

# Temperature-sensitive retinoid isomerase activity of RPE65 mutants associated with Leber Congenital Amaurosis

Received December 7, 2014; accepted January 26, 2015; published online March 9, 2015

Songhua Li<sup>1</sup>, Jane Hu<sup>2</sup>, Robin J. Jin<sup>3</sup>,  
Ashok Aiyar<sup>4</sup>, Samuel G. Jacobson<sup>5</sup>,  
Dean Bok<sup>2</sup> and Minghao Jin<sup>1,\*</sup>

<sup>1</sup>Department of Ophthalmology and Neuroscience Center, Louisiana State University Health Sciences Center, New Orleans, LA 70112 USA; <sup>2</sup>Jules Stein Eye Institute and Department of Neurobiology, University of California, Los Angeles, CA 90095 USA; <sup>3</sup>State University of New York at Buffalo, Buffalo, NY 14214 USA; <sup>4</sup>Department of Microbiology, Immunology and Parasitology, Louisiana State University Health Sciences Center, New Orleans, LA 70112 USA; and <sup>5</sup>Scheie Eye Institute, University of Pennsylvania, Philadelphia, PA 19104 USA

\*Minghao Jin, Neuroscience Center, LSU Health Sciences Center, 2020 Gravier St., New Orleans, LA 70112 USA. Tel: 504-568-2141, email: mjin@lsuhsc.edu

**RPE65 is a membrane-associated retinoid isomerase involved in the visual cycle responsible for sustaining vision. Many mutations in the human RPE65 gene are associated with distinct forms of retinal degenerative diseases. The pathogenic mechanisms for most of these mutations remain poorly understood. Here, we show that three Leber congenital amaurosis-associated RPE65 mutants (R91W, Y249C and R515W) undergo rapid proteasomal degradation mediated by the 26S proteasome non-ATPase regulatory subunit 13 (PSMD13) in cultured human retinal pigment epithelium (RPE) cells. These mutant proteins formed cytosolic inclusion bodies or high molecular weight complexes via disulfide bonds. The mutations are mapped on non-active sites but severely reduced isomerase activity of RPE65. At 30°C, however, the enzymatic function and membrane-association of the mutant RPE65s are significantly rescued possibly due to proper folding. In addition, PSMD13 displayed a drastically decreased effect on degradation of the mutant proteins in the cells grown at 30°C. These results suggest that PSMD13 plays a critical role in regulating pathogenicity of the mutations and the molecular basis for the PSMD13-mediated rapid degradation and loss of function of the mutants is misfolding of RPE65.**

**Keywords:** 26S proteasome non-ATPase regulatory subunit 13 (PSMD13)/retinal degeneration/retinal pigment epithelium/retinoid isomerase/RPE65.

**Abbreviations:** 11cRAL, 11-*cis* retinaldehyde; 11cROL, 11-*cis* retinol; DAPI, 4',6-diamidino-2-phenylindole; DMSO, Dimethyl sulfoxide; DTT, Dithiothreitol; ER, endoplasmic reticulum; HMC, high molecular weight complexes; HPLC, high-performance liquid chromatography; PSMD13, 26S proteasome non-ATPase regulatory subunit 13; RPE, retinal pigment epithelium; SD, standard deviation; UPP, ubiquitin-proteasome pathway.

RPE65 is a key retinoid isomerase (*I–3*) in the visual cycle essential for regenerating 11-*cis* retinaldehyde (11cRAL), the universal chromophore of the visual pigments in both cone and rod photoreceptors. This membrane-associated protein utilizes hydrophobic all-*trans* retinyl esters, such as all-*trans* retinyl palmitate, as its substrate for synthesizing 11-*cis* retinol (*I*, 2). The all-*trans* to 11-*cis* isomerization of retinoid is a rate-limiting step in the visual cycle (4). Mice lacking RPE65 (*Rpe65*<sup>-/-</sup> and *rd12* mice) cannot synthesize 11-*cis* retinoids; therefore, photoreceptors in these mice lose sensitivity to light (5, 6).

More than 70 different missense mutations, including a dominant mutation, in the human *RPE65* gene are associated with retinal degenerative diseases such as Leber congenital amaurosis (LCA), retinitis pigmentosa (RP), and childhood onset retinal dystrophy (Human Gene Mutation Database: www.hgmd.cf.ac.uk/ac/index.php; 7–12). Although most of these mutations have not been studied for their pathogenicity and disease mechanism, some mutations have been shown to abolish isomerase activity of RPE65 (*I*, 3, 13–17). Several mutations have also been shown to reduce expression levels of RPE65 *in vitro* (13–16, 18) and in mouse models (19–21). However, most of these mutations have not been studied for the molecular mechanisms underlying rapid degradation of the mutant proteins. Analysing the molecular pathway leading to rapid degradation of the mutant RPE65s is important for understanding the disease mechanisms of mutations and may lead to the development of a therapeutic intervention for the diseases.

The ubiquitin–proteasome pathway (UPP) is a highly selective proteolytic system that plays a pivotal role in cellular protein quality control by mediating degradation of misfolded proteins (22). Decreased proteasomal activity and proteasome overload are associated with multiple forms of inherited retinal degeneration (23, 24). PSMD13 is a positive regulator of UPP in yeast (25). The mouse PSMD13 is abundantly expressed in the retinal pigment epithelium (RPE; 18), and has been identified as a candidate for a negative regulator of RPE65 (26). We recently have shown that PSMD13 strongly promotes degradation of three disease-associated mutant RPE65s (L22P, T101I and L408P; 18). PSMD13 has been suggested to play a critical role in regulating pathogenicity of these mutations by mediating degradation of misfolded mutant RPE65s (18). Its impact on other RPE65 mutations, however, has not been studied.

Adeno-associated virus mediated therapy trials, which express wild-type RPE65 in patients' RPE, have shown improvement in vision (27–30). However, a subsequent study reported that gene

therapy did not stop the progressive retinal degeneration (31), suggesting that providing enzymatic activity alone may be not enough to block the pathological pathways leading to retinal degeneration. Recent identification of a dominant missense mutation in RPE65 of patients with RP (12) indicates that the mutated RPE65 has a dominant pathogenic effect. Some recessive missense mutations may also result in both loss of function and gain of cytotoxic function due to misfolding, mislocalization and aggregation of mutant RPE65 proteins (13, 14, 18). To prove this, further studies are needed.

Recently, we have shown that some disease-causing RPE65 mutants with mutations in non-active site can be rescued by helping their proper folding (18). In this study, we tried to extend our finding to other pathogenic mutations by testing additional three RPE65 mutants with a R91W, Y249C or R515W substitution. All these mutations are associated with LCA (9, 16, 32–34), the most severe form of retinal degeneration. The R91W substitution is one of the most frequent RPE65 mutations found in patients with LCA (9, 10, 35–38). We investigated these three RPE65 mutants with regard to their potential pathogenic mechanisms and rescue of their enzymatic function.

## Materials and Methods

### Cell culture, transfection and treatment

Cultured primary human RPE, ARPE-19 and 293 T-LC cells were maintained and transfected as described previously (1, 18, 39, 40). Transfected RPE cells were split into a 24-well culture plate without Millicell HA filters and incubated in modified Chee's medium to reduce expression levels of endogenous RPE65 (18, 40). Relative transfection efficiencies were determined by measuring activities of  $\beta$ -galactosidase from pRSV-LacZ (41) co-transfected with a wild-type or mutant RPE65 construct. For low temperature treatment, cells were maintained at 30°C under 5% CO<sub>2</sub>. For chemical treatment, cells were incubated overnight with MG132 (9  $\mu$ M), MG115 (9  $\mu$ M), pepstatin A (5  $\mu$ M) or DMSO in the culture medium.

