Synergistic activity of IDH1 inhibitor BAY1436032 with azacitidine in IDH1 mutant acute myeloid leukemia

Anuhar Chaturvedi,[1] Charu Gupta,[1] Razif Gabdoulline,[1] Nora M. Borchert,[1] Ramya Goparaju,[1] Stefan Kaulfuss,[2] Kerstin Görlich,[1] Renate Schottmann,[1] Basem Othman,[1] Julia Welzenbach,[3] Olaf Panknin,[2] Markus Wagner,[2] Robert Geffers,[4] Arnold Ganser,[1] Felicitas Thol,[1] Michael Jeffers,[5] Andrea Haegebarth[2] and Michael Heuser[1]

[1]Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany; [2]Bayer AG, Berlin, Germany; [3]Institute of Human Genetics, University of Bonn, School of Medicine & University Hospital Bonn, Bonn, Germany; [4]Genome Analytics Research Group, Helmholtz Center for Infection Research, Braunschweig, Germany and [5]Bayer AG, Whippany, NJ, USA

 doi:10.3324/haematol.2019.236992

Received: September 9, 2019.

Accepted: March 26, 2020.

Pre-published: April 2, 2020.

Correspondence: *MICHAEL HEUSER* - heuser.michael@mh-hannover.de

ANUHAR CHATURVEDI - chaturvedi.anuhar@mh-hannover.de

Synergistic Activity of IDH1 Inhibitor BAY1436032 with Azacitidine in IDH1 Mutant Acute Myeloid Leukemia

Supplementary information

Supplementary methods

Compound Preparation

For *in vivo* administration 30 mg/ml of BAY1436032 was prepared in 2-(hydroxypropyl)-β-cyclodextrin (HP-ß-CD, Sigma-Aldrich, Munich, Germany). In brief, 600 mg BAY1436032 was added to 20 ml of 30% HP-ß-CD, pH was made alkaline with 50-100 µl of 10 M NaOH, followed by sonication (Bioruptor, Diagenode, Seraing, Belgium) with 15 seconds pulse on and 15 seconds pulse off until the drug was completely dissolved. Finally, the pH was readjusted to 7.4 with 2 M HCl. The fresh solution was prepared every week for *in vivo* administration. Azacitidine (Celgene, Munich, Germany) diluted in 0.9% NaCl, was purchased from the Pharmacy of Hannover Medical School, and administered within two hours after preparation to the mice. For *in vitro* studies a 1 mM stock solution of BAY1436032, 1 mM of trametinib (Selleckchem, Biozol diagnostics, Eching, Germany), and 1 mM of abemaciclib (Selleckchem) was prepared in DMSO or a 1 mM stock solution of azacitidine was prepared in 0.9% NaCl and stored at -20°C for a maximal period of two weeks. All dilutions were freshly prepared in cell culture medium and used immediately.

Limiting dilution transplantation

For limiting dilution transplantation, primary mice with established leukemia (the proportion of human AML cells in peripheral blood at the start of treatment was 70-80%) were treated for 4 weeks with either vehicle, azacitidine, BAY1436032, or the sequential or simultaneous

combination of BAY1436032 and azacitidine. After 4 weeks of treatment, bone marrow cells were obtained from primary mice and injected intravenously at doses of 2,000,000, 200,000, 20,000, 2,000, 200 or 20 cells (n = 3 mice/dose) into irradiated secondary NSG recipients. Eight weeks after transplantation, the presence of transplanted human cells in peripheral blood was assessed by flow cytometry. Engraftment was determined positive when more than 0.1% $hCD45^+$ cells were detected. The frequency of leukemia initiating cells within the human CD45+ AML cell population was calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions using the ELDA software.[1]

Clonogenic progenitor assay

Colony-forming cell units were assayed in methylcellulose (Methocult H4100; StemCell Technologies, Cologne, Germany) supplemented with 10 ng/mL IL3, 10 ng/mL GM-CSF, 50 ng/mL SCF, 50 ng/mL FLT3-ligand and 3 U/mL EPO (PeproTech, Hamburg, Germany). BAY1436032 and/or azacitidine were added to methylcellulose containing 10^5 human mononuclear cells, which were plated in duplicate. Colonies were evaluated microscopically 10 to 14 days after plating by standard criteria.

Cell culture conditions, cell cycle, and apoptosis

Patient-derived AML cells, freshly isolated or cryopreserved, were cultured in IMDM medium (StemCell Technologies, Cologne, Germany) supplemented with 10% FBS (Sigma-Aldrich, Munich, Germany), 2 mM L-Glutamine (Gibco, Thermofisher Scientific, Bremen, Germany), 20 ng/ml human IL-3, 20 ng/ml human IL-6, 20 ng/ml human GMCSF, 20 ng/ml human GCSF and 50 mg/ml human SCF (all from PeproTech, Hamburg, Germany) and were incubated at 37°C with 5% CO_2 in the humidified atmosphere. Treatment was carried out with 100 nM BAY1436032 and 100 nM azacitidine. 60 hours after the treatment 10 µM of BrdU was added to the cells for 12 hours. Cell cycle analysis was performed according to the manufacturer's

protocol (BD Pharmingen Cat no. 557892). $2x10^5$ cells were acquired per sample. Cell cycle phases were determined according to standard procedures, where BrdU positive cells are in the S phase of the cell cycle. For apoptosis measurement cells were stained with Annexin V-APC and 7AAD after 72 hours of treatment according to the manufacturer's protocol (BD Pharmingen Cat no. 560931) and analyzed on a BD FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Annexin V single positive and Annexin V/7AAD double positive cells were combined to represent apoptotic cells.

Antibodies for flow cytometry

The monoclonal antibody used were CD45-FITC (Clone HI30, Cat no.555482, lot no.7137972), CD14-APC (Clone M5E2, cat no. 555399, Lot no. 7116537) and CD15-PE (Clone W6D3, cat no. 562371) from BD Biosciences, Heidelberg, Germany. Data were collected on a FACS Calibur (BD Biosciences, Heidelberg, Germany).

Gene expression profiling using microarrays

For gene expression profiling RNA was extracted using the RNeasy Plus mini kit (Qiagen) from $hCD45^+$ cells that were sorted from bone marrow of PDX mice 4 weeks after treatment with vehicle, azacitidine (1 mg/kg, s.c., days 1 to 5), BAY1436032 (150 mg/kg, p.o., q.d., 4 weeks) or the simultaneous combination of BAY1436032 and azacitidine.

Quality and integrity of total RNA was controlled on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). 500 ng of total RNA was used for biotin labeling according to the 3´ IVT Express Kit (Affymetrix/ThermoFisher Scientific, Braunschweig, Germany). 7.5 µg of biotinylated cRNA was fragmented and placed in a hybridization cocktail containing 4 biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix GeneChip HG-U133 2.0 for 16 hours at 45°C. Steps for washing and SA-PE staining were processed on the fluidics

station 450 using the recommended FS450 protocol (Affymetrix). Image Analysis was performed on the GCS3000 Scanner and GCOS1.2 Software Suite (Affymetrix).

