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Complementation Test of *Rpe65* Knockout and Tvrm148

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**Purpose.** A mouse mutation, tvrm148, was previously reported as resulting in retinal degeneration. Tvrm148 and *Rpe65* map between markers D3Mit147 and D3Mit19 on a genetic map, but the physical map places RPE65 outside the markers. We asked if *Rpe65* or perhaps another nearby gene is mutated and if the mutant reduced 11-cis-retinal levels. We studied the impact of the tvrm148 mutation on visual function, morphology, and retinoid levels.

**Methods.** Normal phase HPLC was used to measure retinoid levels. *Rpe65*+/+; tvrm148/− (T+/−), tvrm148/tvrm148 (T−/−), *Rpe65*ko/ko (Rpe65−/−), and *Rpe65*−/− mice visual function was measured by optokinetic tracking (OKT) and electrophoretic retinography (ERG). Morphology was assessed by light microscopy and transmission electron microscopy (TEM). qRT-PCR was used to measure *Rpe65* mRNA levels. Immunoblotting measured the size and amount of RPE65 protein.

**Results.** The knockout and tvrm148 alleles did not complement. No 11-cis-retinal was detected in T−/− or Rpe65−/− mice. Visual acuity in *Rpe65*+/− and T+/− mouse was ~0.382 c/d, but 0.037 c/d in T−/− mice at postnatal day 210 (P210). ERG response in T−/− mice was undetectable except at bright flash intensities. Outer nuclear layer (ONL) thickness in T−/− mice was ~70% of *Rpe65*+/− by P210. *Rpe65* mRNA levels in T−/− mice were unchanged, yet 14.5% of *Rpe65*+/− protein levels was detected. Protein size was unchanged.

**Conclusions.** A complementation test revealed the RPE65 knockout and tvrm148 alleles do not complement, proving that the tvrm148 mutation is in *Rpe65*. Behavioral, physiological, molecular, biochemical, and histological approaches indicate that tvrm148 is a null allele of *Rpe65*.

Keywords: RPE65/Rpe65, visual cycle, mutation

Recentaly a catalog of over 160 mouse mutations that cause retinal disease was published.¹ This collection included a brief description of tvrm148, a putative novel autosomal recessive mutation in the *Rpe65* gene. The tvrm148 lesion was mapped between markers D3Mit147 (at 74.02 cM, corresponding to Chr3:148745409-148745542) and D3Mit19 (at 87.60 cM, corresponding to Chr3:157983038-157983067), approximately 9,237,658 bases apart, according to the most recent mouse genome sequence assembly, GRCh38 (Fig. 1A). This region includes 21 validated genes, but surprisingly, does not include *Rpe65*. Further, *Rpe65* is physically located at Chr3:159599181-159625307 by sequence analysis (Fig. 1B). This is approximately 1.6 Mb further telomeric from the distal end of the mapped disease locus (that is, from D3Mit19; Fig. 1B). The region from D3Mit19 to the physical location of the *Rpe65* gene contains another four candidate genes. *Rpe65* is just distal to these genes. Thus, *Rpe65* is not one of the 25 known genes in the mapped locus (Fig. 1B). From these two observations arises the potential complication that the gene harboring the tvrm148 lesion is unknown among 25 candidates.

Another potential complication of the tvrm148 assignment is that it is a missense mutation in *Rpe65* (a single base mutation resulting in the change of a wild type Phe codon to a Ser codon). It is often problematic to assign disease causation to missense mutations in the absence of experimental data; after all, chimpanzee contains a Tyr residue at the same position¹ and presumably retains function. For instance, a counter-hypothesis is that tvrm148 is a mutation in a gene close to *Rpe65*, and due to their proximity, a neutral missense mutation in *Rpe65* might be mistaken for the causative lesion. Because the putative causative mutation being a missense mutation that has not been previously detected in mouse, dog, or man, we thought an independent approach to the mutation assignment and additional experiments of tvrm148 as a candidate mutation in *Rpe65* were warranted.

