Distinct Phenotypic Consequences of Pathogenic C1q–Tumor Necrosis Factor-5 Mutants Associated with Late-Onset Retinal Degeneration

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A pathologic feature of late-onset retinal degeneration caused by the S163R mutation in C1q–tumor necrosis factor-5 (C1QTNF5) is the presence of unusually thick deposits between the retinal pigmented epithelium (RPE) and the vascular choroid, considered a hallmark of this disease. We have previously demonstrated that following its specific expression in mouse RPE, the S163R mutant exhibits a reversed polarized distribution relative to the apically secreted wild-type C1QTNF5, and forms widespread, prominent deposits that gradually increase in size with aging. This study shows that S163R deposits expand to a considerable thickness through a progressive increase in the basolateral RPE membrane, substantially raising the total RPE height, and enabling their clear imaging as a distinct hyporeflective layer by noninvasive optical coherence tomography in advanced aged animals. This phenotype bears a striking similarity to ocular pathology previously documented in patients harboring the S163R mutation. Therefore, we used a similar viral vector-based gene delivery approach to investigate the behavior of two novel pathogenic C1QTNF5 mutants recently reported in patients, P188T and G218C, for which histopathologic data are lacking. We found that both mutants primarily impact the RPE/photoreceptor interface and do not generate basal laminar deposits. The results demonstrate distinct distribution patterns and phenotypic consequences of C1QTNF5 mutants in vivo, and suggest that multiple pathobiological mechanisms contribute to RPE dysfunction and vision loss in this disorder. (Am J Pathol 2022; 190: 1–15; https://doi.org/10.1016/j.ajpath.2022.10.004)

Late-onset retinal degeneration (L-ORD) is a rare autosomal dominant disorder caused by mutations in the C1q–tumor necrosis factor-5 (C1QTNF5) gene, encoding a multimeric protein (CTRP5/C1QTNF5) expressed by the retinal pigmented epithelium (RPE) and ciliary body in the eye.1–4 C1QTNF5 is also secreted by the adipose tissue, and circulates abundantly in human sera.5 L-ORD has primarily been linked to the best characterized p.Ser163Arg (S163R) founder mutation, first reported by Hayward et al1 in 2003. The disease has a long asymptomatic phase, starting with dark adaptation abnormalities in midlife and progressing to widespread retinal degeneration within two decades, ultimately resulting in severe loss of both central and peripheral vision.6–9 Small yellow-white drusen-like dots are initially detected in the temporal macula as the first pathologic change on fundus examination, followed by gradually progressing islands of RPE and chorioretinal atrophy that spread throughout the retina.2,7,10 Interestingly, the disease displays a geographic pattern of temporal-to-nasal...
progression toward the fovea.\textsuperscript{2,10} Full-field electroretinography (ERG) analysis indicates a rod-cone pattern of retinal dysfunction, in which the scotopic rod-driven b-wave is the first to decline, followed by a slower reduction in cone photoreceptor amplitudes.\textsuperscript{2,6} Depending on its stage, L-ORD can be misdiagnosed as early age-related macular degeneration, Sorsby fundus dystrophy, choroideremia, or advanced retinitis pigmentosa.\textsuperscript{9,11}

A distinguishing histologic feature of the disease associated with the S163R mutation is the presence of unusually thick, widespread eosinophilic deposits, sometimes reaching >50 μm in height, accumulating between the basal RPE and Bruch membrane (BrM) with aging.\textsuperscript{1,2,8,11} The hallmark basal RPE deposits in L-ORD are present in all patients carrying the pathogenic S163R mutation in C1QTNF5, and are assumed to represent a key element in the progression of disease pathology and a major contributor to vision loss. In support of this idea, deposit thickness was found to correlate with the degree of photoreceptor cell loss in S163R human donor retinas.\textsuperscript{8} In contrast to human pathology, the C1qtnf5 S163R knock-in mouse models do not display widespread, prominent deposits with aging, or retinal degeneration, as seen in patients with late-stage disease, and may even fail to develop an ocular phenotype, depending on their genetic background.\textsuperscript{1,15} One study showed that both heterozygous and homozygous C1qtnf5 S163R knock-in mice lack any ocular abnormalities throughout their lifespan when compared with age-matched wild-type controls.\textsuperscript{12} Another C1qtnf5 S163R knock-in mouse model was reported to have delayed rod ERG b-wave recovery and subtle morphologic abnormalities in the RPE ultrastructure with aging, including focal basal laminar deposits and retraction of RPE apical processes.\textsuperscript{1,19} This outcome is perhaps consistent with the presence of only mild ophthalmic disturbances in patients with S163R L-ORD until the fifth decade of life, and highlights the need to utilize alternative strategies, such as adeno-associated viral vectors (AAVs) as gene delivery systems to augment the mutant protein expression in RPE and model the disease progression \textit{in vivo}.\textsuperscript{15,16}

Similar to other members of the C1q and tumor necrosis factor--related superfamily, the C1QTNF5 protein has the remarkable ability to self-assemble into trimers, hexamers, and large bouquet-like octadecameric structures.\textsuperscript{1,17,19} It forms a hexagonal lattice associated with the RPE lateral membranes, and it is also secreted by RPE in the apical direction, within the interphotoreceptor matrix.\textsuperscript{1,20} We have previously demonstrated that in stark contrast to the behavior of apically secreted wild-type C1QTNF5, the AAV-expressed S163R mutant is predominantly misrouted toward the basal side of mouse RPE, and generates widespread, prominent eosinophilic deposits that progressively increase in size, reminiscent of those present in patients.\textsuperscript{21} Furthermore, by using tagged constructs, we showed that S163R mutant impairs the apical secretion of wild-type C1QTNF5 \textit{in vivo} and misroutes it toward the basolateral RPE membrane, supporting a dominant negative mechanism of disease.\textsuperscript{22} The gradual accumulation of the S163R mutant in basal laminar deposits was accompanied by changes in the RPE/photoreceptor apical interface, a slow-progressing retinal dysfunction and photoreceptor degeneration, demonstrating the usefulness of the AAV approach to model several key features of L-ORD disease \textit{in vivo}.\textsuperscript{21}

