2'-O-methoxyethyl splice-switching oligos correct splicing from IVS2-745 β-thalassemia patient cells restoring hemoglobin A production and chain rebalance

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Supplementary methods

Statistics

For three or more groups, we compared means with a one-way ANOVA test (for samples with normal distributions and equal variances by the Shapiro-Wilks normality test) or medians with a non-parametric Kruskal-Wallis test. For two groups, we compared means with a t-test (for samples with normal distributions by the Shapiro-Wilks normality test) or medians with a Mann-Whitney test. All tests were done using GraphPad Prism software, version 7.

RNA, RT-PCR, and Quantitative PCR (Q-PCR)

Total RNA was isolated using Trizol (ThermoFisher). Retrotranscription of total mRNA was done using the SuperScript™ III First Strand Kit (ThermoFisher). PCR reactions were performed with the following primers: Fw: 5′-GGCAAGGTGAACGTGGATGAAGTT -3′; Rev: 5′-TAGGCAGAATCCAGATGCTCAAGG-3′. Sequencing on amplified products was completed by using the QIAquick Gel Extraction Kit, Qiagen) and cloned in TOPO® TA Cloning® Kits for Sequencing (Invitrogen, Carlsbad, CA). Q-PCR reactions were performed using the ABI 7900HT or Viia7 systems (Applied Biosystems), with either TaqMan (TaqMan PCR 2X Master Mix from ThermoFisher) or SYBR Green (Power SYBR from ThermoFisher or iTaqTM SYBR® Green Supermix from Bio-Rad) chemistry. Quantitative real-time PCR assays of globin, GAPDH, and glycophorin-A transcripts were carried out using gene-specific double fluorescently labeled probes. The following primer and probe sequences were used (forward, reverse and probe, when used, of each gene, respectively): WT correctly spliced β-globin primers = Fw: 5′-CACCTTTGCCACACTGAGTGA-3′; Rev: 5′-GCCCAG GAGCCTGAAGTTCT-3′; 5′-FAM-CACTGTGACAAGCTGCACGTGGATCC-IOWA BLACK-3′. The following TaqMan inventoried Gene Expression assays from Thermo Fisher were used: GAPDH: Hs02758991_g1; GYPA: Hs00266777_m1. Q-PCR results of WT β-globin were normalized by GAPDH to control for the total amount of cDNA and GYPA to control for the level of differentiation across samples. For PCR of human β-globin cDNA, the following primers were used: Fwd 5′-GTGCGAGAGCGTCAGTATTAAG-3′, Rev 5′-TCCCTGCTTGCCCATACTA-3′.

Droplet digital polymerase chain reactions (ddPCR)

Reactions were performed by Rain Drop plus Digital PCR system (RainDance Technologies). Droplets were generated using RainDance Source chips (RainDance Technologies) from 20 ng of cDNA and deposited in PCR tubes. PCR tubes were transferred into a thermocycler and processed using the following parameters: 10 min 95°C, then 45 cycles of 95°C for 15 sec, and 60°C for 1 min with a ramping speed of 0.6°C/sec, followed by 98°C for 10 min. After PCR completion, tubes were transferred into RainDance Sense chips (RainDance technologies) for fluorescence measurements. The RainDrop Analyst Software (RainDance technologies) was used to analyze the data set to define thresholds and count droplets. Endogenous cDNA amount was quantified by a primer/probe set against human β-actin gene (ThermoFisher Scientific). The wild type of β-globin assay sequences were as follows: 5′- CTGCACGTGGATCCTGAGAA -3′ (forward primer), 5′- GTGATGGGCCAGCACACA -3′ (reverse primer) and 5′-FAM- TTCAGGCTCCTGGGCA -MGBNFQ (probe) (Applied Biosystems).

