The small GTPase ARF6 regulates sphingolipid homeostasis and supports proliferation in acute myeloid leukemia

Helong Gary Zhao,[1*] Nataly Cruz-Rodriguez,[1*] Kent C. Johnson,[2] Anthony D. Pomicter,[3] Briana Bates,[1] Benjamin Bateman,[1] Torsten Haferlach,[4] Tongjun Gu,[1] Jonathan Ahmann,[3] Dongqing Yan,[3] Greg S. Lee,[2] Weiquan Zhu,[2] Jenna Bishop,[1] Shannon J. Odelberg[3] and Michael W. Deininger[1]

[1]Versiti Blood Research Institute, Milwaukee, WI, USA; [2]Program in Molecular Medicine, School of Medicine, University of Utah, Salt Lake City, UT, USA; [3]Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA and [4]MLL Münchner Leukämielabor, Munich, Germany

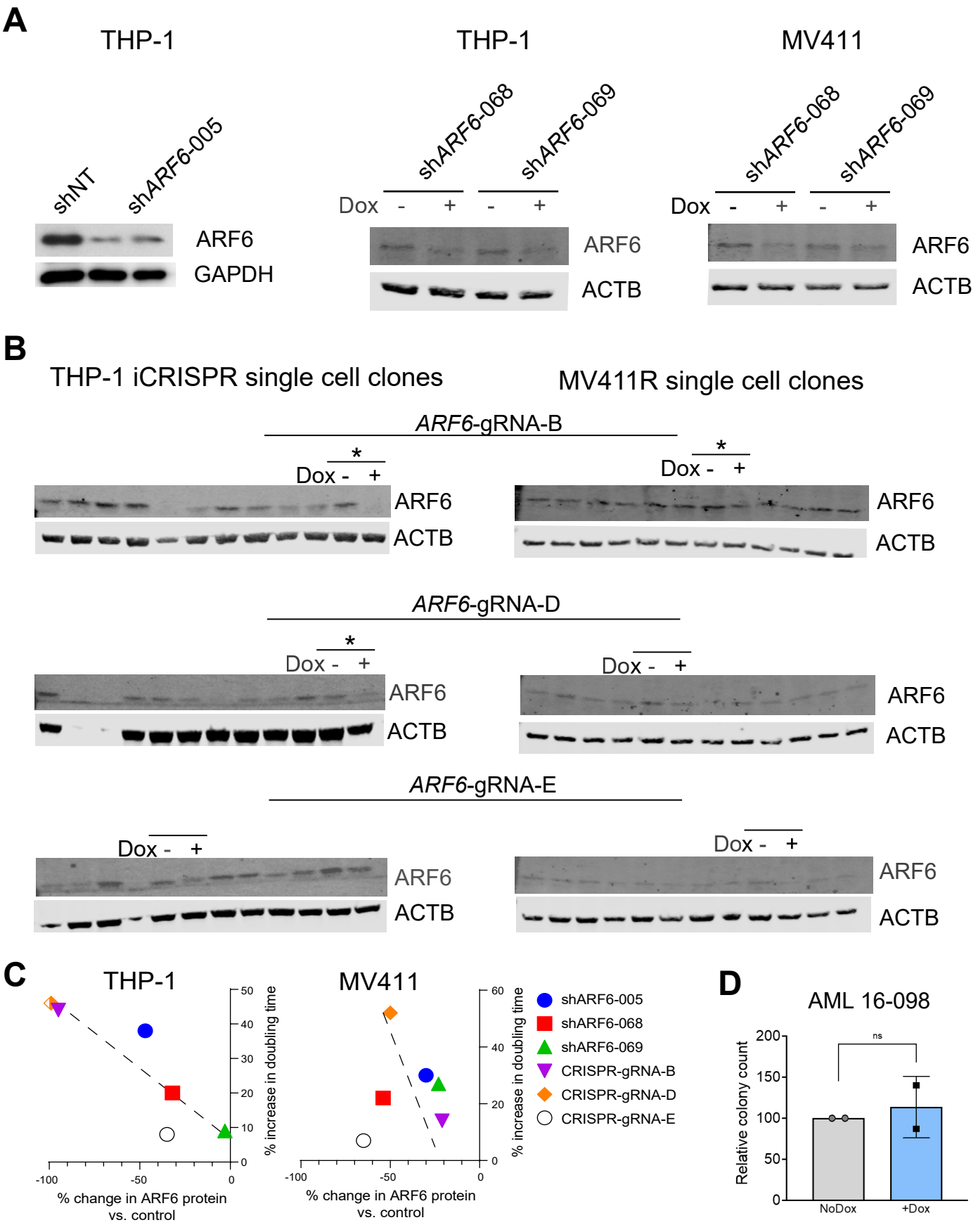
**HGZ and NC-R contributed equally as first authors.*

