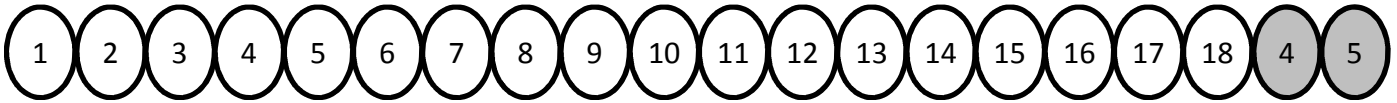
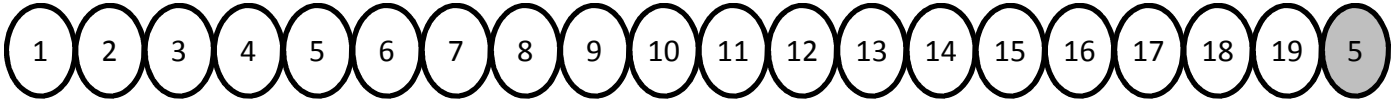
Supplementary figure1

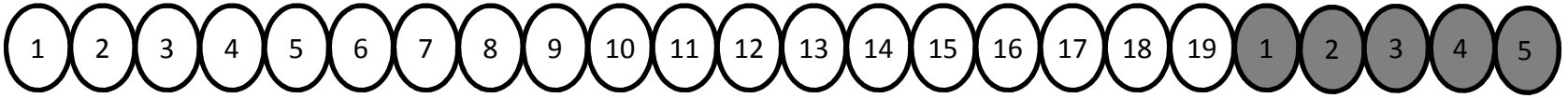
Factor H hybrids



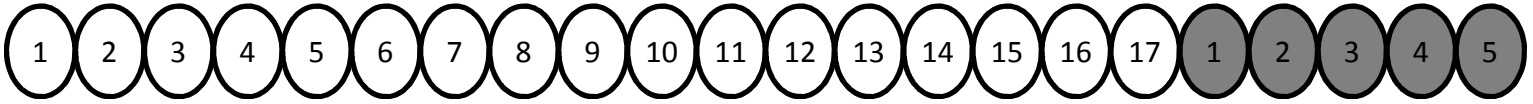
Venables et al



Maga et al



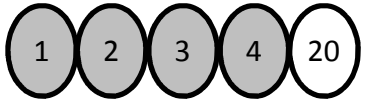
Francis et al



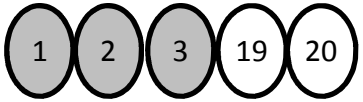
Challis et al

Factor H related 1 hybrids

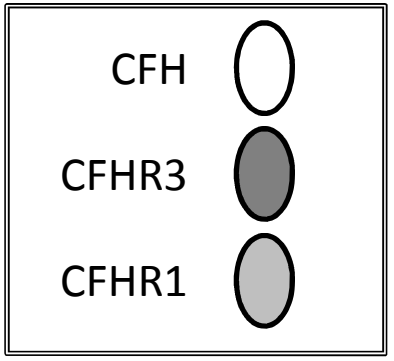
"reverse" hybrids



