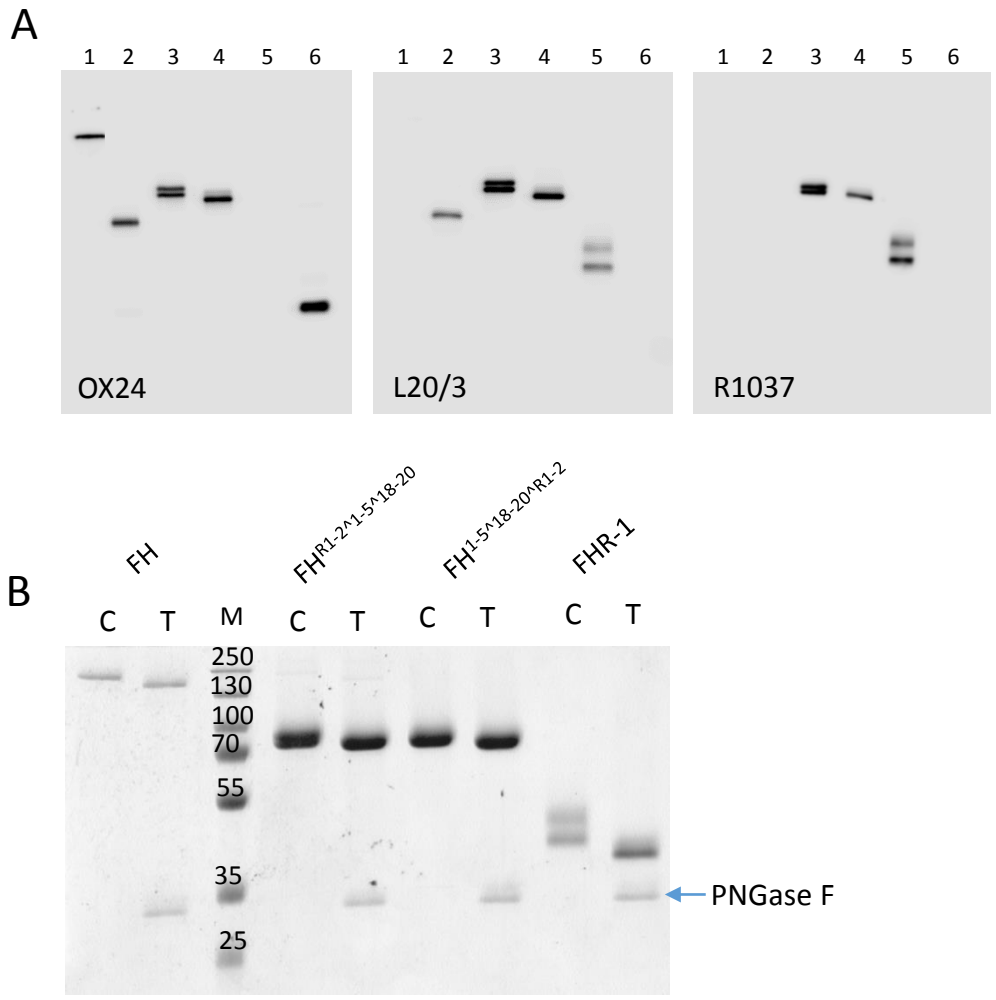
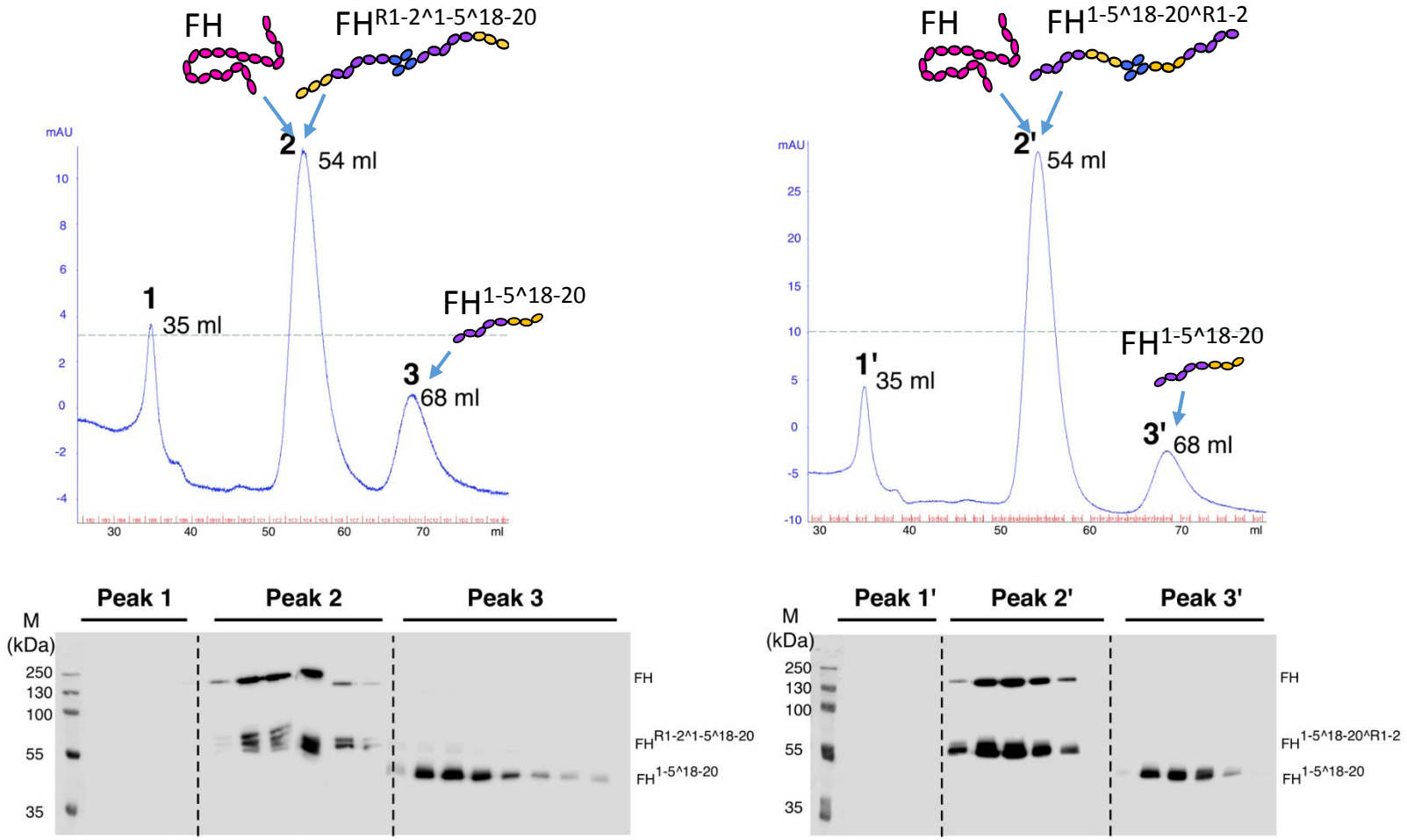
Figure S1



