**SUPPLEMENTAL METHODS**

*Primary cells and functional assays*

Peripheral blood mononuclear cells (PBMCs) were purified from buffy coats in accordance with the Declaration of Helsinki, as anonymized residues of blood donations, used upon signature of specific institutional informed consent for blood product donation. CD3+ or CD4+ T cells were isolated using Pan T cell isolation kit or CD4 T cells isolation kit (Miltenyi Biotech), stimulated and cultured as previously described[1].

For the GMP T cell culture protocol, CD3+ or CD4+ T cells were stimulated using 60 µl/1.5x$10^6$ cells of T Cell TransAct™ reagent (Miltenyi Biotec) and cultured in X-VIVO 15 medium (Lonza) supplemented with 0.5% of Human Serum Albumin (Baxalta), IL-7 (100UI/ml, Miltenyi Biotec) and IL-15 (200UI/ml, Miltenyi Biotec), 1% penicillin/streptomycin (Lonza). Low doses of IL-2 (50UI/ml, Miltenyi Biotec) were supplemented into culture medium 3 days after seeding. 5% human serum (PAN Biotech) was added 24 hours after LNP wash (24 hours after LNP incubation).

Human CB or mPB CD34+ HSPCs were purchased from Stem Express or in-house purified from Mobilized Leukopak (HemaCare), respectively, according to the TIGET-HPCT protocol approved by OSR Ethical Committee, and cultured as previously described[2]. Briefly, cells were seeded at the concentration of 5x$10^5$ cells/ml in serum-free StemSpan SFEM (STEMCELL Technologies) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), 2% glutamine, human early-acting cytokines (SCF 100 ng/ml, Flt3-L 100 ng/ml, TPO 20 ng/ml, and IL-6 20 ng/ml; Peprotech), 1 µM SR-1 (Biovision), 50 nM UM171 (STEMCell Technologies) and 10 µM dmPGE2 (Cayman) added only right-after thawing.

G-CSF or G-CSF + Plerixafor mPB CD34+ HSPCs were purified in house with the CliniMACS CD34 Reagent System (Miltenyi Biotec) from Mobilized Leukopak (AllCells) according to the TIGET-HPCT protocol approved by OSR Ethical Committee and following the manufacturer's instructions. HSPCs were seeded at the concentration of 5x$10^5$ cells/ml in serum-free StemSpan SFEM supplemented with penicillin (100 IU/ml), streptomycin (100 µg /ml), 2% glutamine, human early-acting cytokines (SCF 300 ng/ml, Flt3-L 300 ng/ml, TPO 100 ng/ml; Peprotech), 1 µM SR-1 (Biovision), 35 nM UM171 (STEMCell Technologies) and 10 µM dmPGE2 (Cayman) added only right-after thawing.

Colony-forming-unit HSPCs assay was performed as previously described[2].

For polyclonal T cell stimulation, T cells were removed from PBMCs isolated from three healthy donors using CD3 microbeads (Miltenyi Biotech). T cell depleted PBMCs were equally mixed and irradiated (60Gly). At the end of gene editing procedure edited T cells and controls were harvested and co-cultured in triplicate ($10^5$ cells/well, ratio 1:1) for 72 hours in X-VIVO 15 medium (Lonza), supplemented with 5% human serum and 100U/ml penicillin/streptomycin with irradiated T cell depleted feeder mix ±

anti human CD3 mAb (clone OKT3, 1µg/mL). Proliferation was quantified by thymidine incorporation adding to cell in culture 1 mCi/well [$^3$H]thymidine for additional 16 hours of culture.

Levels of IFN-γ released in the medium were determined by ELISA according to the manufacturer's instructions (BD Biosciences) at the indicated time point.

Allogeneic reactivity determination by Mixed leucocyte reaction (MLR). At the end of gene editing procedure edited T cells and controls were harvested and co-cultured in triplicate ($10^5$ cells/well ratio 1:1) for 72, 96, and 120 hours with irradiated (60Gly) T cell depleted PBMCs of 3 independent healthy donors. Proliferation was quantified by thymidine incorporation. After the indicated time of co-culture, cells were pulsed for 16 hours with 1 mCi/well [$^3$H]thymidine.

*Preparation of LNPs*

GenVoy-ILM T Cell Kit for mRNA (Precision Nanosystems, product number 1000683) was used to prepare LNPs, according to manufacturer's instructions. Briefly, a single reaction of mRNA working solution was prepared by mixing 11 µg of CRISPR/Cas9 mRNA, prepared as described below, or GFP mRNA (5moU – Trilink) with 3.52 µl of Formulation Buffer 10X and filled up to 35.2 µl with sterile water. CRISPR/Cas9 mRNA was prepared by mixing CleanCap® Cas9 mRNA (5moU – Trilink) with sgRNA (Synthego) at a Cas9/sgRNA ratio of 2.49:1. Empty LNPs were prepared by substituting CRISPR/Cas9 mRNA with sterile water. The largest well of the cartridge was then filled with 48 µl of Dilution Buffer, the middle well with 32 µl of mRNA working solution and the smallest well with 16 µl of Lipid Mix. The cartridge was then inserted into the instrument and LNPs formulated with Setting 3. Once the formulation step was completed, the formulated mRNA-LNPs were moved into 96 µl of Dilution Buffer and stored at 4°C for one week at most.

Quantification of encapsulated mRNA was performed using Quant-it™ RiboGreen RNA Assay Kit (Thermo Fisher Scientific), following manufacturer's instructions. Size and polydispersity index were measured by analyzing 40 µl of LNPs diluted in 900 µl of PBS using the ZetaSizer (Malvern) instrument and default parameters.

*Gene editing of primary cells using electroporation*

Primary T cells. After 3 days of stimulation, $5x10^5/10^6$ cells derived from healthy donors for each condition were washed with ten volumes of DPBS and electroporated with 25 pmol or 50 pmol of RNP or with different doses of CRISPR/Cas9 mRNA, prepared as described above (P3 Primary Cell 4D-Nucleofector X Kit, programs DS-130; Lonza). AAV6 transduction was performed 15' after electroporation at a dose of $5x10^4$ vg/cell. 3 µg of GSE56 mRNA[3] was transfected along with RNP when indicated.

CB-/mPB-derived HSPCs. After 3 days of stimulation, $2x10^5$/ $7.5x10^5$ cells for each condition were washed with ten volumes of DPBS and electroporated with 25 pmol or 50 pmol of RNP or with different doses of CRISPR/Cas9 mRNA, prepared as described above (P3 Primary Cell 4D-Nucleofector X Kit, program EO-100; Lonza). AAV6 transduction was performed 15' after electroporation procedure at a dose of $2x10^4$ vg/cell.

*Flow cytometry*

Immunophenotypic and apoptosis analyses were performed as described[2] (Supplemental Table 1). Mitochondrial membrane potential analysis was performed 2 and 24 hours after editing using MitoTracker® Green FM and Red FM (Invitrogen), according to manufacturer's instructions. MitoTracker® Red MFI was normalized on MitoTracker® Green MFI for analysis. Gating strategies are reported in Supplemental Figure 1-2.

