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Lab Resource: Multiple Cell Lines



Generation and genetic repair of two human induced pluripotent cell lines from patients with Epidermolysis Bullosa simplex and dilated cardiomyopathy associated with a heterozygous mutation in the translation initiation codon of *KLHL24*

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A B S T R A C T

Fibroblasts from two patients carrying a heterozygous mutation in the translation initiation codon (c.2 T > G) of the *kelch-like protein 24 (KLHL24)* gene were used to generate human induced pluripotent stem cells (hiPSCs), using non-integrating Sendai virus to deliver reprogramming factors. CRISPR-Cas9 editing was used for genetic correction of the mutation in the patient-hiPSCs. The top-predicted off-target sites were not altered. Patient and isogenic hiPSCs showed typical morphology, expressed pluripotency-associated markers, had the capacity for *in vitro* differentiation into the three germ layers and displayed a normal karyotype. These isogenic pairs will enable *in vitro* modelling of KLHL24-associated heart and skin conditions.

1. Resource Table:

(continued)

Unique stem cell lines identifier	LUMCi045-A LUMCi046-A LUMCi045-A-1 LUMCi046-A-1	Gene/locus	Epidermolysis Bullosa Simplex intermediate with Dilated Cardiomyopathy <i>KLHL24</i> /3q27 Heterozygous <i>KLHL24</i> c.2 T > G (LUMCi045-A and LUMCi046-A)
Alternative name(s) of stem cell lines	LUMCi0145KLHL01 (LUMCi045-A) LUMCi0146KLHL10 (LUMCi046-A) Iso01LUMCi0145KLHL01 (LUMCi045-A-1) Iso02LUMCi0146KLHL10 (LUMCi046-A-1)	Date archived/stock date	Corrected Heterozygous <i>KLHL24</i> c.2 G > T; c.6 A > C (LUMCi045-A-1) Corrected Heterozygous <i>KLHL24</i> c.2 G > T; n.c.-3 A > G (LUMCi046-A-1) 2021-05-03/02
Institution	LUMC hiPSC Hotel, Department of Anatomy and Embryology, Leiden University Medical Center, Einthovenweg 20, 2333 ZC Leiden, The Netherlands	Cell line repository/bank	N/A
Contact information of distributor	Karine Raymond (k.i.raymond@lumc.nl)	Ethical approval	Ethical committees and approval numbers - Comité Ético Científico, Facultad de Medicina, Clínica Alemana - Universidad del Desarrollo (Project number 2013-145). - Medical Ethical Committee at the Leiden University Medical Center (P13.080). - Informed consent was obtained from the patients.
Type of cell lines	hiPSCs		
Origin	Human		
Additional origin info required	Age: 33 (LUMCi045-A) and 25 (LUMCi046-A) Sex: males		
for human ESC or iPSC	Ethnicity if known: Hispanic, Chilean		
Cell Source	Skin Fibroblasts		
Clonality	Clonal		
Associated disease			

(continued on next column)

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2. Resource utility

Patients with mutations in the translation initiation codon of *KLHL24* display Epidermolysis Bullosa Simplex (EBS) associated with high risk of developing Dilated Cardiomyopathy (Has et al., 2020). hiPSCs were generated from two patients with heterozygous c.2 T > G mutation and CRISPR-Cas9 was used to correct the mutation, providing isogenic pairs for *in vitro* disease modelling.

