The *Rpe65*^{rd12} Allele Exerts a Semidominant Negative Effect on Vision in Mice

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Submitted: November 13, 2013

Accepted: March 10, 2014

Citation: Wright CB, Chrenek MA, Feng W, et al. The *Rpe65*^{rd12} allele exerts a semidominant negative effect on vision in mice. *Invest Ophthalmol Vis Sci*. 2014;55:2500–2515. DOI: 10.1167/iovs.13-13574

PURPOSE. The rd12 mouse was reported as a recessively inherited *Rpe65* mutation. We asked if the rd12 mutation resides in *Rpe65* and how the mutation manifests itself.

METHODS. A complementation test was performed by mating *Rpe65*^{KO} (KO/KO) and rd12 mice together to determine if the rd12 mutation is in the *Rpe65* gene. Visual function of wild-type (+/+), KO/+, rd12/+, KO/KO, rd12/rd12, and KO/rd12 mice was measured by optokinetic tracking (OKT) and ERG. Morphology was assessed by retinal cross section. qRT-PCR quantified *Rpe65* mRNA levels. Immunoblotting measured the size and level of RPE65 protein. *Rpe65* mRNA localization was visualized with RNA fluorescence in situ hybridization (FISH). Fractions of *Rpe65* mRNA-bound proteins were separated by linear sucrose gradient fractionation.

RESULTS. The KO and rd12 alleles did not complement. The rd12 allele induced a negative semidominant effect on visual function; OKT responses became undetectable 120 days earlier in rd12/rd12 mice compared with KO/KO mice. rd12/+ mice lost approximately 21% visual acuity by P210. rd12/rd12 mice had fewer cone photoreceptor nuclei than KO/KO mice at P60. rd12/rd12 mice expressed 71% +/+ levels of *Rpe65* mRNA, but protein was undetectable. Mutant mRNA was appropriately spliced, exported to the cytoplasm, trafficked, and contained no other coding mutation aside from the known nonsense mutation. Mutant mRNA was enriched on ribosome-free messenger ribonucleoproteins (mRNPs), whereas wild-type mRNA was enriched on actively translating polyribosomes.

CONCLUSIONS. The rd12 lesion is in *Rpe65*. The rd12 mutant phenotype inherits in a semidominant manner. The effects of the mutant mRNA on visual function may result from inefficient binding to ribosomes for translation.

Keywords: RPE65, rd12, visual cycle

Three mouse models are commonly used for the study of LCA2 pathology and treatment that are reported as having null *Rpe65* mutations. The first model is the *Rpe65*^{KO} (KO) mouse that was artificially constructed and contains a deletion of exons 1 to 3.¹ The second model, the rd12 mouse, was isolated as a spontaneously occurring mutation in the B6.A-H2-T18^a/BoyEg strain that was then repeatedly backcrossed to wild-type (C57BL/6J, referred to as +/+ in this study) to make a congenic inbred strain, which the authors referred to as B6-rd12.² It mapped genetically near *Rpe65*, and a nonsense mutation (R44X) was found in exon 3 of *Rpe65*.² Much as we previously used the complementation test to reconcile the genetic and sequence maps of *Rpe65* with the tvrm148 mutation,³ we performed a complementation test on the rd12 and KO alleles to ask if the rd12 mutation resided in the *Rpe65* locus.

As of November 2013, all 602 mutations listed on the National Center for Biotechnology Information single nucleotide polymorphism (SNP) database identified in human *RPE65*

are autosomal recessive mutations,^{4–11} except *RPE65*^{D477G}, which inherits in an autosomal dominant manner.¹² To date, all known mouse mutations in *Rpe65* inherit in a recessive manner as well.^{1,2,13,14} We studied the retinal degeneration 12 (rd12) mutant because it was thought to be a mutation in *Rpe65*; however, our preliminary studies suggested a dominant characteristic, which is atypical of LCA2 and lesions in *Rpe65*, and called into question whether rd12 was in fact a gene lesion in *Rpe65* or perhaps a nearby gene instead. The differences between the genetic map and the sequence map of the locus for *Rpe65* further caused concern.³ We found this to be a particularly relevant question in light of the fact that the rd12 mutation (*RPE65*^{R44X}) has been recently identified in human patients (refSNP cluster report: rs368088025). Thus, we asked how the rd12 mutation caused disease.

Both the KO and rd12 mouse strains were reported as null *Rpe65* mutants.^{1,2} Immunohistochemical staining for RPE65 in both KO and rd12 mouse models show RPE65 protein is undetectable in the eyes of these mice.^{2,15–18} Knockout mice

do not express detectable *Rpe65* mRNA,¹ and although previous studies speculated the absence of RPE65 protein was attributable to degradation of the *Rpe65* mRNA by nonsense-mediated decay (NMD),² the rd12 allele was later reported to produce *Rpe65* mRNA at a level that was similar to wild-type C57BL/6J mice.¹⁹ This observation caused us to wonder if the rd12 mutation produced any phenotypic variations compared with the KO. The purpose of this study was to ask if the rd12 mutation resides in *Rpe65* and where in the cell that mutation manifests itself at a molecular level (Supplementary Fig. S1). The following questions are addressed in this report: (1) Is the rd12 mutation found in the *Rpe65* gene? (2) Is the rd12 mutation recessive? and (3) If the rd12 mutation is not recessive, what is a potential mechanism for the toxic gain of function of this mutation? Question 1 was addressed by performing a complementation test; Question 2 was addressed by phenotypic assessment of visual function, morphology, and morphometrics; and Question 3 was addressed through molecular characterization of the rd12 mutation.

METHODS

Experimental Animals

Knockout/KO mice¹ were backcrossed to +/+ mice for 10 generations to make the line fully congenic with the +/+ line housed at Emory University for this study. rd12/rd12 mice² congenic to the C57BL/6J mouse strain were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were maintained and bred at Emory University. Knockout/+ mice were bred by crossing +/+ mice with KO/KO mice, rd12/+ mice were bred by crossing +/+ mice with rd12/rd12 mice, and KO/rd12 mice were bred by crossing KO/KO mice with rd12/rd12 mice. Mice were provided food and water ad libitum and maintained in a 12:12-hour light-dark cycle. Mice were killed with CO₂ gas. All procedures and care were approved by Emory Institutional Animal Care and Use Committee and followed ARVO guidelines of animal care and use.

Optokinetic Tracking (OKT)

Visual acuities were measured at 100% contrast at postnatal day (P) 30, P45, P60, P75, P90, P120, P150, P180, and P210 using an OptoMotry 1.7.4 virtual optomotor system^{20,21} (OptoMotry 1.7.4; Cerebral Mechanics, Vancouver, BC, Canada). Initially noted visual acuity measurements between KO/KO and rd12/rd12 mice were confirmed by single-blinded, independent observers (data not shown) prior to longitudinal study.

