Supplementary Materials and Methods

CD34+ cell culture

CD34+ cells from mobilized healthy donors were obtained from Fred Hutchinson Cancer Research Center, Seattle, Washington. The cells were recovered from frozen stocks and incubated for 48 hours in serum free medium (Stemspan H3000, Stemcell Technologies) supplemented with penicillin/streptomycin (Gibco™), the CC110 cytokine cocktail (Stemcell Technologies), and the small molecules StemRegenin1 (SR1, 1 µM) (Cellagen Technology) and Ly2228820 (Ly, 100 nM) (Selleckchem).

HUDEP-2 cell culture

HUDEP-2 cells were cultured in in serum free medium (Stemspan H3000, Stemcell Technologies) supplemented with penicillin/streptomycin (Gibco™), 100 ng/mL stem cell factor (SCF), 3 IU/mL erythropoietin (EPO), 10^{-6} M dexamethasone, and 1 mg/mL doxycycline. Differentiation/maturation was induced in Iscove's modified Dulbecco's medium (IMDM) containing 5% human AB serum, 100 ng/mL SCF, 3 IU/mL EPO, 10 mg/mL insulin, 330 mg/ mL transferrin, 2 U/mL heparin, and 1 mg/mL doxycyclin for 6 days.

RNP transfection

The sgRNAs with the 2′-O-methyl-3′-phosphorothioate modification at the 5' and 3' ends were obtained from Trilink . RNPs were formed after incubation of 32 µg Cas9 protein (PNA Bio) with the respective sgRNA at a molar ratio of 1:6 (unless otherwise annotated) for at least 15 minutes at room temperature, with different RNPs combined after incubation where applicable. Electroporation of CD34+ cells was carried out on an ECM 830 Square Wave Electroporation System (BTX, Holliston, MA) according to manufacturer's instructions. Briefly, after recovery in culture medium described above for 48 hours, 2×10^5 CD34+ cells were washed once with Ca^{2+}/Mg^{2+}-free PBS and resuspended in 100 µl BTXpress electroporation solution, mixed with the respective RNPs, transferred into a 0.2 cm cuvette and electroporated at 250 volts for 5 msec. After electroporation, the cells were incubated in culture medium for 48 h prior to in vitro erythroid differentiation and transplantation into NBSGW mice.

In vitro erythroid differentiation of CD34+ cells

Differentiation of human HSPCs into erythroid cells was done based on a 3 step protocol developed by Douay et al[1]. In step 1, cells at a density of 10^4 cells/ml were incubated for 7 days in a basal medium containing Iscove's modified Dulbecco's medium (IMDM), 5% human plasma, glutamine, Pen-Strep, heparin (2 IU/ml), insulin (10 µg/ml), Holo-Transferrin (330 µg/ml) supplemented with hydrocortisone (1 µM), SCF (100 ng/ml), IL-3 (5 ng/ml) and EPO (3 U/ml). In step 2, cells at a density of 10^5 cells/ml were incubated for 4 days in the same medium supplemented with SCF (100 ng/ml) and EPO (3 U/ml) and in step 3, SCF was removed and the cells were cultured at a density of 10^6 cells/ml cells for 7-10 additional days.

Intracellular flow cytometry detecting human γ-globin expression

Erythroid cells were collected at the end of erythroid differentiation and stained with CD235a (GlyA, ExBio or Dako), a surface marker of late erythropoiesis. To evaluate the HbF expression, erythroid cells were fixed in 4% para-formaldehyde followed by permeabilization in 1:1 acetone/H_2O and stained with anti-human gamma globin (51.7) from Santa Cruz. Enucleated erythroid cells were measured as the cell fraction negative for nuclear red (Thermo Fisher Scientific).

Globin HPLC

Individual globin chain levels were quantified on a Shimadzu Prominence instrument with an SPD-10AV diode array detector and an LC-10AT binary pump (Shimadzu, Kyoto, Japan). Vydac 214TP™ C4 Reversed-Phase columns for polypeptides (214TP54 Column, C4 ,300 Å, 5 μm, 4.6 mm i.d. x 250 mm) (Hichrom, UK) were used. A 38%-60% gradient mixture of 0.1% trifluoroacetic acid in water/acetonitrile was applied at a rate of 1 mL/min.

Insertions and deletions evaluation

For deep sequencing of insertion/deletions (indels), we PCR-amplified a ~250-bp region surrounding the predicted HBG cleavage site and sequenced the products using an Illumina system, as previously described[2]. Genomic DNA was isolated by QuickExtract™(Epicentre). Primary and secondary amplification was performed by AccuPrime Taq HiFi DNA Polymerase (ThermoFisher Scientific) following the manufacturer's recommendations. The primers used are presented in Supp.Table 1. PCR products were cleaned up using a QIAquick PCR Purification Kit (QIAGEN), quantified in Qubit (ThermoFisher Scientific) and loaded on a MiniSeq using Mid Output 300-cycle Reagent Kits (Illumina). Paired end reads were merged and filtered to remove

duplicate tags. Filtered reads were aligned to the respective reference sequence and evaluated for insertions and deletions (indels).

Immunofluorescence imaging of 53BP1

CD34+ cells from mock-treated and nuclease-treated samples were seeded into Poly-L-Lysine (PLL)-coated wells of a sterile 24-well plate (CellVis) and incubated for 20 minutes at RT. Cells were then fixed with 4% PFA (Polysciences Inc, #18814-10), permeabilized with 0.5% Triton X, and then incubated for 2 hours at room temperature with primary antibodies against 53BP1 (MAB3802, mouse, 1:1000 dilution) in 2% BSA/1x PBS. Subsequently, cells were washed 2x with 0.05% Tween-20 (Bio-rad, #161-0781) in PBS, and then incubated with Alexa647 secondary antibody (donkey anti-mouse, #711-166-152, Jackson Labs, 1:500 dilution) in 2% BSA/1x PBS. Lastly, cells were counterstained with DAPI (100ng/mL in 1x PBS) prior to mounting on glass slides using Prolong Gold (Molecular Probes P36930). Samples were imaged using an inverted Nikon Eclipse Ti widefield microscope equipped with a Hamamatsu ORCA-Flash CMOS camera with a 4.2-megapixel sensor and 6.5µm pixel size. 3D image stacks were acquired for all conditions using a 60x Nikon Plan Apo 1.4 NA oil objective. Acquired images were deconvolved using Microvolution software (Microvolution, CA) to minimize the effect of out-of-focus blur. Deconvolved images were processed using in-house Matlab (version 2018B, Mathworks, Natick, MA) scripts to delineate nuclei and 53BP1 protein foci within each nucleus, and count the protein foci thereafter.

ImmunoFISH based off-target profiling analysis

HUDEP2 cells were retrieved from mock-treated and nuclease-treated samples, washed twice with PBS, and resuspended in 1x PBS for a final cell density of 1 million cells per ml. 30 ul of cell solution was seeded in triplicate into Poly-L-Lysine (PLL)-coated wells of a sterile 24-well plate (CellVis) and incubated for 25 minutes at RT. For oligo-DNA FISH labeling of the HBG locus (manuscript in review), cells were then fixed with 4% PFA for 10 minutes at room temperature, washed 2x with PBS, permeabilized with 0.5% triton-PBS for 15 minutes, washed 2x with PBS, treated with 0.1M HCl for 5 minutes, washed 2X with PBS, incubated with RNaseA (25 ug/ml) for half hour at 37C, followed by denaturation in 50% formamide for another half hour at room temperature and then in hybridization buffer containing 250 nM of oligo FISH probes for 10 minutes at ~78C. Cells were subsequently incubated overnight at 37C. Post overnight hybridization, cells were washed successively with 0.2% Tween in 0.2% and 4x SSC respectively

for 5 minutes, and then labeled for 53BP1 by the standard immunofluorescence protocol detailed previously. Cells were then counterstained with DAPI for 10 minutes, and coverslipped with Prolong Gold antifade. Stained cells were imaged in 3D using a 60x 1.4 NA oil objective on a conventional widefield microscope (Nikon Ti) fitted with a Hamamatsu ORCA CMOS camera (pixel size = 6.5µm). Acquired 3D images were processed using Matlab (v2020b, Mathworks, Natick MA) scripts to delineate individual cell nuclei, FISH and 53BP1 protein foci, and additionally, estimate the extent of overlap between the FISH and 53BP1 foci in each cell.

