Supplemental data

Methods

***Cells and Patient Samples*.** All cells were cultured in RPMI-1640 medium supplemented with 10% or 20% FBS, 2mM L-glutamine and 100 U/ml penicillin G-streptomycin in 5% CO_2 at 37°C unless stated otherwise. Murine MOHITO cell line (a generous gift from Jan Cools at the KU Leuven in Belgium) was supplemented with murine IL-7 (10ng/ml) and IL-2 (5ng/ml) (Peprotech). Murine Ba/F3 cells were supplemented with 10 ng/mL mouse IL-3, and BM progenitor cells with murine IL-3 (10ng/ml), IL-6 (10ng/ml), and stem cell factor (50 ng/ml, Peprotech). PER-117 cells were supplemented with (1x) Sodium Pyruvate and Non-essential amino acids (Gibco) and 10 nM b-Mercaptoethanol. Primary cells were cultured as described previously (1).

T cell subsets. RUNX2 expression in human and mouse T-cell subsets were analyzed from (2) and (3), respectively. cKit enrichment on mouse thymocytes was performed using CD117 microbeads (MACS, Miltenyi, #130-091-224).

Lentivirus/Retrovirus production and transduction was performed according to standard procedures. For each RUNX2 shRNA knockdown experiment, two independent target sequences were used, designated shRUNX-B and shRUNX-C (UNM) or shRUNX2-1 and shRUNX2-2 (UGent), each with appropriate scrambled controls. The lentivirus packaging vectors and recombinant expression plasmids were co-transfected into 293FT cells using the Lipofectamine 2000 (Life Sciences, Carlsbad, CA, USA). The culture supernatants were collected after 48 h and subjected to titration. Lentivirus-particles were mixed with 8 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA) and applied to T-ALL cell lines or primary samples at the multiplicity of infection (MOI) of 10 viral particles per cell, and spun at 1150 x g for 90 minutes at 32°C. Following infection, the transduced cells were cultured under puromycin selection (Sigma-Aldrich) for approx. 3 weeks. Lentiviruses-infected cells were analyzed for GFP positivity using the FACSAria flow cytometer (BD Bioscience, San Jose, CA, USA) followed by FACS-sorting.

For bioluminescence in vivo imaging, the PF382 cells expressing RUNX2 and control vector were transduced with pLenti-EF1a (Red-Luciferase)-Rsv (RFP-Blasticidin) lentiviral particles (GenTarget Inc, San Diego, CA, USA). Post infection, the

transduced cells were cultured under blasticidin (4 µg/ml) selection (Gibco) for approx. 3 weeks. Lentiviruses-infected cells were analyzed for GFP^+/RFP^+ using the FACSAria flow cytometer (BD Bioscience) followed by FACS-sorting.

LOUCY cells were transfected with active β-catenin plasmid (pCS2mBCTN533Ac/3) or control plasmid (pCS2+) using Amaxa 4D-Nucleofector (Lonza, Walkersville, MD, USA). Adenoviral particles of Survivin (Ad-BIRC5) or control vector (Ad-CMV-Null) were transduced into LOUCY cells by spinoculation performed in RPMI medium supplemented with polybrene (8 µg/ml). LOUCY cells expressing active β-catenin, survivin and relevant control plasmids were lentivirally co-transduced with shRNA RUNX2 (shRUNX2 B and shRUNX2 C) and scrambled negative control shRNA (shNC).

The pMSCVneo-KMT2A-MLLT4, pMSCVneo-KMT2A-MLLT1 and pMSCVNeo were a generous gift from Dr. Eric So from the King's College London, UK. Each plasmid (10 µg) was transfected into the Phoenix packaging cell line using Lipofectamine 2000. Murine MOHITO and Ba/F3 cells were spinoculated in retroviral supernatants supplemented with 4 µg/ml polybrene and 20% RPMI 1640 followed by selection with 1 mg/ml geneticin (G418). Ba/F3 cells expressing *KMT2A-R* (*KMT2A-MLLT4* and *KMT2A-MLLT1*) or pMSCVNeo plasmid were lentivirally transduced with shRNA RUNX2 (shRUNX2 B and shRUNX2 C) and scrambled negative control shRNA (shNC), if applicable.

RNA extraction RNA was extracted from T-ALL cells using the miRNeasy mini kit (Qiagen) or Direct-Zol™ RNA MiniPrep Kit (Zymo Research), according to the manufacturer's instructions.

Real-time Quantitative PCR (RT-qPCR). Reverse Transcription Reagents were used to synthesize cDNA from 1µg of DNase I treated total RNA. RT-qPCR was performed at UNM using TaqMan® Gene Expression Master Mix for TaqMan assays or with SYBR Green PCR Master Mix for SYBR Green assays on a StepOnePlus™ Real-Time PCR System under standard conditions. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. *KMT2A-MLLT1* and *KMT2A-MLLT4* expression analyses were performed using TaqMan® Gene Expression Assays. TaqMan RT-qPCR was performed according to the manufacturer's specifications. The assay identification numbers were as follows: RUNX2-Hs00231692_m1, RUNX2-Mm00501584_m1, GAPDH-Hs99999905_m1 and GAPDH-Mm99999915_g1. RT-qPCR at UGent was performed

using the SsoAdvanced SYBR Green Supermix (Bio-Rad) and were run on the LightCycler 480 (Roche, model LC480). Every sample was analyzed in duplicate and the gene expression was standardized against at least 3 housekeeping genes. The applied technologies and corresponding primer sequences are listed for each experiment in the reagent table below.

Extracellular and intracellular flow staining. Standard staining protocols were used with antibodies specified in the table below. Intracellular flow cytometry staining of PDX cells was performed upon permeabilization and fixation using FoxP3 staining kit (eBioscience). All flow cytometry was performed using the LSR Fortessa instrument (BD Biosciences) and data was analyzed using Kaluza Analysis Software (Beckmann Coulter, Brea, CA, USA), unless specified otherwise.

***Immunoblotting analysis and Antibodies*.** Cells (10^7) were washed with ice cold PBS and lysed in M-PER® Mammalian Protein Extraction Reagent supplemented with a Halt™ Protease and Phosphatase Inhibitor cocktail. Cell lysates were incubated for 10 min at room temperature (RT) and centrifuged for 15 min at 14,000 x g (4ºC). Equal amounts of total protein were separated on a precast polyacrylamide gradient gel (5-15%) using SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories) using the Bio-Rad Trans-Blot Turbo Transfer System. After blocking, the membrane was incubated with primary antibody at 4°C overnight and then secondary antibody at RT for 1 hour. Blots were developed by chemiluminescence (ECL) reagent and the bands were visualized using the Bio-Rad ChemiDoc™ XRS equipped with Image Lab™ 5.0 Software.

***Cell viability assay*.** Cell viability was monitored by the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA). Absorbance was measured at 490 nm using the Bio-Rad iMark Microplate Absorbance Reader.

***Plasmid constructions*.** A total of 353 bp (623 to 270 bp upstream of the KMT2A coding site) *KMT2A* promoter fragment comprising *RUNX2* binding site was amplified using genomic DNA extracted from LOUCY cell line. The two restriction sites were added to the amplifying primers (For-XHO1 and Rev-HINDIII) for directional cloning into the pGL3 basic luciferase vector. The *KMT2A* promoter primer pair used for cloning into pGL3 basic luciferase vector is listed in the table below.

***Apoptosis and cell cycle assays*.** T-ALL cells (10^6) were cultured in 6-well plates for 48 hours. For apoptosis assays, the cells were washed in binding buffer and stained with 5 µl of Annexin V conjugated to brilliant violet 421 (BV421) and 5 µl of 7-ADD (BD

Pharmingen, San Jose, CA, USA) for 15 minutes at RT in the dark. For cell cycle analyses, the cells were fixed and permabilized in 70% ethanol (2 hrs, -20ºC) and incubated with propidium iodide/RNase Staining Buffer (BD Pharmingen) for 15 min at RT. Stained cells were analyzed by flow cytometry.

Luciferase assay. HEK293 cells were transduced with the lentiviral vectors encoding either *RUNX2*-shRNA (shRUNX2 C) or the scrambled negative control shRNA (shNC). Infected HEK293 cells were co-transfected with pRL and pGL3/KMT2A promoter construct and luciferase activity was determined after 72 h using the Dual-Luciferase Reporter Assay (Promega) and measured with the Synery Neo2 Plate Reader (BioTek).

Animal experiments.

NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice (6-8 week-old) were obtained from the UNM Cancer Center Animal Models Shared Resource and housed in a specific pathogen-free, AAALAC-accredited facility as described previously (4). 10^6 cells per mouse were injected via tail vein (LOUCY cells were transduced with *RUNX2*-shRNAs or with negative control scrambled shRNA) and (PF382 cells were transduced with RUNX2 overexpressing plasmid or control plasmid). For survival experiments, animals (10 mice per group) were euthanized when showing signs of moribund condition or weight loss exceeding 10-15% of total weight. For leukemia burden analyses in RUNX2-depletion model, the animals were injected intravenously with transduced LOUCY cells (10^6 cells per mouse; 4 mice per group). For leukemia burden analyses in RUNX2 overexpression model, the animals (7 mice per group) received 3 x 10^5 transduced PF382 cells via interfemoral injection. All the mice were sacrificed at the same time point. Leukemic cells were extracted from the BM of femurs, blood, meninges, spleen and liver by centrifugation in a Percoll (GE Healthcare) and Ficoll-Paque density gradient. Cells were stained with anti-human APC-CD45+ and anti-mouse BV-421-CD45+ antibody (BD Biosciences) and analyzed by flow cytometry.

Runx2 conditional knockout mice breeding was performed at the Ghent University Hospital animal facility. Runx2 floxed mice were generated carrying a conditional Runx2 allele with exon 4, which encodes the Runt domain, flanked by loxP sites (5). Runx2 flox-neo mice were crossed with FLPe transgenic mice to eliminate the neomycin cassette. To knock out Runx2 in the hematopoietic system, Runx2$^{flox/flox}$ mice were crossed with Vav-iCre transgenic (Vav-iCre$^{tg/+}$) mice which were provided

in-house. Tamoxifen inducible knockout mice were generated by crossing with Cre-ER T2$^{tg/+}$ mice. Genotyping primers are listed below.

