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Supplementary Materials for

Targeting OXPHOS de novo purine synthesis as the nexus of *FLT3* inhibitor–mediated synergistic antileukemic actions

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Other Supplementary Material for this manuscript includes the following:

Data files S1 to S6

Supplementary Text

Supplemental Materials and Methods

Cell culture

MOLM-13 and MV4-11 human AML cell lines were purchased from DSMZ (Germany) in February 2015. Targeted sequencing of our leukemic cell lines revealed that MOLM-13 bears mutations in *NF1, FLT3* and *CBL,* while MV4-11 carries mutations in *TP53* and *FLT3*. HS5-GFP stroma cells were a gift from Dr. William Dalton (H. Lee Moffitt Cancer Center) and MOLM-13 luciferase cells from Dr. Ramiro Garzon (Ohio State University).

Drugs and nucleosides

Gilteritinib, brequinar, EPZ015666, dinaciclib, telaglenastat and midostaurin were purchased from Selleckchem or MedChemExpress. PRT808 was obtained from Prelude Therapeutics. To formulate 6 or 4 nucleoside cocktail, adenosine (A) (Thermo Fisher Scientific; AC164040050), uridine (U) (Sigma-Aldrich; U3003), inosine (I) (Sigma-Aldrich; I4125), cytosine (C) (Sigma-Aldrich;C4654), guanosine (G) (Sigma-Aldrich, G6264) and/or thymidine (T) (Sigma-Aldrich, T1895) were diluted in water and mixed to a final concentration of 50 mM each.

Genome-wide loss-of-function screening

Brunello library plasmids were amplified in NEB 5-alpha electrocompetent cells and plated on LB agar/ampicillin bioassay plates. Plasmids were prepared with endotoxin-free MaxiPrep (Qiagen) following the manufacturer's protocol. Plasmid DNA was packaged into viral particles in HEK293FT cells. Plasmid DNA was PCR-amplified, sequenced on an Illumina MiSeq, and analyzed with MAGeCK in pre-screen experiments. Utilizing a small-scale spin infection and selection with puromycin, optimal multiplicity of infection (MOI) was determined. Puromycin (2 μg/mL) and polybrene (10 μg/mL) were utilized to select for virally transduced and to augment transduction efficiency respectively. To perform the screen, $2.5x10^6$ *FLT3*-ITD AML MOLM-13 cells were plated in each well of 12-well plates (total cell number equals to 400 cells/sgRNA x 76,441 sgRNAs x 100/infection efficiency (%)). Thereafter, cells were incubated with media containing lentiviral particles obtained from HEK293FT cells at the aforementioned ratio with

polybrene and spinoculated at 450xg for 90 minutes. After six hours, cells were centrifuged at 300xg for 10 minutes and resuspended in complete media. Puromycin selection began 48 hours after transduction and continued for seven days of selection to remove essential gene-depleted and non-transduced cells. Cultures were then exposed to 2 nM gilteritinib (MedChemExpress), or 0.00012% dimethyl sulfoxide (DMSO) as a vehicle control for three to five days. Cells were harvested, DNA extracted via Qiagen Blood & Cell Culture DNA Midi Kit following the manufacturer's instructions, and the sgRNA cassette retrieved via PCR as described by the Broad Institute sequencing protocol on Addgene.

The P5 mix and P7 primers design from this protocol were used with the following New England Biolabs NEBNext Indices: 17, 24, 26, 28, 29, 31, 33, 34, 35, 36, and 42. Sample sequencing on an Illumina HiSeq4000 was performed at the Institute for Genomic Medicine at Nationwide Children's Hospital, Columbus, Ohio.

Analysis of resultant data with a Snakemake pipeline for reproducible analyses briefly consisted of FASTQ trimming and demultiplexing with skewer, and MAGeCK for count and robust rank aggregation analysis. Post-puromycin selection levels of sgRNA were compared to plasmid levels for essential gene analysis of MOLM-13 cells. For drugging studies, post-treatment levels of sgRNA were normalized to post-puromycin selection (pre-treatment) levels. Vehicle arms allowed for assessment of late essential genes and mitigated effects of the amplified regions of DNA on false positives.

Ingenuity Pathway Analysis (IPA) was used to predict cellular functions of top candidate genes ranked based on log fold change (LFC). The list of hits in negative selection cohort of CRISPR screen, containing gene symbols, were loaded into IPA analysis software (IPA, Qiagen). The "core analysis" function in the software was used to identify top enriched pathways.

Proliferation and apoptosis analysis

MTS assay was performed to determine cell proliferation under drugging conditions to determine optimal synergy ranges as previously described[11,12]. To evaluate cell apoptosis, shRNA-stable AML cell lines were plated at 5×10^5 cells/mL and were treated with vehicle control (DMSO) or

8 nM gilteritinib. Primary cells were co-cultured with HS5-GFP stromal cells at 1×10^5 cells in 96-well plates in the presence of vehicle, gilteritinib, dinaciclib or combination. Then, cells were washed and stained by Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) (BD Pharmingen). Data acquisition was conducted by using a Cytomics FC 500 Flow Cytometer (Beckman Coulter, Brea, CA), and data analysis was performed with Kaluza Analysis software (Gallios Kaluza, RRID:SCR_016700) (Beckman Coulter, Brea, CA, USA).

Animal Studies

Mice were group-housed under conditions of constant photoperiod (12-hour light/12-hour dark), temperature, and humidity with *ad libitum* access to water and irradiated standard pelleted chow. Aliquots of gelucire were stored at 4°C and warmed each day to 44°◦C in a heat block, then diluted with sterile water to a concentration of 12 mg/mL. Mice were weighed daily prior to treatment to determine appropriate dose and received a maximum volume of 200µL via oral gavage. Mice were monitored by animal technicians who were blinded to treatment groups and determined when mice met Early Removal Criteria (20% weight loss, lethargy, palor, labored breathing, hunching, poor body condition). At 10% weight loss, mice were given a two-day drug holiday.

RNA-seq and bioinformatics pipeline

Scrambled shRNA, shCDK9 or shDHODH-stable cell lines were treated with vehicle or inhibitor for 48 or 96 hours. Then, cells were lysed in Trizol and stored at -80°C. Total RNA was extracted with RNAeasy mini kit (Qiagen). For each mRNA-seq library, the fragment size was evaluated using Agilent BioAnalyzer 2100 HS DNA Kit and library concentration was quantified using Qubit DNA HS Assay Kit. This information was used for library pooling and loading Illumina Novaseq SP flow cell to generate 17-20 million paired-end 150bp clusters per samples.

Sequence results were analyzed using a custom pipeline. First, sequencing reads were quality- and adapter-trimmed using AdapterRemoval (v2.2.0). HISAT2 (v2.0.6) was then used to align trimmed reads to a combined reference of NCBI RefSeq human mitochondrial DNA, human rRNA, and PhiX bacteriophage sequences to remove unwanted reads. Next, filtered reads were aligned to the GRCh38 genome using HISAT2. Post-alignment assessment was performed using QuaCRS. Important parameters included alignment rate, duplication rate, % exonic reads, and

estimated library size. GENCODE (V25) annotated transcript counts were quantified using Subread featureCounts (v1.5.1).

