

1 **Loss of Complement Factor H impairs antioxidant capacity and energy**  
2 **metabolism of human RPE cells**

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11 Keywords

12 Age-related macular degeneration (AMD), complement factor H (CFH), retinal pigment  
13 epithelium (RPE), glycolysis, mitochondria respiration, oxidative stress

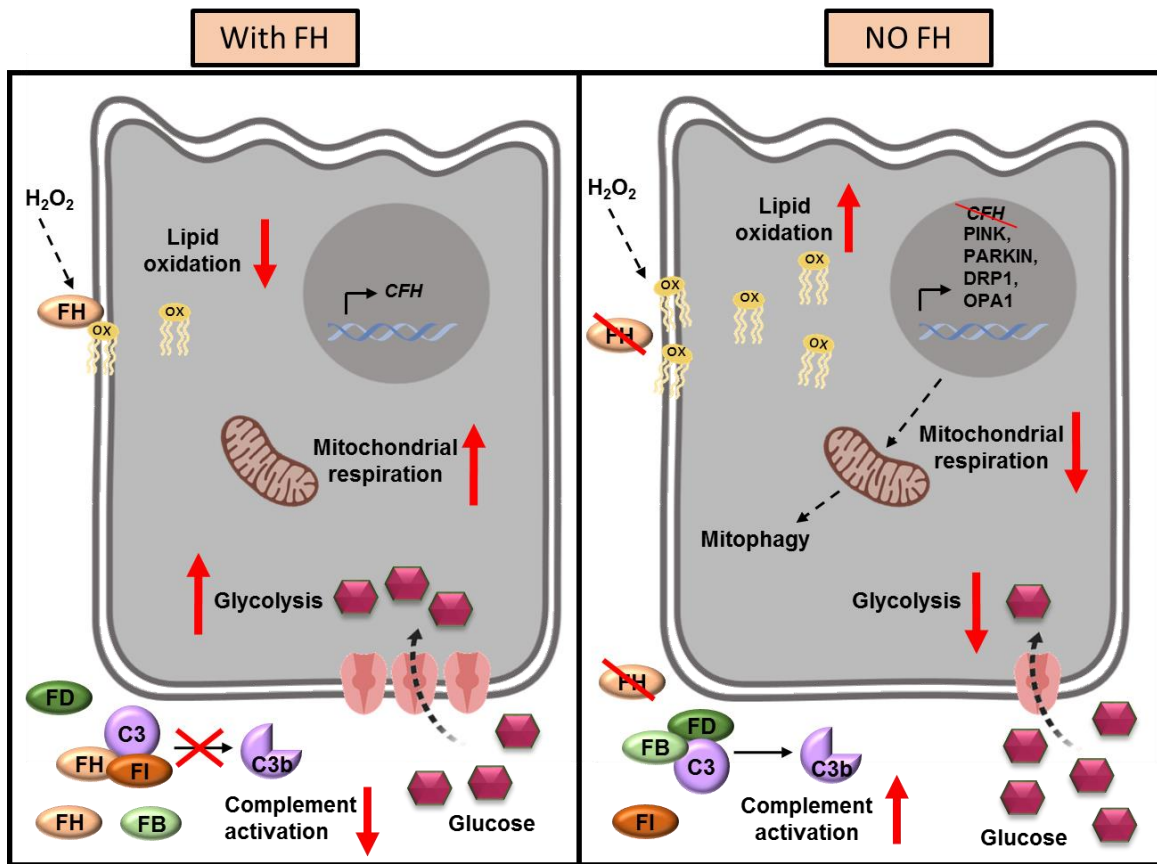
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18 **Abstract**

19 Age-related macular degeneration (AMD) is the leading cause of blindness in the  
20 elderly population. About 50% of AMD patients present polymorphisms in the  
21 Complement Factor H (*CFH*) gene, coding for Factor H protein (FH). AMD-associated  
22 *CFH* risk variants, Y402H in particular, impair FH function leading to complement  
23 overactivation. In AMD, retinal homeostasis is compromised due to dysfunction of  
24 retinal pigment epithelium (RPE) cells. Whether FH contributes to AMD pathogenesis  
25 only via complement system dysregulation remains unclear. To investigate the  
26 potential role of FH on energy metabolism and oxidative stress in RPE cells, we  
27 silenced *CFH* in human hTERT-RPE1 cells. FH-deprived RPE cells exposed to  
28 oxidative insult, showed altered metabolic homeostasis, including reduction of  
29 glycolysis and mitochondrial respiration, paralleled by an increase in lipid peroxidation.  
30 Our data suggest that FH protects RPE cells from oxidative stress and metabolic  
31 reprogramming, highlighting a novel function for FH in AMD pathogenesis.

32 **Graphical abstract**



33

## 34 **Introduction**

35 Age-related macular degeneration (AMD) is characterized by a progressive  
36 degeneration of the macula, leading to central vision loss and ultimately blindness.  
37 AMD is a complex disease, involving ageing, genetic predisposition and environmental  
38 factors, and a full understanding of AMD pathogenesis is lacking, which makes drug  
39 discovery challenging. AMD affects mainly the elderly population and it is estimated  
40 that around 200 million people will be affected by 2020 and 300 million by 2040 [1].  
41 The clinical classification of AMD is based on the appearance of the retina in fundus  
42 imaging, and two forms of AMD are defined: “wet” and “dry” AMD. Fundus  
43 photographs showing traits of neovascularization are distinctive of “wet” AMD. Indeed,  
44 wet AMD is characterized by the formation of new and unorganized blood vessels  
45 network which invade the Bruch’s membrane (BM) and RPE layer, damaging the  
46 retina, a process termed choroidal neovascularisation (CNV). Wet AMD displays a  
47 more severe phenotype, affects circa 10-15% of AMD patients and anti-VEGF therapy  
48 can be applied to slow down the progression of the disease. Dry AMD, for which no  
49 therapy is applicable, affects the majority of AMD patients and it is clinically defined by  
50 the appearance of areas of geographic atrophy with borders of hyperpigmented RPE  
51 cells, as seen in fundus images [2]. Dry AMD is defined by damage and degeneration  
52 of RPE cells, leading to photoreceptors malfunctioning and visual loss. Early stages  
53 of both AMD forms are characterized by the presence of deposits, called drusen,  
54 between the BM and the RPE layer [3]. An intact and well-functioning RPE cell layer,  
55 which provides a barrier between the neuroretina and the choroid capillary network, is  
56 essential for the maintenance of retinal homeostasis. In the presence of drusen or  
57 altered extracellular matrix (ECM) of BM, functionality of RPE cells may be impaired  
58 [4]. In addition, RPE cells fulfil several key functions, such as phagocytosis of the  
59 photoreceptor outer segments, transport of nutrients, preservation of the retinal  
60 structure and, most importantly, due to their high antioxidant capacity, RPE cells  
61 protect the retina from photo-oxidation and oxidative damage [5]. Recently, evidence  
62 in support of the hypothesis that a bioenergetic failure of RPE cells may be at the basis  
63 for AMD pathology has been provided [6]. The retinal microenvironment is highly  
64 oxidized due to a very high energy demand and photo-oxidation. Ageing processes in  
65 combination with external stressors, such as smoking or a high fat diet [7, 8], force  
66 RPE cells to deal with excessive levels of oxidative stress. In fact, energy metabolism

67 of primary RPE cells isolated from AMD patients was found to be strongly impaired  
68 compared to RPE cells from healthy controls [9]. In this model, glycolysis as well as  
69 mitochondrial respiration were reduced in RPE cells from AMD patients [9]. Other  
70 studies showed that mitochondrial dysfunction in RPE cells may represent a relevant  
71 AMD feature. Of the proteins differentially expressed in RPE cells from AMD donors  
72 and healthy controls, many are mitochondrial proteins [10]. Moreover, it has been  
73 shown in a mouse model that conditionally-induced mitochondrial damage in RPE  
74 cells leads to cell metabolic reprogramming and photoreceptors malfunction [11].

75 So far, there is lack of knowledge on the impact of the high-risk genetic variants on  
76 RPE cells homeostasis. A large portion of AMD genetic high-risk variants is located in  
77 genes coding for complement system regulatory proteins (FH, FI, C3, FB/C2) [12]. In  
78 particular, a common polymorphism in the complement factor H (*CFH*) gene, leads to  
79 an amino acid exchange from a tyrosine to histidine at position 402 (Y402H) in the two  
80 proteins encoded by the gene: factor H (FH) and its truncated form factor H-like protein  
81 1 (FHL-1) [13]. This common polymorphism is strongly associated with increased risk  
82 for AMD and may account for ~50% of AMD cases in the United States [14]. The  
83 mechanism by which the FH H402 variant confers predisposition for AMD is not clear.  
84 The FH H402 variant impairs FH and FHL-1 function, leading to uncontrolled  
85 complement system activation *in vitro* [15]. Recent studies highlighted the possibility  
86 that FH is not only contributing to AMD through its classical role in complement  
87 regulation, but it may influence other processes. For example, it has been shown that  
88 the FH H402 variant presents altered binding affinity to C-reactive protein or  
89 malondialdehyde, indicating a possible role in inflammation and lipoprotein  
90 degradation [16, 17], processes both associated with AMD pathogenesis [18, 19].  
91 These defects may influence the ability of RPE cells to cope with oxidative stress. This  
92 study was designed to unravel the impact of endogenous FH loss on RPE cells  
93 metabolism and their vulnerability toward oxidative stress. We employed RNA  
94 interference to decrease FH levels in the hTERT-RPE1 human cell line. Perhaps  
95 unsurprisingly, FH reduction led to activation of the complement system. Using the  
96 Seahorse Extracellular Flux Analyzer to measure bioenergetics, we observed that  
97 knock-down of the *CFH* gene negatively affects mitochondrial and glycolytic function  
98 of RPE cells when compared to controls. The impairment was even more pronounced  
99 when cells were exposed to oxidative stress by pre-treatment with hydrogen peroxide.

