

CRISPR Editing Enables Consequential Tag-Activated MicroRNA-Mediated Endogene Deactivation

Panayiota L. Papasavva ^{1,2}, Petros Patsali ^{1,2}, Constantinos C. Loucari ^{1,2}, Ryo Kurita ³, Yukio Nakamura ⁴, Marina Kleanthous ^{1,2,†} and Carsten W. Lederer ^{1,2,*,†}

¹ Department of Molecular Genetics Thalassemia, The Cyprus Institute of Neurology and Genetics, Nicosia 2371, Cyprus; panayiotap@cing.ac.cy (P.L.P.); petrospa@cing.ac.cy (P.P.); loucari.constantinos@gmail.com (C.C.L.); marinakl@cing.ac.cy (M.K.)

² Cyprus School of Molecular Medicine, Nicosia 2371, Cyprus

³ Research and Development Department, Central Blood Institute, Blood Service Headquarters, Japanese Red Cross Society, Koto-ku, Tokyo 135-8521, Japan; r-kurita@jrc.or.jp

⁴ Cell Engineering Division, RIKEN BioResource Research Center, Tsukuba 305-0074, Japan; yukio.nakamura@riken.jp

* Correspondence: lederer@cing.ac.cy

† Contributed equally to this work.

Table S1. Detected erythromiRs in CD34+ and HUDEP-2 cells sorted by late-erythroid expression

erythromiR	late-erythroid expression ¹	log ₂ fold change (late vs early) ²
hsa-miR-451a	30227.74	4.394422
hsa-let-7a-5p	18887.48	1.275848
hsa-miR-16-5p	18473.67	1.283366
hsa-let-7b-5p	8885.507	2.235738
hsa-miR-22-3p	5006.113	2.003479
hsa-miR-15b-5p	4237.653	2.705584
hsa-miR-1246	4041.64	1.336994
hsa-miR-486-5p	3326.803	1.642032
hsa-miR-12136	713.3783	1.945231
hsa-miR-26b-5p	498.4767	1.162958
hsa-miR-15a-5p	474.665	3.129503
hsa-miR-182-5p	463.9217	3.836165
hsa-miR-183-5p	245.3	3.86204
hsa-miR-194-5p	124.6483	1.744664
hsa-miR-324-5p	85.535	1.186376
hsa-miR-4488	60.32	2.472766
hsa-miR-2110	36.21167	2.465297
hsa-miR-629-5p	34.18667	2.605443
hsa-miR-96-5p	33.9	3.76696
hsa-miR-589-5p	26.34833	1.059306
hsa-miR-320d	20.59	1.941568
hsa-miR-320c	18.29667	2.08572
hsa-miR-505-5p	14.03667	4.395572
hsa-miR-22-5p	11.71667	3.776355
hsa-miR-4732-3p	11.20333	1.479488
hsa-miR-1843	7.275	1.627817
hsa-miR-5010-3p	6.801667	1.747507
hsa-miR-5010-5p	4.846667	4.618168
hsa-miR-194-3p	4.063333	1.714765
hsa-miR-550b-3p	3.81	1.159548
hsa-miR-628-3p	2.996667	1.908415

