Supplemental Rationale: Flow of experimental rationale

Green arrows denote the experimentally supported conclusions in the study. “Y” refers to “yes,” and “N” refers to “no” for the support or rejection of the hypotheses, respectively.

A complementation test determined whether the rd12 mutation resides in the Rpe65 locus (Fig. S1A) and whether it is inherited in an autosomal recessive or autosomal dominant manner (Fig. S1B). Results presented in this study determined the level at which the mutation manifested itself (i.e. RNA or protein) and where in the cell the mutation manifested itself (Fig. S1C).

Supplemental Results: Validation of commercially available Rpe65 primers for qRT-PCR

Commercially available primers specific for Rpe65 were purchased from Qiagen (product number QT00140140). Primer specificity was validated by amplifying Rpe65 mRNA from P60 +/- RPE/choroid whole-cell RNA extract and subcloning the PCR product into a pDRIVE vector (PCR Cloning^Plus Kit, Qiagen). The pDRIVE vector containing the putative Rpe65 PCR product was then sequenced using T7 forward primers for each sequencing reaction (Eurofins MWG Operon, Louisville, KY). DNA sequences were compared to wild type mRNA sequence through BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi). DNA sequencing chromatograms are shown below in Fig. S2. It should be noted that the Qiagen company website advertises that the commercially available Rpe65 primers amplify the exon 12-13 boundary. Our results indicate that the primers are specific to the Rpe65 exon 13-14 boundary instead (as labeled in Fig. S2).
Supplemental Methods: qRT-PCR standard curve

*Rpe65* cDNA was prepared using qRT-PCR with primers specific to *Rpe65* (Qiagen) on whole RNA extracted from +/+ mouse RPE/choroid. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and DNA concentration was calculated by measuring absorbance at 260 nm. Quantitative PCR was performed on eight ten-fold serial dilutions of the purified *Rpe65* cDNA at the indicated concentrations. Cycle number (the point at which the amplification curve crossed threshold) was plotted against RNA concentration, and a four parameter, sigmoidal logarithmic curve of best fit was calculated in SigmaPlot 12.0 (Systat). The logarithmic curve of best fit is presented alongside the standard curve in Fig. S3. This standard curve was used to calculate the amount of *Rpe65* mRNA present in the linear sucrose gradient fractions presented in the text in Fig. 12.

Supplemental Results: ERG traces from genotypes used in this study

Raw average P60 ERG traces recorded at a flash intensity of 137 cd×s/m² from +/+, KO/+; rd12/+, KO/KO, rd12/rd12, and KO/rd12 mice are shown in Fig. S4. ERG traces from +/+, KO/+, and rd12/+ mice look similar despite a slightly smaller response from rd12/+ mice. ERG traces from KO/KO, rd12/rd12, and KO/rd12 mice look similar as well.
Supplemental Results: ERG measurements of heterozygous mutant mice

Scotopic and photopic ERG measurements were taken on +/+ (n=9, 5, 6 at P60, P120, and P180, respectively), KO/+ (n=8, 7, 6 at P60, P120, and P180, respectively), and rd12/+ (n=18, 15, 10 at P60, P120, and P180, respectively) mice at P60, P120, and P180 to determine if only one copy of the rd12 allele was sufficient to drive retinal function loss. rd12/+ mice had mild but statistically significant reductions in scotopic a-wave amplitudes when compared to +/+ and KO/+ mice at P60, P120, and P180 (Fig. S5A). rd12/+ mice also trended toward slightly reduced summed oscillatory potential amplitudes (SOPAs) when compared to +/+ and KO/+ mice at P60 (Fig. S5C) at dim flash intensities, but not P120 (Fig. S5C; \( P = 0.161 \)) or P180 despite trending toward significance (\( P = 0.052 \), Fig. S5C). rd12/+ mice had somewhat reduced scotopic b-wave amplitudes at P120 (Fig. S5B) and P180 (Fig. S5B). Despite reaching statistical significance at some time points, in general and for the most part, rd12/+ mice had only slight reductions in ERG responses. All mice had a progressive loss of amplitude with age but the loss was slightly more pronounced in rd12/+ mice. * \( P < 0.05 \), ** \( P < 0.001 \) +/+ compared to rd12/++; # \( P < 0.05 \), ## \( P < 0.001 \) KO/+ compared to rd12/++; o \( P < 0.05 \), oo \( P < 0.001 \) +/+ compared to KO/+, significance determined through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls testing. Data are presented as mean ± SD.