Correspondence: M. Deininger
mdeininger@versiti.org

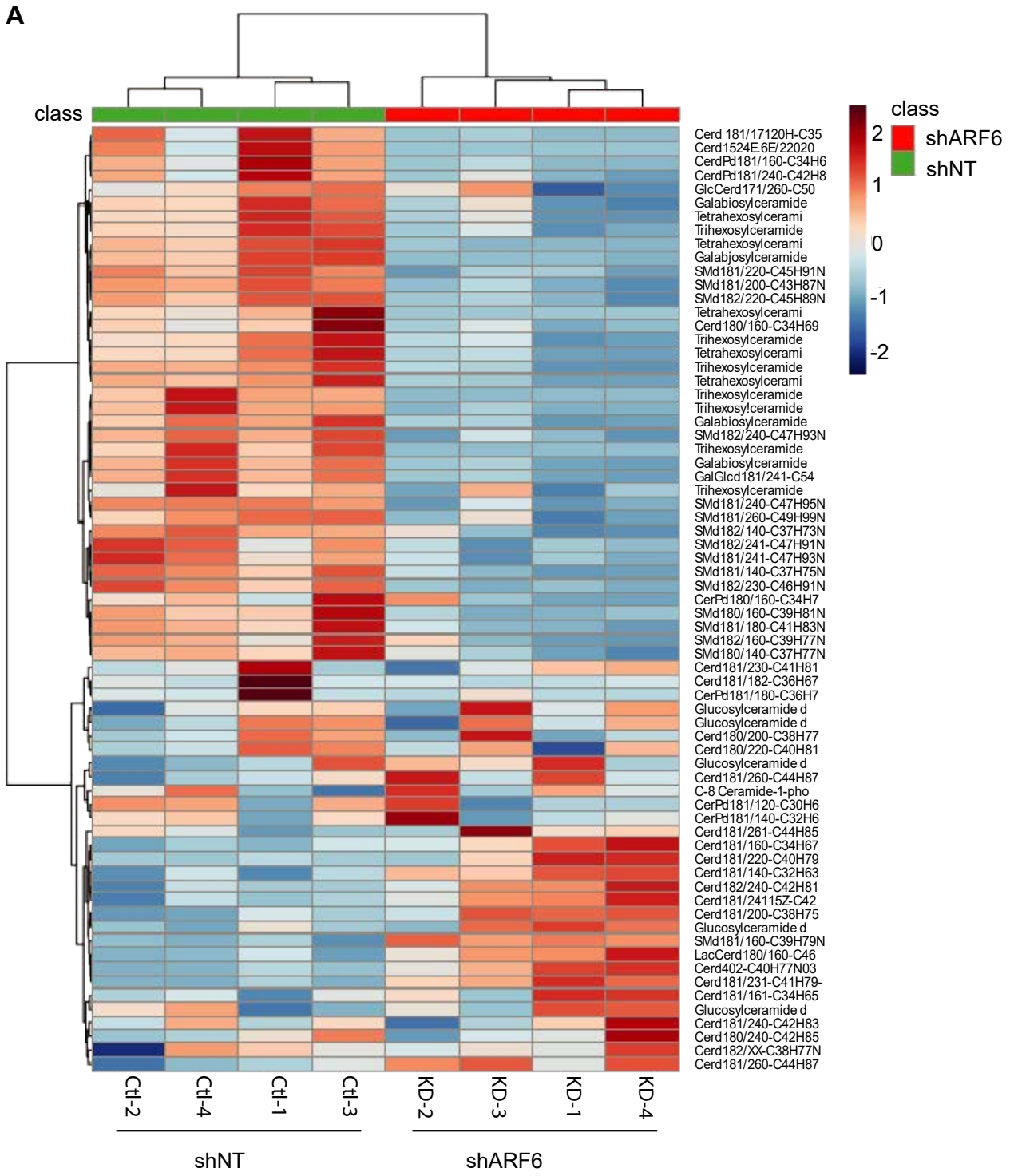
Received: July 24, 2024.
Accepted: June 19, 2025.
Early view: July 10, 2025.

