

Distinct roles for the complement regulators factor H and Crry in protection of the kidney from injury



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Mutations in the complement regulatory proteins are associated with several different diseases. Although these mutations cause dysregulated alternative pathway activation throughout the body, the kidneys are the most common site of injury. The susceptibility of the kidney to alternative pathway-mediated injury may be due to limited expression of complement regulatory proteins on several tissue surfaces within the kidney. To examine the roles of the complement regulatory proteins factor H and Crry in protecting distinct renal surfaces from alternative pathway mediated injury, we generated mice with targeted deletions of the genes for both proteins. Surprisingly, mice with combined genetic deletions of factor H and Crry developed significantly milder renal injury than mice deficient in only factor H. Deficiency of both factor H and Crry was associated with C3 deposition at multiple locations within the kidney, but glomerular C3 deposition was lower than that in factor H alone deficient mice. Thus, factor H and Crry are critical for regulating complement activation at distinct anatomic sites within the kidney. However, widespread activation of the alternative pathway reduces injury by depleting the pool of C3 available at any 1 location.

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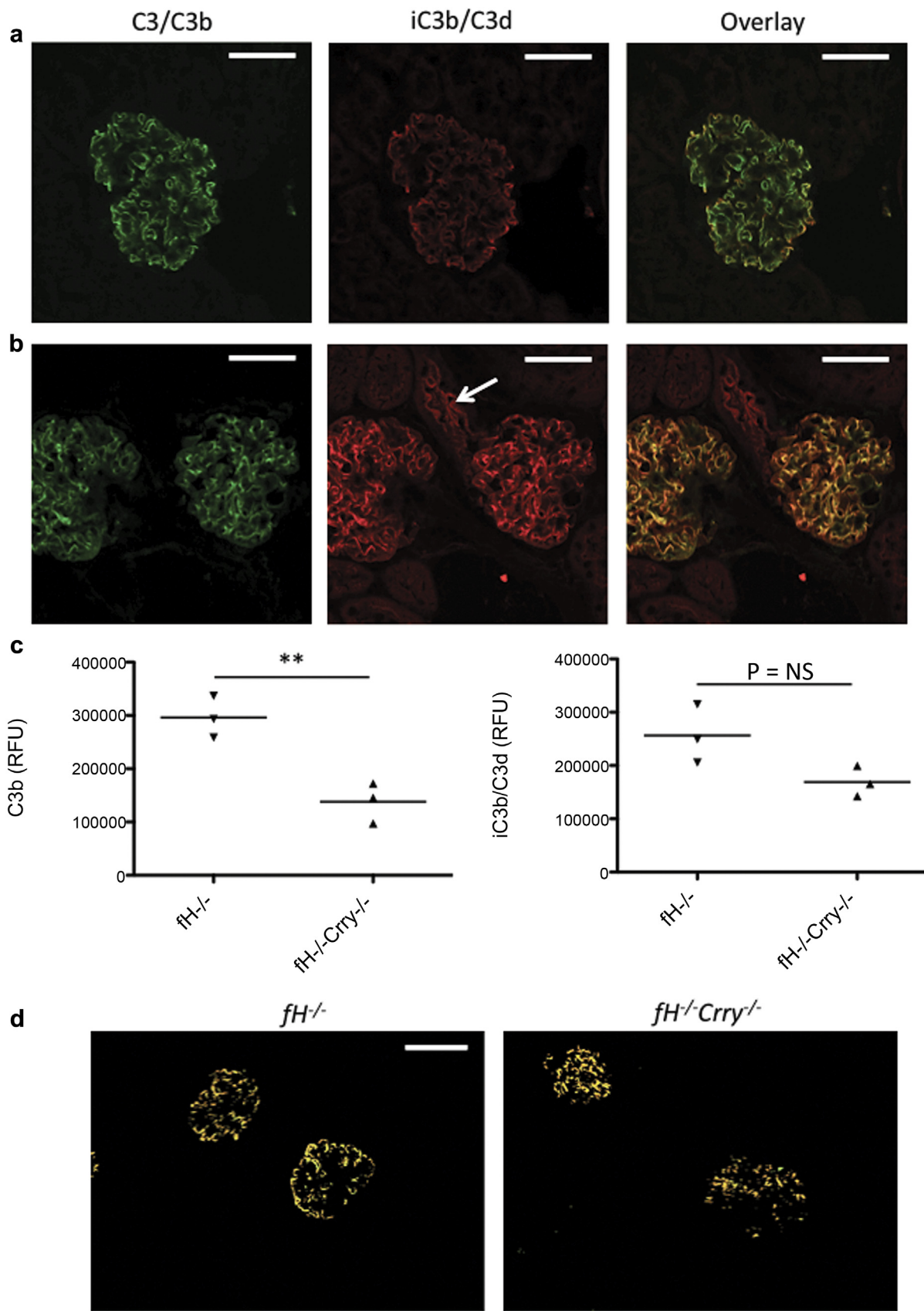
The complement system is an important part of the innate immune system. It defends the host against pathogens and also facilitates the clearance of injured cells and immune complexes.¹ Uncontrolled activation of the complement system can cause inflammatory injury, however, and a group of regulatory proteins controls complement activation on host tissues.² Every cell in the body expresses 1 or more of these regulatory proteins, and soluble regulatory proteins (factor H for the alternative pathway and C4 binding protein for the classical pathway) circulate at high concentrations in plasma. There is redundancy in the control of the complement cascade, and most tissue surfaces are protected by ≥ 1 regulatory protein. Nevertheless, a large number of diseases are associated with genetic mutations and variations in the complement regulators, highlighting the need for the full repertoire of these proteins to prevent pathologic complement activation and autologous injury. Interestingly, systemic defects in complement regulation often manifest with disease that is limited to specific organs, and the kidneys are particularly susceptible to injury in this setting.³

A key example of this is C3 glomerulopathy (C3G), a recently described form of glomerulonephritis in which complement proteins are deposited in the glomeruli in the relative absence of Ig.⁴ C3G is associated with numerous different molecular defects that cause systemic alternative pathway (AP) dysregulation, including mutations in the genes for complement regulatory proteins and autoantibodies that block AP regulation.^{5–9} Although these defects affect complement regulation throughout the body, patients typically present with isolated glomerulonephritis. Most patients with C3G have C3nef, an autoantibody that prevents degradation of the alternative pathway C3 convertase by factor H.¹⁰ Furthermore, the genetic mutations that have been identified in patients with C3G have most frequently involved the gene for factor H.^{5,6,11,12} Mice with a targeted deletion of the gene for factor H (*fH*^{-/-} mice) develop spontaneous glomerulonephritis with the histologic characteristics of C3G, including abundant C3 deposits along the glomerular basement membrane (GBM).¹³

It is not known why the kidney is so frequently and so uniquely affected in patients with systemic defects in regulation of the AP. Factor H controls AP activation both in the

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fluid phase and on tissue surfaces, and there is debate as to whether AP activation in C3G occurs in the plasma or directly on the GBM. Fluid phase complement activation could cause deposition of C3 fragments on the GBM as they are filtered through the glomeruli,¹⁴ and 1 of the genetic defects identified in patients with C3G was shown to impair regulation of the AP in the fluid phase.⁷ On the other hand, the GBM is an acellular surface that is exposed to plasma proteins through a fenestrated glomerular endothelium. The GBM matrix does not contain intrinsic complement regulatory proteins, and this may leave the GBM particularly vulnerable to AP-mediated inflammation in patients with factor H defects.

Mutations in the gene for membrane cofactor protein (MCP) have been rarely identified in patients with C3G,^{5,6} and MCP haplotypes also affect the risk of developing C3G.⁶ Abnormal MCP function would suggest that defective AP regulation on the surface of renal cells contributes to disease. It is also notable that complement regulation on tubular epithelial cells is critically dependent on a single complement regulatory protein, the transmembrane protein Crry.^{15,16} Mice with genetic deletion of Crry develop pathologic complement activation on the basolateral surface of the tubules.^{17,18} MCP is expressed on human tubular epithelial cells and probably functions on this surface in a fashion similar to Crry in rodents.^{15,19} The complete absence of both Crry and factor H in mice would be predicted to cause more extensive injury than deficiency of either protein alone. The closest equivalent in humans, that of combined deficiency of factor H and MCP, has not been reported.

