haematologica
Journal of the Ferrata Storti Foundation

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Received: November 22, 2023.
Accepted: June 20, 2024.

Citation: Mohd Minhajuddin, Amanda Winters, Haobin Ye, Shanshan Pei, Brett Stevens, Austin Gillen, Krysta Engel, Stephanie Gipson, Monica Ransom, Maria Amaya, Anagha Inguva, Maura Gasparetto, Mark J. Althoff, Regan Miller, Ian Shelton, Hunter Tolison, Anna Krug, Rachel Culp-Hill, Angelo D' Alessandro, Daniel W. Sherbenou, Daniel A. Pollyea, Clayton Smith, and Craig T. Jordan. Lysosomal acid lipase A modulates leukemia stem cell response to venetoclax/tyrosine kinase inhibitor combination therapy in blast phase chronic myeloid leukemia. Haematologica. 2024 June 27. doi: 10.3324/haematol.2023.284716 [Epub ahead of print]

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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Running head**:** LIPA modulates venetoclax/TKI response in bpCML

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Data sharing statement: Original data, primer/probe sequences, and protocols are available upon request by contacting the corresponding author.

Authorship Contributions: Contribution: M.M, C.T.J designed research; A.W, H.Y, S.P, B.S, M.A, A.I, B.A, M.G, M.A, R.M, I.S, S.P, A.K, performed and/or analyzed experiments; R.C, A.D performed metabolomic analysis; A.G, K.E, S.G, M.R performed RNA seq analysis; M.M prepared the figures; and M.M and C.T.J. wrote the manuscript with input from A.W, S.P, D.W.S, D.A.P and C.S.

Conflict of Interest Disclosures: DAP has received research funding and served as a consultant to Abbvie. The other authors declare no conflicts of interest.

Acknowledgements: The authors would like to acknowledge the patients who contributed to this research and the outstanding team at the Blood Disorders Center at the University of Colorado.

Funding: CTJ is supported by the Nancy Carroll Allen Chair in Hematology Research, a Leukemia and Lymphoma Society SCOR grant (7020-19), NIH R35CA242376, and Veterans Administration merit award BX004768-01. DAP is supported by the Robert H. Allen MD Chair in

Hematology and the Leukemia and Lymphoma Society Scholar in Clinical Research. AW is supported by 1K08CA279762-01 through NIH

Abstract

The treatment of blast phase chronic myeloid leukemia (bpCML) remains a challenge due at least in part to drug resistance of leukemia stem cells (LSCs). Recent clinical evidence suggests that the BCL-2 inhibitor venetoclax in combination with ABL-targeting tyrosine kinase inhibitors (TKIs) can eradicate bpCML LSCs. In this report, we employed preclinical models of bpCML to investigate the efficacy and underlying mechanism of LSC-targeting with venetoclax/TKI combinations. Transcriptional analysis of LSCs exposed to venetoclax and dasatinib revealed upregulation of genes involved in lysosomal biology, in particular lysosomal acid lipase A (LIPA), a regulator of free fatty acids. Metabolomic analysis confirmed increased levels of free fatty acids in response to venetoclax/dasatinib. Pre-treatment of leukemia cells with bafilomycin, a specific lysosome inhibitor, or genetic perturbation of LIPA, resulted in increased sensitivity of leukemia cells toward venetoclax/dasatinib, implicating LIPA in treatment resistance. Importantly, venetoclax/dasatinib treatment does not affect normal stem cell function, suggestive of a leukemia-specific response. These results demonstrate that venetoclax/dasatinib is an LSC-selective regimen in bpCML and that disrupting LIPA and fatty acid transport enhances venetoclax/dasatinib response in targeting LSCs, providing a rationale for exploring lysosomal disruption as an adjunct therapeutic strategy to prolong disease remission.

Introduction

Chronic myeloid leukemia (CML) is caused by reciprocal translocation of chromosomes 9 and 22, resulting in the generation of a BCR-ABL fusion protein and constitutive activation of the ABL tyrosine kinase(1). The existence of leukemia stem cells (LSCs) in both chronic phase CML (cpCML) and blast phase CML (bpCML) is supported by previous studies(2, 3). ABL tyrosine kinase inhibitors (TKIs) are to date one of the most successful targeted therapies for malignant disease(4). Unfortunately, TKIs do not always eliminate disease initiating LSCs as evidenced by an inability to discontinue TKI therapy in at least 50% of patients who achieve and maintain a deep molecular response for a significant period(5). CML is classified into three phases: chronic, accelerated and blast phase. bpCML, characterized by the acquisition of secondary mutations in addition to t(9;22) and transition to an often treatment-refractory acute leukemia, remains a formidable challenge in the management of CML(6). In bpCML, emergence of TKI resistance frequently occurs through development of tyrosine kinase domain mutations in BCR-ABL. These include the “gatekeeper” T315I mutation, which results from a threonine to isoleucine substitution at position 315 of ABL, leading to first- and second-generation TKI resistance(7-9). Ponatinib and asciminib are next-generation TKIs that can overcome resistance to T315I mutations(10, 11). However, both have the potential for considerable toxicity, (12), and do not always allow for durable responses in bpCML. More effective and safer therapeutic interventions are necessary to improve the cure rate in cpCML (13).

The anti-apoptotic protein BCL-2 has been recently validated as a target in acute leukemia. The BCL-2 family of proteins are key regulators of mitochondrial-mediated apoptosis(14). Work from several groups has demonstrated that inhibition of BCL-2 is cytotoxic to acute myeloid leukemia (AML) cells(15). In addition, we have previously shown that BCL-2 is overexpressed in AML LSCs and is important for their survival(16). The expression of BCL-2 has been reported to be higher in CML compared to normal hematopoietic stem cells and is further increased in bpCML(17). CML cell survival is in part also mediated by upregulation of other BCL-2 family proteins including BCL-X_L and MCL-1(18, 19). We have previously demonstrated that BCL-2 inhibition with venetoclax can eradicate LSC populations in the context of AML via a mechanism involving perturbation of energy metabolism(20) suggesting that similar activities may be relevant to bpCML. Exciting preclinical studies by Carter et al demonstrated in vivo targeting of LSCs in a mouse model of cpCML, as well as in vitro targeting of primitive human bpCML cells, using a combination of venetoclax and the TKI nilotinib(21). Subsequent clinical testing of venetoclax with TKIs in heavily pretreated bpCML patients has shown promising results(22).

Metabolic aberrancies appear to underlie much of venetoclax resistance in AML. One potential culprit could be the enzyme lysosomal acid lipase (LAL), encoded by the lipase A (LIPA) gene, the only known intracellular lipase active at an acidic pH that hydrolyzes cholesteryl ester and triglyceride in the lysosome(23). LIPA-mediated lipid catabolism releases fatty acids for use as an energy source. Recent work has revealed that fatty acids derived from LAL-mediated lipolysis have important functional impacts on macrophage alternative activation(24), metabolic reprogramming of CD8+ memory T cells(25), and lipid mediator synthesis(26). It has also been demonstrated that increased fatty acid metabolism may be a universal mechanism for therapeutic resistance in AML, relevant to conventional chemotherapy(27) and venetoclax (28, 29).

Similarly, metabolic shifts may be important in the development of therapy resistance in bpCML. In the present study, we evaluated the activity of the combination of venetoclax and dasatinib (ven/dasa) in bpCML mouse models and primary bpCML patient samples. Studies were designed to identify pathways that could potentially enhance the relative efficacy of the drug combination in functionally defined LSCs.

Methods

Cell culture: Base media of Iscoves IMDM with 10% fetal bovine serum (FBS) and 1% Pen/Strep was used, supplemented with 10nM human cytokines stem cell factor (SCF), interleukin-3 (IL3), and FLT3.

Human primary bpCML samples: Primary bpCML samples were obtained from patients after informed consent for sample procurement. All specimen acquisition was approved by the University of Colorado Institutional Review Board.

Mouse strains and husbandry: Wild-type C57BL/6J mice, breeders of B6 Cd45.1, Pep Boy mice were purchased from Jackson Laboratory. All mice were housed at the University of Colorado Anschutz Medical Campus Animal Facility in a Specific Pathogen Free facility with individually ventilated cages. Mice were provided ad libitum access to rodent chow diet. All animal experiments were approved by the Office of Laboratory Animal Resources at the University of Colorado Anschutz Medical Campus.

Mouse studies: The genetically induced “GY” mouse model of bpCML, driven by the dual translocations Bcr-Abl (GFP+) and Nup98-Hoxa9 (YFP+), was developed as previously

described(30). A T315I version of this syngeneic mouse model was also generated for this study, using the same protocol as described previously (30), except using T315I mutant version of Bcr-Abl gene. In parallel, patient-derived xenograft studies were performed as previously described(31). Briefly, NSG-S mice were first engrafted with a human primary bpCML sample through tail vain injection. After 4 weeks, they were then treated by oral gavage with either ven alone (100mg/kg/day) or dasa (20mg/kg/day) or the combination.

RNA sequencing: Bcr-Abl^{wt} "GY" syngeneic mice were treated with ven/dasa or vehicle for 4 hours at day 11 post-transplant of the leukemia cells. Mice were sacked after the treatment, bone marrow was isolated and sorted for LSCs (Lin- and Sca1+), and total RNA from LSCs was isolated using the RNeasy Plus kit (Qiagen) using the manufacturer's protocol. Library construction and sequencing were performed according to a previously described protocol(29). Single end reads of 100 nucleotides were generated for each sample on the Illumina HiSeq2500 platform. Methods for RNA sequencing analysis can be found in the supplemental materials.

Metabolomics: Murine LSCs (GY+ Lin-/Sca1+) were isolated using the BD ARIA II cell sorter, treated for 4 hours with ven/dasa or vehicle, and subjected to metabolomics analyses (0.2 million cells/sample) via ultra-high pressure-liquid chromatography-mass spectrometry (UHPLC-MS – Vanquish and Q Exactive, Thermo Fisher) as previously described (32).

Results

Venetoclax and dasatinib target bulk and LSC populations in a bpCML mouse model. To assess the therapeutic efficacy of venetoclax and dasatinib separately and in combination we performed in vitro and in vivo experiments utilizing a mouse model of bpCML. This model employs co-expression of the Bcr-Abl and Nup98-Hoxa9 translocations, independently monitored using green fluorescent protein (GFP) and yellow fluorescent protein (YFP) expression, respectively (henceforth referred to as GY cells). These translocations are detected in human bpCML and the model has previously been described in detail for studies of leukemia genetics and stem cell biology(30, 33, 34). To assess the effect of ven/dasa treatment in vitro,

the GY cells were treated in cell culture with each drug alone or in combination for 24h, and viability was assessed with Annexin V flow cytometry. Bulk cells treated with the two-drug combination demonstrated a significant loss of viability compared to GY cells treated with either drug alone (**Figure 1A**). Furthermore, the percentage of phenotypic leukemia stem cells (LSCs) defined by surface markers (Sca+/lin-) was also significantly reduced in the ven/dasa treated group (**Figure 1B**). To evaluate in vivo activity, syngeneic mice were transplanted with GY cells, 100k cells per mouse through tail vain, and at day five post transplantation, mice were treated for 5 days with vehicle, single agents or the ven/dasa combination. The combination of ven/dasa led to a significant reduction in leukemia burden compared to either drug alone (**Figure 1C**). We also evaluated the effect of ven/dasa on the murine LSC population (Sca1+/Lin-) and observed a significant decrease in the LSC population compared to single agents or vehicle controls (**Figure 1D**).

To further approximate the clinical benefit of ven/dasa therapy, mice with established GY leukemic engraftment were treated for 10 days with single agents or the ven/dasa combination and monitored for survival; mice were sacrificed upon appearance of signs of leukemia-related morbidity. No survival benefit was seen with ven alone relative to vehicle, whereas dasa alone led to significantly increased survival compared to controls. Animals receiving the ven/dasa combination eliminated the leukemic cells and were monitored for up to 80 days with no sign of leukemia recurrence as determined by the absence of any GY cells in their bone marrow upon sacrifice (**Figure 1E-F**).

Kinetics of venetoclax and dasatinib combination in a bpCML mouse model. To better understand the kinetics of drug response, we also performed short-term drug treatment studies. As shown in **Figure 2 A-C**, leukemic mice were treated for 2, 3 and 4 days and leukemia burden as well as effect on LSCs was evaluated. Leukemia burden overall and LSC percentage were significantly decreased as early as 2 days after treatment initiation. Thus, targeting of primitive populations occurs very rapidly. Notably, analysis of normal progenitor cells, (lin-, sca1+, c-kit+, termed LSK), that are co-resident in the same animals showed no detectable reduction in colony-forming assays implicating leukemia specific response (**Figure 2 D**).