### Site-directed mutagenesis

PCR using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Inc.) was carried out to introduce a mutation into the pRK5-RPE65 plasmid encoding the human RPE65 (16). Primers used for generating the mutant RPE65 constructs are shown in Table S1 in the Supplementary Material. Following mutagenesis, all sequences of the constructs were confirmed by DNA sequence analysis.

### Preparation of cell membrane pellets

293 T-LC cells expressing wild-type or mutant RPE65 were resuspended in ice-cold 10 mM HEPES buffer containing protease inhibitors (Roche Life Science) and homogenized in a glass-to-glass tissue grinder. This homogenate was used as the total cell lysate. A portion of the homogenates was centrifuged at 400  $\times g$  for 10 min to remove the cell nuclei. The resulting supernatants were centrifuged for 1 h at 100,000  $\times g$  to pellet the membranes. The membrane pellets were resuspended in HEPES buffer containing 0.1% sodium dodecyl sulfate and used as the membrane fraction.

### Analysis of high molecular weight complexes

ARPE-19 cells expressing WT or mutant RPE65 were incubated with 15  $\mu$ M ubiquitin-activating enzyme (E1) inhibitor (UBEI-41) for 16 h. After washing with phosphate buffered saline (PBS), the cells were incubated in a lysis buffer containing or not containing reducing reagents (100 mM dithiothreitol and/or 2% 2-mercaptoethanol) for 30 min at room temperature or for 10 min at 70°C. The cell lysates were then subjected to immunoblot analysis.

### Immunoblot analysis

Protein samples prepared in the lysis buffer with or without the DTT reducing reagent were separated in a 10 or 12% polyacrylamide gel by electrophoresis. The separated proteins were then transferred onto an Immobilon-P membrane (EMD Millipore Co.). The membrane was incubated in blocking buffer, primary antibody and horseradish peroxidase-conjugated anti-rabbit antibody (PerkinElmer Inc.). Antibodies against RPE65 (42), PSMD13 (Proteintech Inc.),  $\beta$ -actin (Sigma-Aldrich Co.) or E-cadherin (Santa Cruz Biotechnology, Inc.) were used as primary antibodies. Immunoblots were visualized with the enhanced ECL-Prime and quantified as described previously (18). To obtain clear chemiluminescence signals in linear range, exposure times of immunoblot membrane in the ImageQuant LAS 4000 (GE Healthcare Bio-Sciences) were adjusted between 5 and 90 s in 5–20 s intervals.

### Immunoprecipitation

Cells expressing RPE65 and PSMD13-Flag were lysed in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 and protease inhibitors. Immunoprecipitation was carried out using an anti-FLAG M2 affinity gel (Sigma-Aldrich Co.) as described previously (43). The precipitated proteins were used for immunoblot analysis in the presence of reducing reagents.

### Knockdown of PSMD13

PSMD13 depletion in ARPE-19 cells was performed by transfecting PSMD13 siRNA (OriGene Technologies Inc.) using PolyJet (SignaGen Laboratories). Scramble siRNA was used as a negative control. The PSMD13 siRNA targets exon 9 of the human PSMD13 gene. Forty-eight hours post transfection, the cells were used for immunoblot analysis.

### Retinoid isomerase assay

The 293 T-LC cells transfected with construct encoding WT or mutant RPE65 were incubated with 5  $\mu$ M of all-*trans* retinol (atROL) for 16 h at 30 or 37°C. Retinoids extracted from the cells were saponified and analysed by normal-phase HPLC as described previously (1, 26).

### Mapping of mutation sites onto the crystal structure of RPE65

The ID (4F2Z) of the bovine RPE65 structure in a lipid environment (44) was obtained from the RCSB Protein Data Bank. Mapping of mutation sites onto a three-dimensional structure of RPE65 was done using the UCSF Chimera program.

### Immunocytochemistry

ARPE-19 cells grown on a glass coverslip were fixed with 4% paraformaldehyde and incubated with 0.2% Triton X-100 in PBS for 15 min. After blocking with 10% fetal bovine serum (FBS) and 2% goat serum in PBS for 1 h, the cells were incubated with RPE65 antibody overnight at 4°C and then with Alexa Fluor 555-conjugated secondary antibody (Thermo Fisher Scientific Inc.) at room temperature for 1 h. Before and after incubating with antibodies, the cells were washed with 0.1% Tween 20 in PBS three times. Nuclei were labeled with DAPI (Sigma-Aldrich Co.). Images were captured with a Zeiss LSM510 Meta confocal microscope with a 40X oil-immersion objective.

## Results

### PSMD13 promoted degradation of mutant RPE65s in the proteasome

As a first step to analyse the disease mechanisms of the three RPE65 mutations (R91W, Y249C and R515W), we compared expression levels of wild-type and the mutant RPE65s in cultured human RPE cells, which retain many of the functional and morphological characteristics of RPE *in vivo* (40, 45–47). Immunoblot analysis of the RPE cells transfected with wild-type or mutant RPE65 construct showed that expression levels of the three mutant RPE65s were less than

30% of wild-type RPE65 (Fig. 1A). We obtained a similar result in ARPE-19 cells (Fig. 1A). Activities of  $\beta$ -galactosidase expressed from the pRSV-LacZ, which was co-transfected with WT or mutant RPE65 construct, were similar in all transfected cells (Fig. 1B), suggesting that the lower expression levels of the mutant RPE65s were due to post-transcriptional degradation rather than lower transfection efficiency of mutant RPE65s.

To identify the degradation pathway, we treated the cells with proteasome inhibitors (MG115, MG132) or a lysosome inhibitor (pepstatin A). Both proteasome inhibitors, but not the lysosome inhibitor, significantly increased expression levels of all three mutant RPE65s (Fig. 1C), suggesting that these mutant proteins are degraded in the proteasomes. To confirm this result, we tested the effects of PSMD13, a positive regulator of the proteasome (25), on expression levels of the mutant RPE65s. As shown in Fig. 2A, co-transfection of PSMD13 further reduced protein levels of the mutant RPE65s. Conversely, siRNA-mediated knockdown of PSMD13 significantly increased expression levels of the mutant proteins (Fig. 2B). Both overexpression and knockdown of PSMD13 exhibited mild effects on expression levels of wild-type RPE65 (Fig. 2A,B), suggesting that PSMD13 preferentially promotes degradation of mutant RPE65s. In support of this result, immunoprecipitation revealed that PSMD13 strongly interacted with mutant RPE65s (Fig. 2C).

#### **E1 Inhibitor increased accumulation of high molecular weight complexes of mutant RPE65s**

To test whether the mutant RPE65s are misfolded and undergo ubiquitination-dependent proteasomal degradation, we treated ARPE-19 cells expressing wild-type or mutant RPE65 with an inhibitor of ubiquitin-activating enzyme (E1). If ubiquitination is essential for degrading misfolded mutant RPE65, inhibition of ubiquitination should result in accumulation of misfolded RPE65. As shown in Fig 3, high molecular weight complexes (HMC) containing mutant RPE65s is significantly increased in the E1 inhibitor-treated cells compared with DMSO-treated cells. This HMC was significantly reduced when the cell lysates were treated with reducing reagent DTT (Fig. 3), suggesting that the mutant RPE65s formed HMC via disulfide bonds.

#### **Low temperature increased isomerase activity of mutant RPE65s**

By mapping the three mutation sites (R91, Y249 and R515) onto the crystal structure of the bovine RPE65, we found that these mutations are located far from the active site (Fig. 4A,B). We, therefore, tested if low temperature, which has been shown to help proper folding of many mutant proteins (18, 43, 48, 49), can rescue isomerase activity of the three mutant RPE65s. As shown in Fig 5, isomerase activities of the mutant RPE65s in the cells maintained at 30°C were increased at least 2.5-fold compared with their activities in the cells incubated at 37°C. The activity of wild-type

RPE65, however, was not increased in the cells maintained at 30°C (Fig. 5).

