IC_{50} (115 μ M and 34.93 μ 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 μ M
25 and 110 μ M respectively) or CpG (34.93 μ M and 28.22 μ 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 μ M SSZ alone or
3 combined with 55 μ 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 μ 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 μ M SSZ
9 alone or combined with 55 μ M 2-mercaptoethanol, 2 mM N-acetylcysteine, or 10 μ 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 μ M ferrostatin-1 (Fer-1).

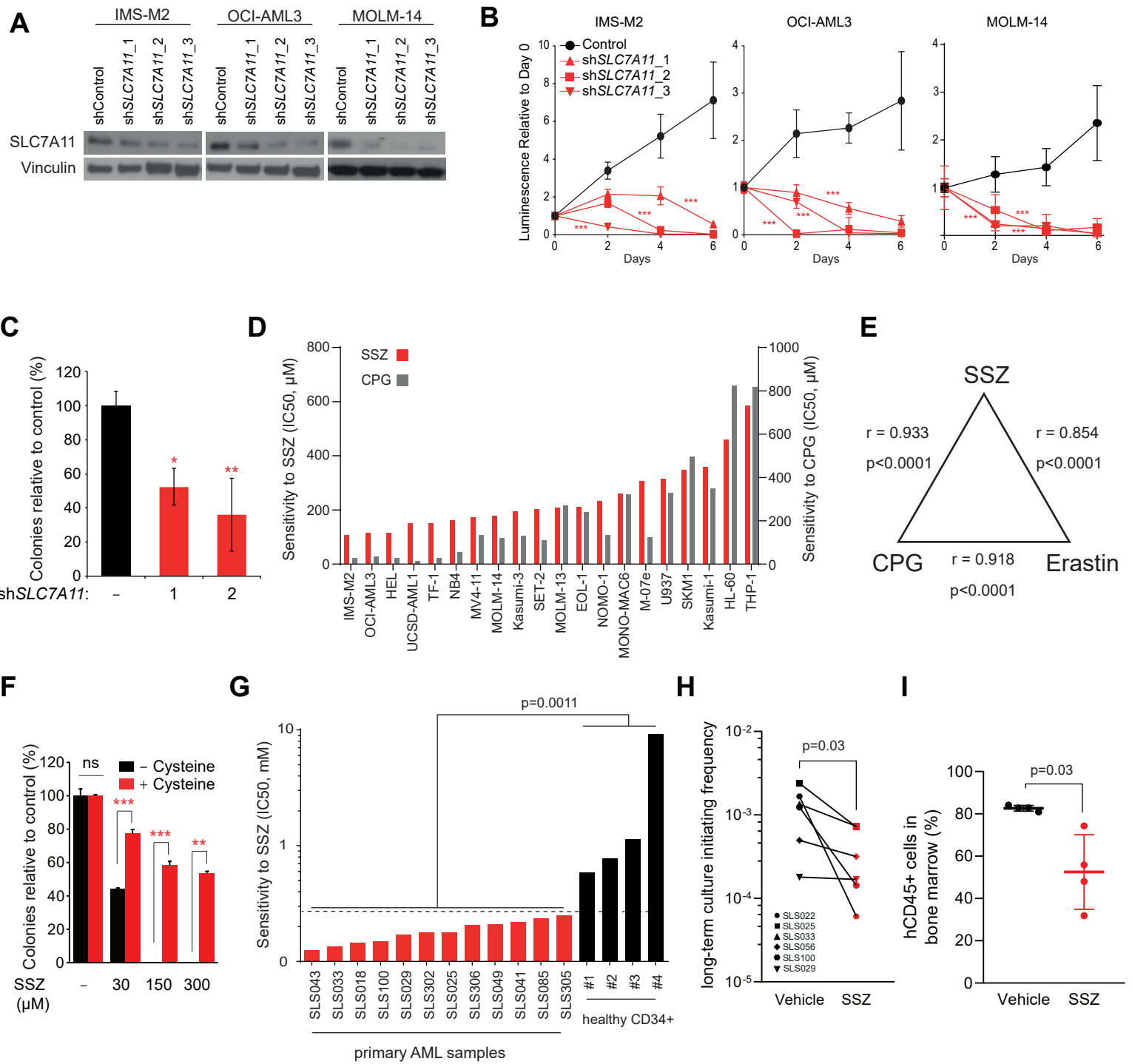
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15 **Figure 5. Sulfasalazine synergizes with anthracycline-based chemotherapies in**
