

# Complement Factor H Deficiency Results in Decreased Neuroretinal Expression of *Cd59a* in Aged Mice

Carsten Faber,<sup>1,3</sup> Jennifer Williams,<sup>2,3</sup> Helene Bæk Juel,<sup>1</sup> John Greenwood,<sup>2</sup> Mogens Holst Nissen,<sup>1</sup> and Stephen E. Moss<sup>2</sup>

**PURPOSE.** The complement system is closely linked to the pathogenesis of AMD. Several complement genes are expressed in RPE, and complement proteins accumulate in drusen. Further, a common variant of complement factor H (CFH) confers increased risk of developing AMD. Because the mechanisms by which changes in the function of CFH influence development of AMD are unclear, we examined ocular complement expression as a consequence of age in control and CFH null mutant mice.

**METHODS.** Gene expression in neuroretinas and RPE/choroid from young and aged WT and *Cfb*<sup>-/-</sup> C57BL/6J mice was analyzed by microarrays. Expression of a wide range of complement genes was compared with expression in liver.

**RESULTS.** An age-associated increased expression of complement, particularly *C1q*, *C3*, and factor B, in the RPE/choroid coincided with increased expression of the negative regulators *Cfb* and *Cd59a* in the neuroretina. Young mice deficient in CFH expressed *Cd59a* similar to WT, but failed to upregulate *Cd59a* expression with age. Hepatic expression of *Cd59a* increased with age regardless of *Cfb* genotype.

**CONCLUSIONS.** While the connection between CFH deficiency and failure to upregulate *CD59a* remains unknown, these results suggest that expression of *CD59* is tissue-specific and that neuroretinal regulation depends on CFH. This could contribute to the visual functional deficits and morphological changes in the *Cfb*<sup>-/-</sup> mouse retina that occur with age. (*Invest Ophthalmol Vis Sci.* 2012;53:6324–6330) DOI:10.1167/iovs.12-10385

AMD is the commonest cause of blindness in the Western world affecting more than 10% of persons above 60 years of age. Although the cellular pathogenesis is not fully understood, disease progression involves degeneration of the RPE. The RPE has several crucial functions. These include supporting the photoreceptors by supplying nutrients, removing shed outer segments, and formation of the outer blood-

retinal barrier (BRB). One of the early clinical hallmarks of AMD is the fundoscopic appearance of drusen, formed by accumulating deposits of lipids and proteins external to the RPE, outside the BRB.<sup>1-4</sup>

Age is the largest risk factor for AMD. Of the known single nucleotide polymorphisms, the Y402H in complement factor H (CFH) confers the highest risk. CFH is the principal fluid-phase inhibitor of the alternative pathway of the complement cascade. The level of complement activation correlates with CFH haplotype, and AMD patients have elevated plasma concentrations of activation split-products.<sup>5-8</sup> Further, several complement components and regulators, including *C3*, *C5*, complement factor B (CFB) and CFH, have been identified in drusen of AMD patients.<sup>3,4,9</sup>

Most complement proteins are primarily synthesized in the liver, but numerous complement genes associated with the alternative and classical pathways are also constitutively expressed in the neuroretina and RPE. As such, the posterior eye expresses its own complement regulatory system, which may function independently in the ocular environment.<sup>10-14</sup> For unknown reasons, ocular complement activation increases with age.<sup>11,13</sup> This may have implications for drusen formation and AMD-pathogenesis. However, the mechanism by which dysregulation of complement via altered regulatory function of CFH leads to retinal injury is not known in detail. Therefore, we analyzed complement gene expression in the neuroretinas and RPE/choroid of young and old WT and *Cfb*<sup>-/-</sup> mice.