The dependence of different renal surfaces on specific regulatory proteins may explain the unique susceptibility of the kidney to AP-mediated injury. Impaired function of factor H may be sufficient to permit AP activation on the GBM, for example, and impaired function of Crry may be sufficient to permit AP activation on tubular epithelial cells. Given the possibility that factor H and Crry protect distinct tissue sites within the kidney, we hypothesized that simultaneous deficiency of both proteins would cause renal injury that is more severe than that caused by either protein alone. To test this hypothesis, we bred mice with targeted genetic deletions of the genes for both factor H and Crry ($fH^{-/-}Crry^{-/-}$ mice) and compared them to mice with single targeted deletion of the gene for factor H ($fH^{-/-}$ mice). We also reconstituted $fH^{-/-}$ mice with purified factor H to determine whether factor H controls AP activation directly on the GBM. The goal of these studies was to identify the contribution of each complement

regulatory protein to the control of AP activation on different surfaces within the kidney and to improve our understanding of the mechanisms by which mutations in these proteins cause glomerular disease.

RESULTS

Breeding of $fH^{-/-}Crry^{-/-}$ mice

Homozygous deficiency of Crry in mice is lethal *in utero* due to uncontrolled alternative pathway activation on the placenta. This can be prevented by maternal C3 deficiency.²⁰ Factor H deficiency leads to a secondary deficiency of C3 due to consumption of the C3.¹³ We intercrossed $fH^{-/-}Crry^{+/+}$ mice and generated $fH^{-/-}Crry^{-/-}$ pups. Once established, the $fH^{-/-}Crry^{-/-}$ mice bred normally, indicating that the depletion of C3 in factor H-deficient mothers was sufficient to prevent loss of loss $Crry^{-/-}$ embryos.

Deficiency of Crry reduces glomerular C3 deposits in $fH^{-/-}$ mice

Complement activation on tissue surfaces causes covalent attachment of C3b.²¹ It has previously been reported that C3d (a downstream degradation fragment of C3b) is deposited in the glomeruli of $fH^{-/-}$ mice.²² To confirm this, we immunostained kidneys from 14-month-old $fH^{-/-}$ mice with an antibody that recognizes the iC3b and/or C3d forms of C3, but does not recognize intact C3 or C3b.²³ We found that iC3b/C3d is deposited in the glomeruli of $fH^{-/-}$ mice in the same pattern as is C3b (Figure 1a), suggesting that the deposited C3b is metabolized to iC3b and C3d *in situ*. We then examined C3 deposition in age-matched $fH^{-/-}Crry^{-/-}$ mice (Figure 1b). We observed less overall C3b in $fH^{-/-}Crry^{-/-}$ mice than in $fH^{-/-}$ mice (Figure 1c). Dual staining of kidney sections with antibodies for C3b and for a component of the GBM showed some areas of colocalization in $fH^{-/-}$ mice, although there were other areas where the proteins did not colocalize (Supplementary Figure S1).

Both factor H and Crry serve as cofactors for the cleavage of C3b by factor I, so we anticipated that deficiency of both proteins would halt C3 metabolism at the level of C3b. There was a trend toward less iC3b/C3d in the glomeruli of $fH^{-/-}Crry^{-/-}$ mice compared with $fH^{-/-}$ mice (Figure 1c). Nevertheless, some iC3b/C3d was detected in all of the glomeruli. To confirm these findings, C3 and C3d deposits were examined in $fH^{-/-}Crry^{-/-}$ mice from a different animal colony (Supplementary Figure S2). C3b and C3d were significantly lower in the glomeruli of those mice. Extraction of images from kidneys double stained for C3b and iC3b/C3d

Figure 1 | C3 metabolism in the glomeruli of mice with targeted deletion of the genes for factor H and Crry. We examined the deposition and metabolism of C3 in the glomeruli of $fH^{-/-}$ and $fH^{-/-}Crry^{-/-}$ mice using confocal microscopy. (a) Kidney sections of $fH^{-/-}$ mice were stained using antibodies to detect C3b (green) and iC3b/C3d (red). Both forms of C3 were detected along the capillary loops. Original magnification $\times 600$. Bar = 50 μ m. (b) In $fH^{-/-}Crry^{-/-}$ mice, C3b and iC3b/C3d colocalized within the mesangium. iC3b/C3d was also detected within the proximal tubules (arrow). Original magnification $\times 600$. (c) The intensities of C3b and iC3b/C3d were measured in the glomeruli of $fH^{-/-}$ and $fH^{-/-}Crry^{-/-}$ mice. Less glomerular C3b was detected in the $fH^{-/-}Crry^{-/-}$ mice. There was a trend toward lower levels of iC3b/C3d in the $fH^{-/-}Crry^{-/-}$ mice, but some iC3b/C3d was seen in all of the glomeruli. $**P < 0.01$. (d) To examine the sites of iC3b/C3d generation, the colocalization function of Olympus FV10-ASW software was used to isolate areas of C3b and iC3b/C3d colocalization in the kidneys of $fH^{-/-}$ and $fH^{-/-}Crry^{-/-}$ mice. Colocalized C3b and iC3b/C3d deposits were seen in the capillary loops of $fH^{-/-}$ mice and in the mesangium of $fH^{-/-}Crry^{-/-}$ mice. Original magnification $\times 200$. Bar = 50 μ m. fH, factor H; RFU, relative fluorescence unit.

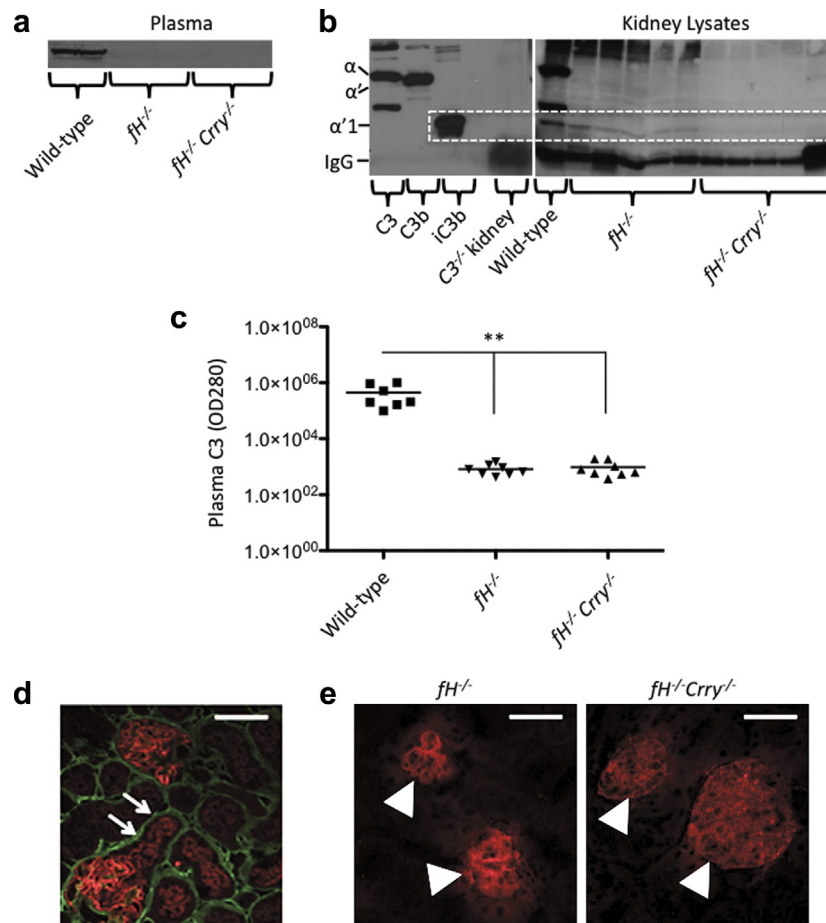


Figure 2 | Complement activation in mice with targeted deletions of the genes for both factor H and Crry. (a) Western blot analysis of C3 fragments in the plasma of *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice demonstrates that intact C3 levels are lower in both strains of mice than in wild-type mice. (b) A Western blot of kidney lysates demonstrates that the C3α'1 fragment of iC3b is more abundant in *fH*^{-/-} mice than in *fH*^{-/-}*Crry*^{-/-} mice. Purified proteins were used to identify the different C3α chain fragments, and the C3α'1 band is indicated with the dashed line. (c) Plasma C3 levels were measured by enzyme-linked immunosorbent assay. C3 levels were significantly lower in the plasma of *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice than in that of wild-type mice. ***P* < 0.01. (d) Kidney sections of *fH*^{-/-}*Crry*^{-/-} mice were stained using antibodies to detect collagen IV (green) and iC3b/C3d (red). Collagen IV was seen in the tubular basement membrane at the basolateral side of the tubules, demonstrating that iC3b/C3d was deposited on the apical surface of the proximal tubules (arrows). (e) Decay accelerating factor was detected in the glomeruli (arrowheads) of both *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice. Original magnification ×600. Bar = 50 μm. fH, factor H; OD280, optical density of 280 nm.

showed a pattern of colocalization in the capillary loops of *fH*^{-/-} mice and in the mesangium of *fH*^{-/-}*Crry*^{-/-} mice (Figure 1d). This indicates that cofactor function is available within the glomeruli, even in the absence of both factor H and Crry. In addition to the glomerular deposits, iC3b/C3d was also seen in the proximal tubules.

