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Supplemental information

Novel lentiviral vectors for gene therapy of sickle cell disease combining gene addition and gene silencing strategies

Mégane Brusson, Anne Chalumeau, Pierre Martinucci, Oriana Romano, Tristan Felix, Valentina Poletti, Samantha Scaramuzza, Sophie Ramadier, Cecile Masson, Giuliana Ferrari, Fulvio Mavilio, Marina Cavazzana, Mario Amendola, and Annarita Miccio

Figure S1

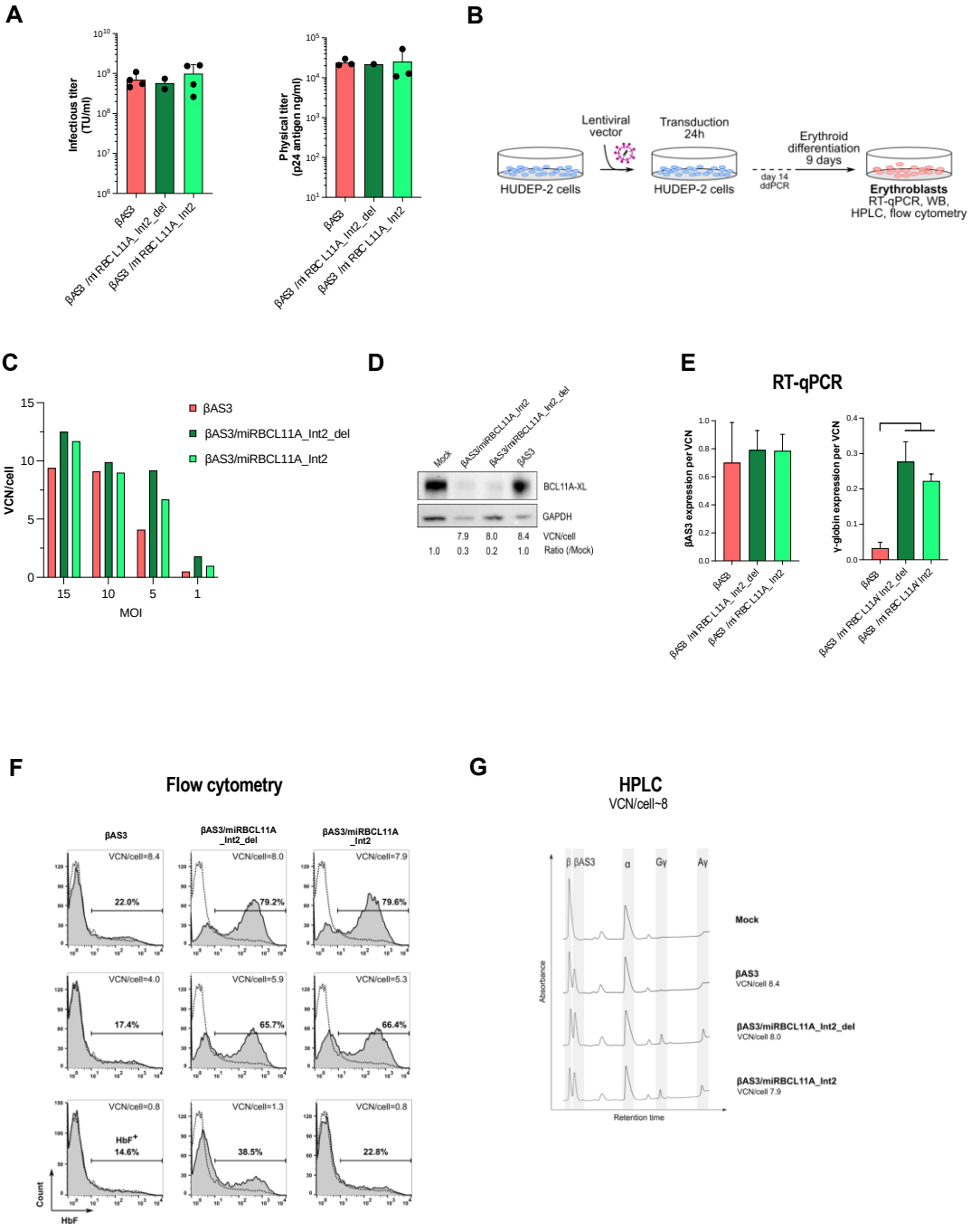

Figure S1: βAS3/miRBCL11A LV reactivates HbF in HUDEP-2 cells.

(A) Infectious [Transduction Unit (TU)/ml] and physical titers of the bifunctional (βAS3/miRBCL11A-LVs) and control (βAS3-LV) vectors. Infectious titers were measured on HCT116 cells, 4 days after transduction (n=1-3 independent LV production). **(B)** HUDEP-2

