A CTRP5 gene S163R mutation knock-in mouse model for late-onset retinal degeneration

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Late-onset retinal macular degeneration (L-ORD) is an autosomal dominant inherited disorder caused by a single missense mutation (S163R) in the CTRP5/C1QTNF5 protein. Early phenotypic features of L-ORD include: dark adaptation abnormalities, nyctalopia, and drusen deposits in the peripheral macular region. Apart from posterior segment abnormalities, these patients also develop abnormally long anterior lens zonules. In the sixth decade of life the rod and cone function declines, accompanied by electroretinogram (ERG) abnormalities. Some patients also develop choroidal neovascularization and glaucoma. In order to understand the disease pathology and mechanisms involved in retinal dystrophy, we generated a knock-in (Ctrp51/2) mouse model carrying the disease-associated mutation in the mouse Ctrp5/C1QTNF5 gene. These mice develop slower rod-b wave recovery consistent with early dark adaptation abnormalities, accumulation of hyperautofluorescence spots, retinal pigment epithelium abnormalities, drusen, Bruch’s membrane abnormalities, loss of photoreceptors, and retinal vascular leakage. The Ctrp51/2 mice, which have most of the pathological features of age-related macular degeneration, are unique and may serve as a valuable model both to understand the molecular pathology of late-onset retinal degeneration and to evaluate therapies.

INTRODUCTION

Late-onset retinal macular degeneration (L-ORD) is an autosomal dominant condition in humans that closely resembles age-related macular degeneration (AMD). L-ORD is caused by a Ser163Arg (S163R) mutation in the Complement 1q Tumour Necrosis Factor 5 gene (CTRP5/C1QTNF5). The clinical features observed in L-ORD include thick and extensive sub-retinal pigment epithelium (RPE) deposits between the RPE and Bruch’s membrane that extend from the central retina to the ora serrata, leading to a loss of both central and peripheral vision. Dark-adaptation abnormalities can precede anatomical symptoms and funduscopic signs in patients with L-ORD. In later stages of the disease, widespread loss of RPE and photoreceptors with choroidal neovascularization (CNV), disciform macular scarring and severe peripheral choriotinal atrophy are observed (1–4). In addition to the retinal abnormalities, patients also exhibit anterior segment abnormalities with long anterior zonules with an abnormal zonule lamella deposited with pigment granules (5,6). The clinical features of L-ORD overlap with other dominantly inherited retinal diseases, including Malattia Leventinese (ML)/ Doyne honeycomb retinal degeneration and dominant drusen (7). The clinical features of L-ORD are mostly mistaken for early AMD in the initial stages and for retinal degeneration in later stages. Its original name was late-onset retinal degeneration, and it is also referred to as autosomal dominant hemorrhagic macular dystrophy, late-onset macular degeneration or L-ORD (1,2,8–12).

Studies on the short chain collagen gene CTRP5 revealed that the gene encodes for a secretory glycoprotein with an N-terminal signal peptide, a short collagen (Gly-X-Y) repeat and a C-terminal globular complement 1q (gC1q) domain. It is highly expressed in RPE and ciliary epithelium layers in...
the eye and at low or minimal levels in other tissues (9,11). The wild-type (WT) CTRP5 exists as a secreted and membrane-associated protein (13). Secretion of the mutant CTRP5 protein is deficient and this is likely to be due to the protein being misfolded and retained within the endoplasmic reticulum (ER) (13,14). Impaired CTRP5 protein secretion because of the mutation may underlie the pathophysiology of L-ORD (13).

CTRP5 is expressed as a dicistronic transcript with the membrane-type frizzled related protein (MFRP) gene in the RPE, ciliary epithelium and the lens (9,11). It has been shown that MFRP and CTRP5 co-localize in the RPE and ciliary body, and they have been reported to directly interact (14,15). Like other dicistronic genes, CTRP5 and MFRP may also participate in a common pathway. The frizzled domain present in MFRP is predicted to function as a receptor for the Wnt proteins (16). Similarly, CTRP5 may also participate in the Wnt pathway through its interaction with MFRP (14). Recently, studies suggested roles for CTRP5 in cellular response to oxidative damage and energy homeostasis (17). Determining the function of CTRP5 and its role in RPE may assist in understanding L-ORD due to a mutation in this gene.

We generated a knock-in mouse model to study the function of CTRP5. In the present study, we describe and characterize the retinal phenotype of a novel heterozygous knock-in mouse model (CTR5S<sup>+/−</sup>) bearing a C>G point mutation (S163R) in the Ctrp5 gene associated with human L-ORD (9). In addition to carrying one copy each of the mutant and WT Ctrp5 alleles, the expression of Ctrp5 in these mice is controlled by the native promoter. The Ctrp5<sup>+/−</sup> mouse model closely mimics the phenotypic features of patients with L-ORD and AMD. The current mouse model we developed is unique in that it exhibits the clinical features of AMD, such as drusen-like deposits, abnormalities in Bruch’s membrane, increased fundus autofluorescence and loss of photoreceptors. In a few mice, the formation of foci of fluorescein leakage in the retinal vasculature was observed. Several transgenic and gene knock-out mouse models that mimic the phenotype of human AMD have been previously described (18). However, none of these models has been shown to develop the range of features, including dark adaptation abnormalities, severe cone photoreceptor loss and accelerated accumulation of autofluorescent spots present in the Ctrp5<sup>+/−</sup> mouse. Having these desired features not only enables the Ctrp5<sup>+/−</sup> mouse to serve as a unique and biologically relevant model for the study of the pathologic consequences of the Ctrp5 S163R mutation but also shows promise as a valuable model for investigation of the pathogenesis underlying L-ORD and AMD, possibly leading to treatment targets.

