Supplemental Figures

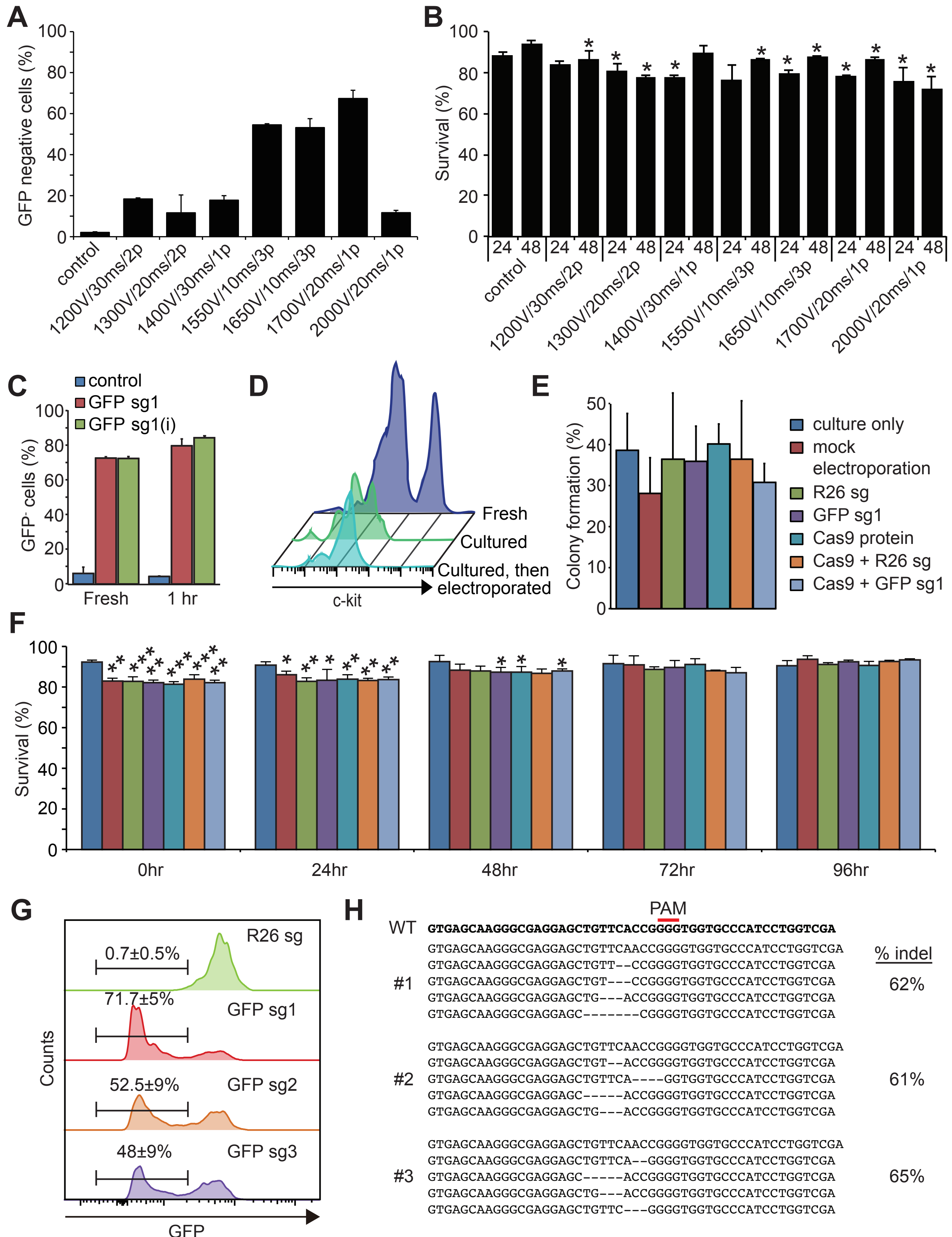


Figure S1. Gene editing in murine HSPCs (related to Figure 1). (**A-B**) Optimization of electroporation conditions. 10^5 c-kit$^+$ cells from Cas9-expressing mice were electroporated with 1 μg of GFP-sg1 using the parameters as indicated. Cells were analyzed for GFP expression at 24 hours after electroporation (**A**) and survival by trypan blue staining at 24 and 48 hours after electroporation (**B**). One pulse at 1700 V, 20 ms had the highest GFP-ablation efficiency with minimum loss of viability (see also Figure 1B), and this condition was used throughout for murine experiments. Comparisons in (**B**) were done with the control at each time points. (**C**) 10^5 c-kit$^+$ cells from Cas9-expressing mice, either freshly isolated or after 1 hour pre-culture, were electroporated with 1 μg of GFP-sg1 with the standard scaffold (GFP-sg) or with the improved scaffold described previously (GFP-sg(i), (Chen et al., 2013)). We did not observe significant increase in GFP ablation efficiency by incorporating the improved scaffold. (**D**) Bone marrow cells enriched for c-kit$^+$ cells were either analyzed freshly, or after 1 hour of pre-culture without electroporation, or after 1 hour of pre-culture with electroporation. 1 hour of pre-culture significantly attenuated c-kit expression. (**E**) We pre-cultured c-kit$^+$ HSPCs for 1 hour and then performed either mock electroporation without sgRNA, electroporated with R26-sg or GFP-sg, or electroporated with Cas9-sgRNA RNP as indicated. Cell were then stained for HSCs and clonally sorted into semi-solid media. HSC pre-cultured and sorted without electroporation had somewhat lower cloning efficiency, but the clonality was not affected by electroporation of sgRNA with or without Cas9 protein. (**F**) Cell survival determined by trypan blue staining at the indicated time points. GFP-expressing HSPCs were either pre-cultured only or electroporated with the sgRNA, Cas9 protein, or Cas9-sgRNA RNP, as indicated in the legend in (**E**). (**G**) Three different sgRNA targeting GFP were electroporated into c-kit$^+$ cells with Cas9 protein. GFP expression was efficiently ablated GFP expression by GFP-sg1, making 71.7±0.5% of cells GFP$^-$, while GFP-sg2 and GFP-sg3 had slightly lower efficiencies. (**H**) High-throughput sequencing of GFP after editing with Cas9-sgRNA RNP. Shown are top 5 GFP-edited sequences discovered by sequencing of 3 samples, and the overall indel frequencies. All data represent mean ± standard deviation; *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$ by Student's t-test.

