

**Deterioration of Phagocytosis in induced Pluripotent Stem Cell-Derived
Retinal Pigment Epithelial Cells established from Patients with Retinitis
Pigmentosa Carrying *Mer Tyrosine Kinase* Mutations**

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¹ Abbreviations: RP, Retinitis Pigmentosa; RPE, retinal pigment epithelium; MERTK, Mer tyrosine kinase; POS, photoreceptor outer segments; iPSC, induced pluripotent stem cells; iPSC-RPE, induced pluripotent stem cells into RPE; MTK, patient with Retinitis Pigmentosa carrying *MERTK* mutations

1 **ABSTRACT**

2 Retinitis pigmentosa (RP) is an incurable retinal degenerative disease with an
3 unknown mechanism of disease progression. Mer tyrosine kinase (*MERTK*),
4 which encodes a receptor of the Tyro3/Axl/Mer family of tyrosine kinases, is one
5 of the causal genes of RP. *MERTK* is reportedly expressed in the retinal pigment
6 epithelium (RPE) and is essential for phagocytosis of the photoreceptor outer
7 segment. Here, we established induced pluripotent stem cells (iPSC) from
8 patients with RP having homozygous or compound heterozygous mutations in
9 *MERTK*, and from healthy subjects; the RP patient- and healthy control-derived
10 iPSCs were differentiated into RPE cells. Although cytoskeleton staining
11 suggested that polarity may have been disturbed mildly, there were no apparent
12 morphological differences between the diseased and normal RPE cells. The
13 internalization of photoreceptor outer segments in diseased iPSC-RPE cells was
14 significantly lower than that in normal iPSC-RPE cells. This *in vitro* disease
15 model may be useful for elucidating the mechanisms of disease progression and
16 screening treatments for the disease.

17
18 **Keywords:** Retinitis pigmentosa, retinal degeneration, iPS cells, retinal pigment
19 epithelium

1. Introduction

Retinitis pigmentosa (RP) is a retinal degeneration that mainly affects the rod photoreceptors or retinal pigment epithelium (RPE), which manifests as progressive night blindness, constriction of the visual field, and eventual blindness (Hartong et al., 2006). It is a rare intractable disease affecting 1/5000 individuals worldwide. To date, approximately 60 genes that are inherited in all Mendelian forms have been implicated in the disease. The mechanisms of RP onset and progression are assumed to vary depending on the causative genes (Hartong et al., 2006). Human intraocular tissues, especially diseased neural retinae or RPE, are difficult to obtain. Therefore, the pathology and underlying mechanisms of RP onset and progression remain unknown.

Mer tyrosine kinase (*MERTK*) is one of the genes implicated in RP (Gal et al., 2000). The gene encodes a receptor of the Tyro3/Axl/Mer (TAM) family of tyrosine kinases (Boye et al., 2013). TAM receptors are expressed by dendritic cells, epithelial cells including RPE cells of the eye, macrophages, and immature natural killer (NK) cells of the immune system, Sertoli cells of the testis, and several other cell types (Lemke and Rothlin, 2008). Within the RPE, *MERTK* is reportedly involved in recycling photoreceptor outer segments (POS) and is necessary for phagocytosis of the POS (Law et al., 2015; Nandrot et al., 2007), an essential function of RPE cells.

Royal College of Surgeons (RCS) rats are a classic model of recessively inherited retinal degeneration with a mutation in *Mertk* (D'Cruz et al., 2000). This rat model is widely used for research on human retinal degenerative disorders (D'Cruz et al., 2000; Nandrot et al., 2000). In this rat model, death of the rods

was first observed at 3 weeks of age, and the outer layers were completely degenerated by 7 weeks of age, when no more rod and cone cells existed (Bourne et al., 1938). However, this rat model has certain limitations in reflecting human pathology of the disease: the degeneration in human patients with *MERTK* mutation progresses slowly, typically over decades; moreover, the rats do not have maculae, which is important for visual acuity and are the main sites where cones are located.

We have previously established a stepwise differentiation of induced pluripotent stem cells (iPSCs) into RPE (iPSC-RPE), enabling the isolation of patient-derived iPSC-RPE cells with high efficiency and purity (Osakada et al., 2009a; Hiram et al., 2009). This allowed us to elucidate the precise mechanisms underlying inherited retinal diseases.

In this study, we established an iPSC-RPE model from the primary cells of patients with RP with *MERTK* gene mutations and analyzed the morphological and functional changes in iPSC-RPE cells to investigate the mechanism of onset and progression of retinal degeneration.

2. Material and methods

2.1. Establishment of iPSCs

This study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Kyoto University Graduate School of Medicine (G259 and R0091). Informed consent was obtained from all patients. Fibroblast cell lines were generated from patients with RP and *MERTK* gene mutations, including one patient with compound heterozygous c.225delA,

p.(Gly76Glufs*3)/c.370C>T, p.(Gln124*) mutations, and one patient with a homozygous c.225delA, p.(Gly76Glufs*3) mutation (MTK), and from three control individuals with normal fundus, without *MERTK* gene mutations (NOR). The method used for human iPSC generation has been described previously (Okita et al., 2013; Nakagawa et al., 2014). We analyzed two cell lines from two patients with MTK (MTK1 and MTK2) and three cell lines from three NOR controls (NOR1, NOR2, and NOR3).

2.2. Culture of Human iPSCs and Differentiation of iPSC-Derived RPE Cells

The methods of human iPSC maintenance and differentiation have been described previously (Osakada et al., 2009a; Hiramami et al., 2009; Kamao et al., 2014; Nakagawa et al., 2014; Hata et al., 2018). Human iPSC-RPE cells were maintained using a previously established method (Kamao et al., 2014). iPSC-RPE cells were cultured in CTS CELLstart (Gibco)-coated dishes in preconfluent medium (1:1 mixture of RPE maintenance medium and RPE differentiation medium). RPE maintenance medium included DMEM/F12 at a 7:3 ratio (Sigma) supplemented with B27 (Invitrogen) and 2 mM L-glutamine (Sigma). The RPE differentiation medium included DMEM/F12 at a 1:1 ratio (Sigma) and 10 % FBS. The medium was changed every 2-3 days. We considered iPSC-RPE progenitor cells as immature cells within one month of passage of differentiated iPS cells, which contain pigments and show hexagonal appearance, and were able to divide.

2.3. Immunocytochemistry

Cells were immunostained as described previously (Hata et al., 2018; Ikeda et al., 2005; Osakada et al., 2009b). The antibodies used were as follows: rabbit anti-ZO-1 (1:200; Invitrogen), mouse anti-EZRIN (1:50; Santa Cruz), mouse anti-CLAUDIN 19 (1:50; Santa Cruz), Alexa Fluor 488 goat anti-rabbit IgG (1:1000; Invitrogen), and Alexa Fluor 488 goat anti-mouse (1:1000; Invitrogen) antibodies. After incubation with the secondary antibody, cell nuclei were counterstained with DAPI (1 µg/mL) (Molecular Probes). Phalloidin (1:50; Invitrogen) was used to stain the actin filaments. Cells were mounted using Pro-Long Gold (Invitrogen) and imaged using a laser-scanning confocal microscope (Leica TCS SP8).

2.4. Proliferation Assay

To analyze the proliferation rate of immature iPSC-RPE cells, the viability of the cells was measured by formazan production (absorbance at 450 nm) with water-soluble tetrazolium salt (WST-8) reagent (Cell Count Reagent SF; Nacalai Tesque), using an ARVO multilabel counter (Wallac) (Hata et al., 2018).

2.5. Transmission electron microscope

Cultured iPSC-RPE cells were first washed with Dulbecco's phosphate-buffered saline (PBS, Nacalai Tesque) and fixed in a solution containing 4 % formaldehyde and 2 % glutaraldehyde. Sections were exposed to a 1 % osmium tetroxide solution in 100 mM phosphate buffer (pH 7.4) for 1 hour at 4 °C and then dehydrated in a series of graded ethanol solutions. The cells were then fixed in Epon 812 resin (Nacalai Tesque) using the inverted beam capsule

procedure and polymerized at 60 °C for 3 days. Ultrathin sections were cut using an ultramicrotome and stained with uranyl acetate and lead citrate. The stained sections were investigated using a transmission electron microscope (model H-7650; Hitachi Co.).

2.6. Western Blot Analysis

iPSC-RPE cells were lysed in radioimmunoprecipitation (RIPA; Pierce) buffer containing a protease inhibitor mixture, and the lysates were centrifuged at 20,400 ×g for 30 minutes at 4 °C. Total protein was quantified using a protein assay bicinchoninate kit (Nacalai Tesque). Protein samples (5–10 µg) were denatured by adding sample buffer solution with reducing reagent (×6) for SDS-PAGE (Nacalai Tesque) at 95 °C for 3 minutes, separated on 12.5 % SuperSep Ace (Wako), and electroblotted onto an Immuno-Blot Poly Vinylidene Difluoride (PVDF) membrane (Bio-Rad). Membranes were incubated in PVDF Blocking Reagent for Can Get Signal (Toyobo) for 1 hour at room temperature, washed twice for 5 minutes in Tris-buffered saline with Tween 20 (TBST), and incubated with a primary antibody overnight at 4 °C. Then, the blots were washed three times in TBST for 10 minutes and incubated with a secondary antibody for 1 hour at room temperature. The blots were then washed four times. Protein bands were detected using ECL Prime western blotting Detection Reagent (GE Healthcare) and visualized using a Molecular Imager ChemiDoc XRS+ system (Bio-Rad). The following antibodies were used: rabbit anti-MERTK (1:500; Abcam, monoclonal antibody developed with a synthetic peptide (100 amino acids of the N-terminal of the MERTK protein), mouse anti-RPE65

(1:10000; Abcam), mouse anti-CRALBP (1:1000; Abcam), mouse anti-BEST 1 (1:200; Abcam), mouse anti-actin (1:5000; Sigma), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000; Cell Signaling), anti-mouse and anti-rabbit secondary antibodies (1:10000; GE Healthcare). ACTIN and GAPDH were used as the loading controls.

2.7. RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA was extracted using an RNeasy Mini Plus Kit (Qiagen) and treated with DNase1 to remove any genomic DNA contamination. cDNA was synthesized from the extracted RNA using a thermal cycler (Takara Bio Inc.). qRT-PCR experiments (40 cycles) were performed using SYBR premix Ex Taq polymerase (Takara Bio Inc.) at an annealing temperature of 60 °C using a 7300 Real-Time PCR System (Applied Biosystems). The primers used were as follows: *RPE65*, 5'CCTGCTGGTGGTTACAAGAAA3' and 5'CCTGCCTGTTACATGAGCTGT3', *CRALBP*, 5'AAGCTGGCTACCCTGGTGT3' and 5'-TGAAGCAATATGCCTGCAAGA3', *BEST1*, 5'CTGGGCTTCTACGTGACGC3' and 5'TTGCTCGTCCTTGCCTTCG3', *MERTK*, 5'GTGCAGCGTTCAGACAATGG3' and 5'TCGATGTAGATGGGATCAGACAC3; and *GAPDH*, 5'ACCACCAACTGCTTAGC3' and 5'GGCATGGACTGTGGTCATGAG3'. *GAPDH* was used as an internal housekeeping gene expression standard.

2.8. Analysis of phagocytosis with FITC-labeled Latex Beads

iPSC-RPE cells were grown to confluence in 24-well dishes and treated with FITC-labeled latex beads (L4655; Sigma, 0.9 μ L/well) in RPE maintenance medium and incubated for 24 hours at 37 °C. The dishes were vigorously shaken, and beads, which were not within the cells, were washed with PBS three times. The cells were photographed using a fluorescence microscope (OLYMPUS IX73). The cells were dissociated with trypsin/EDTA and resuspended in RPE maintenance medium. The proportion of iPSC-RPE cells with FITC-labeled latex beads was measured using a flow cytometer (BD FACS Calibur).