Microarray data was analyzed and presented using R packages and Cluster 3.0 software. [2] Arrays were background corrected, normalized and summarized with the *rma* function of the *affy* package [3]. The *limma* package [4] was used to select differentially expressed genes;the *gplots* package was used for drawing heatmaps. Principal component analysis (PCA) was done with Cluster 3.0 software. Expression values of the top 4000 differentially regulated genes in all arrays were selected for PCA. Expression of the top 500 differentially regulated genes were used for unsupervised hierarchical clustering, the results of which were visualized as a heatmap in Figure 3B. The heatmap in Supplementary Figure S6 used 89 genes with available Affymetrix probeset ID that were differentially expressed between LSC+ and LSC- cell fractions from AML patients (known as the LSC17 gene signature).[5]

The Broad Institute GSEA software package was employed for gene set enrichment analysis using gene ontology gene sets from the Molecular Signatures Database (http://www.broad.mit.edu/ gsea/msigdb/). Gene expression profiling data can be found at the gene expression omnibus database under GEO accession number GSE122428.

Quantification of allele frequencies of mutant IDH1 in vivo

Mutated allele frequencies in blood samples from PDX1 mice were determined using the previously developed MRD protocol.[6] We designed primers to amplify a 114 bp cDNA fragment around the IDH1 mutation site. The fragments were sequenced with the Illumina Miseq sequencer. Paired-end output sequences/reads were aligned to the IDH1 cDNA sequence after combining overlapping forward and reverse reads. Sequences having mouse-specific alterations (there were 11 such positions on the amplicon) were removed. The variant allele frequency was calculated as a ratio of the number of mutated sequences to the number of all aligned sequences.

Gene expression profiling by RNA sequencing

Human AML cells isolated from the spleen and bone marrow of IDH1 R132C (PDX1) mice were cultured in IMDM medium (Stemcell Technology Inc. Vancouver, Canada) supplemented with 10% FBS (Sigma-Aldrich, Munich, Germany), 2 mM L-Glutamine (Life Technologies, Karlsruhe, Germany), 1% Penicillin/Streptomycin (Gibco, ThermoFischer Scientific, Braunschweig, Germany) , 20 ng/ml human GM-CSF, 20 ng/ml human G-CSF, 20 ng/ml human IL-6, 50 ng/µl, human SCF and 20 ng/µl human IL-3 (all from PeproTech, Hamburg, Germany) and were incubated at 37 °C with 5% CO_2 in humidified atmosphere. Treatment was carried out with DMSO (vehicle), 500 nM azacitidine daily from day 1 to day 4, 50 nM BAY1436032 daily from day 1 to day 5, the sequential combination (azacitidine daily from day 1 to day 4 and BAY1436032 daily from day 5 to day 9) and the simultaneous combination (azacitidine daily from day 1 to day 4 and BAY1436032 daily from day 1 to day 5). RNA was isolated on day 6 for cells treated with vehicle, azacitidine, BAY1436032 and the simultaneous combination and on day 10 for the sequential combination using the AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted at 20 µl H2O, and concentration and purity were measured with the NanoDrop 2000 spectrophotometer (ThermoScientific, Darmstadt, Germany). Approximately 150 ng from each sample were sequenced on the Illumina NovaSeq 6000 system with paired-end reads of 50 bp in size. Sequences were aligned to the human genome hg19 with the help of Gencode v.n 25 transcriptome annotations.[7] RNAseq expression data was aligned to GRCh38 / Gencode v.32. A larger dataset of annotated transcripts was used, when more appropriate.[8] The main alignment program was STAR;[9] the program bowtie2 [10] was used for comparison. Mapping to the transcriptome and gene expression analysis was done with TopHat and Cufflinks [11] with the help of Gencode annotation of genes and alternative transcript variants. Gene and transcript expression was quantified by FPKM (Fragments Per Kilobase per Million), TPM (Transcripts Per

Million) values, as well as by fragment counts. The latter was calculated from cufflinks outputs using the program HTSeq [12] and used as input to DESeq2 analysis.[13]

DNA Methylation Microarrays

Genomic DNA (500 ng) isolated from the same samples that were used for RNA sequencing analysis above was bisulfite-treated using the EZ-96 DNA Methylation-Lightning MagPrep Kit (Zymo Research, Freiburg, Germany) according to the manufacturer's instructions. DNA methylation was assessed using the Infinium MethylationEPIC BeadChip Kit (Illumina, San Diego, USA) following the Infinium HD Methylation Assay protocol. Subsequent steps (i.e., amplification, fragmentation, extension, hybridization, staining, and scanning) were performed at the Life & Brain Center (Bonn, Germany). Raw data were processed and quality checked using Illumina's GenomeStudioV2011.1 methylation module (v.1.8).

Cell line

The HT-1080 cell line was purchased from ATCC (ATCC® CCL-121™, Manassas, Virginia, United States) and cultured in Minimum Essential Media (Gibco, ThermoFischer Scientific, Schwerte, Germany). The cells were negative for mycoplasma during routine mycoplasma testings in the lab.

Quantitative RT-PCR

RNA was extracted, reverse transcribed and quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using SYBR green (Invitrogen, ThermoFischer Scientific, Schwerte, Germany) for quantification of double stranded DNA on a StepOne Plus cycler (Applied Biosystems, Darmstadt, Germany). Relative expression was determined with the $2^{-\Delta\Delta CT}$ method, and the housekeeping gene transcript *Abl1* and the gene expression ratio of drug-treated cells relative to vehicle-treated cells was used to normalize the results.

Primers of quantitative PCR

*ELK1*_forward primer	5'-CAGCCAGAGGTGTCTGTTACC-3'
*ELK1*_reverse primer	5'-GAGCGCATGTACTCGTTCC-3'
*MYC*_ forward primer	5'-GGCTCCTGGCAAAAGGTCA-3'
*MYC*_ reverse primer	5'-CTGCGTAGTTGTGCTGATGT-3'
*CREB1*_ forward primer	5'-ATTCACAGGAGTCAGTGGATAGT-3'
*CREB1*_ reverse primer	5'-CACCGTTACAGTGGTGATGG-3'
*ETS1*_ forward primer	5'-GATAGTTGTGATCGCCTCACC-3'
*ETS1*_ reverse primer	5'-GTCCTCTGAGTCGAAGCTGTC-3'
*CCND1*_ forward primer	5'-GCTGCGAAGTGGAAACCATC-3'
*CCND1*_ reverse primer	5'-CCTCCTTCTGCACACATTTGAA-3'
*E2F1*_ forward primer	5'-ACGCTATGAGACCTCACTGAA-3'
*E2F1*_ reverse primer	5'-TCCTGGGTCAACCCCTCAAG-3'
*CCNB1*_ forward primer	5'-AATAAGGCGAAGATCAACATGGC-3'
*CCNB1*_ reverse primer	5'-TTTGTTACCAATGTCCCCAAGAG-3'
*CCNA2*_ forward primer	5'-CGCTGGCGGTACTGAAGTC-3'
*CCNA2*_ reverse primer	5'-GAGGAACGGTGACATGCTCAT-3'
*CCNE1*_ forward primer	5'-AAGGAGCGGGACACCATGA-3'
*CCNE1*_ reverse primer	5'-ACGGTCACGTTTGCCTTCC-3'
*CDC25A*_ forward primer	5'-GTGAAGGCGCTATTTGGCG-3'
*CDC25A*_ reverse primer	5'-TGGTTGCTCATAATCACTGCC-3'
*GABPB1*_ forward primer	5'-TCCACTTCATCTAGCAGCACA-3'
*GABPB1*_ reverse primer	5'-GTAATGGTGTTCGGTCCACTT-3'
*ELF1*_ forward primer	5'-TGTCCAACAGAACGACCTAGT-3'
*ELF1*_ reverse primer	5'-GGCAGGAAAAATAGCTGGATCAC-3'