Retinoid Analysis

Retinoid analyses of mouse retinas and eye cups were performed with normal-phase HPLC in adult mice as previously described.^{3,22,23}

Electroretinography

Electroretinography measurements were taken at P30, P60, and P90 in KO/KO, rd12/rd12, and KO/rd12 mice and at P60, P120, and P180 in +/+, KO/+, and rd12/+ mice using a commercial ERG system (UTAS-E3000; LKC, Gaithersburg, MD, USA), as previously described.^{3,24–27} Oscillatory potentials (OPs) were isolated from scotopic waveforms by filtering using a fifth-order Butterworth filter and performing a Discrete Fourier Transform on the filtered waves.^{28–30} Summed OP amplitudes (SOPAs) were calculated by adding amplitudes

from OP1 to OP6 together^{28–30} in +/+, KO/+, and rd12/+ mice. Rod phototransduction parameters were estimated by fitting the Hood and Birch formulation of the Lamb and Pugh model of phototransduction activation to a-waves from scotopic waveforms.^{31,32}

Histology and Morphometrics

Histology was assessed in +/+, KO/KO, and rd12/rd12 mice at P60 and P210. Retinal sections (prepared by fixing eyes in 2.5% wt/vol glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 [Electron Microscopy Sciences, Hatfield, PA, USA], embedded in LX-112 [Ladd Research Industries, Williston, VT, USA] and section at 1-μm thickness) were stained and analyzed as previously described.³ Cone nuclei were counted at 200-μm areas 250, 1000, and 2000 μm superior and inferior to the optic nerve from sections as previously described.^{33,34}

RNA Extraction and Quantitative RT-PCR

Whole-cell RNA was isolated from P60 RPE/choroid tissue placed in Trizol (Life Technologies Corp., Grand Island, NY, USA) and extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA).³ RPE cells were fractionated and total RNA isolated (SurePrep Nuclear or Cytoplasmic RNA Purification Kit; Fisher Scientific, Pittsburgh, PA, USA). mRNA was amplified with a one-step reaction (Quantitect SYBR Green RT-PCR Kit; Qiagen) under the following 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 a melt curve analysis for the first run of each primer set. Unless stated otherwise, all *Rpe65* amplification reactions were performed with specific primers that were commercially available (Qiagen product number QT00140140) and normalized to 18S RNA. Primers were validated for specificity to *Rpe65* mRNA (Supplementary Fig. S2). Primers specific for *Rpe65* exon boundaries were designed and used as previously described^{3,35} (Table 1). mRNA expression was normalized to 18S ribosomal RNA using the following primers: forward 5'-GTT GGT TTT CGG AAC TGA GGC-3' and reverse 5'-GTC GGC ATC GTT TAT GGT CG-3'.

Immunoblotting

Soluble RPE/choroid extracts were collected at P60 as previously described.³ Tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer (Teknova, Hollister, CA, USA) by vortex mixing vigorously with one new stainless steel set screw for 5 minutes in a 1.5-mL screw-cap microcentrifuge tube. Samples were then centrifuged at 16,000g for 1 minute, and supernatant collected and placed into another tube for storage at -80°C. Pellets left after RIPA-soluble protein removal were then dissolved in 8 M urea and 1 mM dithiothreitol in 1% SDS for 1 hour at 95°C. Samples were then centrifuged at 16,000g for 1 minute, and supernatant removed for storage at -80°C. Protein concentration was determined via bicinchoninic acid protein assay (Novagen, Darmstadt, Germany). A total of 10 μg protein was resolved by SDS-PAGE ("Any kD TGX" Criterion gel; BioRad, Hercules, CA, USA). A custom-made rabbit polyclonal anti-mouse RPE65 N-terminal-specific primary antibody that was raised against the first 43 amino acids of mouse RPE65 protein (Covance, Atlanta, GA, USA) was incubated at 1:2000 dilution for 1 hour at ambient room temperature. Secondary incubation was performed with a goat anti-rabbit-horseradish peroxidase conjugate antibody (Invitrogen, Carlsbad, CA, USA) by 1:5000 dilution for 1 hour at ambient room temperature. Bands were detected by chemilu-

TABLE 1. Primer Sequences for *Rpe65* Exon Boundary Amplification

Exons Amplified	Primer Sequence, 5'–3'
1–3	F: AAA TTG AAC ACC CTG CTG GT R: AGG GCT TGT CCA TGC AAC AG
2–3	F: GGA GGA ACT GTC CTC ACC AC R: TAT GTG ACA TGG CCC TCC TT
3–4	F: TGA AGT TGG ATC TGA GCC TT R: AAC ATA AGC ATC AGT GCG G
4–5	F: TTC ATC CGC ACT GAT GCT TA R: CAA GGG CAT TGT CAG TAA CC
5–6	F: CCC AGA GAC CTT GGA GAC AA R: TGC TTT CAG TGG AGG GAT CT
6–7	F: GTC AAT GGT GCC ACT GCT C R: AGG GGA ACT GCA CAA CAA CT
7–8	F: CTG CAG TGA TCG TTT CAA GC R: CCC ATG CTT TCA TTG GAC TC
8–9	F: TTT CGA GTC CAA TGA AAG CA R: CCA GCA ACA GAG ATC CAC AA
9–10	F: CCT GAC TTC AGG CTG AGG AG R: TCC CCT TTC AAT CTC TTC CA
10–14	F: AGG CTC CTC AGC CTG AAG TC R: AGT CCA TGG AAG GTC ACA GG

Primers designed previously³ for exon boundaries in *Rpe65* are shown. These primer sets were designed using Web-based Primer3 software for whole-cell amplification of *Rpe65* mRNA exon boundaries (in the public domain at http://biotools.umassmed.edu/bioapps/primer3_www.cgi) with *Rpe65* exon sequences. F, forward; R, reverse.

minescence (ECL Plus; Amersham Biosciences, Piscataway, NJ, USA) as previously described.⁵

Fluorescence In Situ Hybridization

Mice were killed at P120 and their eyes enucleated. In situ hybridization was performed as per manufacturer's instructions (QuantiGene ViewRNA ISH Tissue 2-Plex Assay; Affymetrix, Santa Clara, CA, USA). The eyes were fixed in a 1:4 dilution of 16% (wt/vol) paraformaldehyde (Electron Microscopy Sciences) in PBS for 24 hours. Eyes were dehydrated and embedded in paraffin (TissuePrep2; Fisher Scientific). Paraffin-embedded eyes were sectioned at 5- μ m thickness and baked on StarFrost microscope slides (Mercedes Medical, Sarasota, FL, USA) in a hybridization oven at 60°C for 24 hours before in situ hybridization. RNA in situ hybridizations were performed (QuantiGene ViewRNA ISH Tissue 2-Plex Assay kit; Affymetrix) according to manufacturer's protocol. Probes specific for *Rpe65* (Affymetrix product no. VB6-14045) and β -actin (Affymetrix product no. VB1-10350) mRNAs were purchased from Affymetrix for the assay. Nuclei were counterstained with YO-PRO-1 (Invitrogen). Slides were mounted in Vectashield Hard Set (Vector Laboratories, Burlingame, CA, USA) and imaged at $\times 240$ (with a $\times 60$ lens) magnification via confocal microscopy.

