# S-adenosylmethionine addiction confers sensitivity to methionine restriction in *KMT2A*-rearranged acute lymphoblastic leukemia

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# Supplementary Materials

# Supplementary Methods

### Cell culture

The primary human leukemic cell lines used were NALM-6 (RRID:CVCL_0092), Reh (RRID:CVCL_1650), SEM (RRID:CVCL_0095), KOPN-8 (RRID:CVCL_1866), ALL-PO (RRID:CVCL_1069), and BEL-1 (RRID:CVCL_0081). These cell lines were cultured in RPMI1640 medium (Invitrogen, Thermo Fisher Scientific) supplemented with 10% or 20% heat-inactivated fetal bovine serum (FBS, Greiner Bio-One) and 1% penicillin/streptomycin (P/S) (Invitrogen, Thermo Fisher Scientific). Additional human leukemic cell lines used were: NALM-16 (RRID:CVCL_1834), RS4;11 (RRID:CVCL_0093), Jurkat (RRID:CVCL_0065), Karpas-45 (RRID:CVCL_1326), Kasumi-2 (RRID:CVCL_0590), U-937 (RRID:CVCL_0007), and MV4;11 (RRID:CVCL_0064). These were obtained from American Type Culture Collection (ATCC) and cultured in RPMI1640 medium (Invitrogen, Thermo Fisher Scientific, Breda, the Netherlands) supplemented with 10% or 20% heat-inactivated FBS (Greiner Bio-One, Essen, Germany) and 1% penicillin/streptomycin (P/S) (Invitrogen). All cell cultures were kept in a humidified incubator at 37°C and 5% $CO_2$. Cell cultures were tested regularly for the presence of mycoplasma. Refer to Supplementary Table S4 for more information.

For MR experiments, cells were washed once with phosphate buffer saline (PBS, Invitrogen, Thermo Fisher Scientific) to remove any remaining methionine, and then cultured in methionine-free RPMI1640 medium (Invitrogen, Thermo Fisher Scientific, #A1451701) supplemented with 10% FBS and 1% P/S. L-methionine powder (Sigma-Aldrich, #M5308) was dissolved according to manufacturer instructions at 10mg/mL stock in sterile water.

For rescue experiments, cells were cultured in methionine-free or control RPMI1640 medium with the addition of S-adenosylmethionine (SAM, Sigma-Aldrich, #A7007). S-(5'-Adenosyl)-L-methionine chloride dihydrochloride powder was dissolved in water according to manufacturer instructions at a stock concentration of 10mM.

### Cell Viability

Cell viability was determined using amine staining (LIVE/DEAD Fixable Dead Cell Stain Sampler Kit, Thermo Fisher Scientific, #L349630) according to the manufacturer's instructions. Cells were stained at

1:5000 for 30-45 minutes at 4°C and measured by fluorescence-activated cell sorting (FACS) using ZE5 Cell Analyzer (Bio-rad) or CytoFLEX LX (Beckman Coulter), and data were analyzed using Kaluza 2.1 software (Beckman Coulter).

**Metabolic Activity/Cell Proliferation**

Metabolic activity was measured using MTT (Sigma-Aldrich, #475989). Briefly, cells were incubated for a minimum of 4h at a concentration of 1mg/mL until sufficient crystals were formed. The reaction was stopped by the addition of isopropanol with 50mM HCl, and the cells were resuspended until the crystals were fully dissolved. The absorbance was measured at 570nm using a spectrophotometer (SpectraMax iD3), from which the background signal measured at 720nm was subtracted.

**Real-time quantitative-PCR**

Total RNA was extracted using an RNeasy Mini Kit (Qiagen). cDNA was synthesized using an iScriptTM cDNA Synthesis Kit (Bio-Rad). mRNA expression levels were determined using Power SYBR® Green PCR master mix and a CFX96 TouchTM Real-Time PCR detection system (Bio-Rad). Primers were purchased from IDT, and the sequences are shown in Supplementary Table S7.

**Ex vivo culture of patient derived xenografts (PDXs)**

hTERT immortalized mesenchymal stem cells were seeded in a 96-wells plate (14,000 cells/well) 24h prior to the addition of ALL PDXs (140,000 cells/well). Drugs were added immediately after in their respective concentrations. After 72h of treatment, PDX cells were resuspended and transferred to a second 96-wells plate to prevent MSC contamination while measuring. PDX cells were stained with LIVE/DEAD™ Fixable Dead Cell Stain Sampler Kit (Thermo-Fischer, L34960) according to manufacturer instructions.

**Immunoblotting**

For non-histone proteins, cells were lysed in 1X SDS sample buffer containing benzonase nuclease (Sigma-Aldrich, #70746) at 4°C for 30 minutes before denaturation at 95°C for 5 minutes. After lysis, proteins were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in TBS-Tween (0.1%) with 5% low-fat milk (Elk, Campina) and incubated with primary antibodies overnight at 4°C. Refer to Supplementary Table S5 for a complete antibody list.

Membranes were then washed with TBS-Tween and stained with IRDye-conjugated secondary antibodies (Li-cor). Proteins were visualized using the Odyssey® CLx imaging system (Li-cor).
Histone proteins were isolated using an adapted Abcam histone extraction protocol (available online). Briefly, cells were lysed in triton extraction buffer (TEB: PBS containing 0.5% Triton X 100 (v/v), 2mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) NaN3) on ice for 10 minutes. The remaining nuclear pellet was washed and spun down before resuspending in 0.2N HCl to acid extract the histones overnight at 4°C. Afterwards, the supernatant, which now contains the histone proteins, was neutralized with 2M NaOH before quantifying protein content using a Pierce BCA Protein Assay kit (Thermo Fisher, #23225). Protein lysates were mixed with 1X SDS sample buffer and benzonase nuclease and denatured at 95°C for 5 minutes. 3µg of protein were loaded per lane.

**Lentivirus production and transduction**

HEK293T packaging cells (Invitrogen) were co-transfected with a viral backbone encoding the shRNAs and the helper plasmids for virus production (psPAX2 and pMD2.G) using Polyethylenimine (PEI). The plasmids pS-Pax2 (#12260) and pMD2.G (#12259) were obtained from Addgene. KDM2B and KDM4A KD plasmids were purchased from Sigma-Aldrich (pLKO1-Puro). shRNA sequences used are listed in Supplementary Table S6. Virus containing supernatant was collected 48h after transfection and immediately transduced into SEM cells using spinoculation at 700g for minutes at 37°C with 5µg/mL polybrene (Santa Cruz Biotechnology). Puromycin selection was started 72h after transduction (2µg/mL).