Valoti et al

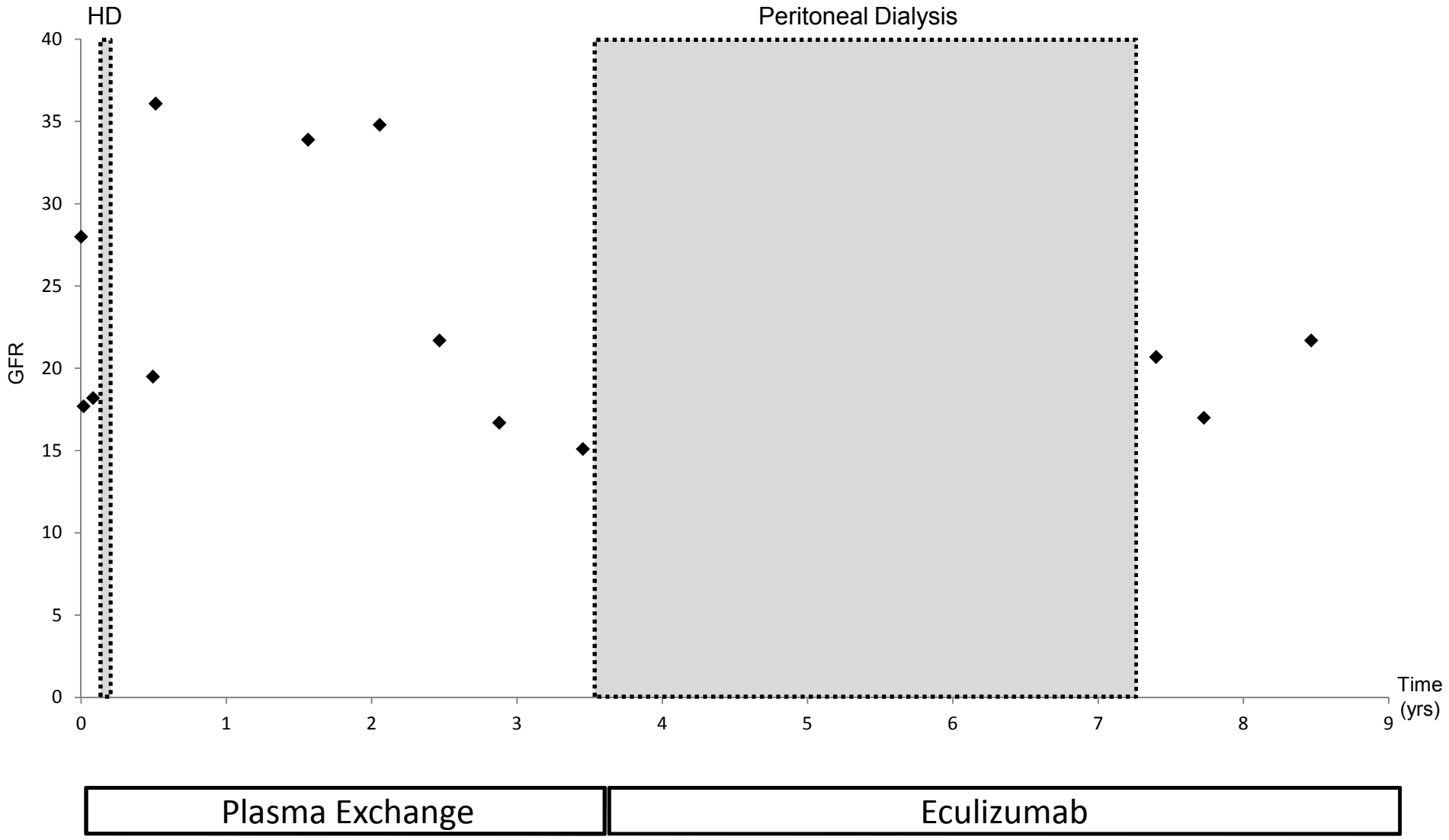


Eyler et al



Supplementary Figure 1 Factor H and Factor H related 1 hybrids described in aHUS

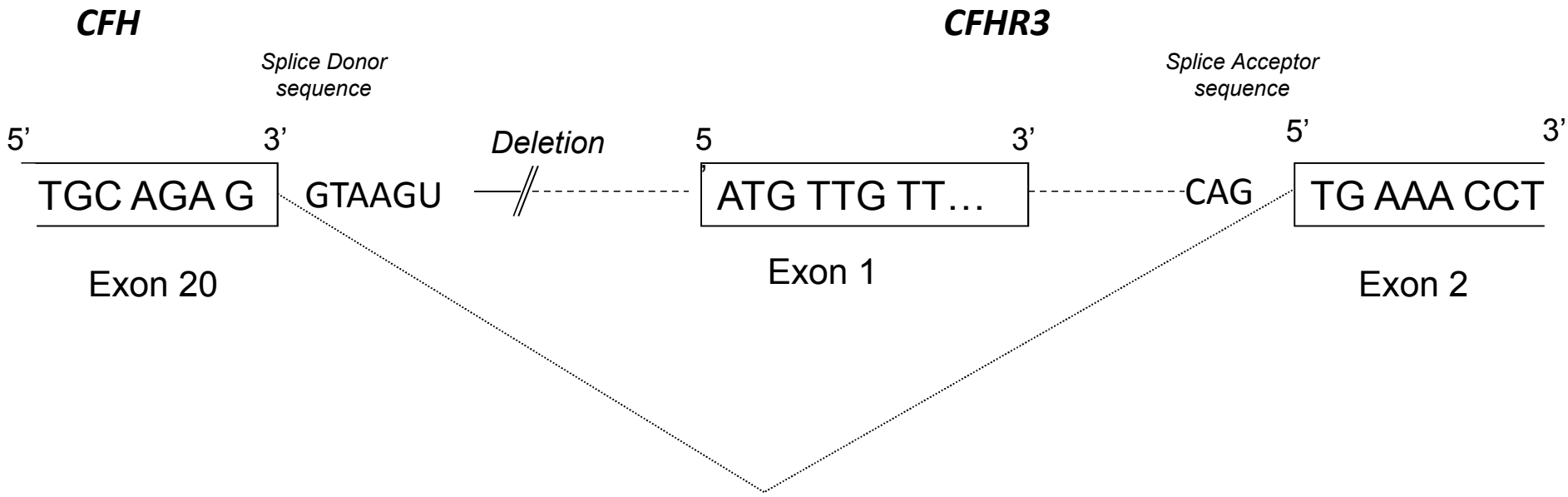
Supplementary figure 2



Supplementary Figure 2- Graph of eGFR against time post presentation. Haemodialysis (HD) was required at the time of the second presentation with aHUS. Over the next ~3.5 years he received weekly plasma exchange with intensified treatment during periods of clinical relapse (>500 PE treatments). After ~3.5 yrs peritoneal dialysis was instituted due to hyperkalaemia, with a creatinine consistently above 250 µmol/L. Plasma exchange was replaced with Eculizumab after one month on dialysis. After ~3.5 years peritoneal dialysis was stopped and creatinines have been in the range 190-230 µmol/L. Since initiation of Eculizumab there have been no episodes of clinical relapse of aHUS.

