Supplementary Material

Protrusion of *KCNJ13* gene knockout retinal pigment epithelium due to oxidative stress-induced cell death

Running title:

Loss of Kir7.1 causes protrusion due to cell death

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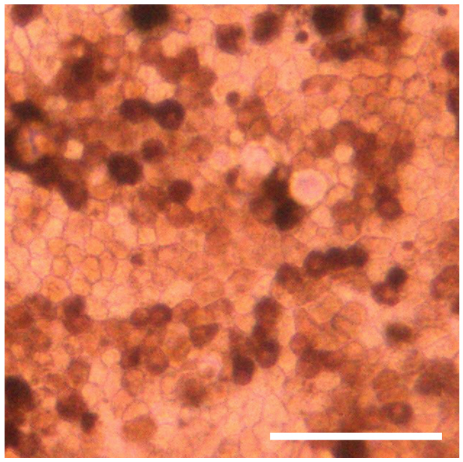
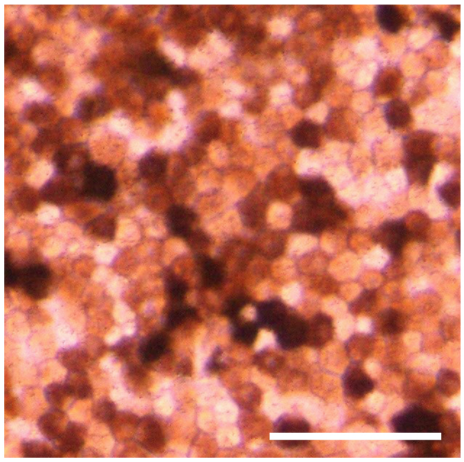
Supplemental Figure 1. WT and *KCNJ13* KO hiPSC-RPE cells are pigmented and show a cobblestone appearance. Scale bars = 100 µm.