Translocation assessment:

HUDEP2/CD34+ cells were retrieved at 72/48h post transfection from mock-treated and nuclease-treated samples, were washed twice with PBS, resuspended in 1x PBS for a final cell density of 1 million cells per ml. 30 ul of cell solution was seeded into Poly-L-Lysine (PLL)-coated wells of a sterile 24-well plate (CellVis) and incubated for 25 minutes at RT. For 2-color FISH labeling of the BCL11a and HBG loci, cells were then fixed with 3:1 Methanol:acetic acid for 10 minutes at room temperature, washed 2x with 2x Sodium Citrate (SSC), incubated with RNaseA (25 ug/ml) for half hour at 37C, followed by denaturation in 50% formamide for another half hour at room temperature and hybridization buffer containing the BCL11a and HBG FISH probes at 250 nM concentration for 10 minutes at ~78C. Cells were then incubated at 37C overnight. Post overnight hybridization, cells were washed successively with 0.2% Tween in 0.2% and 4x SSC respectively for 5 minutes, counterstained with DAPI for 10 minutes, and then coverslipped with Prolong Gold antifade. Stained cells were imaged in 3D using a conventional widefield microscope (Nikon Ti) fitted with a Hamamatsu ORCA CMOS camera (pixel size = 6.5µm). Acquired 3D images were processed using Matlab (v2020b, Mathworks, Natick MA) scripts to delineate individual cell nuclei and FISH foci, and additionally, estimate the extent of overlap between the 2 FISH foci in each cell.

Colony-forming unit (CFU) cultures

$CD34^+$ cells were plated at a density of 1500-2000 cells/ml in semi-solid methylcellulose-based medium containing cytokines, MethoCult™ H4434 (StemCell Technologies), according to the manufacturer's instructions. After 2 weeks of incubation, CFUs were classified and enumerated under a light microscope by trained operators.

Analysis of 4.9kb deletion or inversion

The 4.9kb deletion containing the HBG2 gene and the HBG2/HBG1 intergenic region was analyzed by semiquantitative PCR in HUDEP-2 cells. The KOD Xtreme™ Hot Start DNA Polymerase (Sigma) was used. The primers were Del_forward, 5'-TCCTTCTGTCATTTTGCCTCTGTT-3', and Del_reverse, 5'-ATGCACTAACATCCAACTATACAAAA-3' (indicated by red half arrows in Sup.Fig.6a). PCR cycling conditions were set according to supplier's instructions, with annealing temperature at 60^{o}C, extension time of 10 min and 25 cycles of amplification. Genomic DNA isolated from untreated HUDEP-2 cells (WT) and a HDAd-HBG-CRISPR-transduced HUDEP-2 clone with a bi-allelic 4.9kb deletion were mixed at various ratios and used as template for PCR. WT product was 9.9kb, and a 5.0kb product would indicate a 4.9kb deletion. The signals were quantified by ImageQuant. The percentage of 4.9k deletion used in PCR template was plotted against the percentage of the 4.9kb deletion signal in the product to build a standard curve. The percentage of 4.9kb deletion in samples was calculated using the curve formula generated by Excel. To detect a 4.9kb inversion, we used the following primers: Inversion_forward 5'-GATTTCTCCTGCACCTTTTACTC-3', and Inversion_reverse, 5'-AAGTGTCTTTACTGCTTTTATTTGCT-3' (indicated by green half arrows in Sup.Fig.7a). The primers were validated using a synthesized gBlock as a positive control. A 3.9kb product would indicate a 4.9kb inversion.

For more precise 4.9kb deletion analysis in CD34+ cells we used a method developed by Métais et.al. [4]. Libraries for NGS were made using a 2-step PCR protocol, as described above. Evaluation of the frequency of HBG2 deletion was based on a single nucleotide difference at cDNA position 410 (G>C for HBG2>HBG1, exon 3).

Animal studies

*Xenotransplantation experiments***.** The immunodeficient NOD.Cg-KitW-41J Tyr+ Prkdcscid Il2rgtm1Wjl/ThomJ (NBSGW) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). For HSC transplantation, ex vivo edited 1x10^{6} CD34^{+} cells from healthy donors injected intravenously into NBSGW recipient mice at 48 hours post transfection, without any conditioning. Non-transfected CD34^{+} cells were used as controls. Sixteen weeks post transplantation, NBSGW mice were sacrificed and bone marrow cells were collected, for assessment of multilineage engraftment, HbF expression and evaluation of editing levels.

Mobilization and in vivo editing of thalassemic CD34⁺ cells in a humanized NBSGW mouse model.
A humanized model of thalassemic hemopoiesis was generated by transplanting $CD34^+$ cells from a thalassemia major patient (β^0/β^0) into unconditioned NBSGW mice ($1x10^6$/recipient). Six weeks post transplantation, the mice, having a predominantly human bone marrow chimerism, were mobilized by a 7-day mobilization scheme, including G-CSF 250μg/kg ip (days 1-6) and Plerixafor 5mg/kg ip (former AMD3100 - Mozobil, Genzyme Corp, Cambridge, MA) (days 5-7), as previously described[5]. Sixteen and two hours before Ad-dualCRISPR vector iv injection ($4x10^{10}$ viral particles in two doses, 30 min apart), the animals received Dexamethasone (10 mg/kg) i.p. Nine weeks post *in vivo* transduction, human $CD235a^+$ cells were isolated from the bone marrow and analyzed for HbF expression, editing levels and morphological characterization.
Secondary transplantations were performed as previously described[5], where bone marrow from four long bones of the primary recipients was isolated and after lysis of red blood cells and selection of hDC45+ cells, $2x10^6$ cells were injected in each secondary NSGBW recipient.
All experiments involving animals were conducted in accordance with the institutional guidelines set forth by the University of Washington. The University of Washington is an Association for the Assessment and Accreditation of Laboratory Animal Care International (AALAC)–accredited research institution and all live animal work conducted at this university is in accordance with the Office of Laboratory Animal Welfare (OLAW) Public Health Assurance (PHS) policy, USDA Animal Welfare Act and Regulations, the Guide for the Care and Use of Laboratory Animals and the University of Washington's Institutional Animal Care and Use Committee (IACUC) policies. The studies were approved by the IACUC of the University of Washington (Protocol No. 3108-01) and the George Papanicolaou Hospital (Protocol No. 477957/4580).

T7E1 nuclease assay

Genomic DNA from individual CD34+ cell derived colonies was isolated using a QIAamp DNA Mini Kit (Qiagen). PCR to amplify targeted sites (erythroid bcl11a enhancer or HBG1/2 promoter) was performed for 30-35 cycles using Q5 High-Fidelity DNA Polymerase and the respective set of primers: BCL11A forward, 5'-AGAGAGCCTTCCGAAAGAGG-3', reverse, 5'-GGCAGCTAGACAGGACTTGG-3'; HBG1/2 forward, 5'-CAGGGTTTCTCCTCCAGCATCTTCCACAT-3', reverse, 5'-AGCAGCAGTATCCTCTTGGGG-3'. PCR products was reannealed in NEB2 Buffer and were treated with 10 Units of T7 Endonuclease I (T7EI, New England Biolabs) at 37 °C for 20 min. Reactions were stopped by the addition of EDTA and were resolved by 10% TBE PAGE (Biorad) and stained with ethidium

bromide. 100 bp DNA Ladder (New England Biolabs) were used. Band intensity was analyzed using ImageJ software. % cleavage = (1-sqrt(parental band/(parental band + cleaved bands)) × 100%.

