Inclusion of a short hairpin RNA targeting *BCL11A* into a β-globin expressing vector allows concurrent synthesis of curative adult and fetal hemoglobin

Silvia Pires Lourenco,[1,2] Danuta Jarocha,[1*] Valentina Ghiaccio,[1] Amaliris Guerra,[1] Osheiza Abdulmalik,[1] Ping La,[1] Alexandra Zezulin,[3] Kim Smith-Whitley,[1] Janet L. Kwiatkowski,[1] Virginia Guzikowski,[1] Yukio Nakamura,[4] Tobias Raabe,[3] Laura Breda[1] and Stefano Rivella[1]*

[1]Department of Pediatrics, Hematology, The Children's Hospital of Philadelphia, Philadelphia, PA, USA; [2]Graduate Program in Basic and Applied Biology (GABBA), Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal; [3]Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA and [4]Cell Engineering Division, RIKEN BioResource Research Center, Tsukuba, Japan

**SPL and DJ contributed equally as co-first authors.*

Correspondence: DANUTA JAROCHA - jarochad@email.chop.edu

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Supplementary appendix
Figure legends

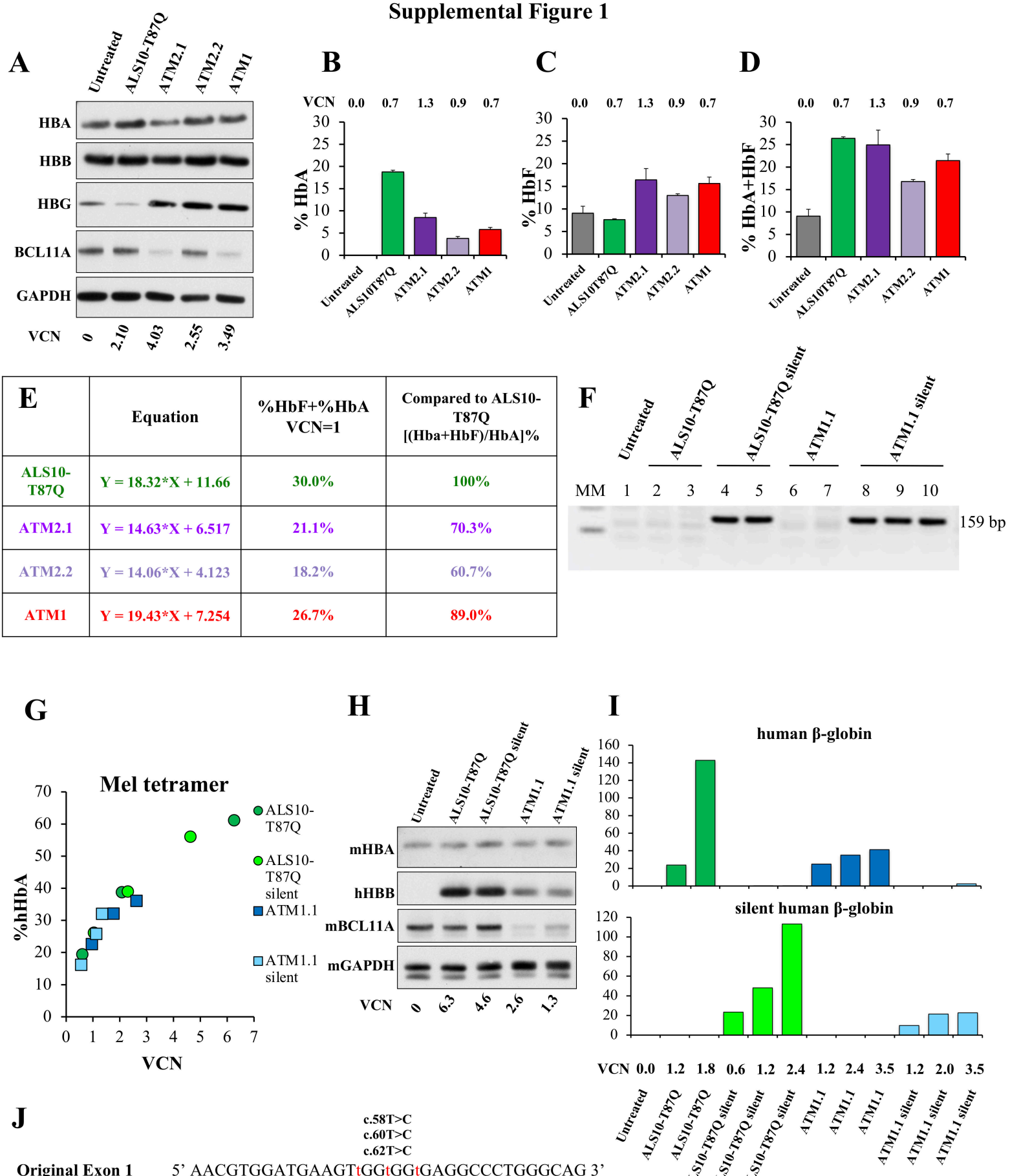
Supplemental Figure 1 – (**A**) Representative Western blot showing HBA, HBB, HBG and BCL11A proteins in differentiated M#9 cells. (**B**) Percentage of HbA^{T87Q} (**C**) HbF and (**D**) HbA^{T87Q}+HbF levels quantified by HPLC over the number of viral integrations per cell after transduction of M#9 with ALS10-T87Q, ATM2.1, ATM2.2 or ATM1. Data are indicated as the mean ± SD, n=3. (**E**) Results of mixed-effects linear regression models to identify the percentage of curative HbA^{T87Q}+HbF produced by ALS10-T87Q, ATM2.1, ATM2.2 and ATM1 at VCN=1. (**F**) Amplification of β-globin sequences carrying silent mutations (as indicated in **Fig.1A**) on plasmids that contain the sequence of ALS10-T87Q, AL10-T87Q-silent, ATM1.1 or ATM1.1-silent. (**G**) Chimeric HbA (human β-globin/mouse α-globin tetramer) production following transduction with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent vector in MEL cells, over range of viral integrations (VCN up to 7 copy/genome). (**H**) Representative Western blot showing mouse HBA (mHBA), human HBB (hHBB) and mouse BCL11A (mBCL11A) protein levels in differentiated MEL cells treated with WT vectors and their counter silent variants. (**I**) Evaluation of gene expression of WT, β-globin, and silent-β-globin in untreated and transduced MEL cells compared to the endogenous control mouse β-actin mRNA at different VCN. (**J**) Modifications of the sequences in exon 1 (top) and exon 2 (bottom) of the β-globin gene to generate the silent version of the β-globin gene.