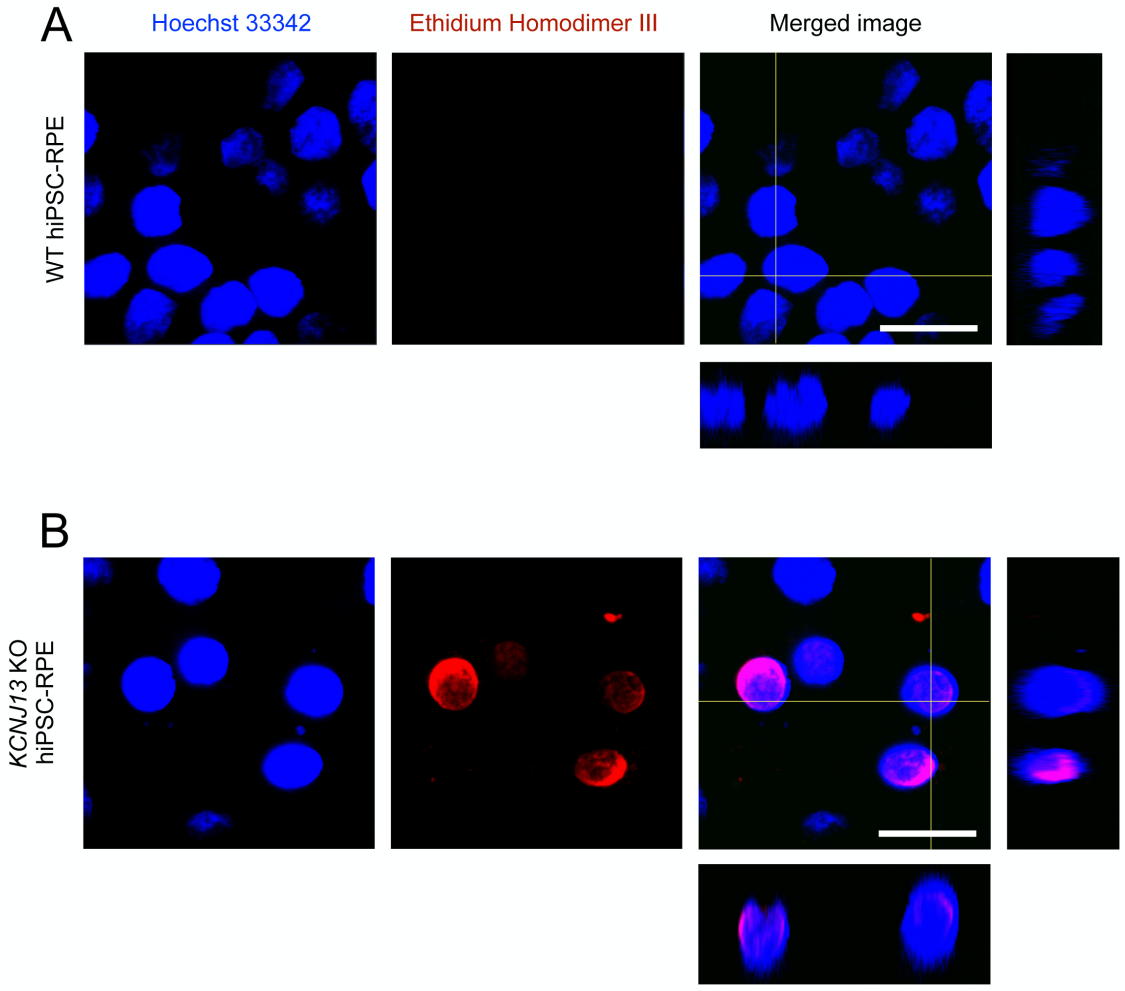
WT hiPSC-RPE



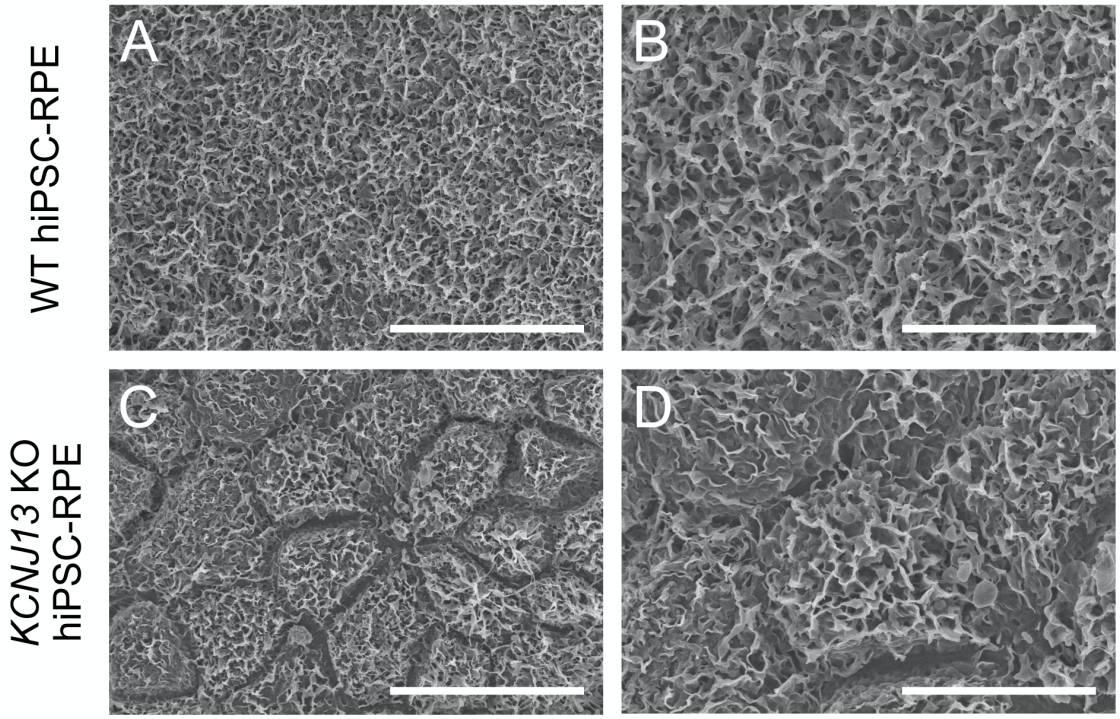
KCNJ13 KO hiPSC-RPE



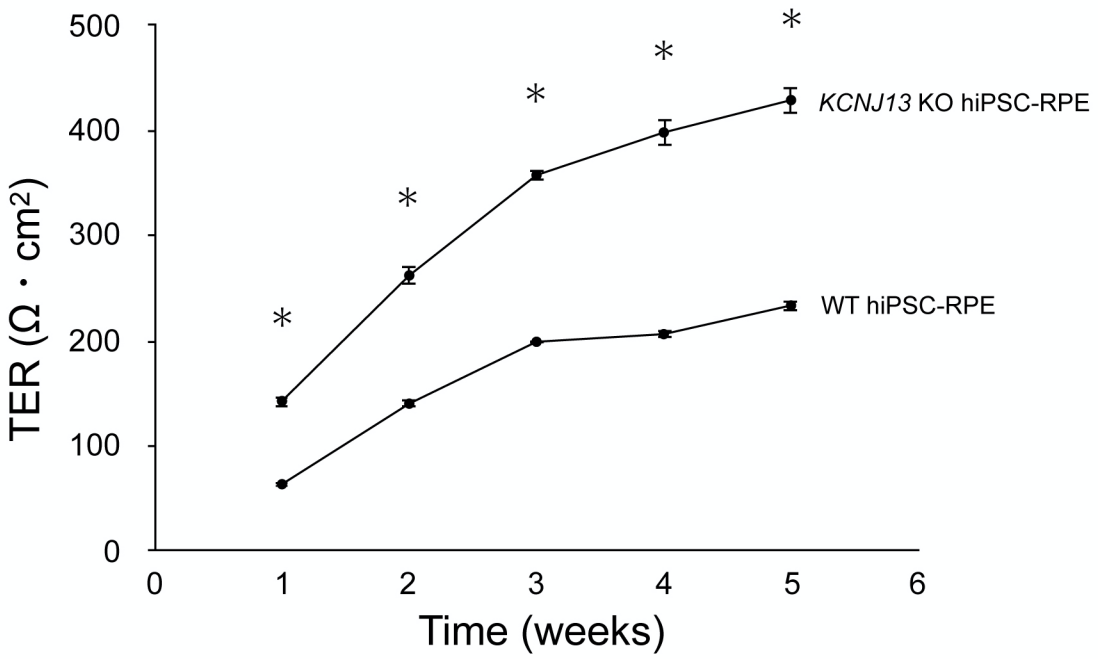
Supplemental Figure 2. Staining for ethidium homodimer III.
Nuclei of *KCNJ13* KO RPE cells are stained. (A) Staining results of the wild type are shown. (B) Staining results of *KCNJ13 KO* hiPSC-RPE cells. Scale bars = 15 μm.