https://doi.org/10.3324/haematol.2024.286228

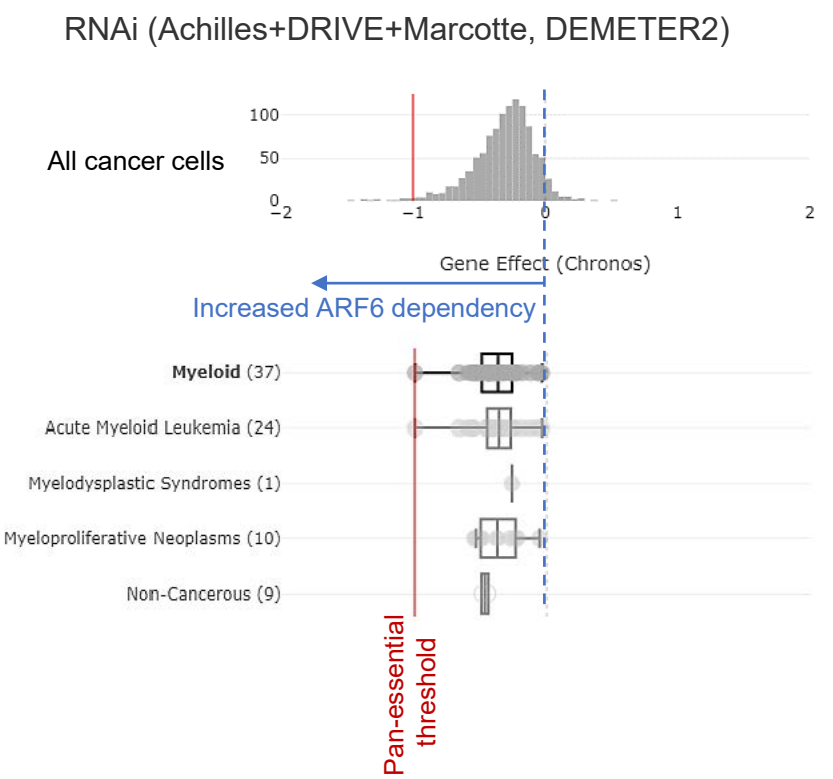
Supplementary Figure 1

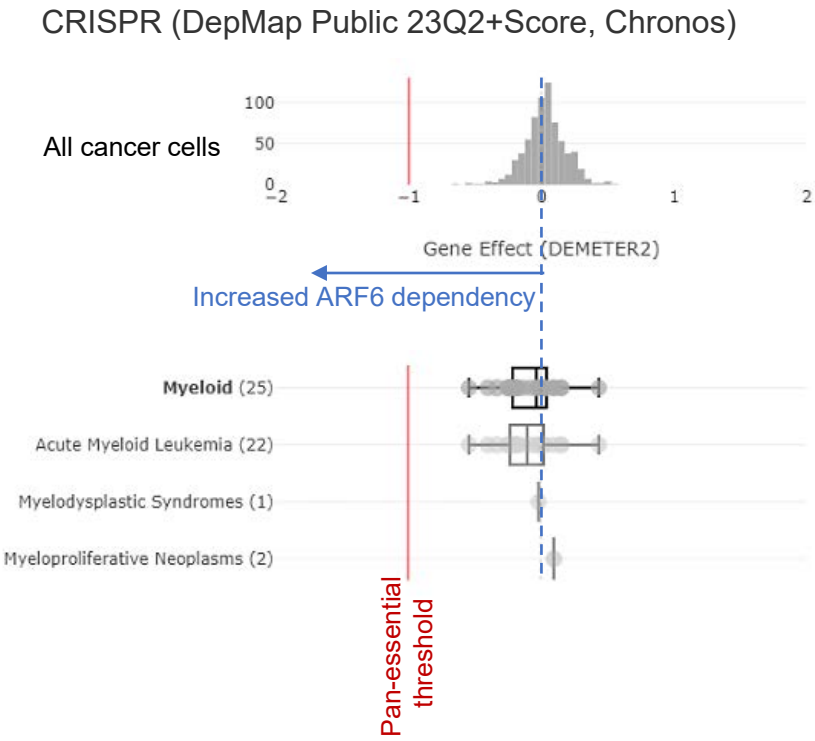


Supplementary Figure 2

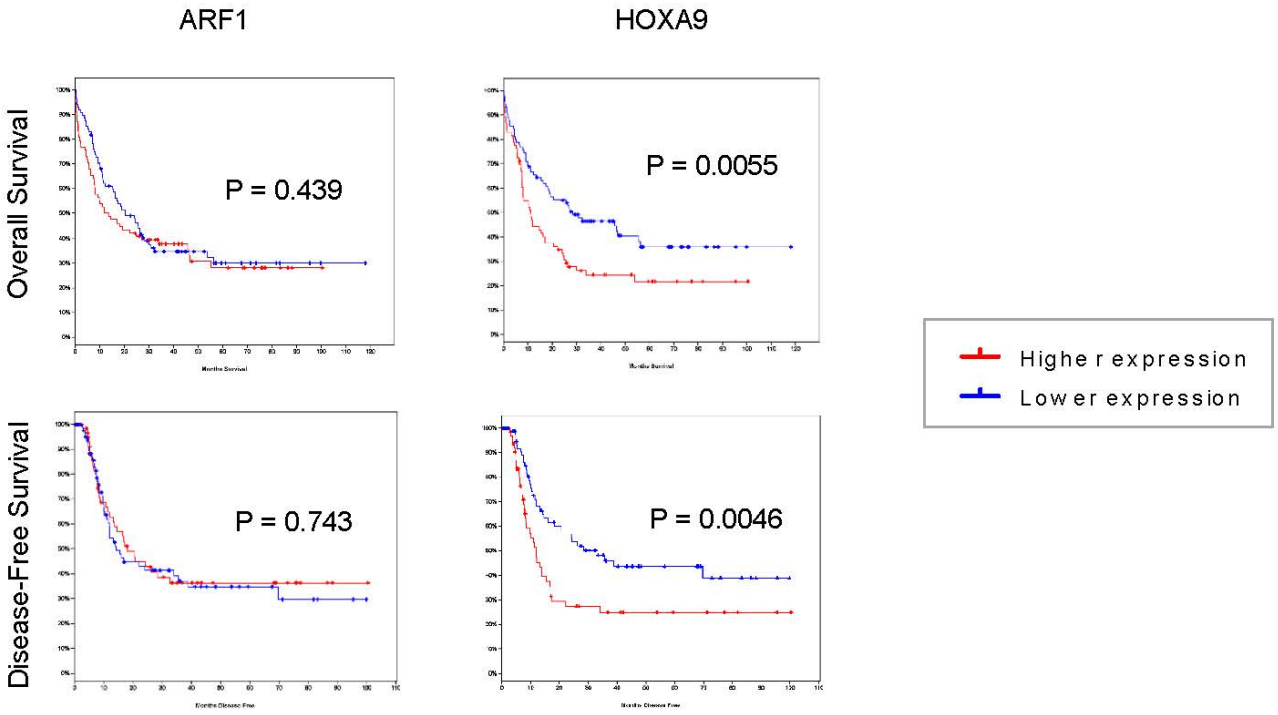


Supplementary Figure 3

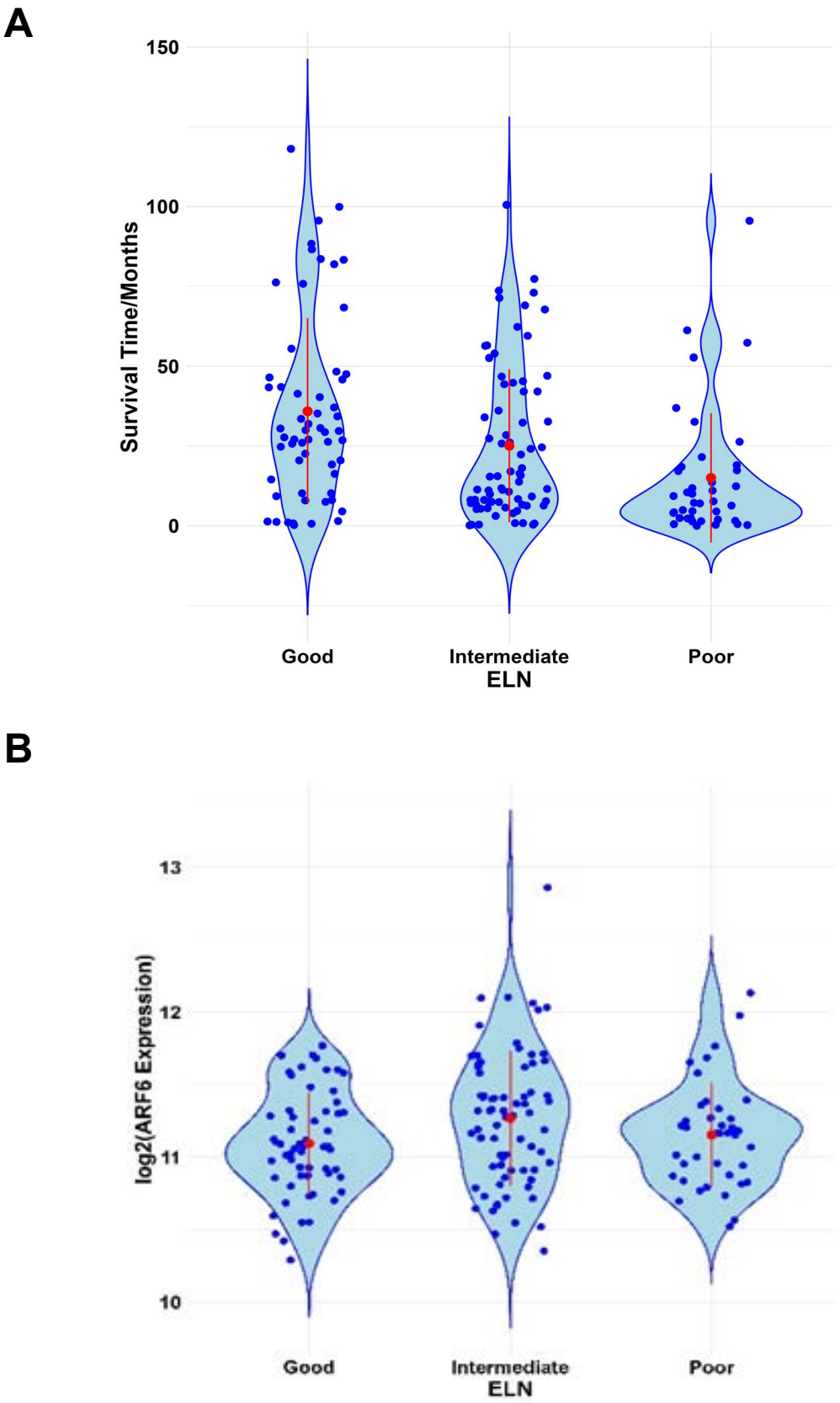




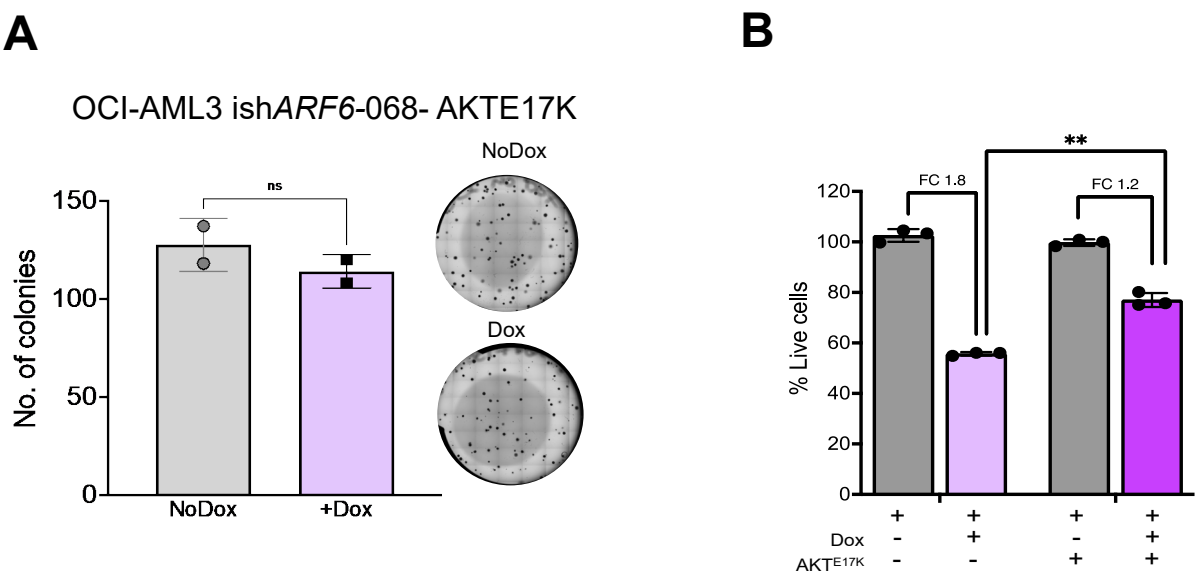
Supplementary Figure 4



