Supplementary information for

Targeting sphingosine kinase 1 induces MCL1 dependent cell death in acute myeloid leukemia

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SUPPLEMENTARY MATERIAL AND METHODS

Constructs

pTRIPZ(PL) was digested with AgeI and EcoRI, blunted and self-ligated to produce pTIPZ(PL). SPHK1(FLAG) IRES mCHERRY and MCL1 IRES EGFP were cloned into pTIPZ(PL) following digestion with EcoRI. shRNA target sequences were from Fellmann *et al* and subcloned into pTRIPZ. Renilla luciferase shRNA sequences (Ren713) were used as non-targeting control sequences. pTRIPZ was modified with the addition of a novel polylinker following digestion with ClaI/MluI and ligation of annealed oligonucleotides 5'-CGATGAATTCGTTAACCTCGAGA-3'and 5'-CGCGTCTCGAGGTTAACGAATTCAT-3'. Primers 5'-TAGAATTCTCGACTTCTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTG ACAGTGAGCG-3' and 5'-TCTCGAATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGC-3' and the following 97-mer oligonucleotide templates were used to amplify the mir30/shRNA target sequences. SPHK1(1) 5'-TGCTGTTGACAGTGAGCGATGGCGTCATGCATCTGTTCTATAGTGAAGCCACAGA TGTATAGAACAGATGCATGACGCCAGTGCCTACTGCCTCGGA-3', SPHK2(1) 5'-TGCTGTTGACAGTGAGCGACCGGTTGCTTCTATTGGTCAATAGTG AAGCCACAGATGTATTGACCAATAGAAGCAACCGGGTGCCTACTGCCTCGGA-3' and Ren713 5'-TGCTGTTGACAGTGAGCGCAGGAATTATAATGCTTATCTATAGTGAAGCCACAGA TGTATAGATAAGCATTATAATTCCTATGCCTACTGCCTCGGA-3'. PCR products were digested with EcoRI and cloned into pTRIPZ(PL) to produce shRNA constructs pTRIPZ-SPHK1(1), pTRIPZ-SPHK2(1) and pTRIPZ-Ren713. Sequencing verified integrity and orientation of the respective shRNA vectors. Transduced cells were tracked by red fluorescent protein (RFP).

RNA-sequencing and bioinformatics

Poly(A) enriched mRNA (New England Biolabs, Ipswich, MA) from control and MP-A08 treated MV4-11 cells in triplicate (biological replicates) were sequenced on the Illumina HiSeq 2500 platform using the stranded single end protocol with a read length of 50. Raw reads were adaptor trimmed and filtered for short sequences using cutadapt v1.3,[2] setting minimum-length option to 18, error-rate 0.2, overlap 5 and a quality score cut-off of 28. The resulting FASTQ files averaging 42 million reads per sample (supplemental Table 2) were analysed and quality checked using the FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The filtered reads were mapped against the human reference genome (Hg19) using the TopHat spliced alignment algorithm[3] (version 2.0.9 with default parameters) returning an average alignment rate of 98% (supplemental Table 2). Counts of reads mapping to each gene were calculated using HTSeq v0.6.1p1[4]. Differential expression analysis was evaluated from TMM normalized gene counts using R (version 3.2.3) and edgeR[5] (version 3.3) following protocols as previously described.[6,7] Functional analyses were performed using the topGO package in R. Alignments were visualised and interrogated using the Integrative Genomics Viewer v2.3.80.[8]

Quantitative RT-PCR

RNA extracted from MV4-11 cells was used to generate cDNA using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's protocol. Quantitative RT-PCR analysis was performed on the cDNA using the QuantiTect SYBR Green PCR master mix (Qiagen). PCR reactions containing 2µl of cDNA (1:5 diluted), 0.5µl of each of forward and reverse oligo primers (100ng/mL), 5µl of QuantiTect SYBR Green PCR master mix, and 2µl of RNAse-free water was performed using a Rotor-Gene 6000 real time PCR machine (Qiagen) with the following thermo-cycling condition: 50ºC for 2 min, 95ºC for 15 min,

followed by 40 cycles of 95ºC for 15 sec, 58ºC for 21 sec, and 72ºC for 15 sec. Melt curve analysis was performed at the end of each PCR reaction to verify the PCR products. Relative expression of the *S1PR2-5* was analysed using the Rotor-Gene Q Series software (Qiagen) using the comparative quantitation method with MV4-11 amplified *S1PR2* used as the calibrator whereas *SPHK1* was quantitated relative to *GAPDH* and *MCL1* was quantitated relative to *ACTB.*

