Supplemental material:

Apolipoprotein C2 - CD36 Promotes Leukemia Growth and Presents a Targetable Axis in Acute Myeloid Leukemia

Authors: Tian Zhang[1], Jiawen Yang[2], Vijaya P. Vaikari[2], John S. Beckford[2], Sharon Wu[2], Mojtaba Akhtari[1,3], Houda Alachkar[2,3]*

Affiliations:

[1] Keck School of Medicine, University of Southern California, Los Angeles, CA
[2] USC School of Pharmacy, University of Southern California, Los Angeles, CA
[3] USC Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA

***Corresponding Author**:
Houda Alachkar, PharmD, Ph.D.
Assistant Professor
University of Southern California, School of Pharmacy
1985 Zonal Avenue John Stauffer Pharmaceutical Sciences Center Room 608
Los Angeles CA 90089
Telephone: 323-442-2696, Email: alachkar@usc.edu

Conflicts of interest statement:

The authors have declared that no conflict of interest exists.

Key Words:
AML, APOC2, CD36, ERK, MLL rearrangement

Running Title:
The role of APOC2-CD36 interaction in AML

Supplemental Methods

Plasmid constructs

The PLVX-APOC2-AcGFP-N1 plasmid was constructed by cloning the APOC2 cDNA from U937 into the *XhoI/BamH1* sites of the PLVX-AvGFP-N1 (Clontech). pLKO.1 - TRC cloning vector was a gift from David Root (Addgene plasmid # 10878 ; http://n2t.net/addgene:10878 ; RRID:Addgene_10878)(1). Tet-pLKO-puro was a gift from Dmitri Wiederschain (Addgene plasmid # 21915; http://n2t.net/addgene:21915 ; RRID:Addgene_21915)(2). Small hairpin RNAs (shRNAs) of APOC2 targeting TGCTGAAGGGAGAGGAGTAAC and AGTTACTGGGAGTCAGCAAAG were inserted into both the pLKO.1-TRC and Tet-pLKO-puro vectors. shRNAs of CD36 targeting CCGACGTTAATCTGAAAGGAA and AGAACCTATTGATGGATTAAA also were inserted into those two plasmids to create lentiviral knockdown CD36 plasmids. mCherry-CD36-C10 was a gift from Michael Davidson (Addgene plasmid # 55011 ; http://n2t.net/addgene:55011 ; RRID:Addgene_55011). The pCDH-EF1-CD36-FHC plasmid was constructed by cloning CD36 cDNA from THP-1 into *EcoRI/NotI* sites of PCDH-EF1-FHC. pCDH-EF1-FHC was a gift from Richard Wood (Addgene plasmid # 64874 ; http://n2t.net/addgene:64874 ; RRID:Addgene_64874)(3). All constructs were confirmed by sequencing (Genewiz, Cambridge, MA, USA).

Cell viability and colony-forming cell assays

Trypan blue assay was used to count the cells either manually or using the Countess II FL automatic cell counter (Life Technologies, Carlsbad, CA, USA) to determine cell viability. The alamarBlue Cell Viability Reagent (ThermoFisher, Waltham, MA, USA) also was used to confirm cell viability. Cells were washed and replaced with fresh medium. For each sample, 100 µl of homogenous cells were subsequently incubated with 10 µl of alarmaBlue dye for 1–4 h at 37°C. The BioTek Synergy H1 Hybrid Multi-Mode Microplate Read Machine (BioTek Instruments, Inc., Winooski, VT, USA) was used to detect fluorescence. All the counting experiments were independently repeated at least three times. Experimenters were blind to conditions during the counting procedures.

Colony-forming cell (CFC) assays were conducted by plating 5×10^4 primary blasts in 0.9% MethoCult (StemCell Technologies, Vancouver, Canada) supplemented with StemSpan CC100. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 10–14 days. Colony-forming units were counted by experimenters blind to the condition. All experiments were performed in duplicate and repeated at least three times.

Immunoblot analysis

To conduct immunoblotting, cells were washed with phosphate buffered saline (PBS) and lysed in Pierce IP Lysis Buffer (ThermoFisher) supplemented with a complete protease inhibitor cocktail (Pierce; ThermoFisher). Cell lysates were microcentrifuged for 10 min at 10,000 × *g* at 4°C, and the supernatants were collected. Cell lysates were measured using the Pierce 660-nm Protein Assay Reagent (ThermoFisher) using the NanoDrop One (Thermo Scientific). Lysates then were resolved by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% non-fat milk or bovine serum albumin for 1 h, and probed with the following antibodies: anti-human APOC2 (Invitrogen, PA1-27196, 1:2,000), anti-phospho-P42/44 (Cell Signaling Technology, Danvers, MA, USA; 9101S, 1:1,000), anti-total-P42/44 (Cell Signaling Technology, 9102S, 1:1,000), anti-Actin (Cell Signaling Technology, 3700S, 1:2,000), and anti-GAPDH (Santa Cruz BioTechnology, Dallas, TX, USA; SC-32233, 1:2,000). Horseradish peroxidase (HRP)-conjugated goat secondary antibodies also were used (Invitrogen, 1:3,000). Immunodetection was achieved using the Pierce ECL Western Blotting Substrate Reagent (ThermoFisher) and ChemiDoc Touch machine (Bio-Rad). Western blot band density was evaluated using ImageJ analysis. For the same batch of samples, the same blot was stripped and probed repeatedly to get the full set of antibody detections. Each western blot was repeated three times independently.

Immunoprecipitation

Cells were washed with ice-cold PBS, lysed in Pierce IP Lysis Buffer (ThermoFisher) supplemented with a complete protease inhibitor cocktail (Pierce; ThermoFisher). After one-hour incubation, cell lysates were centrifuged for 10 min at 10,000 × *g* at 4°C, and the supernatants were collected. Cell lysates were measured using the Pierce 660-nm Protein Assay Reagent (ThermoFisher) using the NanoDrop One (Thermo Scientific). Cell lysates were rotated at 4 °C for at least 30 min. Cell lysates were used for IP with the indicated antibodies. Generally, 1–4 μg commercial antibody was added to 300ug cell lysates, which was then incubated at 4 °C for 3-4 h. After addition of protein A/G agarose beads, incubation was continued for overnight. Immunoprecipitates were extensively washed with IP lysis buffer for 3-5 times and then eluted with SDS–PAGE loading buffer by boiling for 5 min.

RNA extraction, cDNA synthesis, and real-time PCR analysis

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). A total of 1 μg of total RNA was used for cDNA synthesis using the SuperScript® IV First-Strand Synthesis System (Thermo Fisher). Quantitative real-time PCRs were carried out using the Applied Biosystems PowerUp SYBR Green Master Mix (Thermo Fisher). Samples were obtained and analyzed using the Applied Biosystem 7900HT Fast Real-Time PCR System (Thermo Fisher). The gene expression levels were normalized to Actin. The primer sequences of *APOC2* used for qPCR were 5'-CTATAAATCCTCTCTGTGCCCG-3' (forward) and 5'-GGACCTCAAATCCCAATACCAG-3' (reverse). The primer sequences of CD36 used for

qPCR were 5'- GCCAGGTATTGCAGTTCTTTTC-3' (forward) and 5'- TGTCTGGGTTTTCAACTGGAG-3'(reverse).

Lentiviral production

To produce lentivirus, HEK293T cells were transfected with PLVX or PLKO.1 plasmids together with packaging plasmids psPAX and pMD2.G using Calcium Phosphate Transfection Kits (Clontech). Viral particles were collected 72 h after transfection, filtered by a 0.45-μm sterile filter and concentrated in PEG reagent at 4°C overnight. The concentrated viral particles then were centrifuged at 2,000 × *g* for 30 min. Viral pellets were resuspended in complete cell culture medium and stored in -80°C.

Flow cytometry

Cells were harvested from peripheral blood and bone marrow for immunophenotype analysis. After washing with PBS, cells were stained on ice with various antibodies diluted in PBS for 30 min. Subsequently, cells were washed with PBS and resuspended in PBS for flow cytometry analysis. The PE-Cy5.5 anti-human CD45 (eBioscience, 25-0459-41) was used for flow cytometry analysis.