HDAd5/35++ vectors

pBST-sgBCL11AE-miR, pBST-sgHBG1-miR and pBST-sgScr-miR plasmids containing all-in-one CRISPR/Cas9 components targeting the erythroid bcl11a enhancer (5'-CACAGGCTCCAGGAAGGGTT-3'), HBG1 promoter (5'-CAAGGCTATTGGTCAAGGCA-3'), as well as a non-targeting scrambled control sgRNA (5'-ATCGTTTCCGCTTAACGGCG-3'), respectively, were previously described[6]. The 350bp U6-sgBCL11AE sequence was amplified from pBST-sgBCL11AE-miR and inserted into the *AscI* site of pBST-sgHBG1-miR, generating pBST-sgHBG1-sgBCL11AE-miR. To obtain the recombinant adenoviral plasmids, the whole CRISPR cassettes (~8kb) starting from the U6 promoter to the SV40 polyA signal sequence were amplified from the above pBST constructs and ligated with *NheI-XmaI* digested pHCA by Gibson assembly (New England Biolabs), generating the corresponding pHCA-sgBCL11AE-miR, pHCA-sgHBG1-miR, pHCA-sgScr-miR, and pHCA-sgHBG1-sgBCL11AE-miR plasmids. All constructs were confirmed by *HindIII* and *BamHI* digestion as well as sequencing.

For the production of HDAd5/35++ vectors, corresponding plasmids were linearized with *PmeI* and rescued in 116 cells[7] with AdNG163-5/35++, an Ad5/35++ helper vector containing chimeric fibers composed of the Ad5 fiber tail, the Ad35 fiber shaft, and the affinity-enhanced Ad35++ fiber knob[8]. HDAd5/35++ vectors were amplified in 116 cells as described in detail elsewhere[7]. Helper virus contamination levels were found to be <0.05%. Titers were 6-12×10^{12} vp/ml.

Transduction of HUDEP-2 cells and erythroid differentiation

HUDEP-2 cells were grown in StemSpan H3000 (STEMCELL Technologies) supplemented with SCF (100 ng/ml), EPO (3 IU/ml), dexamethasone (10^{-6} M) and doxycycline (1 μg/ml). Cells transduced at various MOIs with adenoviral vectors in low attachment plates for 48 hours and maintained at a concentration of 2.5-5x10^{5} cells/ml for 5 days post transduction. Erythroid differentiation was induced in IMDM (Gibco™) containing 5% human AB serum, SCF (100 ng/mL), EPO (3 IU/mL), Insulin (10 μg/mL), Holo-Transferrin (330 μg/mL), Heparin (2 U/mL) and doxycycline (1 µg/mL) for 6 days.

Transduction of thalassemic CD34$^+$ cells

CD34$^+$ cells from thalassemic patients (previously collected during mobilization clinical trials conducted at G.Papanikolaou Hospital, Thessaloniki, Greece[9,10]) were recovered from frozen stocks and cultured in serum free medium, completed with cytokines as described previously[11]. Thalassemic CD34$^+$ cells were transduced with the Ad-dualCRISPR vector in low attachment plates for 48 hours, at total MOI 4000 and cultured in erythroid differentiation- and methylcellulose-based medium.

Surface staining

To evaluate the HSC phenotype post double-editing, cells were washed and stained with the following fluorochrome-conjugated antibodies: CD34-APC (BD Biosciences), CD38-PE (BD Biosciences) and CD90-PerCP (ExBIO). To assess the multilineage engraftment of CD34$^+$ cells in the bone marrow of NBSGW mice post transplantation, the following antibodies were used: CD45-PerCP (BD Biosciences), CD19-APC (BD Biosciences), CD3-FITC (ExBIO), CD33-PE (ExBIO), CD235a-FITC or CD235a-PE (ExBIO). After wash, cells were resuspended in FACS buffer and analyzed using a FACS-Calibur (BD Biosciences, San Jose, CA). To measure the percentage of HSPCs in peripheral blood of NBSGW mice post mobilization, the following antibodies were used: murine HSPCs: mouse Lineage Cocktail-APC, CD117-PE, LY-6A/E (sca-1)-PerCP (BD Biosciences, San Jose, CA); human HSPCs: CD45-PerCP, CD34-APC, CD46-FITC (BD Biosciences, San Jos bvyie, CA). Debris was excluded using a forward scatter-area and sideward scatter-area gate. Flow cytometry data were then analyzed using FlowJo (version 10.0.8, FlowJo, LLC).

Cytospin slide preparation

Cytospins of 0.3-0.5x10^5 cells were prepared during erythroid differentiation, by cytocentrifugation (ROTOFIX 32, Hettich Zentrifugen) at 500 rpm for 5 minutes. Cytospins were air dried and then stained with Giemsa/May-Grünwald (Merck, Darmstadt, Germany) for 8 and 3 minutes, respectively.

Analysis of Reactive Oxygen Species (ROS) levels

Intracellular ROS levels from erythroid precursors were determined using the General Oxidative Stress Indicator CM-H2DCFDA (#C6827, LifeTechnologies), according to the manufacturer's instructions. Briefly, thalassemic CD34$^+$ cells were incubated with 10μM CM-H2DCFDA in PBS

at 37°C for 1 hour and washed twice with PBS, before analysis. Oxidation of the probe can be detected by the increase of fluorescence (GFP) with flow cytometry.

Magnetic Cell Sorting (MACS)

The human CD235a cells from chimeric bone marrow, were isolated using a CD235a-FITC antibody and the anti-FITC Microbeads (Miltenyi Biotec, San Diego, CA) according to the manufacturer's instructions. The positive fraction was used for analysis of γ-globin mRNA expression post in vivo editing.

Quantitative RT-PCR

Total RNA was extracted from CD235a$^+$ cells (post MACS separation) by using RNA extraction Kit (Qiagen, cat#), following manufacturer's instructions. Quantitect reverse transcription kit (Qiagen, cat# 205311) and power SYBRTM green PCR master mix (Thermo Fisher Scientific, cat# 4367659) were used. Real time quantitative PCR was performed on 7500 Real-Time PCR system (Applied Biosystems). The following primer pairs were used in this work: huActin-b forward, 5'-CCTGCAGAGTTCCAAAGGAGA-3', and reverse, 5'-AGAAAATCTGGCACCACACC-3'; human γ-globin forward, 5'-GTGGAAGATGCTGGAGGAGAAA-3', and reverse, 5'-TGCCATGTGCCTTGACTTTG-3'; human β-globin forward, 5'-GGTGCCCTTGAGGTTGTC-3', and reverse, 5'-ATGAAGTTGGTGGTGAGGC-3'.

Karyotype

Cytogenetic analyses were conducted on unstimulated 0,4-8x106 differentiating erythroblasts on days 7 and 11 of erythroid differentiation culture. Cells were cultured in RPMI 1640 medium with 20% fetal calf serum and incubated at 37 °C, in 5% of CO2, for 24h. Then, 0.10 g/mL colcemid was added and chromosome preparation and G-banding staining performed according to standard protocols. At least twenty metaphases were studied per condition. A clonal cytogenetic abnormality was defined as the same numerical gain or structural abnormalities in at least 2 metaphases or the same numerical loss in at least 3 metaphases, according to the ISCN 2016 criteria (ISCN 2016, An International System for Human Cytogenomic Nomenclature, Karger, Editors: J. McGowan-Jordan, A. Simons, M. Schmid).

