Characterisation of a C1qtnf5 Ser163Arg Knock-In Mouse Model of Late-Onset Retinal Macular Degeneration


Published in:
PLoS ONE

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.
Characterisation of a C1qtnf5 Ser163Arg Knock-In Mouse Model of Late-Onset Retinal Macular Degeneration

**Xinhua Shu**1-3,4, **Ulrich F. O. Luhmann**2, **Tomas S. Aleman**5, **Susan E. Barker**2, **Alan Lennon**1, **Brian Tulloch**1, **Mei Chen**6, **Heping Xu**6, **Samuel G. Jacobson**5, **Robin Ali**2, **Alan F. Wright**1*

1 MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, Edinburgh, United Kingdom, 2 UCL Institute of Ophthalmology, London, United Kingdom, 3 Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Glasgow, United Kingdom, 4 Department of Vision Sciences, Glasgow Caledonian University, Glasgow, United Kingdom, 5 Scheie Eye Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, United States of America, 6 Centre for Vision and Vascular Science, Queen’s University Belfast, Belfast, United Kingdom

**Abstract**

A single founder mutation resulting in a Ser163Arg substitution in the C1QTNF5 gene product causes autosomal dominant late-onset retinal macular degeneration (L-ORMD) in humans, which has clinical and pathological features resembling age-related macular degeneration. We generated and characterised a mouse “knock-in” model carrying the Ser163Arg mutation in the orthologous murine C1qtnf5 gene by site-directed mutagenesis and homologous recombination into mouse embryonic stem cells. Biochemical, immunological, electron microscopic, fundus autofluorescence, electroretinography and laser photoocoagulation analyses were used to characterise the mouse model. Heterozygous and homozygous knock-in mice showed no significant abnormality in any of the above measures at time points up to 2 years. This result contrasts with another C1qtnf5 Ser163Arg knock-in mouse which showed most of the features of L-ORMD but differed in genetic background.

**Introduction**

Late-onset retinal macular degeneration (L-ORMD) is a fully penetrant autosomal dominant disorder associated with a late-onset macular degeneration resembling age-related macular degeneration (AMD) [1,2,3]. L-ORMD shows disease onset in the fifth to sixth decade with impaired dark adaptation associated with both punctate and diffuse sub-retinal pigment epithelium (RPE) deposits, leading to central and later peripheral visual loss and, at late stages, a pan-retinal atrophy often with choroidal neovascularisation (CNV) and disciform scarring. The most striking and consistent pathological feature is a thick (≤50 μm) extracellular sub-RPE deposit, worst in the macula but extending to the extreme retinal periphery [2,3]. The deposits resemble basal laminar deposits that can be seen both in aged and in AMD eyes, with wide-spaced collagen, RPE basal processes penetrating the deposits and appearances consistent with exocytosis of packets of fibrillar material into the deposits [3]. An unusual phenotypic feature is the presence of long ciliary zonules, which extend from the ciliary epithelium to the anterior lens [4,5].

L-ORMD is caused by a single founder Ser163Arg mutation in the Complement 1q Tumour Necrosis Factor 5 (C1QTNF5) gene [1]. C1QTNF5 (formerly called CTRP5) encodes a short-chain collagen which is strongly expressed in RPE, ciliary epithelium and adipose tissue [1,4,6]. The protein is predicted to contain an N-terminal secretory signal, a short helical collagen repeat and a C-terminal globular complement 1q (gC1q) domain which facilitates trimerisation [1,7,8]. The functional consequences of a Ser163Arg mutation in the gC1q domain has been reported to involve destabilisation of the protein as a result of an abnormal surface charge [7,8]. Similar to the closely-related collagens VIII and XI, C1QTNF5 appears to be secreted into the adjacent extracellular matrix but its function is currently unknown. C1QTNF5 protein interacts with the CUB domains of the Membrane type Frizzled Related Protein (MFRP) [8]. MFRP is expressed as a dicistronic transcript with C1QTNF5 [1] and is mutated in human autosomal recessive nanophthalmos and also in the mouse, in the Mfrp vdh mutant, which is associated with retinal degeneration [9,10]. Recently, Park *et al.* reported that the expression of C1QTNF5 is increased in mtDNA-depleted myocytes and that it stimulates the phosphorylation of AMP-activated protein kinase [11]. These authors also showed that serum C1QTNF5 has significantly higher expression in obese/diabetic mice than in controls.

In order to investigate the pathogenic role of the C1QTNF5 Ser163Arg mutation in vivo, we generated a C1qtnf5 Ser163Arg knock-in mouse model of L-ORMD by homologous recombination into mouse embryonic stem cells and analysed the consequences of the mutation on retinal function and morphology.
Results

Generation of C1qtnf5 Ser163Arg mice

Both human C1QTNF5 and mouse C1qtnf5 proteins contain 243 amino acids with 94% identity. In humans, the Ser163Arg mutation is caused by a single point mutation in codon 163 (AGC → AGG) changing the encoded serine to an arginine residue. In the mouse, serine is also encoded by an AGC codon, therefore the same point mutation (AGC → AGG) in mouse C1qtnf5 introduces the mutation found in L-ORMD patients. The targeting strategy and C1qtnf5 Ser163Arg targeting construct are described in detail in Materials and Methods and summarised in Figure 1. The targeting vector contained long (6.8 kb) and short (1.4 kb) genomic fragments from the 

**expression of C1qtnf5, Ser163Arg mice.**

Histological examination of the normal distribution of fluorophores inside the retina and RPE was performed using confocal microscopy (AF-SLO) in three wild-type, C1qtnf5+/Ser163Arg and C1qtnf5+/Ser163Arg/+ Ser163Arg KI mice at 15–16 months of age respectively. There was no significant difference in autofluorescence in inner and outer retina between wild-type and C1qtnf5 Ser163Arg KI mice, although the number of autofluorescence spots in the inner and outer retina of C1qtnf5+/Ser163Arg/+ Ser163Arg mice were slightly greater than those of wild-type mice but this was not statistically significant (Figure 5).

