Pharmacological targeting of actin-dependent dynamin oligomerization ameliorates chronic kidney disease in diverse animal models

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Dysregulation of the actin cytoskeleton in podocytes represents a common pathway in the pathogenesis of proteinuria across a spectrum of chronic kidney diseases (CKD). The GTPase dynamin has been implicated in the maintenance of cellular architecture in podocytes through its direct interaction with actin. Furthermore, the propensity of dynamin to oligomerize into higher-order structures in an actin-dependent manner and to cross-link actin microfilaments into higher-order structures has been correlated with increased actin polymerization and global organization of the actin cytoskeleton in the cell. We found that use of the small molecule Bis-T-23, which promotes actin-dependent dynamin oligomerization and thus increased actin polymerization in injured podocytes, was sufficient to improve renal health in diverse models of both transient kidney disease and CKD. In particular, administration of Bis-T-23 in these renal disease models restored the normal ultrastructure of podocyte foot processes, lowered proteinuria, lowered collagen IV deposits in the mesangial matrix, diminished mesangial matrix expansion and extended lifespan. These results further establish that alterations in the actin cytoskeleton of kidney podocytes is a common hallmark of CKD, while also underscoring the substantial regenerative potential of injured glomeruli and identifying the oligomerization cycle of dynamin as an attractive potential therapeutic target to treat CKD.

Chronic kidney disease (CKD) affects hundreds of millions of people worldwide1,2. It is associated with the appearance of significant amounts of high-molecular-weight plasma proteins in the urine (that is, proteinuria), a symptom of a compromised glomerular filtration barrier (GFB). Renal filter selectivity is maintained by a physical, chemical and signaling interplay between its three core cellular constituents: glomerular endothelial cells, a basement membrane and highly specialized visceral epithelial cells known as podocytes. Injury or functional impairment to any of these three components of the GFB can lead to proteinuria3.

Podocytes are terminally differentiated cells of the glomerulus, which consist of a cell body and primary, microtubule-driven membrane extensions, as well as secondary, actin-based membrane extensions called foot processes. Proteins such as nephrin, CD2-associated protein (CD2AP), α-actinin 4 and inverted formin-2 (INF2) have been implicated in maintaining the podocyte’s complex cytoskeletal structure and function4–8. In addition to genetic mutations in key genes, podocyte injury can also be driven by diabetes or hypertension9.

Irrespective of genetic or disease-based causes, podocyte injury leads to the reorganization of the actin cytoskeleton, which underlies the loss of the actin-based foot processes10–12, a process referred to as foot process effacement13,14. Sustained injury to podocytes results in molecular alterations both within the cell and in its extracellular environment, which ultimately cause podocyte loss and glomerular injury12.

At present, clinical options to treat proteinuria include lowering the glomerular filtration pressure with blockers of the renin-angiotensin-aldosterone system or with immunomodulators such as cyclosporine. However, dysregulation of the actin cytoskeleton upon podocyte injury is a highly dynamic process that involves rapid loss and re-formation of foot processes15, so it has been suggested that interventions that target actin cytoskeleton dynamics would ameliorate kidney dysfunction16.

We and others have shown that the GTPase dynamin is essential for podocyte structure and function17,18. Dynamin regulates clathrin-mediated endocytosis and the actin cytoskeleton19,20. Dynamin has

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a propensity to oligomerize into higher-order structures, which is promoted by lipids21, actin filaments22 and proteins containing the SH3 domain23. Actin-dependent dynamin oligomerization has been implicated in regulating the actin cytoskeleton both by initiating actin polymerization through the removal of the capping protein gelsolin from the barbed ends of actin filaments and by cross-linking actin filaments22. Biochemical analyses using recombinant proteins and fluorescence lifetime imaging microscopy in podocytes recently showed that the small molecule Bis-T-23 promotes actin-dependent dynamin oligomerization24,25.

Given these observations, we investigated the effects of targeting dynamin’s oligomerization cycle with Bis-T-23 in whole animals. Bis-T-23 ameliorated or prevented proteinuria and diminished mesangial matrix expansion in diverse genetic and chronic models of glomerular disease in rodents. Our study suggests the feasibility of treating a diverse range of glomerular kidney diseases by modulating actin dynamics by targeting dynamin’s oligomerization cycle.

RESULTS

Dynamin oligomerization is essential for podocyte function

We first used zebrafish as an experimental model system to test whether actin-dependent dynamin oligomerization has a physiological role in maintaining podocyte function. As previously demonstrated26—depletion of the zebrafish homolog of dynamin, dynamin-2 (Dyn2), by a morpholino-based approach (dnm2 MO) resulted in morphological changes in embryos such as shortened body length, upward curled tails, and pericardial and yolk sac edema compared with treatment with a scrambled control morpholino (control MO) (Fig. 1a), and also in a significant difference in survivorship (Fig. 1b).

The selectivity of the zebrafish glomeruli for proper protein filtration is assessed by measuring the fluorescence intensity of transgenically overexpressed eGFP-tagged vitamin D–binding protein (eGFP-DBP) in the retinal blood vessel of the fish eye as a measure of circulating eGFP-DBP27. A decrease in circulating eGFP-DBP is typically accompanied by foot process effacement and the appearance of eGFP-DBP–mediated fluorescence in the tank water, indicative of a compromised GFB in the manipulated fish28,29. We used this physiological screen in our study; compared with control MO–treated fish, dnm2 MO–treated embryos exhibited foot process effacement (Fig. 1c) and a significant decrease in circulating eGFP-DBP (Fig. 1d). Although the zebrafish genome contains the genes dnm2, dnm1-like and dnm1, which encode three different dynamin isoforms, these observed phenotypes can be attributed to a specific depletion of dnm2—as dnm2 MO treatment did not alter dnm1 or dnm1-like mRNA expression (Supplementary Fig. 1a)—while also suggesting a lack of compensation for depletion of dnm2 expression.

