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5 **Factor H-related protein 5 (CFHR5) interacts with pentraxin 3 and the extracellular**
6 **matrix and modulates complement activation**

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28 **Running title:** CFHR5 binds PTX3 and ECM and modulates complement activation

29 **Abstract**

30 The physiological roles of the factor H-related proteins are controversial and poorly
31 understood. Based on genetic studies, factor H-related protein 5 (CFHR5) is implicated in
32 glomerular diseases, such as atypical hemolytic uremic syndrome, dense deposit disease and
33 CFHR5 nephropathy. CFHR5 was also identified in glomerular immune deposits at the
34 protein level. For CFHR5, weak complement regulatory activity and competition for C3b
35 binding with the plasma complement inhibitor factor H have been reported, but its function
36 remains elusive. Here, we identify pentraxin 3 (PTX3) as a novel ligand of CFHR5. Binding
37 of native CFHR5 to PTX3 was detected in human plasma and the interaction was
38 characterized using recombinant proteins. The binding of PTX3 to CFHR5 is of
39 approximately twofold higher affinity compared to that of factor H. CFHR5 dose-dependently
40 inhibited factor H binding to PTX3 and also to the monomeric, denatured form of the short
41 pentraxin C-reactive protein. Binding of PTX3 to CFHR5 resulted in increased C1q binding.
42 In addition, CFHR5 bound to extracellular matrix in vitro in a dose-dependent manner and
43 competed with factor H for binding. Altogether, CFHR5 reduced factor H binding and its
44 cofactor activity on pentraxins and the extracellular matrix, while at the same time allowed for
45 enhanced C1q binding. Furthermore, CFHR5 allowed formation of the alternative pathway C3
46 convertase and supported complement activation. Thus, CFHR5 may locally enhance
47 complement activation via interference with the complement inhibiting function of factor H,
48 by enhancement of C1q binding and by activating complement, thereby contributing to
49 glomerular disease.

50

51 **Introduction**

52 The human complement factor H-related protein 5 (CFHR5) belongs to the factor H (FH)
53 protein family (1). This protein family includes two splice variants of the factor H gene, FH
54 and FH-like protein 1 (FHL-1), and five CFHR proteins (2). All members of this protein
55 family consist of complement control protein (CCP) domains (also known as sushi domains
56 or short consensus repeats) and show high degree of sequence similarity to each other and to
57 FH. Whereas FH is a well-characterized inhibitor of the alternative complement pathway, the
58 function of the CFHR proteins is controversial (2, 3). In general, the CFHR proteins lack
59 domains and activity related to the complement regulatory CCP1-4 domains of FH. The C-
60 terminal region is highly conserved within the family: all CFHR proteins possess domains
61 similar to CCPs 19-20 of FH. Some CFHR proteins, e.g. CFHR3 contain domains with high
62 similarity to CCP domains 6 and 7 of FH (2).

63 CFHR5 is a 65-kDa plasma glycoprotein produced in the liver, with a reported serum
64 concentration of ~3-6 µg/ml (4). CFHR5 consists of 9 CCP domains that are related to CCPs
65 6-7, CCPs 10-14 and CCPs 19-20 of FH (**Figure 1A**). CFHR5 was originally isolated from
66 human glomerular complement deposits (1) with an antibody generated against preparations
67 of a human kidney with membranoproliferative glomerulonephritis (MPGN) (5). Using this
68 antibody, CFHR5 was detected in glomerular immune deposits in several kidney diseases, e.g.
69 membranous nephropathy, IgA nephropathy, lupus nephritis, focal glomerular sclerosis, and
70 post-infectious glomerulonephritis (6). Variations in the *CFHR5* gene were also found in
71 patients with atypical hemolytic syndrome (aHUS) and MPGN II/dense deposit disease (7-9).
72 Recently, a subtype of C3 glomerulonephritis was linked to a mutation and internal
73 duplication in the *CFHR5* gene and this disease entity was termed CFHR5 nephropathy (10).
74 The function of the CFHR5 protein is not well understood. It was reported that CFHR5 has
75 cofactor activity for factor I in the C3b cleavage and CFHR5 accelerates the decay of the
76 fluid-phase C3bBb convertase. However, these activities were only evident at non-

77 physiological concentrations (4). Analyses of the structural properties of CFHR1, CFHR2 and
78 CFHR5 revealed that these CFHRs form homo- and heterodimers in serum and can deregulate
79 complement by competing with FH for binding to C3b and surface polyanions (11, 12).
80 Recently, a hybrid CFHR2-CFHR5 protein was shown to cause deregulation of complement
81 (13). CFHR5 was also shown to bind to C-reactive protein (CRP), an acute phase protein
82 belonging to the family of pentraxins (4).

83 Pentraxins are pattern recognition molecules of the innate immune system and have
84 the capacity to activate complement by binding C1q (14). CRP can interact with several
85 members of the factor H protein family, namely FH, FHL-1, CFHR4 and CFHR5 (4, 15, 16),
86 but the interaction of FH with the denatured monomeric CRP versus native pentameric CRP is
87 still a controversial issue (16-20). The long pentraxin PTX3 is produced locally by
88 neutrophils, macrophages, myeloid dendritic cells, fibroblasts, endothelial cells and retinal
89 pigment epithelial cells under inflammatory conditions (21, 22). Its plasma level is ~2 ng/ml,
90 which can increase to ~1.5 µg/ml during sepsis, inflammation and infections (23). PTX3 is a
91 45-kDa glycoprotein and forms stable octamers with disulfide bonds (24). It recruits the
92 complement regulators FH, FHL-1, CFHR1 and C4b binding protein (25-27). Both CRP and
93 PTX3 can initiate complement activation and, by binding complement regulators, govern the
94 reaction to opsonization rather than to the lytic terminal pathway (28). A recent report
95 described PTX3 complexes that also contain CFHR5 in sepsis serum/plasma samples (29).

96 The aim of this study was to characterize CFHR5–pentraxin interactions, as well as
97 interaction with the extracellular matrix that could be exposed during kidney endothelial
98 injury, and investigate how they influence the regulatory role of FH and activation of
99 complement.

100

101 **Materials and Methods**

102

103 **Proteins, antibodies and sera**

104 Recombinant human FHL-1, CFHR1, CCPs 8-14 of FH (FH8-14), CFHR4A and CFHR4B
105 were generated using the pBSV-8His Baculovirus expression vector (30), expressed in
106 *Spodoptera frugiperda* (Sf9) cells, and purified by nickel-affinity chromatography as
107 described (31, 32). Recombinant human CFHR5, PTX3, anti-CFHR5 monoclonal and
108 polyclonal antibodies and biotinylated goat anti-human PTX3 antibody were obtained from
109 R&D Systems (Wiesbaden, Germany). Recombinant mutant CFHR5 with CCPs 1-2
110 duplicated was produced as described (11). The N- and C-terminal fragments of PTX3 were
111 obtained as previously described (29). The C-terminal fragments of the CFHR proteins were
112 generated as described (11).

113 Purified human FH, C3, C3b, factor B (FB), factor D, properdin (factor P; FP) factor I
114 (FI), C1q, recombinant human CRP, goat anti-human FH antibody, goat anti-human FB
115 antibody and goat anti-human C1q antibody were obtained from Merck Ltd. (Budapest,
116 Hungary). The anti-FH mAb antibody A254 and the anti-FP mAb A235 were from Quidel
117 (Biomedica, Budapest, Hungary). MaxGel, the goat anti-CRP antibody and the anti-mCRP
118 mAb (clone CRP-8) were from Sigma-Aldrich Ltd. (Budapest, Hungary). The anti-pCRP
119 mAb was purchased from Antibodies-online GmbH (Aachen, Germany). Horseradish
120 peroxidase (HRP)-conjugated goat anti-human C3 was from MP Biomedicals (Solon, OH).
121 HRP-conjugated swine anti-rabbit immunoglobulins, rabbit anti-goat immunoglobulins and
122 goat anti-mouse immunoglobulins were from Dako (Hamburg, Germany).

123 Normal human plasma was collected from healthy individuals after informed consent
124 and pooled.

125

126 **Microtiter plate binding assays**

127 To analyze binding of native CFHR5 from human plasma to PTX3, 25% normal human
128 plasma (NHP), diluted in TBS (10 mM Tris, 140 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂,
129 pH 7.4), was added for 1 h at 37°C to wells coated with 5 µg/ml PTX3 or gelatin. After
130 washing, bound proteins were eluted from the wells with SDS sample buffer (60 mM Tris
131 base, 1% SDS, 10% glycerol, bromophenol blue). Eluted proteins were separated on a 10%
132 SDS-PAGE gel and analyzed by Western blot using CFHR5-specific antibody. Binding of
133 CFHR5 and FH to PTX3- and gelatin-coated wells was measured by ELISA using FH
134 antibody and HRP-conjugated anti-goat Ig. TMB PLUS substrate (Kem-En-Tec Diagnostics,
135 Denmark) was used to visualize binding and the absorbance was measured at 450 nm.

136 To compare PTX3 binding by FH family proteins, Costar microtiter plates (Corning,
137 NY, USA) were coated with 200 nM each of purified FH, recombinant CFHR1, CFHR4A and
138 CFHR5, diluted in TBS in 25 µl, overnight at 4°C. The wells were washed after each step
139 with TBS containing 0.05% Tween-20. After blocking with 4% dry milk in TBS for 2 h at
140 37°C, 5 µg/ml PTX3 was added in TBS for 1 h at 37°C. Bound PTX3 was detected with a
141 biotinylated anti-PTX3 Ab followed by HRP-conjugated streptavidin. Calcium- and pH-
142 dependence of PTX3 binding was analyzed as described previously (27).