To monitor peripheral blood counts at steady state, blood samples were collected from 8- to 12-week-old mice in EDTA-containing Microtainer tubes (BD Biosciences) and analyzed on Cell Dyn-3700 (Abbott Hematology) to measure white and red blood cell counts, platelets, lymphocyte and neutrophil percentages, and hemoglobin levels. Thymocyte counts were performed by manual counting upon total dissection of thymus at 6-10 weeks age. Antibodies used for T-cell subsets are listed below, flow cytometry was performed on a LSRII (BD) cytometer and analyzed with FlowJo for this experiment. To extract lineage-depleted (Lin−) progenitors, BM cells were isolated from femurs and tibias of 8- to 10-week-old mice. Following red blood cell (ACK) lysis, Lin- cells were isolated using the mouse lineage depletion kit (MACS, Miltenyi, 130-110-470).

For engraftment of shRNA transduced PDX (KMT2A-MLLT1), freshly isolated PDX spleen cells were used. pLKO plasmids (shRUNX2-1, shRUNX2-2 or control), with the puromycin selection marker replaced by eGFP by restriction-ligation cloning, were used for lentiviral vector productions. Transductions were performed by spinoculation (90 minutes at 2500 rpm; 8 µg/mL polybrene), and cells were cultured in alpha Minimal Essential Medium supplemented with 5% FCS, 5% human AB serum, human IL7 (10 ng/ml), hFLT3-L (20 ng/ml), hIL2 (100 ng/ml) and hSCF (50 ng/ml). 150,000 eGFP /hCD45 positive cells were sorted and transplanted in NSG mice by tail vein injection and engraftment was monitored by measuring GFP percentage in peripheral blood or organs.

In vitro transformation of mouse progenitor cells was performed on lineage depleted (Runx2$^{fl/fl}$ CreErt2$^{tg/+}$) BM cells that were transduced with KMT2A-MLLT1-eGFP expressing retrovirus. To achieve complete Runx2 knockout, cells were treated with 400 nM 4-hydroxytamoxifen (4-OHT) for 24 hours. Clonal growth of transduced cells was performed on methylcellulose (M3534, STEMCELL Technologies) according to protocol instructions. Cells were harvested and replated every 7 days for 3 consecutive rounds. RNA was collected prior to the first replating (BM progenitors), and after every replating. After the second replating, cells were grown in suspension and considered as KMT2A-MLLT1 (MLL-ENL) transformed cells. For RNA sequencing experiments, RNA from BM progenitors was compared to transformed cells, with

(Runx2 ko) or without (Runx2 wt) 4-OHT treatment. All samples originated from independent biological replicates.

Bioluminescence Imaging. Red Luciferase^{+} leukemic cells (PF382 cells transduced with RUNX2 overexpressing plasmid or control plasmid) were injected intrafemorally into NSG mice (3 x 10^5 cells per mouse; 7 mice per group). Tumor burden was quantified by bioluminescence imaging on the IVIS Spectrum in vivo Imaging System (Perkin Elmer). Mice were injected intraperitoneally with D-Luciferin (Perkin Elmer) at 150 mg/kg and kept conscious for 3 minutes to allow for proper circulation of the D-Luciferin. Mice were then anesthetized via isoflurane inhalation in an induction chamber and transferred to the heated imaging platform. Mice were imaged 10 minutes post-injection in both supine and prone positions using the autoexposure settings to maximize signal acquisition and medium binning to optimize resolution and sensitivity. Bioluminescent signal was analyzed using the Living Image software (Perkin Elmer). To compare signal intensity longitudinally at multiple time points, all images were loaded as a group and analysed using the same settings. Bioluminescent signal is shown as radiance (photons/sec/cm2/steradian).

Microarray and RNA sequencing data sets were from publicly available data resources, including GSE70536 (6), E-MTAB-593 (7) and (8). Data were analyzed as described previously (6).

Chromatin Immunoprecipitation (ChIP). Chromatin immunoprecipitation was performed on T-ALL cells as described in (9). DNA was immunoprecipitated with anti-RUNX2 (D1H7), anti-KMT2A^{N} (A300-086A) and non-specific IgG antibodies (SC-2027 X) (Santa Cruz Biotechnology, TX, USA) and amplified by qRT-PCR using SYBR Green PCR Master Mix on a StepOnePlus™ Real-Time PCR System under standard conditions. Results were quantified by SYBR Green Real-Time PCR analysis. The fold enrichment of immunoprecipitated samples was normalized on INPUT and expressed relative to the mock-treated control (IgG). Results were visualized after separating PCR products on 3% agarose gel stained with GelGreen Nucleic Acid Staining (Biotium, Fremont, CA, USA).

***ChIP sequencing and analysis*.** In brief, 1 × 10^7 cells were cross-linked for each ChIP experiment with 1.1% formaldehyde (Sigma-Aldrich, F1635) at room temperature for 10 min and the cross-linking reaction was quenched with glycine (125 mM final concentration, Sigma-Aldrich, G-8790). Nuclei were isolated and chromatin was purified by chemical lysis. The purified chromatin was fragmented to

200–300 bp fragments by sonication (Covaris, Woburn, MA, USA; S220, Focused-ultrasonicator). Chromatin immunoprecipitation was performed by incubation of the chromatin fraction overnight with 100 µl of protein-A coated beads (Thermo-Scientific, Waltham, MA, USA; catalog number 53139) and 10 µg of the specified antibodies. The next day, beads were washed to remove non-specific binding events and enriched chromatin fragments were eluted from the beads, followed by reverse cross-linking by incubation at 65 °C overnight. DNA was subsequently purified by phenol/chloroform extraction, assisted by phase lock gel tubes (5Prime). DNA obtained from the ChIP assays was adaptor ligated, amplified and analyzed by Illumina sequencing. Raw sequencing data were mapped to the human reference genome (hg38) using Bowtie. Peak calling was performed using MACS2 (10). ChIP seq data have been deposited in the GEO database (GSE151823). HOMER (11) was used for motif analysis, peak annotation, and to produce tag directories and heatmap data matrices of (public) ChIP-seq data. Heatmaps were produced in R. TSS-centered heatmaps were ordered based on average linkage clustering of the rows in the RUNX2 heatmap. RUNX2 ChIP-seq peak heatmaps were ordered by descending RUNX2 peak heights.

Extracellular flux analyses. Sensor cartridge (Agilent Technologies, Santa Clara, CA, USA) was hydrated with XF calibrant (500 µL/well) (Agilent Technologies) at 37°C in a non-CO2 incubator overnight. XF cell culture microplate (Agilent Technologies) was coated with Poly-D lysine (50 µg/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 2 h in a 5% CO2 at 37°C. The wells were washed with H_2O and air-dried. The cells were washed, resuspended with XF media (Agilent Technologies), and plated on poly-D lysine precoated XF cell culture microplate at 0.3 x 10^6 cells per well (200 µl). The plate was centrifuged for 5 min at 400 × g and 300 µL/well XF medium was added to each well (final volume of 500 µL/well). The plate was incubated at 37°C in a non-CO2 incubator for 60 min. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured on an XF24 Analyzer (Agilent Technologies) using he Seahorse XF Cell *Mito Stress*Test and kit and the Seahorse XF *Glycolysis Stress* Test kit, respectively, (Agilent Technologies) according to the manufacturer's protocols. Oxygen consumption rate and extracellular acidification rate were normalized to cell count measured after assay completion.

Migration Assay. Cell migration was tested using the QCM Chemotaxis Cell Migration Assay kit (3 µm) (Millipore Sigma, St. Louis, MO, USA). T-ALL cells were suspended in serum-free RPMI-1640 and loaded into the upper chambers in the transwell system

(0.3 x 10^6 cells per insert). Serum-free RPMI-1640 medium alone or supplemented with CXCL12 (PeproTech) (50ng/µl) was added into the lower chambers. The cells were migrated for 6 hours and the levels of migrated cells were determined by trypan blue staining. For inhibitor studies, the cells were pre-treated with a glucose analog (2-deoxy-glucose, 2DG, 1 mM; Sigma-Aldrich), Drp-1 inhibitor (Mdivi-1, 1 µM; Cayman Chemical, Ann Arbor, MI, USA) and AMPK pathway inhibitor (dorsomorphin, 1µM, Cayman Chemical) for 16 h, prior to loading into the transwell inserts followed by migration assay as described above.

Transendothelial cell migration assay. HUVEC cells (1.5 x 10^5) were seeded into the transwell inserts for 24 h (5 µm, QCM Chemotaxis Cell Migration Assay kit, Millipore Sigma). The HUVEC cells were washed in serum free medium. T-ALL cells were suspended in serum-free RPMI-1640 (0.3 x 10^6 cells per insert) and were loaded onto HUVEC cells monolayer. The migration assay was conducted for 6 h in the presence or absence of CXCL12 (50ng/µl) in the lower chambers. The levels of migrated cells were determined by trypan blue staining.

Cell adhesion assay. Cell adhesion was performed using the ECM Cell Adhesion Array kit (Colorimetric, ECM540, 96 wells, Millipore Sigma) following the manufacturer's instructions. Briefly, the cells were plated into fibronectin coated plates (10^5 cells/100 µl, serum free RPMI 1640) and incubated for 2 h at 37ºC in a 5% CO_2. Non-adherent cells were removed by washing. The cell stain solution (100 µl) was added to each well for 5 minutes at RT. The plate was gently washed 3-5 times with deionized water and the wells were air-dried. The extraction buffer was added to each well (100 µl) and the plate was placed on an orbital shaker for 5-10 minutes at RT. Absorbance was measured at 560 nm using the Bio-Rad iMark Microplate Absorbance Reader.

***Glucose uptake assays*.** Glucose uptake was assessed using a Glucose Uptake-Glo™Assay (Promega). Briefly, 3 x 10^4 cells were washed with PBS and 50µl of 2-deoxyglucose (2DG; 1mM) was added to each well. The cells were incubated for 10 minutes at RT followed by adding 25µl of Stop and Neutralization Buffers. The cells were next incubated with 100µl of 2-deoxyglucose-6-phosphate (2DG6P) Detection Reagent (Component per reaction: Luciferase Reagent 100 µl, $NADP^+$ 1 µl, G6PDH 2.5 µl, Reductase 0.5 µl and Reductase Substrate 0.0625 µl) for 1 hour at RT in dark. Luminescence was measured with the BioTek Synery Neo2 Plate Reader (BioTek).

