Gq signaling causes glomerular injury by activating TRPC6

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Familial forms of focal segmental glomerulosclerosis (FSGS) have been linked to gain-of-function mutations in the gene encoding the transient receptor potential channel C6 (TRPC6). GPCRs coupled to Gq signaling activate TRPC6, suggesting that Gq-dependent TRPC6 activation underlies glomerular diseases. Here, we developed a murine model in which a constitutively active Gq α subunit (GqQ>L, referred to herein as GqQ>L) is specifically expressed in podocytes and examined the effects of this mutation in response to puromycin aminonucleoside (PAN) nephrosis. We found that compared with control animals, animals expressing GqQ>L exhibited robust albuminuria, structural features of FSGS, and reduced numbers of glomerular podocytes. Gq activation stimulated calcineurin (CN) activity, resulting in CN-dependent upregulation of TRPC6 in murine kidneys. Deletion of TRPC6 in GqQ>L-expressing mice prevented FSGS development and inhibited both tubular damage and podocyte loss induced by PAN nephrosis. Similarly, administration of the CN inhibitor FK506 reduced proteinuria and tubular injury but had more modest effects on glomerular pathology and podocyte numbers in animals with constitutive Gq activation. Moreover, these Gq-dependent effects on podocyte injury were generalizable to diabetic kidney disease, as expression of GqQ>L promoted albuminuria, mesangial expansion, and increased glomerular basement membrane width in diabetic mice. Together, these results suggest that targeting Gq/TRPC6 signaling may have therapeutic benefits for the treatment of glomerular diseases.

Introduction

GPCRs linked to Gq activation play a key role in glomerular diseases including receptors for angiotensin II (AT1), endothelins (ETA), thromboxanes (TP), cysteinyl-leukotrienes, and E-series prostaglandins (EPI) (1, 2). These cell surface GPCRs are found in podocytes and regulate pathways involved in cell survival, morphology, motility, and cellular attachment (1–6). As a result, GPCRs are important therapeutic targets for the treatment of glomerular disease processes. In this regard, AT1 receptor blockers (ARBs) are extensively used for the treatment of proteinuric kidney diseases (7–9), and combined therapy using ARBs and ETA blockers is currently being evaluated in clinical trials (10–12).

While the pathways activated by these GPCR systems are diverse, these receptors activate Gq α subunits (1). Gq stimulates phospholipase Cβ (PLCβ) and generates the second messengers diacyl glycerol (DAG) and inositol triphosphates (IP3) (1). DAG is a potent activator of PKC (13), and several PKC isoforms play key roles in the pathogenesis of diabetic kidney disease (14). In contrast, IP3 mobilizes calcium from intracellular stores (13), and DAG and possibly inositol phosphates (IPs) promote activation of transient receptor potential channel C6 (TRPC6) in podocytes (1), which further enhances intracellular calcium levels. The importance of calcium in glomerular disease processes is highlighted by the observation that: (a) Gain-of-function mutations in TRPC6 cause familial forms of focal segmental glomerulosclerosis (FSGS) (15, 16), and (b) TRPC6 is upregulated in primary glomerular diseases (17). Calcium, in turn, can activate additional signaling molecules including calcineurin (CN) (18). In the heart, Gq-dependent signaling cascades are potent activators of CN, and Gq-dependent CN activation promotes cardiac hypertrophy (19, 20) through mechanisms that involve, at least in part, CN-dependent upregulation of TRPC6 (21). In the kidney, CN regulates the stability of the podocyte cytoskeletal protein synaptopodin (SYN) (22) and promotes decreases in the number of podocytes by mechanisms that are dependent on gene transcription and podocyte apoptosis (23, 24). Moreover, recent studies are consistent with the notion that Gq-dependent CN activation and, in turn, TRPC6 induction are relevant to podocyte biology in vivo (25–27).

To investigate the role of Gq signaling in the pathogenesis of glomerular diseases, we expressed a constitutively active Gq α subunit (GqQ>L), herein referred to as GqQ>L specifically in podocytes in a doxycycline-inducible (DOX-inducible) fashion (28). As previously reported, induction of GqQ>L does not cause glomerular injury (28), suggesting that a “second hit” may be required to induce podocyte injury, as has been suggested for...
some familial forms of FSGS (29) such as activating mutations in TRPC6 (30). We therefore determined whether Gq activation in podocytes exacerbated glomerular injury in nephrosis induced by the podocyte toxin puromycin aminonucleoside (PAN) (31). We found that treatment with PAN induced robust albuminuria, foot process (FP) effacement, a decrease in the number of glomerular podocytes, and light microscopic features of FSGS in mice expressing GqQ>L specifically in podocytes compared with what was observed in control animals. These adverse effects were associated with both CN activation in vivo and enhanced expression of TRPC6. Deletion of Trpc6 prevented the development of FSGS and podocyte loss in this model. Similarly, CN blockade with FK506 reduced proteinuria and preserved podocyte numbers but had more modest effects on glomerular and tubular histology. These data support the notion that components of the Gq/TRPC6 signaling cascade are important therapeutic targets for the treatment of glomerular disease processes. Moreover, the data support the concept that a second hit may be required for the development of some familial forms of FSGS such as gain-of-function mutations in TRPC6.