143 To measure PTX3 binding to CFHR protein fragments, wells were coated with 5
144 µg/ml proteins, followed by blocking with 3% BSA in TBS and incubation with 10 µg/ml
145 PTX3. PTX3 binding was detected as described above.

146 To compare binding of PTX3 and C3b to the mutant and wild-type CFHR5, the
147 recombinant proteins were immobilized at 10 µg/ml concentration. Binding of 10 µg/ml
148 PTX3 and 10 µg/ml C3b was measured using the corresponding antibodies. To compare
149 binding of wild-type and mutant CFHR5 from serum, normal serum and patient serum (11)
150 diluted 1:1 in TBS were applied to wells coated with 10 µg/ml PTX3 and CRP for 30 min at
151 37°C. After washing, the eluted proteins were analyzed by Western blot using polyclonal
152 CFHR5 antibody as described above.

153 Binding of C1q to PTX3 was measured by incubating 10 µg/ml C1q added together
154 with increasing amounts of CFHR5 and FH to wells coated with 5 µg/ml PTX3 for 1 h at
155 22°C. C1q binding was detected using anti-C1q. To measure C1q binding to CFHR5-bound
156 PTX3, microplate wells were coated with 5 µg/ml recombinant CFHR5, with PTX3 and, as
157 control, with gelatin, and then incubated with 5 µg/ml PTX3 followed by 25 µg/ml or 50
158 µg/ml C1q. C1q binding was measured using anti-C1q.

159 For inhibition assays, wells coated with MaxGel (diluted 1:30 in TBS containing Ca^{2+}
160 and Mg^{2+}), PTX3 (30 nM) or CRP (87 nM), were incubated with 50 µg/ml FH in the absence
161 or presence of increasing concentrations of CFHR5 for 1 h at 22°C. FH binding was detected
162 with the mAb A254, which does not recognize CFHR5. To detect competition between
163 CFHR5 and FH in serum, heat inactivated (56°C, 30 minutes) human serum was used. Wells
164 were coated with 10 µg/ml PTX3, 10 µg/ml CRP and MaxGel diluted 1:30 in DPBS. After
165 blocking with 4% BSA in DPBS, the wells were incubated for 30 minutes at 37°C with 25%
166 heat inactivated human serum with or without 0.5 µM CFHR5 and CFHR4A. FH binding was
167 detected with mAb A254 and the corresponding secondary antibody.

168 Interaction of CFHR5 with pCRP was measured in TBS containing Ca^{2+} and Mg^{2+} and
169 with mCRP, which was generated from commercially available CRP as described (33), in
170 DPBS (Lonza, Cologne, Germany). CFHR5 and control proteins were immobilized at 5 µg/ml
171 in microplate wells and, after blocking with 3% BSA in the corresponding buffer, incubated
172 with up to 50 µg/ml pCRP or mCRP. CRP binding was detected with the goat anti-human
173 CRP antibody that recognizes both CRP forms (16). In separate assays, CRP was immobilized
174 in microplate wells at 5 µg/ml in DPBS. Under this condition most bound CRP decays into
175 the mCRP form (16). After blocking, 300 nM of CFHR5 (20 µg/ml) and FH (50 µg/ml) were
176 added for 1 h at 22°C and binding was detected using the FH antibody.

177

178 **Cofactor assay for C3b inactivation**

179 To assay FH cofactor activity on surfaces, wells coated with MaxGel (diluted 1:30 in TBS
180 containing Ca^{2+} and Mg^{2+}), gelatin (10 $\mu\text{g/ml}$), PTX3 (30 nM) or CRP (87 nM), were
181 preincubated with 20 $\mu\text{g/ml}$ CFHR5 for 1 h at 22°C. After washing, 50 $\mu\text{g/ml}$ FH was added
182 for 1 h at 22°C, followed by thorough washing. Next, 140 nM C3b and 220 nM factor I,
183 diluted in TBS, were added in 50 μl to the wells, and incubated for 1 h at 37°C. The reactions
184 were stopped by adding reducing SDS-sample buffer. Samples were then loaded onto 10%
185 SDS-PAGE gels, separated by electrophoresis and subjected to Western blot. C3 fragments
186 were detected using HRP-conjugated goat anti-human C3 and an enhanced
187 chemiluminescence detection kit (Merck).

188

189 **Extracellular matrix (ECM) assays**

190 To study the binding of CFHR5, PTX3 and FH to human ECM, MaxGelTM diluted 1:50 in
191 TBS was immobilized on microtiter plate wells overnight at 4°C and used for subsequent
192 binding assays (as described above). Endothelial cell-derived ECM was prepared as described
193 (25, 27) by culturing HUVEC (ATCC; LGC Promochem, Wesel, Germany) on gelatin-coated
194 96-well tissue culture plates (0.2% gelatin) in DMEM (Lonza) supplemented with 10% FCS,
195 1% L-glutamine and 50 $\mu\text{g/ml}$ gentamicin sulfate in a cell incubator with humidified
196 atmosphere containing 5% CO_2 for 7 days at 37°C. Cells were washed and detached from the
197 plate by incubation in DPBS containing 10 mM EDTA at 37°C. Removal of the cells was
198 monitored by microscopy. The cell-free ECM was washed with TBS and used immediately
199 for binding assays as described above. The production of ECM by endothelial cells was
200 confirmed by detecting ECM components after cell detachment, using antibodies against
201 laminin, collagen type IV, and von Willebrand Factor (Sigma-Aldrich).

202

203 **C3 convertase assays**

204 Formation of the C3bBb alternative pathway C3 convertase on surface-bound CFHR5 and
205 detection of the C3 convertase components were performed as previously described (34). The
206 convertase activity was measured by adding 10 µg/ml purified C3 for 1 h at 37°C and
207 quantifying the generated C3a by a C3a ELISA kit (Quidel).

208

209 **Complement activation assays**

210 To measure complement activation due to competition between CFHR5 and FH for ligand
211 binding, Nunc microplate wells were coated with 10 µg/ml PTX3, 10 µg/ml CRP and MaxGel
212 diluted 1:30 in DPBS. After blocking with 4% BSA in DPBS, 12.5% normal human serum
213 (for PTX3 and CRP) and 25% normal human serum (for ECM) were added for 30 minutes at
214 37°C, with or without 20 µg/ml CFHR5 or CFHR4A. Complement activation was detected by
215 measuring deposition of C3 fragments using HRP-conjugated goat anti-human C3.

216 In other experiments, Nunc microtiter plate wells were coated with 5 µg/ml CFHR5,
217 CFHR4A, FH and HSA, and, after blocking with 3% BSA in DPBS, incubated with 10%
218 normal human serum with or without 5 mM Mg²⁺-EGTA or 5 mM EDTA for 30 min at 37°C.
219 Deposition of C3b, FB and FP was detected using the corresponding primary and secondary
220 antibodies.

221

222 **Statistical analysis**

223 Statistical analysis was performed using GraphPad Prism version 4.00 for Windows
224 (GraphPad Software, San Diego California USA). A *p* value < 0.05 was considered
225 statistically significant.

226

227 **Results**

228

229 **CFHR5 binds to PTX3**

230 Previously, we showed that serum FH and CFHR1 bind to PTX3 (27). To investigate binding
231 of CFHR5, we incubated human plasma in PTX3-coated wells, and analyzed the bound
232 proteins after elution and SDS-PAGE by Western blotting using CFHR5-specific antibody.
233 The native CFHR5 protein was detected by this approach in the PTX3-coated wells but not in
234 gelatin-coated control wells (**Fig. 1B**). Direct interaction of CFHR5 with PTX3 was analyzed
235 by ELISA. Recombinant CFHR5 showed dose-dependent binding to PTX3 and the binding of
236 CFHR5 to PTX3 reached saturation at a lower concentration compared to the binding of FH
237 (**Fig. 1C**). To further analyze this, serial dilutions of PTX3 were applied to CFHR5,
238 immobilized in microplate wells, and the amount of bound PTX3 was calculated from a
239 standard curve (**Fig. 1D**). Dose-dependent binding was observed in the 1-10 nM range;
240 however, avidity effects complicate affinity determinations because of the octameric nature of
241 PTX3 and CFHR5 being dimeric, resulting potentially in multivalent binding.

242

243 **Characterization of the CFHR5-PTX3 interaction**

244 To compare the binding of PTX3 to CFHR proteins, equimolar amounts of purified FH and
245 recombinant CFHR1, CFHR4A and CFHR5 were immobilized in microplate wells and PTX3
246 was added in the fluid phase. CFHR1 showed less PTX3 binding capacity compared to FH, in
247 agreement with our previous results (27), whereas CFHR5 showed the strongest binding
248 among the studied proteins (**Fig. 2A**). PTX3 binding to CFHR4A was rather weak, similar to
249 that reported previously for CFHR4B (26).

250 Because the presence of Ca^{2+} is required for several interactions of pentraxins, the role
251 of Ca^{2+} in the binding of PTX3 to CFHR5 was studied. Similar to FH, CFHR5 showed
252 strongly reduced PTX3 binding when Ca^{2+} was absent from the buffer (**Fig. 2B**).