To confirm the lack of *S1PR1* gene expression in MV4-11 cells, the quantitative RT-PCR was performed on the ovarian cancer cell line SKOV3 and resolved on by agarose gel electrophoresis (168bp).

S1PR1 transcript was amplified by the oligo primers: 5'-GAGCACTACGCAGTCAGTCG-3' (forward) and 5'- GGTGGTTCGATGAGTGATCC -3' (reverse); *S1PR2* transcript was amplified by the oligo primers: 5'-ACCATGGGCAGCTTGTACTC -3' (forward) and 5'- TGAGCACCAGAAGGTTTTCC -3' (reverse); *S1PR3* transcript was amplified by the oligo primers: 5'-CTGCCCCTCTACTCCAAGAA -3' (forward) and 5'- AACACGCTCACCACAATCAC -3' (reverse); *S1PR4* transcript was amplified by the oligo primers: 5'-AGCCTTCTGCCCCTCTACTC -3' (forward) and 5'- AGGATCATCAGCACCGTCTT -3' (reverse); *S1PR5* transcript was amplified by the oligo primers: 5'-ACAACTACACCGGCAAGCTC -3' (forward) and 5'- CAGATCCGACAACGTGAGG -3' (reverse); *SPHK1* transcript was amplified by the oligo primers: 5' TCTGGTGGTCATGTCTGGAG-3' (forward) and 5'- GACCTGCTCATAGCCAGCAT -3' (reverse); and *GAPDH* transcript was amplified by the oligo primers: 5'-ACCCAGAAGACTGTGGATGG -3' (forward) and 5'- CAGTGAGCTTCCCGTTCAG -3' (reverse); *MCL1* transcript was amplified by the oligo primers: 5' AAGAGGCTGGGATGGGTTTGTG-3' (forward) and 5'- TTGGTGGTGGTGGTGGTTGG

-3’ (reverse); and *ACTB* transcript was amplified by the oligo primers: 5’-GAGGCACTCTTCCAGCCTT -3’ (forward) and 5’- AAGGTAGTTTCGTGGATGCC -3’(reverse).