RESULTS

Expression of Ctrp5 in Ctrp5<sup>+/−</sup> mouse retina

The knock-in Ctrp5<sup>+/−</sup> mice carrying the S163R mutation in the heterozygous state were generated as described in Figure 1 and are viable and fertile. At 2 months of age, the presence of both WT and mutant Ctrp5 transcripts was detected in the RNA isolated from RPE–choroid tissue of Ctrp5<sup>+/−</sup> mice.
using gene-specific primers by quantitative real-time polymerase chain reaction (qRT-PCR) and restriction digestion analysis (Fig. 2A). AluI restriction enzyme digestion yielded two fragments of 125 and 49 bp for the amplicon from C57BL/6 (WT) mice. The mutation, however, results in the loss of the restriction enzyme site and AluI digestion yielded a prominent 174-bp amplicon for the mutant protein along with faint bands at 125 and 49 bp for the WT copy (Fig. 2A).

Western blot analysis of the RPE–choroid protein extract from 2-month-old Ctrp5+/- mice demonstrated the presence of Ctrp5 (26 kDa, Fig. 2B). β-Actin antibody labelling was used as a loading control. Collectively, these observations indicate that both Ctrp5 transcripts and Ctrp5 protein are produced in the Ctrp5+/- mouse.

Phenotype of the Ctrp5+/- mice

Fundus examination of the heterozygous Ctrp5+/- mice and the WT mice revealed no gross abnormalities from 5 to 21 months of age. Fluorescein angiography (FA) revealed no abnormal findings in WT mice tested from ages 5–21 months (Fig. 3A). In contrast, FA showed dye leakage in two 21-month-old Ctrp5+/- mice (n = 12) at 5 min post-fluorescein injection, indicating the presence of foci of fluorescein leakage in a subset of Ctrp5+/- mice (Fig. 3B).

Electroretinography analysis and rod b-wave recovery

Since the retinal degeneration in human L-ORD patients has a late onset, we chose to test retinal function in mice that were at least 10 months old. Dark and light-adapted electroretinography (ERG) waveforms were similar in shape and timing to those of C57BL/6 mice (WT) (Fig. 4Ai and ii). Although response amplitudes decreased with increasing age, waveform characteristics were unchanged in mice as old as 21 months.

Scotopic b-wave amplitude, which reflects bipolar cell activity, was measured from baseline to the trough of the a-wave. The dark-adapted ERG response was characterized by the rod-saturated b-wave amplitude (Vmax) sensitivity (k), derived from Naka–Rushton fits to the b-wave amplitude (r19), b-wave amplitude (Vbmax), and a-wave amplitude (Vamax) at maximum intensity 1.09 log cd s/m². The photopic ERG was characterized by the b-wave amplitude, Lmax at maximum intensity.

Dark-adapted a and b-wave amplitudes in Ctrp5+/- mice were consistently smaller than in the WT mice at all ages. Reduction in amplitudes from WT mice ranged from 10 to 15% at age 10 months and 15 to 23% at 21 months. The dark-adapted ERG amplitude was significantly lower (Student’s t-test) than WT for Vamax (P = 0.01), Vbmax (P = 0.02), but not for rod-saturated b-wave Vmax (P = 0.06) at all ages (Fig. 4B). The values for k were comparable in the Ctrp5+/- and WT mice from 10 to 16 months. At 18 and 21 months, k in Ctrp5+/- mice was higher than in WT mice by 0.24 log units (about 40% less sensitive than the control mice); the difference was not statistically significant. The cone-mediated b-wave amplitude was also consistently lower in WT mice for all ages (Fig. 4B). The photopic b-wave Lmax was ~10% reduced for WT from 10 months to 18 months. At 21 months, Lmax was significantly reduced by 23% (P < 0.007) (Fig. 4B).

Recovery of rod b-wave from intense bleach in 12-month-old Ctrp5+/- (n = 9) and C57 (n = 5) mice revealed that the recovery was slow in the Ctrp5+/-, and after 60 min, b-wave amplitude recovered to 75% of the pre-bleach amplitude (Fig. 5). The delayed recovery of the rod-b wave observed in the Ctrp5+/- mice is consistent with the dark adaptation abnormalities observed in patients with the S163R mutation.

Accumulation of autofluorescent foci in autofluorescence scanning laser ophthalmoscopy fundus images

To characterize the ocular phenotype during aging and to learn more about potential pathological changes, we examined Ctrp5+/- mice (n = 12) and age-matched WT mice (n = 10) using autofluorescence scanning laser ophthalmoscopy (AF-SLO) at various ages (7–8 months, 12–13 months,
Figure 4. Electoretinography analysis in Ctrp5^{+/-} mice. (A) Representative waveforms for (i) scotopic and (ii) photopic ERGs for 21-month-old C57BL/6 mice and 10-, 16- and 21-month-old Ctrp5^{+/-} mice. (B) Intensity response functions showing change in amplitude with age for Ctrp5^{+/-} and WT mice at 10, 13, 16, 18 and 21 months for (i) rod V_{max} amplitude, (ii) photopic b-wave amplitude, (iii) scotopic a-wave amplitude, and (iv) scotopic b-wave amplitude. Each point represents the mean ± SE.
Electron microscopic evaluation revealed the abnormalities in Bruch’s membrane as early as 5 months of age (Fig. 9B and C). These aberrations were composed of breaks in the inner collagenous zone, outer collagenous layer deposits and small basilar laminar deposits, the frequency and complexity of which increased with the age of the Ctrp5+/- mice (Fig. 9B, C, E, F, K). Beginning at 12 months, the basal portion of the RPE contained vesicular-like or vacuole-like structures that were filled with an amorphous substance that could possibly be lipid (Fig. 9H, I, N, O). Their morphology, grouping and increased frequency with age suggests that vesicular-like profiles may correspond to the autofluorescent spots illustrated in Figure 6. In addition, by 15 months of age, sub-RPE drusen-like deposits were present (Fig. 9K, L), the abundance of which increased with age.

