



Lab Resource: Multiple Cell Lines

Generation of an iPSC line from a retinitis pigmentosa patient carrying a homozygous mutation in *CERKL* and a healthy sibling



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ABSTRACT

Dermal fibroblasts from an autosomal recessive retinitis pigmentosa (RP) patient, homozygous for the mutation c.769 C > T, p.Arg257Ter, in *CERKL* (*Ceramide Kinase-Like*) gene, and a healthy sibling were derived and reprogrammed by Sendai virus. The generated human induced pluripotent stem cell (hiPSC) lines RP3-FiPS4F1 and Ctrl3-FiPS4F1, were free of genomically integrated reprogramming genes, showed stable karyotypes, expressed pluripotency markers and could be differentiated towards the three germ layers in vitro. These hiPSC lines offer a useful resource to study RP pathomechanisms, drug testing and therapeutic opportunities.

Resource table

Unique stem cell lines identifier	ESi066-A ESi067-A
Alternative names of stem cell lines	RP3-FiPS4F1 (ESi066-A), Ctrl3-FiPS4F1 (ESi067-A)
Institution	Research Center Principe Felipe, Valencia, Spain
Contact information of distributor	Dunja Lukovic dlukovic@cipf.es
Type of cell lines	iPSC
Origin	Human
Cell Source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus
Multiline rationale	Disease and healthy sibling control
Gene modification	Yes
Type of modification	Hereditary
Associated disease	Retinitis pigmentosa
Gene/locus	<i>CERKL</i> (NM_201548.4), Chr2:g.182423344 G > A (hg19); Ex 5. c.[769 C > T] (p.Arg257Ter)
Method of modification	N/A
Name of transgene or resistance	N/A

Inducible/constitutive system	N/A
Date archived/stock date	November 5, 2018
Cell line repository/bank	http://www.isciii.es/ISCIII/es/contenidos/fd-el-instituto/fd-organizacion/fd-estructura-directiva/fd-subdireccion-general-investigacion-terapia-celular-medicina-regenerativa/fd-centros-unidades/banco-nacional-lineas-celulares.shtml
Ethical approval	Ethical approval obtained from Valencian Authority for Stem Cell Reseach DGIITC/AMA/pe S-0081/18

Resource utility

Retinitis pigmentosa (RP), a group of inherited retinal dystrophies with a worldwide prevalence of 1:4000, is characterized by photoreceptor degeneration (first rods and later cones) leading to genetic blindness (reviewed in Verbakel et al., 2018). We have generated hiPSC lines from a RP patient homozygous for a nonsense mutation in *CERKL* (p.Arg257Ter), an autosomal recessive RP gene (Tuson et al., 2004). This mutation (also listed as p.Arg283Ter, NM_001030311.2) is the most prevalent *CERKL* mutation in the Spanish population (Ávila-Fernandez et al., 2008). The patient's non-affected sister fibroblasts were also reprogrammed into hiPSCs as a proper control to minimize

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
RP3-FiPS4F1 (ESi066-A)	RP	Male	62	Caucasian	<i>CERKL</i> (NM_201548.4), Chr2:g.182423344 G > A (hg19); Ex 5. c.[769 C > T] (p.Arg257Ter)	Retinitis pigmentosa
Ctrl3-FiPS4F1 (ESi067-A)	Healthy	Female	52	Caucasian	<i>CERKL</i> (NM_201548.4) WT	Healthy

the contribution of genetic background to disease phenotype. The non-integrative reprogramming strategy was applied in order to avoid insertional mutations and generate footprint-free hiPSCs that do not contain detectable vectors or transgenes. The cell lines offer a useful resource for RP pathomechanism studies and drug screening.