Cytokine measurement. Serum was collected from NSG mice (5 per group), 28 days upon tail vein injection of 10^6 cells. Human and mouse cytokines were analyzed using Human Cytokine 42-plex Discovery Assay and Mouse Cytokine 32-Plex Discovery Assay (Eve Technologies; Calgary, AB, Canada).

***Non-competitive and competitive homing*.** For non-competitive homing assay, NSG mice (5 mice per group) were engrafted with 10^7 PF382 cells transduced with *RUNX2* overexpressing plasmid (RUNX2 OE) or control plasmid (NC). The levels of human CD45+/GFP+ cells in BM were assessed 24 h later by flow cytometry. Competitive homing assays were performed similarly. RUNX2 OE cells were labelled for 30 minutes with CellTracker™ Red CMTPX (5 µM, Invitrogen) and NC cells with CellTracker™ Violet BMQC Dye (10 µM, Invitrogen), and injected (10^7 cells/mouse) in 5 NSG mice at a 1:1 ratio.

Mitochondrial membrane potential of T-ALL cells was determined by flow cytometry following 20 minutes staining (37°C, 5% CO2) in pre-warmed PBS with 300 nM of MitoTracker Red (CMXRos, Invitrogen).

Cellular ROS levels were assessed by flow cytometry following 30 minutes incubation of T-ALL cells with 500 nM CellROS Deep red (Invitrogen) in 1 mL growth medium.

Drug sensitivity studies. The synthesis of AI-10-104 (2-(3-methoxypyridin-2-yl)-6-(trifluoromethoxy)-1H-benzo[d]imidazole) was carried out as described previously (12). Analytical data upon synthesis were in full agreement with (12). T-ALL cell lines were incubated for 48 hours in 500 µl medium with 10% FCS to which 5 µL of the appropriate dilution of AI-10-104 or dimethylsulfoxide (DMSO) (0.1% f.c.) was added. For PDX cells treated *ex vivo,* the cells were cultured in alpha Minimal Essential Medium supplemented with 5% FCS, 5% human AB serum, human IL7(10 ng/ml), hFLT3-L (20 ng/ml), hIL2 (100 ng/ml) and hSCF (50 ng/ml). The viability assays were performed with the Celltiter-Glo Luminescent Cell Viability Assay according to the manufacturer's instructions (Promega). The IC50 values were calculated using GraphPad Prism 7.02 software (GraphPad, LaJolla, CA, USA). The experiment was performed 2 or 3 times independently; in every experiment, each concentration was tested in duplicate for every cell line. For RNA sequencing, KARPAS-45 cells were treated with 20 µM AI-10-104 or DMSO control in 4 independent experiments.

RNA sequencing. Total RNA samples (500 ng) were purified using DNAse I kit according to the Rapid out removal DNA kit instructions (Thermoscientific) and converted into cDNA by using QuantSeq 3' mRNA-seq reverse 4 Library Prep Kit

(Lexogen) according to manufacturer's instruction to generate compatible library for Illumina sequencing. The quality of cDNA libraries was determined using a High Sensitivity DNA Assay 2100 Bioanalyzer (Agilent). Sequencing of the cDNA library with 75 bp single end reads was performed using an Illumina HiSeq 2500 system. Quality control of fastq files was done with FastQC and reads were aligned GRCh38 using STARv2.42 and gencode v25 as guide gtf. Counts were generated on the fly by STAR. Differential expression analysis was performed using DESeq2 (13), which used Wald test statistic for p-value calculation. Functional enrichment analysis was performed in R with ReactomePA package, detected genes were used as background and differential up and down genes were used as genesets. All RNA sequencing data are available at GSE151823. For KARPAS-45 shRUNX2 knockdown RNA sequencing experiments, cells were transduced with shRUNX2-1, shRUNX2-2 or scrambled control and sampled 48 hours and 72 hours later. Data obtained from control was compared to pooled data from both RUNX2 specific hairpins for 5 independent experiments.

Supplemental references

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Supplemental list of reagents

Plasmids			
Name	**cat #**	**sequence/specification**	**source**
Lentiviral plasmids			
shRUNX2 B	#TL309683	ACCAAGTAGCAAGGTTCAACGATCTG AGA	Origene
shRUNX2 C	#TL309683	TCCATATCTCTACTATGGCACTTCGTCA G	Origene
shNC	#TR30021	5' GCACTACCAGAGCTAACTCAGATAGT ACT 3'	Origene
RUNX2 OE	RC212884L4	pLenti-C-mGFP-P2A-Puro-RUNX2	Origene
NC	PS100093V	pLenti-C-mGFP-P2A-Puro	Origene
pMD2.G	#12259	lentiviral packaging envelope	Addgene
pMDLg/pRRE	#12251	lentiviral packaging gag/pol	Addgene
pRSVRev	#12253	lentiviral packaging rev	Addgene
shNC	plko-shc002	CAACAAGATGAAGAGCACCAA	Sigma-Aldrich
shRUNX2-1	TRCN0000136 53	GCTACCTATCACAGAGCAA	Sigma-Aldrich
shRUNX2-2	TRCN0000136 55	CAGCACTCCATATCTCTAC	Sigma-Aldrich
pMD2.G	#12259	lentiviral packaging envelope	Addgene
psPAX2	#12260	2nd gen lentiviral packaging	Addgene
Red Luciferase	Q#11641	pLenti-EF1a (Red-Luciferase)-Rsv (RFP-Blast)	GenTarget Inc

pCS2		Control plasmid (for Active β-catenin)	Steven Goossens
pCS2mBCTN533Ac/3		Active β-catenin overexpression	Steven Goossens
Retroviral plasmids	**source**		
pMSCVneo-KMT2A-MLLT4	Eric So	Phoenix cell line packaging	
pMSCVneo-KMT2A-MLLT1	Eric So	Phoenix cell line packaging	
pMSCVNeo	Eric So	Phoenix cell line packaging	
pMSCV-KMT2A-MLLT1-eGFP	Jan De Rijck	pCL-ECO packaging	
pMSCV-PIG	Addgene	pCL-ECO packaging	
Adenoviral plasmids	**cat #**		
Ad-BIRC5	1611	Survivin overexpression	Vectorbiolabs
Ad-CMV-Null	1300	control vector (for Survvin)	Vectorbiolabs

Primers and probes		**Forward primer sequence**	**Reverse primer sequence**
qRT-PCR primers for SYBR-GREEN assays (UGent Samples)			
species	gene		
Human	RUNX2	5'-GTAGCAAGGTTCAACGATCT-3'	5'-GTGAAGACGGTTATGGTCAA-3'
Human	HMBS	5'-GGCAATGCGGCTGCAA-3'	5'-GGGTACCCACGCGAATCAC-3'
Human	TBP	5'-CACGAACCACGGCACTGATT-3'	5'-TTTTCTTGCTGCCAGTCTGGAC-3'
Human	B2M	5'-GCTGTCTCCATGTTTGATGTATCT-3'	5'-TCTCTGCTCCCCACCTCTAAGT-3'
Human	RPL13A	5'-CCTGGAGGAGAAGAGGAAAGAGA-3'	5'-TTGAGGACCTCTGTGTATTTGTCAA-3'
Human	HPRT1	5'-TGACACTGGCAAAACAATGCA-3'	5'-GGTCCTTTTCACCAGCAAGCT-3'
Human	UBC	5'-ATTTGGGTCGCGGTTCTTG-3'	5'-TGCCTTGACATTCTCGATGGT-3'
Mouse	Runx2	5'-GACTGTTATGGTCAAGGTGAA-3'	5'-ACCAAGTAGCCAGGTTCAA-3'
Mouse	Tbp	5'-CCCCACAACTCTTCCATTCT-3'	5'-GCAGGAGTGATAGGGGTCAT-3'
Mouse	Ubc	5'-GCAGATCTTTGTGAAGACCC-3'	5'-GAAGGTACGTCTGTCTTCCT-3'
Mouse	Hmbs	5'-GAATTCAGTGCCATCGTCCT-3'	5'-AATGCAGCGAAGCAGAGTTT-3'
genotyping primers			
Runx2 floxed		5'-TAAATCCAGATGCCCCTGAG-3'	5'-TTGAAACCATCCACAGGTGA-3'
vav-iCre tg		5'-AGATGCCAGGACAT CAGGAACCTG -3'	5'-ATCAGCCACACCAG ACACAGAGATC-3'
Rosa26-CreER T2 tg		5'-CGCCGCATAACCAGTGAAAC-3'	5'-ATGTCCAATTTACTGACCG-3'
control primers		5'-CTGTCCCCAACGGATTTCTA -3'	5'-ACGGTGAGGCCACTTGTATC-3'
Primers used cloning of KMT2A promoter into the pGL3 basic luciferase vector.			
KMT2A- pGL3		5'-AATACTCGAGACCAAGGGCTCTT TTATTCTAGAAGCGTTC-3'	5'-AATAAAGCTTCAACCTGGATA ACTGCATGCCC-3'
ChIP-qRT-PCR primers			
KMT2A promoter		5'-GCGTTCAATTCGGGCTAAC-3'	5'-GAGAGGCATTGCTCGGG-3'
RUNX2 promoter		5'-GATTGATAATAAACTAGA CAGACGTGAT-3'	5'-GCACTATTACTGGAGAGGCAGAATC-3'
Primers and probes for the expression of KMT2A-MLLT4 and KMT2A-MLLT1.			