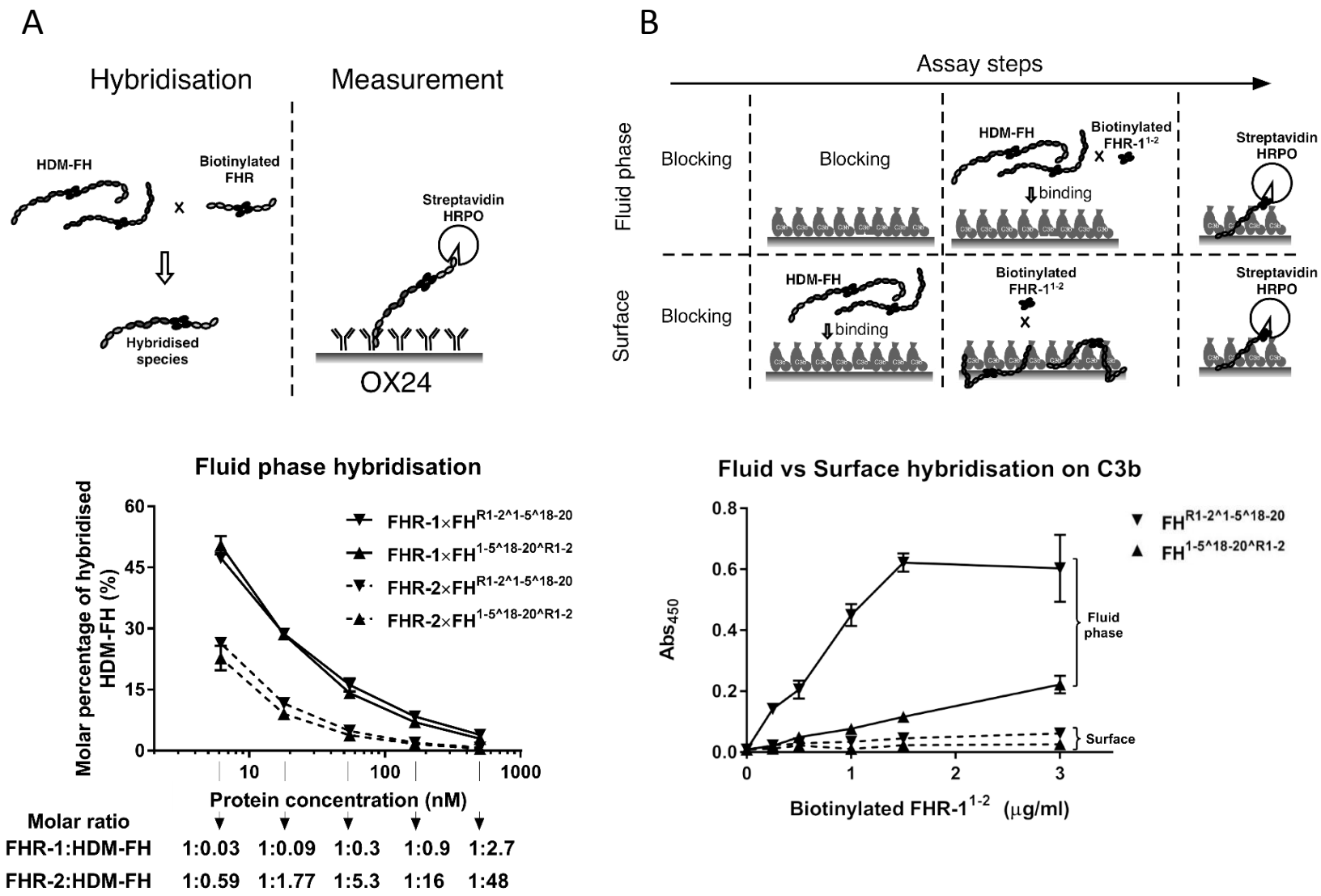
Supplemental Figure S1. (A) Under non-reducing condition and a 12% SDS-PAGE gel followed by western blotting, the immunochemical reactivity of monoclonal antibodies OX24, L20/3 or R1037 (as indicated) to bind FH, FH1-5^18-20, FHR1-2^1-5^18-20, FH1-5^18-20^R1-2, FHR-1 and FH1-5 was examined. (B) PNGase F treatment caused gel mobility change of FH family proteins. A 5 µg protein sample was treated with 500 units of PNGase F or PBS (New England Biolabs, P0704S) under reducing and denaturing conditions according to manufacture's instruction. A 12% SDS-PAGE gel was loaded with 2.5 µg of enzyme treated (T) and PBS treated (C) sample per lane. (B) Estimation of overall protein molecular weight using the DAWN HELEOS II MALS detector, $FH^{R1-2^1-5^18-20}$ (black) and $FH^{1-5^18-20^R1-2}$ (grey). Light scattering (LS) and differential refractive index (dRI) are plotted alongside the fitted total molecular weight (diamonds), and a rough estimation of protein mass (pluses) are shown across each peak.

Figure S2



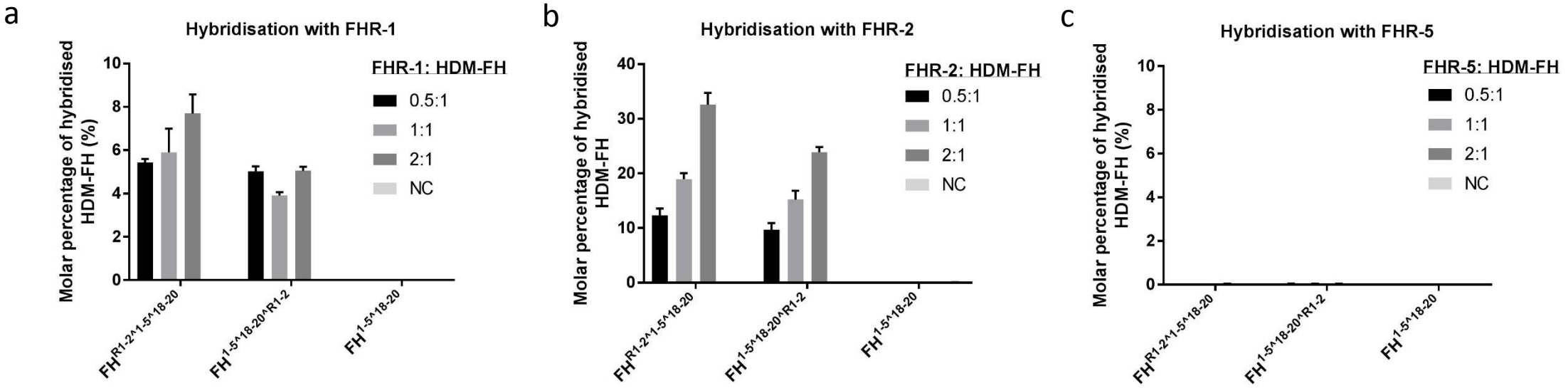
Supplemental Figure S2. Size exclusion chromatography analysis of HDM-FH dimer formation. Factor and $FH^{1-5\text{^}18-20}$ were mixed with $FH^{R1-2\text{^}1-5\text{^}18-20}$ or $FH^{1-5\text{^}18-20\text{^}R1-2}$ and then loaded on a Superdex 200 Hiload 16/600 pg column using an AKTA purifier. Proteins were separated using a flow rate of 0.5 ml/min. The elution peak fractions were analysed by Western blot and goat anti-FH polyclonal antibody followed by a donkey anti-goat-HRPO conjugated antibody. ECL and the Licor used to visualise. Markers are indicated in the left and individual gels indicated by the dashed lines. Representative of 3.

