Supplementary Information

Combined Lentiviral- and RNA-mediated CRISPR/Cas9 Delivery for Efficient and Traceable Gene Editing in Human Hematopoietic Stem and Progenitor Cells

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Supplementary Table 1. List of unique predicted off-target sites for CD45 sg5

Number	Chr position	Score	NGS
1	Chr2:28427250-28427271	0.97	Yes
2	Chr8:144448492-144448513	0.99	Yes
3	ChrX:153573824-153573845	2.43	Yes
4	Chr6:42935481-42935501	3.02	Yes
5	Chr15:90000801-90000821	3.02	PCR failed
6	Chr16:49494734-49494754	4.68	N/A
7	Chr7:98949526-98949547	4.7	N/A
8	Chr16:48447603-48447623	5.64	N/A
9	Chr18:76612153-76612173	5.86	N/A
10	Chr19:8903492-8903513	6.13	N/A

Supplementary Table 2. Off-target editing (% modification normalized to on-target efficiency for each condition) for CD45 sg5

Sample	Off-target site			
	#1 Chr2:28427250-28427271	**#2** Chr8:144448492-144448513	**#3** ChrX:153573824-153573845	**#4** Chr6:42935481-42935501
Untreated	0.03	0.00	0.00	0.00
RNP (3 µg)	0.00	10.15	1.91	1.16
mRNA (1.5 µg)	0.58	4.47	0.63	0.29
LV sg5 + protein (3 µg)	0.00	0.00	0.00	0.00
LV sg5 + mRNA (1.5 µg)	0.13	2.00	0.00	0.00

Supplementary Table 3. sgRNA sequences

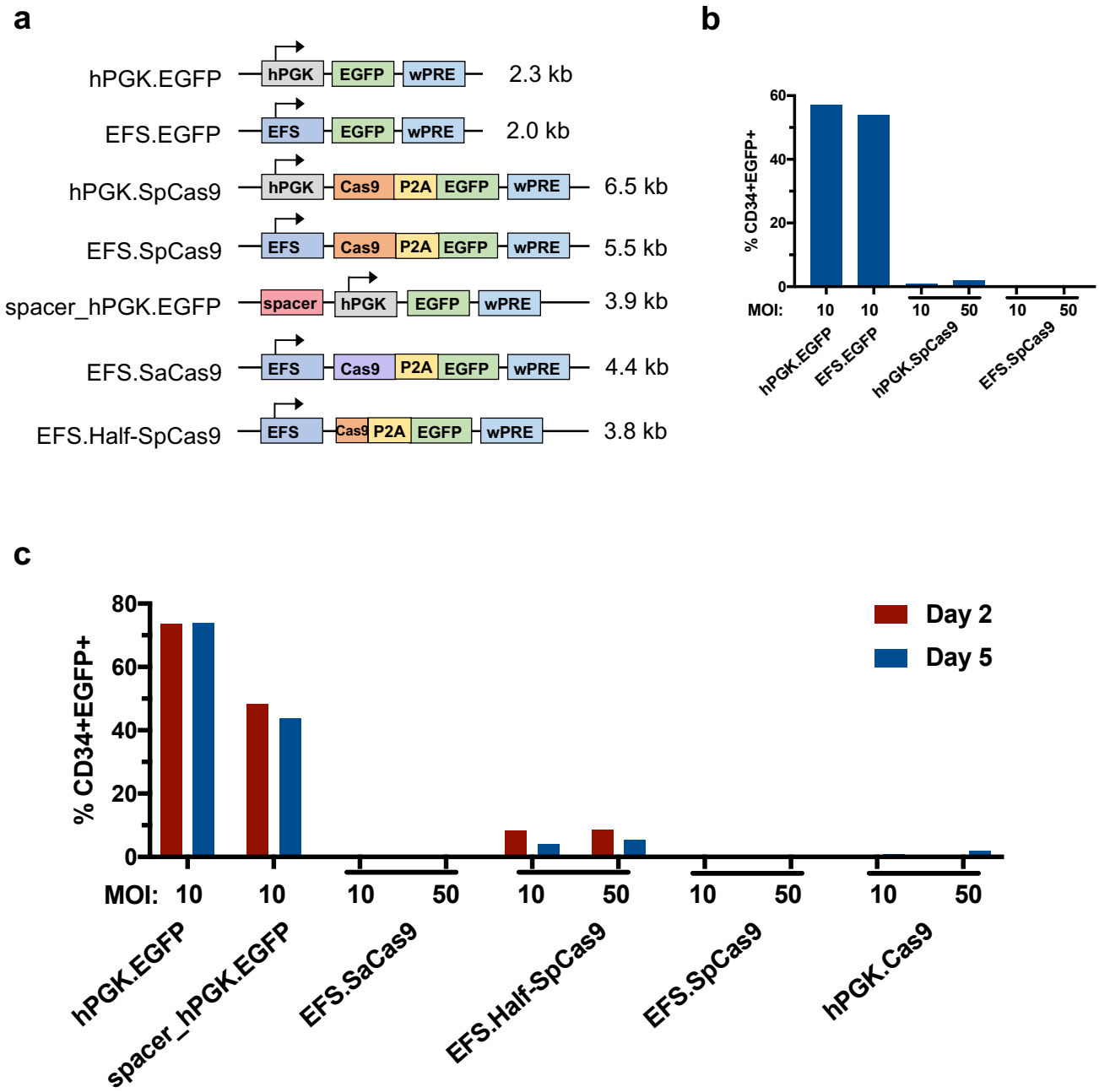
Gene target	Guide	Sequence
CD45	sg5*	GGTGCTGGTGTTGGGCGCAC
CD45	sg8	GGGCGCACAGGAACCTATAT
Non-targeting	ntA	GACGGAGGCTAAGCGTCGCAA
CD44	sg1	GAAGGGCACGTGGTGATTCCC
CD44	sg2	GCAATATGTGTCATACTGGG
CD44	sg3	GTACAGGTATGGGTTCATAGA
CD44	sg4	GCCAGCTATTGTTAACCGTGA
CD29	sg1	GTCATCACATCGTGCAGAAGT
CD29	sg2	GAATGTAACCAACCGTAGCAA
CD29	sg3	GCATAAGGTAGTAGAGGTCAA
CD29	sg4	GACAGCGCATATCTGGAAATT