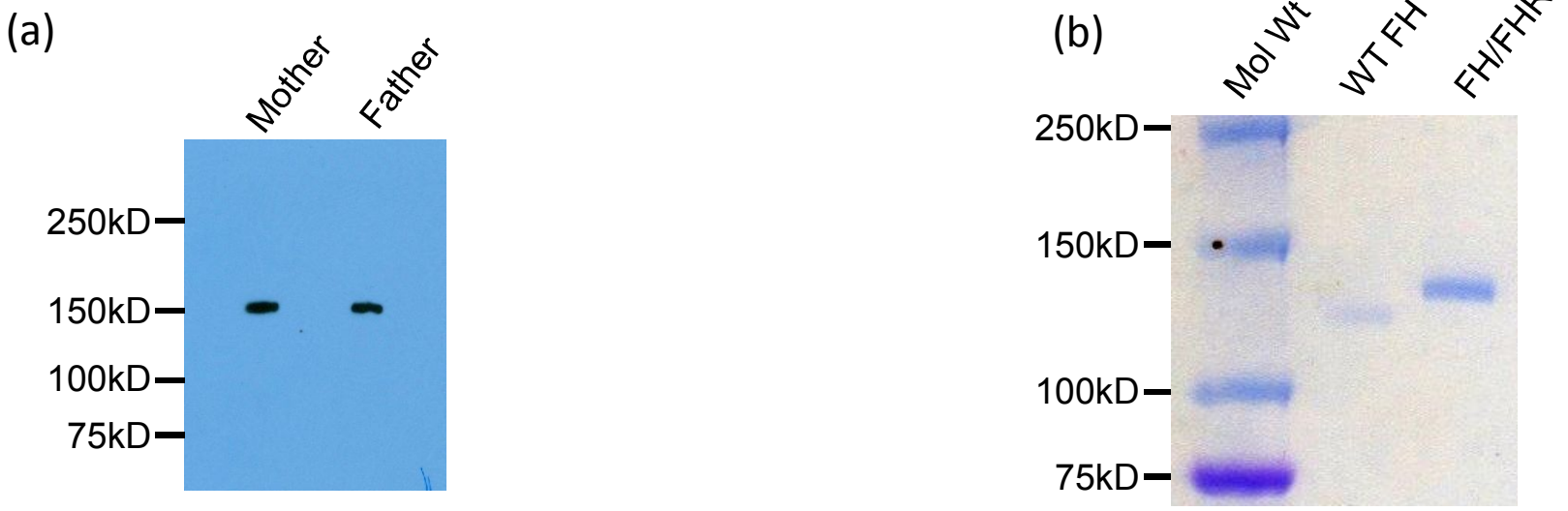
Supplementary figure 3

CFH/CFHR3 splicing



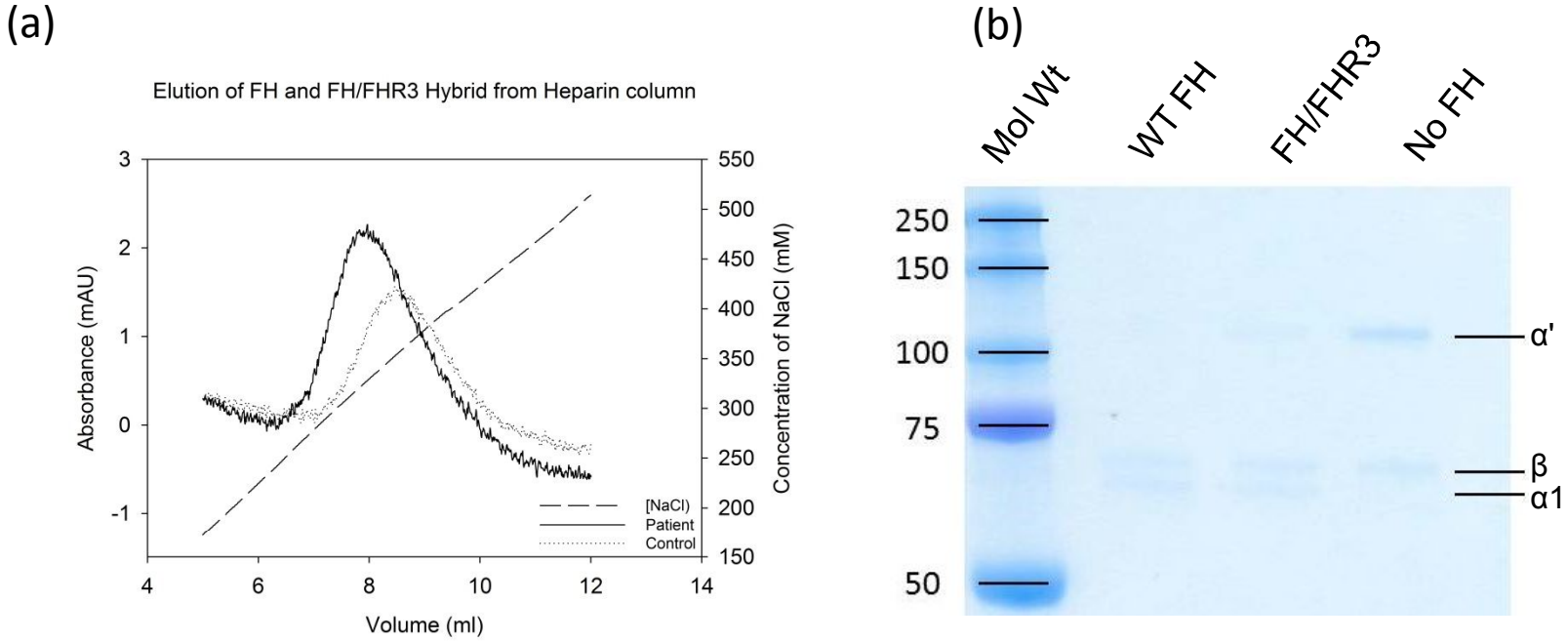
Supplementary Figure 3 Splicing of *CFH* exon 20 into *CFHR3* exon 2

