A Comprehensive Clinical and Biochemical Functional Study of a Novel *RPE65* Hypomorphic Mutation

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Purpose. Later onset and progression of retinal dystrophy occur with some *RPE65* missense mutations. The functional consequences of the novel P25L *RPE65* mutation was correlated with its early-childhood phenotype and compared with other pathogenic missense mutations.

Methods. In addition to typical clinical tests, fundus autofluorescence (FAF), optical coherence tomography (OCT), and two-color threshold perimetry (2CTP) were measured. *RPE65* mutations were screened by SSCP and direct sequencing. Isomerase activity of mutant RPE65 was assayed in 293F cells and quantified by HPLC analysis of retinoids.

Results. A very mild phenotype was detected in a now 7-year-old boy homozygous for the P25L mutation in *RPE65*. Although abnormal dark adaptation was noticed early, best corrected visual acuity was 20/20 at age 5 years and 20/30 at age 7 years. Nystagmus was absent. Cone electroretinogram (ERG) was measurable, rod ERG severely reduced, and FAF very low. 2CTP detected mainly cone-mediated responses in scotopic conditions, and light-adapted cone responses were approximately 1.5 log units below normal. High-resolution spectral domain OCT revealed morphologic changes. Isomerase activity in 293F cells transfected with *RPE65*/P25L was reduced to 7.7% of wild-type *RPE65*-transfected cells, whereas *RPE65*/L22P-transfected cells had 13.5%.

Conclusions. The mild clinical phenotype observed is consistent with the residual activity of a severely hypomorphic mutant RPE65. Reduction to <10% of wild-type RPE65 activity by homozygous P25L correlates with almost complete rod function loss and cone amplitude reduction. Functional survival of cones is possible in patients with residual RPE65 isomerase activity. This patient should profit most from gene therapy. (*Invest Ophthalmol Vis Sci.* 2008;49:5235–5242) DOI: 10.1167/iovs.07-1671

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Human mutations in the gene for the highly preferentially expressed RPE protein RPE65 are associated with a spectrum of retinal dystrophies ranging from more severe early-onset conditions, variously described as Leber congenital amaurosis type 2/autosomal recessive childhood-onset severe retinal dystrophy or early-onset severe retinal dystrophy (LCA2/arCSR, EOSRD) to later onset conditions described as autosomal recessive retinitis pigmentosa (arRP).[1–7] Recently, RPE65 has been established as the isomerase central to the retinoid visual cycle.[8–10] This cycle[11] is crucial for supply of the chromophore 11-*cis* retinal for visual pigment regeneration.

Animal models have contributed greatly to our understanding of the role of RPE65 in the visual cycle, regeneration, and retinal dystrophy. *Rpe65* knockout mice display a biochemical phenotype consisting of extreme chromophore starvation (no rhodopsin) in the photoreceptors concurrent with overaccumulation of all-*trans* retinyl esters in the RPE[10] and are extremely insensitive to light. This insensitivity to light protects *Rpe65*$^{-/-}$ mice from light damage, establishing rhodopsin as the mediator of light-induced retinal damage.[12] There is also a natural mutation in mouse *Rpe65* called *rd12*.[13] In addition, a mutation in the Briard dog *RPE65* gene causes a severe retinal dystrophy in affected animals.[14,15] The utility of gene therapy was established by preclinical trials in these dogs.[16] These are all null mutations and represent one end of a spectrum of disease. Besides these, a hypomorphic animal model has been described: the L450M variant found in C57Bl/6 mice. Although it has been important in elucidating mechanisms in visual pigment regeneration, light damage, and retinal degeneration,[17–20] the M450 form of mouse RPE65 retains approximately 40% of wild-type (L450) activity.[21] This level appears to be more than enough to maintain near-normal function.

In contrast, human *RPE65* EOSRD displays a wide spectrum of severity, age of onset, and progression not seen in animal models.[1–7] Because of the wider range of mutational mechanisms operational (missense, nonsense, deletions or insertions, and splicing anomalies) and the various combinations of these mutations,[22] this diversity promises a wealth of potential clinical details to help understand the mechanisms underlying *RPE65* retinal dystrophy. In practice, however, these details may be obscured by secondary degenerative changes and compounded by delay until a molecular genetics diagnosis is made. Consideration of such details may be necessary when assessing suitability of a patient for gene therapy.

In this article, we present the mild phenotypic consequences of a homozygous P25L missense mutation in a young patient and correlate these with the biochemical effect of this mutation on RPE65 activity. We show that even though the isomerase activity of the mutant RPE65 was quite impaired, the patient had near-normal visual acuity. However, rod function was extremely impaired. In addition, short-wavelength cones appeared more impaired than long-wavelength cones, consistent with findings in other patients with *RPE65* mutations that blue color vision is much more and earlier impaired than is red vision, opposite to the usual case in cone dystrophies. These

findings are discussed in the context of emerging rationales for gene therapy for this disease.

METHODS

Subject

The patient described was identified from a larger study investigating patients with known or suspected childhood-onset retinal dystrophies from the genetics research facility of the Department of Pediatric Ophthalmology, Strabismology and Ophthalmogenetics of the Universitaetsklinikum Regensburg. The patient's parents were informed about the objectives of the examination and volunteered to participate. Informed consent was obtained according to the tenets of the Declaration of Helsinki. The study was approved by the Ethics Committees of the University of Regensburg and of the University of Giessen.