Subsequent testing to measure the proportion of WT and IVS2-745 HBB (Supplementary Figure 6) were performed by Bio-Rad Droplet Digital PCR system, recently acquired by our laboratory. Droplets were prepared using Automated Droplet Generator (Biorad) from 22 ul of PCR mixture, followed by PCR reaction using C1000 Touch Thermal Cycler (Biorad) with the following parameters: 10 min 95°C, then 45 cycles of 95°C for 15 sec and 61°C for 2 min with a ramping speed of 1°C/sec, followed by 98°C for 10 min. Droplets fluorescence measurements were performed using QX200 Droplet Reader and QuantaSoft Software (Biorad). Minimum of 10000 drops were analyzed. Endogenous cDNA amount was quantified by a primer/probe set against human AHSP gene (Hs00372339_g1, ThermoFisher Scientific). The wild type HBB assay sequences were as follows: 5′- GAAGGCTCATGGCAAGAAAG -3′ (forward primer), 5′-CTGGTGGGGTGAATTCTTTG -3′ (reverse primer) and 5′-FAM- ACTTCAGGCTCCTGGGCAACGT -MGBNFQ (probe) (Biorad). The IVS2-745 HBB assay sequences were as follows: 5′- GAGAACTTCAGGGGCAATAATG -3′

(forward primer), 5′- AGCAATATGAAACCTCTTACATCAGT -3′ (reverse primer) and 5′-HEX-TCATGCCTCTTTGCACCATTCTAA -MGBNFQ (probe) (Biorad). The α-globin assay sequences were as follows:5'CGACAAGACCAACGTCAAGG – 3' (forward primer), 5'- ACAGGAACATCCTCTCCAGG-3' (reverse primer) and 5'- HEX-CACGCTGGCGAGTATGGTGC-MGBNFQ (probe) (Biorad).

Vector production and Titering

The human β-globin sequence was mutagenized with the IVS2-745 β-globin sequence, creating a lentiviral vector expressing human IVS2-745 β-globin. Viral stocks were generated by co-transfection into 293T cells (30). Viral supernatants were collected at 24 and 48 hours and filtered through cellulose acetate (0.2 μm). Following ultracentrifugation, serial dilutions of concentrated virus were used to infect 1e5 NIH 3T3 cells (ATCC, Manassas, VA) in 1 mL of transfection buffer complemented with polybrene (Millipore, Billerica, MA) at a final concentration of 8 μg/mL. Genomic DNA was extracted after 3 days using phenol-chloroform-isoamylic alcohol. The multiplicity of infection (MOI) was calculated using the following formula: number of cells (1e5) X dilution factor (1 mL/μL viral preparation) X VCN (measured via real-time PCR, using oligos for WPRE element and Transferrin Receptor gene, see copy number determination) (23).

Copy Number Determination

The number of integrations (VCN) was quantified by Q-PCR using Oligos for (Fw: 5′-GTGCGAGAGCGTCAGTATTAAG-3′; Rev: 5′-TCCCTGCTTGCCCATACTA-3′) for a specific sequence present in the vector (GAG) and compared it to an endogenous control present in two copies within the genome (mouse Transferrin=Fw: 5′-TGTTGTAGTAGGAGCCCAGAGAGA-3′; Rev:5′-AGACCTGTTCCCACACTGGACTT-3′; human ID-1 = Fw: 5′-AAGGTGAGCAAGGTGGAGATTC-3′; Rev: 5′-TTCCGAGTTCAGCTCCAACTG-3′).

Supplementary Table 1

Sequences of β-globin cDNA (WT and mutant 745)
Exons are indicated in different shades of orange. Intronic insertion of 165bp due to the mutation is indicated in green
Stop codon is highlighted in red

cDNA _ β-globin:

CGGCTGTCATCACTTAGACCTCACCCTGTGGAGCCACACCCTAGGGTTGGCCAATCTACTCCCAGGAGCAGG
GAGGGCAGGAGCCAGGGCTGGGCATAAAAGTCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACAC
AACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACT
GCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGCTGCTGGTGGTCTACCC
TTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGT
GAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCA
CCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGCTCCTGGGCA
ACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGA
AAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTC
TATTAAAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTG
GATTCTGCCTAATAAAAAACATTTATTTTCATTGCAA

Extra intron 165bp generated from aberrant splicing in IVS2-745 is a C→G mutation:

GGCAATAATGATACAATGTATCATGCCTCTTTGCACCATTCTAAAGAATAACAGTGATAATTTCTGGGTTAA
GGCAATAGCAATATTTCTGCATATAAATATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTG
CTAATAGCAGCTACAATCCAG

cDNA_ mutant β-globin 745:

CGGCTGTCATCACTTAGACCTCACCCTGTGGAGCCACACCCTAGGGTTGGCCAATCTACTCCCAGGAGCAGG
GAGGGCAGGAGCCAGGGCTGGGCATAAAAGTCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACAC
AACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACT
GCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGCTGCTGGTGGTCTACCC
TTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGT
GAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCA
CCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGGGCAATAAT
GATACAATGTATCATGCCTCTTTGCACCATTCTAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATAGC
AATATTTCTGCATATAAATATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTGCTAATAGCA
GCTACAATCCAGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCCA
CCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGC
TCGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATAT
TATGAAGGGCCTTGAGCATCTGGATTCTGCCTAATAAAAAACATTTATTTTCATTGCAA

cDNA_ β-globin SEQUENCED from M13For after TopoTA cloning (241bp 100%complementary):

GGCTCCATCTATAGGGGCGATTGAATTTAGCGGCCGCGAATTCGCCCTTCTCACCTGGACAACCTCAAGGGC
ACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGCTCCTGGGC
AACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAG
AAAGTGGTGGCTGGTGTGGCTAATGAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGG
GTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCA
CACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAAT
TGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACG
CGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGT
TCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACG
CAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTT
TTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGA

CAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCG
CTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGCGCTTTCTCATAGCTCACGCTGTAGGTATCT
CAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGCTGTGTGCACGAACCCCCCCGTTCAGCCCGACGCTGCGCC
TATCCGGTAACTATCGTCTTGAGTCCAACCCGGGTAAGAACACCGACTTTATCCGCCCACCT

cDNA_ mutant β-globin 745 SEQUENCED from M13For after TopoTA cloning (405bp 100%complementary):

AGGATACATCTATAGGGCGATTGATTTAGCGGCCGCGAATTCGCCCTTCTCACCTGGACAACCTCAAGGGC
ACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGGGCAATAA
TGATACAATGTATCATGCCTCTTTGCACCATTCTAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATAG
CAATATCTCTGCATATAAATATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTGCTAATAGC
AGCTACAATCCAGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCC
ACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGAAGGGCGAATTCGTTTAAACCTGC
AGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAA
ATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAA
TGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAG
CTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTC
ACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTT
ATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGGCCAGGAACC
GTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAGATACCAGGCGTTTCCCCCTGTAGCTCCCTCGTGCG
CCTCCTCCTGTCCGACCCTGCCGCTACCGGATACCTGTCGCTTTCTCCGTCGGAGCGTGCGCTTTCTCATAGC
TCACGCTGTAGATTCTCAGTCGGTGTAGGTCGTCCGCTCCAGCTGGGCTGGGTGGTGACG

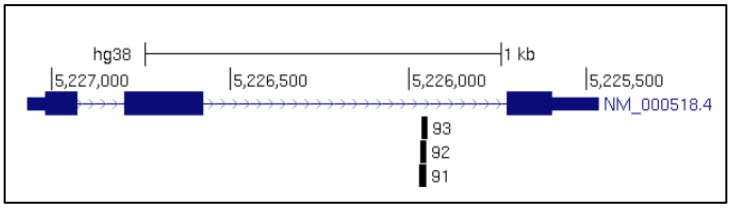
Supplementary Table 2

Sequences of 91-92-93 targeting the IVS2-745 HBB

ID	Sequence
91	CTTTAGAATGGTGCAAAG
92	TTCTTTAGAATGGTGCAA
93	TATTCTTTAGAATGGTGC

Supplementary Table 3

Schematic representation of SSO-target sites in the beta-globin gene



Supplementary Table 4

Results of search for TF binding sites on http://rbpdb.ccbr.utoronto.ca/
The red highlights areas the 3 ASOs bind while the underline marks the splicing factor binding sites

