



Lipofuscin accumulation, abnormal electrophysiology, and photoreceptor degeneration in mutant *ELOVL4* transgenic mice: A model for macular degeneration

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Edited by Jeremy Nathans, Johns Hopkins University School of Medicine, Baltimore, MD, and approved January 27, 2005 (received for review October 15, 2004)

Macular degeneration is a heterogeneous group of disorders characterized by photoreceptor degeneration and atrophy of the retinal pigment epithelium (RPE) in the central retina. An autosomal dominant form of Stargardt macular degeneration (STGD) is caused by mutations in *ELOVL4*, which is predicted to encode an enzyme involved in the elongation of long-chain fatty acids. We generated transgenic mice expressing a mutant form of human *ELOVL4* that causes STGD. In these mice, we show that accumulation by the RPE of undigested phagosomes and lipofuscin, including the fluorophore, 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl]-pyridinium (A2E) is followed by RPE atrophy. Subsequently, photoreceptor degeneration occurs in the central retina in a pattern closely resembling that of human STGD and age-related macular degeneration. The *ELOVL4* transgenic mice thus provide a good model for both STGD and dry age-related macular degeneration, and represent a valuable tool for studies on therapeutic intervention in these forms of blindness.

phagosome | Stargardt disease | photoreceptor | retinal pigment epithelium

Macular degeneration involves the death of photoreceptor cells in the central retina, which is responsible for fine-detail vision. Age-related macular degeneration (AMD) affects $\approx 30\%$ of people over the age of 75 (1, 2), and is becoming a greater health problem with the rapidly growing elderly population of developed countries. There is no treatment to halt or reverse the disease for the dry form, which comprises $\approx 90\%$ of AMD cases. Moreover, there is a lack of suitable animal models for experimentation on therapies for dry AMD. Stargardt macular dystrophy (STGD) shares pathological features with AMD, except that it occurs at a young age. Both AMD and STGD are characterized by the accumulation of high levels of lipofuscin in the retinal pigment epithelium (RPE), which precedes degeneration of the photoreceptors in the macula and RPE atrophy.

The gene responsible for an autosomal dominant form of STGD, STGD3, was identified recently as *ELOVL4* (3, 4). It is predicted to encode an enzyme involved in the elongation of very long-chain fatty acids (hence the name, *ELOVL*), and is highly expressed in rod and cone photoreceptor cells (5, 6). Sequence analysis of human *ELOVL4* cDNA predicts a protein of 314 aa that shares homology with members of the yeast Elo (elongation of long chain fatty acid) family and the human *ELO1* homolog (*HELO1*) (3). *HELO1* and the *ELO* family members possess biochemical features that suggest their participation in reduction reactions occurring during fatty acid elongation (7, 8). Mutational analysis of the *ELOVL4* gene in five large STGD-like macular dystrophy pedigrees revealed a 5-bp deletion, resulting in a frame-shift and the introduction of a stop codon, 51 codons from the end of the coding region (3). Subse-

quently, two 1-bp deletions, 789delT and 794delT, in *ELOVL4* were identified in an independent large Utah pedigree, confirming the role of the *ELOVL4* gene in a subset of dominant macular dystrophies (9). Both the 5-bp deletion and the two 1-bp deletions are predicted to result in a similar truncated *ELOVL4* protein. A third mutation in *ELOVL4*, 270stop, which should also generate a truncated *ELOVL4* protein, has been identified recently in a Dutch family with dominant STGD (10). The importance of the function of *ELOVL4* in the synthesis of polyunsaturated fatty acids is emphasized by the observation that the severity of STGD3 can be alleviated by dietary supplements of EPA and DHA (11, 12).

In the present study, we generated transgenic mice, expressing the 5-bp deletion mutant form of *ELOVL4*, driven by a photoreceptor-specific promoter. Our results demonstrate that the mutant *ELOVL4* exerts a dominant effect on RPE accumulation of undigested phagosomes and lipofuscin, including 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl]-pyridinium (A2E) and its isomers, and on RPE and photoreceptor degeneration, primarily in the central retina. Thus, this animal model represents a good model for STGD and dry AMD, and should be a valuable tool for studies on therapeutic intervention of these forms of blindness.