Expression of photoreceptor-specific genes in Ctrp5+/- mice

To gain insight into the pathology of degeneration, we studied the expression profile of various marker genes in the Ctrp5+/- mice and age-matched control (C57BL/6) mice at ages 5, 8, 12, 15 and 21 months (Fig. 10). The RNA isolated from the RPE–chorioid tissue was used to analyze the expression of Ctrp5, Heme oxygenase 1 (HO-1) and Ceruloplasmin (CP) gene transcripts, whereas the RNA isolated from retina was used to analyze the expression of Rhodopsin and Cone opsins (Opn1sw and Opn1mw). In Fig. 10, we present the expression profile of selected marker genes in Ctrp5+/- retina in comparison to controls, by considering the expression values of these genes in age-matched controls to be 100%. The levels of the Ctrp5 gene expression decreased with age in the Ctrp5+/- mouse model when compared with the control mice. Also, a significant decrease in the levels of the opsins, rhodopsin and both the cone opsins (sw and mw) decreased with age in these mice indicating either significant degeneration or decreased function of cones and rods in our mouse model when compared with the control mice (Fig. 10A). RNA isolated from retinas of 5- and 8-month-old Ctrp5+/- mice showed no significant changes in expression of the cone-specific markers Opn1-sw and Opn1-mw or the rod-specific marker, rhodopsin or the total Ctrp5 (WT and mutant alleles together) levels when compared with the age-matched WT mice (P > 0.1, Fig. 10A).

By 12 months, the expression of total Ctrp5 was reduced by 20% in Ctrp5+/- mice (P < 0.002, Fig. 10A). The expression of rhodopsin was significantly decreased (P < 0.002). Cone-specific genes Opn1-sw and Opn1-mw showed lower levels of expression in Ctrp5+/- (P < 0.002) retinas compared with WT. The expression of total Ctrp5 was reduced by nearly 50% (P < 0.002) and 55% (P < 0.002) in Ctrp5+/- mice compared with the WT mice at 15 and 21 months old, respectively. The rhodopsin levels also reduced by nearly 50% (P < 0.002) in 15-month-old Ctrp5+/- mice and to 40% (P < 0.002) in 21-month-old mice. Nearly 50–60% of the reduction in the levels of cone opsins (Opn1-sw and Opn1-mw) was observed in 15- and 21-month-old Ctrp5+/- mice (Fig. 10A). The Ctrp5+/- mouse manifested a slow degeneration of rods and cones consistent with human L-ORD (8,12).

Retinal structure and outer retinal degeneration in Ctrp5+/- mice

To determine the effect of the S163R mutation on the retina in Ctrp5+/- mice, we evaluated the outer retinal structure from mice aged from 5 to 21 months at the light and electron microscopic levels. Light microscopic evaluation of retinal sections from WT mice showed normal retinal structure across all ages (Fig. 7A, C, E, G, I). In contrast, in Ctrp5+/- mice, there were progressive degenerative changes in the outer retina. The first signs of aberrant photoreceptor structure appeared at 8 months of age (Fig. 7D) with swelling of cone inner segments. At 12 months of age, nuclei of cone photoreceptors appeared pyknotic (Fig. 7F). All inner segments appeared swollen by 15 months, and RPE apical processes were retracted into the sub-retinal space and were no longer surrounding photoreceptor outer segments (OSs) (Fig. 7H). By 21 months of age, the structure of the outer retina continued to deteriorate in the Ctrp5+/- mice as vacuoles appeared throughout the basal portion of the RPE cytoplasm and nuclei of both photoreceptors and RPE were displaced (Fig. 7J). By 21 months, the overall thickness of the retina decreased (Fig. 8). To document the degenerative process in the outer retina, we measured the thickness of the outer nuclear layer. The thickness of photoreceptor nuclei in this layer was significantly reduced compared with controls.
The expression profile of selected stress response genes was also determined in Ctrp5+/−/ mice at various ages (Fig. 10B). The relative expression levels of oxidative stress or the redox-mediated stress markers HO-1 and CP were increased by 12-fold (P < 0.002) and 2.5-fold (P < 0.002), respectively, when compared with the control mice at 12 months. The expression of HO-1 and CP increased further, by 18-fold (P < 0.002) and 3.5-fold (P < 0.05), respectively, in the Ctrp5+/− mice at age 15 months. By 21 months, the level of HO-1 significantly increased to 19-fold (P < 0.002) and CP increased 4-fold in the Ctrp5+/− mice. Subsequently, expression of these genes increased significantly at 15 months, when the retina was further degenerated (Fig. 10B).

In addition to Ctrp5, the expression of the Rpe65 gene was measured as a positive control to determine the extent of degeneration of RPE in the Ctrp5+/− mice when compared with WT mice. The levels of Rpe65 expression decreased in the Ctrp5+/− mice at 15 months (P = 0.25) and further decreased significantly (P < 0.05) in mice at 21 months old, indicating that the Ctrp5+/− mice develop RPE degeneration when compared with WT mice (data not shown). The reduction in the levels of Ctrp5 expression in the Ctrp5+/− mice may be partly due to the degenerating RPE in these mice. We observed the thinning of the OS layer (Supplemental Fig. S1) along with a significant decrease in the expression of rhodopsin and cone opsins (sw, mw) in the later stages of degeneration as the Ctrp5+/− mice aged (Fig. 10A), further indicating the progression of retinal degeneration. A significant increase in the expression of oxidative stress markers like HO-1 and CP in Ctrp5+/− indicates the progression of retinal degeneration and the presence of oxidative stress in the RPE of the Ctrp5+/− mice from ages 5 to 21 months.