The electroretinogram (ERG) was used to assess retinal function in C1qtnf5 Ser163Arg KI mice at 10–12 and 15–18 months of age (Figure 6). Representative ERG responses from 18-month-old mutant mice are compared to responses from a wild-type control in Figure 6A. Maximal rod photoreceptor function was elicited with bright light stimuli in the dark-adapted state (Figure 6A, black traces). Cone function was obtained in dark-adapted mice by recording ERGs within a short interval following rod-suppressing bleaching lights (Figure 6A, bottom traces). Delayed dark adaptation following a photobleach, a prominent feature in human L-ORMD [1,2], was studied in the mice by determining the recovery of the rod ERG photoreponse (a-wave) following a light exposure that completely suppressed it (Figure 6A, gray traces overlapping the DA rod response). ERG responses in mutant animals are similar to those in the wild-type control. a-waves and b-waves were measured conventionally for each of the ERG responses and summary statistics for each group shown (Figure 6B). Rod a-wave amplitudes in homozygous (mean ± SD = 250 ± 53 μV) and heterozygous (238 ± 35 μV) C1qtnf5 Ser163Arg KI mice were similar to each other and to wild-type (276 ± 56 μV) mice (P > 0.05). Cone b-wave amplitudes in homozygous (122 ± 25 μV) and heterozygous (109 ± 20 μV) C1qtnf5 Ser163Arg KI animals were also similar to each other and to wild-type (115 ± 23 μV) mice (P > 0.05).

Recovery (BM) in young (6 months) and old (20 months) wild-type and Ser163Arg KI mice and focused in particular on age-related changes in the retinal pigment epithelium (RPE) and underlying BM. Although aged animals showed amorphous sub-RPE deposits, there was no obvious difference in these deposits between wild-type and Ser163Arg KI mice (Figure 3B). The BM thickness increased with age, so there was a significant correlation between age and BM thickness in each genotype respectively. The values of mean BM thickness of old mice (20–24 months) were approximately twice those from young animals (6 months), but we did not identify a significant difference between age-matched wild-type and Ser163Arg KI mice (Figure 3B, C), indicating that the major cause of the increase in BM thickness was normal ageing.

To assess any possible differences in age-related RPE pathology between wild-type, heterozygous or homozygous mutant C1qtnf5 KI littermates with age, we applied a previously described method for RPE pathology grading in animals between 6 and 24 months of age [12,13]. In Figure 4, the mean sum of the observed RPE damage per section and per animal is shown, estimated from the analysis of three sections per animal. All three genotype groups showed a similar range of RPE damage, that was not significantly different between the three groups (Pearson correlation, ρ = 0.0026, N = 35 r2 = 0.244). This suggests that the observed RPE damage increases with age in all three groups and so is most likely to be due to normal ageing and not to an effect of the Ser163Arg KI allele. These findings are very similar to those in a previous study, in which we also showed a significant increase of RPE damage with age in wild-type mice over a period of 2 to 24 months [13].

Analysis of changes in the normal distribution of fluorophores inside the retina and RPE was performed using confocal microscopy (AF-SLO) in three wild-type, C1qtnf5+/Ser163Arg and C1qtnf5+/Ser163Arg/+ Ser163Arg KI mice at 15–16 months of age respectively. There was no significant difference in autofluorescence in inner and outer retina between wild-type and C1qtnf5 Ser163Arg KI mice, although the number of autofluorescence spots in the inner and outer retina of C1qtnf5+/Ser163Arg/+ Ser163Arg mice were slightly greater than those of wild-type mice but this was not statistically significant (Figure 5).

The electroretinogram (ERG) was used to assess retinal function in C1qtnf5 Ser163Arg KI mice at 10–12 and 15–18 months of age (Figure 6). Representative ERG responses from 18-month-old mutant mice are compared to responses from a wild-type control in Figure 6A. Maximal rod photoreceptor function was elicited with bright light stimuli in the dark-adapted state (Figure 6A, black traces). Cone function was obtained in dark-adapted mice by recording ERGs within a short interval following rod-suppressing bleaching lights (Figure 6A, bottom traces). Delayed dark adaptation following a photobleach, a prominent feature in human L-ORMD [1,2], was studied in the mice by determining the recovery of the rod ERG photoreponse (a-wave) following a light exposure that completely suppressed it (Figure 6A, gray traces overlapping the DA rod response). ERG responses in mutant animals are similar to those in the wild-type control. a-waves and b-waves were measured conventionally for each of the ERG responses and summary statistics for each group shown (Figure 6B). Rod a-wave amplitudes in homozygous (mean ± SD = 250 ± 53 μV) and heterozygous (238 ± 35 μV) C1qtnf5 Ser163Arg KI mice were similar to each other and to wild-type (276 ± 56 μV) mice (P > 0.05). Cone b-wave amplitudes in homozygous (122 ± 25 μV) and heterozygous (109 ± 20 μV) C1qtnf5 Ser163Arg KI animals were also similar to each other and to wild-type (115 ± 23 μV) mice (P > 0.05).