A straightforward way to formally resolve the question of whether tvrm148 is a mutation in the *Rpe65* gene is to perform a complementation test using a deletion mutation solely within the *Rpe65* gene. Here we used the *Rpe65* knockout mouse.² This knockout is well characterized, has been backcrossed against wild type C57BL/6j for 10 generations, and the knockout of the *Rpe65* gene is well understood both genetically and biochemically.² The *Rpe65* gene was partially deleted in the construction of the knockout,² and the resulting gene fragment no longer exhibits any *Rpe65* mRNA² or protein² expression or retinoid isomerase enzymatic activity.²
The fundamental principles of the complementation test are basic in genetics. Here we perform this test asking if the mutation in tvrm148 is in the same gene as the Rpe65 knockout. We compared the phenotypes of the homozygous tvrm148 mutation, tvrm148/Rpe65 heterozygotes, and homozygous Rpe65KO to wild-type C57BL/6J by several histological, behavioral, and biochemical tests. We conclude that tvrm148 does not complement the knockout allele, thus proving unequivocally that this mutation is within the Rpe65 gene. Given this, we hypothesized that tvrm148 should manifest as a retinoid isomerase mutation. This was confirmed on molecular, biochemical, morphological, and functional levels.

METHODS

Experimental Animals

C57BL/6j (Rpe65+/+) mice served as wild type in all experiments. Mutant tvrm148 (T−/−) mutant mice were induced by ENU mutagenesis at The Jackson Laboratory (Bar Harbor, ME) and were purchased for this study; T+/− mice were bred by crossing T−/− mice with Rpe65+/+ mice. Rpe65KO (Rpe65−/−) mice were transferred from the National Eye Institute to Emory University, where they were bred and maintained. T+/− and Rpe65 −/+ mice were bred together to create T+/− mice for the complementation test. Mice were provided food and water ad libitum and maintained in a 12:12 light-dark cycle. Mice were sacrificed with CO2 gas, and all procedures and care were approved by Emory’s Institutional Animal Care Use Committee and all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Computer Predictions

Web-based computer prediction programs were used to predict the effect a putative F229S amino acid substitution could have on RPE65 protein (Sorting Intolerant from Tolerant, SIFT3–7; Polymorphism Phenotyping, polyPhen 2.08–10; Protein Analysis Through Evolutionary Relationships, PANTHER 11; MutationTaster12–19; PMUT 20; Screening for Non-Acceptable Polymorphisms, SNAP 21; SNPs3D 22–24; Predictor of Human Deleterious Single Nucleotide Polymorphisms, PhD-SNP 25–28; and Protein Variation Effect Analyzer, PROVEAN29).

Electroretinography (ERG)

Mice were dark-adapted overnight prior to ERG measurements. Scotopic recordings were performed after mice were dark-adapted, and photopic recordings were performed after mice had light adapted for 10 minutes under a constant illumination of 1.46 log cd·m−2. Measurements were recorded with a commercial ERG system (UTAS-E3000; LKC, Gaithersburg, MD)30–33 on a range of light intensities from 3 × 10−4 cd·s−2 to 137 cd·s−2. The b-wave amplitudes were measured from the trough of the a-wave to the peak of the positive wave; if the a-wave was not present, b-wave amplitudes were measured from the baseline to the top of the positive wave.34–36

Retinoid Analysis

All steps were performed in a darkroom under dim red light. RPE/choroid samples were removed from eyecups and homogenized in 200 μL PBS (pH 7.4, 150 mM NaCl, 1.06 mM KH2PO4, 5.60 mM Na2HPO4) in a disposable homogenizer. Homogenate (10 μL) was taken for the protein assay (Microplate Pierce Coomassie [Bradford] Plus Protein Assay; Thermo Fisher Scientific Inc., Rockford, IL). The remaining homogenate was transferred to a 15 mL screw-top polypropylene tube (BD Falcon; BD Biosciences, San Jose, CA), and 400 μL methanol was added. Retinoids were extracted two times by adding 1.5 mL hexane and vortex mixing at top speed for 1 minute. Samples were centrifuged at 14 240 g for 5 minutes. Hexane was drawn off the upper phase and transferred to a fresh 15 mL screw-top polypropylene tube (BD Falcon; BD Biosciences). Pooled hexane extracts were dried in an evaporation system (TurboVap LV; Zymark Corporation, Hopkinton, MA). Samples were redissolved in 100 μL hexane by vortex mixing for 1
Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Rpe65 mRNA levels were measured by qRT-PCR with P60 Rpe65+/+ and T−/− samples. RNA was isolated from RPE/choroid tissue placed in Trizol (Invitrogen, Grand Island, NY) and extracted using an RNA purification kit (RNasy Mini Kit; Qiagen, Valencia, CA). Rpe65 primers (Qiagen) were used to amplify Rpe65 mRNA with a one-step reaction (Quintetitec SYBR Green RT-PCR kit; Qiagen) under these conditions: 30 minutes at 50°C, 15 minutes at 95°C, 40 cycles at 94°C for 15 seconds, and 55°C for 15 seconds and 72°C for 40 seconds, followed by melt curve analysis. Four technical repeats for each reaction were performed in each run of the instrument (Cycler; Bio-Rad, Hercules, CA), and Rpe65 amplification was normalized to 18S RNA; 18S primers were: forward 5′-GTGAGTTCGGAACATGAGC-3′ and reverse 5′-GTCGCGCAGCTTATGGTCG-3′. There were five mice from different litters per sample group.