RNA Structure Prediction

Predicted secondary centroid structures from mRNA sequences of wild-type, rd12, R91W, and tvrm148 mouse alleles were generated using global RNA structure prediction algorithms (RNAfold v1.4 software; Institute for Theoretical Chemistry, University of Vienna, Vienna, Austria).^{36–38}

Linear Sucrose Gradient Fractionation

Retinal pigment epithelium/choroid tissue was isolated from three P60 mice and pooled together per sample. Tissue was

isolated from three wild-type and three rd12 litters. Retinal pigment epithelium/choroid was dounce-homogenized and incubated at 4°C for 15 minutes to arrest polysome migration in either a polysome-preserving gradient buffer (10 mM Tris, pH 7.5, 100 mM KCl, 100 μ g/mL cycloheximide, 5 μ L/mL RNase inhibitor [Applied Biosystems, Warrington, UK], 5 mM MgCl₂) or a polysome-disrupting buffer that contained 10 mM EDTA instead of MgCl₂ in gradient buffer.^{39–41} Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) was then added to 1% (wt/vol) final concentration and allowed to incubate on ice for 15 minutes to lyse cells. Cellular debris was removed by centrifugation at 20,000g for 30 minutes. Supernatant was loaded on top of a 15% to 45% (wt/vol) linear sucrose gradient for fractionation of mono- and polyribosomes.^{39–41} Sucrose gradients were centrifuged at 39,000g for 90 minutes at 4°C in an SW41 rotor. Ten 1-mL fractions from each gradient were collected into microfuge tubes using a gradient fractionator (Isco, Lincoln, NE, USA). RNA was isolated in Trizol (Invitrogen) as previously described, and *Rpe65* amplified by quantitative RT-PCR (qRT-PCR) using specific primers (Qiagen) and cycle values evaluated against a standard curve^{39–41} (Supplementary Fig. S3).

Statistical Analysis

Graphs were constructed by computer (Microsoft Excel 2008; Microsoft, Inc., Redmond, WA, USA). All graphs represent data as mean $\pm$ SD. Visual acuity, ERG, and morphometric data were analyzed by two-way repeated measures ANOVA (RM-ANOVA) with post hoc Student-Newman-Keuls testing (SigmaPlot 12; Systat, San Jose, CA, USA). qRT-PCR data were analyzed by one-way ANOVA with post hoc Student-Newman-Keuls testing (SigmaPlot 12; Systat). Sample sizes are reported in the results, and each sample group contained mice from at least three different litters unless otherwise noted.

RESULTS

Complementation Test

rd12/rd12 mice were bred to KO/KO mice to determine whether the rd12 and KO alleles complemented each other as measured by OKT, a behavioral measure of visual acuity.^{20,21} KO/KO, rd12/rd12, and KO/rd12 mice had significant reductions in visual acuity compared with +/+ mice at P120 (Fig. 1). There were also significant differences in OKT response among KO/KO, rd12/rd12, and KO/rd12 mice, but not between rd12/rd12 and KO/rd12 mice (Fig. 1). Retinoid analysis of rd12/rd12 mouse eyes showed no detectable amounts of 11-*cis*-retinal or all-*trans*-retinal (Table 2) but showed increased all-*trans*-retinyl ester content (23- to 24-fold +/+ levels), consistent with previous reports of KO/KO,¹ rd12/rd12,² and tvrm148/tvrm148^{3,13} mice.

Visual Acuity

Because the results of the complementation test suggested the rd12 allele might not inherit in a recessive manner, longitudinal OKT measurements were taken to test whether the rd12 allele was inherited in a dominant fashion and caused an earlier loss of visual function than the KO allele. Figure 2 shows that the rd12 allele was inherited in a semidominant manner. Both +/+ and KO/+ mice maintained high visual acuity (0.383 ± 0.001 c/d and 0.381 ± 0.002 c/d, respectively) from P30 until the end of the study at P210 (Fig. 2). rd12/+ mice, on the other hand, had a small but significant reduction in visual acuity compared with +/+ mice at P30 (0.354 ± 0.003 c/d; $P < 0.001$ by two-way RM-ANOVA; Fig. 2) that gradually decreased during the

FIGURE 1. The *Rpe65* knockout and rd12 alleles did not complement. Knockout/rd12 offspring bred from KO/KO and rd12/rd12 parental mice did not have restoration of vision. OKT measurements in P120 +/+ ($n = 11$), KO/KO ($n = 9$), rd12/rd12 ($n = 14$), and KO/rd12 ($n = 20$) are shown. Knockout/KO, rd12/rd12, and KO/rd12 mice had significantly reduced visual acuity measures compared to +/+ mice. Knockout/rd12 and rd12/rd12 mice had significantly reduced responses compared to KO/KO mice but did not differ with respect to one another. ** $P < 0.001$ compared with +/+. Data are presented as mean $\pm$ SD.

study until P210 (0.299 ± 0.005 c/d; $P < 0.001$). Knockout/KO mice exhibited a significant reduction in visual acuity (0.317 ± 0.004 c/d; $P < 0.001$, Fig. 2) compared with +/+ mice at P30 that became undetectable by the end of the study at P210. rd12/rd12 mice had a similar visual acuity compared with KO/KO mice at P30 (0.303 ± 0.003 c/d; $P = 0.145$) but lost all measurable visual acuity by P90 (Fig. 2), much earlier than the KO/KO mice. Knockout/rd12 mice had significantly lower visual acuities than KO/KO mice at all time points tested (except at P210, when all homozygous mutant mice had no detectable visual acuity). In summary, rd12/rd12 and KO/rd12 lost visual acuity early, with their progressive visual losses occurring faster than KO/KO mice. Because a single rd12 allele makes the disease progress faster than the KO mutation, we surmise that the rd12 mutation exhibits at least some dominant characteristics.