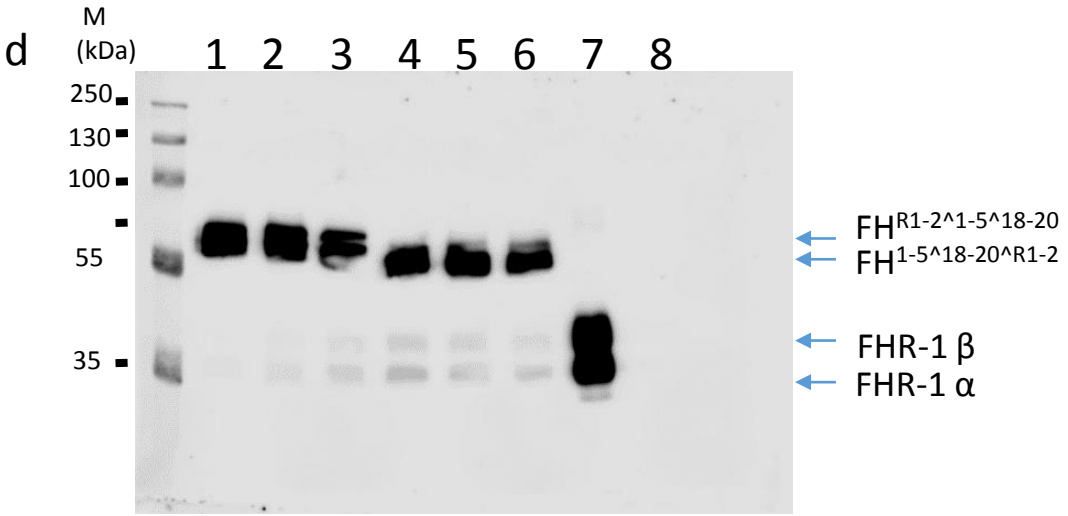
Figure S3



Supplemental Figure S3. Analysis of cross hybridization between the homodimeric mini-FH and human FHRs. (A) A three-fold concentration dilution series of homodimeric mini-FH was incubated with 14.63 or 0.64 μg/ml (i.e. physiological serum concentrations) of biontinylated recombinant FHR-1 or FHR-2, respectively, for 1 hour at 37 °C. The cross hybridised FHR proteins were detected via streptavidin conjugated HRPO, see diagram above (B) On an ELISA plate coated with C3b (5μg/ml), homodimeric mini-FH (300nM) and a concentration series of biotinylated recombinant FHR-1^{1-2} were either applied sequentially or at the same time, see diagram. Again, Hybridised FHR proteins were detected via streptavidin conjugated HRPO. Data shown are a composite of three experiments mean and standard deviation is shown