cells were transduced at increasing MOI (1, 5, 10 and 15) for 24h. VCN/cell was measured 14 days after transduction by ddPCR. Mock- or LV-transduced cells were differentiated into mature erythroblasts for 9 days to evaluate *BCL11A-XL* and globin expression by RT-qPCR, WB, HPLC and flow cytometry. **(C)** Gene transfer efficiency (VCN/cell) obtained for each MOI tested in HUDEP-2 cells transduced with the βAS3-, or βAS3m/miRBCL11A_Int2_del or _Int2 LV vectors. VCN/cell was measured 14 days after transduction. **(D)** BCL11A-XL expression measured by western blot in mock- and LV-transduced HUDEP-2 cells during differentiation. Protein levels were normalized to GAPDH (n=1). **(E)** βAS3- (left panel) and γ-globin (right panel) mRNA levels were measured by RT-qPCR in LV-transduced HUDEP-2 cells after 9 days of differentiation (n=3-4 independent biological replicates for the 3 LVs). Globin mRNA levels were normalized to *HBA* expression. We plotted βAS3-globin and γ-globin mRNA levels per VCN. No significant statistical difference in βAS3 expression was observed between the 3 LVs, while γ-globin mRNA levels were significantly higher in βAS3/miRBCL11A_Int2_del- and βAS3/miRBCL11A_Int2-transduced cells than in βAS3-transduced samples). No significant statistical difference was observed between βAS3/miRBCL11A transduced samples (One-way ANOVA test; *** $P<0.001$ and ns, not significant). **(F)** Flow cytometry analysis of HbF expression in CD235a^{high} HUDEP-2 cells after 9 days of differentiation [mock sample: dotted line (n=1); LV-transduced samples as indicated: solid line (n=3 independent biological replicates per LV)]. **(G)** RP-HPLC chromatographs of globin expression in mock- and LV-transduced cells (VCN/cell~8).

Figure S2

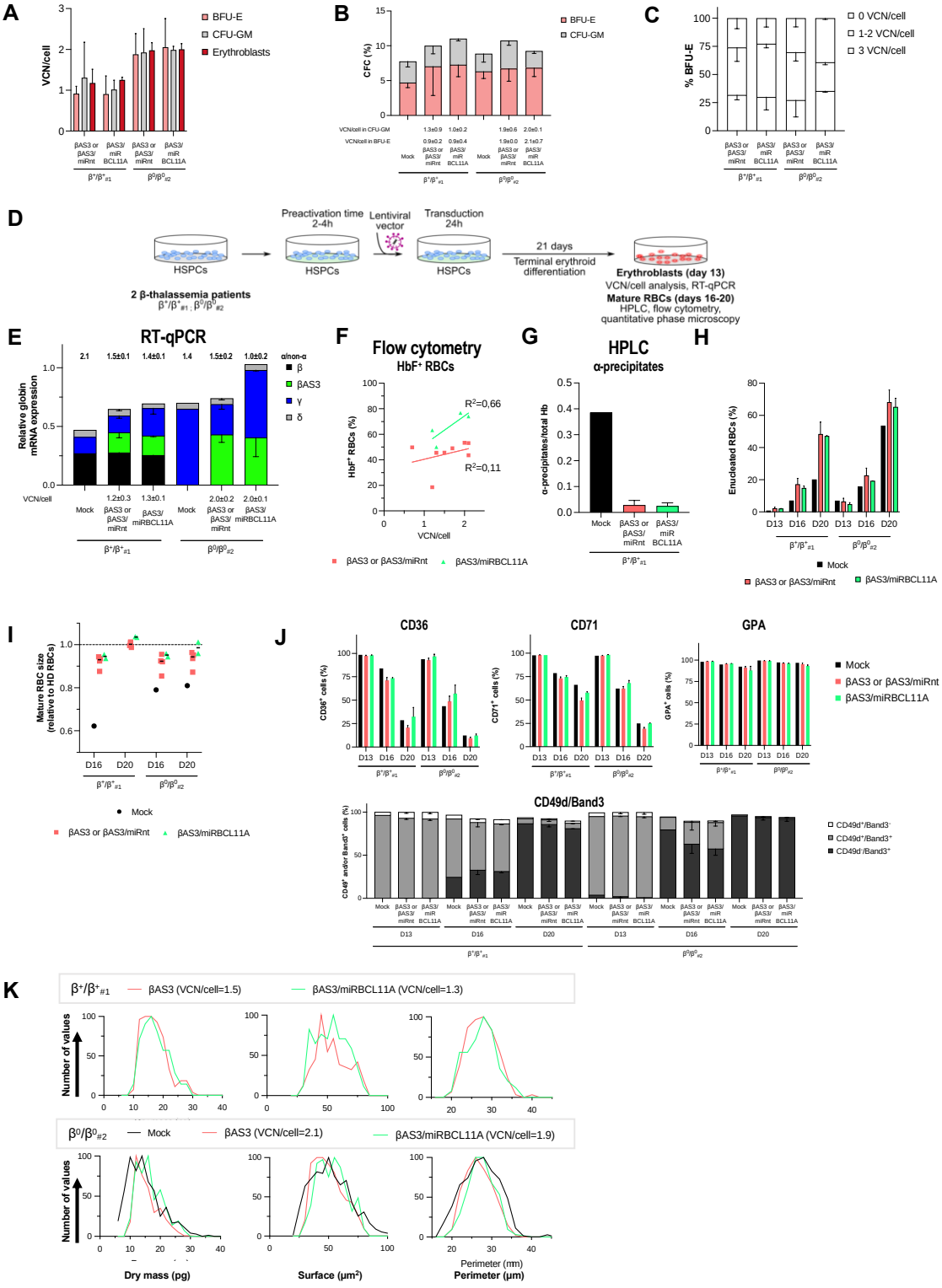

Figure S2: Evaluation of βAS3/miRBCL11A LV efficacy in HSPC-derived erythroblasts from β-thalassemia donors.