ERG Measurements of Homozygous Mutant Mice

Scotopic and photopic ERG measurements were conducted on KO/KO, rd12/rd12, and KO/rd12 mice at P30, P60, and P90 to test whether ERG measurements reflected OKT findings. Electroretinography responses from KO/KO and rd12/rd12 mice were largely consistent with previously published reports.^{1,2} Both rd12/rd12 and KO/rd12 mice had scotopic b-

FIGURE 2. The rd12 allele caused visual acuity loss in a semidominant fashion. Mice harboring at least one copy of the rd12 allele lost visual function at earlier ages than mice that did not harbor the rd12 allele. Visual acuities of +/+ (solid line with diamond points; $n = 8-13$), KO/+ (dotted line with square points largely hidden behind the solid line; $n = 8$), rd12/+ (dotted and dashed line with circle points; $n = 14-21$), KO/KO (dashed line with square points; $n = 6-9$), rd12/rd12 (dotted line with asterisk points; $n = 7-16$), and KO/rd12 mice (dashed line with circle points; $n = 20$) from P30 to P210 are represented on the same graph. ** $P < 0.001$ +/+ compared with rd12/+, ** $P < 0.001$ KO/KO compared with rd12/rd12, oo $P < 0.001$ KO/rd12 compared with rd12/rd12, significance determined through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls testing. There was no difference between +/+ and KO/+ between P30 and P210, and visual acuity measures of the two strains overlap through the duration of the study. Data are presented as mean $\pm$ SD.

wave amplitude reductions compared with KO/KO mice at P60 (Fig. 3B). None of these differences was large (but statistically significant) among rd12/rd12, KO/KO, and KO/rd12, but rd12/rd12 trended toward a consistently lower ERG response at most light levels. Although there were some differences between KO/KO, rd12/rd12, and KO/rd12 mice, all mutant mice were approximately 10^3 - to 10^4 -fold less sensitive to light than wild-type counterparts. Raw ERG traces are shown in Supplementary Figure S4. Mice with one copy of the rd12 mutation had only slight reductions in a-wave (Supplementary Fig. S5A), b-wave (Supplementary Fig. S5B), and SOPAs (Supplementary Fig. S5C), but these reductions were statistically significant at certain time points. Lamb and Pugh a-wave

TABLE 2. Retinoid Levels in +/+, KO/KO, and rd12/rd12 Mice

Genotype*	11- <i>cis</i> -Retinal, pmol/Eye	All- <i>trans</i> -Retinal, pmol/Eye	Retinyl Ester Content, pmol/mg Protein
+/+	233.3 ± 125.9	94.1 ± 24.9	$54.1 \pm 34.1\ddagger$
KO/KO	None detected†	None detected	$1314 \pm 235.8\ddagger$
rd12/rd12	None detected†	None detected	$1261 \pm 394.5\ddagger$

Knockout/KO mice and rd12/rd12 mice do not have detectable levels of 11-*cis*-retinal. Levels of 11-*cis*-retinal, all-*trans*-retinal, and retinyl esters from +/+, KO/KO, and rd12/rd12 retinas and eyecups are shown. These results show mutant mice have buildup of RPE65 substrate and no detectable amounts of product, implying that the retinoid isomerase is inactive. $n = 3$ for each group. Data presented as mean $\pm$ SD.

* Number of animals analyzed per genotype ≥ 3 .

† The lower limit of detection in this assay was 1 pmol.

‡ The lower limit of detection in this assay was 8.8 pmol.

FIGURE 3. Mutant mice had reduced dark-adapted a- and b-wave amplitudes compared with +/+ mice from P30 to P90. Mice that were either homozygous or compound heterozygous for the *rd12* allele trended toward a slightly faster progressive loss of ERG amplitudes than KO/KO mice. Responses from +/+ mice are shown to provide perspective to response losses observed in mutant mice (solid line to the left of mutant mouse responses). (A) Dark-adapted a-wave amplitudes from KO/KO (solid line; $n = 8, 9, 9$ at P30, P60, and P90, respectively), *rd12/rd12* (long dashed line; $n = 7, 7, 6$ at P30, P60, and P90, respectively), and KO/*rd12* (short dashed line; $n = 16, 19, 19$ at P30, P60, and P90, respectively) are shown at P30, P60, and P90. (B) Dark-adapted b-wave amplitudes from KO/KO (solid line), *rd12/rd12* (long dashed line), and KO/*rd12* (short dashed line) are shown at P30, P60, and P90. Based on ERG responses, mutant mice were approximately 10^2 - to 10^4 -fold less sensitive to light than wild-type mice. * $P < 0.05$, ** $P < 0.001$ KO/KO compared with *rd12/rd12*; # $P < 0.05$, ## $P < 0.001$ KO/KO compared with KO/*rd12*; ° $P < 0.05$, °° $P < 0.001$ KO/*rd12* compared with *rd12/rd12*, significance determined through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls testing. Data are represented as mean $\pm$ SD.

modeling indicated no significant reductions in phototransduction kinetic parameters in *rd12/+* mice as compared to either +/+ or KO/+ mice (Supplementary Fig. S6).

Histology and Morphometrics

Retina architecture was compared qualitatively by retina cross sections in +/+, KO/KO, and *rd12/rd12* mice to determine if there were any overt changes in retina architecture or organization between KO/KO and *rd12/rd12* mice (Fig. 4). Retina architecture was relatively preserved in both mutant strains compared with +/+ mice despite some slight retinal

thinning and gaps in the outer segment (OS) (Fig. 4). Quantitative measurements of outer nuclear layer (ONL) and OS thicknesses (Fig. 5) were taken from the retina cross sections. There were no significant differences of ONL or OS thicknesses between KO/KO and *rd12/rd12* mice at either P60 or P210 (Fig. 5). Knockout/KO and *rd12/rd12* mice did not have significant reductions in ONL thickness compared with +/+ mice at P60 ($49.4 \pm 3.7 \mu\text{m}$, $52.3 \pm 5.1 \mu\text{m}$, and $51.2 \pm 3.1 \mu\text{m}$, respectively) but did by P210 when compared with +/+ mice ($41.6 \pm 3.0 \mu\text{m}$, $39.9 \pm 3.7 \mu\text{m}$, and $56.3 \pm 3.6 \mu\text{m}$, respectively). Knockout/KO and *rd12/rd12* mice had signifi-

FIGURE 4. Knockout/KO and rd12/rd12 mice had a thinner retina than +/+ mice. Knockout/KO and rd12/rd12 mice had similar retinal morphology, with only slight retinal thinning compared with wild-type mice at P210. Bright-field images of retina cross sections stained with toluidine blue from +/+, KO/KO, and rd12/rd12 mice are shown at P60 (A–C) and P210 (D–F). Representative images from retina 1 mm superior of the optic nerve are shown. IS, inner segment; INL, inner nuclear layer; GCL, ganglion cell layer.

cant reductions in OS thicknesses compared with +/+ mice at both P60 ($21.3 \pm 1.9 \mu\text{m}$, $23.2 \pm 3.1 \mu\text{m}$, and $31.4 \pm 4.3 \mu\text{m}$, respectively) and P210 ($19.4 \pm 3.0 \mu\text{m}$, $21.5 \pm 4.3 \mu\text{m}$, and $29.0 \pm 3.8 \mu\text{m}$, respectively). rd12/rd12 mice also did not have any significant reductions in ONL nuclear density with respect to KO/KO mice (Supplementary Fig. S7).

rd12/rd12 mice had a slight, but significant, reduction in the number of cone nuclei compared with KO/KO mice in the superior portion of retinal cross sections at P60 (Fig. 6A), despite similar overall ONL thicknesses (Fig. 5). rd12/rd12 mice had 2.0 ± 0.8 cones per 200- μm field in the superior central portion of the retina compared with 4.6 ± 0.9 cones per field in KO/KO mice ($P = 0.04$) and 11.1 ± 1.6 cones per field in +/+ mice ($P < 0.001$) at P60. rd12/rd12 mice had 5.8 ± 1.7 cones per field in the superior peripheral portion of the retina compared with 7.6 ± 1.0 cones per field in KO/KO mice ($P = 0.04$) and 12.8 ± 2.8 cones per field in +/+ mice ($P < 0.001$) at P60. There were no significant differences in cone nuclei number between KO/KO and rd12/rd12 mice at P210 (Fig. 6B).

