Enantiomer-specific and paracrine leukemogenicity of IDH metabolite 2-hydroxyglutarate

Supplementary Materials and Methods

Reduced representation bisulfite sequencing

HoxA9 transduced bone marrow cells expressing CTL vector, IDH1wt or IDH1mut proteins were transplanted in syngeneic recipients. HoxA9 CTL mice were treated with R-2HG daily at a dose of 1 mg/mouse for 4 weeks. Mice were sacrificed after 4 weeks, bone marrow cells were isolated, sorted for GFP and DNA was isolated using the Allprep DNA/RNA kit (Qiagen, Hilden, Germany).

A total of 0.3 μg of DNA was used for RRBS library preparation using a published protocol with minor modifications.(31) Briefly, genomic DNA was digested with MspI (NEB), end-repaired and A-tailed with the Klenow-fragment enzyme (NEB), and ligated (NEB) with 5mC-methylated paired-end sequencing adapters (Illumina). Fragments in a range of 40-220 bp insert size were gel purified (NuSieve 3:1 agarose; Lonza). Libraries were bisulfite converted using the EZ DNA Methylation Kit (ZymoResearch) and amplified using PfuTurboCx polymerase (Agilent Technologies). Each library was sequenced on a separate lane using an Illumina HiScanSQ instrument with Version 3 sequencing chemistry. Libraries were spiked with 45% PhiX DNA to counteract the imbalance in nucleotide representation.(32) Sequencing data can be downloaded from Gene Expression Omnibus.

Analysis of Methylome Data (RRBS-Seq)

Trim Galore (Babraham Bioinformatics) were used in RRBS mode to analyze the quality of the raw data sets (fastq files) and to remove adapter sequence contaminations. Quality trimmed fastq files (phred score >20) were applied as input data for BSMAP aligner (33)

mapping short reads (50mers) to the murine reference genome (mm9) followed by calling methylation sites (RRBS mode, Lister protocol).(34)

Results from methylation calling were further processed using the R package methylKit 0.9.1. methylKit quantifies, compares and annotates methylation calls (CpG) at single nucleotide and genomic interval levels, respectively. For clustering of DMRs, the reads were filtered to contain at least one CpG. Only sequences with a coverage of 20 or more reads were included in the analysis. In addition, all sequences that showed no methylation at all were filtered out.

For determination of DMRs logistic regression and the SLIM-adjustment within the methylkit package was used on the filtered dataset and sequences with at least 40 % difference of methylation were called differentially methylated. For the analysis of DMRs in the vicinity of genes genomic intervals were defined as 500nt windows sliding in steps of 250 nucleotides across the entire genome. The threshold to count for a "CpG" islet is a minimum of 5 CpGs per interval. To estimate the differential methylation status of samples being analyzed, the overall methylation rate was used to calculate a relative difference among these samples. Genomic intervals (CpG islets) with at least 25% difference in their methylation status were called differentially methylated.

Primers for quantitative RT-PCR

The following primers were used for expression analysis (Eurofins MWG Operon, Ebersberg, Germany):

Myadm forward primer: 5'- TGGTGGAGGATGACGTTGTA-3',

Myadm reverse primer: 5'- GAACTTTCTCCTTCCGGTCC-3',

Ccl6 forward primer: 5'- GCGACGATCTTCTTTTTCCA-3',

Ccl6 reverse primer: 5'- CCAGGAGGATGAGAAACTCC-3',

Ccnd3 forward primer: 5'- CCAGGTCCCACTTGAGCTT-3',

Ccnd3 reverse primer: 5'- ACGCCCCTGACTATTGAGAA-3',

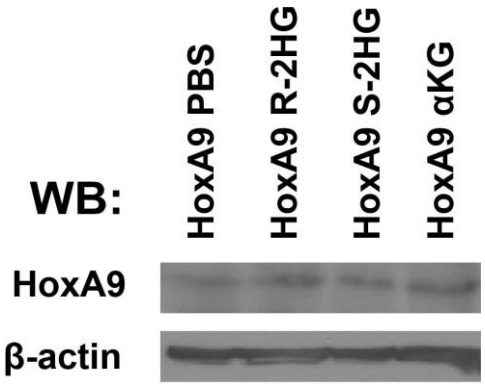
Cdkn2a forward primer: 5'- GCAGAAGAGCTGCTACGTGA-3',

Cdkn2a reverse primer: 5'- CGTGAACATGTTGTTGAGGC -3',

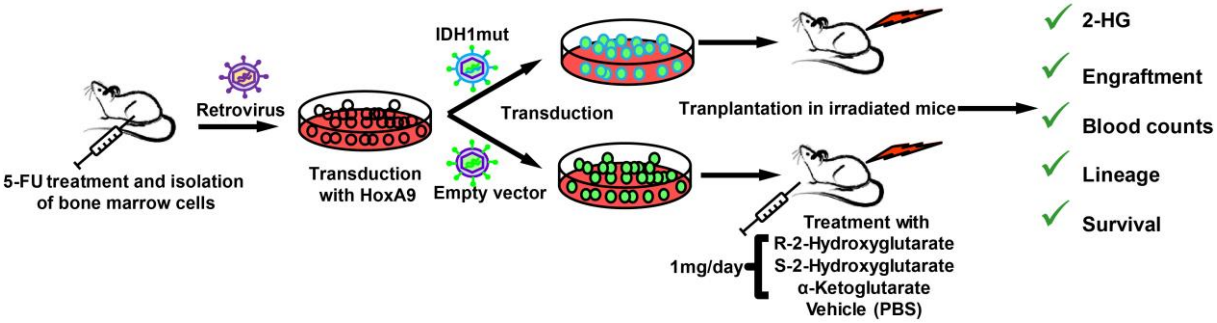
Abl forward primer: 5'- CTGGGGCTCAAAGTCAGATG-3',