Results

To evaluate the role of Gq-coupled signaling cascades in promoting podocyte injury, we expressed a constitutively active Gq α subunit (GqQ>L) specifically in glomerular podocytes in a DOX-inducible fashion (28). As shown in Supplemental Figure 1A (supplemental material available online with this article; doi:10.1172/JCI76767DSI), this system requires 2 transgenic (Tg) animals (32). The first Tg animal expresses reverse tetracycline transactivator (rtTA) under the control of the human podocin promoter (NPHS2) to create NPHS2-rtTA mice (33). The second Tg mouse expresses GqQ>L under the control of tet operator sequence (tetO) and a minimal CMV promoter (PminCMV) to create tetO-GqQ>L mice (32). By breeding the 2 Tg mice, animals were obtained that expressed both transgenes. In these “double-Tg” mice (NPHS2-rtTA tetO-GqQ>L mice, herein referred to as GqQ>L mice), treatment with DOX induced transgene expression. For the studies, we used GqQ>L mice as well as their littermate controls (single-Tg and non-Tg animals), which do not express GqQ>L in the presence of DOX (28). At 3 to 4 months of age, the mice were treated with DOX for 1 week and then received 1 dose of PAN as previously described (34). The mice were then studied as discussed in the Methods.

As shown in Figure 1A and Supplemental Figure 1B, treatment with DOX and PAN induced robust albuminuria following induction of GqQ>L. As shown in Figure 1, B and C, this increase in albuminuria was associated with the development of FSGS in approximately one-third of mice expressing GqQ>L (P < 0.05) as well as of tubular dilation and casts (Figure 1D), which tended to be more severe in GqQ>L mice (Supplemental Figure 1C). These histologic abnormalities were accompanied by a decrease in both the number of glomerular podocytes and podocyte density in GqQ>L mice compared with either untreated controls or controls treated with DOX and PAN (Figure 1E and Table 1) as well as reduced expression of both SYN and WT1 in glomerular preparations as shown by immunoblot analysis (Figure 1, F and G). As previously reported (28), induction of GqQ>L by DOX had no effect on albuminuria or glomerular histology in the absence of PAN treatment (Supplemental Figure 2, A and B), despite efficient expression of the transgene (Supplemental Figure 2C).

Gq activates the serine/threonine phosphatase CN by stimulating an increase in intracellular calcium levels (28). CN activation promotes podocyte injury (23, 24, 35), and these adverse effects are likely mediated in part by dephosphorylation of nuclear factor of activated T cells (NFAT) (23, 24). We therefore determined whether GqQ>L induction in vivo stimulated CN activity using CN reporter mice (36). For these studies, we bred GqQ>L mice with mice expressing an NFAT reporter construct linked to β-gal induction. This created “triple” Tg mice, which express GqQ>L following DOX treatment, and either “single” Tg mice (NFAT reporter mice, NPHS2-rtTA mice, and tetO-GqQ>L mice) or “double” Tg mice (TG NFAT reporter NPHS2-rtTA mice, NFAT reporter tetO-GqQ>L mice, and GqQ>L mice), which either do not express GqQ>L or lack the NFAT reporter construct or both (labeled “other” Tg). We also bred non-Tg mice for use as an additional control. As shown in Figure 2A, treatment with DOX activated the NFAT reporter construct in renal cortices from triple-Tg animals (NFAT reporter GqQ>L mice), with lesser activation in other organ systems and little background activation in either “other” Tg or non-Tg mice. We also evaluated β-gal activity in enriched glomerular preparations from each experimental group in the presence of DOX or sucrose vehicle. As shown in Figure 2B, induction of GqQ>L with DOX caused robust activation of the reporter construct compared with vehicle-treated mice, with little activation of the reporter construct in the presence or absence of DOX in the other experimental groups.

An important gene target of NFAT transcription factors is the ion channel TRPC6 (21). In the heart, CN stimulates cardiac hypertrophy (19, 20), and this hypertrophic effect is mediated in part by enhanced expression of TRPC6 (21). Moreover, recent studies suggest that this pathway is relevant to podocyte biology (26, 27, 37). To determine whether GqQ>L induction enhanced TRPC6 expression by CN-dependent mechanisms, we first determined whether DOX treatment induced Trpc6 mRNA in GqQ>L mice. As shown in Figure 3, A and B, GqQ>L enhanced expression of Trpc6 mRNA in enriched glomerular preparations, and this increase in Trpc6 mRNA levels was inhibited by the pharmacological CN inhibitor FK506. We next determined the effect of GqQ>L induction by immunoblotting glomerular preparations. As shown in Figure 3, C and D, induction of GqQ>L expression by DOX enhanced TRPC6 protein levels compared with those in controls, and this increase was attenuated by FK506.

### Table 1. Effect of PAN nephrosis on glomerular structure

<table>
<thead>
<tr>
<th></th>
<th>Podocytes per glomerular profile</th>
<th>V/glom (×10^5/μm^3)</th>
<th>Nv(P/glom) (×10^7/μm^3)</th>
</tr>
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<tbody>
<tr>
<td>Untreated controls (n = 11)</td>
<td>8.74 ± 0.28</td>
<td>2.24 ± 0.14</td>
<td>56.8 ± 1.7</td>
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<td>PAN controls (n = 9)</td>
<td>7.80 ± 0.19</td>
<td>2.82 ± 0.02</td>
<td>49.7 ± 2.2</td>
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<td>PAN GqQ&gt;L (n = 15)</td>
<td>6.91 ± 0.6a</td>
<td>2.73 ± 0.15</td>
<td>42.4 ± 3.7b</td>
</tr>
</tbody>
</table>