Apoptosis assay

The apoptosis assay was conducted using the eBioscience Annexin V Apoptosis Detection Kit APC (Thermo Fisher Scientific, 88-8007-72). Cells were harvested after starvation synchronization and were then washed once with PBS and binding buffer. Cells were resuspended in binding buffer at a concentration of 5×10^6 cells/ml. Cells were incubated with APC-conjugated Annexin V at a concentration of 100 μl of the cell suspension for 10–15 min at room temperature. Cells then were washed again with binding buffer. Cells then were incubated with 5 μl of propidium iodide staining solution on ice for 5–10 min, and analyzed by flow cytometry.

Seahorse and cellular metabolic analysis

Oxygen consumption rate (OCR: pmol/min/Norm. Unit) and extracellular acidification (ECAR) (mpH/min/Norm. Unit) were determined in MOLM-13 and THP-1 cells using the Agilent Seahorse XF platform and Seahorse XF Real-Time ATP Rate Assay Kit. On the day of assay, 20,000 of overexpression cells were seeded in each well of Poly-D-Lysine (10μg/ml, Sigma, P6407) coated 96-well Agilent Seahorse XF Cell Culture Microplate. The plate was centrifuged at 200g without brake for 1min. Oligomycin and Rotenone+antimycin A were dissolved and diluted in assay medium and loaded in sensor cartridge in final concentration of 1.5μM and 0.5μM respectively.

APOC2 Methylation Patient Data Analysis

We downloaded publicly available Illumina 450K methylation array data from obtained normal hematopoietic stem cells (HSCs) (N = 5) and leukemia $CD34^{+}CD38^{-}$ cells (LSCs) (N = 15) (GSE63409)(4). We analyzed the methylation data to identify APOC2 methylation pattern differences between healthy individuals and individuals with AML.

APOC2 Methylation PCR Analysis

5-Azacytidine was purchased from Sigma-Aldrich (A2385). Cell lines were treated with 5-Aza at 0nM, 250nM, 500nM, 750nM once every day for 72h. DNA samples from 5-Aza treated cells were bisulfite converted using the Zymo EZ DNA Methylation kit for 12 hours using 500ng of DNA as input according to manufacturer protocol. 500ng of Human Methylated DNA Standard and Human Non-Methylated DNA Standard was also bisulfite converted as a control. Methylation specific PCR (MSP) was performed with a primer pair designed to detect methylation in the APOC2 promoter using bisulfite converted DNA as a template. Primers were designed using the MethPrimer online tool(5). Bio-Rad Precision Melt Supermix was used to amplify methylated APOC2 promoter. Left Primer 5' -TTCGTTTATTAAGGTTTGGTTTTTC-3' Right Primer 5' -CGCTATATTACCCAAACTAATCTCG- 3'.

Supplemental Tables:

Table S1. APOC2 methylation CpG island in AML vs Normal

Composite Element REF	CGI_Coordinate	P value	Mean of Normal HSC	Mean of CD34+ AML	Adjusted P Value
cg20090143	CGI:chr19:44954956-44955826	0.549644	0.7395	0.7908	>0.999999
cg25746394	CGI:chr19:44954956-44955826	0.000005	0.7549	0.357	0.000092
cg01958934	CGI:chr19:44954956-44955826	0.000088	0.8279	0.4873	0.001588
cg09555818	CGI:chr19:44954956-44955826	0.000003	0.7855	0.3802	0.000062
cg13119609	CGI:chr19:44954956-44955826	0.000004	0.7862	0.3811	0.000063
cg27436184	CGI:chr19:44954956-44955826	0.391552	0.8308	0.7572	>0.999999
cg10169327	CGI:chr19:44954956-44955826	0.026528	0.7933	0.6023	0.477506
cg14723423	CGI:chr19:44954956-44955826	0.67109	0.8082	0.8447	>0.999999
cg22164781	CGI:chr19:44954956-44955826	0.806455	0.8336	0.8126	>0.999999
cg02912790	CGI:chr19:44954956-44955826	0.84144	0.6801	0.663	>0.999999
cg04347059	CGI:chr19:44954956-44955826	0.355592	0.9151	0.8358	>0.999999
cg04401876	CGI:chr19:44954956-44955826	0.37956	0.8343	0.7589	>0.999999
cg04766076	CGI:chr19:44954956-44955826	0.679093	0.8606	0.8251	>0.999999
cg06736138	CGI:chr19:44954956-44955826	0.963978	0.8452	0.849	>0.999999
cg08656316	CGI:chr19:44954956-44955826	0.7208	0.8275	0.8582	>0.999999
cg17769836	CGI:chr19:44954956-44955826	0.447983	0.8438	0.7786	>0.999999
cg25017250	CGI:chr19:44954956-44955826	0.327566	0.7625	0.6784	>0.999999
cg27353824	CGI:chr19:44954956-44955826	0.569767	0.802	0.7532	>0.999999

Table S2. Clinical characteristics

	Total	APOC2 Low (APOC2 Z<1)	APOC2 High (APOC2 Z≥1)	P Value (APOC2 Low vs High)	Fisher Exact
Sex, No.					0.7247
Female	81	78	3		
Male	92	87	5		
Age, years(range)				0.0837	
Median	58	57	62.5		
Mean	55.23	54.74	65.25		
WBC count				0.2981	
Median	17	18.7	6.2		
Mean	36.63	37.38	21.23		
BM Blast %				0.0099	
Median	72	72	89.5		
Mean	69.09	68.36	84.13		
PB Blast %				0.0033	
Median	39	40	0		
Mean	39.59	40.76	12.29		
NCCN subtype, No.					0.4376
Favorable	33	31	2		
Intermediate	92	89	3		
Poor	45	42	3		
FAB subtype, no					0
M0	16	16	0		
M1	44	43	1		
M2	38	38	0		
M3	16	14	2		
M4	34	33	1		
M5	18	15	3		
M6	2	2	0		
M7	3	3	0		

Table S3. Multivariate cox proportional hazards model analyzing of the effect of high APOC2 (Z >1) expression on overall survival in patients with AML (TCGA DATA: n=153 patients, excluding patients WITH FAB M3 CLASSIFICATION AND FOUR patients without cytogenetic risk information).

	HAZARD RATIO	95% CI		P-VALUE
AGE	1.01	1.00	1.03	0.089
TRANSPLANT STATUS	0.395	0.24	0.64	<0.001
CYTOGENETIC RISK	2.23	1.47	3.37	<0.001
FLT3	1.63	1.04	2.55	0.035
TP53	2.13	1.06	4.28	0.034
APOC2 (Z $\geq$ 1)	2.51	1.03	6.07	0.042

Table S4. Mutation characteristics

Genetic Aberration	Patients with APOC2 Z<1 (n=165)	Patients with APOC2 Z≥1 (n=8)	Fisher's Exact p-value	Patients with wildtype Gene	Patients with mutant Gene	Mann-Whitney U's t-test
	Mutated patients N (%)			Median APOC2 (log2-median centered mRNA)		
APL (M3)	14 (8.48%)	2 (25%)	0.165	5.21	7.93	0.0001
MLL-rearrangements (Histo)	5 (3.09%)	3 (37.5%)	0.003	5.35	9.54	0.0014
FLT3-mut	47 (28.5%)	2 (25%)	>0.999	4.35	6.93	0.0045
TP53-mut	14 (8.48%)	0	>0.999	5.85	1.21	0.0040
DNMT3A-mut	41 (24.8%)	2 (25%)	>0.999	5.28	6.81	0.1962
CEBPA-mut	12 (7.27%)	1 (12.5%)	0.472	5.36	7.34	0.2031
NRAS-mut	12 (7.27%)	0	>0.999	5.50	5.48	0.7103
TET2-mut	15 (9.09%)	0	>0.999	5.62	2.95	0.0860
IDH1-mut	16 (9.70%)	0	>0.999	5.88	1.98	0.0065
IDH2-mut	17 (10.3%)	0	>0.999	5.88	0.75	0.0004
RUNX1-mut	16 (9.70%)	0	>0.999	5.97	0.30	<0.0001
NPM1-mut	45 (27.3%)	3 (37.5%)	0.687	4.24	7.41	<0.0001
WT1-mut	10 (6.06%)	0	>0.999	5.56	4.97	0.5696