RPE65 Protein Levels in rd12 Mice

RPE65 protein was not detected in soluble (Fig. 7A) or insoluble RPE/choroid fractions (Fig. 7B) isolated from rd12/rd12 mice. The 44-amino acid antigen peptide (a synthetic positive control) was 4.7 kDa in size, the expected size of the

truncated protein that could be produced from the nonsense mutant allele. No band of this size was detected (detection limit of 2.5% wild-type RPE65 protein levels; Supplementary Fig. S8) in rd12/rd12 mice (Fig. 7A). Furthermore, preliminary data indicated there was no significant upregulation of endoplasmic reticulum-associated stress markers (*Xbp1*, *Hspa5*, *Ddit3*) in the RPE of +/+, KO/KO, or rd12/rd12 mice (data not shown).

Rpe65 mRNA Level in rd12 Mice

Rpe65 mRNA was present at $0.007\% \pm 0.003\%$ of +/+ levels in KO/KO mice, but surprisingly, *Rpe65* mRNA was present at $70.9\% \pm 26.9\%$ of +/+ levels in rd12/rd12 mice (Fig. 8A). Separation of RPE cells into nuclear and cytoplasmic fractions and relative quantification of mutant mRNA (Fig. 8B) in +/+ and rd12/rd12 mice indicated the mutant mRNA was not sequestered in the nuclei of rd12/rd12 mice. rd12/rd12 mice had $67.4\% \pm 18.5\%$ of wild-type *Rpe65* mRNA levels in the cytoplasm ($P = 0.003$) and $101.1\% \pm 43.2\%$ of wild-type *Rpe65* mRNA levels in the nucleus ($P = 0.959$).

Splicing of Mutant mRNA

All *Rpe65* exons amplified in both rd12/rd12 and +/+ mice and the levels of Rpe65 expression for each amplicon are presented in Supplementary Table S1; across all exons that were amplified, it was found that on average, rd12/rd12 mice expressed *Rpe65* mRNA at 63.8% wild-type levels. Deoxyribonucleic acid sequencing of qRT-PCR products showed no cryptic splice sites, skipped exons, or other mutations with the exception of the nonsense mutation that was previously reported² (Supplementary Fig. S9).

Predicted Rpe65 mRNA Secondary Structures

RNAfold^{36–38} was used to predict the centroid structures that wild-type, rd12, R91W, and tvrm148 alleles could adopt (Fig. 9). The predicted rd12 mRNA centroid structure was virtually identical to the predicted wild-type mRNA structure; tvrm148 and R91W predicted mRNA structures deviated more from wild-type mRNA than the rd12 structure (Fig. 9). Both wild-type and rd12 mRNA structures were predicted to have large regions of both single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA; Fig. 9).

RNA Fluorescence In Situ Hybridization (FISH) of Rpe65 mRNA

In situ hybridization of *Rpe65* mRNA in rd12/rd12 mice qualitatively showed the mutant mRNA localized in similar patterns compared with +/+ mRNA in the RPE cell alone (Fig. 10B). *Rpe65* mRNA had staining throughout the RPE cell, with mRNA being preferentially located in the basal regions of the cytoplasm in both +/+ and rd12/rd12 mice (Fig. 10B). Knockout/KO mice showed no *Rpe65* mRNA in any cell by in situ hybridization (Fig. 10A).

Impaired Association of Mutant mRNA With Ribosomes

We measured the ability of mutant mRNA to be actively translated by fractionating RPE/choroid cytoplasmic extracts into ribosome-free messenger ribonucleoprotein (mRNP)-, monoribosome-, and polyribosome-containing fractions by linear sucrose gradient centrifugation (Supplementary Fig. S10).^{39–41} Under conditions that we used,^{39–41} fractions near the top of the gradient contained mRNP (fractions 1–3) and

FIGURE 5. Knockout/KO and rd12/rd12 had similar ONL (A, B) and OS (C, D) thinning with age. Outer nuclear layer and OS thicknesses were not significantly different between KO/KO and rd12/rd12 at either P60 (A, C) or P210 (B, D). Quantitative measurements of ONL and OS thicknesses were taken at 500-μm intervals superior and inferior of the optic nerve at P60 and P210 in +/+ ($n = 17$ at P60, $n = 12$ at P210, solid line), KO/KO ($n = 18$ at P60, $n = 9$ at P210, dotted line), and rd12/rd12 ($n = 7$ at P60, $n = 18$ at P210, dashed line) mice. * $P < 0.05$, ** $P < 0.001$ +/+ compared with rd12/rd12; # $P < 0.05$, ## $P < 0.001$ KO/KO compared with rd12/rd12, significance determined through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls testing. Data are represented as mean $\pm$ SD.

FIGURE 6. Mice with *Rpe65* mutations had large reductions in the number of cone nuclei. rd12/rd12 mice lost more cones on the superior portion of the retina than KO/KO mice at the same age. Cone nuclei were counted 250 μm, 1000 μm, and 2000 μm superior and inferior of the optic nerve in a 200-μm segment of the retina at P60 (A) and P210 (B). ** $P < 0.001$ +/+ compared to rd12/rd12, * $P < 0.05$ KO/KO compared with rd12/rd12, significance determined through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls testing. Data are presented as mean $\pm$ SD.