Standard DESeq2 (v1.20.0) analysis was used to identify differentially expressed genes (DEG) across treatment and cell line groups. The DESeq2 function was used with default parameters to normalize expression counts and then calculate log2 fold-changes and *p*-values for each gene. To examine transcriptomic differences between groups, Principal Component Analysis (PCA) plots were generated using the log-normalized counts of the top 1000 genes with the highest variance across groups.

For Gene Set Enrichment Analysis (GSEA), DESeq2-normalized counts were used in each analysis against "Hallmark Gene Sets" and "C5 Oncology gene sets". 1000 gene-set permutations were employed to calculate weighted enrichment statistics. Only gene-sets with an FDR adjusted q-value <0.05 were selected for further analysis.

For Cytoscape Enrichment visualization, analysis was conducted according to the Enrichment Map protocol. Briefly, differentially expressed genes present in combination-treated cells (shCDK9+gilteritinib or shDHODH+gilteritinib) and not present in either single-agent treated or control condition were analyzed with GSEA C5 Oncology gene sets. GSEA files were inputted into the EnrichmentMap plugin within Cytoscape program for visualization. Default values (node cutoff FDR q-value<0.05; Jaccard Overlap Combined Index edge cutoff 0.375). Clustered pathway nodes were compared, combined into a common biological process and annotated with AutoAnnotate.

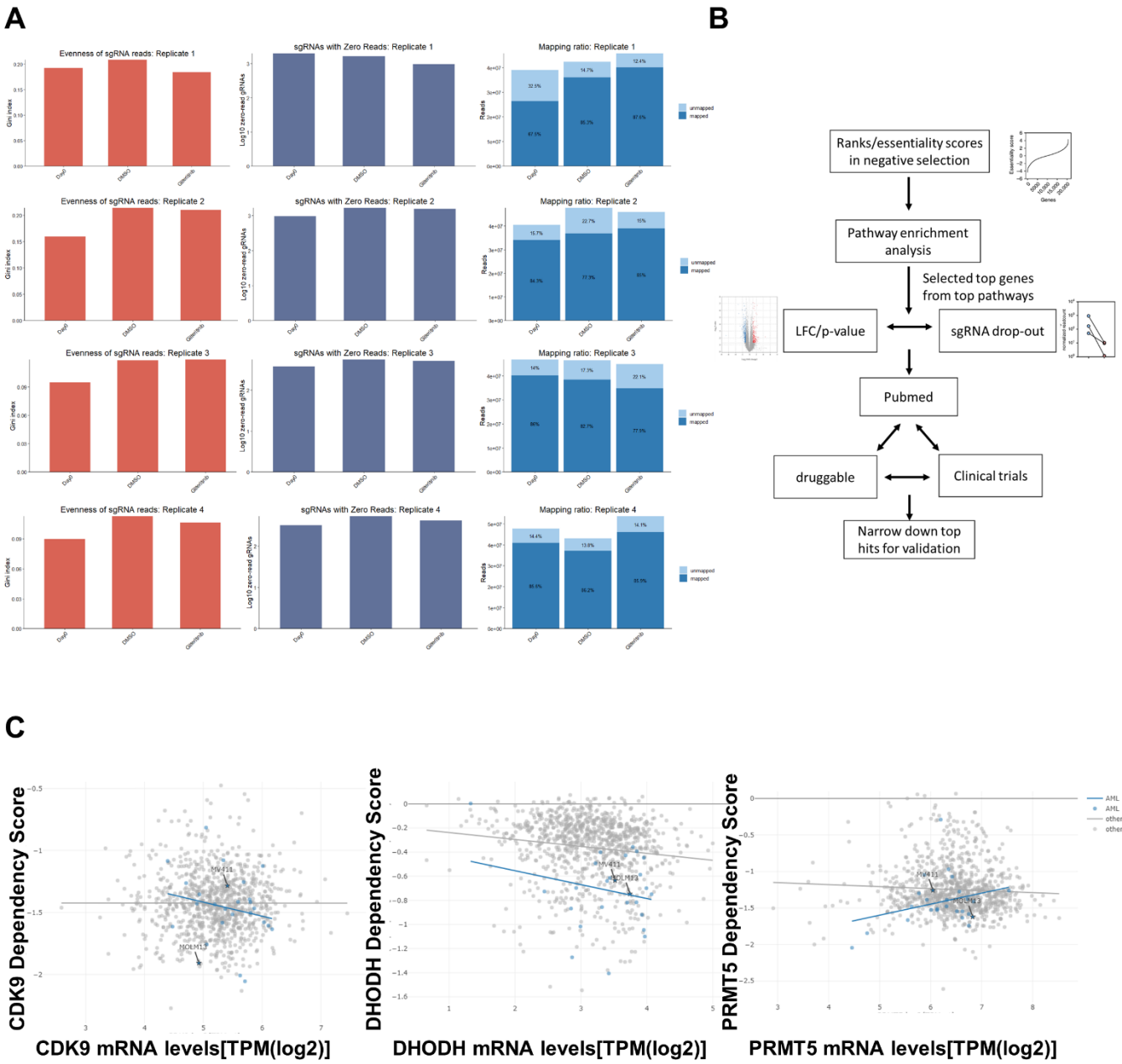
Sample Preparation and MS analysis for Metabolomics

shCDK9-, shPRMT5-, and shDHODH-stable MOLM13 cells were treated with vehicle or 8nM gilteritinib for 48 or 96 hours before metabolites were extracted with MeOH and samples were lyophilized. Lyophilized samples were reconstituted in equal portions of 150 µL of 95:5 H2O:MeOH with 0.1 % formic acid which were then sonicated and centrifuged at 20,000 G for 20 minutes. Blank solvent matrix was also processed as were equal portions of each group to form pooled samples. Following centrifugation, the supernatants were placed into sealed glass LC vials

and analyzed on an Agilent 6545 Quadrupole Time-of-flight mass spectrometer (LC-MS QTOF) in both positive ion mode and negative ion mode with a dual AJS ESI system and an Agilent 1290 Infinity LC system. For separation prior to MS analysis, 5 µL of each sample was injected onto an Agilent C-18 Poroshell column at 40 ^{0}C with Solvent A 100% H2O with 0.1% formic acid and Solvent B 100% MeOH with 0.1% formic acid. A flow rate of 200 uL/min was maintained with an initial gradient of 2% B at 0 min, up to 90% B at 15 min, 95% B at 16 min, back to 2% B at 17 min and held there until the end of the run at 32 min. The mass spectrometer was run in MS^2 scan mode, with a range of 50-1700 amu. Ion source parameters were as follows: gas temp. 250 ºC, source gas flow 8 L/min., nebulizer 25 psi, sheath gas temp. 350 ºC, sheath gas flow 10 L/min, capillary voltage 4000 V, nozzle voltage 500 V for both polarities. Features were selected using data dependent acquisition, in which the top 5 ions at a given time point were retained, used to generate a mass spectral profile, then excluded for a 30 second window.