Intact C3 in the plasma of 14-month-old *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice was lower than that in wild-type mice by Western blot analysis, indicating that it was consumed in both strains of mice (Figure 2a). Lower levels of the α'1 C3 fragment were seen in kidney lysates from *fH*^{-/-}*Crry*^{-/-} mice than in *fH*^{-/-} mice (Figure 2b), which is consistent with the confocal microscopy findings. Plasma C3 levels were also lower in both strains of mice compared with wild-type mice when measured by enzyme-linked immunosorbent assay (ELISA) (Figure 2c). Dual staining of kidney sections for collagen IV and iC3b/C3d demonstrated that the

tubulointerstitial iC3b/C3d in *fH*^{-/-}*Crry*^{-/-} mice was only detected on the apical surface of the proximal tubules (Figure 2d). Crry is expressed in the glomeruli and on the basolateral surface of tubular epithelial cells,¹⁵ so it is not clear why Crry deficiency is associated with deposition on the apical surface. Decay accelerating factor was detected in the glomeruli of both *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice, but was not seen in the tubulointerstitium. The patterns of expression were not detectably different (Figure 2e). Together, these findings demonstrate that there is less overall C3 deposition in the kidneys of *fH*^{-/-}*Crry*^{-/-} mice than of *fH*^{-/-} mice, although there is greater deposition in the mesangium and on the tubules.

Fluid phase AP activation should not cause covalent fixation of C3 fragments to the GBM and does not efficiently form the terminal complement complex (C5b-9).²⁴ We immunostained the kidneys of *fH*^{-/-} mice for C5 (Figure 3a) and C6 (Figure 3b), and both proteins were deposited within

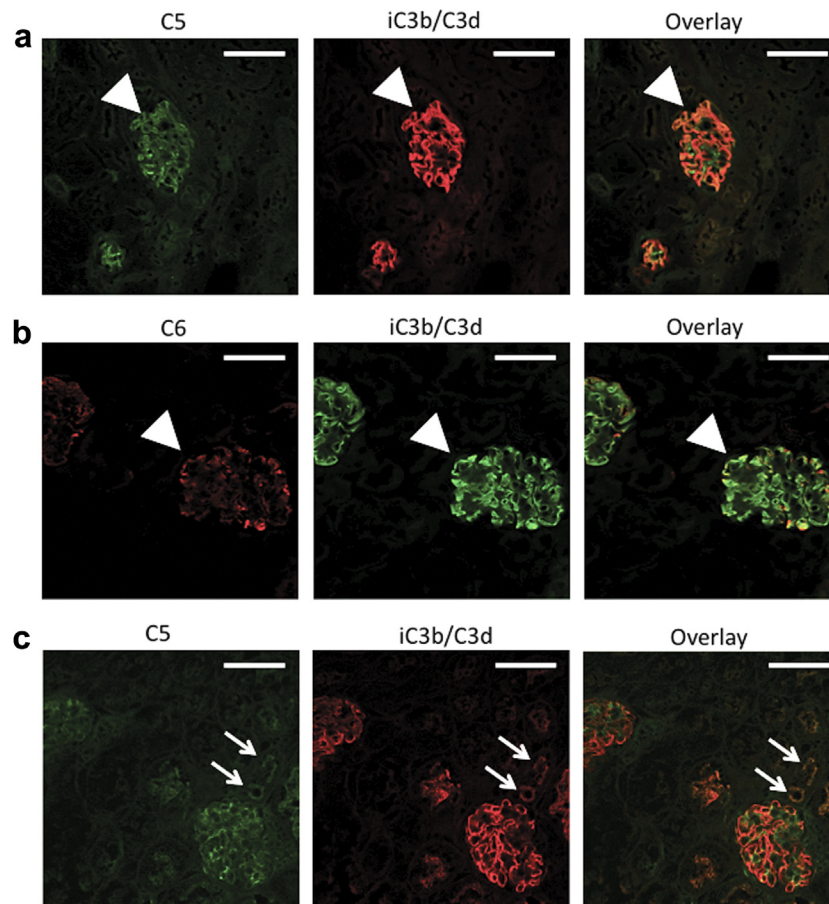


Figure 3 | Terminal complement complex generation in factor H deficient mice. To assess the degree of complement activation in the glomeruli of $fH^{-/-}$ and $fH^{-/-}Crry^{-/-}$ mice, we examined the deposition of C5 and C6 using confocal microscopy. (a) C5 (green) was detected in the glomeruli of $fH^{-/-}$ mice in a pattern similar to that of iC3b/C3d (red). (b) C6 (red) and iC3b/C3d (green) colocalized in the glomeruli and in the proximal tubules of $fH^{-/-}$ mice, indicating that complement activation generated the terminal complement complex at this location. (c) Segmental deposits of C5 (green) were also detected in the glomeruli of $fH^{-/-}Crry^{-/-}$ mice in a pattern similar to iC3b/C3d (red). Glomeruli are indicated with arrowheads. Proximal tubules are indicated with arrows. Original magnification $\times 600$. Bar = 50 μm .

the glomeruli in a pattern similar to the iC3b/C3d deposits. The detection of these proteins in the glomeruli of $fH^{-/-}$ mice indicates that the terminal complement complex is generated, consistent with AP activation directly on this surface. A protein fragment at the expected molecular weight for C5b was also seen by Western blot analysis (Supplementary Figure S3). High-powered views of glomeruli stained for C5 and C6 demonstrate similar, although not identical patterns of deposition (Supplementary Figure S3). C5 deposition was also seen in the glomeruli of $fH^{-/-}Crry^{-/-}$ mice, indicating complete activation of the complement cascade, not simply deposition of C3 fragments generated in the circulation (Figure 3c). C5 was also seen on the apical surface of the proximal tubules, similar to iC3b/C3d.

Deficiency of Crry protects $fH^{-/-}$ mice from glomerular injury

We examined kidneys from 14-month-old $fH^{-/-}Crry^{-/-}$ mice by light microscopy and compared them to kidneys from age-matched $fH^{-/-}$ mice. The glomeruli of the $fH^{-/-}$ mice demonstrated extensive proliferation and hypercellularity in the mesangial and endocapillary compartments, double

contours within the GBMs, and glomerulosclerosis (Figure 4a and b). These histologic markers of injury were significantly lower or absent in the glomeruli of $fH^{-/-}Crry^{-/-}$ mice. Although mesangial hypercellularity and expansion were seen in the glomeruli of some $fH^{-/-}Crry^{-/-}$ mice, no endocapillary proliferation was seen. By electron microscopy, electron dense deposits were seen in the GBM and in the subendothelial space of $fH^{-/-}$ mice, but these abnormalities were not detected in kidneys from $fH^{-/-}Crry^{-/-}$ mice (Figure 4c).

Urine albumin/creatinine was measured in the different mouse strains, and albumin/creatinine levels were significantly lower in urine from 14-month-old $fH^{-/-}Crry^{-/-}$ mice than from $fH^{-/-}$ mice (Figure 5a). Serum urea nitrogen and serum creatinine levels were also significantly lower in the $fH^{-/-}Crry^{-/-}$ mice (Figure 5b and c). These results indicate that, compared with $fH^{-/-}$ mice, simultaneous deficiency of both proteins protects mice from renal injury.

