LAMP-5 is an essential inflammatory-signaling regulator and novel immunotherapy target for mixed lineage leukemia-rearranged acute leukemia

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 doi:10.3324/haematol.2020.257451

Received: April 30, 2020.

Accepted: April 2, 2021.

Pre-published: April 29, 2021.

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Methods:

Cell lines and primary patient-derived xenograft cells

Human leukemia cell lines were maintained in Iscove's Modified Dulbecco medium (IMDM) or Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin. Cell lines were periodically validated by STR genotyping through Genetica Cell Line Testing (LabCorp). Cells were tested and were negative for mycoplasma contamination. None of the cell lines utilized in this study are recognized by the ICLAC as being commonly misidentified.

MLL-AF9 Tet-off human $CD34^+$ cells were a kind gift from Dr. James Mulloy and were generated by transduction of both the pSIN-TREtight-dsRED-MLL/AF9 lentivirus and the MSCV-GFP-IRES-tTA34 retrovirus as described before[1,2].

Fully de-identified primary cells were obtained from the Cincinnati Children's Hospital Medical Center Biorepository. Specimens have been fully de-identified and anonymized. PDX models were generated using residual diagnostic specimens according to an IRB approved protocol (#2008-0021) following proper informed consent. By the time the mice displayed signs of leukemia, the only human cells that remained in the mice were leukemic (MLL-rearrangement was confirmed with FISH). Cells were cultured in IMDM supplemented with 20% FBS and 10 ng/ml human cytokines including SCF, FLT3-Ligand, Thrombopoietin, IL-3, and IL-6.

Animal experiments

For xenograft experiments with MV4;11 and MLL-r primary patient cells, immunocompromised NOD-*Rag1*^{null} *IL2rγ*^{null} (NRG) (Jackson Laboratories, stock no. 007799) recipient mice were conditioned with 30 mg/kg busulfan and transplanted with 2-7.5x10^5 cells 24 hours later. Bone marrow samples were collected four weeks after transplantation; aspirates were analyzed via flow cytometry for the presence

of human CD45$^+$ cells and the presence of shRNA-transduced Venus$^+$ cells. All animals used for this study were 6-12 weeks old. All animal experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC).

Retroviral and lentiviral transductions

Retroviral and lentiviral supernatants were generated by transfection of HEK293T cells using the FuGENE 6 reagent (Promega) or Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's recommendations.

The lentiviral shRNA pLKO.1-Puro plasmids TRCN0000129410 (shLAMP5-1), TRCN0000129378 (shLAMP5-2), and TRCN0000014861 (shIRF7) were purchased from Millipore Sigma. Cells transduced with constructs containing a fluorescent marker (Venus) were isolated 4-5 days after transduction by sorting using MoFlo XDP (Beckman Coulter), FACSAria (BD Biosciences), or a SONY SH800S (Sony Biotechnology).

The retroviral IKBKB S177E S181E (IKBKB-EE) plasmid was a gift from Anjana Rao (Addgene plasmid #11105). FLAG-LAMP5-WT and FLAG-LAMP5-Y276A plasmids were as described previously[3]. FLAG-LAMP5-WT and FLAG-LAMP5-Y276A were cloned into the MSCV-IRES-Puro vector.

ChIP-seq Analysis

ChIP-seq tracks were obtained from the Gene Expression Omnibus from the following references: SEM (GSE74812), MV4;11 and THP-1 (GSE79899), ML-2 (GSE95511), primary patient *MLL-AF4* (GSE83671). Samples with fully analyzed data (available as bedGraph/bigWig,) are presented as-is, with coordinates converted to hg19 using the liftOver function from the UCSC Genome Browser Utilities[4]. For data that only provided genomic intervals (SEM and primary MLL-AF4) FASTQ files were downloaded from the GEO and aligned with bowtie2[5] to hg19 using default parameters. Tracks were

generated by using the bamCompare function from deeptools[6] to compare ChIP targets against input tracks.

RNA-seq Analysis

We performed RNA-seq analysis on MOLM-13 cells that were transduced with shNT or shLAMP5-2. Total RNA was extracted from Venus$^+$ sorted cells using the RNeasy Mini kit (QIAGEN). The RNA-Seq data were aligned to the hg19 reference genome and transcriptome using the software STAR. Produced BAM files were further analyzed in the software AltAnalyze v 2.1.3 and the EnsMart 72 database to produce gene expression estimates as RPKM values from exon-aligning reads. The ranked statistical output of AltAnalyze, from the two groups empirical Bayes moderated t-test (p-value) was used for further analysis in the GSEA 4.0.3 (Broad) with the default MSigDB database, focusing on interferon signaling pathways. For GSEA we multiplied the sign of the expression fold changes (-1 or 1) times the -log10 transformed eBayes p-values as the rank value. RNA-seq data from this study have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE166523.

Cell Viability

MOLM-13, RS4;11, THP-1, and Kasumi-1 cells were plated at 10,000 cells per well in a 96-well plate. Cells were incubated with LAMP-5 therapeutic antibody (TAB-0643CL Creative Biolabs) and Anti-Mouse IgG Fc-DM1 Antibody with Non-Cleavable Linker (AM-103D1-50, Moradec LLC) at 5 ng/uL and 1 ng/uL final concentrations, respectively. To measure cell viability CellTiter-Glo® 2.0 Cell Viability Assay (Promega Cat# G9242 WI, USA) was used following the manufacturer's protocol.

