Lysosomal acid lipase A modulates leukemia stem cell response to venetoclax/tyrosine kinase inhibitor combination therapy in blast phase chronic myeloid leukemia

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SUPPLEMENTAL MATERIALS

Supplemental Methods

Generation of leukemia models

Generation of blast crisis chronic myeloid leukemia (bcCML) model. The mouse model was created as described previously [1]. Briefly, BM cells from 8- to 10-week-old naive C57Bl6J mice were harvested and lineage$^+$ (lin$^+$) cells were depleted. LSK (lin$^-$c-kit$^+$Sca-1$^+$) cells were sorted and cultured in LSK medium (IMDM containing 10% FBS, 10 ng/ml IL-3 and IL-6, 50 ng/ml SCF and Flt3L). The following day, LSK cells were infected with viral supernatant containing virus encoding two leukemic oncogenes Bcr/Abl-GFP or Bcr/Abl-T315I-GFP and Nup98/Hoxa9-YFP twice a day for 3 days and subsequently injected through the retro-orbital sinus into 8- to 10-week-old naive B6 Cd45.1, Pep Boy mice. Bone marrow and spleen cells from leukemic mice were harvested and frozen for generation of 2nd generation leukemic mice. Unless stated in the text, recipient mice received leukemia cells at the dose of 25,000 bulk leukemia cells/mouse.

Xenograft models. Xenograft models were created as described previously[1]. Briefly, 8- to 10-week-old NOD *scid* gamma (NSG-S) mice were transplanted with primary human bpCML or AML samples via tail vein injection (2 million cells /mouse). Mice were subjected to analysis at indicated time points.

Tissue leukemic burden and LSC percentage measurements.

Flow analysis for leukemia cells was performed using BD™ LSR II Flow Cytometer System. Bulk leukemia burden was determined by the percentage of GFP+YFP+ cells (for BCR/ABL+Nup98/HoxA9 model) in bone marrow or spleens. LSC percentage for the BCR/ABL+Nup98/HoxA9 model was defined by the percentage of Sca+/lin- (lineage cocktail consisting of CD3, CD45R, Ter119 and Gr-1) cells within the leukemic population. Flow data were analyzed using flowjo software (https://www.flowjo.com/).

Leukemia cells in vitro treatment. Bulk and lin- GY leukemia cells were treated with indicated agents at indicated time points at a cell dose of 0.5 million cells/ml. LSCs were treated at a cell dose of 0.25 million cells/ml. Primary bpCML patient samples were cultured in IMDM with 10%FBS (1million cells/ml) and treated with indicated agents at indicated time points.

Quantitative *polymerase chain reaction* (qPCR). mRNA from cells was extracted using The RNeasy *Plus Mini* Kit (Qiagen) per manufacturer's instructions. cDNA was synthesized using the iScript cDNA synthesis kit (BioRad) per manufacturer's instructions. cDNA was mixed with primers and PerfeCTa® SYBR® Green FastMix® Reaction Mixes (Quantabio) per manufacturer's instructions. qPCR was performed using a LightCycler® 96 System (Roche). Results were analyzed by LightCycler® 96 SW 1.1 software. GAPDH and ACTIN (ACTB) were used as reference genes. Primers used in this manuscript are shown in **Supplementary**

Gene set enrichment analysis (GSEA). GSEA was performed using the FGSEA package for R (v1.24.0). Genes were ranked using the wald statistic produced by a differential expression test between treatment groups (ven/dasa vs vehicle) with DESeq2 (v1.38.3). Pre-ranked GSEA was run on the ranked list using the multilevel splitting Monte Carlo approach with the KEGG v7.4 C2 (curated) canonical pathways gene sets from MSigDB.

RNA-seq. The total RNA concentration was determined with the NanopDrop 1000 spectrophotometer (NanoDrop) and RNA quality assessed with the Agilent Bioanalyzer (Agilent). The TruSeq Stranded mRNA Sample Preparation Kit (Illumina) was used for next generation sequencing library construction per manufacturer's protocols. Briefly, mRNA was purified from 200ng total RNA with oligo-dT magnetic beads and fragmented. First-strand cDNA synthesis was performed with random hexamer priming followed by second-strand cDNA synthesis using dUTP incorporation for strand marking. End repair and 30 -adenylation was then performed on the double stranded cDNA. Illumina adaptors were ligated to both ends of the cDNA, purified by gel electrophoresis and amplified with PCR primers specific to the adaptor sequences to generate cDNA amplicons of approximately 200-500bp in size. The amplified libraries were hybridized to the Illumina single end flow cell and amplified using the cBot (Illumina). Single end reads of 100nt were generated for each sample using Illumina's HiSeq2500v4. Raw reads generated from Illumina HiSeq2500 sequencing were de-multiplexed using bcl2fastq version 1.8.4. Quality filtering and adaptor removal were performed using Trimmomatic version 0.32 with the following parameters:''Slidingwindow:4:20 Trailing:13 Leading:13 Illuminaclip:adapters.fasta:2:30:10 MINLEN:25.'' Processed/cleaned reads were then mapped to the mouse reference sequence Differential expression analyses and data normalization were performed using the CuffDiff tool from the cufflinks version 2.0.2 package given the following parameters: ''–FDR 0.05 -u -b GENOME.''

