Morphological and Molecular Defects in Human Three-Dimensional Retinal Organoid Model of X-Linked Juvenile Retinoschisis

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Supplemental figure S1. Characterization the XLRS patients. Related to Figure 1.

(A) ERG from patient with C625T RS1 mutation and normal control. The amplitude of dark-adapted (DA) 0.01 and DA 3.0 electroretinogram (ERG) were reduced. An
electronegative ERG was characteristic with retinoschisis which shows the amplitude of b/a ratio<1 in DA 3.0 ERG. The light adapted (LA) 3.0 ERG revealed b-wave amplitude reduced, as well as in the LA 30 Hz flicker responses. (B) Identified the position of mutations and the retinoschisin protein. Exons are represented by gray rectangle (numbers in the rectangle indicate their exon number), introns represented by white rectangle with dotted line, and the numbers around rectangle indicate their size in nucleotides. Dash lines mark the mutations and their localizations in the gene, mRNA and the protein. In the protein diagram, signal sequence is abbreviated SS.
Supplemental figure S2. Characterization the cellular pluripotency of hiPSCs. Related to Figure 1.

(A) Immunofluorescence staining of stemness markers NANOG, Tra-1-60 and Tra-1-81 as well as alkaline phosphatase in the hiPSCs derived from controls, patients, and CRISPR/Cas9-corrected lines. Scale bar: 100 µm. (B) Karyotypes (G-banding) of controls and patients-derived hiPSCs. (C) RT-PCR showing expression of the mRNAs encoding pluripotency markers in the hiPSC clones. GAPDH was used as internal control.
Supplemental figure S3. Characterization of retinal cups (RCs) at different stages.

(A) Immunofluorescence staining of the eye field transcription factors, PAX6, LHX2
and OTX2, at day 13 of retinal differentiation of control, C625T, HDR$^{R1}$ and HDR$^{R2}$ lines. Scale bar: 100 μm. (B) Immunofluorescence microscopy showing expression of retinal progenitor cell marker VSX2 and Ki-67 in 30-day RCs. Scale bar: 100 μm. (C) The 120-day RCs stained with H&E. The lower panel showed the retinal nuclear layer in RCs and the areas (between the two dot lines) that were used for splitting layer quantification. ONL, outer nuclear layer; INL, inner nuclear layer. Scale bar: 100 μm. (D) Whole-mount staining of photoreceptor markers in RCs. Related to Figure 4. Immunofluorescence staining of rhodopsin, G/R opsin, blue opsin and RS1 in 210-day whole-mount RCs. The arrows indicate the positive G/R and blue opsin signal within outer segment layer. Scale bar: 50 μm.
Supplemental figure S4. Schisis phenotype of patient-derived 150-day RCs. Related to Figure 1.

(A) Bright-field images from each of the two clones of control- and patient-RCs at day 150 of differentiation. Scale bar: 1000 µm. (B) 150-day RCs from each of the two clones of control- and patient-RCs stained with H&E.
## Top 5 Off-target Loci

| Off-target Locus location | gRNA sequence (w/o PAM) | PCR product |
|--------------------------|-------------------------|-------------|
| chr12:12908632-12909362  | TGCTCCTCTC CATTGCCATCTGG | 731 bp      |
| chr12:112269703-112269725 (reverse) | AGGAAGTCTGCTTGCCATCAGG | 854 bp      |
| chr16:5117376-5117398 (reverse) | GACACATCTGCATTGCCCAAGG | 964 bp      |
| chr17:8481627-8481649     | GGAAGATCTTCATTGCCATAAGG | 538 bp      |
| chr3:192484074-192484096 (reverse) | GGCTAGTCTTTGCTATCGAG | 748 bp      |

**Figure S5**
Supplemental figure S5. Off-target analysis in CRIPS/Cas9-corrected clones HDR^R1 (#1-11) and HDR^R2 (#2-17-10). Related to Figure 4.

The sequencing results showing no off-target events in the top 5 predicted off-target loci.
Figure S6

A

B

1 52.7%
2 53.1%
3
4
5
6
7
Supplemental figure S6. CRISPR/Cas9 mediated gene correction of RS1 C625T mutation in hiPSCs. Related to Figure 4.