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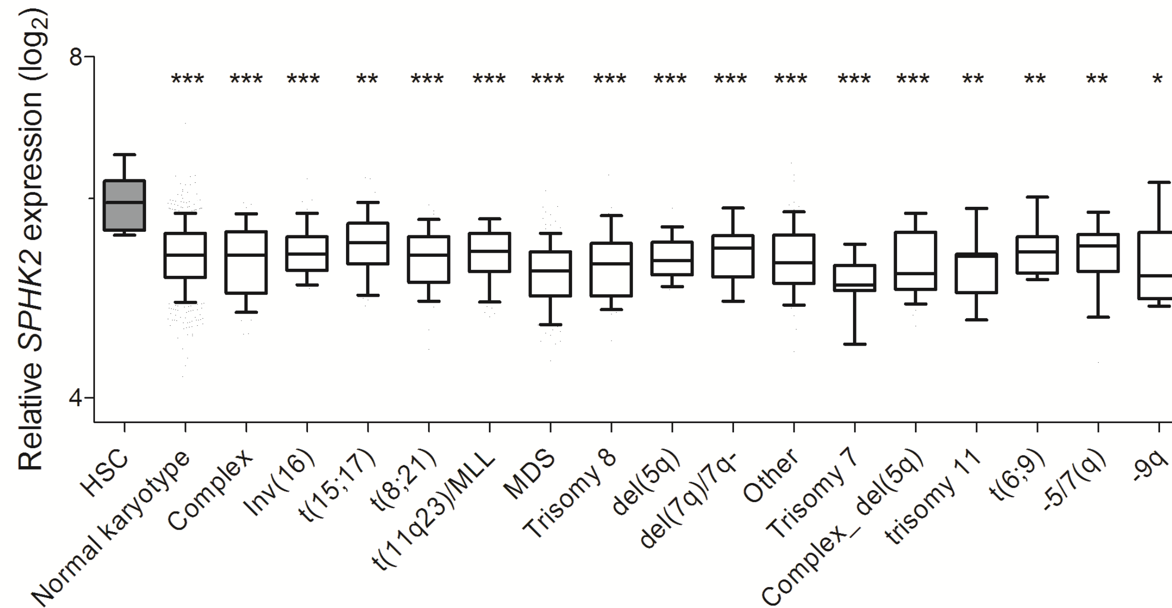
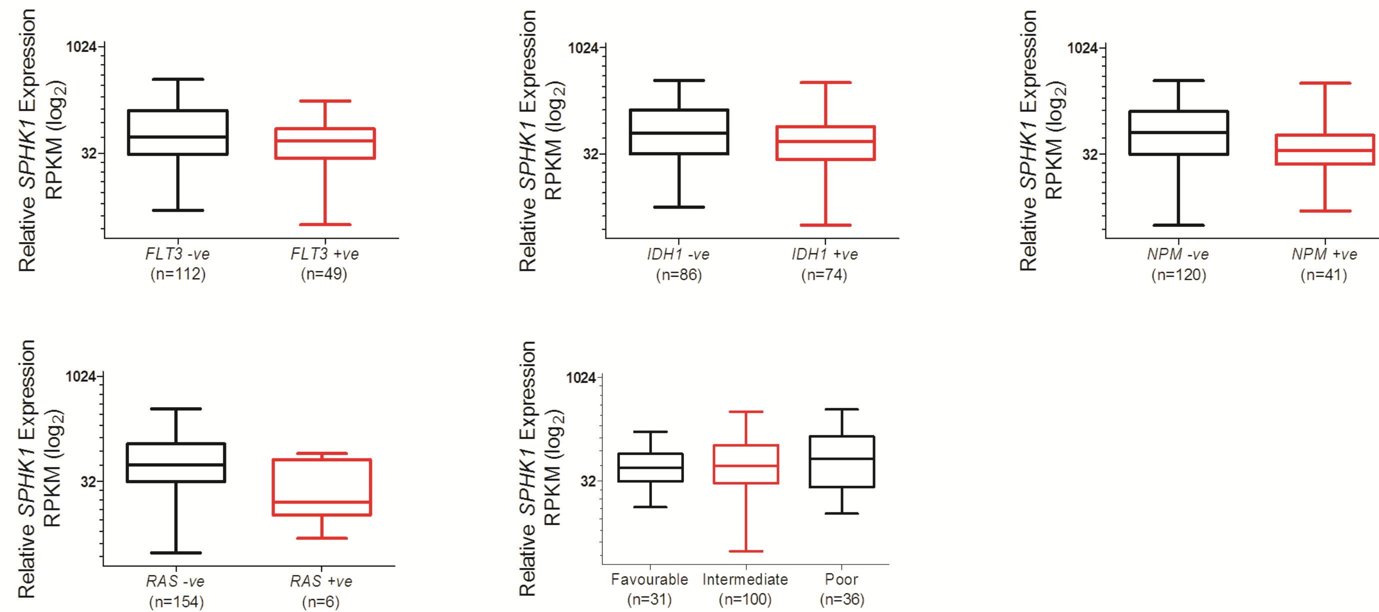
Supplementary Table 2. Summary of RNA-Seq alignment