Due to the known disseminated nature of the GY leukemia model, we also assessed the effect of ven/dasa on leukemic infiltration of extramedullary locations such as the spleen. Treatment of mice with ven/dasa resulted in significant inhibition of bulk disease as well as the LSC compartment compared to either drug alone (**Supplemental Figures 1 A-B**). The average spleen size in the combination treated cohort was reduced to the size of normal spleens

(**Supplemental Figure 1 C**). Overall, the bpCML mouse model showed the enhanced efficacy of the ven/dasa combination over the single drug treatments.

Venetoclax and ponatinib targets bulk and LSC populations in a mouse model of BCR-ABL^{T315I} mutation**.** Despite the success of TKIs, resistance has been identified in some patients due to point mutations in the *BCR-ABL* kinase domain. Ponatinib was developed as a TKI that can inhibit all critical kinase domain mutations including T315I(10). Thus, we investigated the addition of venetoclax to ponatinib in the context of T315I-mutant bpCML. We generated a mouse model of leukemia harboring T315I mutant BCR/ABL-GFP and NUP98/HOXA9-YFP (termed GY-315). As expected, in vitro treatment of GY-315 cells showed resistance to dasatinib. Furthermore, GY-315 cells showed no response to ven/dasa (**Supplemental Figure 2A**). In contrast, both GY and GY-315 cells responded to ponatinib treatment, confirming that ponatinib is active against both the wild type as well as the mutant Bcr-Abl oncogene (**Supplemental Figure 2B**).

To assess the effect of the venatoclax and ponatinib combination (ven/pona) in vitro, the GY-315 cells were treated with each drug alone or in combination for 24h. Treatment with ven/pona resulted in a significant loss of viability of bulk leukemia cells compared to either drug alone (**Figure 3A**). Furthermore, the percentage of phenotypic LSCs (Sca+/lin-) was also significantly reduced in the ven/pona treated group (**Figure 3B**). To evaluate the effect of these drug combinations in vivo, leukemic mice were generated by transplantation of GY-315 cells into recipient animals followed by treatment with each drug alone or the ven/pona combination. Both single agents resulted in decreased leukemia burden in bone marrow, an effect which was more pronounced in mice treated with ponatinib alone, but the combination of ven/pona led to a significant decrease in leukemia burden relative to single agents (**Figure 3C**). Similar results were observed in the LSC (Sca1+/Lin-) population (**Figure 3D**). Survival studies performed using the same experimental approach as shown in Figure 1E demonstrated a significantly prolonged survival for mice treated with the ven/pona combination (**Figure 3E**). Although we observed a significant survival benefit of ven/pona in the T315I mutant GY mouse model we were unable to determine curative outcome due to the toxicity of ponatinib (weight loss and poor grooming). Investigation of lower ponatinib doses in combination with venetoclax may allow eradication of LSCs without treatment-related mortality.

We also assessed the effect of ven/pona on leukemic infiltration of extramedullary locations such as the spleen. Treatment of mice with ven/pona resulted in significant inhibition of bulk

disease as well as the stem cell compartment compared to either drug alone (**Supplemental Figures 2C-D**). The average spleen size in the combination treated mice was reduced to the size of normal spleens (**Supplemental Figure 2E**). Overall, ven/pona and ven/dasa have similar effects and confirm that this approach is translatable to disease that harbors the T315I TKI resistance mutation.

Venetoclax and dasatinib target bulk and primitive populations in bpCML patient samples. To translate these findings to human models of disease, we repeated in vitro and in vivo drug treatment experiments using primary specimens from bpCML patients. Primary leukemia samples (N=4) were treated with single or combination agents in vitro for 24h and assessed using Annexin V flow cytometry. As shown for a representative specimen (**Figures 4A-B**)**,** both the bulk and primitive compartments showed a significant decrease in viability compared to controls, with the most pronounced effect seen in the ven/dasa treated group. Data from three additional primary samples showed a similar response (**Supplementary Figures 3A-D**), confirming the efficacy of ven/TKI as reported previously(21). To directly test the impact of ven/dasa on LSC function in vivo, primary human bpCML cells were transplanted into immunocompromised NSG-S mice(35). At four weeks post-transplant, (10 to 45% human cell engraftment depending on primary sample) mice were treated for 10 days with venetoclax, dasatinib or the combination. A significant reduction in tumor burden was observed in ven/dasa treated mouse cohorts derived from two bpCML patient samples (**Figures 4C**), supporting loss of LSC functionality because of dual drug treatment, compared to either drug alone. We also performed secondary engraftment from the cohort injected with bpCML1. The cells from primary engrafted mice, normalized to the percent of human cells from each treated group, were injected into the tail veins of secondary recipient mice. Mice were sacrificed after twelve weeks, and human hematopoietic engraftment was evaluated by flow cytometry. As can be seen in **Figure 4 D-E**, the cells from the Ven/Dasa treated group failed to engraft in secondary recipients, thereby demonstrating eradication of the leukemia stem cell compartment. To further test the impact of ven/dasa on LSC function in vivo, one more primary human bpCML cells were transplanted into immunocompromised NSG-S mice, as shown in **Figure 4F**, a significant reduction in tumor burden was observed in ven/dasa treated cohort. In contrast, treatment of CD34-enriched normal hematopoietic stem cells (HSCs) with similar doses of single agents or ven/dasa resulted in no significant cell death and no effect on colony formation (**Supplementary Figures 3E-F**), demonstrating a strong LSC-specific effect.

Lysosomal mechanisms influence venetoclax and dasatinib response in murine bpCML.

The collective results from murine models of bpCML show that ven/dasa treatment selectively and directly targets the LSC compartment. Thus, we sought to further define mechanisms of LSC targeting to optimize the efficacy of the ven/dasa regimen. To this end, we performed an analysis of the immediate transcriptional response to ven/dasa treatment in the murine bpCML model. As outlined in **Figure 5A**, leukemic mice were treated with ven/dasa for 4 hours, bone marrow was harvested post-treatment and flow sorted for Lin-/Sca1+ (LSC-enriched populations) and subjected to RNA-seq. For comparison, dasatinib alone and vehicle controls were also included in the transcriptional analyses. Venetoclax conferred no survival advantage over vehicle so was not included in the study. Our rationale in designing this experiment was that genes up regulated by drug treatment, prior to the onset of overt apoptosis, will include mechanisms of protection. As shown in **Figure 5B,** principal component analysis (PCA) indicates distinct gene signatures in the LSCs for each treatment group. Gene set enrichment analyses (GSEA) suggested strong up-regulation of genes involved in lysosomal biology in ven/dasa-treated cells when compared to the expression patterns observed in vehicle controls (**Figures 5C-D**)**.**

To investigate the role of lysosomal activity, we employed the use of Bafilomycin, a specific inhibitor of lysosome function. As shown in **Figure 5E** in vitro pre-treatment of GY cells for one hour with Bafilomycin followed by 24-hour treatment with ven and/or dasa strongly increased sensitivity to ven/dasa treatment, implying a protective role for lysosome activity. Of the genes that were upregulated in the lysosome pathway specifically due to treatment with both venetoclax and dasatinib, we found that lysosomal acid lipase (LIPA or LAL) was consistently elevated (**Figure 4F-G**). This enzyme is also referred to as Cholesterol Ester Hydrolase and is involved in hydrolysis and recycling of cholesterol and free fatty acids for cellular energy(23). The upregulation of LIPA in the ven/dasa treated LSC population was confirmed by qPCR analysis **Supplementary Figure 4I**.

Metabolomics analysis of venetoclax plus dasatinib treated GY cells reveals upregulation of free fatty acids (FFA). Since LIPA is known to positively regulate free fatty acid levels, we performed metabolomic analyses of LSCs isolated after a 4-hour treatment with ven/dasa (**Figure 6A**). We found that several fatty acids were upregulated in the ven/dasa group compared to control (**Figures 6B**). More importantly, supplementing these same fatty acids (e.g., α-Linolenic Acid and Dihomo-g-Linolenic acid) to the media resulted in partial rescue from ven/dasa-mediated cell death, implicating upregulation of fatty acid levels as a protective response to ven/dasa challenge (**Figures 6C-D**).

Modulation of Lipa expression results in altered respose to ven+dasa treatment in a bpCML mouse model . To examine the role of endogenous LIPA in leukemia cells, we utilized CRISPR technology and electroporated Cas9-sgRNA complexes into murine GY leukemia cells to knockout (KO) LIPA (36). As shown in **Figures 7A-B**, this approach achieved strong knockdown of LIPA at both the mRNA and protein level**.** Treatment of LIPA KO cells with ven/dasa resulted in increased sensitivity (**Figure 7C**) further corroborating the concept that upregulation of lysosomal activity serves as a protective response to ven/dasa challenge. We also over-expressed (OE) the gene in murine GY leukemia cells (**Figure 7D-E**) and found treatment of LIPA-OE leukemia cells with ven/dasa resulted in increased resistance compared to cells transduced with vector alone (**Figure 7F**). These data suggest that increased free fatty acids due to higher expression of Lipa results in diminished cytotoxicity of ven/dasa. This implies that utilization of fatty acids by leukemic cells may be important for diminished drug response. Thus, we hypothesized that inhibiting expression of Carnitine palmitoyltransferase 1A (CPT1A), an important free fatty acid mitochondrial membrane transporter, could modulate the ven/dasa response. Indeed, knock-down of CPT1A using siRNA (**Figure 7G**) resulted in increased sensitivity of the GY cells to ven/dasa treatment compared to cells transfected with scrambled siRNA (**Figure 7H**).

Modulation of Lipa expression results in altered response to ven+dasa treatment in human bpCML samples. To determine if the findings from GY mouse cells were also evident in human bpCML cells, we examined the effect of both LIPA knock-down and CPT1A knock-down in primary human bpCML using an siRNA approach. As shown in **Figures 8A-B and Supplementary figure 4C-D**, we confirmed significant knock-down of LIPA expression at mRNA and protein levels and found that treatment of LIPA knock down cells with ven/dasa resulted in increased drug sensitivity compared to scrambled control cells (**Figure 8C and Supplementary figure 4E**). Furthermore, after confirming knock-down of CPT1A expression in primary human samples (**Figure 8D and Supplementary figure 4F-G)** we similarly demonstrate increased sensitivity to ven/dasa compared to scrambled control cells (**Figure 8E and Supplementary figure 4H**). Together these results suggest that treatment with ven/dasa results in upregulation of genes associated with lysosome biology as a protective response, in particular LIPA. LIPA is known to increase production of free fatty acids that can be used as a fuel source, which in part may be responsible for diminished ven/dasa response.

Discussion

The primary goal of this study was to investigate the molecular mechanisms that mediate the LSC-targeting activity of venetoclax in combination with TKIs in bpCML. In agreement with the studies of Carter et al 2016, we observe that venetoclax in combination with a TKI is a highly effective LSC-targeting regimen in both xenograft studies using primary human bpCML samples as well as in a previously described mouse model of bpCML. Survival experiments showed that the ven/dasa combination effectively cured mice of leukemia induced with wild type Bcr-Abl, whereas untreated mice typically succumbed to disease within 10-15 days. We also show the efficacy of venetoclax and ponatinib in a newly created mouse model harboring T315I mutant Bcr-Abl in combination with Nup98/HoxA9. Although we observed a significant survival benefit of ven/pona in the T315I mutant GY mouse model we were unable to determine curative outcome due to the toxicity of ponatinib. Investigation of lower ponatinib doses in combination with venetoclax may allow eradication of LSCs without treatment-related mortality.

Our data further identify a compensatory upregulation of genes associated with lysosome biology upon treatment with ven/dasa. The functional relevance of this finding is clearly evident upon addition of bafilomycin to the ven/dasa regimen, which significantly increased eradication of primitive bpCML cells. In particular, upregulation of *LIPA* seems to be a protective mechanism activated by these cells to attempt survival. Since LIPA is known to regulate free fatty acid levels, we performed metabolomic analysis of LSCs isolated after treatment of ven/dasa for 4 hours. We found several fatty acids were upregulated in the ven/dasa treated cells. Recent work has demonstrated that increased fatty acid metabolism may be a mechanism for AML resistance to conventional chemotherapies such as cytarabine(27, 37) and our lab has previously reported that increased expression of genes involved in fatty acid metabolism correlates with a poor response of AML to venetoclax/azacitidine(28, 29). We hypothesized that activation of fatty acid processing through enhanced LIPA activity may also represent a compensatory response to venetoclax-based therapies that employ TKIs. Accordingly, knockdown of CPT1A, the main mitochondrial transporter of free fatty acids (FFA)(38), resulted in increased sensitivity to ven/dasa combination, confirming the role of FFA in attenuating the ven/dasa response.

