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A De Novo Deletion in the Regulators of Complement Activation Cluster Producing a Hybrid Complement Factor H/Complement Factor H–Related 3 Gene in Atypical Hemolytic Uremic Syndrome

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Abstract

The Regulators of Complement Activation cluster at chromosome 1q32 contains the genes encoding the complement regulator factor H and five factor H related proteins. This is an area of the genome that arose from several large genomic duplications and these low copy repeats can cause genome instability in this region. Genomic disorders affecting these genes have been described in atypical haemolytic uraemic syndrome, arising commonly through non-allelic homologous recombination (NAHR). We describe a novel \textit{CFH/CFHR3} hybrid gene secondary to a \textit{de novo} 6.3kb deletion which arose through microhomology mediated end joining rather than NAHR. We confirm a transcript from this hybrid gene and demonstrate a secreted protein product. Lacking the recognition domain of factor H, this hybrid protein has impaired cell surface complement regulation. That this arose as a \textit{de novo} event suggests that this is a dynamic area of the genome where further genomic disorders may arise.

Key words

Complement factor H, atypical haemolytic uraemic syndrome, non-allelic homologous recombination, microhomology mediated end joining, complement.
The genes encoding complement factor H (CFH) and the factor H related proteins (CFHRs1-5) reside in a 360-kb region in the Regulators of Complement Activation (RCA) cluster on chromosome 1q32 (figure 1a)\(^1\). This is an area of the genome that arose from several large genomic duplications\(^2, 3\) and these low copy repeats can cause genome instability in this region.

Mutations in CFH are the most common genetic predisposition to the thrombotic microangiopathy, atypical haemolytic uraemic syndrome (aHUS)\(^4\). The majority of mutations in CFH occur at the C-terminal end responsible for host polyanion binding\(^5-7\). Several of these CFH mutations (e.g. S1191L; V1197A) have been demonstrated to be gene conversion events between CFH and CFHR1\(^8\). Non-allelic homologous recombination (NAHR) involving CFH and CFHR1 can result in CFH/CFHR1 hybrid genes (supplementary figure 1)\(^9, 10\). A single family with a hybrid CFH/CFHR3 gene (factor H protein (FH) complement control protein modules (CCPs) 1-19 and FHR3 CCPs1-5) has been reported to arise through microhomology mediated end joining (MMEJ)\(^11\) (supplementary figure 1). These genetic variants have all been demonstrated to impair C3b/host polyanion binding on host cells thus impairing local complement regulation\(^5, 8, 11\).

Recently a “reverse” CFHR1/CFH hybrid gene arising through NAHR has been described in aHUS. This encodes a FHR1 where the C terminal CCPs are replaced by the C terminal CCPs of FH\(^12, 13\). Unlike previously reported hybrid proteins, this does not impair FH cell surface binding, but instead acts as a competitive inhibitor of FH\(^13\).

In this study we report a novel CFH/CFHR3 hybrid gene arising through MMEJ which impairs cell surface complement regulation.

An 8 month old boy presented with a diarrhoeal illness; on admission creatinine was 52 µmol/L and urea 11.1 µmol/L. A blood film demonstrated a microangiopathic haemolytic anaemia (MAHA) and lactate dehydrogenase (LDH) was 5747U/L. Stool culture was positive for *E.Coli* O157:H7 and a diagnosis of Shiga toxin-producing *E.coli* (STEC) HUS was made. He did not require renal replacement therapy and did not receive plasma exchange. He was discharged 2 weeks later with a creatinine of 107 µmol/L. He was readmitted 2 weeks later with an upper respiratory tract infection. Creatinine on readmission was 141 µmol/L, urea 20.6 µmol/L, LDH 2398 U/L, platelets 128 x 10\(^9\)/L with evidence of a MAHA on blood film. This relapse suggested a diagnosis of aHUS rather than STEC HUS. Serum complement levels were within the normal range: C3 1.05g/L (0.68-1.80g/L); C4 0.30g/L (0.18-0.60g/L); and FH 0.51g/L.
(0.35-0.59g/L). He commenced plasma exchange and required 3 sessions of haemofiltration. A renal biopsy confirmed a thrombotic microangiopathy (figure 2). Creatinine returned to a baseline of ~100 μmol/L. He was treated with weekly plasma exchange for three and a half years prior to starting Eculizumab on which he has been maintained for three years. Creatinine is currently 200 μmol/L and there have been no further episodes of aHUS while on treatment with Eculizumab (supplementary figure 5).