hsa-miR-3688-3p	2.328333	1.707055
hsa-miR-4732-5p	2.271667	2.883218
hsa-miR-4516	2.133333	2.131863
hsa-miR-548at-5p	1.661667	1.620527
hsa-miR-1306-3p	1.661667	1.217872
hsa-miR-1255b-5p	1.303333	1.093899
hsa-miR-663b	1.203333	2.058942
hsa-miR-3189-3p	1.186667	2.308404
hsa-miR-6513-3p	1.113333	4.453584
hsa-miR-6842-5p	1.075	2.944518
hsa-miR-11401	0.913333	1.181792
hsa-miR-4422	0.843333	1.406704
hsa-miR-4492	0.79	1.737284
hsa-miR-616-3p	0.68	2.021614
hsa-miR-1225-5p	0.656667	2.779737
hsa-miR-133a-3p	0.611833	2.139851
hsa-miR-483-5p	0.573333	4.218646
hsa-miR-3140-3p	0.565	1.681019
hsa-miR-6741-5p	0.556667	1.174868
hsa-miR-3605-5p	0.526667	1.429845
hsa-miR-3688-5p	0.491833	1.953919
hsa-miR-1249-5p	0.490167	1.310045
hsa-miR-4449	0.488333	1.255644
hsa-miR-183-3p	0.460167	3.121828
hsa-miR-4747-5p	0.388333	4.346226
hsa-miR-3619-3p	0.361833	2.460143
hsa-miR-6884-5p	0.356667	2.694188
hsa-miR-3667-5p	0.355	1.494749
hsa-miR-4525	0.3485	3.747597
hsa-miR-6505-3p	0.345	1.684867
hsa-miR-6825-5p	0.330167	6.509475
hsa-miR-664b-5p	0.305333	1.8195
hsa-miR-3191-5p	0.303333	2.856571
hsa-miR-4429	0.296667	3.505062
hsa-miR-4489	0.286833	1.541892
hsa-miR-3190-5p	0.275333	6.671596
hsa-miR-5695	0.271667	1.777872
hsa-miR-184	0.27	2.575449
hsa-miR-3190-3p	0.238333	2.466754
hsa-miR-7155-3p	0.226667	2.269027
hsa-miR-6837-5p	0.135167	3.199413
hsa-miR-6800-5p	0.123333	1.895501

¹: mean normalized miRNA counts in late-erythroid samples and ²: mean log₂ fold change of miRNA expression in late- vs early-erythroid samples, both as calculated by DEGseq [37]

Table S2. Synthetic, modified oligonucleotides used as donor DNA

Type	Oligo_Name	Annotation	Sequence (5'>3')
dsODNs donors for NHEJ-mediated delivery	dsODN451-2MRSSs (ordered as DNA duplex from Metabion)	Sense	5'-P-A*A*CTCAGTAATGGTAACGGTTTGGCCAACTCAGTAATGGTAACGGT*T*T
		Antisense	5'-P-A*A*ACCGTTACCATTACTGAGTTGGCCAAACCGTTACCATTACTGAG*T*T
	dsODN451-4MRSSs (ordered as DNA duplex from Metabion)	Sense	5'-P-A*A*CTCAGTAATGGTAACGGTTTGGCCAACTCAGTAATGGTAACGGTTTCATCAACTCAGTAATGGTAACGGTTTCGATAACTCAGTAATGGTAACGGT*T*T
		Antisense	5'-P-A*A*ACCGTTACCATTACTGAGTTATCGAAACCGTTACCATTACTGAGTTGATGAAACCGTTACCATTACTGAGTTGGCCAAACCGTTACCATTACTGAG*T*T
	dsODN451-2MRSSs (ordered as DNA oligo from IDT)	Sense	5'-P-A*A*CTCAGTAATGGTAACGGTTTGGCCAACTCAGTAATGGTAACGGT*T*T
		Antisense	5'-P-A*A*ACCGTTACCATTACTGAGTTGGCCAAACCGTTACCATTACTGAG*T*T
	dsODN451-4MRSSs (ordered as DNA oligo from IDT)	Sense	5'-P-A*A*CTCAGTAATGGTAACGGTTTGGCCAACTCAGTAATGGTAACGGTTTCATCAACTCAGTAATGGTAACGGTTTCGATAACTCAGTAATGGTAACGGT*T*T
		Antisense	5'-P-A*A*ACCGTTACCATTACTGAGTTATCGAAACCGTTACCATTACTGAGTTGATGAAACCGTTACCATTACTGAGTTGGCCAAACCGTTACCATTACTGAG*T*T
ssODNs for HDR-mediated delivery	GUIDESeq-dsODN (ordered as DNA Oligo from IDT)	Sense	5'-P-G*T*TTAATTGAGTTGTCATATGTTAATAACGGT*A*T
	GUIDESeq-dsODN (ordered as DNA Oligo from IDT)	Antisense	5'-P-A*T*ACCGTTATTAACATATGACAACTCAATTAA*A*C
	ssODN451TS-1MRS	Ultramer DNA Oligo	A*A*G GGA CAA AAA GCA CAC TAC ATA ACA AAC CAA CGA AAC CGT TAC CAT TAC TGA GTT TTA TTA GCT TGC AGT ACT GCA TAC AGT ATG GCA* G*C
	ssODN451NTS-1MRS	Ultramer DNA Oligo	G*C*T GCC ATA CTG TAT GCA GTA CTG CAA GCT AAT AAA ACT CAG TAA TGG TAA CGG TTT CGT TGG TTT GTT ATG TAG TGT GCT TTT TGT CCC* T*T
	ssODN451TS-2MRSSs	Ultramer DNA Oligo	A*A*G GGA CAA AAA GCA CAC TAC ATA ACA AAC CAA CGA AAC CGT TAC CAT TAC TGA GTT GGC CAA ACC GTT ACC ATT ACT GAG TTT TAT TAG CTT GCA GTA CTG CAT ACA GTA TGG CA*G* C
	ssODN451NTS-2MRSSs	Ultramer DNA Oligo	G*C*T GCC ATA CTG TAT GCA GTA CTG CAA GCT AAT AAA ACT CAG TAA TGG TAA CGG TTT GGC CAA CTC AGT AAT GGT AAC GGT TTC GTT GGT TTG TTA TGT AGT GTG CTT TTT GTC CC*T* T
	Alt-R HDR-2MRSSs	Alt-R HDR Donor Oligo	A*A*G GGA CAA AAA GCA CAC TAC ATA ACA AAC CAA CGA AAC CGT TAC CAT TAC TGA GTT GGC CAA ACC GTT ACC ATT ACT GAG TTT TAT TAG CTT GCA GTA CTG CAT ACA GTA TGG CA*G* C