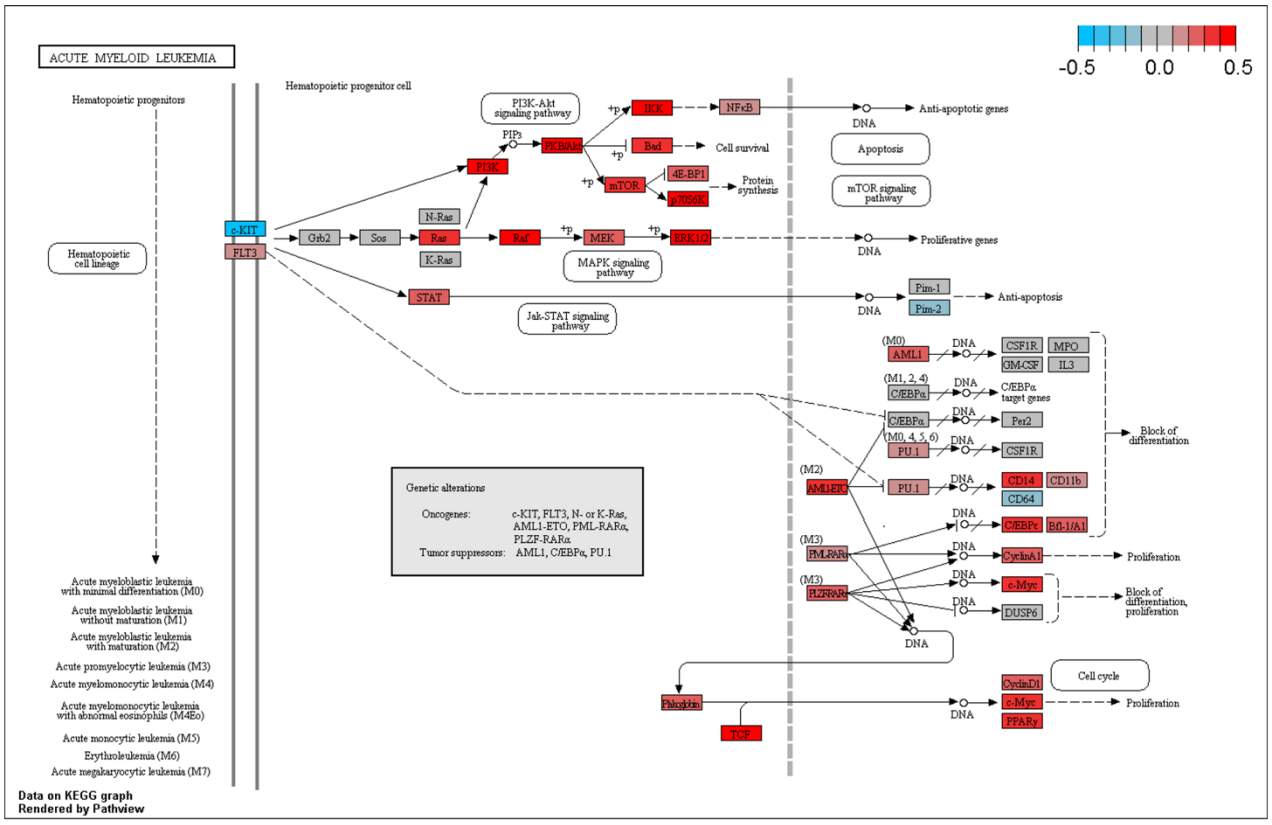
Metabolomics data analysis

Mass spectral data were converted to mzXML using MS convert and then run using Progenesis QI for run alignment using pooled samples for all normalization and peak alignment. Features with a *p*-value of more than 0.05 were removed from each group comparison and all features highest in the blank group were also removed for downstream analyses. Features were identified using Progenesis software and searching against the Human Metabolite Database (HMDB) and LipidMaps metabolite libraries. All features that were tentatively identified with a 10 ppm mas error cut-off were formatted into lists of masses for each group comparisons and run in MetaboAnalysis for feature analysis with the KEGG mouse pathway subset.



Supplementary Figure S1.

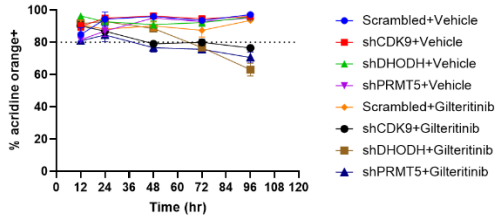
Fig. S1. Gini index for CRISPR screen and Depmap analysis of selected hits. (A) Gini index for evenness of sgRNA reads, sgRNA with zero reads and mapping ratio for Day0, DMSO and gilteritinib samples. **(B)** Workflow of hit selection. **(C)** Dependency scores of *CDK9*, *DHODH* and *PRMT5* across human cancer cell lines based on DepMap analysis are shown with *FLT3*-ITD AML cell lines being highlighted. Vertical line, gene dependency scores; horizontal line, gene TPM (transcripts per kilobase million) based on DepMap. Regression lines in AML (blue) and other cancers (gray) are shown.



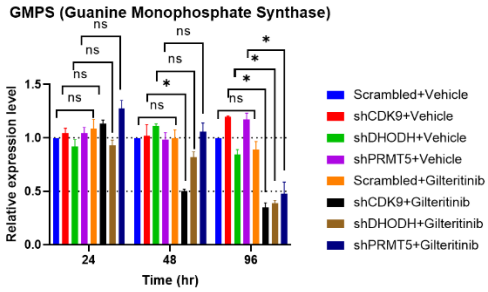
Supplementary Figure S2.

Fig. S2. Hits that are enriched in AML FLT3 signaling pathway. Visualization of positively- (red) and negatively-selected (blue) genes on Acute Myeloid Leukemia FLT3 signaling map. Strength of selection is represented by color saturation.