Recovery (BM) in young (6 months) and old (20 months) wild-type and Ser163Arg KI mice and focused in particular on age-related changes in the retinal pigment epithelium (RPE) and underlying BM. Although aged animals showed amorphous sub-RPE deposits, there was no obvious difference in these deposits between wild-type and Ser163Arg KI mice (Figure 3B). The BM thickness increased with age, so there was a significant correlation between age and BM thickness in each genotype respectively. The values of mean BM thickness of old mice (20–24 months) were approximately twice those from young animals (6 months), but we did not identify a significant difference between age-matched wild-type and Ser163Arg KI mice (Figure 3B, C), indicating that the major cause of the increase in BM thickness was normal ageing.

To assess any possible differences in age-related RPE pathology between wild-type, heterozygous or homozygous mutant C1qtnf5 KI littermates with age, we applied a previously described method for RPE pathology grading in animals between 6 and 24 months of age [12,13]. In Figure 4, the mean sum of the observed RPE damage per section and per animal is shown, estimated from the analysis of three sections per animal. All three genotype groups showed a similar range of RPE damage, that was not significantly different between the three groups (Pearson correlation, ρ = 0.0026, N = 35 r2 = 0.244). This suggests that the observed RPE damage increases with age in all three groups and so is most likely to be due to normal ageing and not to an effect of the Ser163Arg KI allele. These findings are very similar to those in a previous study, in which we also showed a significant increase of RPE damage with age in wild-type mice over a period of 2 to 24 months [13].

Analysis of changes in the normal distribution of fluorophores inside the retina and RPE was performed using confocal microscopy (AF-SLO) in three wild-type, C1qtnf5+/Ser163Arg and C1qtnf5+/Ser163Arg/+ Ser163Arg KI mice at 15–16 months of age respectively. There was no significant difference in autofluorescence in inner and outer retina between wild-type and C1qtnf5 Ser163Arg KI mice, although the number of autofluorescence spots in the inner and outer retina of C1qtnf5+/Ser163Arg/+ Ser163Arg mice were slightly greater than those of wild-type mice but this was not statistically significant (Figure 5).

The electroretinogram (ERG) was used to assess retinal function in C1qtnf5 Ser163Arg KI mice at 10–12 and 15–18 months of age (Figure 6). Representative ERG responses from 18-month-old mutant mice are compared to responses from a wild-type control in Figure 6A. Maximal rod photoreceptor function was elicited with bright light stimuli in the dark-adapted state (Figure 6A, black traces). Cone function was obtained in dark-adapted mice by recording ERGs within a short interval following rod-suppressing bleaching lights (Figure 6A, bottom traces). Delayed dark adaptation following a photobleach, a prominent feature in human L-ORMD [1,2], was studied in the mice by determining the recovery of the rod ERG photoreponse (a-wave) following a light exposure that completely suppressed it (Figure 6A, gray traces overlapping the DA rod response). ERG responses in mutant animals are similar to those in the wild-type control. a-waves and b-waves were measured conventionally for each of the ERG responses and summary statistics for each group shown (Figure 6B). Rod a-wave amplitudes in homozygous (mean ± SD = 250 ± 53 μV) and heterozygous (238 ± 35 μV) C1qtnf5 Ser163Arg KI mice were similar to each other and to wild-type (276 ± 56 μV) mice (P > 0.05). Cone b-wave amplitudes in homozygous (122 ± 25 μV) and heterozygous (109 ± 20 μV) C1qtnf5 Ser163Arg KI animals were also similar to each other and to wild-type (115 ± 23 μV) mice (P > 0.05).
of the a-wave (expressed as a fraction of dark-adapted results) in both homozygotes (0.38 ± 0.08) and heterozygotes (0.45 ± 0.10) reached levels comparable to those of wild-type (0.43 ± 0.09) mice (P > 0.05).

**Figure 1. Introduction of the Ser163Arg mutation into the mouse C1qtnf5 gene.** A) Schematic representation of the murine Mfrp/C1qtnf5 genes. Boxes represent exons, the solid line represents intronic sequence (not drawn to scale). B) The targeting construct showing the long (6.8 kb) homology arm (LA), short (1.4 kb) homology arm (SA) and the central fragment with the Ser163Arg mutation labelled with a *. FRT: Flippase Recognition Target sites, Neo: the neomycin selection cassette. LoxP: sites flanking the introduced mutation and Neo gene, allowing subsequent Cre-recombinase-mediated deletion to generate a knockout mouse. C) Southern blot performed using genomic DNA from two heterozygous mice with a 3’ C1qtnf5 probe showing wild-type genomic DNA digested by AvrII, resulting in a 11.3-kb band, while genomic DNA containing the targeted Ser163Arg mutant showed the expected 6.6-kb band following Flp-mediated excision of the neo cassette. D) Validation of the Ser163Arg point mutation in heterozygous mice by DNA sequencing. The wild-type codon is AGC (serine), the mutant is AGG (arginine), the heterozygous mice show both alleles, highlighted in blue. E) Genotyping of tail biopsy DNA from wild-type and mutant (C1qtnf5 Ser163Arg) mice by PCR amplification of the native wild-type and mutant fragments. The primers anneal close to the FRT-sites flanking the neo cassette (see Figure 1 and Materials and Methods). The wild-type allele corresponds to the lower 432 bp band and the mutant allele to the upper 548 bp band. The gel shows genotypes in a mixture of C1qtnf5 Ser163Arg homozygous mutant (n = 9), heterozygous mutant (n = 4) and wild-type mice (n = 2). A DNA molecular weight marker V (8–587 bp; HaeIII digested pBR322 (Roche)) is shown in the right hand lane.