Table S5. Patient information

Patient ID	Sample Type	FLT3 Mutation
Patient#1	Diagnosis	NA
Patient#2	Diagnosis	NA
Patient#3	Diagnosis	NA
Patient#4	Diagnosis	ITD
Patient#5	Relapse	ITD
Patient#6	Relapse	ITD
Patient#7	Diagnosis	WT
Patient#8	Diagnosis	WT

Supplemental Figures:
Supplementary Figure 1

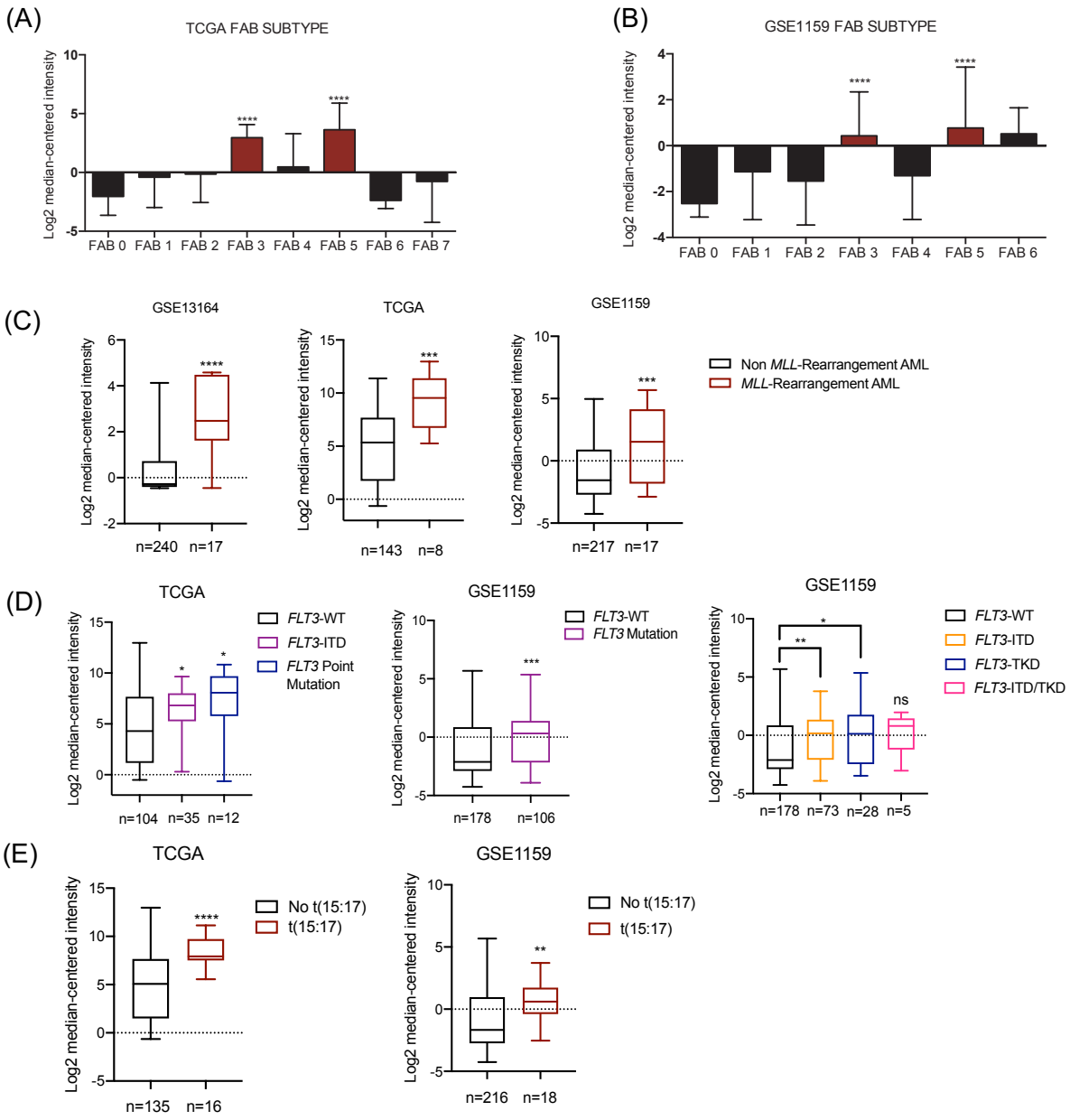

Figure S1. A-B) Relative *APOC2* expression according to the French-American-British (FAB) classification of AML in TCGA and GSE1159 datasets (one-way ANOVA, P<0.0001). **C)** Relative *APOC2* expression in patients with *MLL*-rearranged AML compared with patients without *MLL*-rearranged AML in GSE 13164, TCGA and GSE1159. **D)** Relative *APOC2* expression in patients with *FLT3*-ITD and *FLT3* point mutations AML compared with patients without *FLT3*-WT AML in TCGA and GSE1159. **E)** Relative *APOC2* expression in patients with t(15;17) AML compared with patients without t(15;17) AML in TCGA and GSE1159. The difference between groups was analyzed by Mann- Whitney test (**** P<0.0001; *** P<0.001; ** P<0.01; * P<0.05).

