Published in final edited form as:

Exp Eye Res. 2009 December ; 89(6): 905–912. doi:10.1016/j.exer.2009.07.021.

***Elovl4* 5-bp deletion knock-in mouse model for Stargardt-like macular degeneration demonstrates accumulation of ELOVL4 and lipofuscin**

Vidyullatha Vasireddy¹, Monica M. Jablonski², Naheed W. Khan³, Xiao Fei Wang², Priya Sahu², Janet R Sparrow⁴, and Radha Ayyagari^{1,*}

¹ Shiley Eye Center, University of California San Diego, La Jolla, CA

² UTHSC Hamilton Eye Institute, University of Tennessee, Memphis, TN

³ Kellogg Eye Center, University of Michigan, Ann Arbor, MI

⁴ Department of Ophthalmology, Columbia University, New York, NY

Abstract

The mechanism underlying photoreceptor degeneration in autosomal dominant Stargardt like macular degeneration (STGD3) due to mutations in the elongation of very long chain fatty acids-4 (*ELOVL4*) gene is not fully understood. To evaluate the pathological events associated with STGD3, we used a mouse model that mimics the human STGD3 phenotype and studied the progression of retinal degeneration. Morphological changes in the retina of *Elovl4* 5 bp-deletion knock-in mice (*E_mut*^{+/-}) were evaluated at 22 months of age. The localization of ELOVL4, and the expression pattern of inner retinal tissue marker proteins, and ubiquitin were determined by immunofluorescence labeling of retinal sections. Levels of the retinal pigment epithelium (RPE) lipofuscin fluorophores were measured by quantitative HPLC. Morphological evaluation of the retina revealed an accumulation of RPE debris in the subretinal space. A significant increase in the amount of ELOVL4 was observed in the outer plexiform layer in *E_mut*^{+/-} mice compared to controls. Apart from the accumulation of ELOVL4, *E_mut*^{+/-} mice also exhibited high expression of ubiquitin in the retina. Analysis of lipofuscin fluorophores in the RPE showed a significant elevation of A2E and compounds of the all-*trans*- retinal dimer series in retinas from four and ten month old *E_mut*^{+/-} mice compared to wild-type littermates. These observations suggest that abnormal accumulation of ELOVL4 protein and lipofuscin may lead to photoreceptor degeneration in *E_mut*^{+/-} mice.

Keywords

STGD3; ELOVL4; protein accumulation; lipofuscin

*Corresponding Author: Radha Ayyagari, Ph.D, #206, Jacobs Retina Center, Shiley Eye center, 9415 Campus Point Drive, La Jolla, CA-92037, rayyagari@ucsd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Stargardt-like macular degeneration (STGD3) is inherited in an autosomal dominant manner. Patients affected with STGD3 exhibit progressive central vision loss with onset in the second decade of life. Fundus examination of these patients reveals retinal pigment epithelium (RPE) atrophy and yellowish flecks (Griesinger et al., 2000; Stone et al., 2004). The sequence of elongation of very long chain fatty acids 4 (*ELOVL4*), the gene associated with STGD3 is homologous to mammalian and yeast elongases (ELO). The ELO groups of proteins are known to be involved in the elongation of very long chain fatty acids. Like other ELO proteins, *ELOVL4* contains a distinct iron binding motif, a histidine cluster (HXXHH), and an endoplasmic reticulum (ER) retention signal at its C-terminal end (KXXXX)(Zhang et al., 2001). In the retina, *ELOVL4* is localized predominantly to the ER of rod and cone photoreceptors (Ambasudhan et al., 2004). Based on the sequence similarities and the abundance of *ELOVL4* in retina, it was predicted that *ELOVL4* may be involved in retinal docosa hexaenoic acid (DHA 22:6 (ω -3)) metabolism, but recent studies revealed that *ELOVL4* is critical for elongation of very long chain fatty acids (Vasireddy et al., 2007).