Viability assays

Cells were pelleted and stained in 1 x Annexin V staining buffer containing fluorophore conjugated Annexin V for 15 minutes in 4°C. Stained cells were then re-suspended in 1 x Annexin V buffer containing DAPI and 0.5% FBS and analyzed on a BD FACSCelesta (BD). Viability was determined by percentage of Annexin V-, DAPI- cells within the parent population.

siRNA transfections

A sequence for siLipa (Human), siCPT1A (Human) and siScrambled were purchased directly from Dharmacon's ON-TARGETplus siRNA Reagents collection. The lyophilized siRNA products were re-suspended in RNAse-free water at 5 μmol/L, which was used as a stock solution. $2x10^6$ cells were suspended in 80 μL of Buffer T, and 20μL of siRNA stock solution was added. These cells were then electroporated using the Neon Electroporation Transfection System (Thermo) according to the manufacturer's protocol using the following settings: 1,600 V, 10 ms, 3 pulses.

Metabolomics experiments

Mouse LSCs (GY+;Lin-;Sca1+) were isolated using the BD ARIA II cell sorter (0.2 million cells/sample) and metabolomics analyses were performed via ultra-high pressure-liquid chromatography-mass spectrometry (UHPLC-MS – Vanquish and Q Exactive, Thermo Fisher). Briefly, cells were extracted in ice cold methanol:acetonitrile:water (5:3:2 v/v) at a concentration of 1 million cells/ml of buffer. After vortexing for 30 min at 4°C, samples were centrifuged at 15,000 g for 10 min at 4°C and supernatants processed for metabolomics analyses. Ten microliters of sample extracts were loaded onto a Kinetex XB-C18 column (150 x 2.1 mm i.d., 1.7 µm – Phenomenex). A 3 min isocratic run (5% B) and a 9 min gradient from 5-95% B (phase A: water and B: acetonitrile with + 0.1% formic acid for positive ion mode or with 10 mM ammonium acetate for negative ion mode) were used to elute metabolites.{Nemkov, 2019 #364} The mass spectrometer scanned in Full MS mode (3 min method) or performed acquisition independent fragmentation (AIF - MS/MS analysis – 9 min method) at 70,000 resolution in the 70-900 m/z range, 4 kV spray voltage, 15 sheath gas and 5 auxiliary gas, operated in negative and then positive ion mode (separate runs). Metabolite assignment was performed against an in-house standard library.

LIPA overexpression and LIPA knock-out.

For LIPA overexpression, the template for mouse LIPA ORF was obtained from GeneCopoeia, and subcloned into the pMYs-IRES-Neo retrovirus vector. Retrovirus was produced by transfection of pMYs-LIPG-IRES-Neo or vector plasmid into the Platinum-E (Plat-E) retroviral packaging cells. Leukemia cells were infected with retrovirus for 3 days (1 infection/day), cultured for 24h after infection and then selected by neomycin (2mg/ml) for 3 days. LIPA overexpression was verified by immunoblotting. For knocking out LIPA, sgRNAs targeting mouse LIPA were obtained from Synthego. Electroporating sgRNA-Cas9 complex into leukemia cells was performed as described before [2]. Briefly, each sgRNA including the negative control sgRNA (1 ng sgRNA /0.2 million cells, 1 sgRNA ng/µl) was mix the Cas9 2NLS Nuclease (5 pmol/ng sgRNA, 5 pmol protein/µl) for 30 min at room temperature to generate the sgRNA-Cas9 complexes. Leukemia cells were washed with PBS and resuspended in T buffer (8 µl/0.2 million cells) from the Neon™ Transfection System Kit. Per 2 µl the sgRNA-Cas9 complex was electroporated into 8 µl cell suspension using the Neon Transfection System with the following electroplating condition: 1700 V, 20 ms, 1 pulse. sgRNA are designed and synthesized by Synthego and sgRNA sequences are as following: sg1: a*a*a*A U C A U G C G C U G G G G A U A U C C ; sg2: g*g*c*U C C C C A G C G C A U G A U U A U C U ; sg3: g*c*g*A C C G A G A U A A U C A U G C G C U G sgNon-targeting: g*c*a*CUACCAGAGCUAACUCA (* represents modification to stabilize sgRNA).

Quantitative PCR and table of primers

RNA was isolated according to the manufacturer's protocol (Qiagen) and synthesized into cDNA using iScript (Biorad). Quantitative PCR (qPCR) was performed on cDNA using Power SYBR Green PCR master mix (Thermo Fisher Scientific) on X96 Real time system. Primers sequences used as listed below.

Supplementary Table 1. Quantitative PCR primers used in this study.