2.9. Preparation of FITC-Labeled POS

POS, isolated and purified from fresh cow retinas, were purchased from InVision BioResources. POS (approximately 400 million outer segments, which typically contain 70 mg rhodopsin), were mixed with RPE maintenance medium to the desired POS solution concentration (1 μ g of rhodopsin/ μ L). Then, 1 mL of the POS solution was centrifuged at 10,000 $\times$ g for 3 min at 4 °C. The pellet was suspended in 0.1 M sodium bicarbonate buffer. FITC (Isomer I, Life Technologies; 10 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO). Fifty microliters of the FITC/DMSO solution was added to the POS suspension, and the suspension was stirred for 90 min at room temperature. Then, 4.5 mL of PBS was added to this solution, and the solution was centrifuged at 10,000 $\times$ g for 3 minutes at 4 °C. The pellet was dissolved in 5 mL PBS and centrifuged at 10,000 $\times$ g for 3 minutes at 4 °C. The pellet was dissolved in 5 mL of DMEM F-12 (10 % FBS, 1 % P/S), stirred for 20 minutes at 4 °C, and centrifuged at 10,000 $\times$ g for 3

minutes at 4 °C. The pellet was dissolved in OPTI-MEM, and FITC-labeled POS was achieved (40 µg of rhodopsin/50 µL). We determined the amount of POS used in each experiment according to our previous report (Hata et al., 2018). The description of the unit (POS/cell) used in each experiment is a reference value and may be unreliable because description of rhodopsin amount extracted from the adult bovine eye, which was originally described on the supplier's homepage, has been removed.

2.10. Analysis of phagocytosis with FITC-Labeled POS

iPSC-RPE cells were grown to confluence in 24-well dishes and treated with FITC-labeled POS (20 µg of rhodopsin/well, approximately 0.1 POS/cell) in RPE maintenance medium and incubated for 3 hours at 37 °C. The concentration of POS used on iPSC-RPE cells for FACS analysis was greater than that used in western blot analysis, based on preliminary experiments and our previous studies. The cells were washed three times with PBS to remove the unphagocytosed POS. Cells were photographed using a fluorescence microscope (OLYMPUS IX73). The cells were dissociated using trypsin/EDTA and resuspended in 700 µL of RPE maintenance medium. The proportion of iPSC-RPE cells in which FITC-labeled POS was internalized was measured using a flow cytometer (BD FACS Calibur).

2.11. Western Blot of iPSC-RPE phagocytosed with POS

iPSC-RPE cells were grown to confluence in 24-well dishes and challenged with POS (which contains 10 µg of rhodopsin/well, approximately 0.05 POS/cell) in

RPE maintenance medium and incubated for 3 hours at 37 °C. The POS was washed three times with PBS. The cells were dissociated using trypsin/EDTA and resuspended in 800 µL of RPE maintenance medium. The suspension was centrifuged at 190 ×g for 4 minutes at 4 °C. Cells were lysed in RIPA buffer and analyzed by western blotting as described above using mouse anti-Rhodopsin (1:500; Santa Cruz) and rabbit anti-GAPDH (1:1000; Cell Signaling) antibodies. Anti-mouse and anti-rabbit secondary antibodies were used at a concentration of 1:5000.

2.12. Statistical Analysis

The statistical significance of differences was determined by an unpaired *t*-test. Statistical analyses were performed using SPSS Statistics (version 24.0, SPSS Inc.). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Generation of Patient-Specific iPSCs

We obtained skin biopsy specimens from two patients with RP: a male between 20–30 years of age with compound heterozygous variants in the *MERTK* gene (MTK), consisting of a single base change in exon 2 (c.225delA) and a variant in exon 2 (c.370C>T) (Supplemental Fig. S1), and a female between 50–60 years of age with a homozygous variant in the *MERTK* gene (MTK), consisting of a single base change in exon 2 (c.225delA) (Supplemental Fig. S2) (Oishi et al., 2014). We established iPSC lines from patients with MTK and normal control iPSC lines from three control individuals with normal fundus and without *MERTK*

gene mutation (NOR) (Supplemental Fig. S3). There were no remarkable differences between the MTK and NOR iPSC lines during the establishment of iPSCs.

3.2. Morphological Analyses of iPSC-Derived RPE Cells

The established MTK and NOR iPSCs were differentiated into RPE cells. Differentiated iPSC-RPE cells with a polygonal, cobblestone-like morphology were cultured for over 90 days until high pigmentation appeared, indicating full functional maturity (Fig. 1A and B). Immunocytochemical staining revealed strong expression of zonula-occludens-1 (ZO-1), a tight-junction marker, both in NOR and MTK iPSC-RPE cells (Fig. 1C and D). The polygonal morphology of the cells was confirmed by staining using a membrane marker, EZRIN (Supplemental Fig. S4C and D). Moreover, mRNA and protein expression of EZRIN and actin did not differ between NOR and MTK iPSC-RPE cells (Supplemental Fig. S4A, B, I-K). Cross-sectional staining of the cells using anti-EZRIN antibody and phalloidin revealed that both the apical side as well as the basal side in MTK iPSC-RPE cells was comparable to NOR (Supplemental Fig. S4E-H).

Transmission electron microscopy observations confirmed that NOR and MTK iPSC-RPE cells grew as a monolayer of highly polarized cells with abundant apical microvilli and melanosomes (Fig. 1E-H).

These data showed that although polarity may have been disturbed mildly, there were no apparent morphological differences between NOR and MTK iPSC-RPE cells.

3.3. Gene Expression of iPSC-RPE Cells

To investigate the identity of the generated iPSC-RPE cells, qRT-PCR of RPE-specific genes was performed to compare the expression levels in NOR or MTK iPSC and iPSC-RPE cells (Fig. 1I). The mRNA levels of key RPE-specific markers, *retinal pigment epithelium-specific protein 65 kDa (RPE65)*, *cellular retinaldehyde-binding protein (CRALBP)*, and *bestrophin 1 (BEST1)* (Booij et al., 2010; Liao et al., 2010) were significantly elevated in differentiated NOR or MTK iPSC-RPE cells compared to undifferentiated iPSCs. The expression of RPE65, CRALBP, and BEST1 proteins was confirmed by western blotting (Fig. 1J, Supplemental Fig. S5A). Although there was slight variability among cell lines, there was no significant difference in the expression of RPE-specific proteins among NOR and MTK iPSC-RPE cells (Fig. 1K, Supplemental Fig. S5B). Expression of CLAUDIN19, a major claudin in the tight junctions of the RPE (Peng et al., 2011), was also detected in both NOR and MTK iPSC-RPE (Supplemental Fig. S4L and M). Therefore, we interpreted that the maturity of the iPSC-RPE cells was similar between NOR and MTK.

Next, we examined the expression of *MERTK* in the iPSC-RPE cells. *MERTK* mRNA expression was detected in both NOR and MTK iPSC-RPE cells, and there was no significant difference in the mRNA levels of *MERTK* between MTK iPSC-RPE cells and NOR iPSC-RPE cells (Fig. 1L). Interestingly, *MERTK* protein was detected in NOR iPSC-RPE cells, while full or truncated *MERTK* protein was not detected in MTK iPSC-RPE cells (Fig. 1M and N, Supplemental Fig. S5C and D).

3.4. Proliferation of iPSC-RPE Progenitor Cells

When differentiated cells start expressing pigments and show polygonal appearance, the cells still have proliferative ability, and we defined them as “RPE-progenitor cells” To evaluate the difference in degeneration or cell death in differentiated iPSC-RPE cells between normal and MTK groups, we evaluated the state of cell proliferation using RPE-progenitor cells (Fig. 2). The proliferation ratio of iPSC-RPE progenitor cells was similar between the NOR and MTK groups.

3.5. Phagocytosis in iPSC-RPE Cells

Important functions of RPE cells are phagocytosis and the recycling of discs of the outer segments of photoreceptors (POS). To examine the function of the iPSC-RPE cells, we evaluated the internalization of fluorescein-5-isothiocyanate (FITC)-labeled beads on NOR and MTK iPSC-RPE cells. Fluorescence microscopy showed that there was no significant difference in FITC bead-positive cells in the MTK iPSC-RPE population compared to the NOR iPSC-RPE population (Fig. 3). Orthogonal representations of x-z and y-z cross-sections of x-y confocal stacks of the NOR and MTK iPSC-RPE cells showed internalization of FITC-labeled beads (Fig. 3C, G, K, O, S). Flow cytometry analysis confirmed that there was no significant difference ($P = 0.06$) between the percentage of FITC-positive, bead-internalized MTK iPSC-RPE cells (MTK1; 26.2 ± 1.2 %, MTK2; 33.3 ± 2.6 %; mean $\pm$ standard deviation) and NOR iPSC-RPE cells (NOR1, 28.6 ± 2.7 %; NOR2, 23.0 ± 2.4 %; NOR3, $16.2 \pm$

0.4 %; Fig. 3D, H, L, P, T, U, and Supplemental Fig. S6A-E).

We next evaluated the phagocytosis of POS to more physiologically reflect the RPE function. FITC-labeled POS was added to the iPSC-RPE cells. Fluorescence microscopic images showed that MTK iPSC-RPE cells phagocytosed fewer FITC-labeled POS than NOR iPSC-RPE cells (Fig. 4). Flow cytometric objective studies indicated that percentage of FITC-positive MTK iPSC-RPE cells (MTK1, 9.3 ± 1.4 %; MTK2, 21.7 ± 2.7 %) was significantly lower ($P = 0.046$) than that of NOR iPSC-RPE cells (NOR1, 48.2 ± 1.7 %; NOR2, 16.8 ± 1.2 %; NOR3, 20.0 ± 1.5 %; Fig. 4C, F, I, L, O, P, and Supplemental Fig. S6F-J). These data indicated that phagocytosis of POS in MTK iPSC-RPE cells was reduced compared with that in NOR iPSC-RPE cells.

To confirm these findings, we analyzed the amount of a POS-derived protein, rhodopsin, in iPSC-RPE cells fed with POS. Western blot analysis indicated that rhodopsin was detected in NOR iPSC-RPE cells challenged with POS, whereas it was barely detected in MTK iPSC-RPE cells with POS (Fig. 4Q). Protein levels of rhodopsin, or relative intensity of rhodopsin/GAPDH, in MTK iPSC-RPE cells co-cultured with POS (MTK1, 0.49 ± 0.15 ; MTK2, 0.54 ± 0.20) was significantly lower ($P = 0.016$) than that in NOR iPSC-RPE cells (NOR1, 0.77 ± 0.14 ; NOR2, 0.96 ± 0.12 ; NOR3, 0.65 ± 0.22 ; Fig. 4R). These findings indicated that phagocytosis of POS function was deteriorated in MTK iPSC-RPE cells compared with that in NOR iPSC-RPE cells.

4. Discussion

In the current study, we generated human iPSC-RPE cells derived from patients

1 with RP carrying *MERTK* mutations (sometimes classified as atypical RP
2 because of early macular involvement) and successfully established a human *in*
3 *vitro* model of retinal degeneration with *MERTK* mutation. In this model,
4 significant morphological changes in the diseased iPSC-RPE cells compared
5 with normal iPSC-RPE cells were not detected. Cytoskeleton staining suggested
6 mild disturbances in polarity. In addition, regarding the functional properties, the
7 ability of the diseased iPSC-RPE cells to phagocytose POS was lower than that
8 of normal iPSC-RPE cells.

9 We observed defective phagocytosis in the diseased iPSC-RPE cells using
10 internalized POS detected by flow cytometry or western blotting, which was
11 consistent with previous reports that considered fluorescent images of
12 phagocytosed iPSC-RPE cells from patients with other *MERTK* mutations
13 (Lukovic et al., 2015; Ramsden et al., 2017). In our study, phagocytosis of
14 artificial beads did not deteriorate in diseased iPSC-RPE cells. This result was
15 likely because phagocytosis of the beads is a non-specific,
16 non-receptor-mediated function, as shown in previous reports (Carr et al., 2004,
17 Rejman et al., 2009). FITC-positive iPSC-RPE and rhodopsin protein were
18 detected in the MTK iPSC-RPE cells, as proper binding of POS and non-specific
19 POS internalization by *MERTK*-deficient cells have been reported (Steinberg et
20 al., 1977), although the concentrations were reduced.

