SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

Figure S1: Analysis of HSPC sub-populations (Related to Fig 1)

- **A.** Representative flow cytometry gating strategy for categorizing HSCs, MPPs and progenitors. Live cells were gated based on forward and side scatter, and single cells were selected based on height and width parameters of forward and side scatters. Then, progenitor population was selected as the top 20% of CD38 expression of CD34+ cells, and CD34+38- population was selected as the bottom 5-10%. The CD34+38- population was further subgated to identify HSCs (top 30% of CD90 expression of CD45RA- cells) and MPPs (bottom 30% of CD90 expression of CD45RA- cells).
- **B.** Cell cycle analysis of HSPC sub-populations after two days of prestimulation. N=2. Numbers indicate means.

Figure S2: Assessment of hGemCas9 Nuclease Specificity

- **A.** Cell cycle analysis of sorted cells, performed immediately after (0 hours) and 24 hours after sort. (Related to Fig 3A)
- **B.** Percentage of indels in unsorted PBSCs (from Fig 4B) on target (HBB) and at two most common off-target sites (OT1 and OT2) for this guide. N=3. Data are represented as mean ± SD. ns – not significant, * P≤0.05, based on unpaired t-test.
- **C.** Nuclease specificity, measured as the ratio of nuclease activity on target (HBB) to off-target (OT1+OT2) in unsorted PBSCs, calculated from data in Fig 4B. N=3. Data are represented as mean ± SD. ns – not significant, based on unpaired t-test.
- **D.** Percentage of indels on target (HBB) and at two most common off-target sites (OT1 and OT2) in unsorted PBSCs, electroporated with wtCas9 or hGemCas9, and transduced with AAV6 donor template. N=4. Data are represented as mean ± SD. ns – not significant, * P<0.05, ** P≤0.01, based on unpaired t-test. (Related to Fig 6)
- **E.** Nuclease specificity, calculated as the ratio of nuclease activity on target (HBB) to off-target (OT1+OT2), calculated from data in Fig S2D. N=4. Data are represented as mean ± SD. ns – not significant, based on unpaired t-test.

Figure S3: Synchronization of Human HSPCs with RO (Related to Fig 5)

- **A.** Viability of cells after 20 hours of treatment with DMSO or RO. N=6. Data are represented as bars ± SD. Differences are not significant, based on Wilcoxon Rank Sum test.
- **B.** Clonogenic Potential of PBSCs, pre-treated with DMSO or RO, measured 14 days after plating colonies in methylcellulose medium. N=4. Individual points plotted, line – mean ± SD. Differences are not significant, based on Wilcoxon Rank Sum test.
- **C.** Lineage analysis of CFUs in Fig S3B. CFUs were counted and scored as the following: CFU-granulocyte/ erythrocyte/macrophage/megakaryocyte (CFU-GEMM), burst forming unit-erythroid (BFU-E), CFU-erythroid (CFU-E), CFU-granulocyte/macrophage (CFU-GM), CFU-granulocyte (CFU-G), and CFU-macrophage (CFU-M). N=4. Individual points plotted, line – mean ± SD. Differences are not significant, based on Wilcoxon Rank Sum test.
- **D.** Schema of competitive NSG mouse transplant of RO- and DMSO-treated cells. To evaluate whether RO-pretreated cells retain their hematopoietic potential, a competitive transplant was used. Human mobilized PBSCs were pre-stimulated for 24 hours and split equally into two populations, which were transduced with fluorescent vectors (mCitrine or mStrawberry). Four hours later, RO or DMSO was added to the cells. After 20 hours of treatment, the cells were washed with PBS, counted, and RO- and DMSO-treated cells of opposite colors were mixed in equal proportions of the starting number of cells, and transplanted into mice. Numbers on the bottom indicated the number of cells transplanted (also plotted in Fig S3E). Colors were alternated to account for any potential differences in transduction efficiencies of mCitrine and mStrawberry vectors. DMSO-only mice were used as controls. Mice were harvested 16 weeks post-transplant, and the levels of human engraftment, as well as the percentage of fluorescently transduced cells were evaluate to determine the percent contribution of RO and DMSO treated cells to human engraftment.
- **E.** Numbers of RO- or DMSO-treated cells, competitively transplanted into NSG mice.

F. Levels of human engraftment in NSG mice, 8 or 16 weeks after transplant with RO or DMSO-treated cells. PB – peripheral blood, BM – bone marrow. N=6 for RO + DMSO groups, N=3 for DMSO-only groups. Individual points plotted. Data are represented as geo mean ± SD. Differences are not significant, based on Kruskal-Wallis test.

G. Percentage of DMSO- and RO-treated cells, contributing to all human cells in NSG mice, measured before, during (8 weeks) and after transplant (16 weeks). N=6. Data are represented as mean ± SD. Horizontal dashed lines match the starting percentage of cells. Differences compared to the starting percentages are not significant, based on chi-square test.