Abl reverse primer: 5'- CTGTTTGAAGTTGGTGGGCT-3'

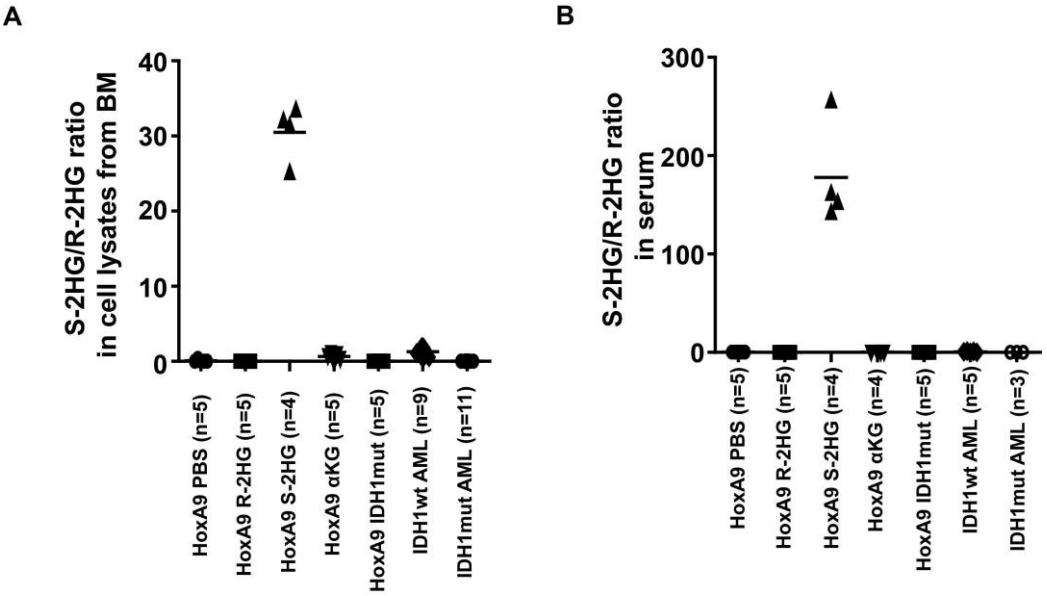
Supplementary Figures



Supplementary Figure S1. Western blot (WB) from bone marrow cells of mice that received transplants of HoxA9 transduced cells and were treated with PBS, R-2HG, S-2HG or αKG for 4 weeks probing for HoxA9 and β-actin.



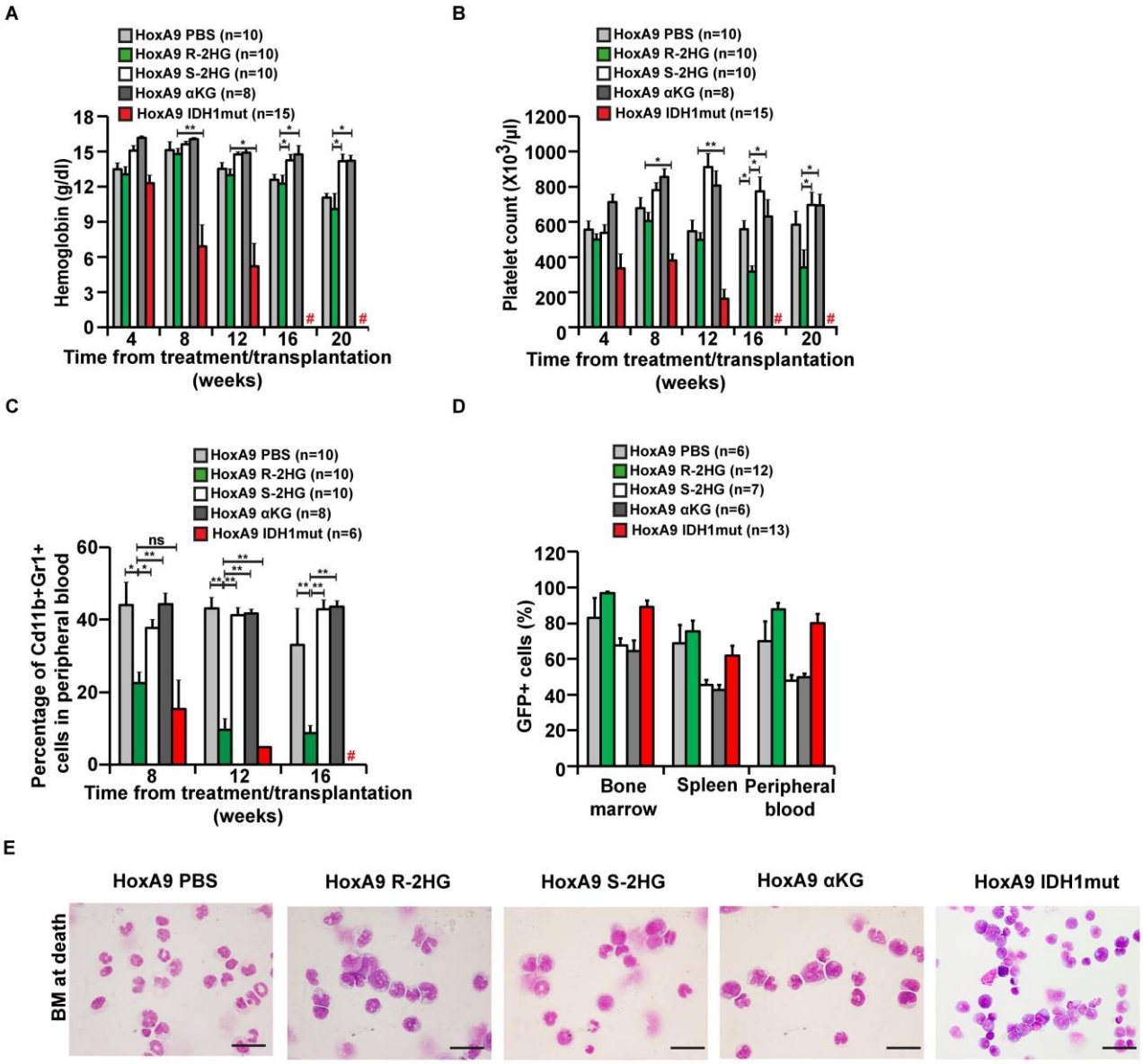
Supplementary Figure S2. Outline of experimental setup to evaluate the effects of R-2HG, S-2HG and αKG in mice overexpressing HoxA9 in vivo.