16 **AML. A-B.** *SLC7A11* gene expression (**A.**) and ssGSEA z-scores of the Cysteine
17 Metabolism Pathway (**B.**) in patients with mutant or wildtype *NPM1* from 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

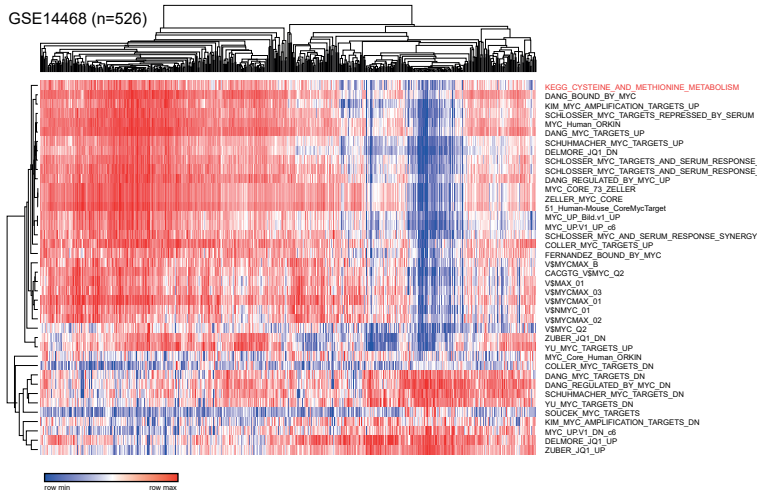
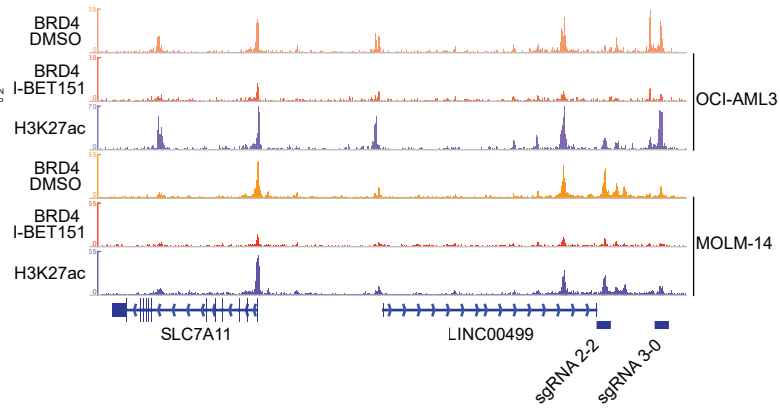
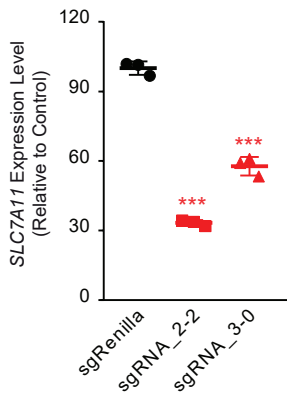
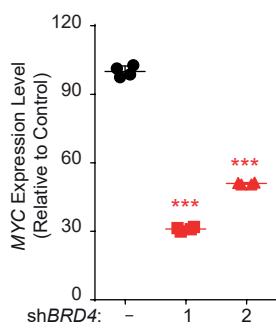
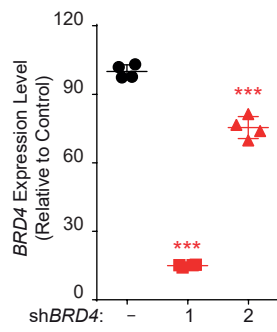
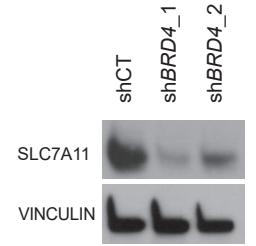
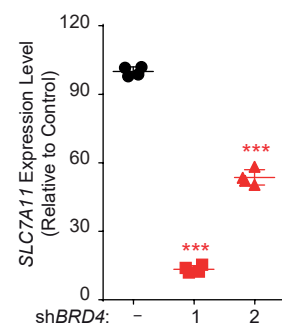
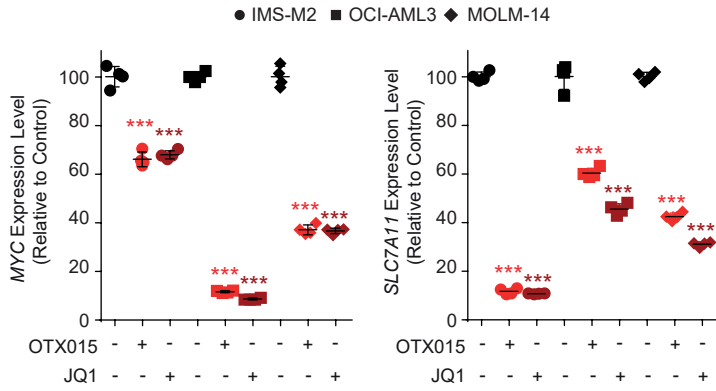
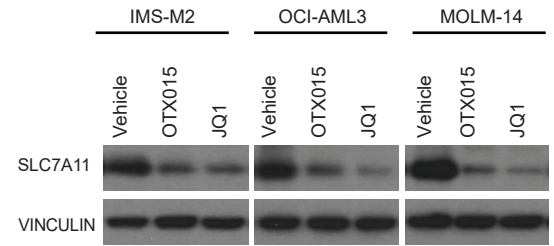
1 acid [ATRA], azacitidine [AZA] and selinexor [SEL]) in OCI-AML3 and IMS-M2 cells.
