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## Supplemental Information

# Optimization of CRISPR/Cas9 Delivery to Human Hematopoietic Stem and Progenitor Cells for Therapeutic Genomic Rearrangements

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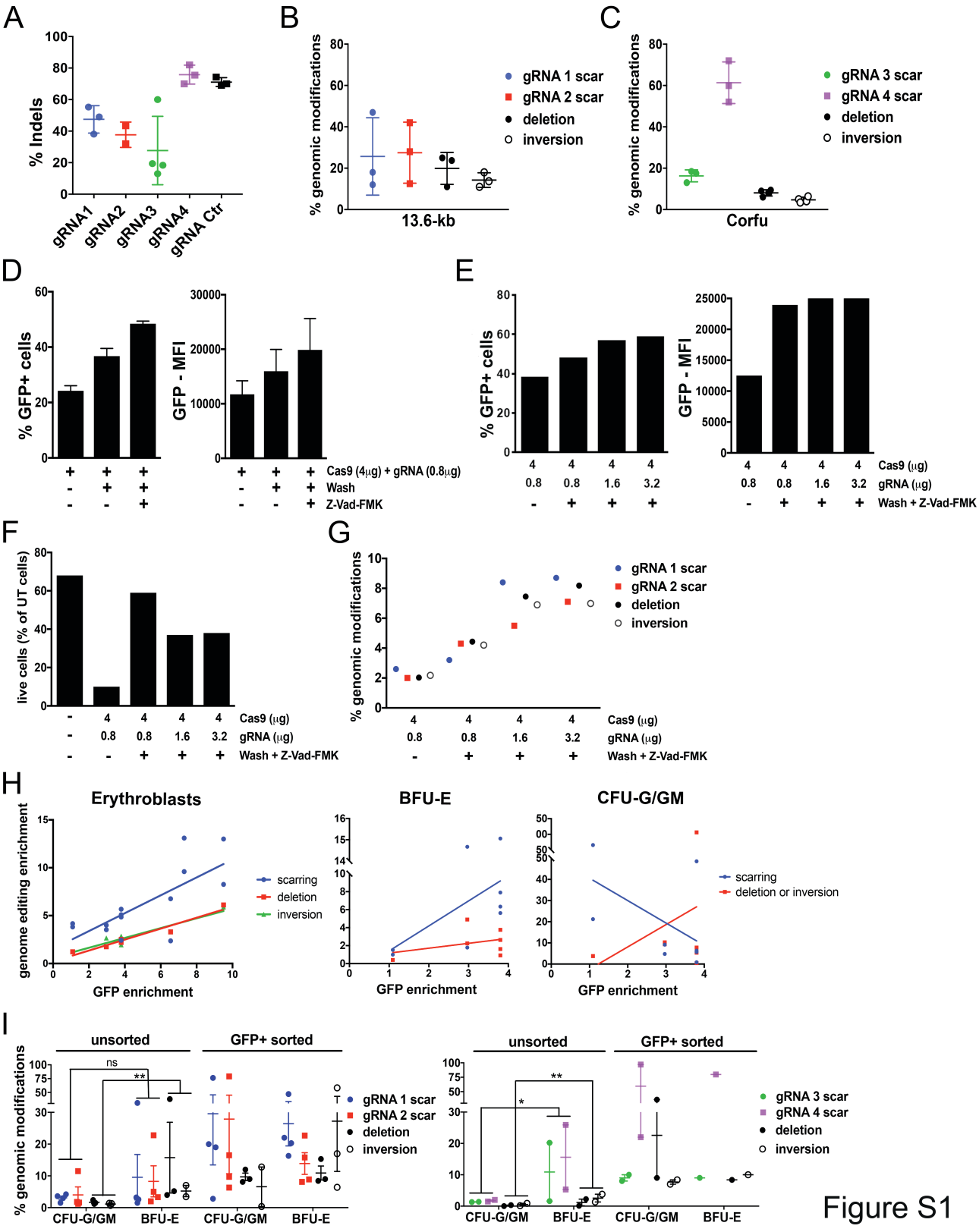
A
% Indels
gRNA1
gRNA2
gRNA3
gRNA4
gRNA Ctr
B
% genomic modifications
13.6-kb
gRNA 1 scar
gRNA 2 scar
deletion
inversion
C
% genomic modifications
Corfu
gRNA 3 scar
gRNA 4 scar
deletion
inversion
D
% GFP+ cells
GFP - MFI
Cas9 (4μg) + gRNA (0.8μg)
Wash
Z-Vad-FMK
E
% GFP+ cells
GFP - MFI
Cas9 (μg)
gRNA (μg)
Wash + Z-Vad-FMK
F
live cells (% of UT cells)
Cas9 (μg)
gRNA (μg)
Wash + Z-Vad-FMK
G
% genomic modifications
gRNA 1 scar
gRNA 2 scar
deletion
inversion
Cas9 (μg)
gRNA (μg)
Wash + Z-Vad-FMK
H
Erythroblasts
BFU-E
CFU-G/GM
genome editing enrichment
GFP enrichment
scarring
deletion
inversion
deletion or inversion
I
unsorted
GFP+ sorted
% genomic modifications
CFU-G/GM
BFU-E
ns
gRNA 1 scar
gRNA 2 scar
deletion
inversion
gRNA 3 scar
gRNA 4 scar