253 The binding of PTX3 to FH and FHL-1 was shown to be increased at lower pH (27).
254 In contrast, we found that the binding of CFHR5 was slightly but significantly reduced at pH
255 5.5 compared with the physiological pH (**Fig. 2C**).

256

257 **Analysis of binding sites involved in the CFHR5-PTX3 interaction**

258 To determine which domain of PTX3 mediates the binding to CFHR5, recombinant N- and C-
259 terminal PTX3 fragments were immobilized on microtiter plates and incubated with
260 recombinant CFHR5. CFHR5 bound to both the N- and C-terminal parts of PTX3 (**Fig. 3A**).
261 FH also bound to both PTX3 fragments, whereas the control recombinant CCPs 8-14 domains
262 of FH did not bind to any PTX3 fragment, in agreement with previous results (26).

263 Because the CCP19-20 domains of FH were shown to be involved in binding PTX3
264 (26, 27), we investigated the capacity of the homologous CCP8-9 domains of CFHR5, and
265 also the homologous domains of the other CFHR proteins, to bind PTX3. PTX3 did not bind
266 to the CFHR5 CCP8-9 fragment, indicating a binding site outside these domains (**Fig. 3B**).
267 PTX3 bound to CCP19-20 of FH and CCP4-5 of CFHR1, as expected from previous studies
268 (26, 27), but it did not bind to CCP3-4 of CFHR2, CCP4-5 of CFHR3 and CCP8-9 of
269 CFHR4A (**Fig. 3B**).

270 A disease-associated CFHR5 protein contains CCPs 1-2 in two copies (**Fig. 1A**). To
271 analyze the role of these domains and the capacity of this disease-associated CFHR5 mutant
272 (mutCFHR5) in PTX3 binding, the binding of PTX3 to immobilized recombinant mutCFHR5
273 protein compared with wild type CFHR5 was measured. The mutant CFHR5 protein bound
274 less PTX3 than the wild type protein, whereas the binding of fluid-phase C3b was comparable
275 to both proteins in the assay (**Fig. 3C**).

276 The interaction was confirmed using serum as a source of CFHR5 (**Fig. 3D**). Normal
277 human serum and a patient serum containing both wild-type CFHR5 and the mutCFHR5 were
278 incubated in wells coated with PTX3 and the related short pentraxin CRP. The bound proteins

279 were analyzed after elution and SDS-PAGE by Western blotting using polyclonal CFHR5
280 antibody. Whereas the amount of the mutCFHR5 was clearly higher in the patient serum
281 compared with the normal CFHR5 (**Fig. 3D**, lane 8), a weaker mutCFHR5 band was observed
282 among both the PTX3- and CRP-bound proteins, confirming the results obtained with
283 recombinant mutCFHR5.

284

285 **Effect of the ligands C3b and C1q on the CFHR5-PTX3 interaction**

286 The main known CFHR5 ligand is the complement fragment C3b. Therefore, we studied if
287 C3b influences the PTX3 binding capacity of CFHR5. To this end, CFHR5 immobilized in
288 microplate wells was preincubated with increasing concentrations of C3b. Then PTX3 was
289 added and PTX3 binding was measured. C3b showed dose-dependent binding to CFHR5 and
290 it did not influence the binding of PTX3 (5 µg/ml) to CFHR5 at concentrations up to 50 µg/ml
291 (data not shown).

292 C1q binding to PTX3 is thought to initiate classical complement pathway activation
293 (35). We investigated whether CFHR5 can interfere with the binding of C1q to PTX3. To this
294 end, wells coated with PTX3 were preincubated with increasing amounts of CFHR5 and C1q
295 binding was measured. CFHR5 could partly inhibit C1q binding to PTX3 (up to ~50% under
296 the tested experimental conditions), whereas FH had no inhibitory effect on C1q binding to
297 PTX3 (**Fig. 4A**). In a reverse setting, we analyzed if CFHR5-bound PTX3 can interact with
298 C1q. C1q showed binding to CFHR5 which was strongly enhanced by PTX3 bound to
299 CFHR5 (**Fig. 4B**). Thus, C1q can bind to CFHR5-bound PTX3.

300

301 **CFHR5 competes with FH for binding to PTX3**

302 The binding of FH to PTX3 is thought to down-regulate PTX3-induced complement
303 activation (26, 27). Therefore, we assessed the ability of CFHR5 to interfere with the binding
304 of the complement inhibitor FH to PTX3. CFHR5 strongly and dose-dependently inhibited

305 FH binding to PTX3, whereas the control protein human serum albumin had no effect on this
306 interaction (**Fig. 5A**). Accordingly, CFHR5 inhibited the cofactor activity of PTX3-bound FH
307 by strongly reducing FH binding to PTX3. When bound to PTX3, FH acted as a cofactor for
308 the factor I-mediated cleavage and inactivation of C3b (**Fig. 5B**, lane 2), which was strongly
309 reduced by CFHR5 added in 1:1 molar ratio to FH (300 nM each) (**Fig. 5B**, lane 3), while
310 CFHR5 itself showed no cofactor activity (**Fig. 5B**, lane 4).

311

312 **CFHR5 binds to denatured, monomeric CRP and inhibits factor H binding and cofactor** 313 **activity**

314 CRP shares a pentraxin domain homologous to that of PTX3. CFHR5 was shown to interact
315 with CRP, but the CRP form which binds to CFHR5 and the functional consequence of this
316 interaction were not investigated (4). We therefore set out to characterize the CFHR5-CRP
317 interaction in more detail. CFHR5 did not bind the native, pentameric form (pCRP) in ELISA
318 under our experimental conditions (**Fig. 6A**), but it readily interacted with the denatured,
319 monomeric CRP (mCRP) (**Fig. 6B**). The control proteins used in these experiments CFHR4A
320 and FH bound pCRP and mCRP, respectively, as expected (16). CFHR5 also bound to mCRP
321 in the reverse setting, when mCRP was generated by immobilization of pCRP on the ELISA
322 plates (**Fig. 6C**).

323 Similar to PTX3, the binding of FH to mCRP was dose-dependently inhibited by
324 CFHR5 (**Fig. 6D**). When bound to mCRP, FH displayed cofactor activity for the factor I-
325 mediated cleavage and inactivation of C3b (lane 2), which was inhibited by CFHR5 (lane 3)
326 (**Fig. 6E**). CFHR5 itself showed no cofactor activity under these conditions (**Fig. 6E**, lane 4).

327

328 **CFHR5 binds to ECM, competes with FH, and recruits PTX3**

329 Previously, we showed that FH, FHL-1 and CFHR1 bind to ECM (27). Therefore, we
330 analyzed the interaction of CFHR5 with the model ECM MaxGel. The binding of native

331 CFHR5 to MaxGel from serum could be detected by Western blot (**Fig. 7A**). Recombinant
332 CFHR5 exhibited a strong, dose-dependent binding to MaxGel (**Fig. 7B**). Similarly to the
333 case of pentraxins (**Figs. 5 and 6**), CFHR5 inhibited the surface-associated cofactor activity
334 of FH by competing with its binding to MaxGel (**Figs. 7C and 7D**). In addition, CFHR5, but
335 not FH, FHL-1 and CFHR1, strongly enhanced binding of PTX3 to both MaxGel and
336 HUVEC-derived ECM (**Fig. 7E**). PTX3 had only minor effect on CFHR5 binding to ECM
337 (not shown).

338

339 **CFHR5 competes with FH in serum**

340 To confirm that the observed competition between CFHR5 and FH occurs also in serum,
341 wells were coated with PTX3, CRP and MaxGel and incubated with heat-inactivated serum,
342 which was used to exclude FH binding through deposited C3 fragments. CFHR5 when added
343 to heat-inactivated serum significantly reduced the amount of bound FH on PTX3, CRP and
344 MaxGel (**Fig. 8A**). As a negative control, recombinant CFHR4A was used, which does not
345 bind well to PTX3 (**Fig. 2A**), immobilized CRP (**Fig. 6**) (16) and MaxGel (27). In line with
346 this, CFHR4A did not inhibit FH binding to any of these ligands (**Fig. 8A**).

347 In addition, C3 fragment deposition on PTX3, CRP and MaxGel was measured in
348 complement active normal serum when CFHR5 was added to increase its concentration. In all
349 cases, addition of CFHR5 but not CFHR4A caused increased C3 deposition, indicating
350 competitive inhibition of FH activity by CFHR5 on these ligands (**Fig. 8B**).

351

352 **The C3bBb alternative pathway C3 convertase assembles on CFHR5**

353 We have previously shown that CFHR4 can activate complement by binding C3b and
354 allowing formation of an active C3bBb alternative pathway C3 convertase (34). We analyzed
355 whether such a C3 convertase can also be formed on CFHR5. To this end, immobilized
356 CFHR5 was incubated with C3b, followed by the addition of purified FB, factor D and FP.

357 Convertase formation was detected by measuring the Bb fragment. Similar to CFHR4A,
358 CFHR5 supported C3bBb formation, but the amount of C3 convertase was less on CFHR5
359 compared with CFHR4A (**Fig. 9A**). The CFHR5-bound convertase was functional as shown
360 by the generation of C3a after the addition of purified C3 to the convertase (**Fig. 9B**). We
361 detected no binding of purified FB alone to CFHR5. We observed weak binding of purified
362 FP to CFHR5, which could be an artefact due to the known in vitro formation of non-
363 physiological FP oligomers (**Supplemental Fig. 1**).