Supplementary Figure 2

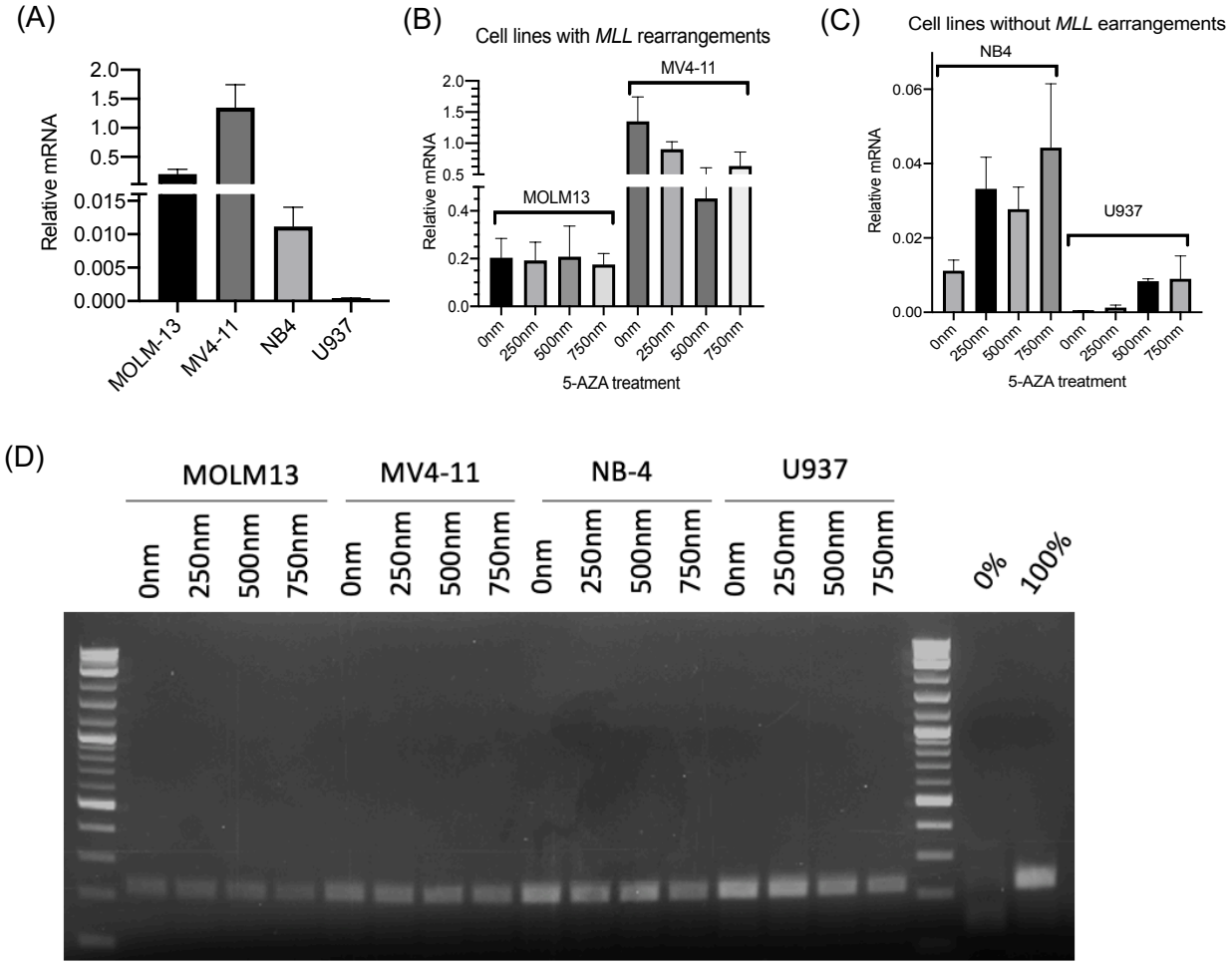

Figure S2: AML cell lines MOLM-13 and MV4-11 (with MLL-rearrangements) and NB4 and U937 (without MLL-rearrangements) were treated with 5-Aza at 0, 250, 500, and 750nm concentrations every day for three days, cells were collected for RNA and DNA were collected on day 3. **A)** qPCR analysis of *APOC2* mRNA levels in MOLM-13, MV4-11, NB4 and U937 at base levels. **B-C)** *APOC2* mRNA level changes post 5-Aza treatment. **D)** *APOC2* promoter demethylation in 5-Aza treated cell lines detected by methylation specific primers (MSP). 0% is the unmethylated control and 100% is fully methylated control. The differences between groups were analyzed by Unpaired T test. (**** P<0.0001; *** P<0.001; ** P<0.01; * P<0.05).

Supplementary Figure 3

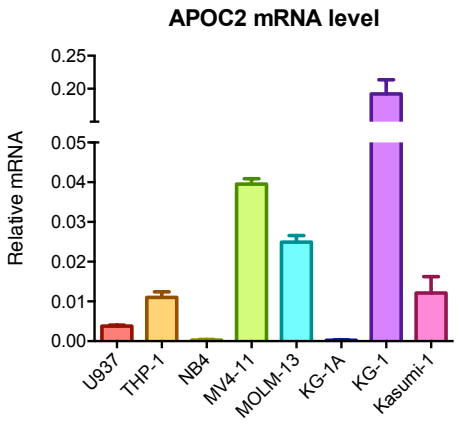

Figure S3: qPCR analysis of *APOC2* mRNA levels in different AML cell lines.

Supplementary Figure 4

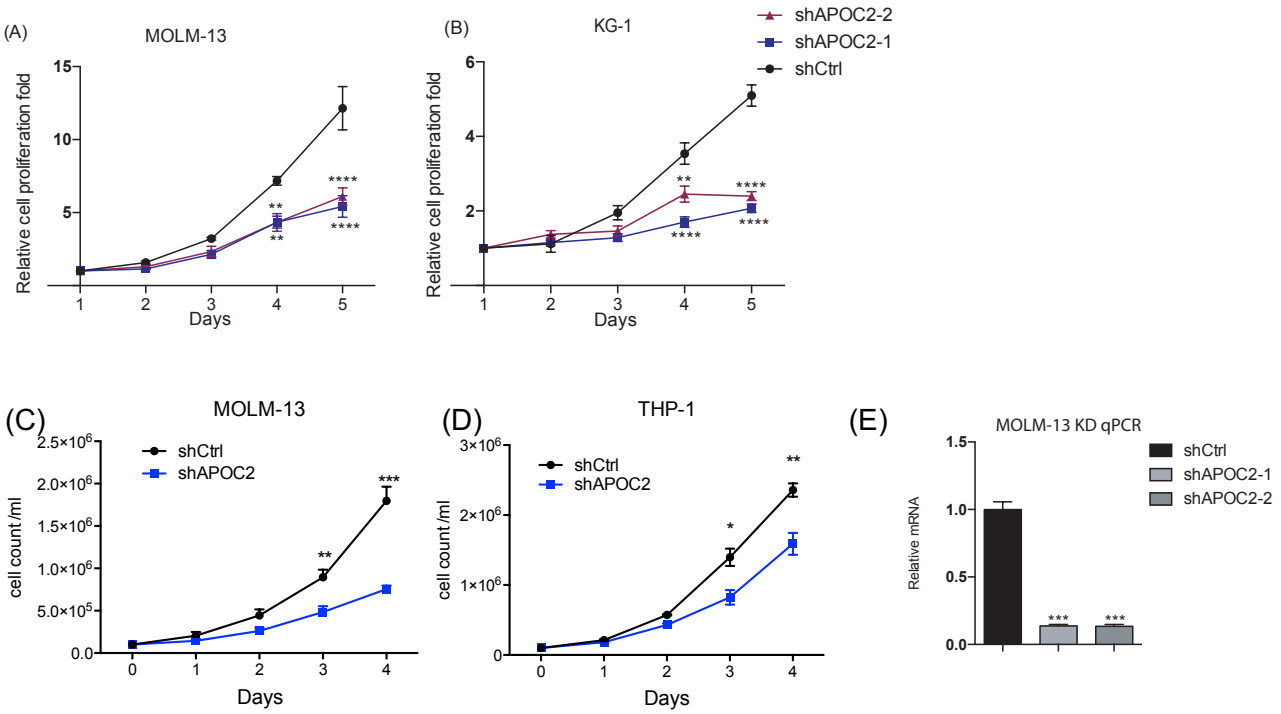

Figure S4. A-D) Cell proliferation assay of **A)** MOLM-13 cells. **B)** KG-1 cells transiently infected with two *APOC2*-shRNAs and control shRNA. Cell proliferation assay of **C)** MOLM-13 cells and **D)** THP-1 cells transiently infected with *APOC2*-shRNA and control shRNA. The differences between groups were analyzed by two-way Anova. (**** P<0.0001; *** P<0.001; ** P<0.01; * P<0.05). **E**) The qPCR results of *APOC2* knockdown in MOLM-13 cell lines. The difference between groups was analyzed by unpaired T test (**** P<0.0001; *** P<0.001; ** P<0.01; * P<0.05).

Supplementary Figure 5

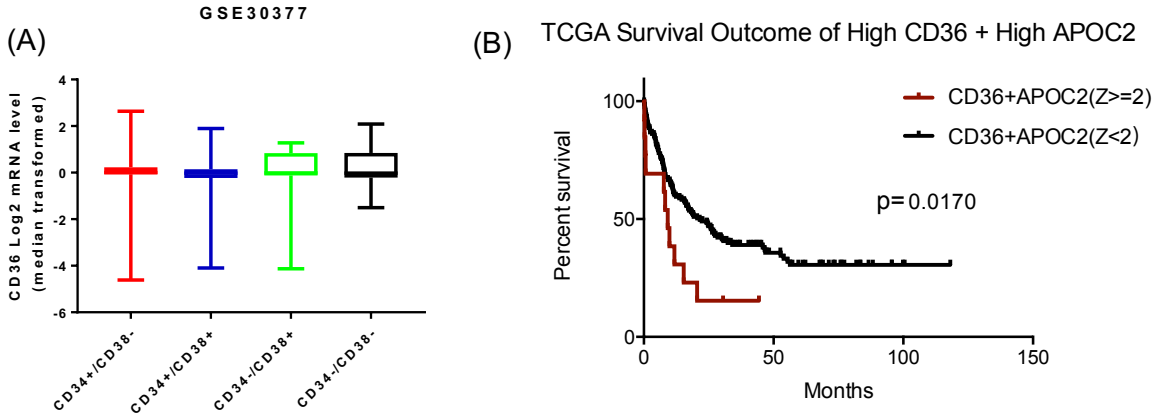

Figure S5: A) *CD36* mRNA levels in CD34+/CD38-, CD34+/CD38+, CD34-/CD38+ and CD34-/CD38- groups. **B)** Patients in the TCGA data were dichotomized based on their *APOC2* and *CD36* mRNA expression Z-score (RNA Seq V2 RSEM) into high (Z-score ≥2) and low (Z-Score <2). Patients with high *APOC2+CD36* levels had significantly shorter OS.