(A) Cas9 double-strand break/homology-directed repair gRNA target region (orange) and PAM sequence (cyan), oligo/plasmid repair template sequence (blue; truncated), ABE7.10 based editing repair gRNA target region (green) and PAM sequence (cyan). (a) and (d) highlight the synonymous SNP nucleotides introduced by Cas9 repair template; (b) is a potential 2nd target for ABE7.10 A:T>G:C conversion within the base editing window (e) (an unintended base conversion at this position would introduce a conservative change (Val>Ala)); (c) is the XLRS-associated c.625C>T (Arg>Cys) SNP.

(B) Sanger sequencing of genomic DNA PCR products of (1) WT hiPSC line, (2) c.625C>T mutant hiPSC line, (3) Cas9-GFP/gRNA/repair template plasmid transfected and GFP sorted hiPSCs (note the high frequency of the three repair-dependent SNPs (a,c,d) as well as the presence of out-of-frame indel alleles (f)); (4) ABE7.10/gRNA/GFP plasmid transfected and GFP sorted hiPSCs (note the high frequency of repair (c), lower frequency of the unintended 2nd base edit (b), and absence of indel alleles); (5) precisely repaired subclone of (4); (6) subclone of (4) with unaltered sequence; (7) subclone of (4) with only the 2nd adenine edited. The percentages represent the average frequencies of repaired RS1 alleles (determined using allele-specific and reference ddPCR assays).
SUPPLEMENTAL MOVIES

Movie S1 to S4. Related to figure 3D and 5E-G.

A normal focal adhesion turnover in 150-day in control-RCs (Movie S1). However, C625T-RCs (Pt1) showed a faster focal adhesion (Movie S2). The normal duration time of paxillin-marked was prolonged and were comparable to those in control-RCs in both CRISPR/Cas9-corrected RCs HDRR1 line (Movie S3) and HDRR2 line (Movie S4).
SUPPLEMENTAL TABLES

Table S1. Information of iPSCs derived from patients and control healthy donors.

Related to figure 1.

| Patient  | Gender | AGE | RS1 Mutation        | Source | Reprogramming method | Clones generated<sup>1</sup> | Clones used<sup>2</sup> |
|----------|--------|-----|---------------------|--------|----------------------|-----------------------------|-------------------------|
| Patient-1| Male   | 43  | c.625C>T (p.R209C)  | PBMCs  | non-viral episomal plasmid protocol | 4                           | 2                       |
| Patient-2| Male   | 16  | c.488G>A (p.W163X)  | PBMCs  | non-viral episomal plasmid protocol | 4                           | 2                       |
| Ctrl-1   | Male   | 34  | Normal              | PBMCs  | non-viral episomal plasmid protocol | 4                           | 2                       |
| Ctrl-2   | Male   | 36  | Normal              | PBMCs  | non-viral episomal plasmid protocol | 3                           | 2                       |

In this study, we collected the blood samples from two XLRS patients and two healthy donors to generate iPSCs. At least three iPSC clones were generated from each patient/healthy donor, and we used two iPSC clones of each to generate RCs and investigate the phenotypes and morphology in this study.

<sup>1</sup>Clone generated: the number of iPSC clones generated from patients’ or control donors’ blood samples.

<sup>2</sup>Clone used: the number of iPSC clones used in the study.

Table S2. CRISPR/Cas9-edited iPSCs from Patient-1 and Control healthy donor-1.

Related to figure 4 and 6.

| HDR correction | Origin          | RS1 Mutation | Source | Reprogramming method                  | Clone generated | Clone used |
|----------------|-----------------|--------------|--------|----------------------------------------|-----------------|------------|
| HDR correction | iPSCs from Patient-1 | Normal      | PBMCs   | non-viral episomal plasmid protocol    | 2               | 2          |
| HDR mutation   | iPSCs from Ctrl-1 | c.625C>T     | PBMCs   | non-viral episomal plasmid protocol    | 2               | 2          |
Table S3. Summary of the phenotypic observation in hiPSC-derived RCs.