Sanger sequencing of aHUS-associated genes (CFH, CFI, CFB, CD46, C3, THBD and DGKE) did not reveal any mutations, although heterozygosity was found for the common Y402H polymorphism (rs1061170) in CFH. Multiplex ligation-dependent probe amplification (MLPA) of CFH and the CFHRs revealed a deletion in the CFH gene (figure 1b). To define the extent of the deletion and to define the breakpoint Sanger sequencing of genomic DNA was undertaken. This showed a 6.3kb deletion extending from CFH intron 20 to the CFH 3’ intergenic region incorporating exons 21, 22 and 23 of CFH (figure 1c). Directly flanking the breakpoint was a 7bp region of microhomology (figure 1d). The deletion was not detected in either parent suggesting that this was a de novo event.

We hypothesised that the loss of CFH exons 21, 22, 23 and the 3’UTR of CFH would lead to aberrant splicing. In silico analysis showed that the next acceptor splice site, following the CFH exon 20 donor site, was 5’ of CFHR3 exon 2, thus potentially producing a transcript for a hybrid CFH/CFHR3 gene (supplementary figure 2). Messenger RNA for this putative hybrid CFH/CFHR3 gene was confirmed by amplifying patient and control cDNA with CFH and CFHR3 specific primers. This demonstrated a product in the patient and not in healthy controls (figure 1e).

To confirm that this hybrid transcript led to the synthesis and secretion of a hybrid protein, western blotting was performed using a series of epitope-defined anti-FH monoclonal antibodies (figure 3a). An initial blot probed with OX24, a FH CCP5-specific antibody, demonstrated, in addition to FH, two additional bands only in the patient (figure 3b). The species at ~160kDa was consistent with the predicted intact hybrid FH/FHR3. A species at ~120kDa, was hypothesised to be a degradation product. No abnormal bands were detected in either parent (supplementary figure 3a). A FH C-terminal specific antibody (L20/3) did not reveal additional aberrant bands, indicating that these protein species lacked FH CCP20 (Fig 3c). Genotyping showed the patient to be heterozygous for the Y402H polymorphism in FH. This allowed the use of monoclonal antibodies specific for histidine or tyrosine in CCP7 of FH. The
normal allele’s product reacted to the histidine specific mAb (MBI7) and gave a single band of ~150kDa consistent with FH. The hybrid allele reacted to the tyrosine specific mAb (MBI6), demonstrating a doublet at ~160Kda (figure 3d). FHR3 is known to be alternately glycosylated\textsuperscript{15-17} and we hypothesise that the doublet is due to an alternately glycosylated hybrid protein. Additionally it can be seen that the presumed degradation product at 120kDa, is only seen using the tyrosine specific mAb, consistent with this only arising from the hybrid protein.

To confirm these findings, FH species were purified from serum using affinity chromatography with an OX24 mAb. These were separated by a 6% SDS PAGE prior to trypsin digestion. Mass spectrometry was then carried out on these three purified bands (figure 4a). Peptide species identified from the ~160kDa band confirmed that this was a hybrid FH/FHR3 protein. Protein fragments from the 150kDa band were consistent with FH. Fragments from CCPs 5, 6, 8, 9 and 14 of FH were seen in the band at ~120kDa. Coverage at CCP7 was insufficient to determine whether there was a tyrosine or histidine at position 402. The western blot analysis and mass spectrometry data together show that breakdown products from only the hybrid protein are seen in serum.

To examine the functional significance, the FH/FHR3 hybrid protein was purified from serum using affinity chromatography with an MBI6 mAb followed by gel filtration (supplementary figure 3b). The FH/FHR3 hybrid protein displayed both impaired cell surface decay acceleration (figure 4b) and cofactor activity (figure 4c).

Thus, in this study we demonstrate a deletion in the RCA cluster resulting in a novel \textit{de novo} CFH/CFHR3 hybrid gene. Microhomology in the sequence flanking the breakpoint suggests that the deletion has occurred through MMEJ. Genomic disorders affecting \textit{CFH} and the \textit{CFHRs} as a result of non-allelic homologous recombination, are found in ~4.5% of aHUS patients. In contrast, only one case of a genomic disorder secondary to MMEJ has been described\textsuperscript{11}.