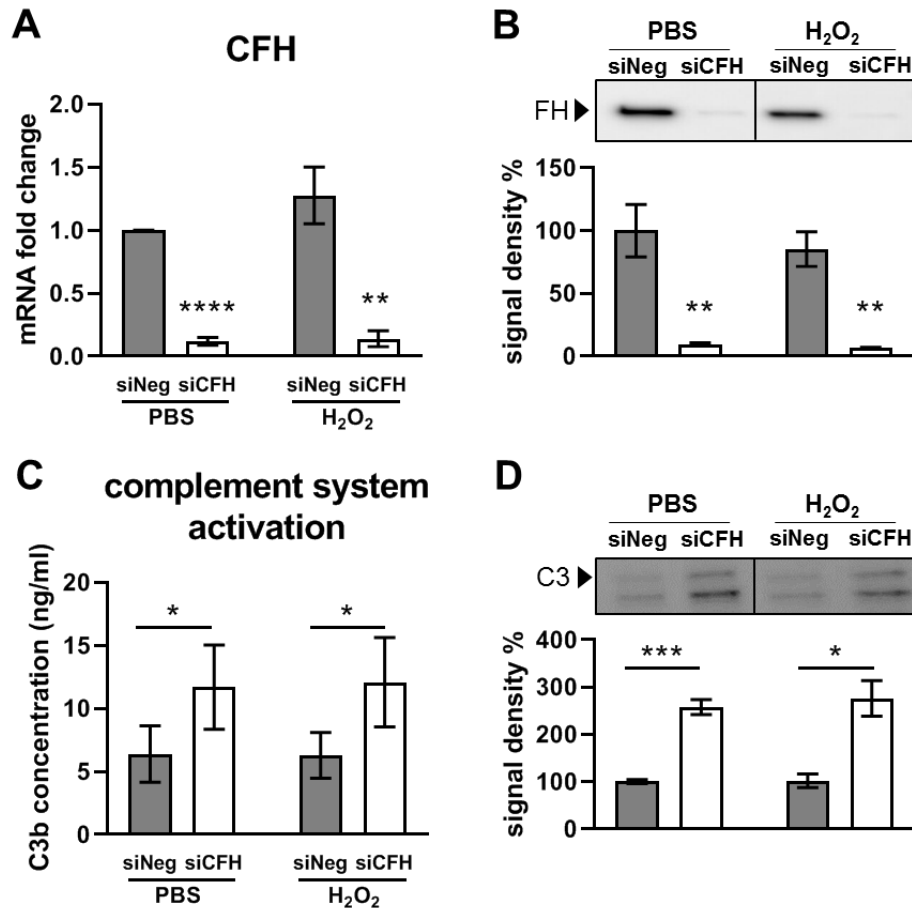
100 The effects of FH reduction on energy metabolism were accompanied by  
101 transcriptional regulation of several glucose metabolism genes as well as genes  
102 modulating mitochondrial stability. RPE cells lacking FH showed a significant increase  
103 in lipid peroxidation, which is a key aspect of AMD pathogenesis and, in parallel, cell  
104 viability was decreased. Our results suggest that endogenous FH, produced by RPE  
105 cells, not only modulates the extracellular microenvironment via its negative effect on  
106 complement activation, but also has an intracellular impact on the antioxidant functions  
107 and metabolic homeostasis of RPE cells, refining the knowledge on how FH is involved  
108 in AMD processes.

## 109 RESULTS

### 110 FH reduction leads to complement activation in RPE cells

111 To investigate the role of FH, we used siRNA to silence the *CFH* gene in hTERT-RPE1  
112 established cell lines and we induced a mild oxidative stress through hydrogen  
113 peroxide pre-treatment (200  $\mu$ M for 90 minutes). This set-up provides the chance to  
114 study *in vitro* the combination of endogenous FH dysregulation and environmental  
115 factors contributing to AMD, which increase oxidative stress. We monitored the  
116 efficiency of *CFH* silencing in all experimental conditions, including PBS and H<sub>2</sub>O<sub>2</sub> pre-  
117 treated cells after 48 hours in culture. Significantly reduced *CFH* mRNA was detected  
118 in *CFH* knock-down cells compared to the siNeg control cells, achieving almost 90%  
119 silencing of the *CFH* gene (Fig 1A). The FH protein was almost undetected in cell  
120 culture supernatants collected at the same time point from the si*CFH* cells compared  
121 to controls (Fig 1B). Based on gene expression levels of RPE markers: Bestrophin 1  
122 (BEST1), Retinoid Isomerohydrolase (RPE65) and Tight junction protein ZO-1 (TJP1),  
123 RPE characteristics in experimental conditions were not altered (Supplementary Fig  
124 1). Depletion of the FH protein led to reduced regulation of complement activation.  
125 C3b is the first cleaved peptide triggering complement activation and denotes the  
126 amplification loop of the proteolytic cascade, characteristic of the alternative pathway  
127 of the complement system [20]. Therefore, we assessed secreted levels of C3b in the  
128 *CFH* knock-down cells by both ELISA and Western blot and found in both cases a 2-  
129 fold increase in detectable C3b (Fig. 1C-D).

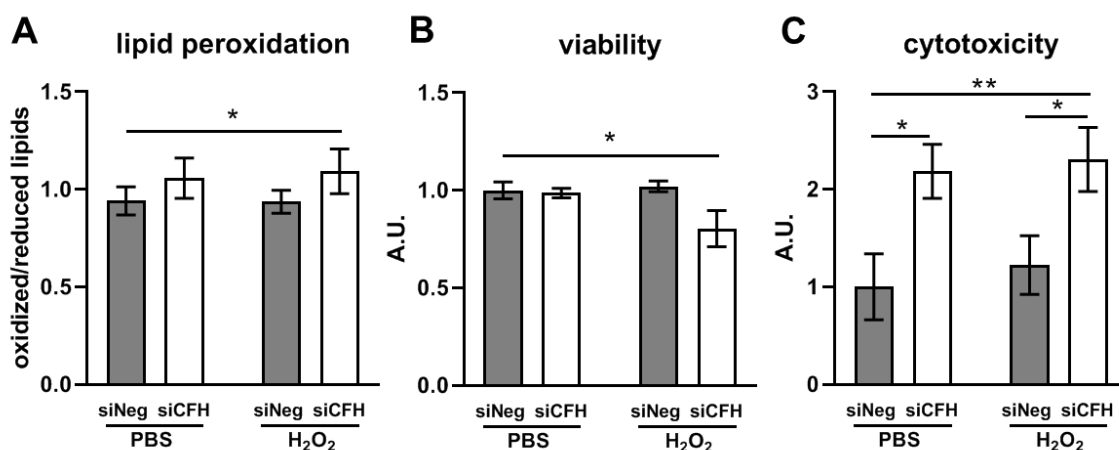




130 **Figure 1. FH reduction leads to complement system activation in RPE cells.**  
 131 hTERT-RPE1 cells were seeded, let attach overnight and silenced for 24 hours with  
 132 negative control (siNeg) or *CFH* specific (siCFH) siRNA. Cells were exposed for 90  
 133 minutes to 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or PBS and cell pellets and cell culture supernatants were  
 134 collected for further processing after 48 hours. **A** Monitoring of *CFH* expression by  
 135 qRT-PCR analyses in silencing negative control (siNeg) and specific *CFH* silenced  
 136 (siCFH) hTERT-RPE1 cells. Data are normalized to housekeeping gene PRPL0 using  
 137  $\Delta \Delta$ Ct method. SEM is shown, n=3. **B** Western blot analyses of FH protein levels in  
 138 cell culture supernatants of hTERT-RPE1 in the same conditions as A. Quantification  
 139 of signal density of 4 independent experiments is shown. **C** C3b ELISA analyses of  
 140 cell culture supernatants of hTERT-RPE1 cells. SEM is shown, n=4. **D** Western blot  
 141 analyses of C3 protein levels in cell culture supernatants of hTERT-RPE1 cells.  
 142 Quantification of signal density of 3 independent experiments is shown. Significance  
 143 was assessed by Student T-test. \*p<0.05, \*\*p<0.01, \*\*\* p<0.001, \*\*\*\*p<0.0001.