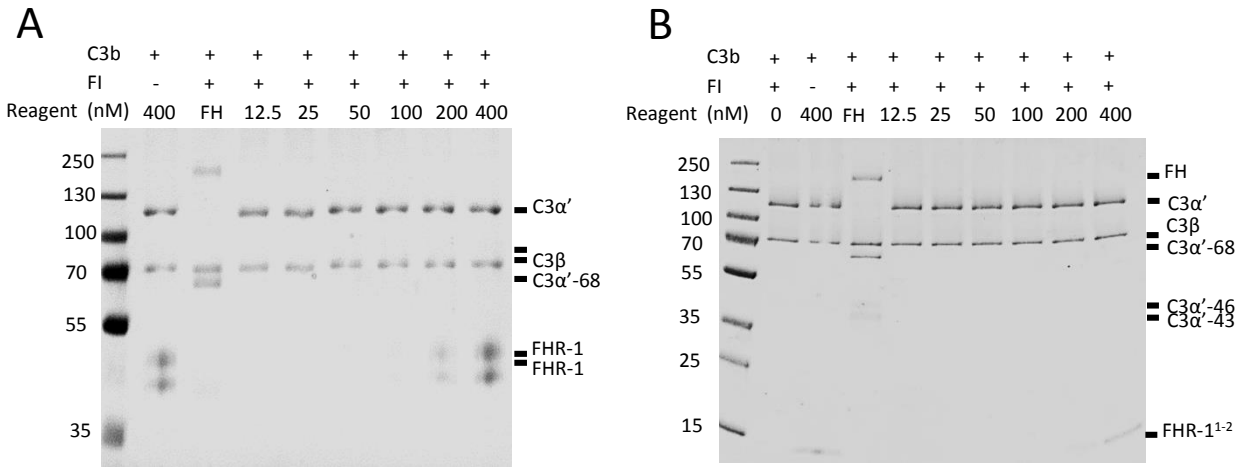
Figure S4





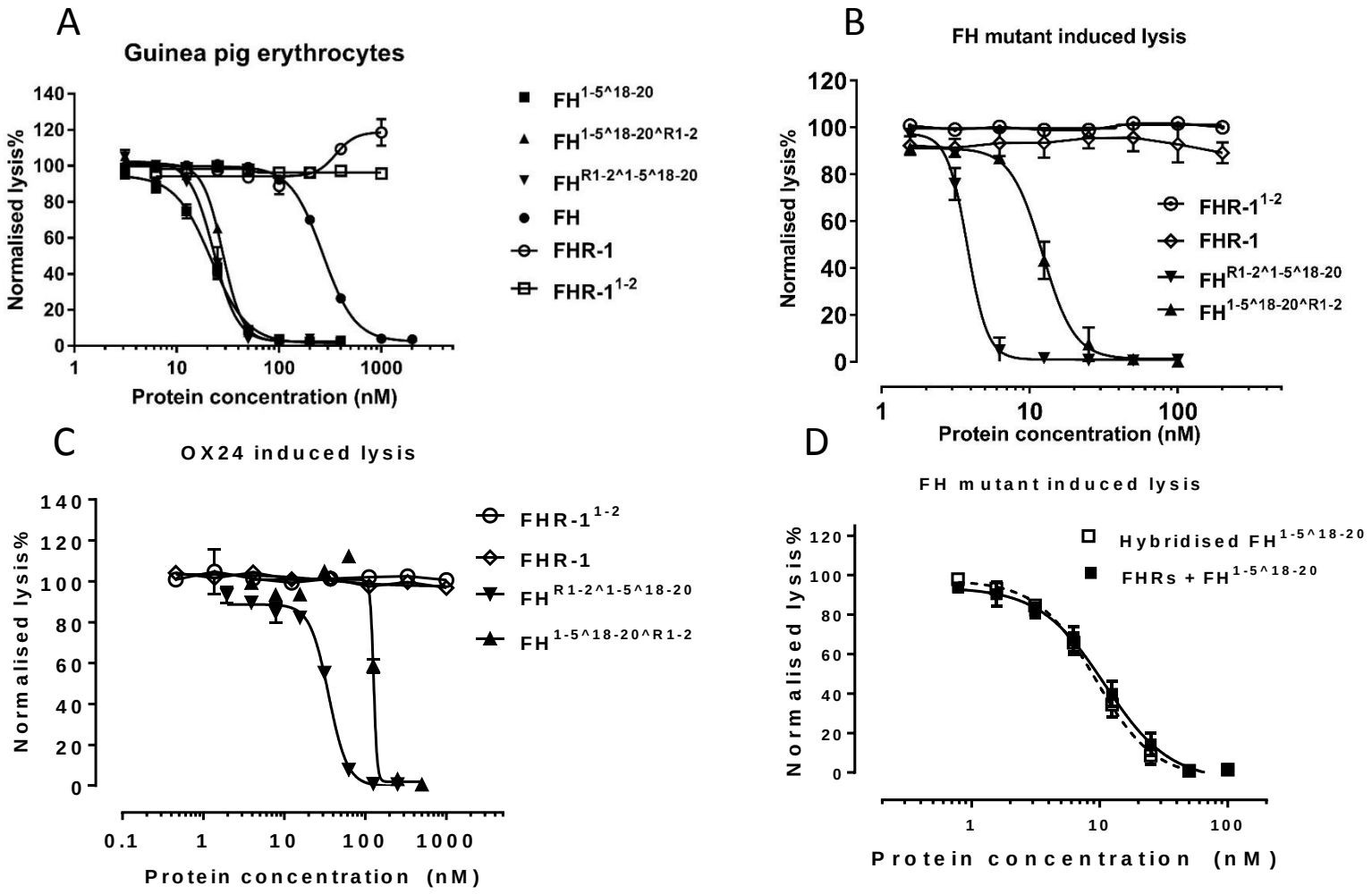
Supplemental Figure S4. Cross hybridizations between HDM-FH and human FHR. After mixing the proteins o/n, the Molar percentages of HDM-FH:FHR1(a) or HDM-FH:FHR2(b) or HDM-FH:FHR-5 (c) were determined by ELISA. In each case, biotinylated recombinant FHR was incubated at 0 (negative control or NC, bar not visible on plot), 0.5, 1 or 2 times their normal physiological serum concentration and 300nM HDM-FH (i.e. molar equivalent to the physiological FHR concentration). Similar cross hybridisation experiments were also performed with FHRs and $FH^{1\text{-}5\text{^}18\text{-}20}$ as further control. As expected, no cross hybridization is noted and so no bars are visible. (D) Western plot using polyclonal anti-FH on samples applied and eluted from a OX23 affinity column after overnight incubation with recombinant FHR-1 (at physiological concentration). Lane (1-3) $FH^{1\text{-}5\text{^}18\text{-}20\text{^}R1\text{-}2}$, Lane (4-6) $FH^{R1\text{-}2\text{^}1\text{-}5\text{^}18\text{-}20}$, Lane (7) purified FHR-1 before mixing (positive control) and lane (8) elute after FHR-1 alone was applied to OX23 column (negative control).

Figure S5



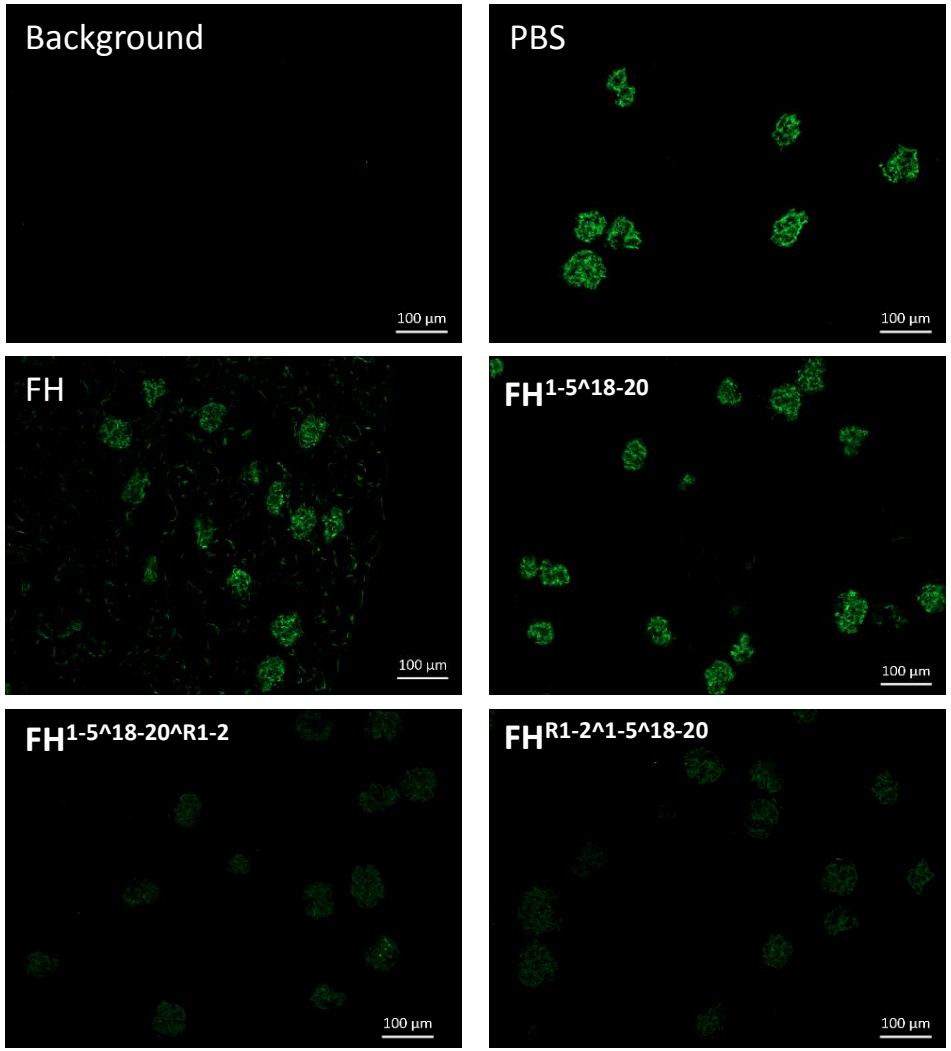
Supplemental Figure S5. Evaluation of fluid phase cofactor activity of the dimerisation domain. C3b (700 nM), FI (20 nM) and either increasing concentrations of FHR-1 (A) or FHR-1^{1-2} (B) were incubated in solution at 37 °C for 1 hour. C3b breakdown was analysed by SDS-PAGE and Coomassie staining. Disappearance of C3α'-110 band and the appearances of C3α'-68, -46 and -43 bands would have been indicative of the C3b proteolytic inactivation, no evidence of such banding change was evident. Densitometry readings, the average of remaining C3b from two experiments, are shown in italics below each lane. One experiment, representative of two independent experiments, is shown.