*Mass-Spectrometry based proteomic analysis*

Protein digestion and peptides clean-up were performed using the PreOmics iST sample preparation kit following manufacturer's instruction. Proteolytic peptides were eluted from cartridges, dried out, re-suspended in 5 µL of Load buffer (PreOmics) and analysed by nLC-MS/MS using an EASY-nLC 1200 (Thermo Fisher Scientific) connected to a Q-Exactive HF instrument (Thermo Fisher Scientific) through a nano-electrospray ion source. The nano-LC system was operated in one column set-up with an EasySpray (S902 Thermo Fisher Scientific) kept at 45°C constant temperature. The separation gradient consisted in a linear increase of solvent B (0.1% formic acid 80% Acetonitrile) from 5 to 20%, over 69 min. The Q-Exactive was operated in the data-dependent acquisition (DDA) to automatically switch between MS and MSMS mode. MS spectra (from m/z 375-1650) were analysed in the Orbitrap detector with resolution R=60,000 at m/z 200. The 15 most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 3e6. MS2 data was acquired at R=15,000 resolution and an ion target value of 1e5. For Higher-energy Collisional Dissociation (HCD) scans 1.4 m/z isolation width and normalized collision energy of 28 were used. The maximum allowed ion accumulation times were 20ms for full scans and 45ms for MSMS. Acquired raw data were analysed using MaxQuant version 1.6.17.0[4], using the Andromeda search engine[5] and a Human Fasta Database downloaded from UniprotKB (74470 Entries). For both group-specific and global parameters, all values were kept as default. The LFQ intensity calculation was enabled, as well as the match between runs (MBRs) feature[6]. All proteins and peptides matching the reversed database were filtered out.

Resulting data were analyzed using the R (v4.0.0)/Bioconductor (v3.12) package DEP (v1.12.0). Protein quantification matrix from MaxQuant was processed and filtered keeping only proteins present in

all replicates of at least one condition. After normalization using a variance stabilizing transformation (vsn), the imputation of missing values was done using random draws from a Gaussian distribution centered around a minimal value. Differential Expressed Proteins (DEPs) among different tested conditions were finally identified and the R/Bioconductor package ClusterProfiler[7] (v3.18.0) was employed to perform pre-ranked Gene Set Enrichment Analysis (GSEA) on the Hallmark categories from the MSigDB.

*HSPC xenotransplantation*

Procedures were performed upon approval of hosting institution and Italian authorities (IACUC 1206). Female NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ mice (The Jackson Laboratory) were held in specific pathogen-free conditions. For xenotransplantation of CB and mPB CD34+ HSPCs, the outgrowths of $5x10^4$ and $7.5x10^5/10^6$ HSPCs, respectively, seeded at the start of the culture (time 0, t0) were injected intravenously 24 h after editing into 8 weeks old female NSG mice, 4 hours after sublethal irradiation (150-200 cGy).

*Statistical analyses*

The number of biologically independent samples is indicated by "n". Data were summarized as median with interquartile range (IQR), unless otherwise specified. Two-tail tests were performed. Standard comparisons of numerical variables between two groups were evaluated either with Mann–Whitney or Wilcoxon's paired test, whilst Kruskal–Wallis or with Friedman test, followed by a post hoc analysis with Dunn's test with Bonferroni's correction, were used in case of three or more groups. Significance threshold was 0.05. "ns" means not significant. Analyses were performed using GraphPad Prism v.9.3.1 (GraphPad).

*Data and code availability*

Sequencing data have been deposited in Gene Expression Omnibus (GSE215250). Mass-spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[8] partner repository (PXD037529). Scripts for RNA-Seq and proteomic analysis are available at http://www.bioinfotiget.it/gitlab/custom/vavassori_lnp2022.