aP < 0.05 versus controls and PAN controls; bP < 0.01 versus untreated controls. V/glom, glomerular volume; Nv(P/glom), podocyte density.
To determine whether TRPC6 plays a role in podocyte injury, we generated GqQ>L mice lacking TRPC6. For these studies, controls, Trpc6+/+ GqQ>L mice, and Trpc6−/− GqQ>L mice (herein referred to as Trpc6-KO GqQ>L mice) were treated with DOX and PAN, as described in the Methods. As demonstrated in Figure 4A and Supplemental Figure 3A, PAN-induced albuminuria was significantly increased in Trpc6−/− GqQ>L mice compared with that seen in either baseline or wild-type (WT) controls treated with PAN. In Trpc6-KO GqQ>L mice, albuminuria induced by PAN was significantly decreased compared with Trpc6−/− GqQ>L mice treated with PAN. Moreover, the absence of TRPC6 completely prevented the development of FSGS in Trpc6−/− GqQ>L mice (Figure 4, B and D). Similarly, tubular injury (dilation and casts) was significantly attenuated in GqQ>L mice lacking TRPC6 compared with Trpc6−/− GqQ>L mice (Figure 4, C and E). Tubulointerstitial (TI) inflammation and fibrosis also tended to be decreased by KO of Trpc6, but these differences did not reach statistical significance (P = 0.07) (Supplemental Figure 3B). As shown in Figure 4F, these light microscopic findings were associated with a decrease in the number of glomerular podocytes in Trpc6−/− GqQ>L mice compared with WT controls, and this decrease in podocyte numbers was prevented by KO of Trpc6 (Trpc6-KO GqQ>L) in mice. A similar pattern was observed when data were expressed as podocyte density (Table 2). Moreover, expression of both SYN and WT1 proteins was preserved by KO of Trpc6 (Figure 4, G and H). As shown in Supplemental Figure 4A, the decrease in SYN protein levels in Trpc6−/− GqQ>L mice treated with PAN was associated with an increase in Syn mRNA levels. In contrast, SYN protein levels in Trpc6-KO GqQ>L mice were preserved without an increase in Syn mRNA expression. Systolic BP (SBP) was similar in Trpc6−/− GqQ>L and Trpc6-KO GqQ>L mice (127 ± 3 [WT] vs. 129 ± 3 [KO]; P = NS).

Quantitative RT-PCR (qRT-PCR) was used to investigate the effect of GqQ>L induction on the CN-responsive genes Trpc6, regulator of CN 1 (Rcan1), and cyclooxygenase 2 (Cox2) (21, 28). As shown in Figure 4I, Cox2 mRNA was significantly increased in GqQ>L mice compared with levels in controls. Similarly, both Trpc6 and Rcan1 tended to be increased in GqQ>L mice compared with controls (Figure 4, I and J). KO of Trpc6 significantly reduced expression of Cox2 mRNA in GqQ>L mice. Similarly, Rcan1 tended to be decreased in Trpc6-KO mice compared with Trpc6−/− GqQ>L mice. A signal for Trpc6 was not detected in the Trpc6-KO mice (Figure 4J). Last, we investigated the effect of TRPC6 on expression of the other TRPC family members Trpc3 and Trpc5. As shown in Supplemental Figure 5, Trpc3 mRNA levels tended to be increased in Trpc6−/− GqQ>L mice treated with PAN, similar to that seen in to mice treated with angiotensin II (AII) (37). In contrast, Trpc3 mRNA levels were significantly decreased in Trpc6-KO GqQ>L mice compared with those in Trpc6−/− GqQ>L mice. Given that KO of Trpc6 may have decreased cytosolic calcium levels, these observations could be consistent with the known stimulatory effects of calcium signal-
The renin-angiotensin system has been suggested to be a disease accelerator in rodent models of diabetic kidney disease (39, 40). In support of this hypothesis, overexpression of a renin transgene was found to promote the development of advanced diabetic kidney disease in Tg rats (39). The enzyme renin initiates an endocrine cascade that results in the generation of the effector peptide AlI and in turn activates the Gq-coupled AT1 receptor (39). Thus, Gq-coupled signaling cascades may provide the second hit that augments kidney damage in rodent models of diabetes. To determine whether the adverse effects of podocyte Gq activation are generalizable to other glomerular disease processes, we determined whether induction of GqG>L exacerbated diabetic kidney disease in a mouse model of type 1 diabetes (FVB/NJ Akita mice) (41). As shown in Figure 7A, podocyte-specific expression of GqG>L enhanced albuminuria in Akita mice (Gq Akita mice) at 16 and 20 weeks of age. Enhanced albuminuria was associated with a significant increase in mesangial expansion (Figure 7, B and C) in either a diffuse (Figure 7B, middle panel) or nodular (Figure 7B, right panel) pattern. The glomerular ultrastructure is shown in Figure 7D. Nodular thickening of the glomerular basement membrane (GBM) was seen on the subepithelial surface in Gq Akita mice, which was associated with a significant increase in average GBM width compared with that seen in age- and sex-matched nondiabetic control group mice (Figure 7E). Focal areas of FP flattening were also observed, which was qualitatively more severe in the diabetic mice (Figure 7D and Supplemental Figure 7). As shown in Figure 7F, there was a significant increase in total collagen content in WT and Gq Akita mice compared with that in the nondiabetic control group. Collagen content tended to be increased in the Gq Akita group compared with WT Akita mice, but this difference was not statistically significant (P < 0.10). Tubular and interstitial injury was similar in both groups by light microscopic examination (Supplemental Figure 8, A and B). Table 4 shows the effects of diabetes on glomerular volume, podocyte density, and podocyte numbers. Glomerular volume and podocyte density were similarly increased and decreased, respectively, in both groups of Akita mice compared with mice in the nondiabetic age- and sex-matched control group. Podocyte numbers also tended to be reduced in both groups of Akita mice, but this difference was not statistically significant.

