SUPPLEMENTARY APPENDIX

Genetic manipulation of primary human natural killer cells to investigate the functional and oncogenic roles of PRDM1

Gehong Dong,[1,2*] Yuping Li,[1*] Logan Lee,[1] Xuxiang Liu,[1] Yunfei Shi,[1,3] Xiaoqian Liu,[1,4] Alyssa Bouska,[5] Qiang Gong,[1] Lingbo Kong,[1] Jinhui Wang,[6] Chih-Hong Lou,[7] Timothy W. McKeithan,[1] Javeed Iqbal[5] and Wing C. Chan[1]

[1]Department of Pathology, City of Hope National Medical Center, Duarte, CA, USA; [2]Department of Pathology, Beijing Tiantan Hospital, Capital Medical University, Beijing, China; [3]Department of Pathology, Peking University Cancer Hospital & Institute, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Beijing, China; [4]Department of Hematology, Affiliated Yantai Yuhuangding Hospital, Qingdao University, Yantai, Shandong, China; [5]Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, USA; [6]Department of Molecular and Cellular Biology, City of Hope, Duarte, CA, USA and [7]The Gene Editing and Viral Vector Core, Department of Shared Resources, Beckman Research Institute of City of Hope, Duarte, CA, USA

**GD and YL contributed equally as co-first authors.*

 doi:10.3324/haematol.2020.254276

Received: April 8, 2020.

Accepted: July 30, 2020.

Pre-published: July 30, 2020.

Correspondence: *WING C. (JOHN) CHAN* - jochan@coh.org

Supplementary Materials and Methods

Cell lines and cell culture

Human embryonic kidney cell line HEK293T was purchased from ATCC, whereas malignant NK cell lines NK-YS[33](DSMZ, Braunschweig, Germany) and KAI3[34] (Health Science Research Resource, Osaka, Japan) were used for Western Blots. HEK293T was cultured in DMEM with 10% fetal bovine serum (FBS), 1% of Penicillin-Streptomycin (10,000 units per ml of penicillin and 10,000 µg per ml of streptomycin, Gibco™). NKYS was cultured in RPMI 1640 supplemented with 10% FBS, 1% of Penicillin-Streptomycin (10,000 U/mL), and 100 units per ml of IL-2 (R&D Bioscience, CA, USA). KAI3 was maintained in RPMI 1640 supplemented with 20% FBS, 1% of Penicillin-Streptomycin (10,000 U/mL), and 200 units per ml of IL-2 (R&D Bioscience, CA, USA).

Isolated primary natural killer (NK) cells from healthy donors (Donor #1 and Donor #2) were cultured in RPMI 1640 (Gibco-Invitrogen, CA, USA) with 10% fetal bovine serum (FBS, Hyclone), 1% of Penicillin-Streptomycin (10,000 U/mL) and 100 units per ml of IL-2 (R&D Systems, USA). The feeder cell K562-Cl9-mb21 was cultured in RPMI 1640 supplemented with 10% FBS, 1% of Penicillin-Streptomycin (10,000 U/mL). Cell suspension was pre-irradiated with 3000 rad (30 Gy) of gamma radiation using a Cesium-source irradiator. The negative selected NK cells were co-cultured with feeder cells at 1:1 to 1:2 ratio. All cells were cultured at 37 °C in 5% CO_2. Cells were periodically tested to exclude mycoplasma contamination.

Plasmids

pSpCas9(BB)-2A-GFP (PX458) plasmid (Addgene plasmid #48138, deposited by Dr. Feng Zhang's lab)[18], was employed for sgRNA cloning. It contains a reading frame of Cas9 from S. pyogenes with

a C-terminal T2A site fused EGFP reading frame, and a cloning backbone with scaffold RNA for sgRNA under a U6 promoter. Each sgRNA was inserted into PX458 by restriction enzyme site BbsI. Cloning vector pUC19 (from ThermoFisher Scientific, Subcloning Efficiency™ DH5α Competent Cells kit No. 18265017) plasmid was used for constructing and sequencing of homologous DNA repairing template (HDRT) and cloning of genomic DNA PCR products.

Design and evaluation of small guide RNAs (sgRNAs)

The workflow of gene editing and single cell cloning is shown in Figure 1. Four small guide RNAs targeting regions within exons 2, 4 and 5 of *PRDM1* were designed using an online CRISPR design tool (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) (Supplementary Table 1). Each sgRNA was cloned into the PX458 vector to generate a corresponding PX458-Cas9-sgRNA plasmid. *PRDM1* sgRNA4 was selected for *PRDM1* KO by plasmid PX458-sgRNA4 electroporation (Figure 1A) and sgRNA2 was chosen for *PRDM1* KO by sgRNA2/Cas9 ribonucleoprotein (RNP) electroporation (Figure 1B). Sequence of sgRNA of *TP53* was from literature[35], and sgRNAs of *DDX3X* and *PTPN6* genes were designed as mentioned above (Supplementary Table 2).

The cleavage efficiency of each sgRNAs was assessed by transfecting each PX458-sgRNA plasmid into HEK293T cells. Genomic DNA PCR products of editing regions from transfected HEK293T and untreated HEK293T cell line were Sanger sequenced. ICE online software (https://www.synthego.com/products/bioinformatics/crispr-analysis) was used to compare the Sanger sequencing results from transfected cells and untransfected cells.

PRDM1 KO mediated by CRISPR/Cas9 with plasmid PX458-sgRNA4 electroporation and single cell cloning

Freshly isolated primary NK cells from a healthy donor (Donor #1) were cocultured with irradiated feeder cells at 1:2 ratio for 7 days. 5 ug of PX458-sgRNA4 plasmid was delivered into 2x10^6 stimulated primary NK cells by electroporation using the Amaxa® Nucleofector® II Device (Lonza, France) according to the manufacturer's suggested U001 protocol. The same amount of empty PX458 vector without sgRNA insertion was used as the control. After 48 hours, NK cells expressing GFP were isolated by FACS and cocultured with feeder cells at a 1:1 ratio until it had fully recovered and started to proliferate vigorously. Expanded sorted cells were seeded by FACS as a single cell per well in 96-well plates, which were pre-plated with 10^4 feeder cells per well. Cell growth in each well was examined using the Nikon microscope (Eclipse, TS100, Japan) three weeks after seeding. We graded the clones into three categories (G1-3) according to the area occupied by the growing cells (Figure S2). G-1 consisted of scattered live cells in the well and G-2 had growing cells occupying less than 50% of the area of the well. G-3 had growing cells occupying more than 50% of the area of the well.

Genome editing screening for single colonies by High Resolution Melting (HRM)-PCR analysis and Sanger sequencing

High Resolution Melting (HRM)-PCR was employed to screen each potential edited single G-3 colony. HRM-PCR primers were designed according to the sequence around the guide RNA (Supplementary Table 4) to produce a PCR products size of about 150 bp for wild type genome. Each of the G-3 clones was transferred to one well of a new 96-well cell culture plate at the end of the third week. Wild type NK cells were also included for negative control in one well of the plate. Five ul of cell suspensions from each well of the whole plate were transferred to a 96-well PCR plate with 50 ul of freshly made Alkaline Lysis Reagent (25 mM NaOH, 0.2 mM EDTA) per well. Cells were lysed at 95℃ for 10 minutes, and 50 ul Neutralization Reagent (40 mM Tris-HCl, pH 5) was

added to each well and mixed thoroughly. One ul of the neutralized supernatant was used as the template for a 20 ul SYBR Green real-time HRM-PCR reaction (Roche, KAPA HRM FAST PCR Kit, KK4202). Clones with different melting temperatures (≥ or ≤0.5℃) than the wile type cells were picked and transferred to another plate (Figure 1A).

These single clones were further expanded (with fresh 1x10^4/well irradiated feeder cells and 100 units per ml of IL-2 in the medium) before their genomic DNA extraction and PCR amplification of editing regions for Sanger sequencing to confirm the genome editing. In each potential single clone, CRISPR editing genome regions were amplified by PCR and then Sanger sequenced to confirm *PRDM1* or other gene loci's alterations (Figure 1A, 2A). TOPO™ TA Cloning™ Kit (Invitrogen, USA, No. 450641) was used to clone the PCR products and to analyze the sequence changes at each allele of the *PRDM1* or other genes. Each identified *PRDM1* modified clone was then expanded for functional studies. We also performed qRT-PCR and Western Blot to confirm the lack of *PRDM1* gene expression in these *PRDM1*$^{-/-}$ NK clones (Figure 2). Clones with different melting temperatures (≥ or ≤0.5℃) than the wild type cells were considered as the potential edited ones (Figure 1A).

Construction of HDRT and sgRNA/Cas9 RNP complex

Double stranded HDRT DNA template was designed as shown in Figure S1. Three fragments, including left and right homologous arms flanking the genome editing site and in-frame inserted GFP or DsRed reading frame plus SV40 poly(A) signal, were obtained by PCR from genomic DNA, plasmid pEGFP-C1 (Clontech, # 6084-1) or pMIDsRed II (pMSCV-IRES-DsRed, Addgene, # 52110). These fragments were assembled by Gibson Assembly method according to manufacturer's instruction (New England Biolabs, Gibson Assembly Master Mix, E2611S). Assembled HDRT template was inserted into pUC19 by BamH I and Kpn I for Sanger sequencing to verify for correct sequence. HDRT DNA was amplified by large scale PCR reaction (New England

Biolabs, Q5 Hot Start High-Fidelity 2x Master Mix, M0494S). PCR products were purified and concentrated using Solid Phase Reversible Immobilization (SPRI) beads (AMPure XP beads, Beckman Coulter, USA, Cat. A63881) according to manufacturer's protocol and used for electroporation with Cas9/sgRNA2 RNP complex. We designed two HDRT templates for sgRNA2, one with GFP and the other with DsRed.

To form the RNP complexes of Cas9-sgRNA, recombinant SpCas9 (from QB3 MacroLab in University of California, Berkeley stored at 40 μM in 20mM HEPES-KOH, pH 7.5, 150 mM KCl, 10% glycerol, 1 mM DTT) was mixed at 1:1 by volume with the 80 μM synthesized single stranded sgRNA2 for *PRDM1* sequence (double ends modified and fused to the scaffold tracrRNA, Synthego, CA, USA) and incubated at 37°C for 15 minutes. A 12.5 ul RNP complex was used (20 μM) for one well reaction of a 24-well plate. The flowchart of HDRT design and preparation was shown in Figure 1B.

10 μg of each HDRT (total 20 μg HDRT DNA template) in 10 μl nuclease free water were mixed with previously formed 12.5 μl Cas9/sgRNA RNP complex. For each electroporation, 100 μl (5x10^6) human primary NK cells (from healthy Donor #2) which had been stimulated by feeder cells at 1:2 ratio for one week were added to the HDRT-RNP complex and mixed gently. Electroporation was performed following Lonza Amaxa's program U001 and electroporation kit (Lonza, VPA-1005). Two days after electroporation, FACS was employed to sort GFP and DsRed double positive cells, which indicated a bi-allelic KO and fluorescent protein replacement of each of the *PRDM1* loci. The CRISPR negative control cells were made by electroporating with only Cas9 protein without sgRNA into NK cells. *PRDM1* gene knock-out was confirmed by Western Blot of PRDM1 and qRT-PCR of target gene *MYC* expression (Figure 3). Genome editing by fluorescent proteins gene in-frame fusion knock-in (KI) was confirmed by flow analysis of GFP/DsRed expression and Sanger

sequencing of PCR products with the primers designed to amplify the junctional sequences of the inserted DNA (Figure 4).