Figure S4: Indel Spectrum Analysis (Related to Fig 6)

A. Indel spectrum of samples in Fig 6E. The top 15 indels are listed. "><" and the dashed blue line indicate nuclease cut site. For wtCas9 – N=10, for hGemCas9 – N=8. Numbers in the table are % indels ± SD. % indel was measured by HTS and calculated as (# of sequence reads containing an indel)/(total # of sequence reads) x 100.

B. A map of beta globin locus illustrating the Cas9 cut site (dashed blue line), and the microhomology region around the cut site (underlined). Red "A" represents the site of the sickle mutation. Numbers indicate the upstream (-) and downstream (+) positions from the cut site. The bottom panel represents DNA sequence after a 9-bp deletion.

Figure S5: Evaluating the Effects of CtIP* mRNA on Gene Editing (Related to Fig 6)

A-B. Levels of gene editing (HDR and NHEJ) in PBSCs pre-treated with RO or DMSO, and electroporated with wtCas9 or hGemCas9 +/- CtIP* IVT mRNA, and subsequently transduced with AAV6 donor template. For wtCas9 – N=10, for hGemCas9 – N=8. Data are represented as mean ± SD. Differences between 0ug and 3ug CtIP* are not significant based on Wilcoxon Rank-Sum test.

C-D. Viability and fold expansion, measured 24 hours post electroporation in S5B. Data are represented as mean ± SD. Differences between 0ug and 3ug CtIP* are not significant based on Wilcoxon Rank-Sum test.

E. Indel spectrum, normalized to %NHEJ for each sample. Numbers in parentheses indicate position of an indel relative to cut site, numbers following parentheses indicate the length of an indel, del – deletion, indel – insertion or deletion. See also Fig S4. For wtCas9 – N=10, for hGemCas9 – N=8. Data are represented as mean ± SD. Differences in indels between +/- CtIP* are not significant based on Kruskal-Wallis test.

Figure S6: Engraftment and Lineage Analysis in NSG Mice (Related to Fig 7)

A. Representative flow cytometry analysis to quantify the levels of human engraftment and lineage distribution in NSG mice. $hCD45^+$ cells were gated as $CD33^+$ (myeloid), $CD34^+$ (hematopoietic), or $CD33^-/34^-$, which were further subdivided into $CD19^+/CD3^-$ (B cells) and $CD19^-/CD3^+$ (T cells).

B. Levels of human engraftment in NSG mice (from Fig 7C), separated by recipient gender. N=3-4 female mice + 3-4 male mice. Individual points are plotted.

C-D. Lineage analysis of engrafted cells in BM (B) and spleen (C) of NSG mice. Analysis was performed by flow cytometry and analyzed using FlowJo software as illustrated in Fig S6A. N=6-7 (3-4 female mice + 3-4 male mice). Individual points are plotted. Line – geo mean ± geo SD. Zero values were not plotted on a log scale, but were used in calculations or SD. Differences are not significant, based on Kruskal-Wallis test.

Figure S7: Gene Editing in NSG mice (Related to Fig 7D)

A. Percentage of gene editing levels maintained after transplant, calculated as (HDR or NHEJ after transplant)/(HDR or NHEJ before transplant)x100. N= 6-7 (3-4 female mice + 3-4 male mice). Individual points are plotted. Line – geo mean ± geo SD. Differences are not significant if not specified, * $P<0.05$, based on Wilcoxon Rank-Sum test.

SUPPLEMENTAL METHODS

Primers

Outer PCR Fwd: 5'-ATGCTTAGAACCGAGGTAGAGTTT-3'
Outer PCR Rev: 5'-CCTGAGACTTCCACACTGATG-3'
Read1: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNGGCAGAGCCATCTATTGCTT-3'
Read2: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGTCTCCTTAAACCTGTCTTG-3'
P5: 5'-AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTT-3'
P7: 5'-
CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'

Flow cytometry

Analysis of HSC/MPP/Progenitor Populations

Marker	***Clone***	***Fluorochrome***
CD34	**581**	APC
CD38	**HIT2**	PE
CD45RA	**HI100**	PerCP-Cy5.5
CD90	**5E10**	PE-Cy7

Competitive Transplant

Marker	***Clone***	***Fluorochrome***
hCD45	**H130**	V450
mCD45	**30F11**	V500
CD34	**561**	APC
CD33	**WM53**	BV711
CD19	**HIB19**	PE Cy7
CD3	**SK7**	APC Cy7
mStraw	**-**	PE
mCitrine	**-**	FITC

Gene Editing Transplant

Marker	***Clone***	***Fluorochrome***
hCD45	**H130**	V450
mCD45	**30F11**	FITC
CD34	**561**	APC
CD33	**WM53**	PE
CD19	**HIB19**	PE Cy7
CD3	**SK7**	APC Cy7

CFU Assay

To evaluate whether cell synchronization has any effect on clonogenic potential, CFU assay was performed. PBSCs were treated with 30uM RO or DMSO. 20 hours after treatment, the cells were washed twice with PBS, and 25 and 100 viable cells were plated in duplicates into MethoCult Optimum methylcellulose-based media (Stem Cell Technologies; Vancouver, Canada) and cultured at 37°C, 5% carbon dioxide incubator. After 14 days, the colonies were characterized and quantified based on their morphology.