Supplementary Figure S3. S-2HG levels in cells and serum of treated mice.

(A) Ratio of S-2HG/R-2HG in cell lysates from bone marrow cells transduced with HoxA9 and a control vector or mutant IDH1 and primary AML cells at diagnosis with wildtype or mutant IDH1. Control vector transduced cells were either treated with PBS, R-2HG, S-2HG or αKG (1 mg intraperitoneally per day). Cells were harvested from bone marrow four weeks after treatment or transplantation (HoxA9 IDH1mut). Bars represent mean ratios.

(B) Concentration of S-2HG in serum as explained in (A).



Supplementary Figure S4. Phenotype of leukemic cells in vivo (HOXA9 model).

(A) Hemoglobin in peripheral blood at different time points after the start of treatment/transplantation (mean ± SEM).

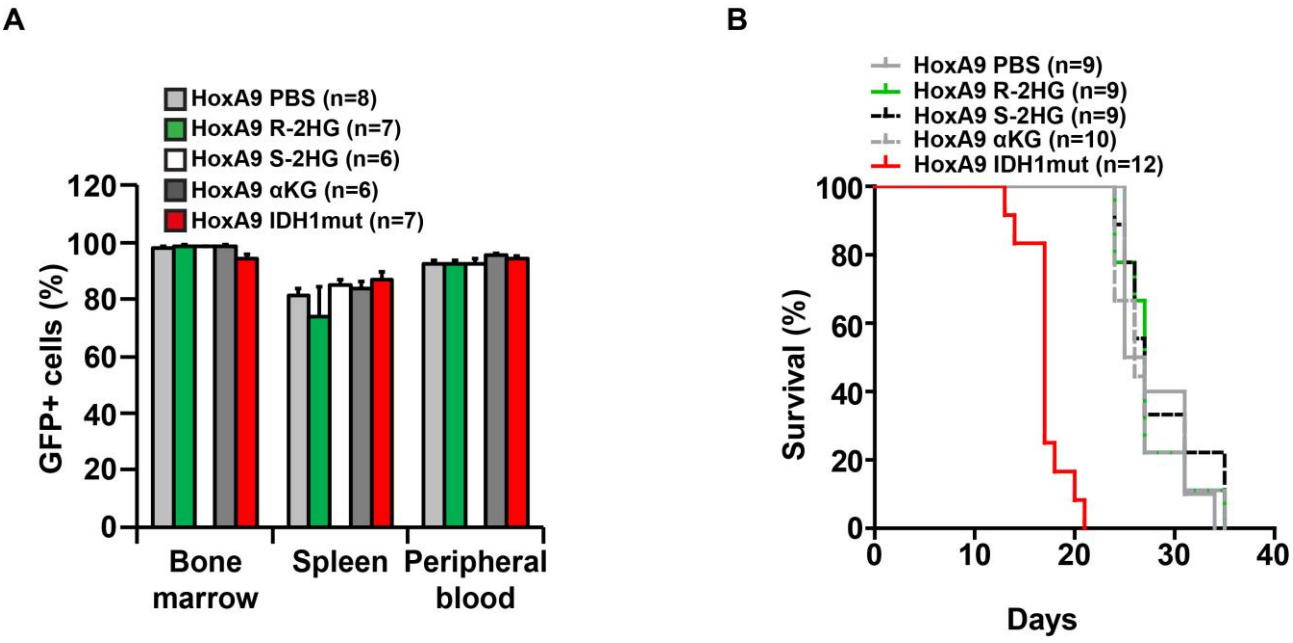
(B) Platelet counts in peripheral blood at different time points after the start of treatment/transplantation (mean ± SEM).

(C) Percentage of mature myeloid cells in peripheral blood at different time points after the start of treatment/transplantation (mean ± SEM).

(D) Engraftment of HoxA9-transduced cells in bone marrow, spleen and peripheral blood of mice that were treated with the indicated metabolites or PBS and HoxA9 IDH1mut cells at the time of death.

(E) Representative Wright-Giemsa stained cytospin preparations of bone marrow cells from moribund mice (scale bars represent 10 μm in the lower panel).