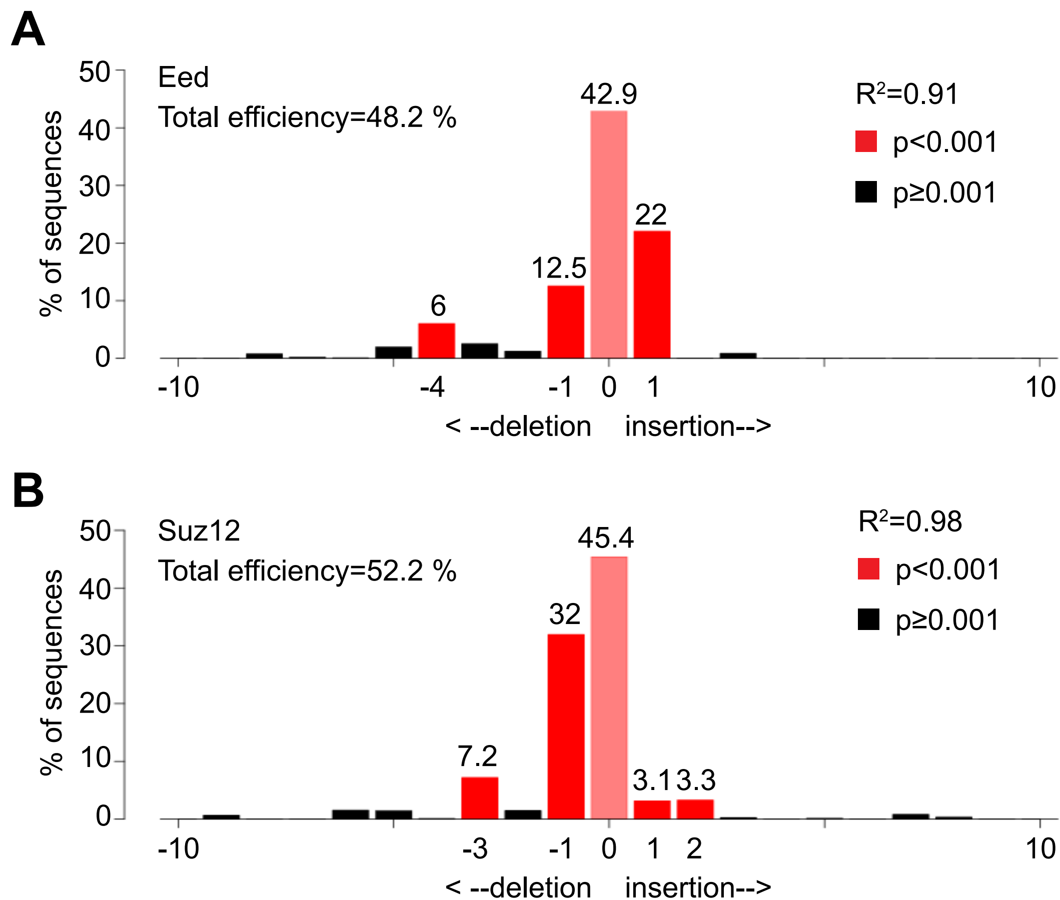


Figure S2. Editing of murine Eed and Suz12 (related to Figure 2).

TIDE assay (Brinkman et al., 2014) was performed 48 hours after electroporation for Eed (**A**) and Suz12 (**B**) (n=3). PCR amplicons spanning the Cas9 cleavage site were subjected to Sanger sequencing and the resulting trace files were analyzed by TIDE. Approximately 50% of the cells at this early time point after electroporation exhibited small indels (n=3).