**SUPPLEMENTAL REFERENCES**


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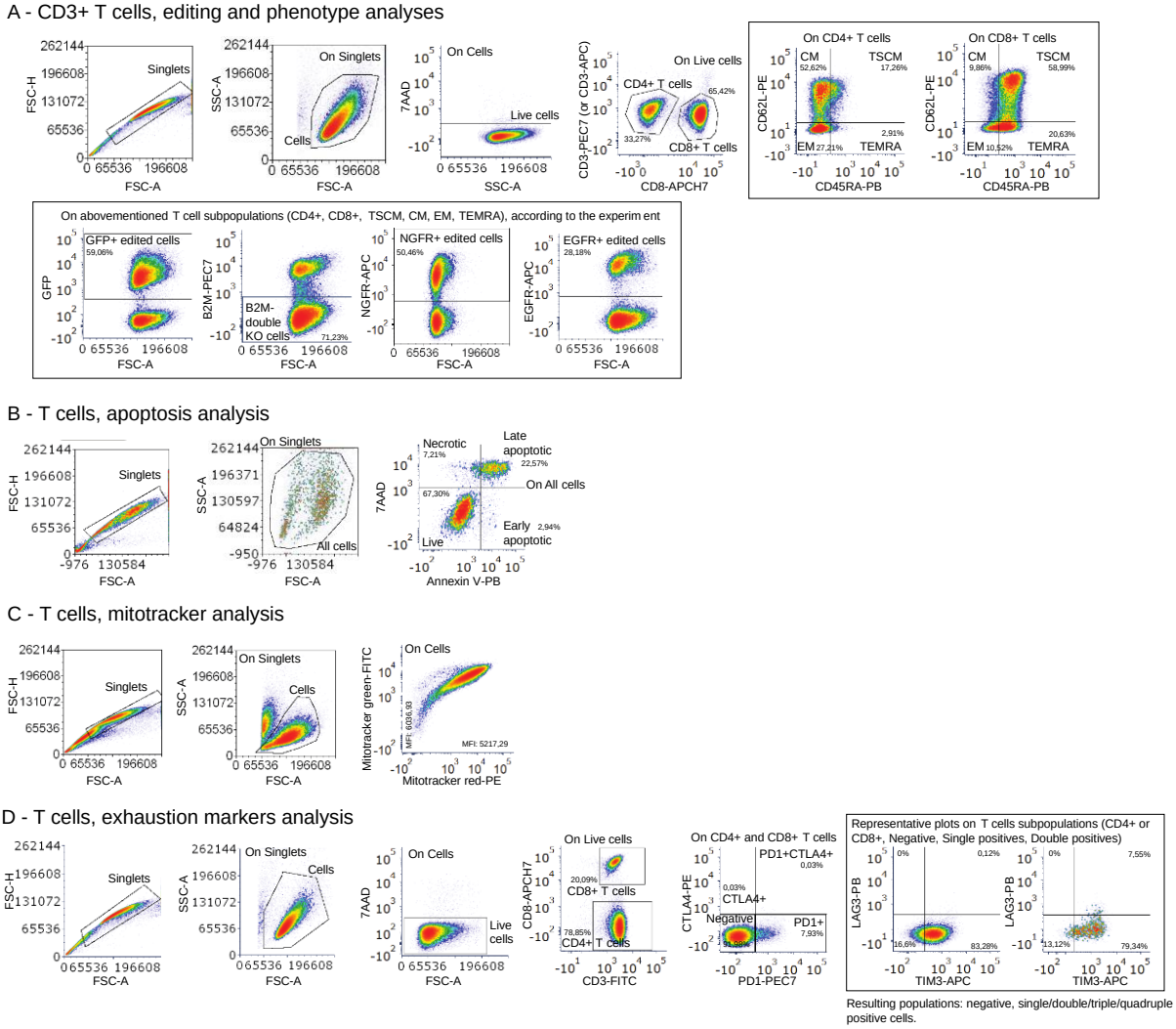
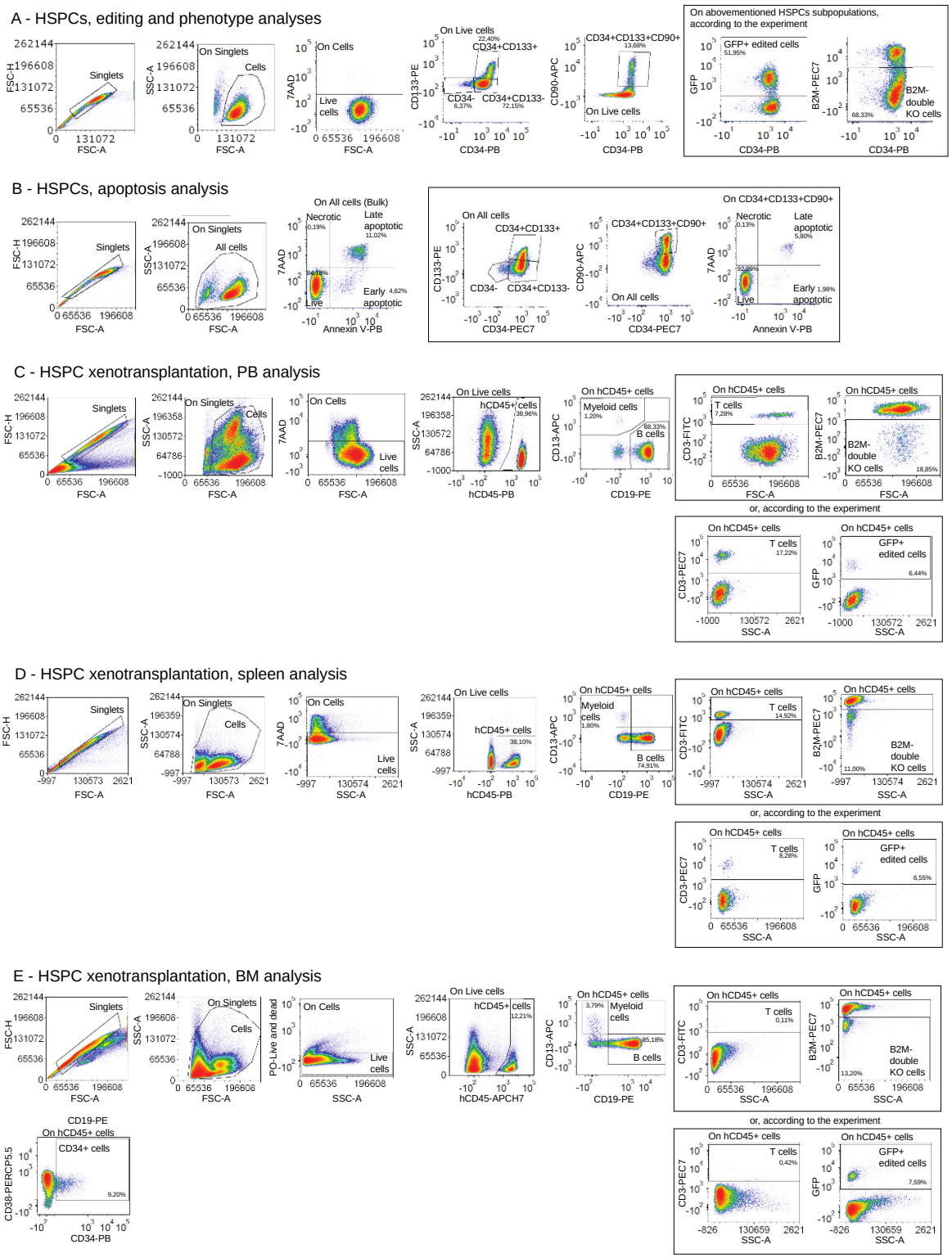
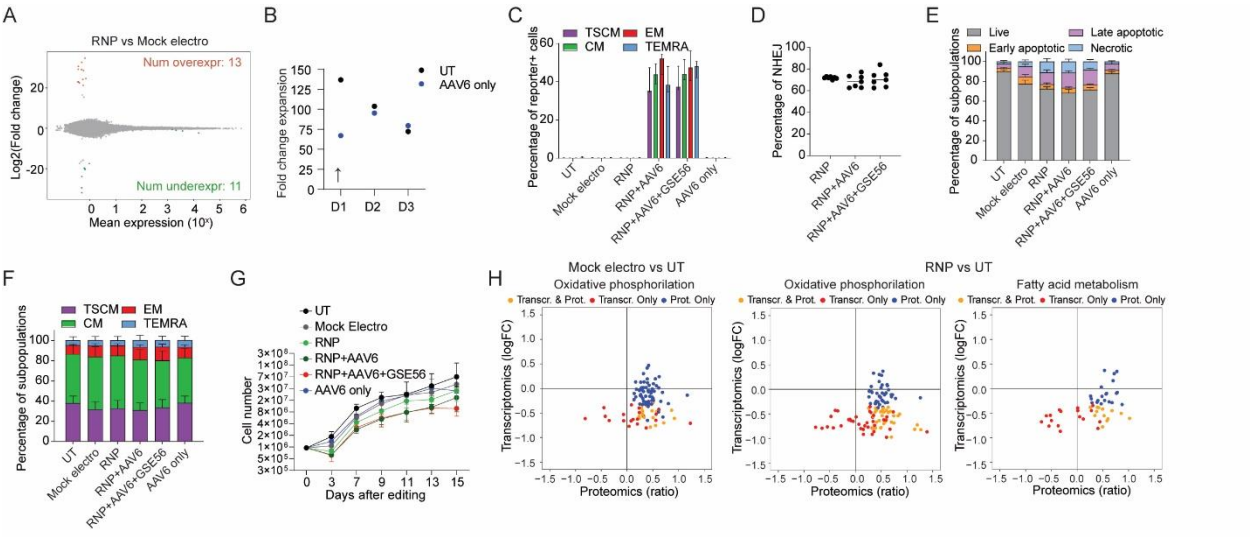
## SUPPLEMENTAL FIGURES