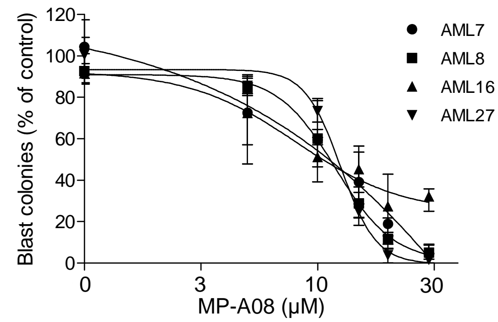
Sample	Raw reads	Reads After Trimming	Reads Mapped	Overall Alignment Rate (%)
Vehicle r1	36,211,011	36,033,330	35,409,280	98.3
Vehicle r2	42,368,430	42,143,911	41,423,555	98.3
Vehicle r3	44,463,363	44,228,054	43,488,610	98.3
MP-A08 r1	39,498,494	39,289,755	38,521,179	98.0
MP-A08 r2	48,959,840	48,678,691	47,691,457	98.0
MP-A08 r3	45,917,107	45,696,515	44,784,747	98.0



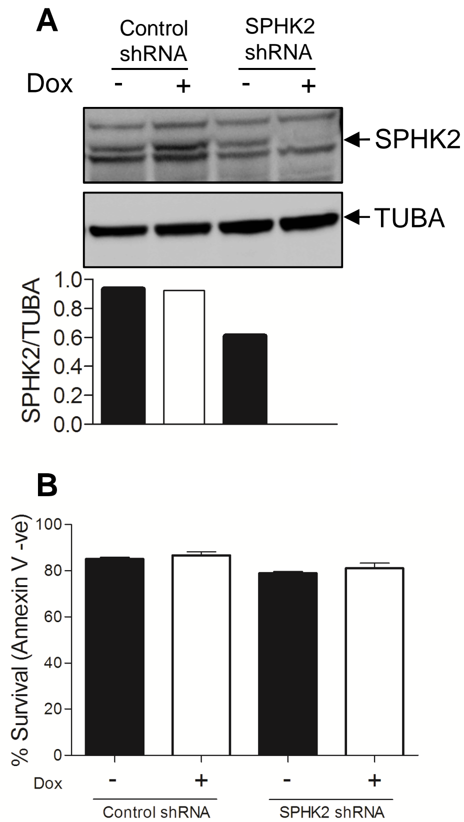
Supplemental Figure 1: *SPHK2* is under-expressed in AML. Microarray analysis of *SPHK2* mRNA levels from FACs purified hematopoietic stem cells (Lin−/CD34+/CD38−/CD90+/CD45RA−) and AML cells from various cytogenetic subgroups obtained from BloodSpot (http://servers.binf.ku.dk/bloodspot/?gene) (median, 25 to 75 percentiles boxed, and 10 to 90 percentiles shown with bars). Significance was assessed by Student's t-test, * $p<0.05$, **$p<0.01$, **$p<0.001$