We have demonstrated that the product of this gene, a 22 CCP domain protein, is secreted albeit with degradation fragments present in the serum suggesting impaired stability of the protein. Functional analysis of the purified FH/FHR3 protein demonstrated impaired cell surface complement regulation.

This FH/FHR3 hybrid protein is similar to that described by Francis \textit{et al.}\textsuperscript{11} in that in both the C-terminal end of FH is replaced by all 5 CCPs of FHR3 (supplementary figure 1). Whilst both hybrids lack CCP20 of FH responsible for cell surface protection, the hybrid reported here also lacks CCPs 18 and 19.
The functional role of FHR3 is unclear, with various regulatory activities being suggested\textsuperscript{16, 18}. Unsurprisingly, given its high homology with CCP\textsubscript{7} of FH, FHR3 binds to heparin\textsuperscript{16}. FHR3 also binds to C3b and C3d through CCP\textsubscript{4-5}\textsuperscript{16, 19}. Competition between FHR3 and FH for surface bound C3b has been described\textsuperscript{18}. The hybrid described by Francis \textit{et al.} demonstrated normal fluid phase complement regulatory activity but a profoundly reduced cell surface complement regulatory activity\textsuperscript{11}.

That such a FH/FHR3 hybrid should lack cell surface regulatory activity is not surprising. Both these hybrids lack the CCP\textsubscript{20} domain of FH responsible for cell surface localisation\textsuperscript{6}. Additionally, structural analysis has revealed a specific hairpin structure suggesting a model whereby cell surface bound C3b is engaged by both CCP\textsubscript{1-4} and CCP\textsubscript{19-20} of FH concurrently\textsuperscript{20}. The elongation of FH by the addition of extra CCPs would prevent such an orientation.

Although the initial presentation of disease was seen in association with a STEC, the rapid relapse suggested aHUS rather than STEC HUS and this was confirmed by the finding of the \textit{CFH/CFHR3} hybrid gene. In individuals carrying mutations in complement genes, additional triggers (e.g. pregnancy\textsuperscript{21}, infection\textsuperscript{22}) are required for disease to manifest\textsuperscript{4, 22}. In this case, STEC served to unmask the genetic predisposition to disease as in 2 previously reported cases of STEC triggered aHUS\textsuperscript{23}.

In summary we describe a deletion in the RCA cluster arising through MMEJ which results in a novel \textit{CFH/CFHR3} hybrid gene. That, this arose as a \textit{de novo} event suggests that this is a dynamic area of the genome where we should expect further genomic disorders.

**Concise Methods**

The study was approved by Newcastle and North Tyneside 1 Research Ethics Committee, and informed consent was obtained in accordance with the Declaration of Helsinki.

**Complement Assays**

C3 and C4 levels were measured by rate nephelometry (Beckman Coulter Array 360). FH levels were measured by radial immunodiffusion (Binding Site). Screening for FH autoantibodies was undertaken using ELISA as described previously\textsuperscript{24}.

**Genetic Analysis and Multiplex ligation-dependent probe amplification**
Mutation screening of CFH<sup>25</sup>, CFI<sup>26</sup>, CFB<sup>27</sup>, MCP<sup>28</sup>, C3<sup>29</sup>, and DGKE<sup>30</sup> was undertaken using Sanger sequencing as previously described. Screening for genomic disorders affecting CFH, CFHR1, CFHR2, CFHR3, and CFHR5 was undertaken using multiplex ligation-dependent probe amplification (MLPA) in both the affected individual and 500 normal controls as previously described<sup>11</sup>. A proprietary kit from MRC Holland (www.mlpa.com; SALSA MLPAkit P236-A1 ARMD) was supplemented by specific in house CFH probes (supplementary). GeneMarker software (Version 1.90) was used to calculate dosage quotients.

**Genomic breakpoint analysis**

To identify the breakpoint of the deletion that resulted in the CFH/CFHR3 hybrid gene, genomic DNA was amplified using a forward primer specific for CFH in exon 20 and a reverse primer in the CFH 3’ region (figure 1c). The sequence of the forward primer was GTAACGTATTCAGTTGATTGC, and the reverse was ACGGATTGCATGTATAAGTG. The product was then sequenced using direct fluorescent sequencing.