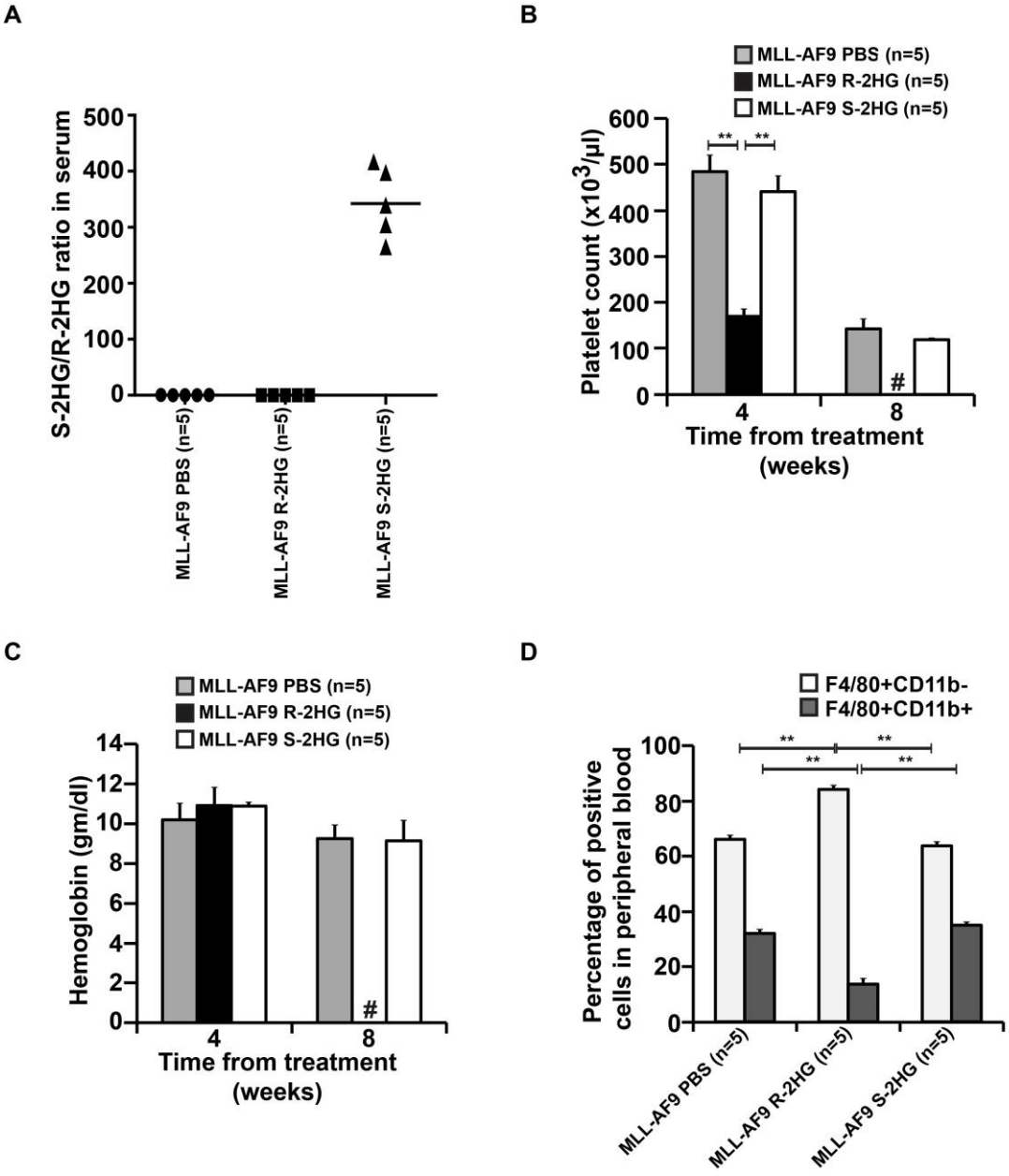
# indicates all mice are dead, * indicates P<.05, ** indicates P<.01, ns, not significant



Supplementary Figure S5. Engraftment of leukemic cells and survival of secondary recipients (HOXA9 model).

A) Engraftment of HoxA9-transduced control and IDH1mut cells in bone marrow, spleen and peripheral blood of secondary recipient mice at time of death. Primary and secondary control transplanted mice had been treated with the indicated metabolites or PBS.

B) Survival of secondary recipient mice at time of death. Primary and secondary control transplanted mice had been treated with the indicated metabolites or PBS.



Supplementary Figure S6. S-2HG levels, blood counts and phenotype of leukemic cells in the MLL-AF9 model.