So far three different mutations in the *ELOVL4* gene have been identified: 1) a 5-bp deletion mutation (Zhang et al., 2001; Maugeri et al., 2004); 2) two 1-base pair mutations; and 3) a nonsense mutation (Zhang et al., 2001; Maugeri et al., 2004). All of these mutations lead to the loss of 51 amino acids from the C-terminal end and premature truncation of the protein. Our previous studies established that mutations in the *ELOVL4* gene exert dominant negative effect (Vasireddy et al., 2005). Further studies on a knock-in mouse model with *Elov4* 5-bp deletion (*E_mut^{+/-}*) in the heterozygous state demonstrated progressive photoreceptor degeneration with a predominant loss of cones at 6–18 months of age. In addition to retinal changes, the *Elov4* 5-bp deletion knock-in mice also showed an accumulation of *ELOVL4* in the inner segments (IS) of the photoreceptors and outer plexiform layer (OPL) at 4 months of age (Vasireddy et al., 2006). Mice carrying the 5-bp deletion mutation in the homozygous state die due to a defective skin permeability barrier. Studies on these mice revealed involvement of *ELOVL4* in the synthesis of fatty acids with chain length longer than C>26 (Vasireddy et al., 2007). To gain insight into the role of *ELOVL4* in the pathophysiology of STGD3 and progression of retinal degeneration, we further characterized *E_mut^{+/-}* mice, and evaluated the changes of retinal pigment epithelium (RPE) and inner retinal layers of *E_mut^{+/-}* mice between 4 and 22 months of age. Electro retinographic response of these mice was measured at age 22 months. Quantification of the RPE lipofuscin fluorophore, A2E and lipofuscin pigments of the all-*trans*-retinal dimer series in eye cups of *E_mut^{+/-}* mice revealed a significant elevation of these fluorophores in *E_mut^{+/-}* retina compared to controls. Immunolabeling of *E_mut^{+/-}* mice retinal sections detected an accumulation of *ELOVL4* in the dendritic tips of rod bipolar cells. No changes were observed in the ERG response and architecture of inner retinal layers at older age. Together these observations suggest that accumulation of *ELOVL4* and lipofuscin in the retina may lead to the progression of photoreceptor degeneration in mice carrying the *Elov4* 5-bp deletion in the heterozygous state.

MATERIALS AND METHODS

Anti-*ELOVL4* antibodies (1:500 dilution, Abcam, Cambridge, MA), The affinity-purified *ELOVL4* antibody was described previously (1:500 dilution (Lagali et al., 2003), anti- PKC alpha antibodies (Sigma-Aldrich, St. Louis, MO), anti-PSD-95 antibody, Alexa fluor-448 (1:1000 dilution, Invitrogen-Molecular probes, Carlsbad, CA); Alexa fluor-555 (1: 2500 dilution, Invitrogen-Molecular probes, Carlsbad, CA), Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), anti-ubiquitin antibody (1:500 dilution, Sigma-Aldrich, St. Louis, MO); paraformaldehyde (EMS), normal goat serum (Vector labs, Burlingame, CA).

Animal maintenance and tissue preparation for immunohistochemistry

Animals were maintained according to the ARVO policy for animal maintenance and following the guidelines of institutional Animal Care and Use Committee. Breeding and genotyping of *Elovl4* knock-in animals was described elsewhere (Vasireddy et al., 2006). All animals were maintained in 12 hours light and 12 hours dark cycles. To genotype for murine *Rpe65* *Leu450Met* variant, DNA derived from mouse tails was PCR-amplified with forward 5'-ACCAGAAATT TGGAGGGAAAC-3' and reverse 5'-CCCTTCCA TTCAGAG CTTCA-3' primers. Digestion of the resulting 545-bp product with 50-fold excess of *Mwo*I restriction enzyme (New England Biolabs, Ipswich, MA) yielded fragments of 180 and 365 bp in the presence of the sequence variant corresponding to Leu450. Undigested 545-bp product was resolved with the Met450 variant and 180, 365 and 545-bp products were indicative of heterozygosity. To avoid variation in lipofuscin content in *E_{mut}^{+/-}* mice due to *Rpe65* Leu450 Met variation, all mice used for lipofuscin estimation were genotyped for this change.

Histology

Twenty two month old *E_{mut}^{+/-}* mice and age matched littermate control animals were perfused with 4% paraformaldehyde in 0.1M phosphate buffer. After enucleation, the eyes were immersed in the same fixative for 24 hours. After fixation, eyes were thoroughly washed with PBS and embedded in OCT and processed for sectioning.

Immunofluorescence labeling of retinal sections

Immunofluorescence labeling of retinal sections was performed as described earlier (Vasireddy et al., 2006). Briefly, 10 μ m thick cryosections were cut from paraformaldehyde (4% w/v)-fixed and OCT-embedded eye cups. The sections were treated with proteinase K, and non-specific binding of the antibody was blocked using 10% normal goat serum in PBS containing 0.1% Triton-X 100 for 1 hour at room temperature (RT), and further incubated overnight at 4°C with diluted primary antibody mixture. Sections were then washed and incubated in appropriate secondary antibody mixture for 1 hour at RT. Following thorough washes with PBS containing Triton X-100 and PBS alone, slides were mounted in mounting media containing DAPI and observed with a Zeiss confocal microscope.