364 Incubation of CFHR5 with 10% normal human serum supplemented with 5 mM Mg²⁺-
365 EGTA confirmed the generation of the alternative pathway C3 convertase as measured by
366 deposition of C3, FB and FP (**Figs. 10A-C**). In serum supplemented with 5 mM EDTA, no
367 complement activation and convertase formation was detected, as expected. The residual C3
368 signal on CFHR4A is due to direct binding of C3 fragment(s) (**Fig. 10A**).

369

370 **Discussion**

371

372 The function of the factor H-related proteins is a controversial issue. Most studies reported
373 lack of FH-like complement inhibiting activity for the CFHR proteins at physiological
374 concentrations (11, 12, 34, 36), as expected from the domain composition of these proteins.
375 However, weak cofactor activity for CFHR3, CFHR4 and CFHR5 (4, 37), and also strong
376 cofactor activity for CFHR3 were reported (38). Complement regulation by other
377 mechanisms, such as inhibition of the terminal complement pathway, were reported for
378 CFHR1 and CFHR2 (39, 40), but others could not detect this activity (11, 41). In addition,
379 enhancement of complement activation by CFHR4 via binding of C3b and CRP was
380 described (32, 34).

381 Despite the suggested complement inhibiting function for the CFHRs, microbes that
382 bound CFHR1, CFHR2 and CFHR5 were not protected by these proteins from complement
383 attack (42, 43). Increased expression of CFHRs under infectious conditions (44) and the
384 described competition of CFHR1 with FH for certain bacterial ligands (45, 46) and for the
385 major opsonin C3b (11) indicate that CFHRs may interfere with the regulatory activity of FH.
386 Cumulatively, these data suggest that instead of inhibition of complement activation, the
387 CFHRs rather cause enhanced activation. This could be advantageous to the human host
388 during infections as it may help the opsonophagocytic removal of microbes, but also that of
389 cellular debris under non-infectious conditions. Depending on the local concentration,
390 oligomeric state, polymorphic and mutant variants, and binding strength of FH, CFHRs and
391 their ligands, the delicate balance between complement activation/deregulation and inhibition
392 could be shifted in favour of activation, resulting in pathologic complement activation.

393 While currently there is no convincing evidence for physiologically relevant
394 complement inhibiting roles of the CFHR proteins, their association with complement-
395 mediated diseases is supported by genetic studies (47). Importantly, three recent reports

396 showed that CFHR1, CFHR2 and CFHR5 deregulate complement by competing with FH for
397 binding to C3b, thus they may rather enhance complement activation in human renal diseases
398 (11-13). Our current study supports a function of CFHR5 as competitive inhibitor of FH and
399 as enhancer of complement activation. We further investigated the role of CFHR5 in
400 complement activation and in modulating the activity of FH, which could be particularly
401 relevant during acute phase response (with increase in pentraxin concentrations) and
402 endothelial injury.

403 We identified novel CFHR5 ligands, such as PTX3, modified CRP and ECM, which
404 are likely relevant in disease-associated roles of CFHR5. CFHR5 was implicated in particular
405 in various kidney pathologies based on genetic and protein-level studies (6-8, 10, 11, 48). Our
406 data suggest that not only binding of C3b or C3b fragments (such as iC3b) by CFHR5 could
407 be important in the context of diseases, but likely also interactions with molecules, such as
408 pentraxins that are upregulated during inflammation and renal endothelial damage, and with
409 host surfaces such as the ECM. This is supported by recent proteomics studies, which
410 identified both CFHR5 and PTX3 as glomerular ECM-associated proteins (49, 50).

411 PTX3 is produced locally at the site of inflammation. Elevated PTX3 levels were
412 described in various infectious and inflammatory diseases, including chronic kidney disease
413 (23, 51, 52). Both protective and detrimental effects of PTX3 have been described, such as in
414 post-ischemic renal injury (23, 53, 54). Systemic PTX3 concentration of up to ~1.5 µg/ml was
415 reported, indicating that locally at inflammatory sites it can reach much higher concentrations,
416 similar to those used in this study, suggesting that the studied PTX3-CFHR5 interaction has
417 physiological relevance.

418 A previous study described cofactor activity for CFHR5, but rather high
419 concentrations of the protein were required for this function (4). In our current study, we
420 found no cofactor activity of CFHR5 at the studied, physiologically relevant concentrations
421 (**Figs. 5-7**). On the other hand, physiological amounts of CFHR5 significantly competed with

422 FH in binding to the pentraxins PTX3 and CRP, as well as to ECM. Importantly, this
423 competition could be detected in serum and caused enhanced complement activation (**Fig. 8**).
424 This represents an indirect means by which CFHR5 deregulates complement, namely, not via
425 direct competition with FH for C3b, but by interfering with FH binding to its other
426 physiological ligands. These data suggest that depending on the FH-binding surfaces and
427 ligands, as well as on local FH and CFHR5 concentrations, CFHR5 can fine-tune local
428 complement regulation by competing with FH.

429 The binding of PTX3 to CFHR5 is the strongest among the tested FH family proteins;
430 this explains the strong inhibitory effect of CFHR5 on FH binding to PTX3 (**Figs. 1, 2 and 5**).
431 The PTX3-CFHR5 interaction is strongly reduced in Ca^{2+} -free buffer (**Fig. 2**), similar to the
432 PTX3 binding to FH, FHL-1 and CFHR1, reported previously (26, 27).

433 Our data show that at reduced pH, which might occur at sites of inflammation, the
434 binding of PTX3 to CFHR5 is reduced. We have previously showed that at lower pH the
435 binding of PTX3 to C1q and CFHR1 is unchanged but the binding of PTX3 to the
436 complement inhibitors FH, FHL-1 and C4b-binding protein is increased (27). Combined,
437 these data suggest that under such conditions complement inhibition is preferred, because the
438 binding of the complement inhibitors increases and that of the deregulator CFHR5 decreases.

439 The PTX3 binding site in CFHR5 appears not to be homologous to the two identified
440 PTX3 binding sites in FH, i.e. FH domains CCP7 and CCP19-20. CCP1-2 of CFHR5 are the
441 dimerization domains and very different structurally and functionally from CCP6-7 of FH.
442 CCP2 of CFHR5 is very similar to CCP2 of CFHR1 (~85% amino acid sequence identity);
443 however, we showed previously that PTX3 binds to CFHR1 via the C-terminus of CFHR1
444 (27). We tested the C-terminal domains of all of the five human CFHR proteins, and only
445 CCP4-5 of CFHR1 bound PTX3 under our experimental conditions. The CCPs 8-9 domains
446 of CFHR5 did not bind PTX3 (**Fig. 3**). In addition, these domains were shown to bind C3b,
447 and C3b did not influence PTX3 binding by CFHR5 in our experiments (not shown).

448 Moreover, the binding site for the related pentraxin CRP was localized to CCPs 3-7 of
449 CFHR5 (4). Thus it is likely, but needs to be tested in the future, that the PTX3-binding site
450 also resides within these middle domains of CFHR5. In addition, the tested disease-associated
451 mutant CFHR5 protein, in which the two N-terminal domains are duplicated, showed reduced
452 PTX3-binding capacity in comparison with the wild-type protein (**Fig. 3**). This could be
453 caused by the restricted availability of PTX3-binding sites in the mutant which has an
454 increased tendency to form oligomers (11).

455 The known PTX3 ligand C1q could partially inhibit PTX3 binding to CFHR5, but not
456 to FH, indicating partially overlapping binding sites or conformational changes of PTX3 when
457 bound to C1q. However, CFHR5-bound PTX3 could still strongly bind C1q (**Fig. 4**). We
458 observed C1q binding to CFHR5 alone, which was strongly and significantly enhanced by
459 PTX3.

460 Interaction of CFHR5 with CRP was first described by McRae et al., but the type of
461 CRP which binds CFHR5 was not studied (4). FH binds preferentially to the modified,
462 monomeric form of CRP via domains CCP7, CCPs 8-11 and CCPs 19-20 (15-17), and at
463 acute phase CRP concentrations it was reported to interact also with the native pentameric
464 form of CRP (18). By contrast, CFHR4 binds primarily to the native pCRP (16, 32). In our
465 assays, CFHR5 bound strongly to mCRP, whereas the binding to pCRP was minor (**Fig. 6**).

466 CFHR5 also strongly and dose-dependently bound to MaxGel, in contrast to the
467 relatively weak binding of FH (27). Accordingly, CFHR5 efficiently inhibited the binding and
468 cofactor activity of FH on this ECM (**Fig. 7**). CFHR5, alone among the investigated FH
469 family proteins, recruited PTX3 to the ECM. These results are in line with recent proteomics
470 data demonstrating the association of both CFHR5 and PTX3 with glomerular EC-derived
471 ECM (49, 50), and suggest that CFHR5 enhances local complement activation via these
472 interactions.

473 We also studied the direct role of CFHR5 in complement activation. In our recent
474 study on CFHR4, we showed that C3b-binding to CFHR4 can result in the assembly of the
475 alternative pathway C3 convertase and activation of C3 (34). In our current study, we found
476 that CFHR5 can similarly promote C3 convertase formation and alternative pathway
477 activation, albeit the effect was weaker compared to CFHR4 (**Figs. 9 and 10**). This activity
478 stands in contrast to the complement alternative pathway inhibitory role of FH.