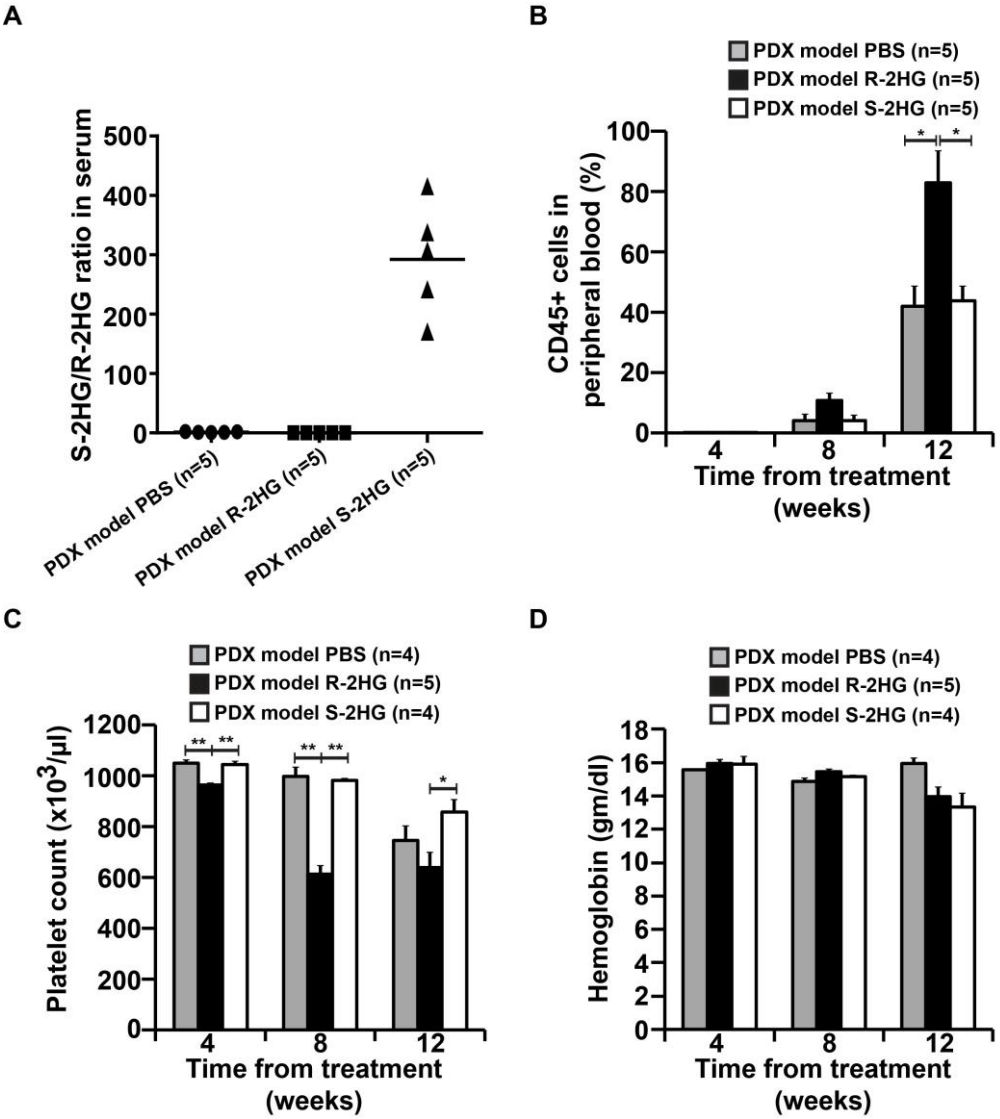
(A) Ratio of S-2HG to R-2HG in serum of mice transplanted with mouse bone marrow cells transduced with MLL-AF9 and either treated with PBS, R-2HG or S-2HG (1 mg intraperitoneally per day). Bars represent mean ratios.

(B) Platelet count in peripheral blood at different time points after the start of treatment (mean ± SEM) in mice transplanted with cells transduced with MLL-AF9 and either treated with PBS, R-2HG or S-2HG (1 mg intraperitoneally per day).

(C) Hemoglobin in peripheral blood at different time points after the start of treatment (mean ± SEM).

(D) Percentage of immature and mature myeloid cells in peripheral blood at 4 weeks after the start of treatment (mean ± SEM).

# indicates all mice are dead, * indicates P<.05, ** indicates P<.01, ns, not significant



Supplementary Figure S7. S-2HG levels, engraftment and blood counts in the human PDX AML model.

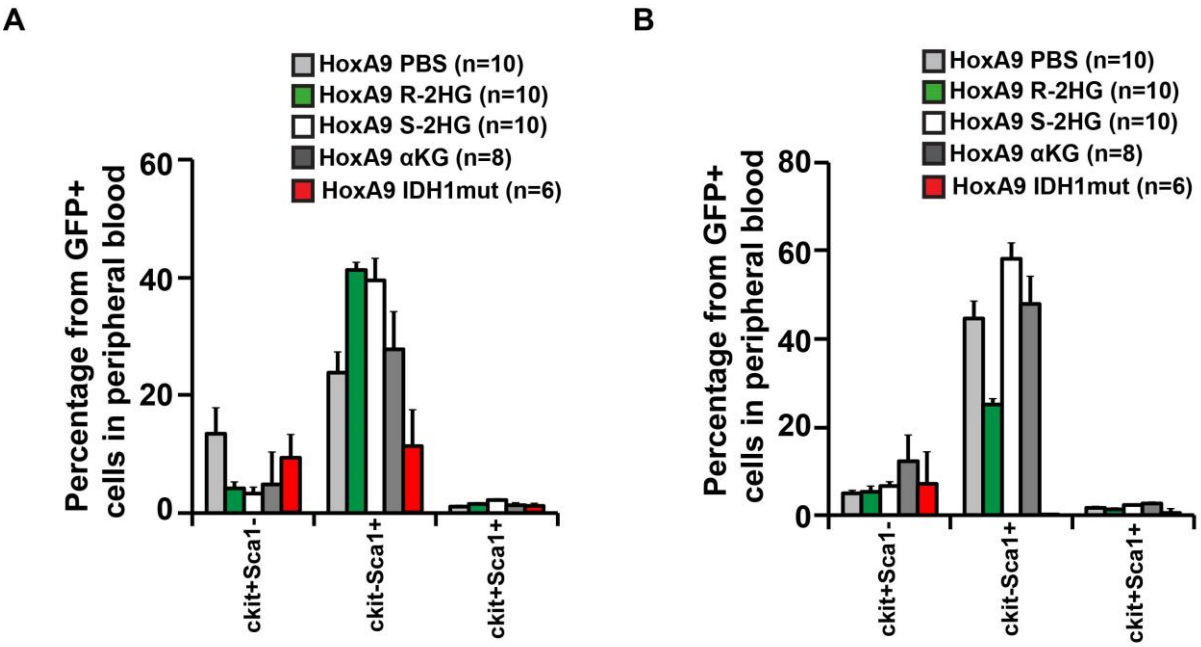
(A) Ratio of S-2HG to R-2HG in serum of mice transplanted with human IDH1wt patient cells and either treated with PBS, R-2HG or S-2HG (1 mg intraperitoneally per day). Bars represent mean ratios.