Resource details

Dermal fibroblast primary cell lines were derived from skin biopsies from a retinitis pigmentosa (RP) patient and his non-affected sister. Fibroblasts were reprogrammed using Sendai virus expressing human OCT4, SOX, KLF4, c-MYC reprogramming factors (Cytotune 2.0; Thermo Fisher). Clonal hiPSC lines from each individual were established and further characterized (Table 1 and Table 2). Sanger sequencing confirmed that the RP patient's fibroblasts (data not shown) and hiPSC line, RP3-FiPS4F1, harboured a nonsense truncating mutation in *CERKL* in homozygosity (Fig. 1A, lower panel), in accordance with previous genotyping data (Tuson et al., 2004). The hiPSCs derived from the healthy sibling, Ctrl3-FiPS4F1, showed the WT sequence in the same region (Fig. 1A, upper panel). Both hiPSC lines showed typical human embryonic stem cell (hESC)-like colony morphology by phase contrast microscopy (Fig. 1B) and were positive for alkaline phosphatase (AP) (Fig. 1C). The expression of pluripotency markers was demonstrated by flow cytometry (SSEA-4 and TRA-1-60) (Fig. 1D), immunofluorescence analyses of OCT4, SOX2, NANOG, SSEA-4 and TRA-1-81 (insets show nuclei staining with DAPI) (Fig. 1E), and RT-PCR for pluripotency markers (Fig. 1F). DNA fingerprinting was used to confirm the origin of the hiPSC clones (Available with authors). Sendai virus was eliminated from the hiPSCs' genome at passage 11 (Supplementary Fig. S1A). To explore genomic integrity, karyotyping was performed and showed that hiPSC lines, RP3-FiPS4F1 and Ctrl3-FiPS4F1, were male and female clones, respectively, and presented normal diploid karyotypes (Supplementary Fig. S1B). The capacity of hiPSCs to differentiate into three germ layers was confirmed by the embryoid body

(EB) assay and expression of ectoderm (TUJ1, PAX6), endoderm (AFP, FOXA2) and mesoderm (VIMENTIN, α -SMA) assessed in differentiating EB cultures (Fig. 1G). Mycoplasma was regularly tested throughout the cell culture and was negative (Supplementary Fig. S1C).

Materials and methods

Reprogramming fibroblasts

The primary fibroblasts derived from the skin biopsies were expanded for four passages in DMEM (10% FBS, 2 mM Glutamax, Penicillin-Streptomycin 1 ×) at 37 °C under 5% CO₂, before being reprogrammed by a Sendai virus containing four genes: OCT3/4, SOX2, KLF4 and c-MYC (Cytotune 2.0, Thermo Fisher) according to manufacturer's instructions. hiPSCs were grown on irradiated (45Gy) human foreskin fibroblasts (ATCC CRL 2429) in hiPSC medium containing Knockout-DMEM, KSR 20%, Glutamax 2 mM, non-essential amino acids 0.1 mM, β -mercaptoethanol 0.23 mM, basic FGF 10 ng/ml, penicillin/streptomycin, at 37 °C/5% CO₂. The hiPSCs were adapted to feeder-free cell culture on plates coated with Matrigel (BD, #354277) using mTeSR1 medium. Matrigel was diluted in DMEM/F-12 according to the dilution factor specified on the certificate of analysis and incubated for 1 h/RT at 1 ml/well of a 6-well plate. Passages were performed using Dispase (STEMCELL Technologies, #07913), every 5–7 days at 1:6–1:10 split ratio.

In vitro differentiation assay

In vitro differentiation was performed by EB formation, namely the hiPSC colonies were lifted manually and cultured in non-adherent conditions in mTeSR1 medium for 24 h, followed by endoderm medium (Knockout-DMEM, 10% FBS, 1% Glutamax, NEAA 1%, β -mercaptoethanol 0.23 mM, Penicillin-Streptomycin 1 ×) for the following 6 days. Thereafter, the EBs were seeded on glass coverslips treated with

