Supplementary Information for

The Interplay of Environmental Luminance and Genetics in the Retinal Dystrophy Induced by the Dominant RPE65 Mutation

Wenjing Wu¹, Yusuke Takahashi¹,², Henry Younghwa Shin¹, Xiang Ma¹, Gennadiy Moiseyev¹* and Jian-Xing Ma¹,²*

¹Department of Physiology, ²Harold Hamm Diabetes Center, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

Corresponding authors

Jian-Xing Ma & Gennadiy Moiseyev, University of Oklahoma Health Sciences Center, BSEB 328B, 941 Stanton L. Young Blvd, Oklahoma City, OK 73104, USA;
Email: Jian-xing-ma@ouhsc.edu
Email: Gennadiy-Moiseyev@ouhsc.edu
Phone number: 1-405-271-4372

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| Antibody       | Host     | Dilution          | Company                | Application       |
|---------------|----------|-------------------|------------------------|-------------------|
| ZO1           | Rat      | IFC 1:200         | Millipore MABT11       | IFC               |
| Calnexin      | Rabbit   | WB 1:1000         | Abcam Ab10286          | WB                |
| Fibrillarin   | Rabbit   | WB 1:1000         | Cell Signaling 2639    | WB                |
| LRAT          | Rabbit   | WB 1:1000         | Abcam ab137034         | WB                |
| RPE65         | Mouse    | WB 1:1000         | Abcam ab13826          | IFC and WB       |
| Crbp1         | Rabbit   | WB 1:1000         | Santa Cruz sc30106     | WB                |
| RDH5          | Mouse    | WB 1:1000         | NOVUS NBP2-15097       | WB                |
| GFAP          | Mouse    | WB 1:1000         | Sigma G-3893           | IFC and WB       |
| β-Actin-HRP   | Mouse    | WB 1:2000         | Santa Cruz SC-1616HRP  | WB                |
| Anti-Mouse-HRP| Horse    | WB 1:4000         | Vector Laboratory PI-2000 | WB            |
| Anti-Rabbit-HRP| Goat   | WB 1:4000         | Vector Laboratory PI-1000 | WB            |
| Anti-mouse-Alexa Fluor-488 | Donkey | IFC 1:400       | Invitrogen A32766      | IFC               |
| Anti-Rabbit-Alexa Fluor-555 | Goat | IFC 1:400       | Invitrogen A32794      | IFC               |
| Anti-Mouse-Alexa Fluor-568 | Goat | IFC 1:400       | Invitrogen A11004      | IFC               |