Immunblotting

RPE/choroid tissue was isolated from P60 Rpe65+/+, T+/−, and T−/− mice in RIPA buffer (Teknova, Hollister, CA). Samples were homogenized by vortex mixing vigorously with stainless steel set screws for 5 minutes. Following homogenization, samples were centrifuged at 16,000g for 1 minute. Supernatants were collected and protein concentration determined via BCA protein assay (Novagen, Darmstadt, Germany). Protein (1 μg) was resolved on a 10% gel (Bis-Tris Criterion Gel; Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad) with a wet transfer system (Bio-Rad). Western blocking reagent (Roche Diagnostics, Indianapolis, IN) was used. A custom-made N-terminal specific antibody to mouse Rpe65 was made by raising antisera against amino acids 1-44 of RPE65 mouse protein. Primary antibody incubation was performed at a 1:5000 dilution for 1 hour at room temperature. A goat antirabbit-HRP conjugate secondary antibody (Invitrogen) was washed with Tris-buffered saline, 0.05% Tween 20, and transferred to a nitrocellulose membrane (Bio-Rad) with a wet transfer system (Bio-Rad). Western blocking reagent (Roche Diagnostics, Indianapolis, IN) was used. A custom-made N-terminal specific antibody to mouse Rpe65 was made by raising antisera against amino acids 1-44 of RPE65 mouse protein. Primary antibody incubation was performed at a 1:5000 dilution for 1 hour at room temperature. A goat antirabbit-HRP conjugate secondary antibody (Invitrogen) was washed with Tris-buffered saline, 0.05% Tween 20, and transferred to a nitrocellulose membrane (Bio-Rad) with a wet transfer system (Bio-Rad). Western blocking reagent (Roche Diagnostics, Indianapolis, IN) was used. A custom-made N-terminal specific antibody to mouse Rpe65 was made by raising antisera against amino acids 1-44 of RPE65 mouse protein. Primary antibody incubation was performed at a 1:5000 dilution for 1 hour at room temperature. A goat antirabbit-HRP conjugate secondary antibody (Invitrogen) was washed with Tris-buffered saline, 0.05% Tween 20, and transferred to a nitrocellulose membrane (Bio-Rad) with a wet transfer system (Bio-Rad). Western blocking reagent (Roche Diagnostics, Indianapolis, IN) was used. A custom-made N-terminal specific antibody to mouse Rpe65 was made by raising antisera against amino acids 1-44 of RPE65 mouse protein. Primary antibody incubation was performed at a 1:5000 dilution for 1 hour at room temperature. A goat antirabbit-HRP conjugate secondary antibody (Invitrogen) was washed with Tris-buffered saline, 0.05% Tween 20, and transferred to a nitrocellulose membrane (Bio-Rad) with a wet transfer system (Bio-Rad). Western blocking reagent (Roche Diagnostics, Indianapolis, IN) was used. A custom-made N-terminal specific antibody to mouse Rpe65 was made by raising antisera against amino acids 1-44 of RPE65 mouse protein. Primary antibody incubation was performed at a 1:5000 dilution for 1 hour at room temperature. A goat antirabbit-HRP conjugate secondary antibody (Invitrogen) was washed with Tris-buffered saline, 0.05% Tween 20, and transferred to a nitrocellulose membrane (Bio-Rad) with a wet transfer system (Bio-Rad). Western blocking reagent (Roche Diagnostics, Indianapolis, IN) was used. A custom-made N-terminal specific antibody to mouse Rpe65 was made by raising antisera against amino acids 1-44 of RPE65 mouse protein. Primary antibody incubation was performed at a 1:5000 dilution for 1 hour at room temperature. A goat antirabbit-HRP conjugate secondary antibody (Invitrogen) was washed with Tris-buffered saline, 0.05% Tween 20, and transferred to a nitrocellulose membrane (Bio-Rad) with a wet transfer system (Bio-Rad). Western blocking reagent (Roche Diagnostics, Indianapolis, IN) was used. A custom-made N-terminal specific antibody to mouse Rpe65 was made by raising antisera against amino acids 1-44 of RPE65 mouse protein. Primary antibody incubation was performed at a 1:5000 dilution for 1 hour at room temperature. A goat antirabbit-HRP conjugate secondary antibody (Invitrogen) was washed with Tris-buffered saline, 0.05% Tween 20, and transferred to a nitrocellulose membrane (Bio-Rad) with a wet transfer system (Bio-Rad). Western blocking reagent (Roche Diagnostics, Indianapolis, IN) was used. A custom-made N-terminal specific antibody to mouse Rpe65 was made by raising antisera against amino acids 1-44 of RPE65 mouse protein. Primary antibody incubation was performed at a 1:5000 dilution for 1 hour at room temperature. A goat ant
PANTHER, MutationTaster, PMUT, SNAP, SNPs3D, PhdSNP, and PROVEAN were used to predict if the putative Rpe65 mutation could negatively impact visual function in mice. With the exception of PROVEAN, all programs predicted the tvrm148 mutation is responsible for RPE65 dysfunction (Table 1). DNA sequencing from Ttvrm148 mice confirmed the presence of the tvrm148 mutation that was previously reported.1 There were no other sequence differences in any of the other exons or regions sequenced comparing tvrm148 to the C57BL/6j reference sequence. See Supplementary Material S1 for a description of the primers and PCR products that were sequenced, and methods used.