Figure S6



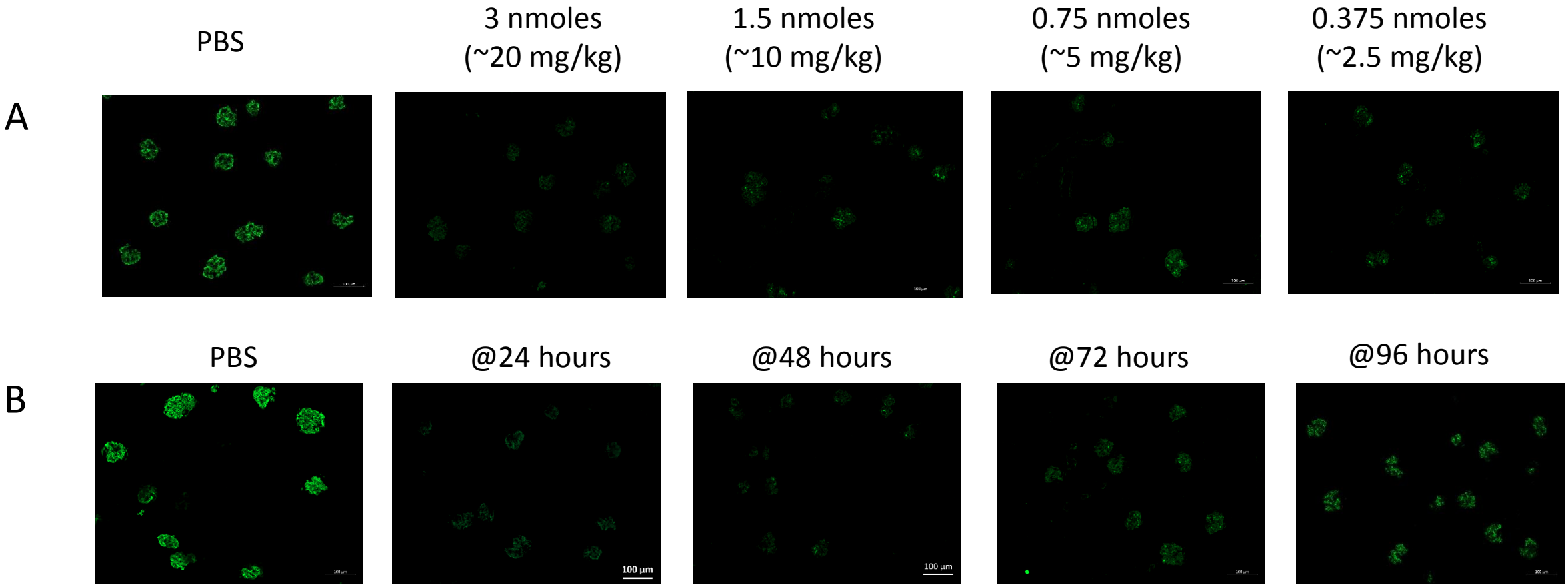
Supplemental Figure S6. (A) The addition of increasing concentrations of FH reagents prevented Guinea pig erythrocyte lysis in 20% normal human serum (NHS). (B) Protection of sheep erythrocyte by addition of increasing concentrations of FH reagents in FH depleted serum supplemented with recombinant human FH S1191A V1197L. (C) Protection of sheep erythrocyte by addition of increasing concentrations of FH reagents in OX24 spiked normal human serum (an autoantibody model serum). (D) The functional impacts of the recombinant FHR were. Sheep erythrocyte lysis was induced in FH depleted serum supplemented with recombinant human FH S1191A V1197L. Before serum was added, a concentration series of doubly diluted $FH^{1-5\text{^}18-20}$ were either pre-incubated for 1 hour (solid-line) or not (dash-line) with a mixture of recombinant human FHR-1 and FHR-2 at their physiological serum concentration. For A, data was normalized against guinea pig erythrocyte lysis with NHS in the absence of FH reagent, while for B-D the data were normalized against sheep erythrocyte lysis with deregulated sera in the absence of FH reagent. A-D show data from four independent measurements of two experiments. The mean and standard deviation for each measurement were calculated for all date sets, curves were fitted using nonlinear regression function in GraphpadPrism.

Figure S7



Supplemental Figure S7. Representative images (n = 3 mice) of glomerular C3 (Green) at 48 hour after a single injection of plasma purified human FH, $FH^{1\text{-}5\text{^}18\text{-}20}$, $FH^{R1\text{-}2\text{^}1\text{-}5\text{^}18\text{-}20}$, $FH^{1\text{-}5\text{^}18\text{-}20\text{^}R1\text{-}2}$ or PBS. Original magnification, ×10, scale bar is shown. Tubule-interstitial staining is observable in plasma purified human FH treated mice.

Figure S8



Supplemental Figure S8. Representative images of glomerular C3 (Green) after a single injection of $FH^{1\text{-}5\text{^}18\text{-}20\text{^}R1\text{-}2}$ or PBS. Row A, groups of 3 mice were treated with PBS or doses from 3 to 0.375 nmoles (≈ 20 – 2.5 mg/kg) as indicated. Kidneys were isolated at 48 hrs and stained for the presence of C3 using goat-anti-C3-FITC (MP biomedical). Row B, groups of 3 mice (for each time point) were treated with PBS (96hr group only) or a 3 nmole injection of $FH^{1\text{-}5\text{^}18\text{-}20\text{^}R1\text{-}2}$ in PBS. Each group was then euthanised at 24, 48, 72 or 96 hours as indicated. Original magnification, ×10, scale bar is shown.