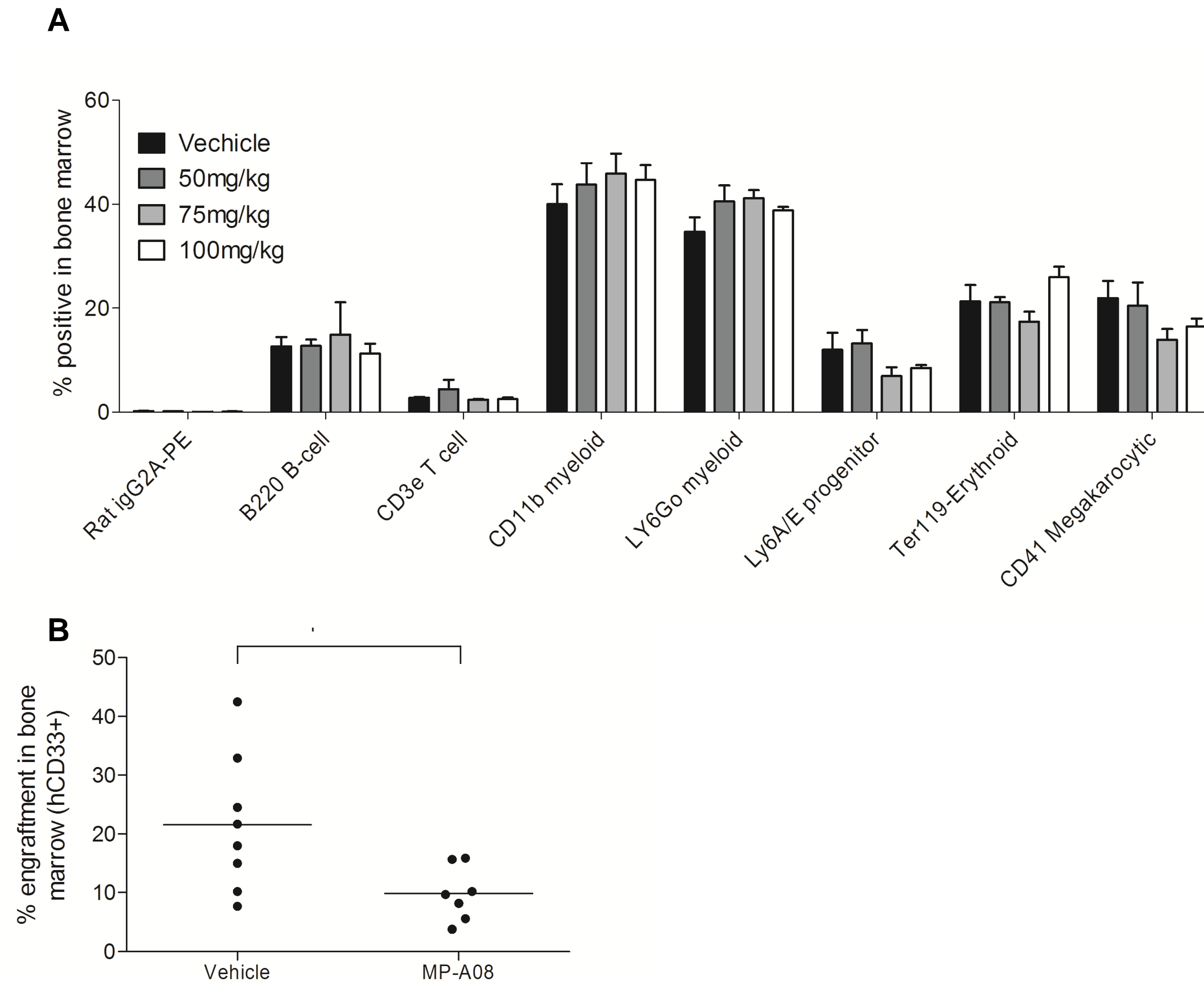
Supplemental Figure 2: ***SPHK1* expression does not correlate with mutational status nor cytogenetic risk group.** Normalized RNA-Seq reads (Reads Per Kilobase per Million mapped reads; RPKM) for *SPHK1* from primary AML patient samples (n=171) obtained from TCGA were stratified by; i) mutational status of *FLT3, IDH1, NPM* and *RAS* and ii) cytogenetic risk group (median, 25 to 75 percentiles boxed, and 1 to 100 percentiles shown with bars). Significance was assessed by Student's t-test.



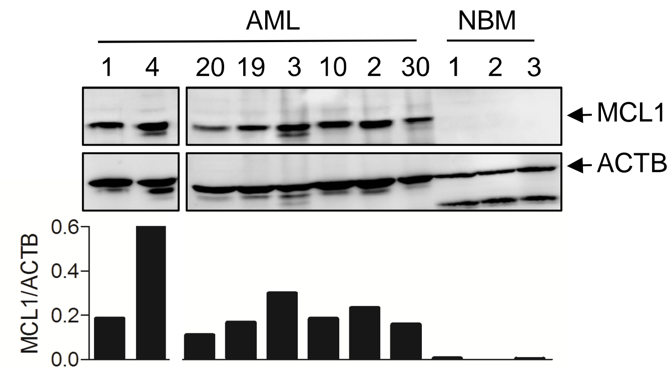
Supplemental Figure 3: **Targeting SPHK1 blocks colony formation in AML.** AML blasts (n=4) were plated in methyl cellulose containing 2ng/ml IL-3 and 50ng/ml GM-CSF with the indicated concentrations of MP-A08 and leukemic (CFU-L) colonies were counted using an inverted microscope. Data represent mean ± SD.