Complementation Test

A complementation test was performed to determine whether the causative mutation driving visual function loss in Ttvrm148 mice resides in the Rpe65 locus. By breeding Ttvrm148 mice and Rpe65+/- mice together, Rpe65+/-, Rpe65+/+ and Rpe65-/- mice were then screened by ERG at 137 cd·s·m⁻² at P30 to assess visual function (Fig. 2). All mutant mice had significantly lower responses (P < 0.001) than Rpe65+/+ mice (264.7 ± 56.4 μV [n = 6]; Fig. 2), but the a-wave responses of Rpe65+/-, Rpe65+/+ and Rpe65-/- mice were not significantly different when compared with each other alongside wild type mice (Rpe65+/- mice, 24.6 ± 16.2 μV [n = 7]; Ttvrm148 mice, 6.1 ± 6.7 μV [n = 5]; Rpe65-/- mice, 35.1 ± 16.4 μV [n = 9]).

Retinoid Levels in Tvrm148, Knockout, and Normal Mouse Eyes

The fact that mutant Ttvrm148 mice did not have appreciable a-wave responses suggested they lack the ability to produce sufficient amounts of 11-cis-retinol (the enzymatic product of RPE65) to support proper photoreceptor function.1 Rpe65+/- mice have also been shown to harbor excessive amounts of retinyl ester,2 and we sought to determine if Ttvrm148 mice shared this canonical chronic loss of 11-cis-retinol and increase in retinyl ester content in the RPE/choroid. Rpe65+/- and Ttvrm148 mice had elevated retinyl esters, while 11-cis retinal was not detected in Rpe65+/- or Ttvrm148 retinas (Table 2). The Ttvrm148 samples had 22X more retinyl esters than Rpe65+/- samples. Retinyl ester content in Rpe65+/- and Ttvrm148 mice did not differ significantly from one another. Retinol isomers in all samples were below the level of detection prior to saponification of mutant samples. For the saponified portion, only all-trans-retinol was detected in the mutant samples and no quantification was attempted.

Inheritance of Tvrm148

We sought to determine how the tvrm148 allele is inherited in mice (alternatively recessive or dominant, autosomal or sex-linked—data not shown). ERG measurements on dark- and light-adapted Rpe65+/-, Ttvrm148, and Ttvrm148 mice were performed at P60 and P180 (Fig. 3). While Ttvrm148 mice did not show any reduction in dark-adapted b-wave amplitudes, Ttvrm148 mice showed depressed dark-adapted b-wave amplitudes at both ages (Figs. 3A, 3C). At P60, in scotopic conditions (0.01551 cd·s·m⁻²), Ttvrm148 mice had no measurable (0 ± 0 μV, P < 0.001) b-wave response, whereas Rpe65+/- and Ttvrm148 mice had an average b-wave amplitude of 197.9 ± 62.4 μV and 221.8 ± 59.6 μV, respectively. At the brightest flash intensity tested, 137 cd·s·m⁻², Ttvrm148 mice had an average b-wave response of 132.4 ± 52.4 μV in comparison to 648.2 ± 196.9 μV and 654.3 ± 177.9 μV in Rpe65+/- and Ttvrm148 mice, respectively (Fig. 3A). By P180, Ttvrm148 mice had an average scotopic (0.01551 cd·s·m⁻²) b-wave amplitude of 0 ± 0 μV compared with 216.0 ± 40.6 μV in Rpe65+/- mice and 191.0 ± 39.1 μV in Ttvrm148 mice; at 137 cd·s·m⁻², Ttvrm148 mice had an average b-wave amplitude of 69.1 ± 50.2 μV compared with 526.1 ± 75.1 μV in Rpe65+/- mice and 541.3 ± 68.6 μV in Ttvrm148 mice (Fig. 3C).