#### **PSMD13 exhibited a reduced effect on degradation of the mutant RPE65s at 30°C**

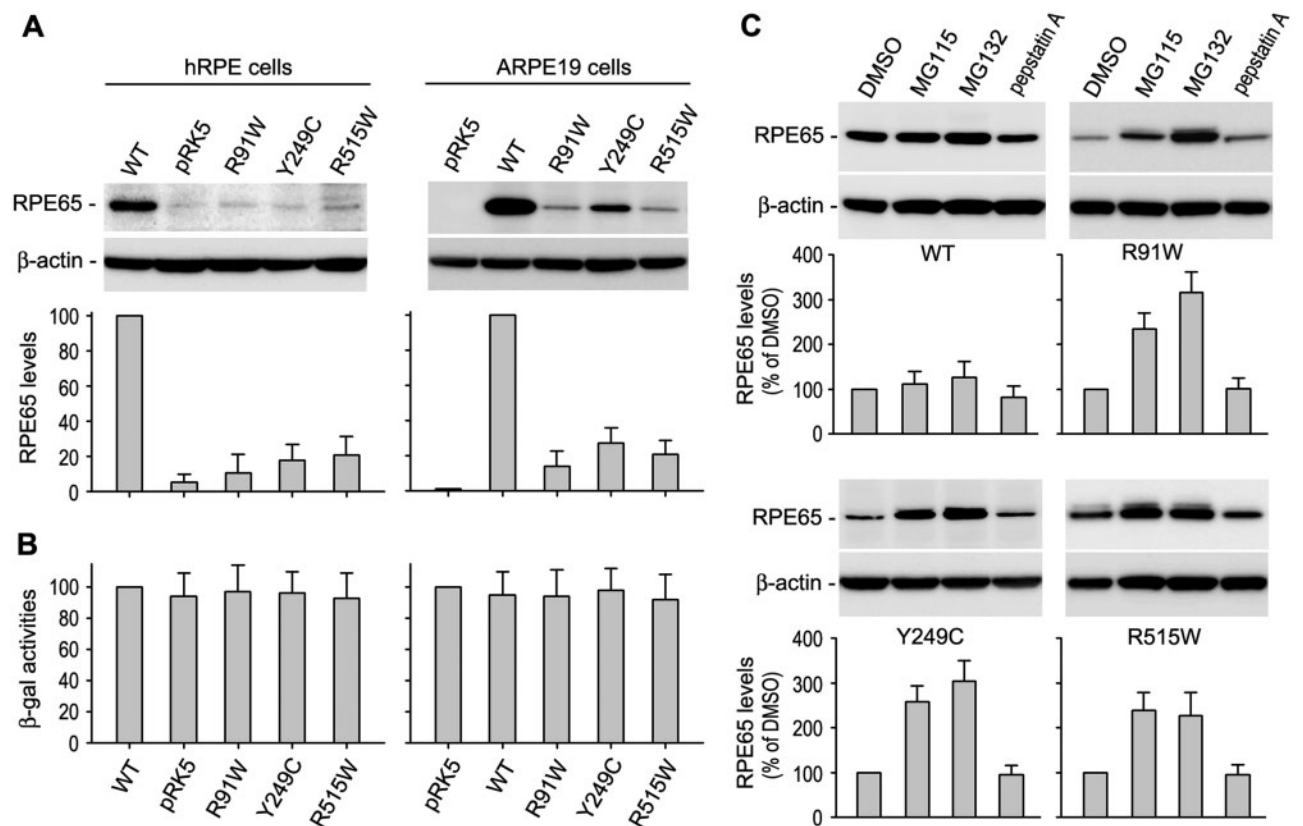
The results described above suggest that low temperature can rescue the non-active site mutant RPE65s by promoting proper folding of RPE65. If PSMD13 mediates degradation of misfolded RPE65 protein, PSMD13 should exhibit a reduced effect on degradation of mutant RPE65s at 30°C. We tested this possibility by co-expressing mutant RPE65 with PSMD13 in ARPE-19 cells grown at 30 and 37°C. At normal temperature, expression levels of the mutant RPE65s in PSMD13-co-transfected cells were reduced at least 60% compared with those in mock vector-co-transfected cells (Fig. 6A). However, expression levels of the mutant RPE65s at 30°C were significantly increased in both cells co-transfected with PSMD13 or mock vector (Fig. 6A). At this low temperature, expression levels of the mutant RPE65s in PSMD13-co-transfected cells were similar to those in mock vector-co-transfected cells (Fig. 6A), suggesting that PSMD13 mediated degradation of misfolded mutant RPE65s and low temperature promoted proper folding of the mutant RPE65s. In support of this possibility, PSMD13 displayed a mild effect on expression levels of wild-type RPE65 at both 37 and 30°C (Fig. 6A).

#### **Low temperature inhibited inclusion body formation of mutant RPE65s**

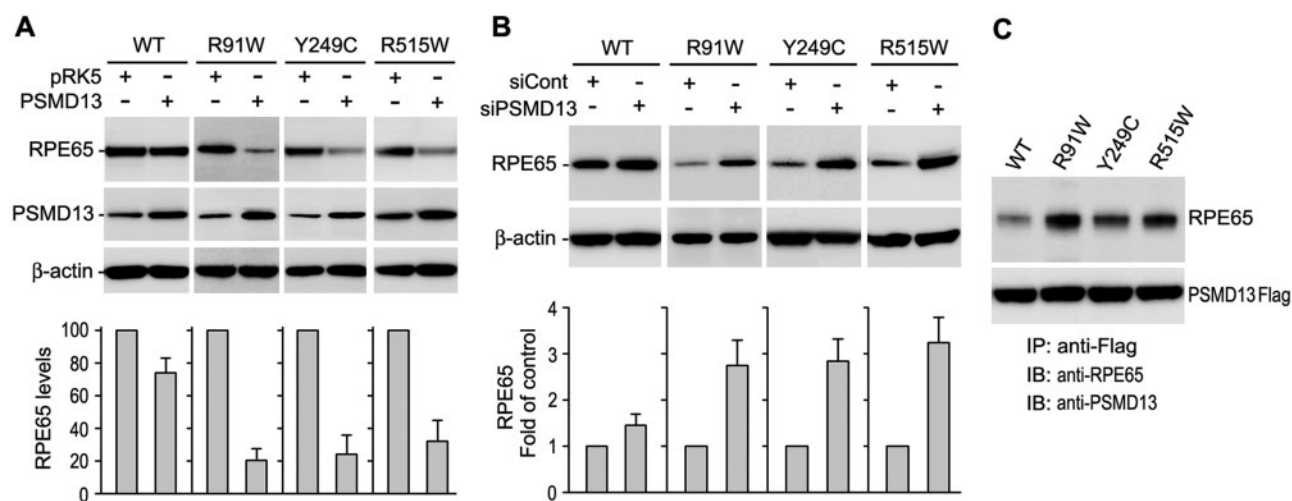
Since the three mutant RPE65s are misfolded at 37°C, we tested whether the mutant RPE65s form aggregates in the cells at 37°C. We expressed the mutant RPE65s in ARPE-19 cells at 37°C and then performed immunocytochemistry. As predicted, the mutant RPE65s formed numerous cytoplasmic inclusion bodies in the cells grown at 37°C (Fig. 6B). To test whether low temperature can reduce the formation of inclusion bodies, we performed the same experiment on the cells maintained at 30°C. Immunocytochemistry showed that cytoplasmic inclusion body formation of the mutant RPE65s were significantly reduced at 30°C (Fig. 6B).