SEAP reporters

THP-1-Blue NF-κB/ISG cells (InvivoGen) carrying a stable integrated NF-κB-inducible or ISG-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct were transduced with shLAMP5-2-Venus and sorted for Venus expression after 48 hours. Cells were then plated at a concentration of

$2x10^4$ cells/well and stimulated with Pam3CSK4 (10 ng/mL) or LPS (100 ng/mL) for 24 hours. Cells were centrifuged and 20 uL supernatant was incubated with 180 uL QUANTI-Blue reagent at 37°C for 30 min for two hours. The levels of NF-κB-induced or ISG-induced SEAP was measured in a microplate reader at 620 nm.

Immunofluorescence

Cells seeded on alcian blue-treated coverslips were fixed with 3.5% paraformaldehyde and permeabilized with 0.05% saponin. Cells were then stained overnight with primary antibodies: anti-LAMP-5 (Thermo Fisher Scientific), anti-MYD88 (R&D Systems), anti-LAMP-1 BV421 (BioLegend) anti-LAMP-2 AF647 (Thermo Fisher Scientific). Immunofluorescence and confocal microscopy were performed with a Zeiss LSM580 63x objective and accompanying imaging software

Flow cytometry

For apoptosis assays, cells were incubated with allophycocyanin (APC)-conjugated Annexin V (BD Bioscience) for 15 minutes at room temperature in 1X Annexin V Binding Buffer (BD Bioscience) followed by staining with 7-aminoactinomycin D (7-AAD) (eBioscience). For surface LAMP-5 detection, cells were incubated with a final concentration of 5 ng/uL of anti-human LAMP-5 therapeutic antibody (Creative Biolabs, NY, USA) overnight and then stained with anti-mouse IgG1-PE (eBioscience). Data were acquired on a FACSCanto analyzer and results were analyzed using FlowJo Version 10 (BD Bioscience).

Colony-forming unit assays

Transduced human cells were sorted 4-5 days after transduction and were cultured in methylcellulose-containing media (StemCell Technologies, H4434). Colonies were scored 10-14 days after plating.

RT- and Quantitative RT-PCR

Total RNA was extracted from human Puromycin-selected or Venus$^+$ sorted cells using the RNeasy Mini kit (QIAGEN). RNA was reversed transcribed into cDNA using iScript Advanced cDNA Synthesis kit (Bio-Rad Laboratories). For Quantitative RT-PCR, 5-10 ng cDNA was analyzed using iTaq Universal SYBR Green Supermix (Bio-Rad) or PowerUP SYBR Green (Thermo Fisher Scientific) in a StepOnePlus Real-Time PCR machine (Applied Biosystems).

Western blotting

Whole-cell lysates were isolated using RIPA buffer (Cell Signaling) and the amount of protein was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Thirty µg of protein was separated by SDS-PAGE on a 4-20% gradient gel (Bio-Rad). After transfer to PVDF membranes, blots were blocked with Odyssey® Blocking Buffer TBS (LI-COR) for one hour and incubated with primary antibodies overnight. After washing, blots were incubated with appropriate secondary IRDye 680RD goat anti-mouse (LI-COR), IRDye 680RD goat anti-rat (LI-COR), and IRDye 800CW goat anti-rabbit (LI-COR) antibodies at a dilution of 1:10,000 for one hour. Images were obtained using the Odyssey CLx Infrared Imaging System (LI-COR).