Factor H provides cofactor activity on the GBM

To investigate whether or not factor H regulates complement activity directly on the GBM, we injected ~ 3 -month-old

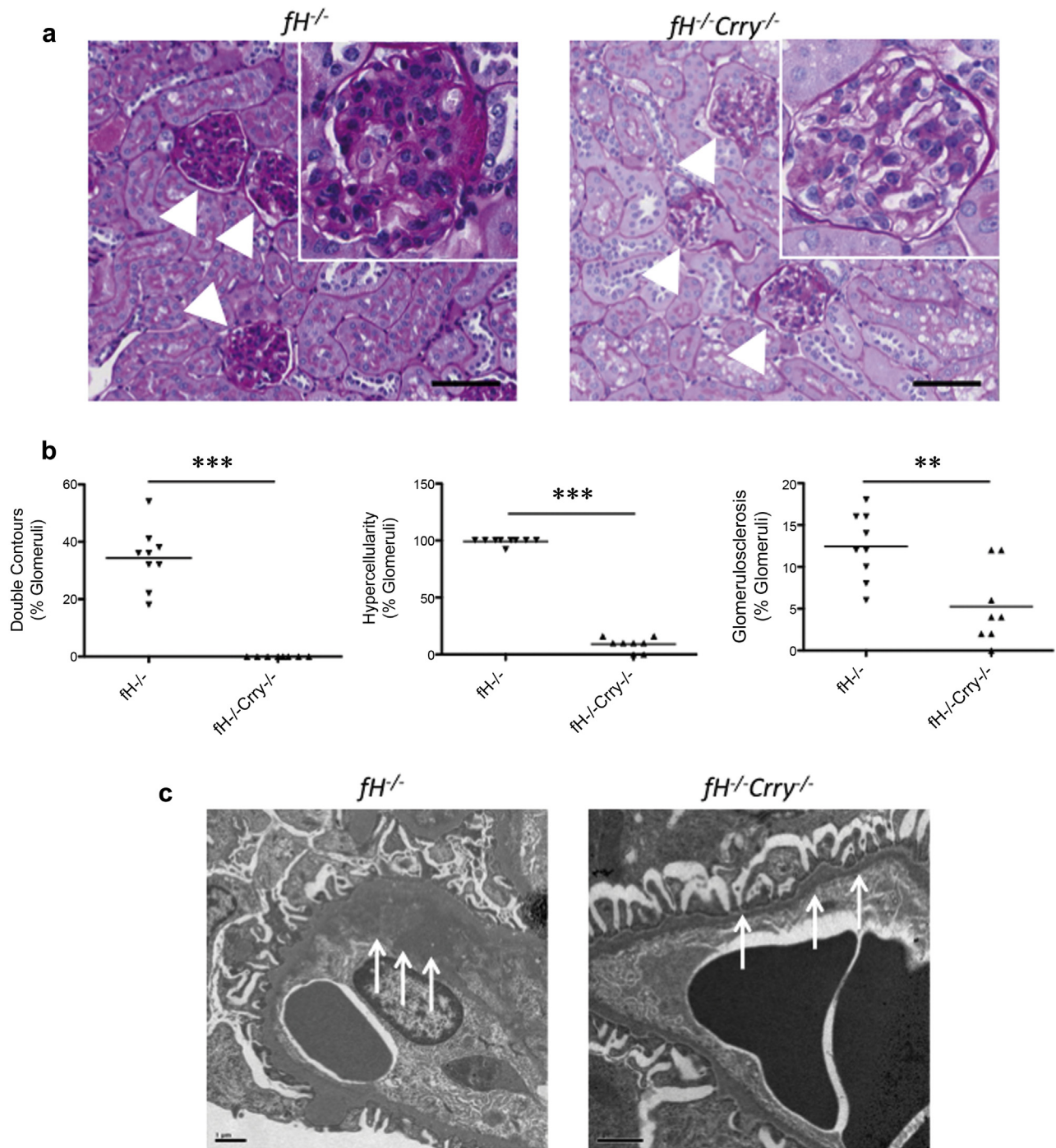


Figure 4 | Mice with targeted deletions of the genes for both factor H and Crry are protected from glomerular injury. (a) Kidney sections from 14-month-old *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice were stained with periodic acid–Schiff and examined for histologic evidence of injury. Glomeruli in *fH*^{-/-} mice demonstrated double contours of the GBM, hypercellularity, mesangial expansion, and glomerulosclerosis. Most of the glomeruli in the *fH*^{-/-}*Crry*^{-/-} mice were normal appearing. Expanded view of a representative glomerulus is shown in the inset. Original magnification $\times 200$ ($\times 400$ for inset). Bar = 100 μ m. (b) Kidney sections from 14-month-old *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice were examined by a renal pathologist in a blinded fashion. Significantly fewer glomeruli in *fH*^{-/-}*Crry*^{-/-} mice demonstrated double contours of the GBM, hypercellularity, or glomerulosclerosis. All of the *fH*^{-/-} mice had both endocapillary proliferation and mesangial expansion, whereas hypercellularity in the *fH*^{-/-} mice was only seen in the mesangium. ** $P < 0.01$, *** $P < 0.001$. (c) Kidney sections from 14-month-old *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice were examined by electron microscopy. The GBMs of *fH*^{-/-} mice were markedly thickened and contained electron-dense material (arrows). The GBMs of *fH*^{-/-}*Crry*^{-/-} mice were normal appearing (arrows). Bar = 1 μ m. GBM, glomerular basement membrane.

fH^{-/-} mice intraperitoneally with 1 mg of purified factor H once per day for 3 successive days and the mice were killed 24 hours after the last injection. As previously reported,^{22,25}

reconstitution of *fH*^{-/-} mice with purified factor H resulted in a decrease in capillary loop C3b and the appearance of C3b deposits in the mesangium (Figure 6a–c). Injection of the

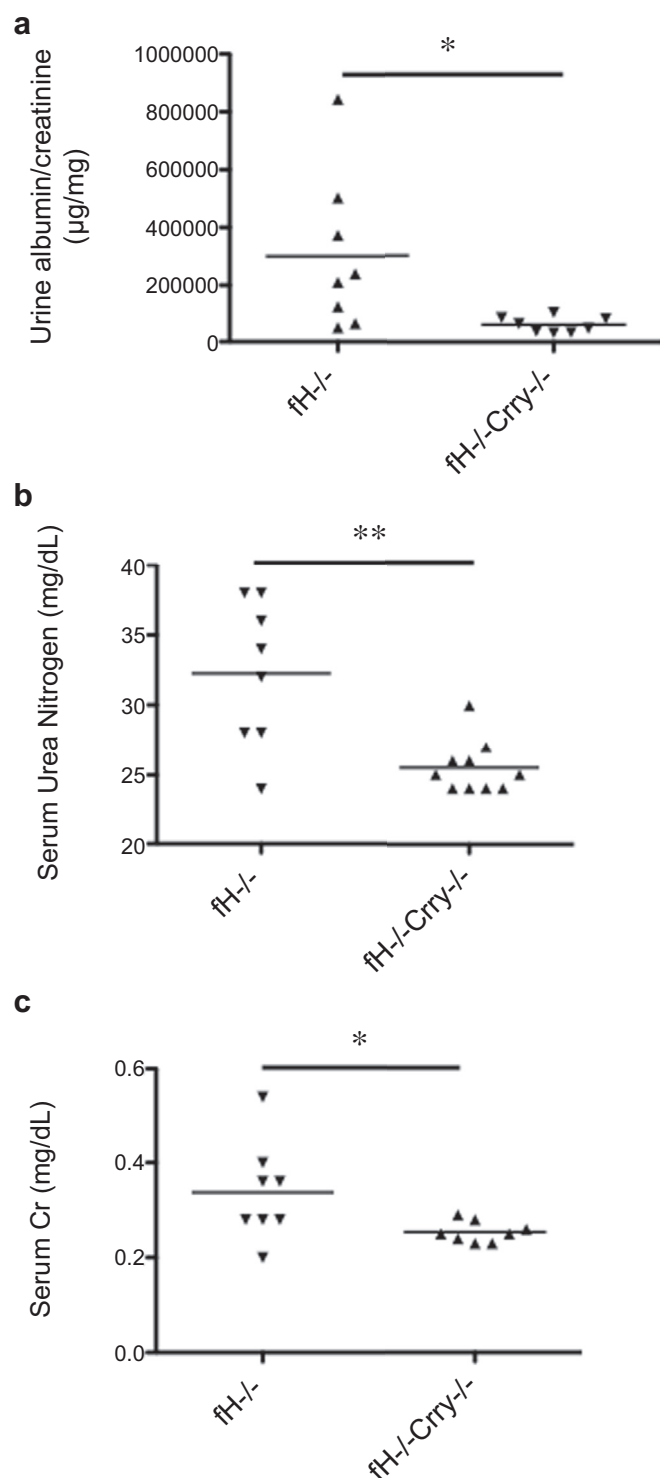


Figure 5 | Mice with targeted deletions of the genes for both factor H and Crry are protected from development of albuminuria and renal failure. (a) Albumin and creatinine levels were measured in the urine from 14-month-old *fh*^{-/-} and *fh*^{-/-}*Crry*^{-/-} mice. The albumin/creatinine content of *fh*^{-/-}*Crry*^{-/-} mice was significantly lower than that of *fh*^{-/-} mice. (b) Serum urea nitrogen and (c) serum creatinine levels were measured in 14-month-old *fh*^{-/-} and *fh*^{-/-}*Crry*^{-/-} mice. Serum urea nitrogen and creatinine were both significantly lower in *fh*^{-/-}*Crry*^{-/-} mice than in *fh*^{-/-} mice. **P* < 0.05, ***P* < 0.01. Cr, creatinine.

factor H may reduce the abundance of glomerular C3b either by reducing the deposition of new C3b and/or providing cofactor activity for conversion of C3b to iC3b/C3d. We found that iC3b/C3d deposition in the glomerular capillary loops did not decrease (Figures 6b). In a previous study, *fh*^{-/-} mice were injected with human factor H. Five successive days of treatment caused a decrease in C3d deposits, indicating that C3d is eventually cleared from this location.²² Injection of the mice with factor H also caused deposition of C3b on the basolateral surface of tubular epithelial cells (Figure 6d), similar to what is seen in wild-type mice.²⁶ By reducing the amount of C3 that is consumed on the GBM and/or in the fluid phase, reconstitution of the mice with factor H likely leaves more intact C3 available to support AP activation in the tubulointerstitium.