## MATERIALS AND METHODS

### Animals

We used young (7–8 weeks of age) and aged (16–17 months of age) WT and *Cfb*<sup>-/-</sup> C57BL/6J mice, which do not harbor the *rd8* mutation in the *Crb* gene.<sup>15</sup> *Cfb*<sup>-/-</sup> mice were provided by Matthew Pickering.<sup>16</sup> All experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Harvest of Tissue and Isolation of RNA

Immediately following cervical dislocation, eyes were removed and hemi-dissected posterior to the ora serrata. The neuroretinas (NR) from both eyes were transferred to an RNA stabilization reagent solution (RNALater; Qiagen, Valencia, CA) for 24 hours, removed, and saved at -80°C. The RPE/choroid remaining in the two eyecups was gently scraped with a 25-gauge needle and transferred to an RNA extraction solution (TRIzol; Invitrogen, Grand Island, NY). Small pieces of liver were transferred to an RNA stabilization reagent solution (Qiagen) for 24 hours, removed, and saved at -80°C. No tissues from different mice were pooled. RNA from both RPE/choroid and NR was isolated using a standard RNA extraction solution (Invitrogen) protocol followed by a cleanup on mini spin columns (RNeasy; Qiagen). Traces of genomic DNA were degraded by treatment with amplification grade DNase I.

From the <sup>1</sup>University of Copenhagen, Faculty of Health Sciences, ISIM, Copenhagen, Denmark; and the <sup>2</sup>UCL Institute of Ophthalmology, London, United Kingdom.

<sup>3</sup>These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Corresponding author: Carsten Faber; carstenfaber@gmail.com.

TABLE. qRT-PCR Primer Sequences

Gene	Accession	Forward (5'-3')	Reverse (5'-3')
<i>Actb</i>	NM_007393.3	TCCAAGTATCCATGAAATAAGTGG	GCAGTACATAATTTACACAGAAGC
<i>C3</i>	NM_009778.2	AGACACAAAGGACCTGGAAGCTGCT	AGGCAGTCTTCTTCGGTGTGTGAA
<i>C1qc</i>	NM_007574.2	GTTCAACAGCAAGCAGGTCA	ACCAGAGAAGACCGTGTGTTGG
<i>Cfb</i>	NM_009888.3	TCCTTTAGGCTGGCAGTTGGATCT	TCATTGATCCACCCATCTGCACCA
<i>Cfb</i>	NM_008198.2	TGATGTGGCCCTAGTCAAGCTCAA	GAGCAACTGTTCCTTGTGCTGCTT
<i>Cd59a</i>	NM_001111060.1	GAGCATGAGCACAGTCACTGGCG	GAACACAGCCAGAAGCAGCAGGAG
<i>Serp1</i>	NM_009776.3	TAGAGCCTTCTCAGATCCCGA	ACTCGTTGGCTACTTTACCCA

RNA from livers was isolated using RNA isolation columns (Nucleospin RNA II; Macherey-Nagel, Bethlehem, PA).

### Microarray

RNA quality and quantity were assessed using a bioanalyzer (Agilent, Horsholm, Denmark). RPE/choroid RNA was linearly amplified using an amplification kit (Ovation WGA; NuGEN Technologies, San Carlos, CA). RPE/choroid and NR-RNA underwent sense target labeling prior to hybridization to miniature arrays (Mouse Gene 1.0 ST Array GeneChips; Affymetrix, Santa Clara, CA). These microarrays (Affymetrix) were washed and stained in a fluidics station (Affymetrix) prior to scanning. All microarray data are MIAME compliant, and raw data have been deposited in the Gene Expression Omnibus repository (accession number GSE38671). The *Cfb* probeset on the miniature arrays (Affymetrix) contains thirty-two 25-mer probes covering 15 of the 22 exons (Integrated Genome Browser 6.6, www.bioviz.org, in the public domain). Therefore, it will also bind to the disrupted *Cfb* transcript in *Cfb*<sup>-/-</sup>, which explains the detection of *Cfb* expression in *Cfb*<sup>-/-</sup> mice.

### qRT-PCR

Ocular RNAs (RPE/choroid, 300 ng; NR, 600 ng) were converted to cDNA using a transcription kit (Qiagen Quantitect RT; Qiagen). RNAs from livers (1 µg) were converted to cDNA using a first strand cDNA synthesis kit (RevertAid; Fermentas, Copenhagen, Denmark). QPCR master mix (Brilliant SYBR; Stratagene, Horsholm, Denmark) was used for qRT-PCR with the primers specified in the Table. For each run, a melting curve was generated to ascertain specificity. Threshold cycle (*Ct*) values were averaged from duplicates differing by less than one cycle. Fold changes between groups of animals were calculated from a beta actin-normalized amount of transcript as  $2^{-(Ct - \beta \text{ actin} - Ct)}$ , target gene).