**Metabolic profiling**

Treated and untreated cells were washed once with PBS and snap frozen before further processing by Metabolon. Metabolic profiling was performed by Metabolon, Inc (Morrisville, North Carolina) using ultrahigh performance liquid chromatograph-tandem mass spectroscopy. Samples were subjected to methanol extraction and then divided into four aliquots for each of the mass spectroscopic methods. The four mass spectroscopic methods used were optimized for acidic positive ion hydrophilic compounds, acidic positive ion hydrophobic compounds, basic negative ions, and negative ionization. For metabolite identification, Metabolon's proprietary software was used. For analysis, the raw metabolomics data were normalized by Bradford protein concentration and rescaled within each batch to set the median value for each metabolite to one. Missing values for metabolites were imputed to be the minimum observed value for that metabolite.

**Chromatin Immunoprecipitation sequencing (ChIP-seq)**

Each condition contained three independent biological replicates immunoprecipitated samples. Per replicate, 40 million leukemic cells were treated with 5µM FIDAS-5 for 24h and crosslinked in 1% formaldehyde for 10 minutes, quenched with glycine and lysed using the SimpleChIP kit (Cell Signaling Technology®, #9003) according to the manufacturer's recommendations. Chromatin fragmentation was conducted using 15 cycles of sonication using a Bioruptor-300 (Diagenode) to generate 150-300 bp fragment size.

Immunoprecipitation was performed using anti-H3K4Me3 (Diagenode, #C15410003-50) or normal rabbit IgG as a negative control (Cell Signaling Technology®, #2729) antibody according to the guidelines of the manufacturer. The library generation and sequencing were performed at BGI genomics (Hongkong, China). Peak calling was performed using MACS2 and visualized in the UCSC genome browser. Over representation analysis of differentially bound genes was performed in R studio using the fgsea package.

**In vivo experiments**

Animal experiments were approved by the Animal Experimental Committee of the Radboud university (RU-DEC-2019-0036). To test the efficacy of a methionine restricted diet SEM cells were intravenously injected into the mice (n=14) and randomized into a control or treated group. One week after transplantation, mice were either placed on a control diet or a 95% MR diet. Both diets were purchased from Bio-services (Schaijk, Netherlands) and given ad libitum. Tumor load was determined weekly by flowcytometric detection of human cells by staining for human CD10, CD45 and CD19 and murine CD45 cells. Mice were sacrificed after the human CD45+ reached 50% of total CD45+ cells. In addition, dried blood spots were collected weekly to measure circulating methionine. See section Methionine Measurements in the Supplementary Methods for additional details.

To test the efficacy of combining MAT2A inhibition with HDAC inhibition, two different PDX samples were injected into the mice. After engraftment, mice were randomized into the different treatment groups. For patient sample 1 (n=24), 10 days after transplantation, mice were orally given a placebo (PBS containing 0.5% Carboxymethylcellulose-Na, 0.2% Tween 80), FIDAS-5 (20mg/kg, MedChemExpress, #HY-136144), Fimepinostat (Selleckchem, #S2759), or combination solution. Fimepinostat was given the first week at 75mg/kg and reduced to 25mg/kg to prevent rapid weight loss. Treatment was given for a

period of three weeks with five days on and two days off. Mice were euthanized after the human CD45+ or CD19+ populations reached at least 50% of total leukocytes in the blood.

For patient sample 2 (n=20), mice were randomized and started on treatment seven days after transplantation. Treatment was extended by a week for a total of four weeks. For this leukemia sample, blood lymphocyte counts were uninformative to estimate tumor load, all mice were sacrificed after 60 days, and spleen weights were used as a measure for Leukemic burden. At this timepoint, the leukemia in untreated mice was well developed, allowing for a dynamic range in leukemia reduction for the treated mice. All mice were given normal chow supplemented with DietGel® Boost (ClearH2O) to minimize weight loss. All mice from all experiments were weighed three times a week and tumor load was measured weekly via tail bleedings.

**Drug screen**

Screening experiments and processing were performed by the high-throughput screening facility of the Princess Máxima Center (https://research.prinsesmaximacentrum.nl/en/core-facilities/high-throughput-screening). The drug library contained 241 drugs. Before the screen, the 384-well drug library plates are shaken for 30 minutes and centrifuged at 500g for 1 minute. 40µl of cells (0.5E6 cells/mL) in RPMI1640 medium are plated in a 384-well plate and using the Echo 550 dispenser, 100nL of the drugs are added from the drug plate to the cell plate to yield final concentrations of 0.1nM, 1nM, 10nM, 100nM, 1µM and 10µM (0.25% DMSO or water). Certain drugs are tested at additional lower concentrations (up to 10 pM) or higher concentrations (up to 200 µM). Cells treated with only DMSO are used as positive controls, whereas cells treated with staurosporine are used as negative controls. The half maximal concentration that inhibits the viability (IC50) and area under the curve (AUC) values are derived from the dose-response curves using the extension package drc in R. IC50 values at 72h were calculated by determining the concentrations of the drug needed to achieve a 50% reduction in cell viability. AUC values were calculated by determining the definite integral of the curve. Quality of the screens was approved after assessment of cell growth, the negative, positive, and empty controls, and the amount of variability between the duplicates.

**Methionine measurements**

Once a week, tail bleedings were made from all the mice in work protocol 2019-0036-003. 15µL of blood were spotted on Whatman® protein saver cards (Sigma-Aldrich, #WHA10531018) while the rest was

processed further for FACS. The blood spots were dried at room temperature for 1-3 hours before being stored at -80°C with silica gels. The protocol for amino acid measurements in blood has been described previously,[14] with the exception that the blood spots were processed differently. Blood spots were resolved by adding 50µL internal standard solution I (IS-I) and 50µL internal standard solution II (IS-II), ultrasonicating for 20 minutes at room temperature, blowing the samples dry at 40°C with nitrogen flow, and dissolving in 140µL UPLC Eluens A2 (10mM ammoniumformiate with 85% acetonitrile and 0.15% formic acid). Chromatographic separation was carried out using 5µL sample volume on an Acquity UPLC BEH Amide column (2.1×100 mm, 1.7 µm particle size) with a Van Guard™ UPLC BEH Amide pre-column (2.1×5mm, 1.7µm particle size) according to the previously described protocol. The column was coupled to the Xevo-TQ MS triple quadrupole mass spectrometer with an electrospray ionization (ESI) source and an Acquity UPLC-system (Waters, Manchester, United Kingdom).