References

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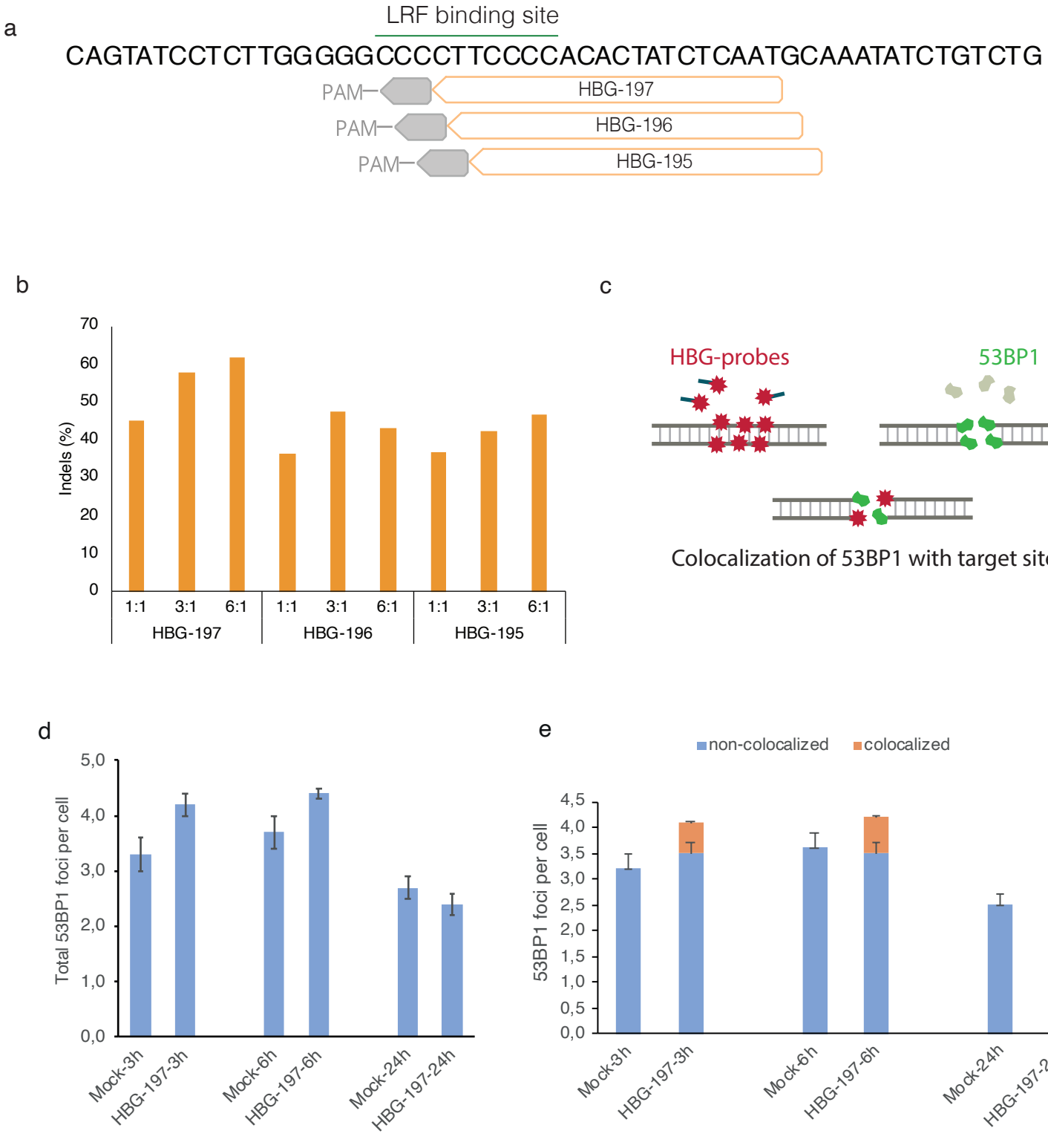
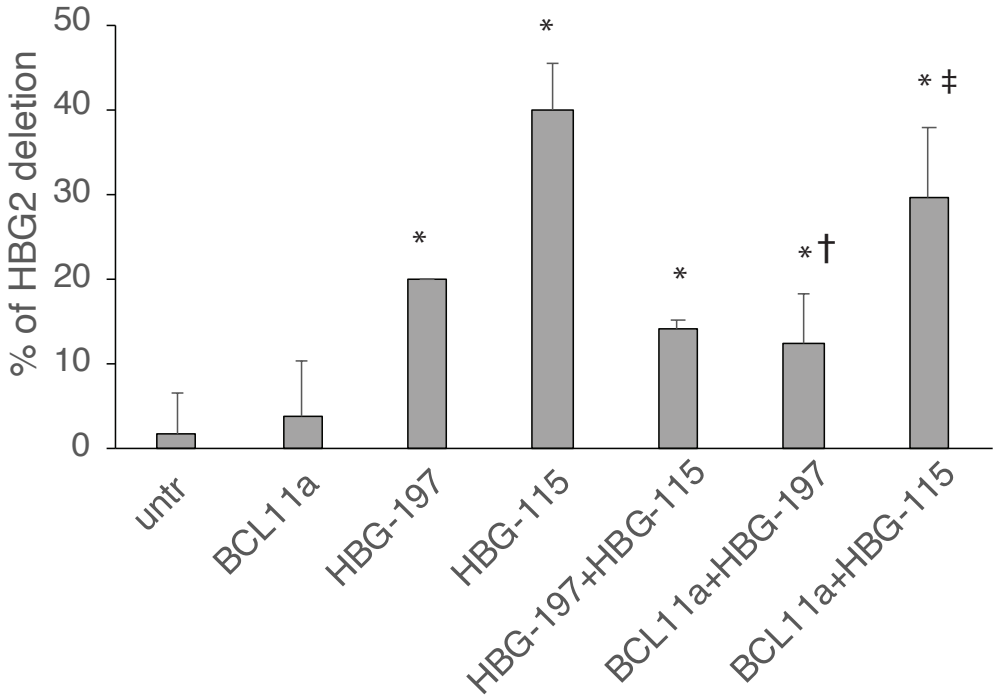
11. Karponi G, Psatha N, Lederer CW, et al. Plerixafor+G-CSF–mobilized CD34+ cells represent an optimal graft source for thalassemia gene therapy. *Blood*. 2015;126:616–619.



Supp.Figure 1. a. Three gRNAs target the LRF binding sites in positions HBG-195, HBG-196 and HBG-197. b. Evaluation of editing efficiency with the three gRNAs targeting the LRF binding site 3 days post transfection. c. Evaluation of potential off-target effects by assessing the colocalized and non-colocalized signal of 53BP1 and HBG-200 site. d. HUDEP-2 cells were transfected with the HBG-197 RNP. Signal for 53BP1 and HBG-200 probes was analyzed in 750 cells in 3 different time points post transfection. e. Separation of non colocalized (blue) and colocalized (orange) signal in the three time points.

