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Lab Resource: Multiple Cell Lines

Generation of three induced pluripotent stem cell lines from an isolated inherited retinal dystrophy patient with *RCBTB1* frameshifting mutations

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A B S T R A C T

Variants in *RCBTB1* have been implicated in inherited retinal disease (IRD). Here, we generated induced pluripotent stem cells (iPSCs) from a 45-year-old female IRD patient harbouring compound heterozygous mutations in the *RCBTB1* gene. Episomal plasmids containing *OCT4*, *SOX2*, *KLF4*, *MYCL*, *LIN28*, shRNA for *TP53* and mir302/367 microRNA were employed to conduct the reprogramming of primary dermal fibroblasts. These iPSC lines provide a useful model for further investigations on the pathophysiological role of mutations in the *RCBTB1* gene in IRD.

Resource utility

Three iPSC lines were generated from a 45-year-old female patient who was diagnosed with late onset retinal dystrophy caused by compound heterozygous mutations in *RCBTB1* (c.170delG and c.707delA). These fully characterized iPSC lines will provide a useful model for investigating disease mechanisms and potential therapies in RCBTB1-associated retinopathy.

Resource details

Mutations in the *RCBTB1* gene located on chromosome 13q14 have been associated with a spectrum of inherited retinal diseases. Heterozygous frameshift mutations in the *RCBTB1* gene were previously identified in two unrelated Taiwanese families with Coats disease and familial exudative vitreoretinopathy (FEVR) (Wu et al., 2016), respectively. In another report, homozygous *RCBTB1* missense mutations were confirmed in cases of isolated and syndromic retinal dystrophy. These families, originating from different ethnic backgrounds including Turkey, Italy, Greece, Algeria and China, revealed diverse retinal phenotypes including retinitis pigmentosa, progressive pattern-like reticular dystrophy and central chorioretinal atrophy with peripheral reticular dystrophy (Coppieters et al., 2016). We recently identified compound heterozygous *RCBTB1* mutations in a Singaporean-Chinese female patient with late onset retinal dystrophy. Intriguingly, the patient involved in our study shared the same frameshifting mutation, c.707delA p.(Asn236Thrfs*11) in the *RCBTB1* gene, as the Coats case (Wu et al., 2016), whilst manifesting a different retinal phenotype that is more similar to the reported homozygous missense cases (Coppieters et al., 2016). To provide resources for investigating the pathophysiological studies of RCBTB1-associated retinopathy, we generated three human iPSC lines from this 45-year old female patient with late onset retinal dystrophy harbouring biallelic frameshifting mutations in *RCBTB1* (NM_018191.4), c.170delG p.(Gly57Glufs*12) and c.707delA (p.Asn236Thrfs*11) (Fig. 1A, Table 1). Targeted Sanger sequencing was performed to demonstrate independent segregation of each variant in family members.

We performed a skin punch biopsy and expanded dermal fibroblasts from the proband patient in culture. Reprogramming was conducted using the Episomal iPSC Reprogramming Plasmid Kit (SC900A-1, System Biosciences) to induce expression of the reprogramming factors *OCT4*, *KLF4*, *SOX2*, *MYCL*, *LIN28*, *TP53* shRNA and the miR-302/367 cluster in patient-derived fibroblasts. Three clonal iPSC cell lines (LEIi011-A, LEIi011-B, LEIi011-C, Table 1) were generated and further characterized (Fig. 1, Table 2). All three lines displayed typical iPSC morphology (Fig. 1A). The c.170delG (p.Gly57Glufs*12) and c.707delA (p.Asn236Thrfs*11) variants in the *RCBTB1* gene were confirmed in all three iPSC lines (Fig. 1A). Analysis of 16 microsatellite markers confirmed that LEIi011-A, LEIi011-B and LEIi010-C were derived from the patient's fibroblasts (Supp Fig. 1). According to the immunostaining