## 144 FH loss increases vulnerability toward oxidative stress in RPE cells

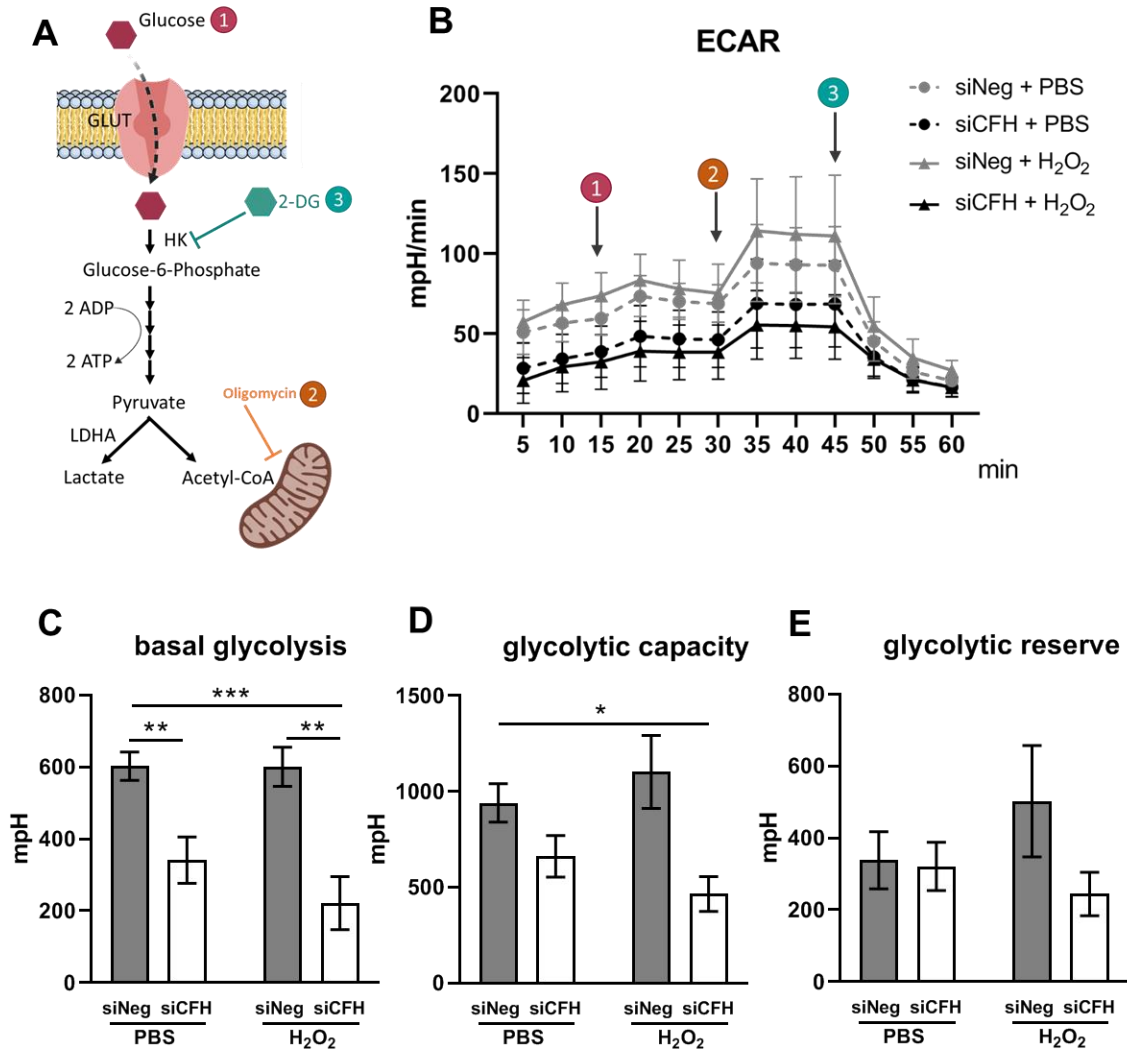
145 In order to assess whether the silencing of *CFH* altered the response of hTERT-RPE1  
146 cells to oxidative stress, we investigated cell lipid peroxidation levels after H<sub>2</sub>O<sub>2</sub>  
147 treatment (Fig. 2A). In our model, lipid peroxidation levels were significantly increased  
148 only in the absence of FH 48 hours after the oxidative treatment (Fig. 2A). As shown  
149 in Fig 2B, cell viability was not affected in the absence of *CFH* expression in PBS  
150 alone, and pre-treatment with H<sub>2</sub>O<sub>2</sub> had no effects on the siNeg control cells,  
151 confirming the known high antioxidant capacity of RPE cells [21]. However, cell  
152 viability was significantly reduced exclusively when RPE cells missing *CFH* expression  
153 were stimulated with H<sub>2</sub>O<sub>2</sub> (Fig. 2B), indicating increased vulnerability toward a short  
154 exposure to oxidative stress in FH deprived RPE cells. In parallel, we investigated cell  
155 membrane damage via a cytotoxicity assay. Silencing of *CFH* in RPE cells led to an  
156 increase in RPE cell damage, irrespective of H<sub>2</sub>O<sub>2</sub>-induced oxidative stress (Fig. 2C).



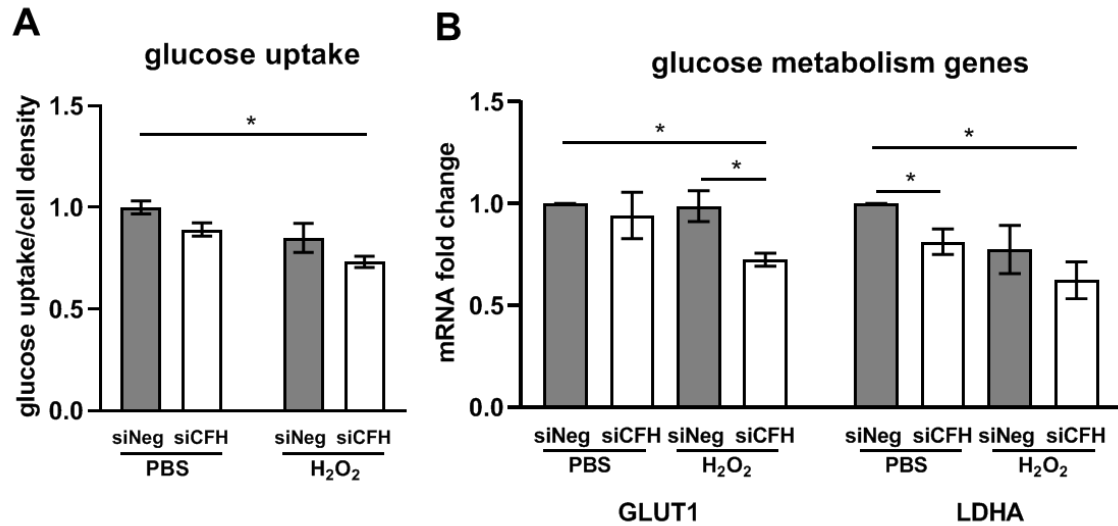
157 **Figure 2. FH loss increases vulnerability toward oxidative stress RPE cells.**  
158 hTERT-RPE1 cells were seeded, let attach overnight and silenced for 24 hours with  
159 negative control (siNeg) or *CFH* specific (siCFH) siRNA. Cells were exposed for 90  
160 minutes to 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or PBS and specific dyes were added after 48 hours. **A** Lipid  
161 peroxidation levels were assessed via BODIPY® 581/591 C11 fluorescent dye.  
162 Fluorescence shift was measured at ~590 nm and ~510 nm. Data are shown as ratio  
163 oxidized/reduced lipids, higher bars indicate higher lipid peroxidation levels. SEM is  
164 shown, n=7 **B** Viability was assessed by cell-permeable fluorescent dye GF-AFC  
165 (glycyl-phenylalanyl-aminofluorocoumarin). SEM is shown, n=5 **C** Cytotoxicity levels  
166 were assessed by cell-impermeable fluorescent dye bis-AAF-R110. SEM is shown,  
167 n=5. A.U. arbitrary units. Significance was assessed by Student t-test (single effect)  
168 and two-way ANOVA (combined effects) as described in the methods section. \*  
169 p<0.05, \*\*p<0.01.

## 170 **FH loss impairs glycolysis in RPE cells**

171 To investigate the influence of FH on RPE cell metabolism, the extracellular  
172 acidification rate (ECAR) was monitored as an indication of glycolytic function using  
173 the glycolysis stress test. Fig 3A shows a schematic representation of glycolysis  
174 pathway highlighting the substances used in the Seahorse analyses. Glucose,  
175 oligomycin and 2-deoxyglucose (2-DG) were sequentially injected (as shown by the  
176 arrows in Fig. 3B) to modulate glycolysis responses and ECAR. Fig. 3B shows ECAR  
177 measurements in siNeg cells and si*CFH* cells pretreated with PBS or 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for  
178 90 minutes. Basal levels of glycolysis were found to be significantly lower by 43% in  
179 RPE cells deprived of FH, compared to siNeg controls (Fig 3C-D). This reduction was  
180 even more pronounced when the *CFH* knock-down cells were pre-treated with H<sub>2</sub>O<sub>2</sub>  
181 (Fig 3C), with glycolysis being reduced by 63% compared to cells treated only with  
182 H<sub>2</sub>O<sub>2</sub> (Fig 3C). Glycolytic capacity was significantly reduced only when *CFH* knock-  
183 down cells were pre-treated with H<sub>2</sub>O<sub>2</sub> (Fig 3C), showing a reduction of 50 %  
184 compared to siNeg control cells (Fig 3D). Also, glycolytic reserve in si*CFH* H<sub>2</sub>O<sub>2</sub>  
185 treated cells was slightly reduced compared to both siNeg controls (Fig 3E).  
186 Consistently, glucose uptake was reduced significantly in si*CFH* cells after H<sub>2</sub>O<sub>2</sub>  
187 exposure compared to siNeg control cells (Fig 4A). In parallel, mRNA expression of  
188 glucose transporter GLUT1 was reduced in H<sub>2</sub>O<sub>2</sub>-treated si*CFH* cells compared to  
189 both treated and untreated controls (Fig 4B). Gene expression of LDHA (lactate  
190 dehydrogenase A), an isoform of LDH which preferentially converts pyruvate to lactate  
191 [22], was also significantly reduced in all si*CFH* cells, more pronouncedly after  
192 peroxide treatment (Fig 4B).