In this study, we extend the phenotypic characterization of S163R basal deposits in aged animals through the combined use of noninvasive imaging techniques, histology, immunofluorescence, and ultrastructural analysis, and provide a direct side-by-side histologic comparison to previously documented human S163R L-ORD pathology. Next, we used a similar AAV-based approach to model the behavior of novel pathogenic C1QTNF5 mutants recently reported in patients, P188T and G216C, for which histopathologic data from donor ocular tissue are lacking.\textsuperscript{23,24} Our understanding of the disease progression and ocular pathology associated with these novel mutants is primarily based on noninvasive optical coherence tomography (OCT) and fundoscopic imaging. Because of the rarity of L-ORD, there are only a few reports describing the ophthalmologic progression of the disease in these cases, and the contribution of subRPE deposits to pathology is unclear. Similar to the classic S163R L-ORD, patients harboring these mutations were reported to display initial dark adaptation abnormalities in midlife, ultimately progressing to a common, severe end-stage phenotype of generalized chorioretinal atrophy resembling advanced RP.\textsuperscript{23,24} Interestingly, a recent study uncovered patients with P188T within the same family presenting with significant differences in terms of fundus appearance, suggesting a spectrum of phenotypes contrasting with the presentation and progression of classic S163R L-ORD.\textsuperscript{25} Furthermore, abnormally long, anteriorly inserted lens zonules, considered an early phenotypic marker of S163R L-ORD, were notably absent in most patients with P188T.\textsuperscript{3,24,25} The present study demonstrates distinct expression patterns and phenotypic consequences of the three C1QTNF5 mutants \textit{in vivo}, and suggests that S163R is uniquely associated with the predominant misrouting of C1QTNF5 toward the RPE basolateral membrane, a behavior integral for the generation of hallmark deposits in this disease.

**Materials and Methods**

**Human Donor Retina Tissue**

The retina from a previously characterized 80-year-old S163R L-ORD donor (Foundation Fighting Blindness, Columbia, MD; donation number 356) was kindly provided by Dr. Joe G. Hollyfield (Department of Ophthalmology, Cleveland Clinic Lerner College of Medicine, Cleveland, OH).\textsuperscript{12} The patient died of myocardial infarction, and the retinal tissue was fixed using 4% paraformaldehyde and 0.5% glutaraldehyde at 5 hours postmortem.\textsuperscript{12} The retina was processed for paraffin embedding, sectioned at a...
thickness of 4 μm, and stained with hematoxylin and eosin (H&E) for comparative histopathologic evaluation to AAV-S163R-injected mouse eyes. Normal ocular tissue from a 79-year-old donor was kindly provided by Dr. Goldis Malek (Duke University School of Medicine, Durham, NC). The eye was fixed in 4% paraformaldehyde, with a short postmortem time of <6 hours. Cause of death was coronary artery disease. H&E-stained retinal sections from human eyes were visualized by light microscopy.

**Animal Care and Use**

All mice were maintained on a 12-hour dim light (50 to 100 lux) and 12-hour dark cycle at the University of Florida Health Science Center Animal Care Services Facility (Gainesville, FL). All experiments were approved by University of Florida Institutional Animal Care and Use Committees, and conducted in accordance with the Association for Research in Vision and Ophthalmic Statement for the Use of Animals in Ophthalmic and Vision Research, and within NIH regulations for Research in Vision and Ophthalmology Statement for the Use of Animals Institutional Animal Care and Use Committees, and conducted in accordance with the Association for Research in Vision and Ophthalmic Research, and within NIH regulations. The 4- to 8-week-old adult C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME; stock number 000664). Albino BALB/cJ mice of similar age were obtained from the University of Florida breeding facility. Noninvasive procedures, including funduscopy, OCT, and ERG, were performed under anesthesia using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). Animals of both sexes were used in all experiments. Pupils were dilated with topically applied Tropi-Phe Ophthalmic Solution (phenylephrine HCl 2.5% and tropicamide 1%; PINE Pharmaceuticals, Tonawanda, NY). Lubricating eye drops (GENTEAL tears; Alcon, Fort Worth, TX) were placed in each eye before and during the procedures to maintain the hydration and clarity of the cornea.

**Recombinant AAV Preparation and Subretinal Vector Delivery**

AAV viral vectors were generated at the Ocular Gene Therapy Core (Department of Ophthalmology, University of Florida). Each vector was subretinally injected in adult C57BL/6J mice, as previously described.24 Albino BALB/cJ mice of similar age were also used for AAV-S163R-hemagglutinin (HA) subretinal injections. The HA-tagged human wild-type and mutant C1QTNF5 coding sequences (S163R, P188T, and G216C), as well as human wild-type myc-tagged C1QTNF5, and S163R–green fluorescent protein (GFP) fusion, all driven by an RPE-specific human bestrophin-1 promoter (previously VMD2), were packaged in scAAV2 quadraple capsid tyrosine mutant (Y272, 444, 500, and 730F) vectors (AAV2quadYF).20 Each right eye received 1 μl vector, at either 1012 vector genome copies (vg)/ml (total 109 vg/eye), or a lower dose of 5 × 1011 vg/ml by diluting each vector with sterile balanced salt solution before injection. These vector doses were previously demonstrated to be nontoxic in retinal gene transfer studies following subretinal delivery to RPE cells.28–30 The left eyes remained uninjected and served as controls. Each AAV vector was delivered to groups consisting of at least 20 mice. Animals that developed surgical complications (cataract) were eliminated from the study.