Supplementary figure 4



Supplementary Figure 4 (a) Reduced Western blot of parental serum using a polyclonal anti FH (Calbiochem) demonstrating a single FH species with no additional bands. The absence of FH/FHR3 hybrid or FH/FHR3 degradation product in either parent is consistent with the genetic data confirming a de novo deletion. (b) Non-reduced SDS PAGE demonstrating purification of the FH/FHR3 hybrid protein. The FH/FHR3 hybrid protein was purified from serum using affinity chromatography with an MBI6 mAb followed by gel filtration. A WT FH control known to carry the tyrosine amino acid at position 402 was purified in an identical manner.

Supplementary figure 5



Supplementary Figure 5 (a) Heparin binding profile of purified FH/FHR3 hybrid protein. Purified proteins from patient (FH/FHR3) and control FH (402Y) were bound to a HiTrap heparin column and eluted with NaCl. The FH/FHR3 hybrid (black) eluted at **325mM** and FH (grey) at **357mM**, indicating reduced binding of the hybrid. Unlike the FH/FHR3 hybrid reported here, the previously reported FH/FHR3 hybrid (Francis *et al.*) had increased heparin binding. This likely reflects the additional loss of CCPs18-19 in the Challis *et al.* hybrid. In both cases there was impaired cell surface decay acceleration and cofactor activity. Dashed line indicates salt gradient. (b) Fluid phase cofactor assay. Equimolar concentrations (50nM) of WT FH and FH/FHR3 were used. In the fluid phase factor I cleaves C3b, in the presence of the FH/FHR hybrid, to iC3b as seen by the generation of the α1 band at equivalent levels to WT FH.