**Supplementary Tables, Figures and Legends (see Excel Supplementary file for Supplementary Tables)**

**Supplementary Table 1**

ANOVA Contrasts of statistically significant metabolites

**Supplementary Table 2**

Characteristics of Patient Derived Xenografts

**Supplementary Table 3**

Compounds and associated targets included in drug library

**Supplementary Table 4**

Characteristics of Cell Lines used in this study

**Supplementary Table 5**

Antibody information

**Supplementary Table 6**

shRNA sequences

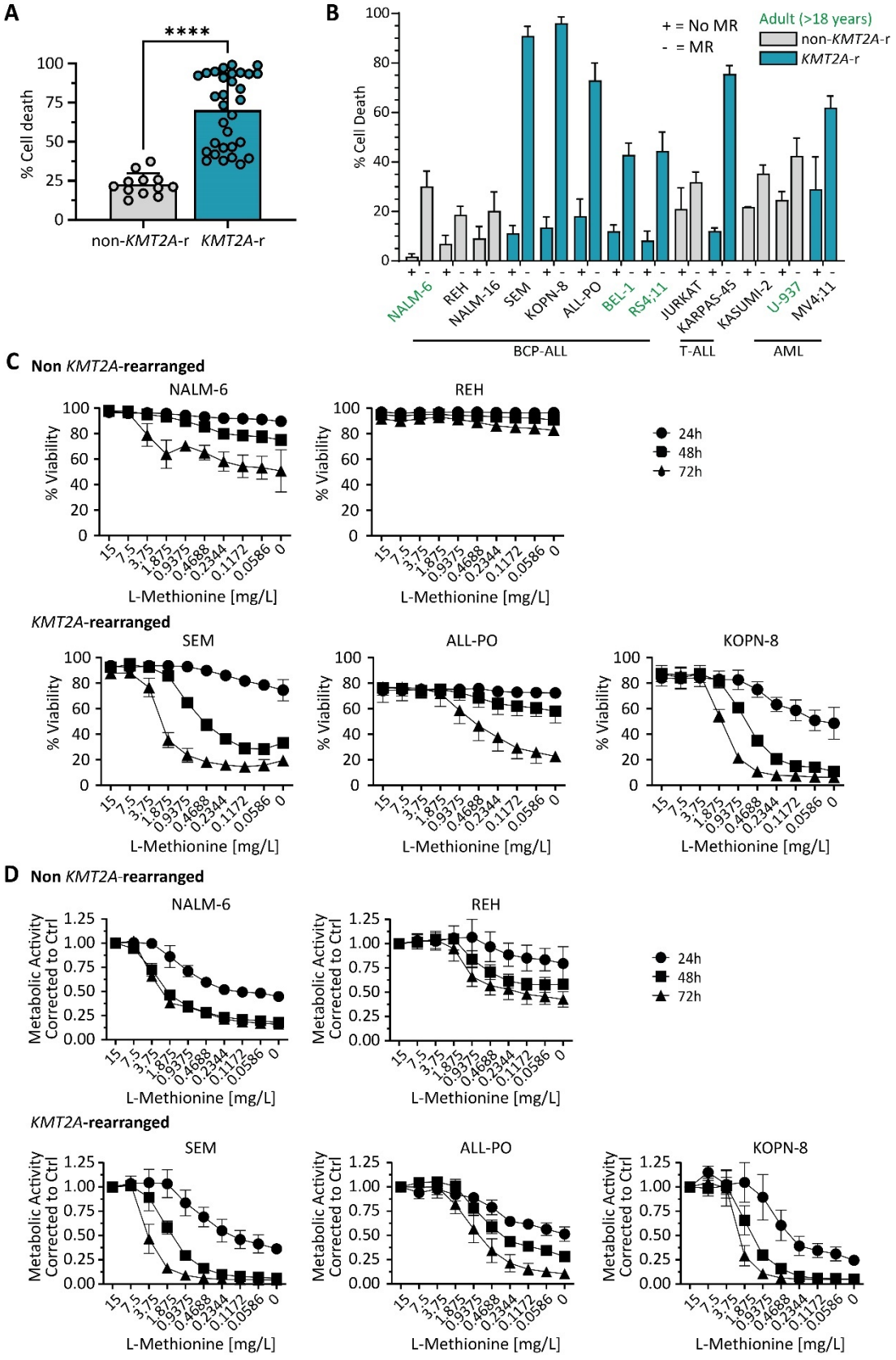
**Supplementary Table 7**

RT-qPCR primer sequences

**Supplementary Table 8**

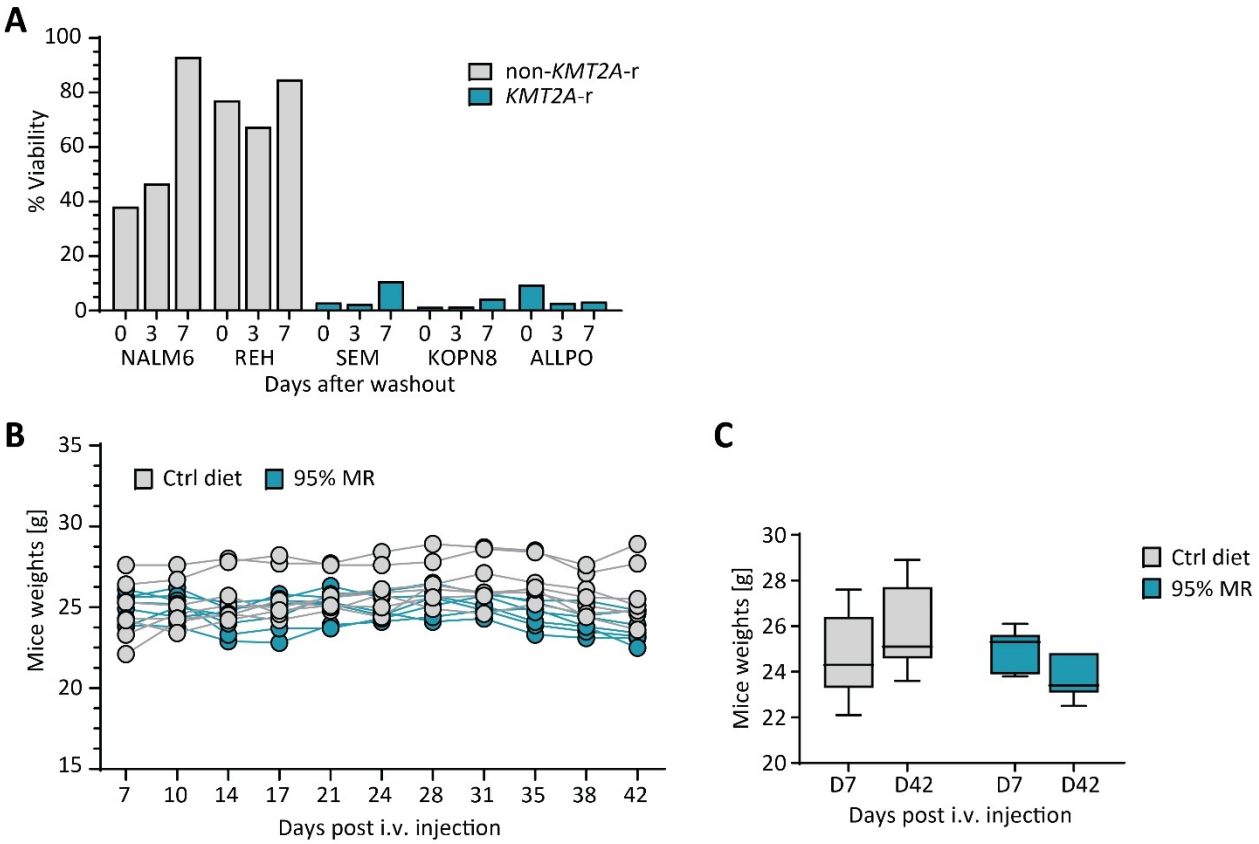
Differentially bound peaks from ChIPseq analysis