Figure S1

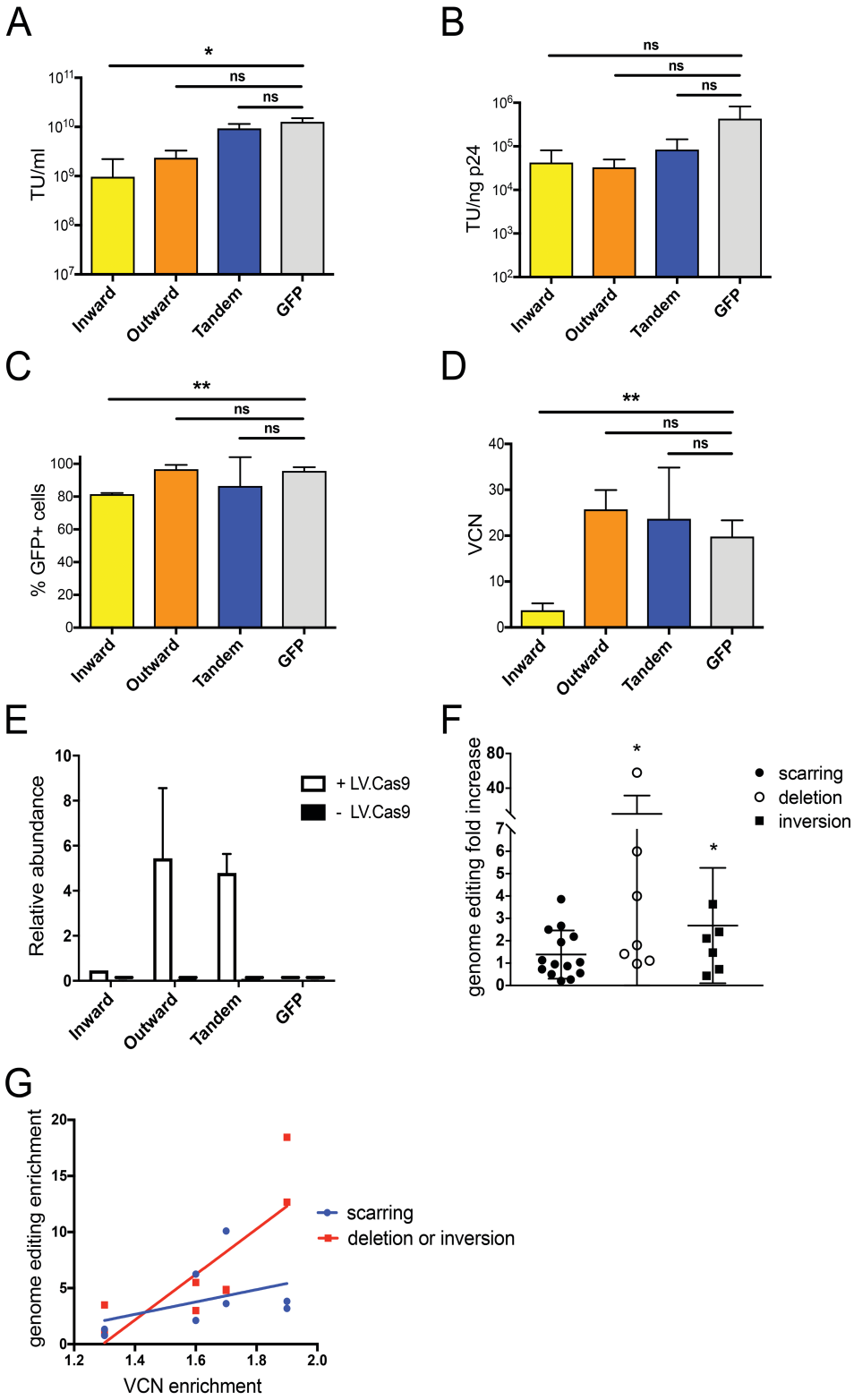
A
*
ns
ns
TU/ml
Inward
Outward
Tandem
GFP
B
ns
ns
ns
TU/ng p24
C
**
ns
ns
% GFP+ cells
D
**
ns
ns
VCN
E
+ LV.Cas9
- LV.Cas9
Relative abundance
F
scarring
deletion
inversion
genome editing fold increase
G
scarring
deletion or inversion
genome editing enrichment
VCN enrichment