doi:10.1371/journal.pone.0027433.g001

**Effects of photocoagulation/laser induced choroidal neovascularisation**

Wild-type and heterozygous or homozygous KI mice at 15–16 month of age were exposed to photocoagulation/laser-induced
C1qtnf5 Knock-in Mouse

Figure 2. C1qtnf5 and Mfrp expression in C1qtnf5 Ser163Arg knock-in (KI) mice. A) Similar expression of C1qtnf5 and Mfrp by reverse transcriptase-PCR in RPE/choroid from wild-type (wt) and heterozygous (wt/ki) or homozygous (ki/ki) C1qtnf5 Ser163Arg KI mice. B) Western blot of eye cups from wild-type and C1qtnf5 heterozygous and homozygous knock-in mice stained with anti-C1QTNF5 antibody (top) and anti-β tubulin antibody (bottom).

doi:10.1371/journal.pone.0027433.g002

Effects of human mutant C1QTNF5 over-expression in mouse retina

We generated lentivirus to over-express human C1QTNF5 wild-type and Ser163Arg mutant proteins. Initial in vivo injection of HeLa cells showed that the wild-type protein was diffusely located in the cytoplasm but most of the mutant protein was retained in the endoplasm reticulum (Figure 8A), consistent with previous reports [8]. Over-expression of wild-type and mutant C1QTNF5 in wild-type mouse retina did not cause any pathological effects on either RPE or photoreceptors (Figure 8B, C).

Discussion

Mouse models of human inherited retinal diseases have proved to be invaluable tools for the analysis of disease mechanisms and, more recently, for evaluating therapy [14,15]. The situation with late-onset or genetically complex disorders, such as age-related macular degeneration, has been less clear-cut due to the presence of anatomical differences (e.g. the mouse has no macula) and the requirement for ageing in addition to inherited and environmental influences, such as diet, genetic background or light exposure. All of these factors may influence both ageing and inflammatory processes, making these disorders intrinsically more difficult to model [16,17]. Some mouse models of inherited macular dystrophies, including the Aker4 model of Stargardt disease, reproduce the biochemical features of the human disease but only manifest a slow photoreceptor degeneration, in contrast to the human disease [18,19]. A transgenic mouse model of Stargardt-like macular degeneration resulting from ELOVL4 mutation on the other hand showed both biochemical and functional features of the human disease [20]. Other macular disease models show few features of the corresponding human disease. For example, a mouse model of Sorsby fundus dystrophy, with a Timp3 Ser136Cys knock-in mutation, showed premature age-related changes in Bruch's membrane and RPE, which were evident at 8 months compared with 30 months in wild-type littermates but lacked the most striking features of the human disease, namely massive sub-RPE deposits (resembling those found in L-ORMD), choroidal neovascularization or retinal atrophy [21]. The lack of overt phenotypic abnormality in C1qtnf5 Ser163Arg knock-in mice up to 24 months is therefore disappointing but by no means unprecedented in view of the mixed success with mouse models of late-onset macular degeneration. The lack of a macula in the mouse seems unlikely to be a problem since L-ORMD becomes a pan-retinal disease in the later stages. Another possible factor is the short lifespan of the mouse compared with humans, in whom it may take 60–70 years to develop the full disease [22]. However, very recently, Chavali et al. [23] reported a very similar knock-in mouse model of L-ORMD in which the same Ser163Arg mutation had been introduced. In contrast to the present study, these mice showed most of the key features of L-ORMD: - ERG changes consistent with early dark adaptation abnormalities, accumulation of hyperautofluorescent spots, RPE and Bruch's membrane abnormalities, drusen, loss of photoreceptors and retinal vascular leakage. The Chavali et al. [23] mouse was made using a similar targeting construct containing introduced LoxP sites (for subsequently making a C1qtnf5 deletion knockout) and FRT sites flanking the neomycin selection cassette but these, together with the S163R mutation, were introduced into a bacterial artificial chromosome (BAC) containing the Mfrp and C1qtnf5 genes rather than our approach in which right and left arms were PCR amplified and cloned separately into a plasmid construct and the mutation introduced by standard site directed mutagenesis. In our study, expression of both Mfrp and C1qtnf5 were found to be unaffected in the resultant knock-in mice compared with wildtype (Figure 2). The expression of C1qtnf5 was unaffected in the Chavali et al. [23] mouse whereas the expression of Mfrp was not checked. One important difference was that Chavali et al. targeted C57BL/6J embryonic stem (ES) cell and maintained the resultant knock-in mice on a 100% C57BL/6J genetic background whereas the present mouse model was constructed using a targeted 129SV ES cell and analysed on a mixed 129SV and C57BL/6J background. Phenotypic differences in mouse models of human disease are commonplace, as are effects of genetic backgrounds (http://phenome.jax.org/) [24]. Many mutations causing human disease show no clinical phenotype in the mouse, even if the targeted protein is completely absent and an appropriate biochemical phenotype is present(e.g. Lesch-Nyhan syndrome, Lowe syndrome, X-linked adrenoleukodystrophy, Fabry disease, galactosemia, Tay-Sachs disease) [25]. Others show mild or partial phenotypes which vary according to the genetic background, including cystic fibrosis, cardiomyopathy, Huntington disease, adenosomatous polyposis, glomerulonephritis, Marfan syndrome and hypertension [25,26,27,28,29,30,31,32,33]. In some cases, the genetic background differences led to the direct identification of modifier genes [34,35] while in others, multiple genes appeared to be involved [36]. Phenotypic differences in ocular mutants on
Figure 3. Retinal structure and ultrastructure in C1qtnf5 knock-in (KI) and wild-type (Wt) mice. A) Retinal sections from mice of the C1qtnf5 wild-type, Ser163 Arg heterozygous (Het) and homozygous (Mut) genotypes at the ages indicated, stained and examined by light microscopy. B) Ultrastructures of the basal site of the RPE and Bruch’s membrane of retinas from wild-type (Wt) and C1qtnf5 Ser163Arg KI (Het, Mut) mice at the ages of 6 months and 20 months, respectively. C) The thickness of Bruch’s membrane increased with age (Pearson correlation Wt: n = 6, p = 0.103, r² = 0.526; Het: n = 22, p = 0.0005, r² = 0.459; Mut: n = 21, p = 0.0039, r² = 0.362) but no significant difference between wild-type and C1qtnf5 Ser163Arg KI mice was found.

