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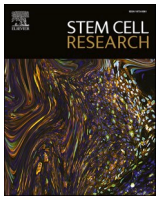

Lab Resource: Genetically-Modified Multiple Cell Lines

Generation and genetic repair of two human induced pluripotent stem cell lines from patients with Epidermolysis Bullosa simplex 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 distinct heterozygous mutation in the *KLHL24* gene (c.1 A>G and c.2T>C) were reprogrammed to obtain hiPSC lines. Non-integrating Sendai virus and CRISPR-Cas9 editing were respectively used to deliver the reprogramming factors and repair the mutation in the patient-hiPSCs to obtain isogenic control pairs. No off-target nuclease activity was detected with the top-predicted sites. Patient and isogenic hiPSCs displayed typical morphology, expressed markers of the undifferentiated state, were able to differentiate into the three germ layers and had normal karyotypes. These isogenic pairs will expand the panel of hiPSC lines to model KLHL24-associated conditions.

1. Resource table

Unique stem cell line identifier	LUMCi058-A (https://hpscreg.eu/user/cellline/edit/LUMCi058-A) LUMCi059-A (https://hpscreg.eu/user/cellline/edit/LUMCi059-A) LUMCi058-A-1 (https://hpscreg.eu/user/cellline/edit/LUMCi058-A-1) LUMCi059-A-1 (https://hpscreg.eu/user/cellline/edit/LUMCi059-A-1)
Alternative name(s) of stem cell line	LUMCi231KLHL03 (LUMCi058-A) LUMCi232KLHL01 (LUMCi059-A) Iso35LUMCi231KLHL03 (LUMCi058-A-1) Iso71LUMCi232KLHL01 (LUMCi059-A-1)
Institution	Leiden University Medical Center, LUMC
Contact information of the reported cell line distributor	Karine Raymond (k.i.raymond@lumc.nl)
Type of cell line	hiPSCs
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	*Age:* 19 (LUMCi058-A); 1 (LUMCi059-A) *Sex:* Female (LUMCi058-A); Male (LUMCi059-A) *Ethnicity/breed/other genetic background information if known:* Caucasian (both donors)
Cell Source	Fibroblasts
Method of reprogramming	Sendai virus, OCT3/4, SOX2, KLF4 and MYC
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	qRT-PCR and immunofluorescence
The cell culture system used	hiPSCs cultured on Vitronectin in TeSR™ E8™ medium
Type of the Genetic Modification	Genetic correction of heterozygous point mutations in the start codon of the *KLHL24* gene
Associated disease	EPIDERMOLYSIS BULLOSA SIMPLEX 6, GENERALIZED INTERMEDIATE, WITH OR WITHOUT CARDIOMYOPATHY; EBS6 (OMIM: 617294)
Gene/locus modified in the reported transgenic line	KLHL24 3q27.1 Corrected heterozygous mutations: c.1G>A (LUMCi058-A-1) c.2C>T (LUMCi059-A-1)

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(*continued*)

Method of modification / user-customisable nucleases (UCN) used, the resource used for design optimisation	CRISPR/Cas9
User-customisable nuclease (UCN) delivery method	Electroporation
All double-stranded DNA genetic material molecules introduced into the cells	N/A
Evidence of the absence of random integration of any plasmids or DS DNA introduced into the cells.	N/A
Analysis of the nuclease-targeted allele status	PCR and sequencing around the editing site
Homozygous allele status validation	N/A
Method of the off-target nuclease activity prediction and surveillance	Targeted PCR/Sequencing
Descriptive name of the transgene	N/A
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	N/A
Inducible/constitutive expression system details	N/A
Date archived/stock creation date	2024-06-07
Cell line repository/bank	LUMCi058-A (https://hpscreg.eu/user/cellline/edit/LUMCi058-A) LUMCi059-A (https://hpscreg.eu/user/cellline/edit/LUMCi059-A) LUMCi058-A-1 (https://hpscreg.eu/user/cellline/edit/LUMCi058-A-1) LUMCi059-A-1 (https://hpscreg.eu/user/cellline/edit/LUMCi059-A-1)
Ethical/GMO work approvals	P13.080 – Medical Ethical Committee (MEC), Leiden University Medical Center (LUMC)
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

The manuscript section expected contents clarification

2. Resource utility

Patients harboring mutations in *KLHL24* suffer from Epidermolysis Bullosa and are at high risk of developing Dilated Cardiomyopathy (Has et al., 2020). Here, hiPSCs were derived from patients with heterozygous c.1A>G or c.2T>C mutation. CRISPR-Cas9 editing was used to repair the mutation, creating isogenic pairs for *in vitro* disease modelling.