## Supplementary Figure 1



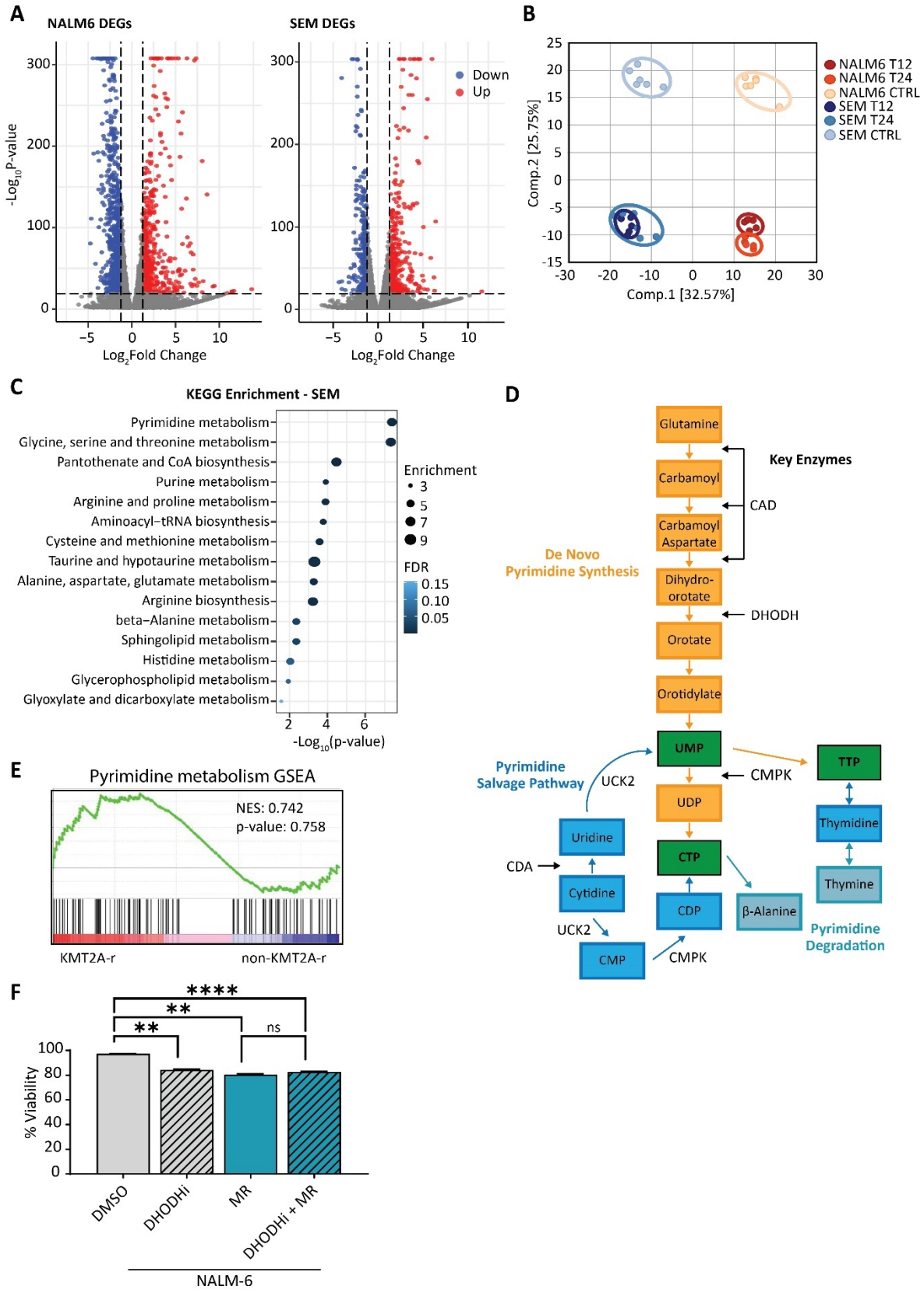
**Supplementary Figure 1**: **(A)** Bar graph comparing the percentage of cell death after complete MR in the *KMT2A*-r cell lines and non-*KMT2A*-r cell lines tested in Figure 1A. P-values were calculated using a two-tailed unpaired t-test (****P < 0.0001). **(B)** A panel of BCP-ALL, T-ALL, and AML cell lines were treated with methionine free RPMI1640 medium. Cell viability was measured after 72h via flow cytometry. Error bars represent standard deviation (SD) of biological replicates. Cell lines in green represent adult patients older than 18. **(C)** Dose response of BCP-ALL cell lines to decreasing amounts of methionine in RPMI1640 medium. Cells were treated for 24h (circle), 48h (square), or 72h (triangle) before cell viability was measured. Results shown represent the mean ± SD from three independent experiments. **(D)** Dose response of the same BCP-ALL cell lines performed in parallel using metabolic activity (MTT) as a read-out after 24h (circle), 48h (square), and 72h (triangle). Results shown are corrected to the fluorescence of the untreated cells and represent the mean ± SD from three independent experiments.