### Statistics

Microarray data were normalized using robust multiarray averaging (RMA). Data were checked for outliers using principal component analysis. ANOVA analysis of data generated a standard *P* value. *P* values were adjusted using the Benjamini & Hochberg method and the false discovery rate was set at  $\leq 0.05$  to create gene lists that showed significant differentially expressed genes and exons between the different groups of mice (Partek Genomics Suite; Partek, St. Louis, MO). The unpaired *t*-test with Welch's correction for unequal variances was used for analysis of the qRT-PCR using graphing software (GraphPad Prism v. 4.03 for Windows; GraphPad Software, La Jolla, CA). *P* values  $\leq 0.05$  were considered statistically significant.

## RESULTS

### Complement Transcriptome in Neuroretina and RPE/Choroid

To characterize the full expression of complement-associated genes in the back of the eye, we isolated RNA from neuroretina

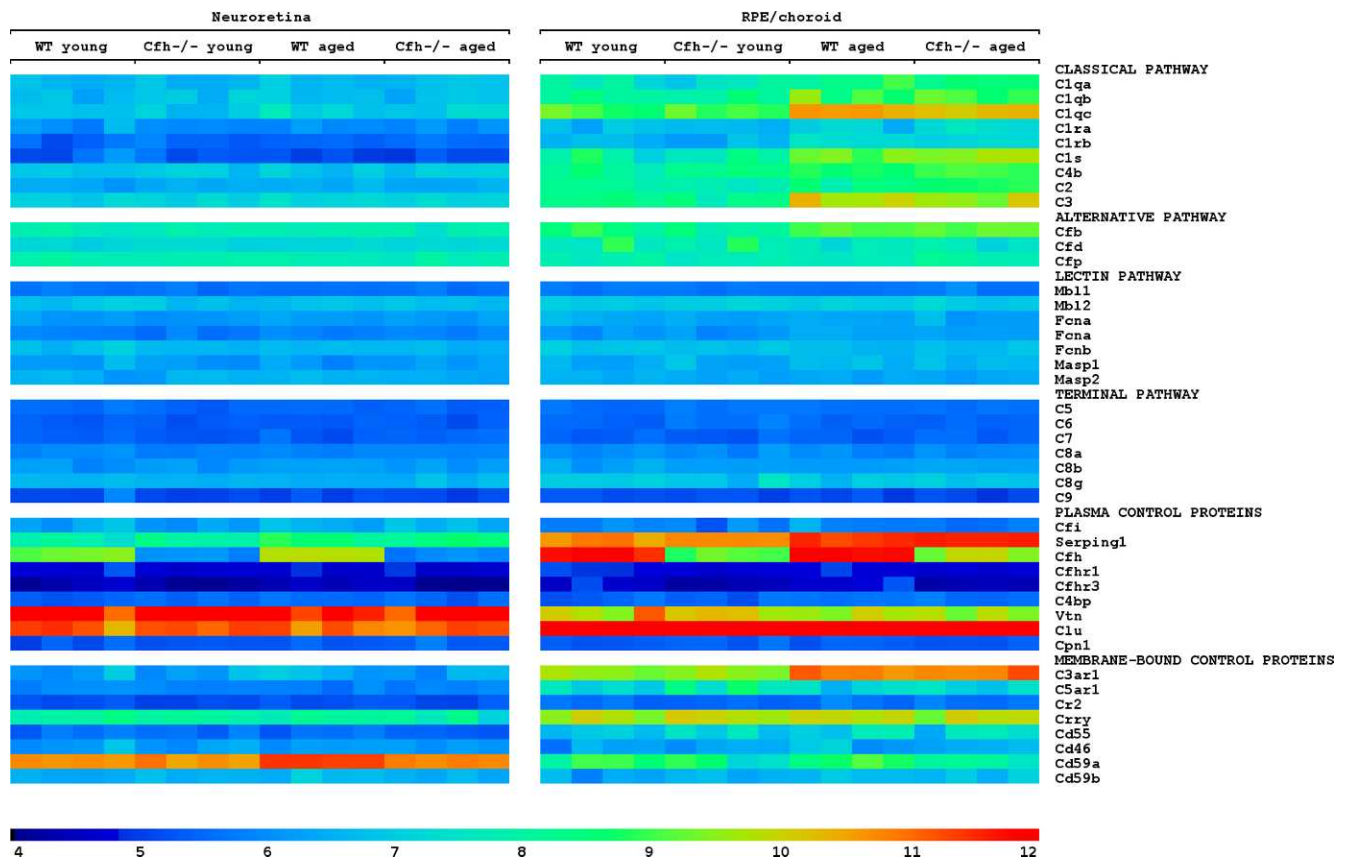
(NR) and RPE/choroid from individual mice and analyzed it using genome-wide Affymetrix microarrays. In RPE/choroid, we did not detect any significant differences in the expression of complement-associated genes between WT and *Cfb*<sup>-/-</sup> mice. With age, however, components of the classical and the alternative pathways including *C1q*, *C1r*, *C1s*, *C3*, and *Cfb* were upregulated in both normal and mutant strains. Genes associated with either the lectin or the terminal pathways were not expressed at detectable levels in the RPE/choroid of young or old mice. The genes encoding the C1-inhibitor, *Serp1*, and the *C3a* receptor, *C3aR1*, were both expressed at high levels and also increased with age. Regardless of age, genes encoding the negative regulators, vitronectin (*Vtn*), *Crry* and in particular clusterin (*Clu*) were expressed at high levels, while *Cd46* and *Cd55* were expressed at low levels (Fig. 1).