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel B
Phenotype	Qualitative analysis (Immunocytochemistry)	Positive staining/expression of pluripotency markers: OCT4, NANOG, SOX2, SSEA-4, TRA-1-81	Fig. 1 panel E
	Quantitative analysis	Healthy: SSEA-4 98,61% TRA-1-60 78,6% RP: SSEA-4 98,87% TRA-1-60 83,7%	Fig. 1 panel D
	Karyotype (G-banding) and resolution	Healthy: 46XX; RP: 46XY Resolution 450–500	Supplementary fig. S1B
Identity	Microsatellite PCR (mPCR) OR STR analysis	N/A 10 STR loci analyzed, all matching	N/A Available with authors
Mutation analysis (if applicable)	Sequencing	<i>CERKL</i> (NM_201548.4) ex 5. [c.769 C > T]	Fig. 1 panel A
Microbiology and virology	Southern Blot OR WGS	N/A	N/A
Differentiation potential	Mycoplasma	<i>Mycoplasma</i> testing by luminescence. Negative.	Supplementary fig. S1C
	Embryoid body formation	Positive TUJ1 and PAX6 for ectoderm, positive α -SMA, VIMENTIN mesodermal staining and positive AFP and FOXA2 endodermal staining	Fig. 1 panel G
Donor screening (optional)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (optional)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