Analysis of lipofuscin pigments

Posterior murine eye cups including sclera, choroid, RPE, and neural retina, were homogenized using a glass tissue grinder, were extracted three times with chloroform/methanol (2:1) and then passed through a reverse phase cartridge (C18 Sep-Pak, Millipore) with 0.1% TFA in methanol. The extract was dried under argon, redissolved in methanol, and analyzed by reverse-phase HPLC using an Alliance System (Waters, Corp, Milford, MA) equipped with 2695 Separation Module, 2996 Photodiode Array Detector, a 2475 Multi π Fluorescence Detector.

Chromatographic separation was achieved on analytical scale Atlantis® dC18 (3 μ m, 4.6 $\times$ 150 mm, Waters, USA) and Delta Pak® C4 (5 μ m, 3.9 $\times$ 150 mm, Waters, USA) columns and an acetonitrile and water gradient with 0.1% trifluoroacetic acid (gradient for dC18; 90–100%, 0–10 min; 100% acetonitrile, 10–20 min; gradient for C4; 75%, 0–5 min; 75–100%, 5–20 min; flow rate, 0.8 ml/min; monitoring at 430 and 510 nm; 10 μ L injection volume). Integrated peak areas were determined using Empower® software, and picomolar concentrations were calculated by reference to external standards of synthesized compounds, the structures of which have been corroborated (Parish et al., 1998; Fishkin et al., 2005; Kim et al., 2007b). For quantitation, A2E, isoA2E and unconjugated all-*trans*-retinal dimer were monitored at 430 nm and all-*trans*-retinal dimer-phosphatidylethanolamine and all-*trans*-retinal ethanolamine were monitored at 500 nm.

RESULTS

Morphology of *E_mut*^{+/-} mice retina

Earlier we reported abnormalities in the RPE and photoreceptor layer of the retina of *E_mut*^{+/-} mice at age 2–15 months. To further evaluate the progression of retinal degeneration in these mice, we investigated the histological changes in the retinas of 22 month old *E_mut*^{+/-} and control mice. Morphological evaluation demonstrated abnormalities in the RPE and photoreceptors. An accumulation of RPE debris in the subretinal space was observed, consistent with our observations in *E_mut*^{+/-} mice at younger ages. Specifically, pigment granules of RPE origin were often found among photoreceptor outer segments. Moreover inner segments were swollen in the *E_mut*^{+/-} mice compared to wild-type littermate controls (Fig. 1). Although these changes were consistent, variation was noted in the amount of RPE debris between animals.

Lipofuscin accumulated in the RPE of *E_mut*^{+/-} mice

Ultrastructural analysis of the RPE of 8 month old *E_mut*^{+/-} mice demonstrated an increased number of electron-dense inclusion bodies compared to age matched controls (Fig. 2). The structure of these electron dense inclusion bodies resembled lipofuscin granules. The number of these lipofuscin granules is significantly higher in *E_mut*^{+/-} mice compared to controls (Fig. 3). Other striking features that are present in the RPE of *E_mut*^{+/-} mice are the presence of many cytoplasmic vacuoles, enlarged melanosomes and the lack of structural definition of the basal infoldings of the RPE (Fig. 2).

Accumulation of lipofuscin pigment in the retina of *E_mut*^{+/-} mice

To further characterize the accumulated lipofuscin in the *Elovl4* 5-bp deletion mutant, we analyzed extracts of posterior eye cups harvested from *E_mut*^{+/-} and wild-type mice for their content of lipofuscin fluorophores (Fig. 4A). Levels of A2E and isoA2E were not significantly different in *E_mut*^{+/-} and wild-type mice at 2 months of age. However, by 4 months, A2E and isoA2E were increased ($p=0.04$) in eye cups of *E_mut*^{+/-} mice, compared to control mice and this difference was also observed at 10 months of age ($p>0.02$).

The RPE lipofuscin pigments of the all-*trans*-retinal dimer series [all-*trans*-retinal dimer, all-*trans*-retinal dimerphosphatidylethanolamine (PE) and all-*trans*-retinal dimer-ethanolamine (E)] were also quantified in eye cups from 4 month old mice and since these compounds are related, picomole levels were summed (Fig. 4B). As with A2E and isoA2E, total levels of the all *trans*-retinal dimer compounds were modestly elevated at 4 months of age, but did not approach the amounts present in the *Abca4* knock-out mouse, a model of recessive Stargardt disease. At 10 months of age levels of A2E and isoA2E were also significantly elevated in *E_mut*^{+/-} mice, compared to controls ($P>0.02$). Comparison of *E_mut*^{+/-} mice with *Abca4*^{-/-} mice, a model for recessive Stargardt disease, revealed that A2E levels were greater in the latter mutant mice. These results indicate that bisretinoid lipofuscin pigments are elevated in the retina of mice carrying the *Elovl4* 5-bp deletion in the heterozygous state.