To test whether the kidney phenotypes were due to loss of dynamin’s role in regulating the actin cytoskeleton, we carried out cross-species comparison of mean survival (P ≤ 0.0001 for comparison of mean survival) and with Bis-T-23 (1 ng per larvae) or with control MO (left) (n = 128 images for control MO and 245 animals for dnm2 MO). Curves represent 180 animals for control MO and 245 animals for dnm2 MO. Scale bar, 2 mm. (b) Survivorship curves of zebrafish larvae injected with either control MO or dnm2 MO. Curves represent 180 animals for control MO and 245 animals for dnm2 MO. Error bars, mean ± s.d. (log-rank: P < 0.0001 for comparison of mean survival time). (c) Representative image of the fluorescence of circulating eGFP-DBP in the retinal vessel plexus of the fish eye 96 h post-fertilization and injected with either control MO or dnm2 MO (left) (n = 128 images for control MO, and n = 94 images for dnm2 MO animals). Scale bars, 100 μm. Transmission electron micrographs of glomeruli analyzed in zebrafish larvae 120 h post-fertilization and injected with either control MO or dnm2 MO (right). Scale bars, 0.5 μm. (d) Intensity of circulating eGFP-DBP (AU, arbitrary units) in the retinal vessel plexus of the fish eye 96 h post-fertilization and treated with the indicated MO and/or expression construct and with Bis-T-23 (1 ng per larvae) or with DMSO as vehicle (20% per larvae). For groups 1–6, 16, and 20, n = 100–150; for all other groups n = 40–100. Black lines represent median intensity in each group (**P ≤ 0.01, ***P ≤ 0.001, unpaired t-test). NS, not statistically significant. (e) A schematic diagram indicating the domain structures of dynamin: GTpase, Middle, PH (pleckstrin homology), GED (GTpase effector domain) and PRD (proline/arginine-rich domain). Indicated mutations: K/E (K-to-E mutations of the indicated amino acid residues in black), E/K (E-to-K mutations of the indicated residues in red) and I690K. (f) A schematic diagram indicating that dimers of dynamin (DynOligo) and tetramers of dynamin (DynTetra) exhibit basal rate of GTp hydrolysis. Oligomerized dynamin (DynOligo), whose formation is promoted by Bis-T-23 (structure shown at right) or through indicated mutations, exhibits increased rate of GTp hydrolysis. DynOligo induces actin polymerization and cross-linking of F-actin, which in turn regulates the structure and function of podocytes. The small arrows in e and f indicate the effect of the mutations on dynamin’s propensity to oligomerize.
Dynamin can be therapeutically targeted by Bis-T-23

We next assessed the effect of Bis-T-23 on circulating eGFP-DBP levels in different genetic backgrounds (Fig. 1d). We first verified that Bis-T-23 promoted oligomerization of zebrafish Dyn2, as shown by an increase in GTPase activity (Supplementary Fig. 2a) and by the presence of dynamin oligomers in the pellet fraction after high-speed centrifugation (Supplementary Fig. 2b). This is expected given the high percentage of homology between mammalian and fish Dyn2 (Supplementary Fig. 2c). Administration of Bis-T-23 had no effect on circulating eGFP-DBP levels in wild-type animals (Supplementary Fig. 2d), control MO– or dnm2 MO–treated fish (Fig. 1d). Moreover, Bis-T-23 did not significantly improve the rescue of eGFP-DBP levels in dnm2 MO–treated fish coexpressing Dyn1 or Dyn1E/K (Fig. 1d). We conclude that Bis-T-23 has no effect in animals that lack dynamin altogether or in animals expressing functional dynamin. In contrast, Bis-T-23 restored circulating eGFP-DBP to control levels in zebrafish expressing dominant-negative Dyn1K/E or Dyn1I690K, but not if endogenous Dyn2 was also lacking (Fig. 1d). Compared to DMSO, Bis-T-23 has no influence on survivorship of fish treated with dyn2 MO and coexpressing either Dyn1K/E or Dyn1I690K (Supplementary Fig. 2e,f). The inability of Bis-T-23 to restore eGFP-DBP levels in the absence of endogenous dynamin provides genetic evidence that this compound directly targets dynamin. These findings suggest that Bis-T-23 targets actin-dependent dynamin oligomerization in podocytes to promote proper GFB function.

We also found greater fluorescent staining in the tail of Bis-T-23-treated wild-type fish compared with DMSO-treated fish (Supplementary Fig. 2g), suggesting the compound results in thicker actin filaments. These findings are consistent with the ability of dynamin oligomerization to increase actin polymerization and to cross-link actin filaments22,23. Moreover, Bis-T-23 increased the number of focal adhesions (FAs) and stress fibers in cultured podocytes (Supplementary Fig. 1c–e), phenocopying the expression of the gain-of-function mutant Dyn1E/K in these cells22 (Supplementary Fig. 1c–e).

Dynamin oligomerization attenuates transient proteinuria

Pharmacokinetic studies showed that Bis-T-23 is rapidly absorbed into the bloodstream of mice after intraperitoneal injection into the body cavity, where it reaches its highest concentration within 15 min