(**A-C**) Mobilized peripheral blood HSPCs from two β-thalassemia donors (β^+/β^+ #1 and β^0/β^0 #2) were either not transduced (Mock; n=1 per donor) or transduced with control (βAS3 or βAS3/miRnt; n=4 independent biological replicates per donor) or bifunctional (βAS3/miRBCL11A; n=2 independent biological replicates per donor) vectors at different MOI for 24 hours. After transduction, cells were plated in clonogenic cultures to evaluate BFU-Es and CFU-GMs after 14 days, or differentiated in erythroid precursors. VCN/cell were measured 14 days after transduction by ddPCR in erythroblasts, BFU-E and CFU-GM pools or in single BFU-Es. βAS3- and βAS3/miRnt-transduced samples were pooled in all the analyses. **(A)** Average VCN/cell (mean±SD) in pools of BFU-Es or CFU-GMs, and in erythroblasts. **(B)** Frequency BFU-Es or CFU-GMs in mock- and LV-transduced HSPCs. Results are represented as % of colonies obtained from 500 plated HSPCs and shown as mean±SD. ns, two-way ANOVA test. **(C)** Clonal analysis of VCN/cell (mean±SD) in single BFU-Es (n=14 for βAS3 and 24 for βAS3/miRBCL11A). ns, two-way ANOVA test. (**D**) HSPCs from two β-thalassemia donors ($\beta^+/\beta^+_{\#1}$ and $\beta^0/\beta^0_{\#2}$) were either mock-transduced (Mock; n=1 per donor) or transduced with control (βAS3 (n=2) or βAS3/miRnt (n=2); in total n=4 independent biological replicates per donor) or bifunctional (βAS3/miRBCL11A; n=2 independent biological replicates per donor) vectors for 24 hours (n=1-4 independent biological replicates per donor). After transduction, cells were differentiated towards the erythroid lineage for 21 days. VCN/cell was measured 14 days after transduction by ddPCR in erythroblasts. Globin expression (RT-qPCR, HPLC, flow cytometry) and RBC properties (flow cytometry and quantitative phase microscopy) were evaluated in erythroblasts or mature RBCs. βAS3- and βAS3/miRnt-transduced samples were pooled in all the analyses (B-G). (**E**) Globin mRNA levels measured by RT-qPCR in mock- and LV-transduced HSPC-derived erythroblasts after 13 days of differentiation. Globin mRNA levels were normalized to *HBA*. α/non-α ratios (mean±SD) are indicated on the top of each histogram. For calculating the α/non-α ratio, globin expression was

normalized to *GAPDH*. (n=1-4 independent biological replicates per donor). **(F)** Proportion of HbF^+ RBCs, quantified by flow cytometry in RBCs after 19 days of differentiation (n=4-8 independent biological replicates). **(G)** Analysis of α-globin precipitates by CE-HPLC in $\beta^+/\beta^+_{\#1}$ patient RBCs. We calculated the proportion of α-globin precipitates over the total Hb tetramers (mock: n=1, βAS3 or βAS3/miRnt: n=4, and βAS3/miRBCL11A: n=2 independent biological replicates). **(H-I)** Enucleation (H) and RBC size (I) measured by flow cytometry along the differentiation (n=1-4 independent biological replicates per donor). RBC size was not analyzed in the $\beta^+/\beta^+_{\#1}$ mock sample because of the low number of enucleated cells. **(J)** Expression of erythroid markers measured by flow cytometry along the differentiation (day 13, 16 and 20) in 2 β-thalassemia donors (n=1, 2 and 4 independent biological replicates per donor for Mock-, βAS3/miRBCL11A-, and βAS3- or βAS3m/miRnt-transduced samples, respectively). In the top panels, we plotted the frequencies of $CD36^+$ (top left panel), $CD71^+$ (top middle panel), and $CD235a^+$ (top right panel) erythroid cells. During erythroid differentiation, cells progressively lose CD36 and CD71 expression. In the bottom panel, we plotted the frequencies of $CD49^+$, $Band3^+$ and $CD49^+Band3^+$ cells among the $CD235a^+$ erythroid cells. During erythroid differentiation, $CD235a^+$ cells lose progressively the CD49 marker and express Band3. βAS3- and βAS3m/miRnt-LVs: VCN/cell=1.2±0.3 and 2.0±0.2 for patient #1 and #2, respectively; βAS3/miRBCL11A-LV: VCN/cell=1.3±0.1 and 2.0±0.1 for patient #1 and #2, respectively. **(K)** RBC parameters [Dry mass (pg), Surface (μm^2), and Perimeter (μm)] were extracted using the BIO-Data software from images taken with the Phasics camera after 19 days of differentiation for $\beta^+/\beta^+_{\#1}$ (upper panel) and $\beta^0/\beta^0_{\#2}$ (lower panel) samples. VCN/cell (mean±SD) is indicated in the legend. Analyses were performed on enucleated RBCs, and data were normalized to the total number of enucleated RBCs and reported as overlaid histograms. Phase microscopy analysis was not performed in the $\beta^+/\beta^+_{\#1}$ mock sample because of the low number of enucleated cells.