WB: Western Blot, IFC: Immunofluorescence-chemistry
Figure S1. Simplified schematic on the mice maintained in DayL. (A) Mice were housed under regular vivarium light until ~3-month old, and then mice were randomly segregated into groups that were maintained 12 hr light/12 hr dark cycle with regular housing or housed in the light shelf. ERG was performed to determine retinal function changes between the mouse groups at the indicated month. (B) Photographs of the home-made light shelf for maintaining the mice under higher light intensity.
Figure S2. Impact of 2000 lux luminance on retinal function in wild-type mice at the ages of 9, 12, 15, and 18 months. (A) Representative dark-adapted (scotopic-top) and light-adapted (photopic-bottom) ERG wave forms. At each tested age the a- (B) and b-wave (C & D) amplitudes between the WT mice maintained in either DimL (~100 lux) or DayL (~2000 lux) were similar. However, we did observe a global age-dependent ERG decline in all the mice groups. The stimulus intensity for scotopic ERG was 4.0 cd.s/m², for photopic ERG was at 10 cd.s/m² with a rod saturating background luminance of 10 cd/m². Data are presented as means ± SEM. Unpaired t-test with Tukey’s post-hoc comparison n = 8 mice/group.
Figure S3. Assessment of retinal function and structure in the 18-month-old wild-type mice maintained under DimL and DayL. (A) Representative scotopic ERG wave forms at increasing luminance intensities and a-wave and b-wave amplitudes (mean ± SEM) (B). (C) Representative photopic ERG wave forms recorded at two light intensities (3 and 10 cd.s/m²) and b-wave amplitudes (mean ± SEM) (D). (E) Representative fundus photographs in the DimL or DayL raised wild-type mice (E, a & b). H&E staining retina cross sections of the DimL or DayL raised wild-type mice (c, d, e & f). Both DimL and DayL-exposed WT mice at 18 months of age showed similar ocular histological features. GCL, ganglion cell layer, IPL, inner plexiform layer, INL, inner nuclear layer, OPL, outer plexiform layer, PR, photoreceptor, RPE, retinal pigmented epithelium. Scale bar 200 µm (black), 40 µm (White). Data are presented as mean ± SEM. Unpaired t-test with Tukey’s post-hoc comparison, n = 8 mice/group.
Figure S4. Under daylight exposure, 6-month-old WT/KI eye exhibited significantly lower 11-cis-retinal chromophore levels. (A) & (C) Representative HPLC chromatograms show the separation of retinoids extracted from the eyes of 6-month-old dim-light (DimL) (A) and daylight (DayL) (C) exposed mice of indicated genotypes. Peaks 1, Retinyl ester; 2, syn-11-cis retinal oxime; 3, syn-all-trans retinal oxime; 4, anti-11-cis retinal oxime; 5, anti-all-trans retinal oxime. (B) & (D) Quantification of 11-cis-retinal chromophore content in the dim-light exposed (B) or daylight exposed (D) mice eyes. Peak 2 (blue) was used for quantification (○, WT/WT; □, WT/KO; Δ, WT/KI). (E) Representative Western blot analysis of opsin content in whole eye extracts of six-month-old mice indicated genotypes under dim-light or daylight exposure. (F) Quantification of opsin content in mouse eyes normalized to actin levels. Each point represents data from a single mouse. n = 4. Data are presented as mean ± SEM. **P < 0.01, ns non-significant in One-way ANOVA with Tukey's post-hoc comparison.
Figure S5. The WT/KI mice retina exhibited GFAP upregulation when compared to the WT/KO retina at 18 months of age. (A) Representative IFC shows fluorescent labeling of RPE65 (Red) and GFAP (Green). (B) Representative Western blot analysis of GFAP in retina extracts from 18-month-old WT/KI and WT/KO exposed to dim-light (DimL) or day-light (DayL) intensity. (C) Quantification of GFAP desitometry of GFAP bands in Western blot in (B) and normalized to actin levels. n = 4. Data are given as mean ± SEM. In One-way ANOVA with Tukey's post-hoc comparison. Inlay- shows integrity of the retina section. RPE; retinal pigmented epithelium, IS/OS; inner segment and outer segment layer of photoreceptor cells, ONL; outer nuclear layer of photoreceptor cells, OPL; outer plexiform layer, INL; inner nuclear layer, IPL; inner plexiform layer, GCL; ganglion cell layer.
Materials and Methods

Light cubicle

The light cubicle is equipped with adjustable LED light panel that operated in 12 h light/12 h dark cycles. The light intensity was confirmed by a light meter (Extech Instruments, Nashua, NH) placed in the mouse cage. A fully assembled mock cage (food tray and water bottle included) was placed in the light cubicle, and the light intensity was measured both inside and outside the cage. The light intensity inside the amber cage was adjusted between 1,800-2,000 lux. Temperature in the shelf during the light exposure was constantly monitored and maintained between 22-24°C by an automated temperature sensor that turns on a fan when above the set threshold (SI Appendix, Fig S1).

Electroretinography (ERG)