**Funduscopy and Spectral-Domain OCT**

The AAV-S163R-GFP-injected eyes were examined with a Micron II fundus imaging system (Phoenix Technology Group, Pleasanton, CA) using either white light or a GFP filter (excitation at 482 nm and emission at 536 nm). All images were taken centered on the optic nerve head. For OCT image acquisition, the AAV-S163R–injected mice were oriented in the holder (AIM-RAS; Leica Microsystems Inc., Buffalo Grove, IL) to consistently place the optic nerve head in the center of the imaging area. A rectangular scanning mode (1.6 × 1.6 mm at 1000 A-scans × 100 B-scans × 10 frames per B-scan) was used to acquire an ultra-high-resolution retinal structure using the Biotig ultra–high-resolution Envisu R2210 Spectral Domain-OCT ophthalmic imaging system (Leica Microsystems Inc.). Histologic H&E sections from the same mice were collected immediately after spectral-domain OCT imaging.

**Electroretinography**

Full-field ERG recordings at increasing flash intensities were performed under dark-adapted conditions using an Espion E2 ColorDome instrument (Diagnosys LLC, Lowell, MA), as previously described.31 Responses from both treated and uninjected eyes were recorded simultaneously.

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism version 9.0.2 for Windows (GraphPad Software, San Diego, CA). Amplitudes of ERG a-waves, b-waves, and c-waves, comparing the AAV-injected eyes with the contralateral untreated controls, were analyzed using the unpaired t-test. All values are expressed as the means ± SEM. P < 0.05 was considered statistically significant.

**Histology and Immunofluorescence**

For morphologic analysis, mouse eyes were fixed in 4% paraformaldehyde for 24 hours at 4°C, processed for paraffin embedding, and sectioned from the dorsal to ventral region at a thickness of 4 μm. Retinal sections were collected at approximately 100-μm intervals from the optic nerve head, from both central and midperipheral areas, stained with H&E, and visualized by light microscopy. Representative images of retinal cross-sections within 400 μm distance from the optic nerve head are shown in high-magnification figures. Immunostaining experiments on mouse retinal sections were performed as previously
described. The following primary antibodies were used: rat monoclonal anti-HA at 1:300 dilution (Roche number 11867423001; Thermo Fisher Scientific, Waltham, MA), rabbit monoclonal anti-glucose transporter type 1 (GLUT1) antibody at 1:1000 dilution (EPR3915 number 115730; Abcam, Cambridge, MA), and goat polyclonal anti-membrane-type frizzled-related protein (MFRP) at 1:500 dilution (number PA5-47490; Invitrogen, Rockford, IL). A mouse monoclonal antibody against rod opsin (Abcam B630 number 230692) was used to label photoreceptor outer segments.21,33 In vitro, secondary antibodies conjugated with AlexaFluor 488 or AlexaFluor 594 were used at 1:400 dilution (Thermo Fisher Scientific). Nuclei were counterstained with DAPI. At least 10 eyes were analyzed for each injected C1QTNF5 construct. The images were acquired by using either a Zeiss Axiophot microscope (Zeiss, Thornwood, NY) or a fully automated wide-field DMi8 Leica fluorescence microscope (Leica AG, Wetzlar, Germany).

**Transient Transfections in Human Embryonic Kidney 293T Cells and Western Blot Analysis**

Human embryonic kidney 293T cells were grown to 80% confluency in 6-well plates at 37°C in Dulbecco’s modified Eagle’s medium (Gibco DMEM+GlutaMAX; number 10569010) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (Gibco; number 15240062). Transient transfections using AAV-C1QTNF5 plasmids (2 μg plasmid per well) were performed using the Jetprime reagent (number 114-07; Polyplus, Illkirch, France). After 48 hours, the growth media and cell lysates were processed for immunoblotting. Western blot analysis was also performed on eye cup homogenates at 1 month following AAV-mediated delivery of S163R-HA, P188T-HA, and G216C-HA. Proteins were resolved by electrophoresis on 4% to 20% Mini Protean TGX gradient gels (number 456-1094; Bio-Rad Laboratories, Hercules, CA) under reducing and denaturing conditions, and transferred to a 0.45-μm Immobilon FL polyvinylidene difluoride membrane (IPFL00010; Millipore-Sigma, Burlington, MA). The membranes were blocked in Odyssey blocking buffer (927 to 40000; LI-COR Biosciences, Lincoln, NE). The following primary antibodies were used: rat monoclonal anti-HA antibody at 1:1000 dilution (Roche; number 11867423001), or rabbit monoclonal anti-HA antibody at 1:3000 dilution (3724S; clone C29F4; Cell Signaling Technology, Danvers, MA); rabbit monoclonal anti-myc at 1:3000 dilution (2278S; clone 7D10; Cell Signaling Technology); and mouse monoclonal anti-z-tubulin at 1:5000 (T5168; clone B-5-1-2; Millipore-Sigma). Secondary antibodies were IRDye 680-conjugated anti-rat (number 926-32219; LI-COR Biosciences) and IRDye 800-conjugated anti-rabbit IgG (number 92532211; LI-COR Biosciences), diluted 1:12,000. Prestained Precision Plus All Blue protein standards (Bio-Rad Laboratories; number 161-0373) are shown on the left-hand side of each image in kilodaltons. Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences). Each experiment was repeated a minimum of four times.