Related to figure 1, 4, 6.

| RC Phenotype                  | hiPSC Lines used in this study | see Figure (and data not shown) |
|------------------------------|---------------------------------|---------------------------------|
|                              | ctrl-1 clone 1 | clone 2 | ctrl-1 clone 1 | clone 2 | pt-1 clone 1 | clone 2 | pt-1 clone 1 | clone 2 | pt-1 clone 1 | clone 2 | ctrl-1 clone 1 | clone 2 | ctrl-1 clone 1 | clone 2 |
| RS1 genotype                 | WT | WT | WT | WT | c.625 G>A | c.625 G>A | c.488 G>A | c.488 G>A | Repaired | Repaired | c.625C>T | c.625C>T |
| Cystic RCs                   | - | - | - | - | + | + | + | + | - | - | + | + |
| Increased split area fraction| - | - | - | - | + | + | + | + | - | - | + | + |
| Reduced GR Opsin expression  | - | - | - | - | + | + | + | + | - | - | + | + |
| Reduced Rhodopsin expression | - | - | - | - | + | + | + | + | - | - | + | + |
| Sensory cilium or ciliary marker defects | - | - | - | - | + | + | + | + | - | - | ND | ND |
| Failure of RS1 to reach the Golgi apparatus | - | ND | - | ND | + | + | + | + | - | - | + | + |
| Reduced expression of multimeric RS1 | - | ND | - | ND | + | ND | + | ND | - | - | + | + |

= not observed (normal, within range of controls)

+= abnormal phenotype observed

ND = not determined

Table S4. The list of the 326 genes that were consistently downregulated in mutant RS1 RCs, compared with control-RCs, on days 90, 120, and 150 post-induction.

Related to Figure 7.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Ethic statements and human samples**

The experimental procedures and protocols involving human samples were conducted according to the tenets of the Declaration of Helsinki and were approved by the Institutional Review Board of Taipei Veterans General Hospital. Human blood samples were obtained after taking informed consent from the patients.

**Reprogramming human PBMCs to iPSC lines with Episomal vectors**

The method of generating hiPSCs from PBMCs was adapted from previously published paper by Okita, K et al. (Okita et al., 2013) with minor modifications. $3 \times 10^6$ PBMCs were used for electroporation (Lonza 2D Nucleofector Technology, U-014 program) with reprogramming plasmids, including 0.83 µg pCXLE-hOCT3/4-shp53, 0.83µg pCXLE-hSK, 0.83µg pCXLE-hUL, and 0.5µg pCXLE-EBNA1 (Addgene), using the Amaxa Human T-cell Nucleofector kit (Lonza) according to the manufacturer’s instructions. Subsequently, PBMCs were seeded onto inactivated mouse embryonic fibroblast (MEF) feeder layer and cultured in X-VIVO 10 medium (Lonza). Four hours after transfection, 30 U/ml IL-2 and 30µl/well of Dynabeads Human T-activator CD3/CD28 were added to PBMCs. An equal volume of human ESC medium (hESCM), containing DMEM/F-12, GlutaMAX, 20 % KnockOut Serum Replacement, 0.1 mM β- mercaptoethanol, 1x MEM Non-Essential Amino Acids Solution (NEAA), 10 ng/mL recombinant human basic fibroblast growth factor (bFGF), and 1% Penicillin/Streptomycin, were added into each well. The emergent colonies were picked after 16-25 days.
Mutational analysis of *RS1*

Mutational analysis of *RS1* was carried out as previously described (D'Souza et al., 2013) with minor modifications. A total of 200 ng of isolated genomic DNA was mixed with 20 pmol of each polymerase chain reaction (PCR) primer and 2x PCR Master mix Solution (i-MAX™ II; iNtRON Biotechnology, Kirkland, WA) in a final reaction volume of 20 µl. To screen the mutation site of *RS1* gene, the primer pairs were adapted from D'Souza et al., and additional PCR primers were designed as followings: 5’-CGGCCACCCAACCTCCAACCTA-3’ and 5’-AAATCCTTATTGGCATTGAATCC-3’ for exon 1; 5’-TGCAAGTTCTCTCGGCCTG ACC-3’ as forward primer for exon 4. The combinations of PCR primers were utilized to amplify 5.4, 1.3, 6.6 and 0.43 kb amplicons covering exon 1, 2-3, 4-5 and 6, respectively. The annealing temperature was 59°C for amplification of exon 1 and 4-5 amplicons, and was 64°C for exon 6 amplification. The PCR reaction for exon 2-3 was thermally cycled using a touchdown strategy: first at 95°C for 2min, followed by 14 cycles of 95°C for 30sec, 63°C (-0.5°C/cycle) for 60sec, and 72°C for 1 min, then 21 cycles of 95°C for 30 sec, 57°C for 60sec, and 72°C for 1 min, followed by a final elongation of 72°C for 7 min before the end of thermal cycling. All PCR amplicons were analyzed on 1% agarose gels and visualized under UV illumination after staining with ethidium bromide (EtBr) to confirm the specificity of PCR prior to Sanger DNA sequencing. DNA sequencing was performed using either PCR primers or primers, 5’-GCATCGTCTCCACCACCGTCAATAG-3’ for exon 1, 5’-CAAAGTGATAGTCCTCTATG-3’ for exon 2, 5’-GAAGCAGGGGCGATTGTTAGA-3’ for exon 3, 5’-GGTGCTTGTGAGTATTGAG-3’ for exon 4, 5’-
TGGGGGAAAGCGCAGATGA TGA TC
- 3' for exon 5 and 5'-CCCCTACGGCCCGCTCTG-3' for exon 6, using the BigDye Terminator Cycle
Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed on ABI
PRISM 3700 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA).