**Confirmation of CFH/CFHR3 RNA product**

mRNA was extracted from peripheral blood lymphocytes of the patient and cDNA was prepared. This was amplified using a forward primer in CFH exon 20 (TGGATGGAGCCAGTAATGTAACATGCAT) and a reverse primer in CFHR3 exon 2 (GAAATAGACCTCCATGTATAATGTCTG) (figure 1c). The PCR product was detected in an ethidium bromide–stained 1.5% agarose gel

**Western blotting**

Detection of abnormal protein products in serum arising as a consequence of the deletion was undertaken by Western blotting. Sera was diluted 1/100 and under non-reducing conditions, electrophoresed on 6% SDS-PAGE gel and transferred onto nitrocellulose. Monoclonal antibodies of known specificity to FH (OX24, CCP 5; L20/3, CCP 20, MBI-6, CCP7 402Y, MBI-7 CCP7, 402H) were used with sheep anti–mouse-Ig HRP (Jackson Immuno Research), at dilutions 1:2000 (primary Ab) and 1:4000 (secondary Ab) (supplementary methods). Following washes in 1xTBST, blots were developed using Pierce ECL Western blotting substrate (Thermo Scientific).

**Purification of factor H species**
FH species (WT FH from normal allele, FH/FHR3 hybrid and FH/FHR3 breakdown product all with CCP5 of FH) were purified from serum with affinity chromatography using immobilised monoclonal antibody to FH (OX24), on a 1 mL HiTrap NHS HP column (GEHealthcare). Following washing with PBS, the bound proteins were then eluted using 0.1M glycine pH2.7. The eluted material was pooled and concentrated for analysis by mass spectrometry.

The FH/FHR3 hybrid protein was purified from patient serum with affinity chromatography using immobilised monoclonal antibody to FH CCP7 402Y (MBI6)\textsuperscript{31}, on a 1 mL HiTrap NHS HP column (GEHealthcare). Following washing with PBS, the bound proteins were eluted using PBS/diethylamine (50mM). Subsequently the FH/FHR3 protein was purified from its degradation product and FHL1 using a Superdex 200 size exclusion column. We also purified WT FH protein from a healthy control. FH or FH/FHR3 hybrid protein purified using the MBI6 column was used for cofactor and decay acceleration assays.

**Mass Spectrometry**

A 6% SDS PAGE was run and the 3 bands identified by coomassie staining were excised from the gel as indicated in figure 4a. Trypsin digest and mass spectrometry were then undertaken (supplementary methods).

**Cell surface decay acceleration assays**

Decay acceleration on sheep erythrocytes was performed as previously described\textsuperscript{32} and in supplementary methods. Briefly, FH/FHR3 hybrid from the patient and FH from control were purified by immuno-affinity chromatography as above and gel filtered into PBS. Alternative pathway convertase (C3bBb) was formed on sheep erythrocytes. Cells were incubated for 15 minutes with 1.24nM to 50nM patient FH/FHR3 hybrid or control FH. The molar concentration of the purified patient FH/FHR3 was estimated using the extinction co-efficient (272170 M.cm\textsuperscript{-1}) while the extinction coefficient of FH (246800 M.cm\textsuperscript{-1}) was used for the control. Lysis was initiated with 4% normal human serum, depleted of factor B and FH (NHS\textDelta B\textDelta H). Maximal lysis was achieved by adding NHS\textDelta B\textDelta H to no FH wells (buffer only). To determine the amount of lysis, cells were pelleted by centrifugation, and haemoglobin release was measured at 420 nm ($A_{420}$). Percentage of inhibition of lysis in the presence of increasing concentrations was defined as: ($A_{420}[\text{buffer only}]-A_{420}[\text{FH}]$) / $A_{420}[\text{buffer only}]$ *100%. 
Cell surface cofactor assays