#### **Low temperature promoted association of the mutant RPE65s with membranes**

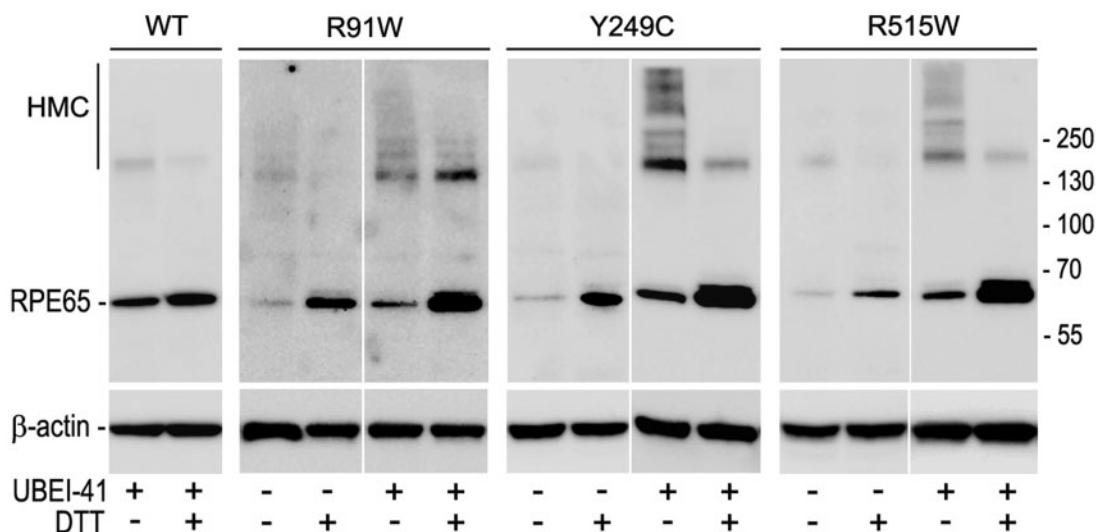
It has been shown that association of RPE65 with membranes is important for its isomerase activity (42, 50–52). We, therefore, tested if low temperature might enhance association of the mutant RPE65s with membranes. We analysed the relative content of wild-type and mutant RPE65s in membrane fractions and total homogenates of 293 T-LC cells grown at 37 or 30°C. Low temperature exhibited a mild effect on association of wild-type RPE65 with membranes (Fig. 7). In contrast, membrane-associated mutant RPE65s were increased 220–340% in the cells maintained at 30°C compared with the cells kept at 37°C (Fig. 7). The three mutant RPE65s were also increased in total homogenates of the cells maintained at 30°C compared with the homogenates of the cells kept at



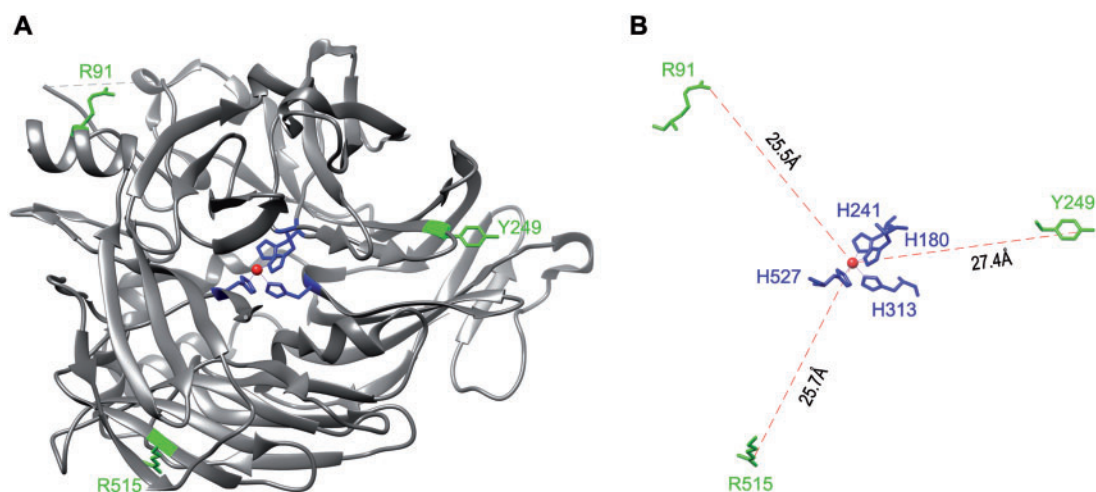
**Fig. 1** Mutant RPE65 proteins are degraded in the proteasome. (A) Immunoblot analysis of RPE65 in primary human RPE (hRPE) and ARPE-19 cells transfected with pRK5 mock vector or construct encoding wild-type (WT) or mutant (R91W, Y249C and R515W) RPE65. Beta-actin was detected as a loading control. Relative intensities of the RPE65 immunoblots were quantified, normalized by the  $\beta$ -actin levels, and expressed as percentage of WT RPE65. (B) Relative  $\beta$ -gal activities from pRSV-LacZ co-transfected with RPE65 constructs into the cells. (C) Immunoblot analysis of WT and the mutant RPE65s in ARPE-19 cells treated with inhibitors of the proteasome (MG115 and MG132) or lysosome (pepstatin A). Relative intensities of the immunoblots were quantified and expressed as percentage of RPE65 in the cells treated with DMSO. All error bars show SD ( $n = 3$ ).



**Fig. 2** PSMD13 mediated degradation of mutant RPE65 proteins. (A) Immunoblot analysis of WT or the indicated mutant RPE65 in ARPE-19 cells co-transfected with pRK5 mock vector or PSMD13 construct. PSMD13 expression was monitored by immunoblot analysis;  $\beta$ -actin was used as a loading control. Relative intensities of the RPE65 immunoblots were quantified and expressed as percentage of WT RPE65 in the histograms. (B) Immunoblot analysis showing increase in expression levels of the mutant RPE65s in ARPE-19 cells co-transfected with PSMD13 siRNA (siPSMD13). Histogram shows relative expression levels of RPE65 in the cells. (C) Immunoprecipitation showing strong interaction of PSMD13 with mutant RPE65 proteins. The 293 T-LC cells expressing PSMD13-Flag fusion protein and WT or mutant RPE65 were immunoprecipitated with a Flag antibody and the precipitates were probed with antibodies against RPE65 or PSMD13.



**Fig. 3** Mutant RPE65s formed high molecular weight complexes (HMC) via disulfide bonds. ARPE-19 cells expressing WT or the indicated mutant RPE65 were treated with inhibitor of ubiquitin-activating enzyme (UBEI-41) and subjected to immunoblot analysis in the presence or absence of reducing reagent (DTT).



**Fig. 4** Mapping of the three mutation sites on the crystal structure of RPE65. (A) A three-dimensional image of the crystal structure of the bovine RPE65. The catalytic site containing  $\text{Fe}^{2+}$  (brown sphere) and four iron-binding histidine residues is in the center of RPE65 structure. The three mutation sites (R91, Y249 and R515) shown in green are mapped in the non-active sites of RPE65. (B) A geometry showing the distances from the iron ion to the mutation sites. H180, H241, H313 and H527 are iron-binding histidine residues.