Clinical Examination

Ophthalmic examination included best-corrected visual acuity BCVA, refraction, orthoptic evaluation, slit lamp biomicroscopy and funduscopy, color vision testing with the Farnsworth-Munsell panel D 15 test, and fundus photography (RetCam120; Massie Laboratory, Pleasanton, CA; model TRC 50X, Topcon Optical, Tokyo, Japan; or Funduscamera FF 450 plus, Carl Zeiss Meditec AG, Jena, Germany).

Perimetry

Kinetic Perimetry. Kinetic photopic visual fields were measured with a Goldmann perimeter (Haag-Streit, Bern, Switzerland), using five targets (V/4e, III/4e, I/3e, I/2e, and I/1e).

Two-Color Threshold Perimetry. Two-color threshold perimetry (2CTP) was performed in scotopic and photopic conditions, to assess the spatial distribution of rod- and cone-mediated function. A modified field perimeter (Humphrey Field Analyzer [HFA], model 640; Carl Zeiss Meditec, modified by Frederick Fitzke, Institute of Ophthalmology, London) was used as reported previously.[23] In the dark-adapted state, a neutral-density (ND) filter) was used for the stimulus generated with a 500-nm cutoff to remain in the effective range of the instrument. The lowest normal sensitivity for each tested locus was determined with the 10th-percentile function.

To determine the photoreceptor system responsible for stimulus detection (mediation), we analyzed the differences between the sensitivities with a 500-nm cut-off filter and a 600-nm cut-on filter, in the dark-adapted state. Photoreceptor mediation was defined as follows: (1) rod mediation of both long- and short-wavelength stimulus for differences of dark-adapted sensitivities above 4 dB; (2) mixed mediation (e.g., rods detect the 500 nm cut-off filter–generated stimulus and cones detect the 600 nm cut-on filter–generated stimulus for differences between 3 and −11 dB); and (3) cone mediation for differences below −12 dB. The negative values for mixed and cone mediation result from the fact that the sensitivities to the 500-nm cut-off filter–generated stimulus are 20 dB lower than the real values, owing to the ND filter used. Dark- and light-adapted photoreceptor sensitivity losses to the 500-nm cut-off and 600-nm cut-on filter–generated stimuli were calculated as the difference between the measured value and the 10th percentile of normal subjects for each test locus.

Electroretinography

Electroretinograms (ERGs) were recorded with DTL electrodes after white Ganzfeld (full-field) stimulation (Spirit; Nicolet, Madison, WI) according to the guidelines of the ISCEV standard for ERG.[24] Artifacts caused by nystagmus were rejected.

Fundus Autofluorescence (FAF)

FAF was recorded with a standard confocal scanning laser ophthalmoscope and the most recent-generation instrument (Heidelberg Retina Angiograph [HRA] and Spectralis HRA-OCT; Heidelberg Engineering, Heidelberg, Germany). A detailed description is given elsewhere.[25] In brief, to amplify the FAF signal, generally 8 to 16 images were aligned using the integrated software and a mean of these images was calculated. With the OCT system, images are averaged automatically. The detector sensitivity of the laser scanning camera was adjusted to the amount of FAF.

Optical Coherence Tomography

OCT scans were performed as previously described.[25] (Stratus OCT; Carl Zeiss Meditec Inc., Dublin, CA), using 512 A-scans over 6 mm transverse scanning length for an optimal sampling rate of 400 A-scans/second. The best images were obtained by using the cross-hair scanning pattern. The fovea and areas of the posterior pole were scanned.

At the latest visit, high-resolution spectral domain OCT, also known as Fourier OCT[26] (Spectralis HRA-OCT, Heidelberg Engineering), using high-resolution–mode 40-kHz scanning, was applied. A series of 1536 A-scans around the fovea were acquired over a B-scan length of 4.3 mm with a series of 25 B-scans over 2.9 mm (volume scan).

Molecular Genetic Analysis

DNA was extracted from peripheral blood lymphocytes[27] and subjected to PCR using oligonucleotide primers specific for *RPE65*[28] and designed in our laboratory as described previously.[25] All coding exons of *RPE65* including 162 bp of the 5′ untranslated region (UTR) and 49 bp of the 3′ UTR were amplified. PCR products were analyzed by single-strand conformation polymorphism (SSCP) analysis on polyacrylamide gels (11 cm) using 10% polyacrylamide (19:1) with a 5-mm overlay of 6% polyacrylamide (19:1) in 1× TBE buffer. The gels were run for 12 to14 hours at 140 to 180 V constant and 10°C. Direct sequencing was applied to amplimers showing aberrant banding patterns in SSCP. Sequencing results were confirmed by a restriction endonuclease assay using Aci I (P25L).

Functional Analysis

Transient Transfection and Cell Culture. Cell culture methods and transient transfection protocols were as previously published.[10] For any given experiment, 3×10^7 293-F cells were transfected with 30 μg of pVitro2 plasmid (containing RPE65 and CRALBP open reading frames [ORFs]) and 30 μg of pVitro3 plasmid (containing LRAT and RDH5 ORFs) in the presence of 40 μL of transfection reagent (293fectin; Invitrogen, Carlsbad, CA), all in a total volume of 30 mL.