*PU.1*_ forward primer	5'-GTGCCCTATGACACGGATCTA-3'
*PU.1*_ reverse primer	5'-AGTCCCAGTAATGGTCGCTAT-3'
*CEBPA*_ forward primer	5'-TTCACATTGCACAAGGCACT-3'
*CEBPA*_ reverse primer	5'-GAGGGACCGGAGTTATGACA-3'
*GABPA*_ forward primer	5'-TTAAACCTGCGGACACTGTTG-3'
*GABPA*_ reverse primer	5'-GTATCCCAAGGCGTTCTTGTT-3'

Immunoblotting

HT-1080 cells were treated with either Vehicle or 1 µM of BAY1436032 or 1 µM of azacitidine alone or in combination at 0 hours and 24 hours. 2 hours after the second treatment, 5 million cells were collected, washed with PBS, resuspended in RIPA buffer (cat no. 89901, Thermo Fischer Scientific, Bremen, Germany), supplemented with the HALT protease inhibitor cocktail (cat no. 78430, Thermo Fischer Scientific, Bremen, Germany) and lysed on ice for 20 minutes with gentle agitation. After centrifugation at 10,000 rpm for 10 minutes at 4°C, the supernatant was collected and the protein concentration was estimated using the Pierce BCA protein assay kit (Thermo Fischer Scientific, Bremen, Germany). 30 µg of protein/sample was separated by SDS PAGE, transferred to a PVDF membrane, blocked and immunoblotted. Clarity Western ECL Substrate (Biorad, Munich, Germany) was used for chemiluminescent protein detection with a ChemiDoc MP Imaging System (Bio-Rad, Munich, Germany).

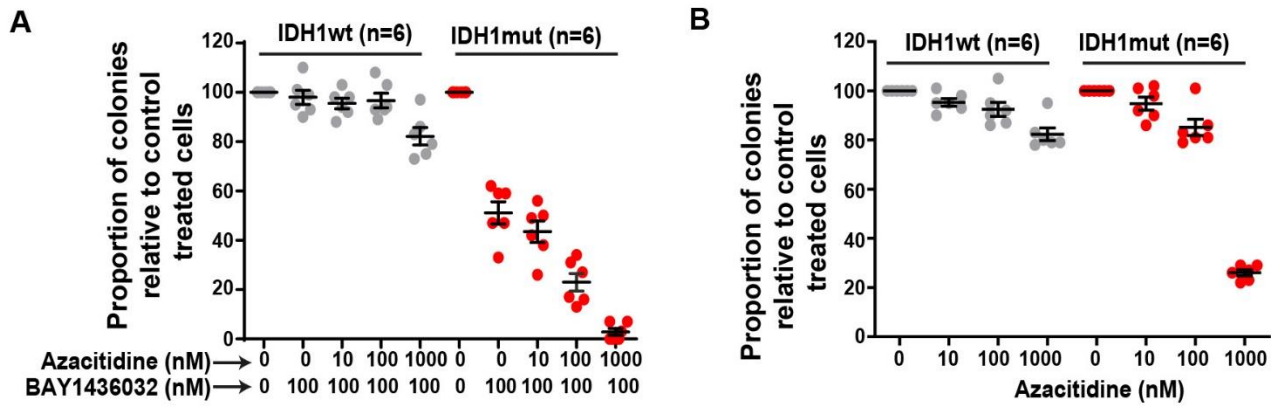
Antibodies for Immunoblotting

The antibodies used for immunoblotting are anti-phosho ERK1/2 (clone: D13.14.4E, cat no. 4370), anti-ERK1/2 (cat no. 9102), anti-ß-actin (clone: 13E5, cat no. 4970) and anti-phospho S807/811 Rb (Cat no.9308) from Cell Signaling Technology, Frankfurt, Germany. Anti-phospho S795 Rb (cat no. ab47474) from Abcam, Cambridge, UK. Anti-ELK1 (clone:E-5, cat no. sc-365876), anti-CyclinD1 (clone:DCS-6, cat no. sc-20044), anti-E2F1 (clone: C-20, cat no. sc-193)

and anti-Rb (clone: M-153, cat no. sc-7905) from Santa Cruz Biotechnology, Heidelberg, Germany.