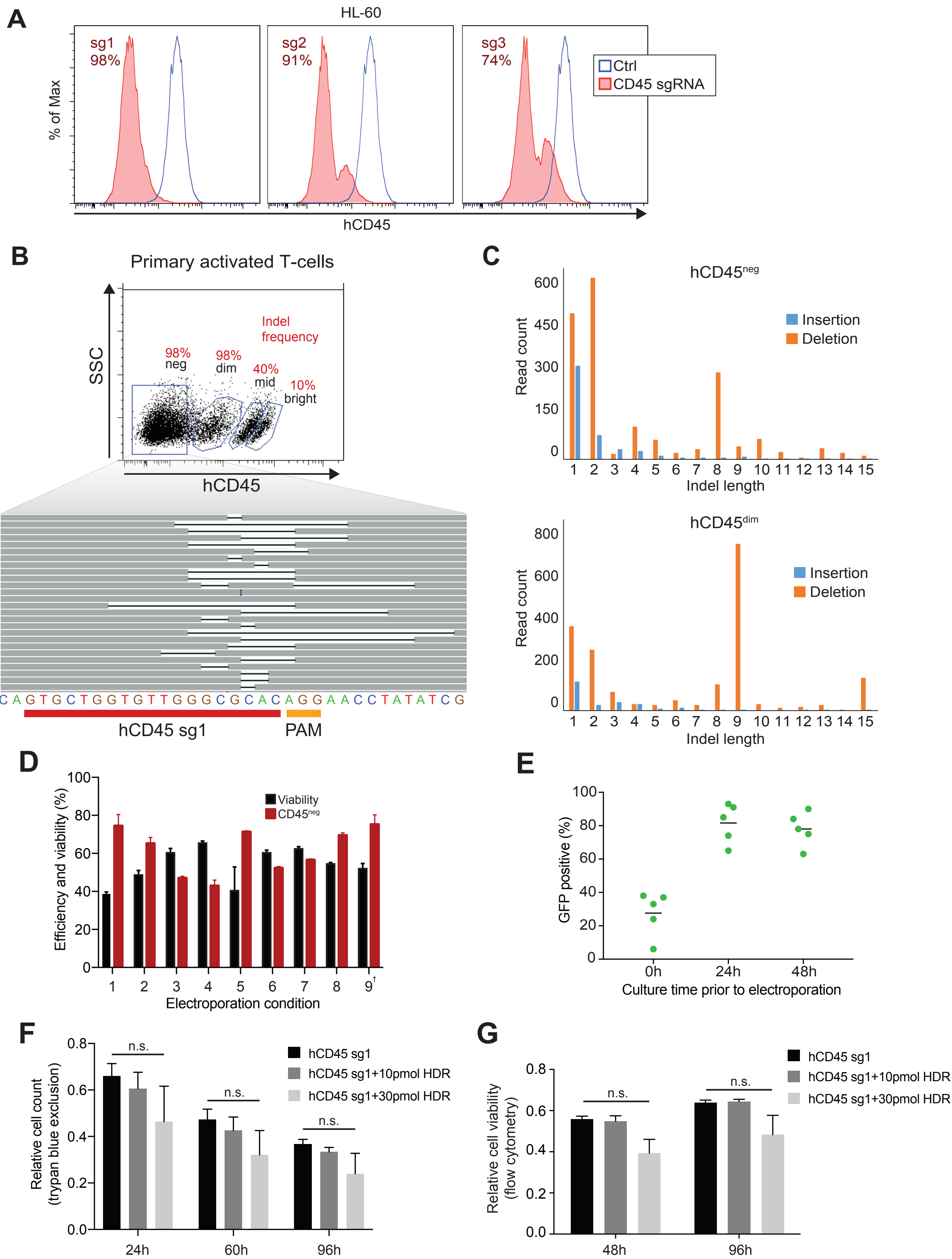
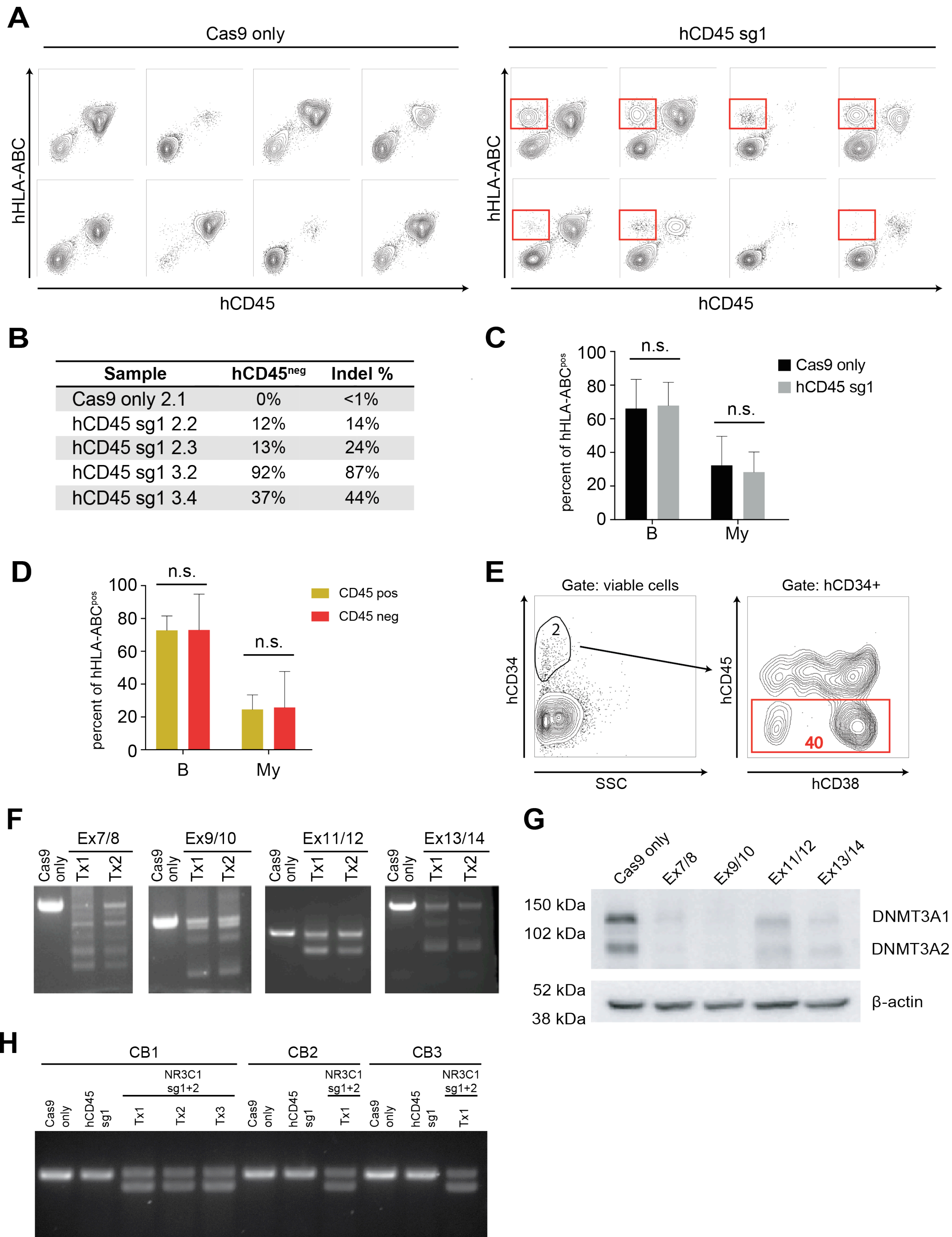