ELOVL4 accumulates in the retina

To examine the localization of ELOVL4 in *E_mut*^{+/-} mice, we performed immunohistochemical labeling of retina from 4 month old *E_mut*^{+/-} mice and age matched controls. Consistent with our earlier observation, intense labeling of ELOVL4 was detected in the OPL region of the retina in *E_mut*^{+/-} mice as compared to controls, suggesting the accumulation of ELOVL4 protein in these animals (Fig. 5).

To further confirm the localization of ELOVL4 in OPL, retinal sections were co-labeled with rod bipolar cell specific marker, protein kinase C- alpha (PKC) (Fig. 6) and PSD-95 (Fig. 7)

antibodies along with anti-ELOVL4 antibodies. Co-immuno labeling of retinal sections of *E_mut*^{+/-} mice and control mice retina with anti-ELOVL4 and anti-PKC antibody showed co-localization of ELOVL4 and PKC in the dendritic tips of rod bipolar cells.

Similarly ELOVL4 showed co-localization with PSD-95. PSD-95 belongs to a family of ion-channel clustering proteins and was reported to be involved in synaptic transmission. Immunohistochemical labeling of *E_mut*^{+/-} mice retinal section with PSD-95 and ELOVL4 antibodies demonstrated the co-localization of ELOVL4 with PSD-95 which labels axon terminals of rods and cones, the rod spherules and cone pedicles (Fig. 7)(Koulen et al., 1998).

Expression of ubiquitin in the retina

In cells, aberrant and/or misfolded proteins are degraded and removed by the ubiquitin proteasomal system. If the cellular burden to remove the aberrant protein increases, unprocessed protein will accumulate and lead to cellular stress. Enhanced expression of ubiquitin and ubiquitination of proteins can serve as an indicator of saturated ubiquitin proteasomal degradation (Alves-Rodrigues et al., 1998; Bence et al., 2001; Saliba et al., 2002). In order to compare the levels of ubiquitin in *E_mut*^{+/-} and control retinas, we evaluated the expression levels of ubiquitin in *E_mut*^{+/-} mice by immunohistochemistry (Fig. 8). Ubiquitin immunoreactivity was observed all through the inner retina in both *E_mut*^{+/-} mice and the control mice. However, the intensity of staining was higher in the IS of the photoreceptors in *E_mut*^{+/-} mice than in age matched controls.

DISCUSSION

The progressive accumulation of high amounts of fluorescent lipofuscin compounds in RPE has been observed in inherited retinal disorders (Eagle, 1984). The bis-retinoid pyridinium compound A2E and its photoisomer isoA2E are major components of lipofuscin and these compounds have been structurally characterized (Parish et al., 1998). A2E forms in photoreceptor outer segments via a multi-step biosynthetic pathway that involves reactions of all-*trans*-retinal and phosphatidyl ethanolamine and the formation of an intermediate, dihydroA2PE (A2PE-H₂) (Parish et al., 1998; Liu et al., 2000; Ben-Shabat et al., 2002; Kim et al., 2007a). Phosphate cleavage of A2PE within RPE lysosomes releases A2E. Enhanced accumulation of A2E and the isomers of A2E was reported in the patients of Stargardt and Stargardt-like macular degeneration. However, the amount of lipofuscin accumulation was observed to be less in Stargardt-like macular degeneration patients compared to Stargardt macular degeneration patients. The accumulation of A2E and isoA2E in RPE cells is increased in *Abca4*^{-/-} null mutant mice (Mata et al., 2000; Kim et al., 2007b; Mata, 2001 #634) and considerably more moderate increases in A2E and isoA2E were also observed here in *E_mut*^{+/-} mice. An additional family of lipofuscin compounds has been characterized, the all-*trans*-retinal dimer series, that includes all-*trans*-retinal dimer-PE, all-*trans*retinal dimer-E and unconjugated all-*trans*-retinal dimer (Fishkin et al., 2005; Kim et al., 2007b). The lipofuscin pigment all-*trans*-retinal dimer-PE is the most prominent member of this series and is notable not only because it is increased in *Abca4*^{-/-} mice, but also because in this mouse model of recessive Stargardt disease, all-*trans*-retinal dimer-PE is more abundant than A2E. While total all-*trans*-retinal dimer pigment was increased in *E_mut*^{+/-} mice relative to wild-type, these pigments were considerably less abundant than in *Abca4*^{-/-} mice. This difference is potentially significant. The mechanism by which *ABCR* (*ABCA4*) mutations predispose to higher lipofuscin levels in autosomal recessive Stargardt disease is understood, the reduction in *ABCR* protein activity making all-*trans*-retinal more readily available for formation of the bis-retinoid compounds that constitute lipofuscin pigments. Conversely, the relationship between mutations in *ELOVL4* and RPE lipofuscin formation is not known.