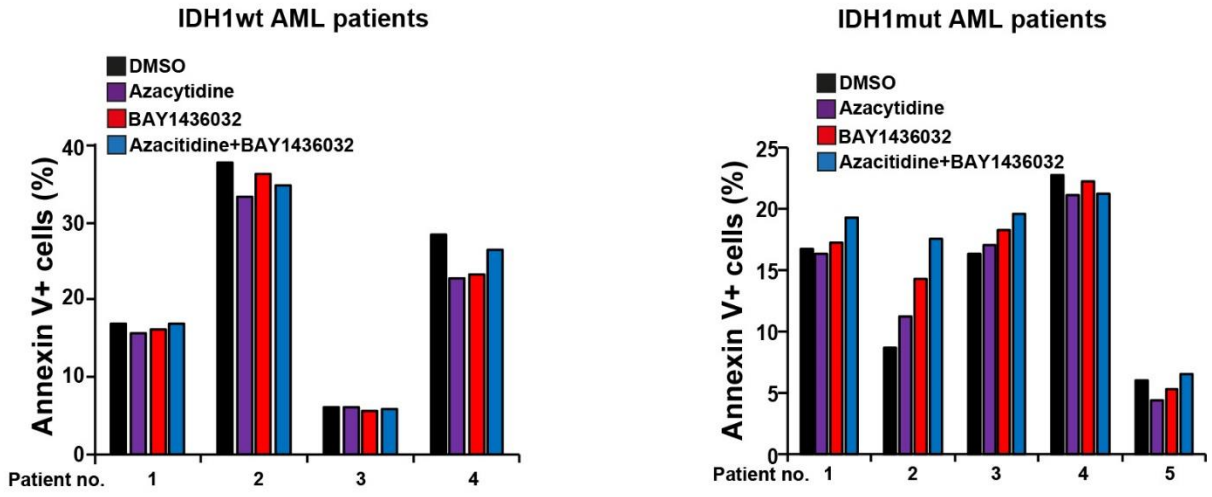
Supplementary Figures

Figure S1



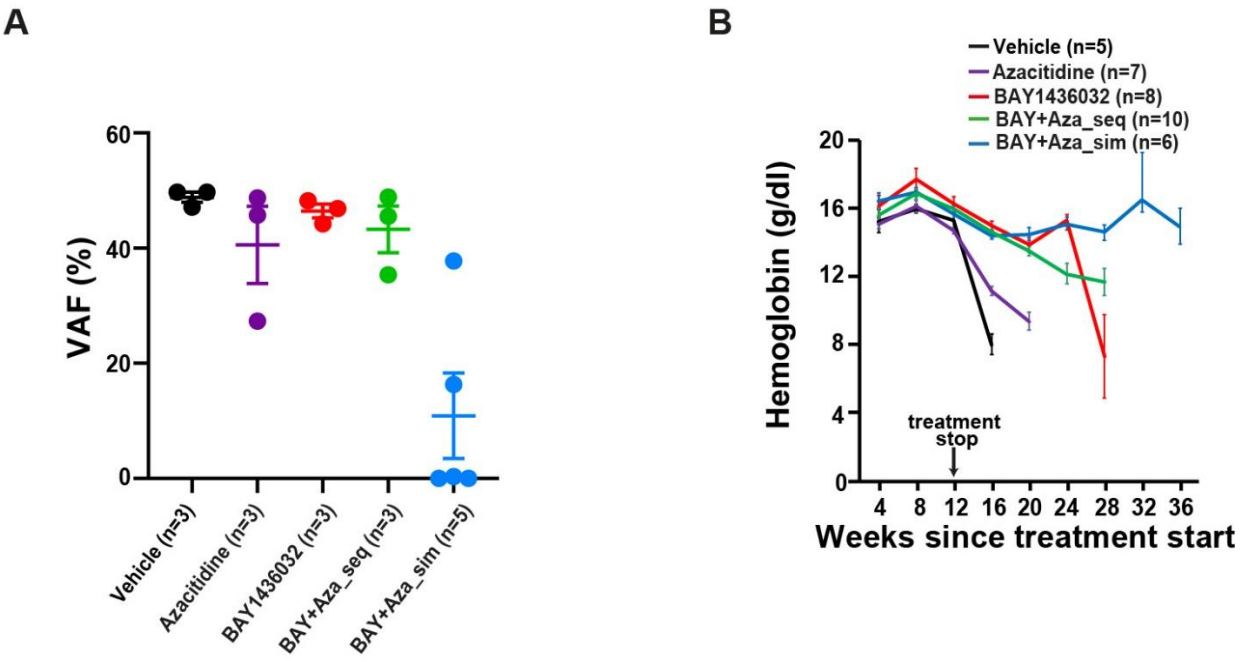
Supplementary Figure S1. Synergistic activity of IDH1 inhibitor BAY1436032 and azacitidine in IDH1 mutant but not in IDH1 wildtype human AML cells relative to control treated cells. (A) Inhibition of colony formation by the simultaneous combination treatment of BAY1436032 (100 nM, corresponding to the IC_{50} in previous experiments) with varying concentrations of azacitidine in colony forming assays using primary human AML cells with wildtype or mutant IDH1. (B) Inhibition of colony formation with varying concentrations of azacitidine in colony forming assays using primary human AML cells with wildtype or mutant IDH1. The graph represents the proportion of colonies relative to DMSO treated cells (mean ± SEM). From the 6 patients with IDH1 mutant AML, 4 harbored an I*DH1R132H* mutation and one each an *IDH1R132C* and *IDH1R132G* mutation.

Figure S2



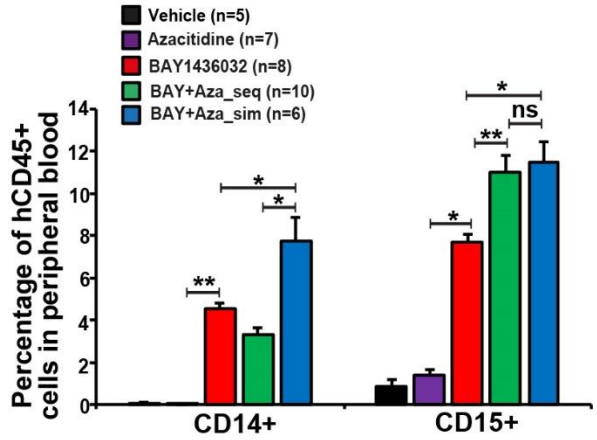
Supplementary Figure S2. The percentage of apoptotic cells did not differ between single agent and combination treatments. Percentage of Annexin V positive cells after treatment with BAY1436032 (100 nM) or azacitidine (100 nM) or the combination of both in IDH wildtype (right) and IDH1 mutant (left) primary AML patient cells. From the 5 patients with IDH1 mutant AML, 3 harbored an *IDH1R132H* mutation and one each an *IDH1R132C* and *IDH1R132G* mutation.

Figure S3



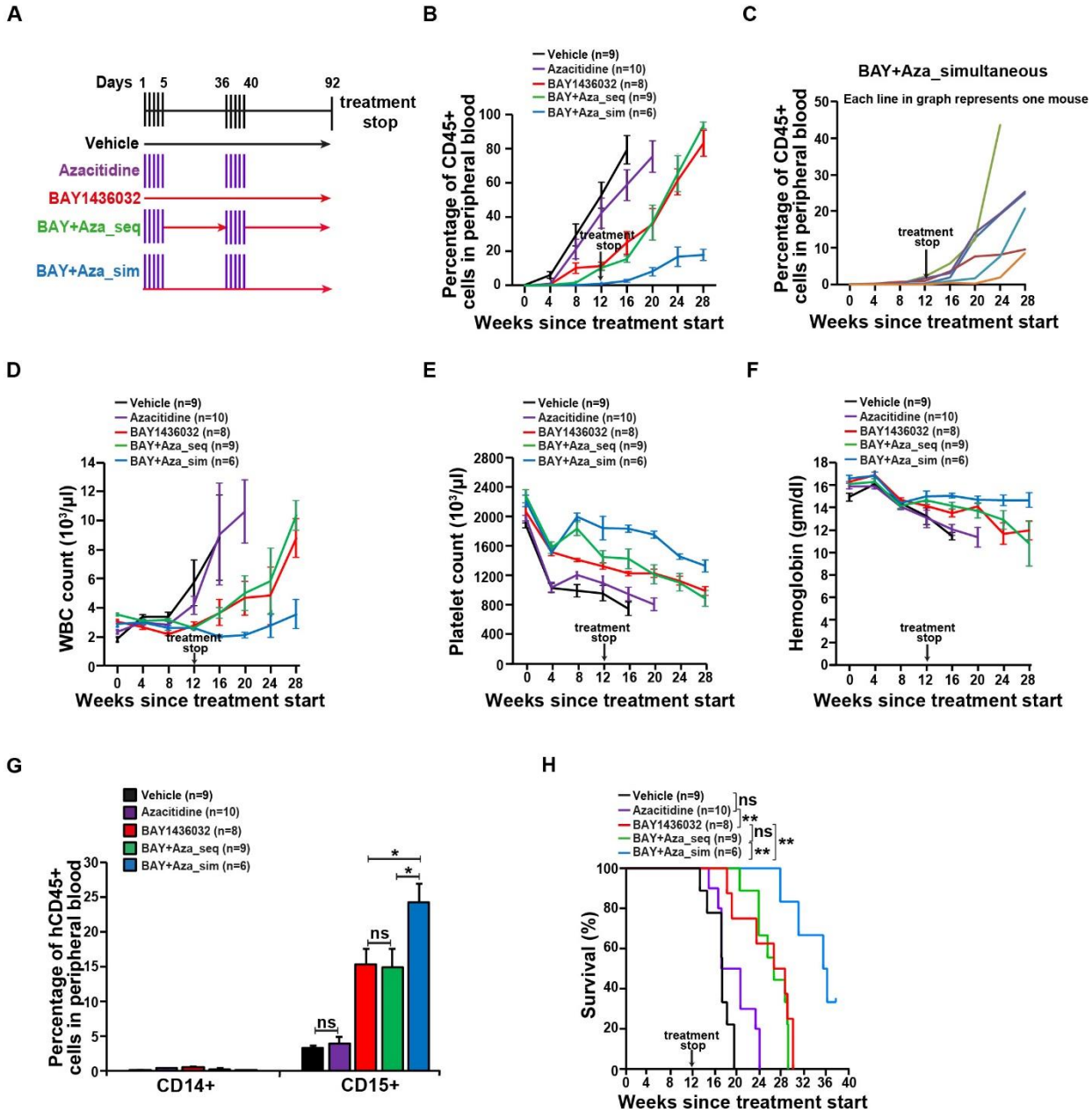
Supplementary Figure S3. IDH1mutant allele fraction is decreased and hemoglobin levels are maintained by the combined simultaneous treatment with BAY1436032 and azacitidine. (A) IDH1 mutant allele frequency assessed by next generation sequencing in peripheral blood of IDH1 mutant PDX mice at 8 weeks after the stop of treatment (20 weeks since treatment start) mean ± SEM for the indicated number of mice. (B) Hemoglobin in the peripheral blood of IDH1 mutant PDX mice at different time points after treatment start with vehicle, azacitidine (1 mg/kg; s.c., days 1-5 and days 29-33), BAY 1436032 (150 mg/kg, p.o., q.d., continuously), or the sequential or simultaneous combination of BAY1436032 and azacitidine according to the treatment regimen shown in Figure 2A.