The clinical implications of our findings are significant. Given that TKIs do not eliminate LSCs in all patients with CML(39) (40), most must be treated with lifelong therapy. ABL-class TKIs are known to have side effects ranging from endocrine dyscrasias to arterial thromboses(41) , and are poorly tolerated in some patients. TKI resistance also develops over time in about 15% of patients(42). One future direction of this work would be to evaluate venetoclax/TKI combinations

in newly diagnosed cpCML to see if more effective LSC targeting can be achieved with lower doses of TKI in the presence of venetoclax, as well as whether addition of venetoclax can reduce evolution of ABL kinase domain resistance mutations. Notably, only 40-50% of patients with cpCML are able to stop single-agent TKIs after prolonged durations of molecular remission, as demonstrated by the prospective STIM1 and TWISTER (43, 44) trials. It would be intriguing to repeat these trials after initial treatment with venetoclax/TKI combinations to see if a higher percentage of cpCML patients are able to discontinue therapy altogether. Furthermore, for bpCML in particular, the only curative treatment available at present is allogeneic hematopoietic stem cell transplant (SCT)(13), where graft-versus-leukemia (GVL) activity can be effective(45) (46). However, GVL often co-occurs with severe graft-versus-host-disease (GVHD) which compounds the toxicity of SCT for patients(47). With more effective LSC targeting, SCT may be required in fewer patients with associated reductions in transplant-related morbidity and mortality, furthermore, patients may be able to go into transplant with lower burdens of disease, which could enhance the survival rates post-SCT.

Finally, an important extension of our current work will be to expand our evaluation of combination therapies with ven/TKIs and fatty acid transport inhibitors or by extension autophagy inhibitors. Triple therapy combinations may enhance LSC targeting and extend durations of response given our preclinical data that blocking these pathways enhances efficacy of ven/dasa against bpCML. Understanding the mechanisms of synergy between venetoclax and TKIs in myeloid malignancies will be important to optimizing clinical use of this regimen.

In summary, we confirm and extend the preclinical utility of Ven/TKI combination therapies for targeting of bpCML LSCs. Furthermore, our data suggest that blocking upregulation of free fatty acids through mechanisms such as inhibition of LIPA activity might synergize with Ven/TKI combinations to eradicate LSCs, allowing for more durable response. Our findings support a therapeutic rationale for blocking pathways involved in free fatty acid generation as a potential strategy for increasing disease-free survival in patients affected by bpCML.

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Figure Legends

Figure 1. Venetoclax/Dasatinib (ven/dasa) combination targets bulk and leukemia stem cell (LSC) compartment in a blast phase chronic myeloid leukemia (bpCML) mouse model. Viability of (A) bulk and (B) LSC compartment (Lin-; Sca1+) GY+ leukemia cells treated in vitro with Ven (100nM), Dasa (100nM), and the combination after 24 hours compared to vehicle control. Leukemic mice were treated with vehicle, Ven alone (100mg/kg/day/oral gavage), Dasa alone (20mg/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 (C) bulk and (D) LSC leukemic burden in bone marrow. (E) Leukemic mice were treated with Vehicle, Ven alone, Dasa alone and the combination as described above. Survival of control and treated leukemic mice (n=8) was monitored and mice were sacrificed upon demonstration of morbid symptoms. For combination-treated mice, no such symptoms appeared so mice were sacrificed 80 days after experiment start. (F) Representative flow plot for engraftment status of leukemia cells (GY Cells) from survival experiment from panel E.

Figure 2. Kinetics of Venetoclax/Dasatinib (ven/dasa) combination in blast phase chronic myeloid leukemia (bpCML) mouse model. (A) Experimental design for 2, 3 and 4-day treatment of leukemic mice, (B) effect on bulk, and (C) effect on the LSC compartment (Lin-Sca+) after 2, 3 and 4-day treatment with Ven/Dasa combination. (D) Colony forming unit (CFU) assay using bone marrow cells (Lin-;Sca1+;cKit+) from normal mice treated for 1 day and 3 days with Ven/Dasa combination. Error bars denote mean ± standard deviation (SD) from triplicates. Statistical analyses were performed using a student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 3. Venetoclax/Ponatinib (ven/pona) combination targets bulk and leukemia stem cell (LSC) compartment in blast phase chronic myeloid leukemia (bpCML) T315I-mutant mouse model. T315I-mutant GY+ leukemia cells were treated In Vitro with Ven (100nM); pona (100nM) and the combination for 24 hours and viability measured relative to vehicle control in (A) bulk leukemia cells and (B) the LSC compartment (Lin-; Sca1+). Similarly, leukemic mice were treated with vehicle, Ven alone (100mg/kg/day/oral gavage), Pona 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 (C) bulk leukemia burden and (D) quantitation of the LSC compartment (Lin-Sca1+) in the bone marrow. (E) Leukemic mice were treated with Vehicle, Ven alone, pona alone and the combination as described above. Survival of control and treated leukemic mice (n=8) was monitored and mice were sacrificed upon demonstration of morbid symptoms. Error bars denote mean ± standard deviation (SD) from triplicates. Statistical analyses were performed using a student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 4. Venetoclax/Dasatinib (ven/dasa) combination targets bulk and leukemia stem cell (LSC) compartment in human primary blast phase chronic myeloid leukemia (bpCML)

patient samples. Viability of a representative bpCML patient sample treated *in vitro* with Ven (100nM); Dasa (100nM) and the combination after 24 hours compared to vehicle control. (A) bulk (B) primitive compartment (CD34+; CD38+). Leukemic NSG-S mice transplanted with human bpCML patient-derived xenografts (C&F) (bpCML1 and bpCML2) were treated with vehicle, Ven alone (100mg/kg/day/oral gavage), Dasa alone (20mg/kg/day/oral gavage) and the combination, starting at day 25 for 10 days. Mice were sacrificed at day 35 post-transplantation. (D) Cells from primary engrafted mice were injected to secondary recipient mice and monitored for 12 weeks and sacrificed after 12 weeks. (E) Flow plot from secondary engraftment experiment. Bone marrow leukemia burden quantified as percentage human CD45 (hCD45+) population is shown. Error bars denote mean ± standard deviation (SD) from triplicates. Statistical analyses were performed using a student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 5. Upregulation of genes related to lysosome biology protects leukemia stem cells (LSCs) from Venetoclax/Dasatinib (ven/dasa) combination therapy. (A) Experimental design for treatment, flow sorting, and RNA isolation from LSCs for total RNA sequencing (RNA-seq). (B) PCA plot for gene expression profile in vehicle, Dasa, and Ven/Dasa treated groups. (C&D) Gene set enrichment analysis (GSEA) showing enrichment of genes related to lysosome biology in combination treated LSCs. (E) Leukemia cells (GY+; lin-) were pre-treated with Bafilomycin (Baf, 10nM) for 1h and then treated in vitro for 24h with Ven alone (100nM), Dasa alone (100nM), or the combination. Viability was measured compared to vehicle control. (F) Heat map showing expression of representative lysosome-related genes in untreated verses Ven/Dasa combination-treated LSCs. (G) Lipa expression in response to dasa alone and Ven/Dasa combination. Error bars denote mean ± standard deviation (SD) from triplicates. Statistical analyses were performed using a student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 6. Venetoclax/Dasatinib (ven/dasa) treatment results in increase of free fatty acids (FFA) in leukemia stem cells (LSCs). (A) Experimental design for treatment followed by metabolomics analysis of free fatty acids in LSCs. (B) Relative amount of different fatty acids in LSCs in response to the treatment: A-Linolenic acid; Myristoleic acid; Hexadecanoic acid and Dihomo-g-Linolenic acid**.** (C&D) Leukemia cells (GY+;lin-) were pre-treated in vitro with (C) linolenic acid (LA) (10uM) or (D) Dihomo-g-Linolenic acid (10uM) for 1h and then treated for 24h with Ven/Dasa combination (100nM). V viability was measured compared to vehicle control. Error bars denote mean ± standard deviation (SD) from triplicates. Statistical analyses were performed using a student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 7. Endogenous LIPA and CPT1A are critical for Venetoclax/Dasatinib (ven/dasa) resistance in mouse leukemia cells. (A) mRNA level of LIPA in LIPA knockout (sgRNA-LIPA) and scramble control (Scr-sgRNA) GY leukemia cells using CRISPR/CAS9 method. (B) Western blot of LIPA in LIPA-KO and scrambled control leukemia cells. (C) Viability of LIPA KO and scramble control GY leukemia cells treated with Ven/Dasa combination (100nM) relative to untreated cells. (D) mRNA level of LIPA in LIPA overexpression (OE) and vector control GY leukemia cells. (E) Western blot of LIPA in LIPA OE and vector control GY leukemia cells. (F) Viability of LIPA OE cells treated with Ven/Dasa combination (100nM) compared to vector control GY leukemia cells, normalized to untreated cells. (G) mRNA level of CPT1A after 48 hours of siRNA knockdown compared to scrambled control in GY 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.

Figure 8. Endogenous LIPA and CPT1A are critical for Venetoclax/Dasatinib (ven/dasa) resistance in human bpCML samples. (A) mRNA level of LIPA after 48 hours of siRNA knockdown compared to scrambled control in human bpCML cells (bpCML2). (B) Western blot of LIPA in LIPA knockdown and scramble control in bpCML2 cells. (C) Viability of LIPA KO and scramble control human bpCML2 cells after treatment with Ven/Dasa combination (100nM) relative to untreated cells. (D) mRNA level of CPT1A after 48 hours of RNA knockdown compared to scrambled control in human bpCML cells (bpCML1). (E) Viability of CPT1A knockdown and scramble control human bpCML1 cells after treatment with Ven/Dasa (100nM) relative to untreated cells. Error bars denote mean ± standard deviation (SD) from triplicates. Statistical analyses were performed using a student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 1