References

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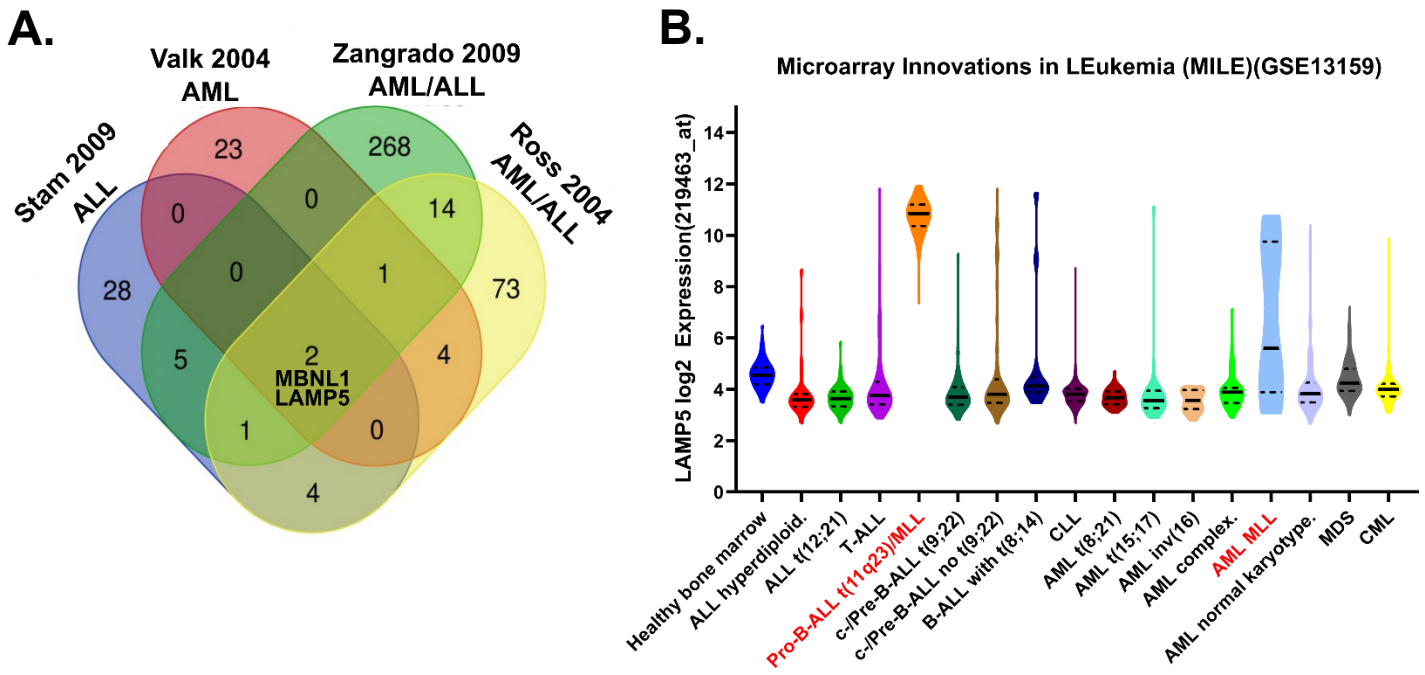
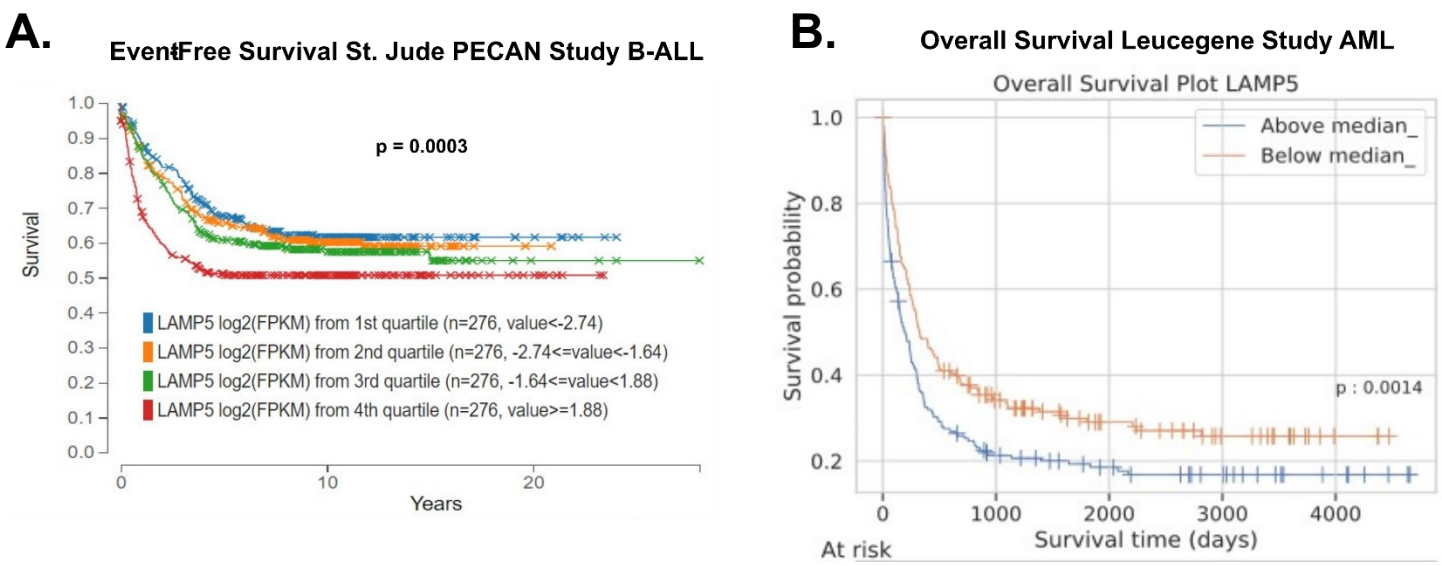
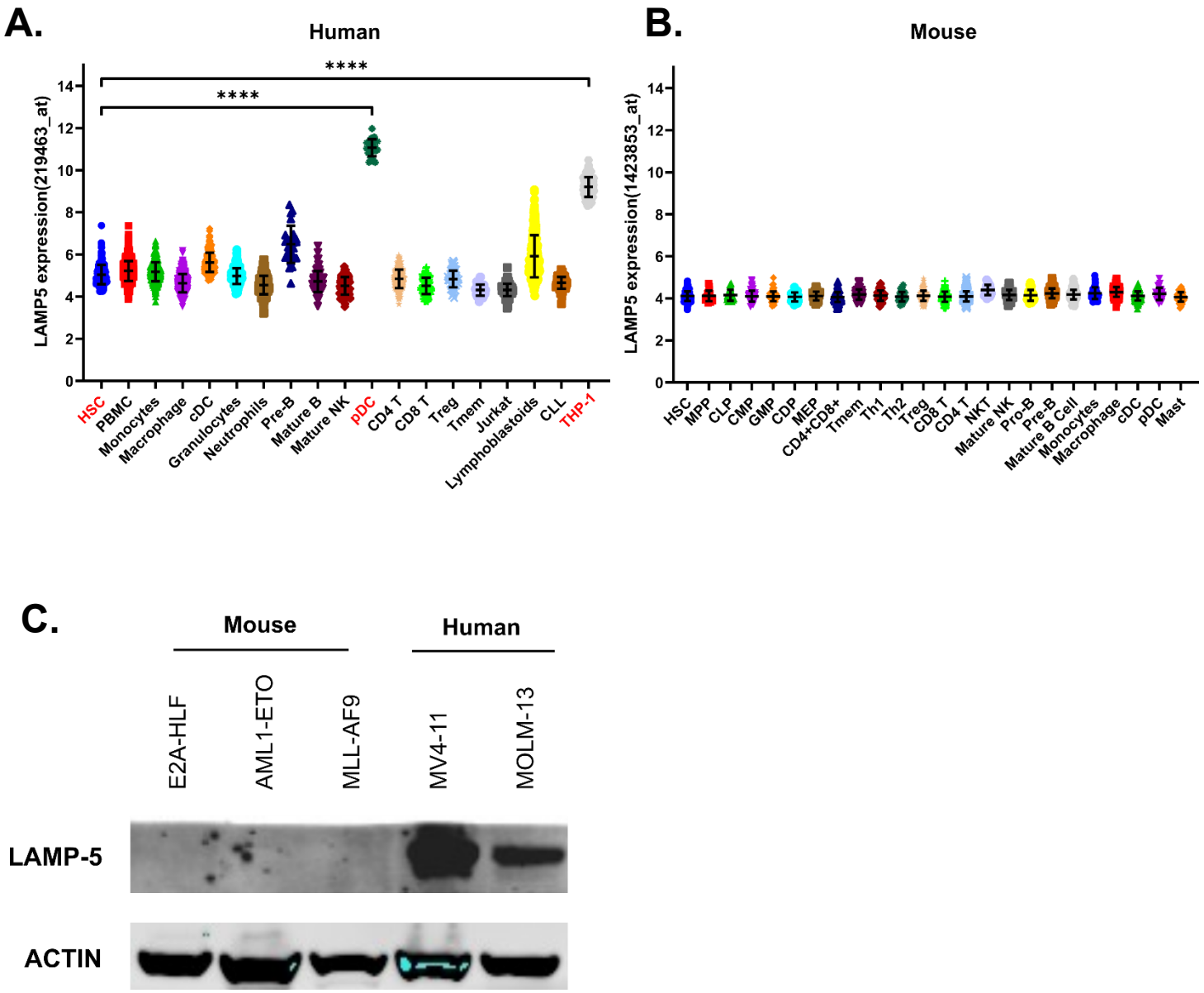
Supplemental Figures