Supplemental Figure 3. SEM images of WT and *KCNJ13* KO hiPSC-RPE. Both RPEs show microvillus development and a cobblestone appearance. (A, B) WT (same images as in Figure 3B, C) and (C, D) KO hiPSC-RPE images are shown. Scale bars = 20 μm in A and C, and 10 μm in B and D.

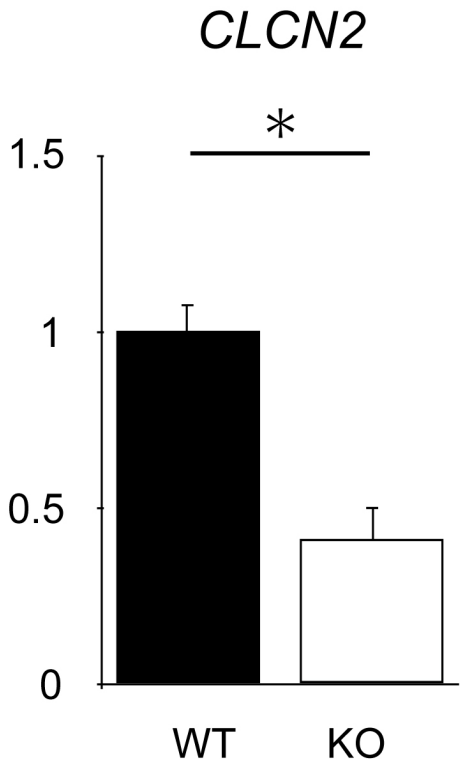


Supplemental Figure 4. TEER measurement of WT and *KCNJ13* KO hiPSC-RPE. *: $P < 0.05$ (Student's t test).