Western blot and antibodies

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Sigma-Aldrich, P8340) before use. Protein concentration was determined by Pierce BCA protein assay (Thermo Fisher Scientific, USA, No. 23225). Twenty to thirty ug of protein per lane were loaded on 12% SDS polyacrylamide gels for electrophoresis. Then gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA, number: IPVH00010) by semi-dry method. The blots were incubated with PRDM1 antibody (Novus, USA, number: NB600-235) at 1:500 dilution overnight at 4°C. The membrane was then blotted with anti-mouse peroxidase-conjugated secondary antibody for 1 hour at room temperature, and analyzed by chemiluminescence (BIO-RAD, USA, No. 170-5060) on X-ray film (Thermo Scientific, USA, No. 34090). Lysates from NK cell lines NKYS and KAI3 were used as positive and negative controls respectively. GAPDH was used as the internal loading control.

Cell proliferation analysis by MTS method and EdU incorperation

MTS assay was used to measure cell proliferation by CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, USA, number: G3581). NK cells were counted on the 8^{th} day after feeder cell stimulation and mixed in a 1:1 ratio with fresh irradiated feeder cells. Mixed NK-feeder cells were seeded into 96-well plates at total 1x10^4 cells per 100 ul per well. Briefly, at each of the desired time points, 20 µl of the CellTiter 96® AQueous One Solution reagent was added into each well and incubated at 37°C for 2 hours. Optical density (OD) was then read at 490 nm using the

automated microplate reader (Tecan Trading AG, Switzerland). *PRDM1*$^{-/-}$ clones #3, #5 and the same cell-age matched wild type parental *PRDM1*$^{+/+}$ control NK cells from Donor #1, or the *PRDM1*$^{-/-}$ (GFP+DsRed+) cells and the same cell-age matched wild type parental *PRDM1*$^{+/+}$ control NK cells from Donor #2 were analyzed for 6 consecutive days (Figure 5). Every day's OD490 reads were compared with the first day's read. Each experimental point was from four replicates.

Cell proliferation was also evaluated by EdU incorporation during 5 consecutive days from the third day after fresh irradiated feeder cells were added. We used Click-iT™ Plus EdU reaction cocktail (Invitrogen, USA, C10634) for the assay. The incorporation of the thymidine analogue, EdU (Invitrogen, USA, No. C10634), into DNA during active DNA synthesis was calculated. All procedures followed the manufacturer's instruction. Briefly, 2 to 3 million cells in cultured were incubated with EdU at 10 μm concentration for 2 hours. The cells were then stained with FITC anti-CD56 antibody to identify NK cells and exclude feeder cells. For *PRDM1* KO by fluorescent protein gene insertion, the GFP+ cells were gated to exclude feeder cells. Cells were then fixed and permeabilized before adding the fluorescent dye Alexa Fluor 647 included in the Click-iT™ Plus reaction cocktail (Invitrogen, USA, C10634). The cells were analyzed by a FACS Fortessa flow cytometer (BD Biosciences, USA) and FlowJo software (Version 10). The negative control consisted of cells from the same population but without EdU treatment. Thirty thousand events were collected and analyzed for EdU-Alexa Fluor 647 intensity to enumerate cells with active DNA synthesis.

Cell cycle analysis and apoptosis assay

Three to five million cells were collected on the third day after fresh feeder cells were added and rinsed twice with ice-cold 1x PBS and fixed with 70% ethanol at 4℃ over-night. After fixation, cells were rinsed twice in ice-cold 1x PBS. The NK cells modified by CRISPR/Cas9-sgRNA4 plasmid electroporation were stained with FITC anti-CD56 antibody to distinguish them from feeder cells.

Then, cells were washed and resuspended in 500 ul Propidium Iodide/RNase staining buffer (BD Biosciences, USA, 550825) and incubated at room temperature for 15 minutes before analysis. NK cells with *PRDM1* KO by Cas9-sgRNA2 RNP and GFP/DsRed insertion were gated by GFP to exclude feeder cells. Cells were stained with 1 µg/ml DAPI just before analysis with flow cytometer Fortessa (BD Biosciences, USA), and 50,000 events were collected and analyzed by FlowJo software (Version 10).

Apoptosis assay was performed during 5 consecutive days from the third day after fresh feeder cells were added. Cells were washed twice with ice-cold 1x PBS, stained with FITC anti-CD56 for 15 minutes, and then washed twice following resuspension in 100 µl 1x binding buffer (Biolegend, USA, No. 422201). For NK cells modified by CRISPR/Cas9-sgRNA4 plasmid electroporation, Cells were then incubated with 5 µl APC Annexin V - (Biolegend, USA, No. 640919) and 10 µl propidium iodide (Biolegend, USA, No. 640919) for 15 minutes. For NK cells with *PRDM1* KO by Cas9-sgRNA2 RNP and GFP/DsRed insertion, cells were similarly stained with Alexa Fluor 647 Annexin V and 200 ng/ml DAPI (Sigma-Aldrich, D9542). GFP positive gate was used to exclude feeder cells. Stained cells were analyzed by flow cytometer Fortessa (BD Biosciences, USA) using FlowJo software. Thirty thousand events were collected and cells in Q3 were considered apoptotic cells.

Quantitative real-time PCR assay

RNA was isolated from fresh cells using RNeasy kit (Qiagen, USA, number: 74106). RNA quality and quantity were measured by NanoDrop 1000 (ThermoFisher Scientific, USA). 500 ng total RNA was transcribed to cDNA with Random Primer Mix and SuperScript III Reverse Transcriptase (Thermofisher Scientific, USA, 11752250) in a 20 ul reaction. qRT-PCR was performed in triplicate using the BioRad Cyler CFX 96. Real-time qPCR experiments were performed with cDNA from 10 ng total RNA in 20 ul volume by Kapa Fast Universal 2XQPCR (Kapa Biosystems, KK4618, USA).

The primer pairs for real-time PCR were designed across exon-exon junctions, and melting curves were used to assess the specificity of the amplifications. The primers for qRT-PCR are listed in Supplementary Table 4. The target gene relative expression levels were normalized by RPL13A in primary NK cells and calculated by 2^-ΔΔCt.

***RNA-seq and data* analysis**

RNA was isolated from fresh cells as mentioned above with additional DNase I treatment. RNA sequencing libraries were prepared with KAPA Stranded mRNA-Seq Kit (Kapa Biosystems, USA, number: KK8421) according to the manufacturer's protocol with minor modifications. Briefly, 100 ng of total RNA from each sample were used for polyadenylated RNA enrichment using oligo dT magnetic beads, and the poly (A) RNA was fragmented with divalent cations under elevated temperature and first-strand cDNA synthesis performed by reverse transcription. After second-strand cDNA synthesis, the double-stranded cDNA underwent end repair, 3′ end adenylation, and ligation to bar-coded adaptors (Illumina, USA). 12 cycles of PCR were performed to produce the final sequencing library. The libraries were validated with the Agilent Bioanalyzer High Sensitivity DNA kit (Agilent). Library templates were prepared for sequencing using cBot cluster generation system (Illumina, USA) with HiSeq SR Cluster V4 Kit (Illumina, GD-401-4001). Sequencing runs were performed in the single read mode. Forty million single reads were generated and the read length for each sample was 51 bp.

Reads were aligned to the NCBI mRNA RefSeq database and reads per kilobase of exon model per million reads (RPKM) were counted for normalization. Differential expression between *PRDM1*$^{-/-}$ and *PRDM1*$^{+/+}$ NK cells was analyzed. Differentially expressed genes (DEGs) were identified, and gene set enrichment analysis (GSEA) was performed between *PRDM1* $^{-/-}$ and matched *PRDM1*$^{+/+}$ NK cells to understand the functional perturbation upon *PRDM1* KO.

Next generation sequencing

Genomic DNA was extracted from the *PRDM1* KO cells as well as 3 additional clones: *PRDM1*$^{-/-}$/*TP53*$^{+/-}$, *PRDM1*$^{-/-}$/*DDX3X*$^{+/-}$, and *PRDM1*$^{-/-}$/*PTPN6*$^{+/-}$ using DNeasy Blood and Tissue Kit (Qiagen, USA, No. 69504). 250 ng of genomic DNA was fragmented using Covaris S220 with the 200 bp peak setting. The fragmented DNA was end-repaired and ligated to Illumina adaptor oligo nucleotides with KAPA Hyper Prep Kit (KAPA Biosystems, Wilmington, MA; number: KK8504). Ligation products were bar-coded, purified and amplified with 7 cycles of PCR. The enriched PCR products were subjected to a custom capture of 334 genes recurrently mutated in lymphomas (Supplementary Table 5) using the SureSelect Target Enrichment Kit (Agilent, Technologies, USA, No. 51904821) according to manufacturer's protocols. The captured products were further amplified with an 8-cycle of PCR, and the purified products were used for cluster generation using cBot cluster generation system with HiSeq PE Cluster Kit V4 (Illumina, USA, number: PE-401-4001). Sequencing runs were performed in the paired end mode using HiSeq2500 platform 12. Raw sequences were aligned to the reference genome (hg19) using BWA (v0.7.5a)[36]. Picard (v1.115) and GATK (v3.1)[37] were used for duplicate marking, local realignment, and base quality recalibration. Variants were called with VarScan (v2.3.6)[38] and then annotated using ANNOVAR (version: 2015-12-14)[39]. We filtered out variants that were recorded in dbSNP database and with a population frequency no less than 1%, and that do not change protein sequence. Variants below 5% frequency will be considered below our threshold to be called a somatic variant.

Statistical analysis

The cloning efficiency of the *PRDM1*-edited NK cells and the normal control NK cells were compared by Fisher Exact test with SPSS13.0 statistical software (SPSS Inc., Chicago, IL, USA). $P<0.05$ indicates the difference is significant.

Supplementary figure legends

Figure S1. Schematic illustration of pUC19-*PRDM1* sgRNA4-DsRed/GFP-HDRT vector construction.

Figure S2. Microscopic pictures illustrating the grading of NK cell clones. Each well was examined and graded into three categories (G1-3) according to cell density and the area of the well occupied. G-1 consisted of scattered live cells in the well and G-2 had growing cells occupying less than 50% of the area of the well. G-3 had growing cells occupying more than 50% of the area of the well.

Figure S3. Sanger sequencing results of other *PRDM1* edited clones by plasmid mediated CRISPR/Cas9 editing. A) Schematic gene structure of *PRDM1* and guide RNA sgRNA4 sequence. **B)** Genome DNA PCR products of clones E8-1, B3-1 and D10-1 amplified from exon 4 were cloned by TOPO cloning. Sanger sequencing results for each single E. coli colony showed: clone E8-1 and clone B3-1 both harbored heterozygous deletion of *PRDM1* (Ref: NM_001198). Clone E8-1: △29bp [409-437]; clone B3-1: △C [420]; clone D10-1 harbored a biallelic deletion (△29bp [409-437]).