3. Resource details

Initially identified as causing genetically unresolved EBS, mono-allelic mutations in the translation initiation codon of *KLHL24* were recently associated with the development of dilated cardiomyopathies (Has et al., 2020). The encoded protein, KLHL24, belongs to the ubiquitin-proteasome system that regulates protein turnover; however, the pathological mechanisms that cause the disease phenotype remain poorly defined. The generation of mutant and genetically corrected isogenic hiPSC lines offers an unlimited source of the different cell types and enables the study the *KLHL24*-associated pathologies in a well-controlled disease model. Fibroblasts were isolated from skin biopsies of two male patients with a heterozygous c.2 T > G mutation in *KLHL24* and were reprogrammed at passage 3 into hiPSCs using replication-defective and persistent Sendai virus carrying *OCT3/4*, *SOX2*, *KLF4*, *MYC* (Nishimura et al., 2011). One hiPSC line was characterized for each patient: lines LUMCi045-A and LUMCi046-A (Table 1). LUMCi045-A and LUMCi046-A were negative for SeV after 7 passages as indicated by qRT-PCR (Supplementary Fig. 1A). They displayed a typical stem cell morphology with high nucleus to cytoplasm ratio (Fig. 1A) and a normal karyotype as assessed by G-banding (passages 22) and KaryoStat assay (passage 15 and 22, respectively) (Fig. 1F,G). Their pluripotency status was confirmed by expression of pluripotency markers: SSEA4, NANOG and OCT3/4 were clearly detected by immunofluorescence staining (Fig. 1C) and SSEA4 and NANOG were found by flow-cytometry (Fig. 1D). The lines were able to differentiate into derivatives of all three germ layers in a ‘spontaneous differentiation’ assay *in vitro*, as illustrated by immunofluorescence staining for the ectodermal marker β III-tubulin (B3-TUB), the endodermal marker α -fetoprotein (AFP) and the mesodermal marker platelet endothelial cell adhesion molecule-1 (PECAM-1) (Fig. 1E). The presence of heterozygous c.2 T > G mutation in exon 4 of *KLHL24* was confirmed by Sanger sequencing (Fig. 1B). The mutated allele was repaired by a Cas9-ribonucleoprotein (RNP) complex, with a mutation-specific single guide RNA (sgRNA) and a single-stranded oligodeoxynucleotide (ssODN) donor template containing the wild-type sequence with a silent mutation either to introduce a *Ava*II restriction site or to disrupt the *Mae*III site (ssODN-1 and -2, for LUMCi045-A-1 and LUMCi046-A-1, respectively; Table 1). Single-cell derived colonies were screened for repair by using the *Ava*II (for LUMCi045-A-1) or *Mae*III (for LUMCi046-A-1) restriction of PCR amplified *KLHL24* start codon region. Correction of the mutation was confirmed by Sanger sequencing (Fig. 1B). The repaired hiPSCs showed the expected morphology and a normal karyotype (passage 6 after gene editing) using G-banding (Fig. 1A,G). Immunofluorescence staining and flow-cytometry analyses revealed expression of the pluripotency markers (Fig. 1C,D) and spontaneous differentiation into the three germ layers was observed *in vitro*, as illustrated by immunofluorescence staining with specific markers as described above (Fig. 1E). All lines were mycoplasma negative (Supplementary Fig. 1B). The origin of the isogenic pairs was confirmed by short tandem repeat (STR) analysis which fully matched the profile of the patient’s fibroblasts. Finally, the absence of off-target mutations was confirmed by Sanger sequencing of the top5 as well as all the exon sites predicted by CRISPOR (crispor.tefor.net; data not shown) (Haeussler et al., 2016). The complete characterization is summarized in Table 1.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Transmission light microscopy	Normal	Fig. 1A
Phenotype	Pluripotency status, qualitative analysis: Immunofluorescent staining	All the lines showed positive staining of pluripotency markers: Oct3/4, NANOG, SSEA4.	Fig. 1C
	Pluripotency status, quantitative analysis: flow-cytometry	Percentage of cells positives for NANOG and SSEA-4: LUMCi045-A (85.9 %) LUMCi046-A (96.3 %) LUMCi045-A-1 (92.2 %) LUMCi046-A-1 (98.3 %)	Fig. 1D
Genotype	Karyotype G-banding, Resolution 5–10 Mb (all four lines)	LUMCi045-A: 46XY LUMCi046-A: 46XY	Fig. 1F,G
	KaryoStat Resolution > 1–2 Mb (two non-edited lines)	LUMCi045-A-1: 46XY LUMCi046-A-1: 46XY	
Identity	Microsatellite PCR (mPCR)	Not performed	N/A
	STR analysis	24 sites tested; all sites matched	Submitted in archive with journal Fig. 1B
Mutation analysis (IF APPLICABLE)	Sequencing	LUMCi045-A and LUMCi046-A : heterozygous c.2 T > G	N/A
		LUMCi045-A-1: corrected c.2 G > T; heterozygous c.6 A > C (silent mutation) LUMCi046-A-1: corrected c.2 G > T; heterozygous n.c.-3 A > G (silent mutation)	
Microbiology and virology	Southern Blot OR WGS	Not performed	N/A
	Luminescence-based mycoplasma testing. Detection SeV by RT-Q-PCR	Negative.	Supplementary Fig. 1B
Differentiation potential	Pluripotency function; spontaneous differentiation	Negative.	Supplementary Fig. 1A
		Expression of ectodermal marker β 3-tubulin (B3-TUB), endodermal marker alpha-fetoprotein (AFP) and mesodermal marker platelet endothelial cell adhesion molecule-1 (PECAM-1) was detected.	Fig. 1E
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