Gene	Forward Sequence 5' – 3'	Reverse Sequence 5' – 3'
LIPA (Mouse)	TGCCCACGGGAACTGTATC	ATCCCCAGCGCATGATTATCT
LIPA (Human)	TCTGGACCCTGCATTCTGAG	CACTAGGGAATCCCCAGTAAGAG
CPT1A (Mouse)	TGGCATCATCACTGGTGTGTT	GTCTAGGGTCCGATTGATCTTTG
CPT1A (Human)	TCCAGTTGGCTTATCGTGGTG	TCCAGAGTCCGATTGATTTTTGC
GAPDH (Mouse)	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
GAPDH (Human)	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Immunoblotting and table of antibodies

Cells were lysed in 2x sample buffer with beta-Mercpatoethanol (1million cells per 100uL). About 500,000 cells per lane were loaded and resolved on 6–12% SDS-PAGE gels, transferred to PVDF membranes, blocked in 5% milk in TBS with 0.1% Triton X-100 (Sigma). After incubation, the PVDF membranes were incubated with primary antibodies at 4°C overnight, washed, incubated with secondary antibodies at room temperature for 1 hour and subjected to imaging. The WB results were imaged on the ChemiDoc Imaging System and visualized in the Image Lab software (BIO-RAD). A table of antibodies used is listed in **Supplementary Table 2**.

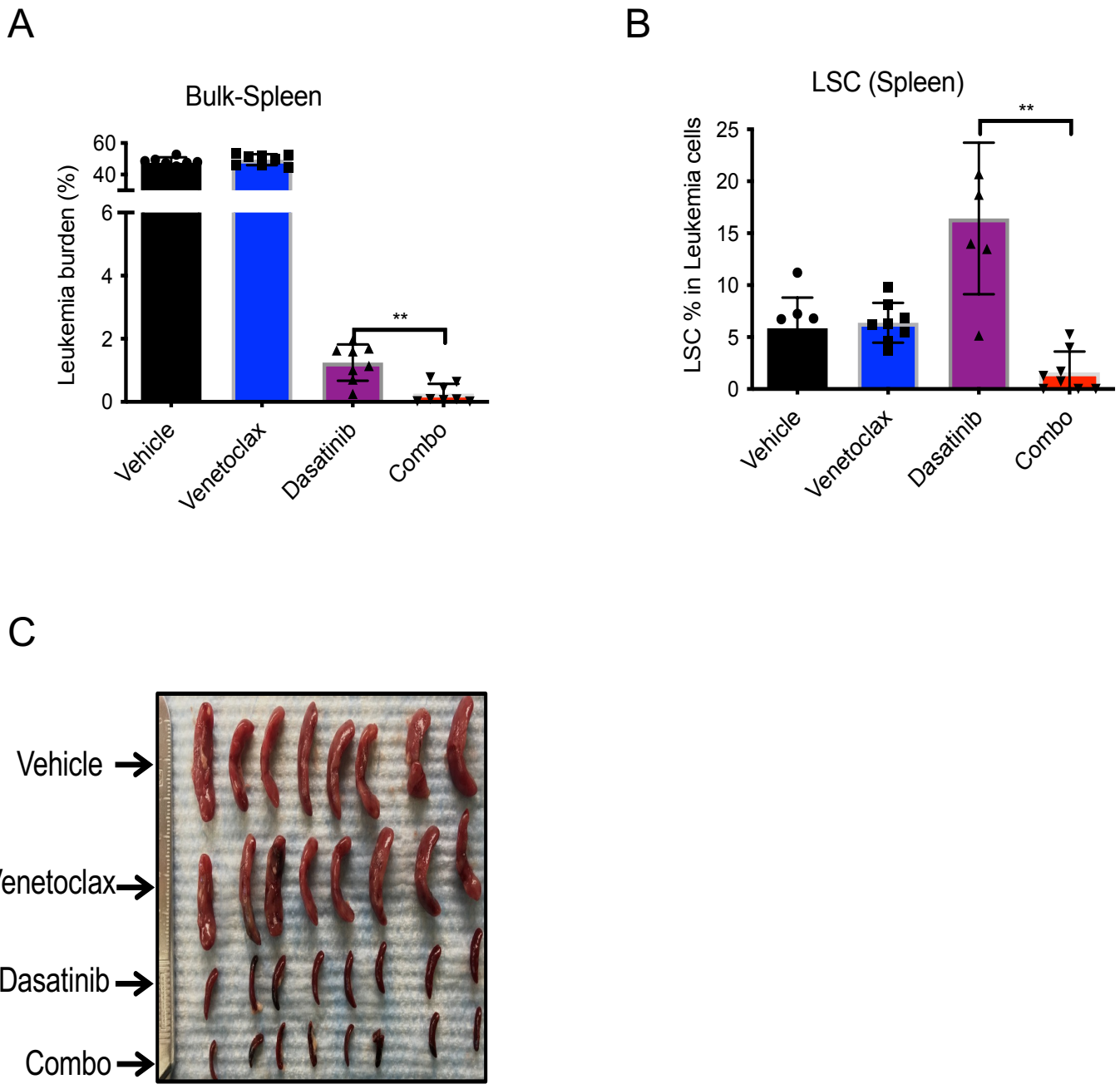
Supplementary Table 2. List of antibodies for Western blot.