In NR, we primarily detected expression of genes encoding negative regulators. Thus, *Vtn*, *Clu*, and *Cd59a* were expressed at high levels. Notably, while *Vtn* and *Clu* remained unchanged with age, the expression of *Cd59a* increased with age, but only in WT mice. Thus, besides the deficient expression of *Cfb*, lack of neuroretinal upregulation of *Cd59a* with age represented the only significant difference between WT and *Cfb*<sup>-/-</sup> mice. Further, in WT mice, the expression of *Cfb* was highest in RPE/choroid, while the expression of *Cd59a* was highest in NR (Fig. 1). The numerical values for all the complement genes can be found in Supplementary Table S1 (see Supplementary Material and Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10385/-/DCSupplemental>) and a full list of all differentially regulated genes identified in the microarray can be found in Supplementary Tables S2–S5 (see Supplementary Material and Supplementary Tables S2–S5, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10385/-/DCSupplemental>).

qRT-PCR of selected complement genes confirmed the lack of neuroretinal upregulation of *Cd59a* with age and showed good agreement with results obtained from microarrays. However, a lower expression of *Cfb* and *C3* and a higher expression of *Serp1* were noted in RPE/choroid of *Cfb*<sup>-/-</sup> mice (Fig. 2).

### Systemic Expression of Complement Genes

To test whether the observed differential expression of complement genes, in particular *Cd59a*, was specific to ocular tissue, we analyzed the expression of selected genes in the liver using qRT-PCR. Because most complement genes are expressed in the liver, we analyzed the same set of genes that were tested in RPE/choroid. Indeed, *Cd59a* increased with age in the liver from both WT and *Cfb*<sup>-/-</sup> mice. Further, an indication of increased activity in the classical pathway with age was noted. As such, with age *C1qc* and *Serp1* increased in WT mice and showed a similar tendency in *Cfb*<sup>-/-</sup> mice. The increased expression of *Cfb* and *C3* with age observed in the RPE/choroid was not recapitulated in the liver (Fig. 3).



**FIGURE 1.** The complement transcriptome in neuroretina and RPE/choroid. Heat map showing that aging resulted in the upregulation of several genes associated with activation of the classical and alternative pathways in RPE/choroid together with upregulation of negative regulators of both early and late pathways in RPE/choroid and neuroretina. Old animals deficient in *Cfb*, however, failed to upregulate *Cd59a*. To visualize differential regulation, normalized expression values are shown in log<sub>2</sub> scale with arbitrary units. Values ranged from 3.5 to 12.5 and 97.0% are within the chosen scale. See Supplementary Table S1 for numeric values (see Supplementary Material and Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10385/-/DCSupplemental>).