Figure S3

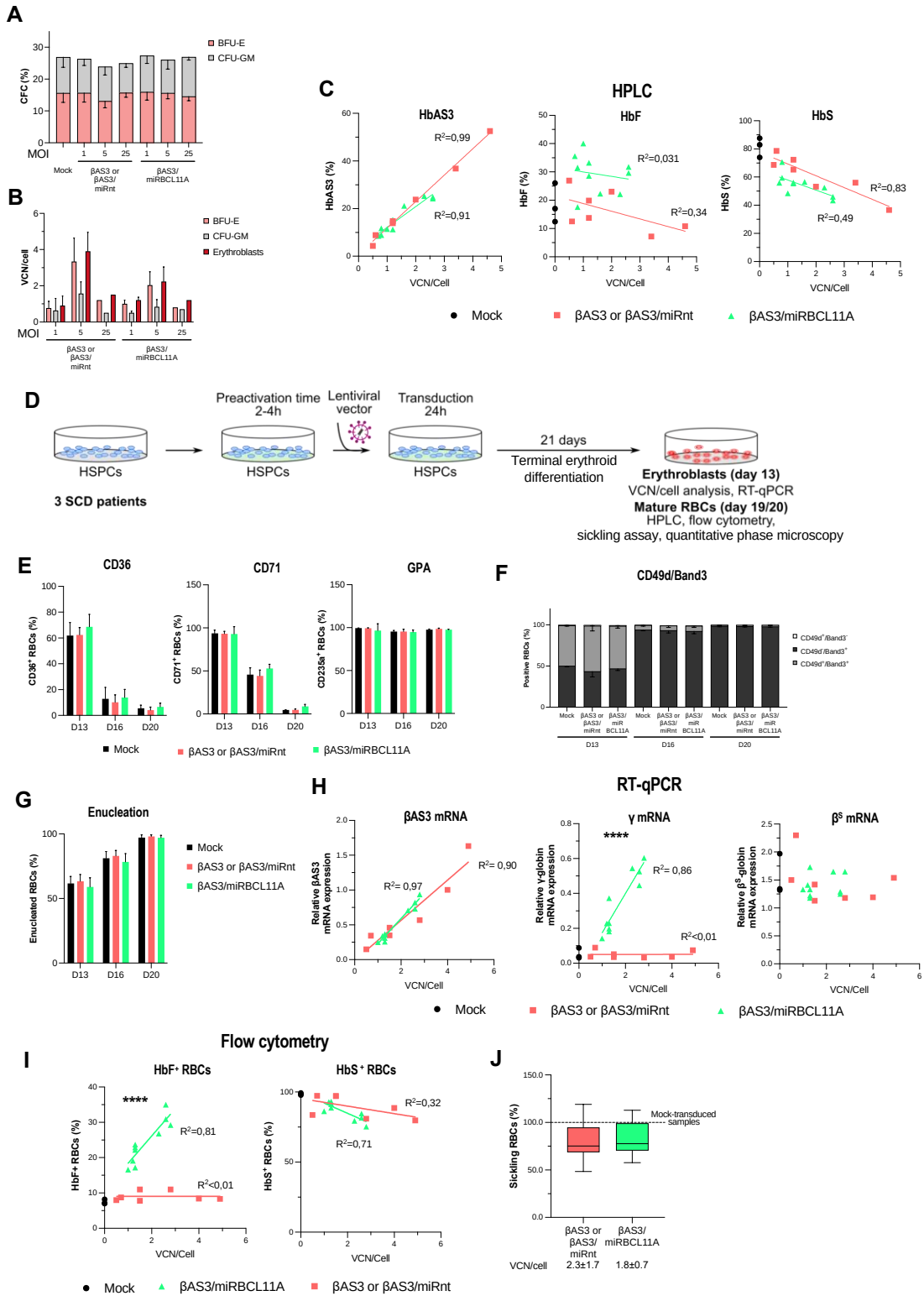

Figure S3: Evaluation of βAS3/miRBCL11A LV efficacy in HSPC-derived erythroblasts SCD donors

(**A-C**) Mobilized (2 donors) or non-mobilized (1 donor) peripheral blood HSPCs from three SCD donors were either not transduced (Mock; n=3 independent biological replicates) or transduced with control (βAS3 or βAS3m/miRnt; n=7 independent biological replicates) or bifunctional (βAS3/miRBCL11A; n=10 independent biological replicates) vectors at different MOI for 24 hours. After transduction, cells were plated in clonogenic cultures to evaluate BFU-Es and CFU-GMs after 14 days, or differentiated in erythroid precursors. VCN/cell were measured 14 days after transduction by ddPCR in erythroblasts and in BFU-E and CFU-GM pools. βAS3- and βAS3m/miRnt-transduced samples were pooled in all the analyses. **(A)** Frequency of BFU-Es and CFU-GMs in mock- and LV-transduced HSPCs. Results are represented as % of colonies obtained from 500 plated HSPCs and shown as mean±SD. ns, two-way ANOVA test. **(B)** Average VCN/cell (mean±SD) in pools of BFU-Es or CFU-GMs, and in erythroblasts. ns, two-way ANOVA test. **(C)** HbAS3 (left panel), HbF (middle panel) and HbS (right panel) expression measured by CE-HPLC in pools of BFU-Es. **(D)** Mobilized (2 donors) or non-mobilized (1 donor) peripheral blood HSPCs from SCD donors were either not transduced (Mock; n=1 per donor) or transduced with control (βAS3 or βAS3/miRnt; n=2-3 independent biological replicates per donor) or bifunctional (βAS3/miRBCL11A; n=3-4 independent biological replicates per donor) vectors at different MOI for 24 hours. After transduction, cells were differentiated towards the erythroid lineage for 21 days. VCN/cell were measured 14 days after transduction by ddPCR in erythroblasts. Globin expression (RT-qPCR and flow cytometry) and RBC differentiation markers and properties (flow cytometry and sickling assay) were evaluated in erythroblasts along the differentiation or in mature RBCs. βAS3- and βAS3/miRnt-transduced samples were pooled in all the analyses (E-J). **(E-G)** Expression of erythroid markers (E-F) and enucleation rate (G) measured by flow cytometry along the differentiation (day 13, 16 and 20) in 3 SCD donors (n=3, 7 and 10 independent biological replicates for mock-, βAS3- or βAS3/miRnt-, and βAS3/miRBCL11A-transduced