We next investigated the effect of diabetes and GqG>L induction on the CN-responsive genes Trpc6, Cox2, and Rcan1. As shown in Figure 7G, both Trpc6 and Cox2 tended to be increased in WT and Gq Akita mice compared with age-matched nondiabetic controls. These differences were statistically significant for Trpc6 and Cox2 in the WT and Gq Akita groups, respectively. As shown in Figure 7H, Rcan1 levels were markedly increased in WT Akita mice compared with levels detected in controls. Rcan1 was also increased ≈3-fold in Gq Akita mice compared with controls.

### Table 2. Effect of Trpc6 KO on glomerular structure

<table>
<thead>
<tr>
<th></th>
<th>Podocytes per glomerular profile</th>
<th>V/glom (×10^5 μm^3)</th>
<th>Nv(P/glom) (×10^7/μm^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAN controls (n = 9)</td>
<td>8.29 ± 0.17</td>
<td>2.59 ± 0.08</td>
<td>48.7 ± 0.7</td>
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<td>PAN GqG&gt;L (n = 9)</td>
<td>6.90 ± 0.33</td>
<td>2.47 ± 0.21</td>
<td>43.2 ± 1.5</td>
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<td>PAN Trpc6 KO (n = 9)</td>
<td>7.67 ± 0.36</td>
<td>2.46 ± 0.22</td>
<td>47.8 ± 1.4</td>
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</tbody>
</table>

*P < 0.05 versus PAN controls; *P < 0.05 versus PAN controls or PAN Trpc6-KO.

### Table 3. Effect of FK506 on glomerular structure

<table>
<thead>
<tr>
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<th>Podocytes per glomerular profile</th>
<th>V/glom (×10^5 μm^3)</th>
<th>Nv(P/glom) (×10^7/μm^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls (n = 11)</td>
<td>8.52 ± 0.17</td>
<td>2.46 ± 0.09</td>
<td>52.9 ± 0.5</td>
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<td>PAN vehicle (n = 13)</td>
<td>6.67 ± 0.42</td>
<td>2.44 ± 0.24</td>
<td>31.0 ± 6.2</td>
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<tr>
<td>PAN FK506 (n = 18)</td>
<td>7.18 ± 0.35</td>
<td>1.90 ± 0.23</td>
<td>48.4 ± 2.3</td>
</tr>
</tbody>
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*P < 0.05 versus untreated controls; *P < 0.01 versus GqG>L mice treated with PAN and vehicle.
but this difference was not statistically significant and was significantly attenuated compared with Rac1 levels in the WT Akita group (P < 0.01). Both blood glucose and glycated albumin levels were similarly elevated in the WT and Gq Akita groups (Supplemental Figure 9).

**Discussion**

In the present studies, we found that podocyte-specific activation of Gq in PAN nephrosis promoted albuminuria, reduced the number of glomerular podocytes, and induced FSGS. KO of Trpc6 both inhibited podocyte loss and completely prevented FSGS induced by PAN nephrosis in GqQ>L mice. These data suggest that TRPC6 is a key downstream target activated by Gq signaling that promotes podocyte injury. Intriguingly, glomerular damage induced by Gq required a second hit (ref. 28 and Supplemental Figure 2). Indeed, a second hit may be required for the development of glomerular disease in some familial forms of FSGS (29).

In this regard, the age at diagnosis is quite broad in patients with FSGS caused by gain-of-function mutations in TRPC6 (15, 16, 42), consistent with the idea that some type of environmental or genetic second hit may be required for the development of glomerular disease in some familial forms of FSGS (29). In this regard, the age at diagnosis is quite broad in patients with FSGS caused by gain-of-function mutations in TRPC6 (15, 16, 42), consistent with the idea that some type of environmental or genetic second hit may be required for the development of glomerular disease (30). In support of this possibility, Gigante et al. (43) described TRPC6 mutations in patients with early-onset nephrotic syndrome. In 2 patients, TRPC6 mutations resulted in an amino acid (N125S) change that had previously been reported to cause the autosomal recessive disease congenital nephropathy of the Finnish type. Thus, the mutant NPHSI allele may have accelerated disease in the pediatric patients with the TRPC6 (N125S) mutation. The concept of a second hit promoting disease progression is further supported by a recent study of autosomal recessive steroid-resistant nephrotic syndrome due to mutations in the NPHS2 gene encoding podocin (44). In this study, the authors suggested that the pathogenicity of 1 NPHS2 allele (R229Q) was dependent on a transassociated mutation in the other allele that caused a different amino acid change, consistent with the idea that a genetic second hit was required to develop a disease phenotype.