Maintenance of human iPSC lines and differentiation to retinal cups

hiPSCs were cultured on Geltrex (Gibco)-coated dish with mTeSR1 medium
(STEMCELL Technologies) according to the manufacturer’s protocol. Cells were
passaged every 5-7 days at about 80% confluence. For differentiation into retinal
organoids, we adapted previously described method with slight modifications
(Ohlemacher et al., 2015; Wahlin et al., 2017; Zhong et al., 2014). In brief, hiPSCs were
detached by treatment with Vercene (Gibco), dissociated into single cells and cultured
in low attachment dish with ROCK inhibitor Y27632 (10 µM, Cayman Chemical
Company) and 3:1 mixture of mTeSR1 and neural induction medium (NIM) containing
DMEM/F12 (1:1, Life Technologies), 1% N2 supplement, 1 × NEAAs and 2 µg/ml
heparin (Sigma) at day 0 to induce embryonic body (EB) formation. EBs were gradually
transitioned into NIM by replacing the medium with a 1:1 ratio of mTeSR1/NIM on
day 3, 1:3 on day 5. On day 7, EBs were seeded to six-well plate containing NIM with
10% FBS at approximate density of 30-40 EBs/well. On day 16, the medium was
changed from NIM to retinal differentiation medium (RDM) containing DMEM/F12
(3:1), 2% B27 supplement without vitamin A (Life Technologies), 1 × NEAAs, and
penicillin/streptomycin. Around day 18-25, the loosely adherent central portions of the
neural clusters were lifted by pipetting with a P1000 pipettor. The collected aggregates
were cultured in suspension with RDM, which allowed them to form three-dimensional
optic cups structures. Between days 30 and 40, the aggregates of optic cups with
“golden circle” morphology were manually separated from the poor quality cell aggregates. For long-term suspension culture, the medium was supplemented with 10% fetal bovine serum (Gibco), 100 mM Taurine (Sigma) and 2 mM GlutaMAX (Invitrogen) beginning on day 35. From days 60-90, the culture medium was supplemented with 1 µM retinoic acid (Sigma) to induce photoreceptor maturation. The medium was supplemented with N2 instead of B27 after day 90. The cell medium was changed every 2-3 days until the desired stage was reached.

**Overexpression of RS1 in HEK293A cell line**

2.5 \times 10^6 HEK293A cells were seeded into 10-cm cell culture dish to get 70% confluence before transfection. On the next day, the culture dish was washed with PBS twice and Opti-MEM medium was added. pLenti-RS1-Myc or pLenti-RS1(C625T)-Myc plasmids (purchased from OriGene) were mixed with TransIT-LT1 reagent and incubated at room temperature for 30 min. The final mixture was added to culture dish, then incubated at 37°C with 5% CO₂ for 72 hours. The cells were harvested by Trypsin-EDTA (0.25%) for further analysis.

**Genomic DNA extraction**

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN), according to the manufacturer’s protocol. The concentration of DNA in each sample was determined by NanoDrop.

**Total RNA extraction, cDNA synthesis and PCR**

Total RNA was extracted from PBMCs, iPSCs and retinal organoids by miRNeasy Mini Kit (QIAGEN), according to the manufacturer’s protocol. The concentration and
quality of each RNA sample was determined by NanoDrop. 1 µg of total RNA was used as a template for reverse transcription using ToolsQuant II Fast RT Kit (BIOTOOLS Co.). All cDNA samples were diluted 1:1 with nuclease-free water and then stored at −20 °C. For PCR, 1µl of synthesized cDNA was used with 10 µM primer and GoTaq Green Master Mix (Promega). Primer sets are showed in Key Resources Table.