Figure S2

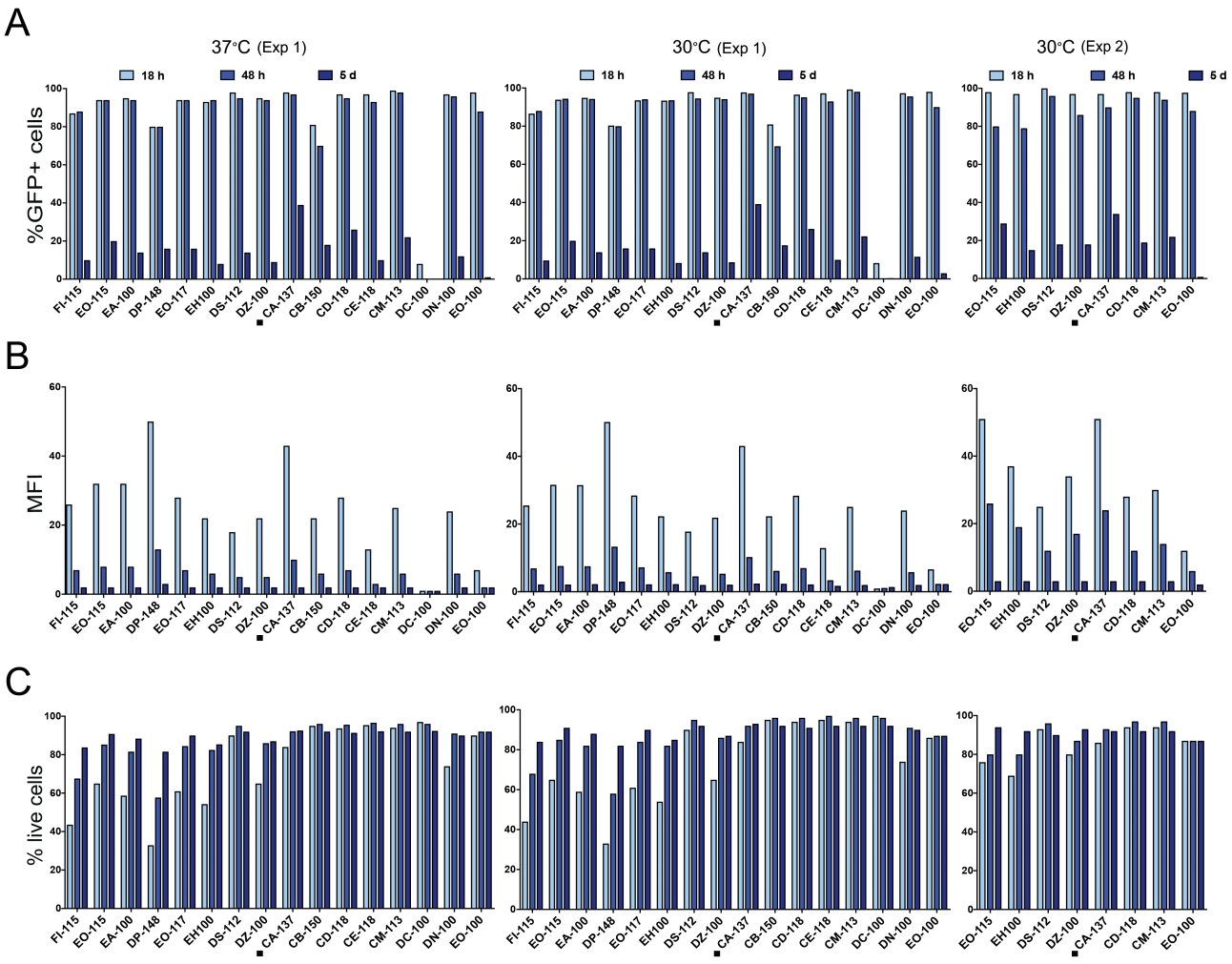
A
37°C (Exp 1)
30°C (Exp 1)
30°C (Exp 2)
18 h
48 h
5 d
%GFP+ cells
B
MFI
C
% live cells
Fl-115
EO-115
EA-100
DP-148
EO-117
EH100
DS-112
DZ-100
CA-137
CB-150
CD-118
CE-118
CM-113
DC-100
DN-100
EO-100