...mean ± s.d. (n = 60 mice per condition. (e) The systolic (SYS) or diastolic (DIA) blood pressure of 129X1/SvJ mice measured invasively using a catheter and stopped urine test after 8 consecutive days of treatment with DMSO (1%, vehicle) or Bis-T-23 (40 mg/kg). Error bars, mean ± s.d. (n = 6 mice per condition. (f) The systolic (SYS) or diastolic (DIA) blood pressure of 129X1/SvJ mice measured invasively using a catheter and stopped urine test after 8 consecutive days of treatment with DMSO (1%, vehicle) or Bis-T-23 (40 mg/kg). Error bars, mean ± s.d. (n = 6 mice per condition. (g) Proteinuria of BALB/c mice determined by spot urine test at indicated times after two consecutive doses of LPS. As indicated, animals were injected with either DMSO (1%, vehicle) or Bis-T-23 (40 mg/kg) (n = 10 mice per condition). (h) Proteinuria of Sprague-Dawley rats treated with PAN and determined by spot urine test. Rats were treated once a day starting 12 d after PAN with DMSO (1%, vehicle) or Bis-T-23 (20 mg/kg) for 6 consecutive days (n = 6 rats per condition. Error bars, mean ± s.d. (P ≤ 0.05; ***P ≤ 0.001, unpaired t-test).
Administration of several different concentrations of Bis-T-23 to wild-type, healthy mice did not induce transient proteinuria as determined by urine albumin/creatinine ratios (ACRs) measured from spotted urine (known as ACR analysis on spot urine) (Fig. 2b), late-onset lasting proteinuria (Supplementary Fig. 3b) or any other obvious toxic effects (for example, discomfort, lack of movement, ruffled fur, physiological abnormalities or signs of toxicity in the liver (data not shown)). In addition, prolonged administration of Bis-T-23 did not produce any alterations in the glomerular filtration rate, as measured by inulin clearance (Fig. 2c), urinary output (Fig. 2d), renal plasma flow as measured by para-aminohippurate clearance (Fig. 2e), blood pressure (Fig. 2f) or blood urea levels (Supplementary Fig. 3c), demonstrating that Bis-T-23 did not induce nephrotoxic effects.

Figure 3  Dynamin oligomerization in podocytes protects against proteinuria. (a) Domain structure of dynamin (top). R725A mutation is situated in the GED, which renders dynamin prone to oligomerize. A schematic diagram (bottom) indicating that the human gene DNM1 carrying R725A mutation (DNM1R725A) was placed under the regulation of a tetracycline-responsive promoter element (TRE; tetO). This transgenic mouse (Tg2) was subsequently bred to a second transgenic strain expressing the reverse tetracycline-transactivator (rtTA) protein under the control of a podocin promoter to allow for podocyte-specific gene expression (Tg1). Expression of DNM1R725A was induced by administration of the tetracycline analog, doxycycline. (b) RT-PCR of DNM1 from wild-type mice (WT), podocin-Cre only transgenic mice (empty) fed with doxycycline and homozygous DNM1R725A/R725A transgenic mice (R725A) fed with doxycycline. Neg, negative control with water as a template; Pos, positive control with plasmid encoding DNM1R725A. nephrin (Nphs1) was used as a positive control. Stand., DNA size standard. (c) Representative electron micrographs of glomeruli (n = 5 or 6 glomeruli per genotype) from empty and R725A transgenic mice fed with either a normal diet (− doxycycline) or doxycycline diet (+ doxycycline). Rows 1 (scale bars, 10 µm) and 2 (scale bars, 1 µm) show scanning electron microscopy. Rows 3 and 4 (scale bars, 1 µm) show transmission electron microscopy (TEM) images. (d) Length of foot processes (FP) determined by analyzing images in c. Doxy, doxycycline. Error bars, mean ± s.d. (**P ≤ 0.001; unpaired t-test). (e) Proteinuria determined by analysis of spot urine samples at indicated times and in the indicated genotypes. Mice were fed with doxycycline (doxy) diet before they were injected with LPS (n = 6 mice per condition). Error bars, mean ± s.d. (***P ≤ 0.001) (unpaired t-test). (f) Representative TEM images of glomeruli (n = 5 or 6 glomeruli per genotype) from empty and R725A transgenic mice 24 h after LPS injection. Animals were fed with either a normal diet (− doxycycline) or doxycycline diet (+ doxycycline). Scale bars, 2 µm (top row) and 1 µm (bottom row).
Figure 4  Dynamin oligomerization targets actin cytoskeleton in podocytes. (a) Proteinuria in wild-type and ACTN4 mice (without treatment or with treatment with either DMSO (1%, vehicle) or with Bis-T-23 (40 mg/kg)) as determined by spot urine test at indicated time points (n = 8 mice per condition). Error bars, mean ± s.d. (**P ≤ 0.01, unpaired t-test). (b, c) Proteinuria in ACTN4 mice determined by spot urine test before and after double injection of a podocin-driven expression vector encoding DNM1\textsuperscript{R725A} mutant protein. Animals were grouped by protein levels before treatment (n = 3 for >1,000 µg/ml ACR; n = 7 for 500–1,000 µg/ml ACR). Individual animals from b are shown in c. Red arrows indicate reduction of proteinuria to control levels. Error bars, mean ± s.d. (***P ≤ 0.01, unpaired t-test). CON, control.

(d) Proteinuria in CD2AP\textsuperscript{KO} mice determined by spot urine test over several days during which animals were treated daily with DMSO (1%, vehicle) or Bis-T-23 (40 mg/kg), starting at postnatal day (P) 18 (n = 5 mice per condition). Error bars, mean ± s.d. (**P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001, unpaired t-test). (e) Coomassie blue staining of SDS-PAGE gel showing protein bands from 2 µl of mouse spot urine at day 22 in d. BSA was used as a standard. (f) Line graph depicting number of live CD2AP\textsuperscript{KO} mice (black lines, n = 20 mice) and CD2AP\textsuperscript{KO} mice injected daily with Bis-T-23 (40 mg/kg) (red lines, n = 7 mice) at indicated time points. Animals exhibited a statistically significant difference in survival rate (log-rank: P < 0.0163).

We next examined the effects of Bis-T-23 on transient proteinuria induced in mice by the administration of lipopolysaccharides (LPS) at time zero and again 24 h later. Bis-T-23 or DMSO (or no treatment) was then given 24 h after the second LPS injection, and ACR analysis on spot urine showed significantly lower proteinuria in the Bis-T-23 treatment group compared with untreated LPS-injected mice or those treated with DMSO, with the most pronounced effect occurring between 2 and 4 h after administration of the compound (Fig. 2g). The timing of this effect was consistent with the timing of Bis-T-23 clearance from the blood (Fig. 2a) and was not a consequence of alterations in blood urea levels (Supplementary Fig. 3c).