Figure S4. Western Blot showed expression of PRDM1 by feeder cells, NKCL cell lines and primary NK cells.

Figure S5. Sanger sequencing of sequential CRISPR/Cas9 KO NK cells of *TP53* on *PRDM1*$^{-/-}$ clone #3. Three single clones were confirmed with both *PRDM1*$^{-/-}$ and *TP53* $^{+/-}$ (Refseq: NM_00001126114) modification. Clone #6: △6 bp (323-328); clone #10: △1 bp (327); clone #23: △107 bp (244-350) of *TP53*.

Figure S6. Sanger sequencing results of sequential CRISPR/Cas9 KO of *DDX3X* on *PRDM1*$^{-/-}$ clone #3. Three single clones were confirmed with both *PRDM1*$^{-/-}$ and *DDX3X*$^{+/-}$

(Refseq: NM_001356) modification. Clone #1: △GG, 2 bp (12-13); clone #6: △22 bp (4-25); clone #8: △5 bp (11-15) of *DDX3X*.

Figure S7. Sanger sequencing results of sequential CRISPR/Cas9 KO of *PTPN6* on *PRDM1*$^{-/-}$ clone #3. One single clone was confirmed with both *PRDM1*$^{-/-}$ and *PTPN6* $^{+/-}$ (Refseq: NM_080549) modification. Clone #4: △64 bp (exon 2, 60-123) of *PTPN6*.

Figure S8. RNA-seq analysis of *PRDM1*$^{-/-}$ NK cells vs. wild type (WT) NK cells. (A) Deletion of *PRDM1* exon-4 sequence noted in RNA-sequence alignment; (B) Knock-in sequence of GFP was noted in exon-5. (C) *PRDM1* transcript level in WT and *PRDM1*$^{-/-}$ NK cells. (D) Gene signature enriched in *PRDM1* KO cells versus *PRDM1* WT cells. (E) Gene signature enriched in *PRDM1* WT cells versus *PRDM1* KO cells. (F) Expression of selected target genes of *PRDM1*. (G) Validation of expression of selected target genes of PRDM1 by RT-PCR. The target gene expression levels were normalized by RPL13A and relative expressions were calculated by 2^-ΔΔCt. Gene expression levels in WT NK cells was set at 1.0. (H) Validation of expression of LAG3 by flow analysis. The cells were stained with APC/Fire™ 750 anti-human CD223 (LAG-3) antibody and then analyzed by a FACS Fortessa flow cytometer (BD Biosciences, USA) and FlowJo software (Version 10).

Figure S9. Replenishing *PRDM1* in NK *PRDM1*$^{-/-}$ cells. 5 ug plasmid pMIG-PRDM1 or pMIG empty vector (EV) were delivered into 3x10^6 NK WT cells or NK PRDM1 $^{-/-}$ cells by electroporation using the Amaxa® Nucleofector® II Device (Lonza, France) according to the manufacturer's suggested U001 protocol. 48 hours post electroporation, cells were counted and aliquot to 3 wells of 24-well plates. Cell numbers were counted again at the 5th day.

Figure S10. ICE online software analysis of indels of the no-fluorecent insertion allele after CRISPR/Cas9 modification of *TP53* gene by RNP electroporation.

Figure S1. Schematic illustration of pUC19-PRDM1 sgRNA2-DsRed/GFP-HDRT vector construction

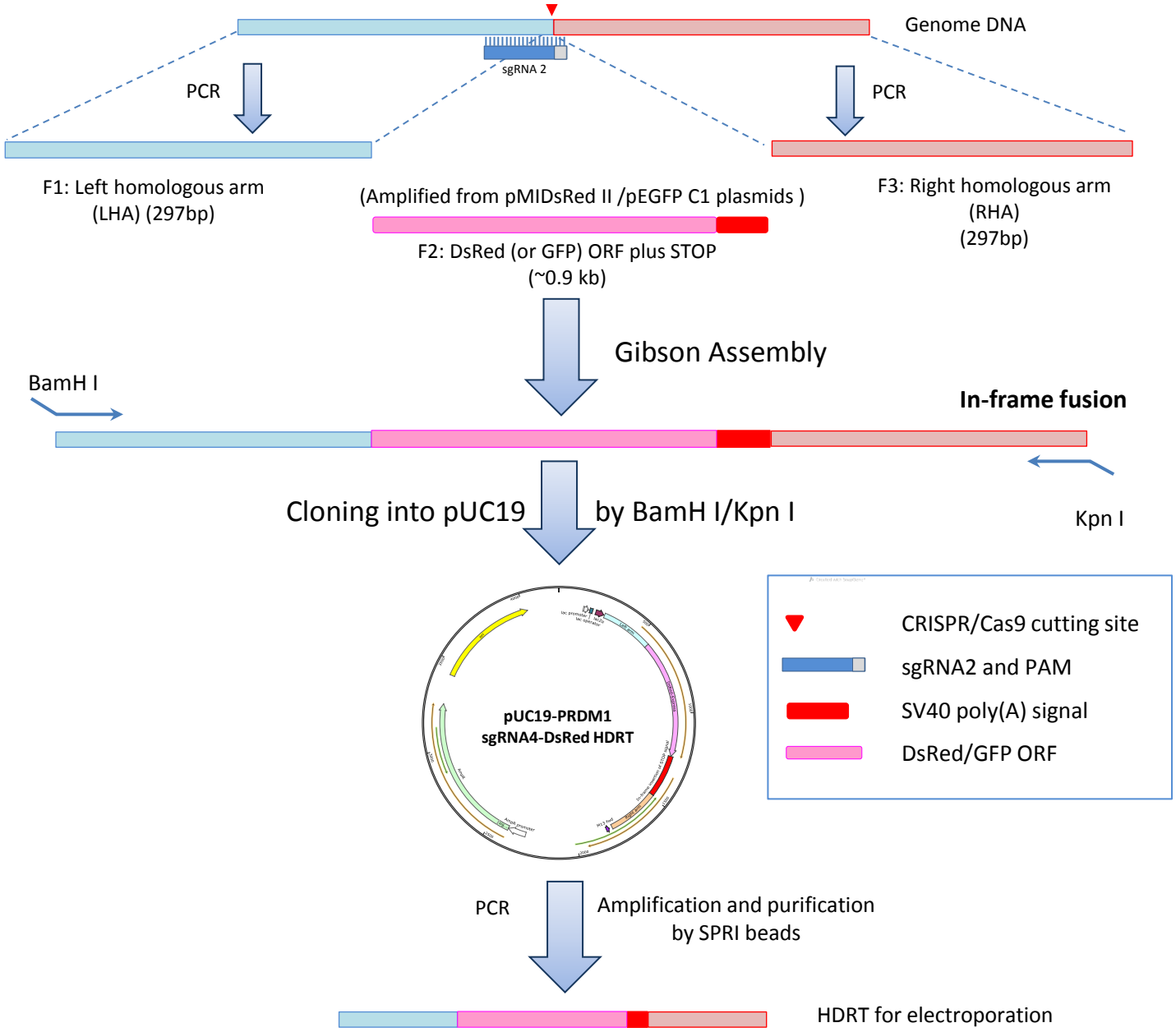
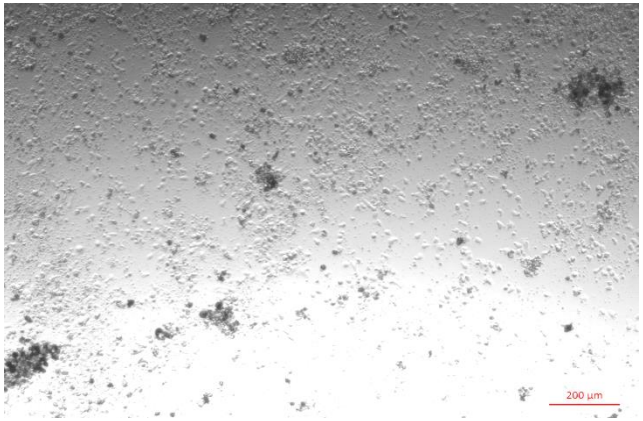
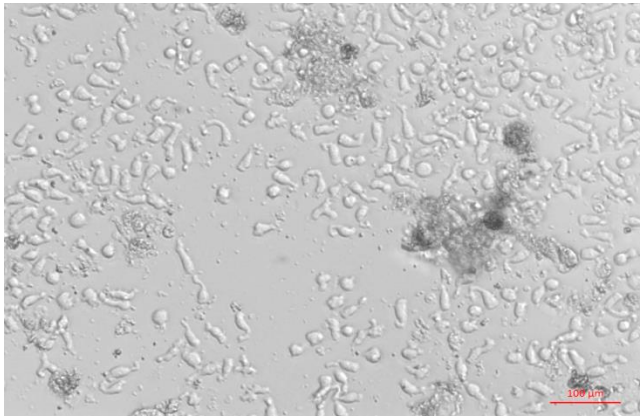


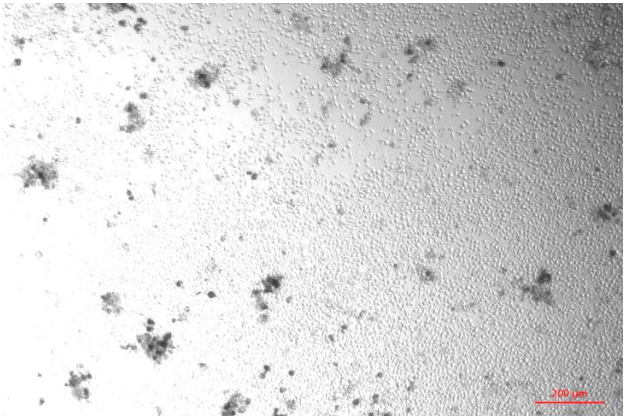
Figure S2. Microscopic pictures illustrating the grading of NK clones 3 weeks after single cell seeding