2 Positive Bliss scores indicate synergism and negative scores antagonism. **E.**
3 Difference in activity of DNR-AraC combination at a fixed 1:20 ratio on the in a 5-point
4 dose-response assay in niche-like conditions with addition of a fixed 4 μ 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^{ITD}*, *DNMT3A^{R88H}* and *IDH1^{R132H}* 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.



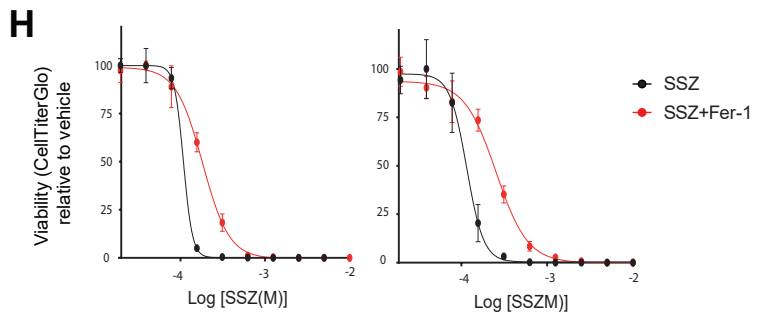
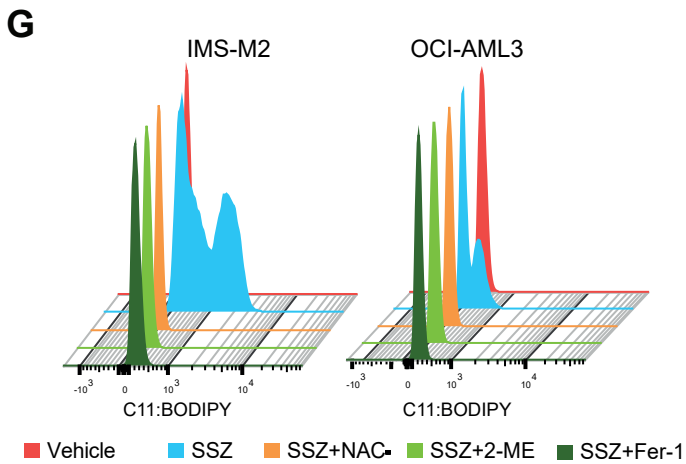
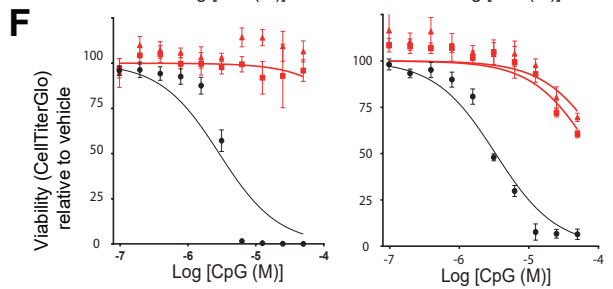
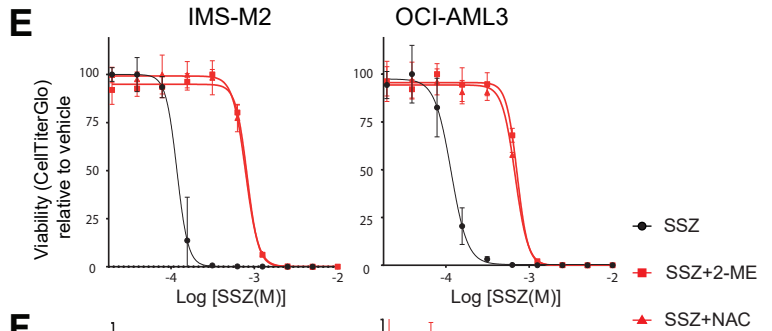
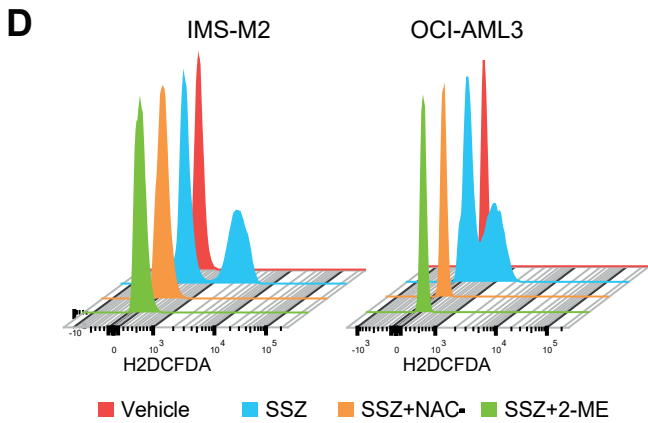
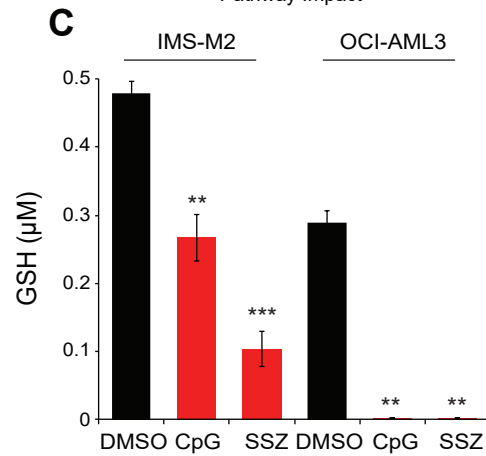