Visual Acuity Affected by Null Mutations

The optokinetic tracking testing, a behavioral measure of visual acuity, was used to determine the effects of the tvrm148 mutation on the visual guided activity of the mouse. A virtual optomotor system99,100 was used to measure visual acuity in Rpe65+/-, Ttvrm148, and Ttvrm148 mice. Rpe65+/- and Ttvrm148 mice maintained a constant high visual acuity (0.381 ± 0.002 c/d) with little variability from P30 through the end of the study at P210 (Fig. 4). Ttvrm148 mice had no detectable reduction in visual acuity. Ttvrm148 mice, on the other hand, had a significant reduction in visual acuity at P30 (0.318 ± 0.003 c/d, and continued to lose visual acuity until the end of the study (Fig. 4). At P210, only a few Ttvrm148 mice retained measurable visual acuity, which produced the average value of 0.037 ± 0.045 c/d (Fig. 4).

Comparison of Slow Loss of Visual Acuity in Ttvrm148 and Rpe65+/- Mice

The visual acuities of Rpe65+/-, Ttvrm148, and Rpe65+/- mice were compared at P30, P120, and P210 to determine if Rpe65+/- and Ttvrm148 mice behaved similarly in early, middle, and late stages of the retinal degeneration (Fig. 5). The slow loss of visual acuity in Ttvrm148 and Rpe65+/- mice was consistent at each age examined. At P30, Ttvrm148 visual acuity was 0.318 ± 0.003 c/d, and Rpe65+/- visual acuity was 0.318 ± 0.003 c/d. At P120, Ttvrm148 visual acuity was 0.225 ± 0.010 c/d, and Rpe65+/- visual acuity was 0.245 ± 0.007 c/d. At P210, Ttvrm148 visual acuity was 0.037 ± 0.045 c/d, and Rpe65+/- visual acuity was 0.000 ± 0.000 c/d.

Histology and Morphometrics Changes—Slight Losses in Mutants

We sought to determine if loss of visual function correlated with a significant loss of retina layer thickness. Eyes were
harvested from P60, P120, P180, and P210 from Rpe65+/+, T−/−, and T+/− mice, and representative sections from P60 and P210 are shown (Fig. 6). The T+/− retina was thinner than Rpe65+/+ or T−/− retinas (Fig. 6). Retina architecture was largely intact even at P210, despite some significant ONL thinning (35.9 ± 2.9 μm in T−/− mice compared with 50.9 ± 4.5 μm in Rpe65+/+ mice) 500 μm on the superior side of the optic nerve (P < 0.001). Some lipid droplets appear in the RPE of T−/− mice (Fig. 7). Images of Rpe65+/+, T−/−, and T+/− retinas taken at P120 and P180 look similar to P60 and P210 (data not shown).

Quantitative measurements of retina cross-sections from P60 and P210 eyes revealed reduced nuclei counts (Figs. 7A, 7B), thinning of the outer nuclear layer (ONL; Figs. 7C, 7D); IS (Figs. 7E, 7F); and OS (Figs. 7G, 7H) in T−/− mice compared with T+/− and Rpe65+/+ mice (Fig. 7). T+/− retina thinning was apparent as early as P60. The ONLs of Rpe65+/+ and T+/− mice were 11 to 12 nuclei thick at both P60 and P210, but T−/− mice had an ONL only 8 to 9 nuclei thick at P60 and 7 to 8 nuclei thick at P210 (Figs. 7A, 7B). By P60, T+/− retina had lost a significant number of cone nuclei (Fig. 8A); Rpe65+/+ retina had 11.8 ± 3.4 cones per 200-μm segment, but T+/− retina had only 6.5 ± 2.3 cone nuclei per 200-μm segment on the superior side of the optic nerve and 3.2 ± 1.4 cone nuclei per 200-μm segment on the inferior side (Fig. 8A). By P210, Rpe65+/+ retina had 10.0 ± 1.8 cone nuclei per 200-μm segment, and T−/− retina had only 2.7 ± 1.4 and 2.0 ± 0.9 cone nuclei per 200-μm segment on the superior and inferior sides of the retina, respectively (Fig. 8B).