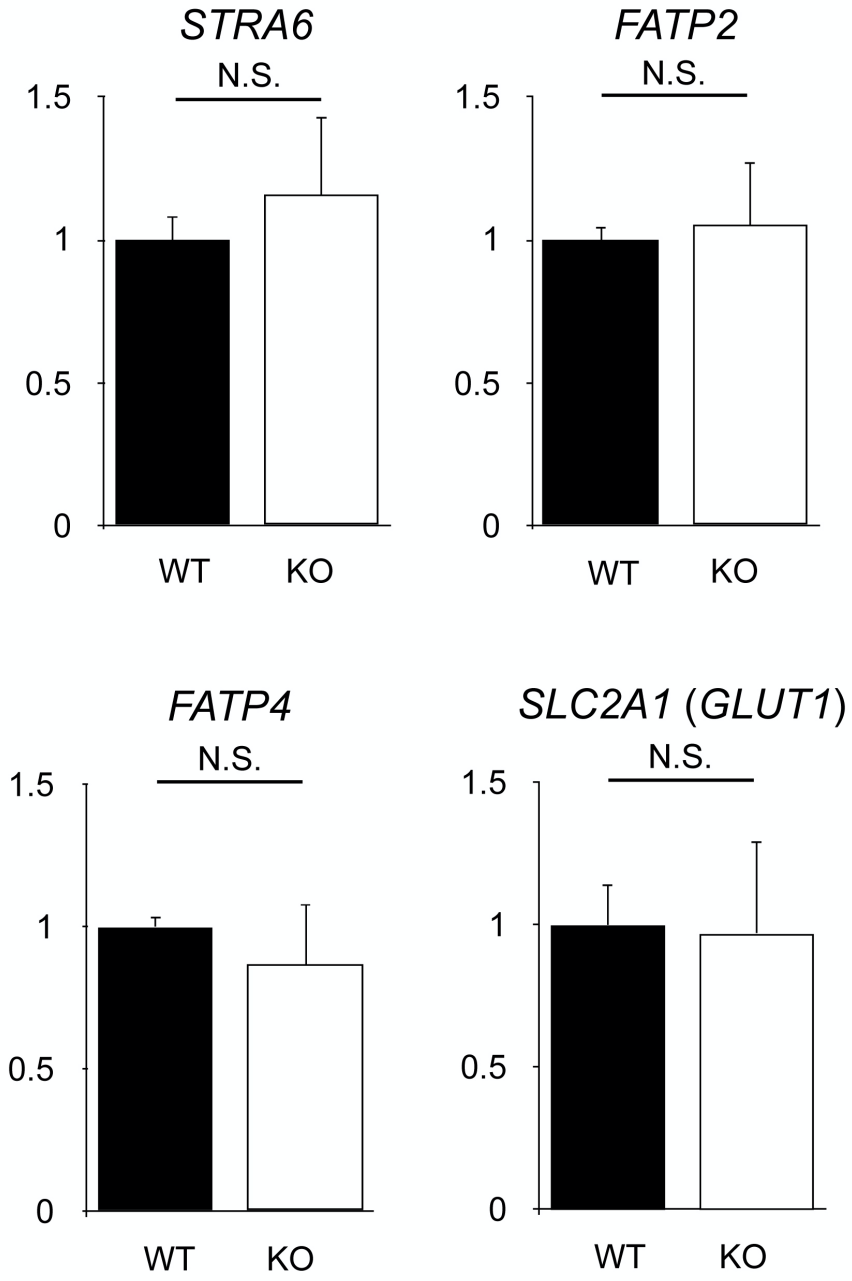
Supplemental Figure 5. Gene expression of a chloride ion channel in WT and KO RPE.