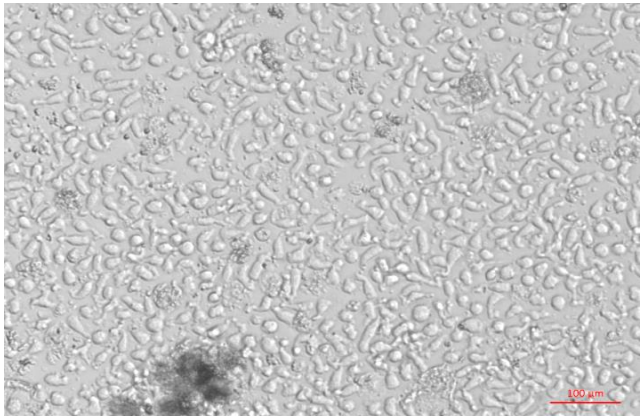
G-1



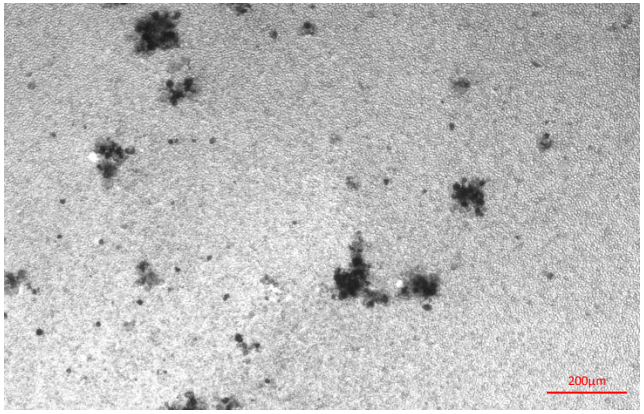


G-2





G-3



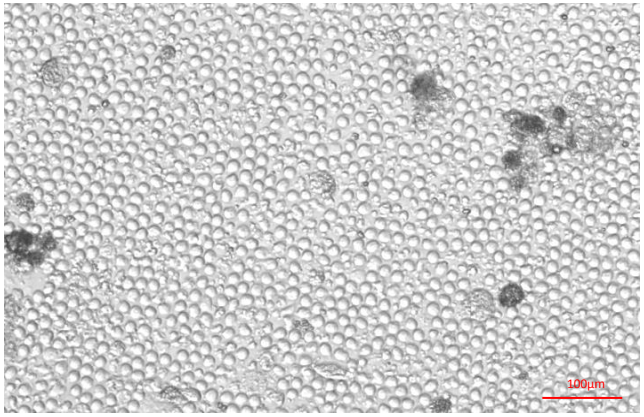


Figure S3. Sanger sequencing results of other *PRDM1 edited* clones by plasmid mediated CRISPR/Cas9

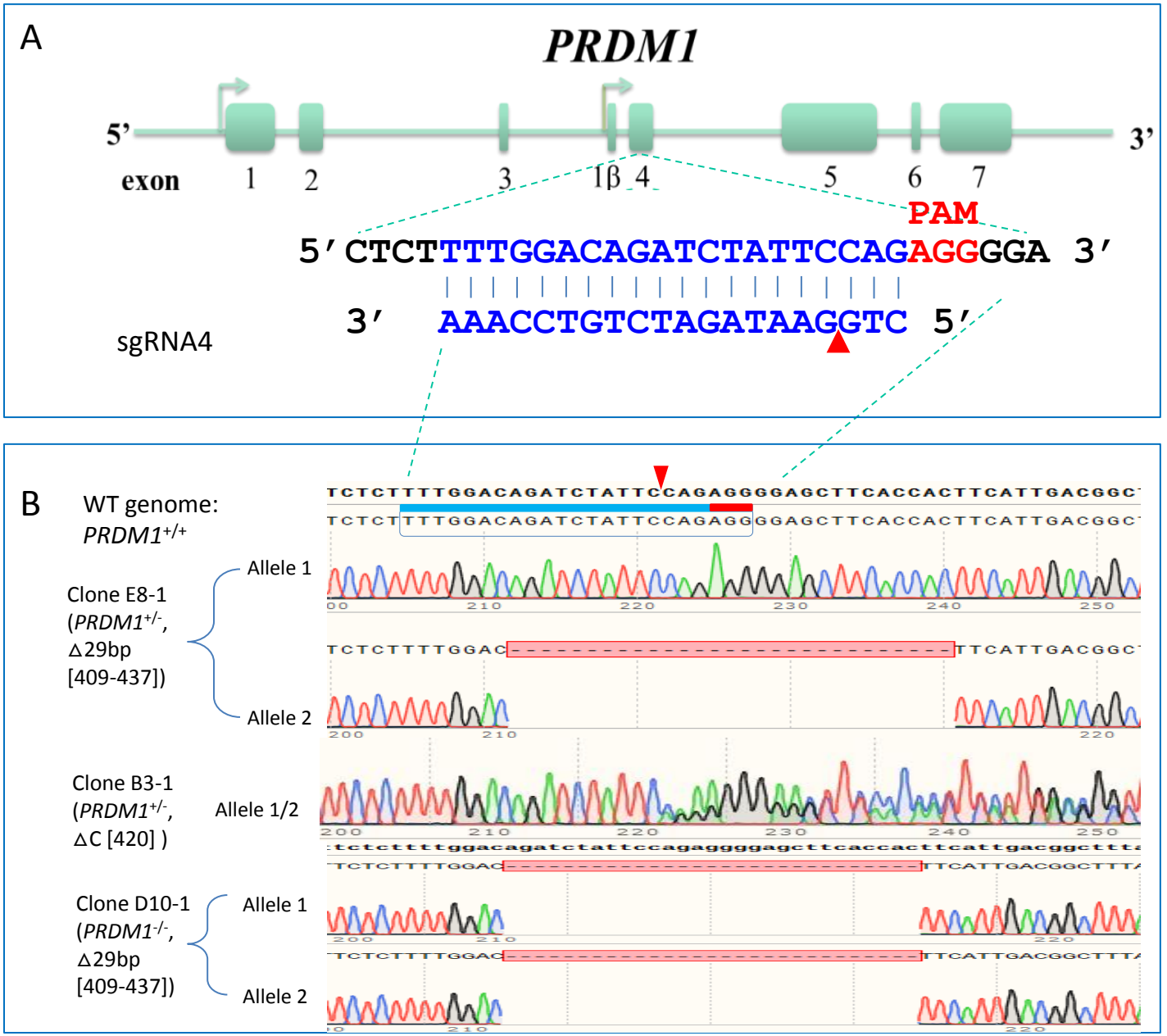


Figure S4. Western Blot shows PRDM1 expression by feeder cells, NKCL cell lines and WT NK cells

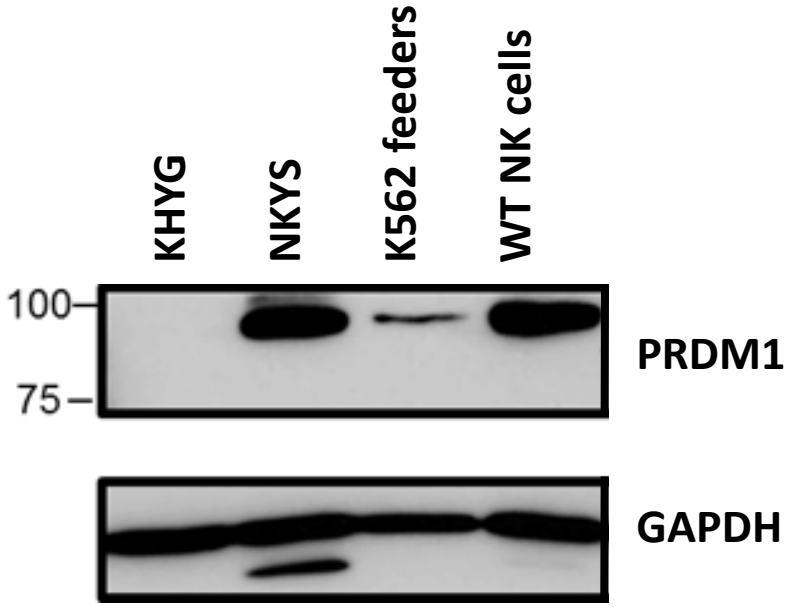


Figure S5. Sanger sequencing results of sequential CRISPR/Cas9 KO of *TP53* on *PRDM1*$^{-/-}$ *clone #3*

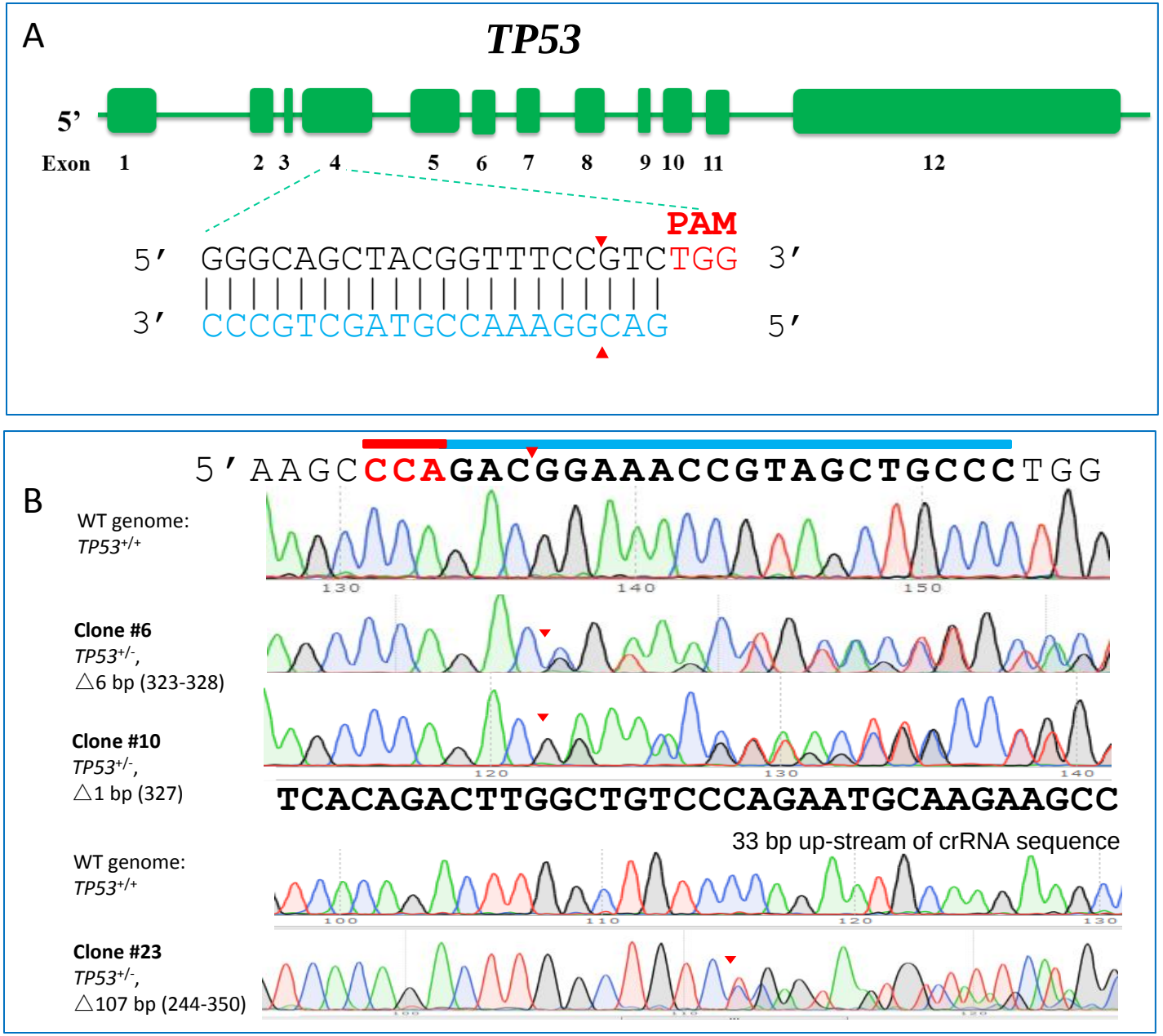


Figure S6. Sanger sequencing results of sequential CRISPR/Cas9 KO of *DDX3X* on *PRDM1*$^{-/-}$ *clone #3*