A
HL-60
sg1
98%
sg2
91%
sg3
74%
Ctrl
CD45 sgRNA
% of Max
hCD45
B
Primary activated T-cells
Indel frequency
98%
neg
98%
dim
40%
mid
10%
bright
SSC
hCD45
CAGTGCTGGTGTTGGGCGCACAGGAACCTATATCG
hCD45 sg1
PAM
C
hCD45neg
Read count
Insertion
Deletion
Indel length
hCD45dim
D
Viability
CD45neg
Efficiency and viability (%)
Electroporation condition
E
GFP positive (%)
0h
24h
48h
Culture time prior to electroporation
F
Relative cell count (trypan blue exclusion)
n.s.
hCD45 sg1
hCD45 sg1+10pmol HDR
hCD45 sg1+30pmol HDR
24h
60h
96h
G
Relative cell viability (flow cytometry)
48h
96h

Figure S3. Gene editing in human hematopoietic cells (related to Figure 3).

(**A**) Flow cytometry analysis of hCD45 expression in the HL-60 cell line 96hrs following electroporation with Cas9 only (blue) or Cas9 and sgRNAs targeting different CD45 exons (red). (**B**) Dot-plot showing four distinct populations of T-lymphocytes based on hCD45 expression levels (above). Sequencing of the targeted CD45 locus in the sorted $CD45^{neg}$ population displayed in an IGV diagram (below). Each horizontal grey bar represents a sequencing read (allele). Deletions are depicted by thin horizontal black lines and insertions are depicted by vertical lines. (**C**) Indel spectra within $CD45^{neg}$ (top panel) and $CD45^{dim}$ (bottom panel) sorted T-lymphocyte populations. (**D**) Optimization of electroporation parameters in human HSPCs: $CD34^{+}$ cells were electroporated with Cas9/hCD45-sg1 RNP using different electroporation parameters. CD45 expression along with viability was measured 96 hours following electroporation. 9 different conditions were tested: 1) 1700V, 20ms, 1pulse; 2) 1400V, 30ms, 1pulse; 3) 1200V, 40ms, 1pulse; 4) 1200V, 20ms, 2pulses; 5) 1400V, 20ms, 2pulses; 6) 1150V, 30ms, 2pulses; 7) 1400V, 10ms, 3pulses; 8) 1500V, 10ms, 3pulses; 9) 1600V, 10ms, 3pulses. Condition 9 (highlighted with †) was used for all further experiments. (**E**) GFP positivity following electroporation of GFP-NPM1 construct in $CD34^{+}$ cells. Cells were cultured for 0, 24 and 48 hours in presence of cytokines (SCF, FLT3L and TPO) and electroporated with GFP-NPM1 mRNA. GFP positivity was measured 24 hours post-electroporation. (**F**) Total viable cell counts examined by trypan blue staining (n=3) of CD34+ cells electroporated either with Cas9/hCD45-sg1 RNP (black), RNP + 10pmol ssODN (dark grey) or RNP + 30pmol ssODN (light grey), relative to non-electroporated cells (n=8). (**G**) Cell viability measured by flow cytometry (n=3) 48 and 96 hours following electroporation with either Cas9/hCD45-sg1 RNP (black), RNP + 10pmol ssODN (dark grey) or RNP + 30pmol ssODN (light grey). The percentages of viable cells (based on scatter plot and PI staining) of Cas9/hCD45-sg1 RNP and RNP + ssODN transfected cells were normalized to the percentage of viable cells of non-electroporated cells. The differences were not statistically significant based on a non-parametric test.



Sample	hCD45^{neg}	Indel %
Cas9 only 2.1	0%	<1%
hCD45 sg1 2.2	12%	14%
hCD45 sg1 2.3	13%	24%
hCD45 sg1 3.2	92%	87%
hCD45 sg1 3.4	37%	44%

Figure S4. CRISPR-Cas9 toolbox applications in human primary $CD34^+$ cells (related to Figure 4).