3. Resource details

KLHL24 is a ubiquitin ligase substrate adaptor that regulates the proteasomal degradation of its targets. Monoallelic mutations in the start codon of the *KLHL24* gene are associated with Epidermolysis Bullosa Simplex, accompanied by the development of cardiomyopathy in patients' early adulthood (Has et al., 2020). These mutations lead to the expression of a KLHL24 variant that lacks the first 28 amino acids of the protein, affecting its own turnover and that of its target substrates (Fig. 1A). hiPSC lines derived from two Chilean adult males harboring such mutations and their isogenic control counterparts have already been reported, enabling the first studies of KLHL24-associated pathologies (Ramovs et al., 2021). Here, we expand the existing toolkit by generating hiPSC lines from two Caucasian patients from Europe, a female carrying a heterozygous c.1A>G and a young male carrying a c.2T>C mutation in *KLHL24* (Fig. 1A), thus providing the means to study sex- and cohort-related pathologies. Patient fibroblasts were reprogrammed at passage 3 via non-integrating Sendai virus delivering OCT3/4, SOX2, KLF4, and MYC (Nishimura et al., 2011). One clone per line was extensively characterized: LUMCi058-A and LUMCi059-A (Table 1). Both lines were negative for SeV after 6 and 10 passages as indicated by immunofluorescence staining and qRT-PCR, respectively (Fig. 1B,C). For each line, an isogenic control was generated by a Cas9-ribonucleoprotein (RNP) complex, with a mutation-specific single guide RNA crRNA-1 and crRNA-2 for lines LUMCi058-A-1 and LUMCi059-A-1 respectively; Table 2) and a common single-stranded oligodeoxynucleotide (ssODN) donor template containing the wild-type sequence with a screening mutation at position −3 that simultaneously disrupts an MaeII restriction site and creates a BsrDI site on the edited allele (ssODN-1; Table 2). The presence of mutated and repaired alleles in each line was confirmed by PCR and Sanger sequencing of the region of interest (Fig. 1D). All patient-derived and isogenic control lines displayed a typical stem cell morphology with high nucleus to cytoplasm ratio (Fig. 1E) and a normal diploid karyotype determined by G-banding (Fig. 1F). The expression of markers of undifferentiated state confirmed their pluripotency status: co-expression of SSEA4 and NANOG was determined by flow cytometry (Fig. 1G) whereas co-expression of SSEA4 and OCT3/4 was detected by immunofluorescence staining (Fig. 1H). The lines were able to give rise to all three germ layers in an *in vitro* directed differentiation assay, as illustrated by immunofluorescence staining for the ectodermal markers Nestin, fatty acid binding protein 7 (FABP7) and Paired box protein 6 (PAX6), the endodermal markers Eomesodermin (EOMES), Forkhead box protein A2 (FOXA2) and GATA binding protein 4 (GATA4), and the mesodermal markers Brachyury, Caudal type homeobox 2 (CDX2) and Vimentin (Fig. 1H). All lines tested negative for mycoplasma (Fig. S1) and the origin of the isogenic pairs was confirmed by short tandem repeat (STR) analysis which completely matched the profile of the patient's fibroblasts (submitted in archive). Finally, the absence of off-target mutations was confirmed by targeted PCR and Sanger sequencing of the 5 most likely off-target regions as predicted by CRISPOR (crispor.tefor.net; Fig. S2) (Haeussler et al., 2016).

4. Materials and methods

4.1. Cell reprogramming and maintenance

This study was approved by an ethical committee (see 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, 1 % Non-Essential Amino Acids, 0.18 % β-mercaptoethanol, 1 % penicillin/streptomycin (all from ThermoFisher Scientific: #31331; #10270; #11140 #31350; #15070) and 10 μg/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/cm^2 on Matrigel coated plates (Corning, #354277) in FM. The next day, cells were refreshed and subsequently maintained in ReproTeSR™ (Stemcell Technologies, #05921, #05922, #05923). At day 18, culture was shifted to TeSR™ E8™ medium (Stemcell Technologies, #05990). After colony picking, hiPSCs were maintained in TeSR™-E8™ 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 an EVOS microscope with 10x magnification.