## Supplementary Figure 2



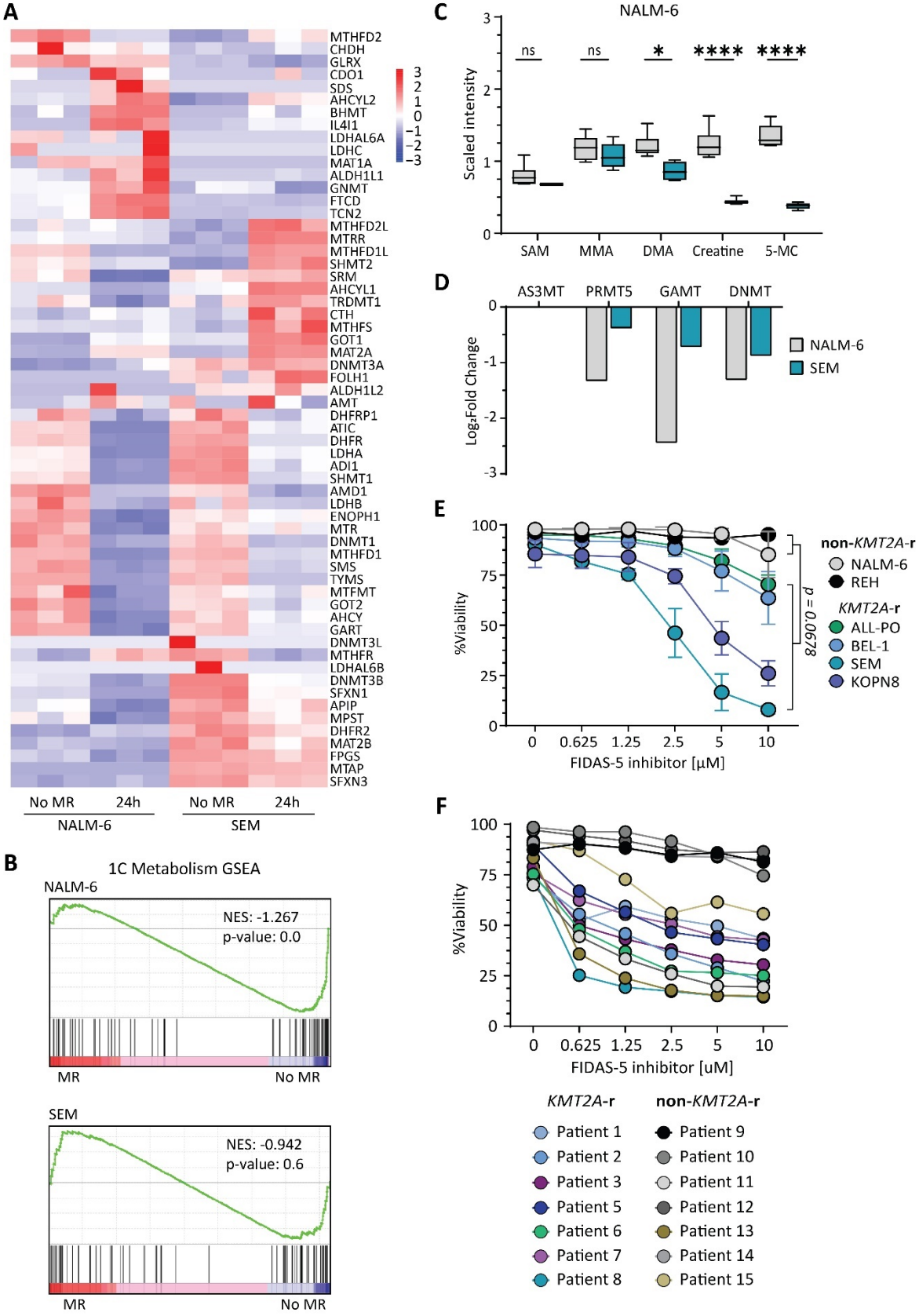

**Supplementary Figure 2**: **(A)** Washout experiment was performed by treating BCP-ALL cells for 72h with complete methionine depletion and re-seeding the cells remaining after treatment back in normal RPMI1640 medium. Viability of the cells were monitored via flow cytometry at day 3 and day 7. **(B-C)** The weights of the mice throughout the experiment (n=14) and the differences in weight at the start of the experiment and on the last day with (n=7) and without 95% MR diet (n=7).