(**A**) Flow cytometry analysis of bone marrows and spleens from NSG mice transplanted with either Cas9 only (left panels) or Cas9/hCD45-sg1 RNP (right panels) treated $CD34^+$ cells. hCD45 negative cells (gated in red) were detected only in mice transplanted with Cas9/hCD45-sg1 RNP electroporated human HSPCs. (**B**) Table showing the percentages of hCD45 negative cells paired with the indel frequencies detected at the targeted locus in engrafted human cells in one Cas9 only control (corresponding to the top left plot of Cas9 only controls on Fig S4A) and four Cas9/hCD45-sg1 RNP electroporated (corresponding to the top four plots of hCD45-sg1 samples on Fig S4A) xenografts. As expected, indel frequencies calculated by high-throughput sequencing are slightly higher than $CD45^{neg}$ cells detected by flow cytometry because populations with two-fold differences in cell surface expression (WT vs heterozygous) are difficult to discriminate by flow cytometry and some of the indels may produce undetectable outcomes at protein level. (**C**) B-cell and myeloid percentages of human cells in Cas9 only (n=8) and in Cas9/hCD45-sg1 RNP (n=8) engrafted mice analyzed two months after transplantation. (**D**) B-cell and myeloid percentages of engrafted human CD45 positive and CD45 negative cells in five mice transplanted with Cas9/hCD45-sg1 CD34+ cells and analyzed two months after transplantation. No differences were detected in terms of lineage differentiation, comparing CD45 positive and CD45 negative human cells. **(E)** Flow cytometry analysis showing engrafted human $CD34^+$ cells in one representative NSG mouse bone marrow two months after transplantation (left panel). 40% of $CD34^+$ cells were hCD45 negative (right panel). hCD45 negative cells were detectable in both $CD34^+CD38^+$ and $CD34^+CD38^-$ populations. **(F)** Agarose gel electrophoresis images of amplified DNA from DNMT3A exons 7-14 in HL-60 cells treated with multiple guides targeting each exon. The multiple bands in each lane represent deletions between two guides. **(G)** Western blot analysis of DNMT3A expression in HL-60 cells 96hrs after electroporation with Cas9 only, Cas9/*DNMT3A* exon 7/8-sg (4 sgRNAs) RNP, Cas9/*DNMT3A* exon 9/10-sg (4 sgRNAs) RNP, Cas9/*DNMT3A* exon 11/12-sg (2 sgRNAs) RNP or Cas9/*DNMT3A* exon 13/14-sg (4 sgRNAs) RNP. **(H)** Agarose gel electrophoresis of amplified DNA from NRC31 exon 3 in human $CD34^+$ cells in Cas9 only controls, Cas9/hCD45-sg1 controls and Cas9/NR3C1 sg1 + sg2 replicates (Tx). CB1, CB2 and CB3 indicate cord blood cells from three different donors (biological replicates). Tx1, Tx2 and Tx3 indicate three technical replicates.

Table S1. Primer and oligo sequences (Related to Figures 1, 2, 3 and 4)

Oligo name	Sequence	Description
sgRNA common reverse primer (overlap PCR)	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	sgRNA production
sgRNA common reverse primer (regular PCR)	AGCACCGACTCGGTGCCACT	sgRNA production
R26 sg	gaaattaatacgactcactataGGGGTCGGCCTCTGGCGGGGgttttagagctagaaatagc	sgRNA
GFP sg1	gaaattaatacgactcactataGGGCGAGGAGCTGTTCACCGgttttagagctagaaatagc	sgRNA
GFP sg1(i)	ttaatacgactcactataGGGCGAGGAGCTGTTCACCGgtttaagagctatgctggaaacagc	sgRNA
GFP sg2(i)	ttaatacgactcactataGGAGGAGCTGTTCACCGGGGgtttaagagctatgctggaaacagc	sgRNA
GFP sg3(i)	ttaatacgactcactataGGCGAGGGCGATGCCACCTAgtttaagagctatgctggaaacagc	sgRNA
mCD45 sg1	ttaatacgactcactataGGAGAGGGCGTCTGCGAGTCgtttaagagctatgctggaaacagc	sgRNA
mCD45 sg2	ttaatacgactcactataGGGAGTCAGGCTGTGGGGACgtttaagagctatgctggaaacagc	sgRNA
mCD45 sg3	ttaatacgactcactataGGTGCTGGTGTTGGGCGTACgtttaagagctatgctggaaacagc	sgRNA
Eed sg	gaaattaatacgactcactataGATGCTTGCATTGGGCAATCgttttagagctagaaatagc	sgRNA
Suz12 sg	gaaattaatacgactcactataGATTGAAGATGAACCTGCTGgttttagagctagaaatagc	sgRNA
hCD45 sg1	ttaatacgactcactataGGTGCTGGTGTTGGGCGCACgttttagagctagaaatagc	sgRNA
hCD45 sg2	ttaatacgactcactataGGGAGCAAGTGAGGATCCTCgttttagagctagaaatagc	sgRNA
hCD45 sg3	ttaatacgactcactataGGGATGCTTGTTCCCTTCAGgttttagagctagaaatagc	sgRNA
DNMT3A ex7 sg1	ttaatacgactcactataGGGGCCCGGGGAGTCTCAGAgttttagagctagaaatagc	sgRNA
DNMT3A ex7 sg2	ttaatacgactcactataGGCCGTGGGGTCCGATGCTGgttttagagctagaaatagc	sgRNA
DNMT3A ex8 sg1	ttaatacgactcactataGGGCCGGGGCTTTGGCATTGgttttagagctagaaatagc	sgRNA
DNMT3A ex8 sg2	ttaatacgactcactataGGGGTCATGTGGTTCGGAGAgttttagagctagaaatagc	sgRNA
DNMT3A ex9 sg1	ttaatacgactcactataGGACTGCAAAACGAGCTCAGgttttagagctagaaatagc	sgRNA
DNMT3A ex9 sg2	ttaatacgactcactataGGTTGTTGTACGTGGCCTGGgttttagagctagaaatagc	sgRNA
DNMT3A ex10 sg1	ttaatacgactcactataGGACACTGCCAAGGCCGTGGgttttagagctagaaatagc	sgRNA
DNMT3A ex10 sg2	ttaatacgactcactataGGGTGGCCAGCAGCCGCGCGgttttagagctagaaatagc	sgRNA
DNMT3A ex11 sg	ttaatacgactcactataGGTGCGTAGGCAGCTGCCTCgttttagagctagaaatagc	sgRNA
DNMT3A ex12 sg	ttaatacgactcactataGGCAGAGCGGCTGGTGTACGgttttagagctagaaatagc	sgRNA
DNMT3A ex13 sg1	ttaatacgactcactataGGGCTCCTACCTTGCAGTTTgttttagagctagaaatagc	sgRNA
DNMT3A ex13 sg2	ttaatacgactcactataGGTAACATTGAGGCTCCCACgttttagagctagaaatagc	sgRNA
DNMT3A ex14 sg1	ttaatacgactcactataGGGTACCAGTACGACGACGAgttttagagctagaaatagc	sgRNA
DNMT3A ex14 sg2	ttaatacgactcactataGGCCGTGAGGTGCTCATGTGgttttagagctagaaatagc	sgRNA
NR3C1 ex3 sg1	ttaatacgactcactataGGTGCTGTTGAGGAGCTGGAgttttagagctagaaatagc	sgRNA
NR3C1 ex3 sg2	ttaatacgactcactataGGAGCACACCAGGCAGAGTTgttttagagctagaaatagc	sgRNA
Cas9-GFP F	GGGGATGTCGAAGAGAATCC	T7E1/Seq
Ubc-GFP F	GGGTTGGCGAGTGTGTTTT	T7E1/Seq