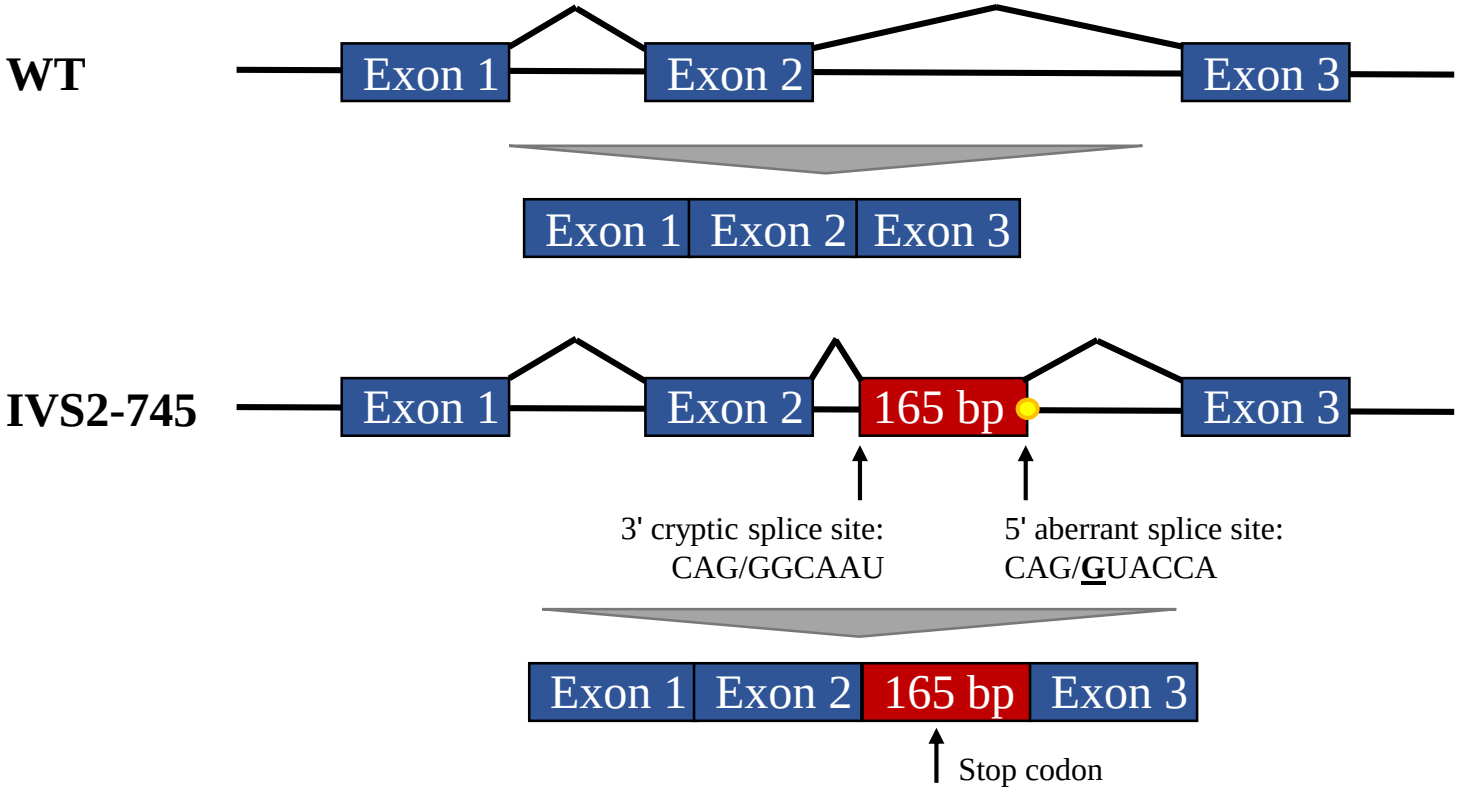
Sequence:

1 AGGGCAAUAA UGAUACAAUG UAUCAUGCCU CUUUGCACCA UUCUAAAGAA UAACAGUGAU

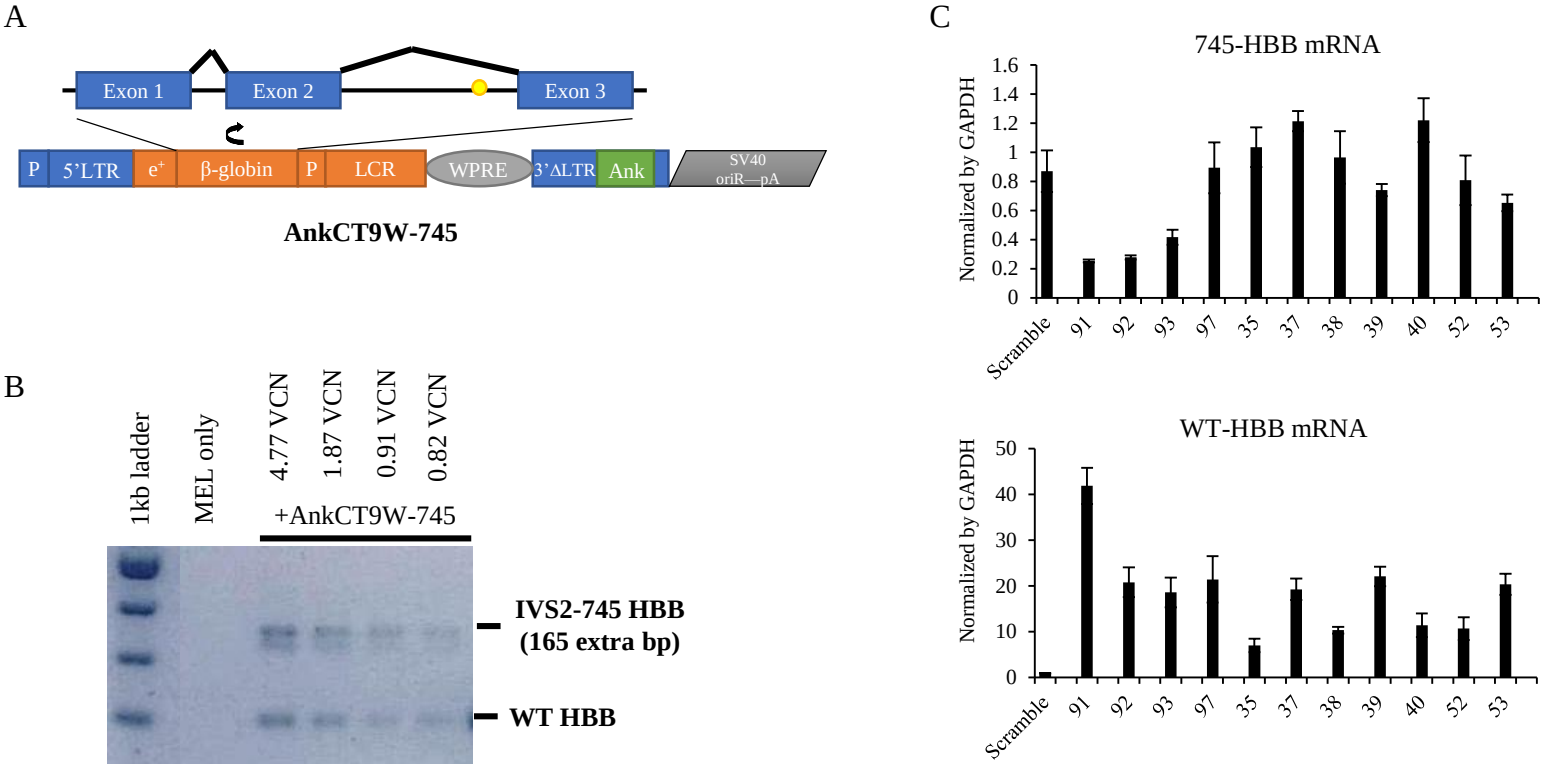
AAUUUCUGGG UUAAGGCAA

Score	Relative Score	RBP Name	Matching sequence
9.795457	81%	SNRPA	UUUGCACC
7.746982	96%	SNRPA	UUUGCAC
4.738923	92%	SFRS13A	AAAGAAU
4.652089	100%	KHDRBS3	AAUAAU
4.449274	95%	KHDRBS3	GAUAAU
4.09405	88%	KHDRBS3	UCUAAA
4.020734	86%	KHDRBS3	AAUAAC