*sgRNA sequence adapted from Gundry *et al.* 2016.

Supplementary Table 4. Primer sequences

Primer	Sequence 5'-3'
CD45_G5_Tag_FWD	CTGGGGCTGACTTTCCTCAG
CD45_G5_Tag_REV	TGAGCATCTCTCCAAACAGACA
CD44_G1t3_Tag_FWD	GTGGGGGAATGGGACATGAG
CD44_G1t3_ Tag _REV	GCACTTTCCCTGACCACCTT
CD44_G4_ Tag _FWD	TCCTAGCCAGAAGTGGGTGT
CD44_G4_ Tag _REV	AGGAGGGCTCTCCCCAAAAT
CD29_G1t2_ Tag _FWD	AGCAGGATGAGGAGGAGAGG
CD29_G1t2_ Tag _REV	ACACTTGGGTCAGTTCTGGG
CD29_G3_ Tag _FWD	TTGGCACACCAGCTCTCAAT
CD29_G3_ Tag _REV	GTGGCATCCCACTACACACA

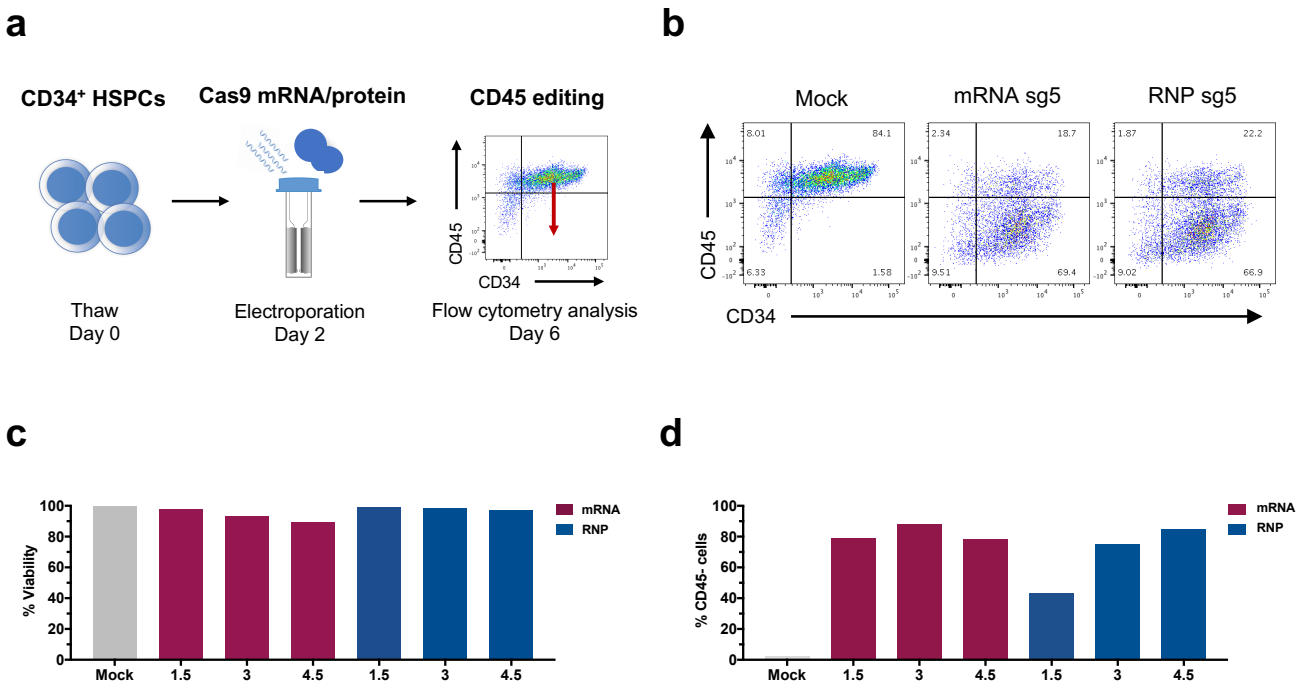
Supplementary Figure S1



Supplementary Figure S1. Challenge of lentiviral Cas9 delivery in human CD34⁺ HSPCs

(**a**) Schematic outline of pLCv2 vectors. (**b and c**) CD34⁺ HSPCs were transduced on day 2 post-thaw. EGFP and CD34 expression was analysed by flow cytometry on day 2 and/or day 5 post-transduction.