In the present study, Gq-dependent TRPC6 activation did not cause glomerular damage in the absence of an additional cell stressor. In this regard, PAN stimulates ROS generation in podocytes (45), and ROS potently activate TRPC6 (46, 47). Moreover, Gq-coupled GPCRs stimulate TRPC6 activation, both directly through the second messengers DAG and possibly IP3 (1), as well as indirectly by stimulating ROS generation (48). In this scenario, TRPC6 may serve as a final common pathway causing podocyte injury by several injury-promoting signaling cascades. These data suggest that, while we often study mediators of glomerular damage as individual entities, it is likely that the biological outcome represents the net effect of the number of injury-promoting pathways activated, the magnitude of activation of each individual mediator, and the genetic predisposition of the individual. Thus, treatment strategies that are currently used for idiopathic forms of FSGS may have beneficial effects in genetic forms of the disease by limiting the number of injury-promoting pathways that contribute to disease progression.

Current treatments for proteinuric kidney diseases include the use of therapies targeting the renin-angiotensin system. Given that these agents inhibit activation of the Gq-coupled AT1 receptor (1), the beneficial effects of the renin-angiotensin sys-
tem blockade in glomerular diseases may be mediated at least in part by inhibition of Gq signaling. In support of this hypothesis, multiple Gq-coupled GPCRs contribute to kidney injury in animal models of glomerular disease including receptors for ETA, TRP, and platelet-activating factor (PAF), cysteiny1 leukotrienes, and EPI (1, 5). Moreover, combined use of AT1 and ETA blockers is an attractive therapeutic strategy in glomerular disease models of glomerular disease including receptors for ETA, TRPC6, and other TRPC family members (54), which further enhances intracellular calcium levels and augments Rho A activation (3, 4). Thus, Gq signaling stimulates multiple signaling cascades (Rho GTPases and CN) that might be targeted to promote podocyte survival and glomerular filtration barrier integrity.

Another therapeutic strategy is CN inhibition (29). Pharmacological CN inhibitors are frequently used for the treatment of primary glomerular disease processes (29, 61). While the beneficial effects of these agents have been attributed to their immunological actions (62), accumulating evidence suggests that this calcium-activated phosphatase has important nonimmunological effects in glomerular diseases, including promoting degradation of the podocyte cytoskeletal protein SYN (22, 62), as well as inducing podocyte apoptosis and podocyte loss (23, 24). In the present studies, GqQ>L induction both activated CN and upregulated TRPC6 in a CN-dependent fashion. These observations may be relevant to kidney pathophysiology, because TRPC6 is induced in several primary glomerular diseases (17). We therefore determined whether the CN inhibitor FK506 attenuated glomerular injury induced by PAN in GqQ>L mice. We found that FK506 attenuated both albuminuria and tubular injury induced by GqQ>L in PAN nephrosis as well as enhanced expression of SYN. Despite these beneficial effects, FK506 had little effect on glomerular histology. The dissociation between effects on proteinuria and renal injury is consistent with several clinical studies suggesting that treatments that inhibit proteinuria do not necessarily translate into reduced disease progression (11, 63). Our findings of the inability of CN inhibition to improve renal histology should, however, be tempered by the observation that current pharmacological CN inhibitors have important off-target effects (64). For example, CN inhibitor therapy has been linked to the development of FSGS in some patients, perhaps by promoting renal ischemia (29, 65). In support of this hypothesis, glomerular volume was reduced in GqQ>L mice treated with FK506 (Table 3), consistent with the idea that FK506 caused renal ischemia and, in turn, a reduction in glomerular volume (66). Moreover, FK506 had little effect on GqQ>L-dependent induction of COX2 (Figure 6J). Indeed, in previous studies, we found that FK506-induced expres-
The mechanisms of this effect cannot be known with certainty. Dong and coworkers (69) reported that CN inhibitors attenuate renal tubular cell (RTC) apoptosis induced by either ischemia or azide-dependent ATP depletion by inhibiting dephosphorylation of the CN substrate dynamin-related protein 1 (DRP1) and by blocking mitochondrial fragmentation and, in turn, RTC apoptosis. Similarly, excessive urinary protein levels promote RTC apoptosis through the mitochondrial apoptotic pathway (70). Thus, it is possible that loss of glomerular filtration barrier integrity induc-
groups using ANOVA, followed by Bonferroni’s post-hoc analysis. The experiments, 2–3 mice were studied in each group. **
P -KO GqQ>L mice. Red blood cells (RBC) are labeled in the capillary loops. For or Trpc6+/+
significantly reduced in Trpc6+/+ apoptosis of RTCs. In this scenario, CN inhibition might attenuate rectly contributes to RTC injury by causing protein overload and apoptosis of RTCs. In this scenario, CN inhibition might attenuate tubular damage by both reducing proteinuria and inhibiting podocyte apoptosis in proteinuric renal diseases.

While off-target actions may have limited the beneficial effects of CN inhibition in the current study, KO of Trpc6 attenuated both glomerular and tubular damage, suggesting that TRPC6 might be an important therapeutic target for the treatment of glomerular diseases. These beneficial effects were associated with a reduction in expression of the CN gene target Cox2, consistent with the idea that some of the beneficial effects may have been mediated by reducing CN activity. In support of targeting TRPC6 for therapeutic benefit, AI-induced albuminuria is reduced in Trpc6-KO mice (37). Moreover, overexpression of TRPC6 in podocytes promotes proteinuria and glomerulosclerosis in mice (71). In contrast, TRPC6 protected podocytes from complement-mediated glomerular disease (72). Thus, the effects of TRPC6 in glomerular disease processes are context dependent, which has important implications if TRPC6 inhibitors are developed for the treatment of glomerular diseases.