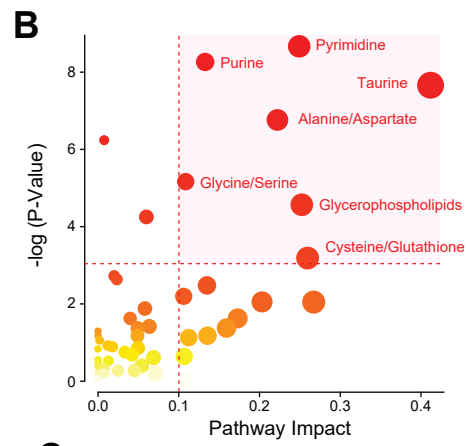
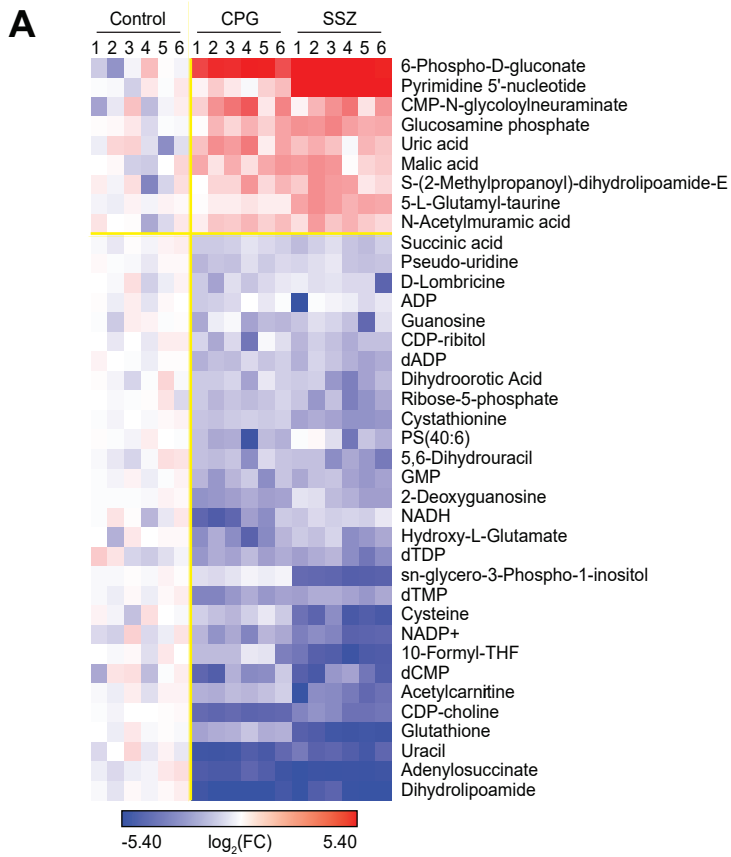
Pardieu et al. Figure 1



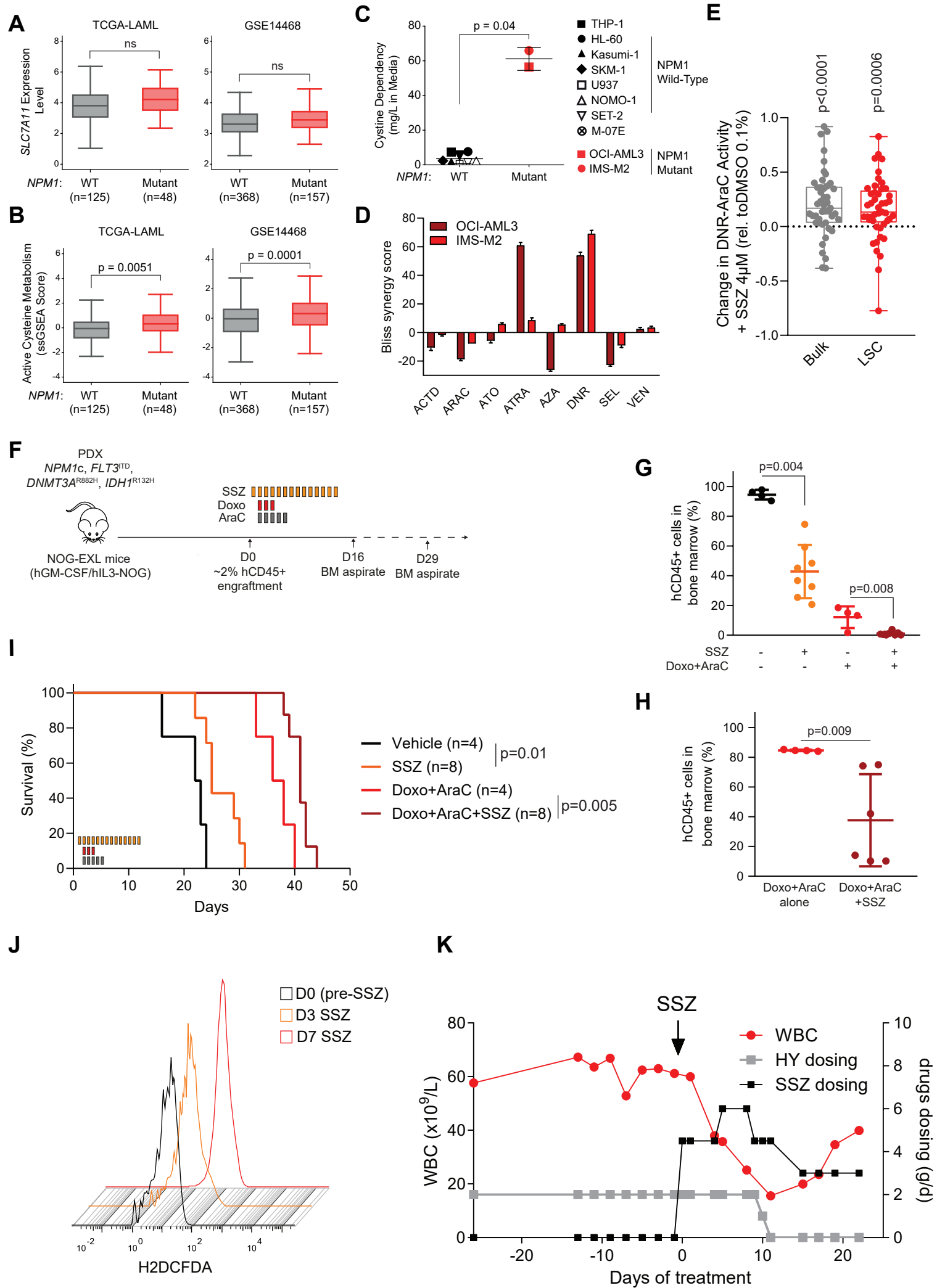
Pardieu et al. Figure 2

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

Pardieu et al. Figure 3



Pardieu et al. Figure 4



Pardieu et al. Figure 5