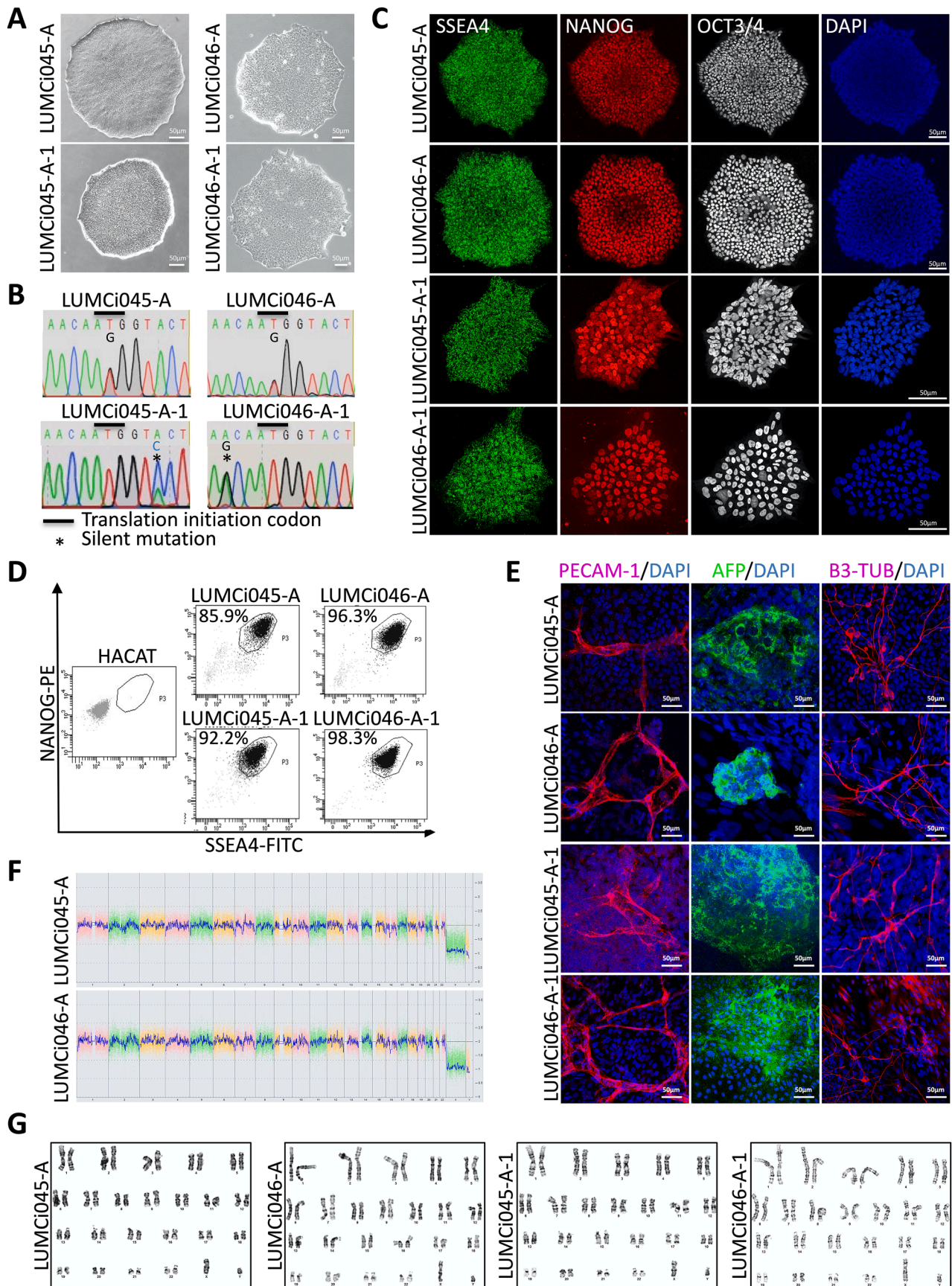


Fig. 1.