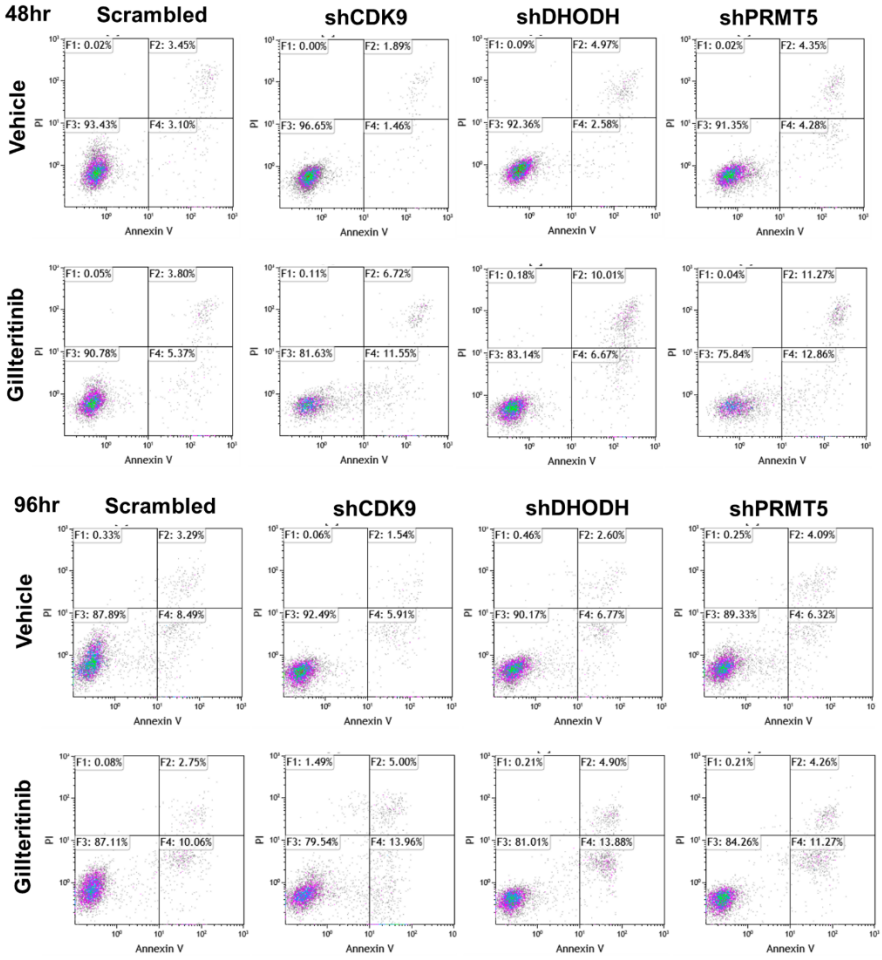
A



B

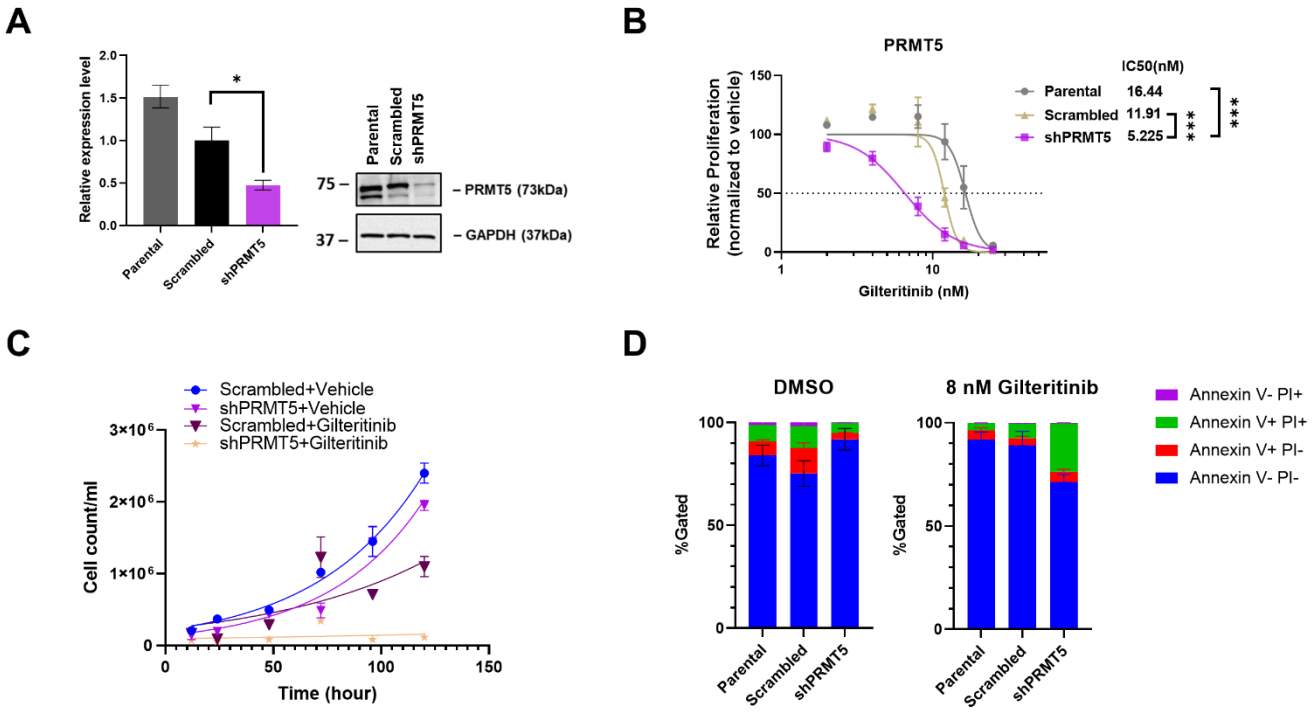


C



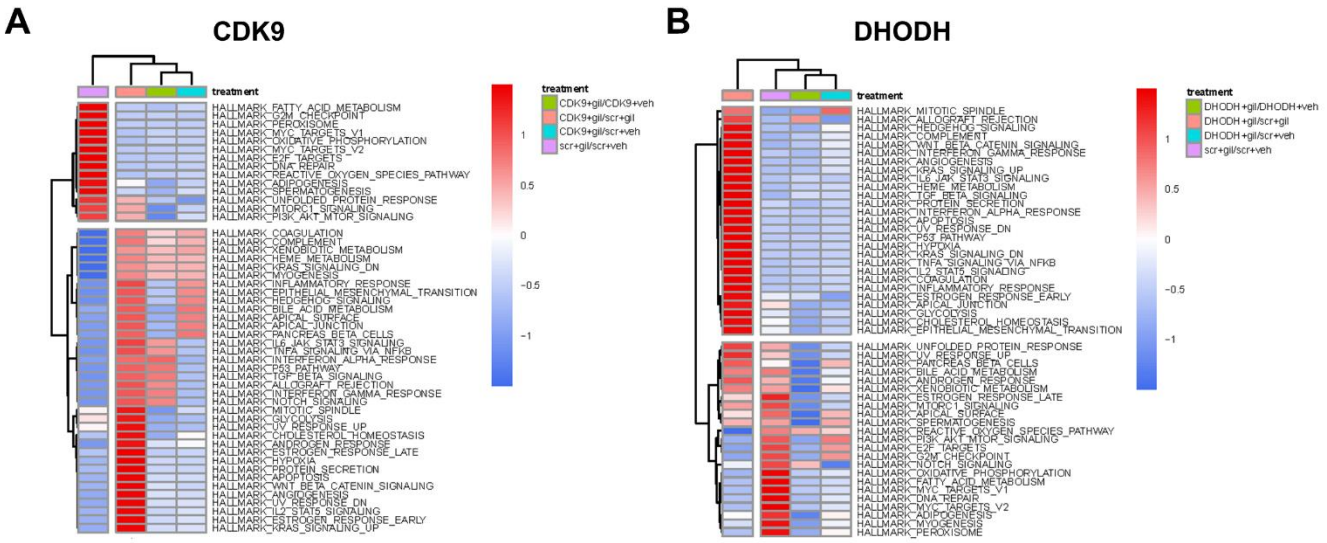
Supplementary Figure S3.

Fig. S3. Timepoint selection for mechanistic studies. (A) Time-dependent changes of the viabilities of scrambled, shCDK9, shDHODH or shPRMT5-stable MOLM-13 cells receiving vehicle control or 8 nM gilteritinib. Cell viability was measured by acridine orange *(*AO*)* and propidium iodide (PI) staining. **(B)** The relative expressions of selected genes (*GMPS*) in scrambled, shCDK9, shPRMT5 and shDHODH-stable MOLM-13 in response to vehicle or 8 nM gilteritinib treatment were measure by real-time PCR with respect to GAPDH. Values are expressed as fold changes (mean±SEM, n=3) relative to vehicle-treated scrambled cells. *$p<0.05$; ns=not significant. **(C)** Scrambled, shCDK9-, shPRMT5- or shDHODH-stable MOLM-13 cells were treated with 8 nM gilteritinib for 48 or 96 hours before being stained with Annexin V- FITC followed by flow cytometric analysis. n=2. Upon 48 and 96 hour treatments, cells did not undergo substantial death, suggesting that 48 and 96 hours are the earliest time points for mechanistic studies.