Materials and Methods

Generation and Characterization of Transgenic *ELOVL4* Transgenic Mice. We made transgenic constructs in which the expression of WT or mutant *ELOVL4* was driven by a human photoreceptor-specific promoter of the gene encoding interphotoreceptor retinoid-binding protein (IRBP) (Fig. 1A). We used a 1.3-kb upstream fragment from the transcription start site of the human *IRBP* gene. This *IRBP* promoter directs expression of a transgene to photoreceptor cells and pinealocytes (13). Human WT *ELOVL4* cDNA was used for the WT transgene. For mutant *ELOVL4* construction, a 5-bp deletion corresponding to the human mutation (delAACTT at 790–794) was introduced by PCR-based site-directed mutagenesis, by using a human WT *ELOVL4* cDNA as a template. The DNA fragment corresponding to the mutant *ELOVL4* was isolated by partial digestion with *EcoRI*, and cloned into the *EcoRI* sites of a

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AMD, age-related macular degeneration; STGD, Stargardt macular dystrophy; RPE, retinal pigment epithelium; IRBP, interphotoreceptor retinoid-binding protein; ERG, electroretinography; A2E, 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl]-pyridinium.

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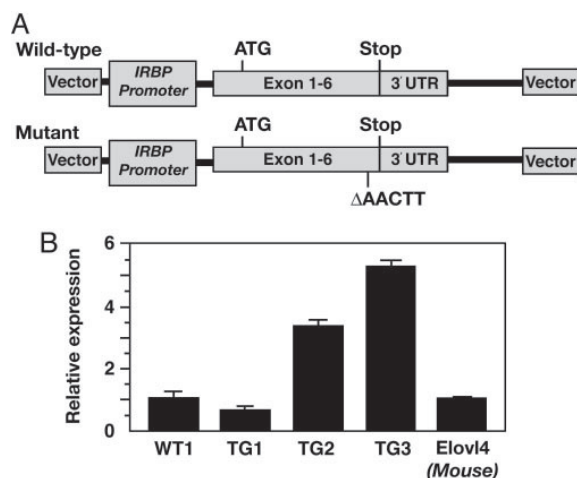


Fig. 1. Transgene constructs and expression. (A) IRBP promoter directs WT and mutant *ELOVL4* cDNA expression. (B) WT and mutant *ELOVL4* expression compared with endogenous mouse *Elov4* expression, as determined by measuring mRNA levels (mean ± SEM) from six separate experiments of semiquantitative PCR (example of gel is shown in Fig. 5). In each experiment, endogenous mouse *Elov4* was normalized to 1.0.

vector containing a human IRBP promoter and bovine poly(A) site. All constructs were verified by direct DNA sequencing after restriction enzyme digestion. The final DNA fragments, containing the entire transgene cassette, including the IRBP promoter, mutant *ELOVL4* cDNA, and 3' poly(A) site, were isolated from the plasmids by digestion with *NorI* and *KpnI*, and then used for microinjection.

We injected the constructs into single C57BL/6 mouse embryos. The embryos were implanted into pseudopregnant foster female mice. Potential transgenic founder mice (F_0) with the integration of transgenes were identified by PCR. We used primer pairs specific to human *ELOVL4* to perform a PCR amplification of genomic DNA from mouse tails. The primer used were 5'-TCATATAATGCGGGATATAGC-3' (forward) and 5'-TTCCACCAAAGATATTTCTG-3' (reverse). Founders of WT1, TG1, TG2, and TG3 were mated to C57BL/6 mice to produce mice of the first and subsequent generations used for analysis. All procedures were approved by appropriate institutional animal care and use committees, and were carried out according to National Institutes of Health guidelines.

Expression of *ELOVL4* Transgene. After removal of the lens, retinas from 2-week-old mice were manually separated from the RPE. Total RNA from retina was extracted with TRIzol (GIBCO/BRL) and 10 μ g of total RNA were converted to cDNA by using RT-PCR. Primers used for RT-PCR were 5'-GTGTGGCTGGGTCCAAA-3' (forward) and 5'-TTGGGGAAGGGG-CAGTC-3' (reverse). This RT-PCR amplified both human *ELOVL4* mRNA expressed from the transgene and mouse endogenous *Elov4* mRNA, and generated a 0.55-kb-long fragment. Endogenous mouse *Elov4* cDNA can be distinguished from transgenic *ELOVL4* because of the presence of an *EcoRI* restriction site in human *ELOVL4* cDNA. To determine the level of transgenic *ELOVL4* expression, digested DNA was separated by agarose gel electrophoresis and measured by densitometry using an EAGLE EYE II and EAGLESIGHT (version 3.2) (Stratagene). Semiquantitative RT-PCR and quantitative real-time RT-PCR (qRT-PCR) was performed to compare the relative expression level of human *ELOVL4* and endogenous mouse *Elov4* genes. The primers used

for *ELOVL4* PCR were 5'-CATGTGTATCATCACTGTACG-3' (forward) and 5-AAAGGAATTCAACTGGGCTC-3' (reverse). The primers used for *Elov4* PCR were 5'-CTTCACGTGTAC-CACCACTGC-3' (forward) and 5-GTGGATGAAAGAGTT-CATCTGG-3' (reverse). The primers used for *Gapdh* PCR were 5'-AAATGGGTGAAGGTCGGTGTG-3' (forward) and 5'-CATGTAGACCATGTAGTTGAG-3' (reverse). Real-time RT-PCR of *ELOVL4* and *Elov4* was performed in each sample by using *Gapdh* as an internal control with the QuantiTect SYBER Green RT-PCR kit (Qiagen, Valencia, CA). Real-time PCR was performed on a DNA Engine Opticon 2 system (MJ Research, Waltham, MA). Normalized gene expression relative to *Gapdh* was calculated with Q-GENE software according to manufacturer's instructions.