Cone loss in Ctrp5+/− mice

To confirm the pathological changes in the cone photoreceptors observed in histology and the decrease in the cone opsin profiles in real-time PCR, we stained the retinal

Figure 6. Accelerated accumulation of autofluorescent spots in Ctrp5+/− mice with age. AF-SLO projection images of 30 frames taken with a 55° angle lens. (A) Representative fundus images obtained by AF-SLO imaging from both Ctrp5+/− mice (n = 12) and age-matched WT mice (n = 10) genotypes at 7–8 months, 12–13 months, 15–16 months and 20–21 months. In both C57Bl/6 and Ctrp5+/− mice, the number of autofluorescent spots was increased with age; however, the accumulation of these spots was accelerated and more pronounced in Ctrp5+/− mice. (B) Quantification of autofluorescent spots in WT (black circles, dashed line) and Ctrp5+/− mice (black squares, continuous line) per AF-SLO images. The number of autofluorescent spots per AF-SLO image is shown as the mean number of spots ± SD of both eyes for each animal. For both genotypes, the mean number of spots was significantly correlated with the age indicating an age-related increase in autofluorescent spots as an age-related normal process (linear regression plot). However, a significantly higher number of autofluorescent spots were observed in 16–21-month-old Ctrp5+/− mice compared with aged-matched WT mice and compared with the young (7–8 months) groups of both genotypes (P < 0.0005).
cryosections of *Ctrp5*+/− mice using both short-wavelength cone (S-cone) opsin and medium-wavelength cone (M-cone) opsin antibodies. The number of cones in the *Ctrp5*+/− mice was found to be less compared with the number of cones in the retina of WT mice starting at 12 months of age. Degenerative changes consequent to loss of both M-cones and S-cones (Fig. 11) were observed in *Ctrp5*+/− mice. The loss of cones appeared to originate in the peripheral retina (both superior and inferior) and subsequently spread into the central retina. Loss of both cones was observed throughout the retina as the *Ctrp5*+/− mice aged; however, the loss of S-Opsin expressing cones (stained red) was predominant among the cones in the *Ctrp5*+/− mice at age 21 months.

To correlate our anatomic evidence of cone loss in *Ctrp5*+/− mice observed by immunohistochemistry with a functional measure of cone function, we examined S-cone and M-cone physiology by measuring light-adapted ERG responses with short wavelength stimuli (21). Results show that both the S-cone and M-cone ERG responses were smaller in amplitude in *Ctrp5*+/− mice than in WT mice (*P* < 0.001 for each) (Fig. 12). There was a statistically significant difference between S- and M-cone ERG amplitudes of the *Ctrp5*+/− mice, with S-cone amplitudes smaller than the M-cone amplitudes (*P* = 0.037). Both S-cone (*P* = 0.0006) and M-cone (*P* = 0.008) amplitudes of *Ctrp5*+/− mice were significantly lower than those of WT mice (Fig. 11). These results indicate the significant loss of S-cones when compared with M-cones in the *Ctrp5*+/− mice at age 18 months and are consistent with immunohistochemical findings.

**DISCUSSION**

In this study, we developed a heterozygous knock-in (*Ctrp5*+/−) mouse model containing the *Ctrp5* S163R mutation that is associated with human L-ORD. The genotype of these mice is identical to that of patients with L-ORD with one allele each of both the WT and mutant *Ctrp5* being expressed. The *Ctrp5*+/− mice show morphological abnormalities of the RPE at 5 months, delayed rod b-wave recovery and mild ERG changes at 12 months. They manifest structural and functional changes beyond the expected age-related changes observed in WT mice. The *Ctrp5*+/− mice develop accumulations within the RPE and extracellular deposits between RPE and Bruch’s membrane, increasing numbers of subretinal hyperfluorescent lesions, progressive reduction in rod and cone ERG amplitudes and loss of rod and cone photoreceptors. The late age of onset, rate of progression of degeneration, severity of disease and the type of lesions

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**Figure 7.** Histological analysis of retinal structure in *Ctrp5*+/− mice by light microscopy. (A, C, E, G, I) Histology of the outer retinas from WT control and *Ctrp5*+/− heterozygote mice from 5 to 21 months. Retinas from control mice show no abnormalities at any age. (B) Retinas from heterozygous 5-month-old *Ctrp5*+/− mice have no apparent pathology at this magnification. (D) At 8 months of age in the heterozygous mice, rod OSs are elongated and properly organized. Cone inner segments appear swollen (black arrows) but no other pathology is visible. (F) By 12 months of age, inner segments of both rods and cones are mildly swollen and the cytoplasm is heterogeneous, suggesting a degenerative process is beginning in the *Ctrp5*+/− mice. In addition, chromatin is condensed in the nuclei of cones (white arrows). (H) In the outer retina of 15-month-old heterozygous mice, all inner segments are markedly swollen (asterisks). In addition, RPE apical processes fill the subretinal space and no longer surround photoreceptor OSs (black arrows). (J) By 21 months of age, there are marked structural abnormalities in both the RPE and photoreceptors: the basal cytoplasm of the RPE is heterogeneous and filled with vesicle-like structures (black arrows); nuclei of both RPE and photoreceptors have migrated into the area of inner and OSs (white arrows); some inner segments remain swollen (asterisk). RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer. Magnification bar = 2 μm.
Figure 8. Comparison of retinal thickness from the eyes of 21-month-old mice. The retina of WT mice (A) is thicker than that of age-matched Ctrp5+/− mice (B). RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar = 10 μm. Direct measurements of the outer nuclear layer (ONL) thickness (μm) in the peripheral, mid-peripheral and central regions of the retina indicate that this layer is significantly thicker in WT mice (P < 0.001) (C).

