Supplemental methods

Factor H gene targeting

We used the pND1 vector to construct the targeting vector, which has neomycin (NEO) and diphtheria toxin (DT) cassettes for positive and negative selection, respectively.[1] This vector has a loxP site and two flippase recognition target (FRT) sites flanking the NEO cassette for potential removal by FLPe recombinase. Factor H gene fragments were amplified from 129/Sv mouse genomic DNA by using the Expand Long Template PCR system (Roche, Indianapolis, IN). The long arm was made from a 7kb fragment of the intron between SCR18 and SCR19 by PCR amplification and ligation into the PCR 2.1 vector (Invitrogen) using the following primers: 5'-GCGGCCGCCTGTGCAAGATGACAAATGTGGA-3' and 5'-CTCGAGCATTCCATGAAGAGATGAGAC-3'. This fragment was then digested with NotI and XhoI and subcloned into the pND1 vector upstream of the NEO cassette. The short arm sequence was 4.7kb in length and contained the 3' end of the intron preceding SCR 19 through the middle of the exon containing SCR20. This sequence was PCR amplified and cloned into the PCR 2.1 vector using the following primers: 5'-GGTACCCTCTTTAGCCTTCTGTAGCA-3' and 5'-GGTACCTCCACCATGATGATAATGGGC-3'. This sequence was then mutated to introduce two early stop codons (TCA-ACA to TAA-TGA) at the beginning of SCR 19 using the Stratagene QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, CA). Once sequence was confirmed, the short arm fragment was digested using KpnI and subcloned into the pND1 vector downstream of the NEO cassette at the same restriction site.

The targeting vector was then linearized by NotI digestion and transfected into TL-1 ES cells.[2] Clones were selected by G418 and screened by BamHI digestion of genomic DNA and

Southern blotting with a 350bp probe near the 3' end of the short arm. ES cell culture, vector transfection, clone selection and chimera mouse production were performed as previously described.[3] For genotyping, the following primers were used for detection of wild-type and mutated DNA by PCR: P1 (fH specific) 5'- ATGTCTCATCTCTTCATGGAATG-3', P2 (NEO specific) 5'-GGGTGGGATTAGATAAATGCC-3' and P3 (fH specific) 5'-CAGATGCCAACGTAGATAGGC-3'.

Generation of mouse fH antibodies

To generate anti-mouse fH polyclonal antibodies, mouse fH and fH SCR19-20 were expressed in HEK cells using the pCAGGS expression vector (kindly provided by Dr. J. Miyazaki at Osaka University, Japan). Mouse fH SCR19-20 was expressed and purified as described.[4] To generate full-length mouse fH, cDNA encoding the full-length mouse fH (kindly provided by Dr. M. Nonaka, University of Tokyo, Japan, nucleotide 110-4361 of NCBI NM_009888.3) in the pBluescript SK(-) vector was digested by EcoRV and AflII and ligated into the pCAGGS vector cassette used to express mouse fH SCR19-20 (reference 4). For both proteins, HEK cells were transfected with the above constructs using Fugene HD (Roche, Mannheim, Germany). After 48h transfection, cells were switched to serum-free medium and cultured for two more days. The cell culture medium was then collected and recombinant fH proteins were purified by Ni^{2+}-chelate chromatography. Rabbit polyclonal antibodies against recombinant mouse fH or fH SCR19-20 were generated by Cocalico Biologicals Inc (Reamstown, PA, USA).

Western blotting

Mouse plasma (0.25-2μl) was diluted with sample buffer and boiled before loading onto 6-8% SDS-PAGE gels under reducing conditions. Samples were then transferred to PVDF membrane and probed with appropriate antibodies. For C3 and fB, HRP goat anti-mouse C3 Ab (1:4000, MP Biomedicals) or affinity-purified goat anti-human fB Ab (cross-reacts with mouse fB; 1:5000, Complement Technology, Inc.) were used as primary antibodies, followed by detection with HRP rabbit-anti goat IgG (1:4000, Bio-Rad). For fH and properdin, rabbit anti-mouse fH or anti-mouse fH SCR 19-20 serum (1:4000, generated in this study), and purified rabbit anti-mouse properdin IgG (2.5μg/ml)[5] were used as primary antibodies, followed by detection with HRP goat-anti rabbit IgG (1:4000, Bio-Rad).

Histology

For light microscopy, kidneys were fixed in 10% formalin-PBS before processing and paraffin embedding. Samples were cut in 4μm sections and stained with H&E or periodic acid Schiff (PAS) reagent. Kidneys or livers were snap-frozen in OCT medium and stored at -80°C. For immunofluorescence studies, 4μm sections were cut and used for staining. For C3, FITC-conjugated goat anti-mouse C3 Ab was used (1:500, MP Biomedicals). Kidneys were stained for IgG by FITC-conjugated goat anti-mouse IgG Fab fragment (1:200, ImmunoResearch Laboratories). C9 was visualized by staining with rabbit anti-rat C9 (1:500, from Dr. Paul Morgan, Cardiff University) followed by staining with FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). For properdin, purified rabbit anti-mouse properdin IgG (5μg/ml)[5] was used followed by Cy3-conjugated goat anti-rabbit IgG (1:100, Abcam). For nephrin staining, guinea pig anti-mouse nephrin Ab (1:100, Progen) was used followed by Alexa Fluor 555-goat anti-guinea pig Ab (1:2000, Invitrogen).

Electron microscopy

Kidneys for electron microscopic examination were collected and immediately fixed overnight at 4°C with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1M sodium cacodylate buffer, pH7.4. After subsequent buffer washes, the samples were post-fixed in 2.0% osmium tetroxide for 1h at room temperature, and rinsed in distilled water prior to *en bloc* staining with 2% uranyl acetate. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in EMbed-812 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software.