479 During kidney endothelial injury, host ligands that are newly exposed (ECM) or the
480 concentration of which is increased (pentraxins), may result in the increased binding of
481 CFHR5 to the site of injury. The oligomeric state of CFHR5 and CRP can further fine-tune
482 these interactions, but this needs to be characterized further.

483 In summary, we show that in addition to competing for C3b binding with FH as
484 reported recently, CFHR5 may promote complement activation and complement-mediated
485 damage/inflammation through three mechanisms: (1) by preventing the binding of the
486 complement inhibitor FH to PTX3, CRP and ECM, (2) by allowing increased binding of the
487 complement activation initiator molecule C1q, and (3) by supporting the assembly of the
488 alternative pathway C3 convertase C3bBb. These functions may explain in part the disease-
489 associated role of CFHR5.

490

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494

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- 685

686 **Footnotes:**

687

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699 ³Abbreviations used in this paper: aHUS, atypical hemolytic uremic syndrome; CCP,

700 complement control protein domain; CFHR, factor H-related; CFHR1, factor H-related

701 protein 1; CFHR5, factor H-related protein 5; CRP, C-reactive protein; DPBS, Dulbecco’s

702 phosphate-buffered saline; ECM, extracellular matrix; FB, factor B; FH, factor H; FI, factor I;

703 FP, factor P; HSA, human serum albumin; mCRP, modified monomeric form of CRP; NHP,

704 normal human plasma; pCRP, native pentameric form of CRP; PTX3, pentraxin 3.

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706 **Author contributions:** M.J. initiated and supervised the study. Á.I.C., A.K., B.U. and M.J.

707 designed the experiments. Á.I.C., A.K., M.Z., Z.B., B.U. and M.J. performed ligand binding

708 and competition assays. Á.I.C., M.Z., B.U. and M.J. performed cofactor assays. Á.I.C.

709 performed convertase and complement activation assays. M.H. generated and provided

710 recombinant CFHR proteins and established the convertase and complement activation

711 assays. J.J.E.C. and S.M.L. generated and provided CFHR fragments and discussed the data.

712 E.G. de J. and M.C.P. generated and provided CFHR5 mutants and discussed the data. K.D.

713 and T.H. generated and provided PTX3 fragments and discussed the data. Á.I.C., B.U. and

714 M.J. wrote the manuscript with the help of the other authors.

715

716 **Figure legends**

717 **Fig. 1. CFHR5 interacts with PTX3.**

718 (A) Schematic drawing of FH, CFHR5 and CFHR4A. FH is built up of 20 CCP domains, of
719 which CCPs 1-4 mediate complement regulatory activity and CCPs 19-20 mediate surface
720 recognition by FH. The CCP domains of CFHR5 and CFHR4A, which was used as a control
721 protein in several experiments, are shown aligned with the corresponding most related FH
722 domains. The numbers above the domains indicate the percentage of amino acid sequence
723 identity between the homologous domains. The mutant CFHR5 protein detected in CFHR5
724 nephropathy and containing a duplication of CCPs 1-2 is also shown. CFHR4B consists of
725 CCPs 1 and 6-9 of CFHR4A (not shown).

726 (B) Microplate wells were coated with gelatin or PTX3, then incubated with 25% normal
727 human plasma (NHP). After washing, bound proteins were removed by adding SDS-sample
728 buffer and subjected to 10% SDS-PAGE and Western blotting using monoclonal anti-CFHR5.
729 The blot is representative of three experiments.

730 (C) Comparison of binding of CFHR5 and FH to PTX3. Immobilized PTX3 and gelatin, used
731 as negative control protein, were incubated with the indicated concentrations of purified FH
732 or recombinant CFHR5. Binding of both proteins was detected by a FH antibody. Data are
733 means \pm SEM derived from four experiments. Both FH and CFHR5 bound to PTX3
734 significantly stronger than to gelatin ($p < 0.001$, two-way ANOVA).

735 (D) Addition of increasing amounts of PTX3 results in a saturable binding to immobilized
736 CFHR5. Specific binding was measured using a standard curve of PTX3. Data are mean \pm
737 SEM from eight experiments.

738

739 **Fig. 2. Comparison of the binding of PTX3 with CFHR proteins, and dependence of the**
740 **PTX3-CFHR5 interaction on calcium concentration and pH.**

741 (A) Binding of PTX3 to recombinant CFHR5 was compared to that of CFHR1, CFHR4A and
742 FH by ELISA. The FH family proteins and bovine serum albumin (BSA), used as negative
743 control, were immobilized in equimolar concentrations (200 nM) in microplate wells, then 5
744 µg/ml recombinant PTX3 was added for 1 h at 37°C. PTX3 binding was determined using a
745 polyclonal anti-PTX3 antibody. The values were normalized for FH binding (100%) and show
746 means + SD derived from three independent experiments. ** $p < 0.01$ and *** $p < 0.001$, one-
747 way ANOVA.

748 (B) The binding of PTX3 to immobilized FH and CFHR5 was compared in DPBS (pH 7.4)
749 with (black bars) and without (white bars) 1 mM Ca^{2+} . The data are normalized to binding in
750 the presence of Ca^{2+} (100%), and represent means + SD from four experiments. *** $p <$
751 0.001, one-way ANOVA.

752 (C) The binding of PTX3 to CFHR5 was compared in TBS with pH 7.4, 6.5 and 5.5. The data
753 shown are normalized to values obtained with TBS pH 7.4 (100%) and represent means + SD
754 from five experiments. * $p < 0.05$, one-way ANOVA.

755

756 **Fig. 3. Characterization of the PTX3-CFHR5 interaction.**

757 (A) Gelatin, full-length PTX3 and the N- and C-terminal PTX3 fragments were immobilized
758 in microplate wells (5 µg/ml). Binding of FH (40 µg/ml), FH8-14 (10 µg/ml) and CFHR5 (10
759 µg/ml) was measured using polyclonal anti-FH antibody. Data are mean absorbance values +
760 SD derived from four independent experiments. ** $p < 0.01$ and *** $p < 0.001$, one-way
761 ANOVA.

762 (B) Binding of PTX3 to the C-terminal CFHR domains that are the homologues of the PTX3-
763 binding CCP19-20 of FH was measured by ELISA. CCP4-5 of CFHR1, CCP3-4 of CFHR2,
764 CCP4-5 of CFHR3, CCP8-9 of CFHR4A, CCP8-9 of CFHR5, CCP19-20 of FH, as well as
765 the full-length FH, CFHR1, CFHR4B, CFHR5 and HSA proteins as controls, were
766 immobilized at 5 µg/ml concentration in microplate wells. Recombinant PTX3 at 10 µg/ml

767 was added and its binding was detected as described for Fig. 2. Data are mean absorbance
768 values + SD derived from three independent experiments. * $p < 0.05$ and *** $p < 0.001$, one-
769 way ANOVA.

770 (C) Binding of PTX3 to the CFHR5 mutant with duplicated CCP1-2 (mutCFHR5) was
771 measured by immobilizing mutCFHR5, and as controls, CFHR5 and gelatin at 10 $\mu\text{g/ml}$ in
772 microplate wells. Binding of 10 $\mu\text{g/ml}$ PTX3, 10 $\mu\text{g/ml}$ C3b and a CFHR5-specific polyclonal
773 antibody was measured by ELISA as described in Materials and methods. The data are means
774 + SD derived from four independent experiments. * $p < 0.05$, one-way ANOVA.

775 (D) Wells coated with PTX3 (lanes 1-3) and CRP (lanes 4-6) were incubated with 50%
776 normal human serum (NHS) (lanes 2 and 5) and a patient serum containing both wild-type
777 CFHR5 and the mutCFHR5 (lanes 3 and 6). The bound proteins were analyzed after elution
778 and 10% SDS-PAGE by Western blotting using polyclonal CFHR5 antibody. Reactivity of
779 the polyclonal antibody with NHS (1 μl , lane 7) and patient serum (1 μl , lane 8) is shown as a
780 control. The blot is representative of two experiments.

781

782 **Fig. 4. C1q binding to PTX3 in the presence of CFHR5.**

783 (A) Binding of C1q to PTX3 was determined in the presence of increasing concentrations of
784 CFHR5 and FH in ELISA. C1q binding was detected using C1q antibody. The normalized
785 values are means \pm SD derived from four independent experiments. * $p < 0.05$ and *** $p <$
786 0.001, two-way ANOVA.

787 (B) C1q binds to CFHR5-bound PTX3. Microplate wells were coated with 5 $\mu\text{g/ml}$
788 recombinant CFHR5 and, as control, with gelatin, and then sequentially incubated with 5
789 $\mu\text{g/ml}$ PTX3 and 25 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$ C1q, as indicated. C1q binding was measured using
790 C1q antibody. The data are means + SD derived from four independent experiments. ** $p <$
791 0.01 and *** $p < 0.001$, one-way ANOVA.

792

793 **Fig. 5. CFHR5 competes with FH binding and activity on PTX3.**

794 (A) The binding of 50 $\mu\text{g/ml}$ (~ 300 nM) FH to PTX3 was measured in the presence of
795 increasing concentrations of CFHR5 (up to 20 $\mu\text{g/ml}$, corresponding to ~ 300 nM CFHR5),
796 using a FH-specific monoclonal antibody for detection. Human serum albumin (HSA) was
797 used as control protein. The normalized values are means \pm SD derived from four independent
798 experiments. The binding of FH was significantly different in the presence of 20 $\mu\text{g/ml}$
799 CFHR5 than in the presence of 20 $\mu\text{g/ml}$ HSA ($p = 0.0005$, unpaired t-test).