(B) Engraftment of human CD45+ cells in mice treated with R-2HG, S-2HG or PBS at different time points of treatment (mean ± SEM).

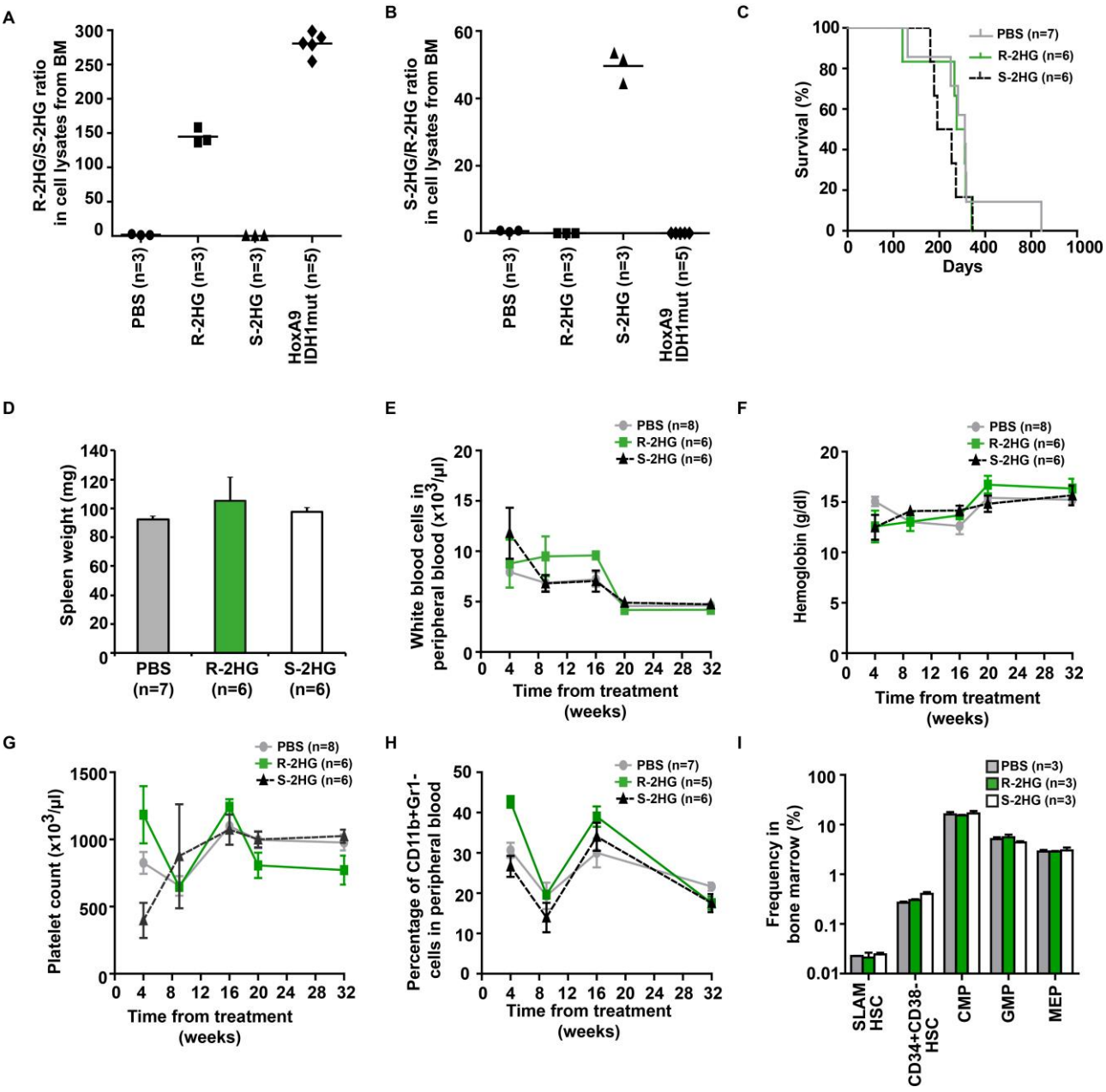
(C) Platelet count in peripheral blood at different time points after the start of treatment (mean ± SEM) in mice transplanted with human IDH1wt AML patient cells and either treated with PBS, R-2HG or S-2HG (1 mg intraperitoneally per day).

(D) Hemoglobin in peripheral blood at different time points after the start of treatment (mean ± SEM).

* indicates P<.05, ** indicates P<.01, ns, not significant



Supplementary Figure S8. Percentage of expression of cKit and Sca1 in peripheral blood cells at 8 weeks (A) and 12 weeks (B) after the start of treatment (mean ± SEM).