Antibody	Source	Catalog #
PE/Cy7 anti-mouse Ly-6A/E (Sca-1)	Biolegend	108114
Alexa Flour 700 Rat Anti-Mouse CD45	Biolegend	560510
APC/Cyanine7 anti-mouse TER-119	Biolegend	116223
APC/Cy7 anti-mouse/human CD45R/B220	Biolegend	103224
APC/Cyanine7 anti-mouse CD3	Biolegend	100222
APC/Cyanine7 anti-mouse Ly-6G/Ly-6C	Biolegend	108424

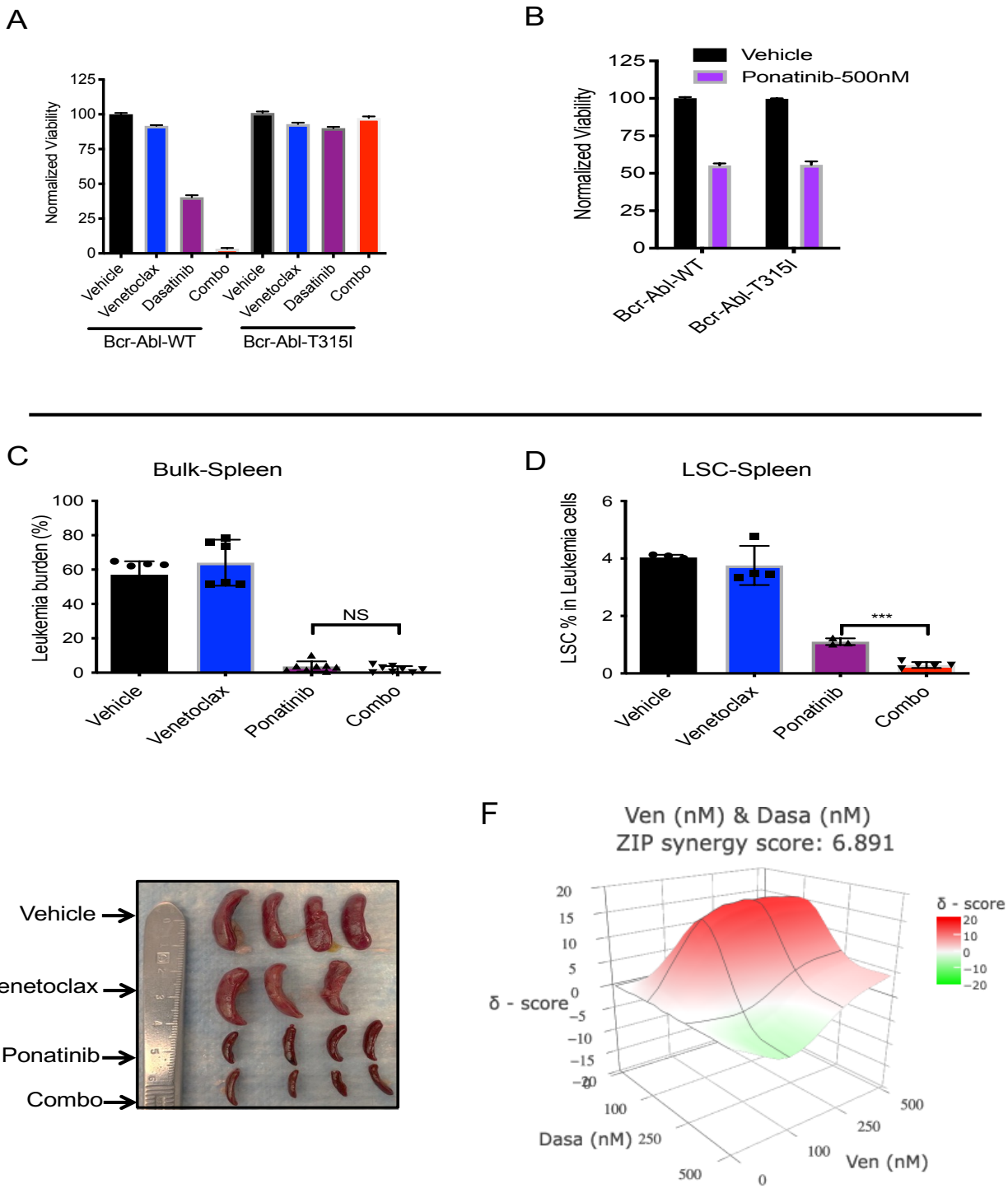
APC Annexin V	BD Biosciences	550475
CPT1A (D3B3) Rabbit mAb	Cell Signaling	12252
GAPDH Mouse monoclonal	Santa Cruz Biotechnology	Sc-32233
Actin	Santa Cruz Biotechnology	(sc-8432 HRP)
LIPA Mouse monoclonal	Santa Cruz Biotechnology	GAPDH Mouse monoclonal

Supplementary Table 3. Other sequences and reagents used

Cytokine SCF	PEPROTech	300-07
Cytokine IL3	PEPROTech	200-03
Cytokine FLT3	PEPROTech	300-19
siLIPA Human	Dharmacon	L-004043-00-0005
siCPT1A Human	Dharmacon	L-009749-00-0005
siCpt1A Mouse	Dharmacon	L-042456-01-0005
Human Methylcellulose Complete Media	R&D Systems	HSC003
Linoleic acid sodium salt	Cayman Chemicals	L8134
Dihomo-γ-Linolenic Acid	Cayman Chemicals	90230
Dasatinib	Sigma-Aldrich	A8774
Ponatinib	Sigma-Aldrich	R8875
Venetoclax	Abbvie	707983