Supplementary Figure 6

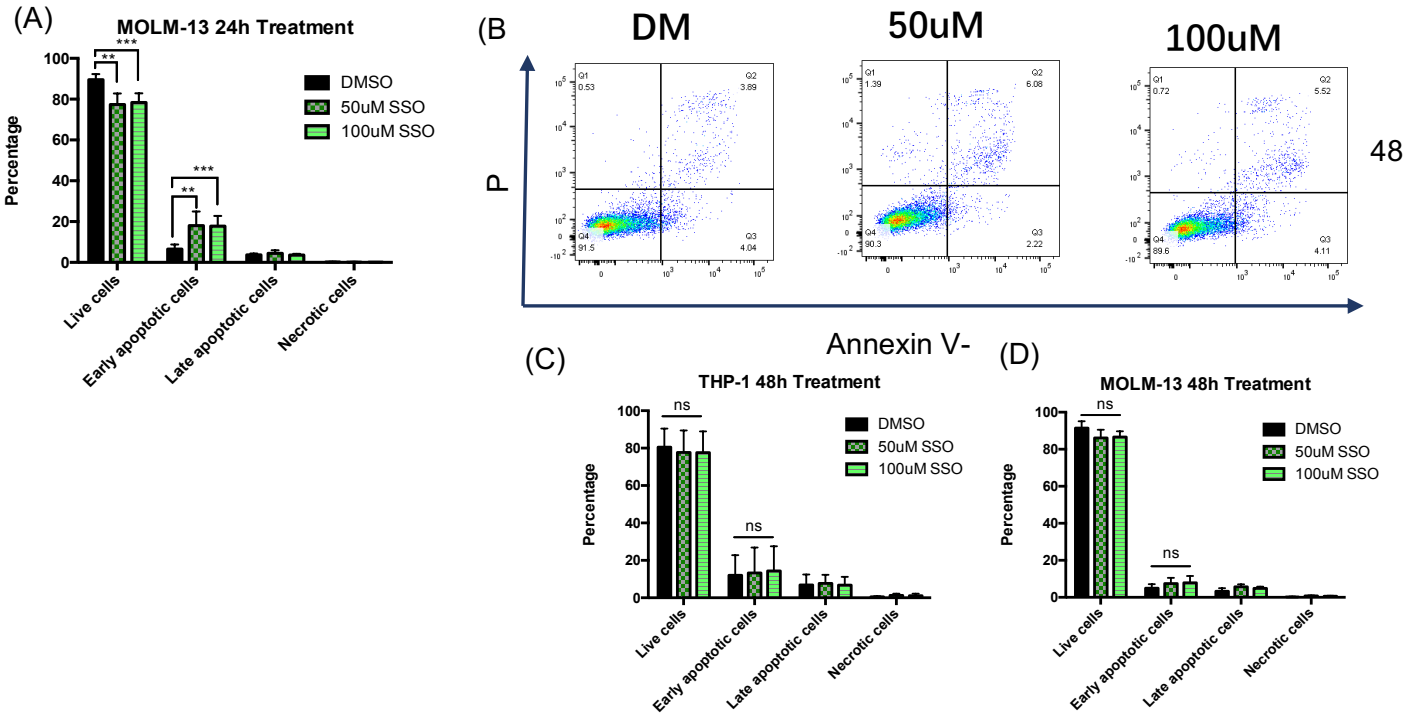

Figure S6: A) Quantification of apoptosis populations in MOLM-13 cells for 24h B) Annexin V and PI staining apoptosis assay in THP-1 cells after 50uM and 100uM SSO treatment at 48h timepoint. C) Quantification of apoptosis populations in THP-1 cells for 48h timepoints. D) Quantification of apoptosis populations in MOLM-13 cells 48h timepoint. The difference between groups was analyzed by unpaired T test (**** P<0.0001; *** P<0.001; ** P<0.01; * P<0.05).

Supplementary Figure 7

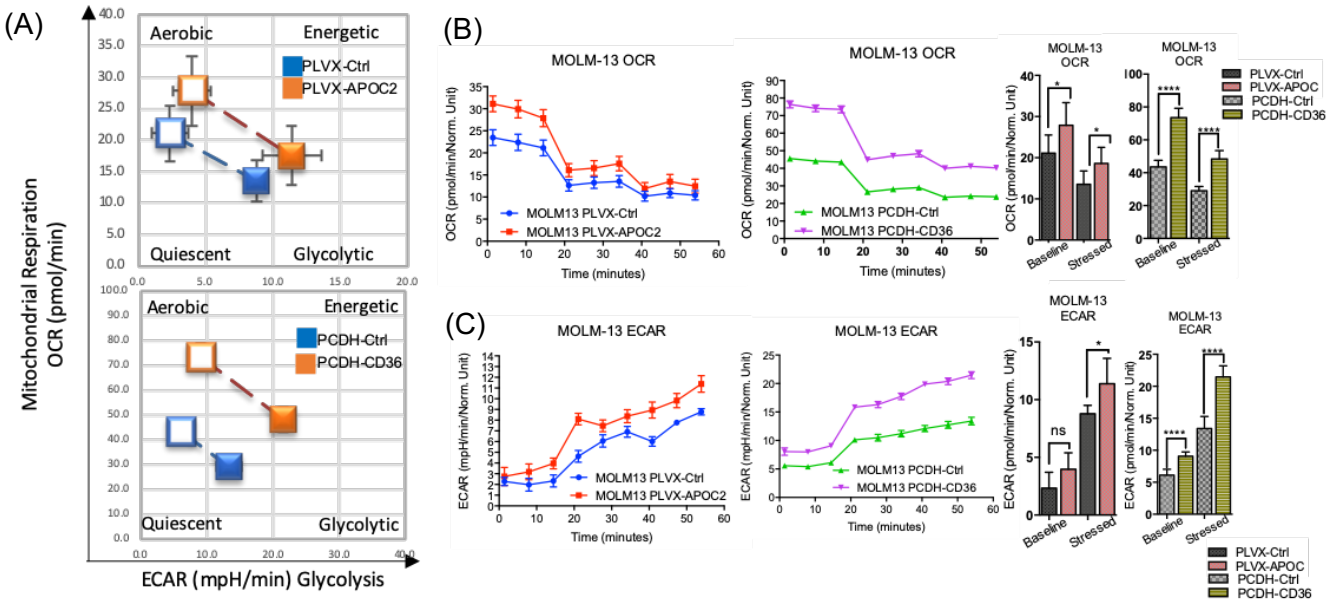

Figure S7: **A)** Cell energy phenotype of PLVX- Ctrl vs OE APOC2 and PCDH-Ctrl vs OE CD36 MOLM-13 cells. Baseline Phenotype is indicated by an open marker. Stressed Phenotype is indicated by a filled marker. **B-C)** OCR, respiration (bar plot), ECAR and glycolysis (bar plot) of OE Ctrl, OE APOC2 and OE CD36 MOLM-13 determined by Seahorse XF cell analysis.