21 *The MERTK* gene is composed of 19 coding exons with two
22 immunoglobulin-like C2-type (Ig-like) domains, two fibronectin type III (FN3)-like
23 domains, a transmembrane (TM) domain, and a tyrosine kinase domain (TK)
24 (Graham et al., 1994; Prasad et al., 2006). The TK domain contains

1 autophosphorylation sites that are required for protein activation. Deletion of A in
2 exon 2 of the *MERTK* gene at position c.225 causes a frameshift, resulting in a
3 stop codon at p.77. The stop codon results in a truncated protein with a part of
4 the first Ig-like domain (Audo et al., 2018). A variant of c.370 C>T in exon 2 of
5 the gene resulted in a stop codon at p.123. The stop codon also results in a
6 truncated protein with part of the first Ig-like domain (Audo et al., 2018). We
7 showed that the expression level of *MERTK* mRNA in iPSC-RPE cells of
8 patients with MTK was comparable to that in the NOR cells. In contrast, *MERTK*
9 protein was hardly detected in iPSC-RPE cells of the patients with MTK in the
10 western blot analysis with an antibody, which should detect the truncated
11 *MERTK* protein (Fig. 1). In mice, *Mertk* is reported to be expressed in RPE cells
12 and is activated by the ligand protein S, which regulates RPE phagocytosis,
13 which is essential for engulfment of POS (Prasad et al., 2006). The patient's
14 truncated *MERTK* proteins are insufficient protein forms without TM and TK
15 domains; thus, these proteins should not be activated by the ligand and their
16 functions are not elicited. We suggest that RPE cells in RP patients with *MERTK*
17 mutations result in a loss of phagocytic function because of low levels of
18 expression, if any, and loss of functions of the mutated *MERTK* protein.
19 Phagocytosis of shed POS from photoreceptors is an important function of RPE
20 cells. The absence of phagocytosis by RPE cells results in toxic accumulation of
21 photoreceptor debris (Prasad et al., 2006), which would lead to the degeneration
22 of photoreceptors in the patients. Studies on chimeric rats using the RCS model
23 in the 1970s suggested that RPE dysfunction leads to primary degeneration of
24 photoreceptor cells (Bok and Hall, 1971; LaVail and Battele, 1975; Mullen and

LaVail, 1976; Edwards and Szamier, 1977). As iPSC-RPE from the patient did not degenerate during the culture period, degeneration of the RPE observed in the patients would be secondary to photoreceptor degeneration or *in vivo* environment around the RPE, as seen in previous studies in the RCS model.

The limitation of our study is that we analyzed iPSC-RPE cells derived from only two patients with *MERTK* mutations. The reason for this is based on retinitis pigmentosa, which is a rare disease, and there are few patients with retinitis pigmentosa caused by *MERTK* mutations. We indeed obtained informed consent from four patients with retinitis pigmentosa caused by *MERTK* mutations and isolated skin fibroblasts. However, we could not establish iPSC lines derived from these two patients. We would like to establish other cell lines to continue to analyze phagocytosis function in iPSC-RPE, if possible. Another limitation is that although we tested two patient-derived iPSC lines, we could not completely exclude any secondary effects on the difference in phagocytic activity between normal and *MERTK*-deficient populations. Reintroduction of the wild-type *MERTK* sequence to the MTK iPSC-RPE cells or introduction of the disease mutation(s) in the NOR iPSC-RPE cells should be performed in future experiments.

5. Conclusions

We successfully generated a human *in vitro* MTK model. In the diseased RPE, *MERTK* expression was barely detected, and phagocytosis deteriorated. The iPSC-RPE cells with *MERTK* mutations can be useful for screening therapeutics for incurable diseases.

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Figure legend

Fig. 1. Phenotypes of MTK patient-specific iPSC-RPE cells.

A-B) Bright-field micrographs of retinal pigment epithelium (RPE) cells differentiated for three months from induced pluripotent stem cells (iPSCs) established from a normal control (NOR) (A) and a patient with retinitis pigmentosa having Mer tyrosine kinase (*MERTK*) mutations (MTK) (B) iPSC-RPE cells. C-D) NOR iPSC-RPE cells (C) and MTK iPSC-RPE cells (D) are stained for zonula-occludens-1 (ZO-1, green) and DAPI (blue). Scale bar: 20 μ m in (A-D). E-H) Transmission electron microscope of NOR (E, G) and MTK (F, H) iPSC-RPE cells cultured for 5 months. Scale bar: 2 μ m (E, F), 50 nm (G, H). I-K) Expression levels of the RPE-specific markers, retinal pigment epithelium-specific protein 65 kDa (RPE65), cellular retinaldehyde-binding protein (CRALBP), Bestrophin 1 (BEST 1). I) mRNA levels of *RPE65*, *CRALBP*, and *BEST1* in NOR and MTK iPSC or iPSC-RPE cells were analyzed with qRT-PCR. The ratios of the mRNA level of each gene to that of *GAPDH* mRNA divided by the average ratio of NOR iPSC are shown as fold change. *RPE65*: *** P < 0.001, NOR iPSC vs. NOR iPSC-RPE and *** P < 0.001, MTK iPSC vs. MTK iPSC-RPE; *CRALBP*: *** P < 0.001, NOR iPSC vs. NOR iPSC-RPE and *** P < 0.001, MTK iPSC vs. MTK iPSC-RPE; *BEST1*: * P = 0.012, NOR iPSC vs. NOR iPSC-RPE and * P = 0.021, MTK iPSC vs. MTK iPSC-RPE. J) Western blot analysis of RPE65, CRALBP, and BEST1 in NOR and MTK iPSC-RPE cells. Complete scans of western blots are shown in Supplemental Fig. S7A and B. K) Statistical analysis of the RPE65, CRALBP, and BEST1 protein levels normalized with ACTIN expression. ns: not significant, unpaired t -test; n = 3. L-N) Expression levels of *MERTK* in NOR and MTK iPSC-RPE cells