### Spatial and Temporal Expression of *Cd59a*

Taking the qRT-PCR data on *Cd59a* together, a pattern of tissue-specific expression and regulation emerges. Thus, the rank order of *Cd59a* expression in young mice (neuroretina > liver > RPE/choroid) showed the highest expression in neuroretinas and was independent of *Cfb* genotype. With age, however, loss of *Cfb* resulted in loss of ocular upregulation of *Cd59a*, whereas the liver displayed increased expression regardless of *Cfb* genotype (Fig. 4).

### DISCUSSION

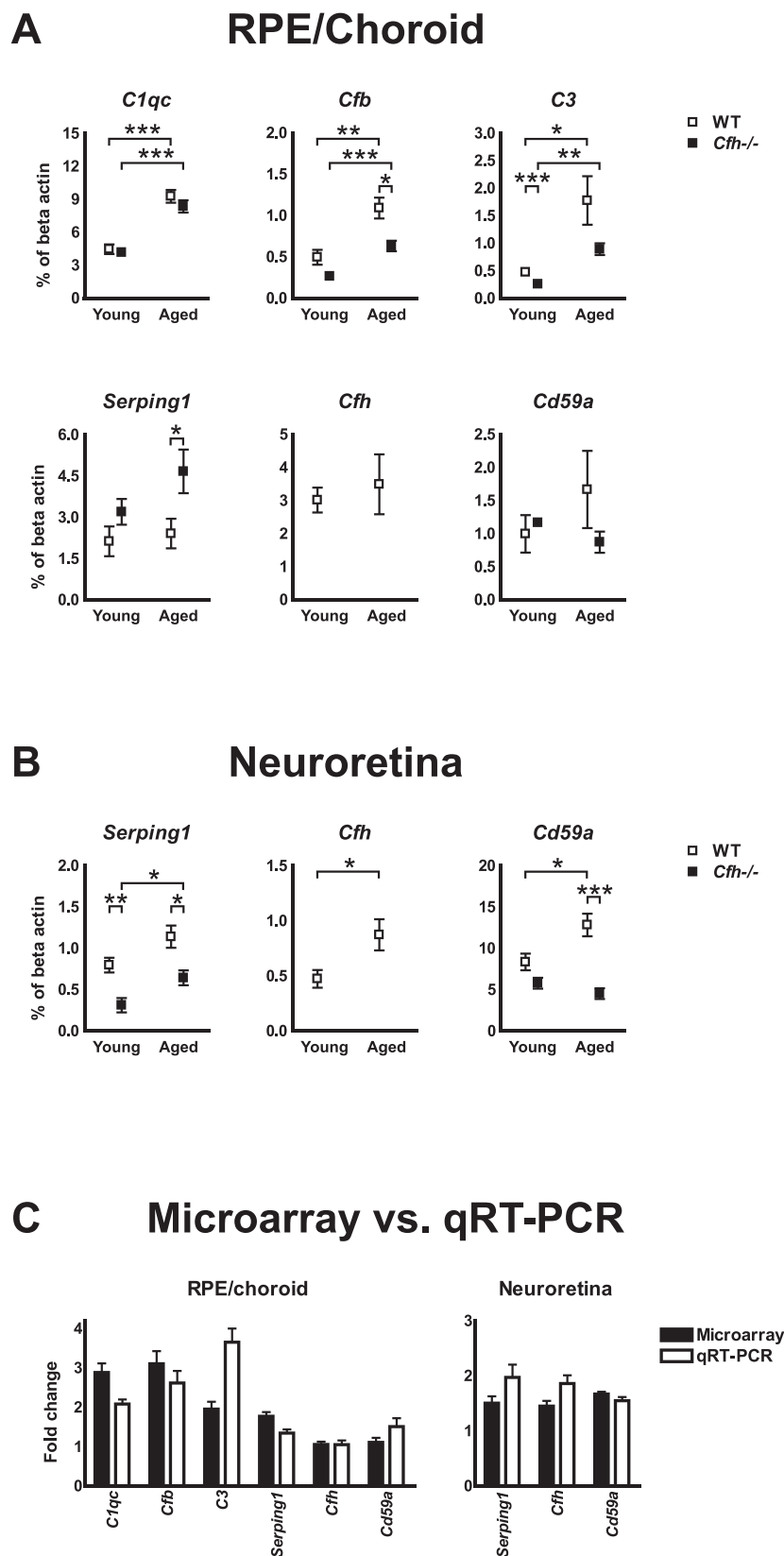
To examine the role of complement factor H in the ocular complement regulatory system, we analyzed the expression of complement-related genes in neuroretinas and RPE/choroid of young and old WT and *Cfh*<sup>-/-</sup> mice. With age, several complement genes were upregulated in both the neuroretina and RPE/choroid of WT mice.