*P denotes a 5' phosphorylation and * indicates a phosphorothioate linkage.*

Table S3. Oligonucleotides for cloning into the gRNA scaffold of lentiCRISPRv2

Target site	Orientation	Oligonucleotides (5'>3')
SC	Sense	CACCGGCTTGC GCGGAGACATGG
	Antisense	AAACCCATGTCTCGCCGCAAGCC
3' UTR 1	Sense	CACCGGTACTGCAAGCTAATAACGT
	Antisense	AAACACGTTATTAGCTTGCACTACC
3' UTR 2	sense	CACCGAGTGTATTTAATTGCGTTCC
	antisense	AAACGGAACGCAATTAAATACACTC
3' UTR 3	Sense	CACCGTTTGTCTCAGCAACGAATTA
	Antisense	AAACTAATTCGTTGCTGAGCAAACC
3' UTR 4	Sense	CACCGTATGATTTATTAGCACAACG
	Antisense	AAACGTTGTGCTAATAAATCATAC
5' UTR 1	Sense	CACCGGACGACGGCTCGGTTACAT
	Antisense	AAACATGTGAACCGAGCCGTCGTCC
5' UTR 2	Sense	CACCGACGACGGCTCGGTTACATC
	Antisense	AAACGATGTGAACCGAGCCGTCGTC
5' UTR 3	Sense	CACCGGAGTCTCCTTCTTTCTAACC
	Antisense	AAACGTTAGAAAGAAGGAGACTCC

Table S4. Primers

Name	Orientation	Sequence (5'>3')
SC	Forward	AAAGCCATGACGGCTCTCCACAAT
	Reverse	CGGCAATGGTTCCAGATGGG
3' UTR 1	Forward	ATTTGGCAGGATAATATAGTGC
	Reverse	CGGGCTTTCTCTTTAATATG
3' UTR 2	Forward	TTGTATTCTCACAACAATGGCTAC
	Reverse	CCTCTTCCTTTCAATATGGTATACA
3' UTR 3	Forward	CCTTTATGTTCTCACCGTTT
	Reverse	CCTCCTCACGTTATAAAATA
3' UTR 4	Forward	CTGGCACCAAAAGAAATAGA
	Reverse	TTTAGGGAGCACAGACATAT
5' UTR 1	Forward	AAGCCATGACGGCTCTCCACAAT
	Reverse	CCTGCGCGCTCTCGTGATTA
5' UTR 2	Forward	AAGCCATGACGGCTCTCCACAAT
	Reverse	CCTGCGCGCTCTCGTGATTA
5' UTR 3	Forward	AAGCCATGACGGCTCTCCACAAT
	Reverse	CCTGCGCGCTCTCGTGATTA
DONOR 451	Forward	GGTTTGGCCAACCTCAGTAATG
lentiCRISPRv2	Forward	GAGGGCCTATTTCCCATGATT