Name	Probe sequence		
KMT2A-MLLT4	[6FAM]AAAATTCCAGCAGATTCGCGAGAGGATTTG[TAM]	5'-GGTCCAGAGCAGAGCAAACAG-3'	5'-GAGGACAGCATTCGCATATCAG-3'
KMT2A-MLLT1	[6FAM]TGGACGGTGCACTCTACATGCCCACTA[TAM]	5'-CAGGGTGGTTTGCTTTCTCTGT-3'	5'-GCGATGCCCCAGCTCTA-3'
TaqMan assay probes			
human RUNX2	RUNX2-Hs00231692_m1		
human GAPDH	GAPDH-Hs99999905_m1		
mouse Runx2	RUNX2-Mm00501584_m1		
mouse Gapdh	GAPDH-Mm99999915_g1		
human LDHA	Hs01378790_g1		
human PGK1	Hs00943178_g1		
human CHCHD2	Hs00853326_g1		
human BIRC5	Hs04194392_s1		
human CTNNB1	Hs00355045_m1		
mouse BIRC5	Mm00599749_m1		
mouse CTNNB1	Mm00483039_m1		

Immunological markers according to EGIL (14) classification

pro-T	CD7+ CD2- CD5-
pre-T	CD7+ CD2+ CD5±
cortical-T	CD1a+
mature	mCD3+ CD1a-

Antibodies used for flow cytometry

Mouse T-cell panel				**Channel used with**	
Dye/target		**Fluorophore**	**Catalog #**	**LSRII**	**company**
CD25		PerCP-Cy5.5;B710	551071	PER-CP	BD Pharmingen
CD3		PE;Y585	12-0031-81	PE	eBioscience
CD8		PE-Cy7;Y780	25-0081-82	Pe-Cy7	eBioscience
LD eFluor 450		eFluor 450; V450	65-0863-14	Pacific Blue	eBioscience
Thy1 (CD90.2)		BV500;V525	561616	Amcyan	BD Bioscience
CD28		APC;R670	17-0281-81	APC	eBioscience
CD4		AF700;R730	56-0042-82	AlexaFluor 700	eBioscience
CD44		APC-Cy7;R780	560568	APC-Cy7	BD Bioscience
Intracellular staining	**clone**				
RUNX2	D1L7F	PE	#98059	PE	CST
Fixable Viability Dye	NA	eFluor 506	65-0866-18	AmCyan	eBioscience
Human targets				**LSR Fortessa**	

CD184 (CXCR4)	2B11/CXCR4	BV421	562738	BV421	BD Biosciences
CD49d (VLA-4)	9F10	PE	555503	PE	BD Biosciences
GlUT1	202915	Alexa Fluor 647	566580	APC	BD Biosciences
BV421 Mouse IgG2a, κ Isotype Control	G155-178	BV421	562439	BV421	BD Biosciences
PE Mouse IgG1, κ Isotype Control	MOPC-21	PE	555749	PE	BD Biosciences
Alexa Fluor 647 Mouse IgG2b, κ Isotype Control	27-35	Alexa Fluor 647	565378	APC	BD Biosciences

Antibodies used for Western blot

RUNX2	D1H7	Cell Signalling Technology
Phospho-Akt (Ser473)	D9E	Cell Signalling Technology
Akt	40D4	Cell Signalling Technology
Phospho-Erk1/2 (Thr202/Tyr204)	9101S	Cell Signalling Technology
Erk1/2	L34F12	Cell Signalling Technology
Bcl-2	2872T	Cell Signalling Technology
p53	7F5	Cell Signalling Technology
Non-phospho β-Catenin (Ser33/37/Thr41)	D13A1	Cell Signalling Technology
Survivin	71G4B7	Cell Signalling Technology
Cleaved Caspase-3 (Asp175)	5A1E	Cell Signalling Technology
Cleaved Caspase-7 (Asp198)	D6H1	Cell Signalling Technology
Cleaved Caspase-9 (Asp315)	9505T	Cell Signalling Technology
Actin	E4D9Z	Cell Signalling Technology
Vinculin	4650T	Cell Signalling Technology
Paxillin	D9G12	Cell Signalling Technology
FAK	3285T	Cell Signalling Technology
Ezrin/Radixin/Moesin	3142T	Cell Signalling Technology
ZEB1	D80D3	Cell Signalling Technology
Snail	C15D3	Cell Signalling Technology
Vimentin	D21H3	Cell Signalling Technology
Phospho-DRP1 (Ser616)	3455S	Cell Signalling Technology
MFF	E5W4M	Cell Signalling Technology
Mitofusin 1	D6E2S	Cell Signalling Technology
Mitofusin 2	D1E9	Cell Signalling Technology
OPA1	D6U6N	Cell Signalling Technology
Tom20	D8T4N	Cell Signalling Technology
PGC-1α	3G6	Cell Signalling Technology
NRF-1	D9K7R	Cell Signalling Technology
Phospho-AMPKα (Thr172)	D4D6D	Cell Signalling Technology
AMPKα	D5A2	Cell Signalling Technology
HRP-linked anti-rabbit IgG		Cell Signalling Technology
HRP-linked anti-mouse IgG		Cell Signalling Technology

KMT2A^{N}	A300-086A	Bethyl laboratories
DRP1	NB110-55288SS	Novus Biologicals
Glut1	NB110-39113SS	Novus Biologicals
FIS1	PA5-22142	Invitrogen
LDHA	3582S	Cell Signalling Technology
PGK1	68540S	Cell Signalling Technology
CHCHD2	NBP1-94106	Novus Biologicals
Phospho-AMPKβ1 (Ser182)	4186S	Cell Signalling Technology
AMPKβ1	12063S	Cell Signalling Technology
Phospho-CaMKK2 (Ser511)	12818S	Cell Signalling Technology
CaMKK2	16810S	Cell Signalling Technology
Phospho-LKB1 (Thr189)	3054S	Cell Signalling Technology
LKB1	27D10	Cell Signalling Technology
Antibodies used for ChIP and ChIP-qPCR		
RUNX2	D1H7	Cell Signalling Technology
KMT2A^{N}	A300-086A	Bethyl laboratories
IgG	SC-2027 X	Santa Cruz
H3K27ac	ab4729	Abcam

Supplementary data figure legends

Supplemental Figure 1. *RUNX2* expression in an independent microarray data set of T-ALL samples (**A**) with *KMT2A-R* (n = 12) compared to the remaining cases (Others, n = 88), (**B**) separated according to ETP status by flow (ETP, n = 12; near-ETP, n = 18; non-ETP, n = 48) (GSE70636) (6). (**C**) *RUNX2* expression in immature (n = 12) and other T-ALL subgroups (TAL, n = 27; TLX, n = 17, HOX, n = 6) by microarray gene expression profiling (7). (**A**) Unpaired two-tailed Mann-Whitney *U*-test and (**B**-**C**) Kruskal-Wallis with Dunn's multiple comparison test were used for statistical analyses (**P* < 0.05, ***P* < 0.005, ****P* < 0.0005, *****P* < 0.0001).

Supplemental Figure 2. (**A**-**B**) Ablation of Runx2 in Runx2$^{fl/fl}$;VaviCre$^{tg/+}$ Lin^{-} compared to Runx2$^{fl/fl}$;VaviCre$^{+/+}$ was confirmed by qRT-PCR and Western blotting, respectively. (**C**-**F**) Peripheral blood cell counts measured in Runx2$^{fl/fl}$;VaviCre$^{+/+}$ (Ctrl) mice, Runx2$^{fl/+}$;VaviCre$^{tg/+}$ (Hez) and Runx2$^{fl/fl}$;VaviCre$^{tg/+}$ (KO) mice (7-10 weeks of age). RBC; red blood cells. (**G**-**L**) Percentages of T-cell subsets measured by flow cytometry. (**C**-**K**) One way ANOVA with Tukey's multiple comparisons test.

Supplemental Figure 3. (**A**) LOUCY and (**B**) KARPAS-45 cells were transduced with *RUNX2* shRNAs (shRUNX2 B and shRUNX2 C) and scrambled control (shNC), and the decrease in *RUNX2* transcript and protein levels was confirmed by qRT-PCR and immunoblotting. (**C**) Annexin V and 7-ADD staining followed by flow cytometry analyses. Shown representative histograms for one of three independent experiments. (**D**, **E**) *RUNX2* mRNA expression levels were assessed by qRT-PCR after transducing Ba/F3 and MOHITO control cells and cells expressing *KMT2A-R* (*KMT2A-MLLT1* or *KMT2A-MLLT4*) with RUNX2 shRNAs (shRUNX2 B and shRUNX2 C) and scrambled control (shNC). (**F**) Ba/F3 cells were subjected to immunoblotting using indicated antibodies. Shown are representative Western blots from at least two independent experiments. (**G**) The levels of *CTNNB1* and *BIRC5* mRNA in transduced T-ALL cell lines (qRT-PCR). Cell growth upon forced expression of (**H**) active β-catenin and (**I**) survivin in LOUCY cells with/without shRNA-mediated *RUNX2* knock-down. Data are means ± SD for one of three independent experiments performed in triplicate; repeated measure ANOVA with Tukey's multiple comparisons test. (**A**, **B**, **D**, **E**, **G**) Data represent the means ± SD of three independent experiments; one-way ANOVA with Tukey's multiple comparisons test; *** $P < 0.0005$, **** $P < 0.0001$.

Supplemental Figure 4. (**A**-**B**) Overlapping binding sites occupancy between RUNX2 in KARPAS-45 (**A**) or RUNX2 in PER-117 (**B**) and RUNX1 and other master regulators of hematopoiesis (ETS1, GATA3) in JURKAT. (**C**) Enrichment of RUNX2 promoter binding in genes that are differentially regulated by RUNX2 knockdown (right) compared to non-differential genes (left). (**D**) Enrichment of CXCR4 signaling pathways in RUNX2 binding peaks in KARPAS-45 based on functional annotation provided by GREAT (3.0.0). (**E**) RUNX2 and H3K27ac binding profile on the MYC enhancer N-ME region in PER-117. (**F**-**G**) *RUNX2* expression is significantly upregulated in both KMT2A-R precursor B-cell acute lymphoblastic leukemia (**E**) and KMT2A-R pediatric acute myeloid leukemia.. (**H**) RT-qPCR (left panel) and western blot analyses (right panel) indicating that forced expression of KMT2A-MLLT4 and KMT2A-MLLT1 induces RUNX2. One-way ANOVA with Tukey's multiple comparisons test (**p < 0.005, ***p < 0.0005). (**I**) Top differentially expressed genes in bone marrow progenitor cells compared to KMT2A-MLLT1 transformed cells (MLL-ENL). (**J**) Public ChIP seq binding profiles of KMT2A-fusion proteins on RUNX2 promoter in both human (upper panel) and mouse (lower panel) context. (**K**) RUNX2 belongs to a small

core set of genes that are directly bound by the three most common KMT2A fusion proteins (KMT2A-AFF1; KMT2A-MLLT3; KMT2A-MLLT1). (**F**-**G**) Unpaired two-tailed Mann-Whitney *U*-test; ****p < 0.0001.