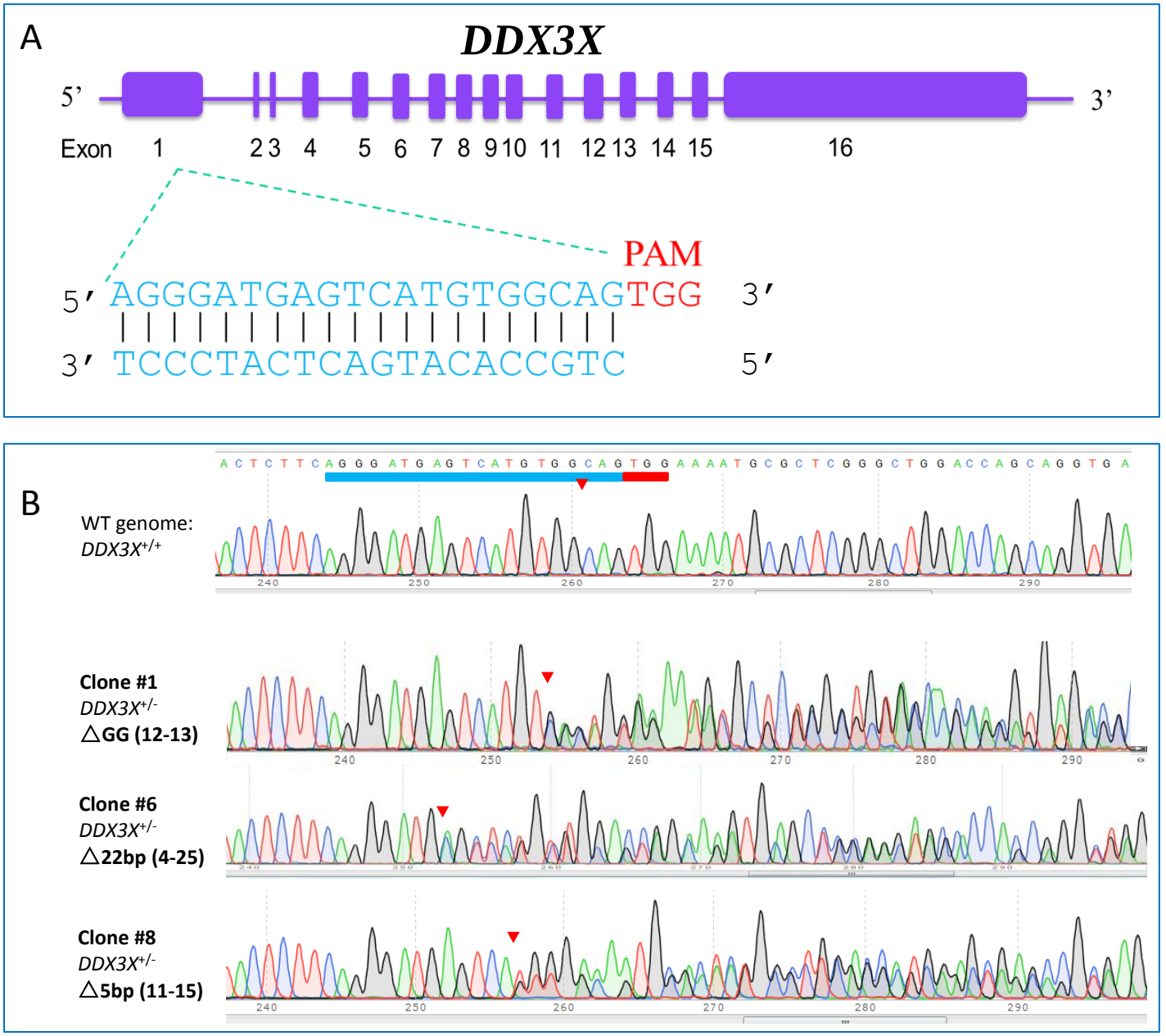


Figure S7. Sanger sequencing results of sequential CRISPR/Cas9 KO of *PTPN6* on *PRDM1*$^{+/-}$ clone #3

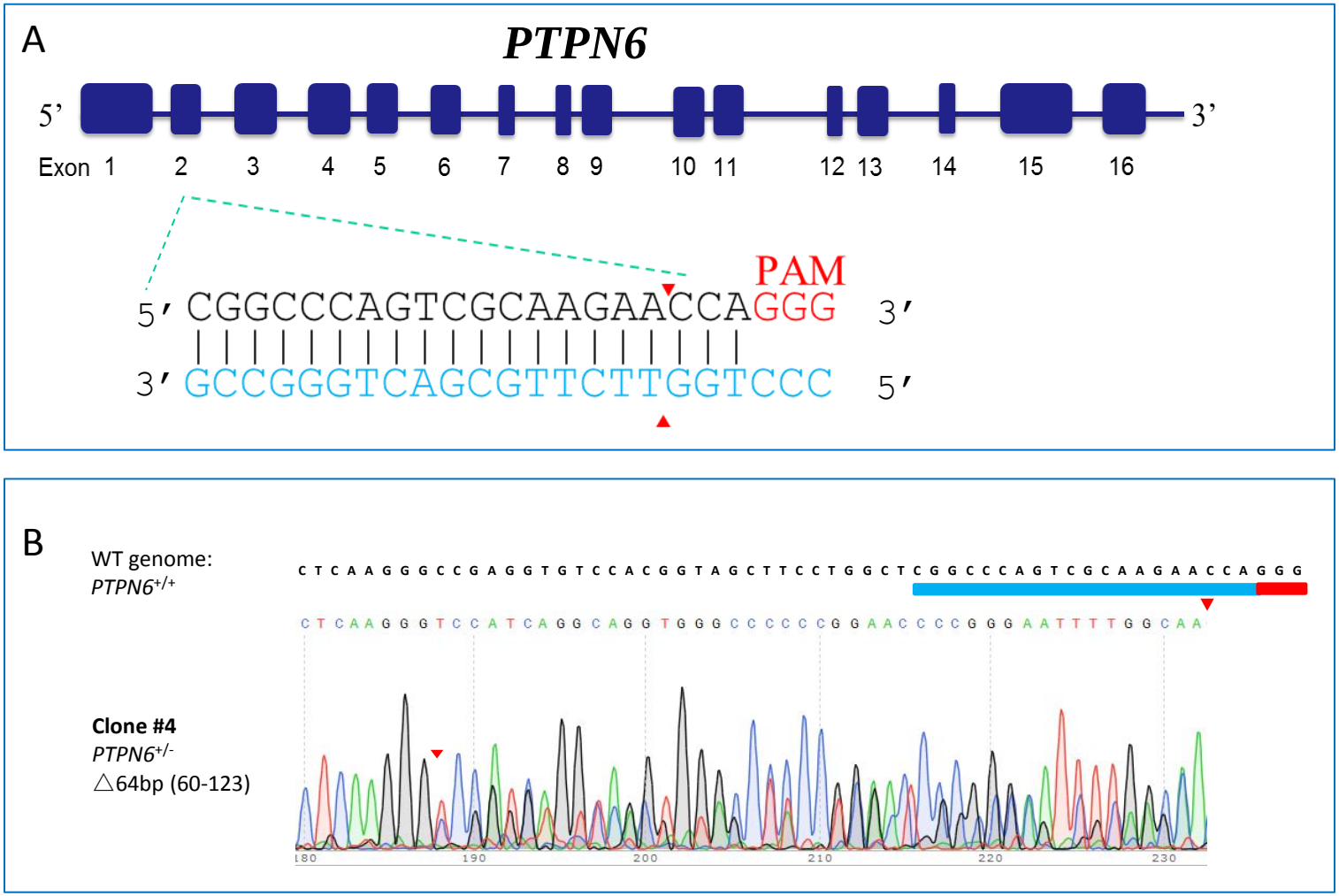
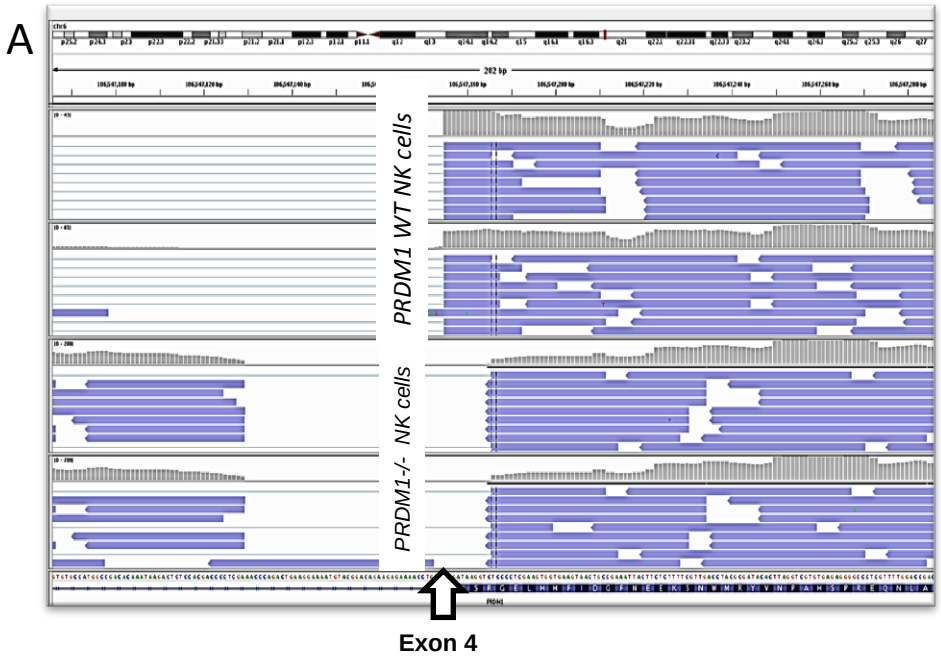
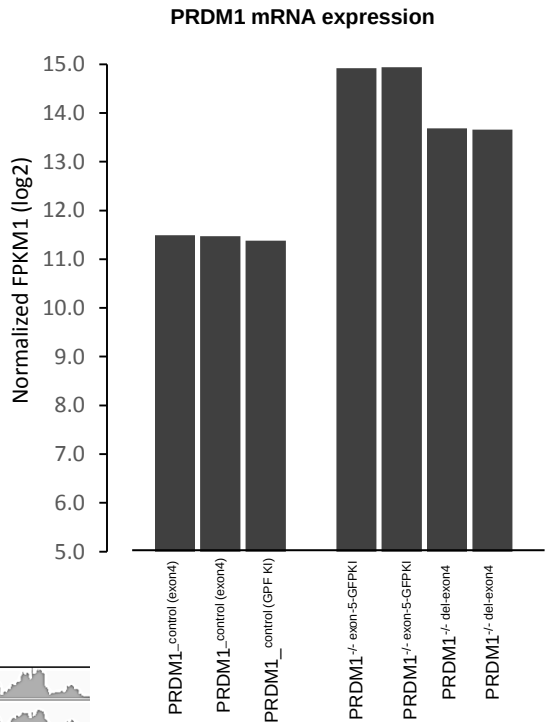


Figure S8. RNA-seq analysis of *PRDM1*$^{-/-}$ NK cells vs. wild type (WT) NK cells