Figure 9. Ultrastructural analysis of RPE and sub-RPE areas from WT control and Ctrp5+/− mice. (A, D, G, J, M) RPE from control mice show no abnormalities at any age. In contrast, the RPE of heterozygous mice have structural aberrations as young as 5 months. (B and C) Subtle disruptions of Bruch’s membrane (black arrows) and basal linear deposits consisting of long collagen and fine granular material are apparent at a low frequency (asterisks). (E and F) After 8 months of age, basal linear deposits are more prominent and more frequent (asterisks). (H, I, K, O) By 12 months, sub-RPE drusen-like deposits remain prominent (asterisks) and the RPE cytoplasm contains vesicular profiles (H, I, N). Also visible by 12 months are vesicular profiles containing an amorphous possibly lipid-like substance (white arrows). Areas enclosed in boxes in (B), (E), (H), (K) and (N) are magnified in the panels (C), (F), (I), (L) and (O), respectively. Magnification bars = 2 μm in first and second columns and 500 nm in third column.
observed in Ctrp5+/− mice resemble the clinical phenotype observed in patients with early stages of L-ORD.

The histopathology of Ctrp5+/− mice also mimics that described in patients with L-ORD and AMD (2,8). In Ctrp5+/− mice, ultrastructural aberrations were present within Bruch’s membrane and the sub-RPE space. Similar pathological changes were observed in patients with L-ORD, AMD and ML (22–24). The eyes of an 82-year-old donor from an L-ORD patient (3) and Ctrp5+/− mice is impeded because human donor eyes with early stages of ocular pathology are extremely rare, several hours often pass before donor eyes are collected and fixed, and other systemic health conditions that may affect the ocular phenotype are not fully known. Taking into consideration all of these caveats, we present this Ctrp5+/− model as an early-stage L-ORD model. Ongoing studies are in progress to determine whether at older ages the Ctrp5+/− mice show a more severe phenotype that more closely mimics human ocular pathology of late stage L-ORD.

Development of basal laminar deposits (drusen) between the RPE and Bruch’s membrane is the earliest change observed in AMD (22,25). RPE vacuoles have also been reported in association with basal deposits in human eyes with AMD (26). In addition, disruptions caused in the basal infoldings due to accumulation of collagen-like material and granular material have been observed in patients with early AMD (26) and L-ORD (2). Similar basal deposits and RPE alterations were also reported in the homozygous Efemp1R345W/R345W mice (27). The basal laminar deposits are thought to form a barrier that impairs the transport of nutrients to the retina, leading to degeneration of retinal tissue in patients with Sorsby’s fundus dystrophy (SFD), L-ORD and AMD (11,28). Development of basal laminar deposits was reported in wild type C57BL/6 mice at older age (29). However, the basal laminar deposits were observed in the Ctrp5+/− mice as early as 5 months and they became prominent with age while these deposits were either absent or minimal in age-matched control mice. Determination of origin and composition of these deposits and their relationship with the retinal pathology observed in Ctrp5+/− mice may assist in understanding the mechanism underlying L-ORD and other diseases with overlapping phenotypes.

We also observed a significant loss of photoreceptors in the Ctrp5+/− mice. The expression profile of photoreceptor marker genes indicated loss of both rods and cones. However, immunohistochemical analysis showed that the degeneration of cones was more apparent than that of rods. It is possible that cone photoreceptors are more susceptible to the damage caused by the mutant Ctrp5 than are rods. Alternatively, the loss of cones may appear to be more evident than the loss of rods due to the cones accounting for only 3% of the total photoreceptors in mice (30).

Age-dependent degeneration of S-cones was more prominent than that of M-cones in Ctrp5+/− mice as assessed by ERGs and immunohistochemistry. The majority of cones in the mouse retina co-express both S- and M-opsins but the expression of S-cone opsin mRNA transcript is three times greater than M-cone opsin mRNA transcripts (30). The ratio of S- to M-cone opsin expression varies in a dorsoventral manner in mice. Greater numbers of S-cones are located in the ventral retina, while M-cones are abundant on the dorsal side (30). Interestingly, the Ctrp5+/− mice also had a predominance of M-opsin-expressing cones among the surviving photoreceptors in the dorsal retina (31). Despite this selective preservation of M-opsin-expressing cones, the photoreceptor loss observed by thinning of outer nuclear layer was found to be uniform throughout the retina in Ctrp5+/− mice. Predominant loss of S-opsin-expressing cones suggests that these
cones may be more sensitive to the effect of mutant Ctrp5 than the M-opsin-expressing cones.

In early stages of the disease, L-ORD patients manifest abnormalities in dark adaptation prior to any visual symptoms or fundus changes and before any changes are noted in the ERG (2). The ERG becomes progressively abnormal and at the end-stage disease, it shows highly reduced amplitudes and delayed implicit times (2). In many aspects, the Ctrp5+/− mice mimicked the course of degeneration in human L-ORD. The onset of degeneration as measured by the ERG was late and differences from WT mice were observed after 13 months for both rod and cone function. However, unlike some L-ORD patients, the ERG amplitudes were still recordable in Ctrp5+/− mice even at age 21 months and were only ∼20–25% reduced from age-matched WT mice. By probing the cones with short wavelength stimuli, we found S-cone degeneration to be markedly higher when compared with M-cones. This is consistent with the pattern of cone loss observed by immunohistochemistry.