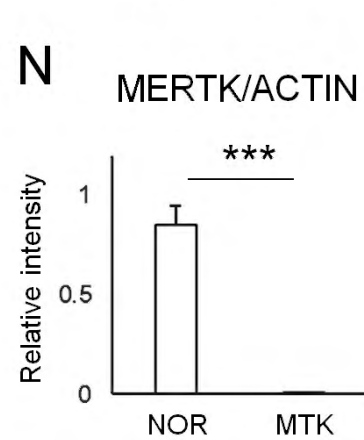
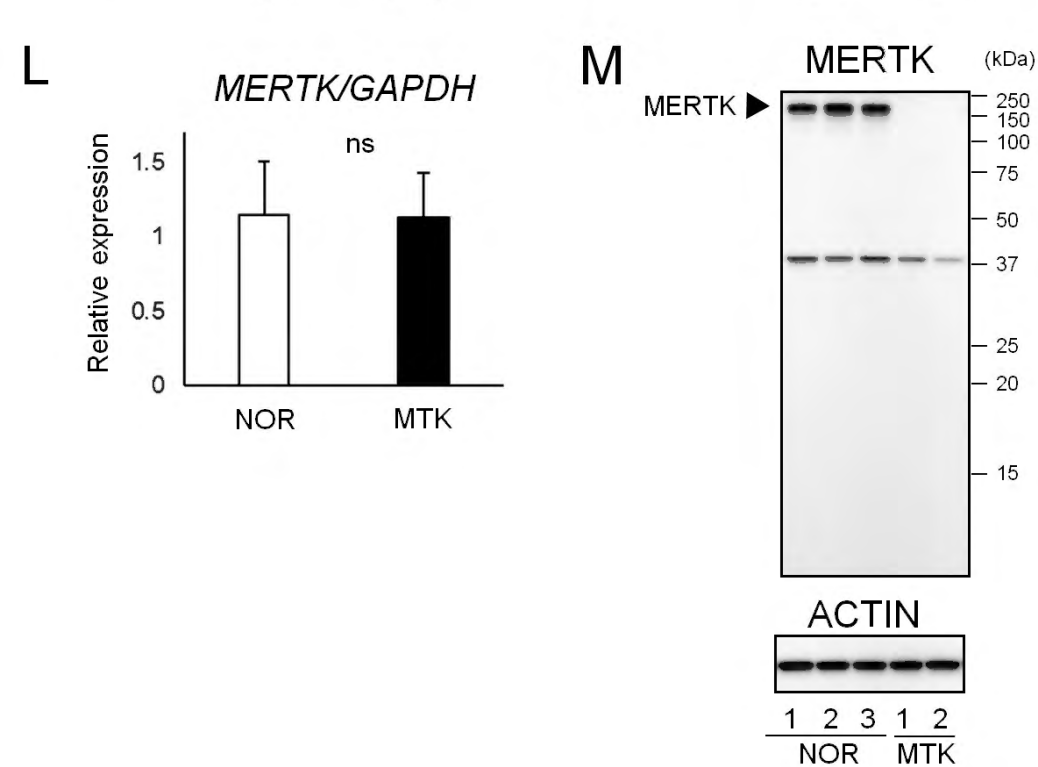
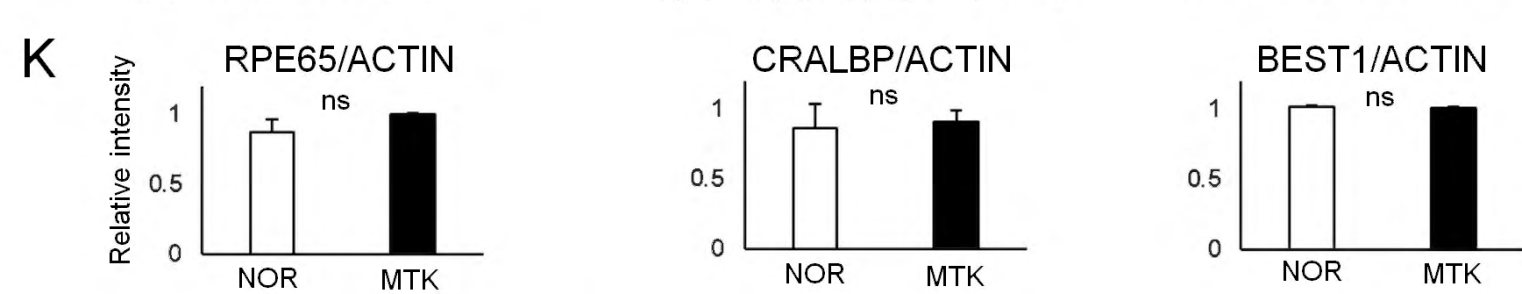
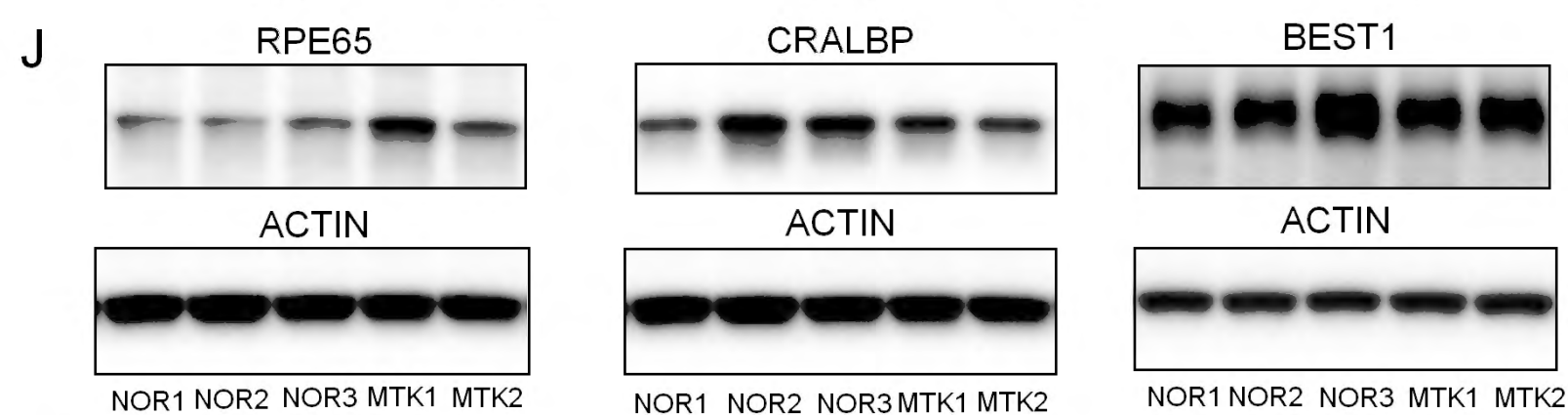
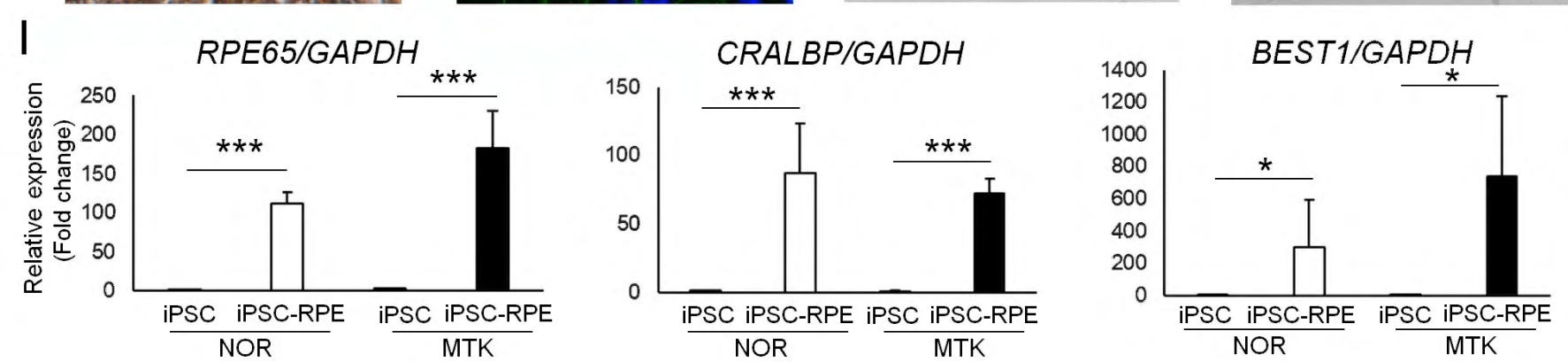
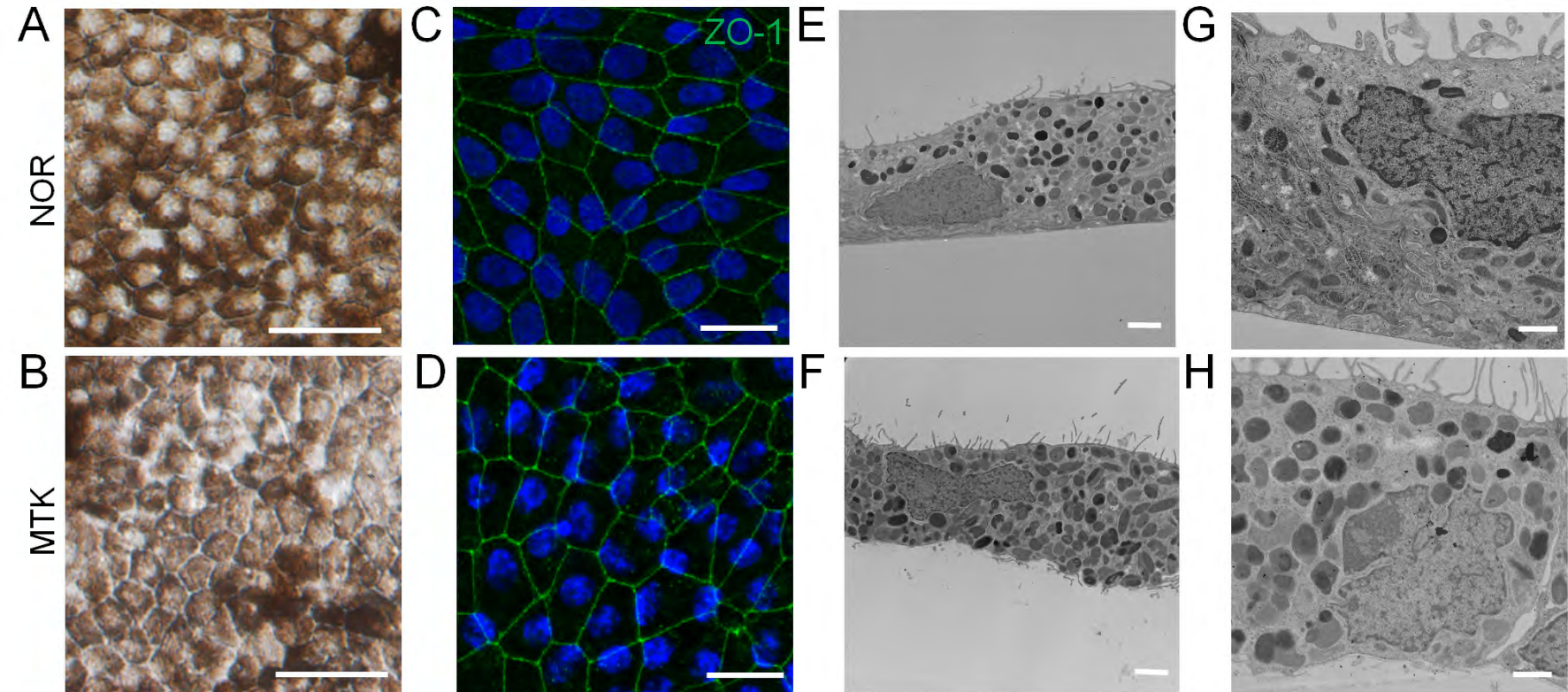
were analyzed. L) The ratio of *MERTK* mRNA level of gene expression to that of *GAPDH* mRNA measured with qRT-PCR is shown. $P = 0.734$, unpaired t -test; $n = 3$. M) Western blot analysis of MERTK in NOR and MTK iPSC-RPE cells. MERTK protein was detected in NOR-1 iPSC-RPE, NOR-2 iPSC-RPE, and NOR-3 iPSC-RPE. Truncated MERTK protein (estimated size: 14 or 22 kDa) was not detected. Western blot scans are shown in Supplemental Fig. S7C. N) Statistical analysis of the MERTK protein levels following normalization with ACTIN expression. $***P < 0.001$, NOR iPSC vs. NOR iPSC-RPE, unpaired t -test; $n = 3$. Error bars indicate standard deviation (SD).

Fig. 2. Evaluation of proliferation of iPSC-RPE progenitor cells. The relative increase of cell number stained with water-soluble tetrazolium salts three days after seeding (day 3) is shown. Measurements of optical density on day 3 were divided with those at day 1. $n = 4$ in each group. Error bars indicate SD.

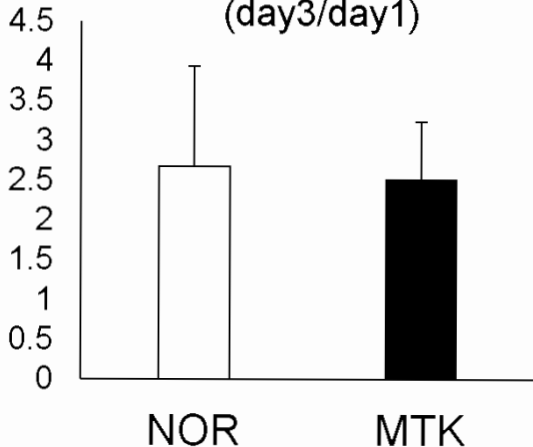
Fig. 3. Internalization of FITC-labeled beads in iPSC-RPE cells. A, B, E, F, I, J, M, N, Q, R) Images of Bright-field (A, E, I, M, Q) and fluorescence (B, F, J, N, R) microscope of NOR (NOR1, A, B; NOR2, E, F; NOR3, I, J) and MTK (MTK1, M, N; MTK2, Q, R) iPSC-RPE cells with Fluorescein-5-isothiocyanate (FITC)-labeled latex beads. Scale bar: 100 μm . C, G, K, O, S) Orthogonal representations of x-z and y-z cross-sections of the NOR iPSC-RPE and MTK iPSC-RPE 24 hours following the addition of FITC-labeled beads. Scale bar: 20 μm . D, H, L, P, T, U) Analysis of internalization of FITC-labeled beads by flow cytometry. U) The percentage of cells in which FITC-labeled beads were

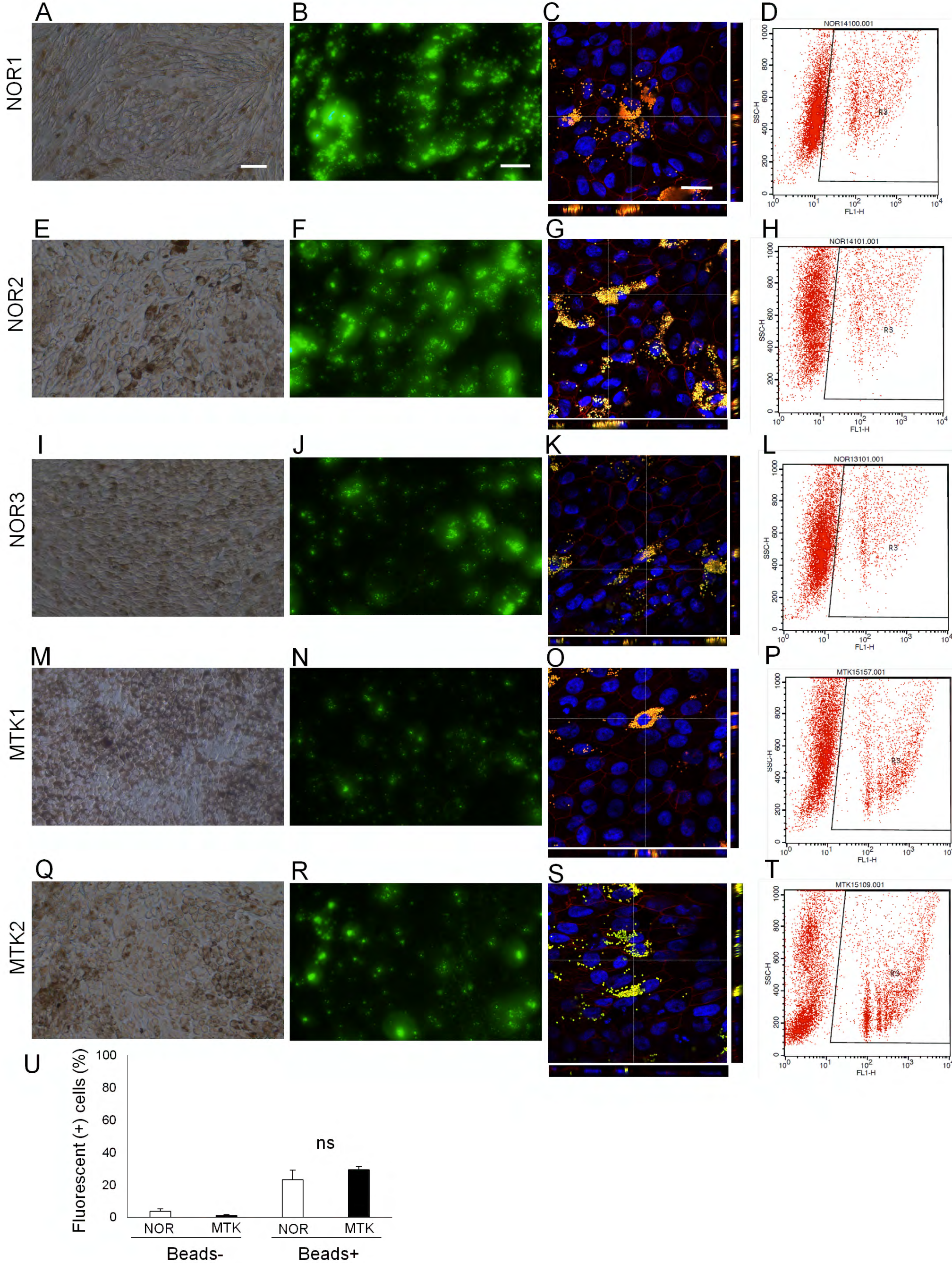
internalized (R3 area in D, H, L, P, T) per total cells was analyzed. $P = 0.06$; unpaired t -test; $n = 3$ for each group. Error bars indicate SD.