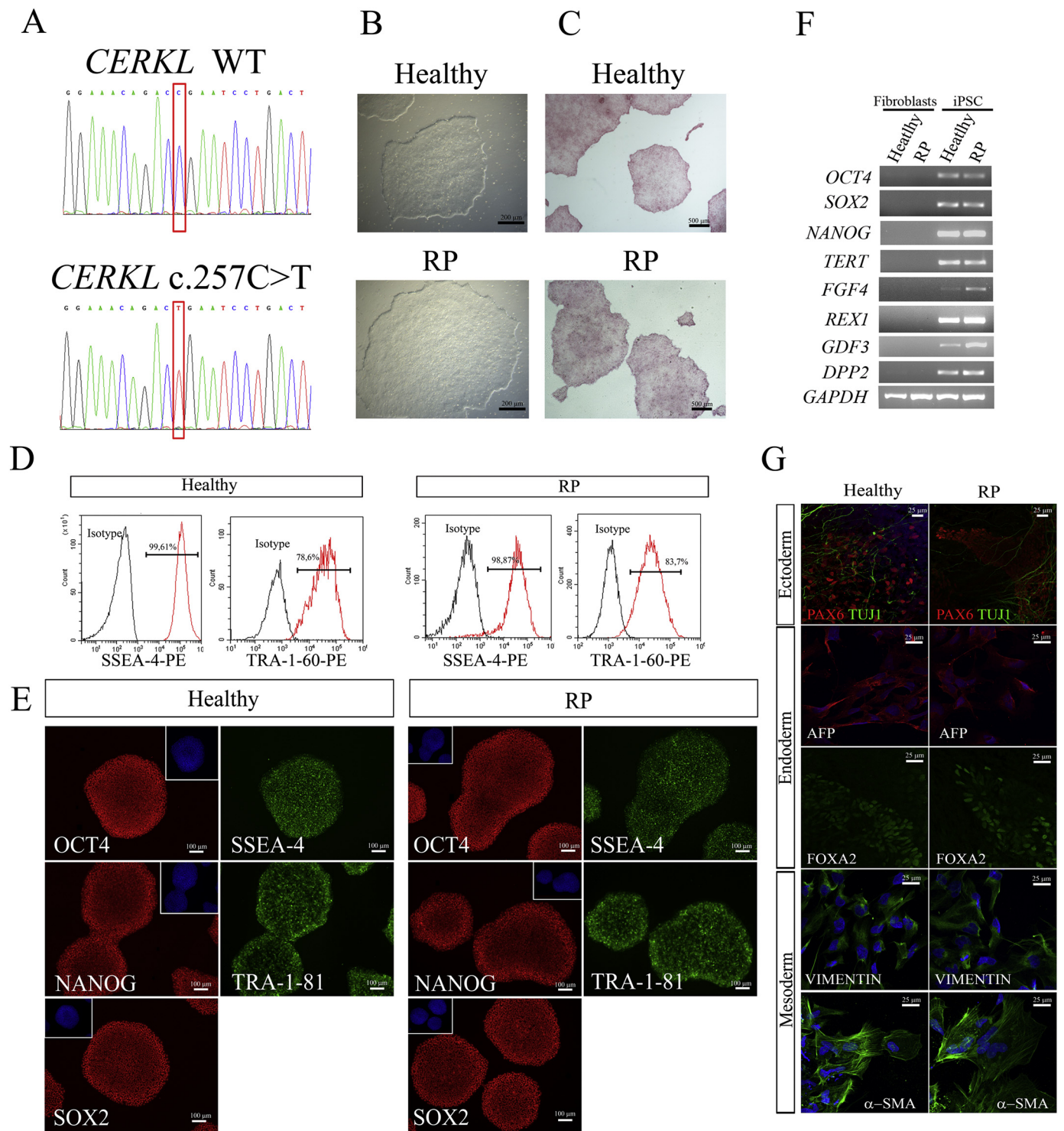


Fig. 1. Characterization of Healthy and RP hiPSC lines. **A.** DNA sequence electropherograms showing the wild type sequence and the c.257C > T mutation in CERKL in Healthy (upper panel) and RP (lower panel) hiPSC lines. **B.** Phase contrast micrographs of Healthy and RP hiPSC colonies cultured in feeder-free conditions. **C.** Alkaline phosphatase positive staining. **D.** Flow cytometry of surface pluripotency markers SSEA-4 and TRA-1-60. **E.** Immunofluorescence for pluripotency markers OCT4, SOX2, NANOG, SSEA-4, TRA-1-81. Nuclei were counterstained with DAPI (blue). **F.** RT-PCR analysis of pluripotency genes in Healthy and RP hiPSC lines. **G.** Immunocytochemistry for ectodermal (PAX6, TUJ1), endodermal (AFP, FOXA2) and mesodermal (SMA, VITRONECTIN) markers. Nuclei were counterstained with DAPI (blue).

0.1% gelatin for 2 h/RT and cultured during 2 weeks in three cell culture media: ectoderm medium (50% Neurobasal medium, 50% DMEM/F12, 1% N2 supplement, 0.5% B27 supplement, penicillin-streptomycin 1 ×), endoderm medium and mesoderm medium (endoderm medium, 0.5 mM ascorbic acid). The coverslips were fixed with 4% PFA for

15 min and analyzed by immunofluorescence (Table 3). Confocal images were taken with a Leica SP8 microscope.

Table 3
Reagent details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency marker	Rabbit anti-NANOG	1:400	Cell Signaling Technology Cat# D73G4 #RRID:AB_10559205
Pluripotency marker	Rabbit anti-OCT4	1:400	Cell Signaling Technology Cat# C30A3 #RRID:AB_2167691
Pluripotency marker	Rabbit anti-SOX2	1:400	Cell Signaling Technology Cat# D6D9 #RRID:AB_2195767
Pluripotency marker	Mouse anti-SSEA4	1:100	BD Pharmingen Cat# 560073 #RRID:AB_1645601
Pluripotency marker	Mouse anti TRA-1-81	1:100	Milipore Cat# MAB4381 #RRID:AB_177638
Pluripotency marker	Mouse anti human SSEA4-PE	1:800	Stem Cell Technologies Cat# 60062PE.1 #RRID N/A
Isotype control	Mouse IgG3 kappa-PE	1:6400	Stem Cell Technologies Cat# 60073PE.1 #RRID N/A
Pluripotency marker	Mouse anti human TRA- 1-60	1:400	Stem Cell Technologies, Cat #60064PE.1 #RRID N/A
Isotype control	PE Mouse IgM	1:400	BD, Pharmingen Cat# 555584 #RRID:AB_395960
Ectoderm marker	Mouse anti-TUJ-1	1:500	Neuromics Cat# MO15013 #RRID:AB_2737114
Ectoderm marker	Rabbit anti-PAX6	1:500	Covance Cat# PRB-278P #RRID:AB_291612
Mesoderm marker	Mouse anti-SMA	1:300	Sigma Cat #A5228 #RRID:AB_262054
Mesoderm marker	Mouse anti-VIMENTIN	1:300	Abcam Cat# ab8978 #RRID:AB_306907
Endoderm marker	Mouse anti-AFP	1:20	RD Cat# MAB 1368 #RRID:AB_357658
Endoderm marker	Goat anti HNF-3 β /FOXA2	1:100	RD Cat# 2400 #RRID:AB_2294104
Secondary antibody	Anti-mouse IgG	1:500	Invitrogen Cat# A11001 #RRID: AB_2534069
Secondary antibody	Anti-rabbit	1:500	Invitrogen Cat# A11002 #RRID:AB_2534070
Secondary antibody	Anti-mouse IgM	1:500	Invitrogen Cat# A21042 #RRID:AB_2535711
Secondary antibody	Anti-goat	1:500	Invitrogen Cat# A11055 #RRID:AB_2534102