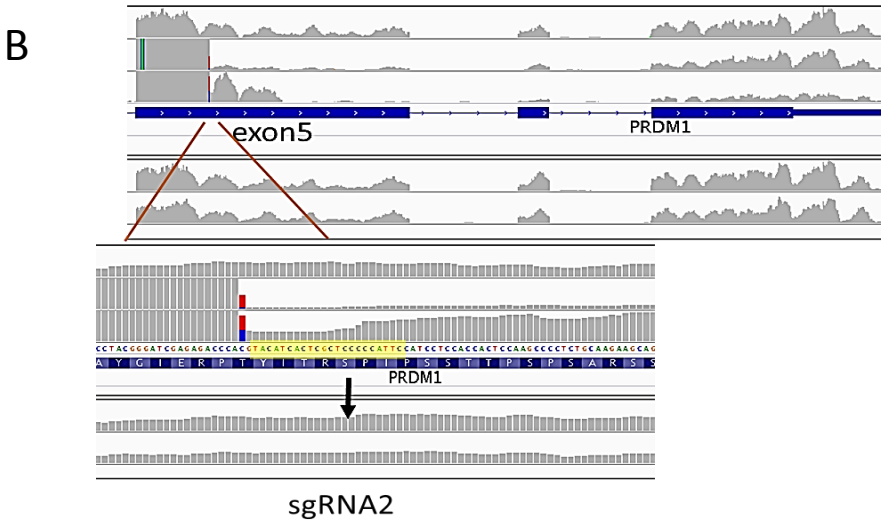


Figure S8D. Gene signature enriched in *PRDM1* KO cells versus *PRDM1* WT cells

D

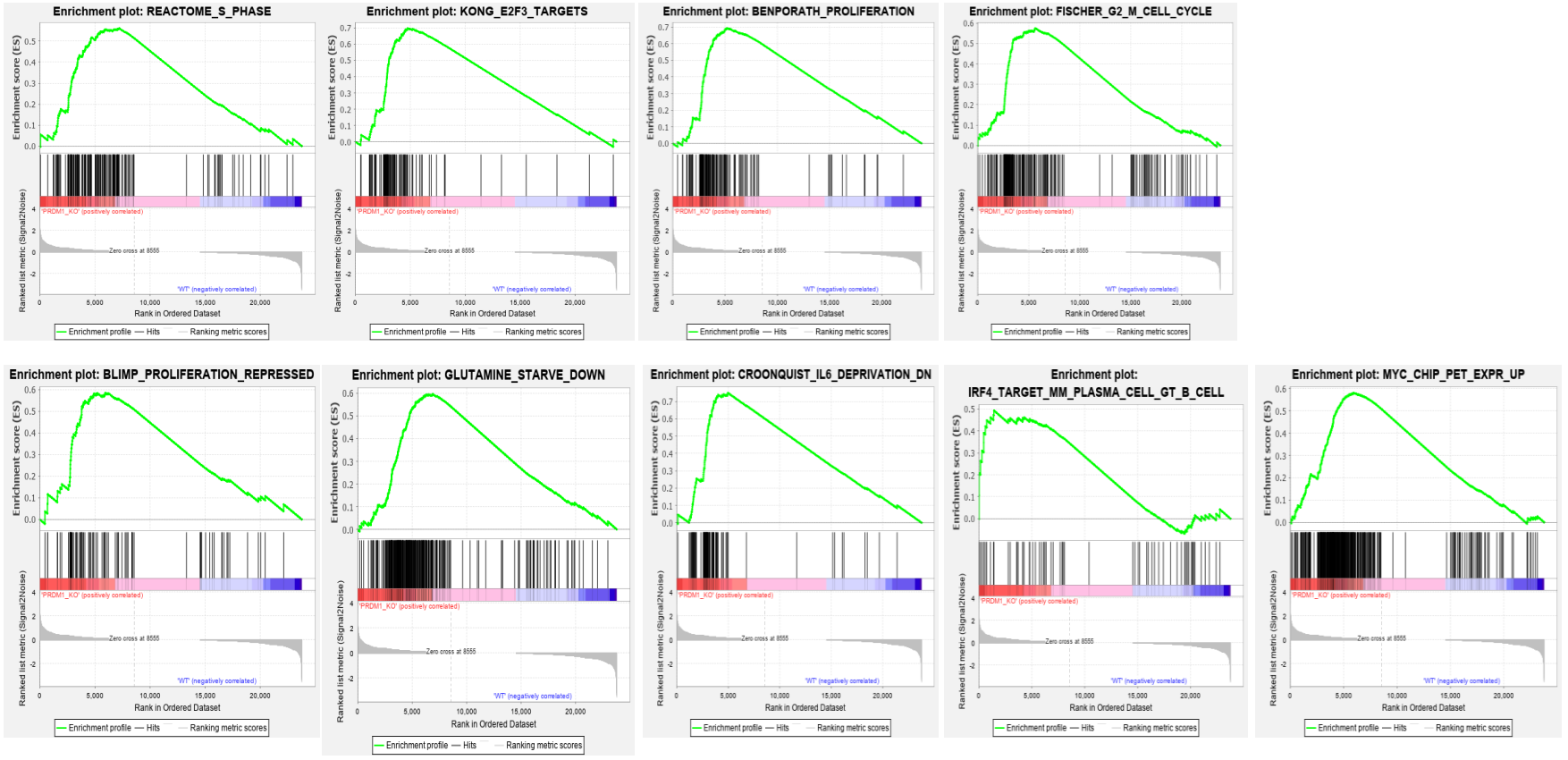
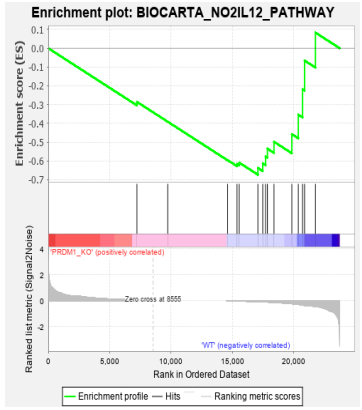
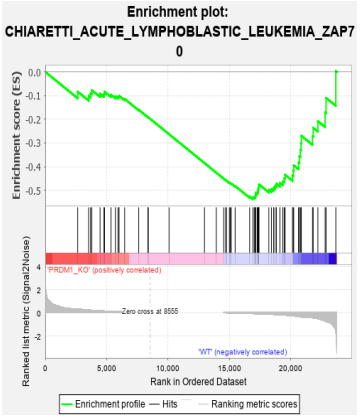
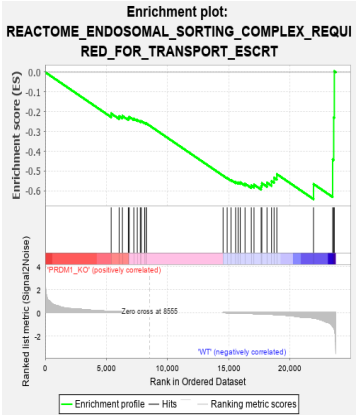


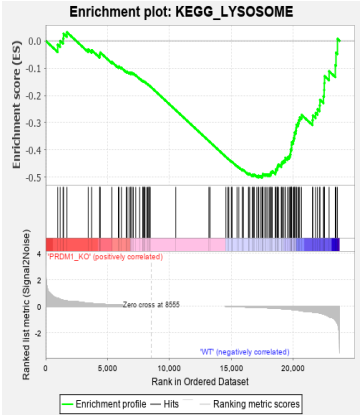
Figure S8E. Gene signature enriched in *PRDM1* WT cells versus *PRDM1* KO cells