Fundus Photography. Fundus photographs were taken from 1.5-year-old control mice and transgenic mice from each line with a handheld Kowa RC-2 fundus camera (Kowa Optimed, Torrance, CA) and Volk 90D lens (Volk Optical, Mentor, OH) (14).

Electroretinography (ERG). Electroretinograms were obtained from mice aged 22 weeks and older in a full-field dome by using methods that are analogous to those common in the clinic and stimuli that are comparable to those specified by the International Society for Clinical Electrophysiology of Vision standard (15). After overnight dark adaptation, the eyes were dilated with scopolamine hydrobromide. Mice were anesthetized with a saline solution containing 40 mg/ml ketamine and 2 mg/ml xylazine. A gold-wire coil placed on one cornea was referenced to a needle electrode in the scalp. A needle electrode in the tail served as ground. Signals were amplified (Tektronix AM502 differential amplifier; $\times 10,000$; 3 dB down at 2 and 10,000 Hz), digitized (sampling rate = 1.25–5 kHz) and averaged. Two different flash stimulators were used. A Grass photostimulator provided short-wavelength 20- μ s flashes (Wratten 47A: max, 470 nm; half-bandwidth, 55 nm) from -3 to 1 log scotopic trolands (scot td-s) in 0.3 log unit steps. Peak-to-peak *b*-wave amplitude–response functions were fit to Michaelis–Menton functions. A Novatron flash unit produced achromatic 1.3-ms flashes from 1 to 3.4 log scot td-s in 0.3 log unit steps. Rod-mediated *a*-waves were fit to a computational model to determine transduction parameters for photoreceptor responses (16). A second Novatron flash unit was mounted in the dome for two-flash studies of photoreceptor inactivation kinetics (17). Mice were exposed to a background (3.2 log Phot-td) for 10 min before eliciting cone-mediated responses with achromatic flashes ranging from 0.2 to 1.4 Phot-td-s.

Histology and Light Microscopy. Mice were maintained in a continuous 12-h light/12-h dark cycle and killed 8–12 h after the onset of the light phase. Anesthetized mice were perfused with 0.1 M PBS and then with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) by intracardiac injection. The superior sclera of each eye was marked. Eyecups were processed for embedment in Epon. From each animal, we obtained 0.5- μ m sections, passing along the dorso-ventral axis of the retina, through the optic nerve head. The width of the photoreceptor outer nuclear layer was determined from sections in which the photoreceptor cells were sectioned along their long axes. In this case, the photoreceptor nuclei appear in columns. The width was expressed in terms of the number of nuclei spanning the layer. Counts were made along the two regions 200–300 μ m dorsal and ventral from the optic nerve head. Five counts were made in each region, giving a total of 10 counts, which were averaged for each eye. For fluorescence microscopy of lipofuscin, thick (8 μ m) cryosections of formaldehyde-fixed retinas were examined by epifluorescence using FITC filters.

Electron Microscopy. Eyes were fixed by whole-animal perfusion, as above, and processed for embedment in Epon 812 resin. Ultrathin