Figure S4



Supplementary Figure S4: The combination of BAY1436032 and azacitidine has additive effects on differentiation of *IDH1* mutant AML cells treated in vivo. Percentage of myeloid differentiation markers CD14 and CD15 from hCD45+ cells in peripheral blood of *IDH1*mutant (R132C) PDX1 mice at 4 weeks after the start of treatment with vehicle, azacitidine (1mg/kg, s.c., days 1-5), BAY1436032 (150 mg/kg, p.o., continuously), or the sequential or simultaneous combination of BAY1436032 and azacitidine (mean ± SEM). * P<0.5; **P<0.01; ns=non-significant

Figure S5

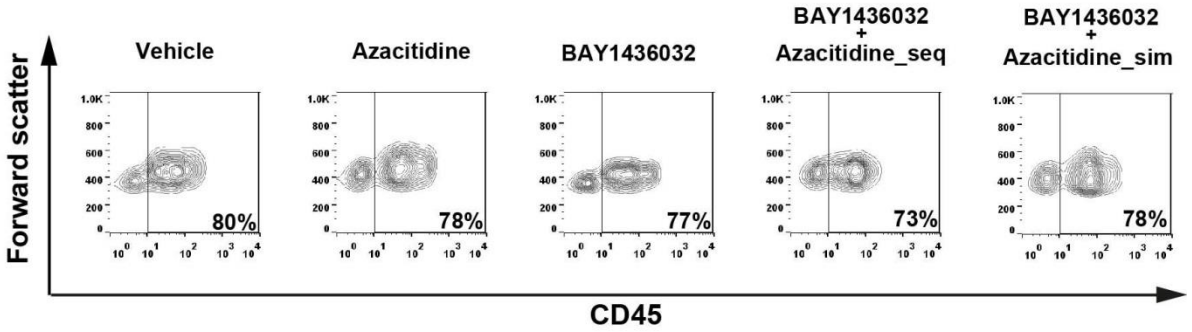


Supplementary Figure S5. BAY1436032 synergizes with azacitidine to exert potent anti-leukemic activity in an independent patient-derived IDH1 mutant AML xenograft model in vivo (PDX2). (A) Schematic representation of the treatment regimens; sim, simultaneous treatment with BAY1436032 and azacitidine; seq, sequential treatment with BAY1436032 and azacitidine. (B) Percentage of hCD45+ leukemic cells in peripheral blood of IDH1 mutant (R132H) PDX2 mice at different time points after treatment start with vehicle, azacitidine

(1mg/kg, s.c., days 1-5 and days 36-40), BAY1436032 (150 mg/kg, p.o., continuously), or the sequential or simultaneous combination of BAY1436032 and azacitidine according to the treatment regimen shown in Figure S5A (mean ± SEM). (C) Percentage of hCD45+ leukemic cells in peripheral blood of individual mice transplanted with human IDH1 mutant AML cells and simultaneously treated with BAY1436032 and azacitidine. (D) White blood cell counts after different time points of treatment (mean ± SEM). (E) Platelet counts after different time points of treatment (mean ± SEM). (F) Hemoglobin after different time points of treatment (mean ± SEM). (G) Kaplan–Meier survival curves of IDH1 mutant PDX2 mice treated with vehicle, azacitidine (1 mg/kg, s.c.), BAY1436032 (150 mg/kg, p.o.), or the sequential or simultaneous combination of BAY1436032 and azacitidine according to the treatment regimen shown in Figure S5A. (H) Percentage of myeloid differentiation markers CD14 and CD15 from hCD45+ cells in peripheral blood of IDH1 mutant (R132H) PDX2 mice at 12 weeks after the start of treatment. (mean ± SEM). * $P<0.5$; ns=non-significant

Figure S6

A

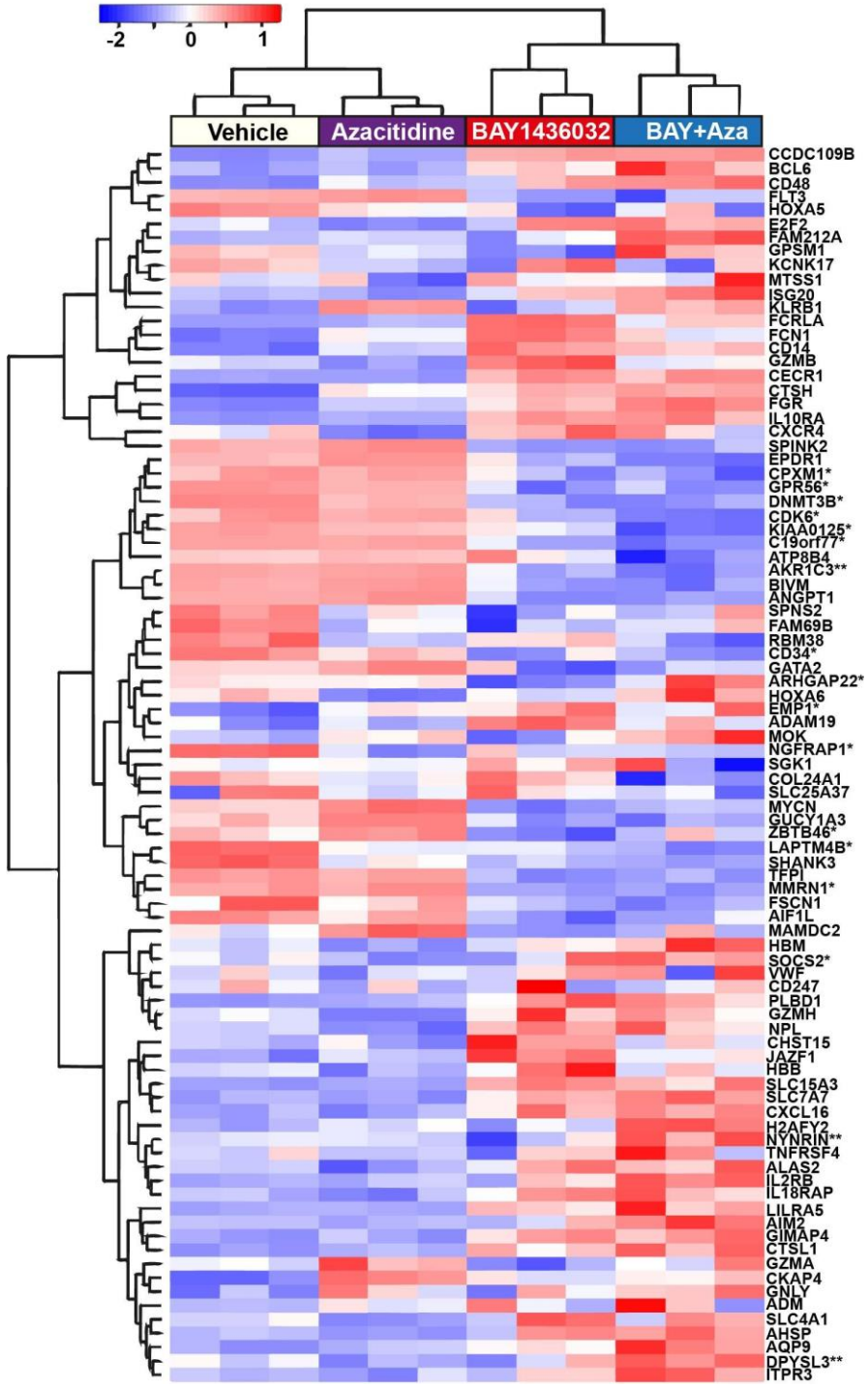


B

Group	Stem cell frequency	±SEM	P Value
Vehicle	1/73	18-287	0.2
Azacitidine	1/304	48-1,994	0.005
BAY1436032	1/8,580	2,440-30,173	0.2
BAY+Aza_seq	1/34,300	5,913-199,114	0.0002
BAY+Aza_sim	1/2,420,000	612,820-9,558,998	