4. Materials and methods

4.1. Cell reprogramming, maintenance and differentiation

This study was approved by ethical committees as indicated in Resource Table.

Fibroblasts were isolated from skin biopsies and cultured in fibroblast medium (FM) composed of DMEM/F12 Glutamax supplemented with 10% Fetal Bovine Serum (FBS), 1% Non-Essential Amino Acids, 0.18% β -mercaptoethanol, 1% penicillin/streptomycin (all from ThermoFisher Scientific: #31331; #10270; #11140 #31350; #15070) and 10 μ g/ml Ascorbic acid (Sigma-Aldrich, #A5960). At passage 3, 10^5 cells were transduced with Sendai virus (SeVdp (KOSM302L), MOI 7.5). 16 h after transduction, cells were plated at the density of 10^3 cells/cm² on Matrigel coated plates (Corning, #354277) in FM. The next day, the cells were refreshed and subsequently maintained with ReproTeSRTM (Stemcell Technologies, #05921, #05922, #05923). At day 18, culture was shifted to TeSRTM E8TM medium (Stemcell Technologies, #05990). After mechanical picking, hiPSCs were maintained in TeSRTM-E8TM on vitronectin-coated plates (Stemcell Technologies, #07180) at 37 °C, 5% CO₂ and 20 % O₂. hiPSCs were passaged once a week as small aggregates using Gentle Cell Dissociation Reagent (Stemcell Technologies, #07174) with a splitting ratio of 1:25. Phase contrast images of live cells were taken 7 days after passage using a Nikon eclipse T1 microscope. To induce differentiation, three days after passage on vitronectin-coated glass coverslips, hiPSCs were cultured in DMEM/F12 containing 20% FBS for 3 weeks with media change every two days.

4.2. qRT-PCR

RNA was isolated with the NucleoSpin® RNA kit (Macherey-Nagel, #740955.50). Reverse transcription of RNA (500 ng) was performed using the iScriptTM cDNA Synthesis Kit (BioRad, #1708891). cDNA was amplified on CFX96 Real-Time PCR Detection System using iQ SYBR Green Supermix (BioRad, # 1708891). Primers are shown in Table 2.

4.3. Gene editing

10^5 cells (LUMCi045-A and LUMCi046-A, passages 15 and 28) were transfected with the Cas9-RNP complex and the ssODN (Integrated DNA Technologies) using the NEON Transfection System (Invitrogen) at 1200 V/30 ms/1pulse. Cells were plated on Corning® Synthmax® II-SC substrate (Merck, # CLS3535-1EA) using TeSRTM-E8TM with CloneR (Stemcell Technologies, #05888). After recovery, 1000 cells were plated on Synthmax II-SC-coated 10 cm dish in TeSRTM-E8TM with CloneR and single cell-derived colonies were screened for the corrected genotype. DNA was isolated using QuickExtractTM DNA Extraction Solution (Lucigen, #QE0905T) and the region of interest was amplified by PCR (Terra PCR Direct Polymerase Mix, TaKaRa; Bio-Rad S1000 Thermal Cycler; program available upon request). Corrected clones were identified by analysis of the restriction pattern obtained after digestion of the PCR fragment with AvaII (LUMCi045-A-1) or MaeIII (LUMCi046-A-1) (New England Biolabs) and confirmed by sequencing.

4.4. Sequencing

Sanger sequencing was performed by Leiden Genome Technology Centre using the ABI3730xl system.

4.5. Immunofluorescence staining

Immunofluorescence staining and imaging were performed as described (Bouma et al., 2019). Primary and secondary antibodies are reported in Table 2.