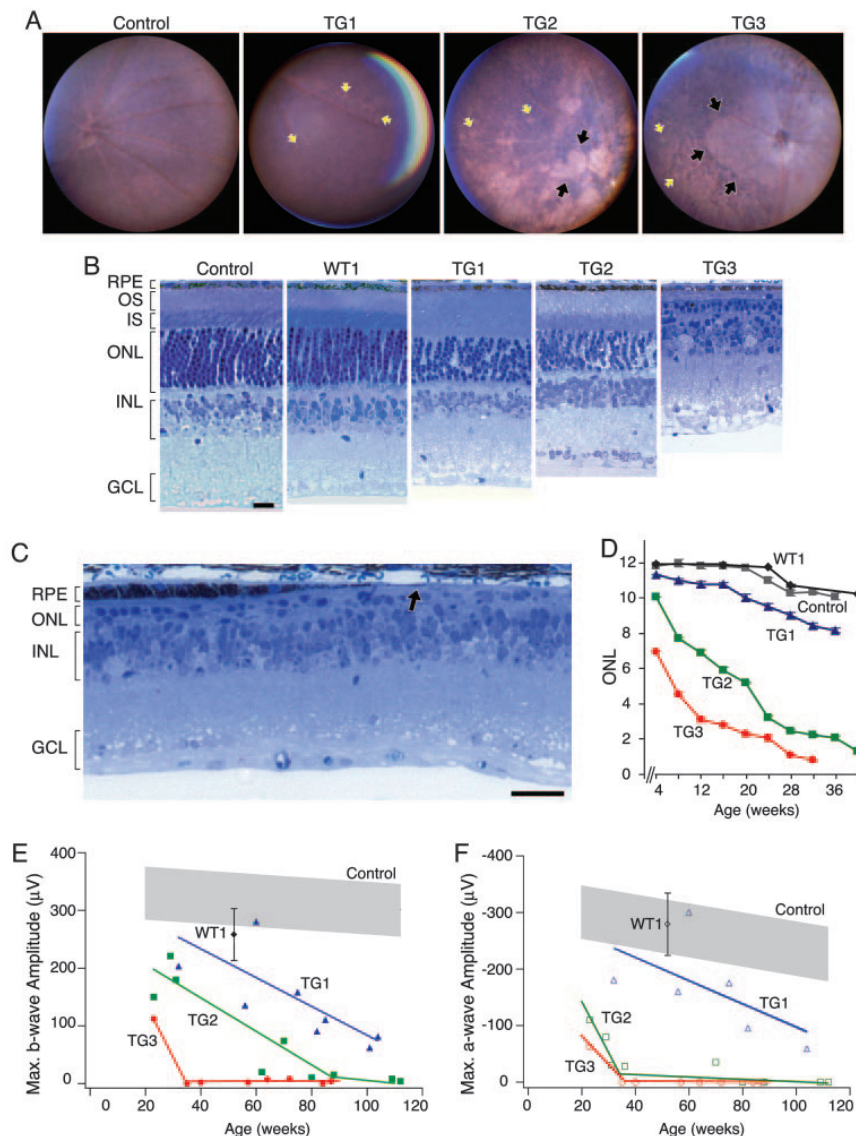


Fig. 2. Characterization of photoreceptor degeneration in *ELOVL4* mutant mice. (A) Fundus photographs from representative mice at ≈ 1.5 years of age. Abnormalities in fundus appearance were more evident with higher levels of mutant *ELOVL4* expression (TG3 > TG2 > TG1). They included depigmented subretinal spots (small yellow arrows) and geographical atrophy (large black arrows). (B) Light microscopy of retinas of WT and transgenic mice. The control retina from a 1-year-old mouse is normal. The retina from a 1-year-old TG1 mouse has lost approximately two layers of photoreceptor cell nuclei. The retina from a 12-week-old TG2 mouse has shortened outer and inner segments and a moderately reduced outer nuclear layer (ONL). The retina from a 12-week-old TG3 mouse has lost a large number of photoreceptor cells, and has perturbed photoreceptor inner and outer segment layers. RPE, retinal pigmented epithelium; OS, photoreceptor outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (Scale bar, 20 μ m.) (C) Lower magnification light microscopy of a 6-month-old TG3 retina, showing peripheral (Left) to central (Right) progression in RPE degeneration. Arrow indicates the margin of complete RPE atrophy. Abbreviations are as in B. (Scale bar, 40 μ m.) (D) Time course of the progressive loss of photoreceptors in mutant *ELOVL4* transgenic mice up to 36 weeks of age. Photoreceptor cell nuclei were counted from the regions that are 200–300 μ m dorsal and ventral from the optic nerve head. Retinas from six mice were counted for each mouse line at each age shown. Error bars (which, in most cases, are too small to be evident) represent $\pm$ SEM. The rate of photoreceptor cell loss was greater with higher levels of mutant *ELOVL4* expression. (E) Progressive loss of the maximal b-wave response of ERG recordings in mutant *ELOVL4* transgenic mice over time. The response declined in all transgenic lines, but was greater with higher levels of mutant *ELOVL4* expression. Mice expressing WT *ELOVL4* (WT1) were indistinguishable from WT mice (90% of WT lie within gray area). (F) Progressive loss of the maximal a-wave response of ERG recordings in mutant *ELOVL4* transgenic mice over time. The response declined in all transgenic lines, but was greater with higher levels of mutant *ELOVL4* expression. Mice expressing WT *ELOVL4* (WT1) were indistinguishable from WT mice (90% of WT lie within gray area)

sections were mounted on copper grids and stained with uranyl acetate and lead citrate before observation in a Philips (model 208) electron microscope.

A2E Extraction. A2E and its isomers were extracted and isolated from 2-month-old mouse eyecups, with their neural retinas removed, by using a modified version of the protocol described in ref.