FIGURE 7. rd12/rd12 mice do not accumulate detectable amounts of RPE65 protein. rd12/rd12 mice did not have detectable amounts of a truncated RPE65 peptide fragment in soluble or insoluble fractions (detection limit defined in Supplementary Fig. S6). (A) Immunoblotting for RPE65 protein in the soluble RPE protein fraction; 1 μ g of soluble +/+ RPE/choroid protein extract, 50 ng of the 44-amino acid antigen, 10 μ g of soluble KO/KO RPE/choroid protein extract, and 10 μ g of soluble rd12/rd12 RPE/choroid protein extract were loaded. Secondary-only controls are shown. (B) Immunoblotting for RPE65 protein in urea-soluble extracts with 8-day exposure to film; 20 μ g protein were loaded in all lanes. Nonspecific binding of primary antibody to molecular weight ladder (Fig. 8A) was noted but was likely an artifact of nonspecific antibody being raised during the polyclonal antibody production.

monoribosomes (where mRNAs are not actively translated; fraction 4) would be translationally dormant, whereas fractions near the bottom of the gradient contained polyribosomes (fractions 5–10).^{39–41} Potential outcomes of this experiment and the interpretation of these outcomes are described in detail in Fig. 11. Most mutant *Rpe65* mRNAs were detected within mRNP-containing fractions (fractions 1–3), whereas most wild-type *Rpe65* mRNAs were present within polyribosome-containing fractions (fractions 5–10, Fig. 12A). On EDTA treatment, polyribosomes were dissociated into subunits,^{39–41} and most

mutant and wild-type mRNAs were released into mRNP fractions (fractions 1–3, Fig. 12B). Approximately 80% of *Rpe65* mRNA is found in mRNP-containing fractions (fractions 1–3, $P < 0.05$). Mutant rd12 *Rpe65* mRNA is found in amounts $174.9\% \pm 9.1\%$ wild-type levels in fractions 1 to 3 (Fig. 13). If rd12 mRNA were actively translating a truncated peptide, most mRNA would be found in fractions 4 to 5 or mostly found in fraction 4 if translation was stalled during the pioneer round of translation (there was only a trend toward significance, $P = 0.106$). Also, if mutant rd12 mRNA contained an internal ribosomal entry site

FIGURE 8. rd12 mutant *Rpe65* mRNA was exported to the cytoplasm. rd12/rd12 mice expressed appreciable *Rpe65* mRNA that was exported to the cytoplasm. (A) +/+, KO/KO, and rd12/rd12 *Rpe65* whole-cell mRNA levels. (B) Cells from RPE/choroid from P60 +/+ and rd12/rd12 mice were fractionated into their cytoplasmic and nuclear components and the RNA extracted for qRT-PCR. *Rpe65* mRNA was present in similar levels in the nuclear fraction. * $P < 0.05$, ** $P < 0.001$ significance tested through Student's unpaired *t*-test. Data are presented as mean $\pm$ SD, $n = 10$.

FIGURE 9. rd12 *Rpe65* mRNA had a predicted secondary structure that closely resembled wild-type mRNA. Aside from an extra bubble (the size of a single base pairing) in the centroid structure (indicated by arrowhead), wild-type and rd12 mRNA structures were predicted to be identical. Predicted centroid structures of *Rpe65* mRNA from wild-type, rd12, R91W, and tvrm148 alleles. Predicted mRNA secondary structures from wild-type and mutant mouse *Rpe65* alleles predicted rd12 mRNA may adopt a structure that more closely resembled wild-type *Rpe65* mRNA than other *Rpe65* alleles.

(IRES), then there would be greater amounts of rd12 mRNA contained in actively translating polyribosomes found in fractions 5 to 10; instead we see very little mRNA contained in those fractions in rd12/rd12 mice. Polyribosome profiles of β -actin in +/+ and rd12/rd12 RPE were similar, suggesting that the lack of *Rpe65* mRNA observed in actively translating fractions was not the result of RNA degradation in fraction samples (Supplementary Fig. S11). As a result, it appears as if most mutant rd12 mRNA is found in mRNP fractions and may not efficiently bind ribosomes for translation.

DISCUSSION

Complementation tests of the rd12 allele with the KO allele showed that the rd12 mutation was in the *Rpe65* gene (Fig. 1).

Also, the rd12 allele induced visual function loss in a semidominant fashion (as illustrated most clearly in the OKTs); the semidominant inheritance was also indicated by several other metrics, including ERGs, histology, and morphometrics, but these effects seem to be more subtle (although consistent and statistically significant). The ERG, histology, and morphometrics data are consistent with previous work with these mice that has been reported in the literature across multiple generations and multiple institutions, making it unlikely the semidominant effects in OKT in mice carrying the rd12 mutation are the result of epigenetic inheritance or genetic drift among colonies. Our laboratory also routinely genotypes our mouse strains for the rd1, rd8, and rd10 mutations, and none of the mouse strains used in these studies harbored these mutations. Granted, there is a remote possibility that the effects we attribute to the rd12 mutation are the result of

FIGURE 10. Ribonucleic acid in situ hybridization showed similar localization of *Rpe65* mRNA in both +/+ and rd12/rd12 RPE cells. There were no noticeable differences in the localization of rd12 mRNA with respect to wild-type mRNA. In situ hybridizations for *Rpe65* (green), β -actin (red) mRNAs, and nuclei (blue; YO-PRO 1 iodide) from paraffin sections taken in +/+, KO/KO, and rd12/rd12 mice. (A) Knockout/KO mice had no detectable *Rpe65* mRNA hybridization. (B) *Rpe65* mRNA was found to localize throughout the cell in similar patterns in +/+ and rd12/rd12 mice, with much of the signal localized around the basal regions of the RPE. Signal was readily apparent in both the nucleus and cytoplasm of the RPE.

another mutation tightly linked to the *Rpe65* locus, but given the lack of genes that are in close proximity to *Rpe65* (based on genetic and sequence maps of chromosome 3), we find this possibility unlikely. We also think it unlikely the variation in visual acuity between mice harboring the KO and rd12 mutations is the result of artifact, as the use of OKT as an accurate, reproducible form of visual function measurement^{42–50} is increasing in the visual research field. Thus, the inheritance pattern in rd12/rd12 is more complex than classic autosomal recessive, and we call it semidominant. This complexity extends to the heterozygous states as well: KO/rd12 mice resembled the more severely affected rd12/rd12 mice and not KO/KO mice with respect to visual acuity (Figs. 1, 2), scotopic a-wave amplitudes (Fig. 3A), and scotopic b-wave amplitudes (Fig. 3B). A single copy of the rd12 allele was sufficient to drive visual deficits in rd12/+ mice as measured by visual acuity (Fig. 2), scotopic a-wave amplitudes (Supplementary Fig. S5A), scotopic b-wave amplitudes (Supplementary Fig. S5B), and SOPA (Supplementary Fig. S5C), but the effect was small (but consistent, and at certain points, significant). We speculate that it is possible the rd12/+

mice have some small degree of cone nuclei loss that could account for the trending loss of ERG amplitudes.

The rd12 mutation abolishes 11-*cis*-retinol product formation, and resulted in retinyl palmitate (substrate) buildup, as is expected when a mutation causes a loss of retinoid isomerase activity (a loss of function). However, the rd12 allele also exerts an additional semidominant action that harms vision in a way that may not be directly related to the visual cycle. Knockout/KO and rd12/rd12 mice have both been reported to be null for 11-*cis*-retinal generation,^{1,2} and modeling of phototransduction kinetics from scotopic a-wave recordings indicated rod photoreceptors in rd12/+ mice had similar maximal a-wave responses (Supplementary Fig. S6A), time delays between flash stimuli and photoreceptor response (Supplementary Fig. S6B), and time constants (a measure of rod sensitivity^{28–30}; Supplementary Fig. S6C) when compared with KO/+ mice, suggesting that phototransduction in rd12/+ mice was normal. Because KO/KO mice possessed no measurable amounts of 11-*cis*-retinal and rd12/+ mice were capable of regenerating functional rhodopsin to the same level as KO/+ mice, these data suggested the separate semidominant action by the rd12 allele might be unrelated to retinoid processing.