4.2. In vitro trilineage differentiation

The ability of hiPSCs to differentiate into the three germ layers (ectoderm, mesoderm, and endoderm) was assessed using differentiation with the Trilineage differentiation kit (Stem Cell Technologies). The differentiation was done according to the manufacturer's instructions on hESC-qualified Matrigel (Corning)-coated coverslips. After 5 or 7 days of culture cells were fixed using 2 % PFA.

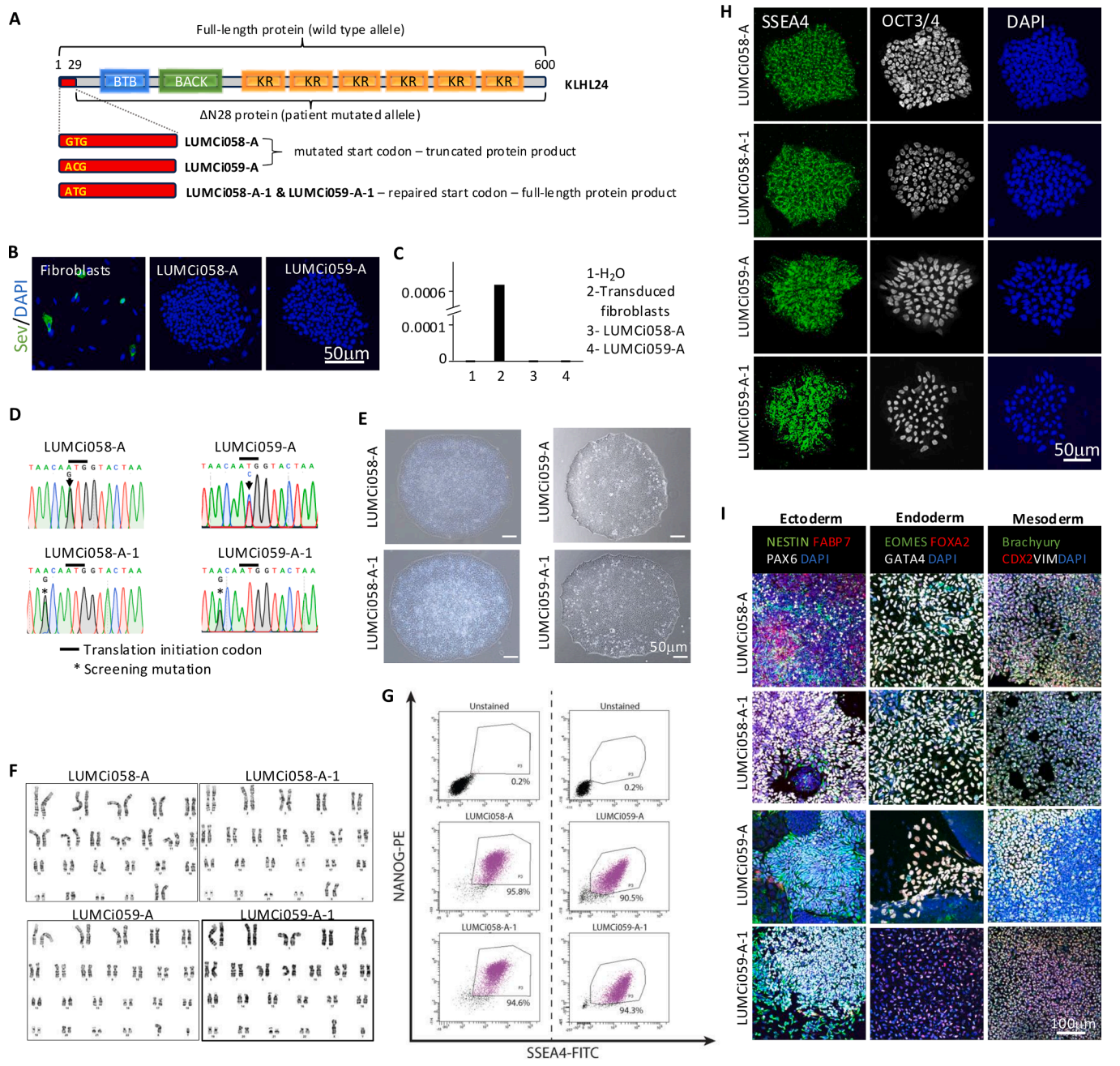

Fig. 1. Characterization of KLHL24 Patient-Derived LUMCi058-A and LUMCi059-A Cell lines and Their Isogenic Repaired Counterparts, LUMCi058-A1 and LUMCi059-A1.