The profile of murine ocular complement gene expression has been analyzed previously, and although there is some disagreement with regards to the neuroretinal up-regulation of complement factors,<sup>11,13</sup> it is generally agreed that RPE/choroid displays increased expression of certain complement factors with age.<sup>11,12</sup> From our results, however, it is evident that this increase coincides with a neuroretinal upregulation of complement inhibitors, possibly to control bystander damage of the photoreceptors.

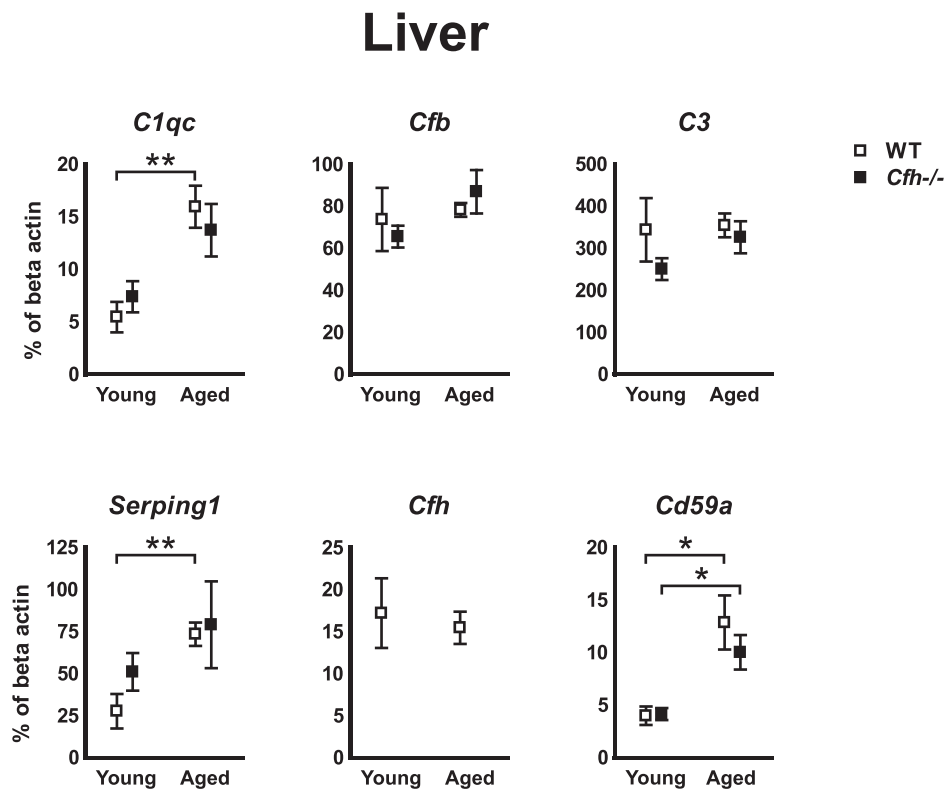
Compared to WT, the CFH-deficient mice displayed most of the same changes in complement expression with age with one notable difference: there was no age-dependent increased neuroretinal expression of *Cd59a*. The murine gene encoding CD59 is duplicated; *Cd59a* represents the main regulator of membrane-attack complex (MAC) assembly and is widely expressed, while *Cd59b* is expressed only in testis.<sup>17</sup> In agreement with our data, the human retina, rat, and murine eyecups have abundant CD59a expression in the photoreceptor and outer plexiform layers, while the RPE expression is weak.<sup>9,18-21</sup> Collectively, this might explain the visual functional deficits and morphological changes in the *Cfh*<sup>-/-</sup> mouse retina with age. Specifically, lack of CFH caused increased deposition of C3 around the photoreceptor outer segments in the neuroretina<sup>22</sup> corresponding to the expression pattern of CD59a in WT mice.<sup>18</sup>