Table S5. Synthetic gRNAs for ribonucleoprotein complex formation

Target site	CRISPR RNA (crRNA) (5'>3')
SC	GCTTGCGGCGAGACATGG
3' UTR 1	GTACTGCAAGCTAATAACGT
3' UTR 4	TATGATTTATTAGCACAACG
5' UTR 1	GACGACGGCTCGGTTACAT
5' UTR 3	GAGTCTCCTTCTTCTAACC

Table S6. Antibodies used in immunoblotting

Type	Antibodies	Dilution	Diluent
Primary antibodies	Mouse Anti-Ctip1/BCL11A antibody; ab19487	1:1,000	1% BSA/1× TBS-0.05% Tween 20
	Hemoglobin α (H-80) rabbit polyclonal IgG; sc-21005	1:1,000	1% BSA/1× TBS-0.05% Tween 20
	Hemoglobin γ (51-7) mouse monoclonal IgG1; sc-21756	1:1,000	1% BSA/1× TBS-0.05% Tween 20
	Anti-β-actin (AC-15) mouse monoclonal IgG1; A-1978	1:10,000	1% skimmed milk/1× TBS-0.05% Tween 20
Secondary antibodies	Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L); 115-035-003	1:8,000	3% skimmed milk/1× TBS-0.05% Tween 20
	Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L); 111-035-003		

TBS: Tris-buffered saline, Tween 20: TWEEN® 20 Detergent from Sigma-Aldrich, Munich, Germany