**Immunohistocytochemistry**

The adherent cells were fixed with ice-cold 100% methanol for 10 min and the whole mount retinal organoids were fixed with 4% paraformaldehyde for 10 min. This was followed by blocking and permeabilization in 5% BSA, 10% fetal bovine serum (FBS) and 0.5% Triton X-100 in PBS for one hour. Immunostaining was performed in 1% BSA and 2% FBS and 0.1% Triton X-100 with the primary antibodies (primary antibodies are listed in Key Resources Table) at 4°C overnight. Cells were washed by PBS and incubated with secondary antibodies and Hoechst 33342 (nuclei stain) for an hour at room temperature. For floating retinal organoids, tissues were collected and fixed with 4% paraformaldehyde for 30 min, embedded in paraffin and sectioned. To observe optic cup morphology, the paraffin-embedded tissue was stained with hematoxylin and eosin (H&E); for immunofluorescence, paraffin-embedded tissue was incubated in Target Retrieval Solution at 100°C for 15 min, followed by blocking and permeabilization steps, then incubating with the primary antibodies and secondary antibodies. Images were acquired with Zeiss LSM 880 or LSM 700 laser scanning confocal microscope.

**Western blotting**

HEK293A cells and retinal organoids were lysed in RIPA lysis buffer with protease
inhibitor cocktail. The total protein concentration was normalized by Bio-Rad Protein Assay Dye Reagent Concentrate. The samples with equal concentrations of protein were denatured in SDS mixture (10 mM Tris pH 6.8, 1% SDS, 10% glycerol) with or without 4% β-mercaptoethanol and resolved in either a 10% (reducing conditions) or 6% (non-reducing conditions) Bis-Tris pre-cast polyacrylamide gels. The gel was transferred to membrane with transfer buffer (25mM Tris, 192 mM glycine and 20% methanol) and transfer device (Mini Trans-Blot cell, Bio-Rad) for 2 hours. The membranes were blocked in 5% skimmed milk (Anchor, power dissolved in PBST) for an hour and incubated with primary antibodies against RS1 at 1:1000 dilution, RCVRN at 1:20000 dilution and β-actin at 1:20000 dilution, overnight at 4°C. The membranes were washed by PBST three times in order to remove primary antibodies, followed by incubation with HRP (horseradish peroxidase-conjugated)-conjugated secondary antibody at 1:1000 dilution in 5% skim milk for 45-60 min at room temperature. The membranes were washed by PBST three times, and the bound antibody was detected using the ECL Western Blotting Reagents (Sigma).

**Transmission electron microscopy**

For transmission electron microscopy, cells were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde with 0.1% tannic acid in 0.1 M sodium cacodylate buffer at room temperature for 30 min. After buffer rinses, samples were postfixed in 1% OsO4 in sodium cacodylate buffer for 30 min on ice, followed by dehydration in ethanol, and infiltration with EPON 812 resin (Electron Microcopy Sciences). Sections (~90-nm thickness) were cut and stained with 1% uranyl acetate and 1% lead citrate. Samples were observed by FEI Tecnai Spirit G2 transmission electron microscope.
Time-lapse total internal reflection fluorescence microscope (Time-lapse TIRFM)

Day 150 retinal cups were collected and dissociated by TrypLE Select enzyme for 15 min. The dissociated cells were seeded onto Geltrex-coated coverslips and transfected with mApple-paxillin by TransIT-LT1 reagent in the next day. Cells were imaged by dual-color time-lapse TIRFM using the iLas multi-modal of TIRF (Roper)/spinning disk confocal (CSUX1, Yokogawa) microscope system, and equipped with a 100 × 1.49NA (Oil-Immersion) Plan objective lens (Nikon). The TIRFM images were captured by using a 512B EMCCD (Photometrics). The time lapse of Paxillin-marked focal adhesions was calculated using MetaMorph software as described previously (Kuo et al., 2011).