Hemolytic assays

Erythrocyte lysis was measured using human and mouse erythrocytes based on previously published methods.[6, 7] Normal human erythrocytes (Valley Biomedical, Winchester, VA) at $5x10^6$ cells/reaction were added to a solution (100 μl final volume) containing Mg^{++}-EGTA GVB^{++} buffer, 50% normal human serum (NHS) in the presence or absence of 30μM human fH19-20 and anti-human CD55 antibody (AbD Serotec, final concentration: 7.5 μg/ml). In assays with anti-properdin antibody, undiluted NHS was incubated with IgG antibody (final concentration: 3 mg/ml) for 30 min at 4°C before use. Goat polyclonal anti-human properdin antibody was purchased from Complement Technologies, Inc. (Tyler, TX) and total IgG purified by ammonium sulfate precipitation. Recombinant human fH19-20 was expressed in *Pichia pastoris* as described previously.[8] For mouse hemolysis assays, $DAF^{-/-}/CD59^{-/-}$ mouse erythrocytes ($5x10^6$ cells/reaction) were mixed with 50% mouse serum diluted in Mg^{++}-EGTA

gelatin veronal buffer (GVB^{++}, final volume: 100 μl) and containing 100 μg/ml anti-Crry Ab in the presence or absence of 30μM murine fH SCR19-20. Rabbit polyclonal anti-mouse Crry antibody was generated as described previously.[9] It was purified by protein A affinity chromatography and found to inhibit Crry function (data not shown). Mouse fH SCR19-20 (mfH19-20) used in haemolytic assays was expressed in yeast. Briefly, the cDNA for mfH19-20 was amplified by PCR using the forward primer 5'GGTACC TCCGAGACTCAACAGGGAAATGTGGGC3' and reverse primer 5'TCTAGAAGTACACAAGTGGGATAATTGATGGTGC3' and a mouse liver cDNA library (Invitrogen) as template. Amplification products were cloned into expression vector PICZαB (Invitrogen) and proteins expressed in *Pichia pastoris* and purified by Nickel chelate chromatography as previously described.[10] EDTA-serum was used as a negative control in all assays. Cells were incubated for 20 min at 37°C, after which cold EDTA-PBS was added to stop the reaction. Reactions were centrifuged and the supernatant OD values were measured at 405nm. Percent lysis was calculated using the following formula, with maximum lysis set as OD value from erythrocytes lysed in water: $(OD_{sample} - OD_{serum\text{-}free\ cells})/OD_{max(water)}$.

Complement deposition on platelets

$DAF^{-/-}/Crry^{-/-}/C3^{-/-}$ mouse platelets were isolated as previously described.[11] Washed platelets ($5x10^6$) were incubated in 100 μl 40% WT mouse serum diluted with modified Tyrode's buffer[11] for 30 minutes at 37°C. Cells were then washed in FACS buffer (PBS with 1% BSA and 1% NaN_3) before staining with FITC-conjugated goat anti-mouse C3 Ab (Fab fragment, MP Biomedicals). Samples were analyzed on a FACS Calibur flow cytometer (Becton Dickinson) and with FlowJo software (TreeStar, Ashland, OR).

Assessment of anti-fH autoantibody production in mice

To assess if autoantibodies against the truncated fH protein (fH SCR1-18) were produced in $fH^{m/m}$ and $fH^{m/m}/fP^{-/-}$ mice, recombinant mouse fH SCR1-18 was expressed and used to test mutant mouse sera by ELISA. Full-length mouse fH cDNA (described above) was used as a template to amplify by PCR SCR1-18 using the following primers: 5'-GCAGCGGCCGCTGAAGATTGTAAAGGTCCTCCT-3' and 5'-TATCCCGGGGTCTCGGCACTTTGG-3' (underlined: NotI site; double underline: SmaI site). The PCR product was amplified using Roche Expand Long Template PCR system (Indianapolis, IN) and cloned into the pCR2.1 vector. The DNA was then subcloned at NotI and SmaI sites into the pCAGGS expression vector cassette used for mfH19-20 expression.[4] HEK cells were transfected using Roche X-tremeGENE HP (Indianapolis, IN). After 48h transfection, cell culture supernatant was collected and recombinant fH SCR1-18 was purified by Ni^{2+}-chelate chromatography (Qiagen). Purified fH SCR1-18 was then dialyzed overnight against PBS.

To confirm the identity of the expressed mouse fH SCR1-18, ELISA plates were coated with 5μg/ml of the protein in 0.1M carbonate buffer for 1h at 37°C. Plates were then blocked with 1%BSA-PBS for 1h at RT and washed 3 times with PBS-T. Then plates were incubated with purified polyclonal rabbit anti-full length mouse fH antibodies for 1h at RT. Following washing, the plates were incubated with HRP-goat anti-rabbit IgG (1:4000, Bio-Rad) for 1h at RT. After washing, plates were developed using OptEIA substrate (BD Biosciences).

To detect murine autoantibodies against fH SCR1-18, ELISA plates were coated with 5μg/ml purified fH SCR1-18 in 0.1M carbonate buffer for 1h at 37°C. Plates were then blocked with 1%BSA-PBS for 1h at RT and washed 3 times with PBS-T, followed by addition of serially

diluted mouse serum in 1% BSA-PBS, starting at 1:100. Following washing, plates were incubated with HRP-rabbit anti-mouse IgG (1:5000, Sigma) for 1h at RT. After washing, plates were developed using OptEIA substrate (BD Biosciences).

Supplemental References

1. Kimura, Y, Miwa, T, Zhou, L & Song, WC: Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. *Blood,* 111**:** 732-40, 2008.

2. Joyner, A: *Gene Targeting: A Practical Approach*, New York, Oxford University Press 2000.

3. Sun, X, Funk, CD, Deng, C, Sahu, A, Lambris, JD & Song, W-C: Role of decay-accelerating factor in regulating complement activation on the erythrocyte surface as revealed by gene targeting. *Proc Natl Acad Sci U S A,* 96**:** 628-633, 1999.