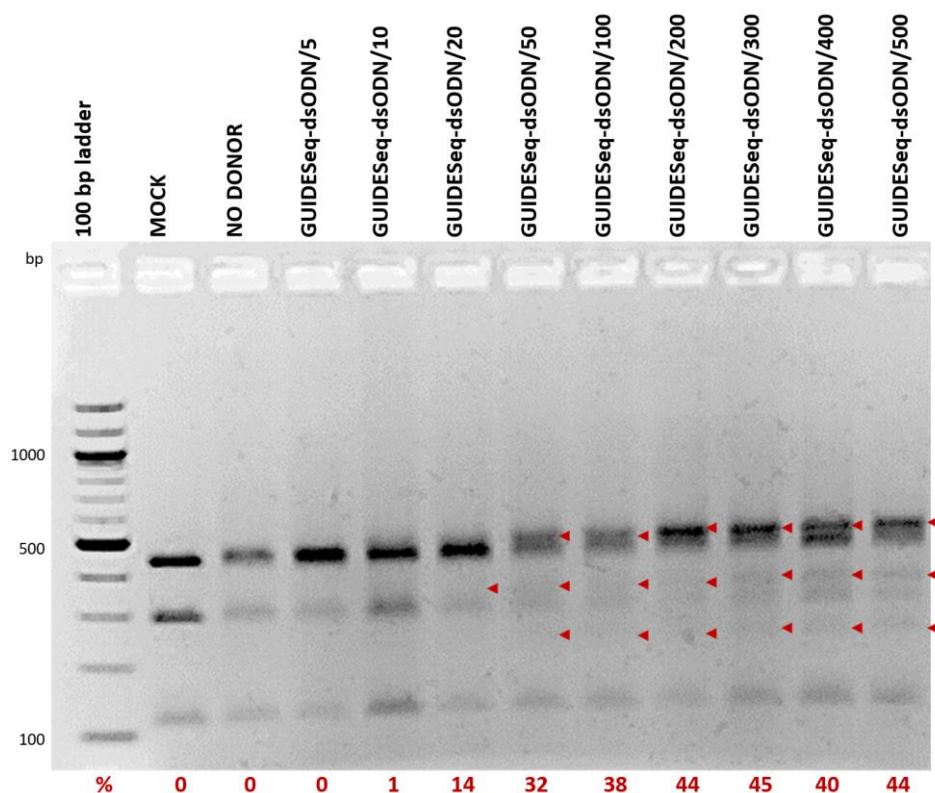


Figure S1. NHEJ-mediated integration of GUIDESeq-dsODN in HEK293T cells. PCR-RFLP analysis of HEK293T cells nucleofected with RNPs and GUIDESeq dsODN was performed to assess integration efficiency. Bands of 436, 288 and 118 bp represent absence of insertion, bands larger than 436 bp represent insertions with incomplete digestion by DdeI, and bands of 323 and 234 bp represent cleavage products of insertions. All bands corresponding to insertions are indicated by arrowheads. Corresponding rates of Dde cleavage (%) (after subtraction of background average cleavage rate of control samples) are reported below the gel. MOCK: mock-nucleofected sample, NO DONOR: cell sample nucleofected only with RNPs, GUIDESeq-dsODN/*: cell samples nucleofected with RNPs and GUIDESeq-dsODN at the indicated picomole quantity. The gel image was processed with an automatic noise filter to remove fluorescent inclusion artefacts.