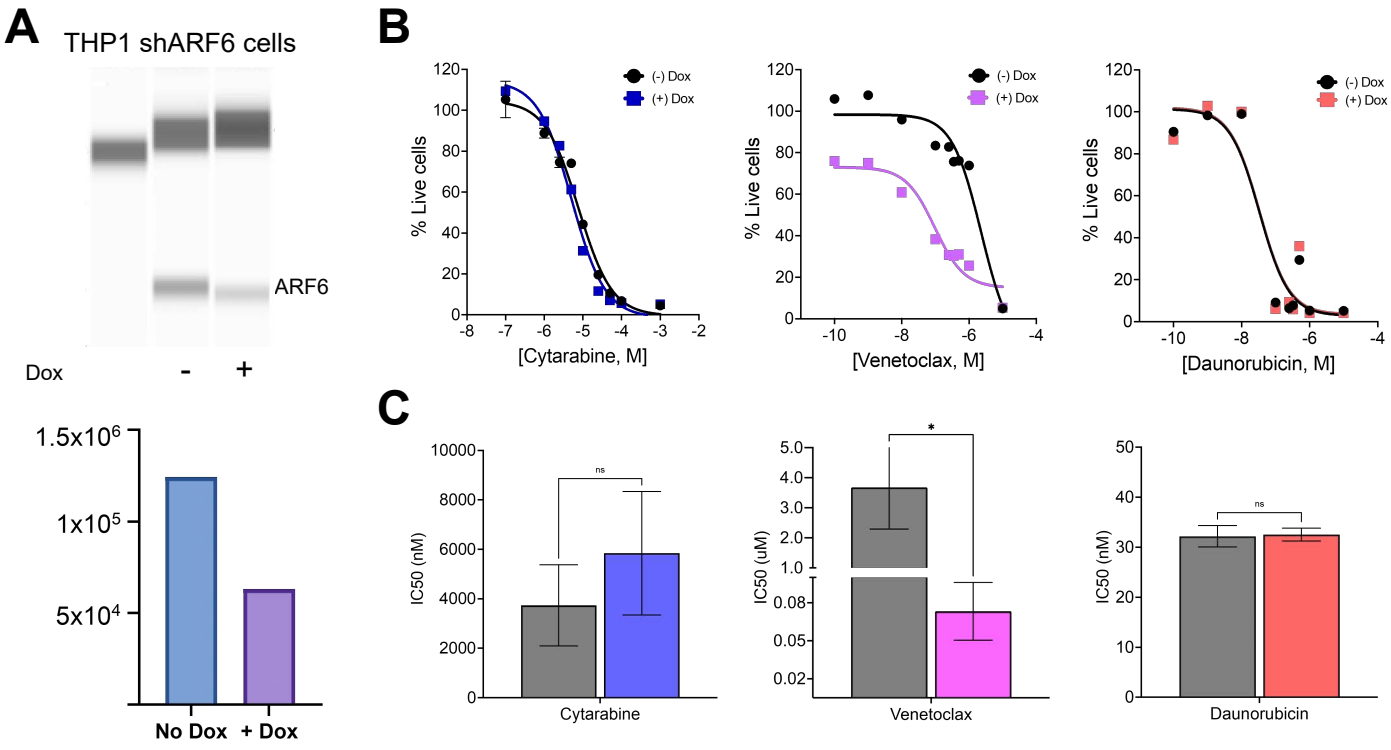
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Supplementary Table 1. Patient characteristics of the AML samples used in this study.

Patient Sample ID	Diagnosis	NGS detected mutations	Karyotype
16-373	Secondary AML	JAK2, DNMT3A	Showed 3q-, 5q-, -17, and -9.
17-268	Secondary AML	JAK2, RUNX1, SRSF2, NRAS, TET2	N.A.
18-367	AML	FLT3ITD, NPM1, IDH2	46,XX [20]
19-002	Secondary AML	N.A.	45,XY,add(5)(q11.2),-18[2] / 44-47,sl, add(4)(q11.2),-7,del(7)(q22q36), der(9;12)(p10q10),-21,?add(21)(p11.2), -22, +1, -4[cp18]
19-007	AML	ASXL1, CBL, SETBP1	N.A.
19-032	AML	NPM1, DNMT3A, FLT3, TET2	46,XY[17]

Supplementary Table 2. Cell line information and DepMap DEMETERS scores of ARF6 perturbation effect.