samples, respectively). βAS3- and βAS3m/miRnt-LVs: VCN/cell=2.3±1.7; βAS3/miRBCL11A-LV: VCN/cell=1.8±0.7. **(H)** βAS3- (left panel), γ- (middle panel) and β^S-globin (right panel) expression measured by RT-qPCR in erythroid precursors after 13 days of differentiation. Linear regression for γ-globin mRNA, ****P<0.0001. (n=3, 7 and 10 independent biological replicates for mock-, βAS3- or βAS3/miRnt-, and βAS3/miRBCL11A-transduced samples, respectively). **(I)** Proportion of HbF^+ (left panel) and HbS^+ (right panel) RBCs evaluated by flow cytometry. Linear regression for HbF^+-RBCs, ****P<0.0001. (n=3, 7 and 10 independent biological replicates for mock-, βAS3- or βAS3/miRnt-, and βAS3/miRBCL11A-transduced samples, respectively). **(J)** Frequency of sickling RBCs after 1-hour incubation at low oxygen tension (0% O_2). VCN/cell (mean±SD) is indicated below each graph. (n=3, 7 and 10 independent biological replicates for mock-, βAS3- or βAS3/miRnt-, and βAS3/miRBCL11A-transduced samples, respectively).

Figure S4

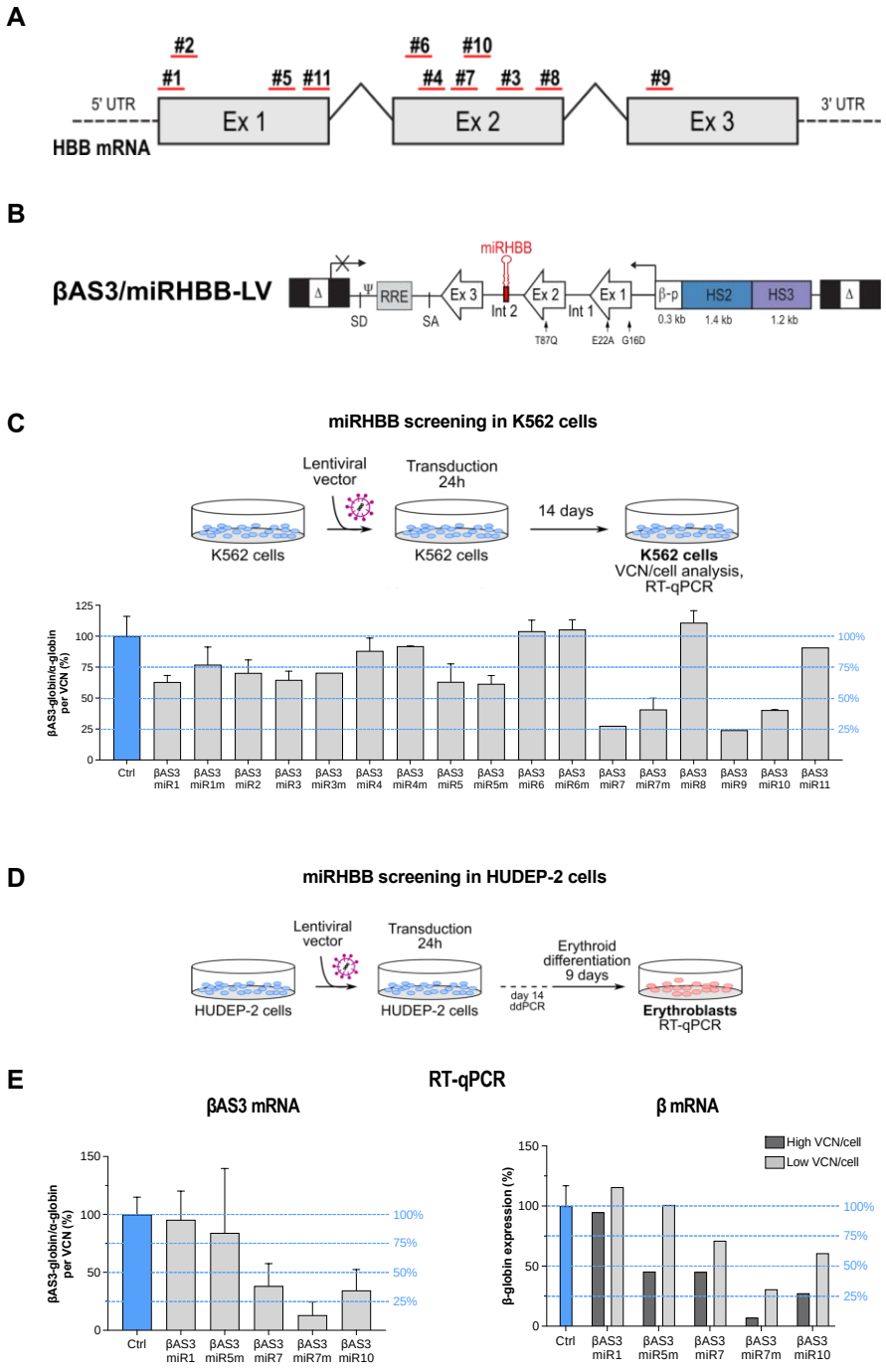

Figure S4: Design of a lentiviral vector expressing the βAS3-globin and an amiR targeting the β-globin.