It has been suggested that LPS-induced transient proteinuria is partly due to proteolytic degradation of dynamin by cytoplasmic cathepsin L (CatL)\textsuperscript{17,31}. As reported, administration of LPS to cultured mouse podocytes led to the loss of stress fibers and FAs\textsuperscript{17}, which was then reversed by either Bis-T-23 or the CatL inhibitor E64 (Supplementary Fig. 4a, b). Notably, Bis-T-23 restored dynamin but not synaptopodin or RhoA levels in LPS-treated podocytes, whereas E64 restored all three proteins (Supplementary Fig. 4c). These results show that Bis-T-23 specifically affects dynamin, and they suggest that the beneficial effects of Bis-T-23 on proteinuria can be explained in whole, or in part, by the assembly of oligomerized dynamin (Supplementary Fig. 4d).

We also examined the effect of Bis-T-23 on puromycin aminonucleoside–induced nephrosis (PAN model)\textsuperscript{32}. We injected rats with puromycin aminonucleoside on day 0, and on day 12, when proteinuria was high, we injected Bis-T-23. Bis-T-23 significantly reduced proteinuria on days 18 and 24 (Fig. 2h). These data suggest that pharmacological induction of dynamin oligomerization can ameliorate transient proteinuria in rodents.

**Dynamin oligomerization protects podocytes from injury**

To further test our hypothesis that dynamin oligomerization exerts a protective role during podocyte injury, we generated transgenic mice expressing the human Dyn1\textsuperscript{R725A} mutant in podocytes. The R725A mutation is situated within the GTPase effector domain (GED) (Fig. 3a) and enhances dynamin’s propensity to oligomerize\textsuperscript{33}. Homozygous DNM1\textsuperscript{R725A/R725A} animals (herein referred to as DYN1\textsuperscript{R725A} transgenic mice) expressed human Dyn1\textsuperscript{R725A} mutant from a doxycycline-inducible, podocin-specific promoter (Fig. 3a). We detected the presence of mRNA encoding Dyn1\textsuperscript{R725A}
in glomerular extracts within 1 week of feeding animals with doxycycline (Fig. 3b).

Although DYN1R725A mice exhibited normal global glomerular morphology (Fig. 3c), the lengths of the foot processes were roughly double those from control animals (podocin-Cre-only transgenic mice) (Fig. 3c,d). The ability of Dyn1R725A to lengthen foot processes is consistent with its effect on FAs and stress fibers in cultured mouse podocytes (Supplementary Fig. 1c–e). In addition, Dyn1R725A mice were significantly protected from LPS-induced proteinuria (Fig. 3e). In these animals, LPS did not induce the transient foot process effacement observed in control animals or in Dyn1R725A transgenic animals in the absence of doxycycline (Fig. 3f). Together with the Bis-T-23 experiments above, these findings show that dynamin oligomerization has a protective role in LPS-induced transient proteinuria.

To directly test our hypothesis that Bis-T-23 was acting by targeting the actin cytoskeleton in podocytes, we next examined the effect of Bis-T-23 on proteinuria using a genetic model of CKD. A point mutation, K256E, in the α-actinin 4 protein causes hyper-bundling of actin filaments in podocytes and focal segmental glomerulosclerosis in homozygous Actn4K256E/K256E animals (herein referred to as ACTN4 mice)3,4. In this model, a single dose of Bis-T-23 caused a transient reduction of proteinuria compared with untreated mice or mice treated with DMSO (Fig. 4a), which correlated with the ability of Bis-T-23 to induce FAs and stress fibers in podocytes from ACTN4 mice (Supplementary Fig. 5a,b). In a complementary approach, we expressed the Dyn1R725A mutant transiently in podocytes using tail vein injection of DNA encoding mutant protein, which we previously showed induces podocyte-specific expression5. Although ACTN4
mice exhibited different levels of proteinuria at baseline (Fig. 4b)\textsuperscript{34}, expression of Dyn1\textsuperscript{R725A} resulted in markedly reduced proteinuria compared with baseline values in every case (Fig. 4b,c), occasionally reducing proteinuria to levels seen in control animals (Fig. 4c). These results show that targeting dynamin oligomerization by two independent means (use of Bis-T-23 or expression of Dyn1\textsuperscript{R725A}) is able to ameliorate proteinuria by altering actin dynamics.

**Dynamin oligomerization ameliorates CKD in diverse models**

We next tested whether targeting dynamin oligomerization using Bis-T-23 is able to reverse proteinuria in diverse genetic models of CKD. Homozygous CD2AP knockout mice (Cd2ap\textsuperscript{−/−}), herein referred to as CD2AP\textsuperscript{KO} mice, are born with intact slit diaphragms, but the animals become mildly proteinuric by postnatal day 18 due to a defect in signaling at the slit diaphragm (Fig. 4d)\textsuperscript{36}. Administration of Bis-T-23 for 6 consecutive days almost completely prevented the onset of high-level proteinuria in the CD2AP\textsuperscript{KO} mice (Fig. 4d,e), which correlated with substantial restoration of the actin cytoskeleton in cultured CD2AP\textsuperscript{KO} podocytes (Supplementary Fig. 5c,d). Furthermore, Bis-T-23 significantly extended the lifespans of CD2AP\textsuperscript{KO} mice (Fig. 4f), suggesting that protection against high-level proteinuria affects the course of the disease and subsequent mortality.