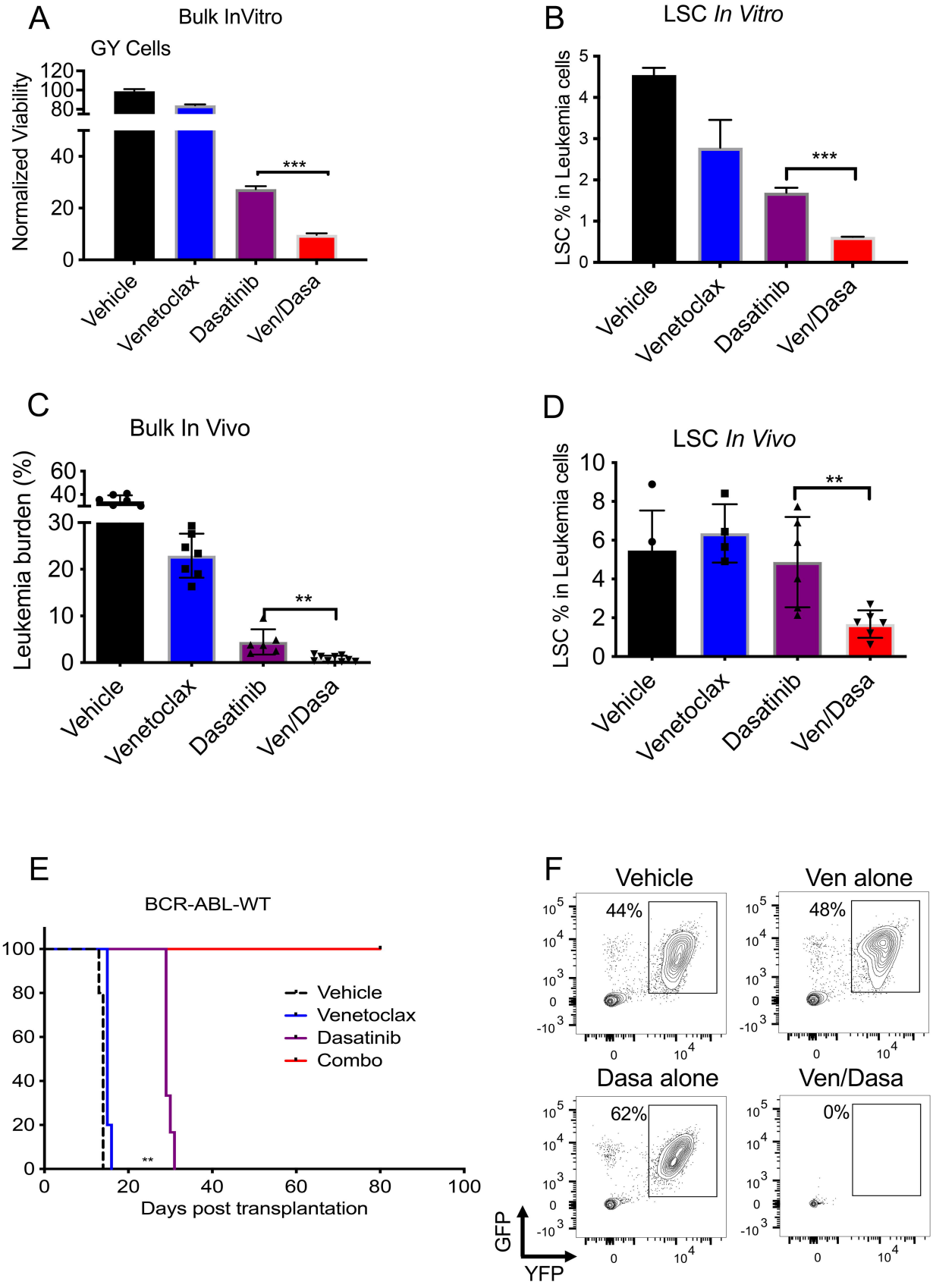

A
Bulk InVitro
GY Cells
Normalized Viability
120
100
80
40
20
0
***
Vehicle
Venetoclax
Dasatinib
Ven/Dasa
B
LSC In Vitro
LSC % in Leukemia cells
5
4
3
2
1
0
***
Vehicle
Venetoclax
Dasatinib
Ven/Dasa
C
Bulk In Vivo
Leukemia burden (%)
60
40
30
20
10
0
**
Vehicle
Venetoclax
Dasatinib
Ven/Dasa
D
LSC In Vivo
LSC % in Leukemia cells
10
8
6
4
2
0
**
Vehicle
Venetoclax
Dasatinib
Ven/Dasa
E
BCR-ABL-WT
Percent survival
100
80
60
40
20
0
**
0
20
40
60
80
100
Days post transplantation
Vehicle
Venetoclax
Dasatinib
Combo
F
Vehicle
44%
Ven alone
48%
Dasa alone
62%
Ven/Dasa
0%
GFP
YFP

Figure 2

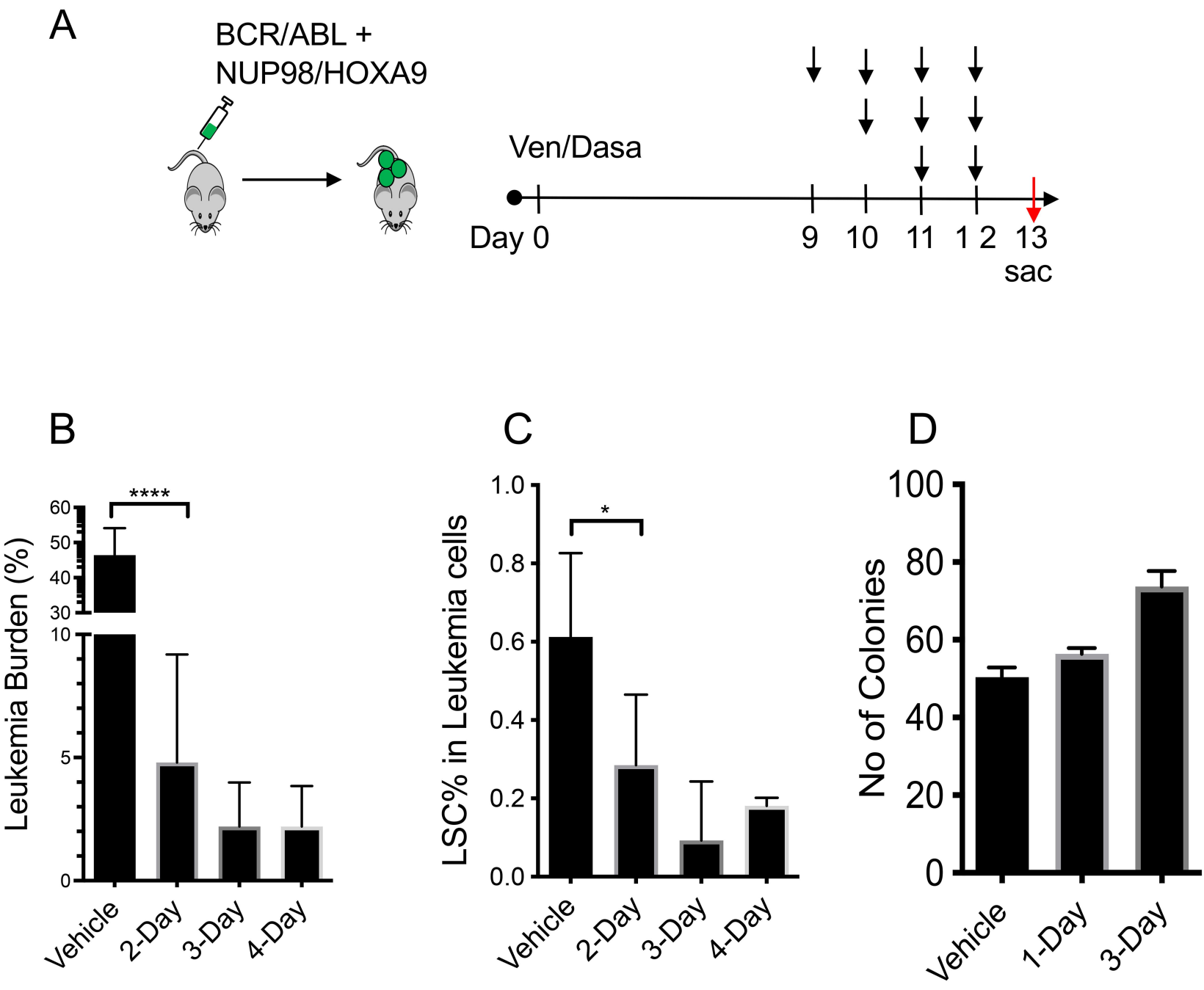

A
BCR/ABL +
NUP98/HOXA9
Ven/Dasa
Day 0
9
10
11
1 2
13
sac
B
****
Leukemia Burden (%)
60
50
40
30
10
5
0
Vehicle
2-Day
3-Day
4-Day
C
*
LSC% in Leukemia cells
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
2-Day
3-Day
4-Day
D
No of Colonies
100
80
60
40
20
0
Vehicle
1-Day
3-Day

Figure 3

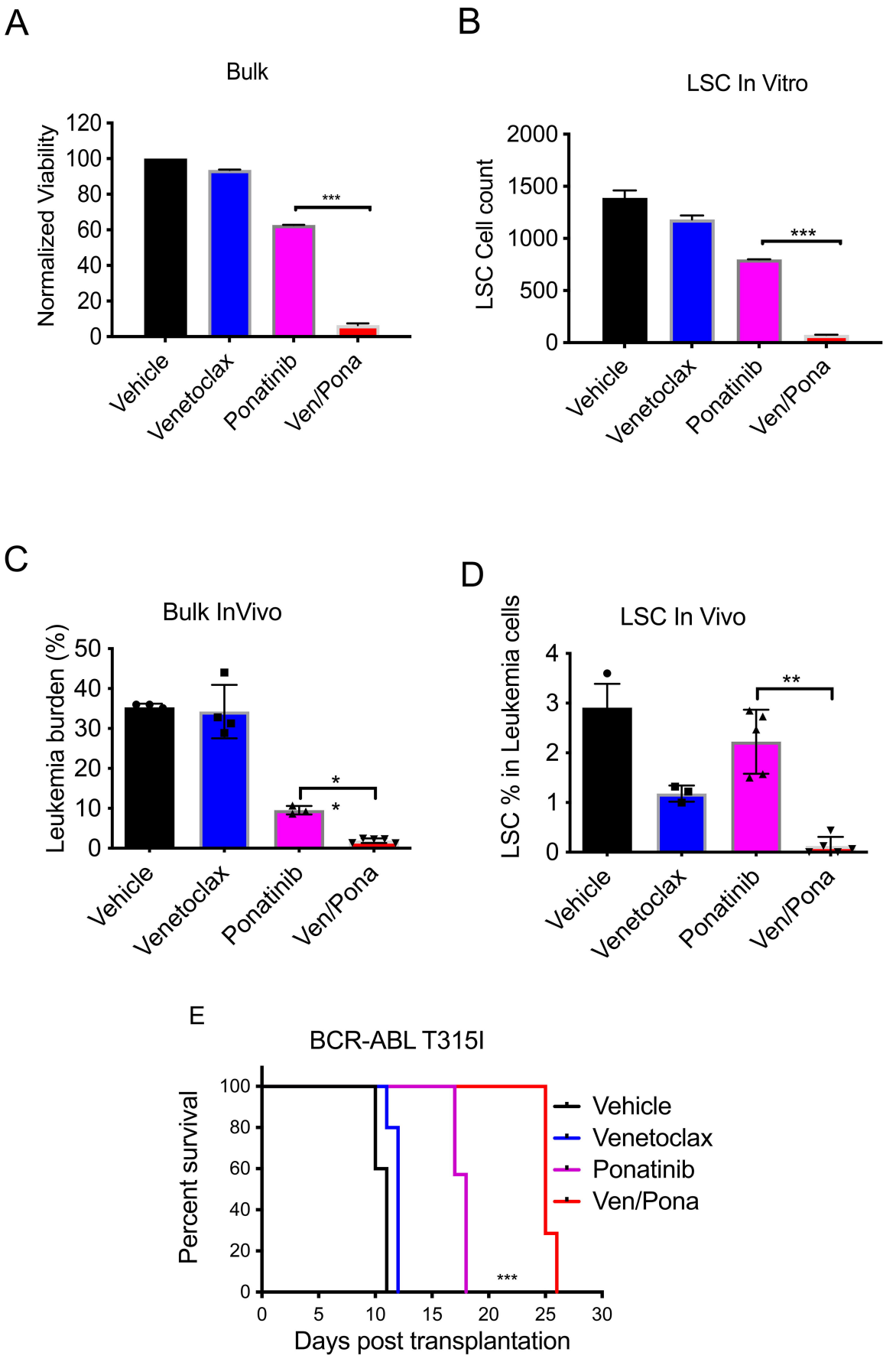

A
Bulk
Normalized Viability
120
100
80
60
40
20
0
***
Vehicle
Venetoclax
Ponatinib
Ven/Pona
B
LSC In Vitro
LSC Cell count
2000
1500
1000
500
0
***
Vehicle
Venetoclax
Ponatinib
Ven/Pona
C
Bulk InVivo
Leukemia burden (%)
50
40
30
20
10
0
*
*
Vehicle
Venetoclax
Ponatinib
Ven/Pona
D
LSC In Vivo
LSC % in Leukemia cells
4
3
2
1
0
**
Vehicle
Venetoclax
Ponatinib
Ven/Pona
E
BCR-ABL T315I
Percent survival
100
80
60
40
20
0
***
0
5
10
15
20
25
30
Days post transplantation
Vehicle
Venetoclax
Ponatinib
Ven/Pona