**Transmission Electron Microscopy**

Electron microscopy was performed on mouse eyes from each AAV-injected group (S163R-HA, P188T-HA, G216C-HA, and S163-GFP), and contralateral uninjected controls, to evaluate the ultrastructure of the RPE/Bruch membrane and RPE/photoreceptor outer segment interfaces. In each case, at least 10 independently processed retinal sections from both eyes of two mice (one male and one female) were analyzed. The sample processing and imaging were performed at the Robert P. Apkarian Integrated Electron Microscopy Core (Emory University, Atlanta, GA). Eyes were placed in freshly made fixative containing 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4). After fixation, the dissected retinas were post-fixed with 1% OsO4 and 1.5% potassium ferrocyanide in 0.1 mol/L cacodylate buffer for 1 hour. After rinsing with de-ionized water for 10 minutes, samples were dehydrated through a graded ethanol series, then infiltrated with a mixture of propylene oxide and Eponate 12 resin (Ted Pella, Inc., Redding, CA) and Eponate 12 resin at a 1:1 ratio overnight. After additional infiltration in propylene oxide and Eponate 12 resin at a 1:2 ratio for 6 hours, the retina samples were placed in pure Eponate 12 resin overnight. Semithin sections (0.5 μm) were stained with toluidine blue for imaging by light microscopy. Grids with ultrathin sections (70 to 80 nm) were stained with 5% uranyl acetate and 2% lead citrate, and imaged on a JEM-1400 transmission electron microscope (JEOL Ltd, Tokyo, Japan).

**Results**

**Detection of S163R Basal Laminar Deposits as a Distinct Layer in Noninvasive OCT Imaging**

Previous OCT imaging studies in patients with S163R L-ORD revealed the presence of abnormal basal laminar deposits as a homogeneous layer of medium internal reflectivity separating a hyperreflective, thickened RPE from the underlying BrM.5,10 Because of the slow developing phenotype in animal models, we used an AAV-based gene delivery approach to investigate the long-term pathologic consequences of S163R expression in mouse RPE. In our earlier study, we documented the effects of S163R expression up until 9 months following subretinal vector delivery in adult C57BL/6J mice, when basal S163R subRPE deposits were detectable by light microscopy following H&E staining.21 To complement and extend previous results, the current study investigates the S163R-associated pathology in aged mice until 22 months after injection, when basal deposits reach a considerable thickness, and are clearly visible in OCT.
Basal Deposits in L-ORD

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Melanin Granules.37,38 The bands distinguished as a distinct layer by noninvasive OCT imaging (Figure 1).

In the control uninjected 24-month—old C57BL/6J mouse eyes, four hyperreflective bands can be detected by OCT in the outer retina/RPE regions (Figure 1A). These bands correspond to the external (or outer) limiting membrane of the retina, the mitochondria-rich ellipsoid zone of the photoreceptor inner segments, the outer segment/apical RPE microvilli interdigitation zone, and the basal RPE/BrM complex.34,35 BrM is discernable from the RPE only under certain pathologic conditions, when the RPE/BrM complex is split by subRPE deposits.35,36 Similar to the human disease, the OCT scan of the AAV-S163R— injected mouse eyes uncovers the presence of a continuous, undulating layer present between an intensely hyperreflective RPE and the BrM, and enables the correlation with histology (Figure 1B).20 The intense hyperreflectivity of the RPE band in AAV-S163R injected eyes may result from a higher concentration of melanosomes in the RPE cytoplasm relative to control (Figure 1C), and an increased scattering from melanin granules.37,38

Light-microscopy examination of retinal sections from the same mouse following H&E staining shows that the hyperreflective OCT layer corresponds to a thick eosinophilic deposit (Figure 1D). The AAV-S163R— injected eye displays shorter and disorganized photoreceptor outer and inner segments compared with control (Figure 1, C and D). However, the injected eye retained regions containing approximately six to eight layers of nuclei, comparable to the uninjected contralateral control retina, indicating a minimal impact on photoreceptor cell loss in pigmented mice containing large deposits in the central part of the retina (Figure 1, C and D, and Supplemental Figure S1A). Previous studies on the effects of normal aging on retinal morphology in C57BL/6J mice showed that the outer nuclear layer thickness is significantly reduced in older animals compared with younger animals, which contain on average 10 to 12 layers of photoreceptor nuclei.39,40 Because there is a lack of reliable commercially available antibodies that recognize the C1QTNF5 protein specifically by immunostaining on paraffin sections, we have used a HA-tagged S163R C1QTNF5 construct, as described in our previous study.21 This enables the positive identification of the basal deposits as consisting of S163R-HA mutant by immunostaining with the anti-HA antibody (Supplemental Figure S1, B and C).

Histologic Comparison of Basal S163R Deposits in Aged Animals to Human Pathology

Because of the rarity of L-ORD, there is limited availability of S163R donor retina tissue for histopathologic analysis. We therefore acquired a peripheral retina sample from one previously documented 80-year—old donor with advanced L-ORD disease (Foundation Fighting Blindness; number 356) and subsequently stained the retinal sections with H&E (Figure 2A and Supplemental Figure S2B). Clinical and histologic details on this patient with S163R L-ORD were previously reported in the literature, and revealed the presence of hallmark prominent subRPE eosinophilic deposits, and advanced retinal degeneration.12 It is estimated that 18- to 24-month—old mice correlate with humans ranging from 56 to 69 years of age.41 Side-by-side comparison of the RPE/choriocapillary interface in aged albino or pigmented AAV-S163R— injected mouse eyes and the 80-year—old L-ORD donor retina shows the striking histologic resemblance to human ocular pathology, with large eosinophilic basal deposits emanating from individual RPE cells, merging into a confluent layer (Figure 2 and Supplemental Figure S3). In advanced aged albino animals (15 months after injection), the prominent deposits consisting of the S163R mutant protein are associated with regions of significant retinal degeneration, similar to the human retina (Figure 2, B and C, and Supplemental Figures S4, S5, and S6). Furthermore, long-term S163R expression in albino mouse RPE causes unusually large, dome-shaped deposits in certain areas (Supplemental Figures S5 and S6). The accelerated disease phenotype in the albino mice is consistent with a previous study showing that rod photoreceptors are susceptible to both aging and lack of pigmentation.40 In addition, a strain—dependent effect on the amount of S163R mutant protein production and rate of deposit formation cannot be ruled out, as the large, dome-shaped deposits were more frequently noted in the albino than pigmented animals. Taken together, these experiments show that the long-term S163R expression in both pigmented and albino mouse RPE triggers an ocular phenotype consistent with human pathology.