4. Barata, L, Miwa, T, Sato, S, Kim, D, Mohammed, I & Song, WC: Deletion of Crry and DAF on murine platelets stimulates thrombopoiesis and increases fH-dependent resistance of peripheral platelets to complement attack. submitted.

5. Kimura, Y, Zhou, L, Miwa, T & Song, WC: Genetic and therapeutic targeting of properdin in mice prevents complement-mediated tissue injury. *J Clin Invest,* 120**:** 3545-54, 2010.

6. Ferreira, VP, Herbert, AP, Hocking, HG, Barlow, PN & Pangburn, MK: Critical role of the C-terminal domains of factor H in regulating complement activation at cell surfaces. *J Immunol,* 177**:** 6308-16, 2006.

7. Heinen, S, Jozsi, M, Hartmann, A, Noris, M, Remuzzi, G, Skerka, C & Zipfel, PF: Hemolytic uremic syndrome: a factor H mutation (E1172Stop) causes defective complement control at the surface of endothelial cells. *J Am Soc Nephrol,* 18**:** 506-14, 2007.

8. Herbert, AP, Uhrin, D, Lyon, M, Pangburn, MK & Barlow, PN: Disease-associated sequence variations congregate in a polyanion recognition patch on human factor H revealed in three-dimensional structure. *J Biol Chem,* 281**:** 16512-20, 2006.

9. Miwa, T, Zhou, L, Kimura, Y, Kim, D, Bhandoola, A & Song, WC: Complement-dependent T cell lymphopenia caused by thymocyte deletion of the membrane complement regulator Crry. *Blood,* 113**:** 2684-94, 2009.

10. Heinen, S, Hartmann, A, Lauer, N, Wiehl, U, Dahse, HM, Schirmer, S, Gropp, K, Enghardt, T, Wallich, R, Halbich, S, Mihlan, M, Schlotzer-Schrehardt, U, Zipfel, PF & Skerka, C: Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. *Blood,* 114**:** 2439-47, 2009.

11. Kim, DD, Miwa, T, Kimura, Y, Schwendener, RA, van Lookeren Campagne, M & Song, W-C: Deficiency of decay-accelerating factor and complement receptor 1-related gene/protein y on

murine platelets leads to complement-dependent clearance by the macrophage phagocytic receptor CRIg. *Blood,* 112**:** 1109-1119, 2008.

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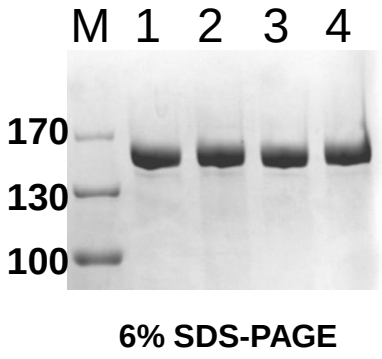

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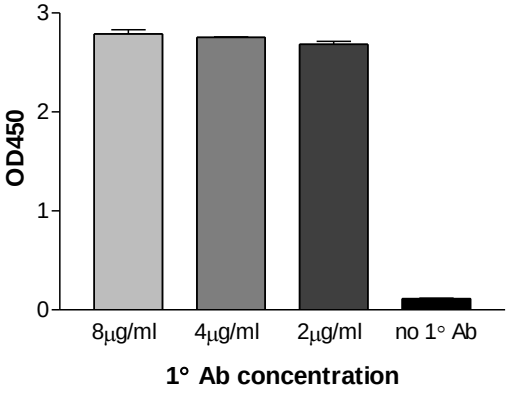

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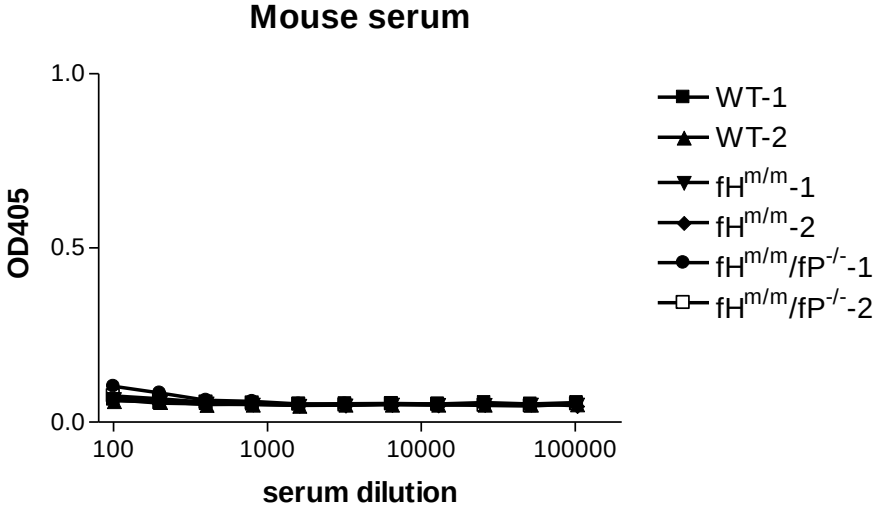