a



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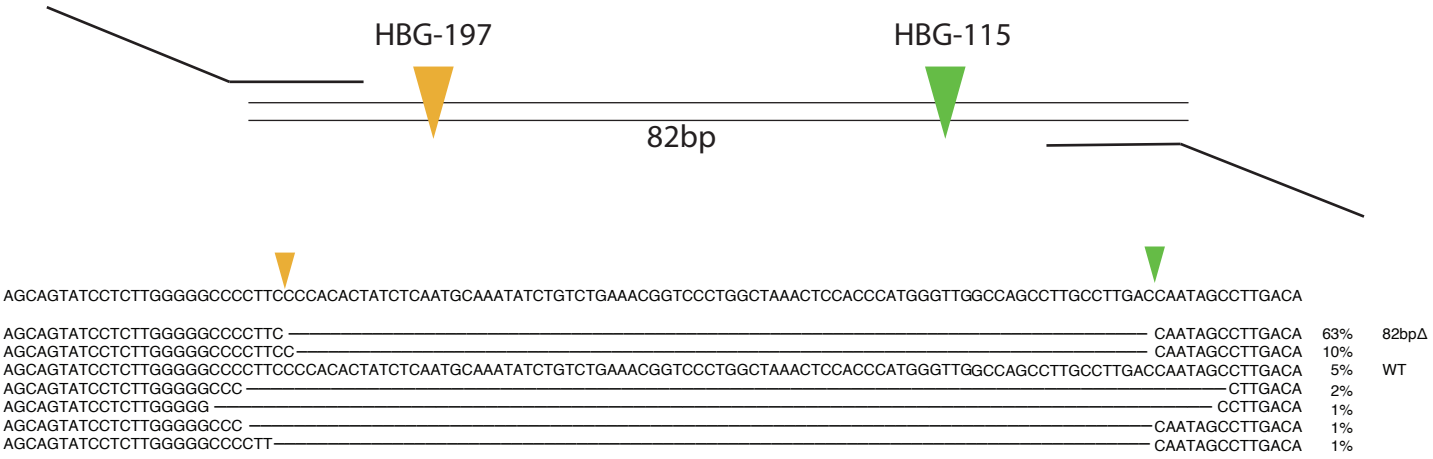
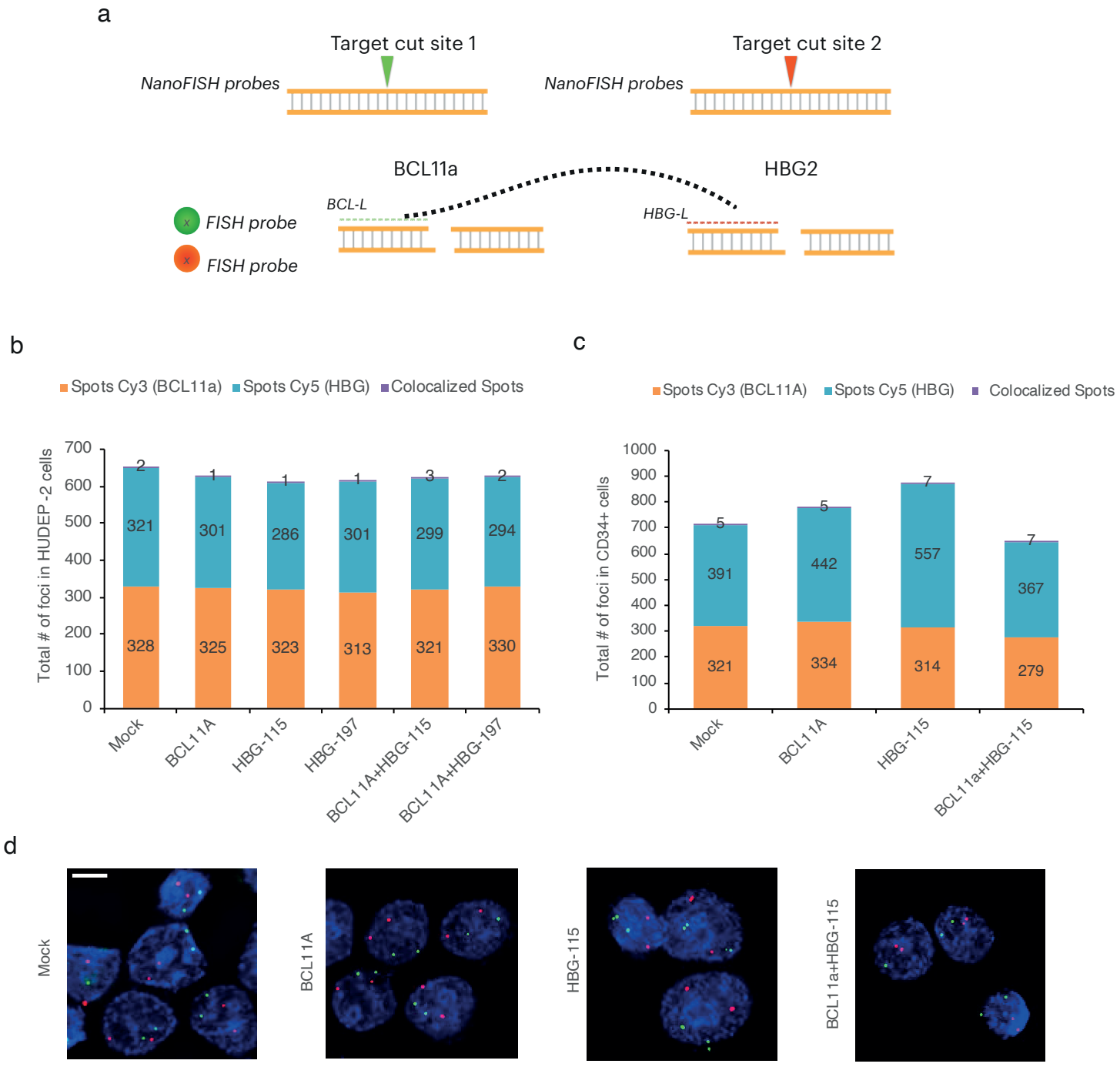
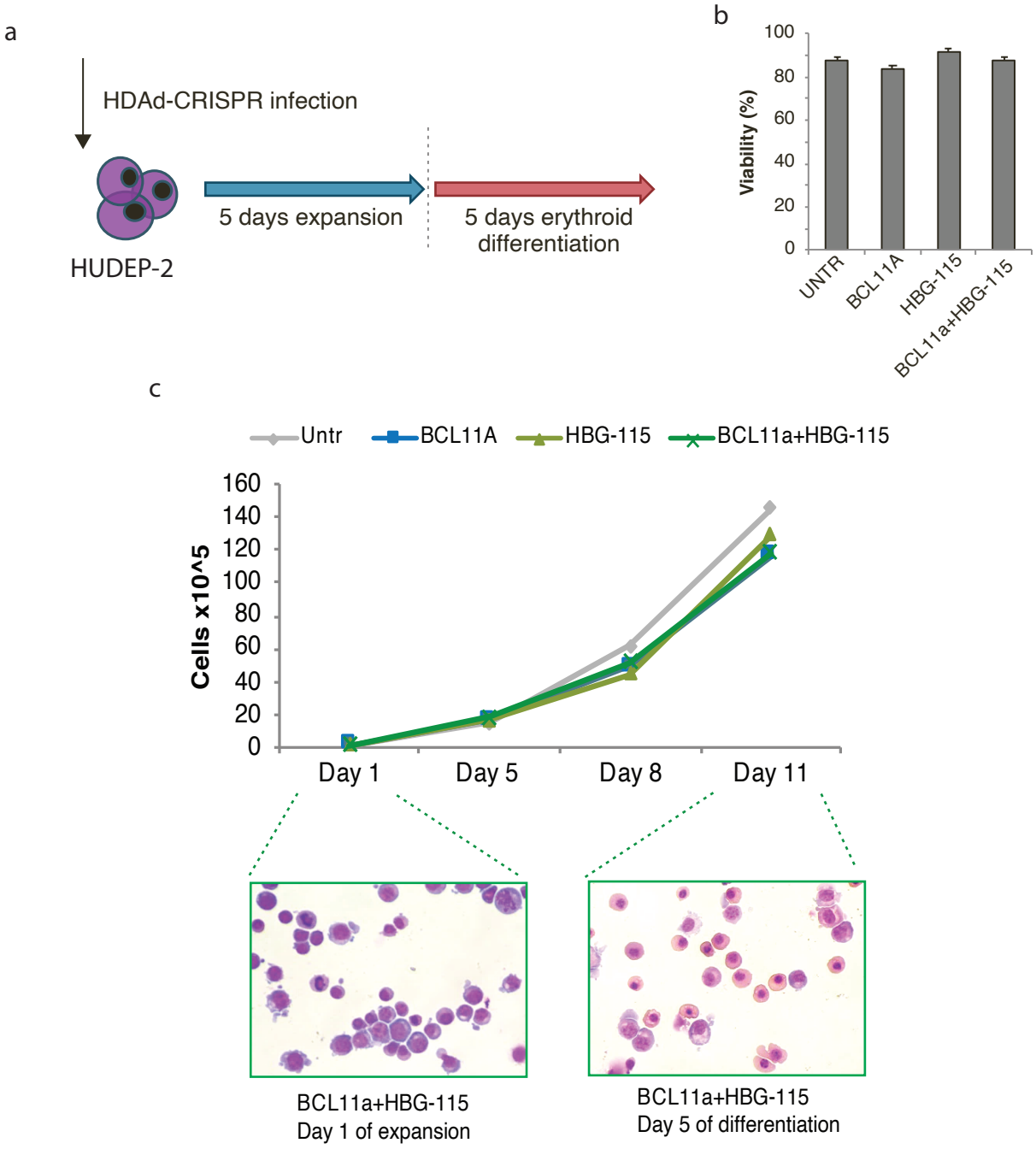