Cofactor activity on sheep erythrocytes was performed as previously described\textsuperscript{32}. Briefly washed C3b-coated sheep erythrocyte cells were resuspended in AP buffer and incubated with an equal volume of a range of concentrations of FH/FHR3 hybrid and WT FH and 2.5 μg/ml factor I (CompTech) for 8 minutes at 25°C. After three washes in AP buffer, AP convertase was formed on the remaining C3b. Lysis was initiated with 4% NHS\textsubscript{ADH}. Again, cells were pelleted and haemoglobin release was measured at 420nm. Percentage of inhibition from lysis was calculated by the formula $(A_{420}[\text{buffer only}]-A_{420}[\text{FH}])/A_{420} [\text{buffer only}] \times 100\%$. 
Figure Legends

Figure 1 (a) Genomic organisation of the RCA cluster region containing the CFH and CFHR genes. (b) Multiplex ligation-dependent probe amplification of CFH, CFHR3, CFHR1, CFHR2 and CFHR5 demonstrating the copy number ratio. The dotted lines represent ratios considered within the normal range. (c) Identification of break point. Genomic DNA was amplified using a forward primer specific for CFH exon 20 (1f) and a reverse primer in the CFH 3’ region intergenic region (1r) and sequenced. The deletion of exons 21, 22 and 23 of CFH is demonstrated (shaded box) and the breakpoint highlighted. (d) The CFH and CFHR3 sequence flanking the breakpoint. The 7 base pair area of microhomology is demonstrated (bold, boxed) (e) Confirmation of hybrid CFH/CFHR3 hybrid mRNA. A message for the hybrid CFH/CFHR3 gene was confirmed by amplifying patient and control cDNA with cross CFH and CFHR3 gene primers (filled arrows 2f and 2r). The agarose gel demonstrates amplified cDNA. The patient lane shows an amplified product consistent with a hybrid CFH/CFHR3 gene, which is not present in either control cDNA.

Figure 2 Renal biopsy of the patient demonstrating thrombi (arrows) on capillary loops (a) H&E (b) Masson trichrome. A developing membranoproliferative pattern of injury can be seen in (c) characterised by capillary loop double contours (arrows, silver counter stained H&E, x400).

Figure 3 (a) The protein product of wild type FH and the predicted FH/FHR3 hybrid protein consisting of CCPs1-17 of FH and CCP1-5 of FHR3. The CCPs originating from FHR3 are highlighted in grey. The patient is heterozygous for a common polymorphism in CCP7 of FH (Y402H). Binding epitopes for the monoclonal antibodies are shown: Ox24-CCP5; MBI-7- CCP7 amino acid 402 histidine variant; MBI-6- CCP7 amino acid 402 tyrosine variant; L20/3-CCP20. (b) Serum Western blot using OX24 demonstrating 2 additional bands in the patient in addition to FH. The upper band is consistent with the predicted size of the FH/FHR3 hybrid. (c) Serum Western blot using L20/3. When using this FH C-terminal specific antibody no additional bands are seen, consistent with the hybrid protein lacking CCP18-20. (d) Serum Western blot using MBI-7. This demonstrates that the patient has a normal allele producing FH with the Histidine at amino acid 402. (e) Serum Western blot using MBI-6. The patient has 3 additional bands and no wild type FH band. This is consistent with the FH/FHR3 hybrid protein carrying the Tyrosine amino acid at position 402. The two additional
upper bands represent differentially glycosylated hybrid protein, consistent with that seen in the native FHR3. There is a faint degradation product only detected with the MBI-6 antibody. This is consistent with the breakdown product being generated only from the hybrid protein and suggests a less stable protein product. The controls were unaffected, unrelated, genotyped samples. In (b) and (c) a sample from an individual heterozygous at Y402H was used. In (d) and (e) samples from individuals homozygous for Y402 (Y/Y); homozygous for H402 (H/H); or heterozygous (Y/H) were used.