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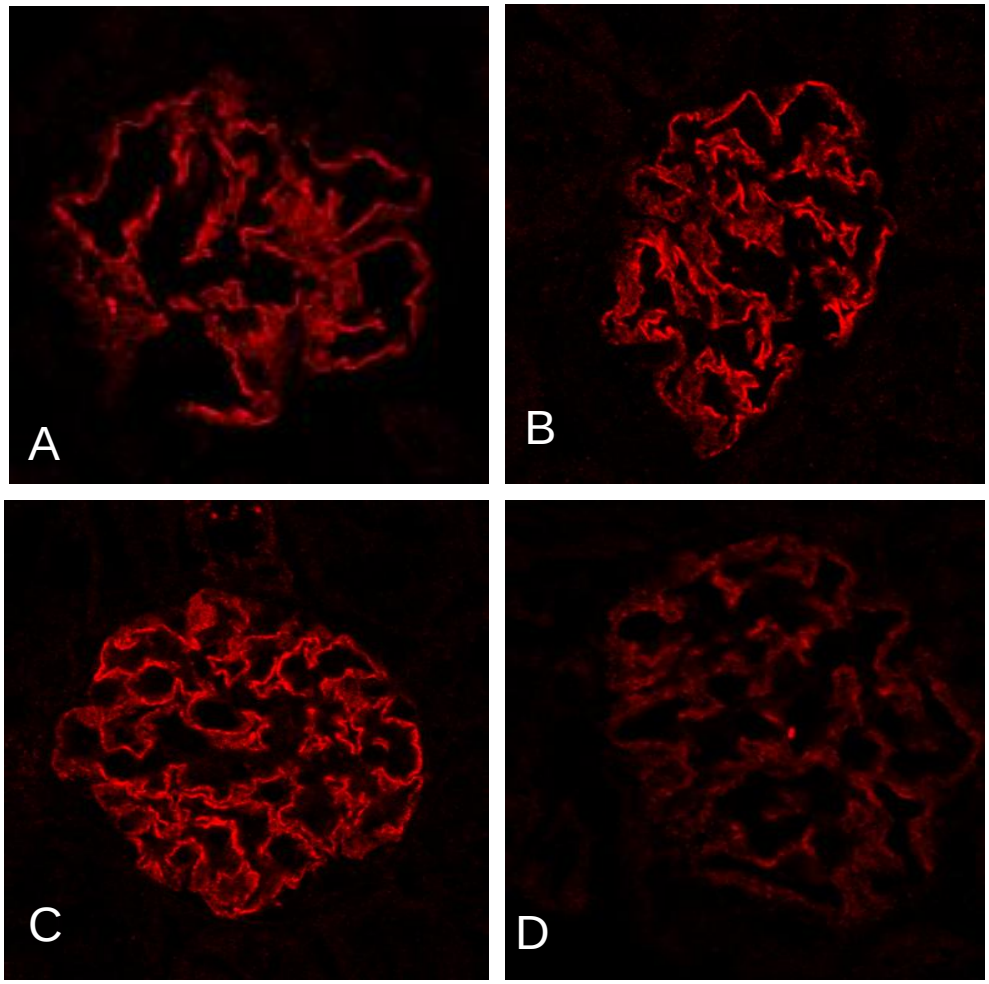

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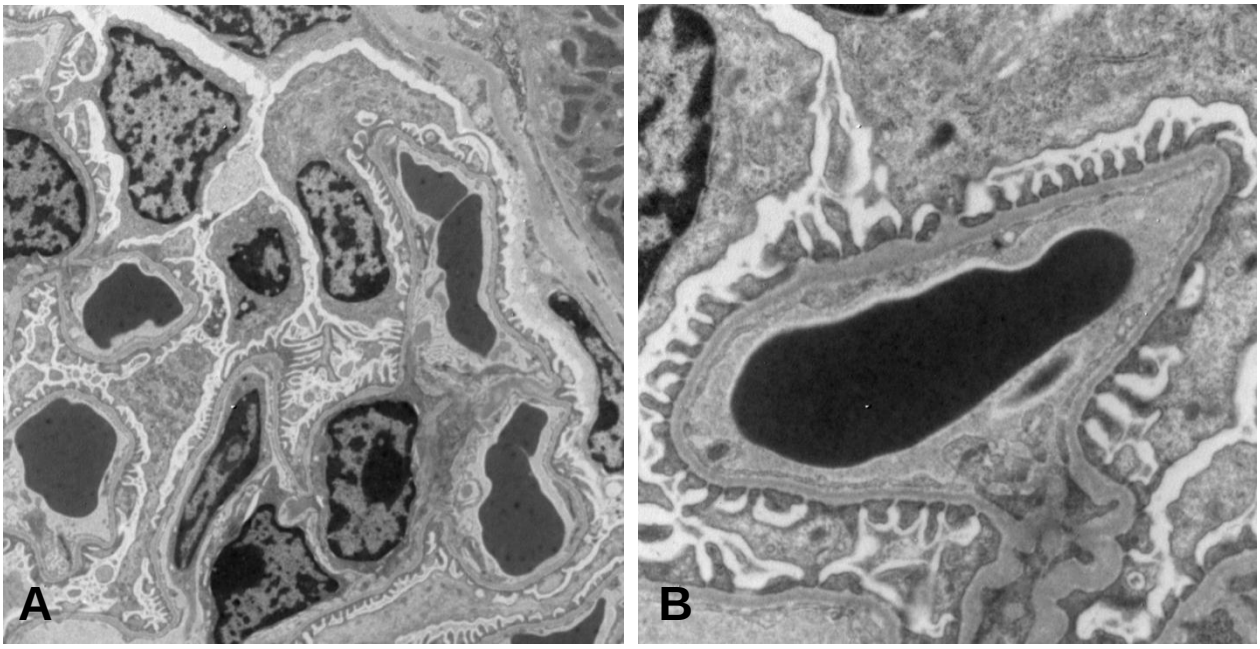