Cell Line Name	FAB subtype	Detected mutations	Primary Disease	RNAi effect score (DEMETER2)	*ARF6* expression (normalized)
OCIAML5	M4	*BCORL1*	Acute Myeloid Leukemia	-0.54016	4.856986
OCIAML2	M4	*MLL-AF6*	Acute Myeloid Leukemia	-0.39919	5.356848
OCIAML3	M4	*DNMT3A, NRAS, NPM1*	Acute Myeloid Leukemia	-0.3305	5.960465
PLB985	M2	*TP53, CDKN2A, NRAS*	Acute Myeloid Leukemia	-0.25373	4.786596
MONOMAC1	M5	*TP53*	Acute Myeloid Leukemia	-0.23972	5.530445
MONOMAC6	?	*MLL-AF9, RUNX1, TP53*	Acute Myeloid Leukemia	-0.23171	6.549823
U937	?	*CALM-AF10, PTEN, PTPN11, TP53, WT1*	Acute Myeloid Leukemia	-0.20767	5.111449
MOLM16	M0	*TP53*	Acute Myeloid Leukemia	-0.19249	5.44427
HNT34	M4	*BCR-ABL1*	Acute Myeloid Leukemia	-0.18255	5.929554
THP1	M5	*CSNK2A, MLL-AF9, NRAS, TP53*	Acute Myeloid Leukemia	-0.17631	5.623223
KASUMI1	M2	*AML1-ETO, KIT, RAD21, TP53*	Acute Myeloid Leukemia	-0.1299	4.907852
MOLM13	M5	*MLL-AF9, FLT3*	Acute Myeloid Leukemia	-0.0754	5.370862
HEL	M6	*JAK2, TP53*	Acute Myeloid Leukemia	-0.03426	4.702658
HEL9217	M6	*JAK2, TP53*	Acute Myeloid Leukemia	-0.01839	5.235344
NB4	M3	*PML-RARA, KRAS, TP53*	Acute Myeloid Leukemia	-0.00028	5.040892
MV411	M5	*MLL-AF4, FLT3*	Acute Myeloid Leukemia	0.017394	5.562548
AML193	M5	*BCORL1, NRAS, TP53*	Acute Myeloid Leukemia	0.020862	5.582556
SKNO1	M2	*AML1-ETO, KIT, TP53*	Acute Myeloid Leukemia	0.027471	4.918386
CMK115	M7	[+21], *TP53*	Acute Myeloid Leukemia	0.091515	4.659925
HL60	M2	*TP53, CDKN2A, NRAS*	Acute Myeloid Leukemia	0.151371	4.947666
NOMO1	M5	*MLL-AF9, EP300, KRAS, TP53*	Acute Myeloid Leukemia	0.152779	5.683416
CMK	M7	[+21], *TP53*	Acute Myeloid Leukemia	0.437513	4.741467

Note: negative RNAi effect score indicates a reduction of cell survival and proliferation compared to control. There does not appear to be a correlation between *ARF6* expression level and the cell line's RNAi effect score in this diverse collection of laboratory-adapted cell lines.

Supplementary Figure Legends

Supplementary Figure 1. (A, B) Immunoblot images showing the reduction of ARF6 protein after shRNA mediated knockdown or CRISPR mediated silencing. In iCRISPR single clone panels, every two adjacent lanes represent a single-cell clone derived from lentivirally infected cell lines, without (-) or with (+) 100 ng/mL doxycycline treatment for 5 days. Asterisks indicate the single cell clones selected for MTS proliferation assays. Note: the guide RNA expression was induced by doxycycline in each clone, here there is variation and incomplete knockdown. **(C)** Effect of inducible shRNAs and CRISPR/gRNAs on the proliferation of THP-1 and MV4-11 cells. Changes in cell proliferation were measured by MTT assay and shown as increase in doubling time. Expression of shRNA and CRISPR/gRNA was induced by addition of 100 ng/mL doxycycline. **(D)** Two primary AML samples were grown in semisolid media with and without 100 ng/mL doxycycline.

Supplementary Figure 2. Enlarged version of the clustered heat map shown in Figure 2A, displaying the full list of sphingolipid species.

Supplementary Figure 3. *ARF6* dependency analysis using DepMap. Upper panel: RNAi screen result summary. Lower panel: CRISPR screen result summary. Dotted blue lines represent a lack of effect upon gene depletion. Negative gene effect scores indicate cell lines' dependency on that gene. Red solid lines represent the threshold for pan-cancer dependency of a gene.

Supplementary Figure 4. Univariate analysis of TCGA AML cohort survival, stratified by ARF1 and HOXA9 expression.

Supplementary Figure 5. (A) Multi-variate analysis of TCGA cohort survival according to ELN risk. **(B)** *ARF6* expression according to ELN risk in the TCGA dataset.

Supplementary Figure 6. Rescue of ARF6 Knockdown by Constitutively Active $AKT1^{E17K}$. (A) Left: Colony formation assay showing the number of colonies generated by OCI-AML3 cells ectopically expressing the constitutively active $AKT1^{E17K}$ mutant. Cells were treated with or without 100 ng/mL Dox for ARF6 knockdown induction. Right: Representative images of colonies. **(B)** Bar graph showing the percentage of cell viability inhibition, normalized to the respective controls. 20,000 OCI-AML3 cells expressing inducible shARF6, with or without the $AKT1^{E17K}$ mutation, were treated with or without Dox for ARF6 knockdown induction over 120 hours. Fold change (FC) represents the comparison between shARF6 +/- Dox and shARF6-AKT1E17K +/- Dox conditions. **p < 0.01.

Supplementary Figure 7. Combined Effect of ARF6 Knockdown and AML Chemotherapeutic Treatments. (A) Top panel: Western blot analysis showing ARF6 expression in THP1 cells treated with 100 ng/mL Dox for 5 days. Bottom panel: Quantification of ARF6 expression normalized to total protein, with and without Dox treatment. **(B)** Sigmoidal curve of the MTS assay displaying the percentage of cell viability inhibition, normalized to vehicle-treated controls. 20,000 THP1 cells expressing inducible shARF6 were treated with gradient concentrations of cytarabine, venetoclax, or daunorubicin, with or without Dox, for 120 hours. **(C)** Bar graph representing the average

IC_{50} values from three independent MTS cell viability experiments for the three chemotherapeutic drugs tested (N=3, mean±SD).