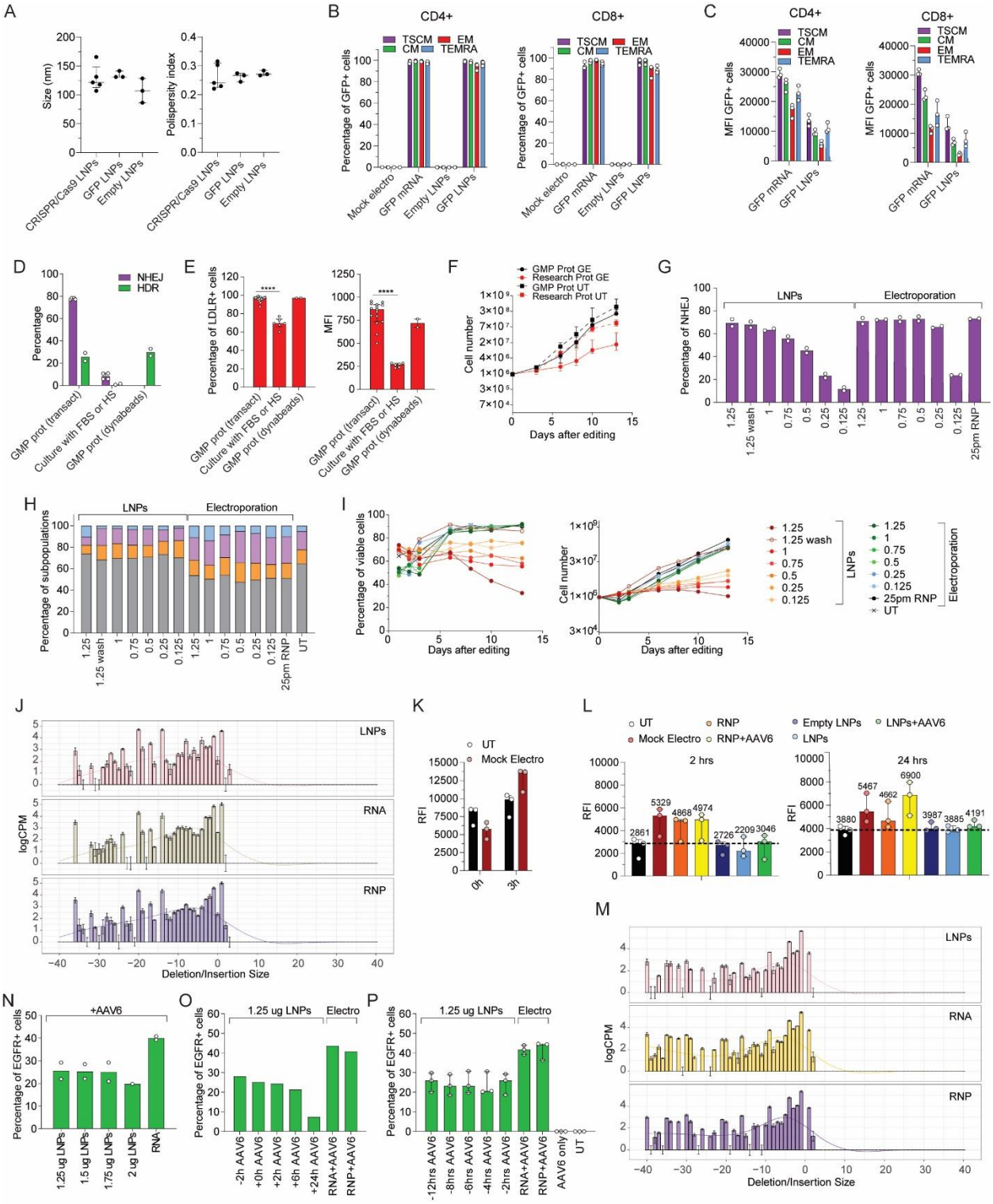

**Supplemental Figure 1. Gating strategy for T cell experiments.**




**Supplemental Figure 2. Gating strategy for HSPC experiments.**

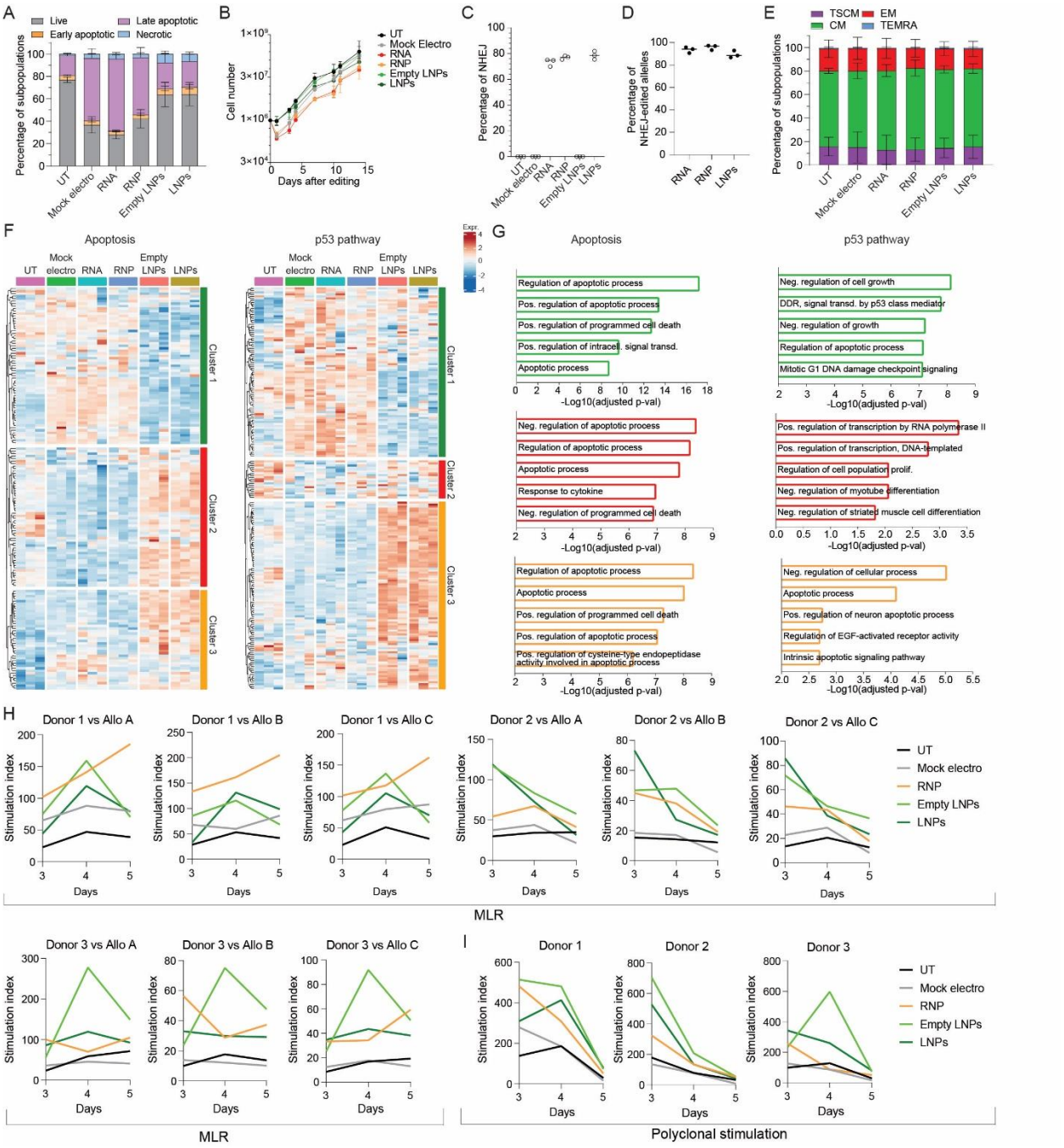



**Supplemental Figure 3. Transient p53 or cell death inhibition do not improve tolerance to editing in CD4+ T cells. A)** MA plot showing significant DEGs in the 'Mock electro vs RNP' comparison from Fig. 1C. **B)** Fold change expansion of UT and 'AAV6 only'-treated T cells measured from day 0 to the end of culture for each donor (D1, D2, D3) in Fig. 1C. **C)** Percentage of reporter+ cells measured by flow cytometry, as surrogate readout of HDR, within subpopulations of male CD4+ T cells (n=7). 3 µg of the p53 inhibitor GSE56 mRNA was co-electroporated, where indicated. Median ± interquartile range (IQR). **D)** Percentage of NHEJ-edited alleles in experiments from C (n=7). Median. **E)** Percentage of live, early/late apoptotic and necrotic cells 24 h after treatments in experiments from C (n = 7). Mean ± SEM. **F)** Cell population composition from C (n=7), 15 days after treatment. Mean ± SEM. **G)** Growth curve from C (n=7). Median ± IQR. **H)** Plots showing changes of expression for genes or proteins belonging to the core enrichment of oxidative phosphorylation (left) or fatty acid metabolism (right) categories from 'Mock electro vs UT' (left) or 'RNP vs UT' (right) comparisons.