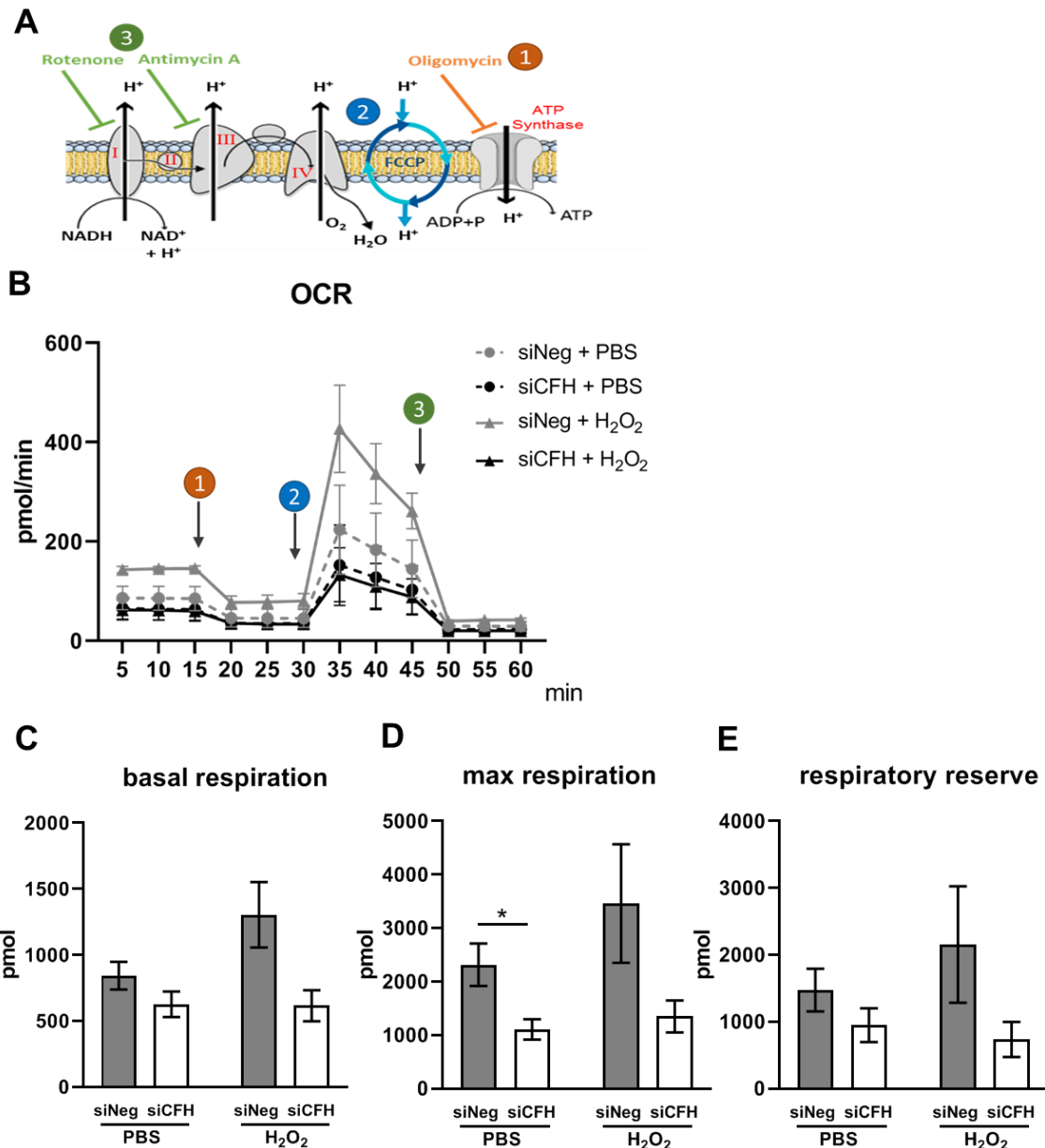
193 **Figure 3. FH loss impairs glycolysis in RPE cells.** **A** Schematic representation of  
 194 glycolysis and steps targeted during Seahorse analyses (1,2,3) **B** hTERT-RPE1 cells  
 195 were seeded, let attach overnight and silenced for 24 hours with negative control  
 196 (siNeg) or *CFH* specific (siCFH) siRNA. 30.000 cells were transferred to Seahorse  
 197 plates overnight and pre-treated for 90 minutes with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or PBS. Curves  
 198 show extracellular acidification rate (ECAR) measured after 48 hours. SEM is shown,  
 199 n=4-8. Arrows indicate injection of glucose (1), oligomycin (2) and 2-deoxyglucose (2-  
 200 DG,3). **C-E** Parameters of glycolytic function are calculated from data shown in B.  
 201 Basal glycolysis (C), glycolytic capacity (D) and glycolytic reserve (E). Significance  
 202 was assessed by Student t-test (single effect) and two-way ANOVA (combined effects)  
 203 as described in the methods section. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001



204 **Figure 4. FH modulates glucose uptake and expression of glucose metabolism**  
205 **genes.** hTERT-RPE1 cells were seeded, let attach overnight and silenced for 24 hours  
206 with negative control (siNeg) or *CFH* specific (si*CFH*) siRNA. Cells were exposed for  
207 90 minutes to 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or PBS **A** Glucose uptake was measured 48 hours after  
208 H<sub>2</sub>O<sub>2</sub> pre-treatment in siNeg control cells and in si*CFH* cells. SEM is shown, n=3. **B**  
209 gene expression analysis by qRT-PCR of glucose transporter 1 (GLUT1/SLC2A1) and  
210 glycolysis enzyme gene lactate dehydrogenase A (LDHA). SEM is shown, n=3. Data  
211 are normalized to housekeeping gene PRPL0 using  $\Delta\Delta$ Ct method. Significance was  
212 assessed by Student t-test (single effect) and two-way ANOVA (combined effects) as  
213 described in the methods section. \* p<0.05.

## 214 **FH loss impairs mitochondrial respiration in RPE cells**

215 The potential influence of FH loss on mitochondrial respiration of RPE cells was  
216 assessed monitoring the oxygen consumption rate (OCR), an indication of  
217 mitochondria respiratory function using the cell mito stress test. Fig 5A shows a  
218 schematic representation of the oxidative phosphorylation (OxPhos) chain,  
219 highlighting the substances used in the Seahorse analyses. Oligomycin, carbonyl  
220 cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and antimycin/rotenone were  
221 sequentially injected (as shown by the arrows in Fig 5B) to assess OCR in different  
222 conditions and calculate parameters of mitochondrial function. Fig 5B shows OCR  
223 measurements in siNeg cells and si*CFH* cells pretreated with PBS or 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for  
224 90 minutes. All parameters of mitochondrial respiration showed a clear trend of  
225 reduction in all si*CFH* groups (Fig 5C-D-E). However, the maximal respiration was  
226 significantly reduced in the absence of FH by 52 % in PBS-treated cells (Fig 5D). A  
227 slight increase in maximal respiration was observed when control cells were treated  
228 only with peroxide (Fig 5D).

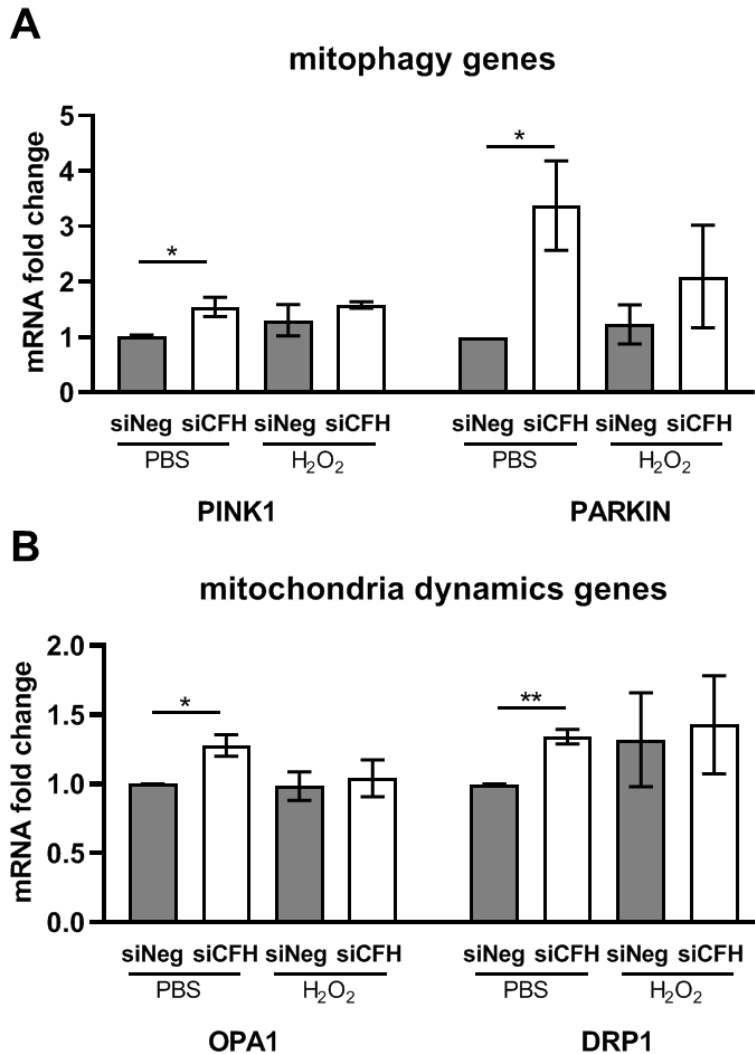


229 **Figure 5. FH loss impairs mitochondrial respiration in RPE cells.** **A** Schematic  
 230 representation of oxidative phosphorylation chain and targeted steps during Seahorse  
 231 analyses (1,2,3) **B** hTERT-RPE1 cells were seeded, let attach overnight and silenced  
 232 for 24 hours with negative control (siNeg) or *CFH* specific (si*CFH*) siRNA. 30.000 cells  
 233 were transferred to seahorse plates overnight and pre-treated for 90 minutes with 200  
 234  $\mu$ M H<sub>2</sub>O<sub>2</sub> or PBS. Curves show oxygen consumption rate (OCR) measured after 48  
 235 hours in hTERT-RPE1. SEM is shown, n=4-8. Arrows indicate injection of oligomycin  
 236 (1), FCCP (2) and antimycin and rotenone (3) **C-E** Parameters of mitochondrial  
 237 function are calculated from data shown in B. basal respiration (C), maximal  
 238 respiration (D) and respiratory reserve (E). Significance was assessed by Student t-  
 239 test (single effect) and two-way ANOVA (combined effects) as described in the  
 240 methods section. \* p<0.05.