doi:10.1371/journal.pone.0027433.g003
different genetic backgrounds are also common, including
abnormalities of ocular development, early and late onset forms of
retinal degeneration and cataract [37,38][39,40][41]. Again,
direct identification of disease modifiers such as variation at the
Rpe65 locus, has proved useful [42]. It remains possible that the
phenotypic differences between the C1qtnf5 knockout mice
reported here and by Chavali et al. [23] result from differences
in the targeting construct, which might for example affect the
expression level of the mutant (or wild-type) allele, but an equally
likely interpretation is the difference in genetic background.
Further work is therefore required to clarify this difference.
Finally, over-expression of the wild-type and mutated forms of
C1QTNF5 did not appear to result in any greater RPE or outer
nuclear layer pathology than was observed following injection with
the control lentiviral construct LNT-GFP or injection with PBS.
This suggests, at least over the time points studied, that the
mutated protein does not exert a gain-of-function pathogenic
effect on either the RPE or ONL, although it is possible that if
much longer time points post-injection were studied, a subtle
phenotype might become apparent. It remains unclear whether L-
ORMD results from a gain-of-function or loss-of-function
although over-expression of C1QTNF5 in vitro does lead to ER
retention [8], as observed with lentivirus over-expression of the
mutant protein in HeLa cells (Figure 8).
Resolving phenotypic differences between our C1qtnf5 KI
mice and that reported by Chavali et al. [23] will be difficult.
Firstly, it will require back-crossing both KI models onto C57BL/6J
or 129SV strains respectively and then repeating the phenotypic
analyses in parallel. If the difference is associated with genetic
background, it may have therapeutic implications but resolving the
mechanism will not be easy since it may be due to polygenic
factors influencing penetrance. In vitro over-expression of the
mutant protein in mouse embryonic fibroblasts from potentially
susceptible compared with non-susceptible strains could help to
clarify whether genetic background effects are due to differences in
the cellular handling of misfolded proteins, but this may vary
between tissues, and many alternative explanations are possible.

Materials and Methods

Ethics statement

All the animal experiments were performed in accordance with
the ARVO Statement for the Use of Animals in Ophthalmic and
Vision Research. Animal work was carried out with the approval
of the Home Office under project licence number PL 6003968.

Generation of C1qtnf5 Ser163Arg knock-in mice

The C1QTNF5 and MFRP genes are located immediately
adjacent to one another in both human and mouse genomes and
are expressed as a dicistronic transcript [1,4]. The mouse Mfrp/ C1qtnf5 gene is located on chromosome 9 (position 44204272-
44211776) and extends for 7.5 kb. The Mfrp/C1qtnf5 gene is
composed of 14 exons which are expressed in a 4.22 kb bicistronic
mRNA. The mouse Mfrp protein is encoded by exons 1 to 13,
while the C1qtnf5 protein is encoded by exons 13 and 14
(Figure 1A). We employed a classical approach of homologous
recombination to replace wild-type C1qtnf5 with a modified
C1qtnf5 allele. In order to make the knock-in targeting construct,
three genomic fragments were PCR amplified using 129SV
genomic DNA as template: the long (5') homology arm which
contained exons 7 to 13 and part of exon 14; the short (3')
homology arm containing the 3' end of exon 14 and the region
located downstream of the Mfrp/C1qtnf5 gene; and a central
fragment, containing a portion of exon 14. The Ser163Arg
mutation (base change AGC to AGG) in C1qtnf5 exon 14 was then
introduced by site-directed mutagenesis into the central fragment

![C1qTNF5 KI line](image)