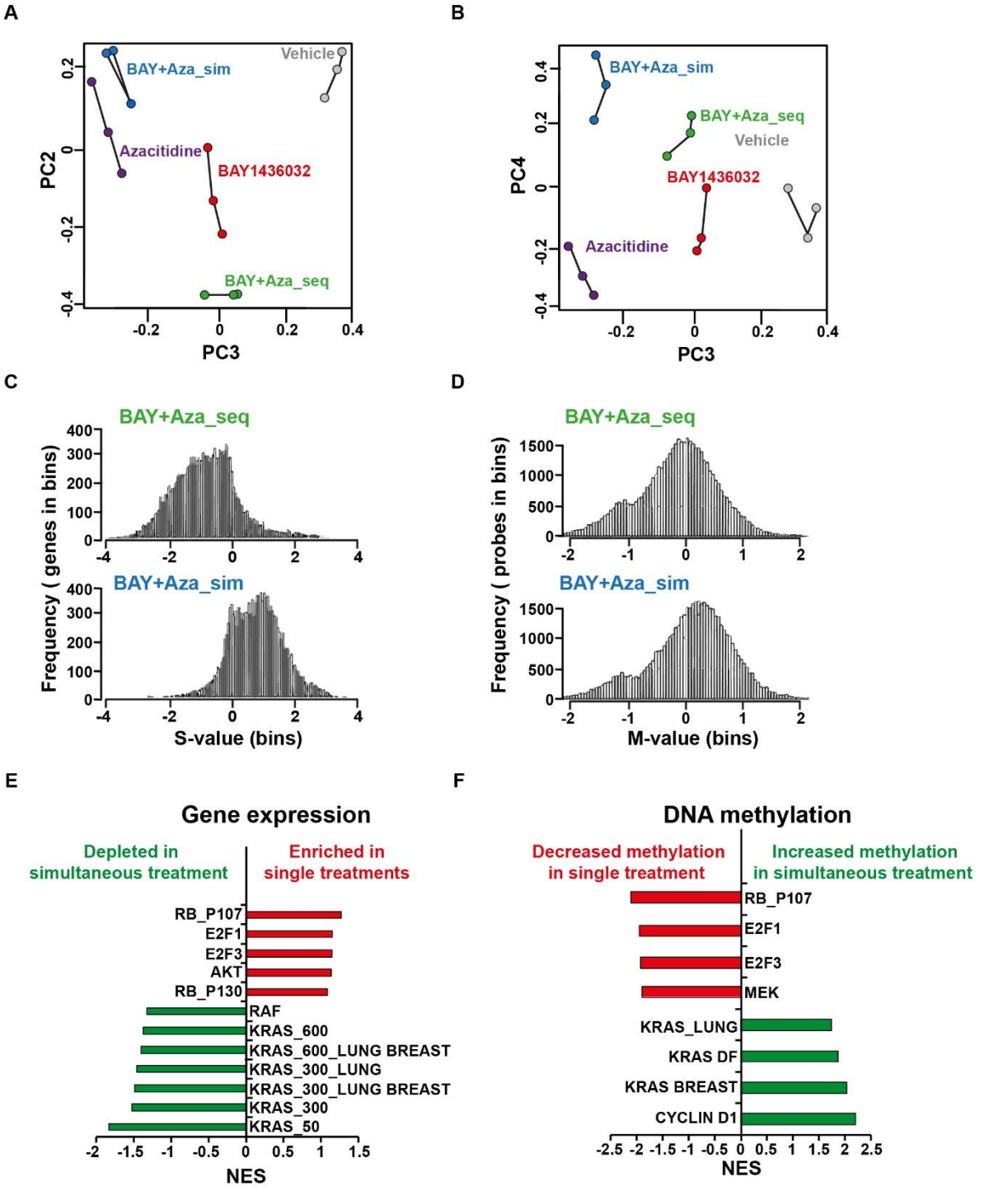
Supplementary Figure S6. Combination treatment with BAY1436032 and azacitidine depletes leukemia stem cells. (A) A representative FACS plot from the peripheral blood of mice before the start of treatment of mice used for the limiting dilution transplantation experiment, which is used to estimate the leukemia stem cell frequency. (B) The estimated leukemia stem cell frequency calculated by limiting dilution transplantation from the bone marrow of mice after treatment with either vehicle, azacitidine, BAY1436032, or the sequential or simultaneous combination of BAY1436032 and azacitidine for four weeks. The bar graph is shown in Figure 3A.

Figure S7



Supplementary Figure S7. Unsupervised hierarchical clustering of genes and cells from bone marrow of *IDH1* mutant PDX1 mice treated with vehicle, azacitidine (1 mg/kg, s.c., days 1 to 5), BAY1436032 (150 mg/kg, p.o., q.d., 4 weeks) or the simultaneous combination of BAY 1436032 and azacitidine, based on 89 genes that are differentially expressed between LSC+ and LSC- cell fractions from AML patients.[7] The genes of the 17-gene LSC score (LSC17) are marked by a star, the genes of the LSC3 score (a subset of LSC17) by 2 stars.