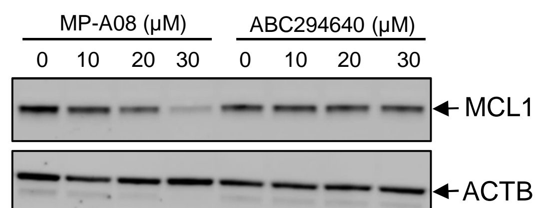
Supplemental Figure 4: Targeting SPHK2 does not induce AML cell death.
(**A**) MV4-11 cells were transduced with doxycycline-inducible shRNA constructs to knockdown SPHK2 and a non-targeting control shRNA. Cells were incubated with 1ng/ml doxycycline and 48 h post-induction immunoblot analysis of whole cell lysates was performed and quantified by laser densitometry (ratio of SPHK2/TUBA) or (**B**) cell survival determined by Annexin V staining (mean, n=3 ± SEM). Significance was assessed by Student's t-test.



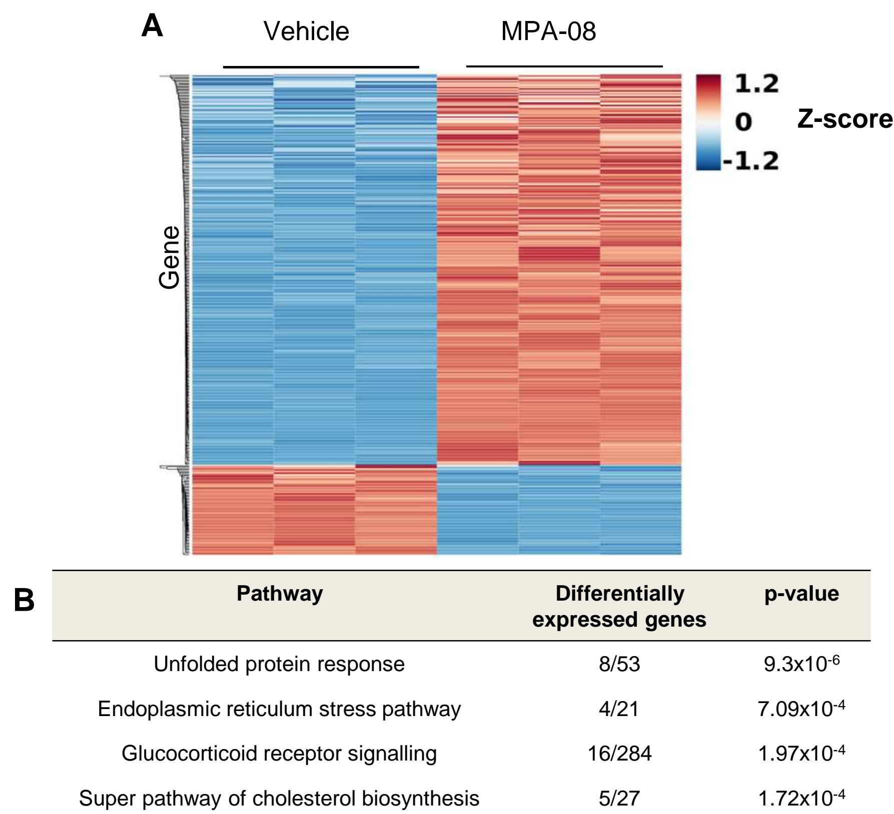
Supplemental Figure 5: **MP-A08 is well tolerated in mice**. (**A**) NOD-SCID mice treated with MP-A08 by i.p at 50, 75 or 100 mg/kg daily for 2 weeks. Following this bone marrow cells were harvested and the major cell lineages quantified by flow cytometry. Lineages analysed included B-cells (B220), T cells (CD3e), myeloid cells (CD11b and LY6Go), progenitor cells (Ly6A/E), erythroid cells (Ter119) and megakaryocytes (CD41). (**B**) NOD-SCID mice were engrafted with primary AML blasts and after disease was established, mice were i.p injected with either vehicle (V) or MP-A08 (100mg/kg) six times a week for two weeks. Engraftment of human myeloid cells was quantified by flow cytometry using anti-CD33



Supplemental Figure 6: MCL1 is over-expressed in AML. Primary AML blasts or normal bone marrow mononuclear (NBM) cells where lysed and subjected to SDS-PAGE followed by Western blot analysis of MCL1 and ACTB. Western blot results were quantified by laser densitometry and expressed as the ratio of MCL1/ACTB.