## 241 **FH loss alters the expression of mitophagy and mitochondria dynamics genes**

242 Several factors contribute to the energy metabolism regulation and antioxidant  
243 capacity of RPE cells, which often both rely on mitochondria function and stability [6,  
244 23]. Impairments in mitochondrial function can be caused by altered oxidative  
245 phosphorylation (OxPhos) chain components as shown for Alzheimer's disease [24],  
246 therefore we investigated by qPCR the expression of OxPhos genes NADH  
247 dehydrogenase 4 (ND4), Cytochrome c oxidase subunit 4 (COX4) and mitochondrial  
248 encoded ATP synthase 6 (ATP6) respectively components of complex 1, complex 4  
249 and ATP synthase [25] (shown in the schematic in Fig 5A). No significant differences  
250 were observed in any of the experimental conditions (Supplementary Fig 2A).  
251 Transcription factors promoting mitochondrial biogenesis like Peroxisome Proliferator-  
252 Activated Receptor Gamma Coactivator 1-Alpha (PGC1a) and Nuclear Factor,  
253 Erythroid 2 Like 2 (NRF2) have been shown to positively influence mitochondria  
254 metabolism and antioxidant response [26, 27]. To test whether FH loss leads to a  
255 dysregulation of those factors, we analyzed gene expression levels of PGC1a and  
256 NRF2 (Supplementary Fig. 2B). No differences were detected for NRF2. On the other  
257 hand, PGC1a levels were higher in absence of FH. This result would suggest an  
258 improvement in mitochondria function, which was not the case in our model. We also  
259 measured transcriptional levels of antioxidant enzymes which are induced by PGC1a  
260 [28], like Peroxiredoxin 3 (PRDX3), Catalase (CAT), Glutathione Peroxidase 1  
261 (GPX1). We found no differences in PRDX3, a slight upregulation of CAT and a  
262 significant increase in GPX1 in the absence of FH (Supplementary Fig. 2C). These  
263 data suggest that RPE cells lacking FH are unsuccessfully trying to respond to an  
264 oxidative stress situation. Another mechanism of mitochondrial quality control is  
265 mitophagy, a mitochondria specific autophagy [29]. We found a significant alteration  
266 in the expression levels of genes regulating mitophagy (Fig 6A), like PTEN induced  
267 putative kinase 1 (PINK1) and E3 Ubiquitin-Protein Ligase Parkin (PARKIN) and  
268 mitochondria dynamics (Fig 6B), like Dynamin-Related Protein 1 (DRP1) and OPA1  
269 Mitochondrial Dynamin Like GTPase (OPA1) when FH was missing (Fig 6B). An  
270 alteration in mitophagy levels and mitochondria dynamics would lead to an increase  
271 in damaged mitochondria or altered mitochondria turnover.





272 **Figure 6. FH modulates expression of mitophagy and mitochondria dynamics**  
 273 **genes.** hTERT-RPE1 cells were seeded, let attach overnight and silenced for 24 hours  
 274 with negative control (siNeg) or *CFH* specific (si*CFH*) siRNA. Cells were exposed for  
 275 90 minutes to 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or PBS and RNA was collected after 48 hours. **A** gene  
 276 expression analysis by qRT-PCR of genes involved in mitophagy processes: PTEN  
 277 Induced Kinase 1 (PINK1) and E3 Ubiquitin-Protein Ligase Parkin (PARKIN) SEM is  
 278 shown, n=3 **B** gene expression analysis by qRT-PCR of genes involved in  
 279 mitochondria dynamics: OPA1 Mitochondrial Dynamin Like GTPase (OPA1) and  
 280 Dynamin-Related Protein 1 (DRP1). SEM is shown, n=3. Data are normalized to  
 281 housekeeping gene PRPL0 using  $\Delta \Delta$ Ct method. Significance was assessed by  
 282 Student T-test \* p<0.05, \*\* p<0.01.

## 283 Discussion

284 The retina is a highly organized multi-layered tissue and, in the early stages of AMD,  
285 Bruch's membrane/RPE layer is the first affected. Bruch's membrane, composed by  
286 overlapping extracellular matrix sheets, together with RPE cells separates the retina  
287 from the choroid capillary network. RPE cells actively transport nutrients, as glucose,  
288 to the retina and eliminate waste material, like oxidized lipids, into the blood stream.  
289 Like every other tissue, also the retina undergoes changes with age, including  
290 variations in collagen types [30], loss in elastin [31] and increase in metalloproteinases  
291 [32]. Bruch's membrane becomes rigid and thickened, where lipids begin to  
292 accumulate underneath the RPE cells [33]. Lipid deposits together with lipofuscin,  
293 melanin and complement proteins are the main constituents of drusen, the hallmark  
294 lesions associated with AMD pathology [3, 34]. These alterations in Bruch's  
295 membrane structure alter physiological conductivity and therefore impede a correct  
296 transport of oxygen or nutrients from the choroidal network to the RPE cells [35-38],  
297 resulting in a condition of hypoxia [39] and starvation. In addition, lifestyle habits, like  
298 smoking or maintaining a high-fat diet, both risk factors for AMD [7, 40], add oxidative  
299 stress to the retina [41, 42], and in particular to the mitochondria of RPE cells and  
300 photoreceptors [11]. With age, and more pronounced in AMD patients, mitochondrial  
301 damage is augmented [10, 43], thus leading to energy misbalance in the RPE cells. A  
302 bioenergetics crisis of RPE cells has been postulated to be at the basis of AMD  
303 pathology, especially in relation to the interplay between RPE cells and photoreceptors  
304 [6]. Since not the entire elderly population (or the population that smoke) is affected  
305 by AMD, other susceptibility factors have to be postulated for RPE cells in AMD  
306 patients. Genetic predisposition plays an essential role in AMD pathogenesis and  
307 carriers of high-risk variants may not be efficiently responding to ageing processes  
308 and oxidative stress. FH risk variants, in particular Y402H, lead to dysregulation of  
309 complement activation and hold differential binding properties to oxidized lipids [44,  
310 45]. In this study, we asked to which extent FH dysregulation may alter mechanisms  
311 relevant to AMD, like energy metabolism and response to oxidative stress. In the early  
312 stages of the disease oxidative stress is limited and can be handled by RPE cells. In  
313 addition, Bruch's membrane is still intact and circulating FH cannot cross  
314 choroid/Bruch's membrane interface due to its size [46]. Therefore, an interplay of  
315 genetics and age-related intraretinal changes are likely to drive onset and progression

316 of early AMD. In consequence, we asked whether RPE cells with reduced FH activity  
317 are more vulnerable to oxidative insult. The *in vitro* system used in this study allowed  
318 us to investigate the effects of endogenous FH specifically on RPE microenvironment  
319 without the influence of systemic alterations present in blood. FH dampens activation  
320 of the alternative complement pathway. FH acts as cofactor for Complement Factor I  
321 (FI), also an inhibitor of complement activation, and displaces Complement Factor B  
322 (FB) from C3. Both activities reduce the levels of C3 turnover and complement  
323 activation [44]. We show here that RPE cells produce FH, as well as C3. FH  
324 downregulation leads to an increase in C3 and C3b levels, prompting complement  
325 activation and turnover. In parallel, we detected a slight reduction of FH after H<sub>2</sub>O<sub>2</sub>  
326 pre-treatment, a phenomenon previously observed in H<sub>2</sub>O<sub>2</sub>-induced senescent  
327 ARPE19 cells [47]. Besides its role as complement regulator, recent studies implicate  
328 FH in lipid metabolism. The AMD-risk-associated FH variant (Y402H) leads in a murine  
329 model to a retinal damage similar to AMD and promotes other aspects of the disease,  
330 including the accumulation of lipoproteins [48]. Moreover, two FH redox forms have  
331 been identified in the circulating blood of AMD patients and those forms hold dual  
332 functions [49]. Indeed, the reduced and oxidized forms of FH, as well as FH-Y402 and  
333 FH-H402, have different binding affinities to oxidized lipids, which accumulate in  
334 drusen [45, 49]. Exogenous FH has been shown to protect ARPE19 cells against  
335 H<sub>2</sub>O<sub>2</sub>-induced stress [49] and recently, also against exposure to oxidized lipids 4-HNE  
336 (4-hydroxy-2-nonenal) [50]. In this study, we show for the first time a protective role of  
337 endogenous *CFH*/FH against oxidative insult. RPE cells, lacking FH, display an  
338 accumulation of oxidized lipids in response to H<sub>2</sub>O<sub>2</sub> pre-treatment. As a consequence,  
339 cell viability of RPE cells is affected only in this condition. Lipid oxidation also affects  
340 membrane permeability [51] and exogenous FH helps maintain a ZO-1 localization in  
341 response to 4-HNE in ARPE19 cells [50]. In our model, we show that reduction of  
342 endogenous FH mediates cell membrane damage in RPE cells. Reduction in  
343 antioxidant capacity, as well as decrease in viability, could underlie a misbalance in  
344 energy metabolism of RPE cells. Several recent evidences show altered bioenergetics  
345 being part of AMD pathology [6, 9]. Mitochondria account for the majority of cell energy  
346 production, via the tricarboxylic acid (TCA) cycle, OxPhos or lipid breakdown.  
347 Nevertheless, mitochondrial metabolism and glycolysis work in a synergetic way, since  
348 by-products of glycolysis, like pyruvate, can fuel the TCA. A disruption of either  
349 mitochondria or glycolytic function can lead to a failure in the metabolic system. Loss