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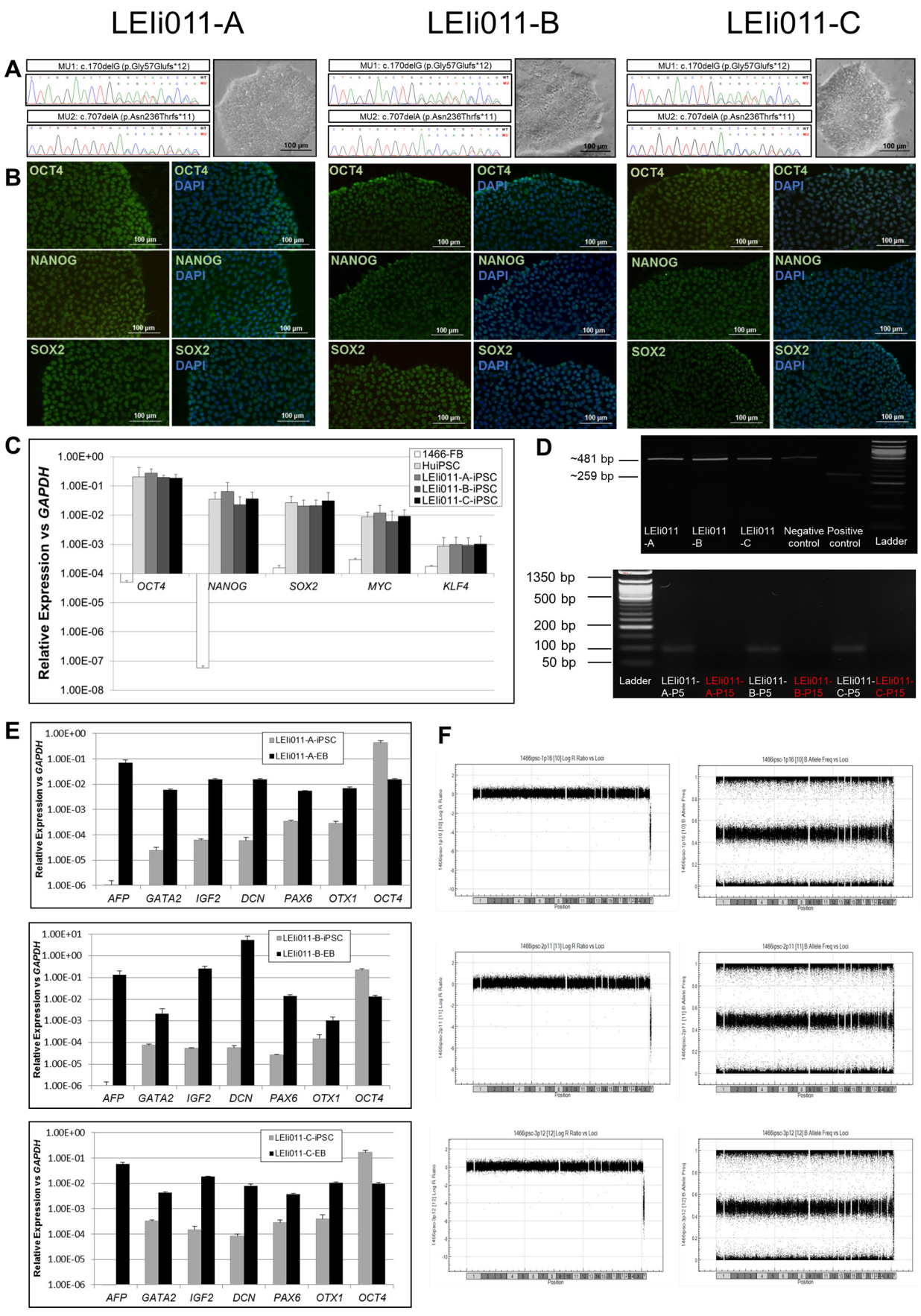

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Fig. 1. A: Direct Sanger sequencing revealed heterozygous frameshifting mutations (c.170delG and c.707delA) in RCBTB1 gene in LEIi011-A, LEIi011-B and LEIi011-C. All the three lines displayed classical iPSC morphology. B: Pluripotency markers (OCT4, NANOG and SOX2) were confirmed to express in LEIi011-A, LEIi011-B and LEIi011-C by immunocytochemistry. Nuclei were counterstained with DAPI (merged images). Scale bars indicate 100 μm. C: Quantitative RT-PCR analysis indicated the expression level of pluripotency markers (OCT4, NANOG, SOX2, MYC and KLF4) in LEIi011-A, LEIi011-B and LEIi011-C was upregulated compared to that of the patient derived fibroblasts (1466-FB) and similar to that of iPSCs derived from a commercial iPSC line. D: PCR screening showed LEIi011-A, LEIi011-B and LEIi011-C were negative for mycoplasma and reprogramming episomes at passage 15. Positive (+) and negative (-) controls yielded bands of the expected sizes as indicated. E: Expression level of markers of endoderm (AFP and GATA2), mesoderm (IGF2 and DCN) and ectoderm (PAX6 and OTX1) lineages was increased, while expression of OCT4 decreased in EBs (black bars) compared to the iPSC lines (grey bars). Error bars represent standard deviation. F: Digital karyotyping demonstrated LEIi011-A, LEIi011-B and LEIi011-C had normal 46, XX diploid karyotypes. LogR values (left panel) and B-allele frequencies (right panel) were plotted against genomic location for 300,000 human SNPs.