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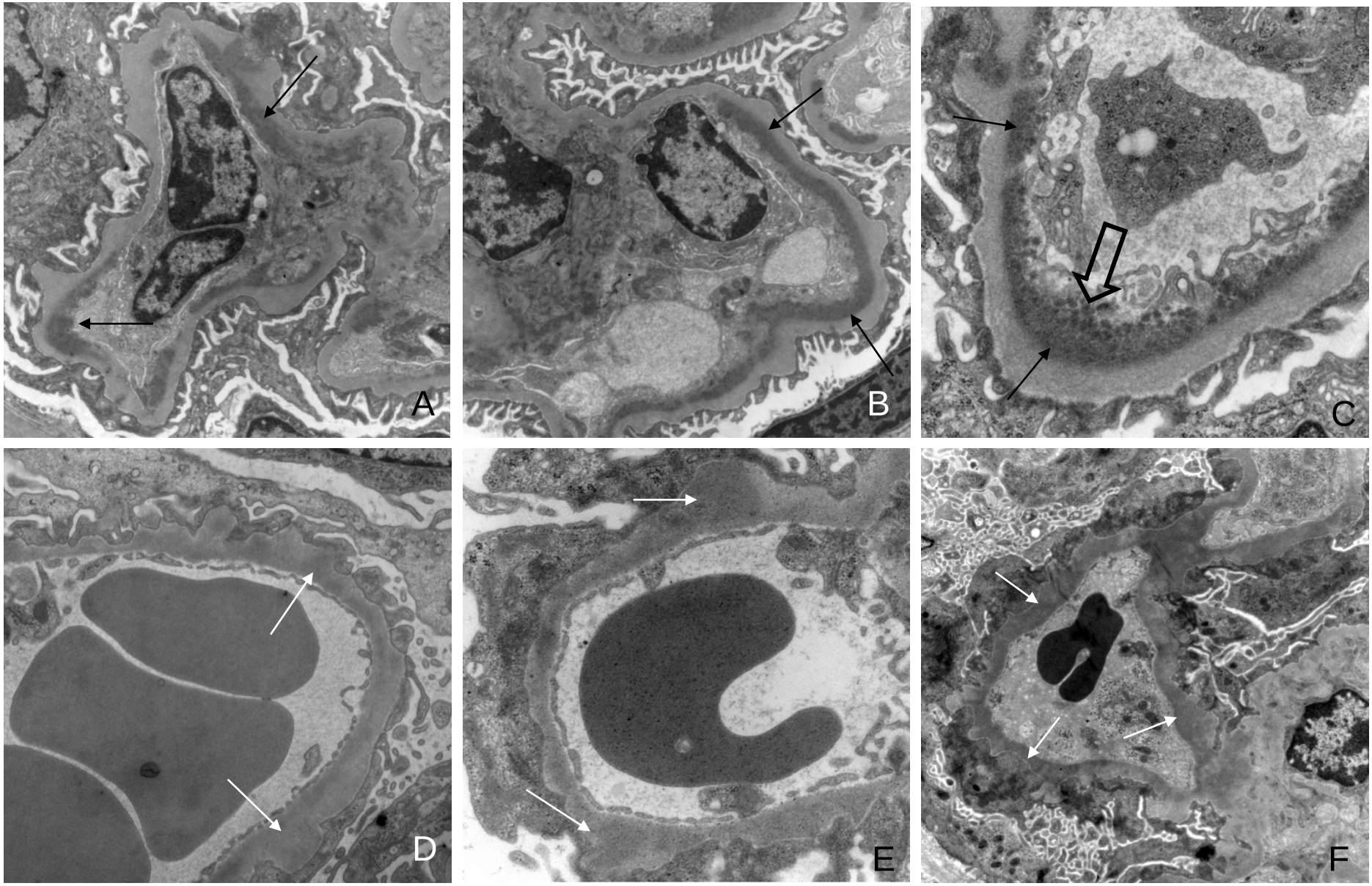

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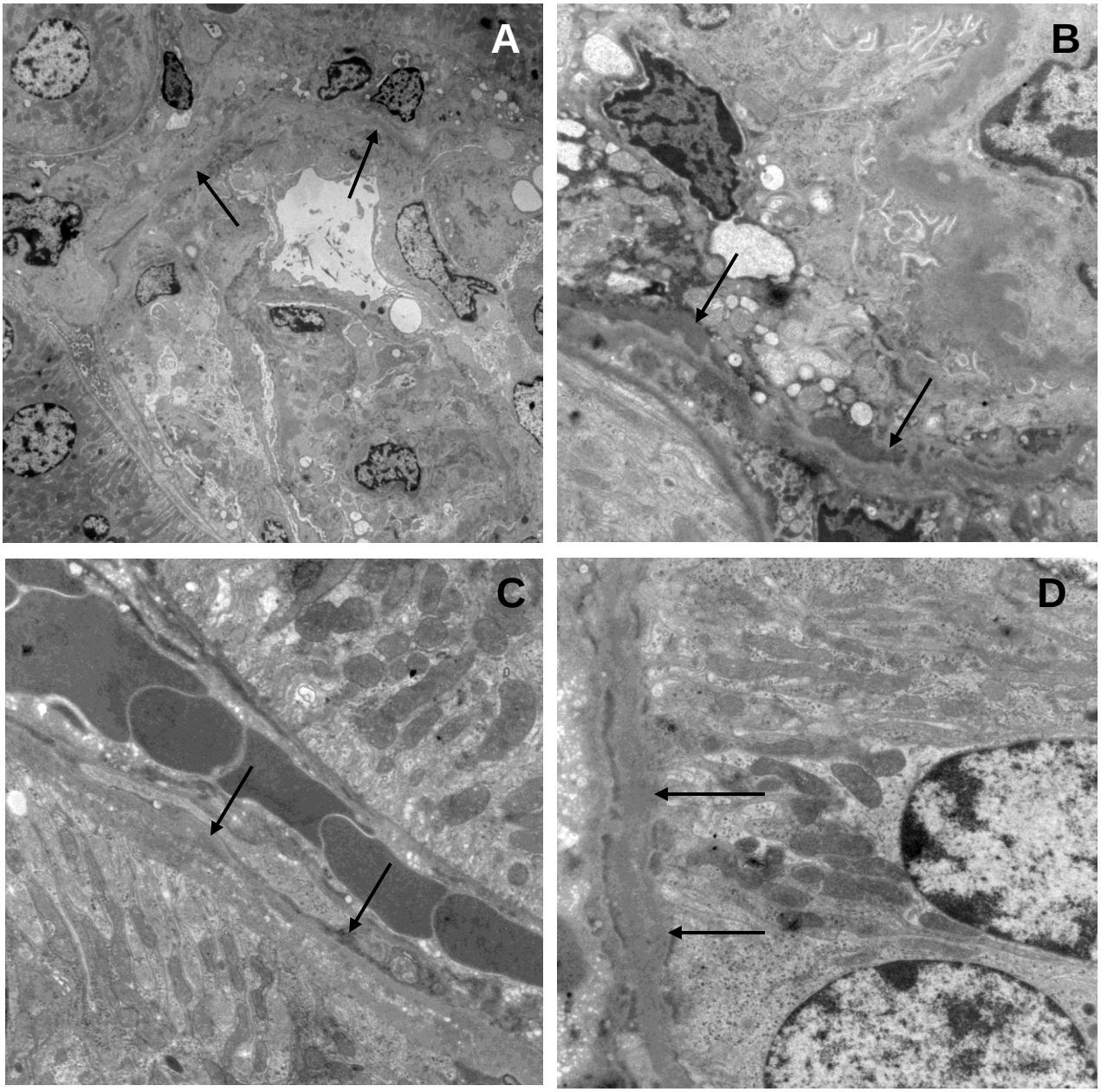