Supplemental Table 1 – (**A**) Sequences of BCL11A miRNAs in ATM1, ATM1.1, ATM1.2, ATM1.3 and ATM1.4 constructs. (**B**) Results of mixed-effects linear regression models of % of curative HbA^{T87Q}+HbF obtained with ALS10-T87Q, ATM1, ATM1.1, ATM1.2, ATM1.3 or ATM1.4 in M#9 treated cells. (**C**) Results of mixed-effects linear regression models of % of curative HbA^{T87Q}+HbF, HbA^{T87Q} and HbF obtained in SCD cells treated with ALS10-T87Q, ALS10-T87Q-silent, ATM1.1 or ATM1.1-silent. (**D**) Sequences of primers used in RT-PCR and qPCR to amplify β-globin, Silβ-globin, γ-globin and β-actin.

Supplemental Figure 2 – (**A**) Quantitative RT-PCR analysis of a SCD patient specimen. The analysis includes quantification of the SCD β-globin mRNA, β–globin transcribed by the vectors, including the Silβ-globin, and induced γ-globin mRNAs and normalized to endogenous GAPDH mRNA adapted from Breda L, et al. Lentiviral vector ALS20 yields high hemoglobin levels with low genomic integrations for treatment of beta-globinopathies Mol Ther. 2021. (**B**) Amplification of the β-globin region encompassing exon 1 and 2, from **Fig.1A**. (**C**) Quantification by ddPCR of vector-derived T87Q β-globin, sickle β-globin and γ-globin mRNAs and normalized to endogenous α-globin mRNA. Bio-Rad ddPCR system was used to perform the analysis using specific primer/probe custom sets detecting vector-derived, T87Q, Sickle or γ-globin expression. The sequences of the oligos used: vector-derived, T87Q β-globin (FW: 5'- CTGGCTCACCTGGACAA-3'; Rev: 5'-CAGGAGCCTGAAGTTCTCA-3'; Probe: 5'- CACCTTTGCCCAGCTGAGTGAG-3'), human SCD β-globin (FW: 5'- CCAGCAGCCTGCCCAG -3'; Rev: 5'-ATCTATTGCTTACATTTGCTTCTGACA-3'; Probe: 5'-

AGACTTCTCCACAGGAGT-3') and human γ-globin (FW: 5'-AGGAGGACAAGGCTACTATCACAAG -3'; Rev: 5'-AGGAGCCTTCCCAGGGTTT -3'; Probe: 5'-CAAGGTGAATGTGGAAGAT-3') referred to human α-globin as an endogenous control (FW: 5'- GCCCTGGAGAGGATGTTCCT-3'; Rev: 5'-TCGAAGTGCGGGAAGTAGGT -3'); Probe: 5'- TCCTTCCCCACCACCAA-3'). (**D**) Representative chromatographic separation (HPLC) of hemolysates from β0/β0 TH25 patient' cells. Control, untransduced (top), ALS10-T87Q (middle) and ATM1.1 (bottom) vector transduced erythroblasts show: HbF, (acetylated and main form), α-aggregates, HbA (acetylated and main form) and HbA_2 peaks at day 7 of differentiation. Dual vector transduced cells demonstrated highest reduction of α-aggregates peak.