RNA Quantitation of Tvrn148 Mutation
RNA was isolated from the RPE of Rpe65+/+ and T−/− mice to determine if the mutant mice expressed similar levels of Rpe65 mRNA. qRT-PCR results (Fig. 10) indicated insignificant differences in steady-state levels of Rpe65 mRNA in T−/− mice compared to Rpe65+/+ mice (P = 0.617). T−/− mice express Rpe65 mRNA at 91.3 ± 54.4% of the level in Rpe65+/+ mice.

**DISCUSSION**

The Rpe65 KO and tvrn148 mutations do not complement one another, proving that the tvrn148 mutation occurs in the Rpe65 locus and is the causative lesion driving visual function loss (Fig. 2; Table 1). The complementation test with the knockout and tvrn148 alleles definitively proves that there is no other mutation contributing to the loss of visual function (Fig. 2). This genetic test also resolves concerns associated with discrepancies we found between the genetic and physical maps of chromosome 5 in the region where the Rpe65 locus and D3Mit147 and D3Mit19 markers reside (Fig. 1).

Retinoid analyses of Rpe65+/+, Rpe65−/−, and T−/− mice, showing undetectable amounts of 11-cis-retinal (downstream enzymatic product) in retina but large amounts of retinyl esters (enzymatic substrate) in RPE/choroid samples from mutant
Figure 3. \( T^{-/-} \) mice, but not \( T^{+/+} \) mice, have a loss of ERG response. Dark- and light-adapted b-wave amplitudes, respectively, from \( Rpe65^{+/+} \), \( T^{+/+} \), and \( T^{-/-} \) mice at P60 (A, B) and P180 (C, D) are shown. \( * P < 0.05 \) \( T^{-/-} \) compared with \( Rpe65^{+/+} \), \( ** P < 0.001 \) \( T^{-/-} \) compared with \( Rpe65^{+/+} \), significance determined through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls testing. There was no difference between \( Rpe65^{+/+} \) and \( T^{+/+} \) ERG responses. Data are presented as absolute values (mean ± SD).

Figure 4. \( T^{-/-} \) mice, but not \( T^{+/+} \) mice, lose visual acuity with age. Visual acuities of \( Rpe65^{+/+} \) (solid line), \( T^{+/+} \) (dotted line: largely hidden behind the solid back line), and \( T^{-/-} \) mouse (dashed line) from P30 to P210 are represented on the same graph. \( * P < 0.05 \) \( T^{-/-} \) compared with \( Rpe65^{+/+} \), \( ** P < 0.001 \) \( T^{-/-} \) compared with \( Rpe65^{+/+} \), significance determined through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls testing. There was no difference between \( Rpe65^{+/+} \) and \( T^{+/+} \) ERG responses. Data are presented as absolute values (mean ± SD).

Figure 5. \( T^{-/-} \) mice have residual visual function similar to \( Rpe65^{-/-} \) mice. Comparison of visual phenotype measured by visual acuity. \( T^{-/-} \) mice have a residual visual function similar to \( Rpe65^{-/-} \) mice. Visual acuities of \( Rpe65^{+/+} \) (black bar), \( T^{+/+} \) (gray bar), and \( Rpe65^{-/-} \) (white bar) mice at P30, P120, and P210 are shown on the same graph. Data are presented as mean ± SD. \( * P < 0.001 \) compared to wild type. \( # P < 0.05 \) \( T^{-/-} \) compared with \( Rpe65^{-/-} \). \( ** P < 0.001 \) \( T^{-/-} \) compared with \( Rpe65^{-/-} \).
mice, confirmed that the tvrm148 mutation results in a biochemically null RPE65 protein product that lacks retinoid isomerase activity (Table 2). Previous reports of mouse Rpe65 mutations show Rpe65KO, Rpe65KOtvrm148, and Rpe65GR91W mice have undetectable scotopic responses at dim flash intensities and bright flash intensities in addition to reduced photopic responses and these previous findings support the importance of RPE65 in 11-cis-retinal production and photoreceptor function. Consistent with other Rpe65 mutants, T+ mice at P50 and P180 had poor dark-mediated vision but relatively appreciable light-mediated vision at early ages (Fig. 3). LCA2 patients suffer from a similar pathology early in their degenerations, so we sought to determine if T−/− mice conform with clinical observations of human patients. T−/− mice, but not T+/− mice, lose visual acuity through P210 (indicating the tvrm148 mutation results in a recessive manner) but have appreciable visual function at early ages (Fig. 4). Rpe65−/− mice have visual acuity losses that parallel with T−/− mice and do not significantly differ from one another (Fig. 5). This is the first reported progressive visual acuity loss in Rpe65 mutant mice and goes well beyond previous work reporting RPE65 visual acuity.