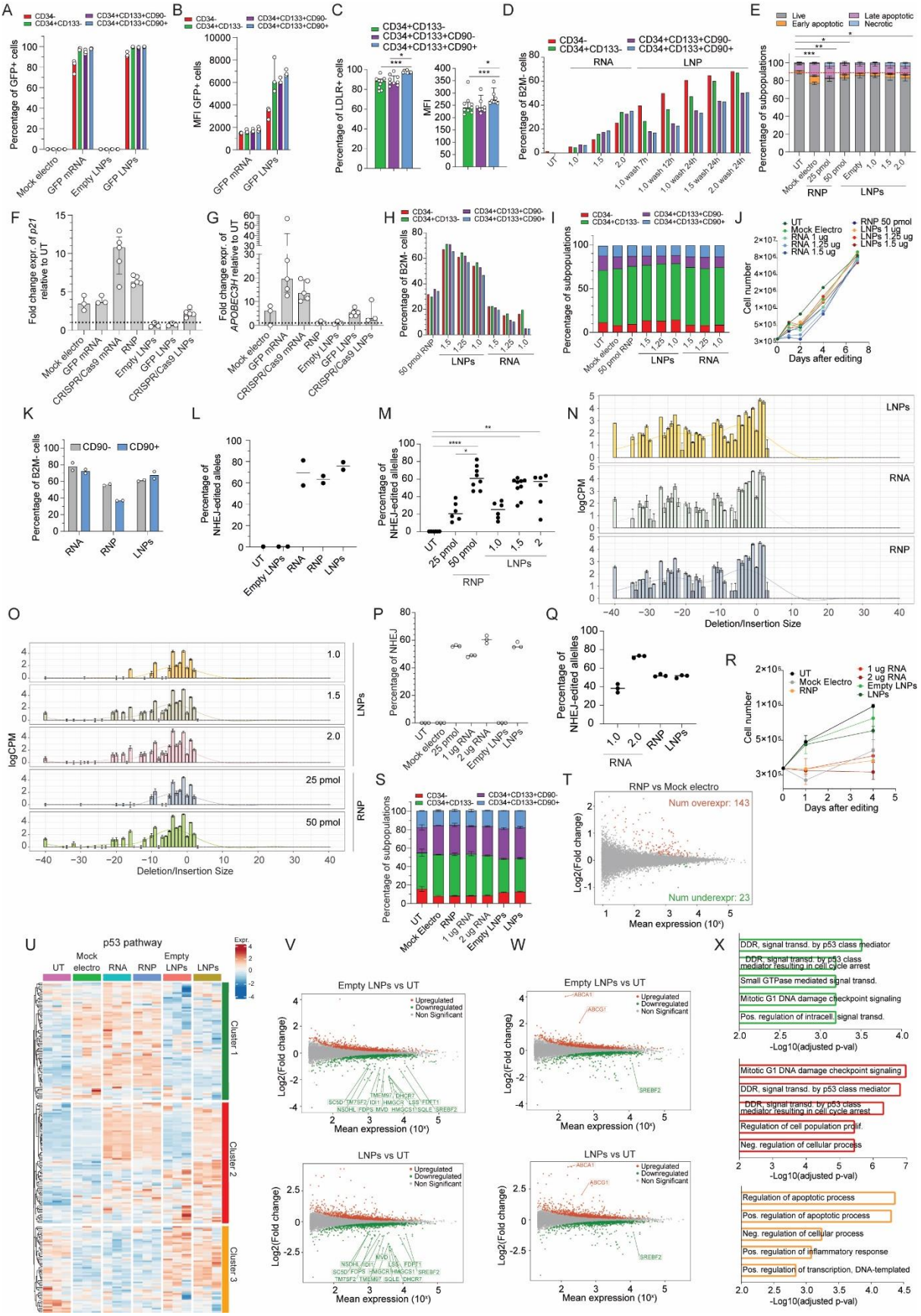

**Supplemental Figure 4. LNP-mediated CRISPR/Cas9 gene editing avoids alterations in mitochondrial potential upon CD4+ T cells editing. A)** Size in nm (left; n=5, 3, 3) and polydispersity index (right; n= 4, 3, 3) of different LNP preparations (Empty LNPs, GFP LNPs, CRISPR/Cas LNPs). Median ± IQR. **B-C)** Percentage (B) and mean fluorescence intensity (MFI) (C) of CD4+ (left) or CD8+

(right) GFP+ cells within T cell subpopulations, 24 hours after GFP mRNA transfection by electroporation or LNPs (1.5 µg for both conditions) (n= 3). Mock electroporation and Empty LNPs were used as controls (n=3). Median ± IQR. **D)** Percentage of NHEJ- and HDR-edited *CD40LG* alleles after editing male CD4+ T cells with LNPs alone (NHEJ) or in combination (HDR) with the cognate HDR template provided by AAV6 in presence of different culture media. Good manufacturing practice (GMP) culture protocol with Transact stimulation (NHEJ: n=5, HDR: n=2); culture protocol with fetal bovine or human serum (NHEJ: n=4, HDR: n=2); GMP culture protocol with Dynabeads stimulation (HDR: n=2). Median ± IQR. **E)** Percentage (left) and MFI (right) of LDLR+ cells measured from A by flow cytometry 3 days after seeding (n=15,6,2). Mann–Whitney test. Median ± IQR. **F)** Growth curve of male CD4+ T cells either UT or edited (GE) at *CD40LG* using different culture protocols (n=8,6,8,6). Median ± IQR. **G)** Percentage of NHEJ-edited *CD40LG* alleles after editing male CD4+ T cells with different doses of LNPs or RNA (n=2). One condition was washed 24 hours after treatment. Electroporation of 25 pmol of RNP was used as control. Median. **H)** Percentage of live, early/late apoptotic and necrotic CD4+ T cells 24h after *CD40LG* editing in experiments from G (n=2). UT cells were used as controls. Mean. **I)** Percentage of viable cells over time (left) and growth curve (right) of CD4+ T cells from H (n=2). Median. **J)** Distribution of indel size at *CD40LG* locus, after editing male CD4+ T cells with LNPs, RNP or RNA (n=3). Mean ± standard deviation. logCPM: logarithm of counts per million reads. **K)** Relative Fluorescence Intensity (RFI) of Mitotracker Red (mitochondrial membrane potential) normalized on Mitotracker Green (mitochondrial mass) measured by flow cytometry 3 hours or immediately (0 h) after electroporation of CD3+ T cells (n=3). UT cells were used as controls. Median ± range. **L)** Normalized mitochondrial potential (RFI) as in K 2 hours (left) or 24 hours (right) after indicated treatments (n=3). Empty LNPs: LNPs formulated in absence of RNA. Median ± range. **M)** Distribution of indel size at *B2M* locus, after editing male CD3+ T cells with LNPs, RNP or RNA (n=3). Mean ± standard deviation. **N)** Percentage of reporter+ CD4+ T cells after *CD40LG* editing using different doses LNPs or 1.25 µg of RNA electroporation in combination with AAV6 (n=2,2,2,1,2). Median. **O-P)** Percentage of reporter+ CD4+ T cells after *CD40LG* editing using 1.25 µg of LNPs in combination with AAV6 delivered at different time points before or after LNPs addition. 25 pmol of RNP and 1.25 µg of mRNA in combination with AAV6 delivered 15 min after electroporation were used as controls, as well as UT cells AAV6 only transduced conditions (left: n=1; right: n=3). Median ± IQR.




**Supplemental Figure 5. LNP- and electroporation-mediated gene editing in CD4+ T cells for transcriptomic analyses. A)** Percentage of live, early/late apoptotic and necrotic CD4+ T cells 24 h after *CD40LG* editing (n=3). Cells were edited with 25 pmol of RNP, 1.25 µg of RNA or 1.25 µg of LNPs. Mean ± SEM. **B)** Growth curve from A (n=3). Median ± range. **C)** Percentage of NHEJ-edited alleles after *CD40LG* editing in conditions from A (n=3), measured by T7 assay. Median ± range. **D)** Percentage of NHEJ-edited alleles after editing in conditions from A (n=3), measured by deep sequencing. Median. **E)** Cell population composition of cells from A (n=3), 14 days after treatment. Mean ± SEM. **F)** Heatmap