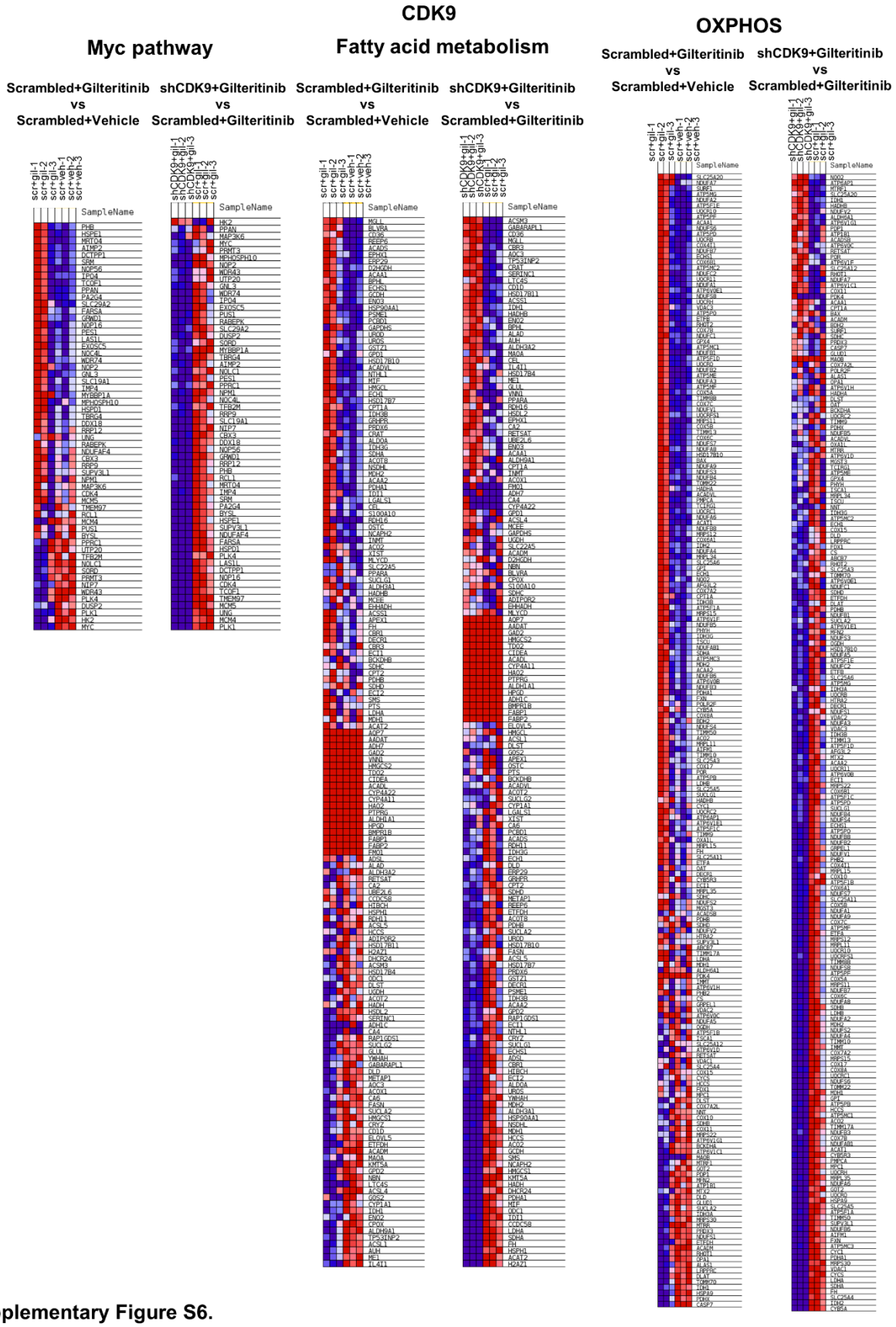
Supplementary Figure S4.

Fig. S4. Genetic depletion of PRMT5 sensitizes AML cells to gilteritinib treatment. (A) Knockdown efficiency of selected targets by shRNA as detected by qPCR and Western blotting; GAPDH serves as loading control. n=2. *p<0.05; **p<0.01. **(B)** Dose-response curves of shPRMT5 -stable MOLM-13 cells in response to 120-hour gilteritinib treatment. Cell viability was measured with MTS. Results are shown as mean±SEM of 4 technical replicates and 3-4 biological replicates. ***p-value<0.0001; ns=not significant. **(C)** Cell counts of MOLM-13 cells at different time points in different treatment group. Results are shown as mean±SEM of 3 biological replicates. **(D)** Knockdown of PRMT5 with shRNA increases the frequency of apoptotic AML cell lines with gilteritinib. Parental, scrambled or gene-targeting shRNA-stable MOLM-13 cells were treated with 8 nM gileritinib for 120 hours and stained with Annexin V/PI for flow cytometry analysis. Data are shown as mean±SEM of % population from triplicates.



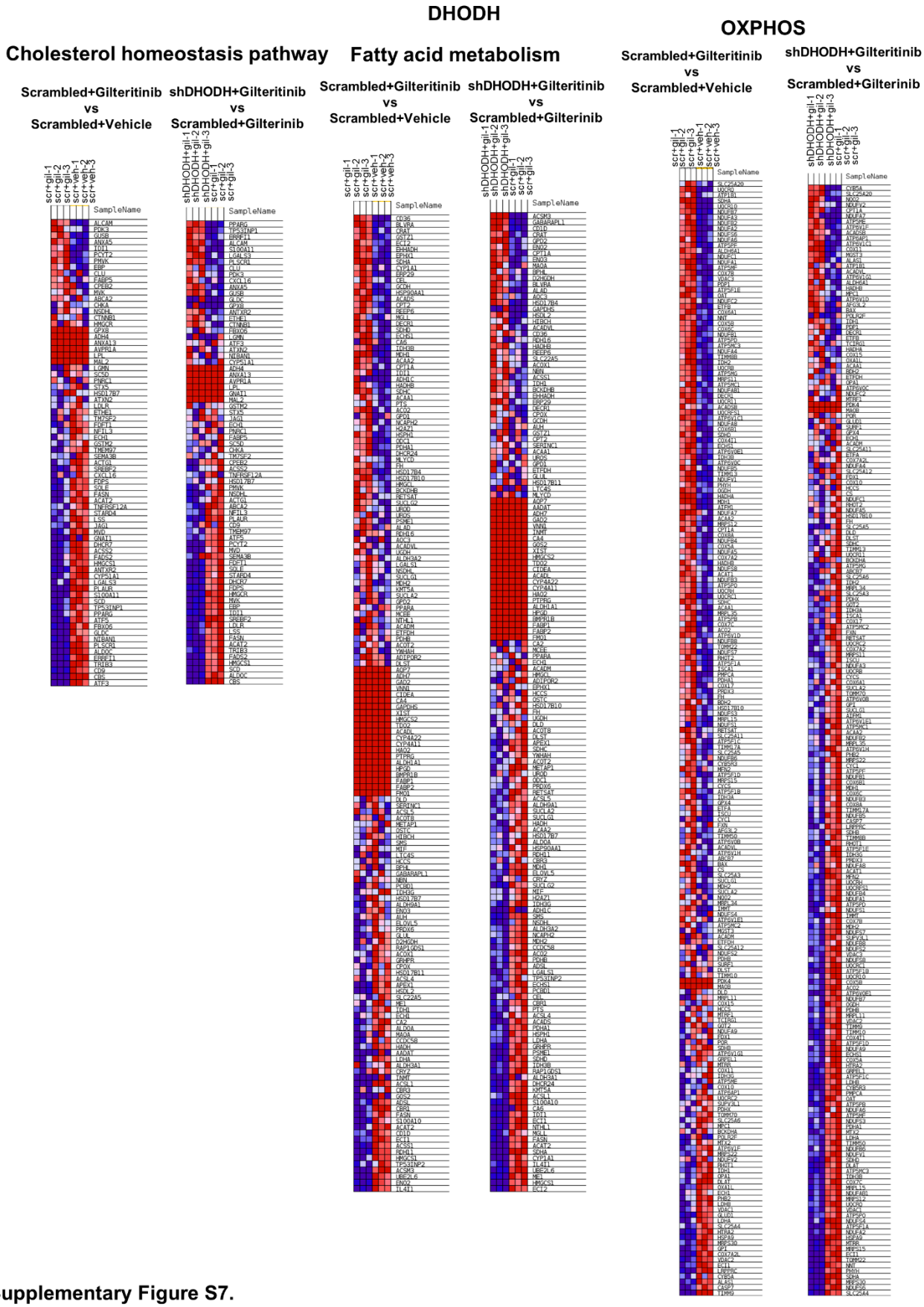
Supplementary Figure S5.