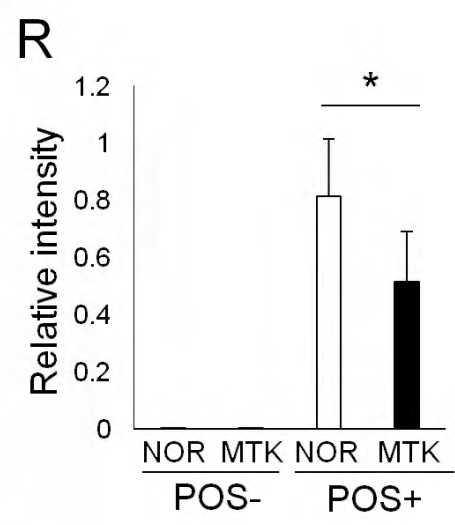
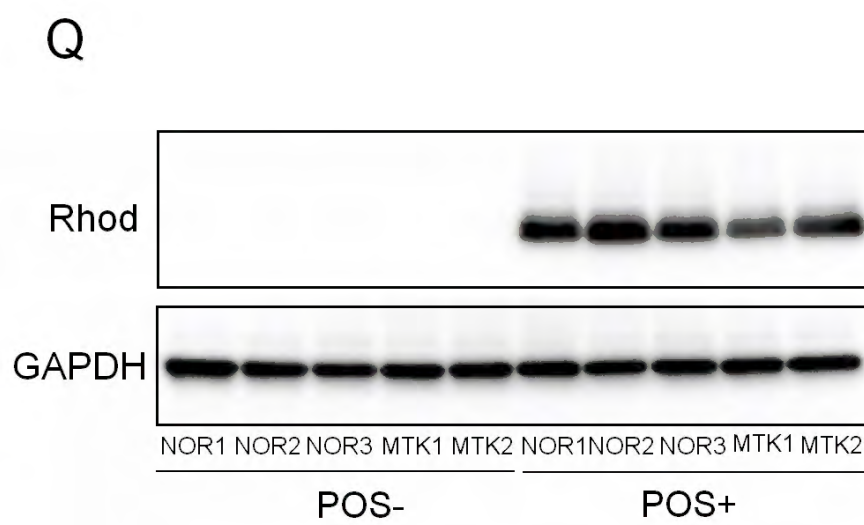
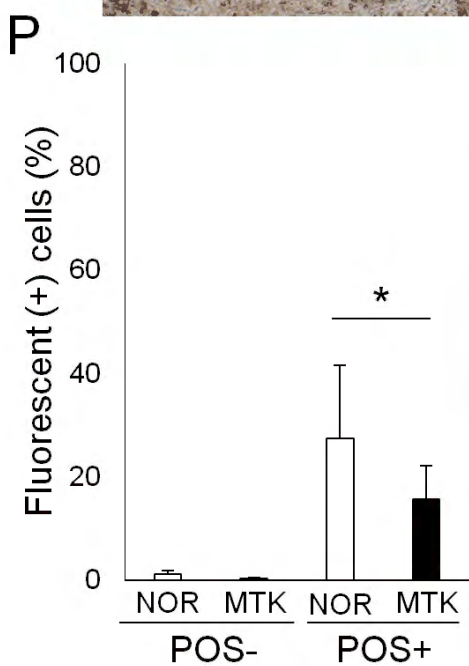
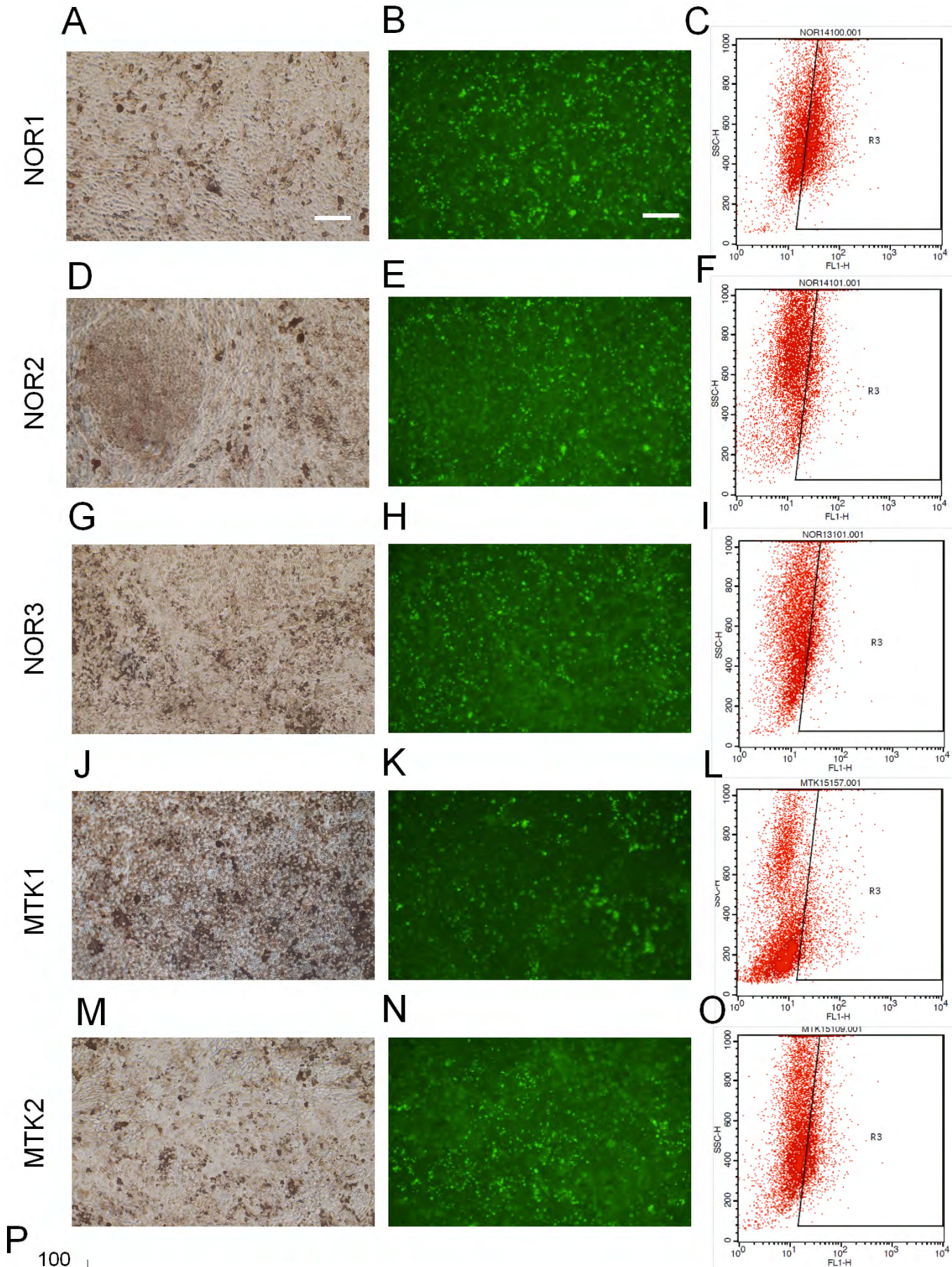
Fig. 4. Phagocytosis of POS in iPSC-RPE cells. A, B, D, E, G, H, J, K, M, N) Images of bright-field (A, D, G, J, M) or fluorescence (B, E, H, K, N) microscope of NOR (NOR1, A, B; NOR2, D, E; NOR3, G, H) and MTK (MTK1, J, K; MTK2, M, N) iPSC-RPE cells fed with FITC-labeled photoreceptor outer segment (POS). Scale bar: 100 μ m. C, F, I, L, O, P) Phagocytosis using FITC-labeled POS was analyzed by flow cytometry. P) The percentage of FITC-positive cells (R3 area in C, F, I, L, O) was significantly lower in MTK iPSC-RPE cells than in NOR iPSC-RPE cells. Q-R) Western blot analysis of rhodopsin protein in MTK and NOR iPSC-RPE cells fed with/without POS. Western blot scans are shown in Supplemental Fig. S8. R) Relative intensity of rhodopsin normalized with GAPDH in iPSC-RPE cells fed with/without POS. $*P = 0.047$, unpaired t -test; $n = 3$ for each group. Error bars indicate SD.



Ratio of proliferation (day3/day1)





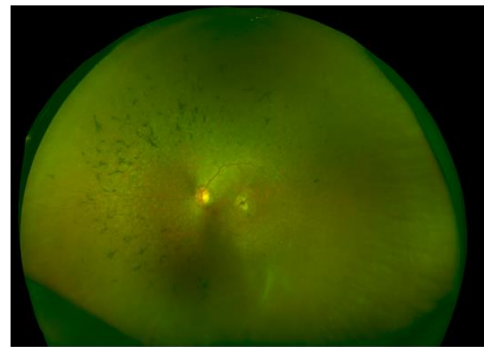
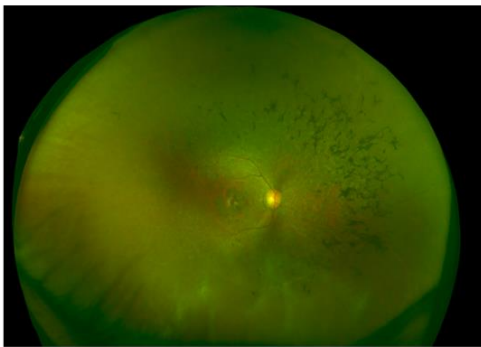


MTK1

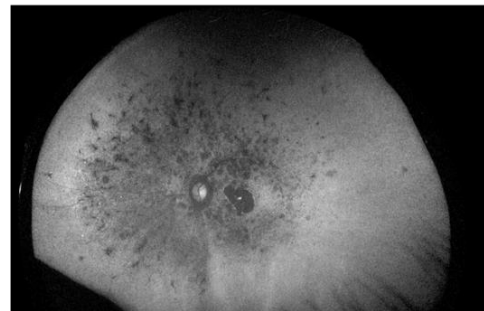
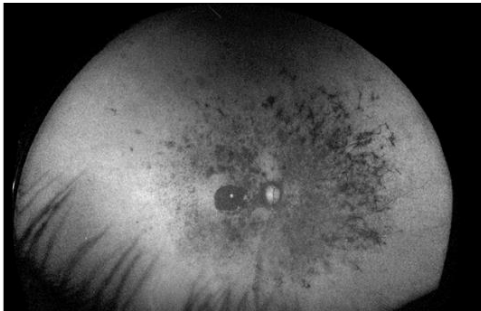
Right

Left

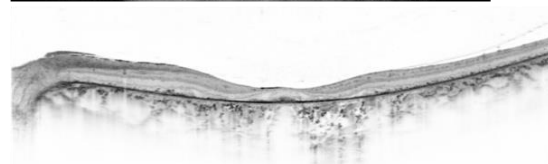
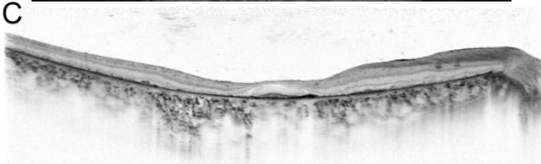
A