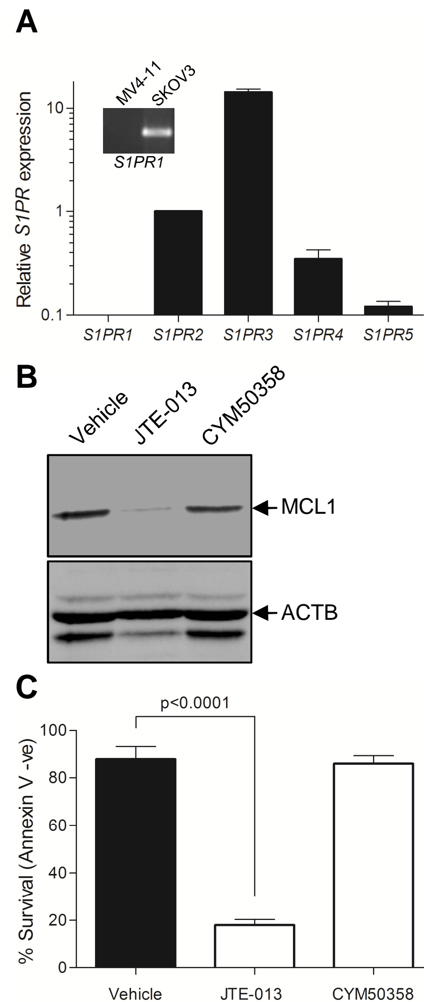


Supplemental Figure 7. Targeting SPHK2 does not alter MCL1 levels in AML.
MV4-11 cells were incubated with either the MP-A08 or ABC294640 for 16 h following which whole cell lysates were immunoblotted with the indicated antibodies.



Pathway	Differentially expressed genes	p-value
Unfolded protein response	8/53	9.3×10^{-6}
Endoplasmic reticulum stress pathway	4/21	7.09×10^{-4}
Glucocorticoid receptor signalling	16/284	1.97×10^{-4}
Super pathway of cholesterol biosynthesis	5/27	1.72×10^{-4}

Supplemental Figure 8. MP-A08 induced transcriptional changes in MV4-11 cells assessed by RNA-Seq.
(**A**) Heatmap of differentially regulated genes in MV4-11 cells induced by 6h treatment of MPA-08 (20μM) as determined by RNA-Seq (n=3, biological replicates). Heat map was generated through hierarchical clustering of gene expression pattern similarities for all control and MP-A08 treated replicate samples using only significant differentially expressed genes identified in the study (N = 410 (333 Up and 77 Down), FDR <= 0.05 and fold change >= 2) (Supplementary Table 1). Z-scores were calculated and graphed for each gene across all samples. Clustering by similar gene expression profiles showed that the samples openly grouped into their respective treatment as was also observed in the multi-dimensional scaling plot (MDS, not shown) for the same data. (**B**) Ingenuity Pathway Analysis of biological pathways enriched for differentially expressed genes upon MP-A08 treatment.



Supplementary Figure 9: **Antagonism of S1PR2 but not S1PR4 induces loss of MCL1 and AML cell death.**
(**A**) quantitative RT-PCR profiling of *S1PR1-5* in MV4-11 cells (mean ± SEM, n=3) and validation of *S1PR1* primers in the ovarian cancer cell line SKOV3 (insert). MV4-11 cells were incubated with either the S1PR2/4 antagonist JTE-013 (10µM) or the S1PR4 selective antagonist CYM50358 (1µM) for 16 h following which whole cell lysates were immunoblotted with the indicated antibodies (B) or for 48h and cell survival determined by Annexin V staining (mean ± SD, n=4). Significance was assessed by Student's t-test.