Relative expression levels of *CLCN2*, a known chloride channel gene expressed in RPE and involved in cell volume expansion. *18S rRNA* was used as an internal control. The expression level of each gene in WT hiPSC-RPE cells was set as 1. Data are shown as the mean ± SEM. *: $P < 0.05$ (Student's *t* test).



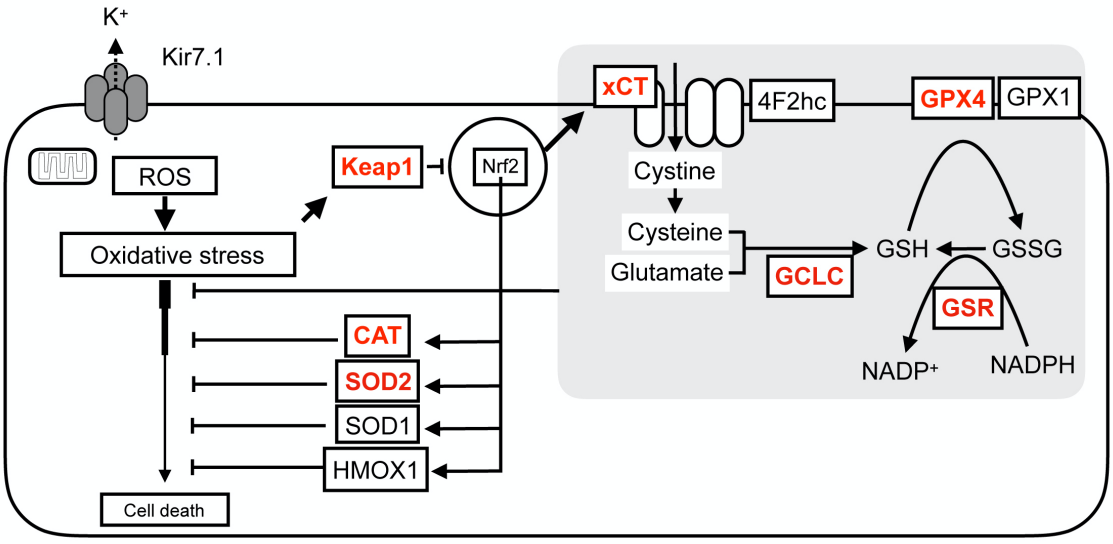
Supplemental Figure 6. Gene expression involved in transepithelial transport.

Relative expression levels of *STRA6* (A), *FATP2* (B), *FATP4* (C), and *SLC2A1* (*GLUT1*) (D), which are involved in transport of retinol, fatty acids, and glucose. *18S rRNA* was used as an internal control. The expression level of each gene in WT hiPSC-RPE cells was set as 1. Data are shown as the mean ± SEM. N.S.: not significant.



Supplemental Figure 7. Schematic diagram showing oxidative stress markers examined in this study.

Glutathione and glutathione-dependent enzymes, which coordinately alleviate oxidative stress, are shown in the shaded region. While a transcription factor, Nrf2 regulates the glutathione system by acting on a cystine/glutamic acid transporter (xCT), it activates transcription of catalase (CAT), superoxide dismutases (SODs), and heme oxygenase 1 (HMOX1) to prevent cellular damage by oxidative stress. Keap1, a repressor of Nrf2, binds to Nrf2 and leads to its degradation by recruiting ubiquitin ligases. Upon oxidative stress, Keap1 is oxidized to release Nrf2 in normal cells. Molecules with altered expression in the *KCNJ13*-deficient hiPSC-RPE are shown in red.