Figure S3

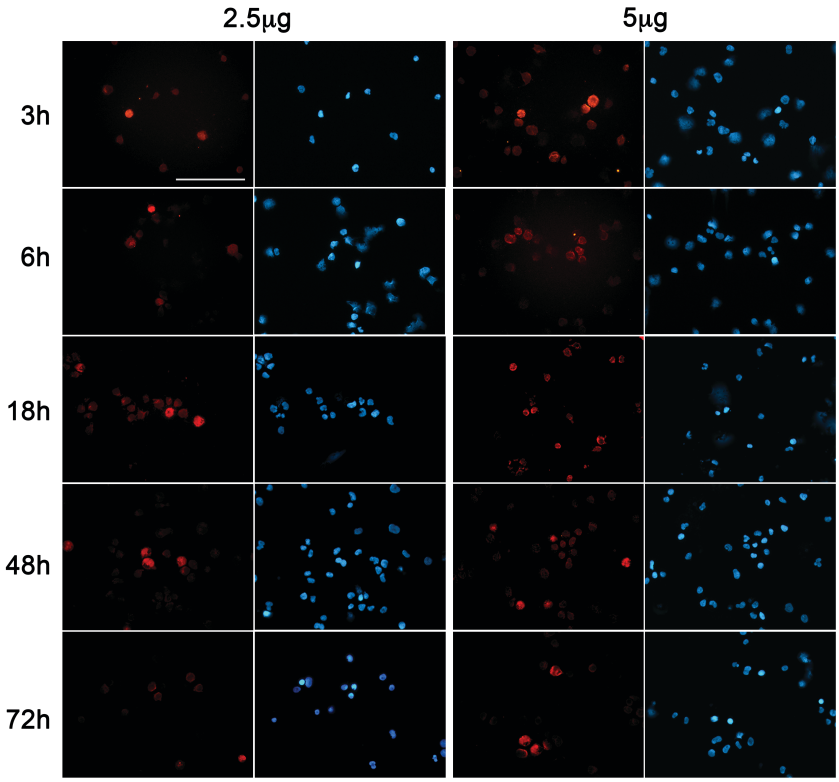


Figure S4

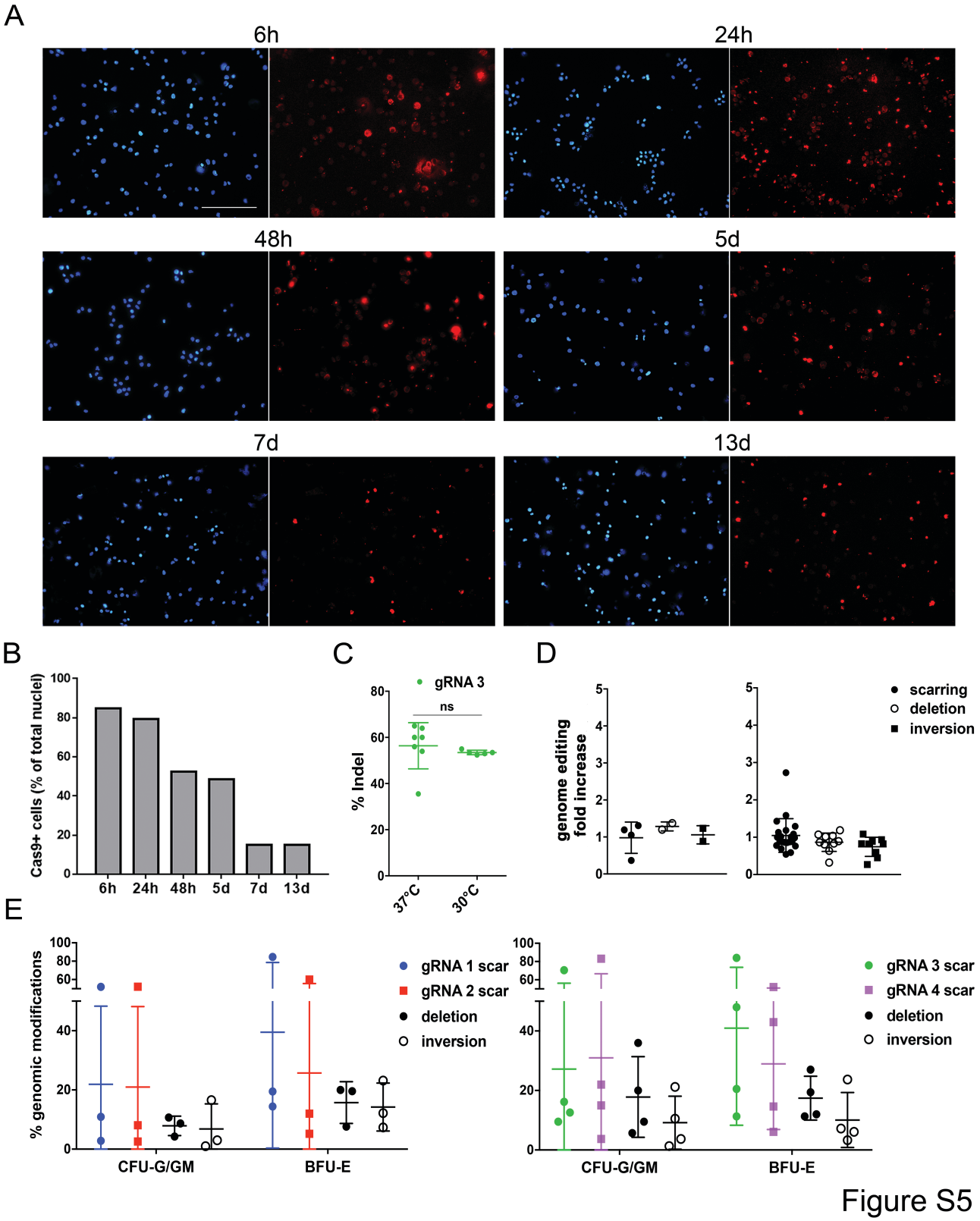
A
6h
24h
48h
5d
7d
13d
B
Cas9+ cells (% of total nuclei)
6h
24h
48h
5d
7d
13d
C
gRNA 3
ns
% Indel
37°C
30°C
D
genome editing fold increase
scarring
deletion
inversion
E
% genomic modifications
gRNA 1 scar
gRNA 2 scar
deletion
inversion
CFU-G/GM
BFU-E
gRNA 3 scar
gRNA 4 scar
deletion
inversion
CFU-G/GM
BFU-E