Primers		
	Target	Forward/Reverse primer (5'–3')
Genotyping	CERKL (583 bp)	GAGCAAACGCTGGTCCAGATG/GCTAGTGGGATGCCAGAAG
Pluripotency marker (RT-PCR)	OCT4 (165 bp)	AAGCCCTCATTTCACCAGG CTTGGAAGCTTAGCCAGGTC
Pluripotency marker (RT-PCR)	SOX2 (181 bp)	TCACATGTCCAGCACTACC CCCATTTCCTCGTTTTTCT
Pluripotency marker (RT-PCR)	NANOG (260 bp)	CCAAATTCTCCTGCCAGTGAC CACGTGGTTTCCAAACAAGAAA
Pluripotency marker (RT-PCR)	REX1 (300 bp)	CAGATCCTAAACAGCTCGCAGAAT GCGTACGCAAATTAAGTCCAGA
Pluripotency marker (RT-PCR)	GDF3 (631 bp)	CTTATGCTACGTAAGGAGCTGGG GTGCCAACCCAGGTCGCCGAAGTT
Pluripotency marker(RT-PCR)	DPPA4 (408 bp)	GGAGCCGCTGCCCTGGAAAATTC TTTTTCCTGATATTCTATCCCAT
House-keeping gene (RT-PCR)	GAPDH (463 bp)	ATCGTGGAAGGACTCATGACCCAT CCCTGTTGCTGTAGCAAATTCGT
Sendai virus detection (RT-PCR)	SeV (181 bp)	GGATCACTAGGTGATATCGAG C* ACCAGACAAGAGTTTAAGAGATATGTATC*
Sendai virus transgene detection (RT-PCR)	KOS (528 bp)	ATGCACCGCTACGACGTGAGCCG ACCTTGACAATCCTGATGTGG
Sendai virus transgene detection (RT-PCR)	Klf (410 bp)	TTCTGCATGCCAGGAGGCC AATGTATCGAAGGTGCTCAA*
Sendai virus transgene detection (RT-PCR)	c-Myc (532 bp)	TAACTGACTAGCAGGCTTGTGC* TCCACATACAGTCTGGATGATGAT

Karyotype analysis

The karyotype was analyzed at passage 13 by G-banding at 400–550 band resolution, 30 metaphases analyzed (Service of Biobanco de Sistema Sanitario Público, Granada, Spain).

Fingerprinting

Genomic DNA (gDNA) from fibroblasts and hiPSCs was extracted using QIAamp DNA Blood mini kit (Qiagen) in the presence of RNase (Roche). Fingerprinting analyses was performed using Promega kit 10 microsatellite markers (TH01, TPOX, vWA, CSF1PO, D16S539, D7S820, D13S317, D5S818 y D21S11, Amelogenin) and analyzed on Abi PRISM 3130 using GeneMapper (Thermo Fisher) by Biobanco de Sistema Sanitario Público, Granada, Spain.