## Supplementary Figure 3



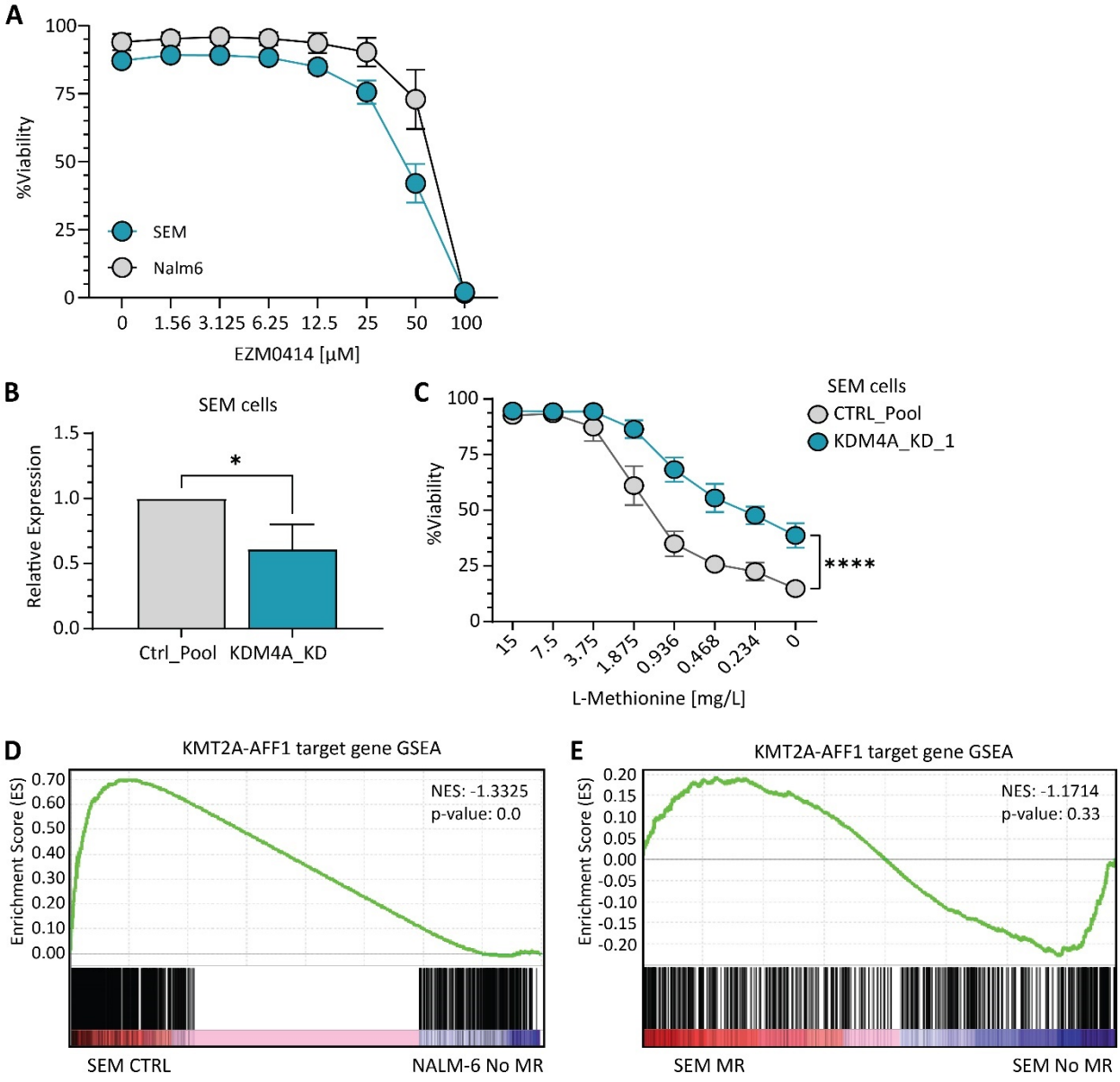
**Supplementary Figure 3**: **(A)** Volcano plots showing up (red) and down (blue) differentially expressed genes (DEGs) with a $log_2$fold change of at least ±1.25 and a p-value less than 10E-20. NALM-6 and SEM cells were bulk RNA sequenced after 24 hours MR treatment in triplicate. **(B)** Principal component analysis of the metabolomics data as calculated by Metabolon comparing NALM-6 and SEM cells treated with complete methionine depletion for 12- and 24h. There are 6 biological replicates per condition. **(C)** KEGG enrichment analysis of SEM and NALM-6 cells using Metaboanalyst 5.0 software on metabolites with a log fold change of at least ±0.75 after 24h MR. **(D)** Schematic showing key components of the pyrimidine synthesis pathway. **(E)** A GSEA plot comparing expression from genes involved in pyrimidine synthesis in 49 KMT2A-r patients and 5 non-KMT2A-r patients. **(F)** NALM-6 cells were treated with 2nM DHODH inhibitor, methionine restriction or the combination thereof for 72h. Cell death was determined by quantification of cells positive for amine-reactive dyes using flow cytometry. P-value was calculated using a one-way ANOVA and Dunnett's multiple comparison tests (**$P < 0.01$, ****$P < 0.0001$).

## Supplementary Figure 4



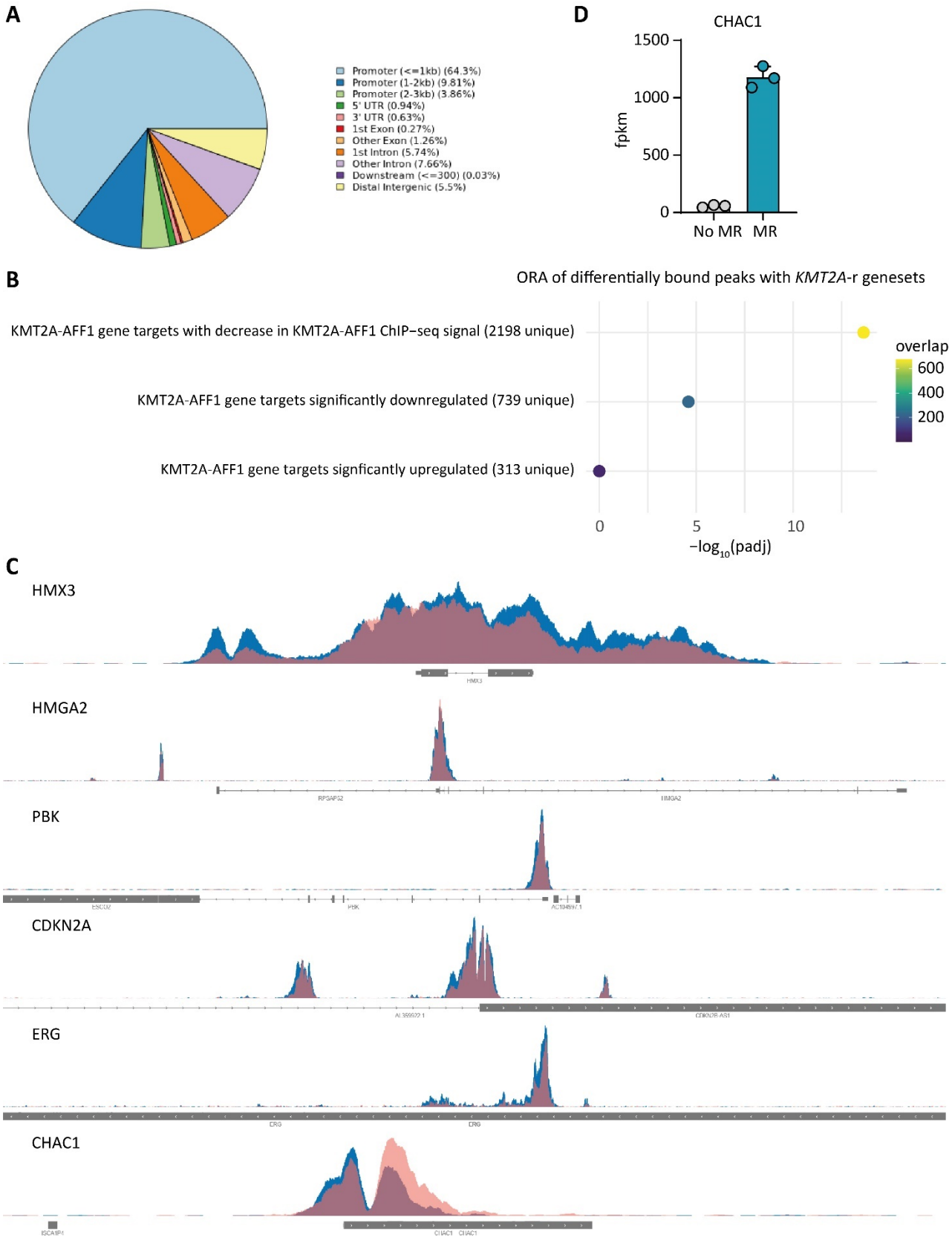
**Supplementary Figure 4**: **(A)** Heatmap of one-carbon metabolism related genes in NALM-6 and SEM cells before and after 24h complete MR. Genes were derived from the gene ontology one-carbon metabolic process and Wikipathways one-carbon metabolism gene sets. **(B)** A GSEA plot comparing MR treated to control in both NALM-6 and SEM cells using a gene set derived from the gene ontology one-carbon metabolic process and Wikipathways one-carbon metabolism gene sets. **(C)** Box plots of normalized and scaled levels, as calculated by Metabolon, of SAM and methyltransferase byproducts measured in NALM-6 cells before and after 24h complete MR. P-values were calculated using a two-tailed unpaired t-test (*P < 0.05, ****P < 0.0001). **(D)** $Log_2$fold change of the methyltransferases that utilize SAM for NALM-6 and SEM cells. **(E)** Dose response of BCP-ALL cell lines to MAT2A inhibitor, FIDAS-5. Cells were treated for 72h before measuring cell viability via flow cytometry. P-value was calculated using a two-tailed unpaired Welch's t-test of the AUCs comparing non-*KMT2A*-r cell lines with *KMT2A*-r cell lines. **(F)** Dose response of PDX samples to MAT2A inhibitor, FIDAS-5. Cells were treated for 72h before measuring cell viability via flow cytometry.