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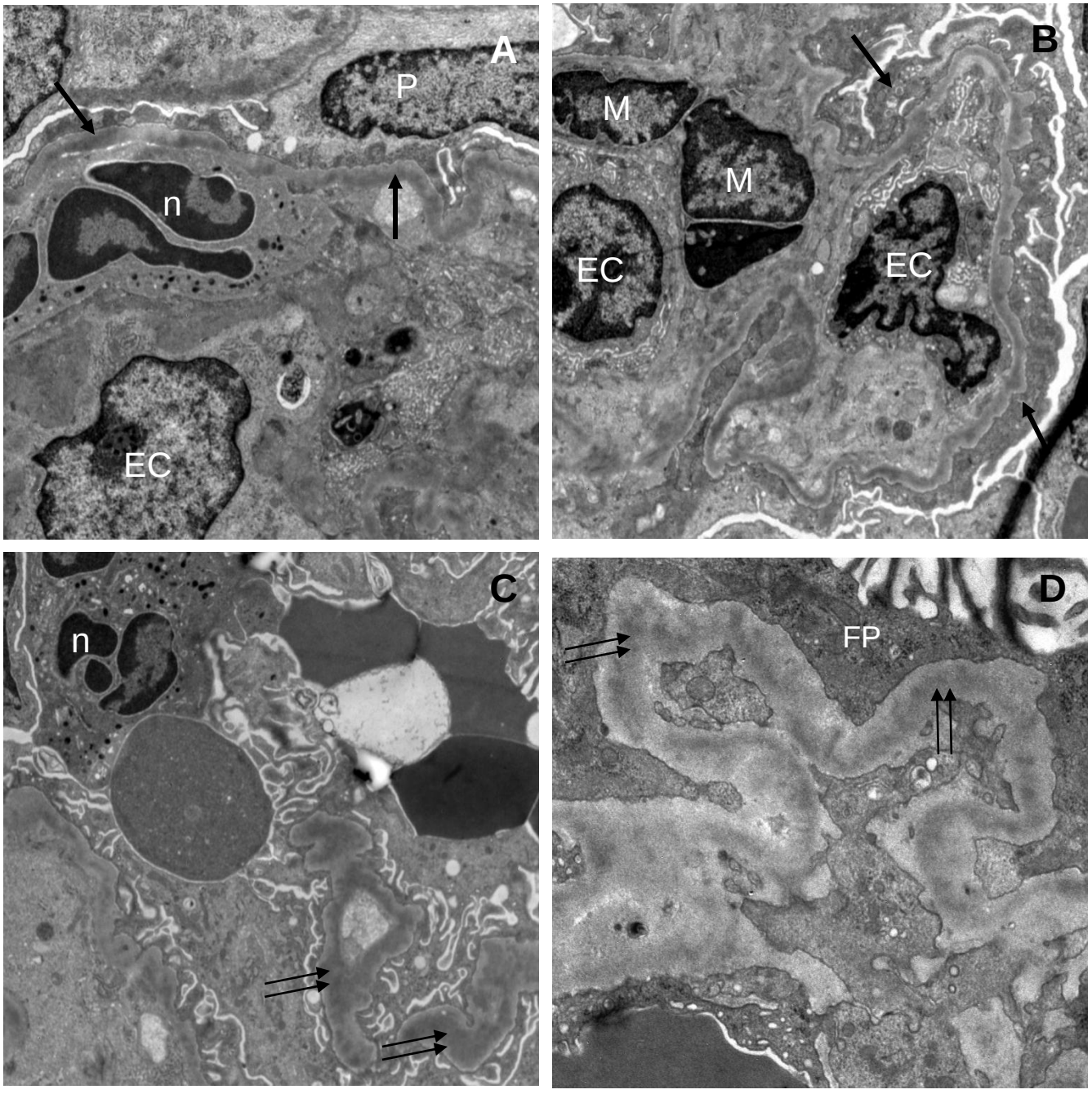