CtIP*

CtIP sequence was obtained from Addgene (Plasmid #71109) and mutated to include T847E change. Gene blocks were ordered from IDT to include homology arms for NEBuilder cloning, and cloned into a T7 plasmid backbone [16].

Competitive Mouse Transplant

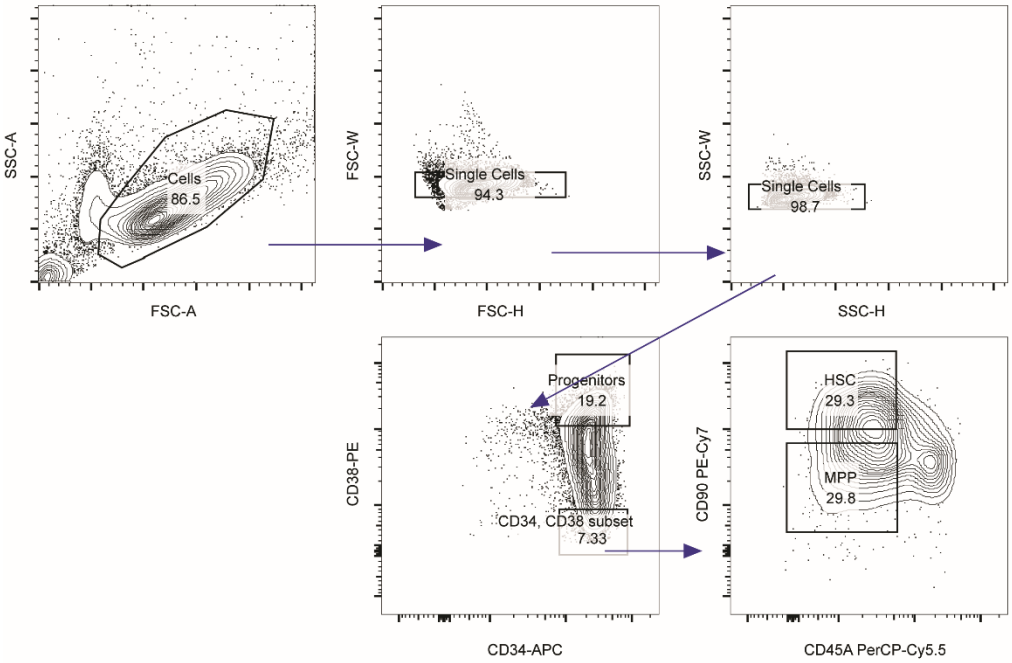
To evaluate whether RO treatment affects engraftment of HSPCs, PBSCs were thawed and prestimulated as described earlier. 24 hours after thaw, the cells were transduced with either UbC mStrawberry or UbC mCitrine lentiviral vectors at MOI 30. Lentiviral vectors were packaged as described previously [51]. 4 hours after transduction, each cell population was split into two fractions and treated with either 30uM RO or DMSO (volume equivalent). 20 hours after treatment, the cells were washed twice with PBS and counted. The cells with opposite colors and opposite treatments (RO^{mCit} + $DMSO^{mStraw}$, and RO^{mStraw} + $DMSO^{mCit}$) were mixed together in equal proportions based to the starting cell numbers. The cells were resuspended in PBS + 1% OKT3 (Biolegend; SanDiego, CA) at $1.8x10^6$ cells per 100ul. Cells were transplanted retro-orbitally into mice ~3-5 hours after 250-cGy total body irradiation. The number of mice to transplant was determined by the number of cells available at the time of injection.

Harvest and Analysis

Retro-orbital bleeding was performed at 8 weeks post-transplant to determine the levels of human engraftment. Reb blood cells were lysed using the using the lysing buffer (BD Biosciences, San Jose, CA). The levels of human engraftment were determined by flow cytometry (detailed flow panels in supplementary methods), and calculated as ($hCD45^+$/($hCD45^+$+$mCD45^+$))x100. Mice were euthanized 16 weeks post-transplant. Two femurs were collected and flushed with MACS buffer. Spleens were squeezed though a 70um filter. Red blood cells were lysed using the lysing buffer (BD Biosciences, San Jose, CA). The levels of human engraftment were determined by flow cytometry (detailed flow panels in supplementary methods), and calculated as ($hCD45^+$/($hCD45^+$+$mCD45^+$))x100. The percent contribution of RO- or DMSO-treated cells was determined using the respective mStrawberry or mCitrine cell population and divided by total number of $hCD45^+$ cells. Harvest and flow cytometry analysis of mice was performed in a blinded manner.