Site-Directed Mutagenesis of RPE65. Site-directed mutagenesis of RPE65 ORF cloned in pVitro2 was performed with a kit (QuikChange XL; Stratagene, La Jolla, CA). The oligonucleotide primer pairs used are listed in Table 1. The mutants were verified by sequence analysis (Northwoods DNA, Inc., Solway, MN) of DNA minipreps. Validated mutant and wild-type plasmids were grown and purified by using kits (Maxi or Mega format, as appropriate; Qiagen, Valencia, CA).

Retinoid Extractions and HPLC. Culture fractions of 19-mL volumes of transfected 293-F cells were centrifuged, and the cells were harvested and retinoids extracted and saponified by the methods previously described.[10] Isomeric retinols were analyzed on 5-μm particle (Lichrospher; Alltech, Deerfield, IL), normal-phase columns (2 × 250 mm) on an isocratic HPLC system equipped with a diode array UV-visible detector (model 1100 series; Agilent Technologies, New Castle, DE), according to the method of Landers and Olson,[29] as modified by us.[10] Data were analyzed with commercial software (ChemStation32 software; Agilent).

Immunoblot Analysis. Cell pellets (~2×10^6 cells) from 1-mL culture aliquots were lysed in 200 μL detergent (CytoBuster; Novagen, Madison, WI), incubated on ice for 10 minutes, and centrifuged at 13,000*g* for 10 minutes, and the supernatant was harvested for SDS-PAGE analysis. Denatured samples were separated on 12% bis-tris gels (NuPage; Invitrogen) and electrotransferred to nitrocellulose mem-

TABLE 1. Oligonucleotide Primers Used to Prepare Mutant Clones

P25L	
Forward	5′-GAA GAG CTG TCG TCG CTG CTC ACC GCC CAC GTG-3′
Reverse	5′-CAC GTG GGC GGT GAG CAG CGA CGA CAG CTC TTC-3′
L22P	
Forward	5′-GAA ACC GTG GAA GAG CCG TCG TCG CCG CTC ACC GCC-3′
Reverse	5′-GGC GGT GAG CGG CGA CGA CGG CTC TTC CAC GGT TTC-3′
Y79H	
Forward	5′-GAA GGA CAC GTC ACC CAT CAC AGA AGG TTC ATC CGC-3′
Reverse	5′-GCG GAT GAA CCT TCT GTG ATG GGT GAC GTG TCC TTC-3′
E95Q	
Forward	5′-GTC CGG GCA ATG ACC CAG AAA AGG ATC GTC ATA ACG-3′
Reverse	5′-CGT TAT GAC GAT CCT TTT CTG GGT CAT TGC CCG GAC-3′

branes. Blots were probed with antibodies by standard procedures and developed in color substrate. Primary antibodies used were: rabbit anti-bovine RPE65 antibody (1:4000) and rabbit anti-CRALBP antibody (1:20,000; gift of John Saari, University of Washington, Seattle). The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000; Novagen). Expression levels of RPE65 were quantitated by fluorescent Western blot. Gels and blots were prepared as just described, by using protein standard markers (Hybond-ECL and ECL-plex Rainbow; GE Healthcare, Piscataway, NJ). Primary antibodies were rabbit anti-RPE65 (1:4000) and mouse monoclonal antibody anti-CRALBP (1:20,000; gift of John Saari). Secondary antibodies used were Cy5-conjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse ECL-plex fluorescent antibodies (both 1:2500). Blots processed according to the manufacturer's protocol were scanned (Typhoon 9410 scanner; GE Healthcare) and quantitated (ImageQuant TL image-analysis software; GE Healthcare). Wild-type and mutant RPE65 levels were normalized to coexpressed CRALBP levels, and mutant levels were calculated relative to wild-type RPE65 expression (set at 100%).

TABLE 2. Clinical, Electrophysiological, and Psychophysical Data

MV, born 2001 *RPE65* Mutation Homozygous P25L	**At 3 Years**	**At 4.5 Years**	**At 6/7 Years**
BCVA OD/OS	1.2/1.0 LEA 3m	1.2/1.0 LEA 3m	0.63/0.63 E
Refraction	OD +3.0 sph OS +3.0 sph	OD +2.5 sph/−0.5 cyl A0° OS +2.75 sph	OD +0.5/−0.25 cyl A32° OS +0.75/−0.25 <A80°
Fundus	Relatively "blond" fundus (Fig. 1A)	"Blond" fundus and increased visibility of choroidal vessels	Small macular reflex (Fig. 1B)
Goldmann Visual Field OD + OS	ND	ND	V/4e, III/4e normal 1/4e slightly constricted
2CT perimetry 30° OS	ND	ND	Dark adapted: mostly cone mediation Light adapted. CSL 1.5 log (Fig. 3)
Full-field ERG			Fig. 4
Scotopic maximum response			
Ampl			
OD	Minimal residual	16%/21%	Minimal residual
OS	Minimal residual	22%/26%	25%/15%
Impl			
OD		√	√
OS		√	√
Photopic 30 Hz-Flicker			
Ampl			
OD	7%	50%	50%
OS	12%	50%	50%
Impl			
OD	√	√	√
OS	√	√	√
Photopic single flash			
Ampl			
OD	9%/15%	32%/34%	41%/51%
OS	16%/23%	30%/33%	15%/26%
Impl			
OD	√	√	√
OS	√	√	√
Lanthony panel D15 OD + OS	ND	ND	Saturated normal Desaturated very few errors along blue axis
FAF OD + OS	Very low (Fig. 1C, top)	Very low	Very low (Fig. 1C, middle)
OCT Stratus	Layering grossly preserved (Fig. 2B)	Layering preserved	Layering grossly preserved (Fig. 2C)
OCT Spectralis HRA-OCT			Altered layering outer retina + thinning (Fig. 2D)