Supplementary Figure 8

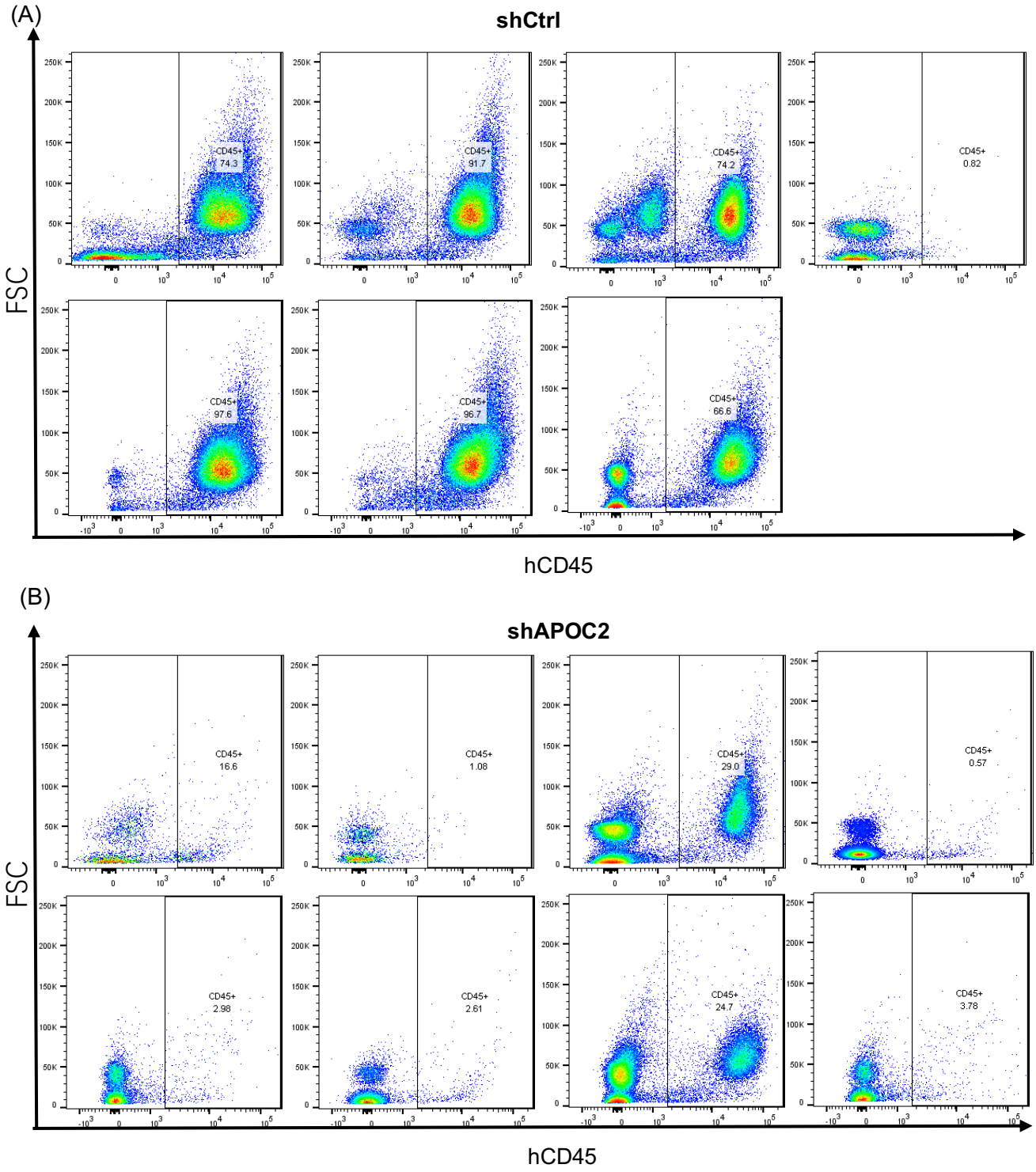

Figure S8. A) Flow cytometry blots showing hCD45 positive engraftment in peripheral blood of HL60 shCtrl group (n=7). **B)**: Flow cytometry blots showing hCD45 positive engraftment in peripheral blood of HL60 shAPOC2 group (n=8).

Supplementary Figure 9

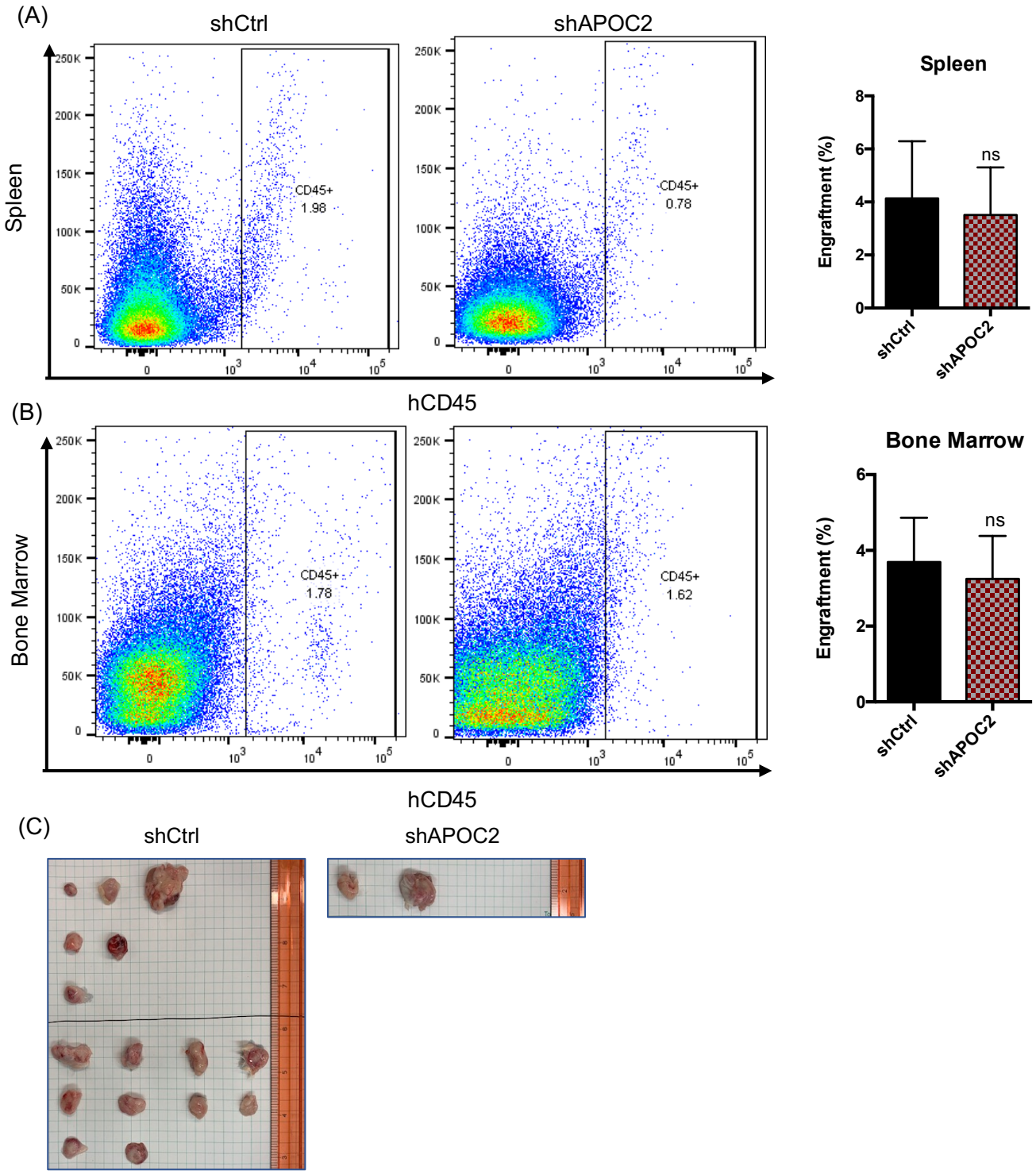

Figure S9. A-B) Representative flow cytometry blots showing hCD45 positive population in bone marrow, and spleen of HL60 shCtrl, and HL60 shAPOC2. Quantitative information of engraftment in spleen and bone marrow was shown in right bar graphs. **C)** Images of tumors collected from the necks, lower abdomens, and legs of engrafted mice.