Figure S5

## SUPPLEMENTARY FIGURES

**Figure S1. Plasmid delivery in K562 cells and primary adult HSPCs.** (**A**) Cleavage efficiency of single gRNAs in K562 (n=3-4). A gRNA targeting the AAVS1 locus was used as control. (**B-C**) Quantification of deletion, inversion and scarring events in K562 cells (n=3). (**D**) Percentage of $GFP^+$ cells (left panel) and Mean Fluorescence Intensity (MFI; right panel) in HSPCs electroporated using plasmids encoding Cas9-GFP and gRNAs 1&2 (n=2-4). (**E-G**) HSPCs were electroporated using plasmids encoding the Cas9-GFP and gRNAs 1&2 (n=1). We plotted the percentage of $GFP^+$ cells (**E**, left panel), the MFI (**E**, right panel), the ratio between transfected and untransfected live cells (**F**), and the editing frequencies (**G**). (**H**) Correlation between the fold enrichment in $GFP^+$ HSPCs and scarring, deletion and inversion fold increase in erythroblasts, BFU-E and CFU-G/GM derived from sorted HSPCs compared to samples obtained from unsorted HSPCs. In erythroblasts, equations that define the best fit lines were: $y= 0.9332x + 1.521$ ($R^2$=0.5338 and $P<0.01$) for scarring, $y= 0.5718x+0.2095$ ($R^2$=0.942 and $P<0.01$) for deletion, and $y= 0.5117x+0.6228$ ($R^2$=0.8934 and $P<0.01$) for inversion. Elevations for regression lines were statistically different (scarring vs deletion or inversion, $P<0.001$). In BFU-E, equations that define the best fit lines were: $y= 2.779x–1.367$ ($R^2$=0.3442 and P=0.13) for scarring, and $y= 0.5428x+0.6291$ ($R^2$=0.1181 and P=0.45) for deletion and inversion. Elevations for regression lines were statistically different (scarring vs deletion and inversion, $P<0.05$). In CFU-G/GM, equations that define the best fit lines were: $y= -10.59x+51.17$ ($R^2$=0.2726 and P=0.18) for scarring, and $y= 10.53x–12.97$ ($R^2$=0.07664 and P=0.54) for deletion and inversion. Regression lines were not statistically different. (**I**) Frequency of genome editing events in CFU-G/GMs and BFU-Es. *$p<0.05$, **$p<0.01$, ns, not significant (paired t test). No statistical differences were observed between erythroblasts and

progenitors derived from sorted HSPCs (two-way ANOVA plus Tukey's multiple comparison test). Error bars denote standard deviation.

**Figure S2. Comparison of LVs expressing gRNA pairs in different configurations.** Titer (**A**) and infectivity (**B**) of LV.Inward, LV.Outward, LV.Tandem and a control GFP-expressing LV (LV.GFP) (n=3). (**C-E**) K562 were co-transduced with LV.Cas9 and LV.Inward, LV.Outward, LV.Tandem or LV.GFP (n=2-4). Percentage of $GFP^+$ cells (**C**) and vector copy number (VCN) (**D**) are indicated. *p<0.05, **p<0.01 (unpaired t test and Kolmogorov-Smirnov test). (**E**) gRNA content in LV-transduced cells, with or without LV.Cas9. (**F**) Fold increase in genome editing efficiency from day 7 to day 13 in 13.6-kb- and Corfu-edited erythroblasts. HSPCs were not subjected to blasticidin selection. *p<0.05 (ratio paired t test). (**G**) Correlation between the fold enrichment in VCN and scarring, deletion and inversion fold increase in selected compared to unselected erythroblasts. Equations that define the best fit lines were: y= 5.505x – 5.043 ($R^2$=0.1774 and P=0.30) for scarring, and y= 20.28x – 26.24 ($R^2$=0.6473 and P<0.05) for deletion and inversion. Regression lines were not statistically different. Error bars denote standard deviation.

**Figure S3. Optimization of RNA electroporation in cord blood-derived HSPCs.** (**A-C**) Testing of 16 Amaxa 4D programs (indicated on the X-axis) with (30ºC, Experiment 1 and 2) or without (37ºC, Experiment 1) a transient cold shock. Histograms display percentage of $GFP^+$ cells (**A**), GFP MFI (**B**) and frequency of live cells (**C**) 18 h, 48 h and 5 d after electroporation.

**Figure S4. Time-course analysis of Cas9$^+$ cells after RNA-mediated HSPC electroporation.** Representative pictures of immunofluorescence staining (red, anti-SpCas9 antibody; blue, DAPI) of cord blood-derived HSPCs after electroporation with 2.5 (left panels) and 5 μg (right panels) of Cas9 mRNA at different time points post-electroporation. 40x magnification. Scale bar, 200 μm.

**Figure S5. Cas9 RNP delivery in HSPCs.** (**A**) Representative pictures (red, anti-SpCas9 antibody; blue, Dapi) from IF staining of cord blood-derived cells at different time points after HSPC electroporation. 40X objective. Scale bar, 200 μm. (**B**) Quantification of Cas9$^+$ cells after HSPC electroporation with Cas9-RNP. (**C**) Editing efficiency upon delivery of Cas9 RNP complexes containing gRNA 3. ns, not significant (Welch t test). (**D**) Fold increase in editing efficiency in Corfu-edited erythroblasts from day 3 to day 7 (left panel) and 13.6-kb and Corfu-edited erythroblasts from day 7 to day 13 (right panel). Increase in genome editing was not significant (ratio paired t test) (**E**) Editing efficiency in CFU-G/GMs and BFU-Es derived from adult HSPCs transfected with Cas9-RNP complexes containing gRNA pairs. No statistical differences were observed between erythroblasts and progenitors (two-way ANOVA plus Tukey's multiple comparison test). Error bars denote standard deviation.