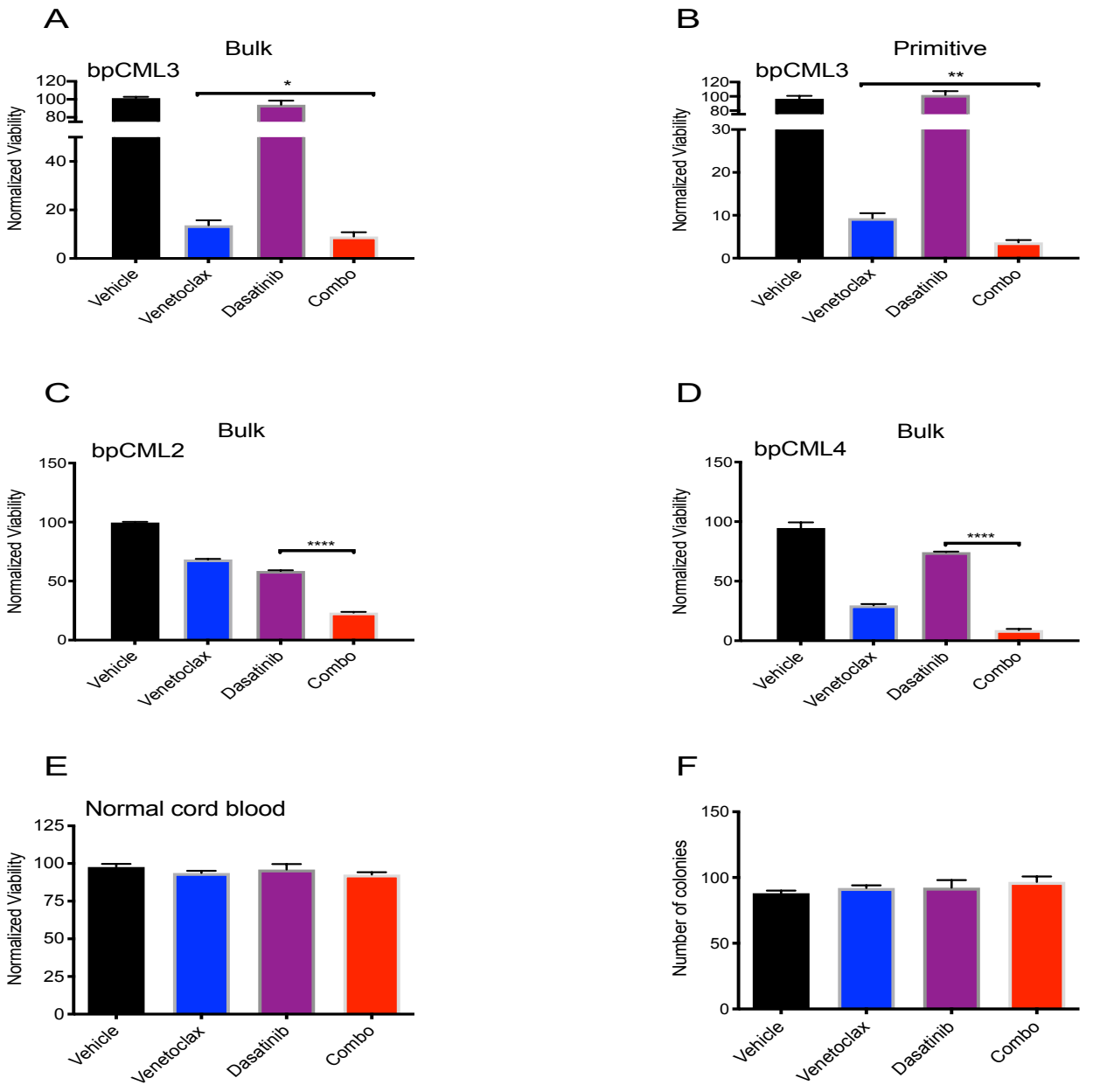


Supplementary Figure 1. Venetoclax/Dasatinib (ven/dasa) combination targets bulk and LSC compartment in an extramedullary site (spleen) in a blast phase chronic myeloid leukemia (bpCML) mouse model. Leukemic mice were treated with vehicle, Ven alone (100mg/kg/day/oral gavage), Dasa alone (20mg/kg/day/OG), and the combination for 5 days starting at day 7 after leukemic transplantation. Mice were sacrificed at day 12 post leukemic transplantation and the tissues were harvested to determine spleen leukemia burden. (A) bulk (B) and LSC compartment (Lin-Sca1+). (C) Image of the spleen size in response to the indicated treatment.