**Figure 4. Retinal pigment epithelium (RPE) damage scores in C1qtnf5 wild-type and Ser163 Arg mutant mice.** RPE damage was scored in wild-type and C1qtnf5 Ser163Arg knock-in (KI) mice at ages between 6 and 24 months old and no significant difference was found between any of the genotypes (n (wt) = 6, n(het) = 15, n(mut) = 14). Overall, there is a significant correlation of RPE damage with age (Pearson correlation n(wt,het,mut) = 35, p = 0.0026, r² = 0.244).
doi:10.1371/journal.pone.0027433.g004
(Figure 1). The above three PCR fragments were cloned into the targeting vector G139 (provided by genOway, Lyon, France) and the linearized construct (Figure 1) was transfected into mouse 129SV ES cells according to genOway’s standard electroporation procedures (i.e. 5 x 10^6 ES cells in presence of 40 μg of linearized plasmid, 260 volt, 500 μF). Positive selection was started 48 hours after electroporation by addition of 200 μg/ml of G418. G418-resistant clones were screened for the correct homologous recombination event by PCR and southern blotting. Three ES clones were used to inject into C57BL/6J blastocysts and a total of 19 chimeric mice were produced from the three injection sessions. Two highly chimeric males (displaying 85% and 98% chimerism) were each mated with two Flp-deleter C57BL/6J females to get heterozygous mice carrying the mutated C1qtnf5 allele devoid of the neo selection cassette. Agouti F1 progeny were screened for assessing the Flp-mediated excision event of the neo cassette on the C1qtnf5 targeted allele by PCR and southern blotting, using genomic DNA isolated from tail biopsies (Figure 1C, E). As there was mosaicism for the Flp C1qtnf5 allele in the F1 mice, the mosaic animals were then crossed with wild-type C57BL/6J mice to generate a pure line of Flp excised heterozygous C1qtnf5 knock-in mice. The resultant germline C1qtnf5 Ser163Arg mutant mice were then inter-crossed to produce stocks of heterozygous and homozygous mice on a mixed 129Sv and C57BL/6J genetic background. Genotyping was performed by PCR amplification of a 432 nucleotide base pair (bp) fragment corresponding to the wild-type allele and a 548 bp fragment for the mutant (Ser163Arg) allele using the following primers:

Forward primer WRI1-F: GAGATGAGTAGTTCCTGAGATGACCACAAGG
Reverse primer WRI1-R: TTGGCAAACCTGGGTGTGTTATCG

The annealing temperature was 65°C (30 secs) and amplification was continued for 35 cycles.

For in vivo procedures, the mice were anesthetized by a single intraperitoneal (i.p.) injection of a mixture of medetomidine and...
hydrochloride (1 mg/kg body weight; Domitor; Pfizer Animal Health, New York, NY), and ketamine (60 mg/kg body weight) in water. Whenever necessary, the pupils were dilated with 1 drop of 1% tropicamide.

Autofluorescence Scanning Laser Ophthalmoscopy (AF-SLO) and semi-quantitative assessment of background autofluorescence

Autofluorescence imaging was performed using a HRA2 scanning laser ophthalmoscope with a 55° angle lens as described previously [13]. We used the autofluorescent mode of the HRA2 to scan the retina with a 488 nm laser that provides the excitation light to stimulate emission of autofluorescence from any possible fluorophores in the retina, including for example lipofuscin in the RPE, which will then be detected by a camera and result in the autofluorescent photographs shown [8]. These photographs are taken systematically with the optic disc positioned at the centre of the image and the image is focussed either on the inner retinal vasculature or the outer retina respectively. Projection images of 30 frames per fundus are shown and were visually evaluated for brighter spots or areas in the image which would indicate the appearance of autofluorescence. Additionally, in an attempt to semi-quantitatively evaluate the diffuse background autofluorescence of the AF-SLO fundus photographs, all images were converted into 16-bit greyscale images and ImageJ software was used to analyse the area of pixels above a set threshold for all images. The mean of AF-pixel per fundus photograph above the threshold was calculated for each animal (n = 3 per genotype) using the values obtained for the right and the left eye.

Figure 6. Electroretinography in wild-type and C1qtnf5 Ser163Arg knock-in mice. A, Dark-adapted (DA) ERGs evoked by 2.2 log scot- c.ds.m^2 flashes (black traces) in 19-month-old heterozygote and homozygote, C1qtnf5 Ser163Arg knock-in mice compared to responses from a representative, wild-type (WT) control. DA cone function (bottom grey traces) was isolated in dark-adapted mice by recording ERGs within a short interval following a rod-suppressing bleaching light; recovery of the rod-mediated ERG response (grey traces overlapping DA rod traces) was assessed by recording ERGs 10 minutes following this bleach. B, Summary statistics of ERG parameters, showing a-wave (rods, left panel) and b-wave (cones, middle panel) in wild-type (WT), C1qtnf5 Ser163Arg heterozygous (Heter) and homozygous (Homoz) knock-in mice. Recovery of the a-wave (photoresponse) after the bleaching exposure is shown (right panel) and expressed as a fraction of the dark-adapted amplitude. The results for both eyes (right eye symbols are slightly displaced to the left) are plotted; circles are the 10–12 month old mice and hexagons are 15–18 month old mice. The grey line in each panel represents 2SD below the mean for WT mice.
doi:10.1371/journal.pone.0027433.g006
Figure 7. Laser-induced choroidal neovascularization in wild-type and C1qtnf5 Ser163 Arg knock-in mice. A, Fundus images from the early and late phase of in vivo fundus fluorescein angiography at 1 and 2 weeks after photocogulation/laser induced choroidal neovascularization (CNV) in 15–16 month old wild-type and C1qtnf5 KI mice. Wt: wild-type mice, Het: heterozygous KI mice, Mut: homozygous KI mice. B, The microglia in laser lesions on RPE flat mounts resulting from CNV laser treated C1qtnf5 KI mice after 2 weeks were labelled with anti-Iba1 (green) and co-labelled with anti-lectin (blood vessels) antibodies. Scale bar, 250 μm. C, D, Quantitative analysis of area of hyperfluorescence in the fundus images from 1 week old animals (C) and 2 week old animals (D) as a measure for CNV lesion size. No significant difference between the hyperfluorescent areas were observed at 1 week (Kruskal-Wallis with Dunn’s multiple comparison test, p = 0.911) or 2 weeks (Kruskal-Wallis with Dunn’s multiple comparison test, p = 0.638). The number of animals for both time points were n (wt) = 4, n (het) = 12, n(mut) = 10.
Figure 8. Effects of Lentiviral expression of wild-type or Ser163Arg mutant C1qtnf5 in mouse retinas. (A) HeLa cells were infected with lentivirus contain wild-type C1QTNF5 (LNT.C1QTNF5-WT) or Ser163Arg mutant C1QTNF5 (LNT.C1QTNF5-Mut). At 72 hr post-infection, cells were stained with anti-CTRP5 (green) and the endoplasmic reticulum (ER) marker anti-BiP (red) antibodies. Cells infected with LNT-CTRP5 or LNT-CTRP5-Mut both showed intracellular staining surrounding the nucleus (a, d) suggesting that both proteins enter the secretory pathway. While the staining on LNT-CTRP5 infected cells is rather diffuse (a), the LNT-CTRP5-Mut showed punctate staining (d) that co-localised with the ER marker BiP (e, f, shown...
Electroretinography (ERG)