CSL, cone sensitivity loss; ND, not done; sph, spherical; cyl, cylinder; LEA, Lea cards; E, E-signs; √, within normal range.

RESULTS

Genotypic Analysis

The patient was born in 2001 to then 36- and 31-year-old consanguineous healthy parents. He had two older healthy siblings, a brother and a sister. The great-grandfathers of the parents were brothers. Molecular genetic analysis showed the patient to have a homozygous C-to-T mutation at nt 128 of RPE65, converting a proline at codon 25 to a leucine. This point mutation resulted in the loss of an Aci I restriction site that was used to confirm the sequencing results (data not shown). The parents were heterozygous for this mutation.

Phenotypic Analysis

The patient was first examined at age 3 years, after the parents had noticed visual problems when the boy moved from daylight to dim light. His visual acuity was normal at age 3 and 4.5 years and had slightly deteriorated at age 6. The clinical, imaging, electrophysiological, and psychophysical data are summarized in Table 2 and Figures 1 through 4. The orthoptic examination was unremarkable, with orthotropia at distance, a small exophoria of 3° at near, and good stereopsis (120 seconds of arc TNO test). The anterior segments were normal. Fundus photography showed some uncharacteristic changes, such as a lightly pigmented appearance, increased visibility of choroidal vessels, reduced macular reflex, and some irregular pigmentation (Figs. 1A, 1B). Full-field ERGs were performed at ages 3, 5, and 6 years and showed severely impaired rod responses, but relatively well-preserved cone responses with amplitudes reduced to approximately 30% to 20% and normal implicit times (Fig. 4). Goldmann perimetry, first performed at age 6 years, showed some constriction to the I/4e isopter.

Fundus autofluorescence FAF was low but detectable and did not change over the time of observation (Fig. 1C).

OCT (Fig. 2) showed well-preserved retinal layering at the initial examinations, but, at age 6 and 7 years, the outer retina showed increased reflectivity and an increased transmission into the choroid. OCT (Spectralis; Heidelberg Engineering) at age 7 detected retinal layer alterations and some thinning, most pronounced in the outer retina (Fig. 2D).

2CTP (Fig. 3) could be performed for the first time at age 6 years. In the dark-adapted state, there was a severe sensitivity loss of 3 to 4 log units to the blue stimulus and of 1 to 1.4 log units to the red stimulus within the test field of 30°. Most responses were cone mediated with only a few mixed responses (i.e., rod mediation for the blue stimulus and cone mediation for the red). Cone sensitivity measured in the light-adapted state showed a diffuse sensitivity loss of approximately 1.5 log units. Of interest, cone sensitivity to red light (mediated by long wavelength cones) was higher than that to blue light (mediated by short-wavelength cones), which is in line with the clinical observation that most patients with RPE65 mutations report on difficulties with blue colors from early on.

Lowered Isomerase Activity of RPE65/P25L

We measured the isomerase activity of cells transfected with mutant constructs of *RPE65*. This activity is a combined phenotypic effect of the mutant of RPE65 on enzymatic activity and stability, comparable to an in vivo homozygous state for each allele. 293-F cells were transfected with pVitro3-LRAT+RDH5 and pVitro2-RPE65/P25L+CRALBP or pVitro2/wild-typeRPE65+CRALBP, grown for 24 hours, and then incubated with 2.5 μM all-*trans* retinol for 6 hours before harvest and analysis of isomeric retinols. Cells transfected with vector harboring the *RPE65*/P25L mutation produced only 7.75% of the 11-*cis* retinol made by cells transfected with pVitro2 containing wild-type *RPE65* (Table 3). We compared the RPE65/P25L mutant isomerase activity with the activities of other missense mutants associated with cases of RPE65 EOSRD previously described as mild,[2,22] choosing to examine L22P, E95Q, and Y79H. L22P was the least severely impaired at 13.5%, E95Q had an intermediate level of 6.1% of wild type, and Y79H activity was almost abolished at 2.5% of wild-type activity (Table 3). Comparison with wild-type *RPE65* of expression of the *RPE65*/P25L mutant was made by immunoblot (Fig. 5) and quantitated (Table 4). We found that the level of P25L RPE65 protein was lowered to 25% of wild type. The expression levels of each of the other mutants, like that of P25L, were reduced compared with wild type. The level of expression of Y79H at 3% of wild type (Fig 5, Table 4) was the most adversely affected of all four mutants, concomitant with the low isomerase activity in cells transfected with its construct.