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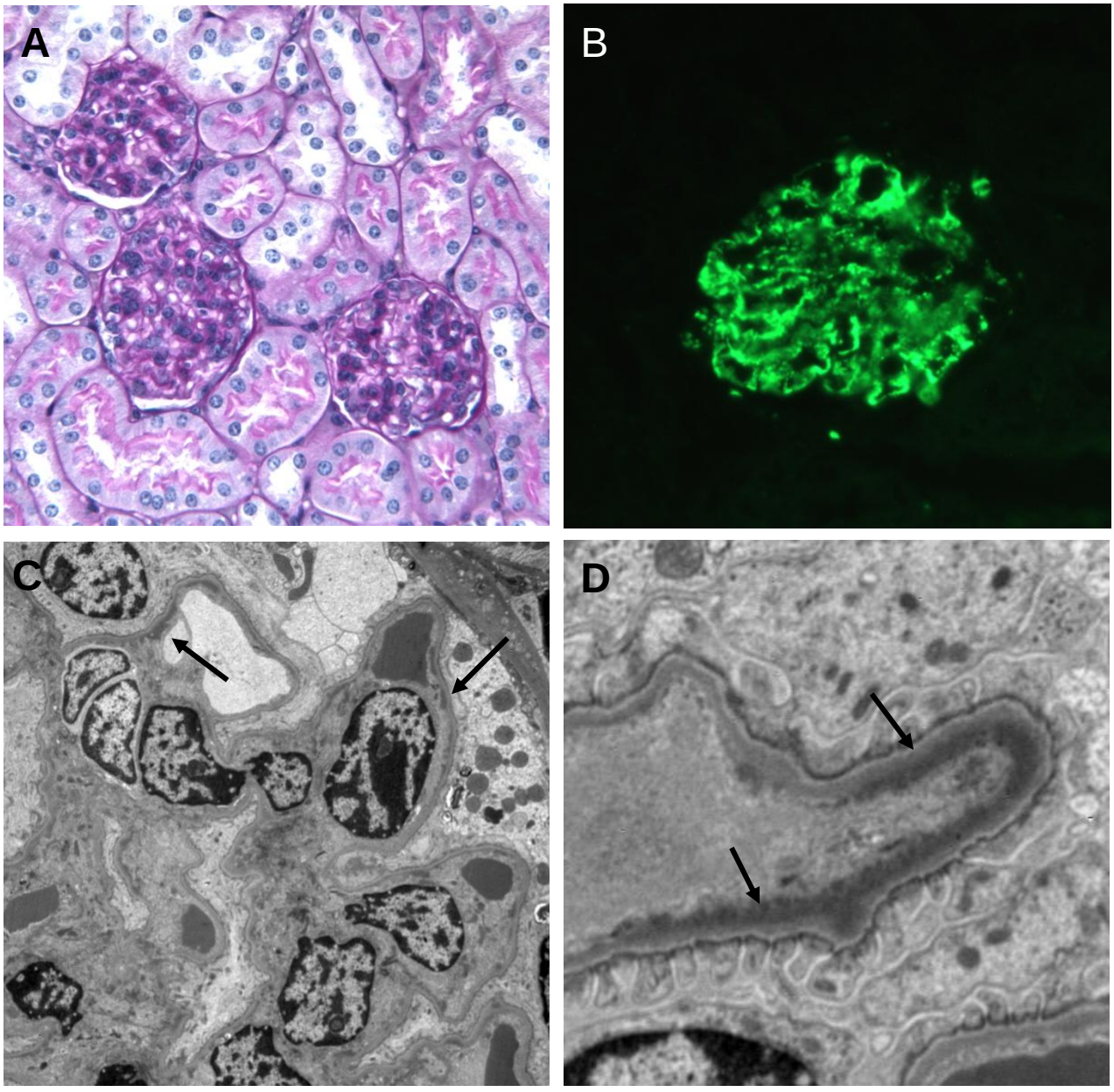

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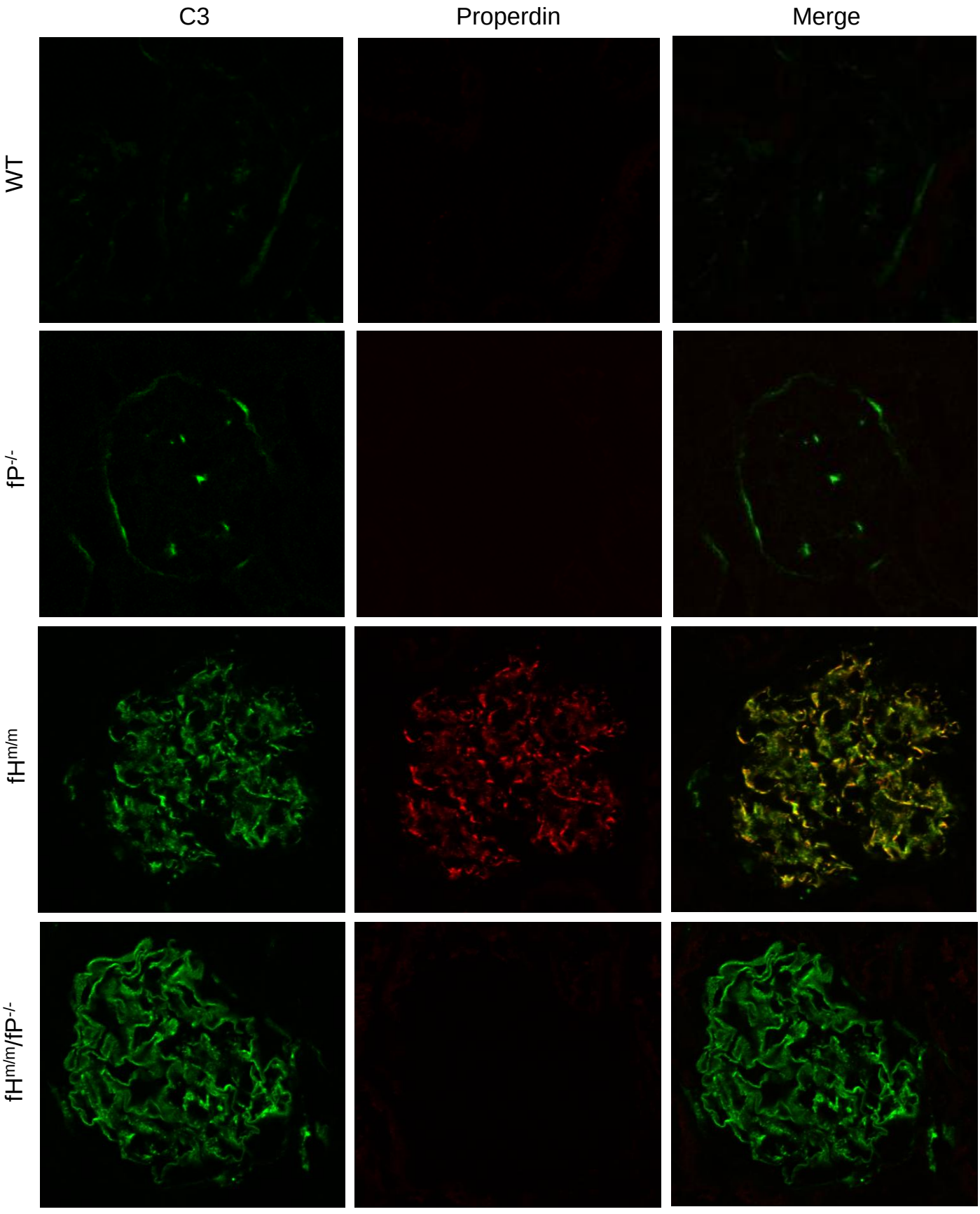