Supplemental Figure 5. (**A**) qRT-PCR and immunoblotting for *RUNX2* mRNA and protein levels in CCRF-CEM and PF382 cells transduced with *RUNX2* expressing plasmid (RUNX2 OE) compared to negative control (NC) plasmid. Data are means ± SD for three independent experiments (****P < 0 .0001; unpaired two-tailed t-test). (**B**) Cells stained with Annexin V and 7-AAD and analyzed for apoptotic cell death by flow cytometry. Representative histograms are shown for one of three independent experiments (**C**) Cell cycle distribution was carried out 48 h post transduction by propidium iodide staining followed by flow cytometry analyses. Data are representative for one of three independent experiments. (**D**) CXCR4 mRNA expression levels upon forced RUNX2 expression (**E**) Ba/F3 cells expressing *KMT2A-R* (*KMT2A-MLLT1* or *KMT2A-MLLT4*), LOUCY and KARPAS-45 cells were transduced with *RUNX2* shRNA (shRUNX2 B and shRUNX2 C) and scrambled negative control (shNC). Cells were lysed and subjected to immunoblotting with the indicated antibodies. Representative blots are shown. (**F**) Total amount of leukemic cells in BM (femurs), meninges, liver, spleen and blood (0.2 ml) of NSG mice (n = 7/group) injected intrafemorally with transduced PF382 cells (3 x 10^5 cells/mouse) at the time of sacrifice (day 34). (**G**) Representative bioluminescence of the tumour load in mice (n = 7/group) engrafted intrafemorally with control (NC) and RUNX2 OE PF382 cells (3 x 10^5 cells/mouse). (**H**) Serum samples analyzed on mouse cytokine array 28 days post-inoculation (i.v.) with PF382 OE and PF382 NC cells (n = 5/group; 10^6 cells/mouse). (**F**) Unpaired two-tailed t-test (**A**, **D**, **H**) with Holm-Sidak's correction for multiple comparisons; *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.0001.

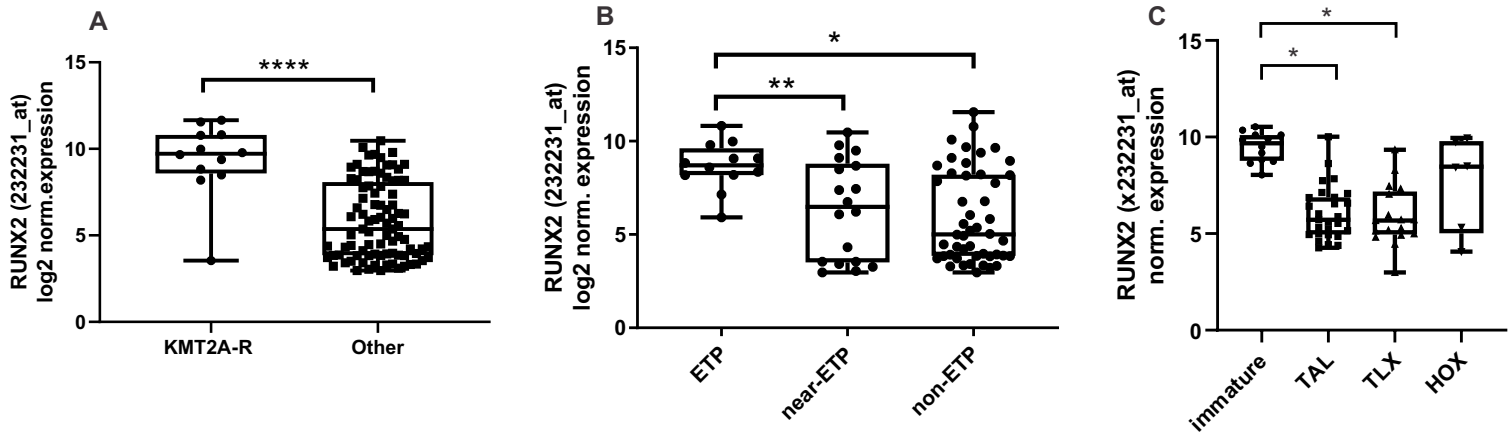
Supplemental Figure 6. Glucose Stress assay performed on the Seahorse XF24 Bioanalyzer in (**A**) CCRF-CEM and (**B**) PF382 cells transduced with RUNX2 expressing plasmid (RUNX2 OE) or negative control plasmid (NC). (**C**) Glucose uptake assay in T-ALL cell lines with forced RUNX2 expression. (**D**, **E**) Extracellular acidification rate (ECAR) analyses, (**F, G**) glucose uptake assay and (**H**) Glut1 expression levels by flow cytometry in MOHITO and/or Ba/F3 cells +/- *KMT2A-R* (*KMT2A-MLLT4, KMT2A-MLLT1*) followed by transduction with shRNAs targeting

RUNX2 (shRUNX2 B, shRUNX2 C) or scrambled control (shNC). (**I**) Glucose stress assay, (**J**) Glut 1 expression levels and (**K**) glucose uptake, in LOUCY cells transduced with shRUNX2 B, shRUNX2 C or scrambled control (shNC). Oxidative respiration rate (OCR) analyses in transduced (**L**) CCRF-CEM, (**M**) PF382, (**N**) MOHITO, (**O**) Ba/F3, (**P**) LOUCY T-ALL cell lines. The ratio of OCR/ECAR in (**Q**) Ba/F3 cells +/- *KMT2A-R* (*KMT2A-MLLT4, KMT2A-MLLT1*) followed by *RUNX2* knock down (**R**) LOUCY with/without RUNX2 depletion. The expression of *LDHA, PGK1 and CHCHD2* mRNA in transduced (**S**) CCRF-CEM and PF382 (**T**) KARPAS-45 and (**U**) LOUCY cells (qRT-PCR). (**V**) Ba/F3 cells +/- *KMT2A-R,* LOUCY and KARPAS-45 cells were transduced with *RUNX2* shRNA (shRUNX2 B and shRUNX2 C) and scrambled negative control (shNC). Shown are representative immunoblots from at least two independent experiments. (**A**, **B**, **D**, **E**, **I**, **L**-**R**) Data are means + SD from one of two representative experiment performed in triplicate. (**C**, **F**-**H**, **J, K, S**-**U**) Data represent the means ± SD of three independent experiments. (**C, S**) Unpaired two-tailed t-test with Holm-Sidak's correction for multiple testing, (**G**, **H**, **F**, **J**, **K**, **T**, **U**) one-way ANOVA with Tukey's multiple comparison test; ***$P < 0.0005$, ****$P < 0.0001$.

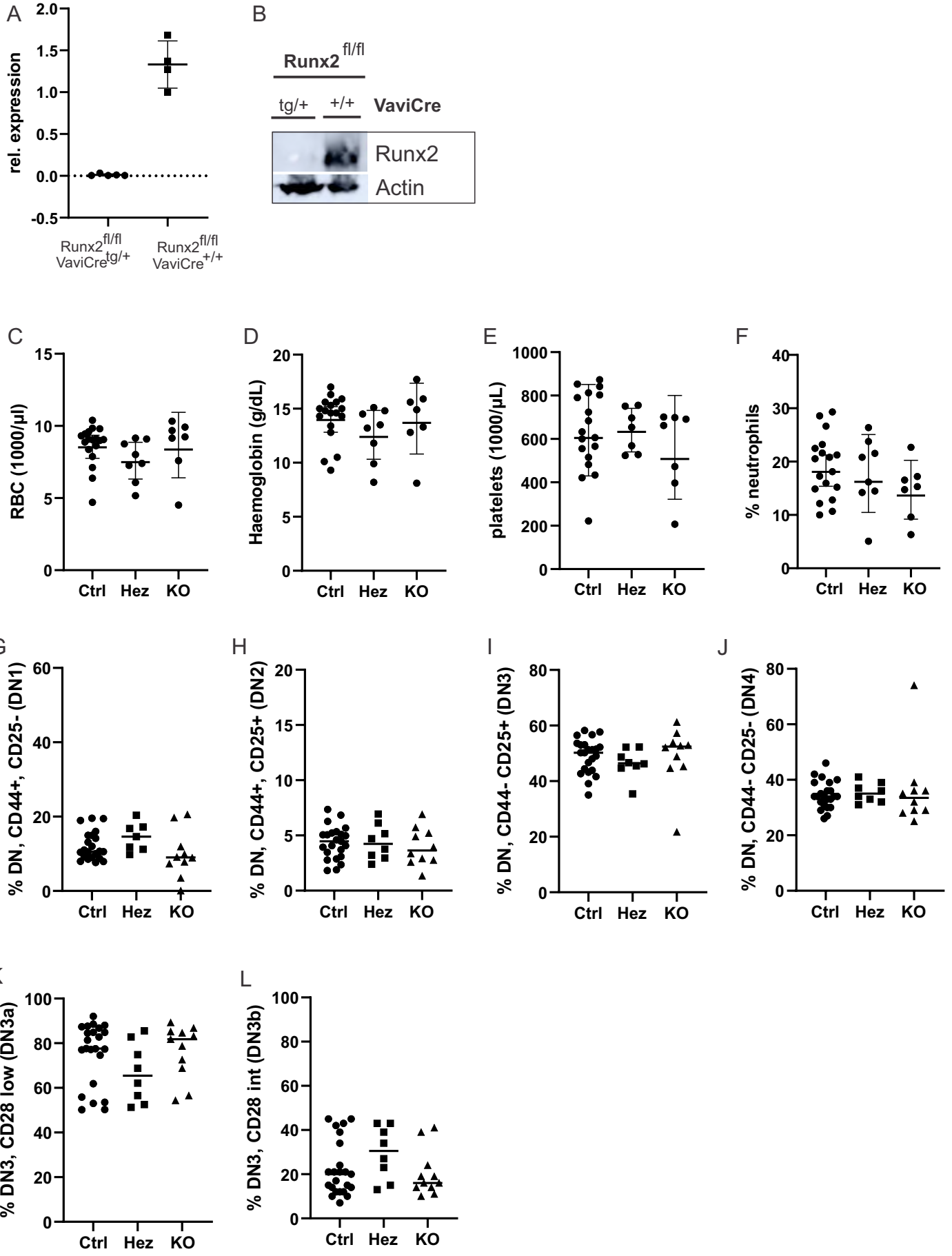
Supplemental Figure 7. (**A, B**) Ba/F3 cells expressing *KMT2A-R* (*KMT2A-MLLT1* or *KMT2A-MLLT4*) and (**C**, **D**) LOUCY cells were transduced with *RUNX2* shRNA (shRUNX2 B and shRUNX2 C) and scrambled negative control (shNC). (**A, C**) Flow cytometric analyses of mitochondrial membrane potential using Mitotracker Red (CMXRos). Shown representative histograms (left) and the median fluorescent intensity (MFI) +/- SD of three independent experiments (right). (**B, D**) Representative histograms for transduced cells stained with CellROS Deep Red (left); the right panel indicates MFI ± SD of three independent experiments.(**E**, **F**) Ba/F3 cells +/- *KMT2A*-R and LOUCY cells transduced with RUNX2 shRNAs (shRUNX2 B and shRUNX2 C) and scrambled negative control (shNC) were lysed and subjected to immunoblotting with indicated antibodies. (**A**-**D**) One-way ANOVA with Tukey's multiple comparisons test; ***$P < 0.0005$, *****$P < 0.0001$).