**TABLE S1**

| gRNA name | sequence | chr | strand | start | end |
|---|---|---|---|---|---|
| gRNA 1 | GGTGCTACATACTTCCTAAGG | 11 | + | 5240482 | 5240501 |
| gRNA 2 | gCAATAGAAACTGGGCATGTGG | 11 | - | 5226876 | 5226895 |
| gRNA 3 | gGTGTGCTGGCCCGCAACTTTGG | 11 | + | 5233049 | 5233071 |
| gRNA 4 | gCCACTCAAGAGATATGGTGAGG | 11 | - | 5240337 | 5240359 |

CRISPR sequences were designed by ZIFIT software as truncated (18-19nt), when needed an initial g (indicated in lower case) was added to the sequence in order to ensure U6-driven gRNA expression. PAM sequence is underlined. For each gRNA we reported the hg38 genomic coordinates.

## TABLE S2

| gRNA1 | Result | Mismatch | Chr Position | Strand | Cut site | Score | Region |
|---|---|---|---|---|---|---|---|
| On-Target | GGTGCTACATACTTCCTAAGG | 0 | Chr11:5240484-5240504 | + | 5240498 | 0 | Intergenic |
| OT1 | GGTGCTCTATACTTCCTAGGG | 2 | Chr22:24981439-24981459 | + | 24981453 | 0.8 | Intergenic |
| OT2 | TGTGCACATACTTCCTAAGG | 1 | Chr2:61285945-61285964 | + | 61285958 | 0.9 | Intronic - USP34 |
| OT3 | GGTCTTCATACTTCCTATGG | 1 | Chr12:95269623-95269642 | + | 95269636 | 1 | Intronic - VEZT |
| OT4 | GGTCTACACACTTCCTAAGG | 1 | Chr1:246194194-246194213 | + | 246194207 | 1.5 | Intronic – SMYD3 |
| OT5 | GGTGCTAAACTACTTCCTATGG | 1 | Chr13:95662010-95662031 | - | 95662016 | 2 | Intergenic |
| **gRNA2** | **Result** | **Mismatch** | **Chr Position** | **Strand** | **Cut site** | **Score** | **Region** |
| On-Target | CAATAGAAACTGGGCATGTGG | 0 | Chr11:5226873-5226893 | - | 5226879 | 0 | Intronic - HBB |
| OT1 | CTAGAGAAACTGGGCATGTGG | 2 | Chr9:73029530-73029550 | - | 73029536 | 0.4 | Intergenic |
| OT2 | CAGGAGAAACTGGGCATGAGG | 2 | Chr17:55376507-55376527 | + | 55376521 | 0.4 | Intergenic |
| OT3 | CAGTGGAAACTGGGCATGGGG | 2 | Chr1:36265156-36265176 | - | 36265162 | 0.4 | Intronic – THRAP3 |
| OT4 | CAATAGATACTGGGCATGAGG | 1 | Chr8:76239868-76239888 | - | 76239874 | 0.5 | Intergenic |
| OT5 | CTTAGAAACTGGGCATGGGG | 1 | ChrX:46682638-46682657 | + | 46682651 | 0.9 | Intronic - SLC9A7 |
| OT6 | CACTAGAAGCTGGGCATGGGG | 2 | Chr1:10058541-10058561 | + | 10058555 | 0.9 | Intronic – UBE4B |
| OT7 | TAATAAAACTGGGCATGTGG | 1 | Chr8:71606742-71606761 | + | 71606755 | 0.9 | Intergenic |
| OT8 | CTATAAAACTGGGCATGAGG | 1 | Chr8:104732647-104732666 | + | 104732660 | 0.9 | Intergenic |
| OT9 | AATAGATACTGGGCATGAGG | 1 | Chr8:76239868-76239887 | - | 76239874 | 1.2 | Intergenic |
| OT10 | CATAGAGACTGGGCATGTGG | 1 | Chr7:106891909-106891928 | - | 106891915 | 1.2 | Intronic – PIK3CG |
| OT11 | AAATAGAAATGGGCATGGGG | 1 | Chr12:4046329-4046348 | - | 4046335 | 1.5 | Intergenic |
| OT12 | CAAGAGAAACTTGGCATGGGG | 2 | Chr9:135352260-135352280 | - | 135352266 | 1.5 | Intergenic |
| **gRNA3** | **Search result** | **Mismatch** | **Chr Position** | **Strand** | **Cut site** | **Score** | **Region** |
| On-Target | GTGTGCTGGCCCGCAACTTTGG | 0 | Chr11:5233049-5233070 | - | 5233055 | 0 | Exonic - HBD |
| OT1 | GTGTGGTGGCACGCAACTTTGG | 2 | ChrX:103212951-103212972 | - | 103212957 | 1 | Intergenic |
| OT2 | GTGTGATGCCCGCAACTTTGG | 1 | Chr15:46065997-46066017 | - | 46066003 | 1 | Intergenic |
| OT3 | GGGTGCTGGCCCGTAACTTGGG | 2 | Chr13:94811220-94811241 | + | 94811235 | 2 | Intergenic |
| **gRNA4** | **Search result** | **Mismatch** | **Chr Position** | **Strand** | **Cut site** | **Score** | **Region** |
| On-Target | CCACTCAAGAGATATGGTGAGG | 0 | Chr11:5240338-5240359 | + | 5240353 | 0 | Intergenic |
| OT1 | CCCCTAAGAGATATGGTGTGG | 1 | Chr10:47376021-47376041 | - | 47376027 | 1 | Intronic - ZNF488 |
| OT2 | CAACTCAAGAGATCTGGTGTGG | 2 | Chr8:1358364-1358385 | + | 1358379 | 2 | Intergenic |

gRNA sequences were analyzed by COSMID [60] and the top predicted (score ≤ 2) off-targets were reported. Low-scoring sites are predicted to be more likely off-targets. None of them occurred within coding or intronic regions of genes involved in HSC and RBC biology. gRNA 1 and gRNA 2 showed minimal off-target activity, as described in Antoniani et al. [10], gRNA 3 and gRNA 4 have few off-targets that show a high score (between 1 and 2).