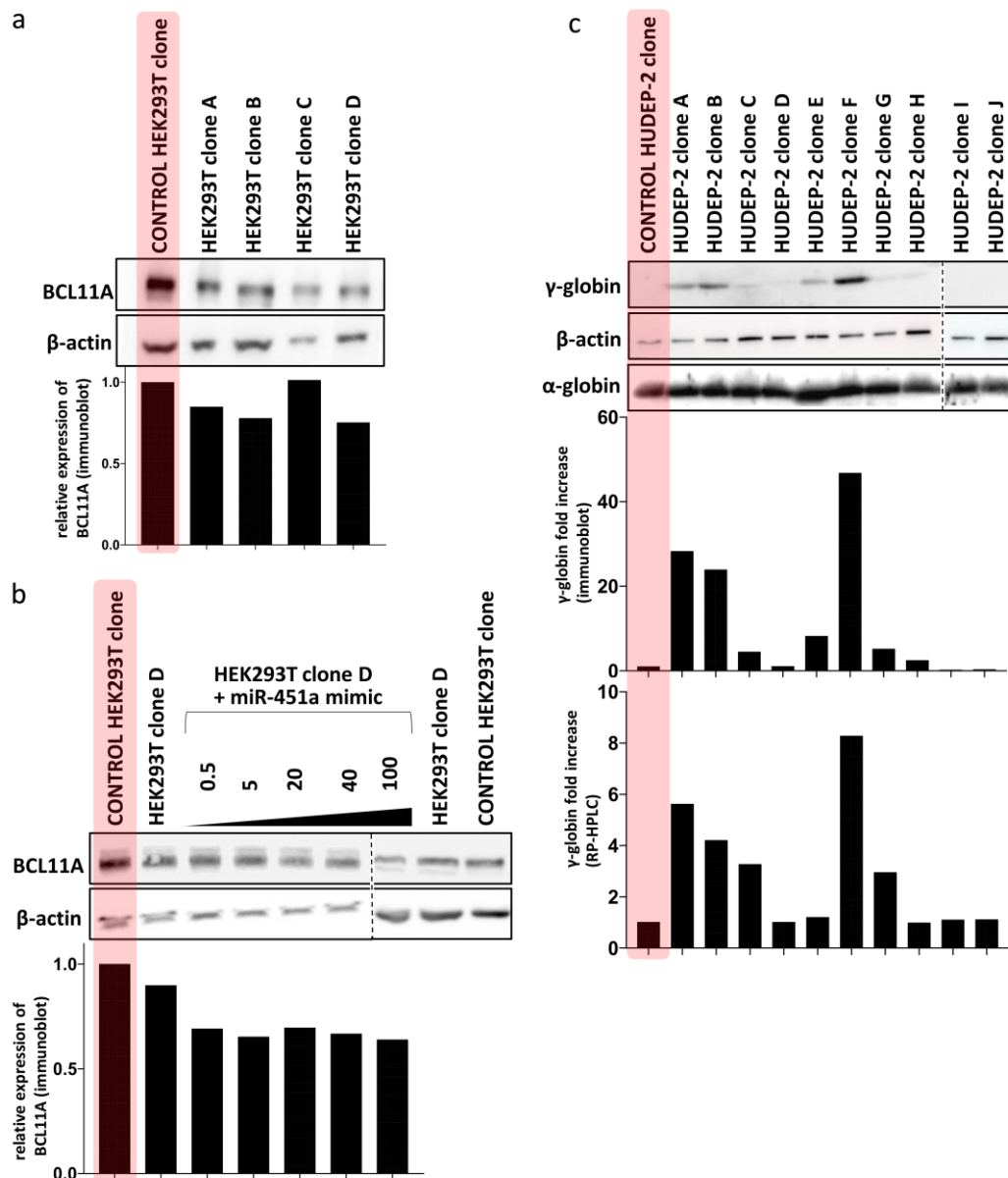


Figure S2. Analysis of monoclonal cell populations at the protein level. One sample per clone (n=1) was analyzed. (a) Immunoblot analysis of HEK293T clones bearing MRSs for miR-451a showed slightly diminished BCL11A expression in clones A (by ~15%), B (by ~22%) and D (by ~25%), relative to expression in a non-transfected wild-type CONTROL HEK293T clone. (b) Transient transfection of 0.5–100 pmole of synthetic hsa-miR-451a miRNA mimic in HEK293T clone D (clone harboring 4 MRSs in homozygosity) resulted in up to ~36% repression of BCL11A expression relative to expression in CONTROL HEK293T clone, and up to 26% compared to the non-transfected HEK293T clone D. (c) Immunoblot analysis of HUDEP-2 clones for γ -globin expression after erythroid differentiation showed 28.3-, 23.9- and 46.8-fold increase in HUDEP-2 clones A, B and F, respectively, and <6-fold increase in clones C, E, G, H, relative to expression in a non-transfected wild-type CONTROL HUDEP-2 clone. Expression of γ -globin was normalised to β -actin (for protein loading) and α -globin (for the level of erythroid differentiation), respectively. Less sensitive RP-HPLC analysis of the same clones showed 5.62-, 4.20- and 8.28-fold increase of γ -globin expression in clones A, B and F, respectively, and <3.5-fold increase in clones C and G. Control bands and values are designated with red overlays. Results for γ -globin expression showed good correlation between the two analysis methods ($r = 0.952$, $R^2 = 0.907$, $p < 0.0001$). Separate blots are indicated by dashed lines. CONTROL HEK293T clone: non-transfected wild-type HEK293T clone, HEK293T clone D: non-transfected HEK293T clone D, HEK293T clone D_miR-451a mimic: HEK293T clone D transfected with miR-451a miRNA mimic with quantities of 0.5–100 pmole.

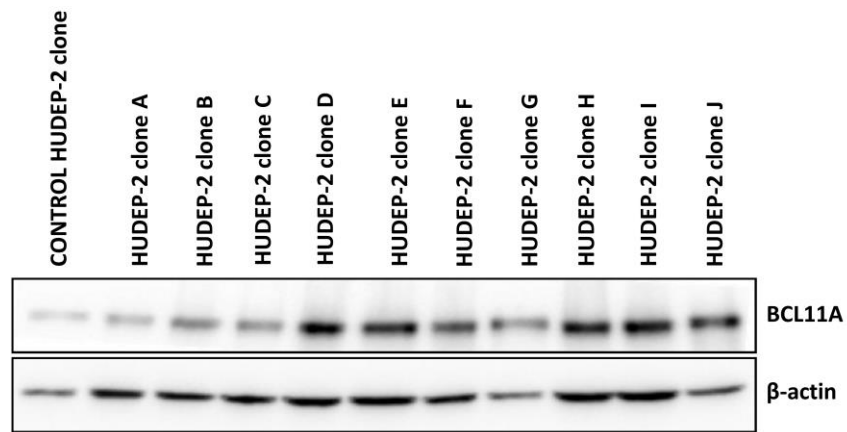


Figure S3. Immunoblot analysis of BCL11A expression in HUDEP-2 clones. The analysis was performed on D4 of erythroid differentiation (intermediate stage) and showed reduction of BCL11A expression only in HUDEP-2 clone A (27% relative to the wild-type CONTROL HUDEP-2 clone) and variably also upregulation in few others (up to 183% for HUDEP-2 clone J).