Figure S1: Analysis of HSPC sub-populations

A Representative Gating Strategy for Analyzing HSPC Sub-Populations



B Cell Cycle Analysis of Immunophenotypic HSPC Subpopulations

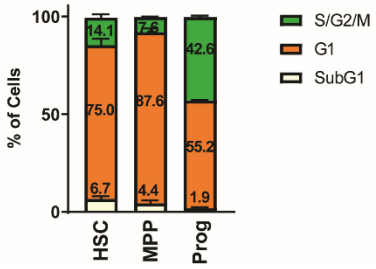


Figure S2: Assessment of hGemCas9 Nuclease Specificity

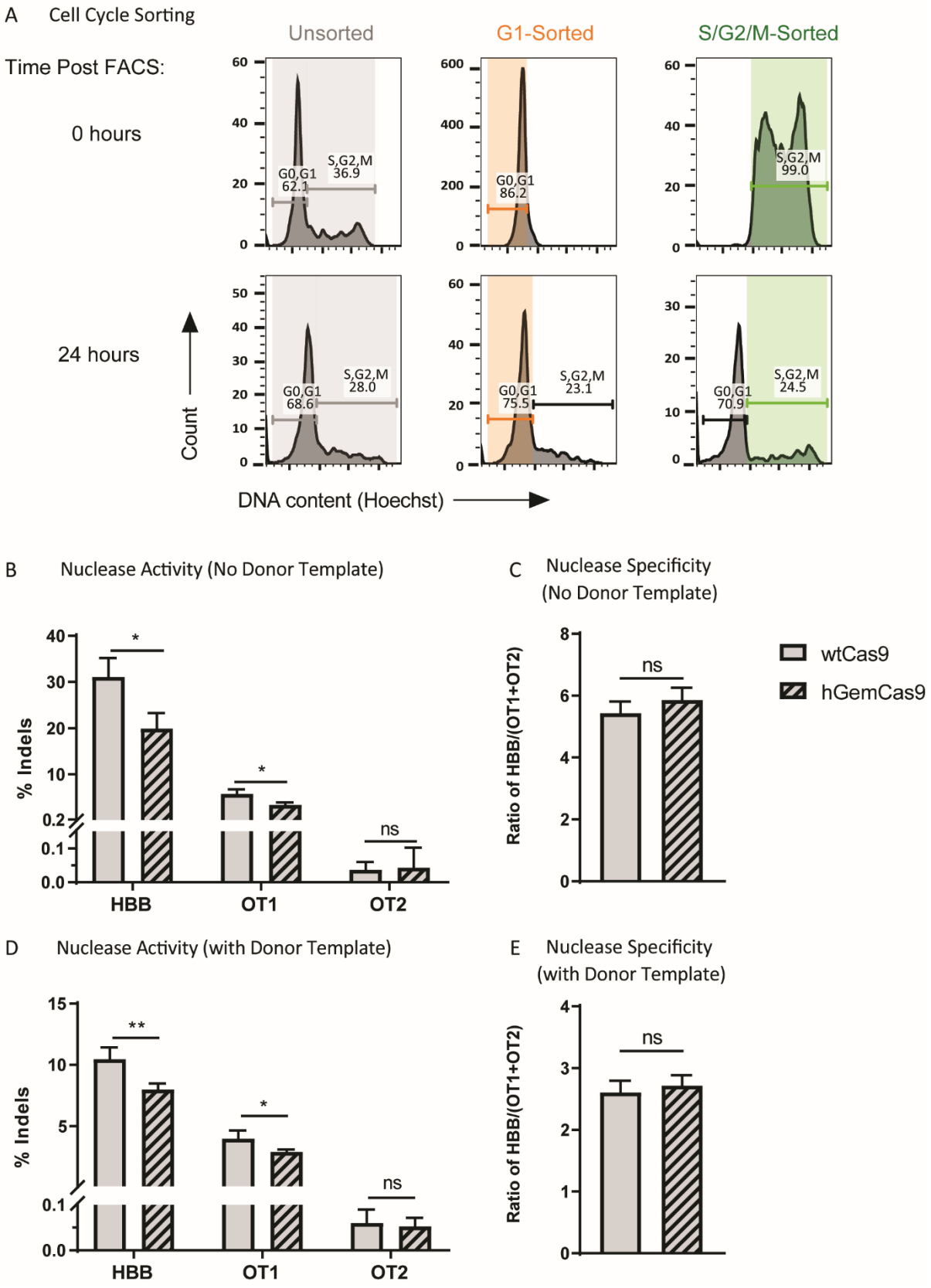


Figure S3: Synchronization of Human HSPCs with RO

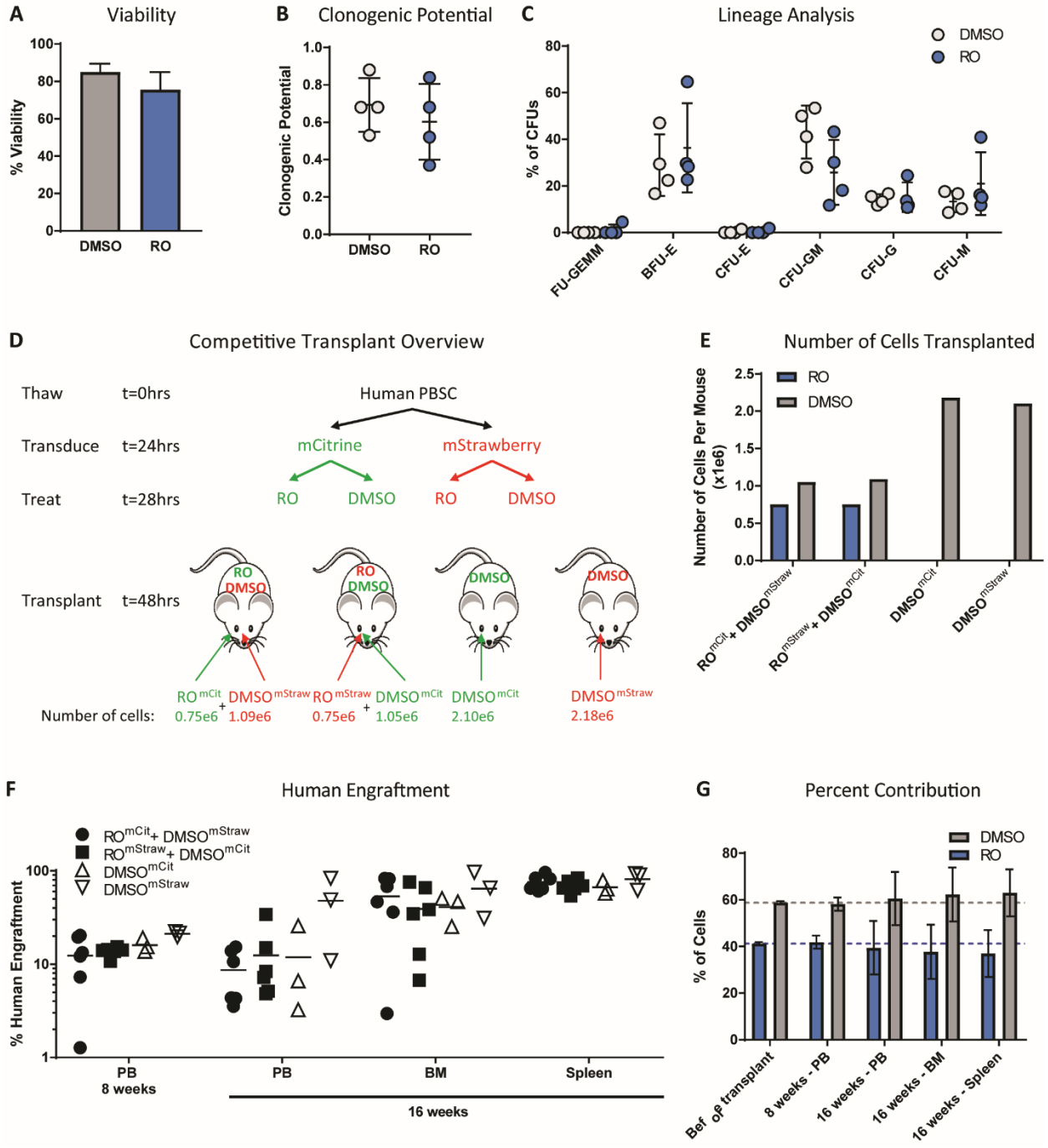


Figure S4: Indel Spectrum Analysis

A Summary of Highest Frequency Indels

--------PAM[-><----------] GAAGTCTGCCGTTACTGCCCTGTGGGGCAAG	indel size	Unsync, wtCas9	RO, wtCas9	Unsync, hGemCas9	RO, hGemCas9
GAAGTCTGCC---------CTGTGGGGCAAG	-9	27.4 ± 3.8	22 ± 8	28.9 ± 1.6	20.5 ± 6.3
GAAGTCTGCCGTaTACTGCCCTGTGGGGCAAG	+1	8.3 ± 0.8	9.4 ± 4.3	7.5 ± 1.3	8.5 ± 3
GAAGTCTGCCGTTA--GCCCTGTGGGGCAAG	-2	5 ± 0.9	5.7 ± 4.6	5.2 ± 0.8	5.8 ± 3.4