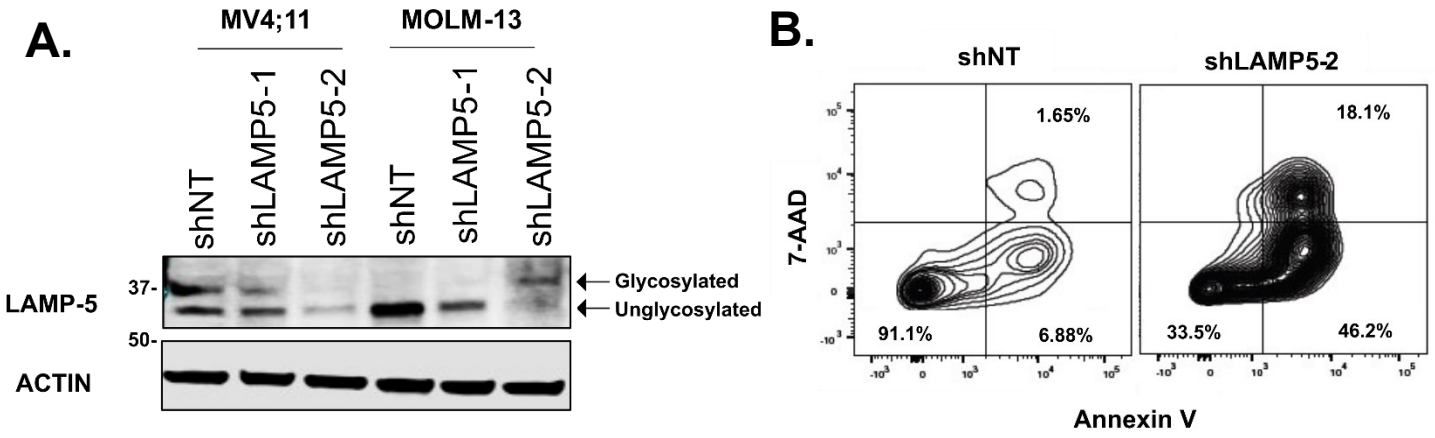
Supplemental Figure 1: *LAMP5* is highly expressed in MLL-r leukemias **(A)** Intersection of published gene expression signatures composed of genes overexpressed in MLL-rearranged AML and ALL when compared to other MLL-germline leukemias. **(B)** Log2 microarray expression of *LAMP5* in multiple molecular subtypes of leukemia from the Microarray Innovations in Leukemia (MILE) Study. Data shown as median values and quartiles. Normalized data from Bloodspot.eu