Targeted AAV-Mediated Expression of S163R-GFP in Mouse RPE Causes Widespread, Fluorescent Basal Lamellar Deposits in Vivo

To readily track the S163R mutant by direct fluorescence imaging without the need for immunostaining, we generated an AAV—bestrophin—1 promoter—S163R-GFP delivery system and visualized the transgene expression following subretinal vector delivery. The S163R-GFP protein is directly visible by noninvasive in vivo fluorescence funduscopic imaging, and is entirely misrouted toward the basolateral RPE side, generating fluorescent basal deposits (Figure 3). The fluorescence emanating from the secreted S163R-GFP protein remains confined in discrete areas corresponding to transduced RPE cells, and does not spread laterally over time (Figure 3, B–D). We next performed immunofluorescence labeling of the AAV-S163R-GFP injected retinal sections with a GLUT1 antibody, to delineate RPE membrane interfaces. GLUT1 protein is strongly expressed on both the apical and basolateral membranes of RPE cells, and represents a useful marker for RPE boundaries (Figure 3, E and G, and Supplemental Figure S7).22,43 As shown by previous studies, GLUT1 is

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also expressed in the ganglion cell layer, the neuronal processes of inner and outer plexiform layers, photoreceptors, and Müller glia (Supplemental Figure S7). Staining for the glucose transporter GLUT1 protein demonstrates that the GFP fluorescence emanating from the S163R basal deposits overlaps with a visibly thicker RPE basolateral membrane as early as 1 month after injection (Figure 3H). The apical/RPE interface is also impacted by the AAV-S163R-GFP expression at early stages (1 month after injection), as demonstrated by the lack of well-defined microvilli labeling by GLUT1 relative to uninjected control eyes (Figure 3 and Supplemental Figure S7).

In aged mice, the S163R-GFP protein accumulates as a thick translucent deposit layer overlapping with the RPE basal side, when retinal cryosections are examined under bright-field microscopy (Supplemental Figure S8). To delineate the precise location of the deposits, the ultrastructure of the RPE/Bruch membrane interface in these
mice was examined by transmission electron microscopy. This analysis demonstrates the presence of extracellular material accumulating within the interspaces of abnormally elongated basal infoldings facing the BrM (Figure 4A–C). The ultrastructure of these deposits is reminiscent of the electron-dense subRPE layer present in L-ORD donor eyes.8,12,13 We have also examined the localization pattern of the MFRP in the AAV-S163R-GFP–injected retinas. Previous studies have shown that in the mouse retina, C1qtnf5 is expressed as a bicistronic transcript, which also includes the Mfrp gene, and MFRP may directly interact with C1QTNF5 at the RPE/photoreceptor interface.18,45,46 We determined that MFRP is normally localized along the RPE apical plasma membrane, on the opposite side of the thick, undulating S163R-GFP basal deposits (Figure 4D).

**Novel C1QTNF5 Pathogenic Mutants Display Distinct Expression Patterns in Vivo and Lead to an Early-Onset RPE Dysfunction**

One study characterized the clinical manifestation of the disease in a few patients harboring novel P188T and G216C mutations, and the behavior of mutant proteins in vitro following transient transfections in hTERT-RPE1 cells.23 In the following experiments, we used the AAV-based gene delivery approach to investigate the in vivo expression pattern and pathologic consequences of P188T and G216C mutants following their expression in mouse RPE (Figure 5). Histologic examination of semithin mouse retina sections following toluidine blue staining at 1 month after injection shows that the P188T mutant (delivered at 10^12 vg/mL) leads to the occasional formation of large aggregates on the apical side of RPE (Figure 5B and Supplemental Figure S9A). This suggests the subretinal deposits are present in isolated areas transduced by a high titer of AAV particles in which the overexpressed mutant protein reaches higher concentrations. The G216C mutant appears to be retained in RPE, leading to photoreceptor outer segments shortening and vacuolization (Figure 5C and Supplemental Figure S9B). A representative section from an aged AAV-S163R-GFP–injected mouse eye is also included to illustrate the presence of a thick S163R deposit layer at the basal side of the RPE, contrasting with the subretinal accumulations in the AAV-P188T–injected eyes (Figure 5D). Notably, the AAV-S163R-GFP–injected mouse retina displays a morphology grossly similar to that of wild-type controls, even at 8 months after injection (Figure 5, A and D). This is consistent with the slow progression of the S163R L-ORD disease phenotype in humans. Immunofluorescence analysis of paraffin retinal sections from AAV-P188T– and AAV-G216C–injected eyes mirrors the histologic data and further demonstrates the...
Figure 4  Evaluation of S163R—green fluorescent protein (GFP) basal deposits by transmission electron microscopy (TEM) and direct fluorescence imaging at 8 months after injection (10-month—old mouse eyes). A: Ultrastructural analysis of the retinal pigmented epithelium (RPE) in the uninjected control eye, showing thin basal infoldings, and long apical microvilli. B and C: Representative TEM images of an adenov-associated viral vector (AAV)—S163R-GFP—infected eye at 8 months after injection. Note the significant elongation and widening of the RPE basal infoldings (red vertical line), and the accumulation of extracellular deposits in the interspaces between infoldings (red asterisk). C: The TEM image includes the RPE/photoreceptor outer segment interface and apical microvilli. D: Visualization of the S163R-GFP basal deposit (green) by direct fluorescence microscopy in formalin-fixed, parafﬁn-embedded ocular tissue sections. Membrane-type frizzled-related protein (MFRP) immunostaining (red) deﬁnes the apical RPE membrane. n = 4 mice evaluated (A–C). Scale bars: 1 μm (A–C); 25 μm (D). BM, Bruch membrane; m, mitochondria; MV, apical RPE microvilli; N, nucleus; OS, outer segment.