Supplementary Methods

Cell Culture

We induced RPE differentiation from wild-type and *KCNJ13* KO iPSCs according to methods in our previous report.[1] Briefly, to differentiate them directly into RPE cells, hiPSCs were cultured on gelatin-coated dishes in differentiation medium (Glasgow minimal essential medium [GMEM]) supplemented with sodium pyruvate, nonessential amino acids, 2-mercaptoethanol and 20% KnockOut Serum Replacement (KSR). At 4 weeks, the differentiation medium was switched to serum-free RPE medium (SFRM) containing Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12) [7:3] supplemented with B-27 supplement and L-glutamine and cultured (in maintenance medium) for 7 days. Pure populations of pigmented cells were obtained by transferring pigmented colonies to SFRM supplemented with FGF2 and SB431542.

Analysis of Cell Morphology

After removing the medium, hiPSC-RPE cells seeded on 12-well Transwells were fixed in 4% paraformaldehyde (PFA) for 30 min and washed with PBS. Cells were permeabilized with 0.3% Triton X-100 for 30 min. After washing three times with PBS, cells were treated with 1% bovine serum albumin (BSA) plus 5% goat serum in PBS for 60 min and incubated with anti-ZO-1 (1:50 dilution in PBS; Thermo Fischer Scientific, Waltham, MA) as a primary antibody at 4 °C overnight. After washing, the cells were incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:1,000 dilution in PBS, Cat. no. A-11011). Immunofluorescence images were taken on an LSM 780 confocal microscope equipped with ZEN 2009 software (Carl Zeiss Microscopy GmbH, Jena, Germany). Only the ZO-1 signal was extracted and converted to grayscale. The area and perimeter of the cells enclosed by ZO-1 signals (white lines in Figure 1A, B) were automatically measured by ImageJ. Lines containing partially broken cell borders were supplemented manually with the paint brush function due to detected by Image J (bold lines). The size threshold was set to 50-2100.

LDH Assay

hiPSC-RPE cells were seeded in 96-well plates (Sumitomo Bakelite). [1] After 4 weeks, the medium (SFRM supplemented with FGF2 and SB431542) was replaced with maintenance medium 2 days before the assay, and on the day of the assay, the medium was collected and diluted 2-fold with SFRM to make

samples. WT hiPSC-RPE cell lysates were prepared as a positive control for LDH release, and maintenance medium was used as a background control. Samples were further mixed with the reaction mixture, and incubated for 30 min at room temperature under dark. The reactions were stopped by addition of stop solution, and the absorbance of the samples was measured at 450 nm (the reference wavelength was 750 nm).

Scanning Electron Microscopy

hiPSC-RPE cells in Transwells were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) overnight. The samples were rinsed in PB for 30 min and then postfixed in 2% osmium tetroxide (OsO4) in PB for 1.5 hours. The samples were dehydrated via an ethanol series and dried with the freeze-drying method using a t-butyl alcohol lyophilizer (ID-2, EIKO, Japan). The dried samples were mounted on aluminum stubs, and OsO_4 vapor deposition was performed (HPC-1S, Vacuum Device Ltd, Japan).

Transepithelial Electrical Resistance (TEER) Assay

TEER was measured according to Kimura et al.[2] Briefly, WT and *KCNJ13* KO hiPS-RPE cells were grown on permeable membrane inserts (5×10^5 cells per insert, 12-well, ThinCerts™, Greiner Bio-One) with a 0.4 μm pore size. TEER of both WT and *KCNJ13* KO hiPSC-RPE cells was measured 4 weeks after seeding using an epithelial voltohm-meter (Millicell-Electrical Resistance System-2, Billerica). The readings for the “blank” were obtained by measuring a well containing only the medium.

References

1. Kanzaki Y, Fujita H, Sato K, et al. KCNJ13 Gene Deletion Impairs Cell Alignment and Phagocytosis in Retinal Pigment Epithelium Derived from Human-Induced Pluripotent Stem Cells. 2020;61(5):38.
2. Kimura S, Morizane Y, Toshima S, et al. Cytotoxic effects of alteplase, a recombinant tissue plasminogen activator, on human retinal pigment epithelial cells. *Jpn J Ophthalmol*. 2021;65(5):731-739.