**TABLE S3**

| gRNA1 | Result | Plasmid | LV | RNA | RNP |
|---|---|---|---|---|---|
| On-Target | GGTGCTACATACTTCCTAAGG | 20.8/24.4/58.4^ | 1.7/1.5/6.5^ | 2.0/1.4/4.8^ | 24.1/20.6/18^ |
| OT1 | GGTGCTCTATACTTCCTAGGG | 0.0004(0.001) | 0.001(0.001) | 0.001(0.001) | **0.011**(0.001) |
| OT2 | TGTGCACATACTTCCTAAGG | **0.004**(0.001) | **0.101**(0.001) | 0.001(0.001) | **0.057**(0.001) |
| OT3 | GGTCTTCATACTTCCTATGG | 0.001(0.001) | **0.011**(0.001) | 0.001(0.001) | 0.001(0.001) |
| **gRNA2** | **Result** | **Plasmid** | **LV** | **RNA** | **RNP** |
| On-Target | CAATAGAAACTGGGCATGTGG | 20.8/24.4/58.8^ | 1.7/1.5/6.7^ | 2.0/1.4/3.1^ | 24.1/20.6/57^ |
| OT1 | CTAGAGAAACTGGGCATGTGG | 0.494(0.587) | **1.042**(0.587) | 0.450(0.587) | 0.478(0.587) |
| OT2 | CAGGAGAAACTGGGCATGAGG | **0.005**(0.001) | **0.061**(0.001) | 0.001(0.001) | **0.002**(0.001) |
| OT3 | CAGTGGAAACTGGGCATGGGG | 0.001(0.001) | **0.007**(0.001) | **0.002**(0.001) | 0.001(0.001) |
| **gRNA3** | **Search result** | **Plasmid** | **LV** | **RNA** | **RNP** |
| On-Target | GTGTGCTGGCCCGCAACTTTGG | 8.8/6.3/7.3^ | 2.9/3.5/3.5^ | 5.0/2.0/6.0^ | 22.6/10.8/19^ |
| OT2 | GTGTGATGCCCGCAACTTTGG | **1.285**(0.291) | **12.009**(0.291) | **0.713**(0.291) | **0.882**(0.291) |
| OT3 | GGGTGCTGGCCCGTAACTTGGG | **0.017**(0.010) | **0.240**(0.010) | 0.004(0.010) | **0.021**(0.010) |
| **gRNA4** | **Search result** | **Plasmid** | **LV** | **RNA** | **RNP** |
| On-Target | CCACTCAAGAGATATGGTGAGG | 8.8/6.3/57.3^ | 2.9/3.5/9.3^ | 5.0/2.0/4.2^ | 22.6/10.8/30.4^ |
| OT1 | CCCCTAAGAGATATGGTGTGG | **0.005**(0.004) | 0.004(0.004) | 0.004(0.004) | 0.004(0.004) |
| OT2 | CAACTCAAGAGATCTGGTGTGG | **0.009**(0.005) | **0.042**(0.005) | **0.006**(0.005) | **0.013**(0.005) |

For each gRNA, the top-predicted off-target (OT) sites identified by COSMID, were amplified in control and genome-edited erythroblasts and subjected to deep sequencing, followed by CRISPRESSO analysis. The background level of InDels measured in non-edited cells is indicated in brackets. Off-target frequencies higher than background are highlighted in red.

^Deletion, inversion and scarring frequency is indicated.

## SUPPLEMENTARY MATERIALS AND METHODS

### List of primers used to evaluate the NHEJ at on-target sites

*gRNA 1*
Forward primer: 5'- AGCACCGCCTATCTATGTGC -3'
Reverse primer: 5'- GGAAACTGGATGCAGAGACCA -3'
*gRNA 2*
Forward primer: 5'- AGGCCATCACTAAAGGCACC -3'
Reverse primer: 5'- AGTCAGGGCAGAGCCATCTA -3'
*gRNA 3*
Forward primer: 5'- GATGGGAATAACCTGGGGATCAGT -3'
Reverse primer: 5'- GTGCTCCCTATCTGTAGAGCC -3'
*gRNA 4*
Forward primer: 5'- CGAGTAAGAGACCATTGTGGCAG -3'
Reverse primer: 5'- GCTTTGTGGGTTATTAGTGGGGAC -3'

NHEJ was measured by PCR using primers annealing upstream and downstream of the gRNA cleavage sites.