(A) Schematic view of the miRHBB target regions within the *HBB* mRNA. Ex1, Exon1; Ex2, Exon2; Ex3, Exon3. **(B)** Structure of the βAS3/miRHBB-LV. Δ, deleted HIV-1 U3 region; SD

and SA, HIV splicing donor and acceptor sites; Ψ, HIV-1 packaging signal; RRE, HIV-1 Rev responsive element; Ex, exons of the human *HBB* gene; β-p, *HBB* promoter of; HS2, 3, DNase I hypersensitive site 2, and 3 of human *HBB* LCR; arrows indicate the mutations introduced in exon 1 (generating the G16D and E22A amino acid substitutions) and exon 2 (generating the T87Q amino acid substitution). **(C)** Screening of the 17 miRHBB in K652 cells. Cells were transduced at a MOI of 3 and 15 with control (βAS3 or βAS3/miRnt LVs, n=12 independent biological replicates) or βAS3/miRBCL11A LV (n=2 independent biological replicates, except for βAS3/miR7 and βAS3/miR9 (n=1). βAS3 mRNA expression was measured by RT-qPCR in K562 cells and normalized to *HBA*. We plotted the levels of βAS3 relative expression per VCN (mean±SD). **(D)** HUDEP-2 cells were transduced with control (ctrl, βAS3- or βAS3/miRnt-LV, n=4 independent biological replicates) or bifunctional (containing miR1, miR5m, miR7, miR7m, or miR10) vectors at low (2) or high (10) MOI for 24h (βAS3/miRHBB-LV, n=2 independent biological replicates per LV). VCN/cell were measured 14 days after transduction by ddPCR. Mock- or LV-transduced cells were differentiated into mature erythroblasts for 9 days to evaluate globin expression (RT-qPCR). **(E)** βAS3- (left panel) and β-globin (right panel) mRNA expression normalized to *HBA* per VCN (C, E) Results are represented as % of the control, which corresponds to the mean of the results obtained with βAS3- and βAS3/miRnt-LVs, and shown as mean±SD.

Figure S5

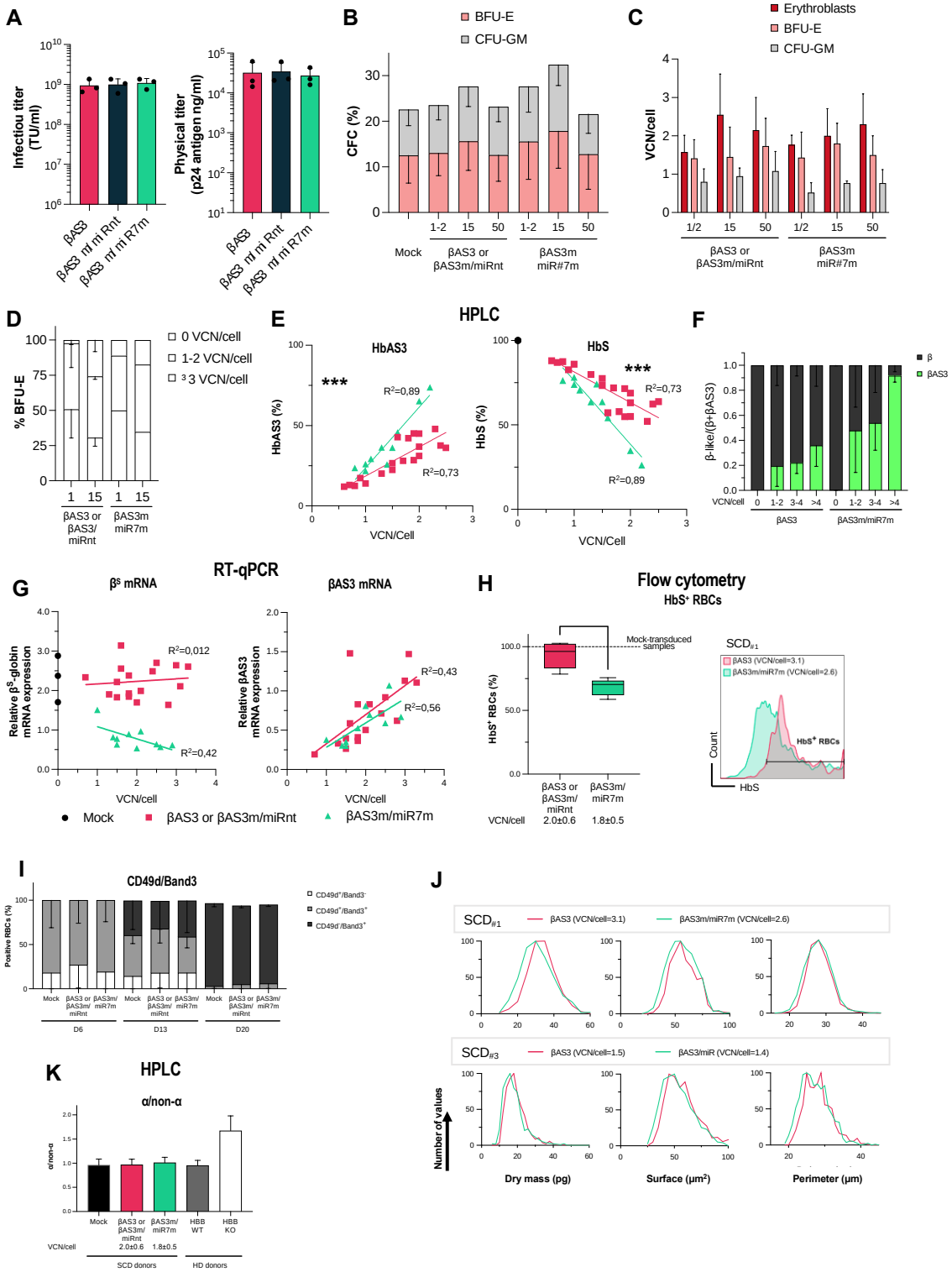

Figure S5: Evaluation of βAS3/miR7m LV efficacy in HSPC-derived erythroblasts SCD donors