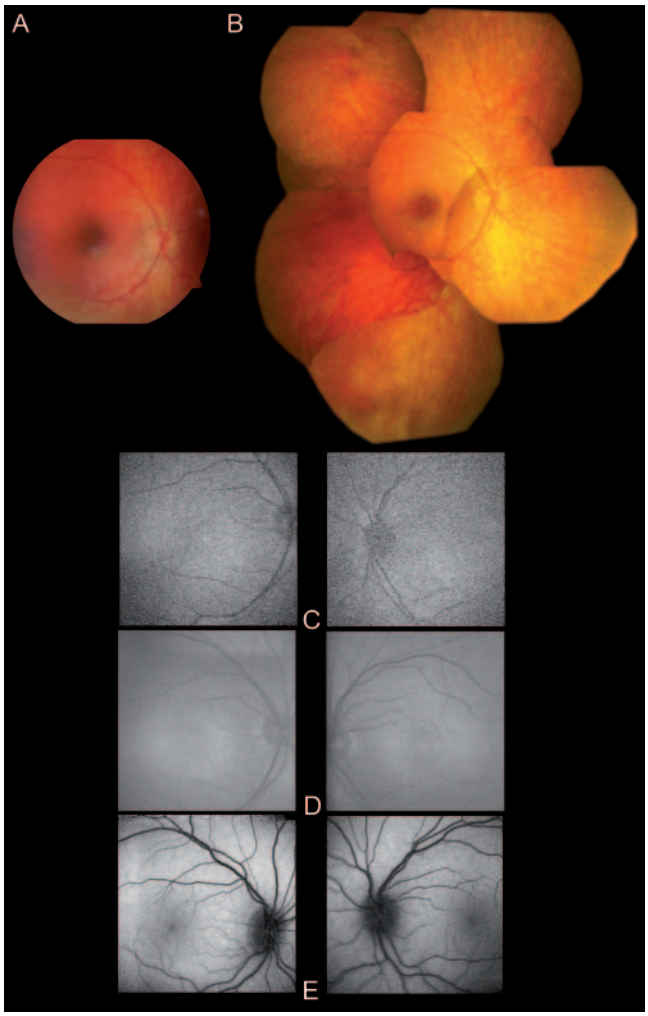

FIGURE 1. Fundus imaging of the patient. (**A**) Fundus photograph of the right eye (RetCam 120; Massie Laboratory) at age 3 years. (**B**) Fundus photomontage of the right eye (FF 450 plus; Carl Zeiss Meditec) at age 7 years. Fundus autofluorescence images of both eyes at age 3 (**C**) and 7 (**D**) years. The low resolution is due to the high level of enhancement needed to record the minimal FAF. For comparison, FAF of an 8-year-old healthy individual (**E**) is shown.

DISCUSSION

We correlated the phenotypic effects of a homozygous P25L mutation in *RPE65* on visual function in a young patient with in vitro measurement of the isomerase activity of RPE65/P25L