4.3. Gene editing and evaluation of off-targets

Gene editing of patient-derived hiPSCs was performed as already described (Ramovs, et al., 2021). Genomic DNA was extracted from iPSCs using QuickExtractTM DNA Extraction solution (Lucigen, #QE09050). PCR was performed by using Terra Taq polymerase (Takara, #639270) according to the manufacturer's instructions by using a Bio-Rad T100 thermal cycler. Corrected clones were identified by analysis of the restriction pattern obtained after digestion of the PCR fragment with restriction enzymes MaeIII and BsrDI (New England Biolabs) and confirmed by Sanger sequencing. The Top5 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.

4.4. 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 previously described (Bouma et al., 2019). Primary and secondary antibodies are reported in Table 2. The parental (LUMCi058-A, LUMCi059-A) and repaired (LUMCi058-A-1 LUMCi059-A-1) lines were analyzed between passage numbers 13–15 and 23–26, respectively.

Table 1
Characterization and validation.

Classification (optional *italicized*)	Test	Result	Data
Schematic of the genetic modification	Schematic illustrating the location of the mutation and the introduced genetic modification	A visual represen tation of the full-lenght and truncated proteins with functional domains	Fig. 1 Panel A
Morphology	Brightfield microscopy	Normal morphology	Fig. 1 Panel E
Pluripotency status evidence for the described cell line	Qualitative analysis of immunocytochemistry staining	Positive immunostaining markers of undifferentiated state: SSEA4. OCT3/4	Fig. 1 Panel H
	Quantitative analysis by Flow cytometry	More than 90 % double-positive cells over negative control for markers of undifferentiated state NANOG and SSEA4	Fig. 1 Panel G
Karyotype	G-banding Karyotyping 5–10 Mb	Normal karyotype – 46, XX for lines LUMCi058-A and LUMCi058-A-1, 46, XY for lines LUMCi059-A and LUMCi059-A-1	Fig. 1 Panel F
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site and Sanger sequencing	*PCR+Sanger sequencing confirmed the heterozygous presence of pathogenic mutations c.1A>G and c.2T>C in parental lines LUMCi058-A and LUMCi059-A respectively, and the correction of those mutations to a homozygous state in lines LUMCi058-A-1 and LUMCi059-A-1.*	Fig. 1 Panel D
	Evaluation of the – (homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s) by PCR and Sanger sequencing	PCR amplification of the region surrounding the edited base pair including a screening mutation introduced in a heterozygous state exclusively to the edited allele.	Fig. 1 Panel D
	Transgene-specific PCR (when applicable)	N/A	N/A
Verification of the absence of random plasmid integration events	PCR/Southern	N/A	N/A
Virology	Detection of SeV by RT-PCR and immunofluoresnce	Negative	Fig. 1 Panels B and C
Parental and modified cell line genetic identity evidence	Microsatellite PCR (mPCR)	Not performed	Submitted in archive
	STR analysis	23 sites tested; all sites matched	
Off-target nuclease activity analysis	PCR across top 5 predicted top likely off-target sites and Sanger sequencing	Demonstration of the lack of mutations in predicted off-target sites	Figure S2
Specific pathogen-free status	Mycoplasma	Luminescence-based testing – negative.	Figure S1
Multilineage differentiation potential	In vitro directed differentiation and IF analysis.	Positive immunostaining of the three germ layer markers: ectodermal (NES, FABP7, PAX6), endodermal (EOMES, FOXA2, GATA4), mesodermal (Brachyury, CDX2, VIM)	Fig. 1 Panel I

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 and FACSDiva sofware (BD). The parental (LUMCi058-A, LUMCi059-A) and repaired (LUMCi058-A-1 LUMCi059-A-1) lines were analyzed between passage numbers 14–17 and 24–26, respectively.

4.7. qRT-PCR

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

4.8. Karyotyping

G-banding analysis was conducted at the Laboratory of Clinical Genetics Leiden (LDGA). A total of 20 metaphases was analyzed at passage numbers 14, 13, 29 and 25 for lines LUMCi058-A, LUMCi059-A, LUMCi058-A-1 and LUMCi059-A-1, respectively.

4.9. Evaluation of off-target nuclease activity

The top 5 most likely 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 Sanger sequencing. Primer sequences can be found in Table 2.

4.10. Mycoplasma test

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

4.11. 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).