Mice with a genetic deletion of the gene encoding PKCe (Prkce\textsuperscript{−/−})\textsuperscript{37}, herein referred to as PKCe\textsuperscript{KO} mice, are defective in multiple signaling pathways that regulate actin cytoskeleton\textsuperscript{38}, and develop proteinuria and visible signs of glomerular injury by \textasciitilde12 weeks of age (Fig. 5a and Supplementary Fig. 6). A single dose of Bis-T-23 administered daily to PKCe\textsuperscript{KO} mice not only prevented the progression of proteinuria but also significantly lowered proteinuria over the course of 8 d compared with untreated mice or those treated with DMSO (Fig. 5a). These animals also exhibited fewer glomeruli with moderate to advanced mesangial expansion compared with untreated or DMSO-treated mice (Fig. 5b,c), and their foot processes appeared more like wild-type processes than did those of the DMSO-treated mice (Fig. 5d).

The effects of Bis-T-23 on proteinuria and morphology of foot processes correlated with the ability of Bis-T-23 to restore the normal global organization of the actin cytoskeleton in cultured podocytes from PKCe\textsuperscript{KO} mice. Loss of PKCe results in a profound loss of FAs and stress fibers, which is almost completely rescued by re-expression of PKCe, but not of a version of the protein harboring a K437R mutation (PKCe\textsuperscript{R437R}), which renders the enzyme kinetically dead (Supplementary Fig. 7a–c). Cell extracts generated from podocytes from PKCe\textsuperscript{KO} mice were defective in actin polymerization, depolymerization, and transforming growth factor-\(\beta\) (TGF-\(\beta\))- and phorbol 12-myristate 13-acetate (PMA, a PKC activator)-induced cycles of cofilin-1 (cfl1, an actin-binding protein that disassembles actin filaments) phosphorylation (Supplementary Fig. 7d–g). Expression of Dyn1\textsuperscript{UK} or addition of Bis-T-23, but not expression of Dyn1\textsuperscript{K437R}, restored FAs and cell size and increased actin polymerization without affecting actin filament depolymerization (Supplementary Fig. 7a–c,h,i) or impairing the phosphorylation cycle of cofilin-1 (Supplementary Fig. 7j). These results strongly argue that Bis-T-23 reverses proteinuria in PKCe\textsuperscript{KO} mice by targeting the actin cytoskeleton even when multiple regulatory pathways have been affected.

To further examine how broadly Bis-T-23 can ameliorate proteinuria, we examined different genetic models of podocyte injury in zebrafish. In addition to knocking down the expression of PKCe (with prkce MO) and CD2AP (with cd2ap MO), we also knocked down the expression of inverted formin-2 (with inf2 MO), an actin-binding protein that severs actin filaments and accelerates their polymerization and depolymerization\textsuperscript{39}, and nephrin (with nphs1 MO), a structural slit diaphragm protein\textsuperscript{40}. The lifespans of the fish treated with cd2ap MO, prkce MO and inf2 MO were all shortened compared with fish treated with a control MO (Supplementary Fig. 8a). Comparable to what was observed earlier in dnm2 MO–treated fish, prkce MO–treated fish exhibited shortened body length, upward curved tails, and pericardial and yolk sac edema compared with control MO–treated fish (Supplementary Fig. 8b,c). In accordance with impaired kidney function, there was a marked lowering of the level of circulating eGFP–DBP in all four models compared with control MO–treated fish (Supplementary Fig. 8d).

Injection of Bis-T-23 was associated with significantly higher circulating eGFP–DBP in cd2ap MO–, prkce MO– and inf2 MO–treated fish (**\(P \leq 0.001\)), but not in nphs1 MO–treated fish, as compared with DMSO injection (Supplementary Fig. 8d). Electron microscopy confirmed that proteinuria in prkce MO–treated fish was due to foot process effacement, whereas the addition of Bis-T-23 was associated with normal foot process morphology (Supplementary Fig. 8e). Concomitant with its effect on proteinuria, Bis-T-23 increased the lifespans of fish treated with cd2ap MO, prkce MO and inf2 MO, but not nphs1 MO, compared with DMSO treatment (Supplementary Fig. 8f–i). Therefore, although Bis-T-23 reversed podocyte injury driven by disruption of multiple signaling pathways, it was unable to reverse injury due to loss of a structural protein such as nephrin.

Finally, we tested the effect of targeting the actin cytoskeleton via dynamin oligomerization in acquired kidney injury due to diabetes. Diabetes was induced in the majority of mice within 2 or 3 d after a single high dose of streptozotocin (STZ)\textsuperscript{41}. Over a period of 16 weeks, the mice developed proteinuria (Fig. 6a). Administration of Bis-T-23, but not DMSO, resulted in lower proteinuria after 8 consecutive days of administration (Fig. 6a). In addition, Bis-T-23, as compared with DMSO, led to improved glomerular histology with less mesangial matrix accumulation, as determined by periodic acid–Schiff (PAS) staining, toluidine blue staining of semi-thin sections and transmission electron microscopy (Fig. 6b). Furthermore, Bis-T-23 treatment, as compared with DMSO treatment, correlated with less expression of collagen IV in sclerotic lesions in diabetic mice (Fig. 6c). We observed no effect of Bis-T-23 on the level of glucose in the blood (Fig. 6d). These findings not only show the ability of Bis-T-23 to lower proteinuria induced by diabetes, but also further underscore the positive effect of Bis-T-23 on glomerular morphology as evidenced by reduced signs of mesangial matrix expansion.

**DISCUSSION**

Podocyte injury accompanied by a dysregulated actin cytoskeleton is observed in almost all cases of proteinuric CKD. In this report, we demonstrated the feasibility of targeting the actin cytoskeleton through dynamin and showed that this strategy has therapeutic potential to combat CKD. Given the role of the cytoskeleton in a number of diverse diseases such as cancer and CKD, earmarking the cytoskeleton as a therapeutic target has been suggested previously\textsuperscript{16,42}. Indeed, compounds aimed at microtubule dynamics are among the most successful anticancer therapies available\textsuperscript{43}. When it comes to targeting the actin cytoskeleton, actin filament-specific therapeutics has never been tested in humans. Our insight that the GTPase dynamin directly regulates actin dynamics via its oligomerization cycle\textsuperscript{22,25} suggested that it might be possible to target the actin cytoskeleton using dynamin as a proxy. Using the small molecule Bis-T-23, which promotes actin-dependent dynamin oligomerization\textsuperscript{25},
we have reported successful targeting of the podocyte actin cytoskel-
eton in diverse animal models of CKD.