SUPPLEMENTAL METHODS AND MATERIALS

Cell culture

All cell lines used in this study were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and streptomycin. For cells stably transfected with lentiviruses carrying a puromycin-resistance gene, the culture medium was supplemented with 0.5 to 2 μg/mL puromycin for continuous selection of transgene-positive cells. For drug treatment of the cells, NAV-2729 (Navigen), A6-4471 (Navigen), or GW4869 (Sigma-Aldrich) dissolved in DMSO were added to cell suspension at indicated concentrations, with equal concentrations of DMSO as control. For ceramide treatment, C22 Ceramide (d18:1/22:0) (Avanti Polar Lipids) dissolved in DMSO was resuspended in culture medium, with an equal concentration of DMSO as control.

Cell proliferation and colony formation assays

Cell proliferation assay was performed using CyQUANT (Invitrogen) reagent according to the manufacturer's instructions. 5,000 AML cells were plated into each well of a 96-well plate with culture medium and indicated treatments. Triplicate wells were assayed for each condition. Cells were incubated for proliferation for 3 days. At the end of each assay, DNA-labelling fluorescent dye was added into each well of cells and detected at excitation/emission wavelength of 485/530 nm with a fluorescent plate reader.

For AML colony formation assay, low-density singular cell suspensions and indicated treatments were added to Human Methylcellulose Base Media (R&D Systems) to a final concentration of 1.27% methylcellulose. Semi-solid cell suspension was injected into 6-well plates to form uniform flat gel layers. Plates were placed in humidified chamber for 12 days to allow singular cells to form colonies. For cord blood $CD34^+$ cell colony formation assay, $CD34^+$ cells isolated from cord blood of two separate donors were suspended in MethoCult™ H4230 (StemCell Technologies) supplemented with cytokine mix CC100 (StemCell Technologies) in duplicates respectively. Indicated concentrations of NAV-2729, A6-4471, or DMSO control were added to the suspensions before they were plated in 35 mm dishes. Granulocyte-macrophage colonies were counted under an inverted microscope after 10 days. The size of colonies was quantified with ImageJ using microscopic images of the cultures.

Drug sensitivity assays

For drug sensitivity assays, 20,000 THP1-shARF6 cells per well were seeded in 96-well plates and treated with gradient concentrations of cytarabine, venetoclax, or daunorubicin, in the presence or absence of 100 ng/mL Dox, for 120 hours. Cell viability was assessed using the MTS assay, and results were normalized to vehicle-treated controls. A sigmoidal dose-response curve was generated to determine the percentage of cell viability inhibition. To compare drug sensitivity, IC_{50} values were calculated from three independent experiments (N=3, mean±SD) and analyzed for statistical significance.

shRNA-mediated gene KD and CRISPR-mediated gene silencing

shRNA constructs and inducible CRISPR-cas9 (iCRISPR) constructs were packaged and delivered into cells as lentiviruses. Lentiviral packaging was done by co-transfecting viral backbone plasmids, psPAX2 packaging plasmid, and VSV-G plasmid into HEK293 cells. Non-inducible shRNAs were inserted in pLKO.1-puro-shRNA plasmid (Sigma-Aldrich), and inducible shRNAs were inserted in pRSIT17-U6Tet-CMV-TetRep-2A-TagGFP2-2A-Puro plasmid. ARF6 shRNAs contain the hairpin sequence of "CCGGGCTCAC-ATGGTTAACC-TCTAACTCGA-GTTAGAGGTT-AACCATGTGA-GCTTTTTG". The control lentivirus expresses a non-targeting (NT)-shRNA (SHC002V). shRNA target sequences are: shARF6-005, GCTCACATGGTTAACCTCTAA (CDS); shARF6-068, CAACAATCCTGTACAAGTTGA (CDS); shARF6-069, CTTGCTGTAGATGGCTTATTT (3'UTR). Dox-inducible guide RNAs were inserted in CRISPR-cas9 plasmid with the following targets: CRISPR-gRNA-B, CATTACTACACTGGGACCCA; CRISPR-gRNA-D, GGCCCAGCTTCAACTTGTAC; CRISPR-gRNA-E, TGCACCGCATTATCAATGAC. For transfection, AML cell lines were infected with 0.1 MOI lentiviruses. 48 hours later, puromycin was added to the culture medium to select for positive clones. For iCRISPR cell lines, single-cell clones of positively transfected cell lines were generated from serial dilution and plating under puromycin selection. Each single-cell clone of iCRISPR was treated with Dox (100 ng/mL) for 5 days to test the efficiency of KO after Dox induced guide RNA expression by immunoblotting. KD was verified by immunoblotting, and cell lines were maintained in puromycin-containing medium. OCI-AML3 and THP1 cell lines constitutively expressing an inducible shRNA targeting ARF6 were transduced with a lentiviral vector containing the pRCDCMUR-CMV-AKT^{E17K}-UbiC-TagRFP construct. This vector encodes the AKT cDNA sequence harboring the E17K mutation under the control of a CMV promoter and co-expresses TagRFP under a ubiquitin C (UbiC) promoter. Lentiviral

transduction was performed in standard culture conditions, and 72 hours post-transduction, double-positive RFP^+ (AKT^{E17K} mutants)/GFP^+ (shARF6-expressing) cells were isolated by fluorescence-activated cell sorting. The sorted populations were subsequently expanded in culture, and downstream experiments were conducted one week after sorting recovery to ensure adequate cell viability and expression stability.