Figure 4

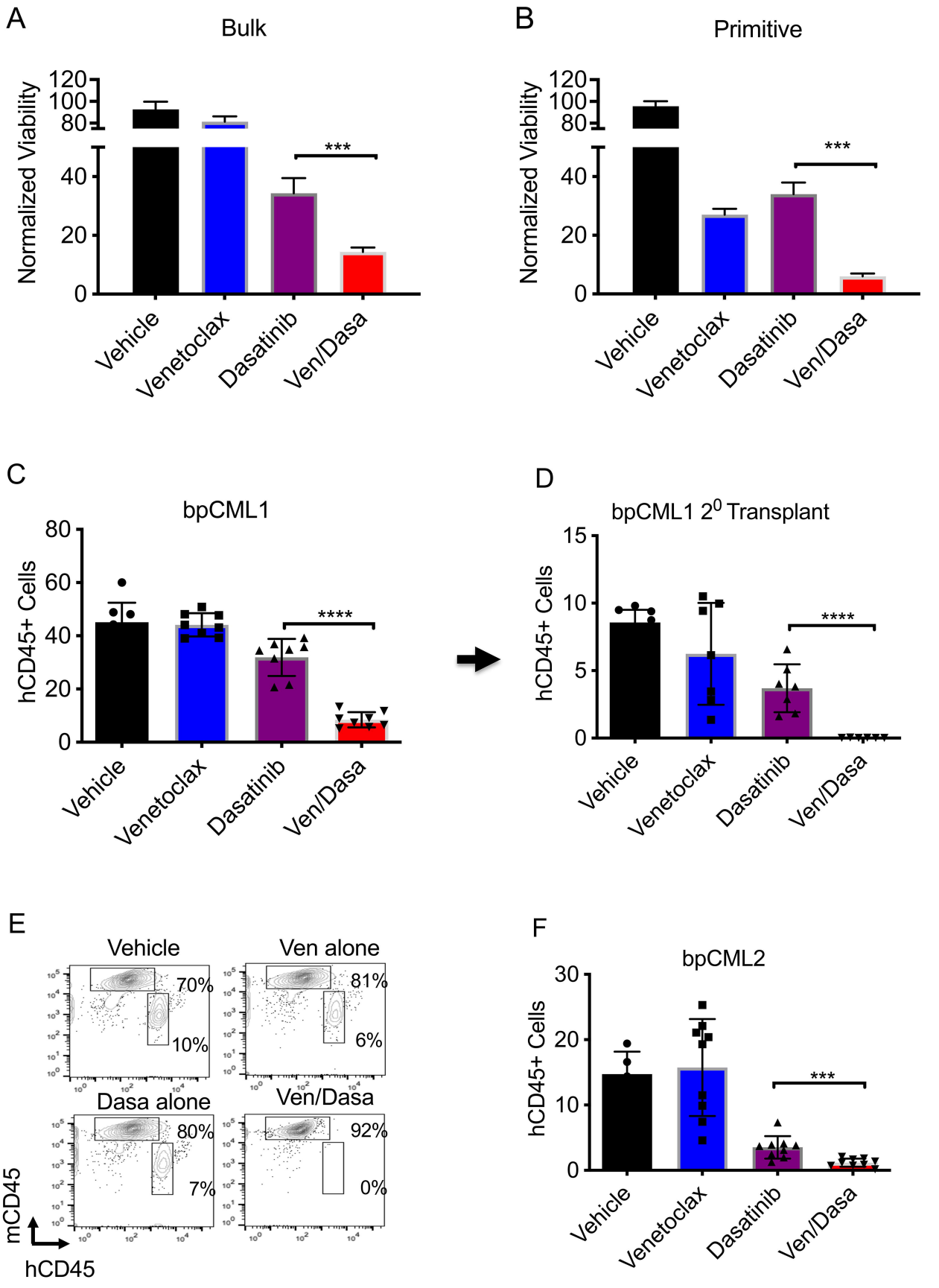

A
Bulk
Normalized Viability
120
100
80
40
20
0
***
Vehicle
Venetoclax
Dasatinib
Ven/Dasa
B
Primitive
Normalized Viability
120
100
80
40
20
0
***
Vehicle
Venetoclax
Dasatinib
Ven/Dasa
C
bpCML1
hCD45+ Cells
80
60
40
20
0
****
Vehicle
Venetoclax
Dasatinib
Ven/Dasa
D
bpCML1 2^0 Transplant
hCD45+ Cells
15
10
5
0
****
Vehicle
Venetoclax
Dasatinib
Ven/Dasa
E
Vehicle
70%
10%
Ven alone
81%
6%
Dasa alone
80%
7%
Ven/Dasa
92%
0%
mCD45
hCD45
F
bpCML2
hCD45+ Cells
30
20
10
0
***
Vehicle
Venetoclax
Dasatinib
Ven/Dasa

Figure 5

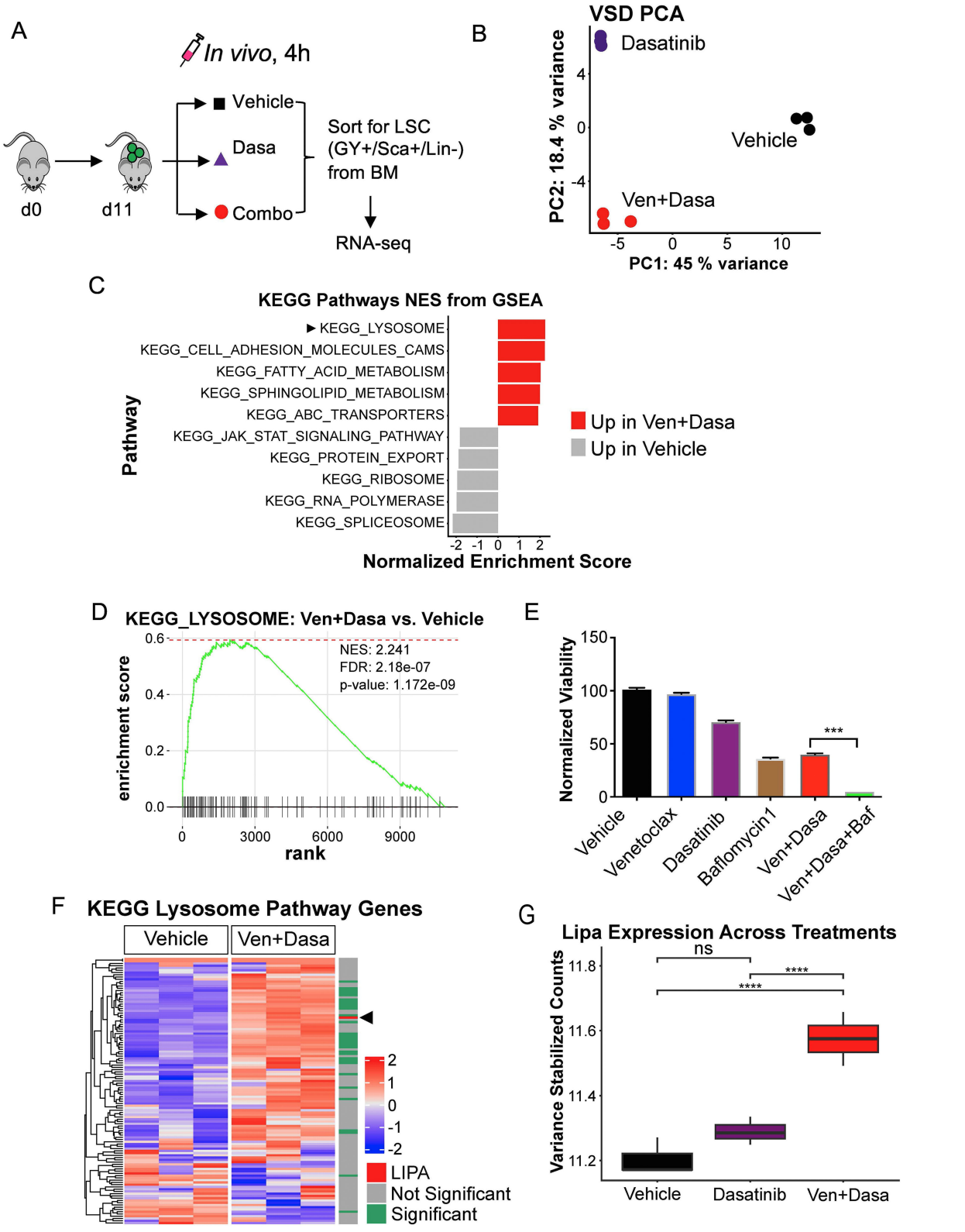
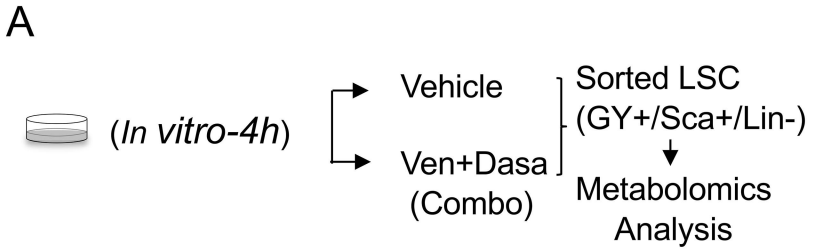
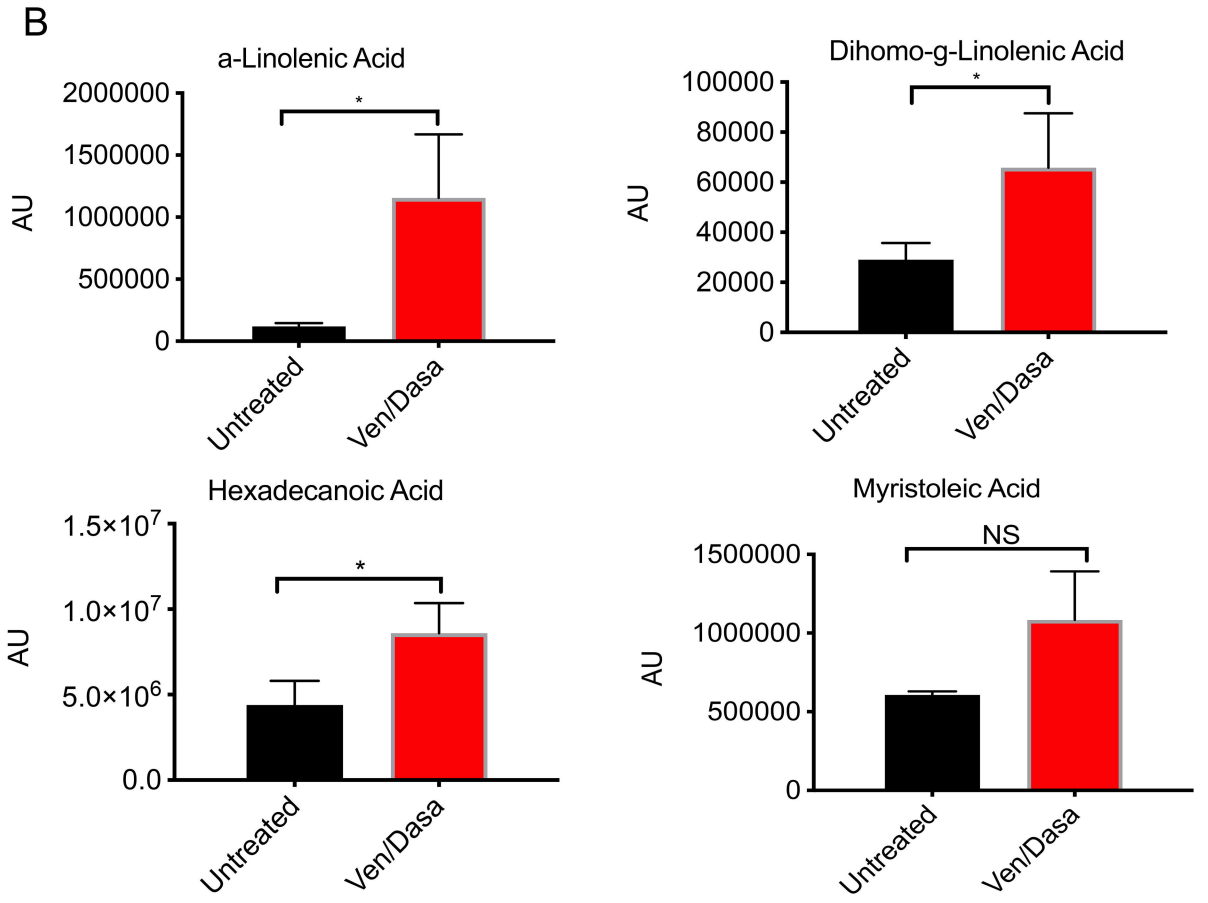
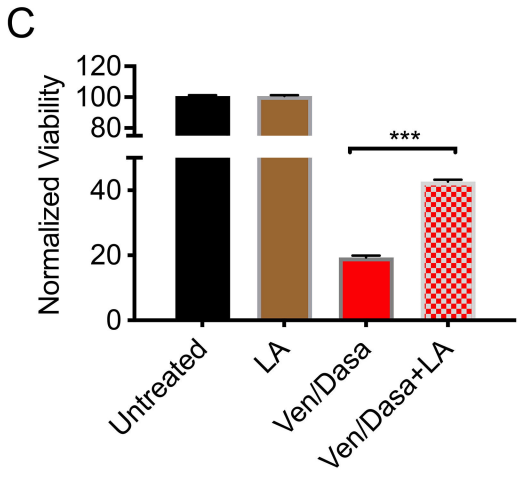