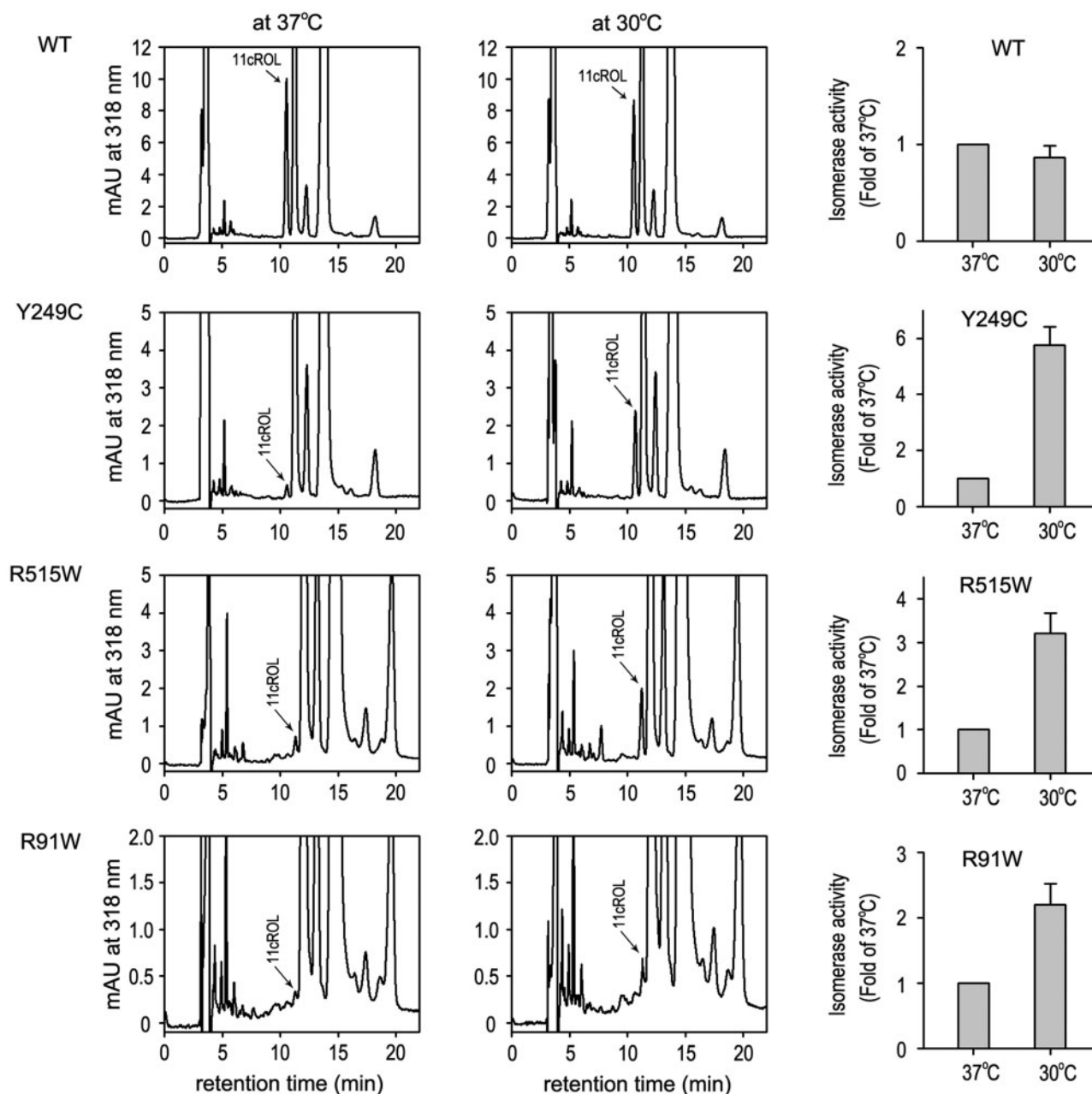
37°C (Fig. 7). However, the increased rates were only 40–80% (Fig. 7), suggesting that membrane-associated mutant RPE65s may be more stable.

## Discussion

Based on our recent study on some disease-causing mutant RPE65s (18), we hypothesized that the majority of pathogenic mutant RPE65s with non-active site mutations can be rescued. The purpose of this study is to consolidate our hypothesis by further analysing additional three RPE65 mutants (R91W, Y249C and R515W) associated with LCA. We observed that all three mutant RPE65s undergo rapid proteasomal degradation most likely due to misfolding. This misfolding induced rapid degradation appears to be a common

and critical molecular basis for their loss of enzymatic function. Consistent with this possibility, retinoid isomerase activity of the three mutant RPE65s was significantly rescued at low temperature, which inhibited degradation of the mutant proteins possibly by helping their proper folding.

RPE65 is an abundant protein in the RPE (53, 54). It has been estimated to account for 10% of total microsomal proteins in the bovine RPE (53). In contrast, its catalytic activity as the retinoid isomerase is significantly lower than those of other visual cycle enzymes (4, 55), partially due to expression of its negative regulators in the RPE (26). The high abundance of RPE65, therefore, is necessary for compensating for its low enzymatic activity in the RPE. In this study, we observed that expression levels of the three



**Fig. 5** Low temperature dependent increase in isomerase activity of mutant RPE65s. Chromatograms of retinoids extracted from 293 T-LC cells expressing WT or the indicated mutant RPE65 at 37°C or 30°C. Peak of 11-*cis* retinol (11cROL) is marked by an arrow. The histograms show relative enzyme activity of WT and the mutant RPE65s at the indicated temperatures. All error bars show SD ( $n = 3$ ).

LCA-associated mutant RPE65s are at least 70% less than that of wild-type RPE65 in both primary human RPE and ARPE-19 cells (Fig. 1A). This lower expression level is one of the reasons causing loss of RPE65 function. Treatment of the cells with proteasome inhibitors (MG115 and MG132), but not a lysosome inhibitor (pepstatin A), significantly increased expression levels of the mutant RPE65s (Fig. 1C), suggesting that the decreased protein levels are mainly due to rapid degradation of mutant proteins in the proteasome. Consistent with the result, co-expression of PSMD13, a positive regulatory subunit of the proteasome (25), further reduced expression levels of the mutant RPE65s (Fig. 2A) most likely through

enhancing the proteolytic function of the proteasome. In support of this possibility, knockdown of PSMD13 significantly increased expression levels of the mutant proteins (Fig. 2B). These results suggest that PSMD13 mediates degradation of the mutant RPE65s in the proteasome of the cells.

Importantly, PSMD13 appears to mediate degradation of misfolded, but not properly folded, RPE65. In yeast, PSMD13 homolog (Rpn9) interacts with multi-ubiquitin receptor (25). Deletion of Rpn9 results in accumulation of multiubiquitinated proteins, suggesting that PSMD13/Rpn9 mediates proteasomal degradation of multiubiquitinated proteins via direct or indirect interaction (25). In this study, we observed

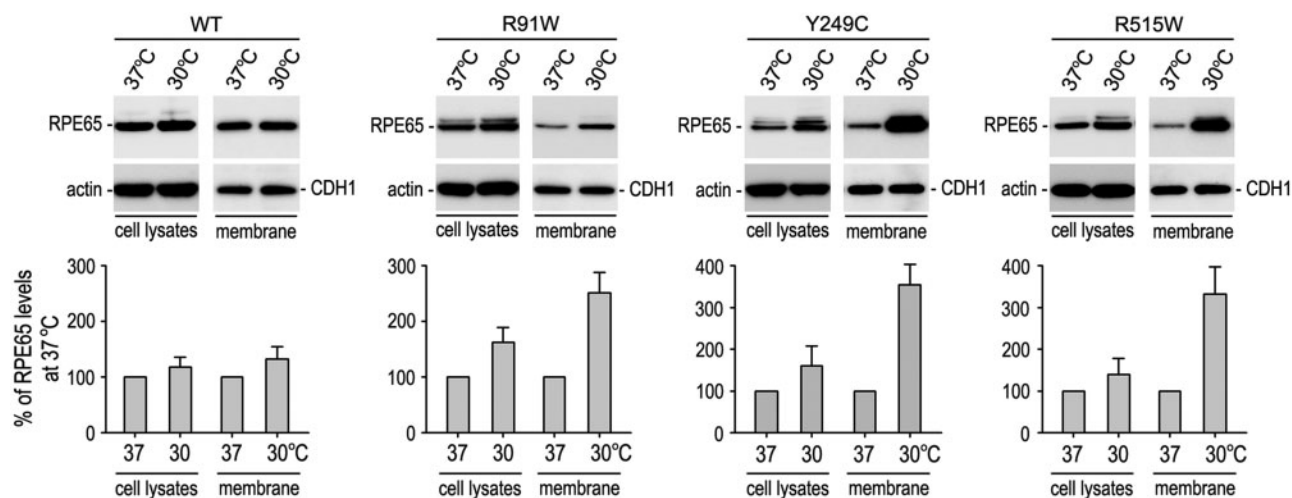


**Fig. 6** Decrease in PSMD13-mediated degradation and inclusion body formation of the mutant RPE65s at low temperature. (A) ARPE-19 cells co-transfected with RPE65 and pPSMD13 or pRK5 were grown at 37 or 30°C and subjected to immunoblot analysis using antibodies against RPE65 or  $\beta$ -actin. Histograms show relative expression levels of WT and the mutant RPE65 proteins in the pPSMD13- or the mock vector-co-transfected cells grown at 37 or 30°C. (B) Representative confocal microscopic images of ARPE-19 cells transfected with WT or mutant RPE65 construct. The cells were incubated at 37 or 30°C, and stained with a RPE65 antibody. Scale bars denote 10  $\mu$ m.