Table S1

	Opsonized C3b surface (250 nM of analytes)			Opsonized iC3b surface (1000 nM of analytes)			Amine couple C3d (1000 nM of analytes)		
Analytes	RU at 270s	MW adjusted RU	Fold changes compared to FH	RU at 270s	MW adjusted RU	Fold changes compared to FH	RU at 270s	MW adjusted RU	Fold changes compared to FH
FH	44.32	44.32	0	8.07	8.07	0	3.15	3.15	0
$FH^{1-5\hat{}18-20}$	81.62	212.85	2.40	83.86	218.69	13.55	105.75	275.77	43.77
$FH^{R1-2\hat{}1-5\hat{}18-20}$	213.7	209.64	2.36	162.90	159.80	9.90	237.47	232.96	36.97
$FH^{1-5\hat{}18-20\hat{}R1-2}$	152.59	151.61	1.71	48.87	48.55	6.01	54.66	54.30	8.61

Supplemental Table S1. Binding responses (RU) were adjusted by multiplying the molecular weight ratio derived by dividing MW of FH (155000 Da) by the MW of $FH^{1-5\hat{}18-20}$ (59436 Da), $FH^{R1-2\hat{}1-5\hat{}18-20}$ (158000 Da) or $FH^{1-5\hat{}18-20\hat{}R1-2}$ (156000 Da). Fold changes of RU were calculated by dividing the MW adjusted RU of a analyte by the RU value of FH.

Table S2

Regulator	IC_{50} values for decay accelerating or cofactor activities on sheep erythrocyte (nM)	
	DAA	CFA
FH	16.19 (R^2=0.98; CI=9.41-22.97)	51.23 (R^2=0.97; CI=40.64-66.91)
$FH^{1-5\hat{}18-20}$	5.85 (R^2=0.98; CI=4.3-6.86)	27.36 (R^2=0.99; CI=87.22-93.64)
$FH^{R1-2\hat{}1-5\hat{}18-20}$	0.758 (R^2=0.98; CI=0.543-0.973)	2.17 (R^2=0.99; CI=1.942-2.436)
$FH^{1-5\hat{}18-20\hat{}R1-2}$	0.484 (R^2=0.98; CI=0.387-0.581)	0.798 (R^2=0.99; CI=0.706-0.895)

Supplemental Table S2. Summary of estimated IC_{50} values for decay accelerating or cofactor activities on sheep erythrocyte IC_{50} values were estimated by fitting the lysis data shown in Figure 4.A-B with the nonlinear regression function in Graphpad Prism

Table S3

Regulator	IC_{50} values (nM) for protection of sheep erythrocyte	
	aHUS model serum	Autoantibody model serum
FH	214.9 (R^2=0.97; CI=197.1-232)	857.6.9 (R^2=0.95; CI=777.5-956)
$FH^{1-5\hat{}18-20}$	11.57 (R^2=0.99; CI=10.77-12.52)	84.47 (R^2=0.96; CI=72.5-92.9)
$FH^{R1-2\hat{}1-5\hat{}18-20}$	2.17(R^2=0.99; CI=2.11-2.24)	35.39 (R^2=0.98; CI=33.15-38.02)
$FH^{1-5\hat{}18-20\hat{}R1-2}$	10.52(R^2=0.97; CI=9.69-11.91)	212.3 (R^2=0.96; CI=192.3-233.2)

Supplemental Table S3. Summary of estimated IC_{50} values for protection of sheep erythrocyte in disease model seraIC_{50} values were estimated by fitting the lysis data shown in Figure 4.C-D with the nonlinear regression function in GraphpadPrism

Table S4

Regulator	IC_{50} values (nM) for protection of sheep erythrocyte		
	Absence of FHR-1 &-2	Presence of FHR-1 &-2	Pre-incubated with FHR-1 &-2
$FH^{1-5\hat{}18-20}$	11.57 (R^2=0.99; CI=10.77-12.52)	10.76 (R^2=0.99; CI=8.99-12.54)	9.16 (R^2=0.99; CI=8.22-10.11)
$FH^{R1-2\hat{}1-5\hat{}18-20}$	2.17(R^2=0.99; CI=2.11-2.24)	2.74 (R^2=0.99; CI=2.63-2.87)	3.509 (R^2=0.99; CI=3.25-3.76)
$FH^{1-5\hat{}18-20\hat{}R1-2}$	10.52(R^2=0.97; CI=9.69-11.91)	9.67 (R^2=0.99; CI=8.94-10.41)	15.17(R^2=0.98; CI=14.93-15.41)

Supplemental Table S4. Functional impact of FHR-1 and-2 on estimated IC_{50} values for protection of sheep erythrocytes in disease model serum. IC_{50} values were estimated by fitting the lysis data shown in Figure 4.C and Figure 4.e with the nonlinear regression function in GraphpadPrism

Supplementary methods

Construct design

To produce recombinant $FH^{R1\text{-}2\text{^}1\text{-}5\text{^}18\text{-}20}$, the DNA encoding the first two SCR domains of FHR-1 were amplified via PCR reaction from a previously cloned and verified pDR2EF1α-CFHR-1 expression vector (GeneBank accession: M65292, in house), using primers to introduce a *XbaI* restriction site at the 5' end and a *SalI* site at the 3' end (fwd 5'-GCTCTAGAATGTGGCTCCTGGTCAGTGTA-3' and rev, 5'-CGCGTCGACGGAAGTGTCAGTGGACCTGC-3', respectively). FH SCRs 1-5 was amplified from pDR2EF1α-P6LNK vector[1, 2] using a forward primer to introduce a *SalI* restriction site at the 5' end and a reverse primer to create a *KpnI* site at the 3' end (fwd 5'-CGCGTCGACGAAGATTGCAATGAACTTCCTCC-3' and rev, 5'-GGGGTACCCCCACCTCCTCCCGAAC-3'). The two PCR products were then digested using restriction enzyme *SalI* and subsequently ligated using T4 DNA ligase. A PCR reaction was performed again using primers aiming to amplify the correctly ligated DNA fragment (fwd 5'- GCTCTAGAATGTGGCTCCTGGTC-3' and rev, 5'- GGGGTACCCCCACCTCC-3'). After an additional gel extraction step, the DNA fragment with the correct size was double digested with *XbaI* and *KpnI*, and ligated with the double digested pDR2EF1α-$FH^{1\text{-}5\text{^}18\text{-}20}$ vector, consequently producing the pDR2EF1α-$FH^{R1\text{-}2\text{^}1\text{-}5\text{^}18\text{-}20}$ expression vector.