Human LCA2 patients have mostly intact retina architecture even at late ages, so we sought to qualitatively and quantitatively examine the retina for similar phenotypes. The architecture of the retina in T−/− mice relative to Rpe65−/− and T+/− mice was strikingly normal considering the visual function deficits (Fig. 6), but these findings were consistent with published literature on RPE65-deficient mouse models. Droplets (Fig. 9) in the RPE in T−/− mice are similar to reports in Rpe65KO, Rpe65KOtvrm148, and Rpe65GR91W mice that are the result of retinyl ester accumulation. T−/− and T+/− mice experienced significant reductions in RPE65 protein level compared to Rpe65+/+ (Fig. 11) even though there was no reduction in Rpe65 miRNA levels in T−/− mice compared with Rpe65+/+ (Fig. 10). T−/− mice had no detectable 11-cis-retinal in neural retina and a 22-fold increases in retinyl esters in RPE samples (Table 2), further indicating the mutant protein is a null mutation that possesses no measurable enzymatic activity.

The visual acuity of T−/− mice is surprisingly high (almost normal) at early ages (Fig. 4) considering the lack of 11-cis-retinal generation in RPE65-deficient mice; neither Rpe65KO nor Rpe65KOtvrm148 mice have detectable amounts of 11-cis-retinal in the retina. Mutations show ~5% the wild type amount of 11-cis-retinal in the retina, and this residual enzymatic product is sufficient to preserve vision significantly longer than what is seen in other Rpe65 mutant mice. We show tvrm148 mice lack 11-cis-retinal altogether (Table 2). The residual visual function in T−/− mice may correspond to residual rod function, not cone function because of reduced photon quantum-catch capability. This, coupled with the fact that Rpe65−/−:Rhodopsin (pure cone function) mice do not retain visual acuity, suggests the residual visual acuity in T−/− mice is driven primarily by rod function. Consistent with previous work, T−/− mice lost 59.9% of cones by P60 (Fig. 8); furthermore, many remaining cones are not functional at that age in Rpe65−/− mice, although they can be restored to activity by reintroduction of 11-cis-retinal.

The source and identity of chromophore for rod photoreceptors in T−/− mice, though, is unclear at this time. However, Rpe65−/− mice may generate and utilize 9-cis-retinal to form isorhodopsin. Knock-in mice generate a small but significant amount of 11-cis-retinal. It is possible Rpe65 mutant mice could obtain enough 11-cis-retinal for residual visual function from photoisomerization. More recently, evidence is emerging that Müller cells may actually provide small amounts of 11-cis-retinal to the retina and it has been proposed this cone opsin mislocalization may have a toxic effect on cone cells. Work with rd12 mice shows most cones are lost from the retina by P30. This is in contrast to most other retinal degenerations reported in mice in which visual function loss is driven primarily by rod photoreceptor loss. Visual acuity measurements are performed in photopic conditions that are dominated by cone photoreceptors in C57BL/6j mice, so it may at first seem counterintuitive that the visual acuity measurements in T−/− mice probably rely on residual rod photoreceptor function. Previous work shows the greatly altered kinetics of rod photoreceptors in Rpe65 knockout mice cause them to function exclusively in photopic conditions because of reduced photon quantum-catch capability. This, coupled with the fact that Rpe65−/−:Rhodopsin (pure cone function) mice do not retain visual acuity, suggests the residual visual acuity in T−/− mice is driven primarily by rod function. Consistent with previous work, T−/− mice lost 59.9% of cones by P60 (Fig. 8); furthermore, many remaining cones are not functional at that age in Rpe65−/− mice, although they can be restored to activity by reintroduction of 11-cis-retinal.