Supplemental Figure 8. Gene set enrichment analysis using top 500 most differentially downregulated (left panel) and upregulated (right panel) genes upon 24h treatment of KARPAS-45 with 20 μM AI-10-104 on the 72 h RUNX2 shRNA knockdown (kd) versus control (ctrl) expression dataset.

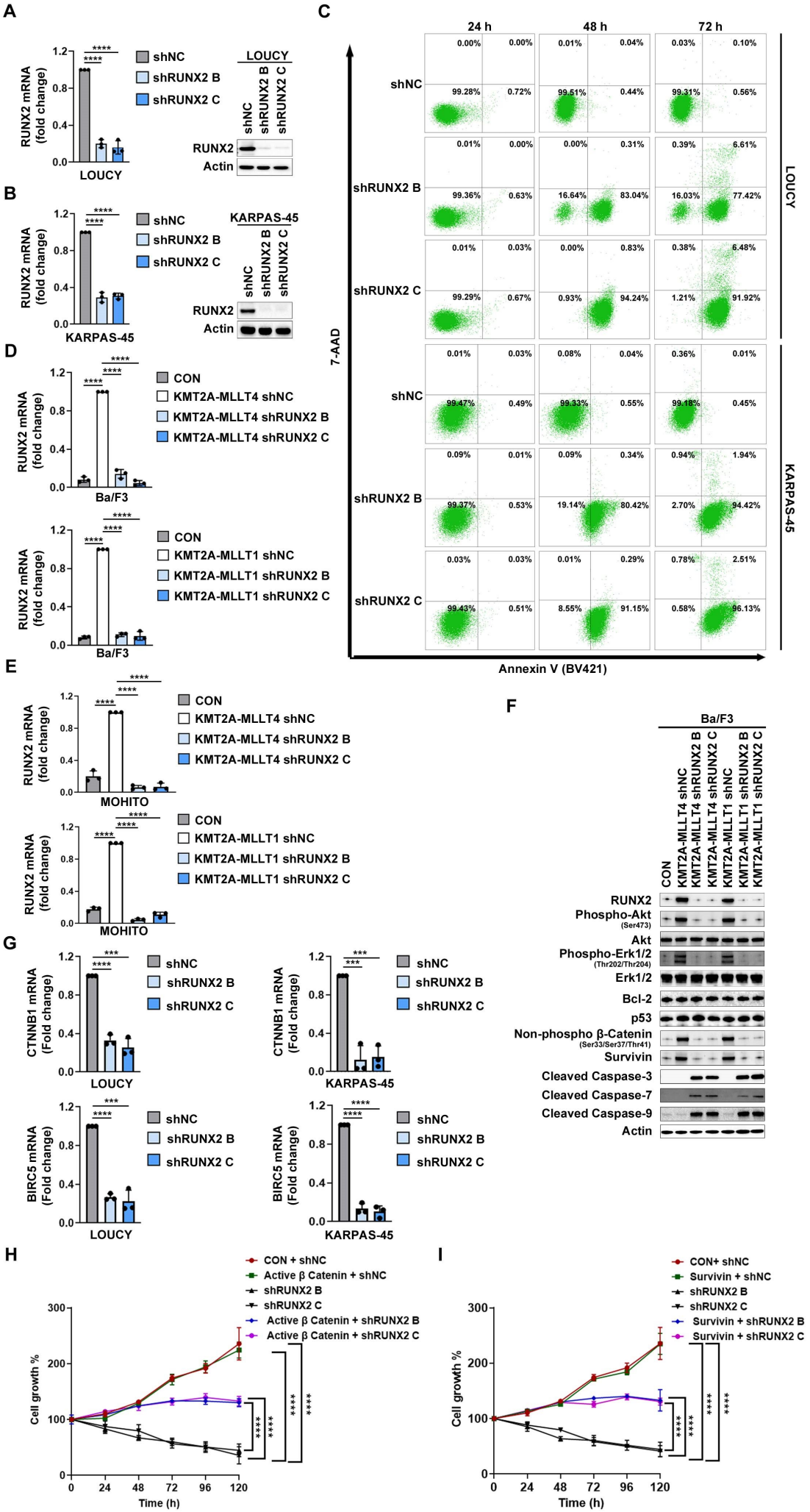
Supplemental Figure 1



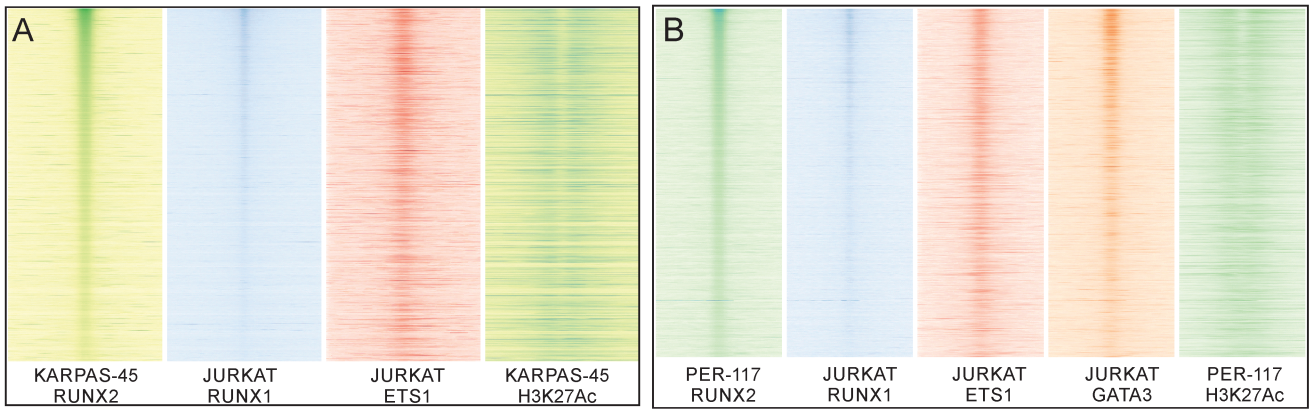
Supplemental Figure 2