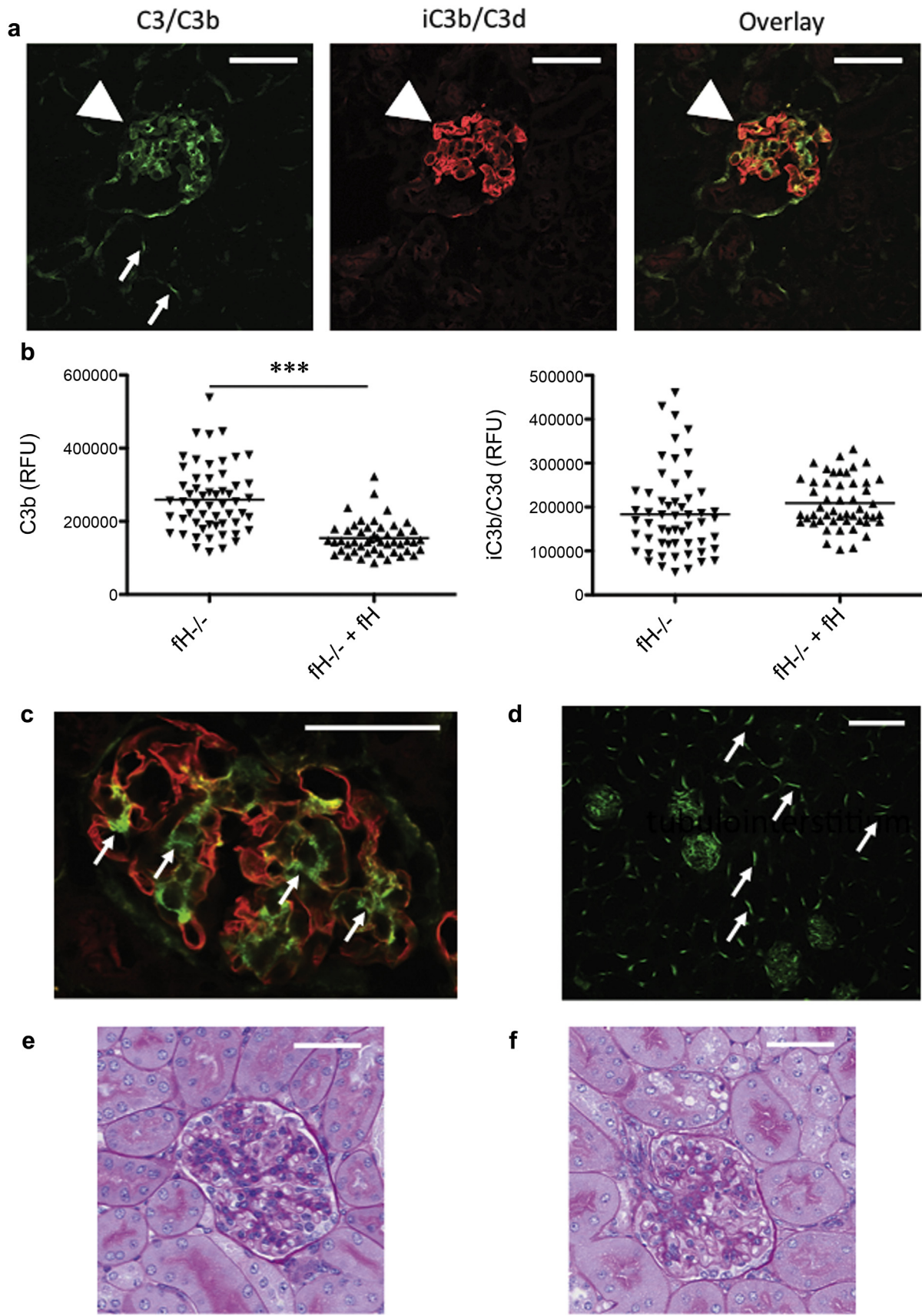
To determine whether the injected factor H binds directly to the GBM, we generated recombinant factor H and labeled it with DyLight 650. We injected ~175 µg of labeled recombinant factor H into *fh*^{-/-} mice and killed the mice 24 hours later. The unfixed kidneys were then analyzed by confocal microscopy. Labeled factor H was detectable in the capillary loops. Factor H did not colocalize with C3b in the mesangium (Figure 7a), whereas the factor H did colocalize with iC3b/C3d along the GBM (Figure 7b). This provides evidence that the factor H serves as a cofactor on the GBM, enabling the conversion of C3b to iC3b/C3d.

Clearance of C3b in the glomerulus requires cofactor activity

We next treated the mice with 2 mg/day of an inhibitory mAb to mouse factor B to block AP activity. This antibody should reduce the deposition of new C3b, but it does not provide cofactor activity for cleavage of preexisting C3b. Although treatment with the anti-factor B antibody reduced AP activation and increased plasma levels of intact C3, the glomerular C3b and iC3b/C3d deposits were unaffected (Supplementary Figure S4). This demonstrates that in the absence of factor H, limited cofactor activity is available for factor I-mediated cleavage of preexisting C3b on the GBM.

DISCUSSION

In the current study, we examine the contributions of factor H and Crry to the regulation of complement activation within the kidney. We initially hypothesized that deficiency of factor H would permit AP activation on the GBM, and that concurrent deficiency of Crry would permit AP activation on tubular epithelial cells and exacerbate injury. Surprisingly, we found that codeficiency of Crry ameliorated glomerulonephritis, and *fh*^{-/-}*Crry*^{-/-} mice were almost completely protected from glomerular injury. C3 fragments were deposited in the glomeruli and the apical surface of the renal tubules of *fh*^{-/-}*Crry*^{-/-} mice, but there was less overall glomerular C3 deposition than in *fh*^{-/-} mice. Furthermore, codeficiency of Crry and factor H protected the mice from fetal loss and tubulointerstitial injury as is seen in *Crry*^{-/-} mice.^{17,18,20,27} Further experiments demonstrated that purified factor H



protein targeted and bound to the GBM after injection into $fH^{-/-}$ mice, suggesting that the injected factor H mediated conversion of C3b to iC3b/C3d along the GBM. In contrast, AP inhibition with a monoclonal antibody to factor B reduced fluid phase C3 consumption, but preexisting C3b in the glomeruli of these mice did not change. This could be due to incomplete normalization of AP activity by the exogenous anti-factor B antibody. Alternatively, the anti-factor B antibody may not inhibit C3 activation along the GBM.

Many systemic defects of AP regulation manifest with isolated inflammation of the kidneys.^{5,6,8,28–30} C3G is associated with genetic variations in factor H, MCP, factor I, C3, and the factor H-related proteins.^{5,6,11,28,29,31,32} Furthermore, activation of the AP causes renal injury in a variety of diseases, including C3G,¹³ ischemic acute kidney injury,^{26,33} atypical hemolytic uremic syndrome,³⁴ focal segmental glomerulosclerosis,³⁵ lupus nephritis,³⁶ and antineutrophil cytoplasmic antibodies-associated vasculitis.³⁷ In spite of these numerous associations, it is not known why dysregulated control of the AP so frequently and so uniquely affects the kidneys. Our data indicate that factor H can provide cofactor activity for the cleavage of C3b that is bound to the GBM. This local action may specifically render the GBM susceptible to complement activation in the setting of factor H abnormalities.

It has been noted that the ultrastructural localization of C3 and electron-dense deposits in the kidney vary among patients with C3G.^{4,5,38} For example, patients can have sub-endothelial, mesangial, and/or epimembranous glomerular deposits.³⁹ Based on our experimental findings, it is possible that the ultrastructural location of C3 deposits in the glomeruli reflects the underlying complement defect, and that different patterns of C3 deposition may be associated with varying degrees of disease severity. Our results also suggest that defective AP regulation on cell surfaces may lead to a milder phenotype than do factor H defects that permit activation directly on the GBM.