350 of *CFH* has been associated with mitochondria impairment in retinal development in a  
351 *CFH* Knock-out mouse model [52] and patients carrying the *CFH* H402 high-risk  
352 variant present increased mitochondrial DNA damage [53]. Whether FH contributes to  
353 metabolic homeostasis of RPE cells has never been investigated. We show that FH  
354 loss alters energy metabolism and lead to a phenotype similar to the one observed in  
355 primary RPE cells derived from AMD patients [9]. After FH reduction, RPE cells show  
356 a decline in basal levels of glycolysis and glycolytic capacity, which were both even  
357 more affected after pre-treatment with hydrogen peroxide. In the same conditions,  
358 GLUT1 expression and glucose uptake were diminished. RPE GLUT1 levels are  
359 particularly important for the preservation of the neuroretina. Indeed, in mice with a  
360 severe reduction of GLUT1 in RPE cells, glucose transport to the retina was severely  
361 hindered and led to photoreceptors cell death [54]. Similar to glycolysis, parameters  
362 of mitochondrial respiration were hampered by FH reduction in RPE cells, with the  
363 maximal respiration to be the most affected. Interestingly, maximal respiration was  
364 increased in control cells after hydrogen peroxide treatment, indicating that RPE cells  
365 may respond to a short oxidative insult by increasing their respiration. Of note, this  
366 phenomenon was completely abolished in si*CFH* cells. Similar to AMD primary RPE  
367 cells, we see in si*CFH* cells upregulation of genes involved in anti-oxidant response,  
368 like CAT and GPX1, as well as transcription factors involved in mitochondria stability  
369 and biogenesis, like PGC1 $\alpha$ . These factors are indicators of a response to oxidative  
370 stress and mitochondria damage, but they are not the only ones contributing to define  
371 whether a cell will successfully escape from excessive oxidative stress. In fact, we do  
372 not observe any improvement in mitochondria function, either in PBS or H<sub>2</sub>O<sub>2</sub> treated  
373 cells, contrarily to AMD primary RPE cells which show a greater resistance toward  
374 oxidative stress after 24 hours [9]. This phenomenon may depend on the experimental  
375 time. Indeed, another study testing the effect of H<sub>2</sub>O<sub>2</sub> after 48 hours, a time point used  
376 in our model, showed more damage in AMD primary RPE cells after oxidative stress  
377 exposure [55]. Cells have developed alternative mechanisms to overcome  
378 mitochondrial dysfunction which are activated in case of damage. Mitophagy is a  
379 specific type of autophagy directed in eliminating unnecessary, damaged or  
380 malfunctioning mitochondria [29]. Mitophagy is classically mediated by the PINK-  
381 PARKIN axes [56], and both genes were upregulated in RPE cells when FH levels  
382 were reduced. PINK1 accumulates on the membrane of damaged mitochondria and  
383 its kinase activity is required for recruitment of PARKIN in order to mediate

384 ubiquitination and organelle removal [56]. PINK1 and PARKIN are also involved in the  
385 transport to the lysosomes of mitochondria-derived vesicles (MDV). MDVs contain  
386 oxidized proteins which are removed from the mitochondrial matrix and their removal  
387 can denote a first effort to rescue mitochondria before engaging in mitophagy [57].  
388 MDV trafficking has been implicated in Parkinson's and Alzheimer's disease through  
389 association of the vps35 protein, which is mutated in the diseases and involved in  
390 MDVs transport [58-60]. In AMD, vesicles accumulation, alterations in autophagy and  
391 lysosomes in RPE cells have been described [61, 62], but a role of MDVs in AMD  
392 pathology has not yet been considered. PINK1 interacts with proteins involved in  
393 mitochondria dynamics including DRP1 and OPA1. Both are fission and fusion genes  
394 [63], and were seen upregulated in *CFH* knock-down cells, highlighting the possibility  
395 that mitochondrial structures and dynamics are compromised by FH reduction. OPA1  
396 and DRP1 work in concert to maintain mitochondrial stability. Indeed, DRP1 loss-of-  
397 function alters OPA1 processing, thus affecting the organization of mitochondrial  
398 membranes [64]. Moreover OPA1 is also involved in mitochondrial contraction and  
399 inner membrane depolarization, leading to proton leak [65]. Thus, the loss of FH  
400 activity likely promotes destabilization of mitochondrial structure and function, followed  
401 by perturbation in mitochondrial energy metabolism, structural maintenance of  
402 mitochondria and an increase in mitophagy.

403 In conclusion, this study provides insight into a new mechanism by which FH  
404 dysregulation could contribute to processes relevant to AMD. FH reduction renders  
405 RPE cells more vulnerable to oxidative stress, with the lipids being particularly  
406 affected. RPE cells lacking functional FH show a reduced bioenergetics profile, in  
407 regard to both, glycolysis and oxidative phosphorylation. We hypothesized the  
408 involvement of mitophagy and mitochondrial dynamics in the process. Taken together,  
409 our results suggest a non-canonical role of FH in AMD and highlight its protective role  
410 in RPE cells against oxidative stress and metabolic reprogramming, which could help  
411 our understanding of the early stages of the disease. Future therapeutic strategies that  
412 systemically target the complement system may consider that simple systemic  
413 inhibition of complement activity alone may be insufficient to successfully treat AMD.

## 414 **Material and Methods**

### 415 **Cell culture**

416 Human retinal pigment epithelium (RPE) cell line hTERT-RPE1 was obtained from the  
417 American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's  
418 modified Eagle's medium (DMEM; Gibco, Germany) containing 10% fetal calf serum  
419 (FCS; Gibco, Germany), penicillin (100 U/mL), streptomycin (100 µg/mL) in a  
420 humidified atmosphere containing 5% CO<sub>2</sub>.

### 421 **Experimental settings**

422 Cells were seeded in complete growth medium without phenol red in 6- or 24-well  
423 plates depending on the experiment and allowed to attach overnight. siRNA mixture  
424 with Viromer Blue reagent was prepared according to the manufacturer (Lipocalyx,  
425 Germany) using a mix of 3 different double strand hairpin interference RNAs specific  
426 for *CFH* and a negative control (Neg), recommended by the provider (IDT  
427 technologies, Belgium). In parallel, a positive fluorescent control was used to monitor  
428 transfection efficiency (data not shown). Culture medium was substituted with fresh  
429 medium and siRNA mixture was added dropwise. After 24 hours, cells were pre-  
430 treated for 90 minutes with medium containing 200 µM H<sub>2</sub>O<sub>2</sub> or PBS as control. Cells  
431 were maintained in serum free medium, unless specified otherwise, for the indicated  
432 experiment duration. Optimal H<sub>2</sub>O<sub>2</sub> concentration was assessed in preliminary  
433 experiments in hTERT-RPE1 cells, where cell density was monitored using Crystal  
434 Violet staining [66] (Supplementary Figure 4). Concentration leading to minimal  
435 damage (200 µM) was used for further experiments.

### 436 **RNA extraction, cDNA synthesis and quantitative RT-PCR**

437 Cell pellets were collected at the indicated time points and were resolved in 1 ml of  
438 TriFAST (PeqLab, Germany), homogenized by inversion and incubated at room  
439 temperature for 5 minutes. Then, 200 µl of chloroform was added and the cell pellets  
440 vortexed for 15 seconds. Samples were left 10 minutes at room temperature and then  
441 centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous phase was transferred  
442 into a new tube and mixed with 500 µl of isopropanol for precipitation. After incubation  
443 for 10 minutes in ice, samples were centrifuged at 12,000 g for 15 minutes at 4°C.  
444 Pellets were rinsed twice with EtOH 75%, dried, resuspended in 20 µl of RNase-free

445 water. RNA purity and concentration were measured using Nanodrop. cDNA was  
 446 synthesized via reverse-transcription of 2-5 µg of RNA using M-MLV Reverse  
 447 Transcriptase (200 U, Promega, Wisconsin, USA), random primers (10 ng/µl,  
 448 Promega, Wisconsin, USA) and dNTPs (0,5 mM) in a total volume of 20 µl. cDNA was  
 449 used to analyse differences in gene expression by qRT-PCR employing SensiMix  
 450 SYBR low-Rox KIT (Bioline, Germany) along with gene specific forward and reverse  
 451 primers (250 nM) listed in Table 1. PCR protocol includes 40 cycles of: 95 °C (15 s),  
 452 57 °C (15 s) and 72 °C (25 s). Relative mRNA expression of each gene of interest  
 453 (GOI) was quantified by using PRPL0 as the housekeeping control gene.

454  $\Delta CT = CT(GOI) - CT(\text{housekeeping})$

455  $\Delta\Delta CT = \Delta CT(\text{sample}) - \Delta CT(\text{control})$

456  $n\text{-fold expression} = 2^{-\Delta\Delta CT(GOI)}$

gene name	fwd	rev
ND4	5'- CCT CGT AGT AAC AGC CAT TCT C -3	5'- CTG TGA GTG CGT TCG TAG TT -3'
COX4	5'- TGT TGG CTA CCA GGG TAT TTA G -3	5'- CTT CGC TCT TCA CAA CAC TTT C -3
ATP6	5'- CAC TAA AGG ACG AAC CTG ATC TC -3	5'- GAT AGT TGG GTG GTT GGT GTA A -3
OPA1	5'- GAG GAC AGC TTG AGG GTT ATT C -3	5'- CTG CAG AGC CTC TTC CAT AAA -3
PINK1	5'- GGC TTG GCA AAT GGA AGA AC -3	5'- CTC AGT CCA GCC TCA TCT ACT A -3
PARKIN	5'- CCA CAC TAC GCA GAA GAG AAA -3'	5'- GAG ACT CAT GCC CTC AGT TAT G -3'
DRP1	5'- GAG CTT CTT TGC AGC CTT TG -3'	5'- CCA GAA TTG GAA GGG CTA TGT -3
LDHA	5'- ACC CAG ATT TAG GGA CTG ATA AAG -3'	5'- CCA ATA GCC CAG GAT GTG TAG -3
SLC2A1	5'- GAT GGG AGT GAG ACA GAA GTA AG -3	5'- CAC TGA TGA GAG GTA CGT GTA AG -3'
PPARGC1A	5'- AGA GCG CCG TGT GAT TTA T -3'	5'- CTC CAT CAT CCC GCA GAT TTA -3'
NFE2L2	5'- TGA TTC TGA CTC CGG CAT TT -3'	5'- GCC AAG TAG TGT GTC TCC ATA G -3
PRDX3	5'- AGC CAT CTT GCC TGG ATA AAT A -3'	5'- GTA GTC TCG GGA AAT CTG CTT AG -3'
CAT	5'- CTG GAG CAC AGC ATC CAA TA -3'	5'- TCA TTC AGC ACG TTC ACA TAG A -3'
GPX1	5'- CAT CAG GAG AAC GCC AAG AA -3'	5'- GCA CTT CTC GAA GAG CAT GA -3'
BEST1	5'- CTC AGT GTG GAC ACC TGT ATG -3	5'- CCC AAC TAG ACA AGT CAG GAA G -3
RPE65	5'- GGA CTT GGC TTG AAT CAC TTT G -3'	5'- AAG ATG GGT TCT GAT GGG TAT G -3'
TJP1	5'- GGC CAG ACA AAG AGC CTA AT -3'	5'- GCT TGA GGA CTC GTA TCT GTA TG -3'
PRPLO	5'- GGA GAA ACT GCT GCC TCA TAT C -3'	5'- CAG CAG CTG GCA CCT TAT T -3'
<i>CFH</i>	5'- CTG ATC GCA AGA AAG ACC AGT A -3'	5'- TGG TAG CAC TGA ACG GAA TTA G -3'