Figure 4. (a) Mass spectrometry of purified proteins. The FH, FH/FHR3 hybrid and the degradation product were purified using affinity chromatography with an Ox24 column. These FH species were separated by 6% SDS PAGE, stained using Coomassie, cut from the gel (left), and submitted for trypsin digest and mass spectrometry. Peptides sequences identified using mass spectrometry are indicated *. The peptides detected in the hybrid degradation product by mass spectrometry are annotated on a full length FH/FHR3 hybrid protein. CCPs which cannot be directly inferred from mass spectrometry data are outlined with a dashed line. A molecular weight of ~120kDa is consistent with a ~17 CCP protein. (b) Decay acceleration assays on sheep erythrocytes. The purified FH/FHR3 hybrid from patient demonstrated impaired cell surface complement regulation compared to wild type (WT) FH purified from control. Alternative pathway convertase (C3bBb) was formed on sheep erythrocytes. Cells were incubated for 15 minutes with dilutions of purified FH/FHR3 hybrid and WT FH before triggering lysis with NHSΔBΔH. Maximal lysis occurs in buffer-only (0 mM FH) conditions. Addition of WT FH caused decay of the C3 convertase and decay of convertase resulting in inhibition of lysis. The FH/FHR3 hybrid was up to 2-fold less efficient at inhibiting lysis. (C) C3b cofactor activity on sheep erythrocytes. WT FH and purified FH/FHR3 hybrid were tested for the ability to act as a cofactor for factor I–catalyzed inactivation of C3b deposited on the surfaces of sheep erythrocytes. The C3 convertase (C3bBb) was formed on residual C3b and lysis was triggered by adding NHSΔBΔH. Maximal lysis occurs in the presence of buffer only (0 mM FH). The addition of WT FH and factor I produces iC3b which decreases convertase formation and subsequent lysis, which is shown as increasing amounts of inhibition of lysis (expressed as percentage of maximal lysis) after incubation with factor I and WT FH.
(black circles) or FH/FHR3 hybrid (white circles). The FH/FHR3 hybrid can be seen to be markedly less active than WT.

Acknowledgments

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Disclosure

Newcastle University has received fees from Alexion Pharmaceuticals for lectures and consultancy undertaken by THJG and DK. AA has received fees from Alexion Pharmaceuticals for lectures. CLH is also employed by GlaxoSmithKline and has shares in this company.
References


19. Hellwage, J, Jokiranta, TS, Friese, MA, Wolk, TU, Kampen, E, Zipfel, PF, Meri, S: Complement C3b/C3d and cell surface polyanions are recognized by


CFH/CFHR3 hybrid gene in aHUS
Figure 1.

(a) 

Genes

(b) 

Copy Number Ratio

(c) 

DNA

(d) 

CFH  CCGTCTCTACTAAA  AATAACA  AAAAATAGGG

CFHR3  AATAAAGATATCAA  AATAACA  CACAATATTATT

CFH/CFHR3  CCGTCTCTACTAAAATAACA  CACAATATTATT
Figure 3.

(a) Wild type FH Protein

Hybrid FH/FHR3 Protein

(b) Control Patient

(c) Control Patient

(d) YY HY HH Patient

(e) YY HY HH Patient
Figure 4.

(a) SDS PAGE

Peptides detected

Hybrid FH/FHR3

FH/FHR3 Hybrid

FH

150 kDa

100 kDa

Hybrid Degradation Product

(b) Decay Acceleration Activity

(c) Cofactor Activity

Protection from Lysis (%) vs Log [FH] nM

Protection from Lysis (%) vs Log [FH] nM
Supplementary Figure 1: 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 Splicing of CFH exon 20 into CFHR3 exon 2
Supplementary Figure 3 (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 4 (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.
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 methods

#### CFH and CFHR5 MLPA probe hybridization sequences

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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 NH₄HCO₃, alkylated with 50 mM iodoacetamide (Sigma) in 100 mM NH₄HCO₃ and digested in gel with 230ng modified trypsin (Promega) in 50 mM NH₄HCO₃, 1 mM CaCl₂. 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: \( A = \varepsilon L c \), (where \( A \)= Absorbance, \( \varepsilon \)= 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 \( \varepsilon \) 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) [5 mM sodium barbitone, pH 7.4, 150 mM NaCl, 7 mM MgCl$_2$, 10 mM 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 40 μg/ml Factor B [prepared in house] and 0.2 μg/ml Factor D [Complement Technology] and incubating for 15 minutes at 37°C. 100 μL of this convertase mixture was then added to 50 μL (1.24 nM – 50 nM) FH/FHR3 hybrid and FH (purified from patient or control), diluted in PBS/20 mM EDTA and left at room temperature for 15 minutes, to allow decay acceleration to take place. 50 μL 4% (vol/vol) NHSΔBΔH in PBS/20 mM 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 1200 rpm for 3 minutes and haemoglobin release was measured at 420 nm (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 50 nM) of FH or FH/FHR3 hybrid and 2.5 μg/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/20 mM 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

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References