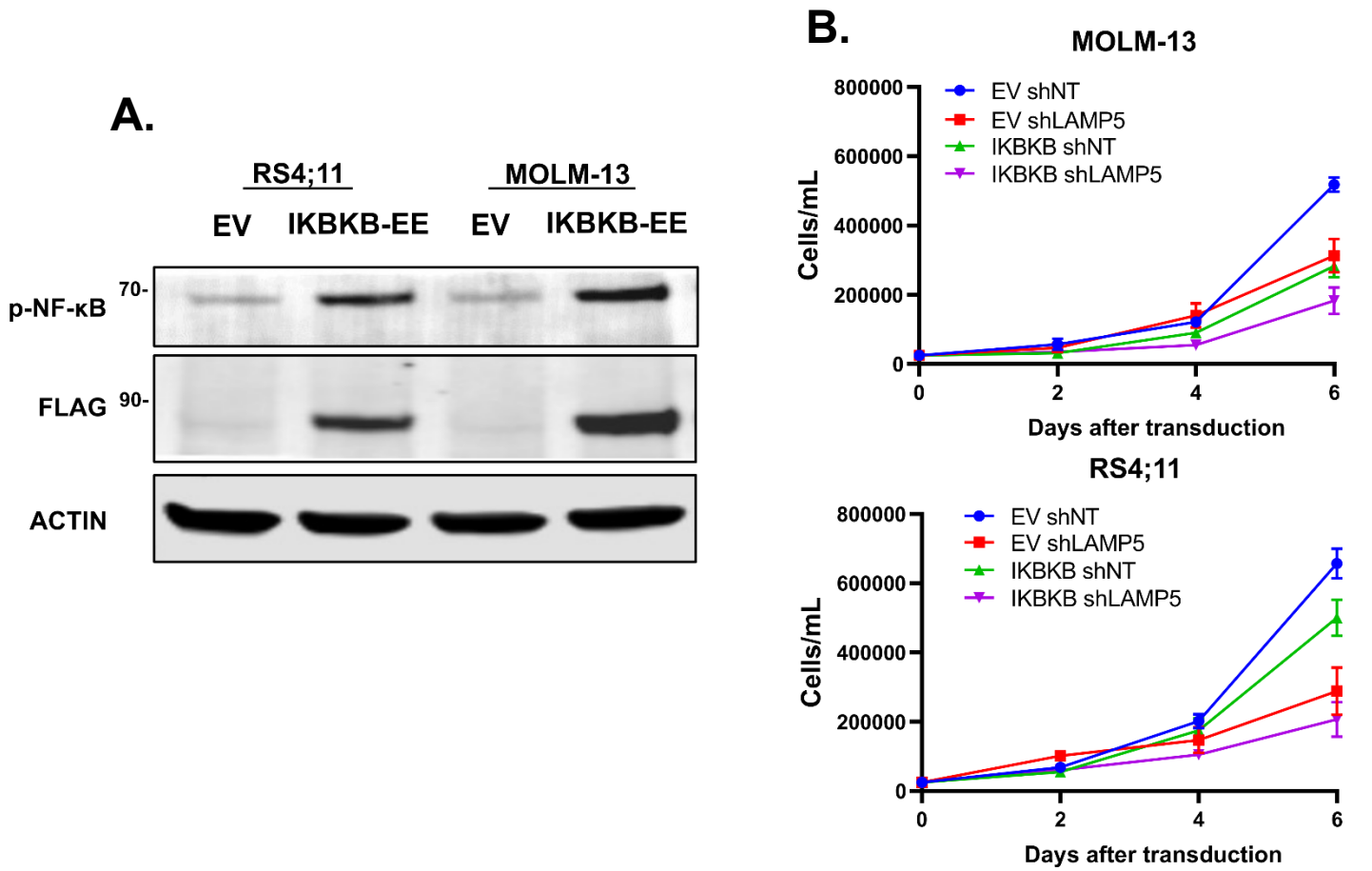
Supplemental Figure 2: High expression of *LAMP5* correlate with poor survival in ALL and AML **(A)** Kaplan-Meier Event-free survival curve of B-ALL patients based on *LAMP5* Log_2(FPKM) expression (n=1,104 two-sided time-stratified Cochran–Mantel–Haenszel test, p=0.003). Data obtained from the St. Jude PeCan Portal. **(B)** Kaplan-Meier Overall survival curve of AML patients based on *LAMP5* (TPM) expression (n=374 two-sided time-stratified Cochran–Mantel–Haenszel test, p=0.0014). Data obtained from the Leucegene Data Portal.



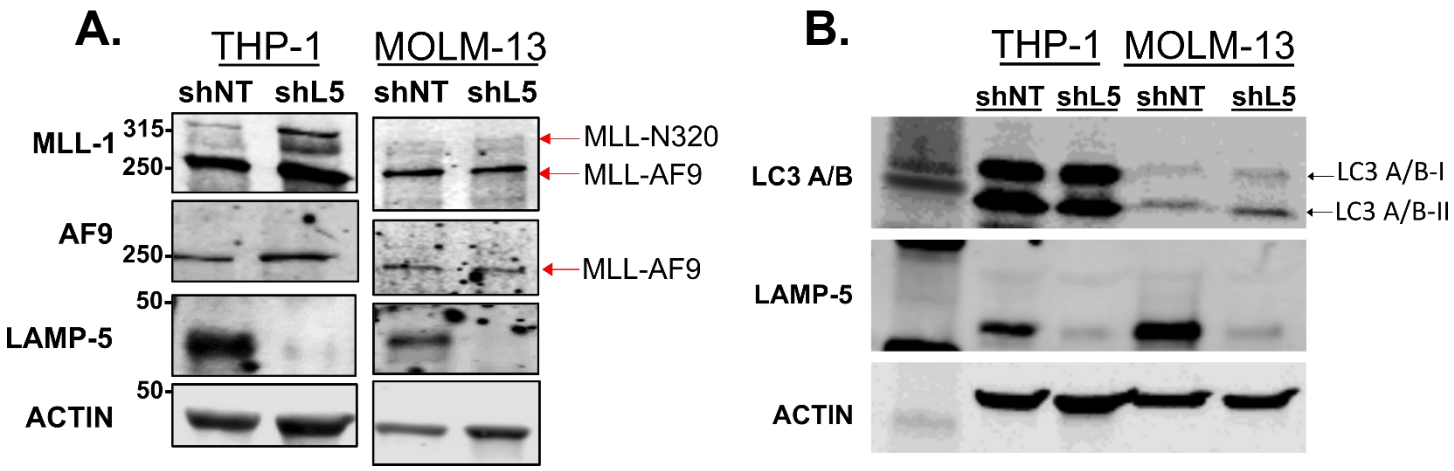
Supplemental Figure 3: LAMP5 is not expressed in healthy blood or leukemia in mice **(A)** Normalized Microarray expression data of *LAMP5* in human blood and leukemia (THP-1 as representative MLL-r leukemia). Data from Immuno-Navigator portal presented as Mean with SD. **(B)** Normalized Microarray expression data of *Lamp5* in mouse blood. Data from Immuno-Navigator portal, presented as Mean with SD. **(C)** Western blot analysis of LAMP-5 expression in Mouse Lin (-) cells transformed with E2A-HLF, AML1-ETO, MLL-AF9. MV4;11 and MOLM-13 cells were used as control.