ERGs were recorded according to previous methodology [43]. Briefly, C1qtnf5 Ser163Arg KI (n = 12; 6 heterozygotes and 6 homozygotes) mice and age-matched wild-type mice (n = 25) were dark adapted (>12 hours) and anesthetized with ketamine HCl (65 mg/kg) and xylazine (5 mg/kg) intramuscularly under dim red light. Pupils were dilated with 1% tropicamide and 2.5% phenylephrine. Medium energy (10-μs duration) and high energy (1-ms duration) flash stimulators with unattenuated luminances of 0.8 and 3.6 log scot-cd · s · m⁻², respectively, were used. Neutral density (Wratten 96; Eastman Kodak Company, Rochester, NY) and blue (Wratten 47A; Eastman Kodak Company) filters served to attenuate and spectrally shape the stimuli. Dark-adapted ERGs were recorded with a single blue 2.2 log scot-cd · s · m⁻² flash. After a 2 minute wait, a series of three white flashes (3.6 log scot-cd · s · m⁻²) were presented in the dark at ~5-sec intervals which completely suppresses the murine, rod-dominated ERG a-wave. The bright blue stimulus was presented again within 30 seconds of this bleaching light and this elicits an ERG dominated by a cone-mediated b-wave. Next, the extent of recovery of the dark-adapted (DA) rod mediated a-wave was assessed 10 minutes later by recording a further response to the bright blue stimulus. The b-wave amplitudes were measured conventionally, from baseline or a-wave trough to positive peak; a-wave amplitudes were measured from baseline to the trough of the a-wave.

Laser-induced choroidal neovascularisation (CNV)

Laser-induced CNV and quantification with fundus fluorescein angiography (FFA) were performed as described previously [44]. Three laser lesions per eye were delivered two to three disc diameters from the papillae with a slit lamp-mounted diode laser system (wavelength 630 nm; laser settings: 210-mW power, 100-μs duration, and 100-μm spot diameter; Keeler, Ltd., Windsor, UK). After 1 and 2 weeks, in vivo FFA was performed by i.p. injection of 200 μl of 2% fluorescein in PBS and imaging of the fundus at 90 seconds (early) and 7 min (late phase) after fluorescein injection using a small animal fundus camera with appropriate filters (Kowa Genesis, Tokyo, Japan). The pixel area of the image was measured in a uniform, randomized way to prevent a potential bias in the observer’s choice. The average BM thickness in each eye was obtained from single images taken at a standard magnification of 5000, positioned approximately 1 mm on each side of the optic nerve. After the calibration of Image J, BM was rotated to the horizontal or vertical, and a grid was superimposed on the image. Ten measurements were taken at points where grid lines passed across BM to yield an average value. Finally, the average measurements for superior and inferior field were added, and a mean value ± SD was obtained for BM thickness in each animal.

Pathology grading

To evaluate pathological effects of the Ser163Arg allele in C1qtnf5 knock-in mice between 6 and 24 months of age and in lentivirus treated C57BL/6 mice between 5 and 16 weeks post injection, we assessed the respective sections (seminithins for the knock-in line and cryosections for the lentivirus treated mice) under bright field microscopy using a 100× objective and a 10× ocular lens, and counted the number of separate individual pathological events, such as cell lysis, pyknosis, swelling, thinning, proliferation and thickening of RPE from the superior to the inferior end of the retina, in each section. From the individual sums of damage per section we calculated the mean sum of RPE alterations from three sections per animal to obtain a representative measure of the RPE damage score per animal, according to a scheme described by Hollyfield et al. [12] that we have also used in previous studies [13]. Similarly, we evaluated retinal damage by counting the drop-out of cells from the outer nuclear layer (ONL) towards the RPE, and thinning of the ONL, to assess retinal damage in virus treated eyes. This provided a mean disease score for the ONL of the retina. For the virus-injected eyes, the area directly around the injection sites was excluded to prevent injection-related damage which might influence the RPE or ONL damage scores.