Supplementary Figure 10

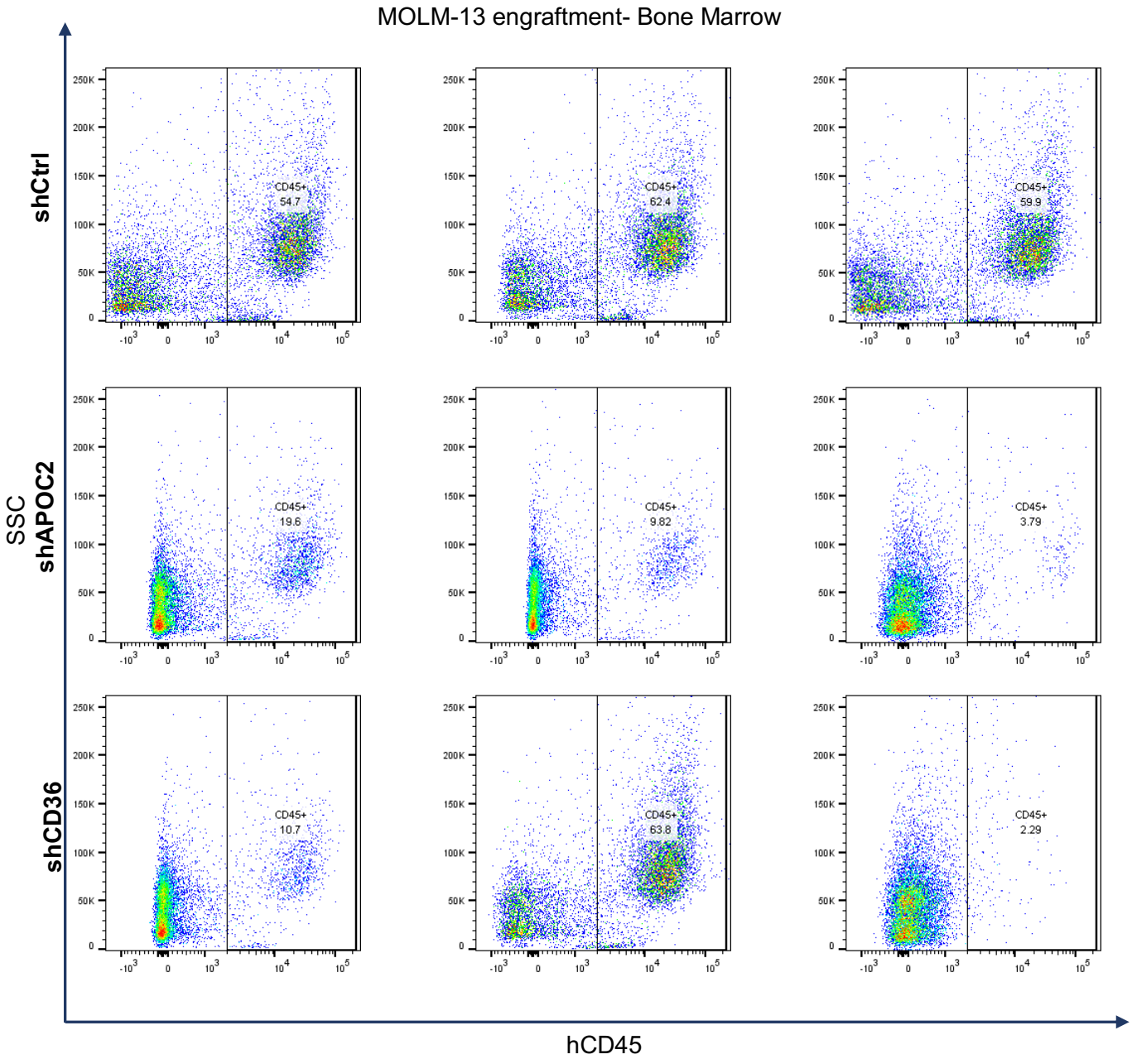

Figure S10: Flow cytometry blots showing hCD45 positive population in bone marrow of MOLM-13 shCtrl, shAPOC2, and shCD36 cells.

Supplementary Figure 11

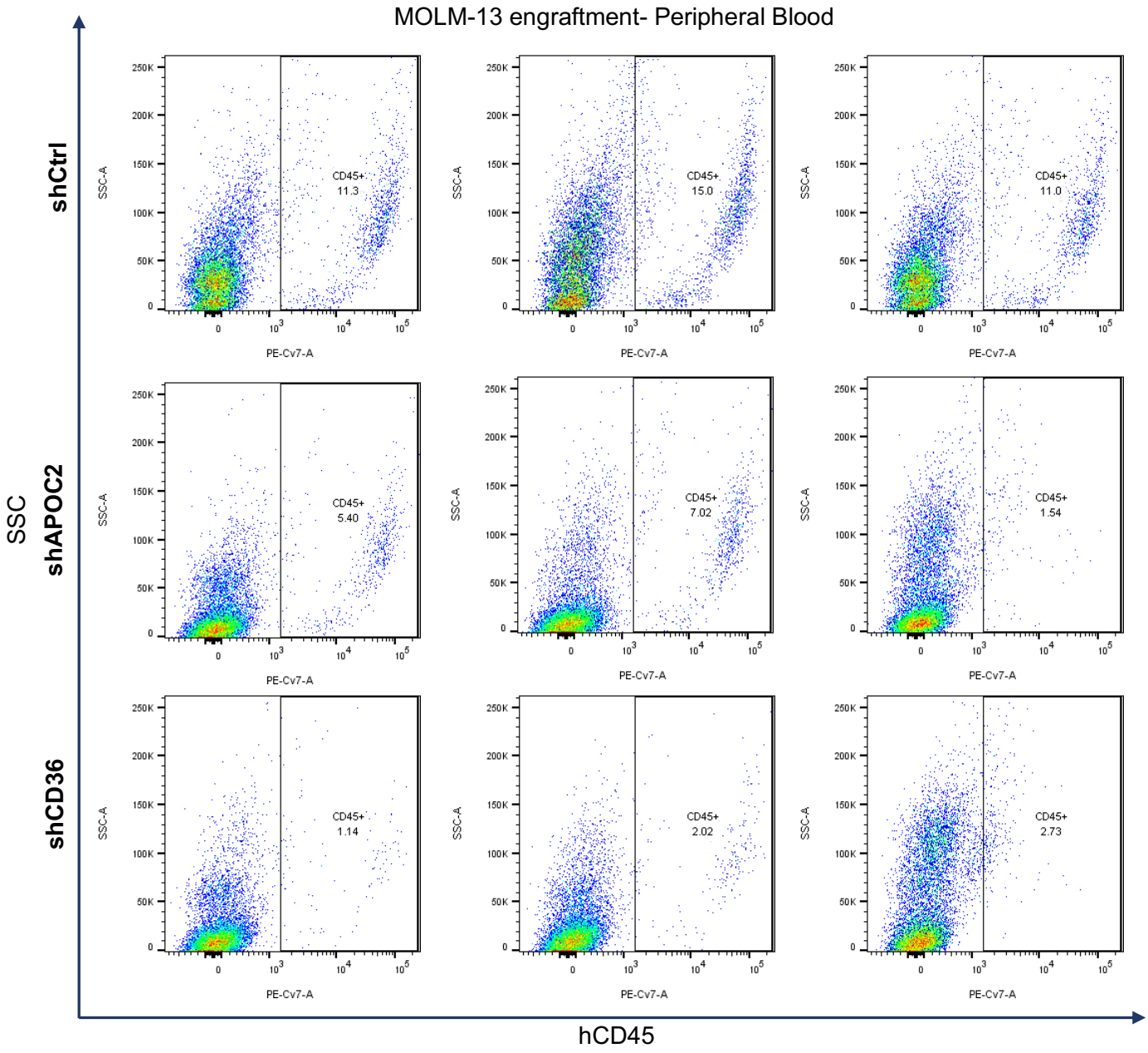

Figure S11: Flow cytometry blots showing hCD45 positive population in peripheral blood of MOLM-13 shCtrl, shAPOC2, and shCD36 cells.