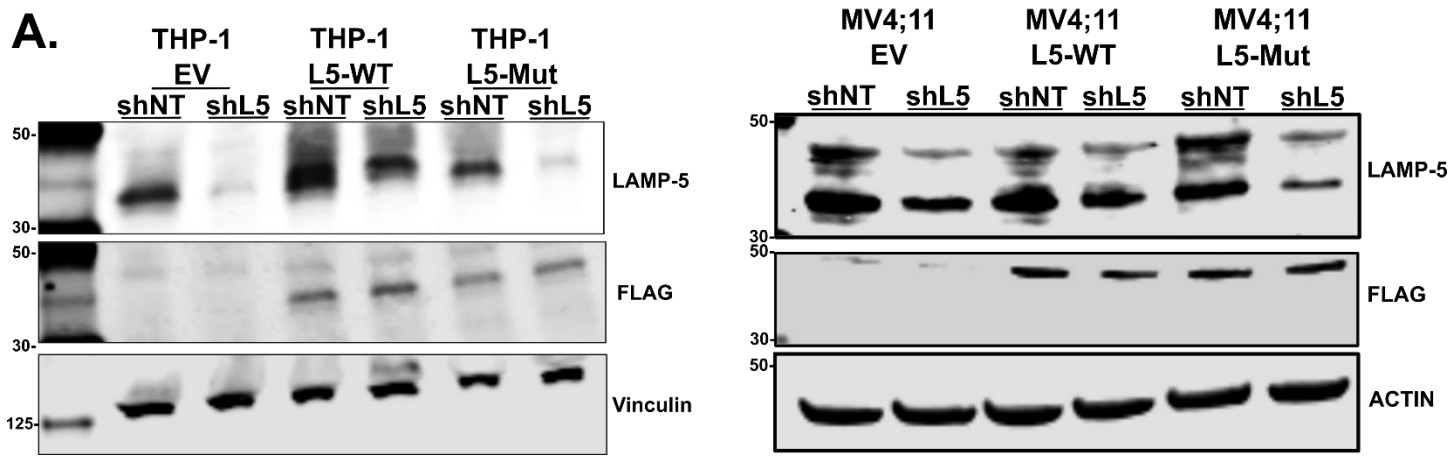
Supplemental Figure 4: Effective knockdown of LAMP5 leads to apoptosis **(A)** Western blot analysis of LAMP-5 expression in MV4;11 and MOLM-13 cells after transduction with shLAMP5-1 and shLAMP5-2. The two bands seen represent both the unglycosylated and glycosylated states of LAMP-5. **(B)** Representative contour flow plots of MOLM-13 cells after transduction with shNT and shLAMP5-2. Labels on the axes represent the respective stains used in flow cytometry detection.



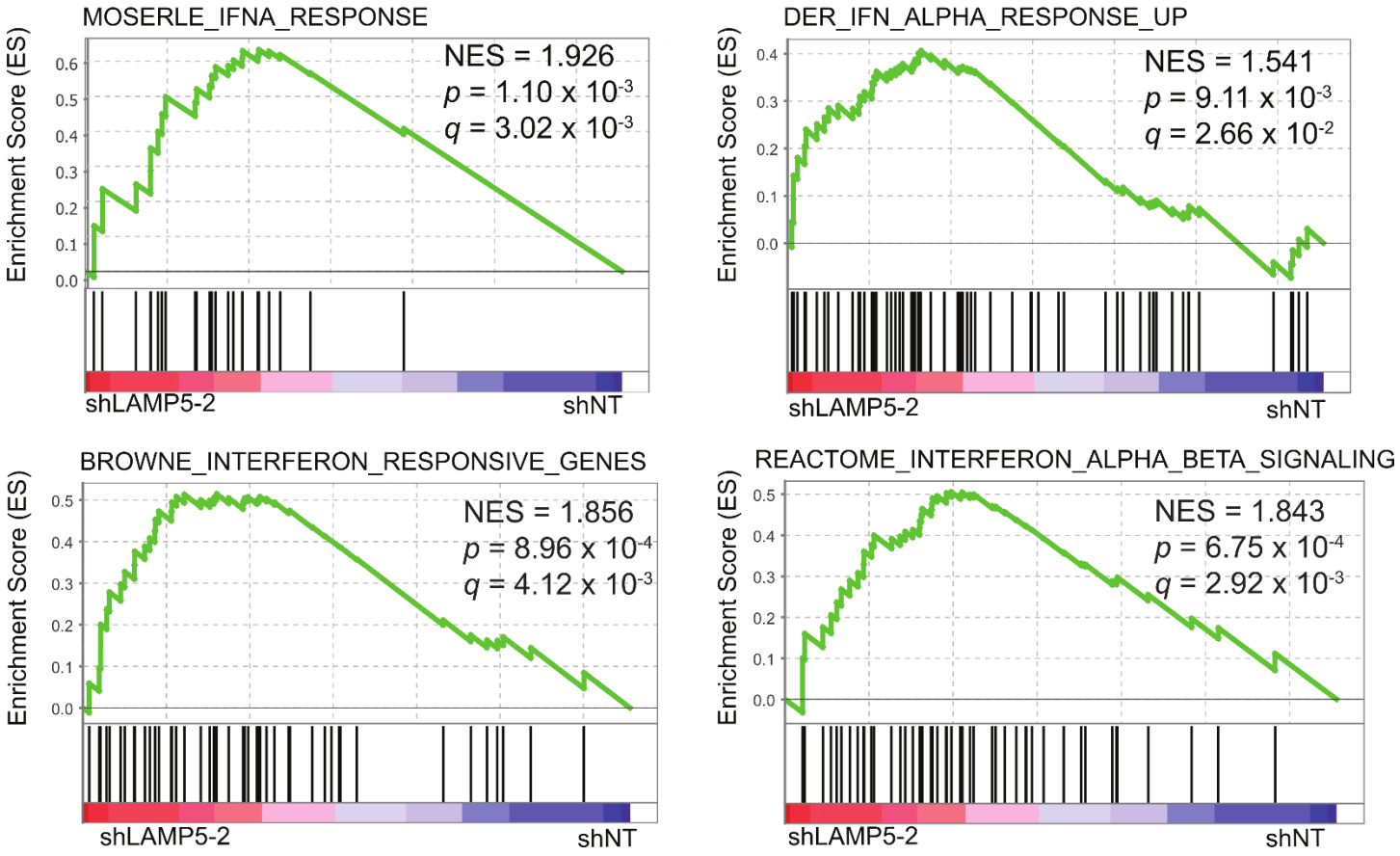
Supplemental Figure 5: NF-κB activation does not rescue LAMP5 depletion effects **(A)** Western blot analysis showing overexpression of IKBKB-EE (FLAG) and activation of p-NF-κB. **(B)** In-vitro cell growth of MOLM-13 and RS4;11 cells transduced with EV or IKBKB-EE followed by LAMP5 downregulation or retention. Data are from 3 technical replicates, representative of 3 independent experiments.