Supplementary methods

CFH and *CFHR5* MLPA probe hybridization sequences

Gene	Exon	Left Primer (5' → 3')	Right Primer (5' → 3')
CFH	1	CACAATTCTTGGAAGAGGAGAACTG	GACGTTGTGAACAGAGTTAGCTGG
	2	TGACAGGTTCCTGGTCTGACCAA	ACATATCCAGAAGGCACCCAGGCT
	3	CGTTTTAGAAAGGCCCTGTGGACATCCTGGA	GATACTCCTTTTGGTACTTTTACCCTTACAGGAGG
	4	GGTATCAATTGCTAGGTGAGATTAATTACCGTGA	ATGTGACACAGATGGATGGACCAATGATATTCCTATATGTGAAGGTAG
	5	GTGCAATGGAACCAGATCGGGAATA	CCATTTTGGACAAGCAGTACGGTTTG
	6	CATGGGTTATGAATACAGTGAAAGAGGAGA	TGCTGTATGCACTGAATCTGGATGGCGTCCGTTGCCT
	7	GGTGACTACTCACCTTTAAGGATTAAACACAGAACTGGAGATGAAATCACGTAC	CAGTGTAGAAATGGTTTTTATCCTGCAACCCGGGGAAATACAGC
	8	CACATTCATTGCACACAAGATGGA	TGGTCGCCAGCAGTACCATGCCTC
	9	GTACAGGGTAAATCTATAGAC	GTTGCCTGCCATCCTGGCTACG
	10	CAGAATGGGAAATGCTAATTCAGCTCCTCCAGGCAGCCCAATGGG	GCTGGTGGCTTTGAGATTATTAAACTCTTTCTCTGCTGC
	11	TGAAACATCAGGATCAATTACATGTGG	GAAAGATGGATGGTCAGCTCAACC
	12	GAATGCCAGAACTAAAAATGACTTCACATGGTTTAAG	CTGAATGACACATTGGACTATGAATGCCATGATGG
	13	ACCTAATTCCGTTCAGTGCTACC	ACTTTGGATTGTCTCCTGACCTCC
	14	CAGTGAAGTGGTGGAATATTATTGCAATC	CTAGATTTCTAATGAAGGGACC
	15	GGAGGAGAGTACCTGTGGAGATATACCTGAACTTGAACATGG	CTGGGCCCAGCTTTCTTCCCCTCCTTATTACTATGG
	16	GGAAAAGAAGGATGGATACACACAGTCTG	CATAAATGGAAGATGGGATCCAGAAGTGAACTGC
	17	GGCACAAATACAATTATGCCCACCTCCACCTCAGATTCC	CAATTCTCACAATATGACAACCACACTGAATTATCG
	18	CCATGTTCACAACCACCTCAGATAGAACAC	GGAACCATTAATTCATCCAGGTCTTCAC
	19	GGAATTGATGGGCCTGCAATTGCAAAATGCTTAG	GAGAAAAATGGTCTCACCCTCCATCATGC
	20	GGATGGAGCCAGTAATGTAACATG	CATTAATAGCAGATGGACAGGAAGGC
	21	CTTATATAGTGTCGAGACAGATGAGTAAATATCCATCTGGTGAG AGAGTACGTTATCAATGTAGGAGC	CCTTATGAAATGTTTGGGGATGAAGAAGTGATGTGTTTAAATGGA AACTGGACGGAAC
	22	CGTAAGTACTTTAATATTCACGTGGCTG	GAAAAATCTCTGTGATGAGTCTGATATTTCACTGTTTG
	23	GGACAGCCAAACAGAAGCTTTATTC	GAGAACAGGTGAATCAGTTGAATTTGTG

Gene	Exon	Left Primer (5' → 3')	Right Primer (5' → 3')
CFHR5	1	GAGACTACCAAGCATGTTGCTCTTATTCAGTGTAATC	CTAATCTCATGGGTATCCACTGTTGGGGGAGAAGG
	2	CCCAGGAACACTTTGTGATTTTCCAAAAATACACCATG	GATTTCTGTATGATGAAGAAGATTATAACCC
	3	CAAATTATTTGCAACACAGGATACAGC	CTTCAAAACAATGAGAAAAACATTTCGTGTG
	4	GTCATGTTCCAATTTTAGAAG	CCAATGTAGATGCTCAGCC
	5	CCACCTCCTCAACTCTCCAATGGTGAAGTTAAG	GAGATAAGAAAAGAGGAATATGGACAC
	6	GGATACATACCTGAACTCGAGTACGGTTATGTTCAGCCGTCTGTC	CCTCCCTATCAACATGGAGTTTCAGTCGAGGTGAATTGC
	7	CAGATGTTCAGACATCTTCAGATACAG	GCACTCAGTCTGTATAAACGGG
	8	GGGAACAATTCTGCCCACCGCCACCTCAGATACCTAATG	CTCAGAATATGACAACCACAGTGAATTATCAGGATGG
	9	CCCATTATCAGTATATCCTCCAGGGTCAACAGTGACGTACCGTTGC	CAGTCCTTCTATAAACTCCAGGGCTCTGTAACTGTAACATGC
	10	GCTGTTGAATTCCAGTGTAAATTCC	CACATAAAGCGATGATATCATCACCACC

In house MLPA probe hybridisation sequences for *CFH* and *CFHR5*.