GAAGTCT--------TGCCCTGTGGGGCAAG	-8	4.1 ± 1.1	3.4 ± 1.7	4.3 ± 1	1.7 ± 1.4
GAAGTCTGCCGTTcACTGCCCTGTGGGGCAAG	+1	3.7 ± 0.7	4 ± 1.8	4 ± 0.9	5.1 ± 4.3
GAAGTCTGCCGTTA-TGCCCTGTGGGGCAAG	-1	3.3 ± 1	3.7 ± 2.5	3 ± 0.4	4 ± 3
GAAGTCTGCCGTTtACTGCCCTGTGGGGCAAG	+1	3.2 ± 0.7	4 ± 2.9	3 ± 0.6	2.8 ± 2.1
GAAGTCTGCCGTT-CTGCCCTGTGGGGCAAG	-1	2.6 ± 0.8	3.7 ± 3.3	2.3 ± 0.9	4.1 ± 4.4
GAAGTCTGCCGTTgACTGCCCTGTGGGGCAAG	+1	2.5 ± 0.7	2.5 ± 1.5	2.5 ± 0.6	4.4 ± 2.6
GAAGTCTGCCGTTA-----CTGTGGGGCAAG	-5	1.9 ± 0.3	2.2 ± 0.9	1.9 ± 0.6	1.4 ± 1.1
GAAGTCTGCCGtaTTACTGCCCTGTGGGGCAAG	+2	1.8 ± 0.3	2.7 ± 2.1	1.9 ± 0.4	1.6 ± 2.9
GAAGTCTGCCGTTA-------GTGGGGCAAG	-7	1.8 ± 0.6	2.4 ± 2.2	1.3 ± 0.2	1.2 ± 1.4
GAAGTCTGCCGTTctACTGCCCTGTGGGGCAAG	+2	1.2 ± 0.3	1.5 ± 0.7	1.3 ± 0.4	2.1 ± 1.5
GAAGTCTGCCGTTccACTGCCCTGTGGGGCAAG	+2	1 ± 0.3	1 ± 1.1	1.2 ± 0.5	1.5 ± 1.7
GAAGTCTGCCGTTA----------GCAAG	-12	0.9 ± 0.3	1.1 ± 0.9	0.8 ± 0.2	1.1 ± 1.2