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
LEIi011-A	LEIi011-A	Female	45	Singaporean Chinese	c.170delG c.707delA	Central chorioretinal atrophy
LEIi011-B	LEIi011-B	Female	45	Singaporean Chinese	c.170delG c.707delA	Central chorioretinal atrophy
LEIi011-C	LEIi011-C	Female	45	Singaporean Chinese	c.170delG c.707delA	Central chorioretinal atrophy

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel A
Phenotype	Qualitative analysis: Immunocytochemistry	Positive for the pluripotency markers: OCT4, NANOG and SOX2	Fig. 1 panel B
	Quantitative analysis: RT-qPCR	Positive for the pluripotency markers *OCT4*, *NANOG*, *SOX2*, *KLF4* and *MYC*	Fig. 1 panel C
Genotype	Karyotype (Infinium HumanCytoSNP-12 Beadchip) and resolution	46,XX Resolution: 0.1 Mb	Fig. 1 panel F
Identity	STR analysis	Matched at 16 loci	Supplementary Fig. 1, Available with the authors
Mutation analysis (If Applicable)	Sequencing	Compound heterozygous c.170delG and c.707delA variants in *RCBTB1* (NM_018191.4)	Fig. 1 panel A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Fig. 1 panel D
Differentiation potential	Embryoid body formation	Upregulation of markers of ectoderm (*PAX6*, *OTX1*), mesoderm (*IGF2*, *DCN*) and endoderm (*AFP*, *GATA2*)	Fig. 1 panel E
Donor screening (Optional)	N/A	N/A	N/A
Genotype additional info (Optional)	N/A	N/A	N/A
	N/A	N/A	N/A

findings, pluripotency markers including OCT4, NANOG and SOX2 were expressed in all of the three lines (Fig. 1B). Supportively, quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) analysis also indicated that the expression level of pluripotency markers (*OCT4*, *SOX2*, *KLF4*, *NANOG* and *MYC*) in the three iPSC lines was similar to that of a commercial iPSC line (HuiPSC; ThermoFisher, Cat#A18945) (Fig. 1C). PCR screening demonstrated all three iPSC lines were mycoplasma-free. A mycoplasma specific 261 bp band was amplified from the positive control but not the negative control or LEIi0011-A-C. The 481 bp internal positive control band was detected in mycoplasma negative samples (Fig. 1D, upper panel). The three patient iPSC lines were reprogramming episome-free after passage 15 as determined by PCR. A 95 bp episomal plasmid specific band was amplified from LEIi011A-C at passage 5, but was not detectable at passage 15 (Fig. 1D, lower panel). Expression of mesoderm (*IGF2* and *DCN*), endoderm (*AFP* and *GATA2*) and ectoderm (*PAX6* and *OTX1*) markers was induced in embryoid bodies, while expression of *OCT4* was downregulated (Fig. 1E), demonstrating the potential of the iPSC lines to differentiate into the three germ layers. Copy number variation (CNV) profiling demonstrated the iPSC lines from the female patient contained normal diploid karyotypes (46, XX). The B allele frequencies (BAF) and LogR ratios of 300,000 single nucleotide polymorphisms (SNP) were analysed, indicating a normal 46,XX karyotype in all three iPSC lines (Fig. 1F). Antibodies and primers used are listed in Table 3.

Materials and methods

Ethics

Patient DNA was collected and genetically diagnosed through the Australian Inherited Retinal Disease Registry and DNA bank. All procedures in this study complied with the Declaration of Helsinki and ethics approval was obtained from the University of Western Australia Human Research Ethics Committee (RA/4/1/7916) and Sir Charles Gairdner Hospital Human Research Ethics Committee (Approval Number 2001–053). Written informed consent was obtained after detailed explanation of the nature of the study from all participants in this project.