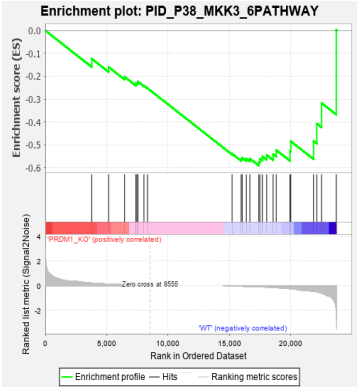
E

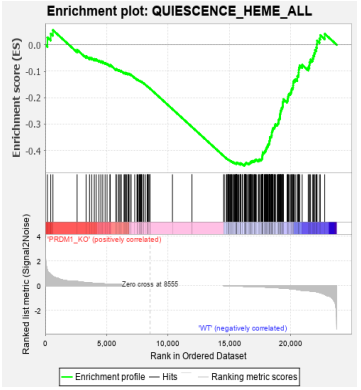


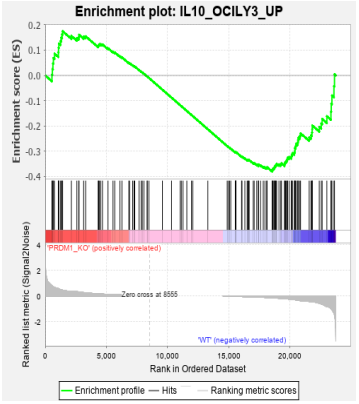












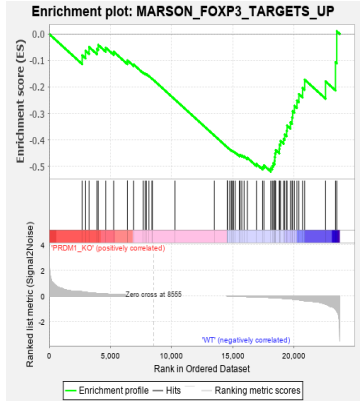


Figure S8F. Expression of selected target genes of PRDM1

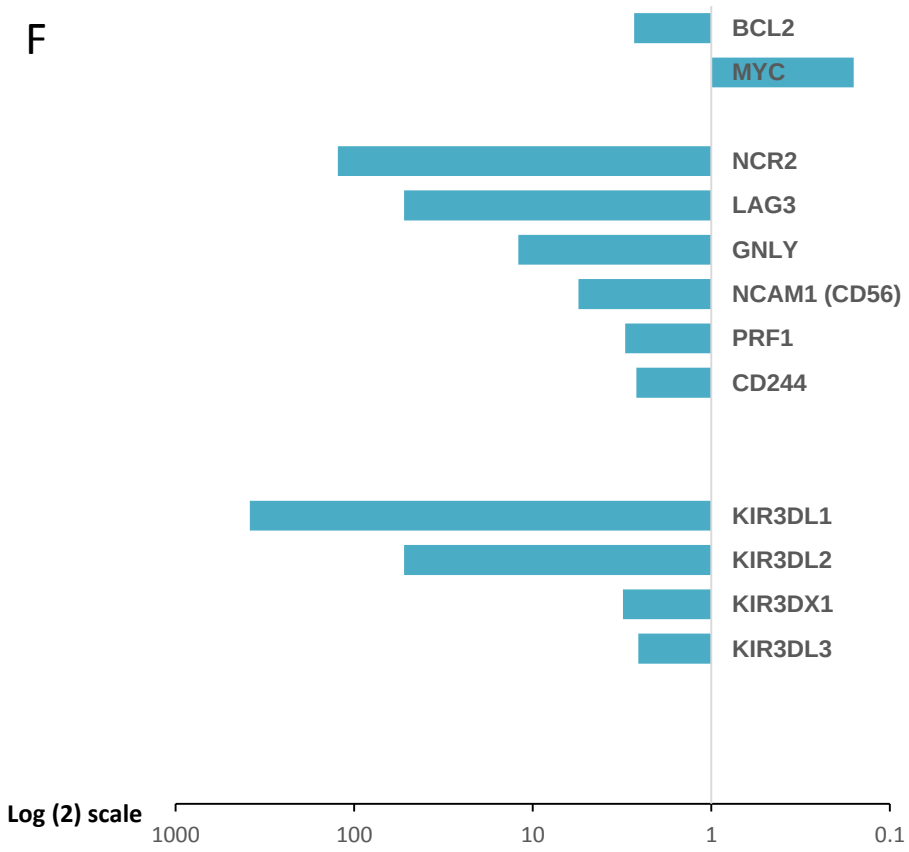


Figure S8G. Validation of expression of selected target genes of PRDM1 by RT-PCR

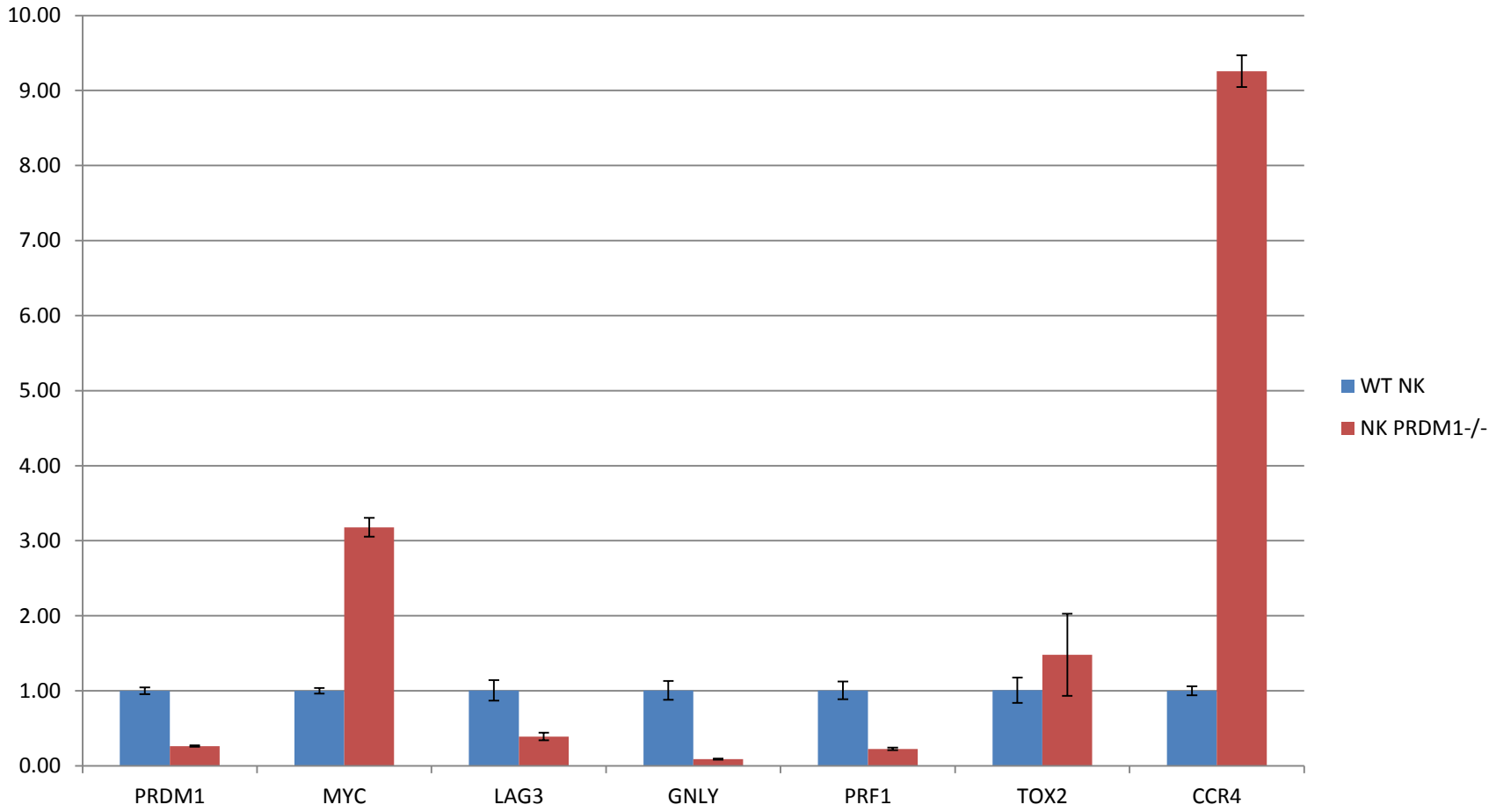


Figure S8H. Validation of expression of LAG3 by flow analysis

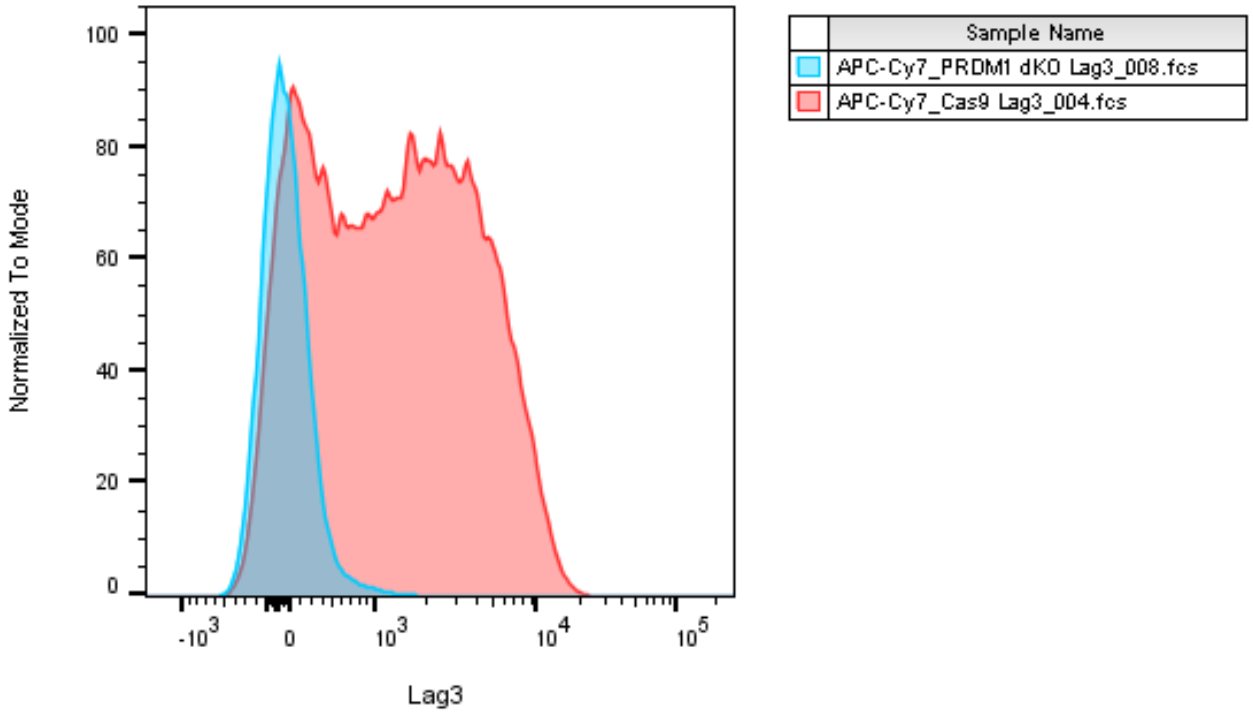


Figure S9. Replenishing *PRDM1* in NK *PRDM1*$^{-/-}$ cells

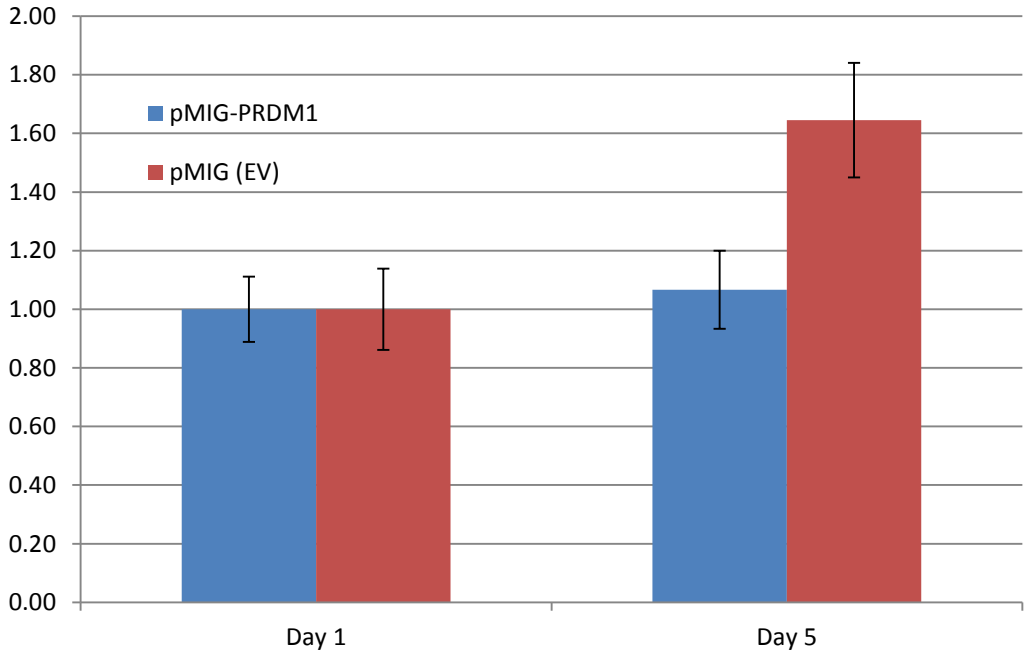
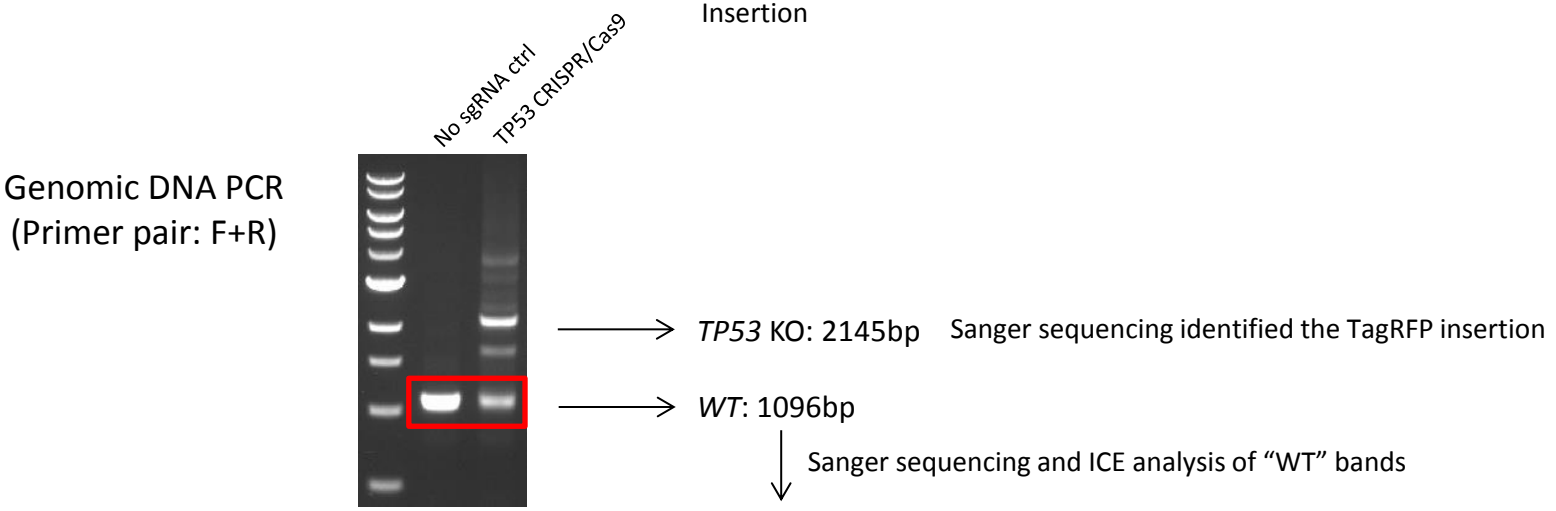