Supplemental Figure 1



E	Equation	%HbF+%HbA VCN=1	Compared to ALS10-T87Q [(Hba+HbF)/HbA]%
ALS10-T87Q	Y = 18.32*X + 11.66	30.0%	100%
ATM2.1	Y = 14.63*X + 6.517	21.1%	70.3%
ATM2.2	Y = 14.06*X + 4.123	18.2%	60.7%
ATM1	Y = 19.43*X + 7.254	26.7%	89.0%

Supplemental Table 1

A

ATM1	...TTGCTGTTGACAGTGAGCG- - - -gatcgagtgttgaataatgataGGGTGAAGCCACAGATGCCtatcattattcaacactcgatc- - - -TGCCTACTGC..
ATM1.1	...TTGCTGTTGACAGTGAGCG- gcgcgatcgagtgttgaataa- - - -GGGTGAAGCCACAGATGCC- - - -ttattcaacactcgatcgcgc -TGCCTACTGC...
ATM1.2	...TTGCTGTTGACAGTGAGCG- - - -agatcgagtgttgaataatgat -GGGTGAAGCCACAGATGCC-atcattattcaacactcgatcc- - - -TGCCTACTGC...
ATM1.3	...TTGCTGTTGACAGTGAGCG- - - -gatcgagtgttgaataatgataTAGTGAAGCCACAGATGTAtatcattattcaacactcgatc- - - -TGCCTACTGC...
ATM1.4	...TTGCTGTTGACAGTGAGCGagcgcgatcgagtgttgaataa - - - -TAGTGAAGCCACAGATGTA- - - -ttattcaacactcgatcgcgccTGCCTACTGC...

B

	Equation	R^2	%HbF+%HbA VCN=1	%HbF+%HbA VCN=2	Compared to ALS10-T87Q [(Hba+HbF)/HbA]%
ALS10-T87Q	Y = 16.18*X + 15.81	0.9919	32.0%	48.2	100%
ATM1	Y = 16.48*X + 9.581	0.9985	26.1%	42.5	81.6%
ATM1.1	Y = 21.85*X + 20.94	0.9996	42.8%	64.6	133.8%
ATM1.2	Y = 16.51*X + 14.2	0.9426	30.7%	47.2	96.0%
ATM1.3	Y = 16.12*X + 14.01	0.9927	30.1%	46.3	94.1%
ATM1.4	Y = 14.06*X + 24.34	0.9972	38.4%	52.5	120.%

C

HbA	Equation	R^2	%HbA VCN=1	Compared to ALS10-T87Q
ALS10-T87Q	Y = 14.76*X + 4.082	0.8823	18.4%	100%
ALS10-T87Q silent	Y = 16.22*X + 9.314	0.9759	25.5%	139%
ATM1.1	Y = 10.99*X + 4.262	0.9592	15.3%	82%
ATM1.1 silent	Y = 7.324*X + 11.61	0.9312	18.9%	103%

HbF	Equation	R^2	%HbF VCN=1	Compared to ALS10-T87Q
ALS10-T87Q	Y = -0.357*X + 8.39	0.0204	8.0%	100%
ALS10-T87Q silent	Y = -1.604*X + 9.08	0.9111	7.5%	109.2%
ATM1.1	Y = 5.101*X + 11.25	0.5878	16.4%	114.1%
ATM1.1 silent	Y = 0.211*X + 19.85	0.0204	20.1%	140.8%

HbF + HbA	Equation	R^2	%HbF+%HbA VCN=1	Compared to ALS10-T87Q [(Hba+HbF)/HbA]%
ALS10-T87Q	Y = 14.45*X + 14.44	0.7692	28.89%	100%
ALS10-T87Q silent	Y = 15.76*X + 17.57	0.9101	33.33%	115%
ATM1.1	Y = 17.39*X + 15.96	0.7865	33.35%	115.4%
ATM1.1 silent	Y = 8.112*X + 31.75	0.7826	39.86%	138.0%

D

Target	Forward Primer	Reverse Primer
Silβ-globin	GTGGATGAAGTCGGCGGC	GGAGAAGGCACCGAGCAC
WTβ-globin	GTGGATGAAGTTGGTGGT	ACTAAAGGCACCGAGCAC
γ-globin	TGGCCTGTGGAGTAAGGTCAA	GAAGCAGAGGACAAGTTCCCA
β-actin	GTTTGAGACCTTCAACCCCAGCC	TAGATGGGCACAATGTGGGTGA

Supplemental Figure 2.