A
In vivo, 4h
Vehicle
Dasa
Combo
d0
d11
Sort for LSC
(GY+/Sca+/Lin-)
from BM
RNA-seq
B
VSD PCA
Dasatinib
Vehicle
Ven+Dasa
PC2: 18.4 % variance
PC1: 45 % variance
C
KEGG Pathways NES from GSEA
KEGG_LYSOSOME
KEGG_CELL_ADHESION_MOLECULES_CAMS
KEGG_FATTY_ACID_METABOLISM
KEGG_SPHINGOLIPID_METABOLISM
KEGG_ABC_TRANSPORTERS
KEGG_JAK_STAT_SIGNALING_PATHWAY
KEGG_PROTEIN_EXPORT
KEGG_RIBOSOME
KEGG_RNA_POLYMERASE
KEGG_SPLICEOSOME
Pathway
Up in Ven+Dasa
Up in Vehicle
Normalized Enrichment Score
D
KEGG_LYSOSOME: Ven+Dasa vs. Vehicle
NES: 2.241
FDR: 2.18e-07
p-value: 1.172e-09
enrichment score
rank
E
Normalized Viability
***
Vehicle
Venetoclax
Dasatinib
Baflomycin1
Ven+Dasa
Ven+Dasa+Baf
F
KEGG Lysosome Pathway Genes
Vehicle
Ven+Dasa
LIPA
Not Significant
Significant
G
Lipa Expression Across Treatments
ns
****
****
Variance Stabilized Counts
Vehicle
Dasatinib
Ven+Dasa

Figure 6


A
(In vitro-4h)
Vehicle
Ven+Dasa
(Combo)
Sorted LSC
(GY+/Sca+/Lin-)
Metabolomics
Analysis


B
a-Linolenic Acid
AU
2000000
1500000
1000000
500000
0
*
Untreated
Ven/Dasa
Dihomo-g-Linolenic Acid
AU
100000
80000
60000
40000
20000
0
*
Untreated
Ven/Dasa
Hexadecanoic Acid
AU
1.5×10^7
1.0×10^7
5.0×10^6
0.0
*
Untreated
Ven/Dasa
Myristoleic Acid
AU
1500000
1000000
500000
0
NS
Untreated
Ven/Dasa


C
Normalized Viability
120
100
80
40
20
0
***
Untreated
LA
Ven/Dasa
Ven/Dasa+LA

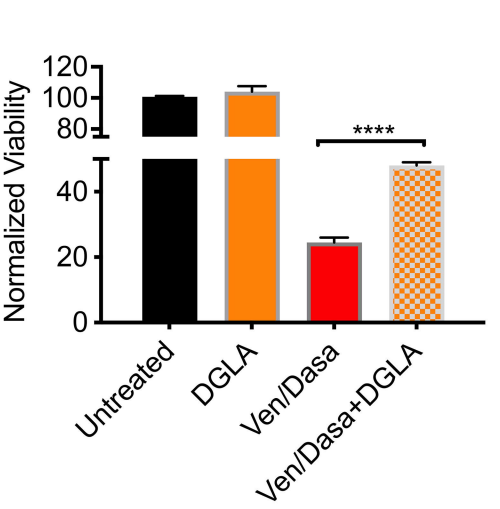

D
Normalized Viability
120
100
80
40
20
0
****
Untreated
DGLA
Ven/Dasa
Ven/Dasa+DGLA

Figure 7

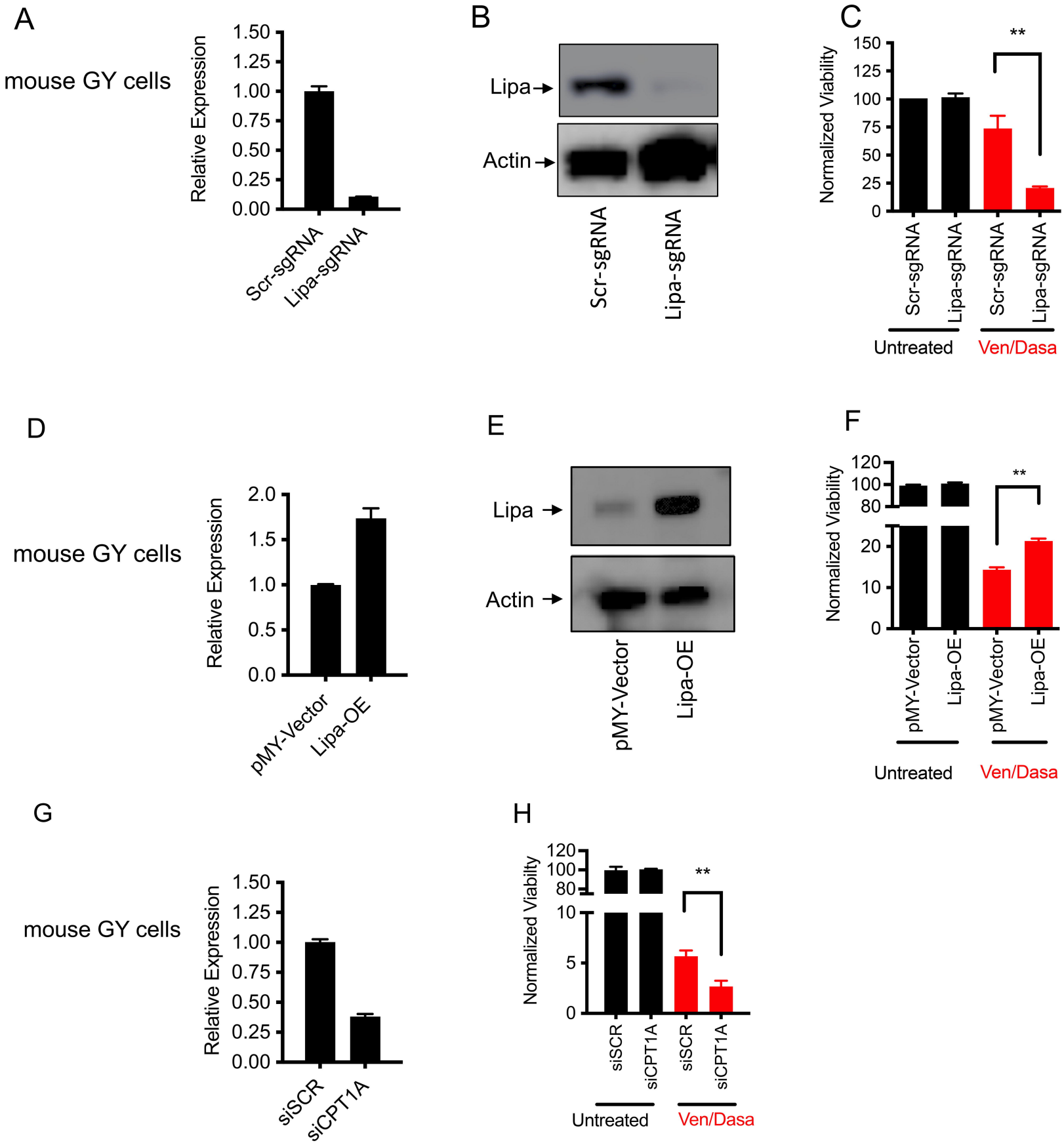
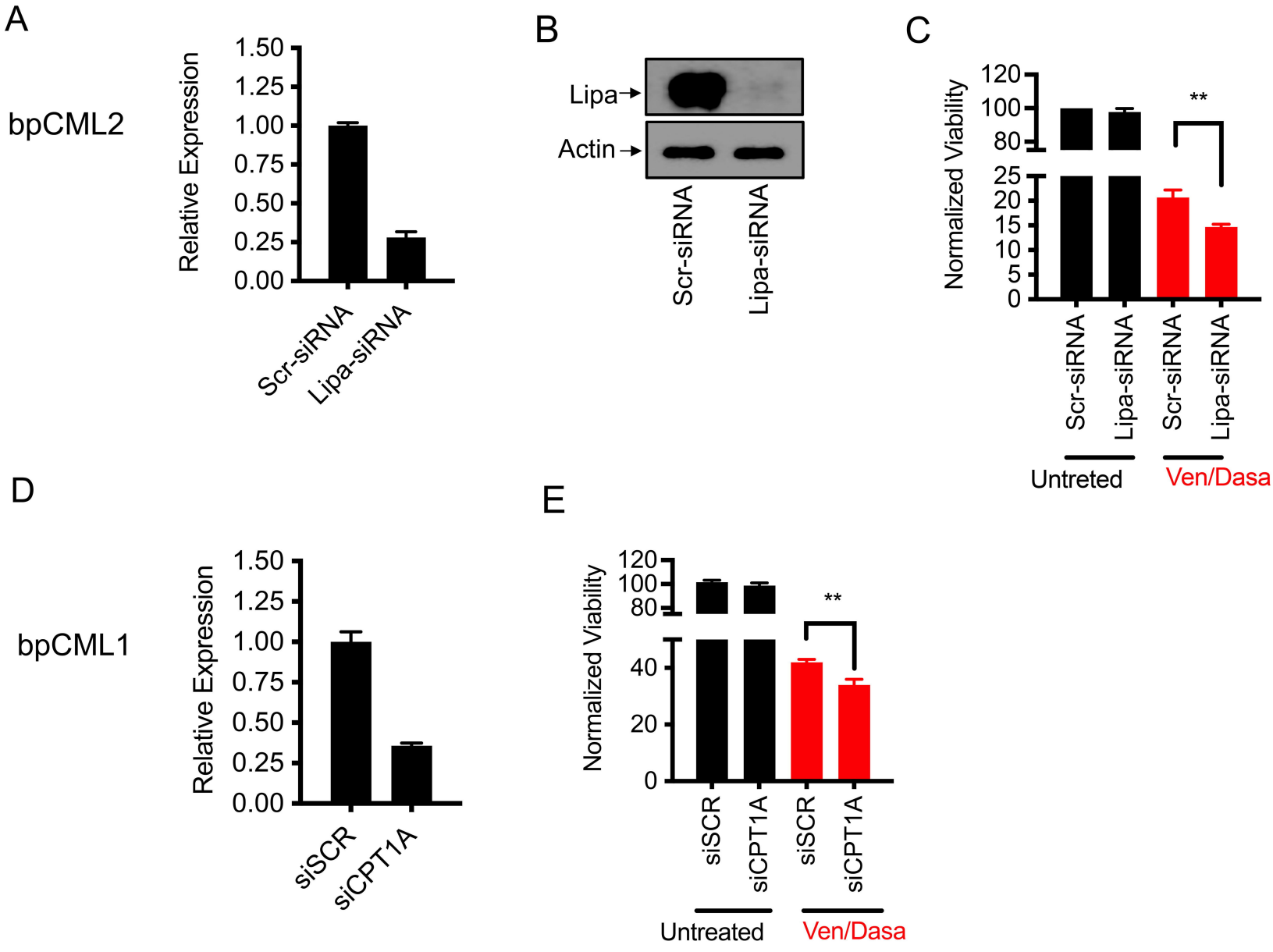

A
mouse GY cells
Relative Expression
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Scr-sgRNA
Lipa-sgRNA
B
Lipa
Actin
Scr-sgRNA
Lipa-sgRNA
C
Normalized Viability
150
125
100
75
50
25
0
**
Scr-sgRNA
Lipa-sgRNA
Scr-sgRNA
Lipa-sgRNA
Untreated
Ven/Dasa
D
mouse GY cells
Relative Expression
2.0
1.5
1.0
0.5
0.0
pMY-Vector
Lipa-OE
E
Lipa
Actin
pMY-Vector
Lipa-OE
F
Normalized Viability
120
100
80
20
10
0
**
pMY-Vector
Lipa-OE
pMY-Vector
Lipa-OE
Untreated
Ven/Dasa
G
mouse GY cells
Relative Expression
1.50
1.25
1.00
0.75
0.50
0.25
0.00
siSCR
siCPT1A
H
Normalized Viabilty
120
100
80
10
5
0
**
siSCR
siCPT1A
siSCR
siCPT1A
Untreated
Ven/Dasa

Figure 8


A
bpCML2
Relative Expression
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1.00
0.75
0.50
0.25
0.00
Scr-siRNA
Lipa-siRNA
B
Lipa
Actin
Scr-siRNA
Lipa-siRNA
C
Normalized Viability
120
100
80
25
20
15
10
5
0
**
Scr-siRNA
Lipa-siRNA
Scr-siRNA
Lipa-siRNA
Untreted
Ven/Dasa
D
bpCML1
Relative Expression
1.50
1.25
1.00
0.75
0.50
0.25
0.00
siSCR
siCPT1A
E
Normalized Viability
120
100
80
40
20
0
**
siSCR
siCPT1A
siSCR
siCPT1A
Untreated
Ven/Dasa

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**.