Although ELOVL4 is observed to be localized in both rod and cone photoreceptors, *E_mut*^{+/-} mice demonstrated progressive loss of cones and STGD3 patients showed central vision loss consistent with cone abnormalities. Accumulation of ELOVL4 protein that was observed in OPL regions is significantly higher in dendritic tips of rod bipolar cells. The relationship between accumulation of ELOVL4 protein in specific regions of OPL and cone degeneration is not understood. It has been demonstrated in various animal models that abnormal accumulation of protein in OPL leads to the degeneration of inner retina and affects the structure and function of second order neurons. The dysfunction in the retina in many of these animal models is evidenced by the altered morphology of the inner retina and reduction in electroretinographic (ERG) b-wave amplitudes. In order to evaluate the alterations in synapses, ERG was recorded in *E_mut*^{+/-} mice and compared with age matched controls. The ERG response of *E_mut*^{+/-} mice did not indicate a significant physiological alteration up to age 24 months (data not shown).

Further evaluation of the inner retina with antibodies specific to outer plexiform layer, amacrine cells, horizontal cells, inner plexiform layer, and ganglionic cell marker proteins did not reveal significant abnormalities in the structure and organization of inner retina in *E_mut*^{+/-} mice (data not shown). These observations suggest that accumulation of ELOVL4 protein in IS and OPL may not cause impairment of inner retinal function. These findings are consistent with the retinal phenotype observed in patients with Stargardt-like macular degeneration.

Abnormal protein accumulation has been reported in different forms of macular degenerations, retinal degenerations and other neurodegenerations (Rajan et al., 2001; Saliba et al., 2002; Soto, 2003; Klenotic et al., 2004; Soto and Estrada, 2008). However the molecular pathology underlying these diseases due to protein accumulation is not clear. Altered protein trafficking and accumulation was observed in retinal tissue due to mutations in the *EFEMP1*, Rhodopsin, *TIMP3*, *CTRP5*, *ABCA4* genes (Marmorstein et al., 2002; Klenotic et al., 2004; Mandal et al., 2006). Among these the rhodopsin P23H mutation which is linked to autosomal dominant retinitis pigmentosa (RP) has been studied in detail. This mutation leads to the formation of perinuclear aggregates, increased ubiquitination and compromise in protein degradation (Illing et al., 2002; Saliba et al., 2002). In *E_mut*^{+/-} mice also increased ubiquitination was observed suggesting a possible compromise in ubiquitin mediated protein degradation pathway. Protein trafficking defects leading to apoptosis have been implicated in other degenerative diseases. The molecular pathology underlying the photoreceptor degeneration in *E_mut*^{+/-} mice with abnormal accumulation of ELOVL4 and recruitment of ubiquitin may be similar to the pathology leading to other neurodegenerations caused by protein trafficking defects.

The studies presented here reveal a possible mechanism underlying initiation of photoreceptor death and atrophy of RPE in STGD3. However, it is not clear whether this pathology is entirely due to the abnormal protein accumulation alone. The ELOVL4 is shown to be involved in elongation of Very long chain fatty acids (VLFA) (C>26) (Vasireddy et al., 2007; Vasireddy et al., 2008) and metabolism of C32-C36 acyl phosphatidyl cholines (McMahon et al., 2007). The mutations observed in STGD3 patients exert dominant negative effect, which result in ELOVL4 aggregate formation, may lead to lack or diminished levels of functional ELOVL4. Consequently, levels of very long chain fatty acids may be depleted in the retina. The effect of depletion of VLFA in the retina is not known. The photoreceptor degeneration observed in STGD3 could be either due to the abnormal accumulation of ELOVL4 or lack of VLFA, while the RPE atrophy could be due to accumulation of lipofuscin. Additional studies are needed to understand the role of ELOVL4 in retinal pathology observed in STGD3. Studies on *E_mut*^{+/-} mice indicate that the STGD3 mutation leads to abnormal structure and function of RPE and predominant cone photoreceptor loss. The retinal pathology observed in these mice is similar to the clinical features observed in patients with STGD3. These mice will serve as a

valuable model to study not only STGD3, but also other retinal degeneration diseases that are associated with abnormal accumulation of mutant proteins and/or lipofuscin.