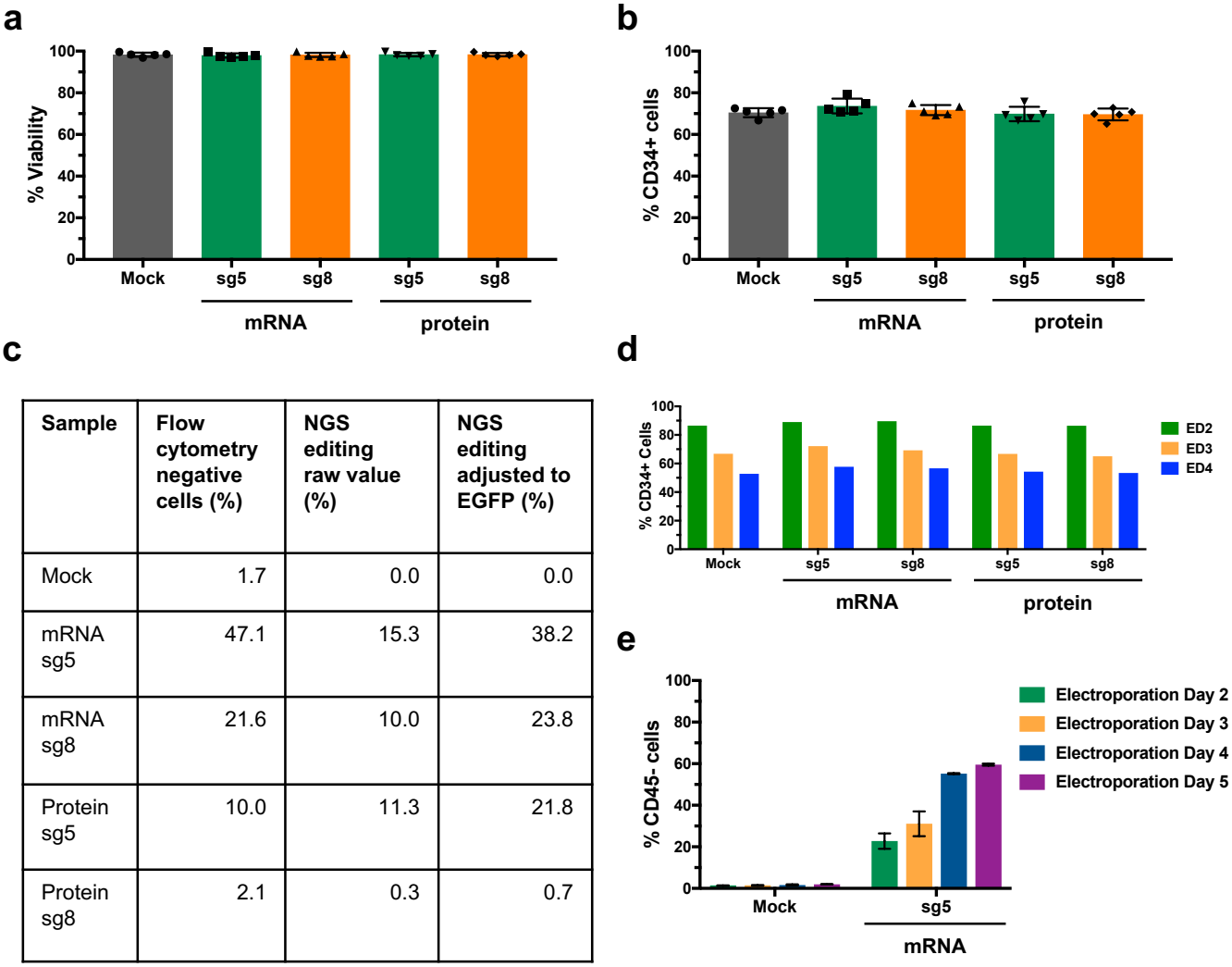
Supplementary Figure S2



Supplementary Figure S2. CD45 editing using Cas9 mRNA or protein with synthetic, modified sgRNA

CD34⁺ HSPCs were treated with Cas9 mRNA or protein at various concentrations, in combination with co-electroporated modified, synthetic sgRNA targeting CD45 (sg5), and analysed by flow cytometry. (**a**) Overview of experimental outline. (**b**) Representative FACS plots of CD45 editing using 1.5 µg Cas9 mRNA or 3 µg Cas9 protein. (**c**) Viability analysis by flow cytometry 7AAD staining. (**d**) Frequency of CD45 edited cells.

Supplementary Figure S3



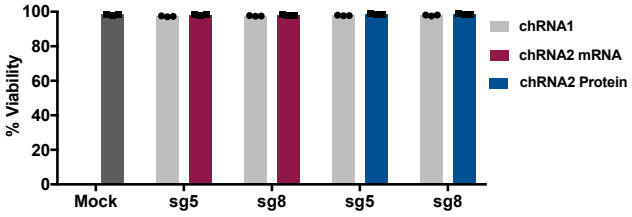
Sample	Flow cytometry negative cells (%)	NGS editing raw value (%)	NGS editing adjusted to EGFP (%)
Mock	1.7	0.0	0.0
mRNA sg5	47.1	15.3	38.2
mRNA sg8	21.6	10.0	23.8
Protein sg5	10.0	11.3	21.8
Protein sg8	2.1	0.3	0.7

Supplementary Figure S3. Effect on viability and CD34 expression from HSPC editing using Cas9 mRNA or protein with lentiviral sgRNA