Supplementary Figure S9. R-2-hydroxyglutarate is insufficient to induce leukemia in normal hematopoietic cells in vivo.

(A) Ratio of R-2HG to S-2HG in bone marrow cells of normal C57Bl6/J mice treated with PBS, R-2HG or S-2HG (1 mg intraperitoneally per day) for 4 weeks and in mice receiving transplants of HoxA9/IDH1 mutant cells at 4 weeks after transplant. Bars represent mean ratios.

(B) Ratio of S-2HG to R-2HG in bone marrow cells of mice in (A)

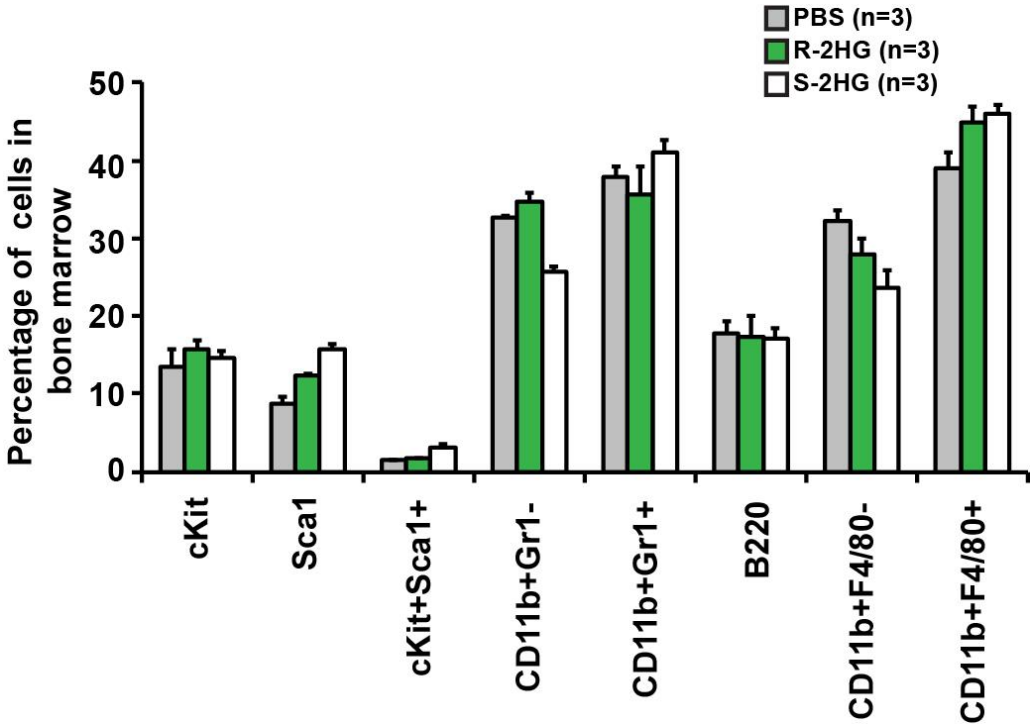
(C) Survival of mice in the different treatment groups.

(D) Average spleen weight in mice treated with PBS, R-2HG or S-2HG at 32 weeks after the start of treatment (mean ± SEM).

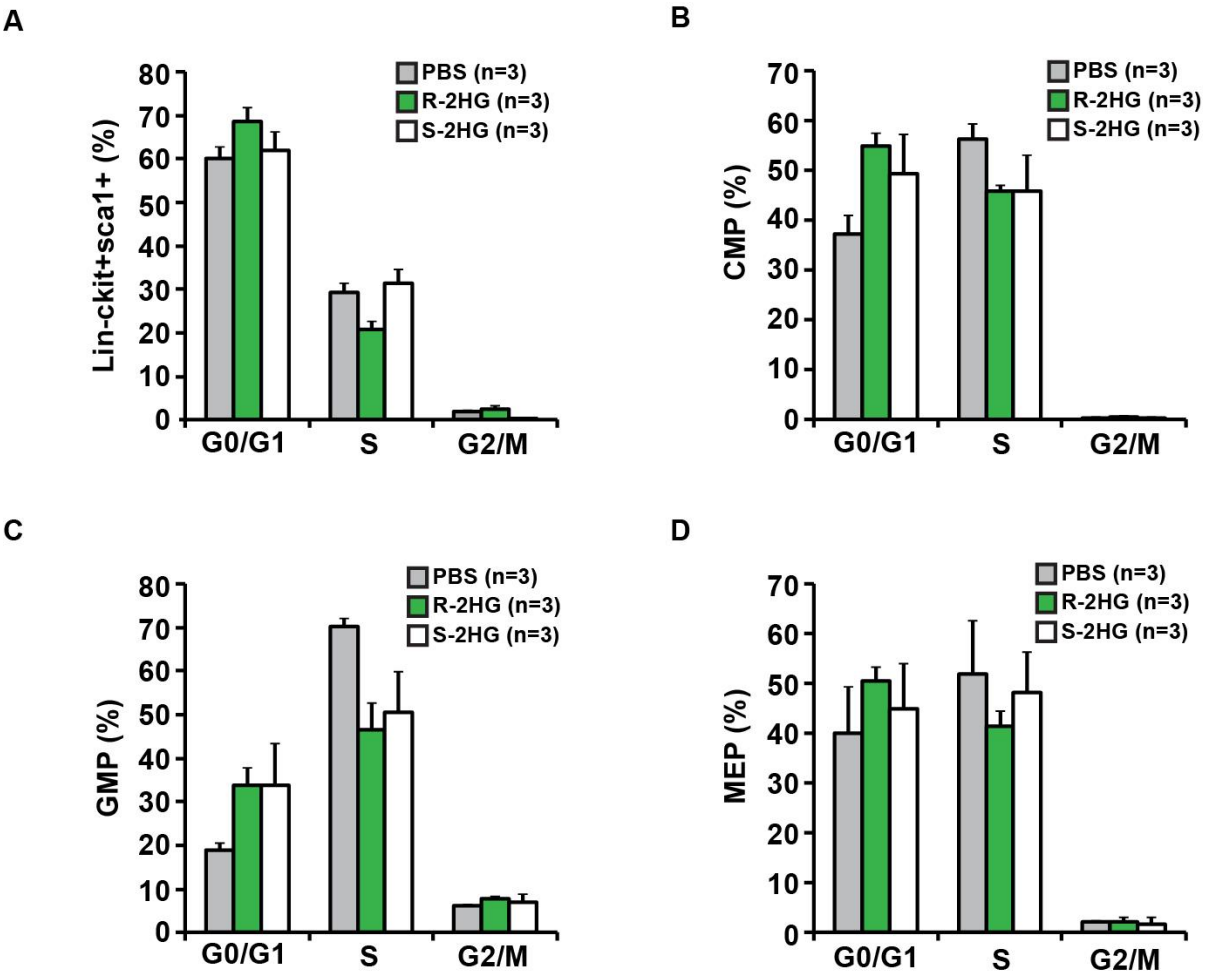
(E) White blood cell count, (F) hemoglobin level, and (G) platelet count in peripheral blood at different time points after the start of treatment (mean ± SEM).