FIGURE 11. Possible outcomes of polyribosome profiling. The goal of this experiment was to determine whether the mutant rd12 *Rpe65* mRNA could be bound by ribosomes, and if so, how many ribosomes bind. Messenger ribonucleic acid-bound species were separated on a linear sucrose gradient, followed by isolation of RNA and qRT-PCR of *Rpe65* mRNA from each fraction. (A) Sucrose gradient fractionation separated RNA binding protein (RBP)-bound RNAs into three possible species: mRNP-bound RNAs (green circles), monoribosome-bound RNAs (black lines with one blue oval), and polyribosome-bound RNAs (black lines with multiple blue ovals). The first three fractions (1–3) contained RNA species mostly bound by mRNPs; the fourth fraction (4) contained RNA species mostly bound by multiple ribosomes. The more ribosomes bound a single mRNA molecule, the further the species migrated down the gradient during centrifugation. (B) Because there were detectable amounts of full-size RPE65 protein from +/+ mice, it would be expected that most wild-type *Rpe65* mRNA would be found in fractions 5 to 10 (this is in fact what was observed). There were four possible outcomes for the mutant mRNA in the cytoplasm: (1) active translation of a full-size peptide (which would require ribosomes to read through the PTC, require an IRES, or another sequence that would allow read through or suppression of termination in the mutant mRNA); (2) inefficient initiation of translation (the mRNA would be bound only by mRNPs, which we observed in rd12/rd12 mice); (3) stalled translation (a single ribosome would stall on the PTC during the pioneer round of translation); and (4) active translation of a truncated peptide (because of the short length of the read frame in the mutant mRNA with the PTC, only one or two ribosomes would bind to the mRNA at a single time). If there were active translation of full-size RPE65 peptides, most of the mRNA would be in fractions 5 to 10 (as was the case in +/+ mice). If there was no (or inefficient) initiation of translation, most mRNA would be found in fractions 1 to 3 (as was the case in rd12/rd12 mice). If there was active translation of a truncated RPE65 peptide in rd12/rd12 mice, most mRNA would be found in fractions 4 to 5.

Both KO/KO and rd12/rd12 mice have been previously reported to have a mostly intact retina architecture despite severe visual function loss,^{1,2} and our findings were consistent with these previous results (Fig. 4). Quantitative measurements of ONL and OS thicknesses in KO/KO and rd12/rd12 mice indicated rd12/rd12 mice did not experience an earlier retina

thinning than KO/KO mice (Fig. 5). Although the overall degrees of retina morphology changes were comparable in KO/KO and rd12/rd12 mice, rd12/rd12 mice lost cone photoreceptor nuclei earlier than KO/KO mice on the superior side of the retina (Fig. 6A). As cone photoreceptors may express *Rpe65* in mice,⁵¹ we speculate that possible expres-

FIGURE 12. rd12 *Rpe65* mRNA was mostly found in ribosome-free mRNPs. Unlike wild-type mRNA, mutant rd12 mRNA was mostly contained in ribosome-free mRNP fractions. (A) Cytoplasmic extracts of RPE/choroid were separated by linear sucrose gradient centrifugation to yield mRNP fractions (1–3), monoribosome fractions (4), and polyribosome fractions (5–10). (B) Mono- and polyribosomal complexes disrupted by the addition of EDTA result in all *Rpe65* mRNA being present in mRNP-containing fractions.

sion of the rd12 allele in the M-cone may directly exert a slightly toxic action on cone photoreceptors. It is also possible the RPE defects produced by the rd12 allele may have some sort of effect on bystander effect on other cells involved in the visual cycle, such as rods, cones, or Müller cells. We tested this hypothesis by looking for *Rpe65* mRNA by FISH (Fig. 10). We detected it only in RPE cells. There was no signal in cones, rods, or other cells in the neural retina. It is possible that *Rpe65* mRNA is expressed at much lower levels in cones and that this level might be below our limit of detection, however.

Previous speculation about the lack of RPE65 protein staining in the RPE suggested that the nonsense rd12 mutation might be subject to degradation by NMD.² This is logical, given that many RNA surveillance pathways, such as Staufen-mediated mRNA decay,⁵² NMD,^{52,53} no-go mRNA decay,⁵³ and nonsense-associated altered splicing^{54,55} exist in the cell to prevent the potential production of a truncated protein with a deleterious effect by a premature termination codon (PTC)-containing mRNA.⁵³ Unfortunately, this speculation was based on immunocytochemistry data that made use of an antibody that recognized an epitope (PETLET) downstream of the nonsense mutation²; even if a truncated protein was produced in rd12/rd12 mice, this antibody² would not be capable of detecting it. However, the absence of anti-PETLET immunoreactivity importantly shows there is no IRES following the rd12 stop codon (at position 44) in the *Rpe65* mRNA.² Significantly, a truncated peptide fragment was not detected in our study with the use of an N-terminal-specific RPE65 antibody (Fig. 7) despite abundant *Rpe65* mRNA being present in rd12/rd12 mice (Fig. 8A).

This study demonstrated that the mutant rd12 mRNA is transcribed (Fig. 8A), spliced (Supplementary Table S1), exported from the nucleus to the cytoplasm (Fig. 8B), and trafficked within the cell normally (Fig. 10), despite having a 29% reduction in mRNA. Deoxyribonucleic acid sequencing of

FIGURE 13. *Rpe65* mRNA is mostly bound by mRNPs in rd12/rd12 mice. The area under the wild-type and rd12 curves in fractions 1 to 3 in the polyribosome profiles of *Rpe65* mRNA were added together to generate the average amount of mRNA present in the mRNP fractions. Because three mRNA-bound species (mRNP-bound, monoribosome-bound, and polyribosome-bound mRNAs) are separated by linear sucrose gradient fractionation in this assay, the areas under the polyribosome curves were added together for each species. Mutant mRNA was much more enriched in mRNP-containing fractions and greatly reduced in actively translating polyribosome fractions in rd12 mice compared with wild-type. There were no significant differences between wild-type and rd12 mice in monoribosome-bound *Rpe65* mRNAs, which could be due to either insufficient resolution in this experiment or because the mutant mRNA is still capable of binding monoribosomes. **P* < 0.05, significance tested through Student's unpaired *t*-test. Data are presented as mean $\pm$ SD.