B Microhomology Around the Cut Site

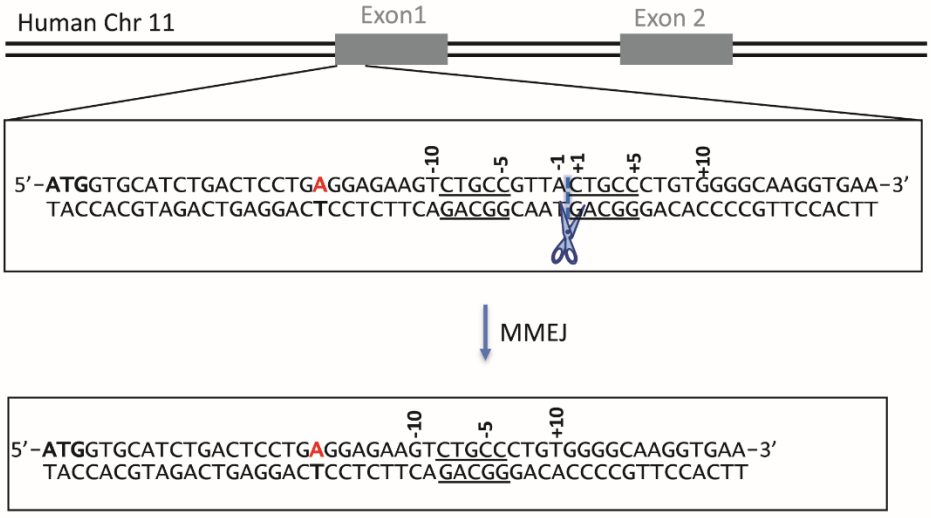
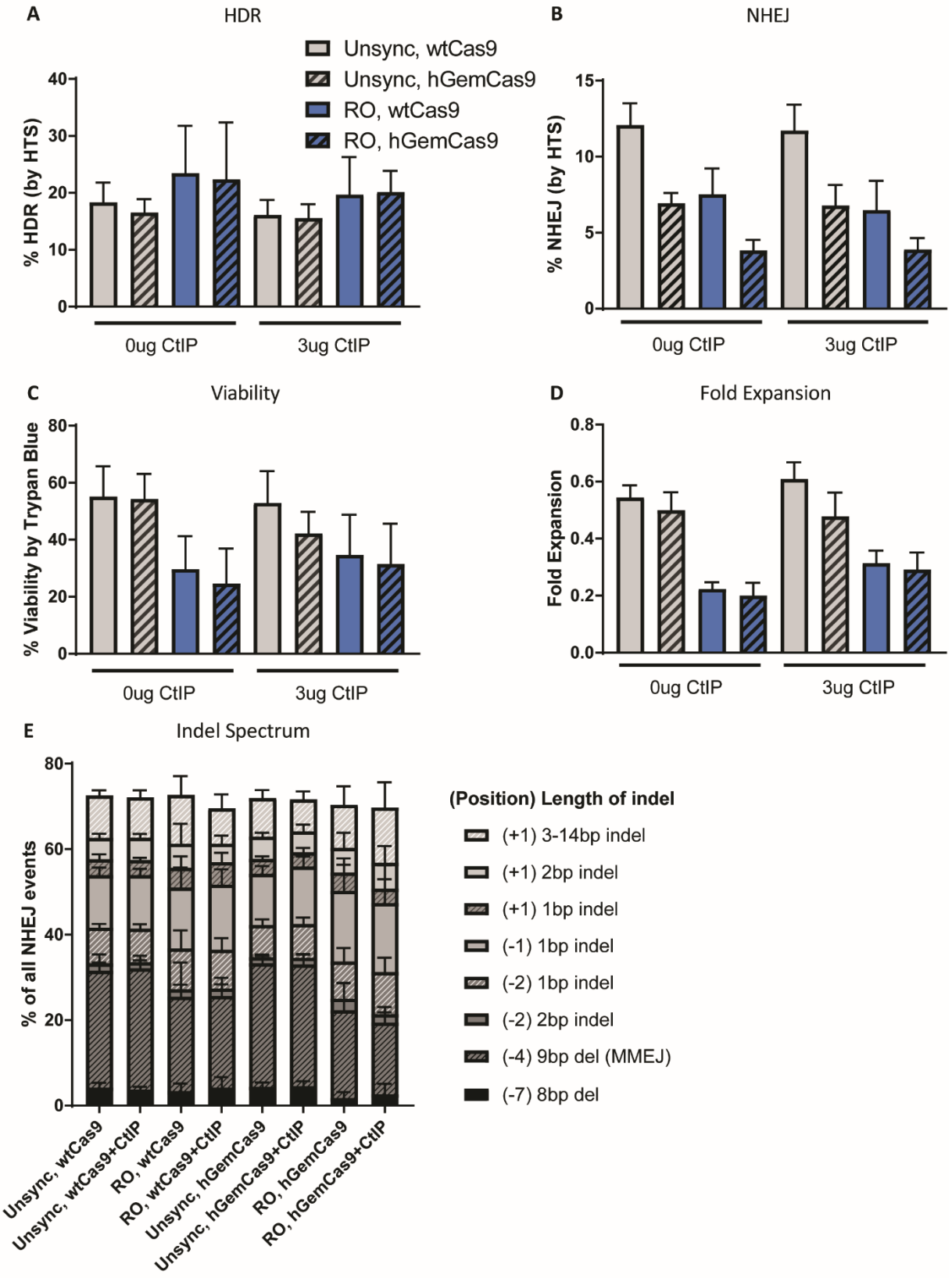