(A) Infectious [Transduction Unit (TU)/ml] and physical titers of βAS3m/miR7m and control (βAS3- and βAS3m/miRnt-LVs) vectors. Infectious titers were measured on HCT116 cells, 4 days after transduction. (n=3 independent LV production). (B, C) Mobilized (2 donors) or non-mobilized (1 donor) peripheral blood HSPCs from SCD donors were either mock-transduced (Mock; n=3 independent biological replicates) or transduced with control (βAS3 or βAS3m/miRnt; n=1-3 independent biological replicates per MOI) or bifunctional (βAS3m/miR7m; n=1-2 independent biological replicates per MOI) vectors at different MOI for 24 hours (n=2-3 per donor). After transduction, cells were plated in clonogenic cultures to evaluate BFU-Es and CFU-GMs after 14 days, or differentiated in erythroid precursors. VCN/cell were measured 14 days after transduction by ddPCR in erythroblasts, BFU-E and CFU-GM pools, and in single BFU-Es. βAS3- and βAS3m/miRnt-transduced samples were pooled in all the analyses. **(B)** BFU-E and CFU-GM frequencies evaluated in mock- and LV-transduced HSPCs. Results are represented as % of colonies obtained from 500 plated HSPCs, performed in duplicates and shown as mean±SD. ns, two-way ANOVA test. **(C)** Average VCN/cell (mean±SD) in bulk BFU-Es, CFU-GMs and erythroblasts. ns, two-way ANOVA test. **(D)** Analysis of VCN/cell in individual BFU-Es (1 donor; n=1-2 independent biological replicates per MOI). Results are shown as mean±SD when applicable. The MOI is indicated below each graph. ns, two-way ANOVA test. **(E)** HbAS3 (left panel) and HbS (right panel) expression measured by CE-HPLC in pools of BFU-Es (3 donors). Linear regression for HbAS3 and HbS expression, ***P<0.001. (n=3, 10 and 19 individual biological replicates for Mock-, βAS3m/miR7m and βAS3- or βAS3m/miRnt-transduced samples, respectively). **(F)** Analysis of β-like globin expression (RT-qPCR) in individual BFU-Es derived from HD (1 donor) HSPCs transduced with βAS3- or βAS3m/miR7m-LV (n=14 and 24 individual BFU-Es from samples transduced with βAS3- or βAS3m/miR7m-LV, respectively). Results are shown as mean±SD. The VCN/cell is indicated below each graph. **(G)** β^S- and βAS3-globin mRNA

expression normalized to *HBA*. (n=3, 17 and 10 independent biological replicates for mock-, βAS3- or βAS3/miRnt-, and βAS3/miRBCL11A-transduced samples, respectively; 3 SCD donors). **(H)** Proportion of HbS^+-RBCs measured by flow cytometry analysis using an antibody recognizing specifically HbS. Mann-Whitney test, ***P<0.001. (n=2, 11 and 5 independent biological replicates for mock-, βAS3- or βAS3/miRnt-, and βAS3/miRBCL11A-transduced samples, respectively; 2 SCD donors). **(I)** Frequencies of $CD49^+$, $Band3^+$ and $CD49^+Band3^+$ cells among the $CD235a^+$ RBCs measured by flow cytometry along the differentiation. **(J)** RBC parameters [Dry mass (pg), Surface (μm^2), Perimeter (μm)] were extracted using the BIO-Data software from images taken with the Phasics camera after 19-21 days of differentiation for two SCD samples, $SCD_{\#1}$ (upper panel) and $SCD_{\#3}$ (lower panel), at a representative VCN/cell. Data were normalized to the total number of mature RBCs and reported as overlaid histograms. (**K**) α/non-α ratios (mean±SD) calculated by measuring globin expression by RP-HPLC. VCN/cell is indicated below the graph. HD samples (HD donors) are HD HSPCs that were either mock-transfected (HBB WT) or modified using a CRISPR/Cas9-gRNA RNP complex disrupting the *HBB* gene (HBB KO) [52]. Cells were differentiated toward the erythroid lineage. βAS3- and βAS3m/miRnt-transduced samples were pooled in all the analyses (A-C, E). (A-C, E) 2 SCD donors, βAS3- and βAS3m/miRnt-LVs: VCN/cell=2.0±0.6; βAS3m/miR7m-LV: VCN/cell=1.8±0.5. (n=2, 11 and 5 independent biological replicates for mock-, βAS3- or βAS3m/miRnt-, and βAS3m/miR7m-transduced samples, respectively). (E) HD (n=2 independent biological replicates).