Acknowledgments

The authors would like to thank Austra Liepa (University of Michigan) for generation, maintenance of the animals Mitchell Gillett (University of Michigan) and Kathy Troughton (University of Tennessee) for sectioning of histological specimens This work is supported by grants to RA from NIH-EY13198; Foundation Fighting Blindness (RA), Research to Prevent Blindness Inc., USA (RA); National Eye Institute grants (EY015208 to MMJ) and EY12951 to JRS; National Eye, Institute core grants (EY013080) to the University of Tennessee Health Science Center; unrestricted grant to the Department of Ophthalmology at the University of Tennessee Health Science Center from Research to Prevent Blindness, Inc., New York, NY. JRS is a recipient of a Senior Scientific Investigator Award from Research to Prevent Blindness.

References

- Alves-Rodrigues A, Gregori L, Figueiredo-Pereira ME. Ubiquitin, cellular inclusions and their role in neurodegeneration. *Trends Neurosci* 1998;21:516–520. [PubMed: 9881849]
- Ambasudhan R, Wang X, Jablonski MM, Thompson DA, Lagali PS, Wong PW, Sieving PA, Ayyagari R. Atrophic macular degeneration mutations in ELOVL4 result in the intracellular misrouting of the protein. *Genomics* 2004;83:615–625. [PubMed: 15028284]
- Ben-Shabat S, Parish CA, Vollmer HR, Itagaki Y, Fishkin N, Nakanishi K, Sparrow JR. Biosynthetic studies of A2E, a major fluorophore of retinal pigment epithelial lipofuscin. *J Biol Chem* 2002;277:7183–7190. [PubMed: 11756445]
- Bence NF, Sampat RM, Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* 2001;292:1552–1555. [PubMed: 11375494]
- Eagle RC Jr. Mechanisms of maculopathy. *Ophthalmology* 1984;91:613–625. [PubMed: 6205340]
- Fishkin NE, Sparrow JR, Allikmets R, Nakanishi K. Isolation and characterization of a retinal pigment epithelial cell fluorophore: an all-trans-retinal dimer conjugate. *Proc Natl Acad Sci U S A* 2005;102:7091–7096. [PubMed: 15870200]
- Griesinger IB, Sieving PA, Ayyagari R. Autosomal dominant macular atrophy at 6q14 excludes CORD7 and MCDR1/PBCRA loci. *Invest Ophthalmol Vis Sci* 2000;41:248–255. [PubMed: 10634627]
- Illing ME, Rajan RS, Bence NF, Kopito RR. A rhodopsin mutant linked to autosomal dominant retinitis pigmentosa is prone to aggregate and interacts with the ubiquitin proteasome system. *J Biol Chem* 2002;277:34150–34160. [PubMed: 12091393]
- Kim LS, Fishman GA, Seiple WH, Szlyk JP, Stone EM. Retinal dysfunction in carriers of bardet-biedl syndrome. *Ophthalmic Genet* 2007a;28:163–168. [PubMed: 17896315]
- Kim SR, Jang YP, Jockusch S, Fishkin NE, Turro NJ, Sparrow JR. The all-trans-retinal dimer series of lipofuscin pigments in retinal pigment epithelial cells in a recessive Stargardt disease model. *Proc Natl Acad Sci U S A* 2007b;104:19273–19278. [PubMed: 18048333]
- Klenotic PA, Munier FL, Marmorstein LY, Anand-Apte B. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is a binding partner of epithelial growth factor-containing fibulin-like extracellular matrix protein 1 (EFEMP1). Implications for macular degenerations. *J Biol Chem* 2004;279:30469–30473. [PubMed: 15123717]
- Koulen P, Fletcher EL, Craven SE, Brecht DS, Wassle H. Immunocytochemical localization of the postsynaptic density protein PSD-95 in the mammalian retina. *J Neurosci* 1998;18:10136–10149. [PubMed: 9822767]
- Lagali PS, Liu J, Ambasudhan R, Kakuk LE, Bernstein SL, Seigel GM, Wong PW, Ayyagari R. Evolutionarily conserved ELOVL4 gene expression in the vertebrate retina. *Invest Ophthalmol Vis Sci* 2003;44:2841–2850. [PubMed: 12824221]
- Liu J, Itagaki Y, Ben-Shabat S, Nakanishi K, Sparrow JR. The biosynthesis of A2E, a fluorophore of aging retina, involves the formation of the precursor, A2-PE, in the photoreceptor outer segment membrane. *J Biol Chem* 2000;275:29354–29360. [PubMed: 10887199]
- Mandal MN, Vasireddy V, Reddy GB, Wang X, Moroi SE, Pattnaik BR, Hughes BA, Heckenlively JR, Hitchcock PF, Jablonski MM, Ayyagari R. CTRP5 is a membrane-associated and secretory protein