Detection of pluripotency markers and vector elimination by RT-PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden,

Germany), and treated with DNase I to remove any genomic DNA contamination. QuantiTect Reverse Transcription Kit (Qiagen) was used to carry out cDNA synthesis from 1 μ g of total RNA according to the manufacturer's instructions. The expression of pluripotency markers was analyzed using the primers described in Table 3. Patient's fibroblasts were used as negative control. For Sendai virus elimination test, fibroblasts collected at day 7 after transduction were used as positive control (C+) for Cytotune 2.0 vector detection by RT-PCR. The PCR reaction was performed with MyTaq DNA Polymerase (Bioline GmbH, Luckenwalde, Germany) using Applied Biosystems Veriti Thermal Cycler with following steps: denaturation 94 °C for 15 s, annealing 50–65 °C for 30 s, extension 72 °C for 45 s (x 35 cycles). *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* expression was used as a control housekeeping gene. Thereafter, PCR products were analyzed on 2% agarose gels.

Immunocytochemistry

hiPSC colonies cultured in plastic dishes were washed in PBS and

fixed in 4% PFA for 15 min at room temperature (RT). Fixed cells were washed twice in PBS and placed in blocking solution (3% serum, 0.5% Triton-X100 in PBS) for 1 h at RT. Cells were then incubated overnight at 4 °C with primary antibodies diluted in blocking solution (Table 3). The following day, cells were washed three times in PBS and incubated with an appropriate secondary antibody at RT for 1 h. Thereafter, cells were stained with DAPI (1:1000) at RT during 5 min, washed three times in PBS and imaged with a Leica DMi 8 microscope coupled with a DFC/7000GT camera.

Flow cytometry

hiPSCs were dissociated using Accutase (Innovative Cell Technologies) for 2–4 min at RT, centrifuged at 300 rcf for 5 min and 100,000 cells resuspended in 200 µl PBS + 2% FBS. Anti-human SSEA-4 and anti-human TRA-1-60 antibodies, both PE conjugated, were added and incubated for 20 min at RT. Isotype negative controls were used at the same final concentration (Table 3). 7-Aminoactinomycin D (7-AAD, Life Technologies) was used to identify live cells. The cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter) and data was analyzed by CytExpert 2.0 software. FACS gates were defined according to isotype controls.

Alkaline phosphatase staining

Alkaline phosphatase staining was carried out using Alkaline Phosphatase Staining Kit II (Stemgent, MA).

Mycoplasma detection

The presence of mycoplasma was tested regularly measuring enzyme activity via luciferase (MycAlert™ PLUS Mycoplasma Detection Kit, Lonza). Ratios of Reading B/ Reading A < 1.0 are considered negative for mycoplasma.

hiPSC nomenclature

The generated hiPSC line was named following Spanish National

Stem Cell Bank recommendations. The lines are registered at <https://hpscereg.eu/> as RP3-FiPS4F1 (ESi066-A) and Ctrl3-FiPS4F1 (ESi067-A).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2019.101455>.

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Author contributions

DL designed study, performed the experiments, data analyses, manuscript writing. ABA, ML, VBF performed experiments, data analyses, GM, RGD genotyped the patient and coordinated patient sample donation, manuscript writing. SE study design, manuscript writing, financial support.

References

- Ávila-Fernandez, A., Riveiro-Alvarez, R., Vallespin, E., Wilke, R., Tapias, I., Cantalapiedra, D., Aguirre-Lamban, J., Gimenez, A., Trujillo-Tiebas, M.J., Ayuso, C., 2008. *CERKL* mutations and associated phenotypes in seven Spanish families with autosomal recessive retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.* 49 (6), 2709–2713. <https://doi.org/10.1167/iovs.07-0865>.
- Tuson, M., Marfany, G., González-Duarte, R., 2004. Mutation of *CERKL*, a novel human ceramide kinase gene, causes autosomal recessive retinitis pigmentosa (RP26). *Am. J. Hum. Genet.* 74 (1), 128–138.
- Verbakel, S.K., van Huet, R.A.C., Boon, C.J.F., den Hollander, A.I., Collin, R.W.J., Klaver, C.C.W., Hoyng, C.B., Roepman, R., Klevering, B.J., 2018. Non-syndromic retinitis pigmentosa. *Prog. Retin. Eye Res.* 66, 157–186. <https://doi.org/10.1016/j.preteyeres.2018.03.005>.