Figure S5: Evaluating the Effects of CtIP* mRNA on Gene Editing



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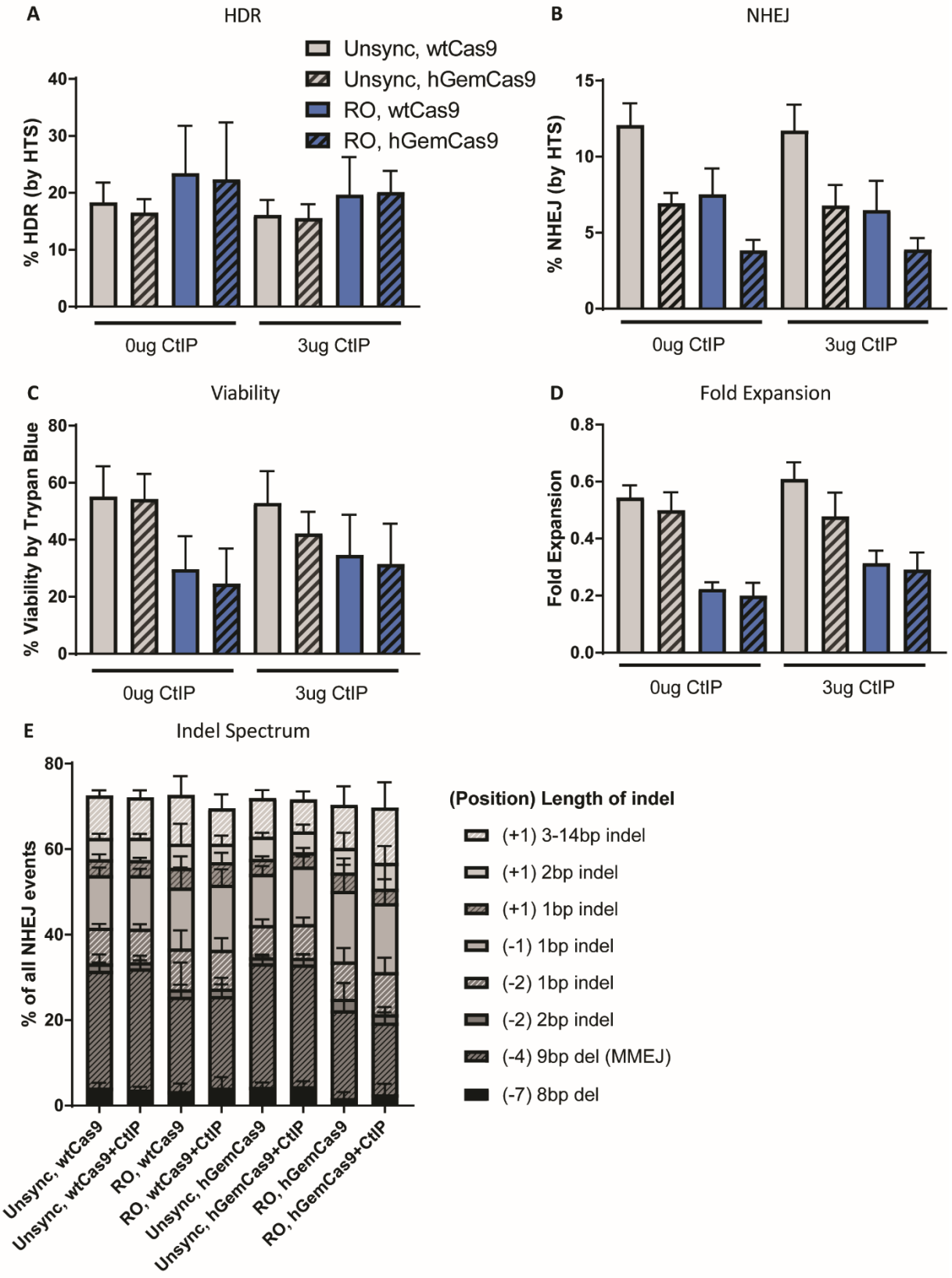