that PSMD13 strongly interacted with mutant RPE65 proteins (Fig. 2C), which might be ubiquitinated due to misfolding (Fig. 3). We also observed that the three RPE65 mutants formed numerous cytoplasmic inclusion bodies (Fig. 6B), suggesting that the mutant RPE65s are misfolded. The interaction of PSMD13 with mutant RPE65s might mediate degradation of the misfolded mutant proteins in the proteasome at normal temperature. At 30°C, however, PSMD13 exhibited a significantly reduced effect on degradation of these mutant RPE65s (Fig. 6A). At this low temperature, not only inclusion body formation is reduced (Fig. 6B) but also enzymatic function of the mutant RPE65s is significantly rescued (Fig. 5), suggesting that low temperature promoted proper folding of these mutant RPE65s. In addition, PSMD13 exhibited a mild effect on degradation of wild-type RPE65 at both 30 and 37°C (Figs 2, 6A). These results suggest that PSMD13 plays an important role in protein

quality control by mediating degradation of misfolded protein. Formation of the HMC and cytoplasmic inclusion bodies (Figs 3 and 6B) suggests that the mutant RPE65 proteins may cause acute or chronic cellular stress. PSMD13 is, therefore, a critical player in eliminating or reducing the cytotoxic effect of misfolded mutant RPE65s.

We recently suggested that whether a mutation is mapped on the active or near the active site is a critical factor in determining if the enzymatic function of the mutant RPE65 can be rescued at low temperature (18). As expected, the isomerase activities of the three mutant RPE65s, whose mutation sites are localized on the non-active sites (Fig. 4), are significantly increased at 30°C (Fig. 5). Inclusion body formation of these mutant RPE65s is also significantly reduced at 30°C (Fig. 6B). These results agree with the effects of low temperature on reversing cellular mislocalization and rescuing the function of some other mutant



**Fig. 7 Increased membrane-association of the mutant RPE65s at low temperature.** Total cell lysates and membrane fractions of 293 T-LC cells expressing WT or mutant RPE65 were analyzed by immunoblot analysis using antibodies against RPE65,  $\beta$ -actin or E-cadherin (CDH1). Histograms show relative contents of RPE65 proteins in the indicated fractions of cells maintained at 37 versus 30°C. Contents of RPE65 in the cell lysates and membrane fractions were normalized by  $\beta$ -actin or CDH1 levels, respectively. Error bars show SD ( $n = 3$ ).

proteins (43, 48, 49). Myocilin and the interphotoreceptor retinoid-binding protein (IRBP) are secretory proteins. However, the glaucoma-causing mutant myocilin and the retinitis pigmentosa-associated IRBP cause endoplasmic reticulum (ER) stress due to misfolding and subsequent accumulation in the ER (43, 56). At low temperature, these mutant proteins can be secreted and the ER stress is significantly reduced (43, 49). Similarly, we recently showed that low temperature not only rescued the isomerase activity but also reduced aggregate formation of three other disease-causing mutant RPE65s (18).

Previous studies have shown that association of RPE65 with membranes is important for its substrate-binding and isomerase function (42, 50–52, 57). Misfolding and subsequent mislocalization of the mutant RPE65s might disrupt their membrane-association, substrate-binding and enzymatic function. We observed that low temperature significantly increased association of the three mutant RPE65s with membranes (Fig. 7). This increased membrane association is most likely due to proper folding and might contribute to the increased isomerase activities of the mutant RPE65s at low temperature (Fig. 5).

Although experimental confirmation is needed, our results strongly suggest that many pathogenic mutant RPE65s with non-active site mutations can be rescued for their enzymatic function by physical and chemical approaches. Since the majority of disease-causing missense mutations in RPE65 are non-active site mutations, this study provides important clues for further investigation of the pathogenic mechanism and functional rescue strategy for these mutant RPE65s associated with retinal degenerative diseases.

## Supplementary Data

Supplementary Data are available at *JB* Online.

## Funding

This work was supported by grants from National Institutes of Health (EY021208 to M. J.; EY017280 to S. G. J.; GM103340 to Louisiana State University Neuroscience COBRE facilities), Macula Vision Research Foundation (to D. B. and to S.G.J.), Research to Prevent Blindness (to Louisiana State University Department of Ophthalmology) and Louisiana Lions Eye Foundation (to Louisiana State University Department of Ophthalmology). DB holds the Dolly Green Chair at the David Geffen School of Medicine, University of California, Los Angeles.

## Conflict of Interest

None declared.

## References

- Jin, M., Li, S., Moghrabi, W.N., Sun, H., and Travis, G.H. (2005) Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. *Cell*. **122**, 449–459
- Moiseyev, G., Chen, Y., Takahashi, Y., Wu, B.X., and Ma, J.X. (2005) RPE65 is the isomerohydrolase in the retinoid visual cycle. *Proc. Natl. Acad. Sci. USA*. **102**, 12413–12418
- Redmond, T.M., Poliakov, E., Yu, S., Tsai, J.Y., Lu, Z., and Gentleman, S. (2005) Mutation of key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle. *Proc. Natl. Acad. Sci. USA*. **102**, 13658–13663
- Winston, A. and Rando, R.R. (1998) Regulation of isomerohydrolase activity in the visual cycle. *Biochemistry*. **37**, 2044–2050
- Redmond, T.M., Yu, S., Lee, E., Bok, D., Hamasaki, D., Chen, N., Goletz, P., Ma, J.X., Crouch, R.K., and Pfeifer, K. (1998) Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat. Genet.* **20**, 344–351
- Pang, J.J., Chang, B., Hawes, N.L., Hurd, R.E., Davisson, M.T., Li, J., Noorwez, S.M., Malhotra, R., McDowell, J.H., Kaushal, S., Hauswirth, W.W., Nusinowitz, S., Thompson, D.A., and Heckenlively, J.R. (2005) Retinal degeneration 12 (rd12): a new, spontaneously arising mouse model for human Leber congenital amaurosis (LCA). *Mol. Vis.* **11**, 152–162