Our results also demonstrate that acquired C3 deficiency in factor H-deficient individuals can be functionally similar to a primary C3 deficiency. Maternal alternative pathway activation within the placenta causes the demise of $Crry^{-/-}$ embryos, but deficiency of complement proteins (C3 or factor B) rescues the embryos.⁴⁰ Our results demonstrate that factor H deficiency also rescues $Crry^{-/-}$ pups, likely due to acquired C3 deficiency in the $fH^{-/-}Crry^{+/-}$ mothers. It is also notable that once $Crry^{-/-}$ mice are established, they become functionally

C3-deficient and breed normally, probably because of increased turnover of plasma C3 and reduced plasma levels of the protein.^{27,41} In other words, widespread consumption of C3 in $Crry^{-/-}$ mothers reduces the availability of C3 for activation in the placenta. It is believed that AP activation on tissues surfaces (possibly the endothelium⁴¹) causes C3 consumption in $Crry^{-/-}$ mice.

There is evidence that C3 consumption in $fH^{-/-}$ mice is caused by uncontrolled AP activation in the fluid phase.^{13,42} It has been proposed that glomerular C3 deposits are due to deposition of iC3b generated in the fluid phase onto the GBM, rather than direct AP activation on the GBM.⁴² However, our results indicate that AP activation may also occur directly on the GBM of $fH^{-/-}$ mice. Consistent with our findings, a recent study demonstrated that a recombinant form of factor H that contained the surface-binding region of factor H (short consensus repeats 19 and 20) reduced the deposition of C3 fragments in the glomeruli of $fH^{-/-}$ mice, whereas a recombinant protein that did not contain this surface-binding region and only contained the complement regulatory region (SCRs 1–5) did not reduce glomerular C3 deposition.⁴³ The anatomic location at which complement activation occurs may have important therapeutic implications. There are recombinant proteins that can deliver the complement regulatory region of factor H to tissue-bound C3d.^{43,44} On the other hand, agents that target complement proteins in solution have also been developed.^{45,46} Our results do not exclude a role for fluid phase AP activation in glomerular injury, but they suggest that therapeutic complement inhibitors that are targeted to anatomic sites of complement inhibition will be protective in C3G.

Factor I is a circulating protease that cleaves (inactivates) C3b, forming iC3b.⁴⁷ The inactivation of C3b by factor I requires cofactor activity, which can be provided by factor H, Crry, CR1, and MCP.⁴⁷ In this regard, $fH^{-/-}Crry^{-/-}$ mice are functionally similar to factor I-deficient ($fI^{-/-}$) mice because they lack cofactor activity (and therefore factor I activity) in the plasma, on the GBM, and on cell surfaces. Rose *et al.*⁴² discovered that C3 fragments are not deposited in the glomerular capillaries of $fI^{-/-}$ mice. The investigators conjectured that conversion of C3b to iC3b by factor I is necessary for the development of glomerular injury and that iC3b is a nephritogenic moiety.⁴² In view of the current study, however, an alternative explanation is that widespread complement activation in both the $fI^{-/-}$ and $fH^{-/-}Crry^{-/-}$ mice reduces the

Figure 6 | Factor H regulates complement activation on the GBM. Factor H-deficient mice were reconstituted with factor H purified from the plasma of wild-type mice, and complement proteins in the glomeruli were examined using confocal microscopy. (a) Kidney sections of reconstituted $fH^{-/-}$ mice were stained using antibodies to detect C3b (green) and iC3b/C3d (red). The pattern of C3b deposits suggested deposition in the mesangium (arrowhead) and along the tubular basement membranes (small arrows). iC3b/C3d was heavily deposited along the GBM. Original magnification $\times 600$. Bar = 50 μ m. (b) The intensities of C3b and iC3b/C3d were measured in the glomeruli of an $fH^{-/-}$ mouse injected with a vehicle control or with purified factor H. Injection with factor H reduced the intensity of glomerular C3b. *** $P < 0.001$. (c) A high-power view of a glomerulus from an $fH^{-/-}$ mouse injected with purified factor H demonstrates that areas of C3b without iC3b/C3d (arrows) are seen in the mesangium, and iC3b/C3d is seen in the capillary loops. Original magnification $\times 600$. Bar = 50 μ m. (d) A low-power view of a kidney section from an $fH^{-/-}$ mouse reconstituted with factor H shows that injection of factor H restored C3b deposition on the tubular basement membranes (arrows). Original magnification $\times 200$. Bar = 100 μ m. Periodic acid–Schiff–stained kidneys from a representative $fH^{-/-}$ mouse injected with (e) vehicle and (f) factor H are shown. Original magnification $\times 600$. Bar = 50 μ m. fH, factor H; GBM, glomerular basement membrane; RFU, relative fluorescence unit.

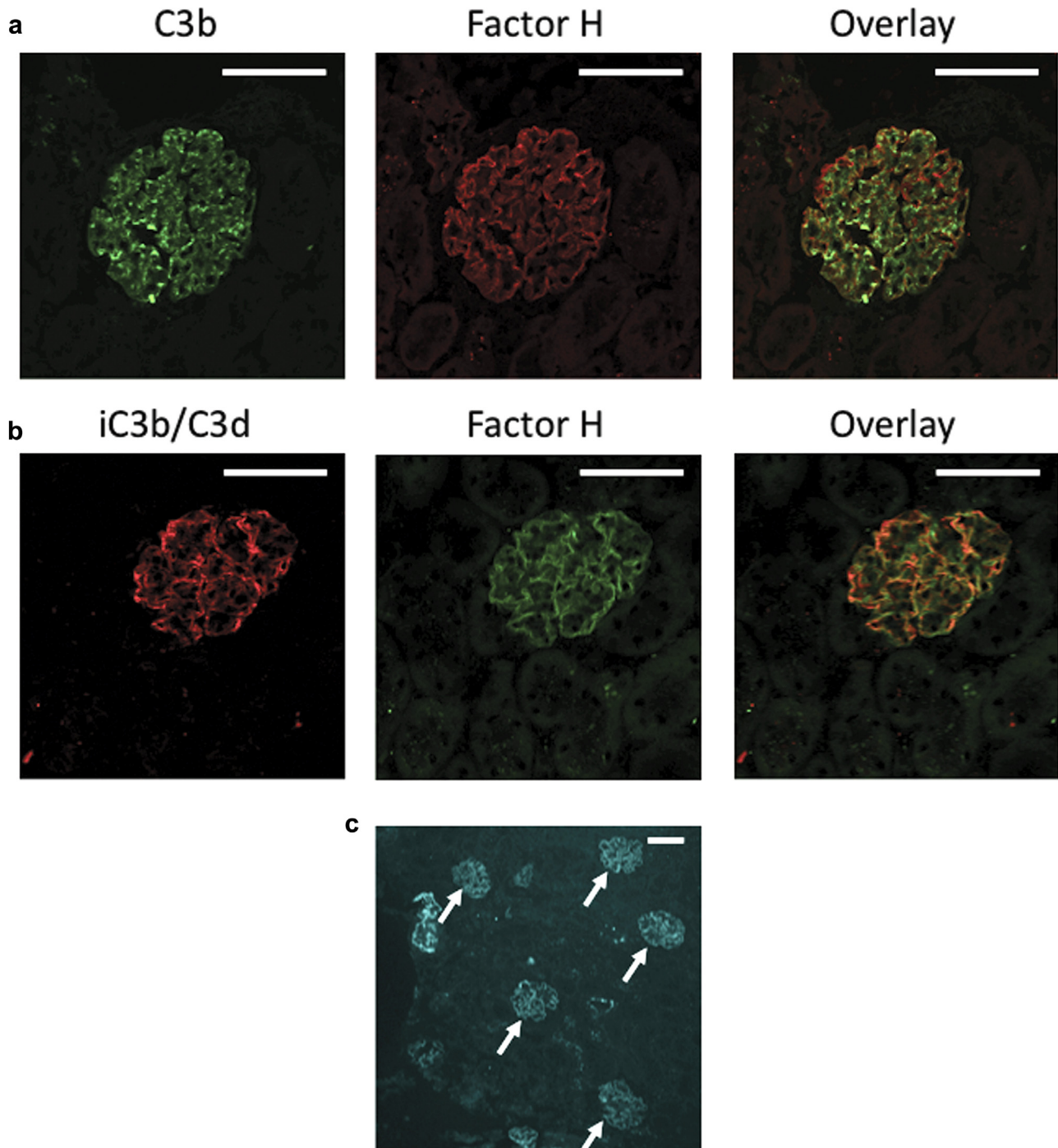


Figure 7 | Factor H binds to the glomerular basement membrane of factor H-deficient mice. The $fH^{-/-}$ mice were injected with DyLight 650-labeled recombinant factor H, and kidneys from these mice were examined by confocal microscopy. (a) C3b was detected in the capillaries and the mesangium of injected mice, and factor H (red) was deposited along the capillary walls. (b) iC3b/C3d was detected along the capillary walls in a pattern similar to the pattern of the injected factor H deposition (green). Original magnification $\times 600$. Bar = 50 μ m. (c) A low-power view of a kidney section demonstrates that factor H primarily localized to the glomeruli of $fH^{-/-}$ mice. Arrows indicate glomeruli. Original magnification $\times 200$. Bar = 50 μ m.

amount of intact C3 available for activation on the GBM (Figure 8). AP dysregulation in both of these strains of mice is more widespread than it is in $fH^{-/-}$ mice, with the net result being less C3 deposition on the GBM and protection from glomerular injury.