- in the RPE and ciliary body and the S163R mutation of CTRP5 impairs its secretion. *Invest Ophthalmol Vis Sci* 2006;47:5505–5513. [PubMed: 17122142]
- Marmorstein LY, Munier FL, Arsenijevic Y, Schorderet DF, McLaughlin PJ, Chung D, Traboulsi E, Marmorstein AD. Aberrant accumulation of EFEMP1 underlies drusen formation in Malattia Leventinese and age-related macular degeneration. *Proc Natl Acad Sci U S A* 2002;99:13067–13072. [PubMed: 12242346]
- Mata NL, Weng J, Travis GH. Biosynthesis of a major lipofuscin fluorophore in mice and humans with ABCR-mediated retinal and macular degeneration. *Proc Natl Acad Sci U S A* 2000;97:7154–7159. [PubMed: 10852960]
- Maugeri A, Meire F, Hoyng CB, Vink C, Van Regemorter N, Karan G, Yang Z, Cremers FP, Zhang K. A novel mutation in the ELOVL4 gene causes autosomal dominant Stargardt-like macular dystrophy. *Invest Ophthalmol Vis Sci* 2004;45:4263–4267. [PubMed: 15557430]
- McMahon A, Jackson SN, Woods AS, Kedzierski W. A Stargardt disease-3 mutation in the mouse Elov4 gene causes retinal deficiency of C32-C36 acyl phosphatidylcholines. *FEBS Lett* 2007;581:5459–5463. [PubMed: 17983602]
- Parish CA, Hashimoto M, Nakanishi K, Dillon J, Sparrow J. Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc Natl Acad Sci U S A* 1998;95:14609–14613. [PubMed: 9843937]
- Rajan RS, Illing ME, Bence NF, Kopito RR. Specificity in intracellular protein aggregation and inclusion body formation. *Proc Natl Acad Sci U S A* 2001;98:13060–13065. [PubMed: 11687604]
- Saliba RS, Munro PM, Luthert PJ, Cheetham ME. The cellular fate of mutant rhodopsin: quality control, degradation and aggresome formation. *J Cell Sci* 2002;115:2907–2918. [PubMed: 12082151]
- Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat Rev Neurosci* 2003;4:49–60. [PubMed: 12511861]
- Soto C, Estrada LD. Protein misfolding and neurodegeneration. *Arch Neurol* 2008;65:184–189. [PubMed: 18268186]
- Stone EM, Braun TA, Russell SR, Kuehn MH, Lotery AJ, Moore PA, Eastman CG, Casavant TL, Sheffield VC. Missense variations in the fibulin 5 gene and age-related macular degeneration. *N Engl J Med* 2004;351:346–353. [PubMed: 15269314]
- Vasireddy V, Sharon M, Salem N Jr, Ayyagari R. Role of ELOVL4 in fatty acid metabolism. *Adv Exp Med Biol* 2008;613:283–290. [PubMed: 18188956]
- Vasireddy V, Vijayasarathy C, Huang J, Wang XF, Jablonski MM, Petty HR, Sieving PA, Ayyagari R. Stargardt-like macular dystrophy protein ELOVL4 exerts a dominant negative effect by recruiting wild-type protein into aggresomes. *Mol Vis* 2005;11:665–676. [PubMed: 16163264]
- Vasireddy V, Jablonski MM, Mandal MN, Raz-Prag D, Wang XF, Nizol L, Iannaccone A, Musch DC, Bush RA, Salem N Jr, Sieving PA, Ayyagari R. Elov4 5-bp-deletion knock-in mice develop progressive photoreceptor degeneration. *Invest Ophthalmol Vis Sci* 2006;47:4558–4568. [PubMed: 17003453]
- Vasireddy V, Uchida Y, Salem N Jr, Kim SY, Mandal MN, Reddy GB, Bodepudi R, Alderson NL, Brown JC, Hama H, Dlugosz A, Elias PM, Holleran WM, Ayyagari R. Loss of functional ELOVL4 depletes very long-chain fatty acids (> or =C28) and the unique omega-O-acylceramides in skin leading to neonatal death. *Hum Mol Genet* 2007;16:471–482. [PubMed: 17208947]
- Zhang K, Kniazeva M, Han M, Li W, Yu Z, Yang Z, Li Y, Metzker ML, Allikmets R, Zack DJ, Kakuk LE, Lagali PS, Wong PW, MacDonald IM, Sieving PA, Figueroa DJ, Austin CP, Gould RJ, Ayyagari R, Petrukhin K. A 5-bp deletion in ELOVL4 is associated with two related forms of autosomal dominant macular dystrophy. *Nat Genet* 2001;27:89–93. [PubMed: 11138005]

Figure 1. Light microscopic evaluation of the *E_{mut}^{+/-}* mice retina in comparison to controls
 Morphological analysis of retinal sections from 22 month old *E_{mut}^{+/-}* mice (B) retina demonstrated accumulation of RPE debris in the subretinal space and swollen inner segments. RPE debris is manifested as pigment granules among outer segments (black arrows). Littermate controls (A) RPE: Retinal pigment epithelium, OS: Outer segments, IS: Inner segments, ONL: Outer nuclear layer, OPL: Outer plexiform layer, INL: Inner plexiform layer. Scale bar = 10 μ m.