Mass Spectrometry

The eluted material was pooled and concentrated to 30uL. This was then run on a 6% SDS PAGE, 3 bands identified by coomassie staining, were excised from the gel as indicated in figure 4a. Proteins were reduced with 10 mM DTT (Sigma) in 100 mM NH_4HCO_3, alkylated with 50 mM iodoacetamide (Sigma) in 100 mM NH_4HCO_3 and digested in gel with 230ng modified trypsin (Promega) in 50 mM NH_4HCO_3, 1 mM $CaCl_2$. Peptides were extracted from the gel pieces and the digests were analysed by LCMSMS using a Dionex U3000 nano-HPLC system (Thermo) coupled to an Orbitrap LTQ XL(ETD) (Thermo) mass spectrometer. Peptides were separated on a 25 cm x 75 µm PepMap column (Thermo) using a 37 min water acetonitrile gradient (0.05% formic acid). Precursor ions were detected in positive mode at 350-1600 m/z with a resolution of 60,000 (at 400 m/z) and a fill target of 500,000 ions and a lockmass was set to 445.120023 m/z. The five most intense ions of each MS scan (with a target value of 10,000 ions) were isolated, fragmented and measured in the linear ion trap. Peaklists in the Mascot generic format (*.mgf) were generated using msconvert (proteowizard.sourceforge.net (Kessner *et al.*, 2008)) and the ensembl human genome (GrCh37.66) was searched using X!Tandem and the gom interface (version 2013.09.01.1(Craig and Beavis, 2004)) with carbamidomethyl set as a fixed modification and Met oxidation set as a variable modification. Two refinement steps were included in the search to include deamidation and methylation artefacts as well

as protein phosphorylation, acetylation, dehydration of Thr and Ser and carbamidomethylation of Gln, His, Asp, Glu and Lys. The protein level false positive rate (as defined in: http://wiki.thegpm.org/wiki/False_positive_rate) in each band was below 1%.

Preparation of serum depleted of factor B and FH

Normal human serum depleted of factor B (FB) and FH (NHSΔBΔH) was prepared by flowing normal human serum over immobilised monoclonal antibody to FB (JC1) and monoclonal antibody to FH (OX24), on separate 1 mL HiTrap NHS HP columns in series. NHSΔBΔH was collected and pooled for use in the cell surface decay and co-factor haemolytic assays.

Cell Surface Decay Haemolytic assay

FH and FH/FHR3 hybrid were purified as described earlier. The concentration of purified proteins was measured at 280nm. Molarities were calculated using Beer's Law: A= ε L c, (where A= Absorbance, ε= Molar extinction coefficient, L= light path and c= Concentration). The extinction coefficients were determined, using ProtParam (Gasteiger *et al.*, 2005), assuming all pairs of cysteines form cystines. The leader sequences for FH (MRLLAKIICLMLWAICVA) and FHR3 (MLLLINVILTLWVSCANG) were excluded from the analysis. The extinction coefficient for FH was 246800 $M.cm^{-1}$. To calculate the extinction coefficient for FH/FHR3, the first 1026 amino acids from FH and the 312 amino acids from FHR3 were analysed, producing an ε of 272170 $M.cm^{-1}$.

Sensitised Sheep erythrocytes (EA) were prepared by incubating 2% (vol/vol) sheep erythrocytes [TCS biosciences] and 1:4000 anti-sheep stromal antibodies [Sigma] in Complement Fixation Diluent (CFD) [Oxoid], at 37°C for 30 minutes. After incubation the mixture was washed using CFD and the cells resuspended to 2% (vol/vol).

C3b was then deposited onto EA (EAC3b) by the addition of 4% NHSΔBΔH and 6µg/ml *Ornithodoros moubata* complement inhibitor (OmCI) in CFD to an equal volume of 2% (vol/vol) EA in CFD and incubated at 37°C for 20 mins. EAC3b were

washed with Alternative Pathway Buffer (AP buffer) [5mM sodium barbitone, pH 7.4, 150 mM NaCl, 7 mM $MgCl_2$, 10mM EGTA] and resuspended to 2%.