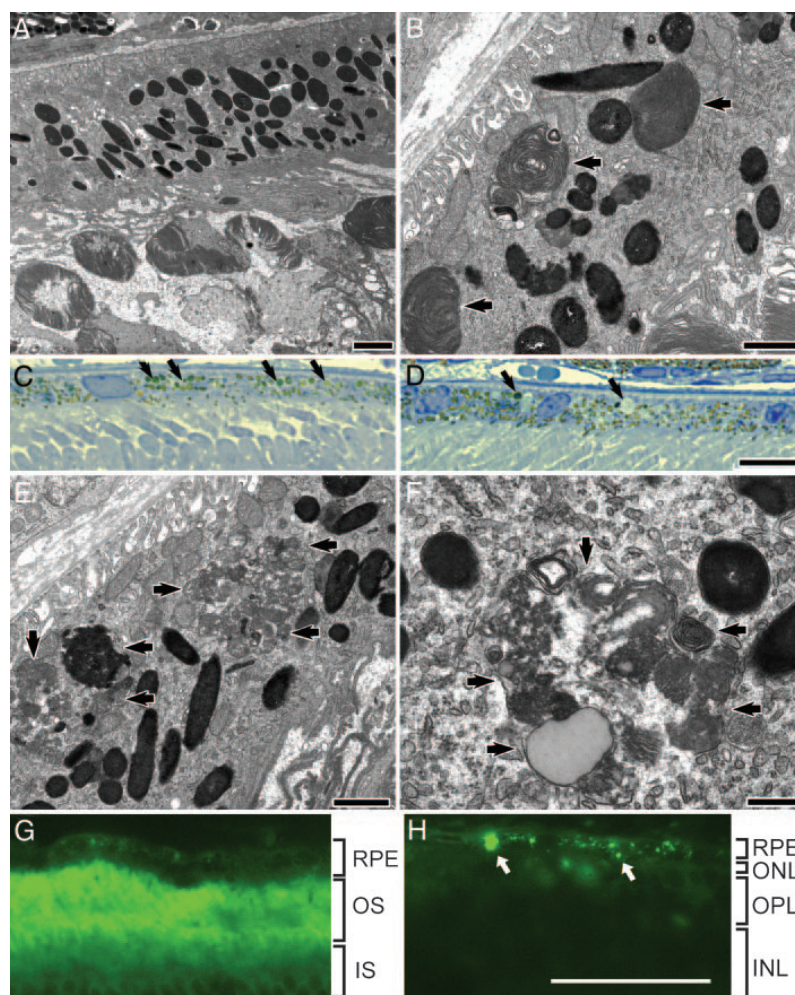


Fig. 3. Ultrastructure of pathology. (A) Photoreceptor outer segments from a 2-month-old TG3 retina, showing disorganized disk membranes. The RPE appears relatively normal. (B–F) Micrographs from 7-month-old TG2 mouse retinas. Arrows indicate phagosomes containing undigested ROS disk membranes (B), bodies in the RPE that correspond to accumulations of lipofuscin material (C and D), more clearly defined by electron microscopy (E and F). In the higher magnification EM image (F), the lipofuscin accumulation is shown to contain undigested membranes, amorphous material, and lipid droplets (lowest arrow indicates a large lipid droplet). (Scale bars, 2 μ m in A; 1 μ m in B and E; 10 μ m in C and D; and 300 nm in F.) (G and H) Lipofuscin accumulation in the RPE cells, as shown by autofluorescence. Cryosections of 10-month-old normal littermate control (G) and TG2 (H) retinas. Several bright dots and clusters of lipofuscin (e.g., arrows) are evident in the RPE cells of the TG2 transgenic mouse, using the FITC filter, whereas only few small dots are present in the normal retina (from a littermate control). Autofluorescence of the photoreceptor outer segments is evident in the control retina. Only a few photoreceptor cells with very short outer segments remain in the transgenic mouse. (Scale bar, 50 μ m.)

18. The RPE was regarded as the origin of all of the A2E and its isomers in the eyecups. TG2 and normal control littermate samples were homogenized in 1:1 CHCl_3 /Methanol (MeOH) (2 ml) and 0.01 M PBS (1 ml). The homogenizer was washed with 1:1 CHCl_3 /MeOH (2 ml), 0.01 M PBS (1 ml), then CHCl_3 (5 ml) and CH_2Cl_2 (5 ml) to remove any remaining material. All solutions were combined, and the organic layer was extracted from the aqueous layer. The aqueous material was then extracted three times with 1:1 CHCl_3 / CH_2Cl_2 (10 ml). The combined organic extracts were dried on Na_2SO_4 and concentrated *in vacuo*, leaving ≈ 2 ml of solution. This solution was eluted with 0.1% trifluoroacetic acid (TFA)/MeOH (6 ml) on a cotton filter and then on a short C18 plug. The filtered material was concentrated *in vacuo* and dissolved in a minimal amount of 1:1.5 MeOH/ CH_2Cl_2 .