Using the similar cloning strategy and the same DNA templates, the DNA fusion for $FH^{1\text{-}5\text{^}18\text{-}20\text{^}R1\text{-}2}$ was constructed. The cDNA of FHR-1 SCRs 1-2 was amplified using forward and reverse primers contained the *KpnI* and *NheI* restriction sites (fwd 5'-GGGGTACCGAAGCAACATTTTGTGATTTTCCA-3' and rev, 5'-CTAGCTAGCTTAGGAAGTGTCAGTGGACCTGC-3'). A subsequent PCR reaction amplified the DNA of $FH^{1\text{-}5\text{^}18\text{-}20}$ utilizing 5' and 3' primers to introduce *SalI* and *KpnI* restriction sites, respectively (fwd 5'-CGCGTCGACACCACCTCCTCATGTGTGAAT-3' and rev, 5'-GGGGTACCTCTTTTTGCACAAGTTGGATACTC-3'). Two PCR products are digested by *KpnI* restriction enzyme and joined by ligation reaction, and then specially designed primers (fwd 5'- CGCGTCGACACCACCTC-3' and rev, 5'- CTAGCTAGCTTAGGAAGTGTCAGTGGACC-3') were used to amplify the correctly ligated DNA fragment. Finally, the DNA fragment and the pDR2EF1α-$FH^{1\text{-}5\text{^}18\text{-}20}$ vector were *SalI* and *NheI* double digested and ligated, through which the SCRs 18^20 DNA in the original vector was replaced by SCRs 18^20^R1-2. CHO cells were cultured in DMEM F-12 medium supplemented with 10% FBS (Biotech) and penicillin-streptomycin solution (1 in 100 dilution, SigmaAlrich UK). Stable transfections were selected in the presence of 0.6 mg/ml Hygromycin B. A single clone of stable expresser was then picked for protein production, which was carried out in the absence of Hygromycin B for 10 days using roller bottle method. The proteins were purified from the over grown media using a 5 ml Hitrap NHS activated HP column (GE Healthcare) coupled with OX-24 monoclonal antibody.

SPR.

All sensorgram curves were acquired at room temperature on CM5 sensor chips, using HBST (10 mM HEPES, 150 mM NaCl, and 0.005% Tween 20, pH 7.4) as running buffer, 1 mM $MgCl_2$ was optionally supplemented to the running buffer when an AP C3 convertase was generated on the chip. To determine the interaction profile on the C3b surface, 150 RU of purified C3b

(CompTech, USA) was amine coupled to the chip surface, subsequently an additional 650 RU of C3b was deposited via AP C3 convertase by repetitive flowing-cross (5 $\mu l/min$) a mixture of Factor B, D and C3, at 20 $\mu g/ml$, $1\mu g/ml$ and 0.5 mg/ml respectively. The C3b deposited on the surface was processed by injection of repetitive cycles of Factor H and I to produce iC3b, the extent of conversion completion was confirmed by the incapacity to assemble AP C3 convertase. The binding affinity? to C3d was evaluated using a separate CM5 chip, where 600 RU C3d (CompTech, USA) were immobilized using standard amine coupling technique. In all SPR experiments, ligands were immobilized to the flow cell 2, and the flow cell 1 was blocked by carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) which served as a reference cell throughout. To determine the binding response, a concentration series of analytes was injected in duplicate (at 15 µl/min for 180 s) followed by running buffer for 400 s and a regeneration phase involving injection of regeneration buffer (10 mM sodium acetate, 1 M NaCl pH 4.5) for 30 s. Binding signals were double-referenced to blank cell and to cell buffer injection.

Heparin chromatography

The glycosaminoglycan binding of Factor H or engineered reagents was evaluated using heparin affinity chromatography. 200 µg of the protein was dissolved in 10 ml Buffer A (20 mM Tris, 50 mM NaCl, pH 7.4) and loaded on a 1ml HiTrap heparin HP column (GE Healthcare) using an AKTA pure liquid chromatograph system. The column bound protein was eluted with a 40 column-volume gradient of Buffer A and B (20 mM Tris, 1 M NaCl, pH 7.4).

Fluid phase FI co-factor activity assay

The complement regulatory activity of FH reagents was measured in a fluid phase FI co-factor activity assay. Briefly, 0.7 µM of C3b and 20 nM of FI were mixed with either FH, $FH^{1\text{-}5\text{^}18\text{-}20}$, $FH^{R1\text{-}2\text{^}1\text{-}5\text{^}18\text{-}20}$ or $FH^{1\text{-}5\text{^}18\text{-}20\text{^}R1\text{-}2}$ in a total volume of 16 µl in PBS buffer at 37°C for 30 min. The proteolytic breakdown of C3b was analysed using a 10% SDS-PAGE gel followed by Coomassie staining.

Sheep erythrocyte assays.

a. Briefly prior to the experiment, Sheep erythrocytes were washed with GVB buffer (5 mM veronal, 145 mM NaCl, 0.1 % (w/v) gelatin) and the cell concentration was adjusted to produce A_{405} reading of 1.0 when completely lysed in water. To set up the reaction, sheep erythrocytes were suspended in 100 µl of 20% of NHS (normal human serum) supplemented with OX-24 (80 µg/ml), FH reagents and Mg^{+}-EGTA (7 mM $MgCl_2$ and 10 mM EGTA) in GVB buffer. Haemolysis was detected after incubating the reaction mixture at 37°C for 30 min, followed by the addition of 150 µl of quenching buffer (GVB supplemented with 10 mM EDTA). The cells were pelleted by centrifugation at 1500*g* for 10 min, and absorbance (A_{405}) of 100 µl of supernatant measured. (b) The aHUS model haemolysis assay was performed in a nearly identical protocol as described above, however the OX-24 supplemented NHS was replaced by the addition of FH depleted serum CompTech, Texas, USA) and recombinant S1191L-V1197A-CFH^{31} to the final concentrations of 20% and 50 nM.
b. To evaluate the functional impact of the heterodimers formed between FH reagents

and FHR-1 and-2 on lysis inhibition. The aHUS model haemolysis assay was modified slightly, before the lytic reaction was set up, a concentration series of doubly diluted FH reagents was either pre-incubated for 1 hour at 37 °C or not with a mixture of recombinant human FHR-1 and FHR-2 at their physiological serum concentrations, 14.64 or 0.64 μg/ml respectively.

c. To assess the decay accelerating function, the antibody sensitized sheep erythrocytes were washed and suspended to 2% (v/v) in VBS buffer (5 mM veronal, 145 mM NaCl, pH 7.4) supplemented with 0.3 mM $CaCl_2$. C3b deposition on sheep erythrocyte surface was achieved by the addition of ΔFBΔFH-NHS (normal human serum subsequently depleted of FB and FH) to the final concentration of 8% in the presence of 1 μM of Eculizumab. To form AP C3 convertase, 2% (v/v) C3b coated sheep erythrocytes were mixed with an identical volume of AP VBS buffer containing 42 μg/ml FB and 0.4 μg/ml FD and incubated at 37°C for 15 min. A stock EDTA solution was added to final concentration of 10 mM to stop further C3 convertase assembly. The cells were washed, suspended to 2% (v/v) in TPB (terminal pathway buffer, PBS supplemented with 20 mM EDTA). Aliquot (50 μl) of cells were incubated with 50 μl either FH, $FH^{1\text{-}5\text{^}18\text{-}20}$, $FH^{R1\text{-}2\text{^}1\text{-}5\text{^}18\text{-}20}$ and $FH^{1\text{-}5\text{^}18\text{-}20\text{^}R1\text{-}2}$ in TPB at 25°C for 10 min. Finally, lysis was developed by adding 50 μl of 4% ΔFBΔFH-NHS prepared in TPB at 37°C for 20 min. To measure haemolysis, cells were pelleted by centrifugation, and the 405 nm absorbance of the supernatant was measured. To measure the FI cofactor activity, 50 μl of 2% (v/v) C3b coated sheep erythrocytes was mixed with equal volume of serial diluted FH reagents containing universally with 2.5 μg/ml FI. After 25 min incubation, cells were pelleted and re-suspended with 100 μl of AP VBS buffer containing 35 μg/ml FB and 0.2 μg/ml FD and incubated at 37°C for an additional 15 min. Finally, the functional C3 convertase was quantified using cell lysis level by adding 50 μl of 4% ΔFBΔFH-NHS prepared in TPB at 37°C for 20 min. To express the cell lysis percentage, control samples included 100% (in water) and 0% (buffer only) lysis. The percentage of inhibition from lysis was calculated as 100-100×((A405 test sample-A405 0% control)/(A405 100%-A405 0% control)).