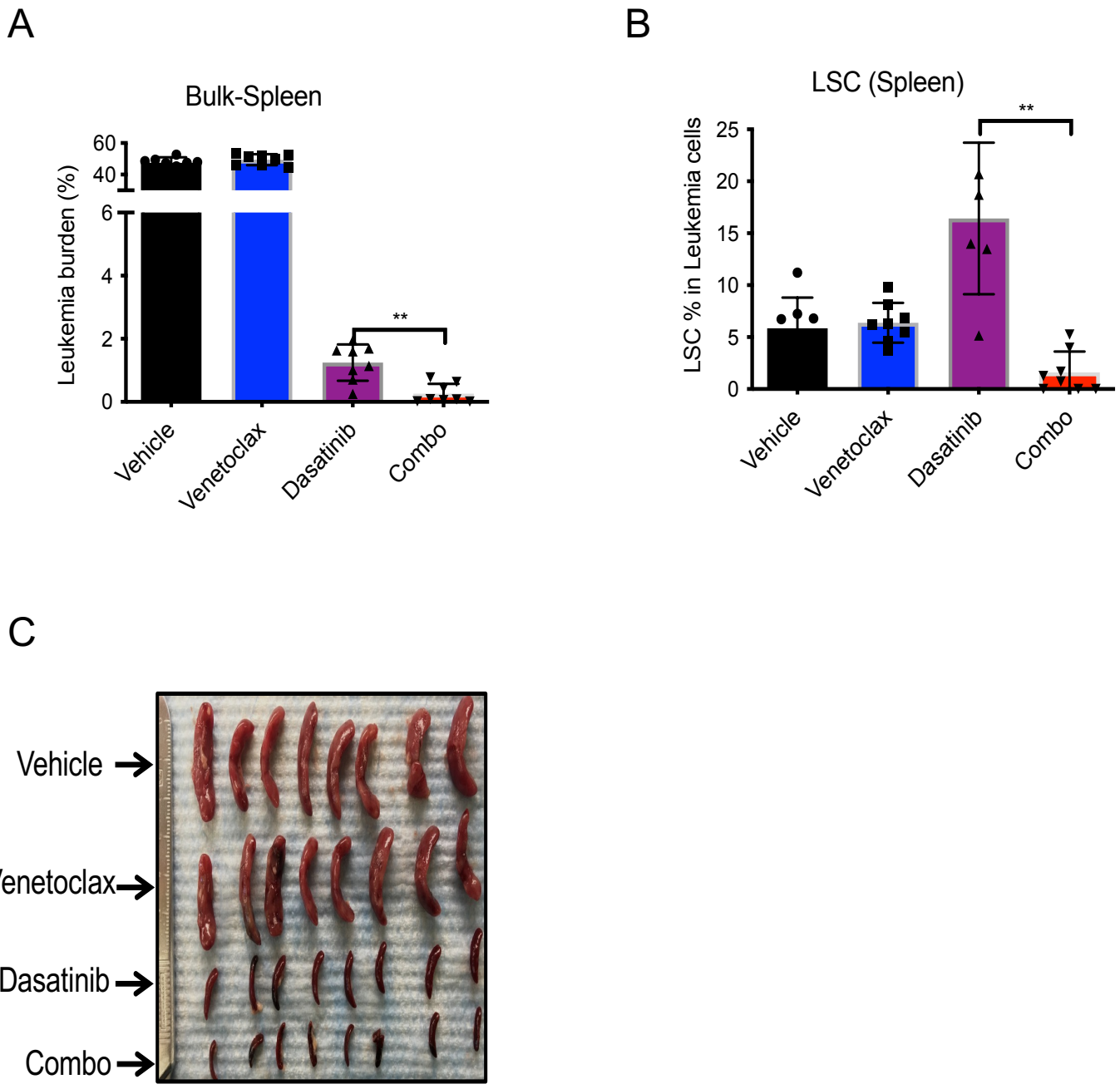
Supplementary Table 2. List of antibodies for Western blot.

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

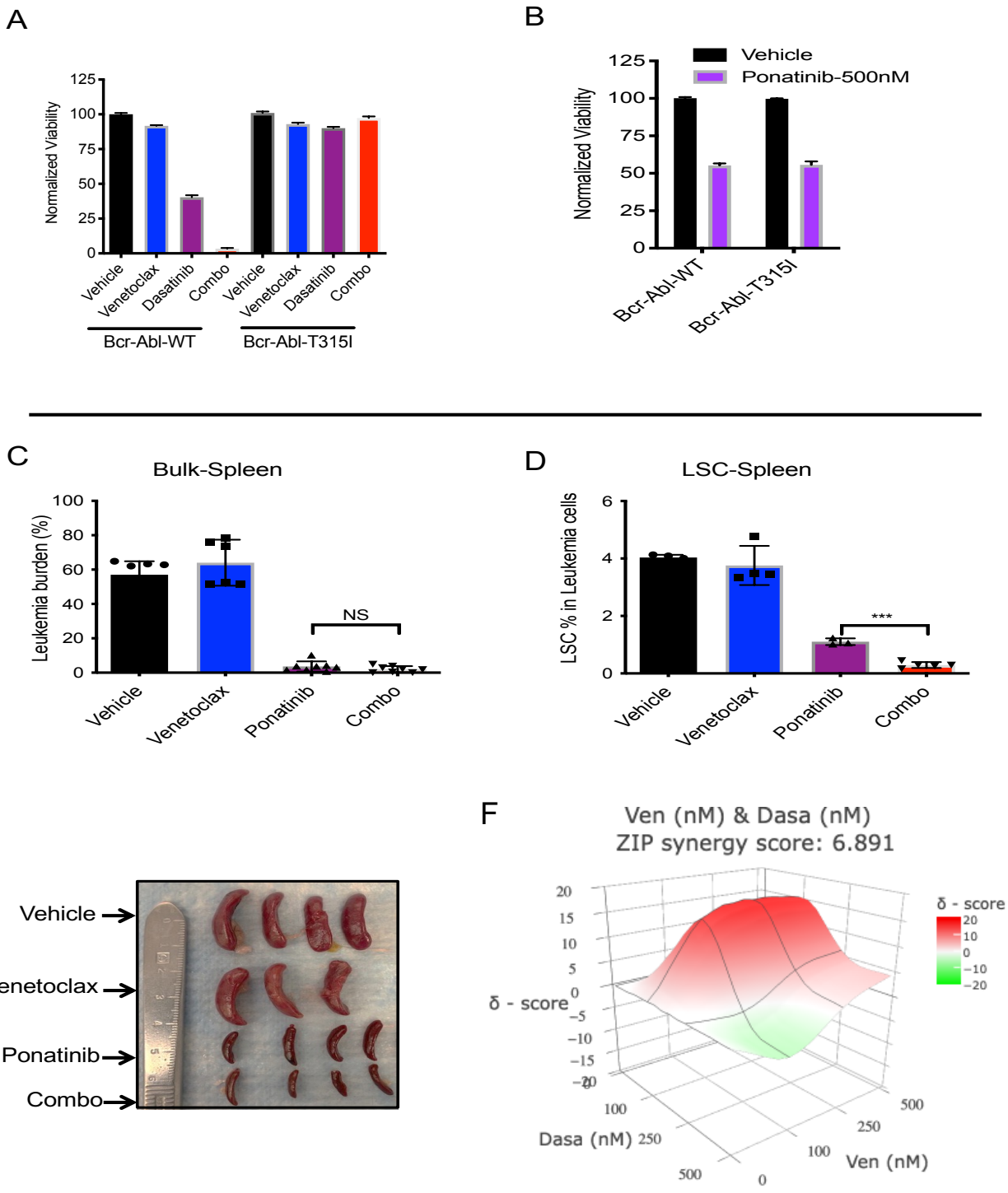
APC Annexin V	BD Biosciences	550475
CPT1A (D3B3) Rabbit mAb	Cell Signaling	12252
GAPDH Mouse monoclonal	Santa Cruz Biotechnology	Sc-32233
Actin	Santa Cruz Biotechnology	(sc-8432 HRP)
LIPA Mouse monoclonal	Santa Cruz Biotechnology	GAPDH Mouse monoclonal

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

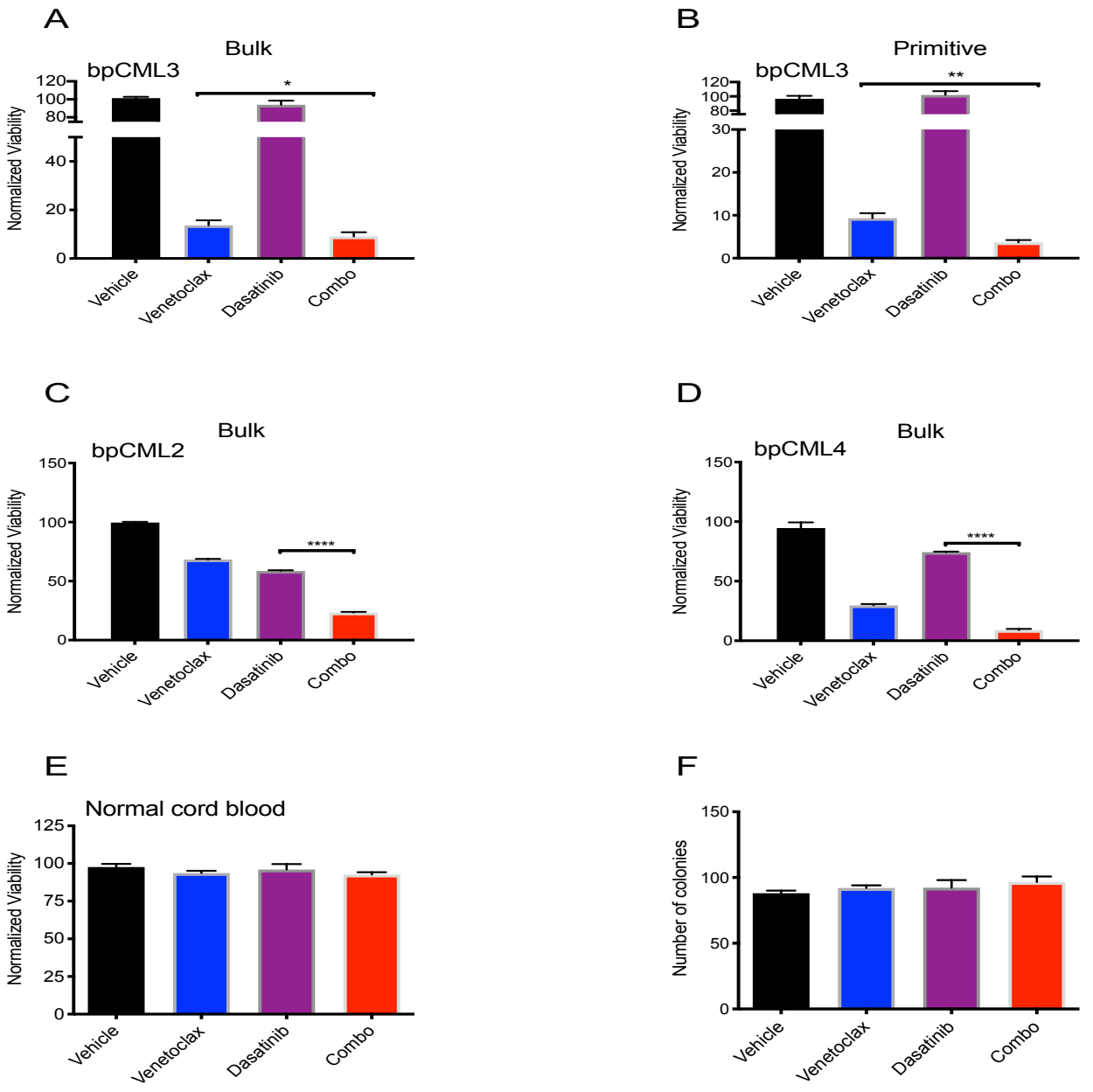


Supplementary Figure 1. Venetoclax/Dasatinib (ven/dasa) combination targets bulk and LSC compartment in an extramedullary site (spleen) in a blast phase chronic myeloid leukemia (bpCML) mouse model. Leukemic mice were treated with vehicle, Ven alone (100mg/kg/day/oral gavage), Dasa alone (20mg/kg/day/OG), 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