distinct in vivo distribution of these mutants (Supplemental Figure S9). P188T is apically secreted into the interphotoreceptor matrix, forming discrete subretinal aggregates in isolated areas, whereas G216C accumulates entirely in RPE (Supplemental Figure S9, C and D). Western blot analysis of eye cup homogenates conﬁrmed the presence of C1QTNF5 monomers and several multimeric species in mouse eyes expressing the mutant proteins (Supplemental Figure S9E). Transient transfection experiments in human embryonic kidney 293T cells show that both P188T and S163R are robustly detected in cell media, indicating that these proteins are secreted, whereas G216C is not, as described in more detail below (Supplemental Figure S9E).

To determine the impact of P188T and G216C expression on RPE and photoreceptor function, ERG analysis was performed at 1 month following subretinal AAV delivery, using a lower vector dose of 5 × 10^11 vg/mL. The c-wave represents a slow ERG component that originates primarily from RPE. Both mutants lead to an early-onset RPE dysfunction, as evidenced by the markedly diminished electroretinogram c-wave amplitudes (Figure 5, E and G). The rod-driven a-wave amplitudes were not signiﬁcantly changed, suggesting that photoreceptor function is not affected at this stage (Figure 5F and Supplemental Figure S10). Furthermore, the b-wave amplitudes reﬂecting the response of second-order retinal neurons in the middle layer of the retina were also normal, although a small delay in the b-wave implicit time was noted in both cases (Figure 5F and Supplemental Figure S10D). In contrast to the early-onset reduction in the c-wave amplitudes associated with P188T and G216C expression, ERG values in AAV-S163R—infected retinas (10^11 vg/mL) were similar to uninjected contralateral controls (Supplemental Figure S10, A and B).

Transmission electron microscopy was used to compare the ultrastructure of RPE cells overexpressing each of the three pathogenic mutant proteins at 1 month after injection (Figure 6). Transmission electron microscopy data show that P188T and G216C expression levels are associated with early-onset structural changes in the RPE and RPE/photoreceptor interface, which are mirrored by functional deﬁcits in the c-wave described above. Representative electron microscopy images from eyes expressing the P188T and G216C proteins reveal the presence of numerous large vesicular structures in the RPE cytoplasm, distinct for each mutant (Figure 6, B and C). The AAV-P188T—infected eyes display numerous spherical vesicles ﬁlling the RPE cytoplasm (Figure 6B). In contrast, RPE cells expressing the G216C mutant contain distinct, irregular-shaped large vesicular structures, reminiscent of distended rough endoplasmic reticulum ﬁlled with mutant collagen deposits (Figure 6C). RPE cells expressing the S163R mutant contain basal laminar deposits within the interspaces of enlarged RPE plasma membrane infoldings facing the BrM.
Figure 5 Mutant C1QTNF5 proteins display distinct phenotypic effects. A–C: Light microscopy images of semithin retinal plastic sections from 3-month-old mouse eyes following toluidine blue staining. A: Uninjected mouse retina. B: Adeno-associated viral vector (AAV)—P188T—induced pathology at 1 month after injection. Note the presence of occasional subretinal deposits (red arrows) and shortening of the photoreceptor outer segments (red asterisk). C: AAV-G216C—induced pathology at 1 month after injection. Note the outer segment vacuolization and shortening (red arrows), and a relatively preserved morphology of the RPE/photoreceptor outer segment interface (red asterisk) compared with B and C. At least four injected eyes were analyzed in each case [AAV vector titer: 1012 viral genome copies (vg)/mL]. E–G: Full-field scotopic electroretinogram (ERG) recordings from 3-month-old control, uninjected, AAV-P188T–, and G216C-injected eyes (1 month after injection; AAV vector titer: 5 × 1012 vg/mL). E: Representative averaged ERG traces showing a dramatic reduction of c-wave amplitudes in AAV-P188T– and AAV-G216C–injected eyes relative to uninjected contralateral controls (1 log cd.s/m² stimulus flash intensity). Note: The c-wave follows the a- and b-waves shown in F; hence, the c-wave ERG recordings are shown on a longer time scale. F: ERG traces showing the a- and b-wave amplitudes, and the slight delay in the b-wave implicit time compared with control. G: Average maximum c-wave amplitudes at increasing stimulus flash intensities. Asterisks indicate statistical significance between controls and AAV-C1QTNF5 mutant injected eyes. Significance is based on the unpaired t-test. Data are shown as means ± SEM (G). n = 8 AAV-P188T and AAV-G216C eyes (G); n = 16 control eyes (G). ***P < 0.001 compared with uninjected controls. Scale bars = 20 μm (A–D), HA, hemagglutinin; IS, inner segment; ONL, outer nuclear layer; OS, outer segment.

AAV-G216C—induced deposits (Figure 6D). In contrast to S163R, the AAV-P188T– and AAV-G216C–injected eyes lack basal deposits, and the regular arrangement of the RPE basal infoldings is disturbed in both cases (Figure 6, B and C).