Cell culture and sequencing

Fibroblasts from the patient were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ThermoFisher) supplemented with 10% fetal calf serum (FBS) and 1× Antibiotic-Antimycotic (ThermoFisher). Fibroblasts were passaged once per week using a split ratio of 1:5. Upon reaching 80–90% confluence, media was removed and fibroblast monolayers were rinsed in PBS then incubated with TrypLE (ThermoFisher) for 5 min at 37 °C. An equal volume of culture media was added to the suspension. Cells were then pelleted by centrifugation

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Mouse anti-OCT4	1:200	StemCell Technologies Cat# 60093, RRID: AB_2561766
	Rabbit anti-SOX2	1:200	Thermo Fisher Scientific Cat# 48–1400, RRID:AB_2533841
	Rabbit anti-NANOG	1:100	Abcam Cat# ab21624, RRID: AB_446437
Secondary antibodies	Goat anti-mouse IgG AlexaFluor-488	1:500	Thermo Fisher Scientific, Cat# A28175, RRID AB_2536161
	Goat anti-rabbit IgG AlexaFluor-488	1:500	Molecular Probes Cat# A-11008, RRID: AB_14165

Primers		
	Target	Forward/Reverse primer (5′-3′)
Episomal plasmids (PCR)	Reprogramming Plasmids	CTTCAACCATCAGGCTTACTTCTA/CTGGTGGGTCAGTAACATCATC
Pluripotency markers (qPCR)	*SOX2*	GCTACAGCATGATGCAGGACCA/TCTGCGAGCTGGTCATGGAGTT
	OCT4	CCTGAAGCAGAAGAGGATCACC/AAAGCGGCAGATGGTCGTTTGG
	NANOG	CTCCAACATCCTGAACCTCAGC/CGTCACACCATTGCTATTCTTCG
	MYC	CCTGGTGCTCCATGAGGAGAC/CAGACTCTGACCTTTTGCCAGG
	KLF4	CATCTCAAGGCACACCTGCGAA/TCGGTCGCATTTTTGGCACTGG
Ectoderm markers (qPCR)	*PAX6*	CTGAGGAATCAGAGAAGACAGGC/ATGGAGCCAGATGTGAAGGAGG
	OTX1	CTACCCTGACATCTTCATGCGG/GGAGAGGACTTCTTCTTGGCTG
Mesoderm markers (qPCR)	*IGF2*	AGACGTACTGTGCTACCCC/TGCTTCCAGGTGTCATATTGG
	DCN	AGAGTACCTGGTGGGCTGG/GTGGGCAGAAGTCACTTGAT
Endoderm markers (qPCR)	*AFP*	TGAGCACTGTTGCAGAGGAG/TTGTTTGACAGAGTGTCTTGTTGA
	GATA2	CTGTCTGCAACGCCTGTG/GTTCCGAGTCTGGATCCCTT
House-keeping genes (qPCR)	*GAPDH*	AGAAGGCTGGGGCTCATTTG/AGGGGCCATCCACAGTCTTC
Targeted mutation analysis/sequencing	*RCBTB1* c.170delG	TCCTGAGCTCTGTAGTTGAAATG/CAGAGTTAGCCCGAAGTGTTTA
	RCBTB1 c.707delA	CAGTTGCAGGAGCATAGAGTAG/GTTAGAGCAAGGAAGGTAGATGG

(200 *g* for 5 min), resuspended in culture media and seeded into new flasks. Human iPSC lines were cultured in StemFlex Medium (ThermoFisher) on Geltrex (ThermoFisher) coated culture plates. IPSC cultures were passaged every 4–5 days using a split ratio of 1:3–1:5. For passaging, culture media was removed and replaced with EDTA solution (0.5 mM EDTA in PBS supplemented with 0.45 g/L NaCl) and incubated at room temperature for 4–5 min. EDTA was then removed and replaced with StemFlex media and iPSC colonies manually dissociated into floating cell clusters using a bent 200 µL tip. The resulting suspension was then seeded onto new Geltrex coated 6 well plates. Both fibroblasts and iPSC cultures were cultured at 37 °C with 5% CO_2. PCR and Sanger sequencing was performed both in DNA isolated from fibroblasts (Supp Fig. 2) and iPSCs (Fig. 1A) to verify the presence of the *RCBTB1* variants using a primer pair (Table 3) flanking the two frameshift mutations.