Briefly, mice were dark adapted overnight (≥ 18 hr). Under dim red light, mice were anesthetized by an intraperitoneal injection of the cocktail (3 μL/g body weight) consisting of 40 mg/ml Ketamine and 3 mg/ml xylazine diluted in saline. The pupils were dilated with 1% cyclopentolate hydrochloride ophthalmic solution (Cyclogyl, Fort Worth, TX), and hypromellose ophthalmic demulcent solution (2.5%; Gonak, Lake Forest, IL) was applied to keep the corneas hydrated. Body temperature was maintained at 37°C with a heating pad. ERG responses were recorded from both eyes using gold wire loop electrodes; the reference electrode was placed into the right cheek, and the ground electrode was inserted subcutaneously into the tail. Scotopic ERGs were performed using light flashes of intensities ranging from -2.4 to 1.6 log cd.s/m². For each intensity, at sufficient intervals (60 to 300s), mice were allowed to readapt to darkness, as to recover from any photobleaching effects. The photopic ERG recordings were performed at 5-minute-intervals in light adaptation at 10 cd.s/m². For photopic ERG, the cone response was measured at two different light intensities (3 and 10 cd.s/m²) in the presence of rod-desensitizing background white light of 10 cd/m², b-wave amplitude was obtained by averaging 15 recordings per luminance intensity. For photobleaching recovery experiments, mice were dark-adapted overnight and then subjected to visual pigment bleach with the background light of a Ganzfeld chamber (1000 cd/m²) for 2 min. Then, a single-flash ERG at 10 cd.s/m² was used to monitor recovery of a-wave amplitude every 5 min for 60 min. The rate of recovery was analyzed by plotting the a-wave amplitude against the initial dark-adapted flash amplitude to obtain a percentage recovery curve.

SD-OCT

Images were captured with the rectangular scan at 1000 A-scans per B scan, and 200 B-scans per frame using image acquisition software provided by the vendor. Total retinal thickness was recorded 500 μm from the center of the optic nerve and averaged at 3 × 3 grid positions and analyzed using the automated InVivo Vue driver software (Bioptigen). OCT measurements of each experimental condition were taken by researchers blinded to the strain information.

RPE65 Isomerase Activity Assay

Briefly, after overnight dark adaptation, mice were euthanized, and their eyes were enucleated. The anterior portion of the globe and the retina were removed, and the remaining eyecup was used for the
assay. Each eyecup was homogenized in a glass grinder, and 125 µg of resulting RPE homogenate was brought to a total volume of 200 µL of the reaction buffer containing 0.2 µM of all-trans [11,12-3H]-retinol radioactively labeled substrate. Reaction buffer composition: (10 mM 1,3-bis-[Tris(hydroxymethyl)-methyl amino propane] (pH 8.0), and 100 mM NaCl), 1% (w/v) bovine serum albumin, and 25 µM cellular retinaldehyde-binding protein. Each sample was incubated at 37°C for 2 hr in dark, and total retinoids were extracted with 300 µL of methanol and 300 µL hexane, and upper layer phase containing the retinoids was collected and analyzed by HPLC. Isomerase activity was calculated from the area of the 11-cis-ROL peak using Radiomatic 610TR software (Perkin Elmer, Waltham, MA) with synthetic 11-cis-[3H]-ROL as the standard.

**HPLC measurement of endogenous retinoids**

Endogenous 11-cis-retinal in the light adapted animals was analyzed using HPLC. Mice were exposed to day light or dim light for 3 hours and sacrificed at the same light conditions. The cornea, lens, and retina were removed, the remaining eyecup was homogenized with a glass grinder in extraction buffer [10 mmol/L NH2OH, 50% ethanol, 50% 2-(N-morpholino) ethanesulfonic acid, pH 6.5], and retinoids were extracted with hexane. The retinoids were dried under argon gas, resuspended in 200 µL of mobile phase (11.2% ethyl acetate, 2.0% dioxane, 1.4% octanol, 85.4% hexane), and injected into HPLC (515 HPLC pump; Waters, Milford, MA) for separation using a normal-phase column (LiChrosphere Si-60; 4.6 × 250 mm; 5 µm) (Alltech, Deerfield, IL) with isocratic elution (1 mL/min). Each retinoid isomer was quantified from the area of its corresponding absorption peak based on synthetic retinoid standards for calibration.

**Immunoblotting**

Briefly, the eyecups were homogenized in RIPA buffer (Cell Signaling Technology, Danvers, MA) containing protease inhibitors (Sigma-Aldrich, St. Louis, MO). The homogenates were centrifuged at 16,000 × g for 15 min, and the supernatant was collected, and protein concentrations were determined with the BCA assay kit (Bio-Rad, Hercules, CA). Total protein (40 µg) from the collected supernatant was resolved by 10% SDS-PAGE, transferred onto a nitrocellulose membrane and blotted with indicated antibodies. Band densitometry analysis was performed on non-saturated blots using the Image Lab Software (BioRad, Temecula, CA). For each quantitative immunoblot experiment (Western blotting, cell fractionation, and velocity sedimentation), experiments were repeated on at least 4 individual mice per genotype.