FIGURE 14. Proposed model for the foundation of the negative semidominant effects exerted by the rd12 allele on visual function. The rd12 allele is (a) transcribed, (b) spliced, (c) processed, and (d) exported to the cytoplasm like the wild-type allele. Once in the cytoplasm, the mutant mRNA is (e) trafficked to the ribosomes much like the wild-type mRNA. Unlike the wild-type mRNA, though, the mutant mRNA (f) becomes sequestered on mRNPs and are unable to bind to ribosomes for the first round (often called the pioneer round) of translation when nonsense RNA surveillance pathways could recognize the rd12 mRNA and degrade it. Consequently, (g) the mutant mRNA-mRNP complexes accumulate and form the foundation for currently unknown downstream mechanisms that cause visual dysfunction in a semidominantly inheriting fashion.

PCR products amplified from mutant mRNA (Supplementary Fig. S9) indicated there are no cryptic splice sites, skipped exons, or any sequence change whatsoever in the mutant mRNA besides the nonsense mutation previously reported². The mutant mRNA is still capable of being trafficked normally, because the localization of the mutant mRNA in the cytoplasm does not differ from wild-type (Fig. 10) but does not efficiently engage with actively translating polyribosomes (Fig. 12). Because there is no appreciable staining of mRNA in extracellular spaces in either +/+ or rd12/rd12 mice, it is also reasonable to conclude that the mutant mRNA is not exerting a semidominant effect on vision because of action in the extracellular space. Because RNA surveillance pathways require nonsense mutation-containing mRNAs to directly interact with ribosomes and stall at the premature stop codon, the sequestration of the mutant mRNA in rd12 mice from ribosomes (Fig. 12A)⁵⁶ may suggest a possibility for the mutant mRNA to evade NMD by evading the pioneer round of translation.

This hypothesis is consistent with previous work attempted to restore visual function in rd12 mice by treating them with gentamicin and G418, which were both being tested for their abilities to allow ribosomes to read through premature stop codons and restore protein function.¹⁹ Although it was found that S334ter-4 rats (rats with a nonsense mutation in the rhodopsin gene) were amenable to treatment with both gentamicin and G418, rd12 mice were resistant to treatment.¹⁹ We speculate that the reason the rd12 mutant was resistant to gentamicin and G418 treatment was because the mRNA was not bound to ribosomes for readthrough of the premature stop codon. We speculate mRNP sequestration of rd12 *Rpe65* mRNA forms the foundation for the semidominant effects on visual function exerted by the rd12 allele (Fig. 14). Future work

could use RNAseq techniques to investigate whether or not the mRNP sequestration of mutant mRNA has an effect on the translation of other mRNAs in the RPE; rd12/+ mice could be examined to test whether the mutant *Rpe65* mRNA interferes with translation of wild-type *Rpe65* mRNA (which could in turn provide clues as to why rd12/+ mice lose visual acuity).

Other mechanisms might be at play with respect to the rd12 allele. Fragile X-associated tremor ataxia syndrome (FXTAS),⁵⁷ a gain-of-function RNA-mediated disorder, has been shown to be mediated by an *FMR1* transcript containing a poly-CAG nucleotide expansion⁵⁸ present in patients with the fragile X syndrome premutation.^{59,60} These toxic *FMR1* transcripts can be found in nuclear inclusion bodies; the repeat expansion is able to sequester RNA-binding proteins in the nucleus,⁵⁸ and this sequestration reduces the pool of splicing factors needed for appropriate splicing of other transcripts,⁵⁸ leading to the semidominantly inherited negative effects seen in FXTAS patients. By analogy, we wonder if it is possible that the mutant rd12 mRNA could exert its effects through sequestration of RNA-binding proteins in the cytoplasm. One such *FMR1* mutation, the FMRP^{1304N} mutation, can sequester mRNAs on mRNPs,³⁹ but to the best of our knowledge, this is the only other known mutation that mirrors our observations. There is precedent in the vision research literature for posttranscriptional mRNA dysregulation as playing a role in visual dysfunction. Tudor Domain Containing 7-deficient mice have defects in posttranscriptional mRNA dysregulation in lens cells,⁶¹ although it should be noted that mRNAs are mistrafficked in the cytoplasm in these mice, an observation we did not see in rd12 mice.

Another possible mechanism for the semidominantly inheriting negative effects seen in mice carrying the rd12 mutation is activation of the innate immune response by RNA

sensors within the RPE. Recent work with Alu RNA in human RPE⁶²⁻⁶⁴ cells indicates Alu RNA can induce an innate immune response through activation of the inflammasome via MyD88.⁶³ Although Alu RNA was found to induce an innate immune response through MyD88 in a Toll-like receptor (TLR)-independent fashion,⁶³ it is possible that the mutant *Rpe65* mRNA could be recognized by ssRNA and dsRNA sensors TLR3 and TLR7/8,⁶⁵ respectively. Future work could involve breeding rd12/rd12 mice to *MyD88*-, *TLR*-, *Nlrp3*-, and *Ifnar*-deficient mice to test for an expected slower loss of visual acuity as in the KO/KO mouse, as the toxic responses of the rd12 mouse would be interrupted by eliminating the mediating inflammasome or interferon pathways.

At the time the rd12 mouse was isolated, the R44X mutation had yet to be identified in humans. Since then, both KO and rd12 mice have been used to develop gene therapy treatments for LCA2 patients,⁶⁶ and some of these treatments have since moved to clinical trials,⁶⁷⁻⁷⁰ where they have been shown to be both safe and effective up to 3 years after treatment⁷¹⁻⁷⁴ despite continuing retinal degeneration.⁷⁵ Recently, the nonsense R44X mutation was identified in humans (refSNP cluster report: rs368088025). The work presented in this study might suggest the patients and presumably unaffected family members of LCA2 patients who are known to carry a single copy of the RPE65^{R44X} mutation may need to be monitored for abnormalities in visual function. The identification of the human R44X mutation makes further study of the rd12 mouse an attractive prospect for the study of LCA2 in a laboratory setting. The rd12/rd12 and rd12/+ mice are models that offer a rare opportunity for future research into RNA-mediated visual deficits, and the implications of future work on the mechanism of action of the rd12 allele may have direct impacts on patients seen in the clinic.

Acknowledgments

We thank the reviewers of this manuscript for the use of their comments and edits and for the opportunity to improve the clarity of our writing so that it may be better understood by the reader.

Supported by National Institutes of Health Grants P30EY006360, R01EY016470, T32EY007092, R01EY014026, R01EY016435, R01EY021592, R01NS070526, and Z01EY000444; an unrestricted Departmental Award from Research to Prevent Blindness; the Intramural Research Program of the National Eye Institute; and a grant from the Abraham J. and Phyllis Katz Foundation. The authors alone are responsible for the content and writing of the paper.

Disclosure: C.B. Wright, None; M.A. Chrenek, None; W. Feng, None; S.E. Getz, None; T. Duncan, None; M.T. Pardue, None; Y. Feng, None; T.M. Redmond, None; J.H. Boatright, None; J.M. Nickerson, None

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