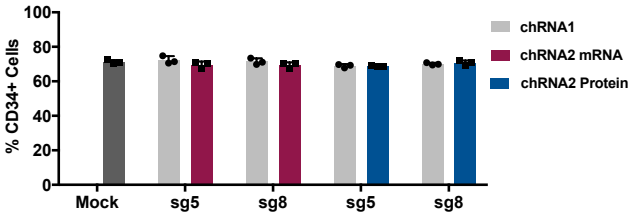
(**a and b**) Primary CD34⁺ HSPCs were transduced with sgRNA targeting CD45 (sg5 or sg8) and subsequently electroporated with Cas9 mRNA or protein (n=5). (**a**) Viability measured by 7AAD staining, (**b**) CD34⁺ expression analysed by flow cytometry 4 days following the electroporation and (**c**) NGS analysis for representative samples, cells were collected 4 days following the electroporation. Note, raw gene editing efficiency scores were divided by percent EGFP⁺ cells to provide an estimate of editing efficiency within the transduced population. (**d**) CD34⁺ cells were transduced on day 1 with sgRNA targeting CD45, electroporated on either day 2, 3 or 4, and subsequently analysed by flow cytometry for CD34 expression 4 days following the electroporation. (**e**) CD34⁺ cells were transduced on day 1 with sgRNA targeting CD45, electroporated with Cas9 mRNA on either day 2, 3, 4 or 5, and subsequently analysed by flow cytometry for CD45- cells 4 days following the electroporation.