Fig. S5. Heatmap of enriched pathways in GSEA Hallmark gene sets. Log2FC preranked lists of DEGs of indicated comparisons were employed to run GSEA against the Hallmark gene sets. Unsupervised hierarchical clustering of normalized enrichment scores (NES) was used to generate a comprehensive heatmap representation of the functional transcriptional outputs of the **(A)** CDK9- and **(B)** DHODH-related treatment comparison sets.



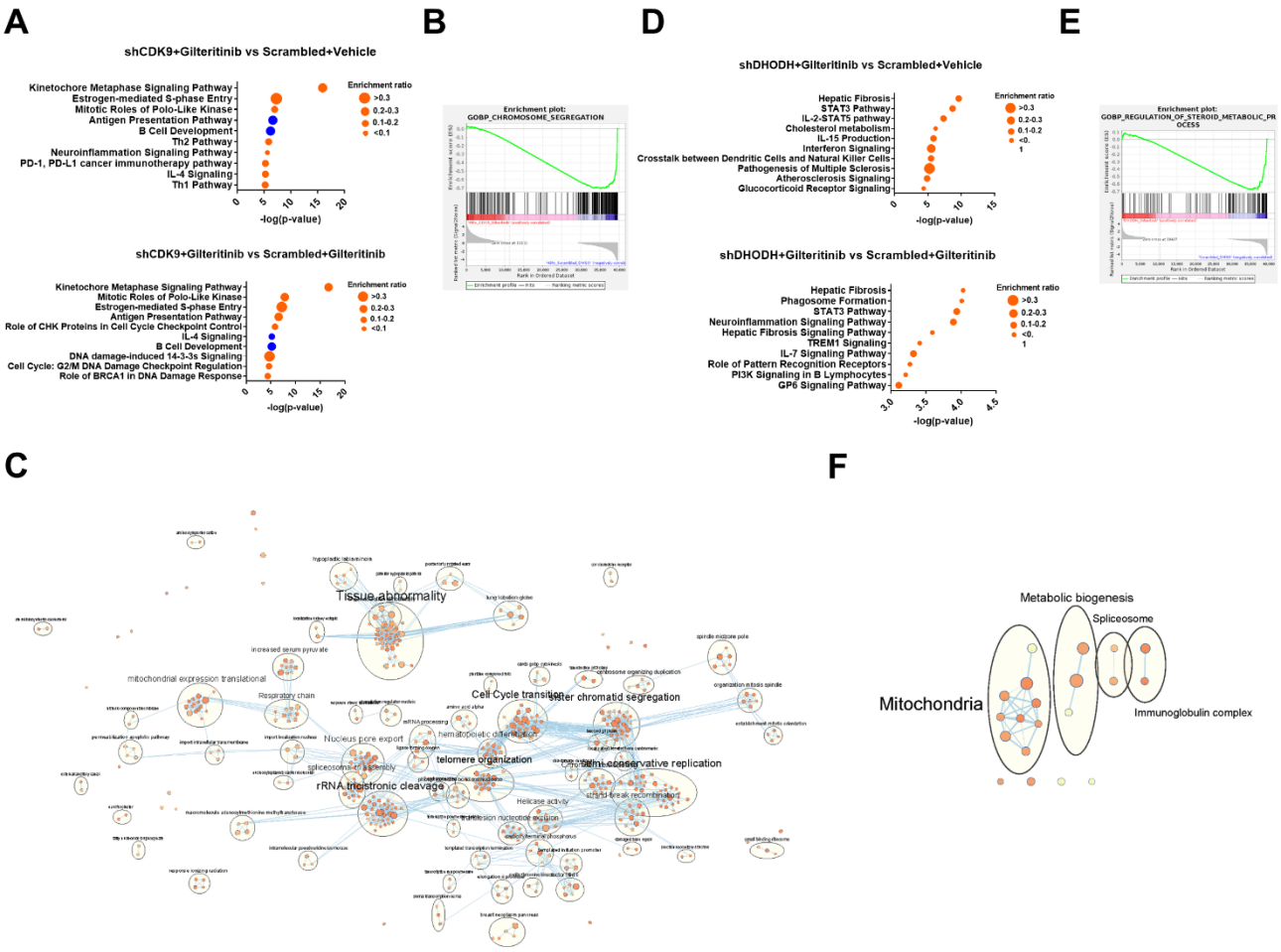
Supplementary Figure S6.

Fig. S6. The heatmaps showing the normalized read counts of gene transcripts of Myc pathway, fatty acid metabolism and OXPHOS pathway in the leading edge subsets across comparisons in shCDK9-mediated synergy.



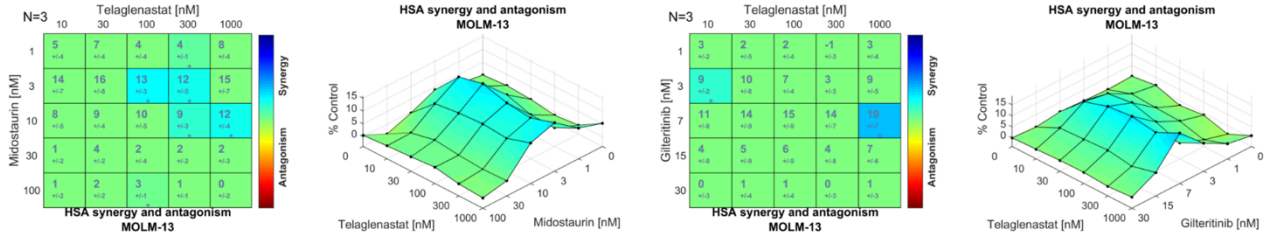
Supplementary Figure S7.