Figure S10. ICE online software analysis of indels of the no-fluorecent insertion allele after CRISPR/Cas9 *modification of TP53* gene by RNP electroporation



Status	Guide Target	PAM Sequence	Indel %	Model Fit (R^2)	Knockout-Score
Succeeded	CGCTATCTGAGCAGCGCTCA	TGG	70	0.86	60

Supplementary Table 1. Small guide RNA (sgRNA) list targeting *PRDM1* gene

	sgRNA name	Target Exon	Strand	sgRNA Target Sequence	Target Context Sequence	PAM Sequence
NM_001198.3	sgRNA1	2	antisense	AGTGCTGGAGTTACACTTGG	TCACAGTGCTGGAGTTACACTTGG**GGG**	GGG
	sgRNA2	5	antisense	GGGGAGCGAGTGATGTACGT	AATGGGGGAGCGAGTGATGTACGT**GGG**	GGG
	sgRNA3	5	antisense	GGACGCGTTCAAGTAAGCGT	CGTAGGACGCGTTCAAGTAAGCGT**AGG**	AGG
	sgRNA4	4	sense	TTTGGACAGATCTATTCCAG	CTCTTTTGGACAGATCTATTCCAG**AGG**	AGG

Supplementary Table 2. Small guide RNA (sgRNA) list *targeting TP53, DDX3X, PTPN6 genes*

	Gene name	Target Exon	Strand	sgRNA Target Sequence	Target Context Sequence	PAM Sequence
NM_00001126114	*TP53*	4	antisense	GACGGAAACCGTAGCTGCCC	GGGCAGCTACGGTTTCCGTC**TGG**	TGG
NM_001356	*DDX3X*	1	sense	CTGCCACATGACTCATCCCT	AGGGATGAGTCATGTGGCAG**TGG**	TGG
NM_080549	*PTPN6*	2	sense	TGGTTCTTGCGACTGGGCCG	CGGCCCAGTCGCAAGAACCA**GGG**	GGG

Supplementary Table 3. Cloning efficiency evaluation

	Number of clones from 96-well plates				
Source of clones	G-1	G-2	G-3	Total wells	Cloning efficiency
FACS sorted GFP$^+$ NK cells after CRISPR	33	44	322	768	47.7% [(44+322)/768]
Control wild type NK cells	47	17	28	384	11.7% [(17+28)/384]

Supplementary Table 4. Primer sequences

Primer Names		Primer sequences
PRDM1 HRM-PCR	Forward	TATTCTGAGAGGTGCTGGGG
	Reverse	GCTGGATTCACATAGCGCAT
MYC-qRT-PCR	Forward	TGCAGCTGCTTAGACGCTGGATTT
	Reverse	GTCGAGGTCATAGTTCCTGTTGGT
RPL13A-qRT-PCR	Forward	ACCGTCTCAAGGTGTTTGACG
	Reverse	GTACTTCCAGCCAACCTCGTG
GAPDH-qRT-PCR	Forward	TCATTGACCTCAACTACATG
	Reverse	TCGCTCCTGGAAGATGGTGAT
PRDM1-exon5,6-qRT-PCR	Forward	ACATGACCGGCTACAAGACC
	Reverse	CCCTTGTTGCAAGTCTGACA
PRDM1-exon2,3-qRT-PCR	Forward	TCAAGTATGCCACCAACAGTG
	Reverse	TTCCTGTTGGCGTTCTTAGG
GFP-HDRT-F1	Forward	GAGGATCCCACAAACACAGAGCAGTCTAAAGC
	Reverse	CGCCCTTGCTCACCATGGGTCTCTCGATCCCG
GFP-HDRT-F2	Forward	ACGGGATCGAGAGACCCATGGTGAGCAAGGGC
	Reverse	GCGAGTGATGTACGTTAAGATACATTGATGAGTTTGGACAAAC
GFP-HDRT-F3	Forward	CTCATCAATGTATCTTAACGTACATCACTCGCTCCCCC
	Reverse	CTCGGTACCCATGCCGTAGGGGGGCAAG
LAG3	Forward	GCGGGGACTTCTCGCTATG
	Reverse	GGCTCTGAGAGATCCTGGGG
GNLY	Forward	CCTGTCTGACGATAGTCCAAAAA
	Reverse	GACCTCCCCGTCCTACACA
PRF1	Forward	GACTGCCTGACTGTCGAGG
	Reverse	TCCCGGTAGGTTTGGTGGAA
TOX2	Forward	AGAGCGAGAACAACGAAGACT
	Reverse	TGGCCTGATAGGAGTAGGCAG
CCR4	Forward	AGAAGGCATCAAGGCATTTGG
	Reverse	ACACATCAGTCATGGACCTGAG
DsRed-HDRT-F1	Reverse	GTCCTCGGAGGAGGCCATGGGTCTCTCGATCCCGTAGG
DsRed-HDRT-F2	Forward	TGGCCTACGGGATCGAGAGACCCATGGCCTCCTCCGAGG
Exon5-HDRT-out	Forward	TTCTCAAGAAGGAGGAGCAAC
	Reverse	CTGGGATGCTCCGGCTG

Supplemental Table 5. List of 334 genes in the custom capture sequencing panel

ABCA7	BCORL1	CDH23	DMXL1	FAS	HIST1H2BD	KDM6A	MEF2B	NT5C3A	PRDM15	RNF213	STAT3	TRAF5	ZFHX3
ACTB	BCR	CDKN2A	DMXL2	FBXO11	HIST1H2BG	KDM6B	MGA	NYAP2	PRKCB	RPN2	STAT5B	TRRAP	ZFP36L1
ADAMTS17	BIRC6	CDKN2B	DNMT3A	FBXW10	HIST1H2BK	KLF2	MIR142	OFD1	PRKCQ	RRAGC	STAT6	TSC22D1	ZFP36L2
ADAMTSL3	BMP7	CECR1	DOCK2	FBXW7	HLA-A	KLHDC7B	MKI67	P2RY8	PRKD2	S1PR1	SYTL3	TYK2	ZFYVE19
AHR	BPTF	CHD1	DOCK3	FMN2	HLA-B	KLHL14	MPEG1	PAPLN	PRKDC	S1PR2	TACC2	TYMP	ZNF335
AKAP8	BRAF	CHD3	DOCK9	FOXO1	HNRNPA2B1	KLHL6	MROH9	PASK	PRKRIR	SAMD9	TAF1	TYRP1	ZNF608
ALMS1	BRD9	CHD8	DPYD	FRMPD1	HUWE1	KMT2A	MS4A1	PAX5	PRPF4B	SBF1	TAGAP	UBE2A	ZNF638
ALPK2	BTG1	CHEK2	DTX1	FTH1	ID3	KMT2C	MSH2	PC	PTEN	SETBP1	TBL1XR1	UBE2O	ZNF717
ANKLE2	BTG2	CIITA	DYNC1H1	FYN	IDH2	KMT2D	MSH5	PCBP1	PTPN1	SETD2	TCF3	UBR5	ZSWIM4
APC	BTK	CNOT1	EBF1	GATA3	IKBKB	KRAS	MSH6	PCMTD1	PTPN13	SGK1	TET1	ULK4	
ARHGEF1	C6ORF48	CREBBP	EEF1A1	GNA11	IKZF3	LAMA2	MTMR8	PDCD11	PTPN2	SH2B3	TET2	USP32	
ARHGEF2	CARD11	CRIPAK	EGR1	GNA13	IL2RG	LILRB1	MTOR	PDE7B	PTPN6	SIGLEC10	TET3	USP9X	
ARID1A	CBLB	CSNK1A1	EGR2	GNAQ	IL6R	LLGL2	MUM1	PDS5B	PTPRC	SLC29A2	TIAM1	VAV1	
ARID1B	CCND1	CSNK2A1	ELP2	GPR183	IL7R	LRRC37A3	MYC	PHIP	PTPRD	SMAD2	TMEM30A	VPS13A	
ARID2	CCND3	CSNK2B	ENKD1	GRIA4	IRF2BP2	LRRK1	MYD88	PIK3CA	RAB14	SMAD3	TMSB4X	VWA7	
ATM	CCR4	CTBP2	ENO2	GTSE1	IRF4	LRRN3	NCOR1	PIK3CB	RAB31	SMAD4	TNFAIP3	WDR90	
ATXN1	CCR7	CTCF	EP300	HDAC7	IRF8	LRWD1	NCOR2	PIK3CD	RANBP6	SMARCA2	TNFRSF13C	WHAMM	
B2M	CCT6B	CTNNB1	ERAP1	HIST1H1B	ITPKB	LYN	NF1	PIK3R1	RAPGEF1	SMARCA4	TNFRSF14	WWP1	
BCAT2	CD28	CUL4B	ERAP2	HIST1H1C	ITPR3	LYST	NFKB2	PIM1	REL	SMARCAL1	TNFSF9	XBP1	
BCL10	CD36	CUL9	ETS1	HIST1H1D	JAK1	MALT1	NFKBIA	PLCG1	RELA	SMARCB1	TNIK	XRCC6BP1	
BCL11A	CD58	CUX1	ETV6	HIST1H1E	JAK2	MAPK1	NLRP12	PLCG2	REV3L	SMARCC1	TOP2A	YTHDF2	
BCL2	CD70	CXCR4	EXOSC6	HIST1H2AC	JAK3	MCL1	NOTCH1	PMS1	RFTN1	SOCS1	TP53	YY1AP1	
BCL6	CD79A	DAB1	EZH2	HIST1H2AG	KANK2	MDN1	NOTCH2	POU2F2	RHOA	SRGAP3	TPST2	ZAP70	
BCL7A	CD79B	DDX3X	FADD	HIST1H2AM	KAT6A	MED12L	NOXA1	PPP6R2	RHOH	SSPO	TRAF2	ZCCHC7	
BCOR	CD83	DHCR7	FANCD2	HIST1H2BC	KDM2B	MED24	NRAS	PRDM1	RHOT2	STAM2	TRAF3	ZEB1	