Table 2

Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Marker	mouse IgG2b anti-OCT3/4	1:100	Santa Cruz, Sc-5279	RRID: AB_628051
Pluripotency Marker	mouse IgG1 anti-NANOG	1:150	Santa Cruz, Sc-293121	RRID: AB_2665475
Pluripotency Marker	mouse IgG3 anti-SSEA4	1:30	Biologend, #330402	RRID: AB_1089208
Pluripotency Markers	PE Mouse IgG1 anti-NANOG	1:5	BDBioscience, AB#560583	RRID: AB_1645522
Pluripotency Markers	FITC Human IgG1 anti-SSEA4	1:25	Miltenyi, #130-098-371	RRID: AB_2653517
Differentiation Markers	mouse IgG2ba anti-b3-tubulin	1:4 000	Covance, #MMS-435P	RRID: AB_2313773
Differentiation Markers	rabbit anti-AFP	1:25	Quartett, #2011200530	RRID: AB_2716839
Differentiation Markers	mouse IgG1 anti-PECAM-1	1:100	DAKO, #M0823	RRID: AB_2114471
Secondary antibodies	Alexa 647 Goat Anti-Mouse IgG2b	1:250	Invitrogen Cat# 21,242	RRID: AB_2535811
Secondary antibodies	Alexa 488 Goat Anti-Mouse IgG3	1:250	Invitrogen Cat# 21,151	RRID: AB_2535784
Secondary antibodies	Alexa 568 Goat Anti-Mouse IgG1	1:250	Invitrogen Cat# 21,124	RRID: AB_2535766
Secondary antibodies	Alexa 568 Goat Anti-Mouse IgG	1:500	Invitrogen Cat# A11031	RRID: AB_144696
Secondary antibodies	Alexa 488 Donkey Anti-rabbit IgG	1:500	Invitrogen Cat# A21206	RRID: AB_2535792
	Primers Target	Size of band	Forward/Reverse primer (5'-3')	
Sendai virus vector (Q-RT-PCR)	SeV	N/A	GCAGCTCTAACGTTGTCTAAA/ CCTGGAGCAAATTCACCATGA	
House keeping gene (Q-RT-PCR)	GAPDH	166 bp	TCCTCTGACTTCAACAGCGA/ GGGTCTTACTCCTTGGAGGC	
mutation site (PCR and Sanger sequencing)	Exon 4: <i>KLHL24</i>	764 bp	TTCAGCTAGAAAAGGGGAGCTT/ AACTTGGCACATGCATCACG	
Off-target site 1 (PCR and Sanger sequencing)	intron: <i>ABCA1</i>	391 bp	TCATAGCTCACTGGAACGTCA/ TGTCATGGGAAATGCTCTCC	
Off-target site 2 (PCR and Sanger sequencing)	intergenic: <i>TRAV3-TRAV4</i>	526 bp	AGGGAGCATAGGTTGTTTCTGA/ CTCAGCAAACAGGACAACCT	
Off-target site 3 (PCR and Sanger sequencing)	intergenic: <i>SAMD13-DNASE2B</i>	554 bp	CCACCCATCTGTATATTGGCTT/ CTCAGCAAGGCATTCTGTGG	
Off-target site 4 (PCR and Sanger sequencing)	intron: <i>CTD-2007A10.1</i>	386 bp	AGTTCACAAAGAGATGGTCCAA/ AGTGGCCTGAACTGTACCTT	
Off-target site 5 (PCR and Sanger sequencing)	intergenic: <i>RNF130-Metazoa_SRP</i>	246 bp	ACCCAAATTCACATACTTTCCA/ TTGATGGTGGTGTGGTGT	
Off-target exon 1 (PCR and Sanger sequencing)	exon: <i>GABRG3</i>	932 bp	CCTTGGGAGCTACAGAGAGG/ ACCTTTTGCTCAGTACCGGA	

(continued on next page)

Table 2 (continued)