800 (B) The cofactor activity of FH bound to PTX3 for the cleavage of C3b in the presence and
801 absence of CFHR5 was measured by Western blot as described in Materials and methods.
802 Microplate wells were coated with 10 $\mu\text{g/ml}$ PTX3 and incubated with 50 $\mu\text{g/ml}$ FH with or
803 without 20 $\mu\text{g/ml}$ CFHR5, as indicated below the blot, then C3b and factor I (FI) were added
804 to each well. The molecular weight marker is indicated in the left, and the C3b chains and the
805 C3b α' -chain cleavage fragments are indicated in the right. The blot was developed using
806 HRP-conjugated C3-specific antibody that recognizes C3b and its fragments but not C3d. The
807 blot is representative of three independent experiments.

808

809 **Fig. 6. Interaction of CFHR5 with CRP.**

810 (A) FH, CFHR4A and CFHR5 were immobilized in microplate wells at 5 $\mu\text{g/ml}$. After
811 blocking, binding of 50 $\mu\text{g/ml}$ pCRP was measured in TBS containing 2 mM CaCl_2 and 1
812 mM MgCl_2 using a polyclonal CRP-specific antibody. Data are mean absorbance values + SD
813 from three experiments. ***, $p < 0.001$, one-way ANOVA.

814 (B) In parallel experiments, binding of 25 $\mu\text{g/ml}$ mCRP, generated from pCRP by
815 urea/chelation treatment, was measured in DPBS without Ca^{2+} using the same polyclonal anti-
816 CRP as in (A). Data are mean absorbance values + SD from three experiments. ***, $p <$
817 0.001, one-way ANOVA.

818 (C) To measure CFHR5 binding to surface-bound CRP, 5 $\mu\text{g/ml}$ CRP was immobilized in
819 microplate wells, which results in the generation of mCRP. Equimolar amounts (300 nM) of
820 CFHR5 and FH were added and their binding was detected using FH antibody. Data are mean
821 absorbance values + SD from four experiments. ***, $p < 0.001$, one-way ANOVA.

822 (D) The binding of 50 $\mu\text{g/ml}$ (~ 300 nM) FH to CRP, immobilized in microplate wells, was
823 measured by ELISA in the presence of increasing concentrations of CFHR5 (up to 20 $\mu\text{g/ml}$,
824 corresponding to ~ 300 nM CFHR5), using a FH-specific monoclonal antibody for detection.
825 Human serum albumin (HSA) was used as control protein. The normalized values are means
826 \pm SD derived from three independent experiments. The binding of FH was significantly
827 different in the presence of 20 $\mu\text{g/ml}$ CFHR5 than in the presence of 20 $\mu\text{g/ml}$ HSA ($p <$
828 0.0001, unpaired t-test).

829 (E) The cofactor activity of FH bound to CRP for the cleavage of C3b in the presence and
830 absence of CFHR5 was measured by Western blot. Microplate wells were coated with 10
831 $\mu\text{g/ml}$ CRP and incubated with 50 $\mu\text{g/ml}$ FH with or without 20 $\mu\text{g/ml}$ CFHR5, as indicated
832 below the blot, then C3b and factor I (FI) were added to each well. The blot was developed as
833 described for Fig. 5., and is representative of three independent experiments. The molecular
834 weight marker is indicated in the left, and the C3b chains and the C3b α' -chain cleavage
835 fragments are indicated in the right.

836

837 **Fig. 7. Interaction of CFHR5 with the extracellular matrix.**

838 (A) Microplate wells were coated with MaxGel or HSA, and incubated with normal human
839 serum. After washing, bound proteins were removed by adding SDS-sample buffer and
840 subjected to 10% SDS-PAGE and Western blotting using polyclonal anti-CFHR5. The blot is
841 representative of three experiments.

842 (B) Dose-dependent binding of recombinant CFHR5, added in the indicated concentrations, to
843 MaxGel and gelatin (both immobilized at 20 $\mu\text{g/ml}$) was measured by ELISA using

844 polyclonal antibody to human CFHR5. The data are means \pm SD of absorbance values derived
845 from four independent experiments.

846 (C) The binding of 50 $\mu\text{g/ml}$ (~ 300 nM) FH to MaxGel was measured by ELISA in the
847 presence of increasing concentrations of CFHR5 (up to 20 $\mu\text{g/ml}$, corresponding to ~ 300 nM
848 CFHR5), using a FH-specific monoclonal antibody for detection. Human serum albumin
849 (HSA) was used as control protein. The normalized values are means \pm SD derived from three
850 independent experiments. The binding of FH was significantly different in the presence of 20
851 $\mu\text{g/ml}$ CFHR5 than in the presence of 20 $\mu\text{g/ml}$ HSA ($p = 0.0027$, unpaired t-test).

852 (D) The cofactor activity of FH bound to MaxGel for the cleavage of C3b in the presence and
853 absence of CFHR5 was measured by Western blot. Microplate wells were coated with
854 MaxGel (diluted 1:30) and incubated with 50 $\mu\text{g/ml}$ FH with or without 20 $\mu\text{g/ml}$ CFHR5, as
855 indicated below the blot, then C3b and factor I (FI) were added to each well. The blot was
856 developed as described for Fig. 5., and is representative of three independent experiments.
857 The molecular weight marker is indicated in the left, and the C3b chains and the C3b α' -chain
858 cleavage fragments are indicated in the right.

859 (E) Binding of 5 $\mu\text{g/ml}$ PTX3 to MaxGel (white bars) and HUVEC-derived ECM (black bars)
860 preincubated with the indicated FH-family proteins at 20 $\mu\text{g/ml}$ (recombinant CFHR1 and
861 CFHR5) or 100 $\mu\text{g/ml}$ (recombinant FHL-1 and purified FH) concentration was measured by
862 ELISA as described for Fig. 2. The normalized values are means \pm SD derived from four
863 independent experiments. **, $p < 0.01$ and ***, $p < 0.001$, one-way ANOVA.

864

865 **Fig. 8. CFHR5 competes with FH in serum for binding to PTX3, CRP and MaxGel and**
866 **causes enhanced C3 fragment deposition.**

867 (A) Wells were coated with 10 $\mu\text{g/ml}$ PTX3, 10 $\mu\text{g/ml}$ CRP and MaxGel diluted 1:30 in
868 DPBS. After blocking, the wells were incubated for 30 minutes at 37°C with 25% heat
869 inactivated human serum with or without 0.5 μM CFHR5 and CFHR4A. FH binding was

870 detected with mAb A254 and the corresponding secondary antibody. The normalized values
871 are means + SD derived from three independent experiments. ***, $p < 0.001$, one-way
872 ANOVA.

873 (B) Nunc microplate wells were coated with 10 $\mu\text{g/ml}$ PTX3, 10 $\mu\text{g/ml}$ CRP and MaxGel
874 diluted 1:30 in DPBS. After blocking, 12.5% normal human serum (for PTX3 and CRP) and
875 25% normal human serum (for ECM) were added for 30 minutes at 37°C, with or without 20
876 $\mu\text{g/ml}$ CFHR5 or CFHR4A. Complement activation was detected by measuring C3 fragment
877 deposition using HRP-conjugated goat anti-human C3. Data represent mean absorbance
878 values + SD from three independent experiments. **, $p < 0.01$ and ***, $p < 0.001$, one-way
879 ANOVA.

880

881 **Fig. 9. Assembly of the alternative pathway C3 convertase on CFHR5.**

882 (A) Assembly of the C3bBb convertase on CFHR5. Recombinant CFHR4A, CFHR5 and, as
883 negative control, HSA were immobilized in microplate wells, followed by incubation with 10
884 $\mu\text{g/ml}$ C3b. The alternative pathway C3 convertase was built up by adding purified FB, factor
885 D and FP for 30 min at 37°C. The convertase was detected with polyclonal anti-FB antibody.
886 The data are mean absorbance values + SD derived from five independent experiments. **, p
887 < 0.01 and ***, $p < 0.001$, one-way ANOVA.

888 (B) Activity of the CFHR5-bound convertase was measured by adding 10 $\mu\text{g/ml}$ C3 to the
889 wells for 1 h at 37°C. C3a generation was measured by Quidel's C3a ELISA kit. Data are
890 mean absorbance values + SD from three experiments. ***, $p < 0.001$, one-way ANOVA.