## Supplementary Figure 5



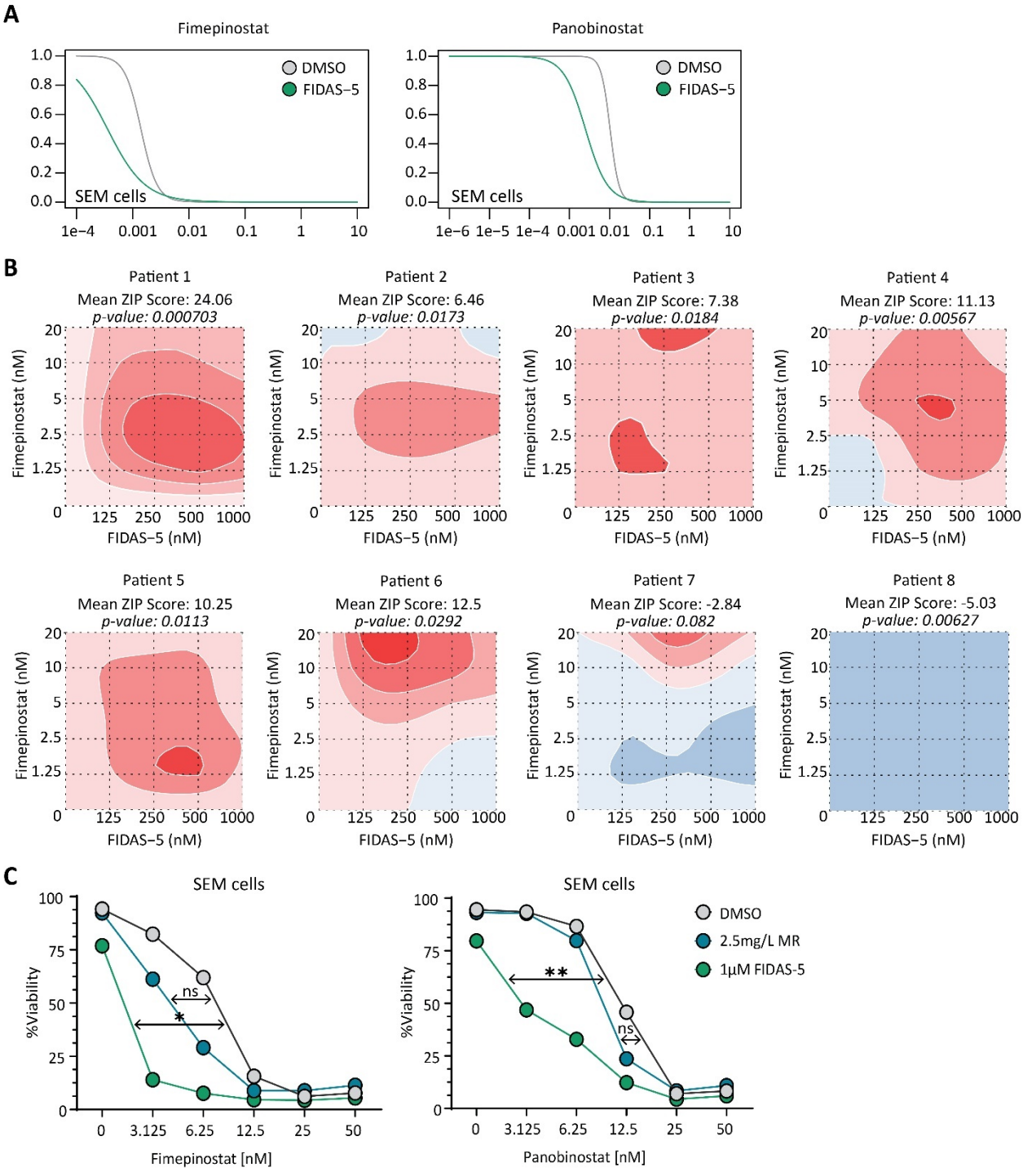
**Supplementary Figure 5**: **(A)** Dose response of SEM and NALM-6 cells to increasing levels of lysine transferase SET domain containing 2 (SETD2) inhibitor EZM0414. Viability was measured via flow cytometry 72h after treatment. Results shown are the mean ± SD from three independent experiments. **(B)** Relative *KDM4A* mRNA expression determined by qPCR in *KDM4A* KD and control (non-targeting shRNA) SEM cells. Results shown are the mean ± SD from three independent experiments. P-value was calculated using a two-tailed unpaired t-test (*, $P < 0.05$). **(C)** Dose response of *KDM4A* KD SEM cells and control SEM cells to decreasing levels of methionine in RPMI1640 medium. Viability was measured via flow cytometry 72h after treatment. Results shown are the mean ± SD from three independent experiments. P-value was calculated using a one-way ANOVA of the AUCs (****, $P < 0.0001$). **(D)** A GSEA plot comparing steady state control NALM-6 and SEM cells using a gene set derived from *KMT2A*-AFF1 target gene reported by Kerry et. al.[29] **(E)** A GSEA plot comparing MR treated to control in SEM cells using the same *KMT2A-AFF1* target gene set.