Supplemental Results: rd12/+ mice have normal rod phototransduction kinetics

Hood and Birch formulation of the Lamb and Pugh phototransduction activation models generated by dark-adapted a-wave responses in +/+ (solid line), KO/+ (dotted line), and
rd12/+ (dashed line) are shown in Fig. S6. Maximal a-wave responses (R_{m,PII}, Fig. S6A),
time delay between flash and rod response (t_{d}, Fig. S6B), and phototransduction cascade
time constant (S, Fig. S6C) are shown at P60, P120, and P180. Significance tested
through two-way repeated measures ANOVA with post hoc Student-Newman-Keuls
testing. Phototransduction parameters in the three strains do not differ significantly. Data
are presented as mean ± SD.

Supplemental Methods: DNA sequencing of \textit{Rpe65} mRNA
qRT-PCR with primers specific to overlapping regions of \textit{Rpe65} mRNA isolated from
+/+ and rd12/rd12 mice (Table 1, main text) was performed to create DNA samples for
sequencing. \textit{Rpe65} mRNA expression in rd12/rd12 mice is shown relative to levels in +/+ mice. N=5 for each genotype. DNA sequences of amplified regions were identical
between rd12/rd12 and +/+ mice, with the exception of the nonsense mutation (a single
nucleotide change) present in mutant mice in exon 3 (Fig. S8). Statistical significance
determined through student’s unpaired t-test, \textit{P} values of rd12/rd12 compared to +/+ are
listed in the table. These results are consistent with \textit{Rpe65} mRNA seen in whole-cell
extracts (i.e. that rd12/rd12 mice have a 30-50% reduction in mRNA levels compared to
+/+ mice, Fig. 9). The samples were sequenced using the forward primers from each
amplification reaction (Eurofins MWG Operon, Louisville, KY). +/+ and rd12/rd12
samples were compared through BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi).

Supplemental Results: There are no significant differences in ONL density between
KO/KO and rd12/rd12 mice.
ONL density of +/-, KO/KO, and rd12/rd12 mice at P60 and P210 were measured by counting the number of nuclei that are found directly on a line moving orthogonally through the RPE/choroid every 500 µm superior and inferior of the optic nerve on toluidine blue-stained retina cross sections. There were no significant differences in ONL density between KO/KO and rd12/rd12 mice at either P60 (+/+ n=22, KO/KO n=10, rd12/rd12 n=7) or P210 (+/+ n=10, KO/KO n=9, rd12/rd12 n=11; Fig. S7), but there was a slightly higher ONL nuclear density at P210 in rd12/rd12 mice compared to KO/KO mice that did not reach statistical significance. +/- mice had a significantly denser ONL at P210 than either KO/KO or rd12/rd12 mice (P <0.05).

Supplemental Results: The N-terminal specific RPE65 antibody can detect as little as 2.5% wild type levels of RPE65 peptide. Serial dilutions of wild type RPE/choroid protein extract were separated by SDS-PAGE and stained with the N-terminal specific antibody used in this study (Fig. S8). Assuming 100% wild type expression at 20 µg of RPE/choroid protein extract, as little as 2.5% that amount is detectable with the antibody.

Supplemental Results: Rpe65 mRNA from rd12/rd12 mice contains only one mutation. DNA sequencing results from Rpe65 mRNA isolated from +/- and rd12/rd12 mice showed the rd12/rd12 mice had only one mutation, a C to T nucleotide substitution (c.130C>T) that created a premature termination codon (PTC; Fig. S9, below). This transition mutation results in an R44X nonsense mutation.
Supplemental Rationale: Linear sucrose gradient fractionation. Cycloheximide treatment of homogenate pauses translation with any ribosomal proteins (blue ovals; Fig. S10) left bound to mRNA so that centrifugation can separate mRNA molecules based on how many ribosomes are bound to them. During centrifugation in the sucrose gradient, mRNA molecules bound by multiple ribosomes will sediment more than mRNAs bound by one or two ribosomes. mRNAs bound by no ribosomes will remain near the top of the gradient, but are bound by ribosome-free messenger ribonucleoproteins (mRNPs; green circles; Fig. S10) instead. When the fractions are drawn out of the gradient, absorbance is measured at 254 nm. A higher absorbance corresponds to a greater amount of protein-bound mRNA.