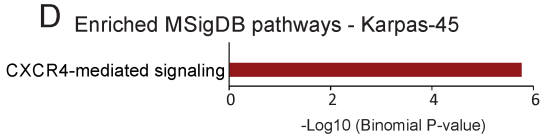


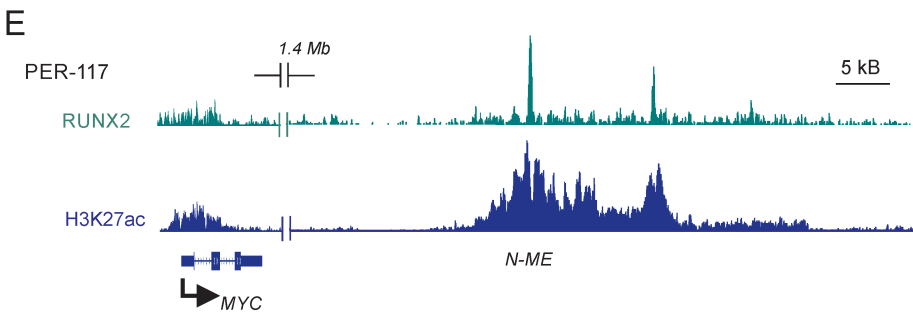
Supplemental Figure 3

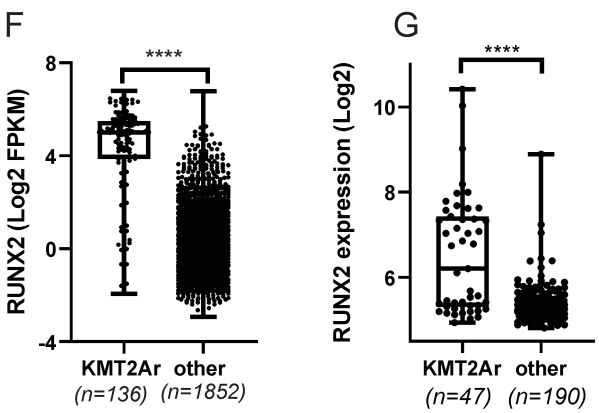


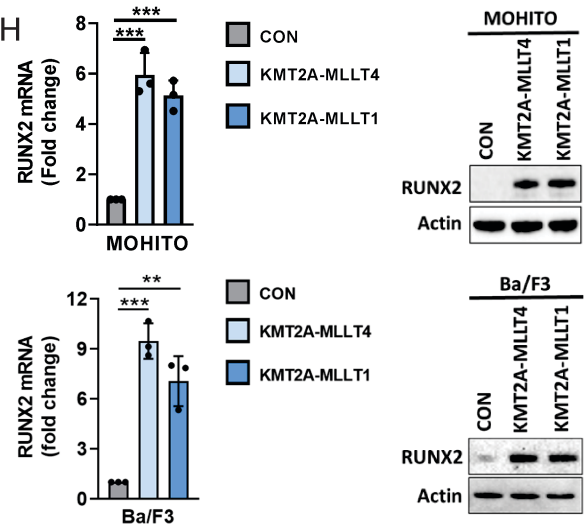
Supplemental Figure 4

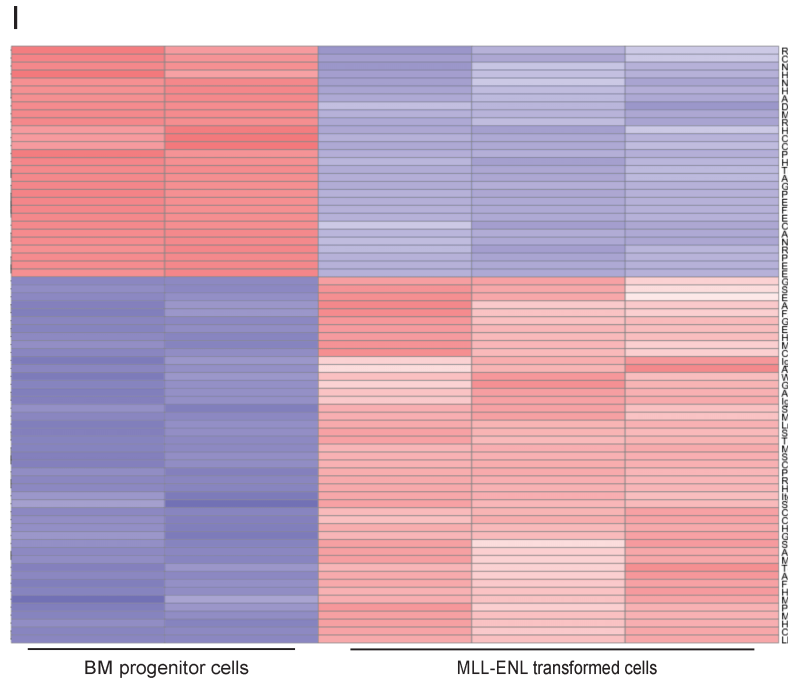


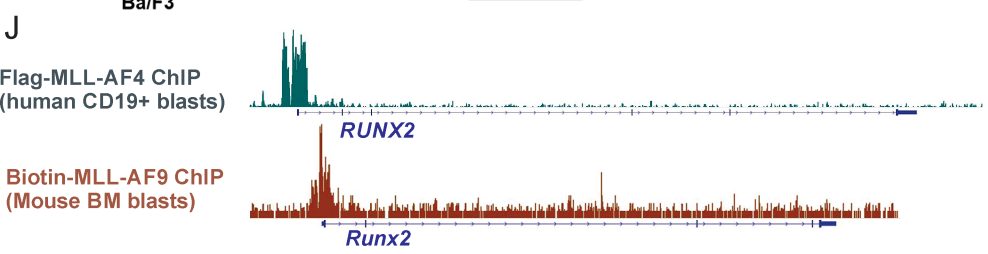


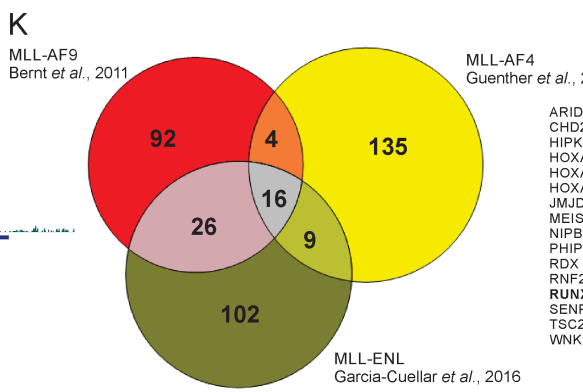




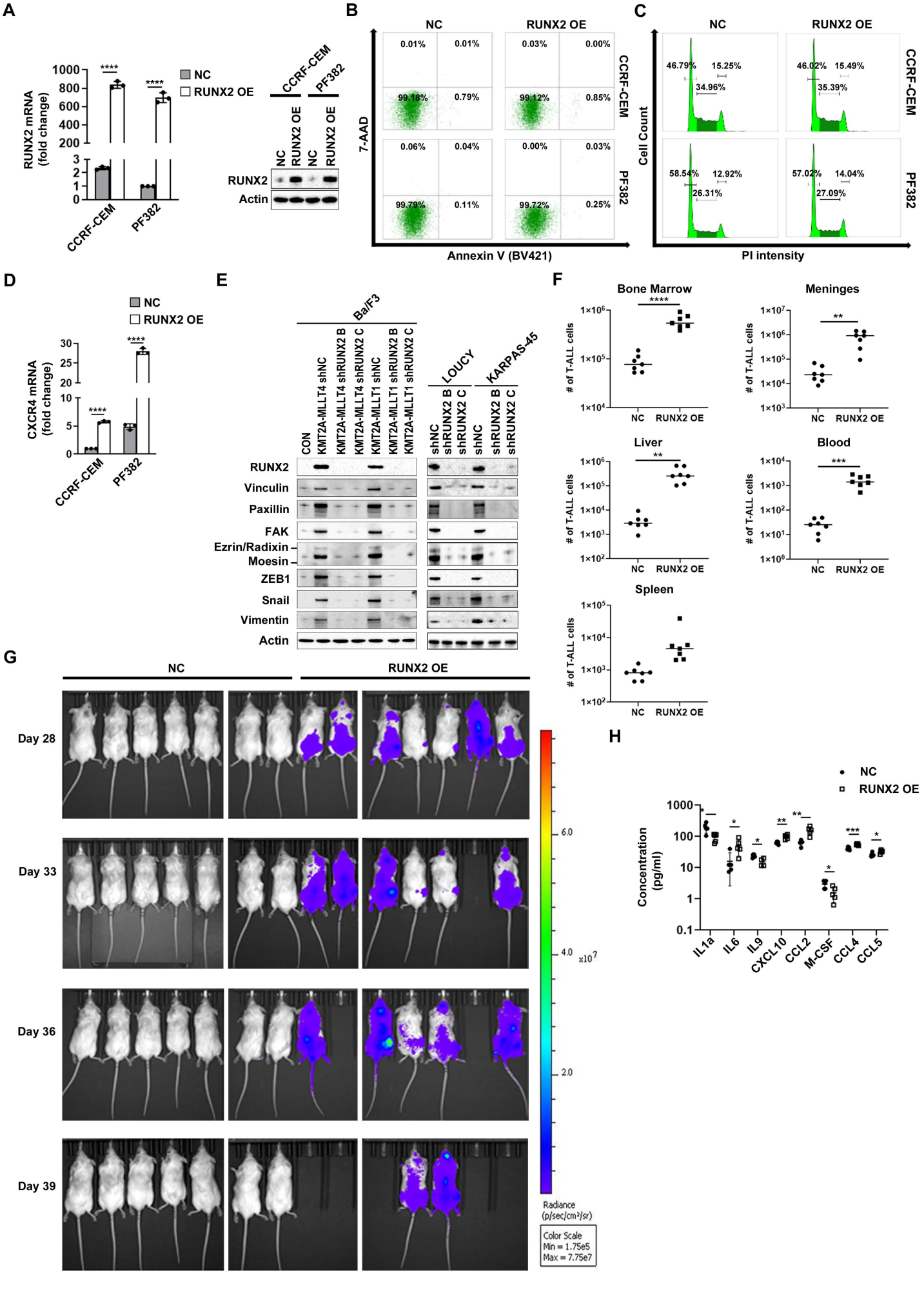




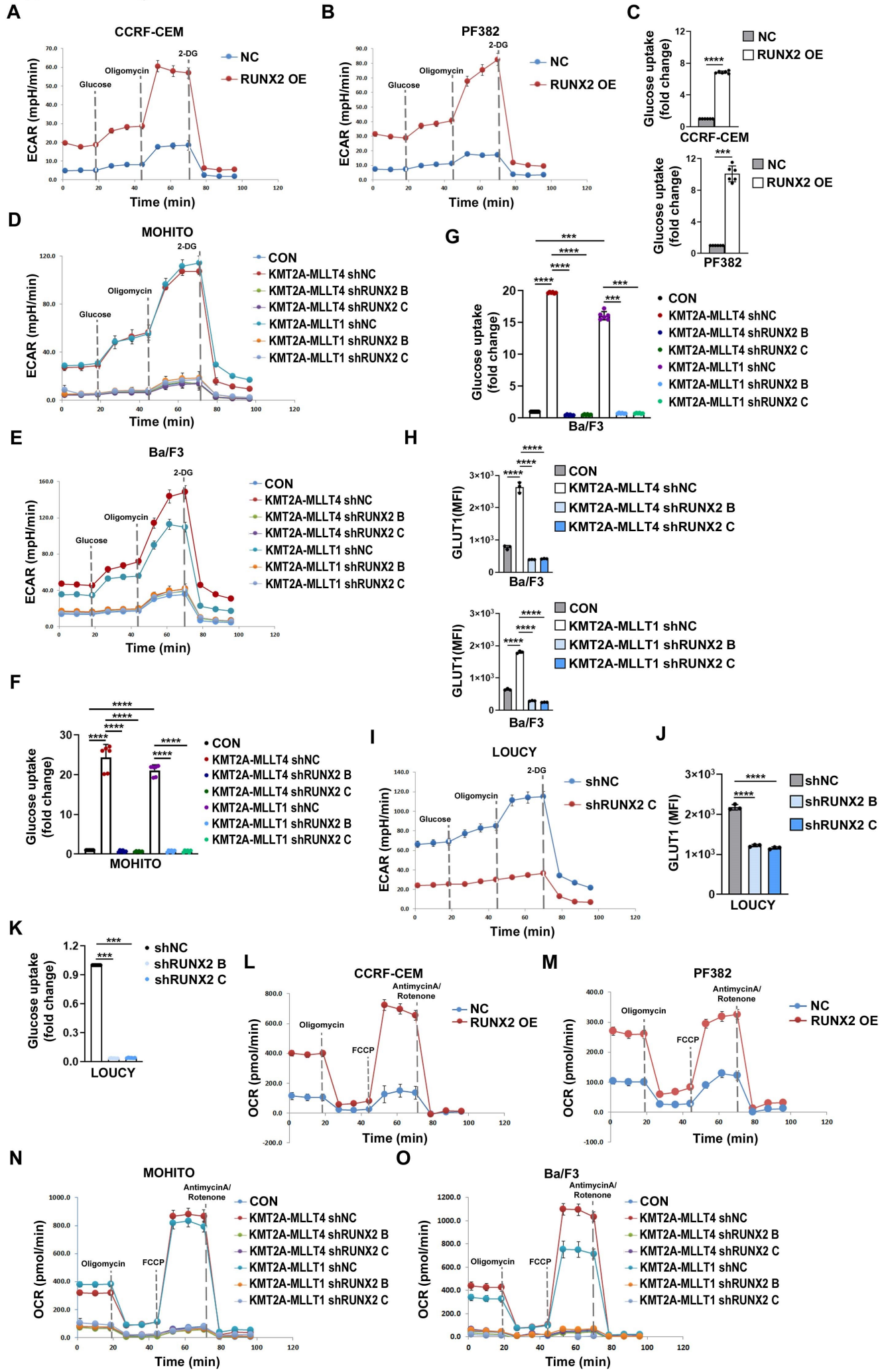