457 Table 1. list of qPCR primers

## 458 **Western Blot**

459 Cell culture supernatants were collected 48 hours after H<sub>2</sub>O<sub>2</sub> pre-treatment. Following  
460 cell debris removal by centrifugation, supernatants were precipitated using ice-cold  
461 acetone. Proteins were re-suspended in NuPAGE™ LDS Sample Buffer containing  
462 reducing agent (Invitrogen, California, USA), separated on 8-16% or 4-12% SDS-  
463 PAGE gels and transferred on PVDF membranes. Membranes were exposed  
464 overnight to the primary antibodies (anti-FH, Santa Cruz, Texas, USA; anti-C3,  
465 Invitrogen, California, USA) and for 1 hour to HRP-conjugated anti-mouse or anti-  
466 rabbit secondary antibody (1:2.000, Cell Signaling, Massachusetts, USA)  
467 Immunoreactivity was visualized with Pierce™ ECL Western Blotting Substrate  
468 (Thermo Scientific, Massachusetts, USA) and detected with FusionFX instrument  
469 (Vilber Lourmat, France).

## 470 **C3b ELISA**

471 C3b concentration was evaluated in cell culture supernatants by ELISA assay  
472 according to the manufacturer's instructions (Abcam, UK). Samples were loaded  
473 undiluted along with standards and controls in 96 well-plate coated with specific C3b  
474 antibody. Absorbance was read at a wavelength of 450 nm immediately after the assay  
475 procedure at Spark multimode microplate reader (Tecan, Switzerland). Subtraction  
476 readings at 570 nm were taken to correct optical imperfections.

## 477 **Cytotoxicity and viability assay**

478 Cytotoxicity and viability were assessed using ApoTox-Glo™ Triplex Assay (Promega,  
479 Wisconsin, USA) according to the manufacturer's instructions. Briefly, two fluorogenic  
480 dyes were added to the cell culture media. Viability was assessed by cell-permeable  
481 GF-AFC (glycyl-phenylalanyl-aminofluorocoumarin) dye, which is cleaved by live-cell  
482 proteases and fluorescence signal is read at 400<sub>Ex</sub>/505<sub>Em</sub>. Cytotoxicity, defined by cell  
483 membrane damage, was assessed by cell-impermeable bis-AAF-R110 (bis-  
484 alanylalanyl-phenylalanyl-rhodamine 110) dye, which is cleaved by dead-cell  
485 proteases released in the cell culture supernatants after membranes damages.  
486 Fluorescence is read at 485<sub>Ex</sub>/520<sub>Em</sub>. Spark multimode microplate reader (Tecan,  
487 Switzerland) was used for fluorescence measurements. The cleaved products have  
488 different excitation/emission readouts; therefore, simultaneous measurements of



489 viability and cytotoxicity were possible. Data are normalized to untreated siNeg  
490 controls.

#### 491 **Lipid peroxidation detection**

492 Lipid peroxidation in live cells was measured via Image-iT® Lipid Peroxidation Kit  
493 (Thermo Fischer, Massachusetts, USA), based on BODIPY® 581/591 C11 fluorescent  
494 dye. At the indicated time point, the dye was added in cell culture media at the final  
495 concentration of 5  $\mu$ M. Following incubation and washing steps, fluorescence was  
496 measured at Spark multimode microplate reader (Tecan, Switzerland). Upon  
497 oxidation, the reagent shifts fluorescence emission peak from ~590 nm to ~510 nm.  
498 Data are shown as ratio of oxidized/reduced lipids.

#### 499 **Mitochondrial respiration**

500 Mitochondrial function was assessed in live cells using an XFp Extracellular Flux  
501 Analyzer (Agilent Technologies, California, USA). After 24 hours silencing (siNeg vs  
502 si*CFH*) in 6-well-plates, hTERT-RPE1 cells ( $3 \times 10^4$  cells/well) were seeded in at least  
503 duplicates in XFpSeahorse microplates and allowed to adhere overnight. Cells were  
504 pre-treated for 90 minutes with medium containing 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or PBS. Following  
505 medium change, cells were grown for further 48 hours. Measurements of oxygen  
506 consumption rate (OCR) were performed in freshly prepared assay medium, pH 7.4  
507 (Cell Mito Stress Test Assay Medium), according to the manufacturer's protocol.  
508 Mitochondrial function was evaluated after serial injections of 10  $\mu$ M oligomycin, 10  
509  $\mu$ M carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and 2  $\mu$ M Antimycin  
510 A / 1  $\mu$ M Rotenone (all Sigma-Aldrich; Missouri, USA). The data were analyzed using  
511 Wave 2.6 Software. All measurements from different experiments were normalized to  
512 the mean of the baseline values of siNeg controls.

#### 513 **Glycolysis**

514 Glycolysis function was assessed in live cells using an XFp Extracellular Flux Analyzer  
515 (Agilent Technologies, California, USA). After 24 hours silencing (siNeg vs si*CFH*) in  
516 6-well-plates, hTERT-RPE1 cells ( $3 \times 10^4$  cells/well) were seeded in at least duplicates  
517 in XFpSeahorse microplates and allowed to adhere overnight. Cells were pre-treated  
518 for 90 minutes with medium containing 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or PBS. Following medium  
519 change, cells were grown for further 48 hours. Measurements of extra-cellular

520 acidification rate (ECAR) were performed in freshly prepared assay medium, pH 7.4  
521 (Glycolysis Stress Test Assay Medium), according to the manufacturer's protocol.  
522 Glycolysis was assessed by serially injecting 10 mM Glucose, 10  $\mu$ M Oligomycin and  
523 50 mM 2-Deoxy-D-Glucose (all Sigma-Aldrich). The data were analyzed using Wave  
524 2.6 Software. All measurements from different experiments were normalized to the  
525 mean of the baseline values of siNeg controls.

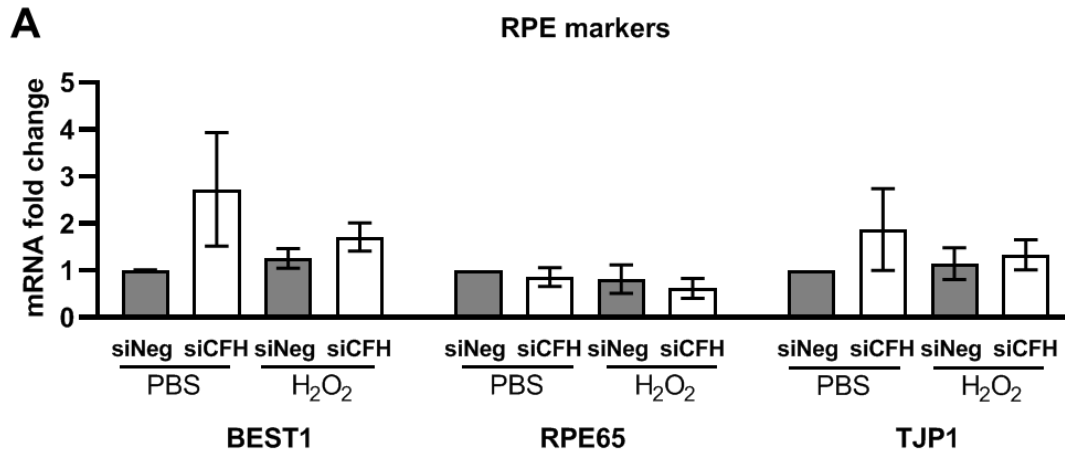
## 526 **Glucose uptake**

527 Glucose uptake was assessed using Glucose Uptake-Glo Assay (Promega,  
528 Wisconsin, USA) according to the manufacturer's instructions. Briefly, at the desired  
529 time point cells were washed with PBS and incubated 10 minutes with 1 mM glucose  
530 analogue 2-deoxyglucose (2DG), which can be phosphorylated into 2-deoxyglucose-  
531 6-phosphate (2DG6P), but not furthered processed by the glycolysis enzymes. After  
532 addition of Stop Buffer and neutralization Buffer, 2DG6P Detection reagent containing  
533 glucose-6-phosphate dehydrogenase (G6PDH), NADP+, reductase, Glo-luciferase  
534 and luciferin substrate was added to allow detection. Luminescence was recorded  
535 using Spark multimode microplate reader (Tecan, Switzerland).