7. Gu, S.M., Thompson, D.A., Srikumari, C.R., Lorenz, B., Finckh, U., Nicoletti, A., Murthy, K.R., Rathmann, M., Kumaramanickavel, G., Denton, M.J., and Gal, A. (1997) Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy. *Nat. Genet.* **17**, 194–197
8. Marlhens, F., Bareil, C., Griffoin, J.M., Zrenner, E., Amalric, P., Eliaou, C., Liu, S.Y., Harris, E., Redmond, T.M., Arnaud, B., Claustres, M., and Hamel, C.P. (1997) Mutations in RPE65 cause Leber's congenital amaurosis. *Nat. Genet.* **17**, 139–141
9. Morimura, H., Fishman, G.A., Grover, S.A., Fulton, A.B., Berson, E.L., and Dryja, T.P. (1998) Mutations in the RPE65 gene in patients with autosomal recessive retinitis pigmentosa or leber congenital amaurosis. *Proc. Natl. Acad. Sci. USA.* **95**, 3088–3093
10. Thompson, D.A., Gyurus, P., Fleischer, L.L., Bingham, E.L., McHenry, C.L., Apfelstedt-Sylla, E., Zrenner, E., Lorenz, B., Richards, J.E., Jacobson, S.G., Sieving, P.A., and Gal, A. (2000) Genetics and phenotypes of RPE65 mutations in inherited retinal degeneration. *Invest. Ophthalmol. Vis. Sci.* **41**, 4293–4299
11. Lotery, A.J., Namperumalsamy, P., Jacobson, S.G., Weleber, R.G., Fishman, G.A., Musarella, M.A., Hoyt, C.S., Heon, E., Levin, A., Jan, J., Lam, B., Carr, R.E., Franklin, A., Radha, S., Andorf, J.L., Sheffield, V.C., and Stone, E.M. (2000) Mutation analysis of 3 genes in patients with Leber congenital amaurosis. *Arch. Ophthalmol.* **118**, 538–543
12. Bowne, S.J., Humphries, M.M., Sullivan, L.S., Kenna, P.F., Tam, L.C., Kiang, A.S., Campbell, M., Weinstock, G.M., Koboldt, D.C., Ding, L., Fulton, R.S., Sodergren, E.J., Allman, D., Millington-Ward, S., Palfi, A., McKee, A., Blanton, S.H., Slifer, S., Konidari, I., Farrar, G.J., Daiger, S.P., and Humphries, P. (2011) A dominant mutation in RPE65 identified by whole-exome sequencing causes retinitis pigmentosa with choroidal involvement. *Eur. J. Hum. Genet.* **19**, 1074–1081
13. Chen, Y., Moiseyev, G., Takahashi, Y., and Ma, J.X. (2006) Impacts of two point mutations of RPE65 from Leber's congenital amaurosis on the stability, subcellular localization and isomerohydrolase activity of RPE65. *FEBS Lett.* **580**, 4200–4204
14. Takahashi, Y., Chen, Y., Moiseyev, G., and Ma, J.X. (2006) Two point mutations of RPE65 from patients with retinal dystrophies decrease the stability of RPE65 protein and abolish its isomerohydrolase activity. *J. Biol. Chem.* **281**, 21820–21826
15. Bereta, G., Kiser, P.D., Golczak, M., Sun, W., Heon, E., Saperstein, D.A., and Palczewski, K. (2008) Impact of retinal disease-associated RPE65 mutations on retinoid isomerization. *Biochemistry.* **47**, 9856–9865
16. Philp, A.R., Jin, M., Li, S., Schindler, E.I., Iannaccone, A., Lam, B.L., Weleber, R.G., Fishman, G.A., Jacobson, S.G., Mullins, R.F., Travis, G.H., and Stone, E.M. (2009) Predicting the pathogenicity of RPE65 mutations. *Hum. Mutat.* **30**, 1183–1188
17. Nikolaeva, O., Takahashi, Y., Moiseyev, G., and Ma, J.X. (2010) Negative charge of the glutamic acid 417 residue is crucial for isomerohydrolase activity of RPE65. *Biochem. Biophys. Res. Commun.* **391**, 1757–1761
18. Li, S., Izumi, T., Hu, J., Jin, H.H., Siddiqui, A.A., Jacobson, S.G., Bok, D., and Jin, M. (2014) Rescue of Enzymatic Function for Disease-associated RPE65 Proteins Containing Various Missense Mutations in Non-active Sites. *J. Biol. Chem.* **289**, 18943–18956
19. Samardzija, M., von Lintig, J., Tanimoto, N., Oberhauser, V., Thiersch, M., Reme, C.E., Seeliger, M., Grimm, C., and Wenzel, A. (2008) R91W mutation in Rpe65 leads to milder early-onset retinal dystrophy due to the generation of low levels of 11-cis-retinal. *Hum. Mol. Genet.* **17**, 281–292
20. Won, J., Shi, L.Y., Hicks, W., Wang, J., Hurd, R., Naggert, J.K., Chang, B., and Nishina, P.M. (2011) Mouse model resources for vision research. *J. Ophthalmol.* **2011**, 391384
21. Wright, C.B., Chrenek, M.A., Foster, S.L., Duncan, T., Redmond, T.M., Pardue, M.T., Boatright, J.H., and Nickerson, J.M. (2013) Complementation test of Rpe65 knockout and tvrm148. *Invest. Ophthalmol. Vis. Sci.* **54**, 5111–5122
22. Shang, F. and Taylor, A. (2012) Role of the ubiquitin-proteasome in protein quality control and signaling: implication in the pathogenesis of eye diseases. *Prog. Mol. Biol. Transl. Sci.* **109**, 347–396
23. Lobanova, E.S., Finkelstein, S., Skiba, N.P., and Arshavsky, V.Y. (2013) Proteasome overload is a common stress factor in multiple forms of inherited retinal degeneration. *Proc. Natl. Acad. Sci. USA.* **110**, 9986–9991
24. Ando, R., Noda, K., Tomaru, U., Kamoshita, M., Ozawa, Y., Notomi, S., Hisatomi, T., Noda, M., Kanda, A., Ishibashi, T., Kasahara, M., and Ishida, S. (2014) Decreased proteasomal activity causes photoreceptor degeneration in mice. *Invest. Ophthalmol. Vis. Sci.* **55**, 4682–4690
25. Takeuchi, J., Fujimuro, M., Yokosawa, H., Tanaka, K., and Toh-e, A. (1999) Rpn9 is required for efficient assembly of the yeast 26S proteasome. *Mol. Cell. Biol.* **19**, 6575–6584
26. Li, S., Lee, J., Zhou, Y., Gordon, W.C., Hill, J.M., Bazan, N.G., Miner, J.H., and Jin, M. (2013) Fatty acid transport protein 4 (FATP4) prevents light-induced degeneration of cone and rod photoreceptors by inhibiting RPE65 isomerase. *J. Neurosci.* **33**, 3178–3189
27. Bainbridge, J.W., Smith, A.J., Barker, S.S., Robbie, S., Henderson, R., Balaggan, K., Viswanathan, A., Holder, G.E., Stockman, A., Tyler, N., Petersen-Jones, S., Bhattacharya, S.S., Thrasher, A.J., Fitzke, F.W., Carter, B.J., Rubin, G.S., Moore, A.T., and Ali, R.R. (2008) Effect of gene therapy on visual function in Leber's congenital amaurosis. *N. Engl. J. Med.* **358**, 2231–2239
28. Cideciyan, A.V., Aleman, T.S., Boye, S.L., Schwartz, S.B., Kaushal, S., Roman, A.J., Pang, J.J., Sumaroka, A., Windsor, E.A., Wilson, J.M., Flotte, T.R., Fishman, G.A., Heon, E., Stone, E.M., Byrne, B.J., Jacobson, S.G., and Hauswirth, W.W. (2008) Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proc. Natl. Acad. Sci. USA.* **105**, 15112–15117
29. Hauswirth, W.W., Aleman, T.S., Kaushal, S., Cideciyan, A.V., Schwartz, S.B., Wang, L., Conlon, T.J., Boye, S.L., Flotte, T.R., Byrne, B.J., and Jacobson, S.G. (2008) Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. *Hum. Gene Ther.* **19**, 979–990
30. Maguire, A.M., Simonelli, F., Pierce, E.A., Pugh, E.N. Jr., Mingozzi, F., Bennicelli, J., Banfi, S.,