GFP R	GAACTTCAGGGTCAGCTTGC	T7E1/Seq
Eed F	CCTTAGAGCCTGGTGCTCTGT	T7E1/Seq
Eed R	GGATGGCATAAGAAAGCTACAA	T7E1/Seq
Suz12 F	GTGCACTCTGAACTGCCGTA	T7E1/Seq
Suz12 R	CCACTGTAACCTGGGCAGA	T7E1/Seq
mCD45 1F	CTGTGGCACCTTTGTGTCAT	primer
mCD45 1R	TTGGCTGCTGAATGTCTGAG	primer
mCD45 2F	GGTTCCTTCACCCACTGAGA	primer
mCD45 2R	GACACCTCTGTCGCCTTAGC	primer
EGFP F	TTGAAACAAGCAGGGGATGT	primer
EGFP R	GAACTTCAGGGTCAGCTTGC	primer
hCD45 ex24-25 F	AGTACATGCAAGTCCTGCACAATATC	primer
hCD45 ex24-25 R	TTTGTGGTCATCTTTCTTCCACAGT	primer
hCD45 ex25 F	CACACCTGACAGCTTTTCCATGA	primer
hCD45 ex25 R	CATCCACTTTGTTCTCGGCTTCC	primer
DNMT3A ex7 F	TTTCACGGCAAGGCAGCTGGTTG	primer
DNMT3A ex7 R	AGAGGAGAGCAGGACGGGAGGAG	primer
DNMT3A ex8 F	GATCAGGGTGGCAGGGCCTCGT	primer
DNMT3A ex8 R	CACCACAGGCAGAGTAGGGGTGA	primer
DNMT3A ex9 F	GCCAGTTGCAAGGCATGGGGTG	primer
DNMT3A ex10 R	TTGCCTGTGCCACCCTCACTACTC	primer
DNMT3A ex11 F	TACTCTGCCCCATGCCACACTA	primer
DNMT3A ex12 R	ACCCCACTGTAAGGAGGGTGGG	primer
DNMT3A ex13 F	GGAGAGGCCCTTCGGTGGTACT	primer
DNMT3A ex14 R	GAGGCCAAGGTGTGCTACCTGGA	primer
NR3C1 ex3 F	GCCCCAGCATGAGACCAGAT	primer
NR3C1 ex3 R	CACACACTACCTTCCACTGCTCT	primer
hCD45 symmetric HDR template	TCGCCTTAGCTTGACAACATAACCATAAACATCCACTTTGTTCTCGGCTTCCAGGCCTTCTAGCATGGCATCAATTCCGATATAGGTTGCCGTACGACCAACACCAGCACTGGCAAACCAAGCAAAAAAAAGCAGAGGCATGATCATGGAAAAGCTGTCAGGTGTGAAGCCAATATAAGAATCTAAG	ssODN
hCD45 asymmetric HDR template	TCGCCTTAGCTTGACAACATAACCATAAACATCCACTTTGTTCTCGGCTTCCAGGCCTTCTAGCATGGCATCAATTCCGATATAGGTTGCCGTACGACCAACACCAGCACTGGCAAACCAAGCAAAAAAAAGCAG	ssODN

Table S2. Most commonly observed indels and large deletions in mouse and human HSPCs (Related to Figures 1, 3 and 4)

Supplementary Experimental Procedures

Mice

The mice used in this study were C57BL/6, Cas9 knock-in (Platt et al., 2014) and Vav-iCre (de Boer et al., 2003) mice. NOD/SCID/IL2Rγ-/- (NSG) mice (The Jackson Laboratory) were fed autoclaved food and water. Mice were housed in AAALAC-accredited, specific-pathogen-free animal care facilities at Baylor College of Medicine (BCM). All procedures were approved by BCM Institutional Animal Care and Use Committees.

Production of CRISPR sgRNA

Protospacer sequences for each target gene were identified using the CRISPRscan algorithm (www.crisprscan.org) (Moreno-Mateos et al., 2015). DNA templates for sgRNAs were made using the protocol described by Li et al (Li et al., 2013). In brief, primers containing a T7 promoter, the protospacer sequence, and a 21-nt sequence corresponding to the 5′ end of the sgRNA scaffold sequence were obtained from Integrated DNA Technologies (IDT). Full-length DNA templates were then produced by amplifying the sgRNA scaffold off of the PX458 plasmid (Addgene #48138) using each custom forward primer and a universal reverse primer (see primers list) specific for the 3' end of the sgRNA scaffold. The PCR products were purified and in vitro transcribed with the HiScribe T7 High Yield RNA Synthesis Kit (NEB) following manufacturer instructions. In vitro transcription products were purified using RNA Clean & Concentrator-25 (Zymo Research) and eluted in nuclease-free water. To generate sgRNA containing the improved scaffold sequences, a forward primer containing a T7 promoter, a reverse primer specific for the 3' end of the improved scaffold, and pKLV-U6gRNA-EF(BbsI)-PGKpuro2ABFP plasmid (Addgene # 62348) were used in a PCR reaction.