Reprogramming and differentiation

Reprogramming was conducted using Episomal iPSC Reprogramming Plasmid Kit (SC900A-1, System Biosciences), according to the manufacturer's instructions. Three colonies were chosen for iPSC expansion at Day 25. To differentiate EBs, we seeded iPSC into suspension culture plates in StemFlex medium supplemented with 10 µM Y27632 (Sigma-Aldrich). At Day 2, medium was changed to DMEM medium supplemented with 20% knockout serum replacement (KSR, ThermoFisher), 1% non-essential amino acids (MEM-NEAA, ThermoFisher) and antibiotic- antimycotics (ThermoFisher). At Day 7, EBs were transferred to adherent plates coated with geltrex and maintained for another 7 days in the same media. Finally, RNA was collected for transcript analysis for markers of three embryonic layers at Day 14 of EBs culture.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was harvested from fibroblasts and from iPSCs with Trizol reagent (Invitrogen). The Nanodrop 2000 spectrophotometer (ThermoFisher) was used to quantify the extracted RNA. 0.5–1 µg of RNA was reverse-transcribed to generate first strand cDNA using RT2 First Strand Kit (Qiagen) according to the supplier's protocol. qRT-PCR analysis was then performed with primers in the CFX Connect Real-Time System (BioRad), with the PowerSYBR Green PCR Master Mix (Applied Biosystem). Data was analysed using delta-CT method. Gene expression levels were measured and normalized to *GAPDH* expression. Primers used are listed in Table 3.

Immunocytochemistry analysis

IPSC were initially seeded into a 24 well plate with a Geltrex coated glass coverslip in each well and cultured for 1–2 days. Cells were then fixed in 4% paraformaldehyde phosphate buffered saline (PBS) for 15 min at 37 °C, then washed three times with PBS before incubation with blocking buffer (PBS containing 0.1% Triton X-100, 5% bovine serum albumin) for 1 h at room temperature. Primary antibodies were diluted in blocking buffer then added to the cells for overnight incubation at 4 °C, followed by three washes with PBS. Secondary antibodies were diluted in blocking buffer containing DAPI (1 µg/mL) and incubated with cells for 2 h at room temperature in the dark. Coverslips were then washed three times in PBS and mounted onto slides. Stained cells were visualized using the Olympus BX60 fluorescence microscope equipped with DP-Controller 3.1.1.267 acquisition software (Olympus Corporation, Tokyo, Japan) and merged using ImageJ software (version 1.43).

Digital karyotyping

Digital karyotyping was conducted on the three iPSC lines (LEIi011-A, LEIi011-B and LEIi011-C, (passage 16, 11 and 12, respectively) using the Infinium HumanCytoSNP-12 Beadchip to identify genomic insertions or deletions. Balanced translocations are not detected using this method. DNA was collected from iPSC lines using FlexiGene DNA kit (Qiagen) and CNV analysis was performed on GenomeStudio 2.0 software using the CNVpartition 2.0 plugin (Illumina) (D'Antonio et al., 2017).

Microsatellite analysis

Promega PowerPlex 16H system (Promega, performed at the Australian Genome Research Facility) was used for the analysis of microsatellite markers in the DNA collected from LEIi011-A, LEIi011-B and LEIi011-C cell lines. We identified identical phenotypes at sixteen loci including D18S51, D21S11, TH01, D3S1358, Penta E, FGA, TPOX, D8S1179, vWA, CSF1PO, D16S539, D7S820, D13S317, D5S818, Penta D and Amelogenin (Supp Fig. 1), indicating all three iPSC lines originated from the same individual.

Episomal and mycoplasma screening

Mycoplasma testing by PCR screening was performed on all three iPSC lines using the LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich, Fig. 1D). PCR screening for reprogramming episomes was performed using primers listed in Table 3.

Key resources table

Unique stem cell lines identifier	LEIi011-A LEIi011-B LEIi011-C
Alternative names of stem cell lines	1466ips1 (LEIi011-A) 1466ips2 (LEIi011-B) 1466ips3 (LEIi011-C)
Institution	Lions Eye Institute, Nedlands, Western Australia, Australia
Contact information of distributor	Dr Samuel McLenachan: smclenachan@lei.org.au Dr. Fred K Chen: fredchen@lei.org.au
Type of cell lines	iPSC
Origin	Human
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Episomal plasmids
Multiline rationale	Isogenic clones
Gene modification	Yes
Type of modification	Hereditary
Associated disease	Central chorioretinal atrophy
Gene/locus	RCBTB1/13q14
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	4/06/2016
Cell line repository/bank	https://hpscreg.eu/cell-line/LEIi011-A https://hpscreg.eu/cell-line/LEIi010-B https://hpscreg.eu/cell-line/LEIi010-C
Ethical approval	Human Research Ethics Office, University of Western Australia (RA/4/1/7916)

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Inherited Retinal Diseases Registry and DNA Bank).

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101549.

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