Figure S6: Engraftment and Lineage Analysis in NSG Mice

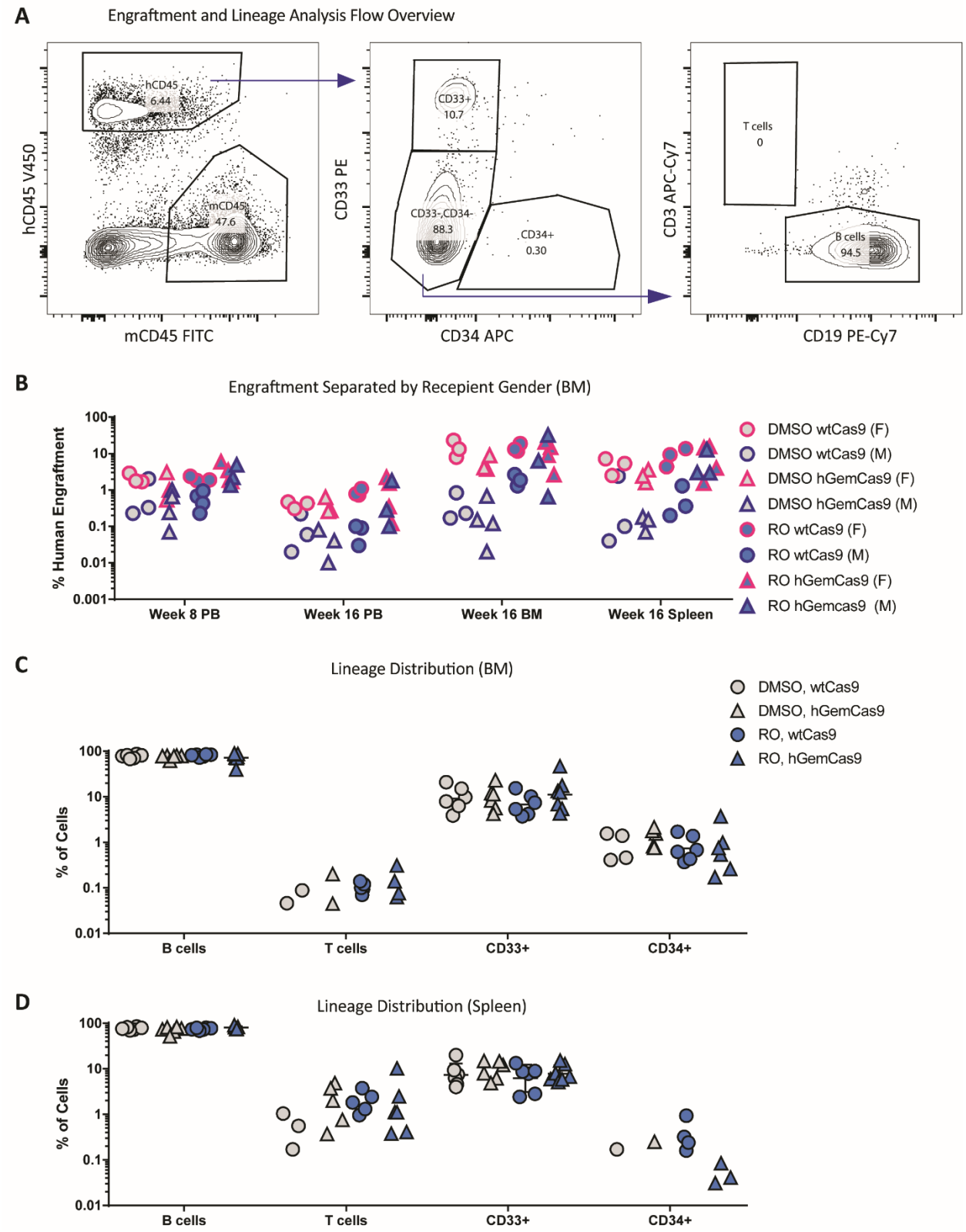

Figure S7: Gene Editing in NSG Mice