Murine cell isolation, culture and transfection

Murine bone marrow cells were isolated by flushing tibias and femurs in HBSS supplemented with 2% heat-inactivated bovine serum (Gibco, Grand Island, NY). To isolate c-kit$^+$ cells, bone marrow cells were stained with biotin conjugated c-kit antibodies followed by anti-biotin microbeads (Miltenyl Biotec) and separated using autoMACS (Miltenyl Biotec). Cells were cultured in either

X-Vivo 15 media (Lonza) supplemented with 2% FBS, 50 ng/ml SCF, 50 ng/ml TPO, 10 ng/ml IL-3, and 10 ng/ml IL-6, or StemPro-34 Serum Free media (Thermo Fisher Scientific) supplemented with L-Glutamine (2 mM), SCF (10 ng/mL), and TPO (100 ng/mL) with similar results. Cytokines were purchased from Peprotech or Miltenyi Biotec.

For experiments using Cas9-expressing mice, cells were either electroporated directly or after culturing for the indicated time. 1×10^5 cells were resuspended into 10 µl of Buffer T (Invitrogen), mixed with sgRNA and electroporated using the Neon transfection system (Thermo Fisher Scientific). For experiments using sgRNAs pre-complexed with Cas9 protein, 200 ng to 1 µg of sgRNA was incubated with 1 µg Cas9 protein (PNA Bio, 1 µg/µL in Buffer T or PBS) for 10-15 minutes at room temperature in a final volume of 2 µl. 1×10^5 cells cultured for 1-3 hours were resuspended in 10 µl of Buffer T and added to the Cas9-sgRNA RNP for a total volume of 12 µl. After optimizing the electroporation conditions as shown in Figure S1A, we used the optimal condition, which is 1700V, 20ms, 1 pulse for all other murine experiments. Electroporated cells were either directly used to sort HSCs, plated on Methocult M3434 (Stem Cell Technologies), or incubated in StemPro-34 with cytokines for 16-20 hours before plating in Methocult M3434 media.

Murine HSPC culture

To examine the serial replating capacity of HSPCs after gene editing, 5,000 cells were plated into Methocult M3434 (StemCell Technologies) after electroporation. Colony counting and replating were performed every 7 days. Media with colonies were resuspended in PBS and cells were counted to replate 5,000 cells into fresh media. Single colonies were picked and sequenced by Sanger sequencing.

To examine the clonality of murine HSPCs after electroporation, HSPCs were precultured for 1 hour and then either left without manipulation or electroporated with the components shown in Figure 2D. Cells were then stained with antibodies to identify HSCs (see "Flow cytometry" below) and sorted. Clonality was calculated based on the numbers of colonies formed 10-12 days after plating and the numbers of HSCs sorted. To examine GFP disruption in HSCs, cells were electroporated and stained as above, and single HSCs were sorted individually into a well of a 96-well plate. Colonies were individually resuspended in PBS 10-12 days later and analyzed by flow cytometry to examine GFP expression.

T7E1 and TIDE assays

PCR amplicon spanning the Cas9-sgRNA cleavage site was diluted 1:4 in 1x Buffer 2 (NEB) and hybridized slowly in a thermal cycler. Hybridized fragments were then digested with 1.25 U of T7 endonuclease I (NEB) for 10 minutes at 37°C. Digested fragments were separated by polyacrylamide gel electrophoresis. Band intensities were analyzed using imageJ software by plotting band intensities of each lane. % cleavage was calculated by the ratio of the intensities of the cleaved bands to uncleaved bands. TIDE assays were performed as described previously (Brinkman et al., 2014).

Human cell culture, and transfection

Human acute myeloid leukemia (AML) cell lines (HL60, Kasumi and OCI-AML2) were obtained from ATCC and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2% L-glutamine (cRPMI) (Thermo Fisher Scientific). Human cord blood and peripheral blood mononuclear cells (PBMCs) were processed by Ficoll gradient (Lymphoprep, StemCell Technologies). Human primary T- cells were selected and activated by culturing PBMCs from individual donors on anti-CD3/CD28 coated plates in RPMI/Click's 1:1 Medium supplemented with 10 ng/ml IL-7 and 5 ng/ml IL-15 for four days prior to electroporation. $CD34^+$ cord blood cells from individual donors were enriched from mononuclear cells using CD34 microbeads and Auto-MACS Pro (Miltenyi Biotec) following manufacturer's instructions. Purity of sorted human cord blood $CD34^+$ cells was assessed in each sample immediately after sorting. Sorted $CD34^+$ cells were cultured in StemSpan SFEM II (Stem Cell Technologies) supplemented with 100ng/ml FLT3L, 100ng/ml thrombopoietin and 100ng/ml stem cell factor (Peprotech) for a maximum of 48 hours prior to electroporation.

Incubation of Cas9 with sgRNAs was performed as described for mouse cells, except AML cell lines were electroporated in Buffer R (Invitrogen). In each replicate 150,000-250,000 cells were electroporated. Experiments with multiple guides in the same reaction had a total of 1 µg sgRNA (e.g. 500 ng of each sgRNAs when 2 guides were tested). Electroporation was performed using the Neon Transfection System (Thermo Fisher Scientific). Electroporation conditions used for AML cell lines were based on manufacturer instructions (1350V, 35ms, 1pulse). T cells were electroporated as previously described by Schumann et al (Schumann et al., 2015) (i.e. 1600V, 10ms, 3 pulses). For the human HSPCs electroporation optimization, cord blood derived CD34+ cells were expanded for 96 hours prior to electroporation. Following conditions were tested: 1) 1700V, 20ms, 1pulse; 2) 1400V, 30ms, 1pulse; 3)

1200V, 40ms, 1pulse; 4) 1200V, 20ms, 2pulses; 5) 1400V, 20ms, 2pulses; 6) 1150V, 30ms, 2pulses; 7) 1400V, 10ms, 3pulses, 8) 1500V, 10ms, 3pulses; 9) 1600V, 10ms, 3pulses. Condition 9 was used for all further experiments.