Ultrastructural examination of the RPE/photoreceptor interface shows that P188T and G216C expression is associated with severe loss of RPE apical microvilli (Figure 6, F and G). Secreted P188T forms discrete deposits within the apical RPE/outer segments interface, suggesting a higher propensity to aggregate (Figure 6F). Taken together, these data show that both P188T and G216C mutants significantly impact the RPE/photoreceptor interface, and do not generate basal laminar deposits in vivo. The AAV-S163R–injected eyes display long, disorganized apical microvilli that no longer interdigitate normally with photoreceptor outer segments (Figure 6H). This indicates an early-onset impact of S163R expression on the RPE/photoreceptor outer segment interface, consistent with observations from previous studies.10,15,21

S163R and P188T Mutants Are Efficiently Secreted into the Extracellular Medium, Both in the Absence and Presence of Wild-Type C1QTNF5 Protein

In previous in vivo experiments using HA and myc epitope tagging constructs, we showed that wild-type C1QTNF5 protein displays an apical distribution pattern following its specific AAV-mediated expression in adult mouse RPE cells, a behavior consistent with that of the endogenous protein, and that co-expression of wild-type and S163R mutant misroutes both proteins toward the basolateral RPE membrane.21,22 In contrast to S163R, the P188T mutant displays a unidirectional apical distribution, similar to the AAV-expressed wild-type protein (Figure 7, A and B). It is therefore expected that this mutant does not impair the secretion of wild-type C1QTNF5. To determine the effects of specific pathogenic mutations on wild-type C1QTNF5 secretion, the HA-tagged mutants S163R, P188T, G216C, and wild-type C1QTNF5 were expressed either alone or in combination with a myc-tagged wild-type protein in human embryonic kidney 293T cells (Figure 7C). Western blot analysis shows that P188T is present in cell culture media, in both the absence and the presence of wild-type protein (Figure 7C). In contrast, the G216C mutation abolishes C1QTNF5 secretion, and the mutant protein is only detected in cell lysates (Figure 7C and Supplemental Figure S1). These results are consistent with a previous study performed in hTERT-RPE1 cells using untagged constructs.23 The HA-tagged G216C protein is detectable at low levels in the media following its co-expression with the myc-tagged wild-type (Figure 7C). This suggests that a small fraction of G216C mutant is secreted in the presence of wild-type protein. Qualitatively, we noticed that G216C consistently led to a significant reduction of the myc-tagged wild-type protein. This finding was confirmed by quantitative analysis of the Western blots (Figure 7D).
C1QTNF5 secretion into the media following cotransfection experiments, suggesting that the G216C mutant traps the wild-type protein in the endoplasmic reticulum by hetero-oligomerization (Figure 7C). In contrast to previous studies, robust expression of S163R mutant protein was detected in the cell culture media in both the absence and the presence of the myc-tagged wild-type protein, indicating that this mutant is secreted (Figure 7C). Furthermore, similar to P188T, the S163R mutant does not impair the myc-tagged wild-type C1QTNF5 protein secretion into the media (Figure 7C). Taken together, these experiments suggest that the substitution of serine at position 163 for a positively charged arginine does not affect C1QTNF5 expression in mouse RPE, the S163R mutant exhibits a reversed polarized distribution relative to the apically secreted wild-type C1QTNF5, and forms a thick, widespread eosinophilic layer between the RPE and the vascular choroid. The present study shows that S163R deposits reach a considerable thickness through a progressive expansion in the basolateral RPE membrane, resulting in a dramatic increase in the total RPE height in advanced aged animals. This phenotype bears a striking histologic resemblance to human ocular pathology, and provides further evidence that an RPE source of misrouted S163R mutant C1QTNF5 protein is sufficient to generate prominent deposits in vivo. In contrast, the AAV-expressed P188T mutant is apically secreted into the interphotoreceptor matrix, and forms discrete subretinal aggregates, whereas G216C is entirely retained in RPE. These results demonstrate the existence of distinct strategies by which the RPE handles the presence of C1QTNF5 mutant proteins in vivo, which may influence the disease progression and clinical manifestation with aging.

Structurally, C1QTNF5 has been compared with collagen VIII and X, which contain the globular gC1q signature domain and have the ability to self-assemble into threedimensional networks, forming hexagonal lattices. The long-term basolateral secretion of the S163R mutant by RPE and its propensity toward molecular self-assembly may
drive the generation of unusually large subRPE deposits in vivo, as described in this study. Interestingly, two other monogenic dominant inherited disorders caused by mutations in genes that maintain the integrity of the extracellular matrix result in the formation of widespread, confluent deposits between the RPE and choroid. These include Sorsby fundus dystrophy, caused by mutations in TIMP3, and Malattia Leventinese-Doyne honeycomb retinal dystrophy, caused by mutations in the EFEMP1 gene. TIMP3 is a member of the family of endogenous tissue inhibitors of extracellular matrix metalloproteinases, and is primarily expressed by RPE and normally deposited in the BrM. Patients with Sorsby fundus dystrophy display an unusual thickening of BrM, and accumulations of TIMP3 in the subRPE deposits. TIMP3 is one of the binding partners for epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (EFEMP1), also known as fibulin-3. Electron microscopic examination of the RPE/Bruch membrane interface in the Efemp1 knock-in mice carrying the p.Arg345Trp (R345W) pathogenic mutation revealed the presence of extensive basal laminar deposits that increased in size with aging. Abnormal accumulation of EFEMP1 protein in patients with R345W Malattia Leventinese-Doyne honeycomb retinal dystrophy was detected on the basal RPE side, overlaying the subRPE deposits. Primary RPE cells from the homozygous Malattia Leventinese-Doyne honeycomb retinal dystrophy mouse model containing the R345 mutation in Efpem1 also recapitulate the generation of basal deposits, and display an increased accumulation of mutant EFEMP1 in the extracellular matrix, consistent with patient histologic data. Recent studies using Sorsby fundus dystrophy and Malattia Leventinese-Doyne honeycomb retinal dystrophy patient-derived induced pluripotent stem cells further demonstrate that RPE dysfunction in these monogenic disorders is sufficient to generate subRPE deposits, highlighting the importance of these in vitro systems for investigating RPE cell-autonomous molecular triggers that contribute to the development of pathology.