Fig. S7. The heatmaps showing the normalized read counts of gene transcripts of cholesterol homeostasis, fatty acid metabolism and OXPHOS pathways in the leading edge subsets across comparisons in shDHODH-mediated synergy.



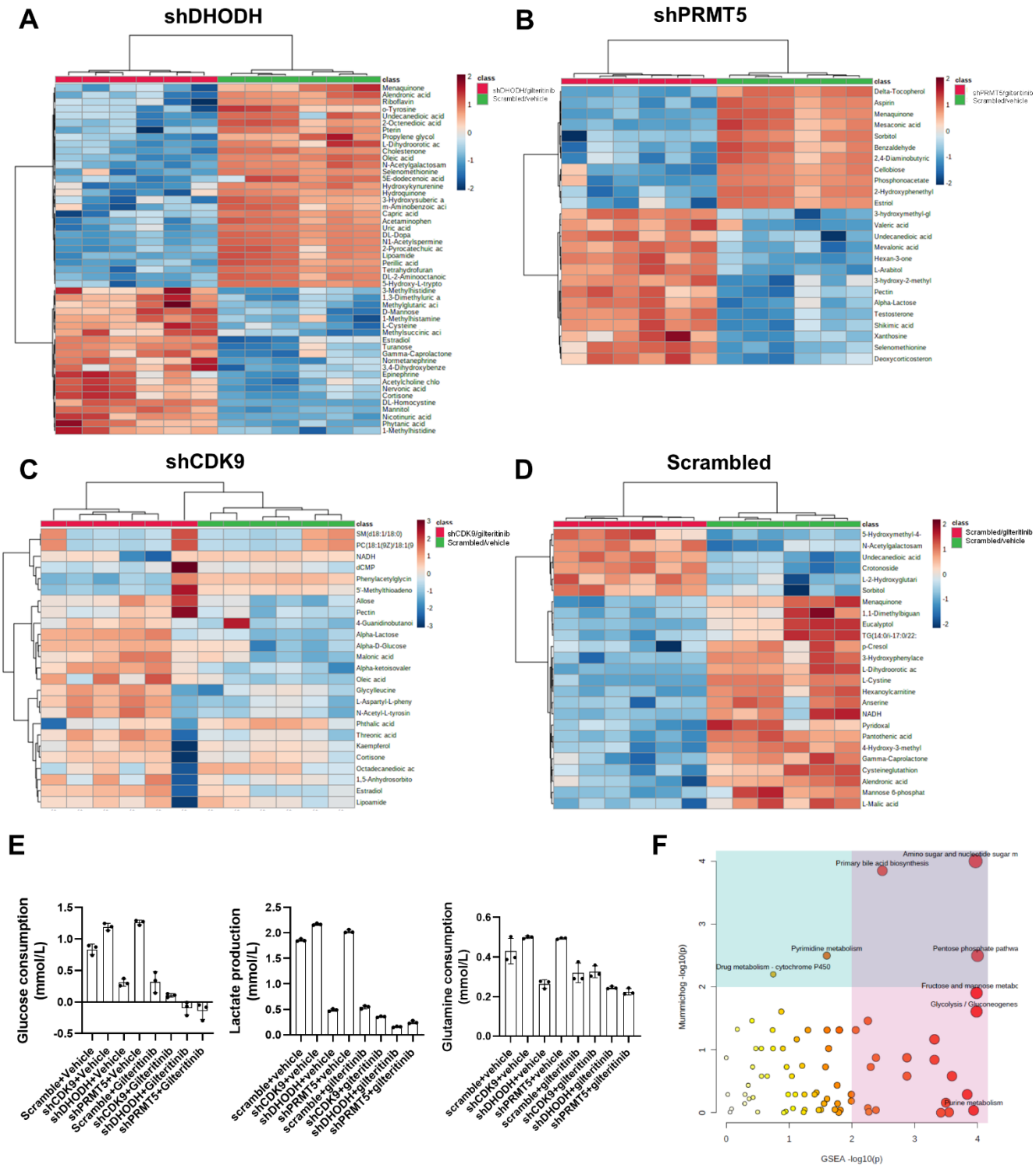
Supplementary Figure S8.

Fig. S8. Top enriched pathways by IPA, GSEA C5 gene sets and Cytoscape. (A) Top enriched pathways of DEGs (FDR<0.05 and LFC>2.0) of shCDK9+gilteritinib vs scrambled+vehicle comparison predicted by IPA. Orange: downregulated; Blue: upregulated. **(B)** GSEA plot of chromosome segregation which is one of the top significantly downregulated pathways in C5 gene sets for shCDK9+gilteritinib vs scrambled+vehicle comparison. **(C)** Cytoscape enrichment map of top gene programs in shCDK9+gilteritinib vs scrambled+gilteritinib comparison. **(D)** Top enriched pathways of DEGs (FDR<0.05 and LFC>2.0) of shDHODH+gilteritinib vs scrambled+vehicle comparison predicted by IPA. **(E)** GSEA plot of steroid metabolism which is one of the top significantly downregulated pathways in C5 gene sets for shDHODH+gilteritinib vs scrambled+vehicle comparison. **(F)** Cytoscape enrichment map of top gene programs in shDHODH+gilteritinib vs scrambled+gilteritinib comparison.



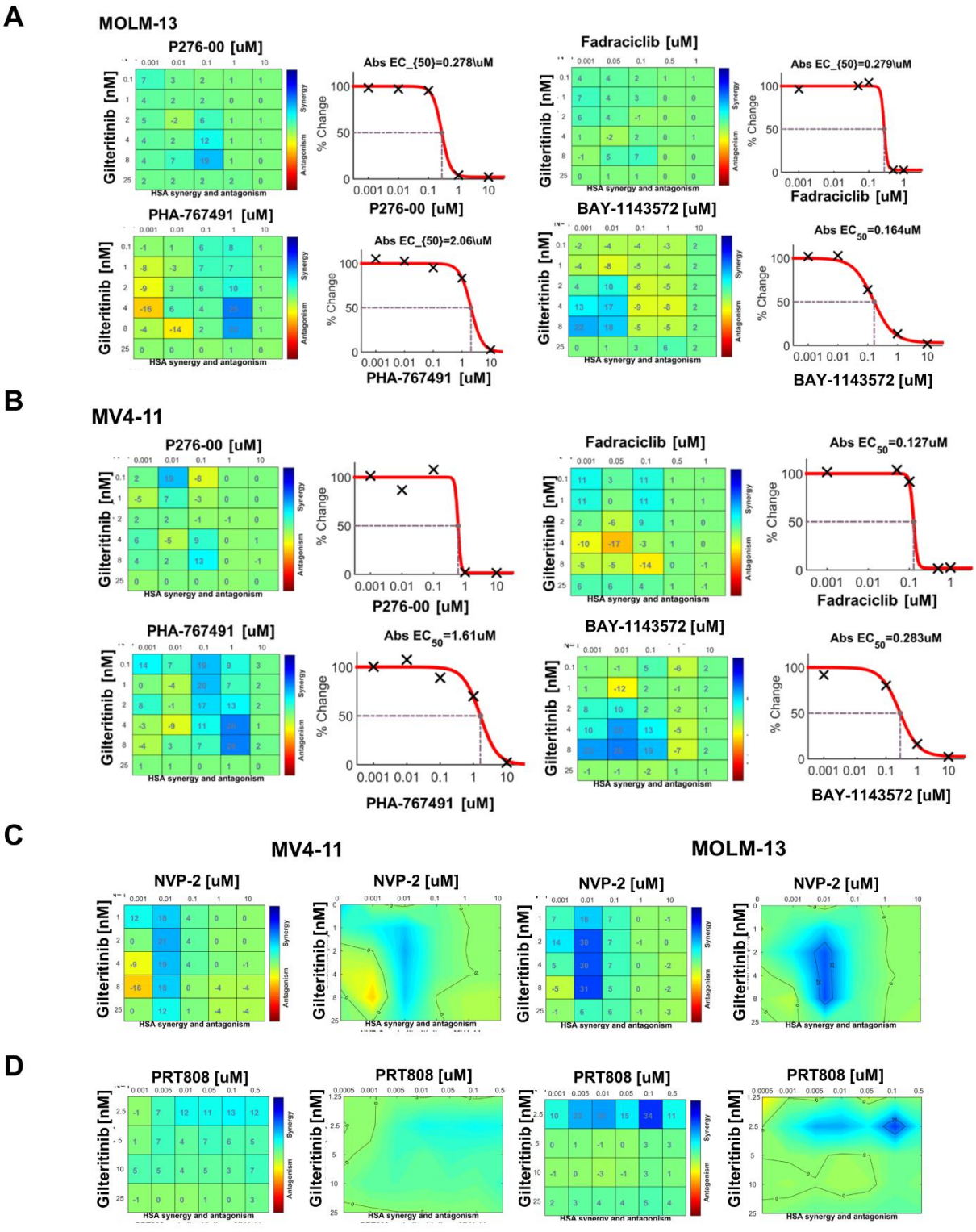
Supplementary Figure S9.