Figure S6

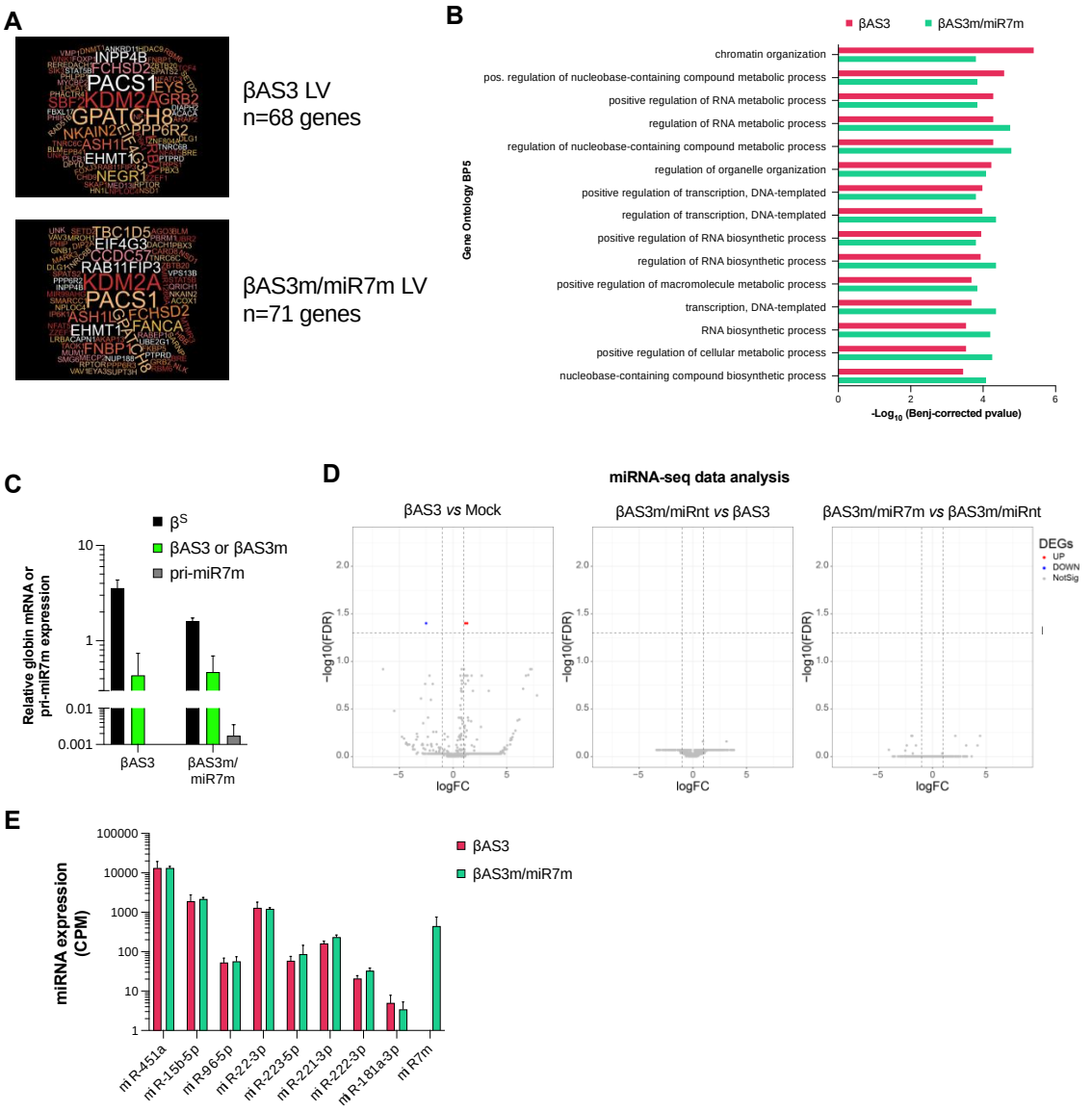

Figure S6: Safety analysis of the βAS3/miR7m LV

(A) Top targeted genes in βAS3-LV and βAS3m/miR7m-LV-transduced HSPCs (n=1 per LV). βAS3-LV integration sites were retrieved from Poletti et al. [61]. **(B)** Gene ontology analysis of LV- (βAS3 or βAS3m/miR7m) targeted genes defined by a read count > 95th percentile. BP, biological function (1 healthy donor; n=1 per LV). (**C**) Globin (*HBB* and βAS3) mRNA and pri-miR7m expression measured by RNA-seq and normalized to *HBA* in βAS3- and βAS3m/miR7m-LV transduced samples (n=3 independent biological replicates per LV). **(D)**

miRNA-seq-based analysis comparing mRNA or miRNA expression between two sample groups: βAS3 and Mock (left), βAS3m/miRnt and βAS3 (middle), βAS3m/miR7m and βAS3m/miRnt (right) (2 SCD donors were used; n=2 and 3 independent biological replicates for Mock and LV-transduced samples, respectively). Differentially expressed miRs (DEGs) with an FDR < 0.05 and an absolute log2 fold-change (logFC) ≥ 1 are highlighted in red (up-regulated, UP) or in blue (down-regulated, DOWN). miRs that are not differentially expressed are represented in grey (NotSig). (**E**) Expression of selected endogenous miRs involved in erythroid differentiation, which are upregulated (e.g., miR-451a, miR15b-5p, miR-96-5p, and miR-22-3p) or downregulated (e.g., miR-223-5p, miR-221-3p, miR-222-3p, and miR-181a-3p) along differentiation [30], and the artificial miR7m in βAS3- and βAS3m/miR7m-transduced cells measured by miRNA-seq and expressed in CPM (count per million reads) (n=3 independent biological replicates per LV).