Supplementary Figure 12

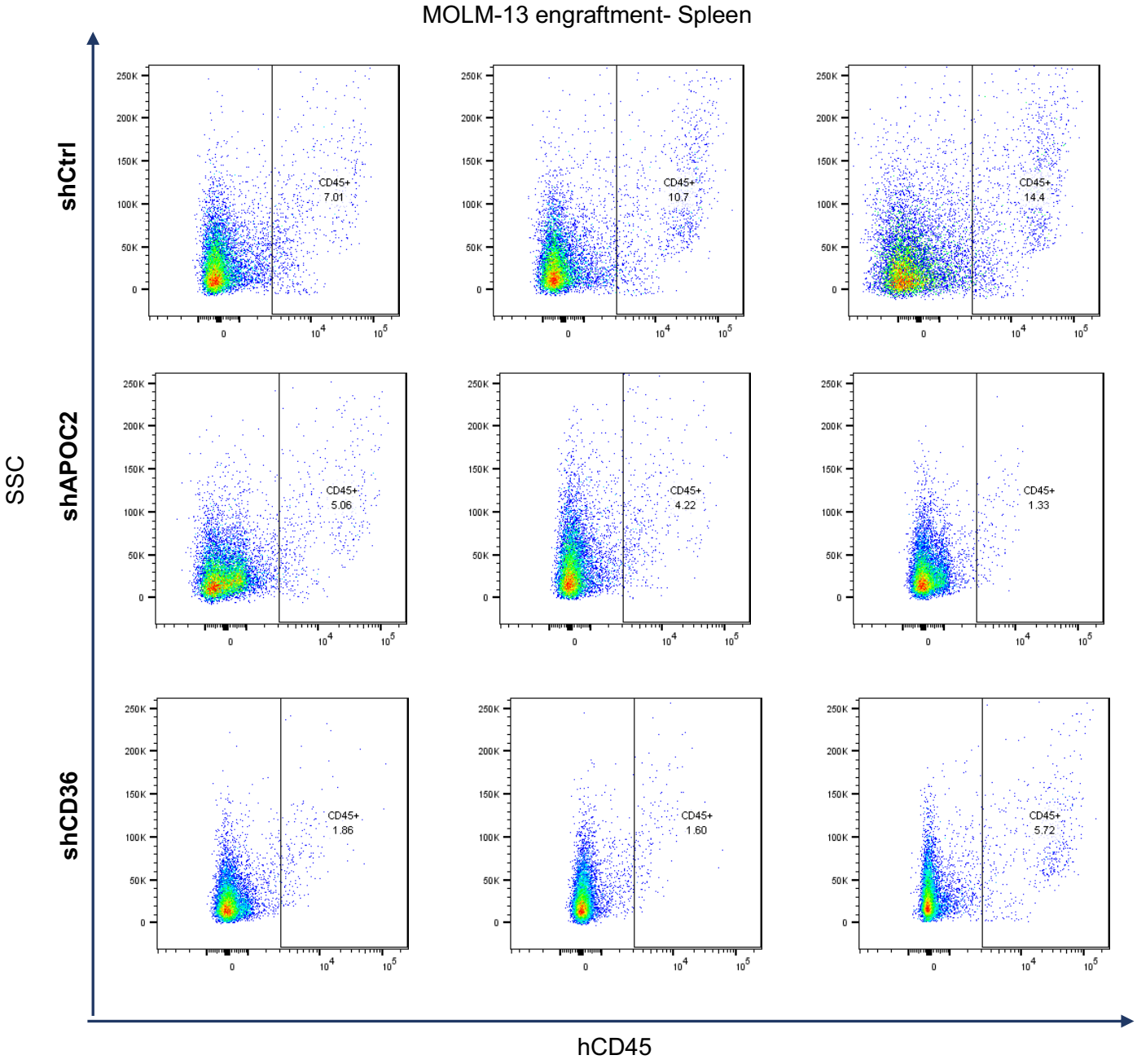

Figure S12: Flow cytometry blots showing hCD45 positive population in spleens of MOLM-13 shCtrl, shAPOC2, and shCD36 cells.

Supplementary Figure 13

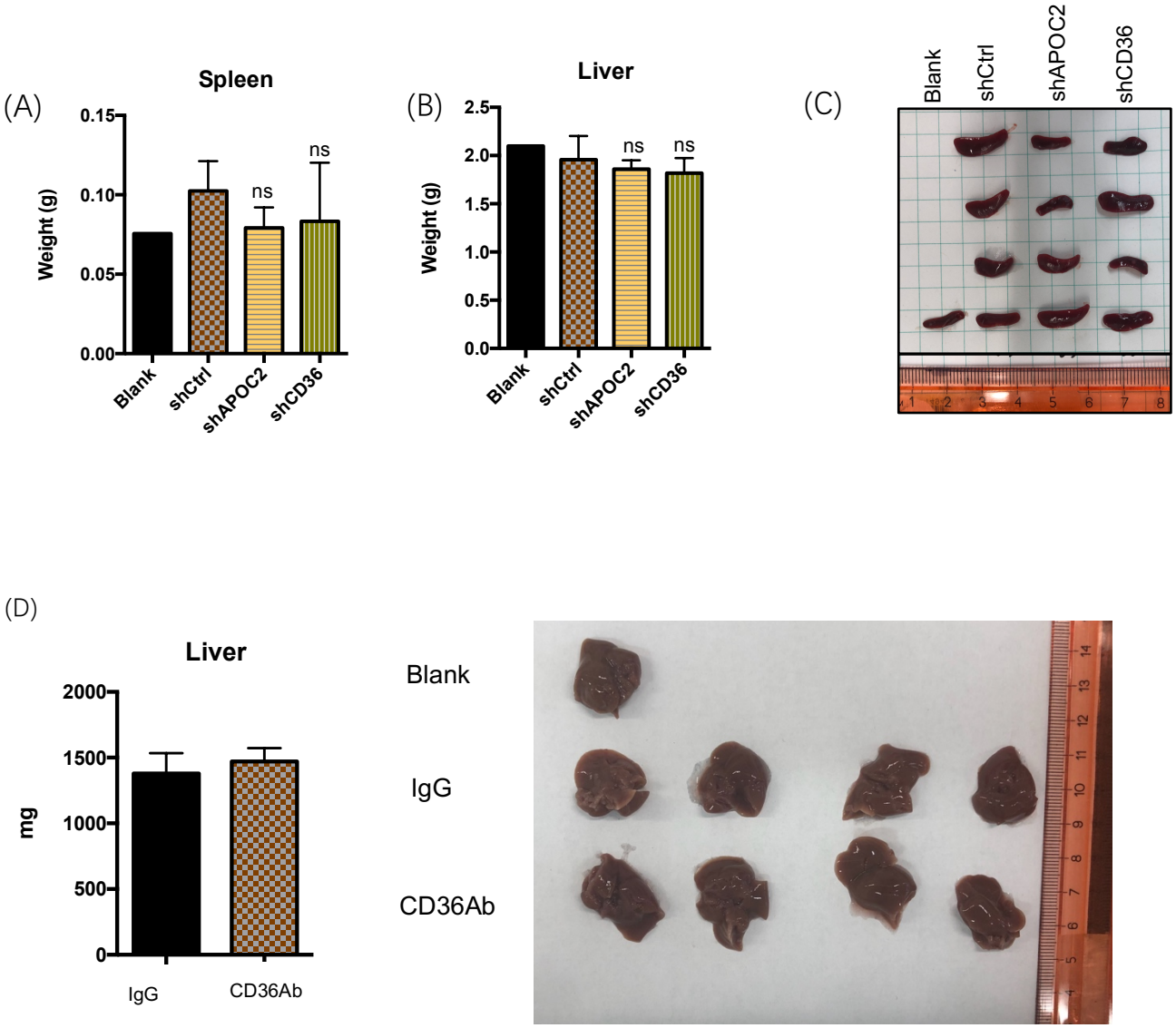

Figure S13. A-B) Weights of spleens and livers collected from mice engrafted with MOLM-13 shCtrl, MOLM-13 shAPOC2, and MOLM-13 shCD36 luciferase cells. **C)** Spleens collected from mice engrafted with MOLM-13 shCtrl, MOLM-13 shAPOC2, and MOLM-13 shCD36 luciferase cells. **D**) Weights and sizes of livers collected from mice engrafted with MOLM-13 and treated with IgG and CD36 antibody.

References

1. Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepfer AM, Hinkle G*, et al.* A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell **2006**;124(6):1283-98 doi 10.1016/j.cell.2006.01.040.
2. Wiederschain D, Wee S, Chen L, Loo A, Yang G, Huang A*, et al.* Single-vector inducible lentiviral RNAi system for oncology target validation. Cell Cycle **2009**;8(3):498-504 doi 10.4161/cc.8.3.7701.
3. Yousefzadeh MJ, Wyatt DW, Takata K, Mu Y, Hensley SC, Tomida J*, et al.* Mechanism of suppression of chromosomal instability by DNA polymerase POLQ. PLoS Genet **2014**;10(10):e1004654 doi 10.1371/journal.pgen.1004654.
4. Jung N, Dai B, Gentles AJ, Majeti R, Feinberg AP. An LSC epigenetic signature is largely mutation independent and implicates the HOXA cluster in AML pathogenesis. Nat Commun **2015**;6:8489 doi 10.1038/ncomms9489.
5. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics **2002**;18(11):1427-31 doi 10.1093/bioinformatics/18.11.1427.