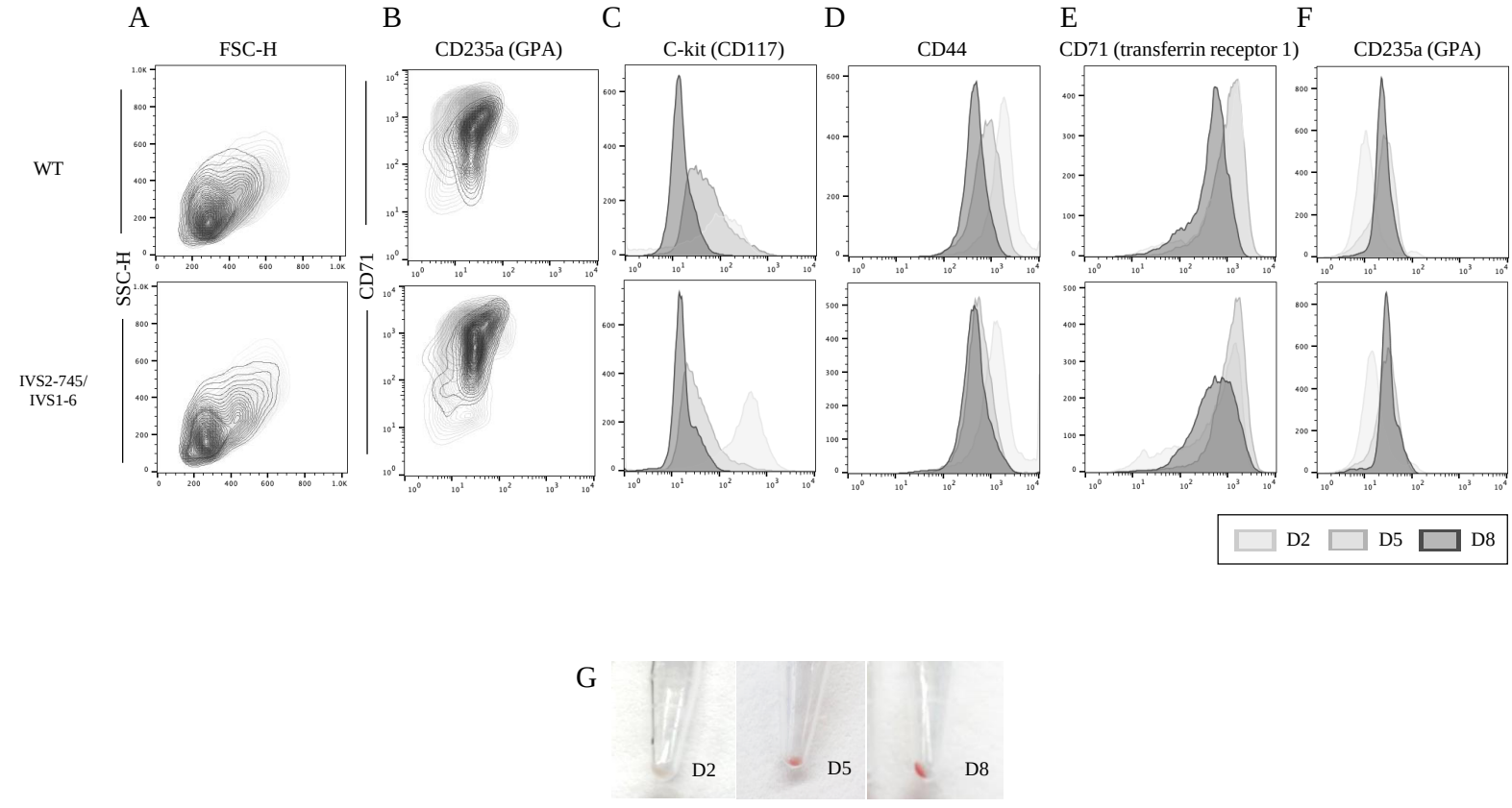
Supplementary Figure 1. Splicing of WT human β-globin and aberrant splicing on mutant IVS2-745. IVS2-745 occurs in the intravenous sequence, or intron 2, of the beta-globin gene. It generates an aberrant 5' splice site at nucleotide 745 (indicated by the yellow circle). A corresponding cryptic 3' splice site at nucleotide 579 is activated, resulting in the inclusion of a 165nt sequence in the aberrant pre-mRNA. The IVS2-745 mRNA does not translate into functional beta-globin protein, given that the extra intronic sequence introduces a stop codon 48nt after exon 2.



Supplementary Figure 2. Pre-screening of 2'-MOE-SSOs in mouse erythroleukemia (MEL) cells expressing the aberrant IVS2-745 HBB for splicing correction (A) The AnkCT9W lentivirus, which expresses wild-type beta-globin was modified with mutagenesis (yellow circle) to express the IVS2-745 beta-globin. (B) MEL-745 cells were created by infecting MEL cells with the AnkCT9W-745 lentivirus. C) Eleven 2' MOE-SSOs targeting the aberrant mRNA and a scramble dose (all 5μM) were originally tested for their ability to correct splicing in MEL cells expressing the aberrant IVS2-745 mRNA.