To determine whether the adverse effects of podocyte Gq activation were generalizable to other glomerular disease processes, we investigated the effect of GqQ>L induction on diabetic kidney disease in Akita mice. The rationale for studying Gq activation in this model is based on the observations that: (a) the renin-angiotensin system is a disease accelerator in rodent models of diabetic kidney disease (39, 40); and (b) the beneficial actions of AT1 receptor blockers in diabetic nephropathy are mediated by blocking the Gq-coupled AT1 receptor (8, 9). In this scenario, signaling cascades activated by Gq may provide the second hit that augments kidney damage in diabetic kidney disease. In support of this hypothesis, induction of GqQ>L promoted albuminuria, enhanced mesangial expansion, and increased GBM width in Gq Akita mice compared with WT Akita animals. In WT Akita mice, the diabetic milieu was also associated with a significant increase in expression of mRNA for the CN-responsive genes TrpC6 (P < 0.05) and Rcan1 (P < 0.01), consistent with published studies suggesting that renal CN activity is enhanced in rodent models of diabetes (23, 73). Surprisingly, induction of GqQ>L was not associated with a significant increase in mRNA for either TrpC6 or Rcan1. We speculate that these observations are the result of compensatory mechanisms that limit the duration of Gq activation, including enhanced expression of the negative regulator of Gq signaling RGS2 (regulation of G protein signaling 2) (74) and modulation of IP3 receptor expression and activity (75, 76). In support of this hypothesis, IP3 receptor expression tended to be decreased in both groups of Akita mice compared with that in controls, and RGS2 levels tended to be higher in Gq Akita mice compared with those in WT Akita mice (Supplemental Figure 10). Regulation of Gq-dependent calcium signaling is, however, complex and includes not only modulation of mRNA and protein levels of the downstream signaling molecules, but also posttranslational modifications, accessory proteins, and proteasomal degradation (74–77). These regulatory mechanisms are an ongoing and evolving area of investigation, as they are incompletely understood. Further study will be required to better understand these counterregulatory pathways.

In the present study, systemic BP was similar in Trpc6+/+ GqQ>L and Trpc6-KO GqQ>L mice. This finding contrasts with the higher systemic BP reported by Dietrich et al. (78) in the initial description of Trpc6-KO mice. While we can only speculate on the reason for this difference, genetic background may play a role. In the study by Dietrich et al. (78), the mice were on a mixed 129Sv/C56BL/6J background; in the present study, the mice were on an inbred FVB/NJ background. Consistent with an effect of genetic background on the BP phenotype, Eckel et al. (37) reported no differences in systemic BP by either tail-cuff manometry or radiotelemetry in mice on an inbred 129SvEv background. Moreover, a significant increase in systemic BP would likely have minimized the beneficial effects of TrpC6-KO in the PAN nephropsis model, given the adverse effect of increased BP on the progression of kidney disease. Finally, glomerular disease in mice is generally less severe compared with glomerular disease processes in other rodent models (45). Thus, the lack of a phenotype in GqQ>L mice might not be generalizable to other species. In addition, the adverse effects of Gq activation in the present study are likely cell-type specific. With regard to the latter, P2Y2 purinergic receptors are potent activators of TRPC6 in podocytes (46, 79), but whole-body KO of the gene encoding the P2Y2 receptor was found to aggravate kidney injury following subtotal nephrectomy (80). In that study, the authors attributed the adverse effects of P2Y2 KO to higher systemic BP and ineffective compensatory hypertrophy (80). Indeed, the P2Y2 receptor has complex effects on salt and water metabolism (81)
that caused a significant increase in systolic BP following subtotal nephrectomy compared with that in WT animals (80). Additional studies will therefore be required to determine whether targeting Gq signaling using pharmacologic agents is a potentially beneficial strategy for treating glomerular disease processes.

In summary, we found that induction of GqQ>L specifically in podocytes caused robust albuminuria, FP effacement, decreased numbers of glomerular podocytes, and light microscopic features of FSGS. Moreover, Gq activation stimulated CN activity and upregulated TRPC6 expression in a CN-dependent fashion. KO of Trpc6 ameliorated albuminuria, FP effacement, and the decrease in glomerular podocyte numbers as well as prevented the development of FSGS and inhibited tubular injury. CN inhibition had similar beneficial effects on albuminuria, but was less effective in reducing renal histologic damage. Taken together, these data suggest that components of the Gq/CN/TRPC6 signaling cascade are important therapeutic targets for the treatment of FSGS and likely other glomerular disease processes. In support of the latter,
obtained from Jeffery Kopp (33) at the NIH and are now available through The Jackson Laboratory. \textit{Trpc6}-KO mice were obtained from Lutz Birnbaumer (78) through a collaboration with Michelle Winn.

**Experimental protocol.** All experiments were performed using mice backcrossed onto an FVB/NJ background for more than 10 generations. In pilot studies, age- and sex-matched 3- to 4-month-old littermates received DOX in their drinking water (with 2% sucrose to enhance palatability) for 1 week, and then nephrosis was induced by a single i.p. injection of PAN (500 mg/kg) as previously described (34). DOX was continued for the next 2 weeks, and urine was collected on the day prior to the PAN injection (day 0) and on days 7, 10, 14, and 28.
after injection. As shown in Supplemental Figure 11, these pilot studies suggested that the most robust albuminuria occurred 10 days after PAN injection in GqQ>L mice. For the FSGS studies, we therefore collected urine on days 0 and 10. Mice were sacrificed and their kidneys harvested for examination by light microscopy, transmission electron microscopy (TEM), and fluorescence microscopy and for preparation of enriched glomerular preparations as previously described (28).