**Sucrose Velocity Sedimentation Assay**

In brief, the eyecups were homogenized in a solubilization buffer (PBS, pH 7.0, containing 1% (v/v) Triton X-100, 5 mM EDTA, 5 mg/ml N-ethylmaleimide (NEM) and protease inhibitors). The homogenates were then centrifuged at 158,000 × g on a bench top ultracentrifuge (Beckman Coulter Optima L-110K, rotor TLA110, Palo Alto, CA) for 25 min to remove all insoluble debris. Equal amounts (400 µg) of proteins from each sample were gently layered onto the top of the sucrose gradient. Continuous density gradient of 5-20% sucrose was prepared by sequentially layering of 1 ml each of PBS buffered 20, 15, 10, and 5% sucrose containing 0.1% Triton X-100 and 10 mM NEM (Sigma-Aldrich, St. Louis, MO) into an ultracentrifugation tube with dimensions of 14×89 mm (Beckman-Palo Alto, CA). Gradients were allowed
to become continuous by diffusion at room temperature for 1 hr and then chilled on ice for at least 30 min prior to sample loading. The loaded column was centrifuged at 104,000 × g for 18 hr at 4°C in a Beckman LB70M centrifuge with a swinging bucket rotor (SW41; Beckman Instruments, Palo Alto, CA). Equal volume of each fraction of the resolved gradient was collected by pipetting from the top of the column. The samples were then analyzed using reducing SDS-PAGE followed by Western blotting of RPE65. Summary graphs for sucrose density sedimentation experiments were prepared by comparing RPE65 densitometry intensity in each fraction as a percentage of total of RPE65 signal from all fractions.

**Subcellular Fractionation**

Mice were euthanized, and their eyes were enucleated. The cornea, lens, and retina were removed, and the remaining eyecup was fractionated into cytosolic, membrane, nuclear, and cytoskeletal fractions using FractionPrep™ (BioVision, Mountain View, CA) following the manufacturer’s protocol. Equal number of eyecups were used for each sample preparation, and equal volume of each fraction was resolved by SDS-PAGE and analyzed by Western blotting using an antibody for RPE65. Distribution of RPE65 in each fraction was analyzed by densitometry, and the ratio of RPE65 in the membrane fraction to that in the cytosolic fraction was plotted. Calnexin and fibrillarin were used as markers to identify fraction purity of ER membrane and the nucleus, respectively.

**Construction of Pvitro-2 heterozygous RPE65-WT/D477G.**

RPE65-WT-His and D477G-FLAG in pBluescript SK(+) were separately generated by conventional cloning and using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as previously described (1). To generate expression vectors of WT RPE65 or D477G alone, each cDNA in SK(+) was digested with EcoRI and HindIII sites and subcloned into an expression vector, pcDNA3.1(+) (Invitrogen). Further, to mimic human patient condition (heterozygous WT-RPE65 /D477G), WT-RPE65 -His and D477G-FLAG were subcloned in pVitro2-blasticidin-mcs (InvivoGen, San Diego, CA). The WT RPE65-His in SK(+) was digested with BgIII and Nhel, while the D477G-FLAG in SK(+) was digested with BamHI and Clal. Both fragments were then purified from 0.8% agarose gel and were separately subcloned into the pVitro2 vector. Sequence of inserted fragments in the vector was confirmed by DNA sequencing. Then the plasmid was purified using a QIAfilter Maxi-Prep Kit (Qiagen, Germany) following the manufacturer’s instructions. The plasmid concentration for every construct was determined using a NanoDrop 2000 spectrophotometer (Thermo, USA). All constructs were stored at -20°C until further use.