## Supplementary Figure 6



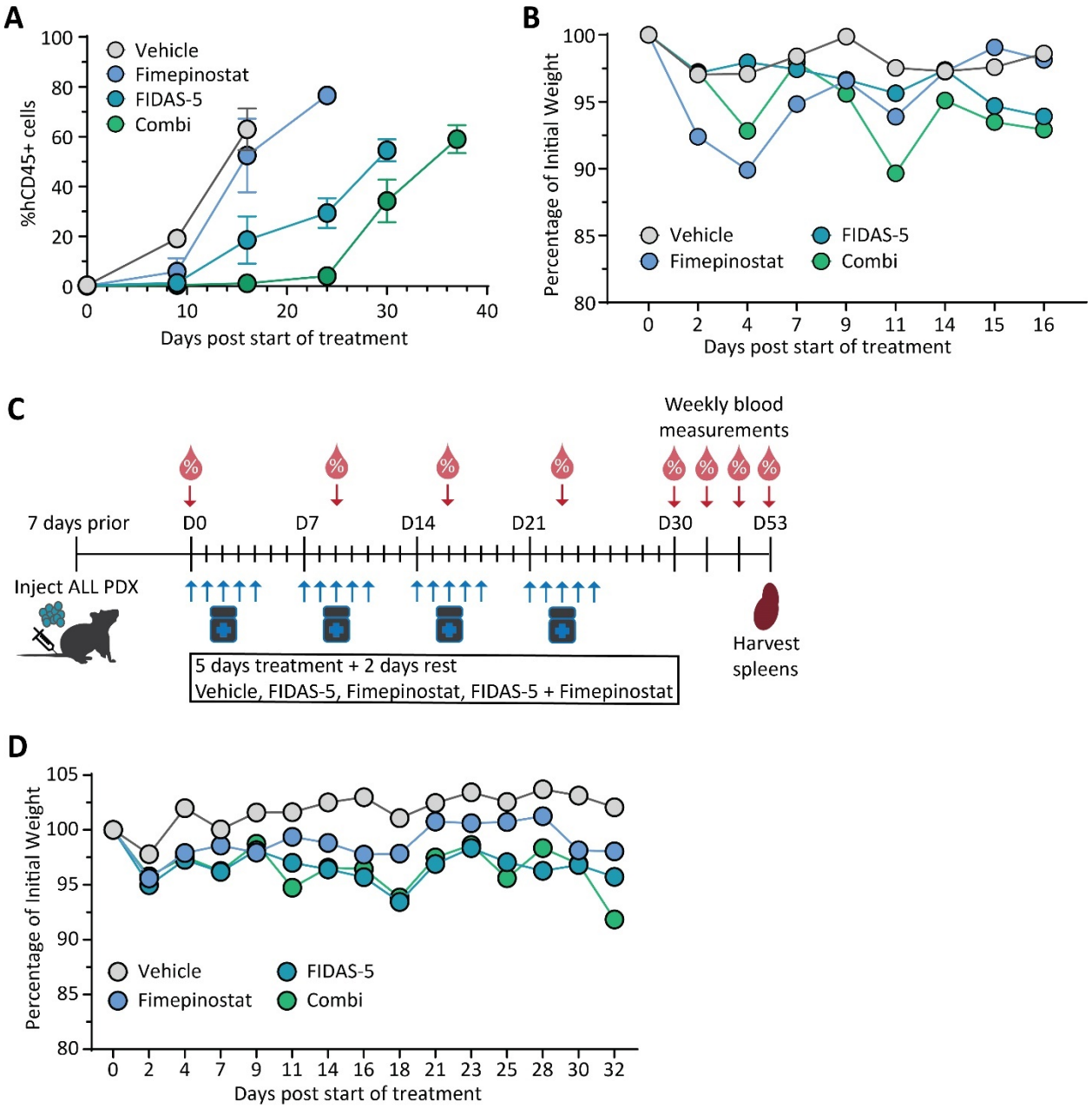
**Supplementary Figure 6: (A)** Venn diagram showing the proportion of H3K4Me3 ChIP-seq reads mapping to different structural elements in the gene. **(B)** Visual representation of the overrepresentation analysis of H3K4Me3 presence on *KMT2A-AFF1* target genes. **(C)** ChIPseq tracks showing the presence of H3K4Me3 at the same locus in DMSO control (blue) and FIDAS-5 treated (red) cells. **(D) E**xpression of ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1 (CHAC1) in control or MR-treated SEM cells, obtained using RNA sequencing.

## Supplementary Figure 7



**Supplementary Figure 7**: **(A)** Dose titration of the top two HDAC inhibitors Fimepinostat and Panobinostat in combination with 1µM FIDAS-5 as measured by the fully automated high-throughput screening facility. Cell proliferation was measured 72h after treatment using MTT. **(B)** 8 different patient derived xenografts (PDXs) were tested *ex vivo* with five doses of FIDAS-5 against six doses of Fimepinostat in a synergy matrix. Cells were seeded on mesenchymal stem cells and treated for 72h before measuring cell viability via flow cytometry. Mean synergy ZIP Scores were calculated using SynergyFinder. **(C)** Dose titration of Fimepinostat and Panobinostat in combination with 1µM FIDAS-5 or 2.5mg/L MR. Cell viability was measured via flow cytometry after 72h treatment. Results shown are the mean ± SD from three independent experiments. P-value was calculated using a one-way ANOVA of the AUCs and Dunnett's multiple comparisons test (*$P < 0.05$, **$P < 0.01$).

## Supplementary Figure 8



**Supplementary Figure 8**: **(A)** Disease progression measured by the average percentage of human CD45+ cells in the peripheral blood of mice treated with control diet (n=6), FIDAS-5 (n=6), Fimepinostat (n=6) or combination (n=6). Mice were taken out of the experiment when the leukemic burden reached 50%. **(B)** The average percentage weight loss of the mice throughout the experiment for each group (n=24) compared to the weight at the start of treatment. **(C)** PDX patient sample 2 was transplanted via intravenous injection seven days prior to the start of treatment. Mice (n=20) were then randomized and given an oral vehicle, FIDAS-5 (20mg/kg), Fimepinostat (25mg/kg), or combination dose for four weeks, with five consecutive days of treatment followed by two days of rest. Leukemic burden was monitored in peripheral blood via weekly flow cytometry measurements. Spleens were harvested on the final day of the experiment and weighed accordingly. **(D)** The average percentage weight loss of the mice throughout the experiment for each group (n=20) compared to the weight at the start of treatment.