ABE7.10 RS1 C625T base editing repair agents

For base editing, the Cas9-GFP plasmid was replaced with ABE7.10 and GFP expression plasmids (pCMV-ABE7.10 (Addgene #102919) and pmaxGFP (Lonza)), no template was used, and the gRNA expression plasmid expressed a gRNA with the target sequence (g)ATGCAGACGTGCAGCCCGAG.

Genome Editing of C625T mutant hiPSCs

The patient-specific iPSCs were dissociated into single cell by treatment with TrypLE Select (Thermo). 1 × 10⁶ single iPSCs were transfected under different conditions (Plasmid template directed repair: 5 µg pCas9-GFP, 10 µg pRS1-HR-gRNA; Oligo-directed repair: 5 µg pCas9-GFP, 5µg pRS1-gRNA and 10µg RS1 Oligo; ABE7.10 mediated repair: 7µg pCMV-ABE7.10, 1µg pmaxGFP, and 2µg of the ABE7.10 RS1 repair gRNA plasmid) using the Lonza 2D Nucleofector Technology and the Amaxa
Stem Cell Kit V (cat. #VCA-1003) with program B-016 according to the manufacturer’s instructions. Cells were plated onto a Matrigel-coated dish in mTeSR1 medium with 10 μM Y27632. 48 hr after transfection, iPSCs were detached by TrypLE Select in the presence of 10 μM Y-27632. The top 50% GFP positive iPSCs were sorted with the BD FACSAria II flow cytometer (BD Biosciences). The cells were plated (~2000 cells on one six-well plate) onto MEF feeder layers with half MEF-conditioned hESC medium and half fresh hESC medium with 10 μM Y27632. The medium was changed every day until the colonies formed. Colonies were picked, sub-cloned to ensure clonality, and expanded using clump. Clonality and genotype were confirmed by copy-number ddPCR and Sanger sequencing.

Quantification of targeting efficiencies by droplet digital PCR

To quantify targeting efficiencies, genomic DNA was extracted from genome editing reagent transfected cells. For repair-template transfected cells a 1.1kb RS1 locus specific amplicon (primers CAAGTGGCATTTTCTTAGCAGCACCCCTCC and CACGTATAGCCTCTCAGGGCAG) was generated and purified in order to eliminate contaminating repair template molecules prior to ddPCR analysis. The number of RS1 locus molecules, template-repaired RS1 locus molecules, and ABE7.10-repaired RS1 locus molecules was determined using ddPCR assay #3, #1, and #2 (see ddPCR assays table), respectively, in appropriate 2-color copy number assay combinations (assays #1+#3 for template-dependent repair and assays #2+#3 for ABE7.10-mediated repair) on a Biorad QX200 ddPCR system.

Off-target analysis

gRNA sequence was used as a query for the Zhang lab CRISPR design tool
The top 5 off-target hits with canonical PAM sequence were:

TGCTCCTCTCCATTGCCATCTGG (chromosome 12 plus strand),
AGGAAGTCTGCCTGCCATTCAAGG (chromosome 12 minus strand),
GACACATCTGCATTGCCACCAGG (chromosome 16 minus strand),
GGAAAGTCTTCAATTGCCAAGG (chromosome 17 plus strand),
GGCTAGTCTTCATTGCTATCAGG (chromosome 3 minus strand). The PCR primers for these regions were designed accordingly.

**RNA-Seq analysis on in vitro retinal cups samples**

RNAs from two independent experiments of hiPSCs or RCs were extracted with Trizol (Invitrogen), following the manufacturer’s recommendations. Ten micrograms of total RNA were subjected to two rounds purification using Dynaloligo-dT beads (Invitrogen). Purified RNA was fragmented with 103 fragmentation buffer (Ambion) and used for first-strand cDNA synthesis, and used random hexamer primers (Invitrogen) and SuperScript II enzyme (Invitrogen). Second strand cDNA was obtained by adding RNaseH (Invitrogen) and DNA Pol I (New England BioLabs). Subsequently, the short fragments are connected with adapters. The resulting double-stranded cDNA was used for Illumina library preparation and sequenced with Illumina Genome Analyzer. Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used for quantification and qualification of the sample library. Afterward, the library was sequenced using Illumina HiSeqTM4000. The original image data was transferred into sequence data via base calling, which was defined as raw data or raw reads and saved as FASTQ file. The reads were aligned to the genome using TopHat (v2.0.10, [http://ccb.jhu.edu/software/tophat/index.shtml](http://ccb.jhu.edu/software/tophat/index.shtml)) and Bowtie2 (v2.1.0, [http://bowtie-bio.sourceforge.net/index.shtml](http://bowtie-bio.sourceforge.net/index.shtml)) with the default parameter settings. Followed by eXpress
conversion, the summed RPKM (reads per kilobase per million mapped reads) of transcripts sharing each gene id was calculated.