Supplementary Figure 3. Characterization of samples by flow cytometry at day-2, 5 and 8 of differentiation (in WT and IVS2-745 IVS1-6 specimen specimens). (A**)** Cell size and density is reduced throughout erythroid maturation, as indicated by reduction of SSC-FSC. As expected, (B) maturation of erythroid cells is accompanied by increased expression of the CD235a (GPA) marker and progressive reduction of CD71 in both WT and BT IVS2-745/ IVS1-6 cells. Maturation of erythroid cells is accompanied by decreased expression of CD117 (C), CD44 (D) and CD71 (E) markers and increased expression of the CD235a (GPA) (F). Accordingly, cell pellets become more red (G).



Supplementary Figure 4. Cell proliferation, mRNA and protein expression in WT and IVS2-745/IVS1-6 specimen specimens). (A) Cell proliferation and proportion of benzidine positive cell in erythroblasts isolated from healthy individual and from a patient with IVS1-6 / IVS2-745 BT, at different stages of erythroid maturation. (B) Abundance of WT beta-globin mRNA and (C) separation of tetrameric hemoglobins in specimens from A and (D) relative HbA proportion versus HbA absolute concentration calculated from C in 1E+06 benzidine positive cells collected at D8. ***p< 0.001, **** p< 0.0001.

A

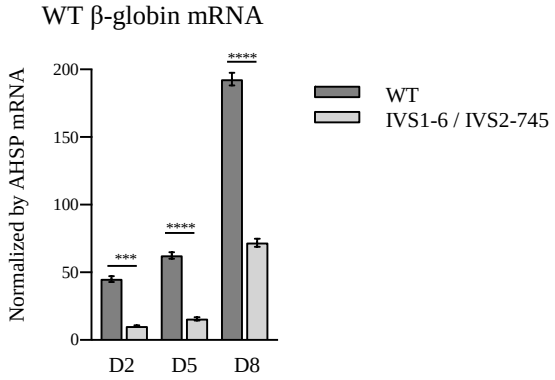
WT

Day	Proliferation	Benzidine count
D2	1.4-fold	0.1%
D5	4.4-fold	83.1%
D8	10.2-fold	93.7%

IVS1-6 / IVS2-745

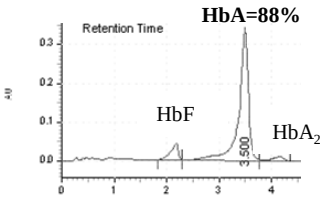
Day	Proliferation	Benzidine count
D2	0.9-fold	0.1%
D5	3.9-fold	97.2%
D8	12.1-fold	82%