Figure 2. Electron micrographs of *E_mut*^{+/-} mice retina in comparison to controls

Ultrastructural analysis of the retina of *E_mut*^{+/-} mice (B) revealed an increased number of electron dense lipofuscin granules in the RPE (white arrows) compared to that of age matched litter mate controls (A). The RPE of *E_mut*^{+/-} mice also contains many cytoplasmic vacuoles (black arrows). Moreover, the basal infoldings of the RPE lack structural detail (asterisk) Scale bar = 2 μ m.

Figure 3. Quantitation of the lipofuscin granules in *E_mut*^{+/-} mice retina

Quantitation of number of lipofuscin granules in each retinal section of *E_mut*^{+/-} mice and age matched control animal retinal section demonstrated a significant increase in the number of lipofuscin granules in *E_mut*^{+/-} mice retina compared to controls. Values (mean ± SD) are based on examining 25 contiguous electron micrographs from eyes from 2 wild type and 5 *E_mut*^{+/-} mice. P value was determined by an unpaired t-test.

Figure 4. Quantitation of the lipofuscin pigments in *E_mut*^{+/-} mouse

A. A2E and isoA2E in posterior eye cups were measured by quantitative HPLC with monitoring at 430 nm. Levels of A2E and isoA2E were summed. Values (mean $\pm$ SEM) are based on 2–5 replicates per age and each sample was obtained by pooling 4 eyes. Genotyping for the *Rpe65* Leu450Met variant: *, *Rpe65* 450Leu (homozygous) and 450LeuMet (heterozygous) were pooled in equal numbers in wild-type and *E_mut*^{+/-}; **, *Rpe65* 450 Leu (homozygous); *** not genotyped for *Rpe65* variant **B.** Levels of A2E and isoA2E (summed) together with total (summed) all-*trans*-retinal dimer series compounds [all-*trans*-retinal dimer, all-*trans*-retinal dimer-phosphatidylethanolamine and all-*trans*-retinal –ethanolamine] in wild-type versus *E_mut*^{+/-} mice and in *Abca4*^{-/-} mice. All mice were *Rpe65*Leu450 and 4 months of age. Values (mean $\pm$ SEM) are based on 2–3 replicates per age and each sample was obtained by pooling 4 eyes. Pigments were identified by co-injection with authentic standard. P values determined by unpaired t-test.

Figure 5. ELOVL4 protein accumulates in the retina of *E_mut*^{+/-} mice
Immunofluorescence analysis of retinal sections from 4 month old controls (A) and *E_mut*^{+/-} mice (B) demonstrated an accumulation of ELOVL4 protein (red) in the OPL of *E_mut*^{+/-} mice retina. Nuclei are labeled with DAPI. ONL: Outer nuclear layer, OPL, Outer plexiform layer, INL: Inner Nuclear layer. Scale bar =10μm.

Figure 6. Accumulation of ELOVL4 protein in synaptic terminals of bipolar cells

Cross sections of retina from 4 month old *E_mut*^{+/-} mice (A) and Wt control (B) double stained with the bipolar cell marker PKC-alpha antibody (green) and ELOVL4 antibody (red). ELOVL4 was observed to be co-localized with PKC-alpha antibody at the dendritic tips of rod bipolar cells. Panel C is a higher magnification image showing the localization of ELOVL4 in rod bipolar cells. Scale is 50 μM.

Figure 7. Accumulation of ELOVL4 in the OPL of *E_mut*^{+/-} mice

Immunofluorescence images of 4 month old control (A,C,E) and *E_mut*^{+/-} mice (B,D,F) retinal sections labeled with anti-ELOVL4 antibody (red) and PSD-95 (green). Immunofluorescence of ELOVL4 labeling (red) is co-localized with the PSD-95 antibody (green) labeling in the outer plexiform layer region in *E_mut*^{+/-} mice compared to age matched controls. Scale is 50 μ M.

Figure 8. Accumulation of ubiquitin in *E_mut*^{+/-} mice

Immunohistochemical analysis of retinal sections from 4 month old controls (A) and *E_mut*^{+/-} mice (B) revealed an increased labeling of ubiquitin (green) in *E_mut*^{+/-} mice retina compared to controls. Red fluorescence indicates ELOVL4. Nuclei were stained with DAPI. Scale is 50 μ M.