An unexpected finding of the current study is that iC3b/C3d fragments were generated in the mesangium and tubules of $fH^{-/-}Crry^{-/-}$ mice. It is not clear what is serving as the cofactor in these mice. CR1 and MCP have cofactor activity, but are not expressed on the GBM or the apical surface of

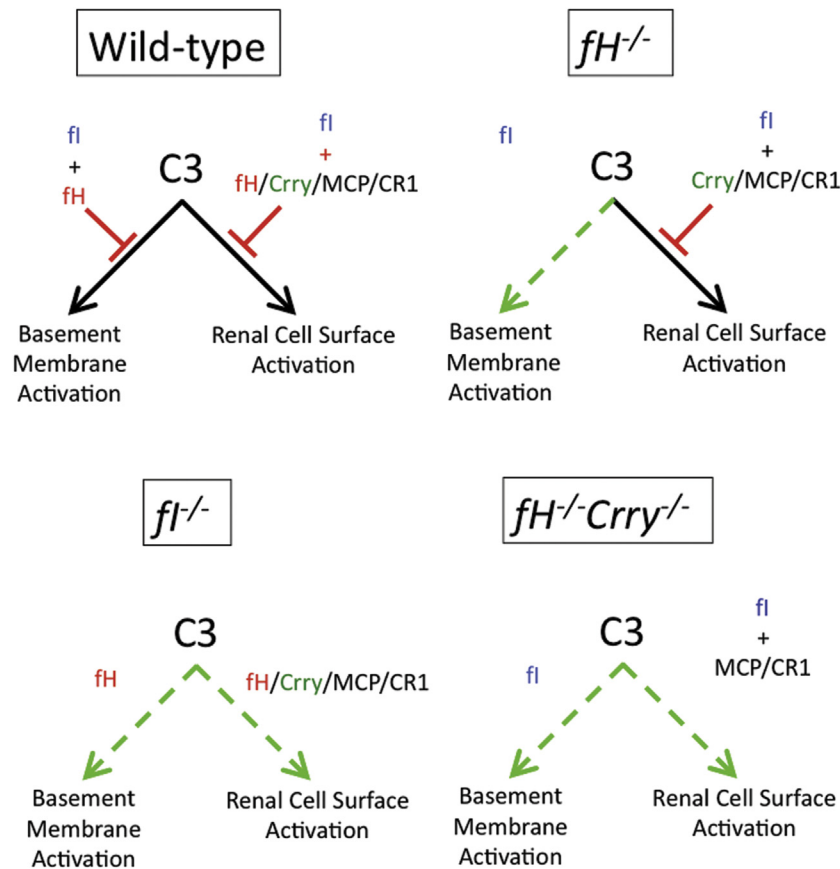


Figure 8 | Model of glomerular complement regulation. Factor I and factor H together control AP activation on the GBM. Factor I also controls AP activation on renal cell surfaces. Cofactor function on cell surfaces is provided by factor H, MCP, CR1, and Crry (in rodents). Deficiency of factor H in $fH^{-/-}$ mice permits uncontrolled AP activation on the GBM. Factor I deficiency permits uncontrolled activation on the GBM and on cell surfaces. Combined factor H and Crry deficiency also permits activation on the GBM and on cell surfaces. AP, alternative pathway; fH, factor H; fl, factor I; GBM, glomerular basement membrane; MCP, membrane cofactor protein.

tubular epithelial cells.¹⁹ Other proteases can cleave C3b, including plasmin,⁴⁸ thrombin, and cathepsin.⁴⁹ These proteases may contribute to C3 metabolism in the kidneys of $fH^{-/-}Crry^{-/-}$ mice. Nevertheless, reconstitution of $fH^{-/-}$ mice with factor H increased C3b degradation, confirming that factor H is the primary AP regulator on the GBM and that the other available cofactors and proteases do not fully compensate for factor H.

Complement-mediated injury occurs when regulatory proteins do not adequately protect host surfaces. We have shown that factor H directly inhibits complement activation on the GBM, and that deficiency of factor H renders this particular surface susceptible to complement activation. Cell surface complement regulatory proteins, such as Crry, do not function on the GBM, so deficiency of these proteins creates other anatomic sites that compete with the GBM for C3. Our results demonstrate that if complement activation is too widespread, the depletion of intact C3 can reduce injury at particular tissue sites. A large number of complement defects have been discovered, including genetic variations, mutations, autoantibodies to complement proteins, as well as the identification of new proteins that interact with the complement system. Greater

understanding of how these defects contribute to complement activation on particular tissues will improve our understanding of complement-mediated disease and will help in the design of effective new complement-inhibitory therapies.

MATERIALS AND METHODS

Animals

Mice with targeted deletion of the genes for factor H¹³ and Crry²⁰ were generated as previously described. The mice have been backcrossed onto a C57BL/6 background for more than 9 generations. The mice were intercrossed until we had established $fH^{-/-}Crry^{+/+}$ breeding sets. Pups were genotyped by the polymerase chain reaction using the primers shown in Supplementary Table S1, and a colony of $fH^{-/-}Crry^{-/-}$ mice was then established. Eight male 14-month-old mice from 2 founder pairs were compared with an age-matched cohort of 8 male $fH^{-/-}$ mice. In addition, a cohort of $fH^{-/-}Crry^{-/-}$ mice was set up in the laboratory of Dr. Pickering, and C3 deposition in the glomeruli of these mice was analyzed (Supplementary Figure S1). For experiments involving the reconstitution of $fH^{-/-}$ mice with purified factor H, 3 3.5-month-old mice were used. Control C57BL/6 mice were obtained from Jackson Laboratories. Mice were housed and maintained in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*,

and all experiments were approved by the University of Colorado Center for Laboratory Animal Care.

Reagents

The following antibodies were used in these studies: Unconjugated and fluorescein isothiocyanate-conjugated goat IgG to mouse complement C3 (MP Biomedicals, Santa Ana, CA), a mAb that only recognizes the iC3b and C3d fragments of C3 (mAb 3d29),²³ a mAb that recognizes all of the C3 fragments by Western blot analysis (mAb 3d11),²³ monoclonal murine antimouse C5,⁵⁰ a mAb that recognizes the α 3 chain of collagen IV (8D1),⁵¹ rabbit antimouse C6 (Hycult Biotech, Uden, the Netherlands), rabbit polyclonal antibody to collagen IV (EMD Millipore, Billerica, MA), Alexa-647-conjugated Armenian hamster antimouse CD55, clone RIKO-3 (Biolegend, San Diego, CA), horseradish peroxidase-conjugated goat IgG to mouse complement C3 (MP Biomedicals), and 4',6-diamidino-2-phenylindole nuclear stain (Sigma Aldrich, St. Louis, MO). Secondary antibodies include streptavidin conjugated with Alexa-555 and Alexa-488 (Life Technologies, Grand Island, NY), horseradish peroxidase, Cy3, and fluorescein isothiocyanate-conjugated goat antimouse IgG (Jackson ImmunoResearch, West Grove, PA), antigen-presenting cell-conjugated goat antirabbit IgG, Alexa 594-conjugated donkey antirabbit IgG (Invitrogen, Grand Island, NY), and fluorescein isothiocyanate-conjugated donkey antirabbit IgG (Jackson ImmunoResearch). ChromePure Rabbit IgG, fluorescein isothiocyanate-conjugated ChromePure Goat IgG (Jackson ImmunoResearch, West Grove, PA), and mouse antihuman factor I (Quidel, San Diego, CA) were used as isotype controls. The mAb 1379 was used to block mouse alternative pathway activity *in vivo*.⁵²