HPLC Analysis. A gradient (84–100% acetonitrile/ H_2O in 0.05% TFA) was used to separate A2E on a Waters 600 HPLC with photodiode-array detector, by using a reverse-phase C18 column (Cosmosil 4.6 $\times$ 250 mm, Sorbent Technologies, Atlanta). A2E and isomers were quantified by using external A2E standards.

Results

Human *ELOVL4* Gene Expression in Mouse Retina. To gain insight into molecular mechanism of macular degeneration caused by mutant *ELOVL4*, we generated transgenic mice carrying the 5-bp deletion mutant *ELOVL4*. Expression of this transgene was driven by an IRBP promoter, so that it was specific for photoreceptor cells (Fig. 1A). Photoreceptor cell expression of the human transgene was verified by *in situ* hybridization using a probe that was specific for

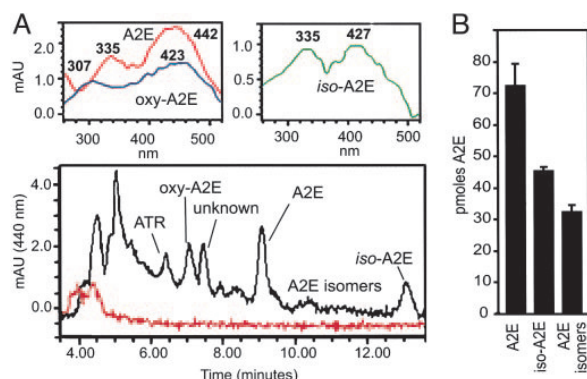


Fig. 4. Analysis and quantification of A2E in the RPE of TG2 and littermate control mice. (A) (Upper) UV-visible spectra of A2E, with λ_{max} of 442, 335 (red), oxy-A2E, with λ_{max} of 423, 307 (blue), and iso-A2E, with λ_{max} of 427, 335 (green). (Lower) HPLC chromatogram of 2-month-old TG2/littermate RPE extracts (red, littermate; black, TG2). Peaks of interest are labeled as ATR (all-trans retinal), oxy-A2E (oxygenated A2E), an uncharacterized bis-retinoid, A2E (all-trans), A2E isomers (additional isomers of A2E), iso-A2E (13-cis). ATR was presumably resulted from residual photoreceptor outer segments during eyecup preparation. (B) Quantification of A2E and isomers in TG2 mouse eyecups: all-trans-A2E, 72.45 pmol per eye $\pm$ 7.9; iso-A2E, 45.7 pmol per eye $\pm$ 2.1; and remaining A2E isomers, 0.65 nmol per eye $\pm$ 0.04. No detectable amount of A2E was found in littermate RPE samples.

the human *ELOVL4* mRNA. There was no apparent difference in the expression of the transgene between photoreceptors in central and peripheral retina (data not shown). To assess expression levels of the mutant *ELOVL4* transgene in different mouse lines semi-quantitative RT-PCR was carried out by using total retinal RNA. Levels of *ELOVL4* (WT or mutant) expression in WT1, TG1, TG2, and TG3 were found to be 1.0, 0.6, 3.3, and 5.3 times the level of endogenous mouse *Elov4*, respectively (Fig. 1B and Fig. 5, which is published as supporting information on the PNAS web site). The levels of WT and mutant *ELOVL4* expression were verified by real-time quantitative RT-PCR using *Gapdh* as an internal control (data not shown).

Photoreceptor Degeneration in Mutant *ELOVL4* Transgenic Mice. Fundus images and light microscopy of retinal sections from the transgenic mice revealed progressive retinal degeneration (Fig. 2A–C). Abnormalities in fundus appearance related to the expression level of the mutant *ELOVL4*; higher level of expression resulted in a more abnormal fundus (Fig. 2A). Nevertheless, even the TG1 mice, with less expression of the mutant *ELOVL4* than of the normal endogenous *Elov4*, underwent retinal deterioration, with subretinal deposits similar to retinal flecks seen in patients with STGD (TG1 in Fig. 2A). The fundus images also show that the deterioration of the retina is localized, as found in patients with a severe form of dry AMD, called “geographical atrophy” (e.g., TG3 in Fig. 2A). These localized regions of the retina appear lighter because of depigmentation in areas of RPE atrophy and underlying photoreceptor degeneration (Fig. 2A, especially TG3). Thus, they resemble fundus images of patients with STGD and the dry form of AMD (compare images in ref. 3). In contrast, expression of WT *ELOVL4* at a higher level than that of TG1 does not cause retinal degeneration (Fig. 2A, WT1).