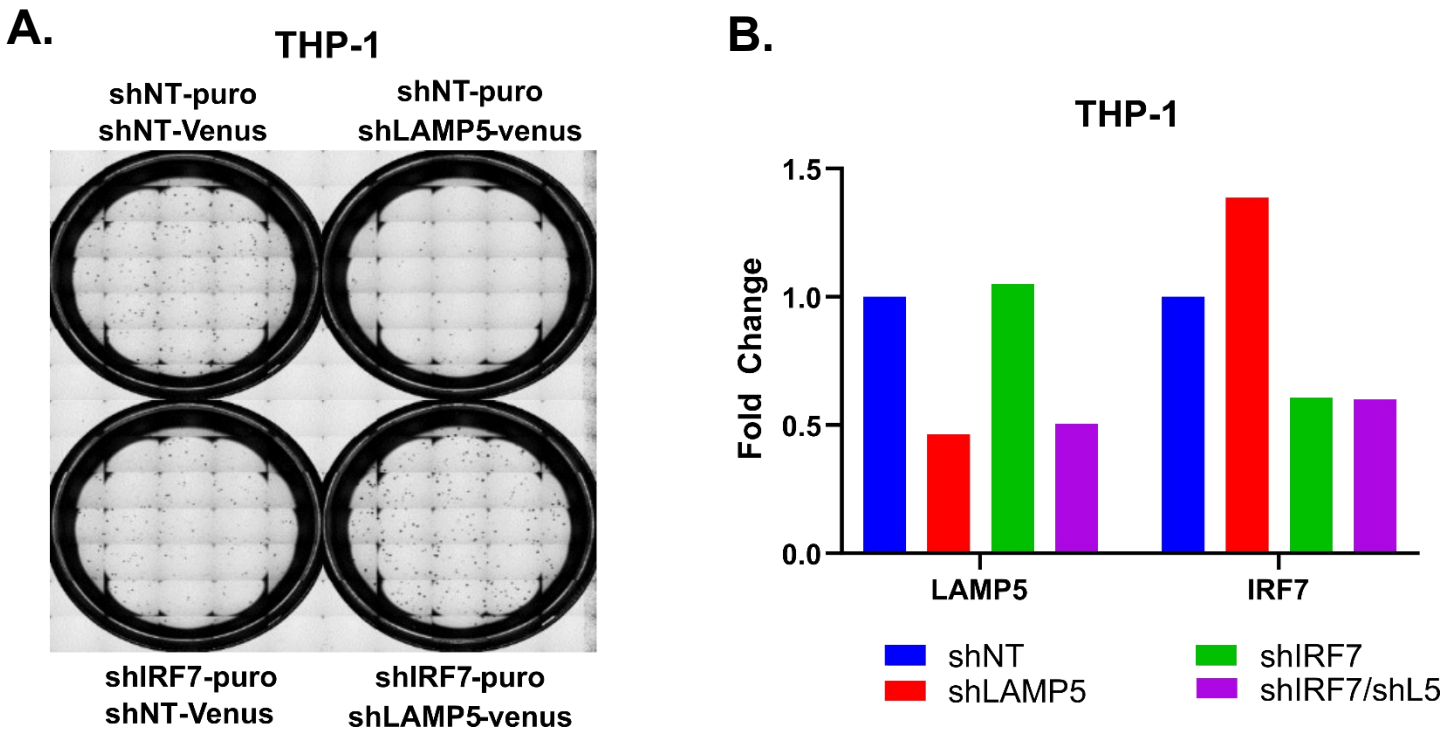
Supplemental Figure 6: Depletion of LAMP-5 does not affect the MLL-FP or activate autophagy **(A)** Western blot analysis showing the expression of the Germline MLL1 and MLL-AF9, C-term AF9, LAMP5 and ACTIN after LAMP5 knockdown in MOLM-13 and THP-1 cell lines. **(B)** Western blot analysis showing LC3 A/B expression after LAMP5 knockdown in THP-1 and MOLM-13 cell lines



Supplemental Figure 7: (A) Western blot analysis showing expression of the endogenous and overexpressed (FLAG) LAMP-5 upon knockdown of the endogenous LAMP-5 in both THP-1 and MV4;11 cells**.**



Supplemental Figure 8: LAMP5 knockdown leads to enrichment of IFN related gene signature**.** GSEA analysis of MOLM-13 cells after transduction with shNT or shLAMP5-2.