Experimental laser photocoagulation of Bruch's membrane in mice results in choroidal neovascularization (CNV) via complement activation. Thus, C3 and MAC were reported deposited in sites of experimental CNV, and loss of complement function or delivery of CD59a reduced CNV formation in studies using this model.<sup>23-26</sup> Notably, laser-induced acute complement activation included a transient decrease in CD59a expression.<sup>24</sup> Thus, expression of CD59a may decrease in the context of increased complement activation.

In ARPE19 cells, CD59 expression was shown to increase in response to T cell-derived mediators.<sup>27</sup> Primary cultures of human RPE cells also upregulated CD59 expression in



**FIGURE 2.** Validation of deficient neuroretinal upregulation of *Cd59a* with age. qRT-PCR analysis of selected genes that were differentially regulated in microarrays. (A) RPE/choroid. (B) Neuroretina. Average expression normalized to beta actin; 5–6 mice in each group. \**P* < 0.05. \*\**P* < 0.01. \*\*\**P* < 0.001. (C) Comparison of age-dependent fold change from microarray and qRT-PCR. Data pooled from WT and *Cfh*<sup>-/-</sup> mice except values for *Cfb* and *Cd59a*, where only data from WT mice are shown. All error bars represent SEM.

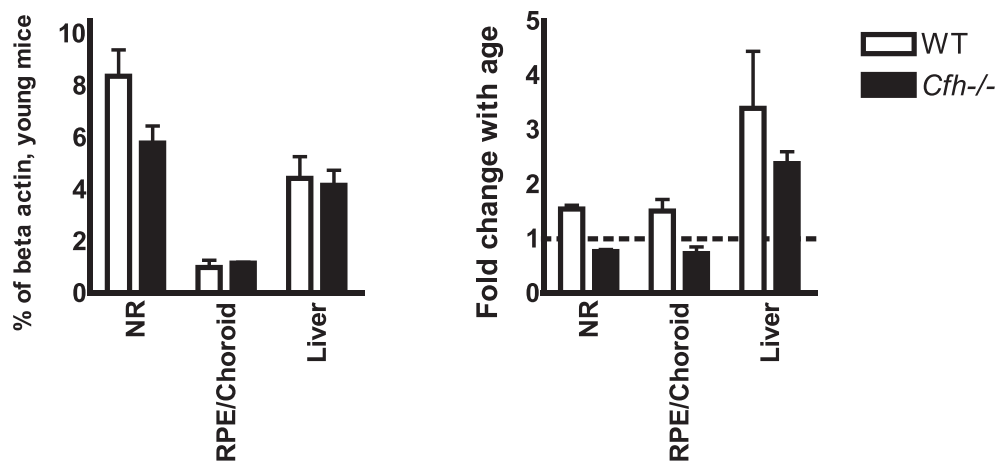


**FIGURE 3.** Hepatic expression of *Cd59a* increases with age independently of *Cfb*. qRT-PCR analyze of selected complement genes in the liver revealed increased expression of *Cd59a* with age in both WT and *Cfb*<sup>-/-</sup> mice. The classical components *C1qc* and *Serping1* also increased with age (only tendency in *Cfb*<sup>-/-</sup> mice), while there was no differential expression of *Cfb* or *C3*; 5 to 6 mice in each group. Error bars represent SEM. \**P* < 0.05. \*\**P* < 0.01.