CRediT authorship contribution statement

Spyridon T. Pachis: Formal analysis, Writing – review & editing. **Veronika Ramovs:** Formal analysis, Writing – review & editing. **Christian Freund:** Methodology. **Cristina Has:** Resources. **Karine Raymond:** Conceptualization, Funding acquisition, Writing – original draft.

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.

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	mouse IgG2b anti-OCT3/4	1:100	Santa Cruz, Sc- 5279, RRID: AB_628051
Pluripotency markers	mouse IgG3 anti-SSEA4	1:30	Biolegend, #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	Nestin-Alexa488	1:200	Cell Signaling Technology Cat#35884. RRID:AB_2799037
Differentiation markers	PAX6-Alexa647	1:200	Cell Signaling Technology Cat#60433. RRID: AB_2797599
Differentiation markers	FABP7-Alexa555	1:100	Cell Signaling Technology Cat#13347. RRID: AB_2572210
Differentiation markers	FOXA2-Alexa555	1:500	Cell Signaling Technology Cat#50079. RRID: AB_10891055
Differentiation markers	GATA4-Alexa647	1:200	Cell Signaling Technology Cat#66309. RRID: AB_2799108Cell Signaling
Differentiation markers	EOMES-Alexa488	1:100	Cell Signaling Technology Cat#96823. RRID: AB_2798791
Differentiation markers	Brachyury-Alexa488	1:200	Cell Signaling Technology Cat#94663. RRID: AB_2799983
Differentiation markers	Vimentin-Alexa647	1:400	Cell Signaling Technology Cat#9856. RRID: AB_10834530
Differentiation markers	CDX2-Alexa555	1:500	Cell Signaling Technology Cat#84638. RRID: AB_2943240
Site-specific nuclease			
Nuclease information	Cas9 nuclease V3	IDT - 1081058	
Delivery method	Electroporation	Neon Transfection System, program 6	
Selection/enrichment strategy	Single cell dispensing + restriction site analysis		
Bioinformatic gRNA on– and –off-target binding prediction tool	CIRSPOR	*http://crispor.tefor.net/crispor.py?batchId=6a6XDCzstBaa2Mhvyv3z* *http://crispor.tefor.net/crispor.py?batchId=eW4gTjQuUOdA7AlzgBXb*	

Primers and Oligonucleotides used in this study

	Target	**Size**	**Forward/Reverse primer (5′-3′)**
crRNA-1	KLHL24	N/A	ATAGTCAACTGATGTAACAG
crRNA-2	KLHL24	N/A	ATAGTCAACTGATGTAACAA
ssODN-1	Template for wild type KLHL24 + screening mutation	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
Genotyping primers	PCR to determine mutation status of KLHL24	760 bp	TTCAGCTAGAAAAGGGGAGCTT/AACTTGGCACATGCATCACG
Sendai virus vector (q-RT-PCR)	Sev	N/A	GCAGCTCTAACGTTGTCAAA/CCTGGAGCAAATTCACCATGA
Housekeeping gene (q-RT-PCR)	GAPDH	166 bp	TCCTCTGACTTCAACAGCGA/GGGTCTTACTCCTTGGAGGC
Off-target site 1 primers	ABCA1	391 bp	TCATAGCTCACTGGAACGTCA/TGTCATGGGAAATGCTCTCC
Off-target site 2 primers	TRAV3-TRAV4	526 bp	AGGGAGCATAGGTTGTTTCTGA/CTCAGCAAAACCAGGACAACT
Off-target site 3 primers	CTB-1I21.1-CTB-1I21.2	429 bp	CATCTGTAGACTCAGCAACCTGG/GGATAGTACCTGGGTATGCC
Off-target site 4 primers	RPS12P15-KB-1980E6.3	489 bp	GAAGAGCCAACAGCCTTTTG/CTGGAGGGAAATATACACAAGGGG
Off-target site 5 primers	RNF130-RPS15AP18	246 bp	GTTTCCCTGGCAATAGGAGTAG/GATGGAGTTTTGCTTTTGTTGCCC
Off-target site 6 primers	SAMD13/RP11-376 N17.4-UOX	554 bp	CCACCCATCTGTATATTGGCTT/CTCAGCAAGGCATTCTGTGG
Off-target site 7 primers	CTD-2007A10.1	386 bp	AGTTCACAAAGAGATGGTCCAA/AGTGGCCTGAACTGTACCTT

Data availability

Data will be made available on request.

Acknowledgements

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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.2024.103551.

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