Supplementary Figure S4

a



b



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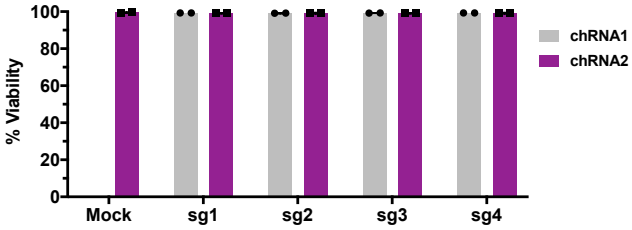

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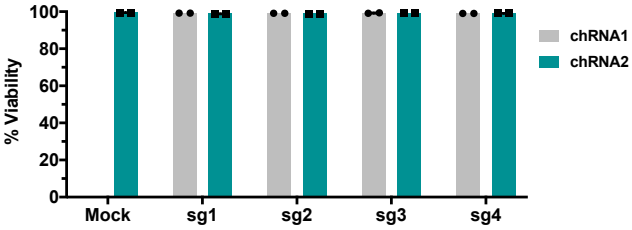


e

Sample CD44	Flow cytometry negative cells (%)	NGS editing raw value (%)	NGS editing adjusted to EGFP (%)
Mock	5.9 ± 1.5	0.0 ± 0.0	0.0 ± 0.0
sg3 chRNA1	22.3 ± 3.7	17.7 ± 3.8	32.1 ± 6.6
sg3 chRNA2	56.8 ± 14.8	36.6 ± 15.6	67.5 ± 30.4
sg4 chRNA1	76.6 ± 2.8	43.2 ± 14.4	95.8 ± 5.9
sg4 chRNA2	87.2 ± 1.7	51.7 ± 2.5	100 ± 0.0

f

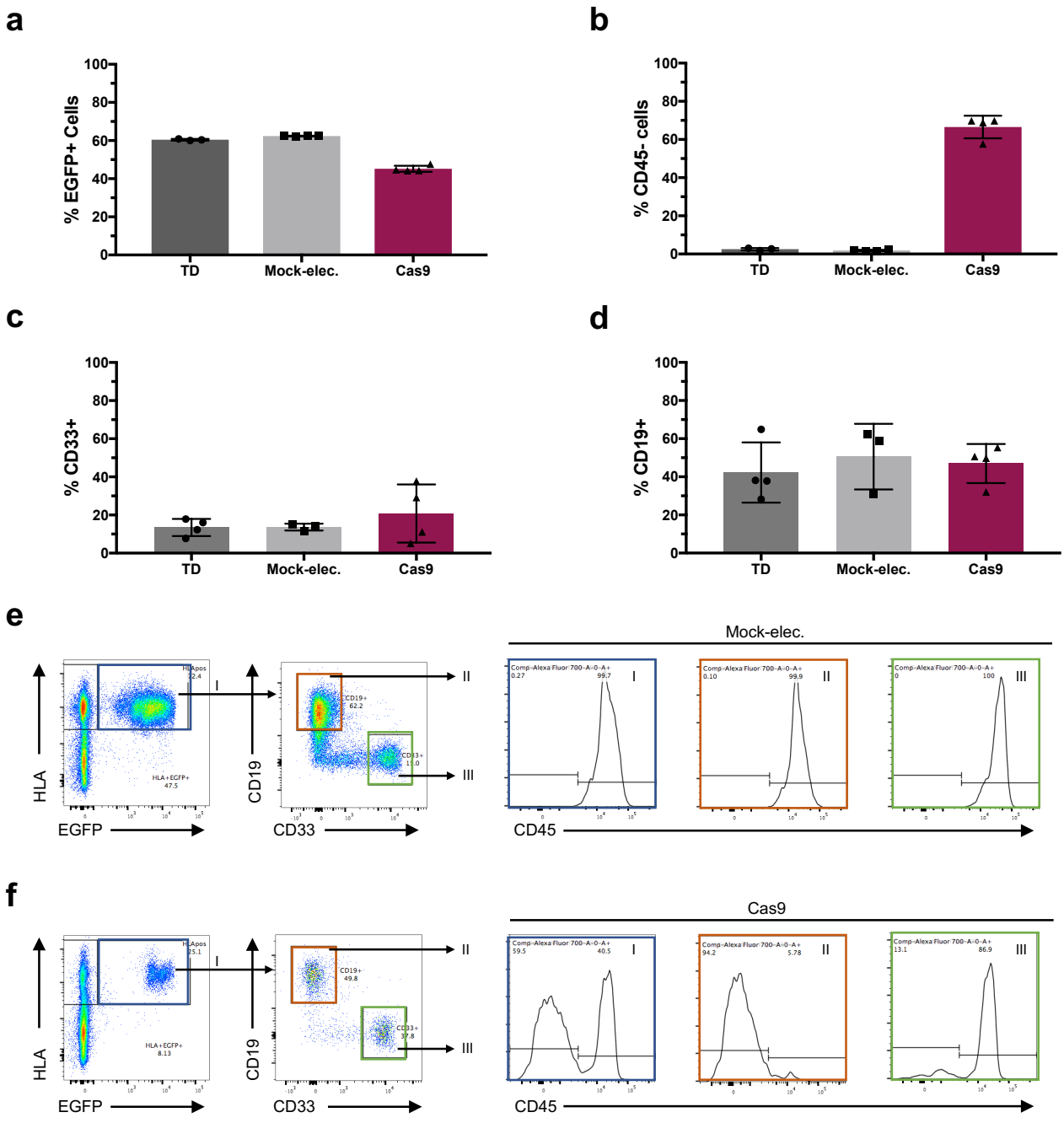
Sample CD29	Flow cytometry negative cells (%)	NGS editing raw value (%)	NGS editing adjusted to EGFP (%)
Mock	5.9 ± 1.5	0.0 ± 0.0	0.0 ± 0.0
sg1 chRNA1	53.5 ± 3.7	33.2 ± 1.6	91.4 ± 2.2
sg1 chRNA2	61.5 ± 1.2	41.6 ± 7.4	91.7 ± 11.7
sg3 chRNA1	30.8 ± 10.2	21.9 ± 5.7	54.9 ± 13.7
sg3 chRNA2	61.4 ± 17.6	42.9 ± 9.3	87.3 ± 18.0