Histological sections of the retinas support the fundus images. Photoreceptor cell degeneration is evident by the loss of photoreceptor nuclei (from the outer nuclear layer). The extent of this loss is greater in the mice with higher expression of mutant *ELOVL4* (Fig. 2B). In the different lines, 50% of the photoreceptors were lost from the regions, 200–300 μ m dorsal and ventral from the optic

nerve head, at 6 weeks, 16 weeks, and 18 months for the TG3, TG2, and TG1 mice, respectively (Fig. 2D). Also, like the fundus images (Fig. 2A), histology showed that the photoreceptor degeneration was not uniform across the retina. The degeneration was typically more severe in the center of the retina rather than in the periphery (Fig. 6A–E, which is published as supporting information on the PNAS web site). Similarly, RPE atrophy was typically more severe in the center of the retina than in the periphery (Fig. 2C).

Deterioration of Electrophysiological Responses. ERG recordings were performed to assess retinal function in the different lines of transgenic mice. In mice expressing WT *Elov4* (WT1), we found no reduction in the average maximum rod *b*-wave response at 1 year of age ($258 \pm 45 \mu$ V) relative to WT ($304 \pm 58 \mu$ V). In lines expressing mutant *ELOVL4*, the rate of deterioration of maximal rod *b*-wave responses, like the alterations in fundus images and histology, was found to correlate with the level of expression (Fig. 2E; see also Fig. 7 and Supporting Text, which are published as supporting information on the PNAS web site). Mice from the lowest expressing line (TG1) had maximum *b*-wave amplitudes toward the lower end of the normal range at 22 weeks of age and showed a decline to about one-third the normal amplitude by 84 weeks (1.5 years) of age. TG2 mice showed a more rapid decline so that responses were not detectable by 84 weeks. TG3 mice showed greatly reduced responses at 22 weeks and were not detectable by 35 weeks. Cone *b*-wave obtained in the presence of a rod-saturating background showed declines in amplitude that closely paralleled those in rod *b*-waves (Fig. 7).

Electron Microscopy of Photoreceptor Outer Segments. Before photoreceptor degeneration, the ROS disk organization appeared normal in the TG1 and TG2 retinas. However, in retinas expressing the higher level of mutant *ELOVL4* (TG3), failure to form a completely normal ROS structure was evident as early as 3 weeks of age. The ROSs were severely stunted. They contained disk membranes, but the disks were clustered in relatively small groups and not stacked as in normal outer segment structure (for example, see Fig. 3A).

Accumulation of Lipofuscin in the RPE. Photoreceptor cells in TG1 and TG2 mice degenerated much more slowly than the rapidly degenerating cells in TG3 mice (Fig. 2). Because of the longer period for the degeneration to unfold in the TG1 and TG2 retinas, we were able to observe events that take longer to manifest themselves. More undigested phagosomes containing whorls of membrane were evident in these mutant retinas than in controls (Fig. 3B) irrespective of the time of day, and by $\approx$ 7 months of age in TG2 retinas, large accumulations of lipofuscin were observed throughout the RPE. The approximate frequency of these accumulations in the central retina is indicated by the lower-power light micrographs in Fig. 3C and D. Electron micrographs show that the accumulations contain membranous debris and lipid droplets, typical of lipofuscin (Fig. 3E and F). Fluorescence images depicting autofluorescence also show the increased accumulation of lipofuscin in the RPE of mutant *ELOVL4* retinas (Fig. 3H and D). Lastly, we measured the levels of A2E, iso-A2E, and additional A2E isomers (the major blue-light absorbing fluorophores in lipofuscin) in the RPE of the TG2 and control mice at 2 months of age. We found large amounts of these compounds in TG2 mice, whereas levels in control mice were below the level of detection (Fig. 4). In addition, the RPE damaging oxy-A2E, as well as an unknown amino-retinoid compound and all-trans retinal, were all found in significant quantities in TG2 mice (Fig. 4A). Together, these results indicate an increased level of lipofuscin in the RPE of mutant *ELOVL4* transgenic mice before significant photoreceptor cell death.

Discussion

In the present study, we generated transgenic mouse lines, each expressing different levels of WT or mutant *ELOVL4*. As a result of mutant *ELOVL4*, we observed and characterized photoreceptor degeneration, which possessed the hallmarks of macular degeneration, including accumulation of lipofuscin in the RPE, development of abnormal electrophysiology, and localized atrophy of the RPE and photoreceptors. The *ELOVL4* transgenic mice thus represent an appropriate model for STGD and dry AMD.