Supplemental Results. Polyribosome profiles of β-actin are similar between wild type and rd12 mice. Polyribosome profiles of β-actin are similar in both wild type and rd12 mice (Fig. S11), indicating that other mRNAs are not affected by the inefficient binding of Rpe65 mRNA to ribosomes in rd12 mice. This also suggests the lack of Rpe65 mRNA observed in actively-translating ribosome fractions in rd12 mice (main text) is not the result of RNA degradation of samples.
Supplemental Figure 1. Flowcharts of hypotheses and experiments in this study.

A

Purpose
Test if the rd12 mutation is in Rpe65.

Hypothesis
The rd12 resides in the Rpe65 gene.

Conclusion
The rd12 mutation is in the Rpe65 gene.

B

Purpose
Test if the rd12 allele has a recessive mode of inheritance.

Hypothesis
The rd12 allele is recessive.

Conclusion
The rd12 mutation is dominant.

Result
YES

Result
NO
Purpose
Determine the molecular level where the mutation's effects manifest

Hypothesis
The rd12 allele produces a toxic truncated peptide

Conclusion
The mutation acts on the RNA level

Result
NO

Conclusion
The truncated peptide is toxic

Result
YES

Hypothesis
The mutant mRNA is abnormally processed in the nucleus

Conclusion
The mutation acts in the cytoplasm

Result
YES

Conclusion
The mutant mRNA exerts effects in nucleus

Result
NO

Hypothesis
The mutant mRNA is abnormally trafficked in ribosomes

Conclusion
Ribosomes stall and cannot release mutant mRNA

Result
YES
Supplemental Figure 2: Commercially available primers for mouse *Rpe65* amplify exons 13-14 in the gene.
Supplemental Figure 3: Standard curve of *Rpe65* amplification by qRT-PCR

\[ y = 5.3142 + \frac{24.4641}{1 + (\frac{x}{-1.3991 \times 10^{-4}})^{0.34}} \]
Supplemental Figure 4: Raw ERG traces of genotypes in this study.
Supplemental Figure 5. One copy of the rd12 allele causes small reductions in dark-adapted ERG amplitudes.
Supplemental Figure 6: Lamb and Pugh modeling of a-wave responses show rd12/+ mice have normal rod phototransduction kinetics.
Supplemental Figure 7. There are no significant differences between radial nuclei densities between KO/KO and rd12/rd12 mice.
Supplemental Table 1. All Rpe65 exons were amplified in the mutant rd12 mRNA.

<table>
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<tr>
<th>Exons Amplified</th>
<th>Percent Wild Type Expression</th>
<th>P-Value (compared to +/-)</th>
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<tr>
<td>1-3</td>
<td>63.7 ± 17.8</td>
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<tr>
<td>2-3</td>
<td>65.2 ± 9.3</td>
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<td>3-4</td>
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<td>4-5</td>
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<td>5-6</td>
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<td>6-7</td>
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<tr>
<td>7-8</td>
<td>65.5 ± 9.0</td>
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<tr>
<td>8-9</td>
<td>52.3 ± 12.0</td>
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<tr>
<td>9-10</td>
<td>54.5 ± 9.0</td>
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</tr>
<tr>
<td>10-14</td>
<td>54.7 ± 5.6</td>
<td>0.024*</td>
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</table>
Supplemental Figure 8. The lower limit of detection of the N-terminal specific antibody is 2.5% wild type RPE65 protein level.
Supplemental Figure 9: Chromatograms of DNA sequences from wild type and rd12

Rpe65 mRNA showed a nonsense mutation in rd12/rd12 mice.

### wild type

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### rd12

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Supplemental Figure 10: Linear sucrose gradient fractionation.
Supplemental Figure 11. β-actin polyribosome profiles in +/+ and rd12/rd12 RPE.