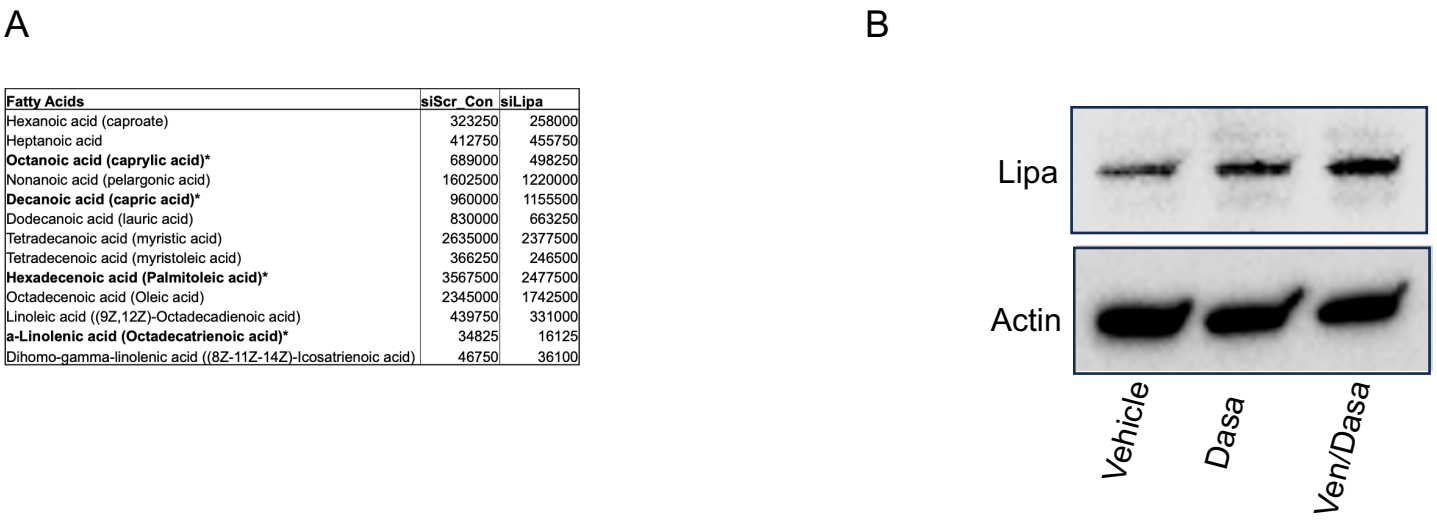


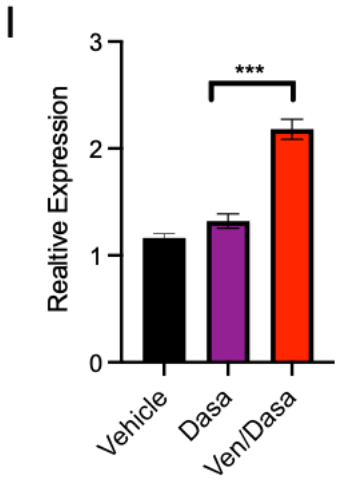
Supplementary Figure 3. Venetoclax/Dasatinib (ven/dasa) combination targets bulk and leukemia stem cell (LSC) compartments in human primary blast phase chronic myeloid leukemia (bpCML). Viability of bpCML patient samples after In Vitro treatment with Ven (100nM), Dasa (100nM), and the combination after 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

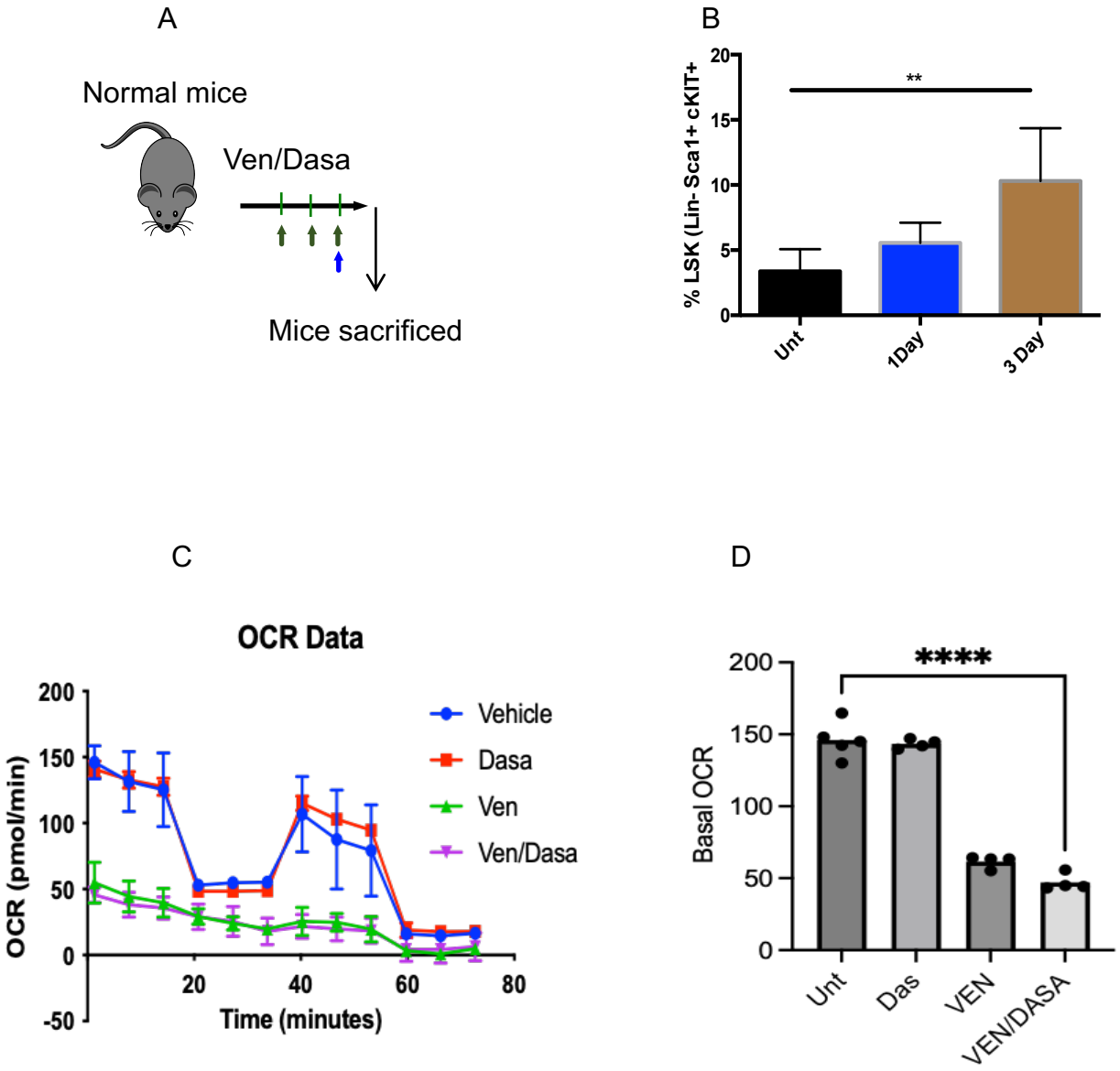
Fatty Acids	siScr_Con	siLipa
Hexanoic acid (caproate)	323250	258000
Heptanoic acid	412750	455750
Octanoic acid (caprylic acid)*	689000	498250
Nonanoic acid (pelargonic acid)	1602500	1220000
Decanoic acid (capric acid)*	960000	1155500
Dodecanoic acid (lauric acid)	830000	663250
Tetradecanoic acid (myristic 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





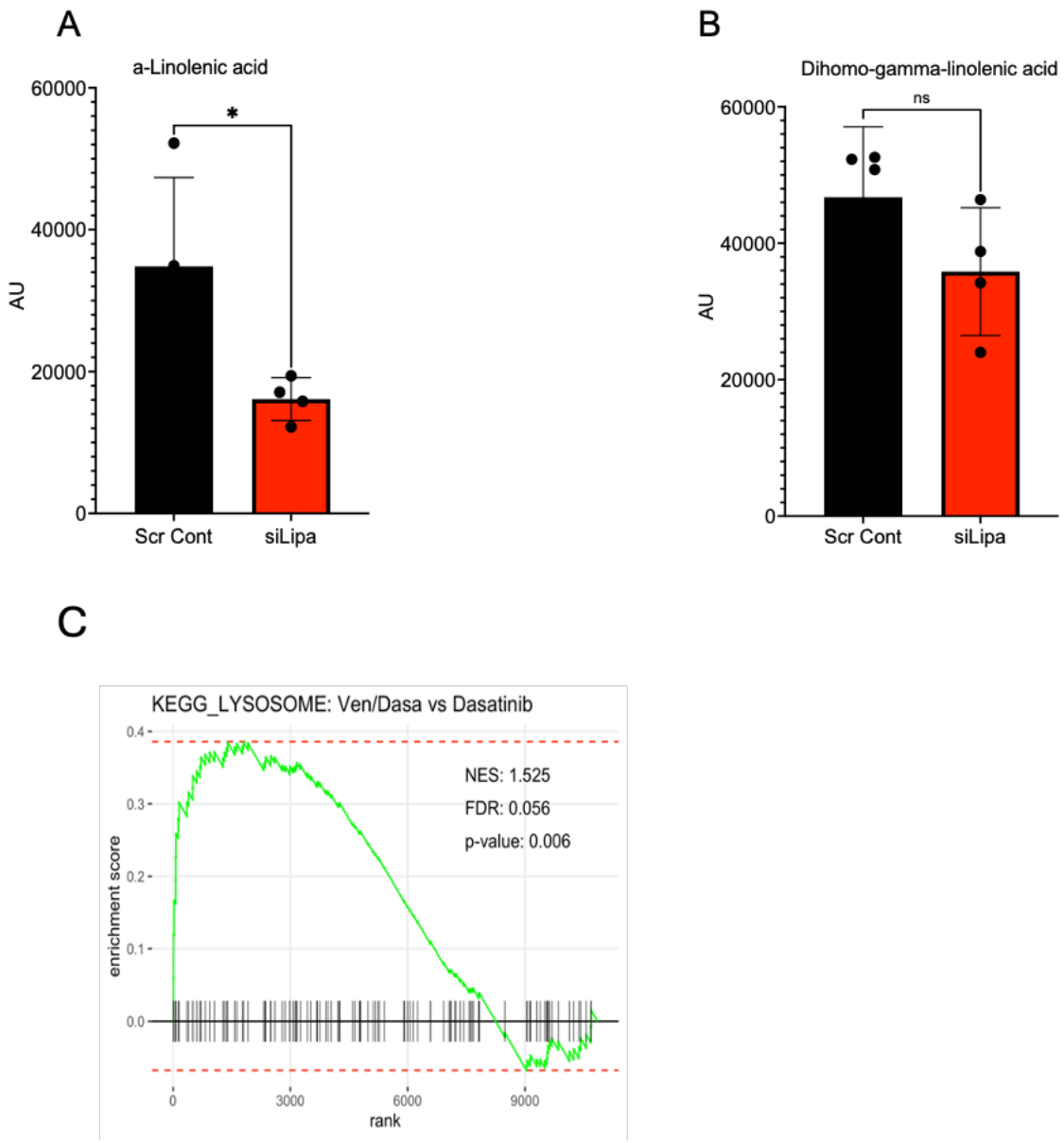
Supplementary Figure 4. (A) Leukemia cells were electroporated with siRNA against Lipa or siScr as a control. After 24 hours cells were analyzed by mass spectrometry to determined levels of free fatty acids. Bold and stared(*) free fatty acids were significantly decreased (AU) after lipa knockdown.(B) 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.