showing normalized read counts for all genes belonging to the apoptosis (left) and p53 (right) pathways GO category in conditions Fig. 3A. **G)** Adjusted p-values for the top-5 enriched GO categories (Biological Processes), when computing genes belonging to clusters from F. **H, I)** Proliferative responses of CD4+ T cells isolated from healthy donors (n=3), in resting phase after the indicated treatments, driven by (H) co-culture with CD3-depleted PBMCs (MLR, mixed leucocytes reaction) isolated from allogeneic donors (n=3), or (I) polyclonal stimulation (anti-CD3), were evaluated in time by [$^{3}$H]thymidine incorporation and expressed as stimulation index (cpm stimulated / cpm unstimulated).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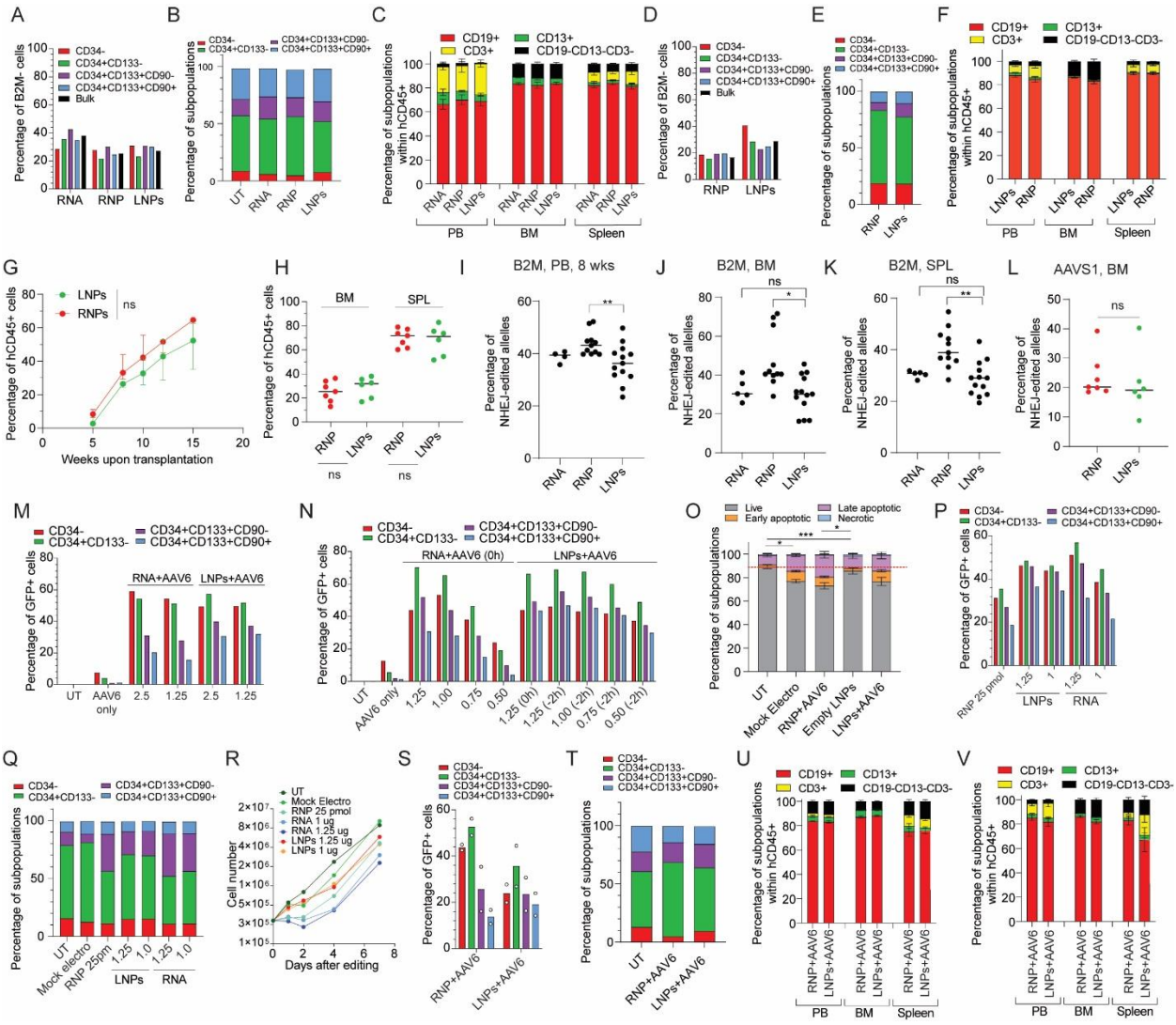
Percentage of GFP+ cells
MFI GFP+ cells
Percentage of LDLR+ cells
MFI
Percentage of B2M- cells
Percentage of subpopulations
Fold change expr. of p21 relative to UT
Fold change expr. of APOBEC3H relative to UT
Cell number
Days after editing
Percentage of NHEJ-edited alleles
logCPM
Deletion/Insertion Size
Mean expression (10^x)
Log2(Fold change)
-Log10(adjusted p-val)
p53 pathway
Empty LNPs vs UT
LNPs vs UT
RNP vs Mock electro
Num overexpr: 143
Num underexpr: 23
DDR, signal transd. by p53 class mediator
Mitotic G1 DNA damage checkpoint signaling
Regulation of apoptotic process
Pos. regulation of apoptotic process
Neg. regulation of cellular process
Pos. regulation of inflammatory response
Pos. regulation of transcription, DNA-templated

**Supplemental Figure 6. Optimization of LNP-mediated CRISPR/Cas9 editing for gene disruption in human HSPCs. A-B)** Percentage (A) and MFI (B) of GFP+ cells within HSPC subpopulations, 24 hours after GFP mRNA transfection by electroporation or LNPs (1.5 µg for both condition) (n=3). Mock electroporation and Empty LNPs were used as controls. Median ± IQR. **C)** Percentage (left) and MFI (right) of LDLR+ cells within mPB HSPC subpopulations 3 days after seeding (n=9). Friedman test with Dunn's multiple comparisons. Median ± IQR. **D)** Percentage of B2M- cells within mPB HSPC subpopulations edited with different doses (µg) of RNA or LNP and then washed at different times (n = 1). **E)** Percentage of live, early/late apoptotic and necrotic bulk mPB HSPCs from Fig. 4D (n=6). Friedman test with Dunn's multiple comparisons performed on live cells. Mean ± SEM. **F-G)** Fold change expression of *p21* (F) and *APOBEC3H* (G) relative to UT 24 hours after treatment (n=3). HSPCs were electroporated with 1.5 µg GFP mRNA or CRISPR/Cas9 mRNA/RNP; or treated with 1.5 µg LNPs carrying GFP mRNA or CRISPR/Cas9 targeting *B2M*. Mock electroporation and Empty LNPs were used as controls. Median ± IQR. **H)** Percentage of B2M- cells within CB HSPC subpopulations edited with different doses (µg) of RNA or LNP, or with 50 pmol of RNP (n=1). **I)** Cell population composition of CB HSPCs from H (n=1), 4 days after treatment. UT and Mock Electro cells were used as control (n=1). **J)** Growth curve from I (n=1). **K-L)** Percentage of B2M- cells (K) and NHEJ-edited *B2M* alleles (L) in human HSPCs, 4 days after editing with a *B2M* sgRNA targeting exon 1 (n=2). Median. **M)** Percentage of NHEJ-edited alleles in human HSPCs 4 days after editing with the *B2M* sgRNA targeting exon 2 (n=6, 6, 8, 6, 9, 6). Kruskal-Wallis test with Dunn's multiple comparisons. Median. **N-O)** Distribution of indel size at *B2M* locus, after editing HSPCs with LNPs, RNP or RNA targeting exon 1 (N, n=2) or exon 2 (O, n=6, 6, 8, 6, 9, 6), at the indicated doses. Mean ± standard deviation. **P-Q)** Percentage of NHEJ-edited *AAVS1* alleles after editing HSPCs with 1 µg of LNPs, 25 pmol of RNP, or 1 or 2 µg of RNA (1-2 ug mRNA), measured by T7 assay (P) or by deep sequencing (Q) (n=3). UT, Mock Electro and Empty LNPs were added as controls (n=3). Median. **R)** Growth curve from P (n=3). Median ± IQR. **S)** Cell population composition from P (n=3), 4 days after treatment. Mean ± SEM. **T)** MA plot showing significant DEGs in 'RNP vs Mock electro' comparison from Fig. 4I. **U)** Heatmap showing normalized read counts for all genes belonging to the p53 pathway GO category in conditions Fig. 4I. **V-W)** MA plot showing significant DEGs in 'Empty LNPs vs UT' (top) or 'LNPs vs UT' (bottom) comparisons from Fig. 4I. Labels highlight (V) DEGs belonging to the core enrichment of cholesterol homeostasis category, and (W) *SREBF2*, *ABCA1*, *ABCG1*. **X)** Adjusted p-values for the top-5 enriched GO categories (Biological Processes), when computing genes belonging to clusters from U.