Fig. S9. Inhibition of glutaminolysis sensitizes AML cells to gilteritinib treatment. Inhibition of glutaminolysis sensitizes AML cells to gilteritinib treatment. Proliferation assay of a range of doses of midostaurin or gilteritinib with telaglenastat on MOLM-13 cells treated for 48 hours.



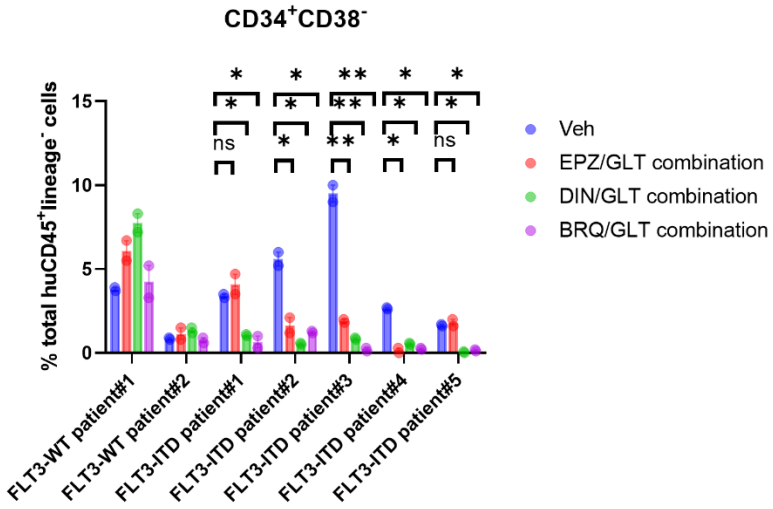
Supplementary Figure S10.

Fig. S10. Metabolic rewiring induced by combined treatments. (A-D) Heatmaps showing the relative abundances of metabolites in scrambled/gilteritinib, shCDK9/gilteritinib, shPRMT5/gilteritinib and shDHODH/gilteritinib groups with respect to scrambled/vehicle control. **(E)** Glycolysis and glutaminolysis activities of shRNA-stable MOLM-13 cells being treated with vehicle or 8nM gilteritinib for 48 hours. The levels of glucose consumption, lactate production and glutamine consumption in cell culture supernatants were measured with Bioanalyzer. **(F)** Top enriched metabolic pathways of vehicle-treated shDHODH-stable MOLM-13 cells as predicted by Mummichog analysis and GSEA analysis. The size of the circle is correlated with the amounts of metabolites being identified in the pathway.



Supplementary Figure S11.

Fig. S11. Inhibition of CDK9 or PRMT5 with selective inhibitors sensitizes AML cells to gilteritinib treatment. (A-B) Proliferation assay of a range of doses of gilteritinib with P276-00, Fadraciclib, PHA-767491 or BAY-1143572 on MV4-11 and MOLM-13 cells treated for 48 hours. Highest single agent (HSA) analysis was used to determine regions of synergy. **(C-D)** Proliferation assay of a range of doses of gilteritinib with CDK9-specific inhibitor, NVP-2, and PRMT5-specific inhibitor, PRT808, on MV4-11 and MOLM-13 cells treated for 48 hours. Highest single agent (HSA) analysis was used to determine regions of synergy.



Supplementary Figure S12.

Fig. S12. Combination treatments suppressed self-renew potentials of *FLT3*-ITD primary AML patient cells by eliminating leukemia stem cells. The percentage of $CD34^{+}CD38^{-}$ LSC-like cells after 1st CFU plating upon different combination treatments.

Table S1. Top glycolysis-related hits in positive selection of CRISPR screen upon gilteritinib treatment

Symbol	LFC	P-value	FDR
HK2	0.50927	0.20872	0.974033
HK3	0.26627	0.26627	1
ADPGK	0.22316	0.17689	0.954762
GCK	0.12237	0.53816	1
GALM	0.10276	0.019535	0.767946
FBP1	0.14261	0.30509	1
FBP2	0.04007	0.23063	1
ALDOB	0.32733	0.37422	1
PFKFB1	0.20998	0.12818	0.893543
PFKFB3	0.4452	0.011619	0.690842
PFKL	0.077715	0.12334	0.893543
PFKP	0.087083	0.27318	1
PGAM2	0.1604	0.47914	1
ENO3	0.15734	0.007891	0.650115
LDHAL6B	0.2094	0.69973	1
LDHC	0.47032	0.15974	0.954762
ADH1B	0.4235	0.11353	0.893543
ADH1C	0.44118	0.36371	1
ADH6	0.2873	0.32511	1
PANK1	0.059618	0.1818	0.954762
ALDH3A1	0.21865	0.084481	0.893543
AKR1A1	0.14113	0.69853	1
ALDH1A3	0.019469	0.28247	1
PCK1	0.19596	0.37008	1
ALDH3B2	0.039132	0.6925	1
ALDH9A1	0.28067	0.08448	0.893543
ALDH7A1	0.30553	0.15001	0.948913
ALDH3A2	0.26762	0.27531	1

Data S1. (separate file)

Data file S1. Top genes in CRISPR screen

Data file S2. Gene set enrichment of top hits in CRISPR screen

Data file S3. Venn analysis of shared genes by CRIPSR screen, shCDK9 RNA-seq and shDHODH RNA-seq

Data file S4. Gene set enrichment of shared genes among CRISPR screen and RNA-seq

Date file S5. Top DEG table for shDHODH+gilteritinib vs scrambled+vehicle-96hour

Date file S6. Top DEG table for shCDK9+gilteritinib vs scrambled+vehicle-48hour