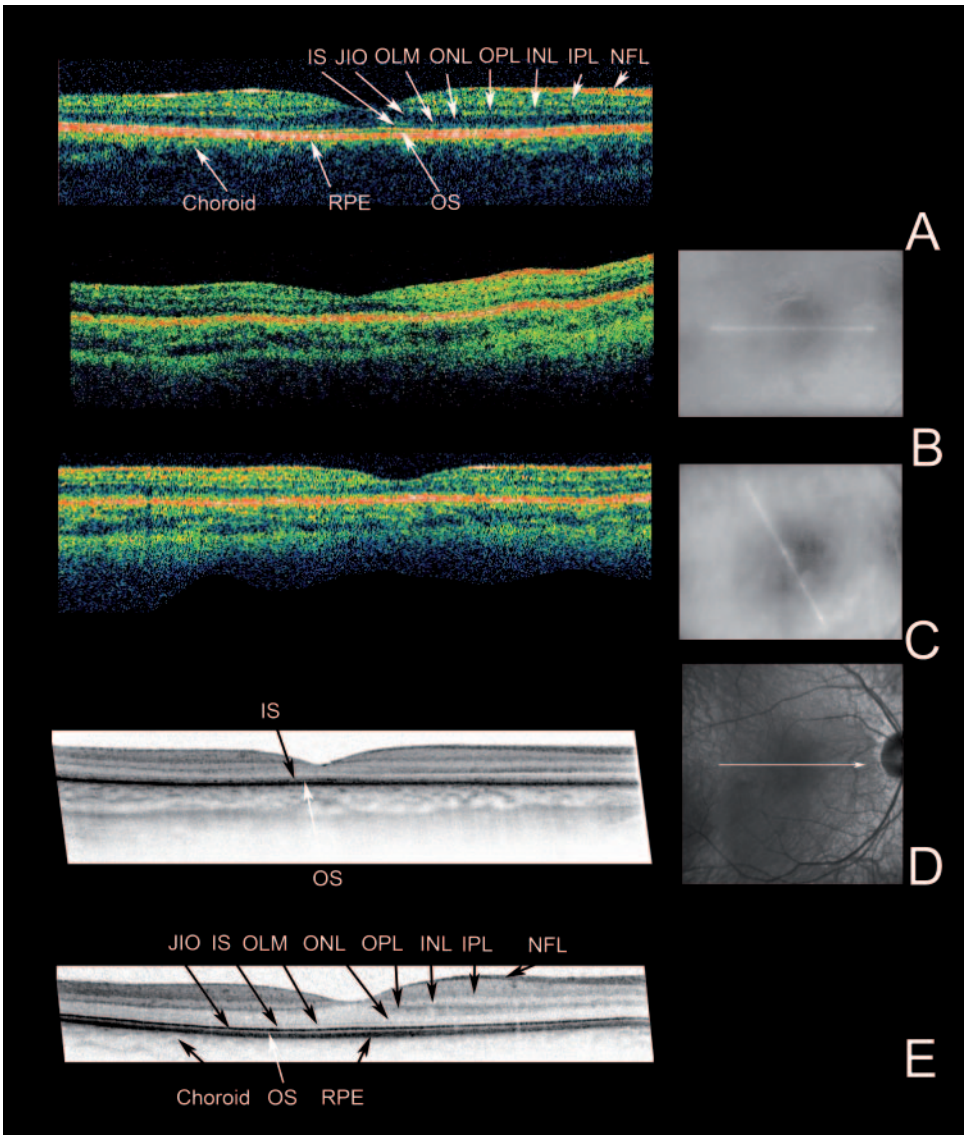

FIGURE 2. OCT imaging. For each eye the OCT image is accompanied by a fundus image showing the position of the scan. The findings were similar in both eyes and thus only the right eyes are shown. (**A**) OCT recording (Stratus OCT; Carl Zeiss Meditec) of a 20-year-old normal proband. (**B**, **C**) OCTs of the patient at ages 3 and 7 years. (**D**) At the age of 7 years, a high-resolution OCT was recorded (Spectralis HRA-OCT; Heidelberg Engineering). For comparison (**E**), an OCT recording of the 20-year-old normal proband with the same system is included. Note the overall reduced thickness of the retina (**B**–**D**). The imaging (**D**) shows that this loss of thickness was due to the loss of stratification in the inner and outer segment layers and thinning of the outer nuclear layer. NFL, nerve fiber layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OLM, outer limiting membrane; IS, inner segment layer; JIO, junction between inner and outer segments; OS, outer segment layer; RPE, retinal pigment epithelium.

mutant protein. The mild clinical phenotype not yet complicated by long-term degeneration was consistent with residual activity of a severely hypomorphic mutant enzyme. When tested in vitro, P25L RPE65 had only approximately 8% of wild-type activity. Yet, in vivo this residual activity maintained a physiologically relevant level of regeneration in psychophysical testing. These findings have relevance to understanding the natural history of *RPE65*-EOSRD and in providing rational indicators for the application of therapies.

The most important primary effect of RPE65 deficiency is its impact on visual pigment regeneration.[30,31] Various combinations of null and missense mutations can give a spectrum of

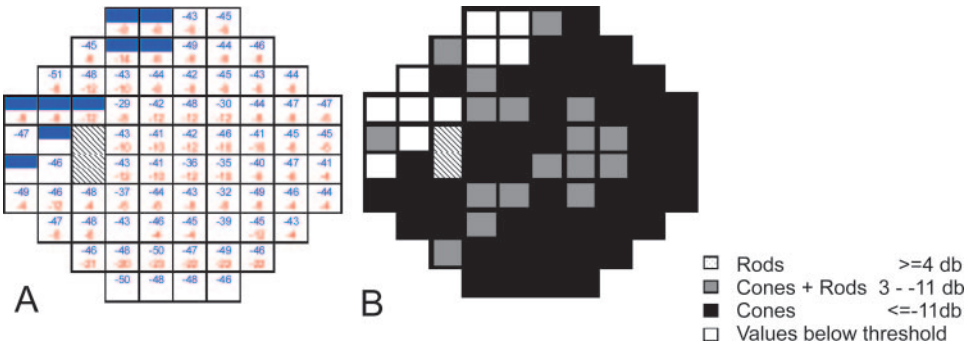

FIGURE 3. 2CTP of a 30° visual field of the left eye of the patient at age 6 years. Hatched fields indicate the optic nerve head. (**A**) *Blue*: dark-adapted sensitivity loss to 500 nm cut-off filter–induced stimulus; *red*: light-adapted sensitivity loss to 600-nm cut-on filter–induced stimulus for each tested target. *Filled fields*: sensitivity below threshold; *empty fields*: sensitivities within normal range: *shaded fields*: sensitivity below the 10th percentile of normal values. Numbers indicate sensitivity losses in decibels. (**B**) Mediation: *black*: cone-mediated responses at both wavelengths (pure cone mediation); *gray*: rod mediation with a 500-nm cut-off filter and cone mediation with a 600-nm cut-on filter (mixed response). Dark-adapted responses were mostly cone mediated. Values were close to those of pure cone mediation (12 loci, 10-dB difference; 7 loci, 8-dB difference).