Supplementary Figure 2. Leukemic cells harboring mutant T315I-Bcr-Abl do not respond to dasatinib treatment. (A) Viability of leukemia cells harboring Bcr-Abl-WT or Bcr-Abl-T315I mutant to Ven (100nM), Dasa (100nM) or Ven/Dasa combination. (B) Viability of leukemia cells harboring Bcr-Abl-WT or Bcr-Abl-T315I mutant to Ponatinib (100nM).
Leukemic mice were treated with vehicle, Ven alone (100mg/kg/day/oral gavage), Ponatinib alone (10mg/kg/day/oral gavage), and the combination for 5 days starting at day 7 after leukemic transplantation. Mice were sacrificed at day 12 post leukemic transplantation and the tissues were harvested to determine spleen (C) bulk and (D) LSC (Lin-Sca1+) leukemia burden. (E) Image of the spleen size in response to the indicated treatment. (F) Venetoclax and Dasatinib dose curve to calculate ZIP synergy score.

Supplementary Figure 3

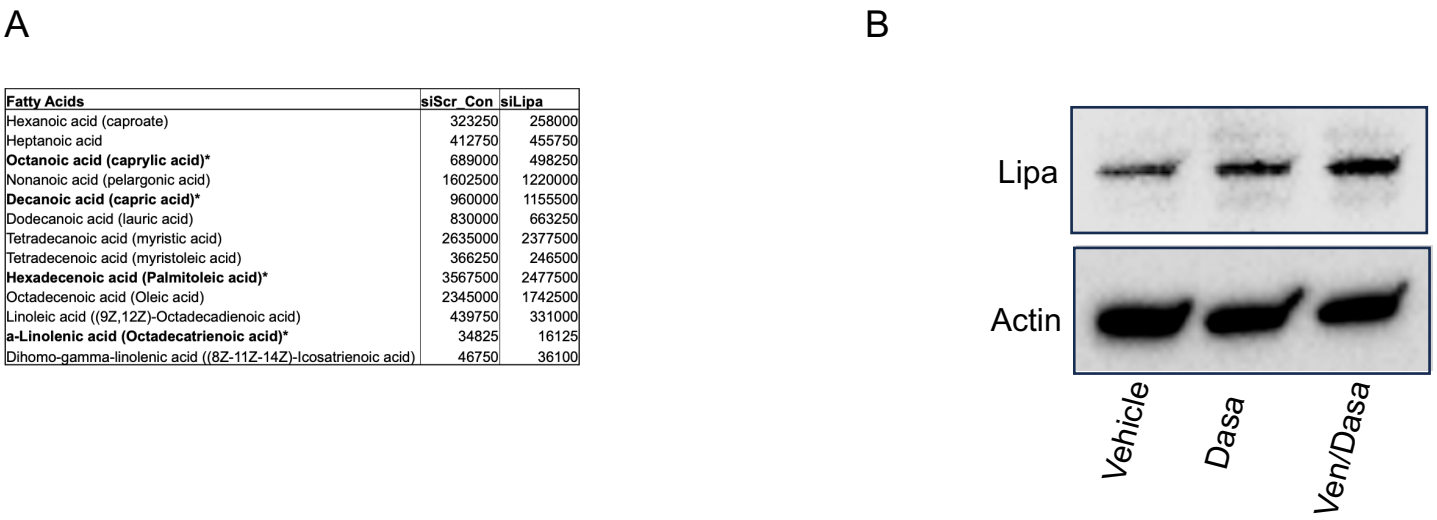


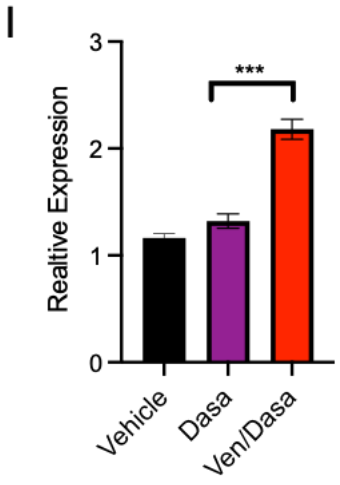
Supplementary Figure 3. Venetoclax/Dasatinib (ven/dasa) combination targets bulk and leukemia stem cell (LSC) compartments in human primary blast phase chronic myeloid leukemia (bpCML). Viability of bpCML patient samples after In Vitro treatment with Ven (100nM), Dasa (100nM), and the combination after 24 hours compared to vehicle control. (A) bulk (B) primitive compartment (CD34+; CD38+) (bpCML3). (C), bpCML2 (bulk). (D) bpCML4 (bulk). Error bars denote mean ± standard deviation (SD) from triplicates. (E) Viability of CD34-enriched normal cord blood cells in response to Ven (100nM), Dasa (100nM), and the Ven/Dasa combination. (F) Colony forming assay from CD34-enriched normal cord blood cells in response to Ven alone, Dasa alone, and the Ven/Dasa combination.