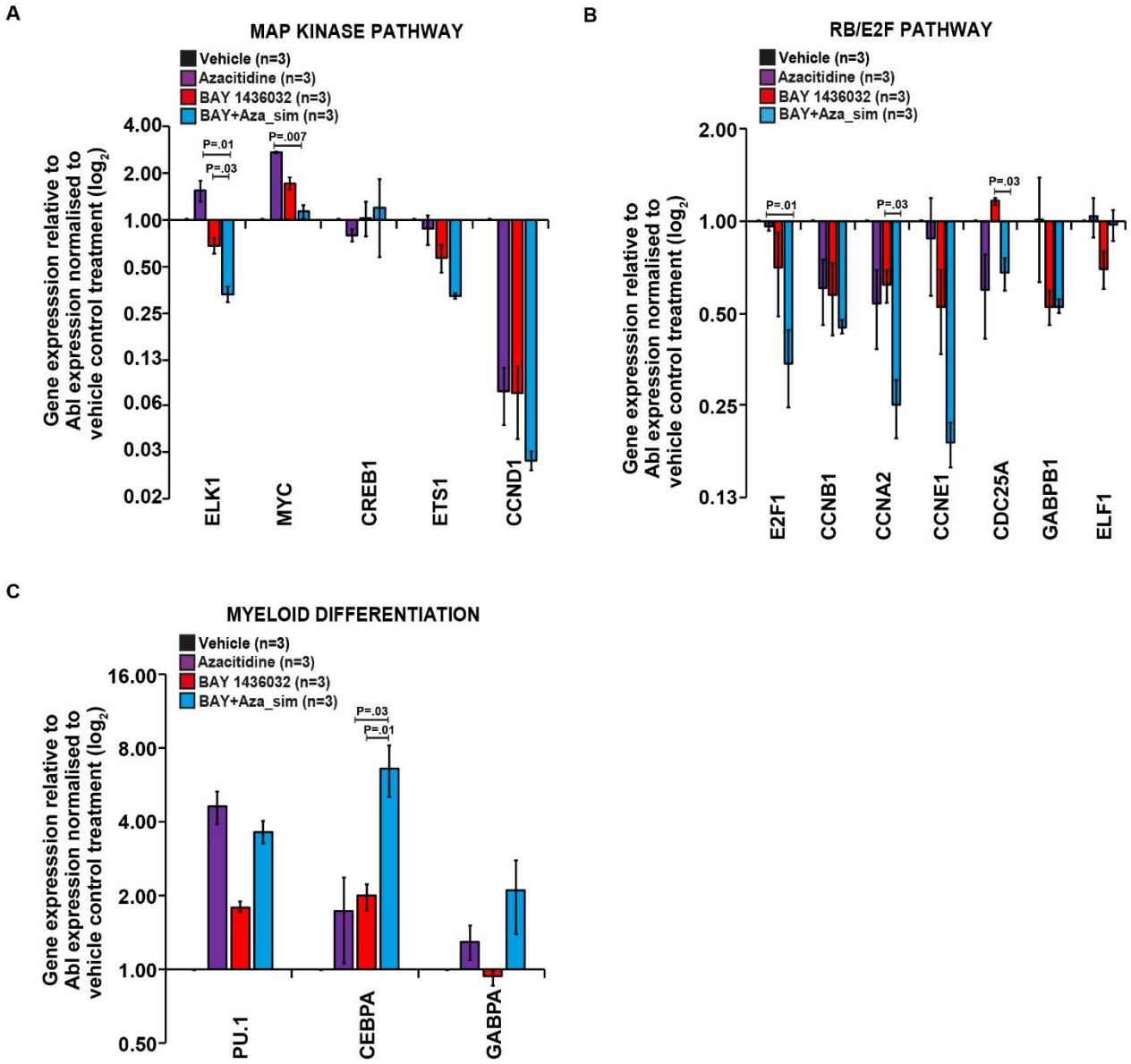
Figure S8


A
Vehicle
BAY+Aza_sim
Azacitidine
BAY1436032
BAY+Aza_seq
PC2
PC3
B
BAY+Aza_sim
BAY+Aza_seq
Vehicle
BAY1436032
Azacitidine
PC4
PC3
C
BAY+Aza_seq
BAY+Aza_sim
Frequency (genes in bins)
S-value (bins)
D
BAY+Aza_seq
BAY+Aza_sim
Frequency (probes in bins)
M-value (bins)
E
Gene expression
Depleted in simultaneous treatment
Enriched in single treatments
RB_P107
E2F1
E2F3
AKT
RB_P130
RAF
KRAS_600
KRAS_600_LUNG BREAST
KRAS_300_LUNG
KRAS_300_LUNG BREAST
KRAS_300
KRAS_50
NES
F
DNA methylation
Decreased methylation in single treatment
Increased methylation in simultaneous treatment
RB_P107
E2F1
E2F3
MEK
KRAS_LUNG
KRAS DF
KRAS BREAST
CYCLIN D1
NES

Supplementary Figure S8. Comparison of gene expression between sequential and simultaneous treatment groups and correlation of gene expression changes with changes in DNA methylation.

(A) Results of principal component analysis of all treatment groups using principal components 2 and 3. Read counts from RNASeq analysis were converted to Counts Per Million mapped reads. (B) Results of principal component analysis of all treatment groups using principal components 3 and 4 as above. (C) Distribution of gene expression synergy S-values, S=AB-A-B, where AB, A, and B are expression changes under combined (sequential or simultaneous, AB) or separate treatments [azacitidine (A) or BAY1436032 (B)]. (D) Distribution of methylation levels of Illumina MethylationEPIC probes in sequential (top) and simultaneous (bottom) treatment groups. Average_beta (B) values were converted to M-values as M=log2(B/(1-B). The top 50.000 most differentially methylated probes were selected, and normalized M-values were used to prepare the histogram. (E) Gene set enrichment analysis (MSigDB version 6.0) showing the most enriched transcription factor target gene sets from RNASeq analyses, and (F) gene set enrichment analysis (MSigDB version 6.0) showing the most enriched transcription factor target gene sets from DNA methylation analysis in primary human IDH1 mutant AML cells that were isolated from the PDX1 mouse. Simultaneous treatment refers to the simultaneous treatment of the cells with BAY1436032 and azacitidine in vitro. Single treatment refers to the treatment of the cells with either BAY1436032 alone or azacitidine alone in vitro. The two single treated groups were combined to one group and compared to the simultaneously treated group.

Figure S9



Supplementary Figure S9. Validation of gene expression changes in BAY1436032 and azacitidine treated cells by qRT-PCR. Gene expression levels of MAP kinase signaling genes (A), RB/E2F signaling genes (B) and myeloid differentiation genes (C) from the bone marrow of IDH1 mutant PDX1 mice treated with vehicle, azacitidine (1 mg/kg, s.c., days 1 to 5), BAY1436032 (150 mg/kg, p.o., q.d., 4 weeks) or simultaneous combination of BAY1436032 and azacitidine. Gene expression was determined by quantitative RT-PCR relative to the housekeeping gene ABL and was normalized to gene expression in vehicle-treated mice/cells (mean ± SEM, n= three independent experiments).

Supplementary References

1. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods. 2009;347(1-2):70-78.
2. de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. Bioinformatics. 2004;20(9):1453-1454.
3. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics. 2004;20(3):307-315.
4. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43(7):e47.
5. Ng SW, Mitchell A, Kennedy JA, et al. A 17-gene stemness score for rapid determination of risk in acute leukaemia. Nature. 2016;540(7633):433-437.
6. Thol F, Gabdoulline R, Liebich A, et al. Measurable residual disease monitoring by NGS before allogeneic hematopoietic cell transplantation in AML. Blood. 2018;132(16):1703-1713.
7. Harrow J, Frankish A, Gonzalez JM, et al. GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res. 2012;22(9):1760-1774.
8. Iyer MK, Niknafs YS, Malik R, et al. The landscape of long noncoding RNAs in the human transcriptome. Nat Genet. 2015;47(3):199-208.
9. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21.
10. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-359.
11. Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc. 2012;7(3):562-578.
12. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31(2):166-169.
13. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.

Supplementary Tables

Supplementary Table S1: Top 50 gene sets from transcription factor collection depleted in azacitidine treated cells compared to vehicle.