Lipid analysis by HPLC-MS

Lipid extraction

Frozen cell pellets were resuspended in 400 µL ice-cold CHCl3/MeOH (1:1) then homogenized. 500 pmol of internal standard (C17 Ceramide (d18:1/17:0) N-heptadecanoyl-D-erythro-sphingosine, Avanti Lipids) were added. After centrifuging at 15,000xg for 5 min at 4 °C, the supernatants were added to 30 µL of 1 M KOH in MeOH and then incubated for 4 hours at 50 °C. The samples were dried down by ~1 mL in speedvac, then 25 µL glacial acetic acid was added, followed by 300 µL chloroform and 600 µL dd-H2O. Samples were centrifuged at 15,000xg for 2 min at 4 °C. The lower phase was transferred and dried in speedvac. Lipid samples were reconstituted in 100 µL ACN:H2O:IPA (1:1:2) + 0.1% formic acid and transferred to an LC/MS vial with insert for analysis. A pooled quality control (QC) sample was prepared by taking 10 µL aliquots from each sample. Concurrently a process blank sample is prepared.

HPLC-MS analysis

Lipid extracts were separated on an Acquity UPLC CSH C18 1.7 µm 2.1 x 100 mm column maintained at 60 °C connected to an Agilent HiP 1290 Sampler, Agilent 1290 Infinity pump, equipped with an Agilent 1290 Flex Cube and Agilent 6520 Accurate Mass Q-TOF dual ESI mass spectrometer. The source gas temperature was set to 350 °C, with a gas flow of 11.1 (L/min) and a nebulizer pressure of 24 psig. VCap voltage was set at 3000 V, fragmentor at 80 V, skimmer at 65 V and Octopole RF peak at 750 V. Reference masses in positive mode (m/z 121.0509 and 922.0098) were infused with nebulizer pressure at 2 psig. Samples were acquired with the scan range between m/z 100 ~ 1700. Mobile phase A consisted of ACN:water (60:40 v/v) in 10 mM ammonium formate and 0.1% formic acid, and mobile phase B consisted of IPA:water (90:10 v/v) in 10 mM ammonium formate and 0.1% formic acid. The chromatography gradient started at 15% mobile phase B then increased to 30% B over 4 min, 52% B from 4-5 min, 82% B from 5-22 min, 95% B from 22-23 min, 99% B from 23-27 min. From 27-38 min it was held at 99%B, then decreased to 15% B from 38-38.2 min and was held there from 38.2-44 min. Flow was 0.3 mL/min throughout. Injection volume was 5 µL. Tandem mass spectrometry was conducted using the same LC gradient and at collision energies of 10 V, 20 V and 40 V.

Data analysis

Results from HPLC-MS experiments were collected using Agilent Mass Hunter Workstation and analyzed using the software packages, Mass Hunter Qual and Mass Hunter Quant. Separately, molecular ions were also evaluated using METLIN (Scripps) and LipidMaps on-line metabolite databases. Only lipids with relative standard deviation (RSD) less than 30% in QC samples were used for data analysis. Additionally, compounds identified in blanks or double blanks (AUC target blank > 50% of AUC target QC) were removed from analysis. Sphingolipids were quantitated based on peak area ratios to the standard added to the extracts. Enrichment analysis was performed as previous described(63). Multidimensional scaling and grouping were performed using the MetaboAnalyst(64) engine.

RNA extraction and qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Life Technologies) and purified with Direct-zol RNA miniprep kit (Zymo research). Extracted RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative real-time PCR (qRT-PCR) was then performed on Applied Biosystems 7900HT Fast Real-Time PCR System using Power SYBR Green Master Mix (Life Technologies). Data analysis was performed using the standard "ΔΔCt method", with GAPDH as the housekeeping control.

Flow cytometry

Apoptosis analysis was performed using a commercial staining kit (BioLegend). AML cells treated with indicated drugs were harvested and washed with cold Cell Staining Buffer. Cells were stained with FITC Annexin V and Propidium Iodide in Annexin V Binding Buffer at a concentration of 0.25-1.0 x 10^7 cells / mL for 15 min at room temperature (25°C) in the dark. For analyzing ceramide content in live cells, a monoclonal anti-ceramide antibody (mouse, clone MID 15B4, Millipore-Sigma) was used to stain the cells, followed by PE-conjugated secondary

antibody labelling and cytometer analysis. For analyzing AML cells in xenografted mouse circulation, mice were bled from the tail vein and blood was collected in PBS containing 1% EDTA. PE-conjugated anti-human CD45 and APC-conjugated anti-mouse CD45 antibodies (BioLegend) were added to the blood samples and staining for 1 hour in dark. Red blood cells were then lysed by adding 20 x volume of ACK lysis buffer. Cells were washed once with 1 mL PBS and then analyzed. All samples were analyzed immediately after staining with a FACS Canto flow cytometer using standard machine settings.

Mouse xenograft studies Adult NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG, The Jackson Laboratory, Bar Harbor, ME) mice were myelodepleted by 9 Gy whole-body irradiation with an RS 2000 X-ray irradiator (Rad Source, Suwanee, GA) or by intraperitoneal injection of 20 mg/kg busulfan(65). Myelodepleted mice were injected via tail vein with 10^6 THP-1 human AML cell line. Starting from week 2 post-xenograft, mice were monitored and bled weekly to determine circulating human AML cell percentage in the total white blood cells. All animal studies were performed under an approved protocol by the University of Utah Institutional Animal Care and Use Committee.