Supplementary Figure 4

A

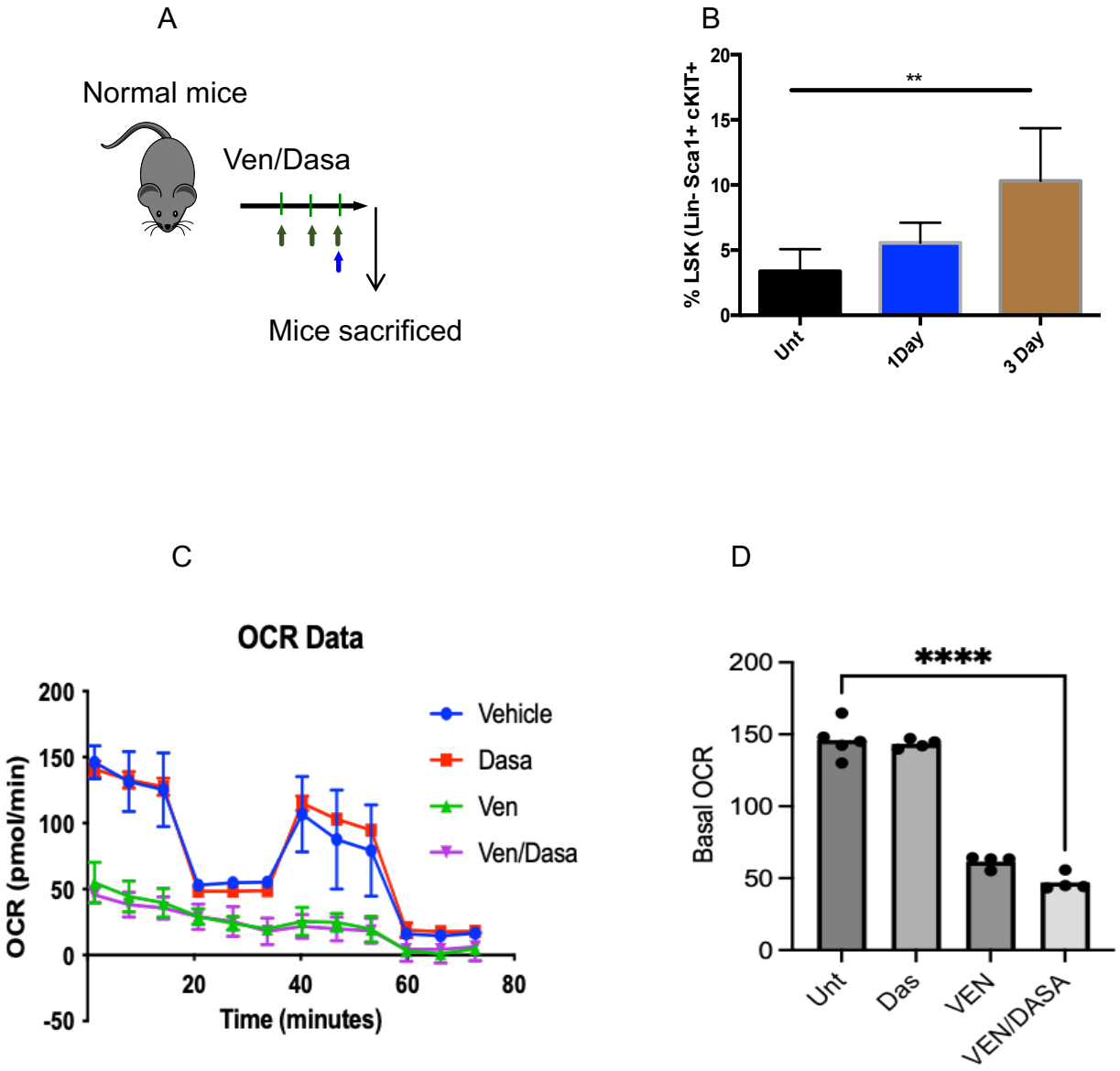
Fatty Acids	siScr_Con	siLipa
Hexanoic acid (caproate)	323250	258000
Heptanoic acid	412750	455750
Octanoic acid (caprylic acid)*	689000	498250
Nonanoic acid (pelargonic acid)	1602500	1220000
Decanoic acid (capric acid)*	960000	1155500
Dodecanoic acid (lauric acid)	830000	663250
Tetradecanoic acid (myristic acid)	2635000	2377500
Tetradecenoic acid (myristoleic acid)	366250	246500
Hexadecenoic acid (Palmitoleic acid)*	3567500	2477500
Octadecenoic acid (Oleic acid)	2345000	1742500
Linoleic acid ((9Z,12Z)-Octadecadienoic acid)	439750	331000
a-Linolenic acid (Octadecatrienoic acid)*	34825	16125
Dihomo-gamma-linolenic acid ((8Z-11Z-14Z)-Icosatrienoic acid)	46750	36100