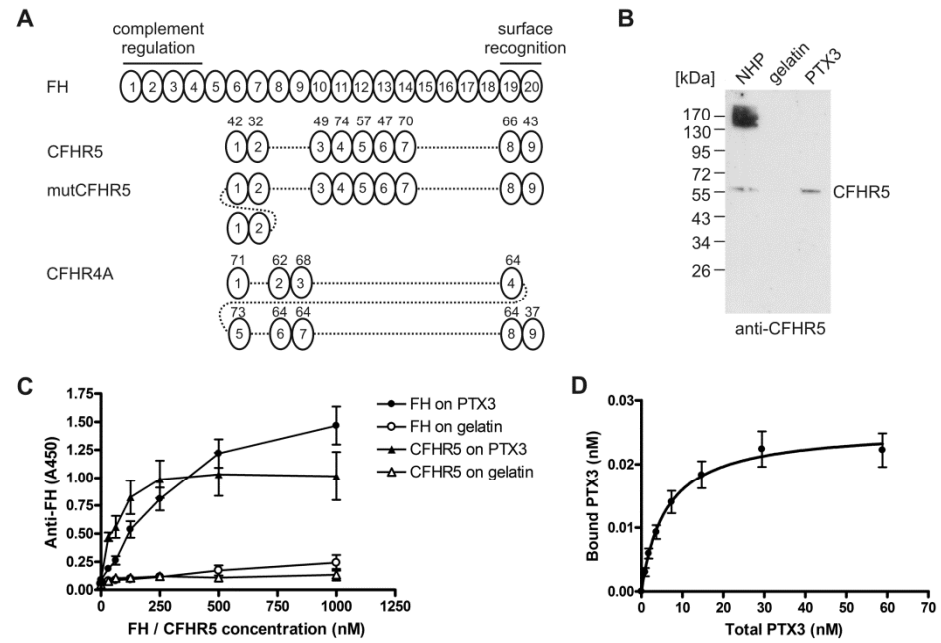
891

892 **Fig. 10. Complement activation by CFHR5.**

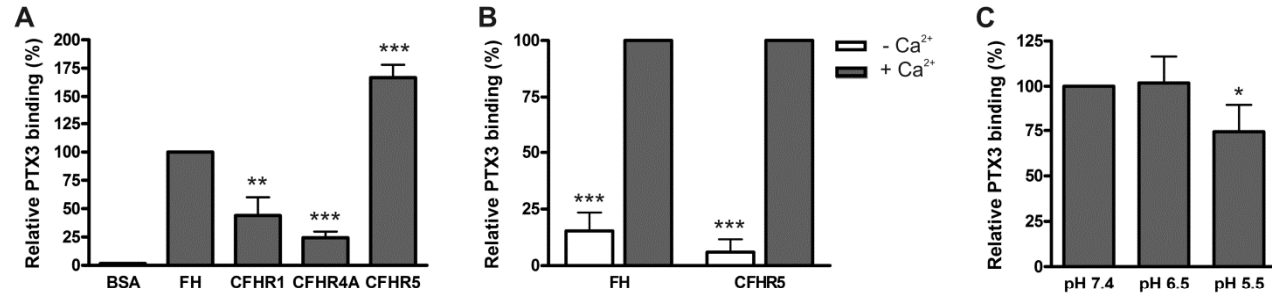
893 CFHR5 was immobilized on microplate wells and incubated with 10% normal human serum
894 in 5 mM Mg^{2+} -EGTA buffer to allow only alternative pathway activation or with 10% serum
895 containing 5 mM EDTA to inhibit complement activation. Deposition of (A) C3b, (B) factor

896 B (FB) and (C) properdin (FP) was detected using the corresponding antibodies. Immobilized
897 CFHR4A was used as positive control and factor H and human serum albumin (HSA) were
898 used as negative controls. The data are means + SD derived from three independent
899 experiments. **, $p < 0.01$ and ***, $p < 0.001$, one-way ANOVA.