The presence of extracellular protein and lipid-containing deposits within the RPE/choroid interface is considered central to the pathogenesis and severity of many ocular disorders, and may also predispose the patients to an increased risk of developing choroidal neovascularization. Histopathologic analysis has shown that there is a strong relationship between the presence of basal laminar deposits in the macula and the exudative form of age-related macular degeneration. Previous studies have reported that CIQTNF5 interacts with HTRA1, a serine protease associated with age-related macular degeneration, implicating this enzyme and the dysregulation of extracellular matrix in L-ORD pathology. The distinct expression pattern of the three pathogenic CIQTNF5 mutants, as described in the present study, suggests that multiple pathobiological mechanisms may contribute to RPE dysfunction and vision loss in this disorder, and prominent, hallmark basal RPE deposits similar to those found in the S163R pathology are not a universal characteristic of L-ORD. Depending on their unique structural properties and distribution patterns, the accumulation of distinct mutant CIQTNF5 hetero-oligomeric species may cause a spectrum of clinical phenotypes. This multifaceted nature of L-ORD is supported by recently published clinical studies, in which patients harboring pathogenic P188T or Q180E CIQTNF5 mutations lack hypomelanotic markers of classic S163R L-ORD. In patients with P188T, OCT imaging uncovered a hyperreflective focal thickening of the outer segment/apical RPE microvilli interdigitation zone, consistent perhaps with changes in the RPE/photoreceptor interface induced by the apical accumulation of the P188T mutant. In severe cases, there was loss of interdigitation zone, ellipsoid zone, and RPE, with preservation of BrM. Another recent study showed that the pathogenic Q180E variant is associated with an autosomal dominant gyrate
atrophy-like choroidal dystrophy, further expanding the clinical spectrum of C1QTNF5-inherited retinal diseases.70 In contrast to S163R-induced pathology, patients harboring the Q180E mutation were found to display a more severe loss of ERG function at a younger age, and lacked the characteristic prominent basal subRPE deposits, even in areas with significant loss of both photoreceptors and RPE cells. Abnormally long anterior zonular insertions and choroidal neovascularization were also absent in these patients.70

The current study and previous research suggest both dominant negative and toxic gain-of-function disease mechanisms, in which the C1QTNF5 mutants affect the wild-type protein function and apical secretion by trapping it within aberrant hetero-oligomeric structures. All C1QTNF5 pathogenic mutations are therefore expected to gradually induce changes in the RPE-photoreceptor interface, with relevance for future translational studies. An important aspect for future research is to determine how various changes in mutant/wild-type ratio within C1QTNF5 hetero-oligomers could impact the manifestation and severity of the emerging phenotype. This could be achieved with AAV-based systems by co-expressing tagged constructs to differentiate them from the endogenous wild-type protein.22 Although viral vectors have successfully been used as tools to generate models of several late-onset neurodegenerative human disorders, their use is also associated with disadvantages, such as the inability to faithfully mimic the patient stoichiometry of mutant/wild-type protein expression.71 As a complementary system to in vivo AAV-based modeling, patient-specific induced pluripotent stem cells, human iPSC-RPE, have recently provided additional key insights into L-ORD pathology, and represent a platform for testing therapeutic strategies.72 Recent studies support the utility of these in vitro models to investigate the molecular mechanisms of age-related and inherited RPE-based ocular disorders and develop treatments.63,72–74 In contrast to the in vivo AAV-S163R-based model, no accumulation of C1QTNF5 was detected in the subRPE space using an iPSC-derived RPE from patients with L-ORD as an in vitro system.75 Potential factors that account for this difference are the longer time frame required for the basolateral accumulation of the S163R mutant, the antibodies used for C1QTNF5 protein detection, and the presence of a native RPE/BrM interface in vivo, which may be needed to model this specific aspect of the disease.

In conclusion, this study predicts the existence of distinct histologic phenotypes associated with various pathogenic C1QTNF5 mutations in L-ORD. The long asymptomatic phase of this disease suggests a potentially large window of opportunity for therapeutic interventions. Treatment strategies aimed at reducing the expression of mutant C1QTNF5 protein accumulation before the RPE/photoreceptor interface is significantly altered may be necessary to delay the onset of RPE dysfunction and retinal degeneration. Future studies focused on investigating the detailed biophysical properties of C1QTNF5 mutants, influencing their self-assembly, oligomeric structures, aggregation state, and relative protease resistance, have the potential to elucidate the mechanistic basis of their distinct behavior and pathobiology.

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Supplemental Data

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Author Contributions

A.D. wrote the original draft, conceived and designed the study, and reviewed and edited the final manuscript; L.X. participated in experiment design, performed data analysis, and reviewed and edited the manuscript; A.D., L.X., S.N.B., M.K., W.N.R., W.A.B., C.P.S., and F.M.D. performed experiments and interpreted and analyzed data; W.N.R., M.K., W.A.B., C.B.R., and W.C.S. analyzed data and reviewed and edited the manuscript. All authors agreed with the submission in its final form.

References


35. Wilk MA, Huenkaphaler AL, Collery RF, Link BA, Carroll J: The effect of retinal melanin on optical coherence tomography images. Transl Vis Sci Technol 2017, 6:8


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Xu et al

Zhang N, Boatright JH, Nickerson JM: Age-related retinal changes in wild-type C57BL6/J mice between 2 and 32 months. Invest Ophthalmol Vis Sci 2021, 62:9

Gresh J, Goletz PW, Crouch RK, Rohrer B: Structure-function analysis of rods and cones in juvenile, adult, and aged C57Bl/6 and Balb/c mice. Vis Neurosci 2003, 20:211–220


Garland DL, Pierce EA, Fernandez-Godino R: Complement C5 is not critical for the formation of sub-RPE deposits in Epm1 mutant mice. Sci Rep 2021, 11:10416


Della NG, Campochiaro PA, Osborne FLA 5.6.0 DTD # AJPA3806_proof 7 November 2022 3:20 pm EO: AJPA-D-22-