We found no evidence from our analysis that expression of the WT *ELOVL4* caused photoreceptor degeneration. The development of mutant phenotypes, including photoreceptor and RPE degeneration, was related to the expression level of the mutant *ELOVL4* transgene. It is important to note that even though the phenotypes developed more slowly in the TG1 mice, they still occurred. In this line of mice, the expression level of the mutant *ELOVL4* was not greater than that of the endogenous *Elovl4* (it was significantly less). From the published data on human patients, it is unclear whether STGD3 results from a dominant gain of function or dominant negative effect of the mutant *ELOVL4*, or simply from haploinsufficiency. Our observations of faster development of the mutant phenotypes with increased transgene expression demonstrate a dominant effect of the mutant gene.

AMD and STGD share some important clinical and pathologic features including lipofuscin accumulation in RPE and photoreceptor death. The *ELOVL4*-mutant mouse phenotypes closely mimic not only those of STGD, but also many aspects of AMD, so that the mice may have a more general application. By microscopy, mutant *ELOVL4* mice were found to exhibit significant lipofuscin accumulation and photoreceptor and RPE atrophy in a pattern closely resembling the human counterpart; the central region of retina surrounding the optic nerve head had a more severe phenotype than that of the periphery. Although the mouse retina does not have a macula *per se*, there is a higher concentration of photoreceptors in the center versus the periphery. The histopathology of the mutant retinas was consistent with the fundus appearance, which resembles the geographic atrophy found in STGD and dry AMD patients. It was also consistent with the ERG changes, which reflected localized areas of both rod and cone photoreceptor loss.

ELOVL4 is expressed in rod and cone photoreceptor cells (5) and encodes a protein with similarities to proteins involved in elongation of long chain fatty acids (3). ROS membranes have an unusual fatty acid composition, with very high levels of docosahexaenoic acid (19). Blood levels of docosahexaenoic acid and other long-chain fatty acids are often lower in patients (20, 21) and animals (22) undergoing photoreceptor degeneration. ROS fatty acid composition may be important for the well defined lamellae structure of the

ROS disks. Interestingly, however, it was only in mice of the highest expressing line (TG3), with a 5-fold overexpression of mutant *ELOVL4*, that we observed any irregularities in disk membrane organization. It may be that in the TG3 photoreceptors, the fatty acid composition was so perturbed that instability of the ROS disk membranes is the primary defect. In the TG2 and TG1 photoreceptors, where expression of mutant *ELOVL4* was more moderate, the primary pathology appears in the RPE cells. The abnormal accumulation of undigested phagosomes suggests a problem with the disposal of ingested disk membranes, which probably underlies the accumulation of lipofuscin. It has long been speculated that the deposition of lipofuscin in the RPE results from incomplete digestion of phagocytosed ROS membranes (23).

It has been proposed that RPE pathology is a major contributor to photoreceptor cell death in macular degeneration, stemming from excessive accumulation of lipofuscin, and in particular, A2E and its isomers, the major blue-light absorbing fluorophores of lipofuscin in the RPE (24, 25). Our results show large amounts of A2E accumulation in *ELOVL4* mutant mice compared with undetectable amounts in littermate controls. In the *Abca4* knockout mouse, which is a model for recessive Stargardt macular degeneration, A2E-containing lipofuscin is also present in the RPE (26, 27). The *ELOVL4*-transgenic mouse thus represents a second model of Stargardt macular degeneration showing a deleterious RPE accumulation of A2E-containing lipofuscin; although the *ELOVL4*-transgenic mice appear to accumulate manyfold more A2E than *Abca4* knockout mice. In addition to emphasizing the importance of lipofuscin accumulation in Stargardt macular degeneration, our results provide evidence of its relevance to macular degeneration in general. Together with lipofuscin accumulation, the *Abca4* knockout mouse has a delay in dark adaptation, as measured by ERG, but does not undergo photoreceptor death or severe ERG changes (26) like those we have observed in *ELOVL4* transgenic mice. Thus, the *ELOVL4* transgenic mice have a somewhat closer phenotype to human STGD and AMD. The progressive decline in retinal function of the *ELOVL4* transgenic mice makes them an attractive model for tests of therapeutic intervention.

We thank Drs. Kim Howes, Wolfgang Baehr, and Jeanne M. Frederick for advice and critical reading of the manuscript. This research was supported by National Institutes of Health Grants R01EY14428, R01EY14448, and GCRC M01-RR00064 (to K.Z.), R01EY13408 and core P30EY12598 (to D.S.W.), and R01EY05235 (to D.G.B.), the Knights Templar Eye Research Foundation (to G.K. and Z.Y.), Brigham Young University (to H.R.V.-S.), and grants (to K.Z.) from the Ruth and Milton Steinbach Fund, Ronald McDonald House Charities, the Macular Vision Research Foundation, Research to Prevent Blindness, Inc., Grant Ritter Fund, American Health Assistance Foundation, the Karl Kirchgessner Foundation, the Val and Edith Green Foundation, and the Simmons Foundation.

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