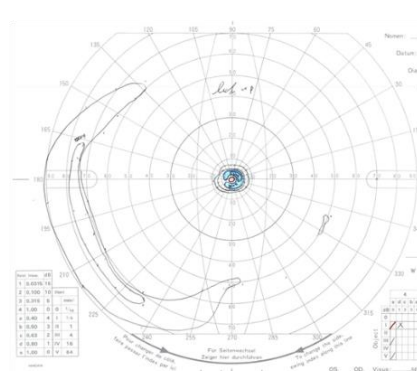
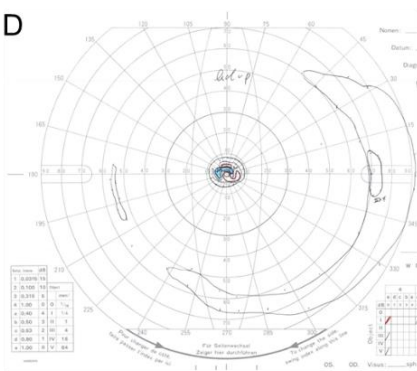
B



C

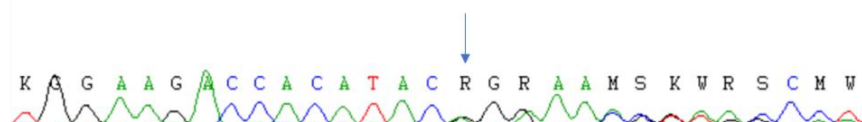


D

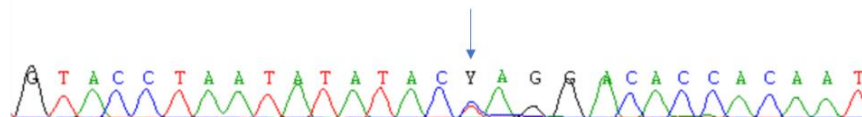


E

MERTK c.225delA
p.(Gly76Glufs*3)



MERTK c.370C>T
p.(Gln124*)



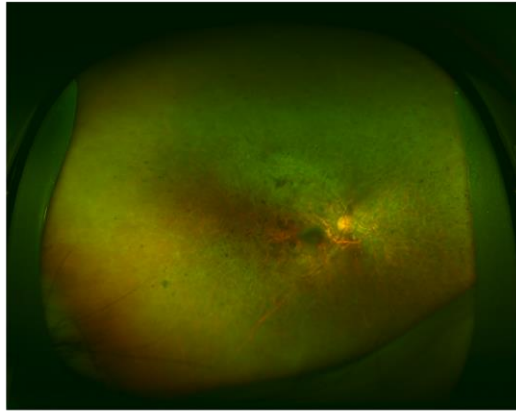
Supplemental Fig. S1. Clinical findings and the *MERTK* DNA sequence of patient MTK1, whose fibroblasts were used to establish iPSCs. A) Widefield Optos color images show narrow blood vessels and mild to widespread osteoclast pigmentation. B) Widefield Optos autofluorescence images showing diffuse RPE atrophy. C) Spectral-domain optical coherence tomography also showed an atrophic outer retina and RPE. D) The visual field test indicates concentric contraction of the visual field and detects paracentral scotomas in both eyes. E) Patient MTK1 is a compound heterozygote for *MERTK* mutations. The variants found in the patient were located in exon2. R, M, S, K, W, and Y are the mixed bases. R, A, or G. M, A, or C. S, G, or C. K, G, or T. W, A, or T. Y, C, or T.

MTK2

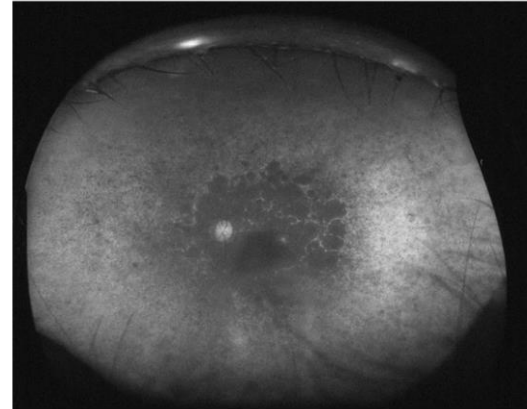
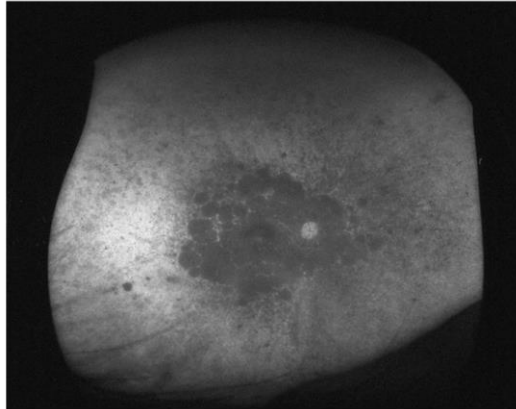
A

Right

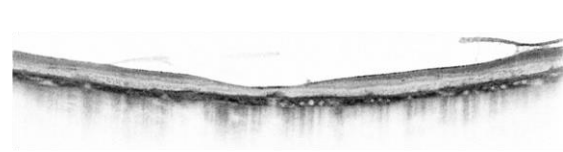
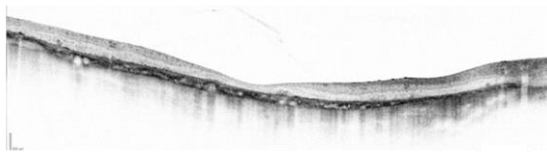
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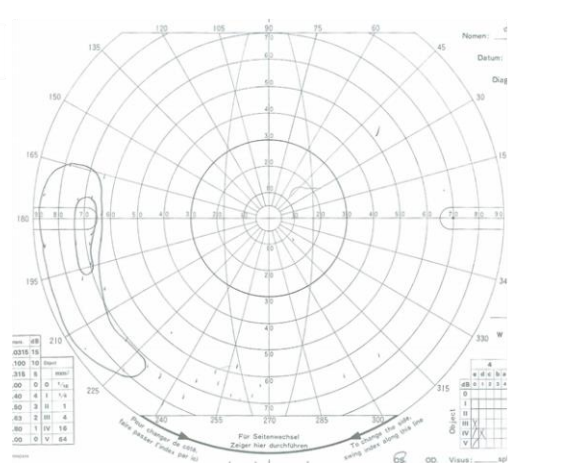
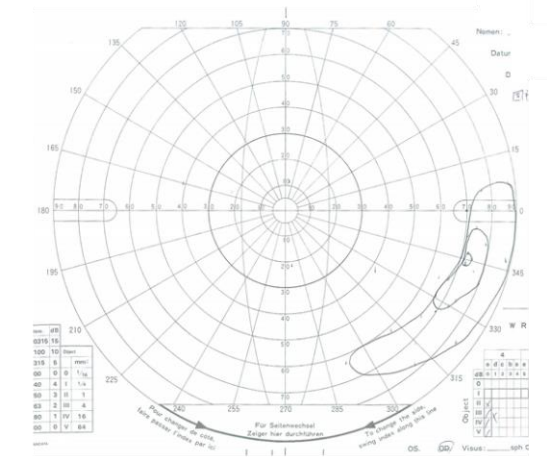
B



C



D

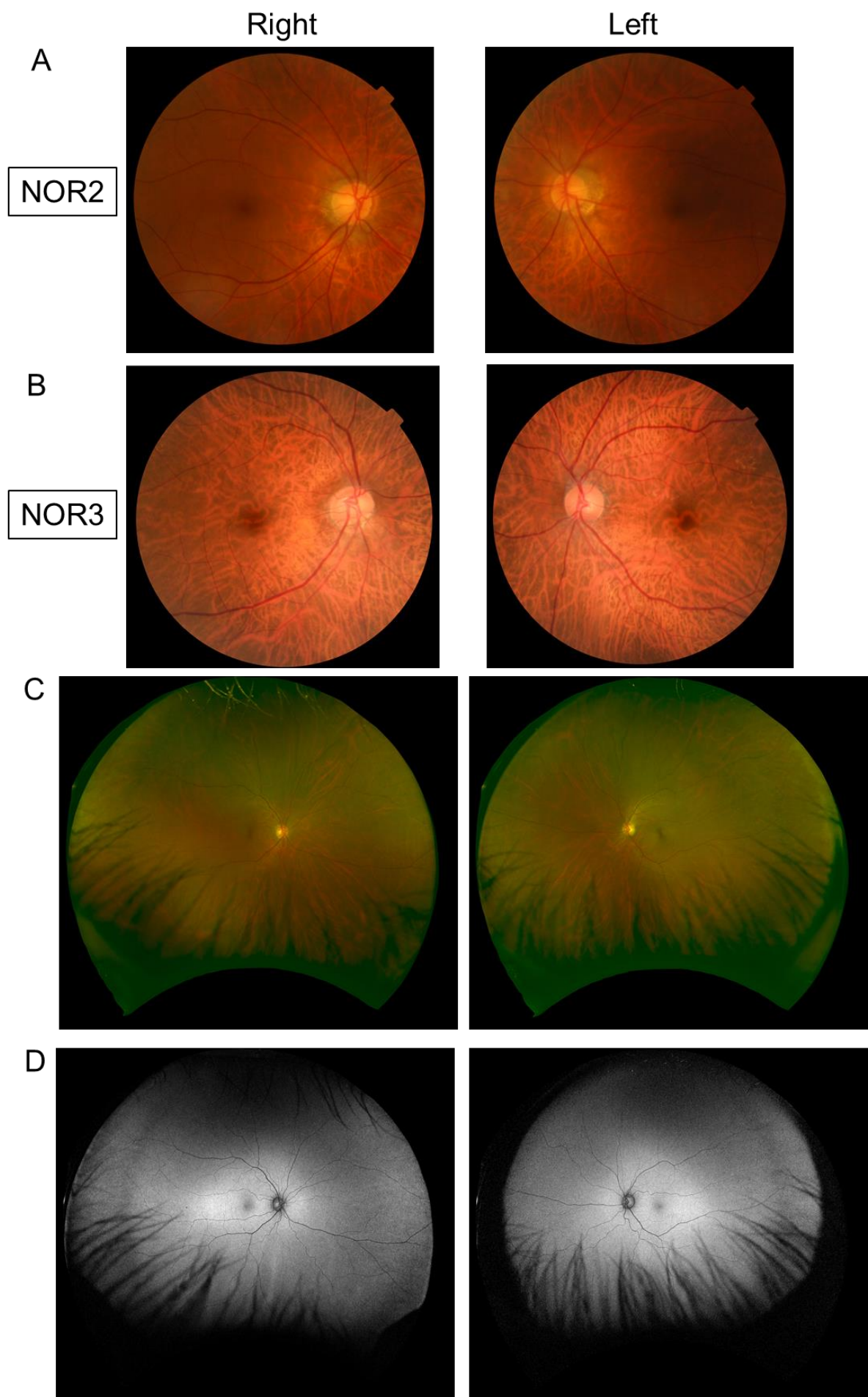


E

MERTK c.225delA
p.(Gly76Glufs*3)