Low RPE65 protein levels in T−/− mice could be the result of reduced translation of the Rpe65 transcript in T−/− mice or the result of RPE65 protein instability. Recent work concerning the three dimensional structure of B. taurus RPE65 protein provides insight into reasons why the RPE65 cispropeller protein may be nonfunctional and possibly unstable. The Rpe65transcript in exon 7 of RPE65 and results in a substitution of Ser for Phe at amino acid position 229. Because Phe 229 is conserved in RPE65 from a wide variety of species, it is possible that a missense mutation at this position could be pathogenic even though the residue is not thought to participate in the active site of RPE65. The pathogenicity (Table 2) of the F229S substitution might be attributed to two other factors. First, RPE65 is a seven-bladed β-propeller that folds to form a hydrophobic tunnel leading to the active site. Mutations interfering with β-propeller folding may perturb the...
FIGURE 7. T−/− mouse retina layers progressively thin with age. Quantitative measurements every 500 μm superior and inferior of the optic nerve in Rpe65+/+ (solid line); T+/− (dotted line); and T−/− (dashed line) retina were taken for the number of stacked nuclei in the ONL (A, B); ONL thickness (C, D); IS thickness (E, F); and OS thickness (G, H) at P60 (Rpe65+/+ n = 11, T+/− n = 8, T−/− n = 12) and P210 (Rpe65+/+ n = 7, T+/− n = 6, T−/− n = 14). *P < 0.05 T−/− compared with Rpe65+/+. **P < 0.001 T−/− compared with Rpe65+/+. +P < 0.05 Rpe65+/+ compared with T+/−. Significance determined through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls testing. Data are presented as mean ± SD.
**FIGURE 8.** T<sup>+/−</sup> mice have significant cone nuclei loss at P60 and P210. Cone nuclei were counted at distances of 0.25, 1, and 2 mm superior and inferior of the optic nerve in a 200-μm segment of the retina at (A) P60 and (B) P210. **P < 0.001** Rpe65<sup>+/−</sup> compared with T<sup>+/−</sup>, significance determined through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls testing. Data are presented as mean ± SD.

**FIGURE 9.** TEM shows lipid droplets indicative of retinyl ester accumulation in the RPE of T<sup>+/−</sup> and T<sup>−/−</sup> mice. TEM was performed on sections of RPE 1000 μm superior to the optic nerve in Rpe65<sup>+/+</sup>, T<sup>+/−</sup>, and T<sup>−/−</sup> mice at P60 and P210. (A–C) TEM images of RPE from Rpe65<sup>+/+</sup>, T<sup>+/−</sup>, and T<sup>−/−</sup> mice at P60, respectively, at ×2900 magnification are shown. (D–F) TEM images of Rpe65<sup>+/+</sup>, T<sup>+/−</sup>, and T<sup>−/−</sup> mice at P210, respectively, at ×2900 magnification are shown. (G–I) TEM images of Rpe65<sup>+/+</sup>, T<sup>+/−</sup>, and T<sup>−/−</sup> mice at P210, respectively, at ×10,000 magnification are shown. N, nucleus; L, lipid droplet.
The tvrm148 mutation abolishes residual RPE65 enzymatic activity. The RPE65 tvrm148 mouse, a knock-in model for the most common missense mutation seen in humans, retains residual RPE65 enzymatic activity. The RPE65 tvrm148 mouse, though, is the first model of RPE65 deficiency to present a null phenotype while retaining the ability to produce a mutant RPE65 protein (albeit low; Fig. 11). Because this model has a missense mutation in a region of the protein known for recessively inheriting, disease-causing missense mutations in human subjects while still retaining a null phenotype, the RPE65 tvrm148 model may have unique utility for studying future treatments for LCA2.

Future work with this mutant mouse model could involve RPE65 gene augmentation to see if visual function rescue in these mice resemble rescue seen in other RPE65-deficient mice. Other future work could examine cone photoreceptor loss in T−/− mice to see if the loss resembles that seen in other RPE65-deficient mice. Like the knockout mouse, the tvrm148 mutation acts as an autosomal recessive, null mutation. Compared with the knockout, almost all other properties were very similar based on visual optokinetic testing (Fig. 4) and ERGs (Fig. 2). Molecular properties of the tvrm148 mRNA are remarkable in its nearly normal abundance (Fig. 10), but with little accumulated protein under steady-state conditions (Fig. 11). We might speculate that the mRNA is processed normally but that the protein is unstable and rapidly degraded.

In summary, a complementation test proves that tvrm148 is a mutation in the RPE65 gene. This mutation results in a null RPE65 mouse that makes no 11-cis-retinal. The tvrm148 mice have a visual phenotype resembling that found in RPE65KO mice, losing visual acuity and ERGs in a similar manner. Protein is present in low amounts and lacks activity, despite wild type levels of mRNA. This work suggests that the tvrm148 mouse may serve as a model for LCA2 and can thus help in the understanding of the basic biology of the visual cycle and aid in the future development of treatment strategies for the disease. Although our work does not resolve the apparent difference in the genetic map and current gene sequence assembly, it does demonstrate the tvrm148 mutation as a causative lesion in RPE65.
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