Supp.Figure 2.. HBG2 deletion detected by deep sequencing, presented as imbalance of the HBG2/HBG1 ratio. b. Targeting HBG-197 and HBG-115 simultaneously creates an 82bp deletion in the majority of alleles.



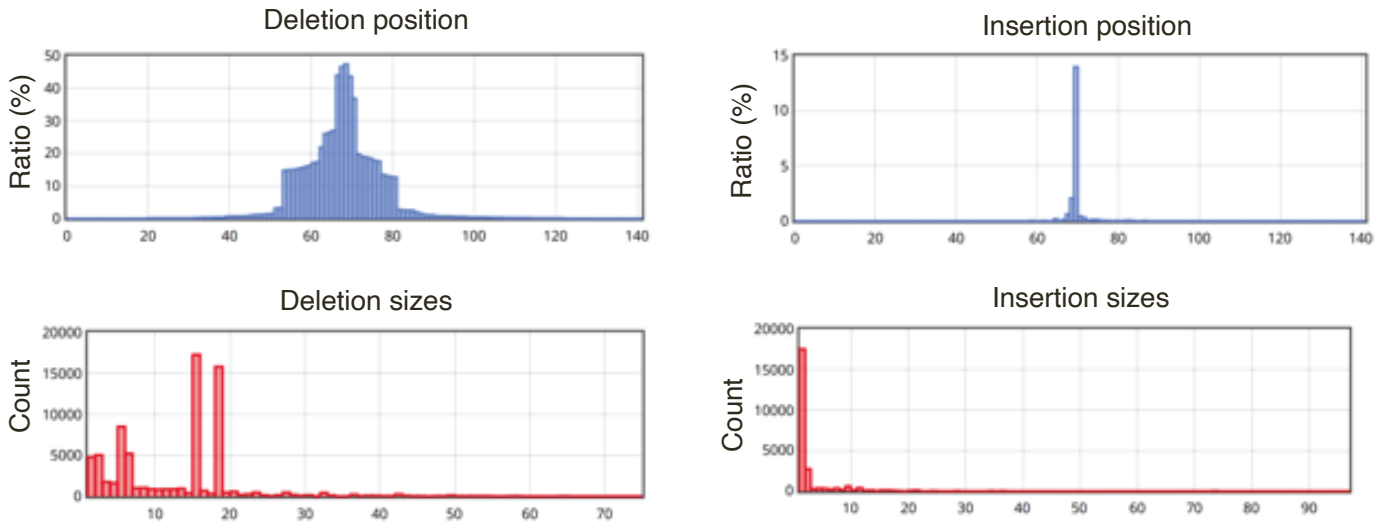
Supp.Figure 3. Oligo-FISH based translocation analysis. a. Experimental design. NanoFISH probes were designed for thetwo targeted sites. In case of translocation events the two signals should be colocalized. b. Total numbers of spots per region and colocalized spots in mock transfected and edited cells with all described RNPs and RNP cominations in HUDEP-2 cells, 72 hours post transfection. c. Total number of spots in CD34+ cells 48 hours post transfection. d. Representative pictures of CD34+ cells in 5um scale. 200 nuclei were evaluated per condition.



Supp.Figure 4. a. HUDEP-2 cells were transduced with the single or dual HD-Ad-CRISPR vectors during their expansion phase. Cell viability, growth and HbF levels were assayed throughout expansion and differentiation. b. Viability of HUDEP-2 cells after transduction with HD-Ad-CRISPR (single or dual) at MOI of 500. c. Cell growth throughout expansion and differentiation. HD-Ad-dualCRISPR transduced cells grow, differentiate and mature efficiently, identically to the control samples.

Day 3 HUDEP population infected with Ad-dualCRISPR, Indels for HBG = 72.7%

DELETIONS:		# of reads	% of reads
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTCAAGGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	WT	31687	24.3%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTC-AGGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-1	905	0.7%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTCAA-GCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-1	2690	2.1%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTC--GGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-2	765	0.6%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTCA--GCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-2	2841	2.2%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTCAAG--AAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-2	265	0.2%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTC---GCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-3	1021	0.8%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGT----GCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-4	624	0.5%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGT-----CAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGAAC	-5	6375	4.9%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTG-----GGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-5	896	0.7%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATT------GGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-6	3216	2.5%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATT------GTCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-6	365	0.3%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTCAA------GCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-6	375	0.3%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATT-------GCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-7	394	0.3%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTAT-------GGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-7	324	0.2%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTAT--------GCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-8	430	0.3%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCT---------GGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-9	213	0.2%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTA---------GCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-9	327	0.3%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATT----------GGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-11	239	0.2%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGG----------TGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-12	246	0.2%
ACTATTGGTCAAGTTTGCCTT-------------GTCAAGGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-13	583	0.4%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGT---------------CAACCCATGGGTGGAGTTTAGCCAGGAAC	-15	3797	2.9%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTAT---------------TGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-15	4222	3.2%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGT---------------CAACCCATGGGTGGAGTTTAGCCAGGGAC	-15	7489	5.7%
ACTATTGGTCAAGTTTGCCTT------------------GGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-18	688	0.5%
ACTATTGGTCAAGTTTGCCTTGT------------------CAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	-18	13156	10.1%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGT--------------------CATGGGTGGAGTTTAGCCAGGGAC	-20	362	0.3%
INSERTIONS:			
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTCAAGGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	WT		
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTCAAAGGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	+1	429	0.3%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTCAAGGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	+1	14547	11.1%
ACTATTGGTCAAGTTTGCCTTGTCAAGGCTATTGGTCAAGAGGCAAGGCTGGCCAACCCATGGGTGGAGTTTAGCCAGGGAC	+2	2318	1.8%



Supp.Figure 5. Analysis of insertions and deletions in the HBG1/2 promoter in the Ad-dualCRISPR transduced cells.