(H) Percentage of immature myeloid cells in peripheral blood at different time points after the start of treatment (mean ± SEM).

(I) Frequency of hematopoietic stem and progenitor cells in bone marrow of mice at 16 weeks after the start of treatment. SLAM HSC (CD48-CD150+CD244+), LSK HSC, hematopoietic stem cells (Lin-cKit+Sca1+); CMP, common myeloid progenitor cells (Lin-cKit+Sca1-CD34+CD16/32-); GMP, granulocyte macrophage progenitor cells (Lin-cKit+Sca1-CD34+CD16/32+); MEP, megakaryocyte-erythroid progenitor cells (Lin-cKit+Sca1-CD34loCD16/32-).



Supplementary Figure S10. Immunophenotype of bone marrow of C57BL/6 mice which were treated for 8 months with the indicated metabolites.



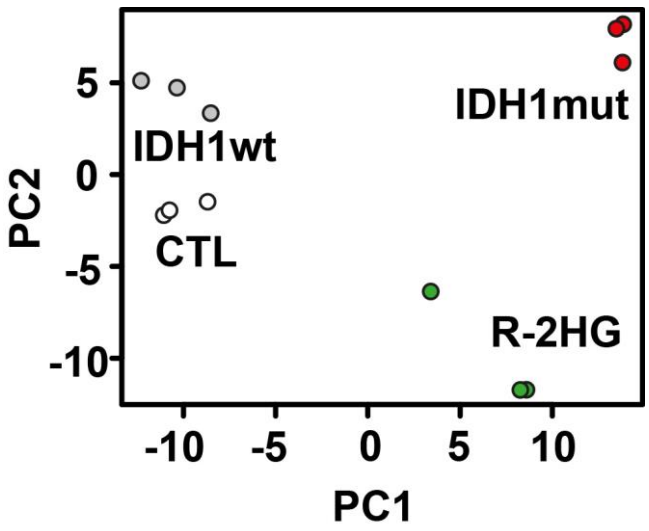
Supplementary Figure S11. Cell cycle analysis in hematopoietic progenitor cells from mice which were treated for 4 months with the indicated metabolites.

A) Cell cycle distribution in LSK progenitor cells (lin-ckit+sca1+)

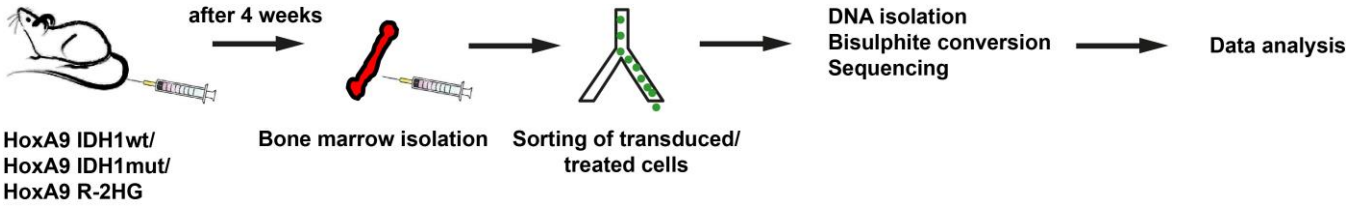
B) Cell cycle distribution in CMP progenitor cells (Lin-cKit+Sca1-CD34+CD16/32-).

C) Cell cycle distribution in GMP progenitor cells (Lin-cKit+Sca1-CD34+CD16/32+).

D) Cell cycle distribution in MEP progenitor cells (Lin-cKit+Sca1-CD34loCD16/32-).



Supplementary Figure S12. Principal component analysis of all experimental groups using differentially expressed genes between IDH1 mutant and IDH wildtype cells.



Supplementary Figure S13. Outline of experimental setup for RRBS analysis of transformed bone marrow cells.