Previous studies of mouse models of AMD report a broad spectrum of ERG changes, while the ERG response of AMD patients was found to decline progressively with age (32–34). Aging studies in WT mice showed age-related reduction in the ERG amplitudes with preservation of waveform characteristics (35). Although subtle changes were reported in the RPE and Bruch’s membrane of heterozygous and homozygous Timp3 knock-in mice, the scotopic and photopic ERGs were not altered in their lifespan (36). Knock-out models of both Ccl2 and Ccv2 which develop drusen and other features of AMD did not show significant alteration in the ERG response.

Figure 11. Localization of cone opsins in the mice retina. (A) Dorsoventral distribution of S-cones (red) and M-cones (green) by immunohistochemical localization in the retinas from WT mice at 21 months, and Ctrp5+/− mice at 12 and 21 months. Blue: nuclei.
Figure 12. Spectral ERG responses recorded from 18-month-old Ctrp5\(^{+/−}\) mice. Means ± SEM of light-adapted S- (360 nm) and M- (510 nm) ERG amplitudes. Dashed horizontal lines represent ± SD for the WT S-cone responses. S-cone amplitude in Ctrp5\(^{+/−}\) mice is significantly lower than M-cone responses.

(37–40). Although the RPE abnormalities observed in the model for ML due to the Efemp1 gene mutation are severe and closely resemble the phenotype of AMD and the Ctrp5\(^{+/−}\) mice, the ERGs of these mice were found to be unaltered even at older age (41). The ERG response of the C\(\beta\)h gene knock-out model also showed compromised rod photoreceptor function with minimal changes in cone function (42). In comparison to the other mouse models with phenotypic features resembling AMD in patients, the Ctrp5\(^{+/−}\) is the only mouse model showing alteration in both rod and cone photoreceptor ERG response consistent with the phenotype of AMD.

Dark adaptation abnormalities were noted at the early stages of disease in SFD, ML, AMD and L-ORD (8,23,43,44). In fact, the delayed dark adaptation has been suggested as a phenotypic marker for pre-symptomatic detection of L-ORD and AMD (8). In patients with ML or EFEMP1 retinal dystrophy with confluent macular deposits, scotopic sensitivity is reduced and dark adaptation kinetics are prolonged over the macular deposits but are normal elsewhere (23). In previously reported mouse models for AMD, the dark adaptation kinetics were not reported. Our studies in Ctrp5\(^{+/−}\) mice indicated delayed rod-b wave recovery consistent with the dark adaptation abnormalities observed in the patients with AMD and L-ORD, thus making this a good model to study early onset retinal changes associated with this phenotype.

Progressive accumulation of a significantly large number of hyperautofluorescent spots was observed in Ctrp5\(^{+/−}\) mice with age. Commonly, the fundus autofluorescence is derived from LF at the level of the RPE (45). In the RPE cell layer, the progressive LF accumulation is a result of constant phagocytosis of shed photoreceptor OS disks (46–48). Previous studies have shown that various LF compounds such as A2-E (N-retinylidene-N-retinylethanol-amine), a dominant fluorophore, possess toxic properties which may interfere with normal RPE cell function, causing RPE dystrophy (49–52). Similar to the Ctrp5\(^{+/−}\) mouse model, mice deficient in C\(\beta\)h and Ccl2 showed accelerated accumulation of autofluorescent spots with age (42). In other mouse models and in AMD, phagosomes and LF inclusions in macrophages were reported to contribute to autofluorescence (40,53,54). The Ctrp5\(^{+/−}\) mice showed increased accumulation of autofluorescent spots as they aged. These spots appear to be distributed uniformly. Unlike the other mouse models described, the hyper-autofluorescent spots in the Ctrp5\(^{+/−}\) mice may come from RPE cells as the macrophages were not observed in the vicinity of RPE by immunohistochemistry using Mac-1 and F4/80\(^{+}\) antibodies (data not shown). The increase in the number of autofluorescent foci in the Ctrp5\(^{+/−}\) mice with age may suggest the presence of LF in the RPE of these mice. Determination of the composition of autofluorescent spots in these mice may help in understanding their origin.

The mechanism of RPE degeneration in AMD is likely to be multifactorial, with the potential involvement of oxidative stress, environmental factors, intense light exposure and gene mutations (55). Similar oxidative stress may induce mitochondrial damage (56). It was previously reported that the mutant CTRP5 is not secreted and that it is retained within the ER and tends to form aggregates in vitro causing ER stress (13,14). CTRP5 was also reported to stimulate AMP-Kinase which is involved in mediating oxidative stress response in RPE cells (57). The expression of C1QTNF5/CTRP5 is reported to be drastically increased following depletion of mtDNA in myocytes (17). It is previously reported that the dominant negative mutation in the CTRP5 gene may lead to aggregation of the protein in the ER, causing ER stress (14). Prolonged ER stress leads to organelle damage and dysfunction, and ultimately results in cell death. ER stress is linked to the pathogenesis of neurodegenerative disorders, such as AMD (58). ER stress is also reported to be involved in oxidative injury to the RPE (58). The oxidative injury in turn may result in mitochondrial damage in the RPE cell (56). Accumulation of the mutant CTRP5 protein, which is also deficient in secretion, may result in RPE cells having compromised ability to phagocytose photoreceptor debris and function. Therefore, during aging either ER stress or oxidative stress or a combination of both may result in compromised RPE function leading to photoreceptor degeneration in patients with L-ORD and in our mouse model.

CNV is a hallmark pathologic feature common to AMD and L-ORD (10,59,60). Although the phenotype of Ctrp5\(^{+/−}\) mice is fairly uniform, leaky retinal vessels were developed in only 2 out of 12 mice tested at ages 8–21 months indicating variation in this feature. This is similar to the variation observed in the development of CNV in L-ORD patients. Among the other mouse models with phenotypic features of AMD, only Ccl2\(^{−/−}\) and Ccr2\(^{−/−}\) were reported to develop CNV as they aged (59).