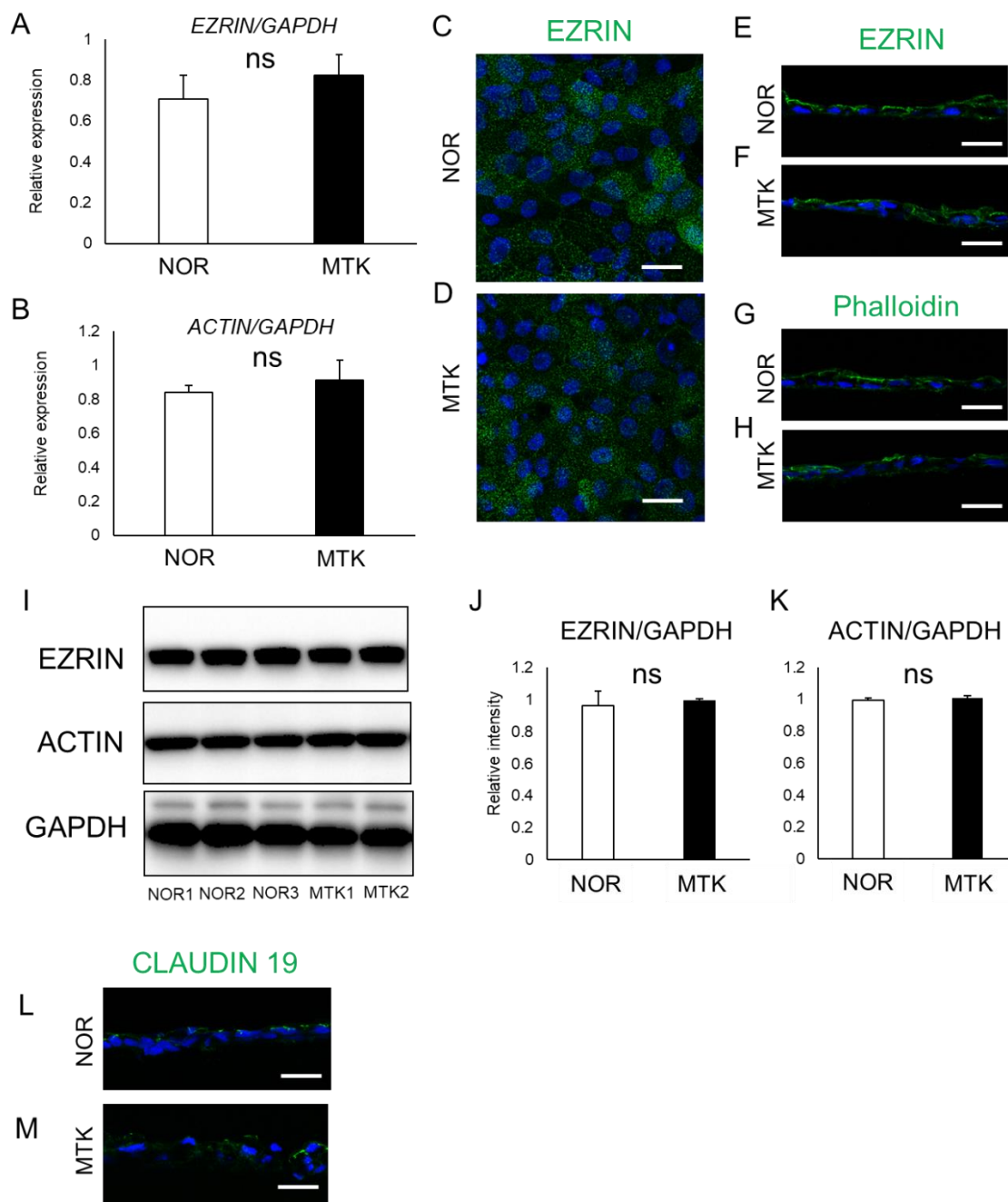


Supplemental Fig. S2. Clinical findings and the *MERTK* DNA sequence of patient MTK2, whose fibroblasts were used to establish iPSCs. A) Widefield Optos color images. B. Widefield Optos autofluorescence images C) Spectral-domain optical coherence tomography images D) The visual field test showed a central visual field defect and only a residual peripheral visual field in both eyes. E) Patient MTK2 is a homozygote for a *MERTK* mutation. The variants found in the patient were located in exon2.

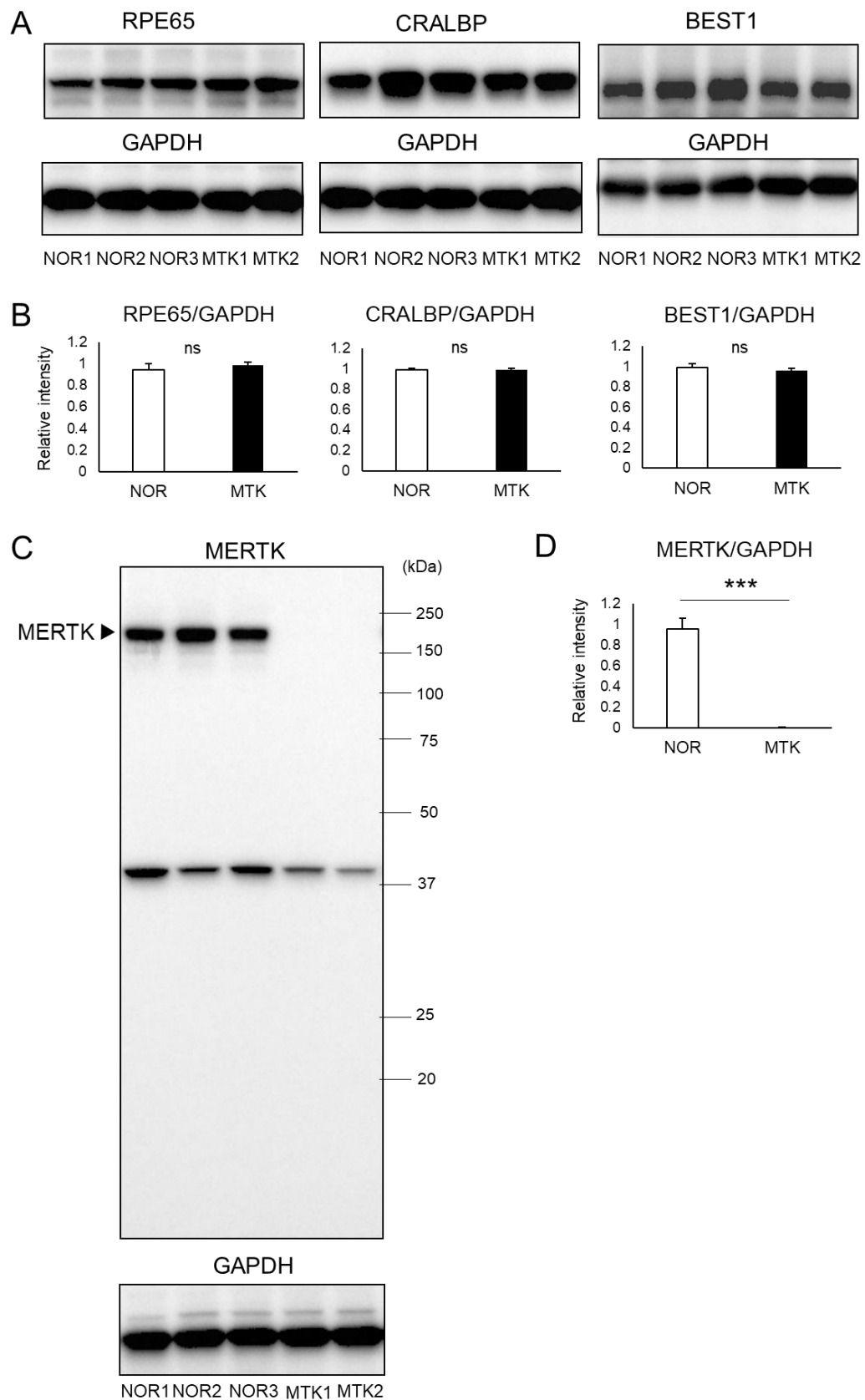


Supplemental Fig. S3. Clinical findings of NOR2 and NOR3 and normal fundus.

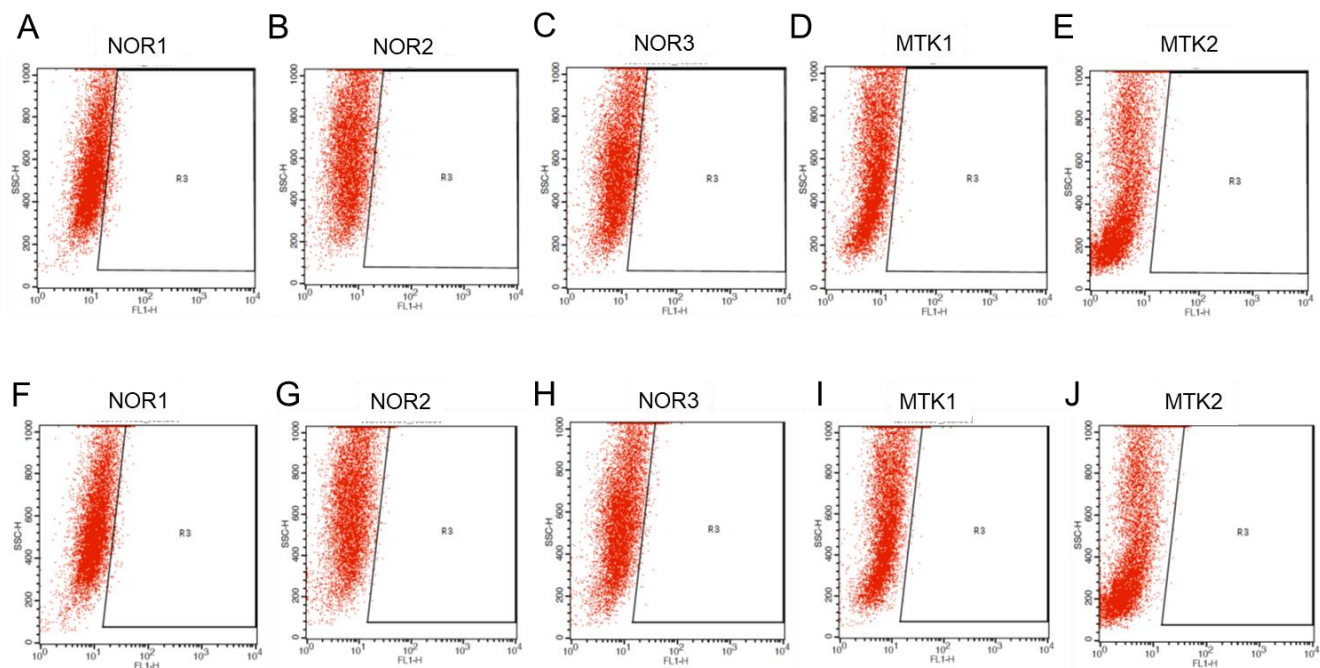
A, B) Color fundus images show no abnormalities in NOR2 (A) or NOR3 (B). C, D) Widefield Optos color images (C) and widefield Optos autofluorescence images of normal eyes for reference.



Supplemental Fig. S4. Expression of EZRIN, ACTIN, and CLAUDIN 19 A, B) mRNA levels of *EZRIN* and *ACTIN* in NOR and MTK iPSC-RPE cells were analyzed by qRT-PCR. The ratios of the mRNA level of each gene to that of *GAPDH* mRNA are shown. (A) *EZRIN*: $P = 0.082$, NOR iPSC-RPE vs. MTK iPSC-RPE; (B) *ACTIN*, $P = 0.224$; NOR iPSC-RPE vs. MTK iPSC-RPE. ns: not significant. Unpaired t -test; $n = 3$. Error bars indicate SD. The primers used were as follows: *EZRIN*, 5'CATCACTGAGGCAGAAGAAC3' and 5'TGTCATTGTGGGTCCTCTTATTC3'; *ACTIN*, 5'CAAAGACCTGTACGCCAACAC3' and 3'CTCCTGCTTGCTGATCCACAT5'; Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'ACCACCAACTGCTTAGC3' and 5'GGCATGGACTGTGGTCATGAG3'. *GAPDH* was used as an internal housekeeping gene expression standard. C-H) NOR iPSC-RPE cells (C, E, G) and MTK iPSC-RPE cells (D, F, H) were stained for EZRIN (green, C-F) or phalloidin (green, G, H) and DAPI (blue). I) Western blot analysis of EZRIN, ACTIN, and GAPDH in NOR and MTK iPSC-RPE cells. Complete scans of western blots are shown in Supplementary Fig. 9A. J, K) Statistical analysis of EZRIN and ACTIN protein levels normalized to GAPDH expression. ns: not significant. Unpaired t -test; $n = 3$. Error bars indicate SD. L, M) NOR iPSC-RPE cells (L) and MTK iPSC-RPE cells (M) were stained for CLAUDIN 19 (green) and DAPI (blue). NOR iPSC-RPE cells (E, G, L) and MTK iPSC-RPE cells (F, H, M) were cultured on a porous membrane, and the cryosections were used for staining. Scale bar: 20 μ m for C-H, L, and M.