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat #	RRID
Off-target exon 2 (PCR and Sanger sequencing)	exon: <i>AMER1</i>	722 bp	TCATTCGGAGCTCAACACT/ATGGCCACGTTCAACTCAAG	
Off-target exon 3 (PCR and Sanger sequencing)	exon: <i>HUWE1</i>	640 bp	AGGGAGGGATGCTTCAAACA/AAGAGCAGCATTTTGAGGCC	
Off-target exon 4 (PCR and Sanger sequencing)	exon: <i>RMND5A</i>	447bp	CTCTCCAGGTAGTCTCGCAG/AAACCTGCCACACCTAGACA	
Off-target exon 5 (PCR and Sanger sequencing)	exon: <i>WDHD1</i>	267 bp	TGCACCTCCCAATGTGCTAG/TTAGGCCAAAGACCGGGTTC	
Off-target exon 6 (PCR and Sanger sequencing)	exon: <i>TBC1D30</i>	527 bp	CCCTGTGAACACTCTCCTCT/TTAGGCCAAAGACCGGGTTC	
Off-target exon 7 (PCR and Sanger sequencing)	exon: <i>SRP72</i>	535 bp	CACCTTGCTCCACATCACTG/GGGCAGAGTGAGAGAAATGG	
Off-target exon 8 (PCR and Sanger sequencing)	exon: <i>NANOGP1</i>	313 bp	GATTTCTCAACCCCTTTTGG/GCCACCACATTCAGCTTTTT	
Off-target exon 9 (PCR and Sanger sequencing)	exon: <i>TNRC6B</i>	585 bp	TGCAGCCTCTTCTTTGTCT/CGACCTCCTCTGCTCTTTCT	
crRNA (gene editing)	Translation initiation codon <i>KLHL24</i> (c.2 T > G)	N/A	ATAGTCAACTGATGTAACAA	
ssODN-1 for repair c.2 T > G line LUMCi045-A (gene editing)		N/A	TGA TTT GAA TAC TGA ATT TTT TGC ATA TTG AAA TGT TTT CCT TTT TTT ACT TTT AGC CAC ATA AAG AAG ATC CCT AAT AGT CAT TTC TCA ACA ATT ATA TAG TCA ACT GAT GTA ACA ATG GTC CTA ATA TTG GGA CGC AGA CTA AAC AGA GAG GAT CTT GGG GTG CGT GAT TCC CCA	
ssODN-2 for repair c.2 T > G line LUMCi046-A (gene editing)		N/A	TGA TTT GAA TAC TGA ATT TTT TGC ATA TTG AAA TGT TTT CCT TTT TTT ACT TTT AGC CAC ATA AAG AAG ATC CCT AAT AGT CAT TTC TCA ACA ATT ATA TAG TCA ACT GAT GTA GCA ATG GTA CTA ATA TTG GGA CGC AGA CTA AAC AGA GAG GAT CTT GGG GTG CGT GAT TCC CCA	

RRID Requirement for antibodies: use <http://antibodyregistry.org/> to retrieve RRID for antibodies and include ID in table as shown in examples.

4.6. Flow-cytometry analysis

Cells were dissociated into single cells with Gentle Cell dissociation reagent (Stemcell Technologies, #07174) and fixed and permeabilized using the FIX & PERM™ Cell Permeabilization kit (ThermoFisher, #GAS004). Cells were incubated with the antibodies (Table 2) for 1 h in the dark at RT and analyzed with a LSRII flow-cytometer (BD). The HACAT keratinocyte line was used as a negative control.

4.7. Karyotyping

G-banding analysis was conducted at the Laboratory of Clinical

Genetics Leiden (LDGA). A total of 20 metaphases was analyzed. The KaryoStat assay was performed according to manufacturer's instructions (ThermoFisher Scientific).

4.8. Evaluation of off-target effects

The Top5 and exonic off-target sites were predicted using the CRISPOR online tool (crispo.tefor.net) (Haeussler et al., 2016). PCR products of the predicted sites were analyzed by sequencing.

4.9. Mycoplasma detection

The mycoplasma status was assessed using the MycoAlert™ mycoplasma detection kit (Lonza, #LT07-418) following the manufacturer's protocol.

4.10. STR analysis

Cell line authentication was performed by the Department of Human Genetics, LUMC, by using the PowerPlex® Fusion System 5C autosomal STR kit (Promega) as previously described (Westen et al., 2014).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2021.102582>.

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