Using a combination of diverse dynamin mutants, experiment-
al organisms and Bis-T-23, we established the physiological role of
dynamin oligomerization in regulating actin cytoskeleton. Morpholino-mediated knockdown of dnm2 in zebrafish decreased
circulating eGFP-DBP, a phenotype that was rescued by presence of
wild-type Dyn2 but not by Dyn1K14E (impair actin binding) or
Dyn1609K (impaired in oligomerization). Overexpression of both
mutants induced proteinuria by forming hetero-oligomers with
endogenous dynamin22,36,44. This effect was reversed by Bis-T-23,
but only in the presence of endogenous zebrafish Dyn2, providing
direct evidence that Bis-T-23 promotes actin-dependent dynamin
oligomerization in the kidney. In a complementary approach, we
showed that DYNI12T23A mice exhibited unusually long foot processes,
Further providing evidence that dynamin oligomerization regulates
actin dynamics in podocytes. Accordingly, DYNI12T23A mice were pro-
tected from LPS-induced proteinuria. In further support of Bis-T-23
targeting the actin cytoskeleton, this compound transiently lowered
proteinuria in ACTN4 mice.

The ability of Bis-T-23 to ameliorate proteinuria and improve
glomerular morphology in extremely diverse models of transient kid-
ney disease (LPS and PAN-induced proteinuria) and CKD (ACTN4,
PCKeKO, CD2APKO and STZ-induced diabetes) further validates the
idea that actin dysregulation is a common downstream manifesta-
tion of podocyte injury. Although actin cytoskeleton dynamics is
classically linked to foot process effacement and reformation, it has
been suggested that active remodeling of cortical actin regulates the
spatiotemporal organization of cell surface molecules45. Given the
unique characteristics of dynamin as a regulatory GTPase that can
bind membranes and directly cross-link actin filaments and stimu-
late actin polymerization, we suggest that dynamin oligomerization
might break a vicious cycle of actin dysregulation triggered by
injury. Our study suggests that targeting dynamin’s oligomerization
processes, Bis-T-23 prevented and reversed early signs of glomerular
proteinuria. Is this, reformation of proper foot processes) and signaling properties.
Signaling can take the form of positive cross-talk between podocytes,
endothelial cells, mesangial cells and maybe even progenitor cells of
the glomerulus46–48. Supporting this rationale, it has been shown that
alterations in the signaling within podocytes initiated proteinuria
to cross-talk between podocytes and endothelial cells, and that pro-
teinuria preceded effacement of foot processes49. Further supporting
a role for actin dynamics beyond morphological changes within foot
processes, Bis-T-23 prevented and reversed early signs of glomerular
injury. Our study suggests that targeting dynamin’s oligomerization
cycle might break a vicious cycle of actin dysregulation triggered by
missing or defective upstream signals.

In light of dynamin’s central role in regulating podocyte actin
dynamics, our study suggests that dynamin oligomerization repres-
sents a viable target in treating CKD. Currently no specific therapeu-
tic option exists for proteinuria in CKD in general, and no specific
treatment option exists for genetic forms of focal segmental
glomerulosclerosis in particular. Given that the majority of podocyte
injury in CKD can be traced back to the dysregulation of actin10–12,
the direct targeting of actin cytoskeleton dynamics in podocytes
seems to be an attractive therapeutic approach for human protei
nic kidney diseases.

METHODS

Methods and any associated references are available in the online
version of the paper.

Note: Any Supplementary Information and Source Data files are available in the
online version of the paper.

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AUTHOR CONTRIBUTIONS

M.S., H.H., J.R. and S.S. designed the research; B.T., C.G., V.A.S., M.K., N.H., P.S.,
L.S., L.T., J.-K.P., S.E., D.H.-K., C.W., S.M., C.C., N.T., S.H., S.R., M.K.S., A.V. and
F.G. performed the research. B.T., C.G., M.K., S.S., and S.S. analyzed the data.
M.S., T.B., C.G. and S.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online
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ONLINE METHODS

General experimental approaches. No samples, mice or data points were excluded from the reported analysis. Samples were not randomized to experimental groups. All analyses were performed unblinded except the following: measurement of intensity of circulating eGFP-DBP in the retinal vessel plexus of the fish eye and record of survivorship for all reported analyses, quantification of mesangial matrix expansion in glomeruli isolated from PKCε23 mice, semiquantitative analysis of collagen IV staining in STZ experiments, and the length of the foot processes in DYN1R725A animals.

Materials, antibodies and standard techniques. Bis-T-23 (2-cyano-N-3-[2-cyano-3-(3,4,5-trihydroxyphenyl) acryloylamino][propyl]-3-(3,4,5 trihydroxy phenyl)acrylamide) (Aberjona Laboratories, Inc., Beverly, MA) was prepared as 30 mM stock solutions in DMSO and stored frozen. The required amounts were added to the reaction vessel, achieving final DMSO concentrations of 1% or less. DMSO and GTP were from Sigma-Aldrich (St. Louis, MO). DMSO was used as a control vehicle (0.1%-1%) in all experiments. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and reconstituted in deionized water, unless otherwise stated.