- Marshall, K.A., Testa, F., Surace, E.M., Rossi, S., Lyubarsky, A., Arruda, V.R., Konkle, B., Stone, E., Sun, J., Jacobs, J., Dell'Osso, L., Hertle, R., Ma, J.X., Redmond, T.M., Zhu, X., Hauck, B., Zeleniaia, O., Shindler, K.S., Maguire, M.G., Wright, J.F., Volpe, N.J., McDonnell, J.W., Auricchio, A., High, K.A., and Bennett, J (2008) Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N. Engl. J. Med.* **358**, 2240–2248
31. Cideciyan, A.V., Jacobson, S.G., Beltran, W.A., Sumaroka, A., Swider, M., Iwabe, S., Roman, A.J., Olivares, M.B., Schwartz, S.B., Komaromy, A.M., Hauswirth, W.W., and Aguirre, G.D. (2013) Human retinal gene therapy for Leber congenital amaurosis shows advancing retinal degeneration despite enduring visual improvement. *Proc. Natl. Acad. Sci. USA.* **110**, E517–525
  32. Henderson, R.H., Waseem, N., Searle, R., van der Spuy, J., Russell-Eggitt, I., Bhattacharya, S.S., Thompson, D.A., Holder, G.E., Cheetham, M.E., Webster, A.R., and Moore, A.T. (2007) An assessment of the apex microarray technology in genotyping patients with Leber congenital amaurosis and early-onset severe retinal dystrophy. *Invest. Ophthalmol. Vis. Sci.* **48**, 5684–5689
  33. Kondo, H., Qin, M., Mizota, A., Kondo, M., Hayashi, H., Hayashi, K., Oshima, K., Tahira, T., and Hayashi, K. (2004) A homozygosity-based search for mutations in patients with autosomal recessive retinitis pigmentosa, using microsatellite markers. *Invest. Ophthalmol. Vis. Sci.* **45**, 4433–4439
  34. Katagiri, S., Hayashi, T., Kondo, M., Tsukitome, H., Yoshitake, K., Akahori, M., Ikeo, K., Tsuneoka, H., and Iwata, T (2014) RPE65 Mutations in Two Japanese Families with Leber Congenital Amaurosis. *Ophthalmic Genet.* 1–9
  35. Lorenz, B., Gyurus, P., Preising, M., Bremser, D., Gu, S., Andrassi, M., Gerth, C., and Gal, A. (2000) Early-onset severe rod-cone dystrophy in young children with RPE65 mutations. *Invest. Ophthalmol. Vis. Sci.* **41**, 2735–2742
  36. El Matri, L., Ambresin, A., Schorderet, D.F., Kawasaki, A., Seeliger, M.W., Wenzel, A., Arsenijevic, Y., Borruat, F.X., and Munier, F.L. (2006) Phenotype of three consanguineous Tunisian families with early-onset retinal degeneration caused by an R91W homozygous mutation in the RPE65 gene. *Graefes Arch. Clin. Exp. Ophthalmol.* **244**, 1104–1112
  37. Jacobson, S.G., Aleman, T.S., Cideciyan, A.V., Heon, E., Golczak, M., Beltran, W.A., Sumaroka, A., Schwartz, S.B., Roman, A.J., Windsor, E.A., Wilson, J.M., Aguirre, G.D., Stone, E.M., and Palczewski, K. (2007) Human cone photoreceptor dependence on RPE65 isomerase. *Proc. Natl. Acad. Sci. USA.* **104**, 15123–15128
  38. Seong, M.W., Kim, S.Y., Yu, Y.S., Hwang, J.M., Kim, J.Y., and Park, S.S. (2008) Molecular characterization of Leber congenital amaurosis in Koreans. *Mol. Vis.* **14**, 1429–1436
  39. Dunn, K.C., Aotaki-Keen, A.E., Putkey, F.R., and Hjelmeland, L.M. (1996) ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp. Eye Res.* **62**, 155–169
  40. Hu, J. and Bok, D. (2001) A cell culture medium that supports the differentiation of human retinal pigment epithelium into functionally polarized monolayers. *Mol. Vis.* **7**, 14–19
  41. Shirasawa, H., Jin, M.H., Shimizu, K., Akutsu, N., Shino, Y., and Simizu, B. (1994) Transcription-modulatory activity of full-length E6 and E6\*1 proteins of human papillomavirus type 16. *Virology.* **203**, 36–42
  42. Jin, M., Yuan, Q., Li, S., and Travis, G.H. (2007) Role of LRAT on the retinoid isomerase activity and membrane association of Rpe65. *J. Biol. Chem.* **282**, 20915–20924
  43. Li, S., Yang, Z., Hu, J., Gordon, W.C., Bazan, N.G., Haas, A.L., Bok, D., and Jin, M. (2013) Secretory defect and cytotoxicity: the potential disease mechanisms for the retinitis pigmentosa (RP)-associated interphotoreceptor retinoid-binding protein (IRBP). *J. Biol. Chem.* **288**, 11395–11406
  44. Kiser, P.D., Farquhar, E.R., Shi, W., Sui, X., Chance, M.R., and Palczewski, K. (2012) Structure of RPE65 isomerase in a lipidic matrix reveals roles for phospholipids and iron in catalysis. *Proc. Natl. Acad. Sci. USA.* **109**, E2747–2756
  45. Carlson, A. and Bok, D. (1992) Promotion of the release of 11-cis-retinal from cultured retinal pigment epithelium by interphotoreceptor retinoid-binding protein. *Biochemistry.* **31**, 9056–9062
  46. Carlson, A. and Bok, D. (1999) Polarity of 11-cis retinal release from cultured retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* **40**, 533–537
  47. Liao, J.L., Yu, J., Huang, K., Hu, J., Diemer, T., Ma, Z., Dvash, T., Yang, X.J., Travis, G.H., Williams, D.S., Bok, D., and Fan, G. (2010) Molecular signature of primary retinal pigment epithelium and stem-cell-derived RPE cells. *Hum. Mol. Genet.* **19**, 4229–4238
  48. Denning, G.M., Anderson, M.P., Amara, J.F., Marshall, J., Smith, A.E., and Welsh, M.J. (1992) Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature.* **358**, 761–764
  49. Vollrath, D. and Liu, Y. (2006) Temperature sensitive secretion of mutant myocilins. *Exp. Eye Res.* **82**, 1030–1036
  50. Nikolaeva, O., Takahashi, Y., Moiseyev, G., and Ma, J.X. (2009) Purified RPE65 shows isomerohydrolase activity after reassociation with a phospholipid membrane. *FEBS J.* **276**, 3020–3030
  51. Takahashi, Y., Moiseyev, G., Ablonczy, Z., Chen, Y., Crouch, R.K., and Ma, J.X. (2009) Identification of a novel palmitoylation site essential for membrane association and isomerohydrolase activity of RPE65. *J. Biol. Chem.* **284**, 3211–3218
  52. Golczak, M., Kiser, P.D., Lodowski, D.T., Maeda, A., and Palczewski, K. (2010) Importance of membrane structural integrity for RPE65 retinoid isomerization activity. *J. Biol. Chem.* **285**, 9667–9682
  53. Bavik, C.O., Busch, C., and Eriksson, U. (1992) Characterization of a plasma retinol-binding protein membrane receptor expressed in the retinal pigment epithelium. *J. Biol. Chem.* **267**, 23035–23042
  54. Hamel, C.P., Tsilou, E., Pfeffer, B.A., Hooks, J.J., Detrick, B., and Redmond, T.M. (1993) Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated in vitro. *J. Biol. Chem.* **268**, 15751–15757
  55. Saari, J.C. and Bredberg, D.L. (1988) CoA- and non-CoA-dependent retinol esterification in retinal pigment epithelium. *J. Biol. Chem.* **263**, 8084–8090

56. Joe, M.K., Sohn, S., Hur, W., Moon, Y., Choi, Y.R., and Kee, C. (2003) Accumulation of mutant myocilins in ER leads to ER stress and potential cytotoxicity in human trabecular meshwork cells. *Biochem. Biophys. Res. Commun.* **312**, 592–600
57. Mata, N.L., Moghrabi, W.N., Lee, J.S., Bui, T.V., Radu, R.A., Horwitz, J., and Travis, G.H. (2004) Rpe65 is a retinyl ester binding protein that presents insoluble substrate to the isomerase in retinal pigment epithelial cells. *J. Biol. Chem.* **279**, 635–643