To determine the role of TRPC6 in PAN nephrosis, Trpc6-KO mice (Trpc6<sup>−/−</sup> mice) were bred onto an FVB background for more than 10 generations and then crossed with tetO-rtTA and NPHS2 GqQ>L mice. Heterozygous offspring were then bred to create both double-Tg mice lacking Trpc6 as well as controls (single-Tg and non-Tg Trpc6<sup>−/−</sup> mice). Mice were then studied using the PAN protocol described above.

To determine the role of CN in PAN nephrosis, GqQ>L mice were treated with an injectable form of FK506 by diluting the drug in saline (0.9% sodium chloride) and then administering twice-daily s.c. injections of either saline vehicle or 10 mg/kg/day FK506 beginning the day of PAN treatment and continuing until harvest. Mice were then studied using the PAN protocol described above.

For the NFAT reporter studies, NPHS2-rtTA mice and tetO-GqQ>L mice were bred with mice expressing an NFAT-responsive promoter driving expression of β-gal (36) to create mice with 2 different transgenes. Double-Tg offspring were then bred to create mice that expressed all 3 transgenes. In these triple-Tg mice, treatment with DOX induced GqQ>L and activated CN, which promoted expression of β-gal. Controls included double- and single-Tg mice (labeled “other Tg” mice) as well as non-Tg animals. To measure NFAT promoter activity, cortical and glomerular homogenates were prepared, and β-gal activity was measured using the Galacto-Star Chemiluminescent Reporter Gene Assay (Applied Biosystems). Data are expressed as β-gal activity per milligram protein.

To determine the effect of GqQ>L induction on TRPC6 expression in vivo, mice expressing GqQ>L and control mice were treated for 1 week with DOX in the drinking water, and then enriched glomerular preparations were prepared as previously described (28). The glomerular preparations were then either: (a) snap-frozen in liquid nitrogen and saved at −70°C for preparation of total cellular RNA; or (b) solubilized in NP-40 lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 2 mM EDTA, 1% IGEPAL CA-630 [NP-40]) with protease inhibitors (Sigma-Aldrich) by sonication and frozen at −70°C for immunoblot analysis. In some studies, mice received 10 mg/kg/day FK506 or vehicle, as described above, during the DOX administration.

For the Akita studies, FVB/NJ Akita mice (41) were bred with both FVB/NJ tetO-GqQ>L mice and NPHS2-rtTA mice to create double-Tg FVB/NJ Akita animals using male Akita mice and nondiabetic female mice, because fertility is reduced in diabetic mothers (9.4 ± 2.4 [nondiabetic] vs. 5.8 ± 2.8 [diabetic] pups/litter; P < 0.001). For the studies, only male mice were examined, because kidney disease is mild in diabetic female animals (41). Age- and sex-matched single-Tg Akita and non-TG Akita mice were used as controls. For these studies, the transgene was induced by daily s.c. injections of the DOX analog anhydrotrercycline (10 mg/kg; Cayman Chemical) to avoid potential problems of giving DOX in sucrose water to diabetic mice and to standardize the drug dosage. Moreover, DOX may have beneficial effects in diabetes (82), perhaps by inhibiting MMP activity (82). In contrast, anhydrotrercycline has minimal MMP inhibitory effects (82). Treatment with anhydrotrercycline was initiated in mice at 4 weeks of age, and urine was collected at 12, 16, and 20 weeks of age. Mice were then sacrificed and kidneys harvested for examination by light microscopy, TEM, and fluorescence microscopy and for preparation of enriched glomerular preparations.

Isolation of mouse glomeruli. Enriched glomerular preparations were prepared using previously described methods (28). Portions of the glomerular pellet were then used to either: (a) prepare total cellular RNA using the TRizol reagent according to the manufacturer’s directions (Life Technologies); or (b) solubilize in NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% IGEPA L CA-630 [NP-40]) with protease inhibitors (Sigma-Aldrich) by sonication. RNA and protein samples were then frozen at −70°C. By light microscopy, the purity of the glomerular preparations ranged from 60% to 70%.

Reverse transcription followed by qPCR. Total cellular RNA was prepared using glomerular preparations and TRizol reagent (Life Technologies) according to the manufacturer’s directions. RNA was treated with RNase-Free DNAase (QIAGEN) and then reverse transcribed with SuperScript Reverse Transcriptase (Invitrogen) and oligo (dT) primers. Real-time quantitative PCR was performed using an iCycler (Bio-Rad) and the universal SYBR Green PCR Master Mix Kit (Applied Biosystems, Life Technologies). The amplification signals were normalized to endogenous cyclophilin mRNA levels. The primer sequences used for qRT-PCR can be found in the Supplemental Methods.