A convertase was formed on the sheep erythrocytes by adding 2% (vol/vol) EAC3b cells to an equal volume of AP Buffer, containing 40µg/ml Factor B [prepared in house] and 0.2 µg/ml Factor D [Complement Technology] and incubating for 15 minutes at 37°C. 100µL of this convertase mixture was then added to 50 µL (1.24nM – 50nM) FH/FHR3 hybrid and FH (purified from patient or control), diluted in PBS/20nM EDTA and left at room temperature for 15 minutes, to allow decay acceleration to take place. 50µL 4% (vol/vol) NHSΔBΔH in PBS/20mM EDTA was then added and incubated at 37°C for 30 minutes to initiate lysis. To determine the amount of lysis, cells were pelleted by centrifugation at 1200rpm for 3 minutes and haemoglobin release was measured at 420nm (A_{420}). All values were blank-corrected, using the mean A_{420} of no serum controls. Maximal lysis was achieved by adding NHSΔBΔH to no FH wells (buffer only). Percentage of inhibition of lysis in the presence of increasing concentrations of FH was defined as: (A_{420}[buffer only]-A_{420}[FH]) / A_{420}[buffer only] *100%.

Cell Surface Co-factor assay

FH and FH/FHR3 hybrid were purified as above. EAC3b was prepared as above. 2% EAC3b in AP buffer were then incubated with an equal volume of AP buffer containing a concentration range (0.6 to 50nM) of FH or FH/FHR3 hybrid and 2.5µg/ml Factor I (Complement Technology) for 8 minutes at 25°C. After three washes in AP buffer, a 50 µl aliquot of cells (2%) was mixed with 50 µl AP buffer containing FB (40 µg/ml) and FD (0.2 µg/ml) and then incubated for 15 minutes at 25°C to form AP convertase on the remaining C3b. Lysis was developed by adding 50 ml 4% NHSΔBΔH in PBS/20mM EDTA and incubating at 37°C for 30 minutes. Percentage of inhibition from lysis was calculated by the formula (A_{420}[buffer only]-A_{420}[fH])/A_{420}[buffer only]*100%

Fluid phase co-factor Assay

Cofactor activity for factor I–mediated proteolytic cleavage of C3b in the fluid phase was analysed. 3µl of C3b (5.68 µM) (Comptech), 4.5 µl of factor I (0.14µM) (Comptech), and 5 µL of WT FH or FH/FHR3 hybrid (0.15 µM) were made up to a final volume of 15 µl in PBS. A negative control using PBS instead of FH was also performed. The mixture was incubated at 37°C for 60 minutes and the reaction was stopped by the addition of 2X lamelli reducing buffer to a final volume of 30 ml and heated to 95°C for 5 minutes. The products of the reaction were then separated by SDS-PAGE and visualised by Coomassie stain.

Factor H Antibodies used for protein detection in figure 3

Antibody	Epitope	Source	Reference
Ox24	CCP5	Sigma-Aldrich	*J Exp Med.(1998) 87:451*
L20/3	CCP19-20	Santa Cruz Biotechnology	*J Immunol.(2012)189:3528*
MBI6	CCP7 402Y	In house	*Kidney Int. (2010) 78:782*
MBI7	CCP7 402H	In house	*Kidney Int. (2010) 78:782*

References

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Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S.e., Wilkins, M., Appel, R. and Bairoch, A. (2005) 'Protein Identification and Analysis Tools on the ExPASy Server', in Walker, J. (ed.) *The Proteomics Protocols Handbook*. Humana Press, pp. 571-607.

Kessner, D., Chambers, M., Burke, R., Agus, D. and Mallick, P. (2008) 'ProteoWizard: open source software for rapid proteomics tools development', *Bioinformatics*, 24(21), pp. 2534-6.