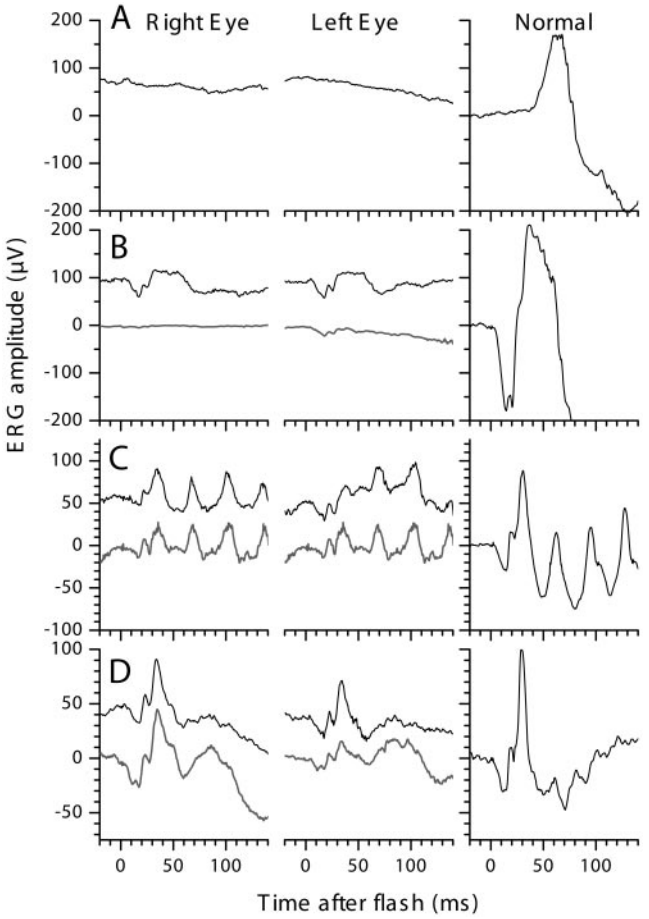

FIGURE 4. Ganzfeld ERG recordings of both eyes of the patient at 5 (*top traces*) and 6 (*bottom traces*) years. With a 2.75-log-attenuated ISCEV standard flash after 40 minutes of dark adaptation (**A**), hardly any rod response was obtained. Compared with a normal recording (*right*), average rod-and-cone responses to ISCEV standard flashes at 5 (**B**, *top trace*) and 6 (bottom trace) years were greatly reduced but not delayed. Responses to 30-Hz flicker after 10 minutes of light adaptation (**C**) were about half the normal size and within normal implicit time ranges. Light-adapted responses to single standard flashes (**D**) were approximately two thirds the normal size. Most responses obtained with the patient under general anesthesia at age 3 years were even smaller (see Table 2).

disease from mild to severe. How RPE65 activity governs the dynamic range of visual pigment regeneration will become clearer as less-severe late-onset forms of *RPE65*-related retinal dystrophy disease are described. To put the biochemical data in context, we compared the RPE65/P25L mutant isomerase activity with *RPE65*-EOSRD missense mutations previously described as mild, including L22P, E95Q, and Y79H. Unlike the P25L patient, however, early clinical data are almost completely lacking. L22P was a compound heterozygote with H68Y[2] and the patient had "a milder phenotype in childhood

TABLE 3. Effect of Mutations on RPE65 Isomerase Activity

Mutant	Mean Activity	SD	*n*
P25L	7.75	1.4	10
L22P	13.5	1.6	6
Y79H	2.5	0.6	7
E95Q	6.1	1.1	6

Activity is represented as percentage of wild-type RPE65 isomerase activity, with the SD and number of replicates.

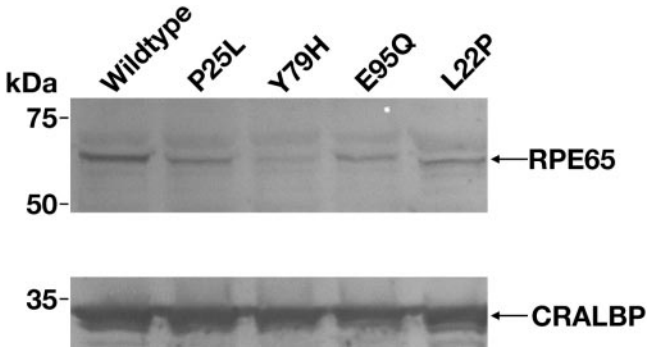

FIGURE 5. Immunoblot analysis of RPE65 expression in 293-F cells transfected with wild-type, P25L, Y79H, E95Q, and L22P mutants of RPE65 (*top*) and CRALBP expression in the corresponding cells (*bottom*). Each of the mutants showed less expression than did the wild-type construct. Y79H showed the lowest level of expression. Each lane was equivalently loaded with extract from 10^5 cells.

and a slower progression of the disease," but visual function was severely affected at the time of molecular diagnosis (at 40 years).[2] We have previously shown that H68Y essentially abolishes RPE65 isomerase activity (<2%).[10] We estimate the average level of RPE65 isomerase activity ((13.5%+2%)/2=7.75%) of the L22P/H68Y patient to be similar to that of the P25L patient (7.75%). In the Y79H/E95Q[22] patient, there was also a later time of onset (night blindness at 3 years, RP at age 20, and residual vision at age 58[22]). The average level of RPE65 isomerase activity [6.1%+2.5%)/2 = 4.3%] of Y79H/E95Q is estimated to be less than P25L/P25L. Overall, however, these residual levels (<10%) of RPE65 activity may be enough to delay time of onset and delay, but not prevent slow cumulative progression/degeneration.