Guinea pig erythrocyte assay

Briefly prior to the experiment, Sheep erythrocytes were washed with GVB buffer and the cell concentration was adjusted to produce A_{405} reading of 0.8 when completely lysed in water. To set up the reaction, Guinea pig erythrocytes were suspended in 100 μl of 20% of NHS, FH reagents and Mg^{+}-EGTA (7 mM $MgCl_2$ and 10 mM EGTA) in GVB buffer. Haemolysis was detected after incubating the reaction mixture at 37°C for 60 min, followed by the addition of 150 μl of quenching buffer (GVB supplemented with 10 mM EDTA). The cells were pelleted by centrifugation at 1500*g* for 10 min, and absorbance (A_{405}) of 100 μl of supernatant measured

Measurement of mouse plasma C3 or FH reagent by ELISA

Intact C3 and FH reagent levels in the mouse serum samples were determined by ELISA. To determine mouse C3 level, 50 μl of the diluted sample (1 in 800) was used for analysis. 20 ng/well of mAb 11H9 (Hycult Biotech via Cambridge Bioscience, UK) were immobilized on a NUNC Maxisorp flat-bottom ELISA plate to capture mouse C3, then detected by an HRP-conjugated goat polyclonal anti-mouse C3 antibody (1 in 25000, MP Biomedicals, Santa Ana, CA catalog no. 0855557). The C3 concentrations were interpreted based on a standard curve

generated using purified mouse C3 with known concentration. Similarly, to measure concentration of FH reagents, 100 µl of diluted serum sample was added to an ELISA plate coated with 0.5 µg/well of OX-24 mAb. Following the wash, 100 µl of polyclonal sheep anti-human FH antibody (1 in 20000; PC030, The Binding Site, UK) was applied. After further washing, bound antibody was detected using a donkey anti-Sheep HRP (1 in 20000; 713-035-147-JIR (Jackson immunoResearch), via Stratech Scientific, UK). For each FH reagent, a corresponding standard curve was generated using the purified protein.

Immunostaining of mouse renal sections for murine C3 and human FH epitope

Five micrometer cyrosections from mouse kidneys were mounted on a SHANDON Colorfrost Plus microscope slide (Thermo Scientific), before fixing in 100% ice cold acetone and stored in -80°C. The thawed tissue sections were blocked for 1 hour with 60 µl of 20% (v/v) goat serum in PBS, then detected with 60 µl of FITC-conjugated goat polyclonal anti-mouse C3 Ab (1 in 200, MP Biomedicals, Santa Ana, CA catalog no. 0855500) and Dylight 650 (Innova Bioscience) directly conjugated OX24 monoclonal antibody (20 µg/ml) diluted in PBS for 30 min. After repetitive wash with PBS, the tissue sections were stained with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories; Burlingame) and covered with glass coverslips. Fluorescent images were taken at ×10 or ×20 magnification utilizing a Zeiss Axio imager II.

FHR Cross hybridization analysis

Quantification of fluid phase hybridized HDM-FH via ELISA

Human recombinant FHR-1^{1-2}, FHR-1, FHR-2 and FHR-5 were produced in house via CHO expression system and purified to homogeneity via metal affinity chromatograph. Purified proteins were biotinylated using Lightning-Link Rapid Type A Biotin antibody labeling Kit (Innova Bioscience), and buffer exchanged to PBS.

On an NUNC Maxisorp flat-bottom ELISA plate, three dilution series of biotinylated FHR were immobilized overnight at 4°C, the rest of the plate was immobilized with 50 µl of 2 µg/ml of OX-24 monoclonal antibody. Fluid phase hybridization was carried out at 37°C for 1 hour in a PCR machine, biotinylated FHR was mixed with FH reagent in PBS+4% BSA. The prepared ELISA plate was washed with PBST and blocked with PBST+1%BSA. 50 µl of the hybridized sample (1:500, 1:1000, 1:2000, and 1:4000) was applied to the OX-24 coated wells in PBS 2% BSA, while biotinylated FHR standard curve wells received PBS 2% BSA instead. After 1 hour, the plate was washed three times with PBST and bound b-FHRs detected with 50 µl of Streptavidin-HRPO (Jackson ImmunoResearch, 1:20000). After an hour incubation, the plate was washed and developed with 50 µl of TMB solution for 5 min. 50 µl of 10 % H_2SO_4 was used to stop the color development, and the plate was read at 450 nm. The hybridized FHR concentration was interpreted using the biotinylated FHR standard curve. The molar percentage of hybridised FHR was calculated as ((Hybridised FHR Concentration/homodimeric FH concentration)*molecular weight ratio*100).

Comparison of cross hybridization on ligand bound or free homodimeric mini-FH

On an NUNC Maxisorp flat-bottom ELISA plate, three dilution series of biotinylated FHR^{1-2}

were immobilized overnight at 4°C, the rest of the plate was immobilized with 50 µl of 5 µg/ml of human C3b (Comptech USA). The plate is washed and blocked. The homodimeric mini-FH (300 nM) and a concentration series of biotinylated recombinant FHR-1^{1-2} (3-0.25µg/ml) were either applied step-wisely or at the same time. FHR-1^{1-2} was also applied separately to acquire the background binding level. The plate was wash three times with PBST and detected with 50 µl of Streptavidin-HRPO (Jackson ImmunoResearch, 1:20000). After an hour incubation, the plate was washed and developed with 50 µl of TMB solution for 5 min. 50 µl of 10 % H_2SO_4 was used to stop the color development, and the plate was read at 450 nm.

immuno-affinity pull-down

FHR-1 was incubated with $FH^{1-5\text{^}18-20}$, $FH^{1-5\text{^}18-20\text{^}R1-2}$ or alone. The post-incubation samples were purified by OX23 antibody coated on a pre-blocked Maxisorb plate. The plate was washed, the captured molecules were eluted using Laemmli sample buffer and analyzed by non-reducing polyclonal anti-FH western blot.

References.

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