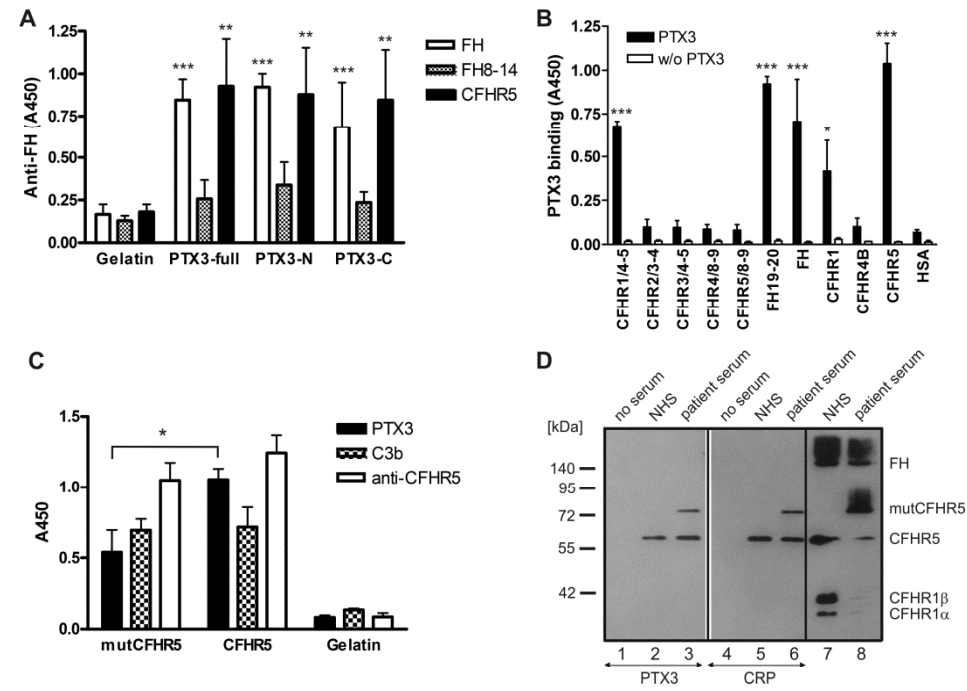
Csincsi et al., 2014
Figure 1



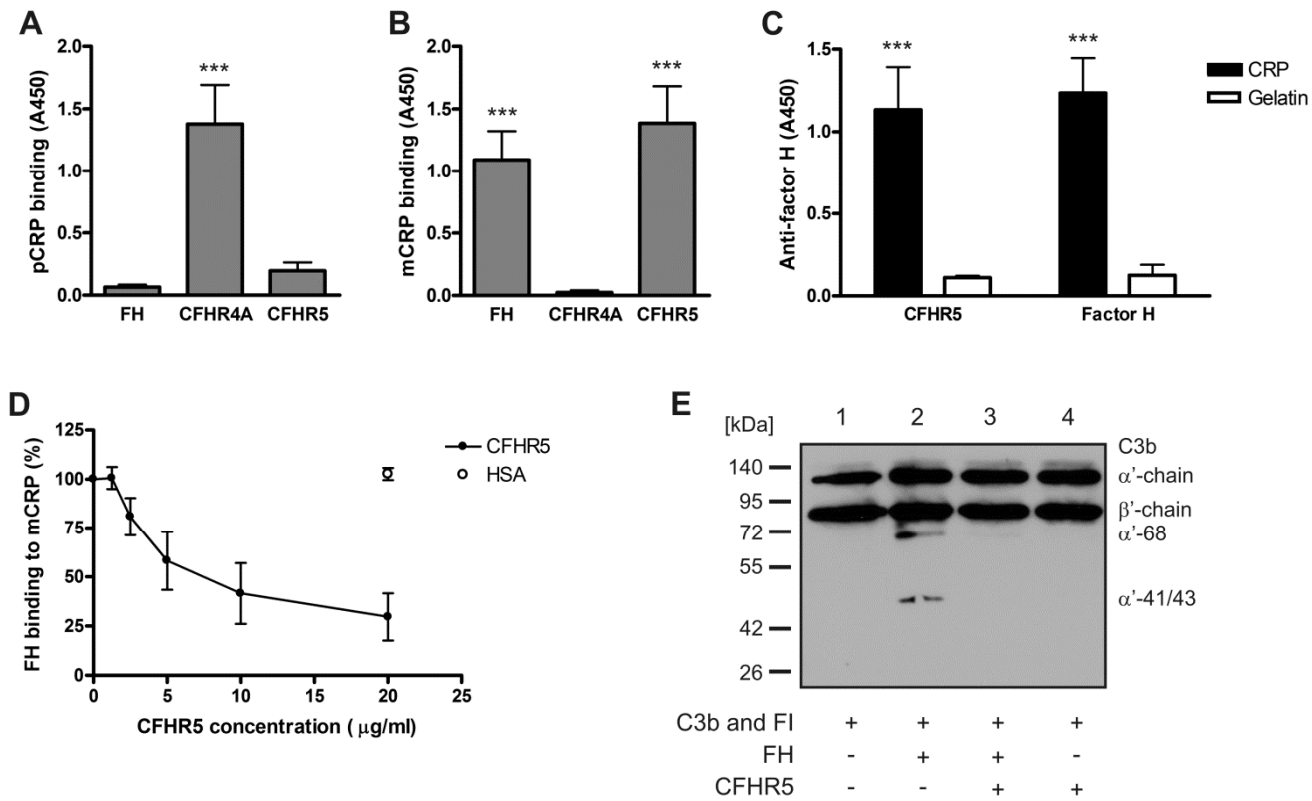
Csincsi et al., 2014
Figure 2



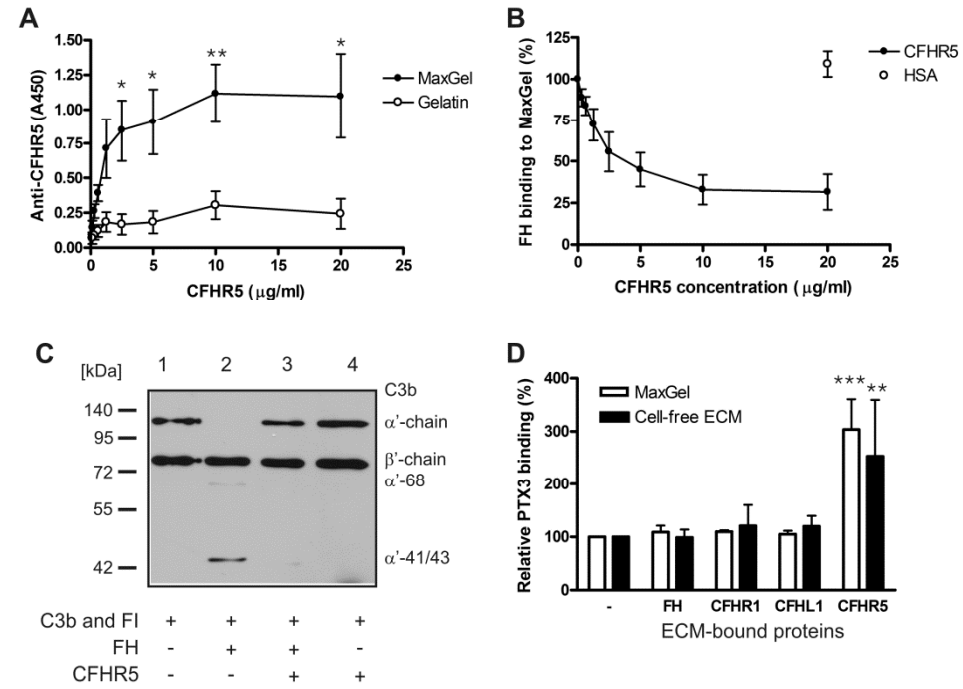
Csincsi et al., 2014
Figure 3



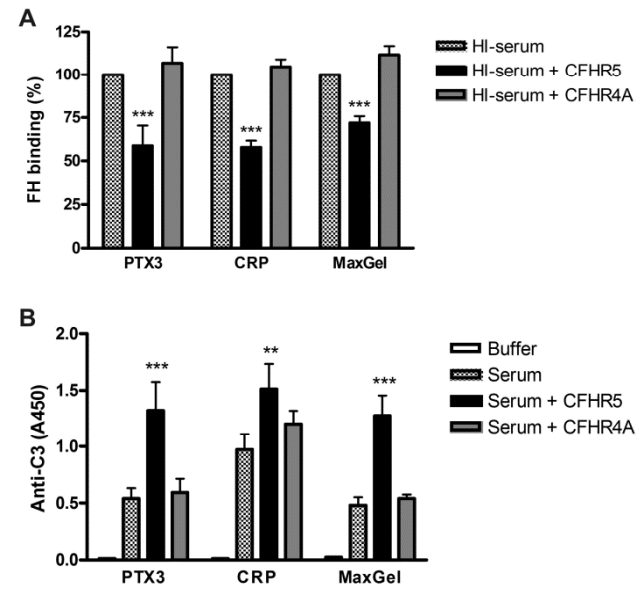
Csincsi et al., 2014
Figure 6



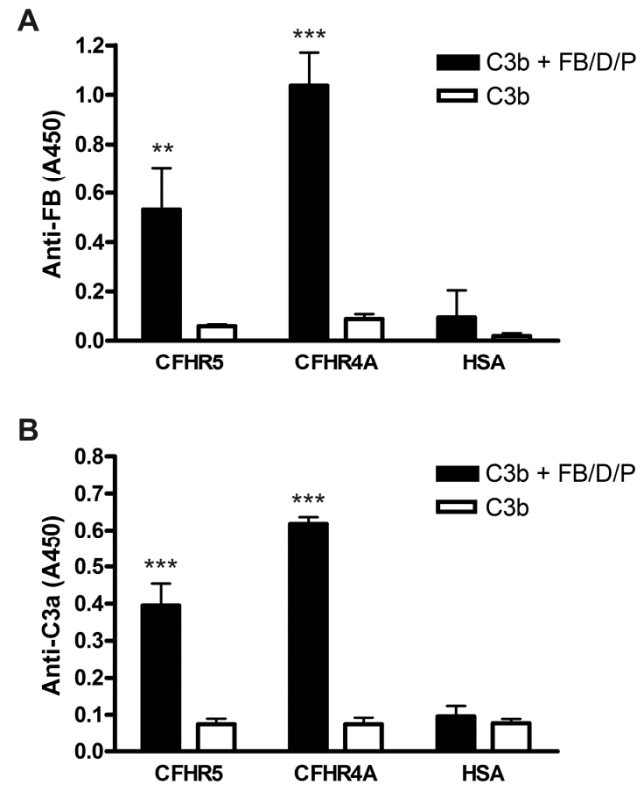
Csincsi et al., 2014
Figure 7



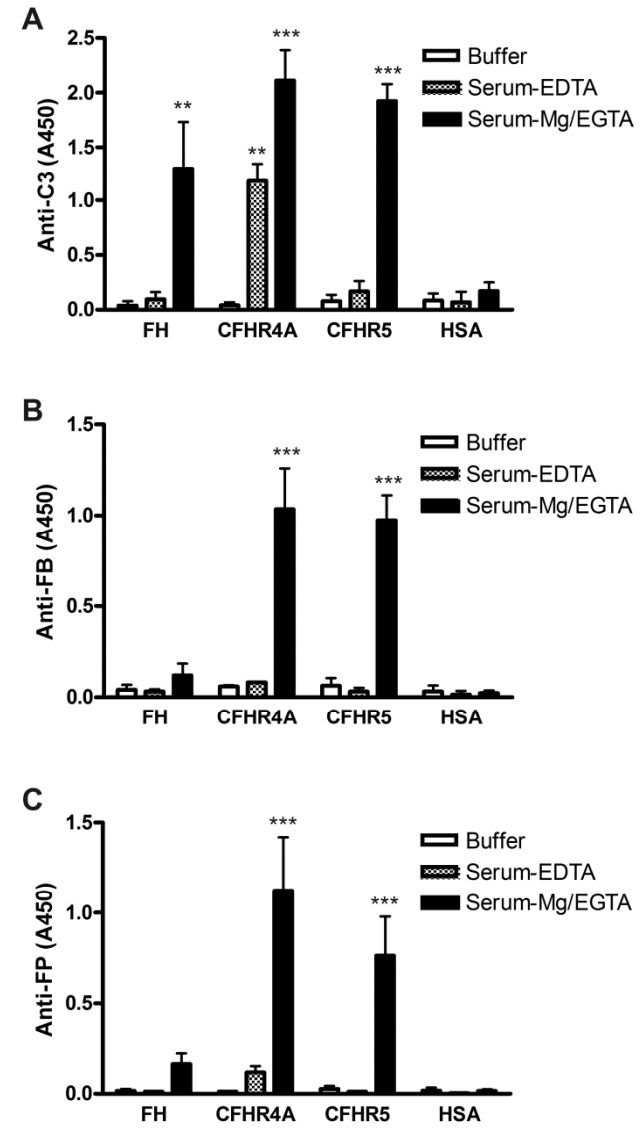
Csincsi et al., 2014
Figure 8



Csincsi et al., 2014
Figure 9



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Figure 10



Supplemental Figure 1

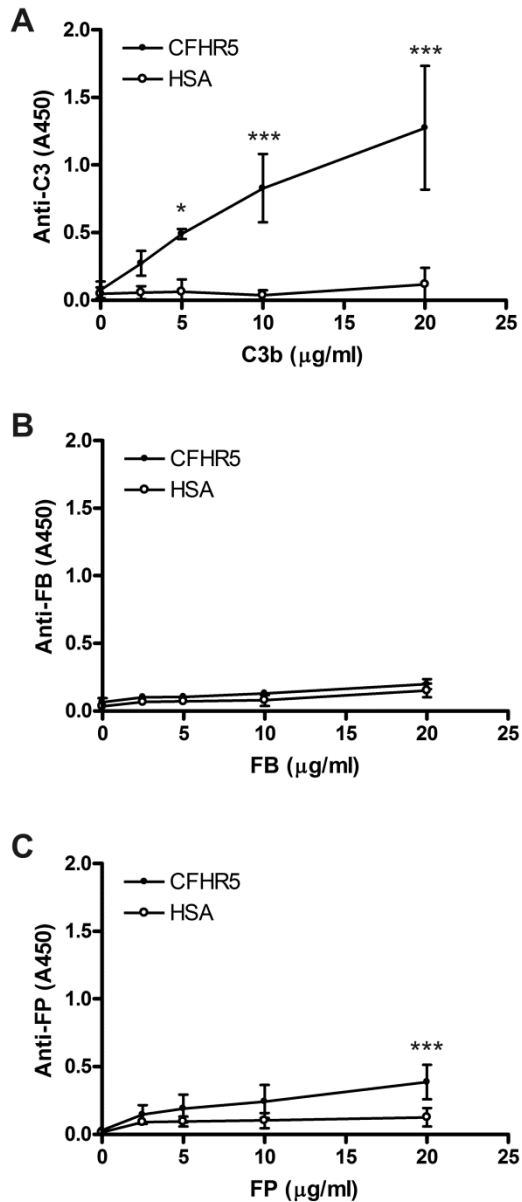


Figure S1. Binding of purified C3b, factor B (FB) and properdin (FP) to CFHR5.

Dose-dependent binding of C3b (A), FB (B) and FP (C), added in the indicated concentrations, to CFHR5 and HSA, both immobilized at 5 µg/ml, was measured by ELISA using the corresponding antibodies. Data are means ± SD from at least three independent experiments. *, $p < 0.05$ and ***, $p < 0.001$ (two-way ANOVA).