Recombinant murine factor H was generated using the codon-optimized (*Homo sapiens*) DNA sequence for murine factor H (comprising residues 19–1234; Uniprot identifier: P06909). The gene was subcloned into the pDONR221 entry vector (Life Technologies; construct purchased from GeneArt, Grand Island, NY). The engineered sequence additionally contained DNA encoding an Ig kappa chain leader sequence to facilitate secretion of the target protein, a Gly-Ala-Gly-Ala-Gly-Ala linker region, a hexa-histidine (His₆) fusion tag, a second linker region (Asp-Tyr-Asp-Ile-Pro-Thr-Thr), and a Tobacco Etch Virus nuclear inclusion A endopeptidase cleavage site (Glu-Asn-Leu-Tyr-Gln-Gly), all of which were located upstream to the factor H gene. The synthetic gene was recombined into a pcDNA3.2/V5-Dest expression vector using Gateway LR clonase II enzyme mix (Life Technologies) according to the manufacturer's instructions. Chinese hamster ovary cells were transfected with the vector using Lipofectamine 2000 (Invitrogen), and protein was produced under geneticin 418 (ENZO Life Sciences, Farmingdale, NY) selection. The cells were grown in Dulbecco's modified Eagle's medium supplemented with fetal calf serum and geneticin 418. Protein was purified using a HiTrap His column (GE Healthcare, Uppsala, Sweden) and purity of the protein was confirmed by electrophoresis on a 12.5% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA) followed by Coomassie staining and Western blot analysis. In some experiments, the recombinant protein was fluorescently labeled using a DyLight 650 Labeling Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

Confocal microscopy

Kidneys were snap frozen in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) at the time of harvest and stored at -80°C . Four micrometer sagittal sections were cut using a cryostat, warmed to room temperature, and fixed with acetone.

Nonspecific binding was blocked with 10% heat-inactivated goat serum. Primary antibodies were diluted in 2% heat-inactivated goat serum and incubated overnight at 4°C . Autofluorescence was blocked with 0.05% Sudan Black B in 70% ethanol for 20 minutes at room temperature.⁵³ High-resolution images were obtained with an Olympus FV1000 FCS/RICS confocal microscope (Olympus Scientific Solutions Americas Corp, Waltham, MA). The localization of deposits in the mesangium or in peripheral capillary loops was assessed based on the pattern of deposition. The intensity and colocalization of proteins were measured by drawing regions of interest around glomeruli and analyzing the regions of interest with Olympus FV10-ASW software. Staining using isotype control antibodies is shown in [Supplementary Figure S5](#).

Western blot analysis

C3 and C5 were detected in plasma and kidney lysates by Western blot analysis. Plasma samples diluted 1:10 in phosphate-buffered saline or 200 μg of tissue lysate were reduced by boiling with 0.15 M dithiothreitol for 10 minutes at 100°C . The proteins were resolved by electrophoresis with a 10% Tris-HCl gel (Bio-Rad) and transferred to a polyvinylidene fluoride membrane. C3 fragments were detected using mAb 3d11 diluted 1:1000 in 2.5% milk in phosphate-buffered saline. C5 fragments were detected using BB5.1 diluted 1:500 in 2.5% milk-phosphate-buffered saline. In both cases, the secondary antibody was horseradish peroxidase-conjugated goat antimouse IgG (H+L) (Jackson ImmunoResearch). The secondary antibody was detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Purified human C3, C3b, and iC3b (Comptech, Tyler, TX) were used as size controls.

C3 ELISA

Plasma C3 levels were compared using an ELISA. ELISA plates were coated with goat IgG fraction to mouse complement C3 (MP Biomedicals) diluted 1:100 in carbonate-bicarbonate buffer as a capture antibody and incubated for 1 hour at 4°C . The plasma samples were diluted 1:3200 in carbonate-bicarbonate buffer and added to wells. Bound C3 was then detected with a horseradish peroxidase-conjugated Goat IgG fraction to mouse complement C3 (MP Biomedicals) diluted 1:800 in phosphate-buffered saline with 0.1% Tween20. The ELISA plates were then developed using 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific), and the optical density at 280 nm was measured. All of the samples were included on the same plate, and optical density at 280 nm values were compared.

Albumin, serum urea nitrogen, and creatinine measurements

Spontaneously voided urine samples were collected by placing mice onto ELISA plates or collected directly into Eppendorf tubes. Urine albumin was determined by ELISA according to manufacturer's instructions (Bethyl Laboratories, Inc, Montgomery, TX). Serum urea nitrogen and serum and urine creatinine were determined by automated analysis with the Alfa Wassermann ACE Clinical Chemistry Analyzer (Alfa Wassermann, West Caldwell, NJ).

Renal histology

Kidney tissue was fixed in formalin, embedded in paraffin, cut into 4- μm sections, and stained with periodic acid-Schiff. Sections were assessed for the extent of hypercellularity, double contours, and glomerulosclerosis as previously described.⁵⁴ Histologic analysis was performed by a renal pathologist blinded to the sample identity. Fifty glomeruli per animal per section were examined. The glomerular sections were assessed for hypercellularity and mesangial expansion

as previously described.^{42,54} The glomerular scores for each animal were averaged, and kidneys from 8 to 9 animals per group were included in the analysis. Additional kidney tissue for electron microscopy was fixed in 2% glutaraldehyde/2% paraformaldehyde with 0.1 M cacodylate/3% sucrose, embedded in resin, and cut into ultrathin sections. Five animals per group and 10 to 12 glomeruli per animal were analyzed.

Statistical analysis

Statistical analysis between groups was assessed by unpaired *t*-test using GraphPad Prism software (GraphPad Prism, La Jolla, CA). A *P*-value <0.05 was considered statistically significant.

DISCLOSURE

JMT and VMH receive royalties from Alexion Pharmaceuticals, Inc. All other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Colocalization of C3b with collagen IV in the glomeruli factor H-deficient mice. We stained kidneys from *fH*^{-/-} mice for C3b (green) and the α 3 chain of collagen IV (red). Areas of colocalization in the capillary loops are indicated with arrows. Original magnification \times 600. Bar = 50 μ m.

Figure S2. C3 deposition in the glomeruli of an independent cohort of mice with targeted deletion of the genes for factor H (*fH*) and *Crry*. We examined the deposition of C3 fragments in the glomeruli of an independent cohort of *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice (generated in the laboratory of MCP). The intensities of C3b and iC3b/C3d were measured in the glomeruli of *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice using confocal microscopy. Less glomerular C3b and iC3b/C3d was detected in the *fH*^{-/-}*Crry*^{-/-} mice. ***P* < 0.01, ****P* < 0.001. RFU, relative fluorescence unit.

Figure S3. Detection of C5 fragments. (A) Western blot analysis was performed on samples from wild-type plasma and a lysate made from the kidney of an *fH*^{-/-}*Crry*^{-/-} mouse. The blot was probed with a monoclonal antibody to C5 (mAb BB5.1). Bands at the correct molecular weight for intact C5 (~190 kD), the α chain of C5 (~115 kD), and the α' chain of C5 (~105 kD) were detected. (B) We stained kidneys from *fH*^{-/-} mice for C5 (red) and C6 (green). Areas of colocalization in the capillary loops of the overlay image appear yellow. Glomeruli are indicated with arrowheads. Original magnification \times 600. Bar = 50 μ m.

Figure S4. Factor H-deficient mice treated with an inhibitory antibody to factor B. Factor H (*fH*)-deficient mice were treated with an inhibitory monoclonal antibody to factor B (*fB*). (A) Western blot analysis of C3 fragments in the plasma of treated mice demonstrates that intact C3 levels were higher in anti-factor B-treated *fH*^{-/-} mice than in untreated mice. (B) Plasma C3 levels were measured by enzyme-linked immunosorbent assay and were higher in anti-factor B-treated *fH*^{-/-} mice than in untreated mice. (C) Kidney sections of *fH*^{-/-} mice treated with the anti-factor B antibody were stained using antibodies to detect C3b (green) and 9C3b/C3d (red). C3b and iC3b/C3d were detected along the glomerular basement membrane in a pattern identical to that of *fH*^{-/-} mice. Original magnification \times 600. Bar = 100 μ m. OD280, optical density at 280 nm.

Figure S5. Representative isotype control staining for confocal microscopy. Original magnification \times 600. Bar = 50 μ m.

Table S1. Primers used to genotype *fH*^{-/-} and *fH*^{-/-}*Crry*^{-/-} mice.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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