**Immunoprecipitation**

Briefly, 200 μL of the aforementioned protein aliquot was analyzed as the input fraction. The rest of the extract was incubated with anti-His or anti-Flag antibodies and protein A/G sepharose beads (Santa Cruz, Dallas, TX) overnight under constant, mild agitation at 4°C. The supernatant was collected and analyzed as the unbound fraction. The beads were washed three times in PBS containing 0.1% (v/v) Tween-20 by gentle pipetting. Proteins were eluted from the beads by incubation with 2% SDS and analyzed as the bound fraction. The input, unbound, and bound (eluent from the beads) fractions were analyzed by SDS-PAGE/Western blotting.
Protein Stability Assay

To determine the half-lives of RPE65 and in the presence and absence of D477G mutant, 293A-LRAT cells were cultured in Dulbecco’s modified Eagle’s Medium supplemented with 5% fetal bovine serum until 70-80% confluent. The cells were transfected with pVitro vector containing 2 copies of WT-RPE65 or 2 copies of D477G mutant or one copy of each gene. After transfection of pVitro2-WT/WT (contains 2 copies of WT RPE65), pVitro2-WT/D477G (contains 1 copy of WT RPE65 and 1 copy of D477G mutant cDNA) or pVitro2-D477G/D477G (contains 2 copies of D477G mutant cDNA) expression constructs, protein expression was allowed for 18 hr followed by the addition of 50 µg/ml cycloheximide (CHX; Sigma-Aldrich, St. Louis, MO) to interrupt all protein translation. The cells were harvested at the 0, 6, and 12 hr following the addition of CHX. The expression levels of the proteins were confirmed by Western blot analysis and semi-quantified with densitometry. Apparent half-lives were averaged from 3 independent experiments and expressed as mean ± SEM. Half-live values were calculated using the half-life equation.

\[ T = \frac{\ln(2)}{\lambda} = \tau \ln(2) \]

To evaluate the effect of ubiquitin proteasome pathway (UPP) on the D477G variant half-life, the cells were co-transfected with the plasmids as described above, and 18 hr after transfection, MG132 5 µM (Sigma-Aldrich, St. Louis, MO) and CHX were added to the cells. Cells were harvested at 12 hr following the addition of MG132 and CHX and processed for Western blot analysis.

Histology

Enucleated eyes were fixed in Davidson’s fixative (20 mL of 10% neutral buffered formalin, 10 mL of glacial acetic acid, 30 mL of 95% (v/v) ethanol, and 30 mL of distilled water) for 36 hr. The eyes were then washed three times in 70% ethanol and stored in 70% ethanol for subsequent serial dehydration and embedment in paraffin. As previously described (2), eyes were sectioned (5 µm thickness) on a standard microtome and then annealed onto glass slides at 60°C. Following hematoxylin/eosin staining of tissue sections gross retinal morphology was assessed by Olympus Provis Ax-70 light microscope. Morphometric measurements were made on 40× micrographs using Adobe photoshop CS6 (Berkeley, CA, Peachpit Press). The thickness of the outer nuclear layer, was measured on central retina sections at equidistant positions from the optic disc towards the periphery of both superior and inferior retinal halves.

Immuno-staining and confocal imaging

For immune-staining of retinal flat-mounts, mouse eyeballs were fixed in phosphate-buffered 4% paraformaldehyde (PFA) for 30 min on ice before dissection. Muscle and fat surrounding the eye globe were cleaned off. The anterior portion of the cornea and the lens were dissected away, and the retina was removed to expose underlying RPE. The dissected eye-cup (sclera, choroid, and RPE) was washed three times in PBS-T (PBS with 0.2% Triton X-100), and permeabilized and blocked for 1 hr in PBS containing 10% FBS and 0.1% Triton X-100. Following overnight incubation at 4°C with a primary antibody diluted in the blocking solution, the eyecups were washed three times with PBS containing 0.1% Triton X-100, and incubated with appropriate fluorophore-conjugated secondary antibodies and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies) for 2 hr. The eyecup was flattened on a slide, and a
total of 4 straight cuts from the limbal edge were made to form 4-petal flower-like structure and mounted in Vectashield mounting media (Vector Laboratories; Burlingame, CA). Fluorescence images were captured under a laser scanning confocal microscope (FV 1000, Olympus, Center Valley, PA).

**SI References**

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2. Y. Shin *et al.*, A novel RPE65 inhibitor CU239 suppresses visual cycle and prevents retinal degeneration. *Biochim Biophys Acta Mol Basis Dis* **1864**, 2420-2429 (2018).