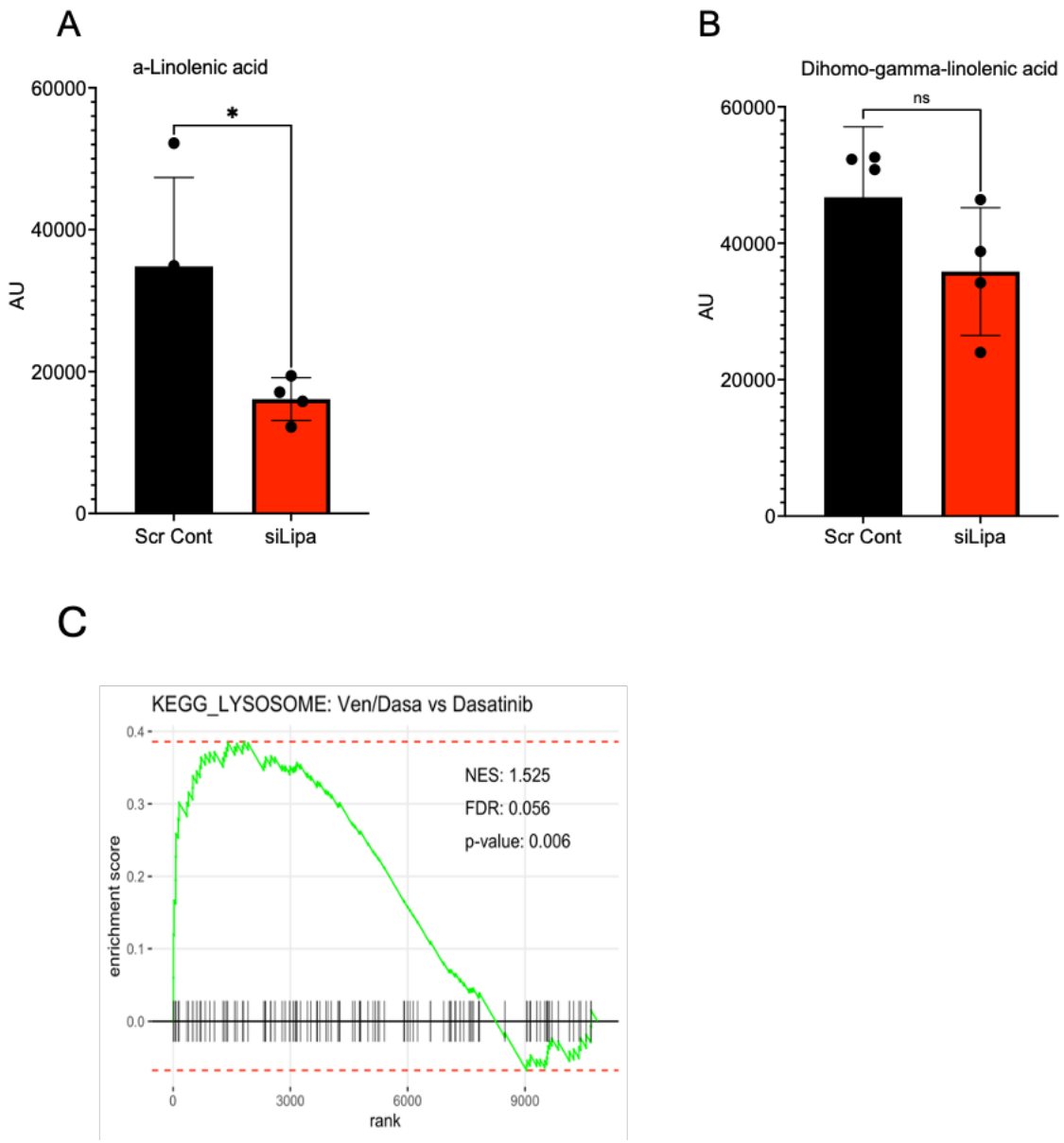
Supplementary Figure 4. (A) Leukemia cells were electroporated with siRNA against Lipa or siScr as a control. After 24 hours cells were analyzed by mass spectrometry to determined levels of free fatty acids. Bold and stared(*) free fatty acids were significantly decreased (AU) after lipa knockdown.(B) Leukemia cells were treated with ven/dasa combination for 4hrs, after the treatment cells were lysed in laemmli buffer and subjected to western blot analysis and probed for Lipa protein, actin was used as loading control. (C) mRNA level of LIPA in LIPA knockout (siRNA-LIPA) and scramble control (Scr-siRNA) (D) Western blot of LIPA in LIPA-Knockdown and scrambled control leukemia cells. (E) Viability of LIPA knockdown and scramble control leukemia cells treated with Ven/Dasa combination (100nM) relative to untreated cells. (F) mRNA level of CPT1A after 24 hours of siRNA knockdown compared to scrambled control in leukemia cells. (G) Western blot of CPT1A in CPT1A-Knockdown and scrambled control leukemia cells. (H) Viability of siRNA mediated CPT1A knockdown and scramble control GY leukemia cells after treatment with Ven/Dasa combination (100nM) relative to untreated cells. (I) mRNA expression of LIPA (quantitative RT-PCR) in LSCs in response to Dasa alone and ven/dasa combination compared to vehicle-treated controls.

Supplementary Figure 5



Supplementary Figure 5. (A) Experimemntal plan for normal mice treatment (B) Percent of LSK (Lin-,Sca1+, cKit+) population in normal mice treated with ven/dasa combination, for 1 day and 3 days with Ven/Dasa combination. (C-D) Basal oxygen consumption rate (OCR) in total bpCML as evaluated by the Seahorse XF96 extracellular Flux analyzer 5 replicate wells of 3×105 leukemic cells were analyzed.

Supplementary Figure 6



Supplementary Figure 6 . (A) a-Linolenic acid (B) Dihomo gamma-Linolenic acid, FFA levels (AU), in response to Lipa knockdown in leukemia cells compared to Scr controls. (C) Gene set enrichment analysis (GSEA) showing enrichment of genes related to lysosome biology in ven/dasa Vs dasa-alone treated LSCs.