The data presented in this study establish that a single-point mutation in the Ctrp5 gene causes a retinal phenotype in mice that is remarkably similar to that of L-ORD and AMD. In addition, unlike the other models of AMD, Ctrp5\(^{+/−}\) mice develop delayed rod-b wave recovery, altered rod and cone photoreceptor ERG response, severe cone photoreceptor loss and accelerated accumulation of hyperautofluorescent spots. Therefore, the Ctrp5\(^{+/−}\) mice serve as a unique model to study both L-ORD and AMD. These mice also show promise as a valuable model for investigation of the
mechanism underlying late-onset retinal degeneration. The presence of the features described in this article makes the Ctrp5+/− mice unique among the existing models of AMD.

MATERIALS AND METHODS

Antibodies used for the study

The following antibodies were used in our studies: anti-CTRP5 polyclonal antibody (1:500 dilution; described elsewhere (13)); anti-β-actin antibody (1:500 dilution; Sigma-Aldrich, St Louis, MO, USA), anti-S-opsin antibody (1:200 dilution; Chemicon, Temecula, CA, USA), anti-M-opsin antibody (1:200 dilution; Chemicon); anti-rabbit Alexa Fluor 555 (1:2500 dilution) and anti-mouse Alexa Fluor 488 (1:2500 dilution) (Invitrogen-Molecular Probes, Carlsbad, CA, USA).

Construct design for the Ctrp5+/− mice

To construct a targeting vector, a 11.5 kb mouse genomic DNA fragment containing exons 1–3 of Ctrp5 was cloned from a positively identified C57BL/6 (RPCI-23) BAC clone (82, D10). The clone contained the sequence between 2.21 kb downstream to exon 3 and 7.64 kb upstream to exon 2. The LoxP/FRT flanked Neo cassette was inserted 693 bp 3′ to the C−>G point mutation engineered into exon 3 using a Red/ET recombination kit (Gene Bridges, Heidelberg, Germany). Overlap extension PCR was used to generate the point mutation. The single loxP site was 5′ to exon 2. The targeting vector was confirmed by restriction analysis after each modification step. The total size of the targeting construct (including vector backbone and Neo cassette) was 15.71 kb (Fig. 1).

The targeting vector was linearized by NotI and electroporated into C57BL/6 embryonic stem cells which were subjected to selection with G418 antibiotic and ganciclovir. PCR and southern blot analyses were used to identify recombinant clones. Correctly targeted embryonic stem cells were injected into BALB/c blastocysts and implanted into pseudo-pregnant mice, to generate chimeras. These chimeras were mated with C57BL/6J mice to transmit the targeting allele. Mice carrying the Ctrp5 693 C−>G mutation were identified by amplification of the tail DNA followed by sequencing (Fig. 1). The Ctrp5+/− mice were generated with assistance from iNgenious Targeting Laboratory, Inc. (Stony Brook, NY, USA).

Animal maintenance

Mice were maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with protocols approved by UCSD Institutional Animal Care and Use Committee. All studies were performed using Ctrp5+/− mice and WT littermate mice as controls. Animals were genotyped by amplification of mouse tail genomic DNA using PCR and testing for the presence of a C−>G point mutation by AluI restriction digestion analysis (9). The genotypes were determined by analyzing the digested products on a 1.5% agarose gel. Mice were maintained in a 12 h dark−light cycle. All tissues used for analysis were collected from animals at the end of the dark cycle. Ctrp5+/− and WT mice of ages 5–21 months were used to study histology, ultra structure and photoreceptor gene expression. ERG was performed on these mice from ages 10 to 21 months.

Protein isolation and western blot analysis

Total protein extract was prepared from the mouse RPE–choroid in 1 × RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich). The extracts containing ~30 μg of protein per lane were subjected to western blot analysis, as described earlier with anti-Ctrp5 antibodies and anti-β-actin antibodies (3,61). The expression levels of Ctrp5 in different samples were compared by loading equal amounts of protein and normalizing the intensity of immunopositive bands with that of β-actin.

Fundoscopy and FA

In vivo fundus FA of CNV lesions was conducted using a SPECTRALIS® high-resolution, spectral domain optical coherence tomography (HRA+OCT) imaging system (Heidelberg Engineering, Inc., Vista, CA, USA). Animals were anesthetized with an intra-peritoneal loading dose of ketamine (93 mg/kg) and xylazine (8 mg/kg). Pupils were dilated with topical 1% atropine and 0.5% tropicamide. Body temperature was maintained at 37°C with a heating pad during the course of the experiment.

The fundus of the mice was examined for abnormalities by fundoscopy. Subsequently, FA was performed on the mice by injecting fluorescein (0.02 ml of 25% fluorescein, Hub Pharmaceuticals, Rancho Cucamonga, CA, USA). Early and late-phase fundus angiograms were obtained at an interval of 5 min till 15 min after injection. The early phase angiogram was obtained nearly 1 min after injection; timing started immediately after fluorescein injection.

Electroretinography

To evaluate the retinal function, full field ERGs were recorded in heterozygous Ctrp5+/− mice and littermate C57BL/6 control mice (WT) at ages 10 (n = 12), 14 (n = 12), 16 (n = 9), 18 (n = 9) and 21 (n = 8) months. Mice were dark-adapted overnight and were prepared under dim red illumination. Animals were anesthetized as described above. Corneal ERGs were recorded from both eyes using gold wire loops with 0.5% tetracaine eye drops for topical anaesthesia and a drop of 2% methylcellulose for corneal hydration. A gold wire loop placed in the mouth was used as reference and the ground electrode was on the tail.