**Supplemental Figure 7. Optimization of LNP-mediated CRISPR/Cas9 editing for HDR-mediated editing in human HSPCs. A-B)** Percentage of B2M- cells within mPB HSPC subpopulations (A) and cell population composition (B) 4 days after treatment, for xenotransplantation experiment of Figure 4J. (n=1). **C)** Lineage composition of hCD45$^{+}$ in PB, BM and spleen of mice from Fig. 4J at the endpoint (n=5, 5, 6). B cells: CD19+; Myeloid cells: CD13+; T cells: CD3+. **D-E)** Percentage of B2M- cells within CB HSPC subpopulations (D) and cell population composition (E) 4 days after treatment, for xenotransplantation experiment of Fig. 4N (n=1). **F)** Lineage composition of hCD45$^{+}$ in PB, BM and spleen of mice from Fig. 4N at the endpoint (n=6,7). B cells: CD19+; Myeloid cells: CD13+; T cells: CD3+. **G)** Percentage of circulating hCD45+ cells over time in mice transplanted with mPB-HSPCs edited with 25 pmol of RNP or 1.5µg of LNPs (n=7,6), targeting *AAVS1*. Mann-Whitney test. Median ± IQR. **H)** Percentage of hCD45+ cells in BM and spleen of mice from G (n=7,6). Mann-Whitney test. Median. **I-K)** Percentage of NHEJ-edited *B2M* exon-2 alleles in circulating human cells at 8 weeks after transplant (I; n=4, 11, 13; Mann-

Whitney test), in BM (J; n=5, 11, 13; Kruskal-Wallis test) and in spleen (K; n=5, 11, 13; Kruskal-Wallis test) of mice from experiments in Fig. 4J, N. Median. **L)** Percentage of NHEJ-edited *AAVS1* alleles within human cells in BM of mice from G. Mann-Whitney test. Median. **M-N)** Percentage of GFP+ cells within mPB HSPC subpopulations using different doses of LNPs or RNA, and different timings of AAV6 transduction (n=1). UT and AAV6 only were added as controls (n=1). **O)** Percentage of live, early/late apoptotic and necrotic bulk mPB HSPCs from Fig. 5C (n=6). Friedman test with Dunn's multiple comparisons performed on live cells. Mean ± SEM. **P)** Percentage of GFP+ cells within CB HSPC subpopulations using different doses of RNA or LNP, in combination with AAV6 transduction performed immediately after electroporation or 2 hours before transfection, respectively. Cells edited with 25 pmol of RNP were used as control (n=1). **Q)** Cell population composition from P (n=1), 4 days after treatment. UT cells were used as control. **R)** Growth curve from Q (n=1). **S-T)** Percentage of B2M- cells within mPB HSPC subpopulations (S, Median) and cell population composition (T, Mean) 4 days after treatment for xenotransplantation experiment of Fig. 5I, K (n=2). **U-V)** Lineage composition (as in C) of hCD45+ in PB, BM and spleen of mice from Fig. 5I (I; n=4,5) or 5K (J; n=7) at the endpoint.

**SUPPLEMENTAL TABLES**

| Specificity | Fluorochrome | Clone | Supplier | Dilution |
|---|---|---|---|---|
| Anti-human CD16/32 | N/A | N/A | Miltenyi Biotec | 1:50 |
| Anti-human CD271 (LNGFR) | APC | ME20.4-1.H4 | Miltenyi Biotec | 1:50 |
| Anti-human EGFR | Biotin conjugated | HU1 | R&D Systems | 1:100 |
| Anti-biotin | APC | Bio3-18E7 | Miltenyi Biotec | 1:100 |
| Anti-biotin | FITC | Bio3-18E7 | Miltenyi Biotec | 1:100 |
| Anti-human CD62L | PE | DREG-56 | BD | 1:50 |
| Anti-human B2M | PEC7 | 2M2 | Biolegend | 1:100 |
| Anti-human CD45RA | VioBlue | T6D11 | Miltenyi Biotec | 1:50 |
| Anti-human CD133/2 | PE | REA816 | Miltenyi Biotec | 1:50 |
| Anti-human CD34 | VioBlue | AC136 | Miltenyi Biotec | 1:50 |
| Anti-human CD90 | APC | 5E10 | BD | 1:33 |
| Anti-human CD45 | PB | HI30 | Biolegend | 1:50 |
| Anti-human CD45 | APC-Vio770 | 2D1 | BD | 1:50 |

| | | | | |
|---|---|---|---|---|
| Anti-human CD19 | PE | SJ25C1 | BD | 1:50 |
| Anti-human CD3 | PEcy7 | HIT3a | Biolegend | 1:50 |
| Anti-human CD3 | APC | UCHT1 | BD | 1:50 |
| Anti-human CD3 | FITC | SK7 | Supplier:BD | 1:50 |
| Anti-human CD13 | APC | WM15 | BD | 1:50 |
| Anti-human CD38 | PerCP5.5 | HB7 | Biolegend | 1:20 |
| Anti-human CD4 | PB | RPA-T4 | Supplier:BD | 1:50 |
| Anti-human CD8 | APCH7 | SK1 | BD | 1:33 |
| Anti-human LDLR | PE | P01130 | R&D Systems | 1:20 |
| Anti-mouse CD16/32 | none | 2.4G2 | BD | 1:100 |
| Anti-human CD279 (PD1) | PeVio770 | REA1165 | Supplier:Miltenyi Biotec | 1:50 |
| Anti-human CD152 (CTLA4) | PE | REA1003 | Miltenyi Biotec | 1:50 |
| Anti-human CD366 (TIM3) | APC | REA635 | Miltenyi Biotec | 1:50 |
| Anti-human CD223 (LAG3) | VioBlue | REA351 | Miltenyi Biotec | 1:50 |

**Supplemental Table 1. List of antibodies used in this study.**

| **Description** | **Orientation** | **Sequence (5'-3')** |
|---|---|---|
| NHEJ T7 *CD40LG* | FW | CTTCACAAGCACTGATTGTAGTTGC |
| | RV | CCAAACACAAATAACCAACCAGACC |
| NHEJ T7 *AAVS1* | FW | CTTCAGGACAGCATGTTTGC |
| | RV | GGACTAGAAAGGTGAAGAGCC |
| B2M exon 2 NGS PCR1 | FW | AAGATGAGTATGCCTGCCGT |
| | RV | TGGGATGGGACTCATTCAGG |
| B2M exon 2 NGS P5 1 | FW | AATGATACGGCGACCACCGAGATCTACACGTAAGG AGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AAGATGAGTATGCCTGCCGT |

| | | |
|---|---|---|
| B2M exon 2 NGS P5 2 | FW | AATGATACGGCGACCACCGAGATCTACACTGCATATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGATGAGTATGCCTGCCGT |
| B2M exon 2 NGS P5 3 | FW | AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGATGAGTATGCCTGCCGT |
| B2M exon 2 NGS P5 4 | FW | AATGATACGGCGACCACCGAGATCTACACCCTAGAGTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGATGAGTATGCCTGCCGT |
| B2M exon 2 NGS P5 5 | FW | AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGATGAGTATGCCTGCCGT |
| B2M exon 2 NGS P5 6 | FW | AATGATACGGCGACCACCGAGATCTACACTCGACTAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGATGAGTATGCCTGCCGT |
| B2M exon 2 NGS P5 7 | FW | AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGATGAGTATGCCTGCCGT |
| B2M exon 2 NGS P7 1 | RV | CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGATGGGACTCATTCAGG |
| B2M exon 2 NGS P7 2 | RV | CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGATGGGACTCATTCAGG |
| B2M exon 2 NGS P7 3 | RV | CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGATGGGACTCATTCAGG |
| B2M exon 2 NGS P7 4 | RV | CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGATGGGACTCATTCAGG |
| B2M exon 2 NGS P7 5 | RV | CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGATGGGACTCATTCAGG |

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|---|---|---|
| B2M exon 2 NGS P7 6 | RV | CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGATGGGACTCATTCAGG |
| B2M exon 2 NGS P7 7 | RV | CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGATGGGACTCATTCAGG |
| B2M exon 1 NGS PCR1 | FW | CGCGTTTAATATAAGTGGAGGC |
| | RV | GGAGAACTTGGAGAAGGGAAGT |
| B2M exon 1 NGS P5 1 | FW | AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCGTTTAATATAAGTGGAGGC |
| B2M exon 1 NGS P5 2 | FW | AATGATACGGGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCGTTTAATATAAGTGGAGGC |
| B2M exon 1 NGS P5 3 | FW | AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCGTTTAATATAAGTGGAGGC |
| B2M exon 1 NGS P5 4 | FW | AATGATACGGCGACCACCGAGATCTACACCCTAGAGTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCGTTTAATATAAGTGGAGGC |
| B2M exon 1 NGS P5 5 | FW | AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCGTTTAATATAAGTGGAGGC |
| B2M exon 1 NGS P7 1 | RV | CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAGAACTTGGAGAAGGGAAGT |
| B2M exon 1 NGS P7 2 | RV | CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAGAACTTGGAGAAGGGAAGT |
| B2M exon 1 NGS P7 3 | RV | CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAGAACTTGGAGAAGGGAAGT |

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|---|---|---|
| B2M exon 1 NGS P7 4 | RV | CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCT CGTGGGCTCGGAGATGTGTATAAGAGACAGGGAGA ACTTGGAGAAGGGAAGT |
| B2M exon 1 NGS P7 5 | RV | CAAGCAGAAGACGGCATACGAGATCTAGTACGGTC TCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAG AACTTGGAGAAGGGAAGT |
| CD40LG NGS P5 1 | FW | AATGATACGGCGACCACCGAGATCTACACTAGATCG CNNWNNWNNACACTCTTTCCCTACACGACGCTCTTC CGATCTCACTGAGGAGTATAATTGGCTGGT |
| CD40LG NGS P5 2 | FW | AATGATACGGCGACCACCGAGATCTACACCTCTCTA TNNWNNWNNACACTCTTTCCCTACACGACGCTCTTC CGATCTCACTGAGGAGTATAATTGGCTGGT |
| CD40LG NGS P5 3 | FW | AATGATACGGCGACCACCGAGATCTACACTATCCTC TNNWNNWNNACACTCTTTCCCTACACGACGCTCTTC CGATCTCACTGAGGAGTATAATTGGCTGGT |
| CD40LG NGS P5 4 | FW | AATGATACGGCGACCACCGAGATCTACACAGAGTA GANNWNNWNNACACTCTTTCCCTACACGACGCTCT TCCGATCTCACTGAGGAGTATAATTGGCTGGT |
| CD40LG NGS P5 5 | FW | AATGATACGGCGACCACCGAGATCTACACGTAAGG AGNNWNNWNNACACTCTTTCCCTACACGACGCTCT TCCGATCTCACTGAGGAGTATAATTGGCTGGT |
| CD40LG NGS P5 6 | FW | AATGATACGGCGACCACCGAGATCTACACACTGCAT ANNWNNWNNACACTCTTTCCCTACACGACGCTCTT CCGATCTCACTGAGGAGTATAATTGGCTGGT |
| CD40LG NGS P7 1 | RV | CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAAGACCA GAAGCCCCCTCTTTT |
| CD40LG NGS P7 2 | RV | CAAGCAGAAGACGGCATACGAGATCTAGTACGGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAAGACCA GAAGCCCCCTCTTTT |
| CD40LG NGS P7 3 | RV | CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTGA CTGGAGTCCTCTCTATGGGCAGTCGGTGAAGACCAG AAGCCCCCTCTTTT |

| | | |
|---|---|---|
| CD40LG NGS P7 4 | RV | CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAAGACCA GAAGCCCCCTCTTTT |
| CD40LG NGS P7 5 | RV | CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAAGACCA GAAGCCCCCTCTTTT |
| CD40LG NGS P7 6 | RV | CAAGCAGAAGACGGCATACGAGATCATGCCTAGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAAGACCA GAAGCCCCCTCTTTT |
| AAVS1 NGS PCR1 | FW | CGAGAGCTCAGCTAGTCTTCTT |
| | RV | GAGATGGCTCCAGGAAATGGG |
| AAVS1 NGS P5 1 | RV | AATGATACGGCGACCACCGAGATCTACACTAGATCG CNNWNNWNNACACTCTTTCCCTACACGACGCTCTTC CGATCTCGAGAGCTCAGCTAGTCTTCTT |
| AAVS1 NGS P5 2 | RV | AATGATACGGCGACCACCGAGATCTACACCTCTCTA TNNWNNWNNACACTCTTTCCCTACACGACGCTCTTC CGATCTCGAGAGCTCAGCTAGTCTTCTT |
| AAVS1 NGS P5 3 | RV | AATGATACGGCGACCACCGAGATCTACACTATCCTC TNNWNNWNNACACTCTTTCCCTACACGACGCTCTTC CGATCTCGAGAGCTCAGCTAGTCTTCTT |
| AAVS1 NGS P5 4 | RV | AATGATACGGCGACCACCGAGATCTACACAGAGTA GANNWNNWNNACACTCTTTCCCTACACGACGCTCT TCCGATCTCGAGAGCTCAGCTAGTCTTCTT |
| AAVS1 NGS P5 5 | RV | AATGATACGGCGACCACCGAGATCTACACGTAAGG AGNNWNNWNNACACTCTTTCCCTACACGACGCTCT TCCGATCTCGAGAGCTCAGCTAGTCTTCTT |
| AAVS1 NGS P5 6 | RV | AATGATACGGCGACCACCGAGATCTACACACTGCAT ANNWNNWNNACACTCTTTCCCTACACGACGCTCTT CCGATCTCGAGAGCTCAGCTAGTCTTCTT |
| AAVS1 NGS P7 1 | FW | CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAGAGATG GCTCCAGGAAATGGG |

| | | |
|---|---|---|
| AAVS1 NGS P7 2 | FW | CAAGCAGAAGACGGCATACGAGATCTAGTACGGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAGAGATG GCTCCAGGAAATGG |
| AAVS1 NGS P7 3 | FW | CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTGA CTGGAGTCCTCTCTATGGGCAGTCGGTGAGAGATGG CTCCAGGAAATGGG |
| AAVS1 NGS P7 4 | FW | CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAGAGATG GCTCCAGGAAATGGG |
| AAVS1 NGS P7 5 | FW | CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAGAGATG GCTCCAGGAAATGGG |
| AAVS1 NGS P7 6 | FW | CAAGCAGAAGACGGCATACGAGATCATGCCTAGTG ACTGGAGTCCTCTCTATGGGCAGTCGGTGAGAGATG GCTCCAGGAAATGGG |
| Custom index 1 seq primer | | CTCACCGACTGCCCATAGAGAGGACTCCAGTCAC |
| Custom read 2 AAVS1 seq primer | | GTGACTGGAGTCCTCTCTATGGGCAGTCGGTGAG |
| Custom read 2 CD40L seq primer | | GTGACTGGAGTCCTCTCTATGGGCAGTCGGTGA |
| *CD40LG* 5' HDR junction ddPCR | FW | TTAGGAGGGGGTCTGATACA |
| | RV | TCCTCGATCTGTGGGAGGAAGAGAA |
| | Probe (FAM) | TCAGTCTCCCTCTGAGATGT |

**Supplemental Table 2. List of primers and probes used in this study.**