Pyrene actin polymerization assay: The differentiated podocytes were lysed with G-buffer (5mM Tris-Cl pH 8.0, 0.2 mM CaCl2, 200 µM ATP). Protein concentration was measured using BCA reagent. G-actin stock was prepared by adding 225 µl of ice cold G-buffer to 0.1 mg pyrene actin to get a final concentration of 0.4 mg/ml and mixing well and leaving on ice for 1 h to depolymerize actin oligomers. The G-actin stock was centrifuged at 14,000 rpm at 4 °C for 30 min and the supernatant was transferred into a new tube on ice. 200 µl G-actin stock was added to each well of a 96-well plate. The fluorescence intensity of pyrene actin was measured in a TECAN plate reader with extinction of 360 nm and emission of 420 nm. Base line was established by measuring the fluorescence intensity for 5 min. At least 30 µg podocyte lysate with 20 µl actin polymerization buffer (500 mM KCl, 20 mM MgCl2, 10 mM ATP) was added to each well and the increased fluorescence intensity was measured every 60 s for 50 min.

Pyrene actin depolymerization assay: For the depolymerization assay, pyrene F-actin was prepared by quickly thawing frozen actin on ice. Pyrene actin was diluted by adding G-buffer to a final concentration of 1 mg/ml. Actin was polymerized after adding 10x actin polymerization buffer (0.25x final strength) and incubated at RT for 1 h. 200 µl F-actin stock was added in each well of a 96-well plate. After measuring the baseline of fluorescence intensity, the podocyte lysate was added to each well and the decreased fluorescence intensity was measured every 60 s for 50 min.

Cell culture and immunofluorescence. Wild-type and all other mouse podocyte cell lines were grown as described25. Immortalized PKCε22,23 podocytes, immortalized ACTN4 podocytes25, and immortalized CD2AP24 podocytes have been described. Adenoviral infections of cultured podocytes were performed as described25.

Cultured podocytes were stained with anti-paxillin antibody and rhodamine-phalloidin. Images were captured with a Zeiss LSM 5 PASCAL laser scanning microscope under a 40x objective lens. For quantification of actin, cells were imaged with the aforementioned microscope using a fixed exposure for phalloidin. The intensity of the actin staining was measured from whole cells by Image J (v1.47m) software. Total fluorescence from control (DMSO-treated or wild-type Dyn1 infected) cells and mutant Dyn1-infected and/or drug-treated cells was analyzed separately, with staining intensity normalized to the control cells. The experiment was repeated at least three times. Number of FAs was determined by integrated morphometry analysis performed using Image J (v1.47m) on thresholded images to select classified objects of a size range of >1 pixels as FAs, based on anti-paxillin staining. The analyzed particles command was used to measure number of FAs. When indicated, data were further analyzed using GraphPad Prism (v 4.03) for Windows (GraphPad Software, San Diego, CA) to perform statistical analysis using two-tailed unpaired t-tests. Based on this analysis, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 were considered statistically significant, and P > 0.05 was considered not significant.
Morpholino injection: The following splice donor morpholinos were designed and ordered from GeneTools (Philomath, OR): Control MO: 5′-CCTCTTACCTGATTACAATTATA-3′, prke MO: 5′-TCAGCCAGACGTTTAAACACCACT-3′, cd2ap MO: 5′-CATACTCAGCACCACCTGACACACC-3′, inf2 MO: 5′-AGAGTTAAGGTCTACACTGCTTTG-3′, nphs1 MO: 5′-CCTGTTGCTTAATCCCTGGAGTCTCC-3′, dnm2 MO: 5′-CAACCCGACTGTCCTACACCGGATTCT-3′.

Morpholino injections were carried out with concentrations ranging from 25 to 250 µM, with an injection volume of 4.6 nL in 100 mM KCl and 0.1% phenol red. Embryos were monitored for the development of the phenotype until 120 h post-fertilization. The phenotype was scored P1 to P4, based on the amount of edema present in the yolk sac or the presence of pericardial effusion. Rescue experiments were performed using mRNA injections expressing rat Dyn2 or human Dyn1, Dyn1/E/K, Dyn1/K/E, and Dyn1/I690K. Rescue plasmids were linearized and transcribed using the T7 Message Machine kit (Ambion, Grand Island, NY). The integrity and concentration of mRNA was determined by lab-on-chip technology in a bioanalyzer (Agilent Technologies, Santa Clara, CA). The integrity and concentration of mRNA was determined by lab-on-chip technology in a bioanalyzer (Agilent Technologies, Santa Clara, CA).

real-time PCR: Real-time PCR (RT-PCR) was carried out using the Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) system. The cDNA was amplified with fast start Taq polymerase, SYBR green (Invitrogen, Darmstadt, Germany), primers for target genes of interest and the following PCR conditions: 5 min at 95 °C, 45 cycles; 10 s at 95 °C; 10 s at 60 °C; and 10 s at 72 °C. Fast start Taq polymerase, buffer, MgCl2, and dNTPs were from the fast start Taq DNA polymerase dNTP pack (Roche Diagnostics). Each PCR test sample was pipetted with Master Mix (1 ml 10× buffer, 0.8 ml MgCl2 (25 mM), 0.2 ml dNTPs (10 mM), 0.5 10x SYBR green, 1 ml 0.1% Tween-20, 1.5 ml DEPC water) into 96-well plates, sealed with foil, briefly centrifuged, and measured in Light Cycler 480. The reaction was composed of 10 µl master mix, 1 µl forward primer and 1 µl backward primer, 0.1 µl fast start Taq polymerase, and 6.9 µl DEPC water. The specificity of each primer set was proved by analyzing the melting curve. All the samples were measured in triplicate and normalized with the housekeeping gene hypoxanthin phosphoribosyltransferase 1. The following primer pairs were used to examine the expression level of dynamin isoforms in zebrafish: dnm2 fwd: 5′-CTCTCATGCTTCCTGGAAGA-3′, dnm2 rev 5′-TCTCGATGTCCTCCTCAGGA-3′, dnm1 fwd 5′-AGAACGCAATTGTTTACCT-3′, dnm1 rev 5′-AAGCTGACAGAAGGGGAC-3′, dnm1-like fwd 5′-TCAAATGGCTGTGGTTGGGA-3′, dnm1-like rev 5′-GTCTGCCCTGGGTTACATC-3′, hprt fwd 5′-TCAATCATGACCGAACTGTA-3′, hprt rev 5′-AGACGGCCTTCTCATGTTT-3′.