Dark-adapted ERGs were recorded with a Ganzfeld configuration using the Espion e2 recording system to brief xenon white flashes from −5.8 to +1.09 log cd s/m²/flash in steps of 0.5 log units. Responses were amplified at 1000 gain at 1.25 to 1000 Hz, and digitized at a rate of 2000 Hz. A notch filter was used to remove 60 Hz line noise. Responses were recorded at 3–60 s intervals depending upon the stimulus intensity and were computer averaged. To examine the scotopic or rod driven b-wave, responses to flash energies from
ERGs in
These stimuli were then used to record S- and M-cone emitting diodes (510 nm peak) by adjusting the flash energy. White light for 2 min in a Ganzfeld dome (62). Recovery were dark-adapted overnight and were exposed to 1000 lux over a 2 log unit range in steps of 0.3 log units.

To study and quantify the response between the S- and M-cones, we examined the photopic ERG of 18-month-old mice (WT: n = 3; Ctrp5+/−: n = 5) by isolating S- and M-cone components as described previously (21). ERG response amplitudes in the WT mice to a Xenon UV flash at 360 nm were matched to responses elicited with green light-response amplitudes in the WT mice to a Xenon UV flash at 360 nm were matched to responses elicited with green light-emitting diodes (510 nm peak) by adjusting the flash energy. These stimuli were then used to record S- and M-cone ERGs in Ctrp5+/− mice.

Rod b-wave recovery after a photobleach
Thirteen-month-old WT (n = 5) and Ctrp5+/− (n = 9) mice were dark-adapted overnight and were exposed to 1000 lux white light for 2 min in a Ganzfeld dome (62). Recovery from bleaching light was tracked by recording the dark-adapted rod b-wave ERG to a dim −2.82 log cd s/m² white flash every 3 min for 60 min. After an initial short period of network adaptation, rod b-wave tracked receptor potential and recovered in parallel with rhodopsin regeneration. The stimulus intensity was sufficiently dim to exclude cone contribution (63). Responses were normalized by the pre-bleach rod b-wave amplitude.

Autofluorescence imaging
Autofluorescence imaging was performed using the SPECTRALIS® HRA+OCT scanning laser ophthalmoscope (Heidelberg Engineering, Inc.) as previously described (20,64). Using a 55° angle lens, projection images of 10 frames per fundus were taken after positioning the optic disc in the centre of the image and focusing on the outer retina.

Retinal histology and ultrastructure
For evaluation of the retinal histology, eyes from 5-, 8-, 12-, 15- and 21-month-old Ctrp5+/− mice and age-matched littermate control animals were fixed by immersion fixation in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer. Eyes were dehydrated with a graded series of ethanol and protected in 30% sucrose and embedded in OCT tissue freezing medium before sectioning. For evaluation of cone cell survival, and localization of Ctrp5, immunohistochemistry was performed using short (S)- and medium (M)-wavelength cone opsins-specific antibodies and anti-Ctrp5 antibody as described earlier (3,13). Sections were mounted in mounting media containing DAPI (4’,6-diamidino-2-phenylindole), observed under an Olympus FV1000 Confocal microscope, and images were captured with the use of appropriate filters and lasers.

Images were captured using image-capturing software (Advanced Microscopy Techniques, Danvers, MA, USA).

The retinal sections stained with Toluidine Blue O were scanned using the Aperio ScanScope CS system (Aperio Technologies, Inc, Vista, CA, USA). Quantitative analyses to determine the number of nuclei were performed with the Aperio ImageScope software Ver. 10.2.2.2319 program and the Aperio Immunohistochemistry Nuclear Algorithm (IHC Nuclear Ver. 10) (65–67). Number of nuclei present at the peripheral, central and mid-peripheral regions of both dorsal and ventral retina were determined independently. At least three measurements each were made on a single region of the retinal section. The mean and standard deviation of the readings obtained from three animals (two sections per animal from the region that is close to the optic nerve and 100 microns apart) of each genotype were tabulated and presented.

Expression of Ctrp5 and photoreceptor-specific genes in Ctrp5+/− retinas
Total RNA from RPE–choroid and retina was isolated separately from 5-, 8-, 12-, 15- and 21-month-old animal retinas (RNesy kit; Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s guidelines, and the reverse transcription reaction was performed (SuperScript-II; Invitrogen). The comparative Ct method was used to calculate the expression of total Ctrp5, and photoreceptor-specific gene expression in different samples of mouse retina, as described earlier (13). The expression of photoreceptor-specific genes was normalized against the RpL19 gene, as described previously (11).

Immunohistochemistry
For immunolocalization of antigens, eyes from 8-, 12-, 15- and 21-month-old Ctrp5+/− mice and age-matched littermate control animals were fixed by immersion fixation in 4% paraformaldehyde in 0.1 m phosphate buffer. Eyes in fixative were thoroughly washed with phosphate-buffered saline, cryoprotected in 30% sucrose and embedded in OCT tissue freezing medium before sectioning. For evaluation of cone cell survival, and localization of Ctrp5, immunohistochemistry was performed using short (S)- and medium (M)-wavelength cone opsins-specific antibodies and anti-Ctrp5 antibody as described earlier (3,13). Sections were mounted in mounting media containing DAPI (4’,6-diamidino-2-phenylindole), observed under an Olympus FV1000 Confocal microscope, and images were captured with the use of appropriate filters and lasers.

Statistical analysis
Data are presented as means ± SD. Comparisons of means between two experimental groups were performed using the two-tailed, independent Student’s t-test.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.
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Conflict of Interest statement. None declared.

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