Supplemental Figure 5



Supplemental Figure 6



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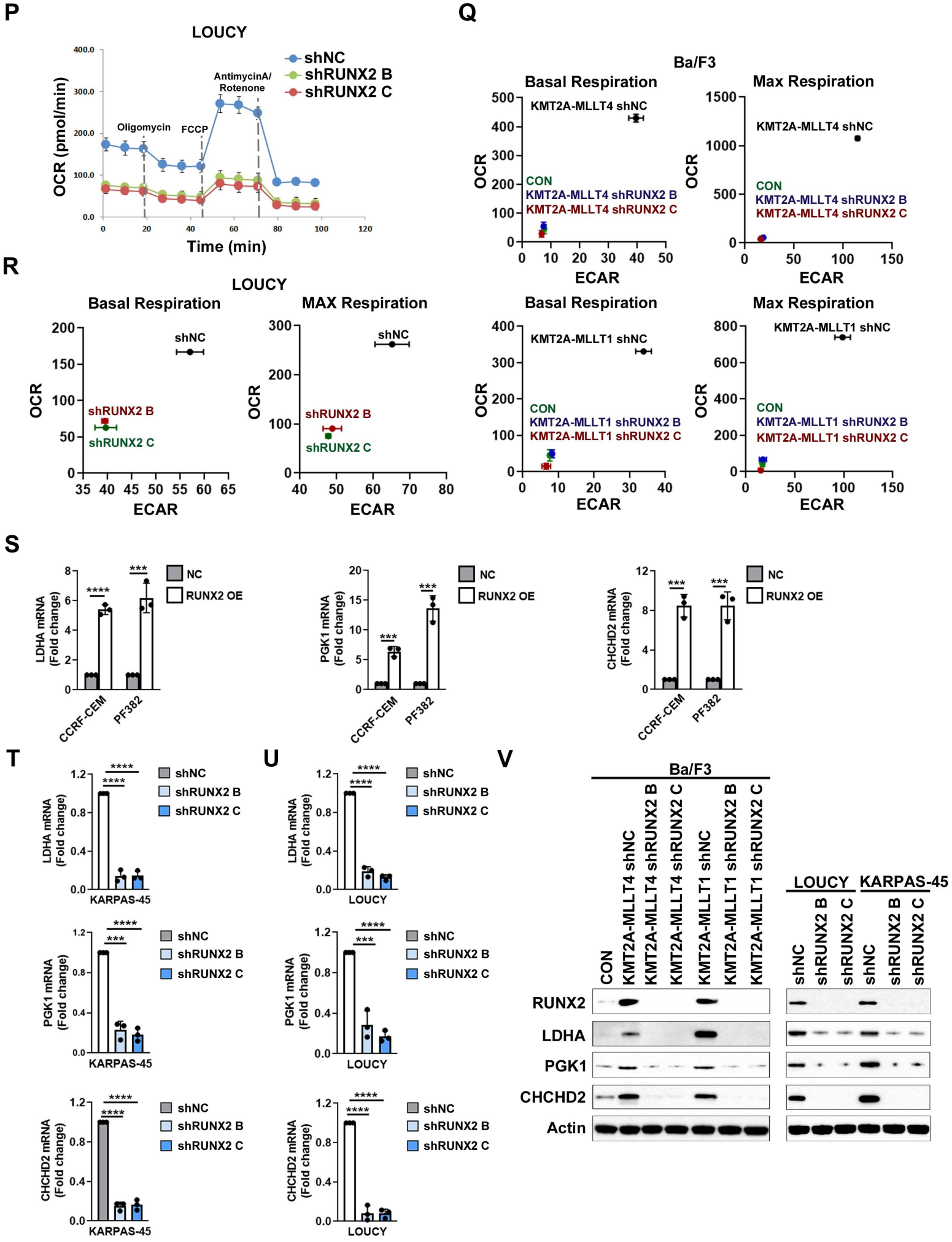
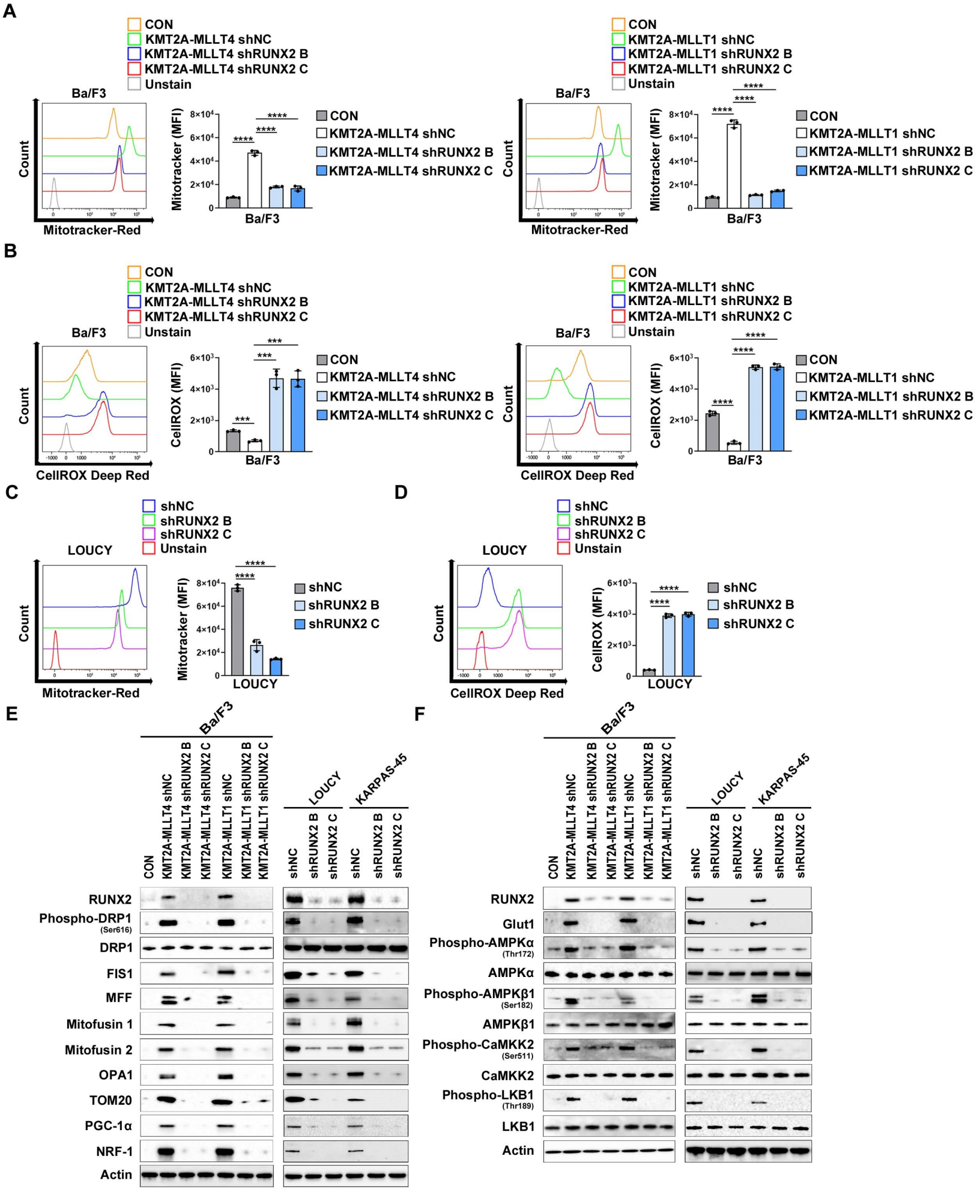
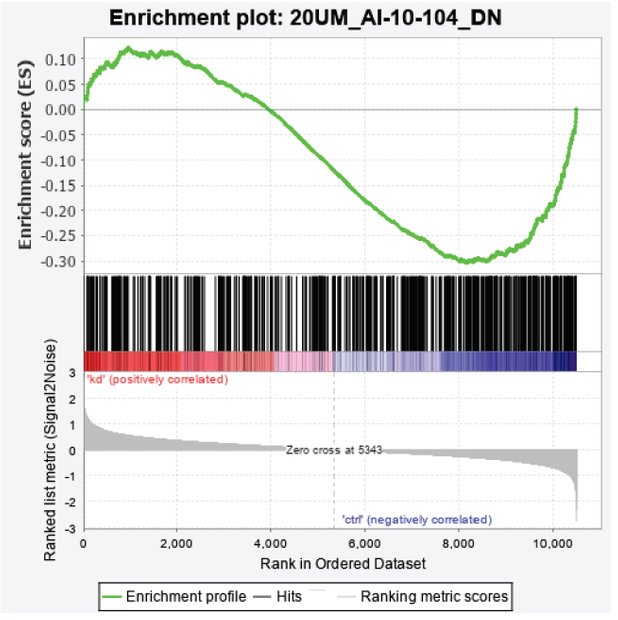


Supplemental Figure 7



Supplemental Figure 8



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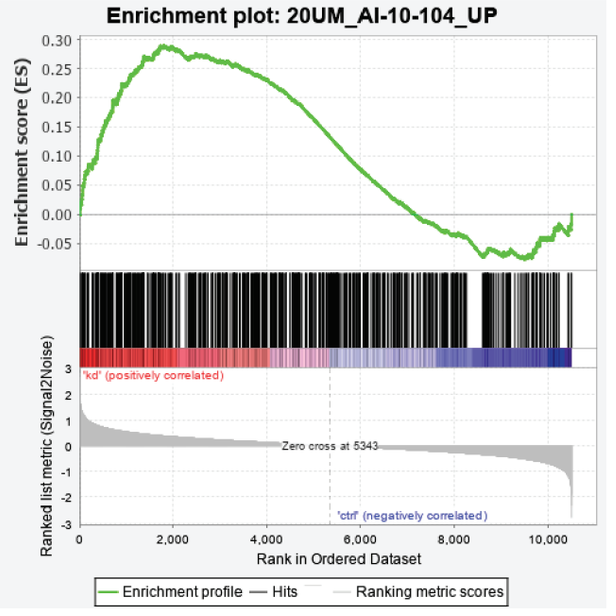

NES: 1.44 p = 0.001 FDR = 0.001