Immunocytochemistry

8×10⁵ HeLa cells were plated in DMEM+10% FCS+antibiotic/antimycotic (D10) in 96-well plates and incubated overnight.
On the following day, cells were infected with either LNT-CTRPN5-WT or LNT-CTRPN5-Mut in D10. At 72 h post-infection, cells well fixed with ice-cold methanol, washed with PBS containing 0.05% Tween-20 (PBS-T). Cells were blocked with 3% BSA in PBS-T for 1 hr at room temperature (RT) before they were incubated with rabbit-anti-CTRPN5 (1:250 in block solution, antibody provided by A.F. Wright [8]) for 1 hr at RT° C. Cells were washed 4 times in PBS-T and then incubated with goat anti-rabbit-Alexa488 (1:400 in block, Invitrogen, Paisley, UK) for 45 minutes at RT. For double labelling with the ER-marker BIP, the cells were then washed 4 times in PBS and re-blocked for 1 hr before incubation with the rabbit anti-BIP (1:100 in block, C8918, Sigma, Gillingham, UK) antibody for 1 hr at RT. After 4 additional wash steps, the goat anti-rabbit-Alexa546 antibody (1:400 in block, Invitrogen, Paisley, UK) was applied for 45 mins, the cells washed again 4 times before they were counterstained with Hoechst (1:1000 in PBS-T) and washed once more. For imaging, the cells were kept hydrated with blocking solution in the 96-well plate and imaged with Q-Capture software (Q Imaging, Maidenhead, UK) on an inverted Axio Oberver.Z1 microscope (Carl Zeiss, Jena, Germany).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs from mouse eye cup tissues were extracted using RNAeasy Mini kit (QIAGEN) according to the manufacturer’s instructions. Two micrograms of the resulting RNA was reverse transcribed with random primers using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The cDNAs of C1qtnf5 and Mftp were then amplified using platinum Taq DNA polymerase (Invitrogen) as previously described [45]. PCR primers were manufactured by Sigma Aldrich. Primer sequences are available on request.

Western blotting

Eyecups from wild-type, heterozygous and homozygous C1qtnf5 Ser163Arg knock-in mice were lysed in a buffer containing 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.05% SDS, 2 mM EDTA, 1 mM sodium-vanadate, 5 mM sodium-fluoride and 10 mM iodoacetamide with a protease inhibitor cocktail (Roche). The lysates were centrifuged for 10 min at 13,000 rpm and the post-nuclear supernatants were collected. Equal amounts of protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The primary antibodies were used at 1:1,000 dilution for anti-C1QTNF5 and 1:2,000 dilution for anti-β-tubulin antibodies. HRP-conjugated secondary antibodies were used at 1:5,000 dilution. The membrane-bound antibodies were detected by ECL (Amersham Biosciences).

Lentivirus production

Second generation HIV-1-based self-inactivating lentiviral vectors pseudotyped with the vesicular stomatitis virus glycoprotein envelope contained either the murine C1qtnf5 wild type or the murine C1qtnf5 Ser163Arg mutant cDNA or the GFP reporter gene (Lenti.GFP). All vectors contain deletions in the 3’ long-terminal repeats, making them self-inactivating, and include the central polypurine tract (cPPT), which is necessary for second-strand DNA synthesis, and may enhance the nuclear transport of the provirus. All cDNAs are driven by the spleen focus-forming virus (SFFV) promoter and each vector contains the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to stabilize mRNA and increase expression. Vectors were produced using transient triple transfection of 293T cells as described previously [46]. At 28–72 hr post-transfection, supernatant was harvested and filtered through a 0.45 μm filter. The virus particles were concentrated by ultracentrifugation at 90,000 g for 2 hr and the pelleted virus was resuspended in OptiMEM and stored at −80°C. Virus was titred using a Reverse Transcriptase activity ELISA kit (Roche Diagnostics, Burgess Hill, UK) according to the manufacturer’s instructions. Transgene expression was detected by immunocytochemistry on infected HEK293 cells. Cells were infected with 1 μl of virus in a 24 well plate. Three days post-infection, cells were washed and fixed with ice-cold methanol and stained with a polyclonal rabbit anti-human C1QTNF5 antibody [8] and goat anti-rabbit AlexaFluor546 antibody. Cells were counterstained with Hoechst, imaged using a Zeiss Axioplan II fluorescent microscope and analysed using QCapture Pro software (QImaging, Maidenhead, UK).

Subretinal injections

Subretinal administration of vectors was performed in anesthetized 12 to 16-week-old C57BL/6 mice (Harlan UK Ltd.), under direct retinoscopy through an operating microscope as previously described [47]. A 1.5-cm 34-gauge hypodermic needle (Hamilton, UK) was used to inject 2 microlitres of virus suspension (titer of 10^9 particles/ml) into the subretinal space to produce a bullous retinal detachment in the superior hemisphere and a second in the inferior hemisphere. Mice received C1qtnf5-wild type virus in one eye and C1qtnf5-Ser163Arg mutant virus in the other eye. Control mice received PBS or titre-matched Lenti.GFP. Retinal vessels remained patent during and following the injections. All animals received chloramphenicol 1% eye ointment to the cornea.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 for Windows (GraphPad Software Inc, La Jolla, USA). Changes in the thickness of Bruch’s membrane or in RPE damage with age were assessed using Pearson correlation statistics, a parametric measure of association for two continuous random variables. Differences in autofluorescence were assessed using a Kruskal-Wallis with Dunn’s multiple comparison test. RPE and outer nuclear layer disease scoring was assessed on randomized images using a one-way analysis of variance.

Acknowledgments

We thank gentOway (Lyons, France) for help with vector design and targeting strategy, mouse ES cell injections and targeting experiments. We also thank Craig Nicol for help with the artwork.

Author Contributions

Conceived and designed the experiments: AFW RA SGJ. Performed the experiments: XS UL TSA SEB AL BT MC HX. Analyzed the data: XS UL TSA SEB AL BT MC HX. Wrote the paper: AFW XS UL SGJ.

References