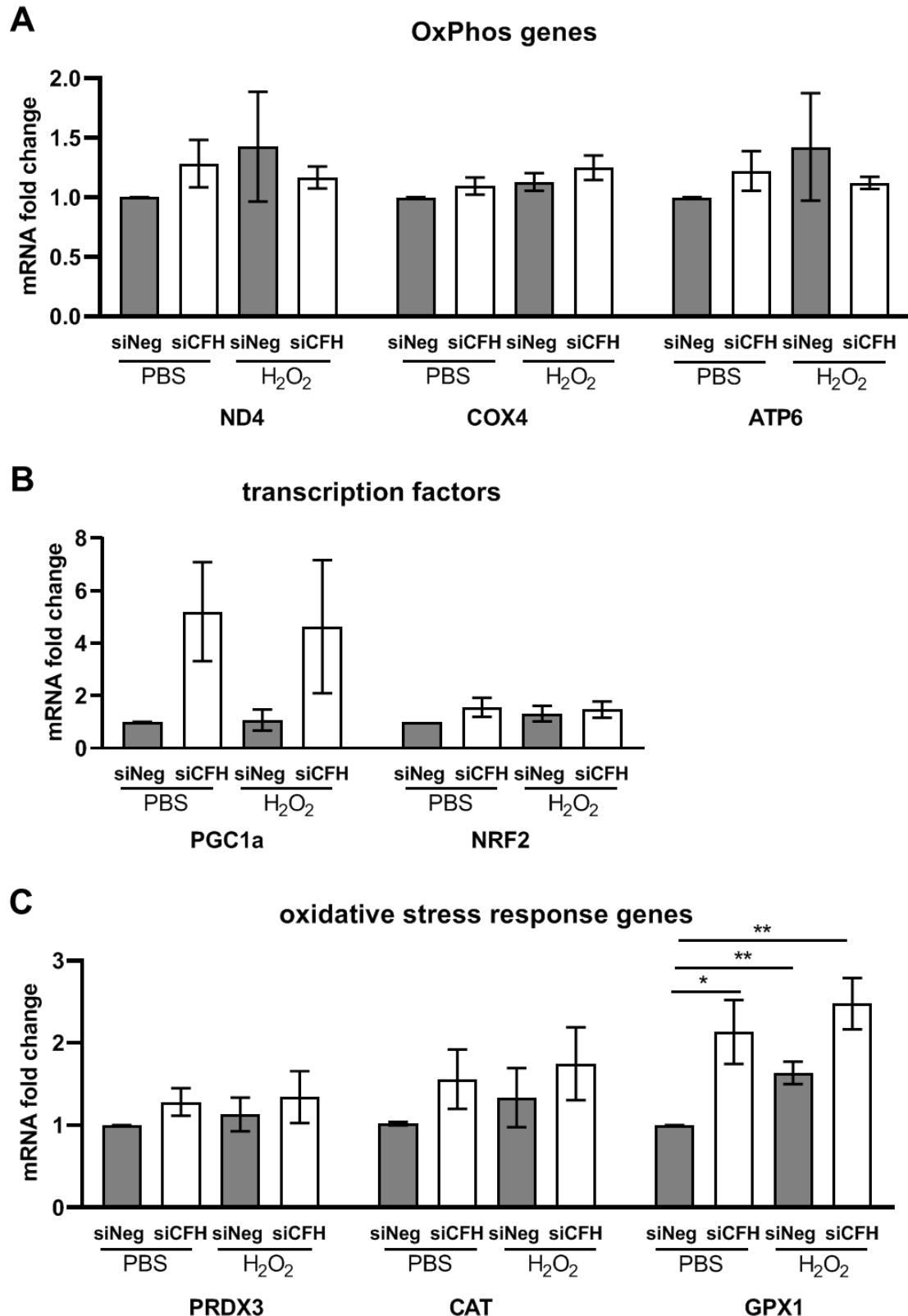
## 536 **Statistical analysis**

537 All data sets were tested for normal distribution. The combined effects of FH reduction  
538 and peroxide pre-treatment on lipid peroxidation, viability; cytotoxicity, glucose uptake  
539 and gene expression were assessed with two-way analyses of variance (ANOVA). An  
540 unpaired Student T-test was used to compare data from control cells (siNeg) versus  
541 siCFH cells and H<sub>2</sub>O<sub>2</sub>-treated cells, as well as to compare siNeg and siCFH cells after  
542 pre-treatment with H<sub>2</sub>O<sub>2</sub>. ELISA data were analyzed using a paired Student t-test.  
543 Bioenergetics data were subjected to outliers' identification via ROUT method. An  
544 unpaired Student T-test (or Mann-Whitney test in case the dataset did not pass a  
545 normality test) was used to compare data from control cells (siNeg) versus siCFH cells  
546 and H<sub>2</sub>O<sub>2</sub>-treated cells, as well as to compare siNeg and siCFH cells after pre-  
547 treatment with H<sub>2</sub>O<sub>2</sub>. Combined effects of FH reduction and peroxide pre-treatment on  
548 metabolic parameters were analyzed using two-way ANOVA. Analyses were  
549 performed using GraphPad Prism 8 software. Western Blot images were analyzed for  
550 signal quantification using Fiji (ImageJ). Significance level was set at  $p < 0.05$ .

551 **Supplementary Figures**

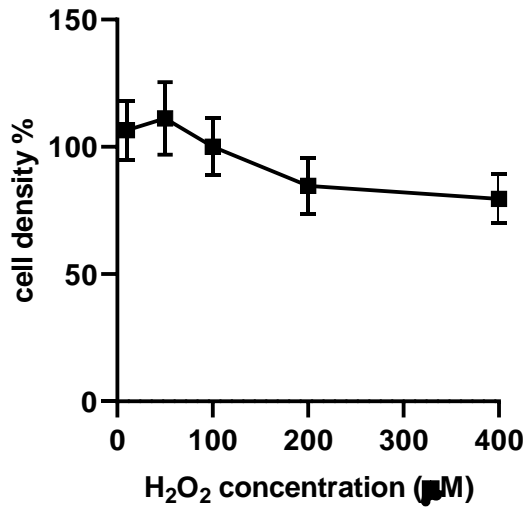


552 **Suppl. Figure 1. Expression of RPE markers in experimental conditions.** Cells  
553 were seeded, let attach overnight and silenced for 24 hours with negative control  
554 (siNeg) or *CFH* specific (si*CFH*) siRNA. Cells were exposed for 90 minutes to 200  $\mu$ M  
555 H<sub>2</sub>O<sub>2</sub> or PBS and after 48 hours RNA was collected. **A** Gene expression analysis by  
556 qRT-PCR of RPE markers: Bestrophin 1 (BEST1), Retinoid Isomerohydrolase  
557 (RPE65), Tight junction protein ZO-1 (TJP1). SEM is shown, n=3. Data are normalized  
558 to housekeeping gene PRPL0 using  $\Delta\Delta$ Ct method.

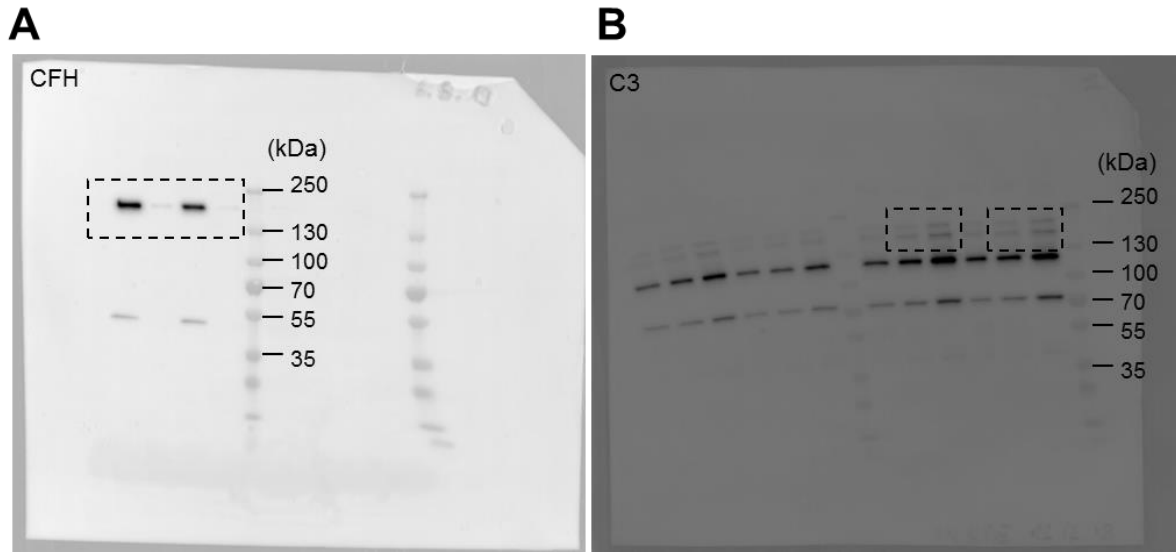


559 **Suppl. Figure 2. Expression of OxPhos genes, transcription factors and**  
 560 **oxidative response genes in experimental conditions.** hTERT-RPE1 cells were  
 561 seeded, let attach overnight and silenced for 24 hours with negative control (siNeg) or  
 562 *CFH* specific (si*CFH*) siRNA. Cells were exposed for 90 minutes to 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or  
 563 PBS and after 48 hours RNA was collected. **A** Gene expression analysis by qRT-PCR

564 of OxPhos genes: NADH dehydrogenase 4 (ND4), Cytochrome c oxidase subunit 4  
565 (COX4) and mitochondrially encoded ATP synthase 6 (ATP6). SEM is shown, n=3 **B**  
566 Gene expression analysis by qRT-PCR of transcription factors: Peroxisome  
567 Proliferator-Activated Receptor Gamma Coactivator 1-Alpha (PGC1a/PPARGC1A)  
568 and Nuclear Factor, Erythroid 2 Like 2 (NRF2/NFE2L2 ). SEM is shown, n=3 **C** gene  
569 expression analysis by qRT-PCR of genes involved in oxidative stress response:  
570 peroxiredoxin 3 (PRDX3), catalase (CAT), Glutathione Peroxidase 1 (GPX1). SEM is  
571 shown, n=3. Data are normalized to housekeeping gene PRPL0 using  $\Delta \Delta Ct$  method.  
572 Significance was assessed by Student t-test (single effect) and two-way ANOVA  
573 (combined effects) as described in the methods section. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



574 **Suppl. Figure 4. Assessment of optimal concentration for H<sub>2</sub>O<sub>2</sub> pre-treatment.**  
575 hTERT-RPE1 cells were seeded, let attach overnight and exposed for 90 minutes to  
576 increasing concentrations of H<sub>2</sub>O<sub>2</sub> and after 48 hours cell density was analyzed via  
577 Crystal Violet staining.



578 **Suppl. Figure 5.** Full images of Western Blots in main Figure 1. A corresponds to Fig  
579 1B. B corresponds to Fig 1D.

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