For data analysis, we removed the adapter sequence using PEAT (v2, https://github.com/jhhung/PEAT/issues) and retained junction reads by aligning the clean reads to the human genome hg19 (Harrow et al., 2012) genome with RNAStar (v2.5.2, https://code.google.com/archive/p/rna-star/). Transcript assembly and differential expression analysis were performed using Cufflinks (v2.1.1, http://cole-trapnell-lab.github.io/cufflinks/). The top 2000 genes with at least two-fold changes of the RPKM value of each gene between RC samples were used to identify up- or down-regulated genes over the control.

The data mining, PCA analysis, similarity analysis and heatmap were performed in the Orange platform (V3.13) (Demšar et al., 2013). The RNA-seq reads were submitted to GEO: GSE137910.

**RC retinal split area fraction calculation**

For RC splitting area quantification, the organoid section images were captured using an Olympus DP73 camera, driven by cellSens software. To quantify the split area, we applied H&E staining and ImageJ software for the measurement. Each of the H&E staining of RC was first determined the neural retinal layer by cropping the region between outer nuclear layer (ONL) and inner nuclear layer (INL) (as shown in Figure S2C). The images then converted to gray scale and measured the white area by setting threshold to exclude the region of nuclear and cytosol staining. The % of splitting layer was calculated by the ratio of white (gap) area and the total area between ONL and INL of each RC. Twenty images of H&E staining sections of each group (Ctrl-1-RCs, Ctrl-
2-RCs, Pt-1-RCs, Pt-2-RCs, R1-RCs, R2-RCs, M1-RCs, and M2-RCs.), were used for the analysis. The images come from 3 biological replicates. The definition of the smallest split area was 50 µm. The measurement of colocalization of RS1 with GRP94 or Golgi97 was performed using ZEN 2.1 SP3 software (ZEISS). The protein expression level and the fluorescence cell count were measured by ImageJ.

**Statistical analysis**

The statistical analysis was performed as the mean± standard error of the mean (SEM) and compared by using the Student’s t-test or one-way ANOVA. The statistics and images were generated using Microsoft Excel. P-values less than 0.05 were considered significant (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). To assess the degree of statistical significance of the differences between the frequencies of RCs with cysts we used Fisher’s exact test.
## KEY RESOURCES TABLE

### List of Antibodies

| Antibodies                                           | Source          | Identifier |
|------------------------------------------------------|-----------------|------------|
| Rabbit polyclonal anti-NANOG                         | Cell Signaling  | 3580       |
| Mouse monoclonal anti-TRA-1-81                      | Cell Signaling  | 4745       |
| Mouse monoclonal anti-TRA-1-60                       | Cell Signaling  | 4746       |
| Mouse monoclonal anti-SSEA4                         | Cell Signaling  | 4755       |
| Mouse monoclonal anti-LHX2                          | Thermo Fisher   | MA5-15834  |
| Rabbit polyclonal anti-PAX6                          | Thermo Fisher   | PA5-25970  |
| Mouse monoclonal anti-OTX2                          | Thermo Fisher   | MA5-15854  |
| Rabbit polyclonal anti-RECOVERIN                     | Cell Signaling  | 2056       |
| Rabbit monoclonal anti-COX IV                        | Millipore       | AB5585     |
| Mouse monoclonal anti-RHODOPSIN                      | Millipore       | MABN15     |
| Rabbit polyclonal anti-OPSIN (Red/Green)             | Millipore       | AB5405     |
| Rabbit polyclonal anti-OPSIN (Blue)                  | Millipore       | AB5407     |
| Mouse monoclonal anti-RS1                            | Abcam           | AB167579   |
| Rabbit monoclonal anti-GRP94                         | Cell Signaling  | 20292      |
| Rabbit monoclonal anti-Golgi-97                      | Cell Signaling  | 13192      |
| Mouse monoclonal anti-β-actin                        | Sigma           | A5441      |
| Mouse monoclonal anti-Arl13b                         | Abcam           | AB136648   |
| Mouse monoclonal anti-Polyglutamylation Modification (GT335) | AdipoGen       | AG-20B-0020-C100 |
| Rabbit polyclonal anti-E-Cadherin                    | GeneTex         | GTX100443  |
| Mouse monoclonal anti-Paxillin                       | BD Biosciences  | 610052     |
| Goat polyclonal anti-CHX10 (VSX2)                    | Santa Cruz      | Sc-21690   |
| Mouse monoclonal anti-Ki-67                          | Cell Signaling  | 9449       |
| Alexa Fluor™ 488 anti-Phalloidin                     | Thermo Fisher   | A12379     |
| Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 546 | Thermo Fisher | A-11003    |
| Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 | Thermo Fisher | A-11001    |
| Donkey anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 | Thermo Fisher | A-21202    |
| Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 546 | Thermo Fisher | A-11035    |
| Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 | Thermo Fisher | A-11034    |
| Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 | Thermo Fisher | A-21206    |
| Donkey anti-Goat IgG (H+L) Secondary Antibody, Alexa Fluor 488 | Thermo Fisher | A-11055    |
| Horse anti-mouse IgG, HRP-linked                      | Cell Signaling  | 7076       |
| Goat anti-rabbit IgG, HRP-linked                      | Cell Signaling  | 7074       |