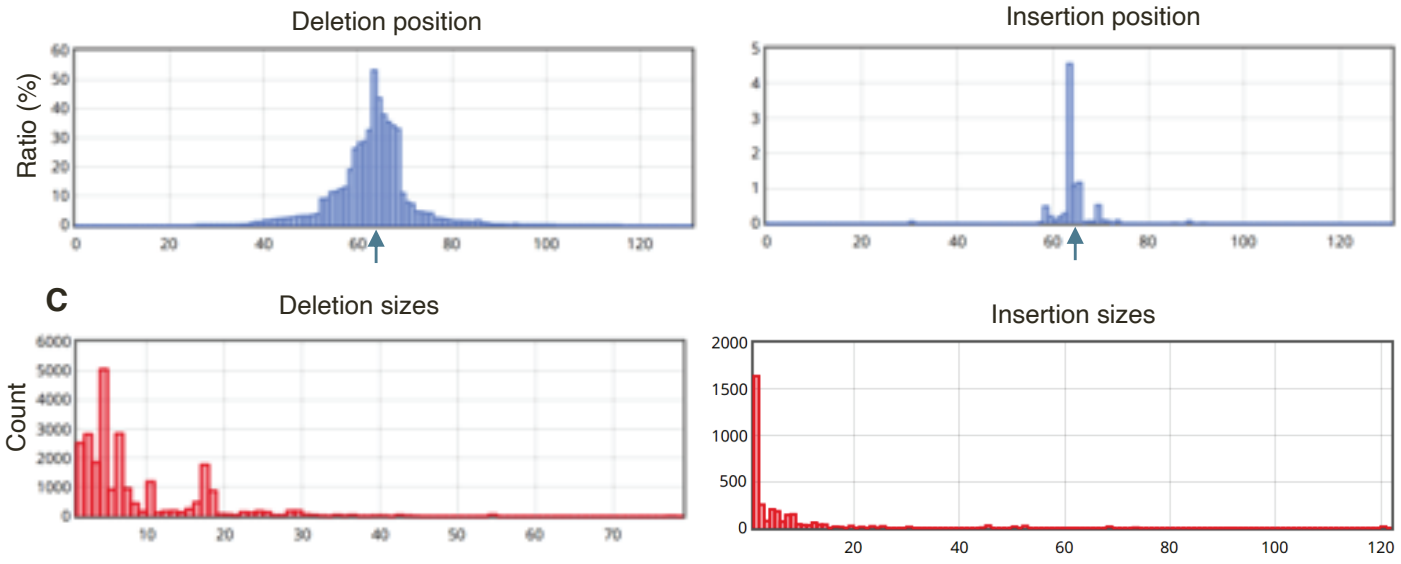
Day 3 HUDEP population infected with Ad-dualCRISPR, Indels for BCL11a-e = 84.1%

DELETIONS:

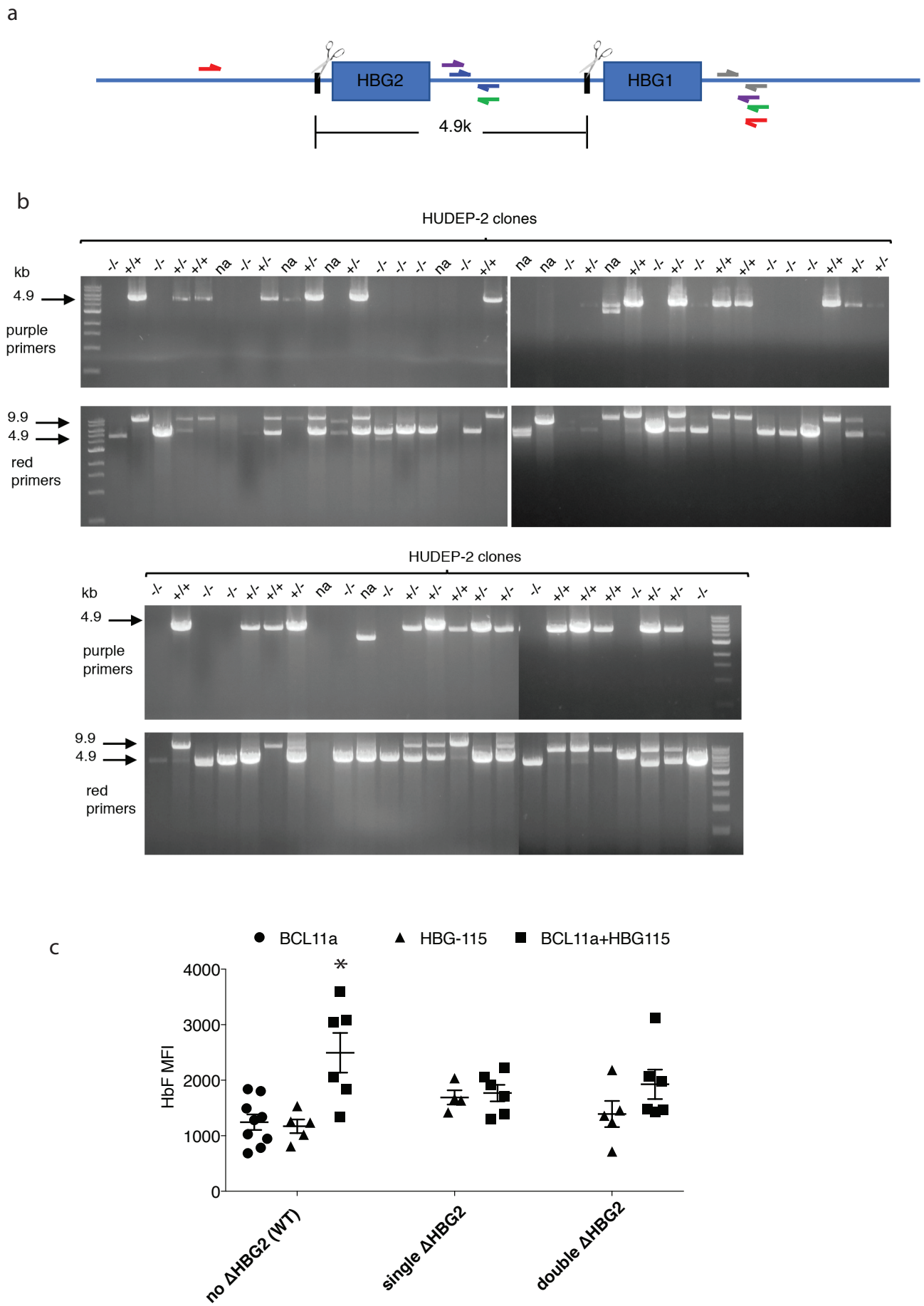
		# of reads	% of reads
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAAGGGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	WT	5561	15.9%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAA-GGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-1	1536	4.4%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAA-AGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-1	81	0.2%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGA--GGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-2	297	0.9%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAA--GTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-2	1802	5.2%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAG---GGGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-3	67	0.2%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGA---GTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-3	1232	3.5%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCC----AGGGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-4	1402	4.0%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGA----TTAGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-4	408	1.2%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAAGG----AGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-4	1906	5.5%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCA-----GGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-5	477	1.4%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGA-----TTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-5	80	0.2%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAA------GGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-6	1725	4.9%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCA------GTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-6	335	1.0%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCC-------GTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-7	123	0.4%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAA-------GCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-7	425	1.2%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAAGG-------CTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-7	67	0.2%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGA--------GCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-8	165	0.5%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCA----------GGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-10	851	2.4%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAAG----------GATTAGGGTGGGGGCGTGGGTGGGGTAGA	-12	63	0.2%
TGCAAGCTAACAGTTGCTTTTATCACA----------GTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-13	65	0.2%
TGCAAGCTAACAGTTGCTTTTATCACAGGCT----------CCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-15	148	0.4%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAG----------GATTAGGGTGGGGGCGTGGGTGGGGTAGA	-16	293	0.8%
TGCAAGCTAACAGTTGCTTTTATCACA----------GGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-17	1212	3.5%
TGCAAGCTAACAGTTGCTTTTATCACAGG----------CTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-18	528	1.5%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCC----------AGGGTGGGGGCGTGGGTGGGGTAGA	-22	62	0.2%
TGCAAGCTAACA----------GTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	-28	102	0.3%

INSERTIONS:

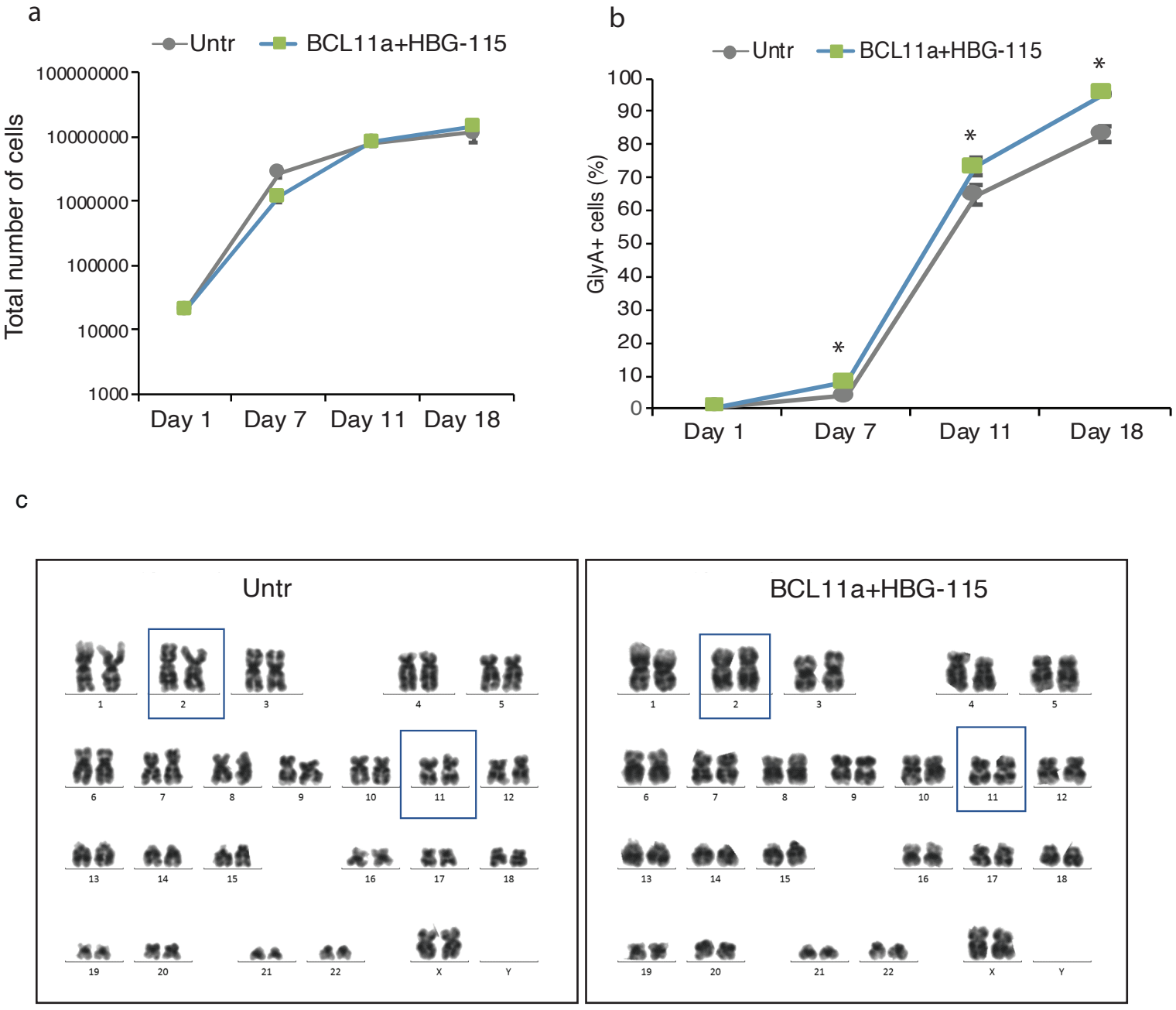
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAAGGGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	WT		
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAAGGGGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	+1	1233	3.5%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAAGGGGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	+2	100	0.3%
TGCAAGCTAACAGTTGCTTTTATCACAGGCTCCAGGAAGGAAGGGTTTGGCCTCTGATTAGGGTGGGGGCGTGGGTGGGGTAGA	+4	135	0.4%



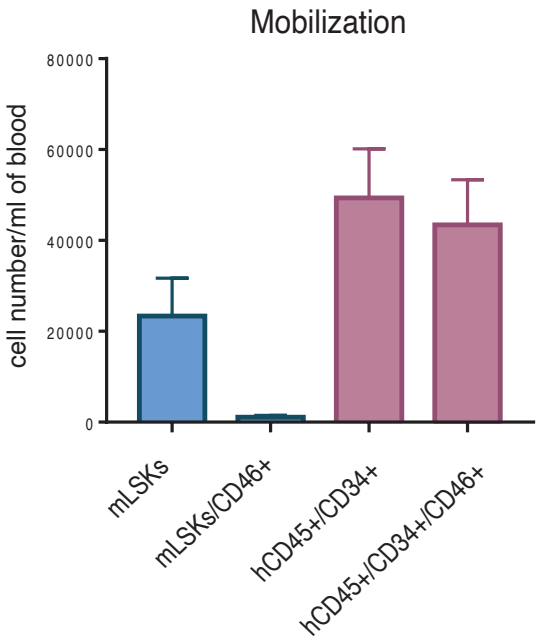
Supp.Figure 6. Analysis of insertions and deletions in the BCL11a enhancer in the Ad-dualCRISPR transduced cells.



Supp.Figure 7. Semi-quantitative PCR to identify HUDEP-2 clones with HBG2 (4.9kb) deletion. a. Primer position and experimental design. b.PCR showing deletion of one (+/-) or both (-/-) HBG2 alleles. c. HbF expression as mean fluorescence intensity (MFI) in HUDEP-2 clones transduced with one of the two HD-Ad-singleCRISPR (BCL11a, HBG-115) or the HD-Ad-dualCRISPR vector and separated based on presence of both (WT), one (Single ΔHBG2) or no (double ΔHBG2) HBG2 genes.



Supp.Figure 8. a. Cell growth during erythroid differentiation of edited and unedited thalassemic cells. b. Frequency of GlyA+ cells in different time points during erythropoiesis. c. Karyotype of untreated (Untr) and HD-Ad-dualCRISPR treated (BCL11a+HBG-115) thalassemic cells.



Supp.Figure 9. Efficiency of G-CSF+AMD3100 mobilization in NSGW mice xenotransplanted with β0/β0 thalassemic cells.

Patient	Genotype	Phenotype	Mobilization scheme
P01	CD39/IVSI-110	β^0/β^+	G-CSF+Plerixafor
P02	CD39/CD39	β^0/β^0	G-CSF
P03	IVSI-110/IVSI-110	β^{+}/β^+	Plerixafor

Supp.Table 1

Miniseq Primers				
BCL11A enhancer	Forward	5'	ACACGACGCTCTTCCGATCTNNNNGTCTGCCAGTCCTCTTCTACC	3'
	Reverse	5'	GACGTGTGCTCTTCCGATCTNNNNCACACCAGGGTCAATACAACTT	3'
HBG-197	Forward	5'	ACACGACGCTCTTCCGATCTNNNNAGAAAAATTGGAATGACTGAATCGG	3'
	Reverse	5'	GACGTGTGCTCTTCCGATCTNNNNCCTTGTCAAGGCTATTGGTCAAGG	3'
HBG-115	Forward	5'	ACACGACGCTCTTCCGATCTNNNNCCCTTCCCCACACTATCTCAATG	3'
	Reverse	5'	GACGTGTGCTCTTCCGATCTNNNNCGTCTGGACTAGGAGCTTATTGATAACC	3'
HBG-197 to HBG-115	Forward	5'	ACACGACGCTCTTCCGATCTNNNNAGCAGTATCCTCTTGGGG	3'
	Reverse	5'	GACGTGTGCTCTTCCGATCTNNNNTCTAAGACTATTGGTCAAGTTTGC	3'
ΔHBG2	Forward	5'	ACACGACGCTCTTCCGATCTNNNNGGTGGAAGCTTGGTGTGTAG	3'
	Reverse	5'	GACGTGTGCTCTTCCGATCTNNNNAAAGCCTATCCTTGAAAGCTCTG	3'

Supp.Table 2