Immunoblot analysis. Immunoblotting was performed as previously described (28) using the Invitrogen Bis-Tris mini-gel system and the following Abs: (a) a mouse mAb against SYN (catalog 65194; Progen Biotechnik); (b) a mouse mAb against WT1 (immunoblotting) (catalog sc-7385; Santa Cruz Biotechnology Inc.); and (c) a rabbit polyclonal Ab against TRPC6 (catalog ACC-017; Alomone Labs). As a positive control for the TRPC6 immunoblotting experiments, human embryonic kidney cells (HEK293 cells) were obtained from the Duke Cell Culture Facility and were transfected with a TRPC6 cDNA (83). HEK293 cell lysates were then prepared using NP-40 lysis buffer. To assess protein loading, the immunoblots were stripped according to the manufacturer’s instructions, and immunoblotting was performed using a mouse mAb against β-actin (catalog MAB1501R; Chemicon International). For densitometry, the immunoblots were converted into a digital format using an Epson Perfection 1670 Scanner (Seiko Epson) and then analyzed using ScanAnalysis 2.5 software (Biosoft). Densitometric data were normalized by dividing the protein signals for TRPC6, SYN, or WT1 by the matched signal for β-actin. When multiple blots were required to analyze a large number of samples, all gels were run and transferred simultaneously to PVDF membranes. The proteins were then detected by immunoblotting using the Abs described above. For these studies, ECL (Thermo Scientific) was performed by placing all PDVF membranes into a film cassette and exposing the x-ray film to all immunoblots for the same duration. The full, uncit gels are shown in the supplemental material.

IHC. Mouse kidney cortex was embedded in OCT compound, snap-frozen in liquid nitrogen, and stored at −70°C until sectioning. Expression of SYN and WT1 was identified by indirect immunofluorescence using a mouse mAb against SYN (catalog 65194; Progen Biotechnik), a rabbit polyclonal Ab against WT1 (catalog sc-192; Santa Cruz Biotechnology Inc.), or a rat mAb (clone 5F10) against the HA tag in the GqQ>L construct (catalog 11-867-423-001; F. Hoffmann-La Roche). Briefly, frozen sections were fixed in 2% paraformaldehyde for 10 minutes, air dried, treated for 10 minutes with 1% Triton-X in Dulbecco’s
PBS (D-PBS), and then blocked for 1 hour in 20 mM Tris-HCl, 137 mM NaCl, pH 7.6 (TBS), with 0.2% Tween-20 (T-TBS) and 5% nonfat dry milk. The SYN, WT1, and HA Abs were added at a dilution of 1:200 in T-TBS with 5% nonfat dry milk. After incubating overnight, slides were washed 3 times in D-PBS and then incubated for 1 hour with either an Alexa Fluar 488 donkey anti-rabbit Ab (catalog A21206; Life Technologies), a goat CY3 anti-rat Ab (catalog A10522; Life Technologies), or a fluoresceinated donkey anti-mouse Ab (catalog ab98554; Abcam), all at a dilution of 1:100 in T-TBS with 5% nonfat dry milk. Slides were then washed 3 times in D-PBS, coverslips were applied using adhesive containing DAPI, and slides were examined using a Nikon Eclipse TE2000-S fluorescence microscope.

BP measurements. Systolic BP was measured using a computerized tail-cuff system (Hatteras Instruments) in conscious mice as previously described (34). This technique has previously been shown to correlate closely with intra-arterial measurements (84).

Histopathology. Light microscopic sections were stained with H&E and periodic acid Schiff (PAS) and then evaluated by a pathologist (A.F. Buckley) blinded to the genotype. FSGS, tubule dilation, and T1 inflammation and fibrosis were graded using a semiquantitative scale of 0 to 3 (0 = normal, 1 = mild, 2 = moderate, 3 = severe). For the Akita studies, mesangial expansion was assessed using the same grading scale as that described previously (41).

Albuminuria. Urine was collected for 24 hours in metabolic cages specifically designed for collection of urine in mice (Hatteras Instruments). Urinary albumin concentrations were measured using a kit from AssayPro, and urine creatinine levels were measured using a kit from Exocell. Urinary albumin excretion was expressed as both the albumin/creatinine ratio.

TEM and GBM width analyses. TEM was performed as previously described (55) at the Duke Electron Microscopy Core Facility. Analysis at the ultrastructural level was performed in a qualitative fashion, and areas of interest were selected in semithin sections for preparation of ultrathin sections to be examined by a pathologist (A.F. Buckley) blinded to the genotype. Digital images (3 animals from each group) were analyzed for density of patent slit diaphragms using Adobe Photoshop CS6 Extended Software (Adobe Systems) as previously described (34). Data were expressed as the number of patent slit diaphragms per micrometer of GBM length.

Quantitation of GBM width, podocyte numbers, podocyte density, and glomerular volume. See the Supplemental Methods.

Statistics. Data are presented as the mean ± SEM, and statistical analyses were performed using Prism software, version 5.0 (GraphPad Software). For comparison of continuous variables, a Kolmogorov-Smirnov test of normality was performed prior to assessing statistical significance using the following statistical methods: (a) a 2-tailed t test for variables passing the normality test; or (b) a Mann-Whitney U test for variables that were not normally distributed. For comparisons between more than 2 groups, statistical analysis included either: (a) a 1-way ANOVA, followed by a Bonferroni’s multiple comparisons post test for normally distributed variables; or (b) a Kruskal-Wallis test, followed by a Dunn’s multiple comparisons post test for variables that were not normally distributed. For noncontinuous variables, data were calculated using either Fisher’s exact test or χ² analysis. Histologic data were analyzed as a noncontinuous variable using the number of mice with the specified histologic abnormality. P values of less than 0.05 were considered significant. Graphs of the histologic findings are presented as the percentage of mice with the specified abnormality to permit a more effective comparison of the differences between the experimental manipulations in studies with an imbalance in the number of mice in each group.

Study approval. All animal care and experiments conformed to NIH guidelines (Guide for the Care and Use of Laboratory Animals. 8th ed. The National Academies Press. 2011.) and were approved by the IACUC of the Duke University Medical Center.

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