Transcription factors depleted in azacitidine treated cells compared to vehicle			
Rank	**NAME**	**NES**	**P**
1	HMX1_01	-1.5813	0.0000
2	AHR_Q5	-1.4931	0.0000
3	ZIC3_01	-1.4407	0.0000
4	SREBP1_Q6	-1.4345	0.0000
5	CREB_02	-1.4230	0.0000
6	HEN1_01	-1.3367	0.0000
7	SMAD_Q6	-1.3367	0.0000
8	NFMUE1_Q6	-1.3331	0.0000
9	HEN1_02	-1.3288	0.0000
10	MAZR_01	-1.3269	0.0000
11	SREBP1_02	-1.3043	0.1026
12	MYB_Q3	-1.2937	0.0000
13	CP2_02	-1.2859	0.0000
14	E2F_Q4_01	-1.2723	0.0808
15	AREB6_03	-1.2616	0.0000
16	VDR_Q3	-1.2542	0.0000
17	AP1FJ_Q2	-1.2534	0.0000
18	FAC1_01	-1.2531	0.0000
19	E2F1_Q6_01	-1.2501	0.0000
20	MZF1_02	-1.2459	0.0000
21	CREBP1_Q2	-1.2423	0.1882
22	SPZ1_01	-1.2332	0.0000
23	ZF5_B	-1.2319	0.0889
24	PAX4_01	-1.2288	0.0832
25	PAX4_03	-1.2256	0.0000
26	ETS_Q4	-1.2241	0.1149
27	SP1_Q6	-1.2239	0.1075
28	SP3_Q3	-1.2215	0.0000
29	STAT3_01	-1.2197	0.2122
30	E2F1_Q3_01	-1.2182	0.0848
31	PPAR_DR1_Q2	-1.2177	0.0000
32	EGR_Q6	-1.2170	0.2024
33	CEBPB_01	-1.2151	0.0000
34	STAT5A_02	-1.2123	0.0000
35	AP2REP_01	-1.2072	0.0873
36	CEBPDELTA_Q6	-1.2066	0.0000

37	CREB_Q2_01	-1.2037	0.0000
38	E2F4DP1_01	-1.2033	0.0000
39	CREB_Q4	-1.2030	0.0851
40	LFA1_Q6	-1.2020	0.0000
41	ETS1_B	-1.1995	0.0000
42	ATF4_Q2	-1.1978	0.0861
43	DR1_Q3	-1.1927	0.0000
44	CMYB_01	-1.1894	0.0000
45	TEL2_Q6	-1.1855	0.1191
46	PXR_Q2	-1.1853	0.0000
47	PPARA_02	-1.1818	0.1071
48	ZID_01	-1.1754	0.0000
49	AREB6_01	-1.1732	0.1394
50	MYB_Q5_01	-1.1728	0.2162

Supplementary Table S2: Top 50 gene sets from transcription factor collection depleted in BAY1436032 compared to vehicle.

Transcription factors depleted in BAY1436032 treated cells compared to vehicle			
Rank	**NAME**	**NES**	**P**
1	E2F1_Q6	-1.5809	0.0000
2	E2F_Q4	-1.5759	0.0000
3	E2F4DP1_01	-1.5494	0.0000
4	E2F1DP2_01	-1.5459	0.0000
5	E2F1DP1RB_01	-1.5154	0.0000
6	PPARG_01	-1.5117	0.0000
7	MAX_01	-1.4863	0.0000
8	MYCMAX_03	-1.4522	0.0000
9	E2F_03	-1.4455	0.0000
10	MYCMAX_B	-1.4322	0.0000
11	E2F_Q3	-1.4300	0.0000
12	NFMUE1_Q6	-1.3755	0.0000
13	USF_01	-1.3676	0.0000
14	PAX6_01	-1.3667	0.0000
15	USF_Q6	-1.3628	0.0000
16	NFY_01	-1.3468	0.0000
17	STAT1_02	-1.3412	0.0857
18	ARNT_01	-1.3389	0.0000
19	CETS1P54_01	-1.3317	0.0854
20	YY1_02	-1.3283	0.0854
21	SP1_Q6	-1.3232	0.0000
22	IRF2_01	-1.3050	0.0000
23	NRF1_Q6	-1.3045	0.0868
24	WHN_B	-1.2910	0.0000
25	NMYC_01	-1.2893	0.0000
26	HIF1_Q3	-1.2879	0.0000
27	EGR3_01	-1.2838	0.0000
28	ARNT_02	-1.2836	0.1184
29	AHR_Q5	-1.2484	0.1146
30	USF_C	-1.2003	0.1594
31	HIF1_Q5	-1.1786	0.0768
32	USF_Q6_01	-1.1696	0.2045
33	SP1_Q4_01	-1.1695	0.2004
34	CREB_Q3	-1.1639	0.1620
35	TAXCREB_01	-1.1571	0.3051
36	TEL2_Q6	-1.1558	0.2560
37	ELK1_01	-1.1525	0.1839
38	STAT3_01	-1.1369	0.2182

39	USF_02	-1.1311	> 0.0001
40	NFY_Q6	-1.1218	0.1730
41	USF2_Q6	-1.1198	0.2825
42	MYCMAX_02	-1.1053	0.2008
43	RFX1_01	-1.0893	0.2900
44	NFY_Q6_01	-1.0864	0.2769
45	NFY_C	-1.0842	0.3122
46	EGR1_01	-1.0768	0.3152
47	CREB_01	-1.0744	0.1585
48	CMYB_01	-1.0723	0.2872
49	CREB_02	-1.0673	0.2544
50	ALPHACP1_01	-1.0518	0.2004

Supplementary Table S3: Top 50 gene sets from transcription factor collection depleted in BAY1436032+azacitidine treated cells in combination compared to single agent treated cells with BAY1436032 or azacitidine or vehicle

Transcription factor collection depleted in BAY1436032+azacitidine treated cells in combination compared to single agent treated cells with BAY1436032 or azacitidine or vehicle			
Rank	**NAME**	**NES**	**P**
1	TEL2_Q6	-1.4806	0.0000
2	NRF2_01	-1.4528	0.0000
3	STAT1_02	-1.4504	0.0230
4	CETS1P54_01	-1.4393	0.0000
5	USF_C	-1.4233	0.0124
6	GABP_B	-1.4224	0.0132
7	ELK1_02	-1.4009	0.0485
8	ELK1_01	-1.4002	0.0000
9	NRF1_Q6	-1.3888	0.0106
10	XBP1_01	-1.3734	0.0000
11	ETS_Q4	-1.3423	0.0000
12	PEA3_Q6	-1.2693	0.0955
13	AR_02	-1.2662	0.1125
14	USF_Q6	-1.2348	0.0751
15	ARNT_02	-1.2179	0.1245
16	TAXCREB_01	-1.1946	0.2124
17	NFMUE1_Q6	-1.1944	0.2978
18	YY1_Q6	-1.1883	0.2018
19	USF2_Q6	-1.1865	0.1486
20	HTF_01	-1.1838	0.2541
21	USF_02	-1.1203	0.2243
22	NFY_Q6_01	-1.1196	0.2353
23	E2F1_Q6	-1.1087	0.3866
24	STAT1_03	-1.0921	0.3358
25	AHR_01	-1.0917	0.2455
26	E2F4DP1_01	-1.0893	0.4101
27	PAX2_01	-1.0801	0.2749
28	E2F1_Q6_01	-1.0727	0.4387
29	E4F1_Q6	-1.0690	0.3424
30	E2F1_Q4	-1.0657	0.4000
31	E2F1DP1RB_01	-1.0640	0.4543
32	NFY_C	-1.0613	0.3341
33	USF_01	-1.0589	0.3564
34	MYC_Q2	-1.0556	0.3535
35	NMYC_01	-1.0538	0.2346
36	PPARG_01	-1.0523	0.3776
37	E2F_02	-1.0478	0.5012

38	E2F1DP1_01	-1.0361	0.5203
39	E2F1DP2_01	-1.0361	0.5203
40	E2F4DP2_01	-1.0361	0.5203
41	E2F_Q6	-1.0358	0.4715
42	E2F_Q4	-1.0346	0.4736
43	E2F1_Q3	-1.0175	0.5201
44	E4BP4_01	-1.0135	0.4281
45	E2F_Q3	-1.0097	0.5553
46	ARNT_01	-1.0095	0.4567
47	MAX_01	-0.9983	0.3861
48	WHN_B	-0.9906	0.4354
49	ATF6_01	-0.9735	0.5466
50	MYCMAX_B	-0.9724	0.4007