B

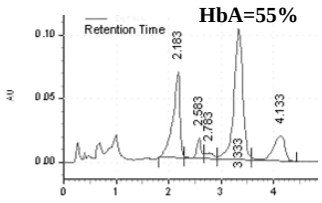


C

WT



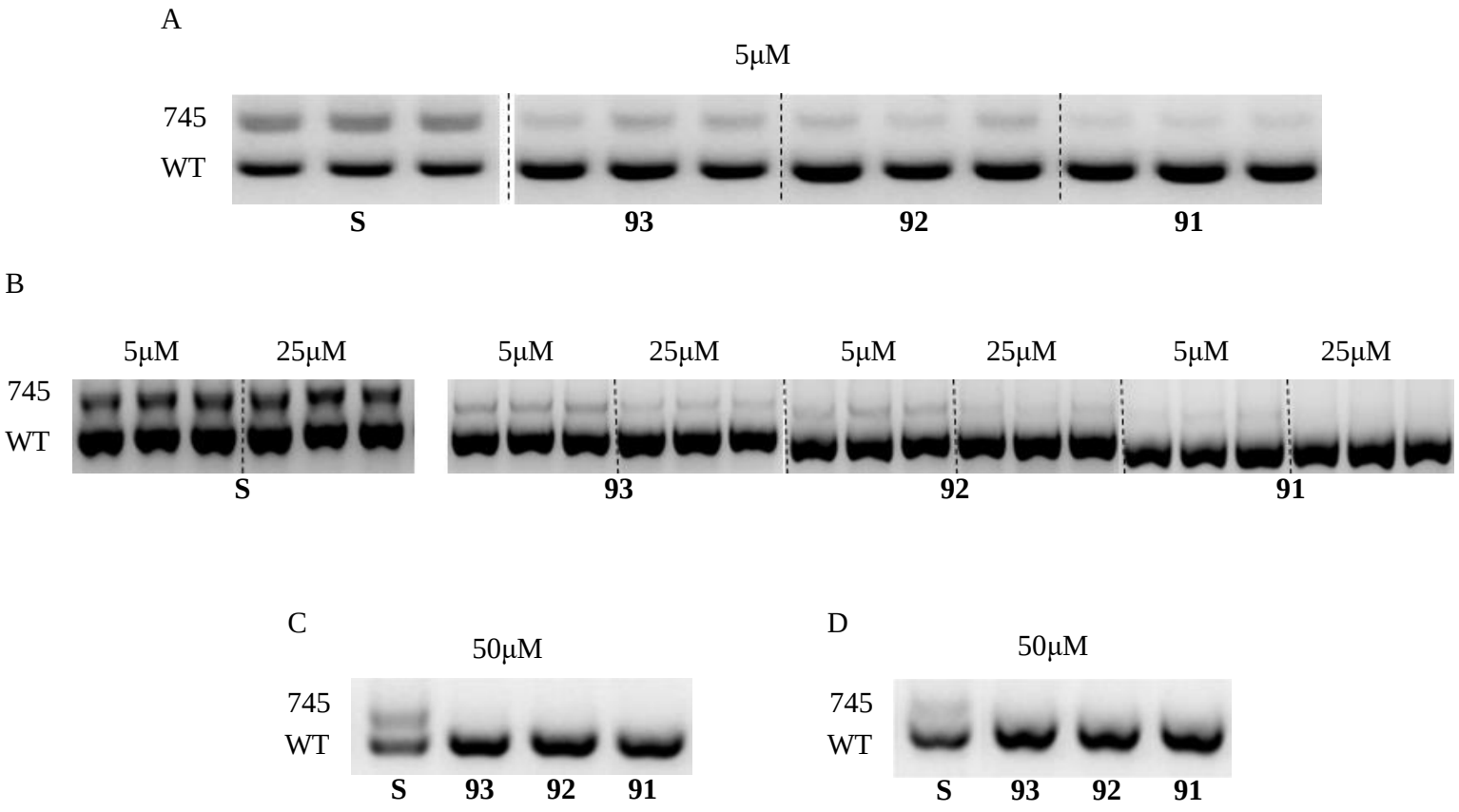
IVS1-6 / IVS2-745



D

Proportion of HbA% in WT/BT specimen	Proportion of absolute HbA (area under curve) in WT/BT specimen
1.6-fold	3.35-fold

Supplementary Figure 5. Electrophoresis of PCR products of cDNA obtained from erythroblasts from patients with the IVS2-745 mutation. Bands relative to WT and aberrant IVS2-745 β-globin cDNA amplification of the two distinct messengers are indicated. (A) P1 specimen treated with scramble and with the 2'-MOE-SSOs 91, 92 and 93 at a 5μM dose. (B) P1 specimen treated with scramble and with the same oligos at a 5μM and a 25μM dose. (C) and (D) Specimen P3 and P4 treated with scramble and with the three 2'-MOE-SSOs at a 50μM dose.



Supplementary Figure 6. 2'-MOE-SSOs 91 greatly increases amount of WT over IVS2-745 β-globin mRNA. In the ddPCR analysis in A), y axes indicates relative expression of WT β-globin normalized to the IVS2-745, while in B) the cDNA concentration in the generated droplets of the same sample analyzed with WT or the IVS2-745 probe and primer sets. IVS-2-745/β0 heterozygous sample, P4. Statistics (ANOVA/Kruskal-Wallis) compared scramble control and non-treated sample to 2'-MOE-SSO 91 treatment were at the dose of 50μM. n=3. **** p< 0.0001. In B) statistic indicated by * represent the different WT mRNA abundance while # represent the different IVS2-745 mRNA abundance.