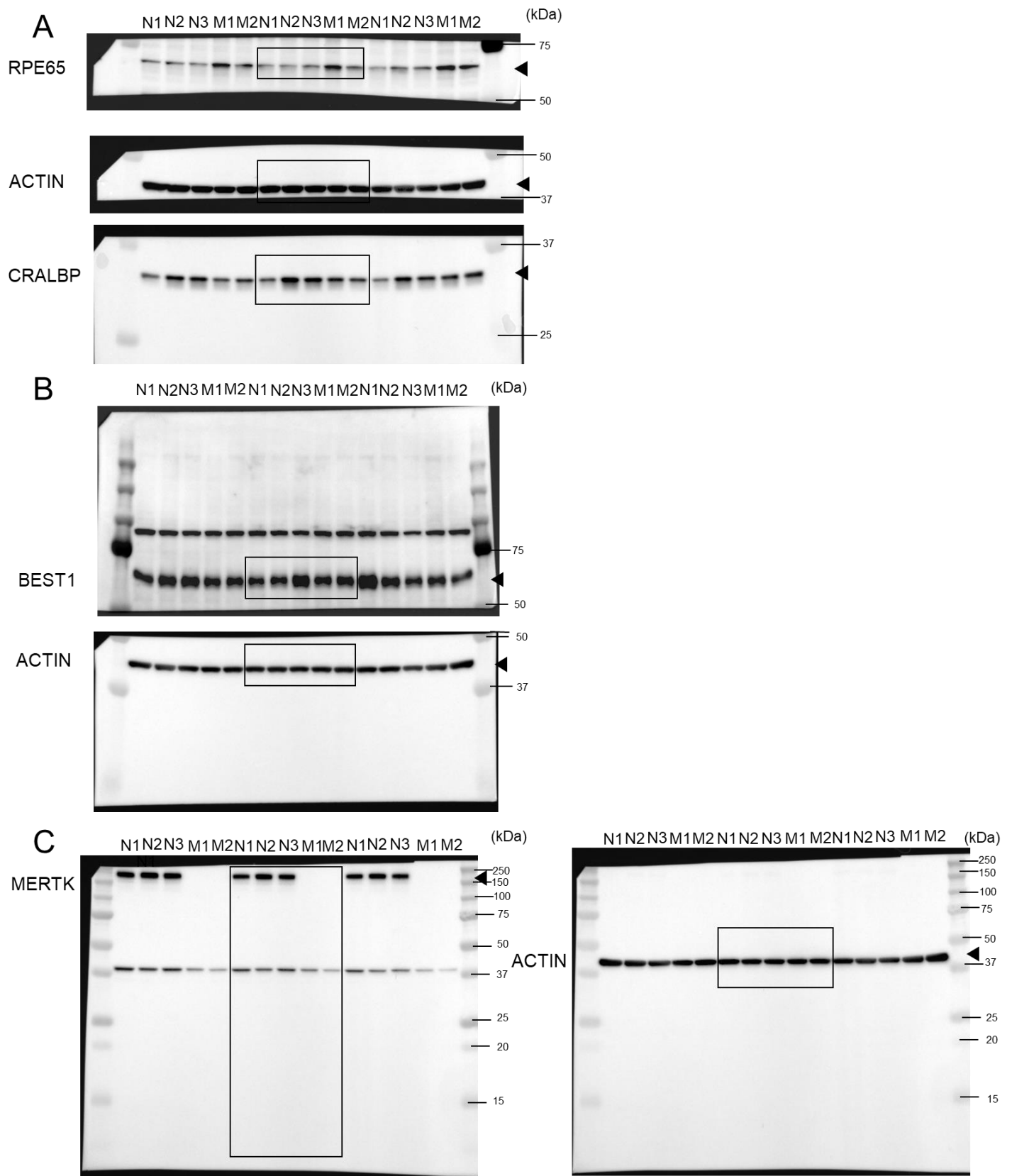


Supplemental Fig. S5. Western blot analysis of RPE65, CRALBP, BEST1, and MERTK expression in NOR and MTK iPSC-RPE cells. Statistical analysis of RPE65, CRALBP, BEST1, and MERTK protein levels was performed after normalization with GAPDH expression. ns: not significant. *** $P < 0.001$. Unpaired t -test; $n = 3$. Error bars indicate SD.



Supplemental Fig. S6. Negative controls of FACS experiments in Fig. 3 and Fig. 4.

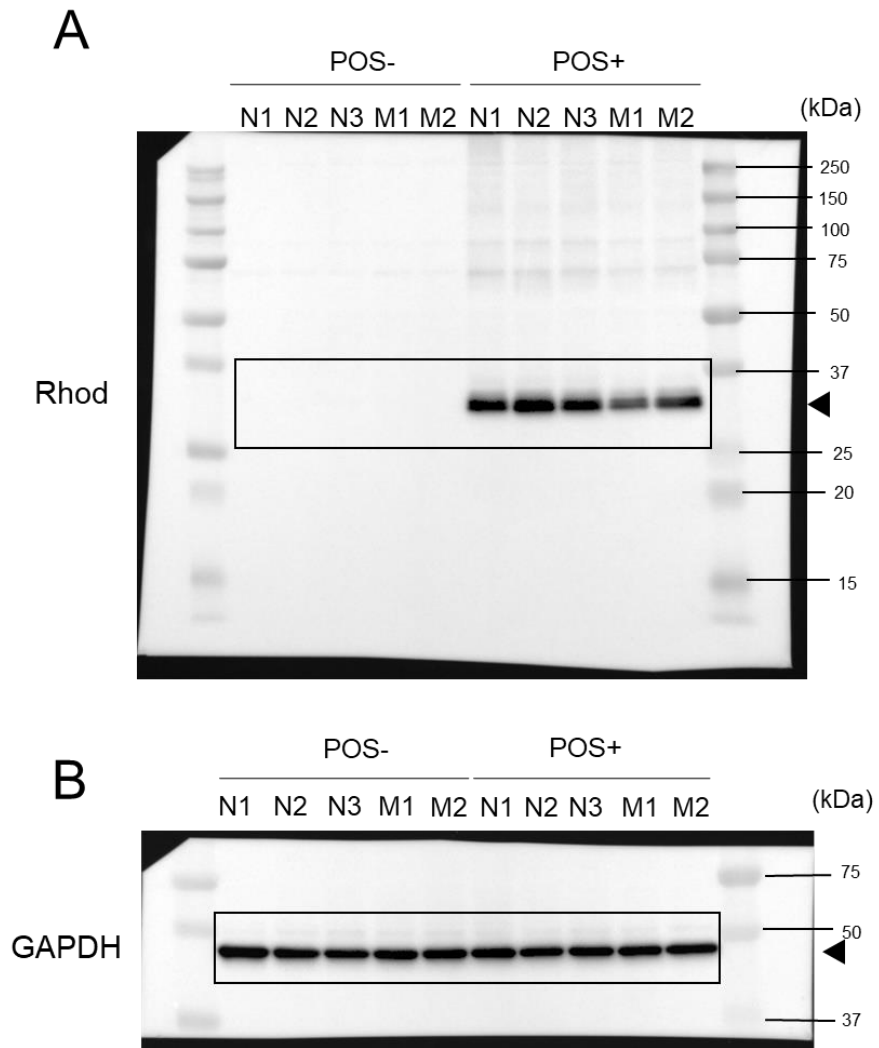
A-E) FITC-labeled beads (-) on NOR and MTK iPS-RPE cells: NOR1 (A), NOR2 (B), NOR3 (C), MTK1 (D), and MTK2 (E). F-J) FITC-labeled POS (-) on NOR and MTK iPS-RPE cells: NOR1 (F), NOR2 (G), NOR3 (H), MTK1 (I), and MTK2 (J).



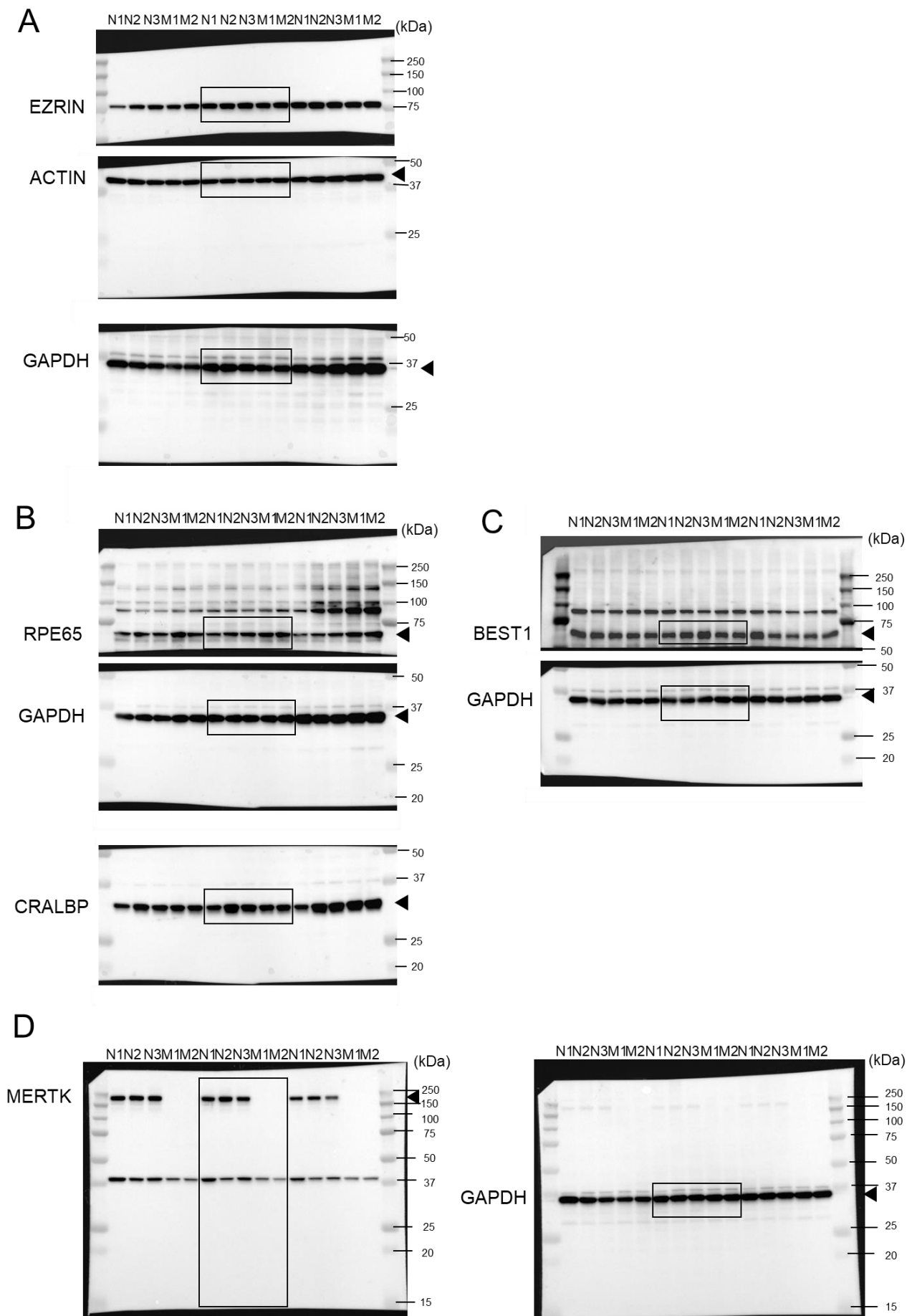
Supplemental Fig. S7. Complete scans of the western blots presented in Fig. 1J and 1M

RPE65 (A), CRALBP (A), BEST1 (B), and MERTK (C) were analyzed, and ACTIN was used as a loading control.

N: NOR iPSC-RPE; M: MTK-iPSC-RPE.



Supplemental Fig. S8. Complete scans of western blots are shown in Fig. 4Q. Rhodopsin (Rhod) intensity was analyzed and GAPDH was used as a loading control. N: NOR iPSC-RPE; M: MTK-iPSC-RPE.



Supplemental Fig. S9. Complete scans of western blots are shown in Supplemental Fig. S4I, S5A, S5C. EZRIN, ACTIN (A), RPE65, CRALBP (B), BEST1 (C), and MERTK (D) expressions were analyzed, and GAPDH was used as a loading control. N: NOR iPSC-RPE; M: MTK-iPSC-RPE.