response to TNF $\alpha$  or IL1 $\beta$ , but cultured murine RPE cells failed to do so.<sup>18</sup> Further, oxidative stress increased expression of CD59 in an epithelial cell line derived from human lung,<sup>28</sup> whereas the expression was decreased in ARPE19.<sup>29</sup> LPS induced upregulation of CD59 in human monocyte-derived dendritic cells<sup>30</sup> and in human monocytes,<sup>31</sup> whereas murine bone marrow-derived macrophages stimulated with IFN $\gamma$  and LPS or immune complex and LPS downregulated expression of *Cd59a*.<sup>32</sup> It is not known, whether these differences are caused by different stimulation, demands for costimulation, or

actually reflect differences in the regulation of CD59 between human and mouse. Together, the reports do support a tissue-specific regulation of CD59, which may be a consequence of complex control at the transcriptional level.<sup>33</sup>

Although the total loss of CFH-function in *Cfb*<sup>-/-</sup> mice does not accurately reflect the relative loss of function in the AMD risk-conferring CFH<sup>Y402H</sup> genotype, similarities have been reported. As such, it was shown that RPE from donors with the CFH<sup>HH402</sup> genotype had a tendency to decreased expression of CD59 compared with RPE from the CFH<sup>YY402</sup> donors,<sup>18</sup>



**FIGURE 4.** Only ocular tissue from *Cfb*<sup>-/-</sup> mice displays deficient up-regulation of *Cd59a* with age. Comparison of qRT-PCR data from different tissues, revealed a tissue-specific basal expression and regulation of *Cd59a*. The expression in neuroretinas of young mice was highest, whereas the upregulation with age was more marked in the liver. The dotted line represents cutoff between positive and negative fold change.

while there was an increased content of MAC in human RPE/choroid from individuals with the CFH<sup>HH402</sup> genotype.<sup>34</sup> And in a gene expression study on a few donor eyes, a tendency to decreased expression of CD59 in retina and RPE/choroid from AMD patients was shown.<sup>9</sup> Notably, several studies have demonstrated increased plasma levels of complement regulators and split-products in AMD patients, in particular with the CFH<sup>HH402</sup> genotype,<sup>5-8</sup> which thus might act to decrease ocular expression of CD59 and/or increase ocular deposition of MAC. In support of this, systemic virus infection in mice resulting in acute complement activation induced a transient 2-fold decrease in *Cd59a* expression in RPE/choroid (Faber C. unpublished data) and a decreased density of CD59 on peripheral monocytes have been reported in patients with neovascular AMD.<sup>35</sup> Collectively, the retinal damage in AMD could be the result of a local decrease in CD59 expression in response to increased systemic complement activation.

Increased density of MAC in human choroid has previously been associated with loss of RPE and AMD severity.<sup>36</sup> Unlike MAC and the negative regulators MCP/CD46, CFH, vitronectin, and clusterin, CD59 is one of a few complement-related molecules, which, to the best of our knowledge, has not been identified in drusen.<sup>3,4,9</sup> While negative results have to be interpreted with caution, it does support our hypothesis that down-regulation of CD59 is an early event in AMD pathogenesis.

Deficiency of CFH was inconsequential to hepatic expression of the complement genes tested. Specifically, *Cd59a* was upregulated with age both in WT and *Cfb*<sup>-/-</sup> mice, thus validating that the lack of neuroretinal upregulation of *Cd59a* is not caused by a systemic defect of *Cd59a* regulation in the *Cfb*<sup>-/-</sup> mice. Unlike the RPE/choroid, the hepatocytes did not increase expression of *C3* and *Cfb*, which suggests that ocular expression of complement genes is influenced by changes in the local microenvironment. This might include accumulation of waste products from the visual cycle<sup>37</sup> or oxidative damage.<sup>38</sup> Nevertheless, hepatic complement gene expression probably also changes in response to stimuli, accordingly it has been reported that expression of several complement genes, including *C3* and CFB, is higher in adult compared with fetal human liver.<sup>9</sup>

In summary, we have shown that the age-related increased expression of complement in RPE/choroid is upheld by an increased neuroretinal expression of negative regulators. The lack of neuroretinal upregulation of *Cd59* with age constitutes the main difference between WT and *Cfb*<sup>-/-</sup> mice, which might explain the functional deficits and altered retinal morphology of aged *Cfb*<sup>-/-</sup> mice.

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