supplemental Fig 8

Supplemental Figure legends

Supplemental Figure 1. $fH^{m/m}$ and $fH^{m/m}/fP^{-/-}$ mice do not develop autoantibodies against mutant fH. (A) Coomassie blue staining of purified recombinant mouse fH SCR1-18 on 6% SDS-PAGE gel showing a single band at the expected molecular weight. (B) Detection of plate-coated recombinant mfH SCR1-18 by ELISA using a rabbit polyclonal anti-mouse fH Ab, confirming the protein identify of mfH SCR1-18. n=2 wells for each concentration of 1° (primary) antibody. (C) Plate-coated mfH SCR1-18 was not detected by WT, $fH^{m/m}$ or $fH^{m/m}/fP^{-/-}$ mouse serum, suggesting no anti-fH autoantibodies were present in these mice (n=2 mice for each genotype).

Supplemental Figure 2. Nephrin expression was reduced in the glomeruli of $fH^{m/m}/fP^{-/-}$ mice, indicating podocyte injury. Frozen sections of kidney from 8-week old WT (**A**), $fP^{-/-}$(**B**), $fH^{m/m}$ (**C**) and $fH^{m/m}/fP^{-/-}$ mice (**D**) were stained for nephrin (red) and examined by confocal immunofluorescence microscopy. Original magnification 400x for all panels. Results are representative of two independent experiments.

Supplemental Figure 3. Electron microscopy of $fP^{-/-}$ mouse kidneys showing normal architecture and tissue integrity. The glomeruli of an 8-week old $fP^{-/-}$ mouse had normal cellularity and patent capillary loops (**A**, 6000x), as well as normal GBM with intact foot processes (**B**, 20,000x).

Supplemental Figure 4. Electron microscopy showing different patterns of dense deposits in the kidneys of $fH^{m/m}$ and $fH^{m/m}/fP^{-/-}$ mice. Glomeruli from 12-month old $fH^{m/m}$ mice had defined segmental and linear subendothelial deposits (**A**-**C**, black arrows) while glomeruli of 2-month old $fH^{m/m}/fP^{-/-}$ mice had irregular basement membrane thickening with intramembranous dense deposits (**D**-**F**, white arrows). In some $fH^{m/m}$ mouse kidney sections, electron dense granules were seen near the subendothelial deposits (**C**, open arrow). Original magnification: **A**-**B**, 12,000x; **C**, **E**, 30,000x; **D**, 15,000x; **F**, 10,000x.

Supplemental Figure 5. Electron microscopy showing focal dense deposits on Bowman's capsule and tubular basement membrane in kidneys of $fH^{m/m}/fP^{-/-}$ mice. (**A, B**) Dense deposits on Bowman's capsule presented as intramembranous (**A,** 3000x) or sub-capsular (**B,** 10,000x). (**C, D**) Focal dense deposits were also found both within (**D**, 10,000x) and adjacent (**B**, 10,000x) to tubular basement membranes of normal or increased thickness. Black arrows indicate locations of dense deposits.

Supplemental Figure 6. Electron microscopy showing $fH^{m/m}$ mice treated with anti-fP mAb had similar glomerular pathology to $fH^{m/m}/fP^{-/-}$ mice. (**A, B**) Glomeruli had significant podocyte injury and foot process effacement (black arrows) in addition to leukocyte (neutrophil, n) infiltration. Endothelial cell (EC) activation, and mesangial cell (M) and matrix proliferation were also evident. P, podocyte. (**C, D)** Considerable GBM

thickening and intramembranous dense deposits (double arrows) were also observed, especially in areas of podocyte foot process effacement (FP). Original magnifications: 10,000x for **A-C** and 20,000x for **D**.

Supplemental Figure 7. Light, immunofluorescent and electron microscopy of kidneys from $fH^{m/m}$ mice treated for 18 weeks with an irrelevant control mouse mAb. (**A)** PAS staining shows some membrane thickening and mesangial proliferation, but no crescent formation or interstitial injury was evident (400x). (**B)** Glomerular C3 deposition was predominantly granular and was detected both on mesangial cells and in capillary loops (400x). (**C-D)** EM showed subendothelial dense deposits (black arrows), as seen in untreated $fH^{m/m}$ mice of similar age, rather than intramembranous. Original magnifications: 6,000x for **C**, 25,000x for **D**.

Supplemental Figure 8. Colocalization of C3 and fP in the kidney of $fH^{m/m}$ mice by confocal fluorescence microscopy. WT and $fP^{-/-}$ kidneys showed no glomerular C3 (green) or fP (red) staining, or had only trace C3 staining on the Bowman's capsule and the mesangium as is often observed in naïve WT mice. In the $fH^{m/m}$ mouse kidney, glomerular C3 staining was granular and mesangial and largely colocalized with fP (yellow); In the $fH^{m/m}/fP^{-/-}$ mouse kidney, C3 staining was linear and localized to the capillary loops, and as expected no fP staining was detected. Original magnification 400x.