### List of primers used for ddPCR-based measurement of deletion and inversion frequencies

*Control primers at Chr11*
Forward primer: 5'-CCCTTCCGAGAGGATTTAGG-3'
Reverse primer: 5'-AGTCGGGATCTGAACAATGG-3'
*Primers to detect the 13.6-kb deletion*
Forward primer: 5'-GTAGACCACCAGCAGCCTAA-3'
Reverse primer: 5'-AAATGCCTACAAGCCCCCTG-3'
*Primers to detect the 13.6-kb inversion*
Forward primer: 5'-GTAGACCACCAGCAGCCTAA-3'
Reverse primer: 5'-AATGAAACTGGAGAAGAAAGGGT-3'

*Primers to detect the Corfu deletion*

Forward primer: 5'- ACACCAGCCACCACCTTCTG -3'

Reverse primer: 5'- GCACCCTCAAACCTAAAACCTCAAAGAAAG -3'

*Primers to detect the Corfu inversion*

Forward primer: 5'- ACACCAGCCACCACCTTCTG -3'

Reverse primer: 5'- AATTTCAGAAGCTGTTAGATGGTAGCACCG -3'

Deletion events were detected by PCR using primers upstream and downstream of the target regions. Inversion junctions were amplified using two primers in the same orientation, one inside and one outside the targeted sequences[10].

**List of primers used to evaluate the NHEJ at off-target sites**

*gRNA 1 OT1*

Forward primer: 5'- GCACACCCTGGTGTGTGTCT -3'

Reverse primer: 5'- TCTGAAGCTCCCCAGGGAGT -3'

*gRNA 1 OT2*

Forward primer: 5'- GTATATACTTGTGTTAACCATGTTTTCTGTGGCTG -3'

Reverse primer: 5'- CAGTTCTAGTTCTTCCTCATATAAGGGGAGAAA -3'

*gRNA 1 OT3*

Forward primer: 5'- CACTATGCTTGCTAACATATATTAGAGAAGAGCTAC -3'

Reverse primer: 5'- GACCAAAATATGATCAGTGAACATATGTGATGAACG -3'

*gRNA 2 OT1*

Forward primer: 5'- GTCTTGGTTTACTCAGCTCTAAAATGTTTAGCAG -3'

Reverse primer: 5'- GCCACTTTAATGCCACTGCCC -3'

*gRNA 2 OT2*

Forward primer: 5'- GATTTTGTTTCACTCATTGTGACTCATATACCATCC -3'

Reverse primer: 5'- GCCACTGTACCCAGCCTTTC -3'

*gRNA 2 OT3*

Forward primer: 5'- CATACTGGTTCATTAATTGGGACAAATGTACCATACT -3'

Reverse primer: 5'- CTGAGGTACTAGGGGTTAGGAC -3'

*gRNA 3 OT2*

Forward primer: 5'- TTGTAACTAACTACAAAAGACCTTGAATACCCAAAGC -3'

Reverse primer: 5'- CTGTTCTAGTAGTGTATATGTGTTTTATGTCAATGCC -3'

*gRNA 3 OT3*

Forward primer: 5'- AGCCCAGGATAATGTGGATGCC -3'

Reverse primer: 5'- CCCGTCATCACAGCTGCAAG -3'

*gRNA 4 OT1*

Forward primer: 5'- GGAGCAATACTTCCATGCTATTCATCCTG -3'

Reverse primer: 5'- CAGTGACAAGAGTGGGTTAGACG -3'

*gRNA 4 OT2*

Forward primer: 5'- AAATCTACCTCCTTAACCAAAACCCCGATC -3'

Reverse primer: 5'- ACGTCTTCATTTCCGATCAGCAGC -3'

**List of primers and probes used for qRT-PCR to quantify Cas9 mRNAs and gRNAs**

Cas9 Forward primer: 5'- GGACTCCCGGATGAACACTAAG -3'

Cas9 Reverse primer: 5'- GTTGTTGATCTCGCGCACTTT -3'

Cas9 Probe: 5'- FAM–TGGTGTCCGATTTCCGGA -3'

sgRNA Forward primer: 5'- GTTTTAGAGCTAGAAATAGCAAGTTAA -3'

sgRNA qPCR Reverse primer: 5'- AAAAGCACCGACTCGGTG -3'

sgRNA probe: 5'- FAM-CTAGTCC$^{+}$G$^{+}$T$^{+}$T$^{+}$A$^{+}$T$^{+}$CAACTTGA-IBFQ -3' ($^{+}$indicates LNA nucleotide)

GAPDH Forward primer: 5'- CTTCATTGACCTCAACTACATGGTTT -3'

GAPDH Reverse primer: 5'- TGGGATTTCCATTGATGACAAG -3'

GAPDH Probe: 5'- VIC-CAAATTCCATGGCACCGTCAAGGC -3'

Cas9 and gRNA qRT-PCR results were normalized to GAPDH mRNA levels.

**List of primers and probes used for qRT-PCR to quantify globin expression**

HBG1 and HBG2 Forward primer: 5'- CCTGTCCTCTGCCTCTGCC -3'

HBG1 and HBG2 Reverse primer: 5'- GGATTGCCAAAACGGTCAC -3'

HBB Forward primer: 5'- GCAAGGTGAACGTGGATGAAGT -3'
HBB Reverse primer: 5'- TAACAGCATCAGGAGTGGACAGA-3'
HBA Forward primer: 5'- CGGTCAACTTCAAGCTCCTAA -3'
HBA Reverse primer: 5'- ACAGAAGCCAGGAACTTGTC -3'

HBG1/2 and HBB qRT-PCR results were normalized to HBA mRNA levels and the fold change in HBG1/2 and HBB expression in edited erythroblasts was calculated in comparison to control samples.