Supplemental Figure 9: IRF7 knockdown rescue LAMP5 knockdown effects **(A)** Representative images of THP-1 cells transduced with shNT/shIRF7/shLAMP5/shCombo (shIRF7/shLAMP5) after one week in methylcellulose. **(B)** Relative expression of *LAMP5* and *IRF7* after knockdown of LAMP-5, IRF7, or LAMP-5 and IRF7 together.

Supplemental Table #1: Genes overlapping between Guo et al. and Lavallée et al.

GOLGA8M	golgin A8 family member M
SKIDA1	SKI/DACH domain containing 1
CASC10	cancer susceptibility candidate gene 10
CLSTN2	calsyntenin 2
PPP1R27	protein phosphatase 1 regulatory subunit 27
IL22RA2	interleukin 22 receptor subunit alpha 2
MPPED1	metallophosphoesterase domain containing 1
ZNF521	zinc finger protein 521
HOXA9	homeobox A9
UPK3A	uroplakin 3A
PENK	proenkephalin
SCUBE1	signal peptide, CUB domain and EGF like domain containing 1
FEZ1	fasciculation and elongation protein zeta 1
HOXA7	homeobox A7
TGM5	transglutaminase 5
LPAR3	lysophosphatidic acid receptor 3
VAT1L	vesicle amine transport 1 like
ADRA2C	adrenoceptor alpha 2C
HOXA6	homeobox A6
HOXA5	homeobox A5
KCNE1L	Potassium Voltage-Gated Channel Subfamily E Member 1-Like
CTGF	Connective Tissue Growth Factor
PRL	prolactin
DES	desmin
HOXA11	homeobox A11
LAMP5	lysosomal associated membrane protein family member 5
DAPL1	death associated protein like 1

Genes predicted to be in the surface by the In-silico Human Surfaceome

Supplemental Table 2: List of reagents

Reagent or Resource	Source	Identifier
Antibodies		
Anti-LAMP5 (34.2)	Thermo Fisher Scientific	#14-9778-80; RRID:AB_2573029
anti-MYD88	R&D Systems	AF2928; RRID:AB_2297977
anti-LAMP-1 BV421	Biolegend	#328626; RRID:AB_11203537
anti-AF9	Bethyl	A300-597A; RRID:AB_495520
anti-MLL1 (D2M7U)	Cell Signaling Technology	#14689; RRID:AB_2688009
anti-JNK	Cell Signaling Technology	#9252; RRID:AB_2250373
anti-phospho-JNK (81E11)	Cell Signaling Technology	# 4668; RRID:AB_823588
anti-p38 MAPK (D13E1)	Cell Signaling Technology	#8690; RRID:AB_10999090
anti-Phospho-p38 MAPK (D3F9)	Cell Signaling Technology	# 4511; RRID:AB_2139682
anti-Phospho-NF-kB p65 (93H1)	Cell Signaling Technology	# 4025 RRID:AB_10827881
anti-NF-kB p65 (L8F6)	Cell Signaling Technology	#6956; RRID:AB_10828935
anti-Actin (13E5)	Cell Signaling Technology	#4970; RRID: AB_2223172
anti- β-tubulin (9F3)	Cell Signaling Technology	#2128; RRID: AB_823664
Anti-Vinculin	Cell Signaling Technology	#13901; RRID:AB_2728768
anti-Phospho-IRAK1	Assay Biotech	A1074; RRID:AB_10683572
Anti-LAMP5, Clone D1	Creative Biolabs	TAB-0643CL
anti-mouse IgG1-PE	Thermo Fisher Scientific	#12-4015-82; RRID:AB_10717258
Anti-Mouse IgG Fc-DM1	Moradec LLC	AM-103D1-50
IRDye 680RD Goat anti-Rat IgG	Li-Cor Biosciences	Cat# 925-68076, RRID:AB_2814913
IRDye 680RD Goat anti-Mouse IgG	Li-Cor Biosciences	Cat# 925-68070, RRID:AB_2651128
IRDye 800CW Goat anti-Rabbit IgG	Li-Cor Biosciences	Cat# 925-32211, RRID:AB_2651127

shRNA		
LAMP5(shLAMP5-1)	Millipore Sigma	TRCN0000129410
LAMP5 (shLAMP5-2)	Millipore Sigma	TRCN0000129378
IRF7 (shIRF7)	Millipore Sigma	TRCN0000014861

Primer sequences		Sequence
Human LAMP5	Forward	TACGACTCCTCGGAGAAAACC
	Reverse	TGACACTCATAGGACTTCCCAG
Human ACTB	Forward	CTCTTCCAGCCTTCCTTCCT
	Reverse	AGCACTGTGTTGGCGTACAG
Human IFNA2	Forward	GCTTGGGATGAGACCCTCCTA
	Reverse	CCCACCCCCTGTATCACAC
Human IFNB	Forward	GTCACTGTGCCTGGACCATAG
	Reverse	GTTTCGGAGGTAACCTGTAAGTC
Human MLL-AF9	Forward	AATAAGCAGGAGAATGCAGG
	Reverse	TGCCTTGTCACATTCACCAT
Human IRF7	Forward	CCCACGCTATACCATCTACCT
	Reverse	GATGTCGTCATAGAGGCTGTTG