Supplementary Figure S4. Modified chimeric guide RNA backbone enhances editing efficiency in HSPCs without compromising viability or CD34 expression

CD34⁺ cells were transduced with sg5 or sg8 targeting CD45 encoding either chimeric guide RNA backbone 1 or 2 on day 1 post-thaw, electroporated with Cas9 mRNA or protein on day 4, and analysed by flow cytometry on day 8 (n=3). (**a**) Viability by 7AAD staining and (**b**) CD34⁺ expression.

CD34⁺ cells were transduced on day 1 post-thaw with 4 different lentiviral sgRNAs per target gene encoding either chimeric guide RNA backbone 1 or 2, electroporated with 2 μg Cas9 mRNA on day 4, and analysed by flow cytometry on day 8. At this time point, cells were also collected for NGS (n = 2). (**c**) Viability by 7AAD staining in CD44 edited cells and (**d**) in CD29 edited cells. (**e**) NGS analysis of the CD44 locus and (**f**) of the CD29 locus in edited cells ± standard deviation (s.d.). Note, raw gene editing efficiency scores were divided by percent EGFP⁺ cells to provide an estimate of editing efficiency within the transduced population.

Supplementary Figure S5



Supplementary Figure S5. Edited CD34⁺ HSPCs retain reconstitution capacity in vivo
(**a and b**) A portion of edited CD34⁺ cells from each group was not used for transplantation and maintained in culture and analysed by flow cytometry 4 days after electroporation. (**a**) Frequency of EGFP⁺ cells. (**b**) Frequency of CD45- cells in the EGFP⁺ population. (**c-f**) BM from transplanted NSG mice harvested 9-12 weeks post-transplantation analysed by flow cytometry. n=3-4 mice per group. (**c**) Frequency of CD33⁺ cells within the HLA⁺EGFP⁺ population. (**d**) Frequency of CD19⁺ cells within the HLA⁺EGFP⁺ population. (**e and f**) Gating strategy to determine CD45 editing in the HLA⁺EGFP⁺ population (I), CD19⁺ population (II) and in the CD33⁺ population (III), showing examples for (**e**) mock-electroporated and (**f**) Cas9-electroporated cells.