Efficient regeneration is required under conditions of high ambient light when photoisomerizations are at peak level.[31] Insufficient supply of 11-*cis* retinal causes night blindness due to insufficient formation of rhodopsin. Ligand-free opsin constitutively activates the phototransduction cascade and causes photoreceptor degeneration by mimicking a constant light-adapted state.[32] Thus, any activity of RPE65 supports survival of photoreceptors by formation of rhodopsin and control of its state of activity. Although faster regeneration is beneficial under conditions of higher ambient light, it confers a higher susceptibility to light-induced damage,[33] due to greater turnover of activated phototransduction species and greater A2E accumulation,[18,34] both of which are linked to retinal degeneration in animal models.[35,36]

FAF detects lipofuscin accumulation in the RPE and lipofuscin/A2E accumulation is correlated with RPE65 activity: *Rpe65*$^{-/-}$ mice accumulate less than 10% of lipofuscin fluorophore as wild-type mice.[34] Similarly, FAF, as a clinically accessible surrogate for A2E/lipofuscin accumulation in the RPE, measures RPE65 activity. This patient's reduced FAF correlates with the lower RPE65 activity of P25L mutant RPE65. Evalua-

TABLE 4. Effect of Mutations on RPE65 Protein Expression

Mutant	Mean Expression	SD	*n*
P25L	24.33	5.06	7
L22P	32.4	7.64	4
Y79H	3.31	0.38	4
E95Q	28.07	6.1	5

Mutant expression level was measured by fluorescence immunoblot and is normalized to and represented as percentage of wild-type RPE65 expression, with SD and number of replicates.

tion of FAF plays an important role in the differential diagnosis of RPE65 EOSRD/LCA. Of paramount importance is the association of normal fundus with low or no autofluorescence.[25] In severe RPE65 mutations, FAF is nondetectable from early on. Lowered FAF also occurs with severe retinal changes on ophthalmoscopy, and normal FAF occurs with abnormal fundus, but neither of these situations occurs in *RPE65*-EOSRD/LCA. Of interest, in this regard, Simonelli et al.[37] present a lower than normal FAF image from an *RPE65* patient, in association with a normal fundus, thus supporting our interpretation. In their own interpretation of the data, more weight is given to the actual existence of FAF than to its relative diminishment in the *RPE65* patients, underestimating its importance in *RPE65*-EOSRD diagnosis.

Cone vision in many non-null cases of human *RPE65*-EOSRD is relatively spared compared with rod vision (night blindness and lack of rod ERG), in contrast to the knockout mouse model, which is by definition null. In the *Rpe65*$^{-/-}$ mouse, cones degenerate much more rapidly than do rods, and this raises concern as to the timing of rescue.[38] Is the situation observed in mice directly relevant to humans? Certainly, both cone and rod visual functions are severely affected in many patients,[7,22] but the chronology of rod versus cone demise is still not known. Greater preservation of foveal structure concomitant with relatively spared cone-mediated function is seen in patients with a variety of RPE65 mutations.[39] Thus, the low chromophore level in the P25L patient, and possibly in other patients, may spare cone function. Conversely, how is cone survival affected in mice with a knockin missense mutation? Recently, the phenotype of an *RPE65* R91W missense knockin mouse has been published.[40] This mouse makes 5% of wild-type level of 11-*cis* retinal and has less severe morphologic and physiological changes, including better cone function, than the *Rpe65* knockout. Another strikingly consistent finding is that blue cone vision is more markedly affected in *RPE65*-EOSRD patients than is red cone vision.[7] Why is this so? It may be due to a combination of the biochemistry of cone pigments in general, combined with the abundance/distribution of blue cones in particular. Cone opsins make a reversible Schiff's base with 11-*cis* retinal that does not necessitate photoisomerization to break it,[41,42] whereas in rod opsin, the bond is essentially irreversible without photoisomerization. Thus, cone pigments more easily release chromophore, which if captured by rod pigment is not relinquished. The second consideration is the abundance and distribution of blue cones, comprising only approximately 10% of all cones. In the fovea, where blue cones are excluded from the central fovea,[43] when chromophore is limited, blue cones may be outcompeted by the abundance of red/green cones. In the peripheral retina, where rod cells are overwhelming, the biochemical properties of rod opsin will ensure that blue cones are outcompeted when chromophore is limited. However, that RPE65 is more abundant centrally than peripherally[39] may further reduce peripheral rod and blue cone access to chromophore compared with central red/green cones and may help explain the serious rod deficit as well as the blue cone deficit.

In conclusion, this study represents the earliest time point yet comprehensively analyzed in the natural history of RPE65-associated severe retinal dystrophy in humans. The residual activity of the hypomorphic P25L RPE65 can maintain some, but not all, features of normal vision. Of note, despite relative normal vision, morphologic changes are already detected at that age by high-resolution spectral domain OCT. In the Briard dog, *Rpe65* knockout, and *rd12* mice, morphologic changes are also already evident at early ages. Nevertheless, this does not prevent rescue by gene therapy.[44,45] The combination of clinical measurements with functional biochemical data in this patient strongly reinforces the concept that functional survival of cones is possible in patients with residual RPE65 activity and is a practical target for therapy. Although the future outcome of this patient is not clear, the condition is likely to be slow to progress. The relative sparing of cone function may make this patient an ideal candidate for RPE65 somatic gene therapy, trials for which are currently in progress.[46,47]

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