### List of Reagents and Commercial Assays

| Reagents and Resources for cell culture | Source          | Identifier |
|----------------------------------------|-----------------|------------|
| X-VIVO™ 10                             | Lonza           | 04-743Q    |
| Opti-MEM™                              | Thermo Fisher   | 51985091   |
| mTeSR™1                | StemCell Technologies | 85850 |
|------------------------|-----------------------|-------|
| DMEM/F-12, GlutaMAX™   | Thermo Fisher         | 10565 |
| Advanced DMEM          | Thermo Fisher         | 12491 |
| Advanced DMEM/F-12     | Thermo Fisher         | 12634 |
| Geltrex                | Thermo Fisher         | A1413302 |
| IL-2                   | PeproTech             | 200-02 |
| Dynabeads™ Human T-Activator CD3/CD28 | Thermo Fisher | 11132D |
| Fetal Bovine Serum (FBS) | HyClone         | SH30071.03 |
| Trypsin-EDTA (0.25%), phenol red | Thermo Fisher | 25200072 |
| Versene solution       | Thermo Fisher         | 15040066 |
| TrypLE™ Select         | Thermo Fisher         | 12563011 |
| KnockOut™ Serum Replacement | Thermo Fisher | 10828028 |
| β-mercaptoethanol      | Thermo Fisher         | 21985023 |
| MEM Non-Essential Amino Acids Solution | Thermo Fisher | 11140 |
| Recombinant human basic fibroblast growth factor (bFGF) | PeproTech | 100-18B |
| Penicillin-Streptomycin| Thermo Fisher         | 15140 |
| Y-27632                | Selleck Chemicals     | 17502048 |
| N-2 Supplement         | Thermo Fisher         | 12587010 |
| B-27™ Supplement, minus vitamin A | Thermo Fisher | 15140 |
| Heparin                | Sigma                 | 1784 |
| Taurine                | Sigma                 | 12825 |
| GlutaMAX™ Supplement   | Thermo Fisher         | 35050061 |
| Retinoic acid          | Sigma                 | 12625 |
| TransIT®-LT1           | Mirus                 | 2300 |
| **Commercial Assays**  | **Source**            | **Identifier** |
| QIAnamp DNA Blood Mini Kit | QIAGEN            | 51106 |
| miRNeasy Mini Kit      | QIAGEN                | 217004 |
| Human T Cell Nucleofector® Kit | Lonza         | VPA-1002 |
| Cell Line Nucleofector® Kit V | Lonza       | VCA-1003 |
| **Reagents and Resources** | **Source**            | **Identifier** |
| Target Retrieval Solution, Low pH | Agilent        | DM829 |
| RIPA Lysis Buffer      | Millipore             | 20-188 |
| cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail | ROCHE | 04693159001 |

| Gene name | Forward primer | Reverse primer | Size (bp) |
|-----------|----------------|----------------|-----------|
| OCT4      | GACAGGGGGAGGGAGGAGCTA | CTTCCCTCCACCCAGTTGCCCCAA | 144       |
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