GFP-NPM1 construct was kindly provided by B. Falini (University of Perugia, Italy). GFP-NPM1 coding sequence was cloned adjacent to the T7 promoter contained in the pcDNA 3.1 plasmid (Invitrogen). GFP-NPM1 mRNA was in vitro transcribed using the T7 ARCA mRNA kit (NEB) and 1.2 µg mRNA were transfected in CD34$^+$ cells at different culture times (0, 24, and 48 hours of culture) using the previously described optimized conditions.

For knock-in experiments, ssODN templates oriented in an anti-sense direction relative to the target locus were ordered from IDT. The symmetric ssODN template (S) contained 90bp homology arms distal and proximal to the PAM sequence. The asymmetric ssODN template (A) contained a 36bp homology arm on the PAM distal side and a 90bp homology arm on the PAM proximal side (Richardson et al., 2016). Either 10 pmol or 30 pmol of ssODN templates (1 µl volume) were added to the Cas9-sgRNA RNP mixture (11.5 µl volume) immediately prior to electroporation.

In vivo transplantation of CD34$^+$ cord blood cells

Adult (6-8 weeks of age) NSG mice were conditioned with sub-lethal (2.5 Gy) total-body irradiation. The conditioned recipients were transplanted with 150,000-250,000 CD34$^+$ cord blood cells. Cells were isolated from individual cord blood donors, expanded for 48 hours and split into one Cas9 only and one Cas9/hCD45 sgRNA1 RNP electroporated aliquot. Each aliquot was injected into recipients 6 hours post electroporation for a total time of culture of 54 hours. At 2 months post-transplantation, the mice were euthanized and bone marrow and spleen were collected for identification of hematopoietic cell chimerism by flow cytometry. To confirm the engraftment of knock-out cells at genomic level, human CD45 exon 25 was amplified by PCR from harvested bone marrow cells and sequenced for analysis of indels at the targeted CD45 locus.

Flow cytometry

Murine GFP expression levels were measured 24 to 48 hours after electroporation. To sort murine HSCs after electroporation of c-kit$^+$ HSPCs, we stained electroporated cells with CD150-PE-Cy5 (Biolegend), CD48-PE-Cy7, Sca-1-APC, Streptavidin-APC-Cy7 (to label Biotin-conjugated c-kit antibody used to enrich HSPCs), and PE-conjugated lineage antibodies (B220, CD2, CD3, CD8, Gr-1,

Ter119). All murine antibodies were from eBioscience unless otherwise noted. To measure human CD45 expression in AML cell lines and primary cells, transfected cells were cultured for 4 days and stained with FITC-conjugated anti-human CD45 antibody (BD Biosciences). Sorting of $CD45^{neg}$, $CD45^{dim}$, $CD45^{mid}$, $CD45^{bright}$ activated T-cells was performed four days after electroporation. Purity of sorted human cord blood $CD34^{+}$ cells was assessed in each sample using PE-conjugated anti-CD34 antibody (BD Biosciences). Engraftment of human cells in NSG mice was evaluated in bone marrows and spleens using a specific PE-conjugated anti-human HLA-ABC antibody (BD Biosciences). Bone marrow cells were also stained with FITC-conjugated anti-human CD45, PerCP-Cy5.5 conjugated anti-human CD19 and APC conjugated anti-human CD3 antibodies (BD Biosciences).

For human xenograft lineage assessment B cells were gated as $hHLA\text{-}ABC^{+}hCD19^{+}$, T cells as $hHLA\text{-}ABC^{+}hCD3^{+}$ and myeloid cells as $hHLA\text{-}ABC^{+}hCD19^{-}hCD3^{-}$ viable events. Staining of engrafted HSPCs was performed using a combination of anti human CD45-FITC, anti human CD34-PE and anti human CD38-PE (BD Biosciences). In all experiments viable cells were selected applying a first gate on scatter parameters and a second gate on DAPI or propidium iodide negative cells. Flow cytometry was performed with FACSAria II, LSR II, or LSRFortessa flow-cytometers (BD Biosciences).

Western Blotting

5×10^{5} cells were dissolved directly in 50 µl of 1x Laemmli sample buffer and boiled at 95°C for 5 minutes. 15 µl of lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 4-20% gradient gels (BioRad) and transferred onto PVDF membranes (GE Healthcare). Membranes were incubated with primary rabbit anti-DNMT3A (H-295, Santa Cruz) followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz).

High throughput sequencing

Sequencing libraries were prepared using Illumina Nextera XT Kit (Illumina) and sequenced with the Illumina MiSeq or NextSeq instruments (Illumina). Indel frequencies were computed by using a custom aligner/script.

Identification of off-target sites

CRISPRscan (Moreno-Mateos et al., 2015) was used to predict possible off-target sites for hCD45-sg1. Three candidate loci were indentified:

- OT1: chr7:98949525 (TRAPP gene, exon 34)
- OT2: chr8:144448491 (intergenic)
- OT3: chr2:28427248 (intergenic)

Indel frequencies at predicted loci were calculated through high-throughput sequencing of amplicons spanning the off-target sites in T-cells and CD34+ cells targeted with Cas9/hCD45-sg1 RNP.

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