Transmission electron microscopy of zebrafish: Morphant larval zebrafish were sampled at 120 h post-fertilization and fixed in 1.5% glutaraldehyde/1% PFA and 70 mM Na3PO4, pH 7.2. After fixation, the embryos were washed three times in 0.2 M cacodylate buffer and then post-fixed in 1% osmium tetroxide for 1 h at room temperature. After rinsing with cacodylate buffer, all specimens were dehydrated in a graded ethanol series and infiltrated and embedded with epoxy according to manufacturer’s protocol (Hard Plus Resin 812, Electronmicroscopy Sciences, Hatfield, PA). Thin-sections of 0.5 and 1 µm were generated with a Leica RM2165 rotary microtome and stained with 0.5% toluidine blue in a 1% sodium tetraborate solution. When the pronephros was identified on toluidine blue staining, ultra-thin (80-100 nm) thick sections of the kidney were cut and mounted on slot grids (Luelx, Friday Harbor, WA). The sections were stained with 2% uranyl acetate in distilled water and contrasted with lead citrate. Sections were viewed and photographed on a JEOL-1230 transmission electron microscope (Eching, Germany) and an attached CCD-camera.

Number of animals per experiment in Figure 1d: wild type, n = 144; control, n = 128; dnm2 MO, n = 94; dnm2 MO + Dnm2, n = 167; dnm2 MO + Dyn1, n = 106; dnm2 MO + Dyn1/E/K, n = 114; dnm2 MO + Dyn1/K/E, n = 40; dnm2 MO + Dyn1/I690K, n = 43; wild type + Bis-T-23, n = 57; dnm2 MO + Bis-T-23, n = 57; dnm2 MO + Dyn1 + Bis-T-23, n = 39; dnm2 MO + Dyn1/K/E + Bis-T-23, n = 57; dnm2 MO + Dyn1/I690K + Bis-T-23, n = 55; dnm2 MO + Dyn1K/E + Bis-T-23, n = 50; Dyn1, n = 73; Dyn1, n = 94; Dyn1/E/K, n = 74; Dyn1/K/E, n = 95; Dyn1 + Bis-T-23, n = 40; Dyn1/I690K + Bis-T-23, n = 119; Dyn1K/E + Bis-T-23, n = 44.

Number of animals per experiment in Supplementary Figure 8d: Control MO, n = 296; control MO + DMSO, n = 265; prke MO, n = 92; prke MO + DMSO, n = 78; prke MO + Bis-T-23, n = 88; cd2ap MO, n = 40; cd2ap + DMSO, n = 91; cd2ap + Bis-T-23, n = 90; inf2 MO, n = 92; inf2 MO + DMSO, n = 99; inf2 MO + Bis-T-23, n = 74; nphs1 MO, n = 114; nphs1 MO + DMSO, n = 64; nphs1 + Bis-T-23, n = 70.

The animal protocol for zebrafish experiments was approved by the Animal Care Committee of the Mount Desert Island Biological Laboratory, Bar Harbor, ME.

Animal studies: Animal strain: BALB/c mice (8 weeks old, males), C57BL/6 (12 weeks old, males), and 129X1/Sv mice (8 weeks old, males) were from The Jackson Laboratories. Sprague-Dawley rats (8 weeks old, males) were from Charles River Laboratories. PKEGrKO mice (12 weeks old, mixed genders)37, CD2APKO mice (18 days old, mixed genders)37, ACTN4 mice (12 weeks old, mixed genders)38 have been described. Dyn1K/EKO mice (12 weeks old, mixed genders) have been generated in this study (see below).

Pharmacokinetic studies were performed using C57BL/6 male mice by GenScript (Piscataway, NJ). Mice experiments using BALB/c, C57BL/6, and ACTN4 mice have been approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee, Boston, MA. Mice experiments...
using PKCεKO mice, CD2APKO mice and Sv/129 mice have been approved by the Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany. Mice experiments using DYN1R725A and rats have been approved by the Rush University Medical Center Institutional Animal Care and Use Committee, Chicago, IL.

STZ-induced type 1 diabetic mice: 129X1/Sv mice (8 weeks old, males) received intraperitoneal injections of streptozotocin (Sigma-Aldrich, St. Louis, MO) (125 mg/kg body weight) in 50 mmol/l sodium citrate buffer (pH 4.5) or just sodium citrate buffer (vehicle) on day 1 and 4. Blood glucose level was measured every other day with Glucose Elite (Bayer, Leverkusen, Bayer). Animals with glucose levels > 16 mmol/l were regarded as hyperglycemic/diabetic. After 16 weeks of diabetes, the animals were subjected to Bi-T-23 treatment for 8 days.

Urine albumin/creatinine ratio measurements: Spot urine was used to determine levels of albumin and creatinine using either mouse/rat albumin ELISA kit (Bethyl Laboratories, Montgomery, TX) or mouse/rat creatinine ELISA kit (Cayman Chemical, Ann Arbor, MI), respectively, in all animal models except transgenic DYN1R725A mice. Urine samples from Empty (podocine-only transgenic) animals and transgenic DYN1R725A mice were collected by placing them into urine collection containers for 4 to 6 h. A physical barrier prevented the mixing of solid and liquid wastes, allowing urine samples to be collected free of fecal contamination. Samples were diluted to 1:5, 1:10, or 1:50 with distilled water before total protein was measured using a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX) and urine creatinine measurements were taken using an ELISA kit (Cayman Chemical, Ann Arbor, MI). Absorbances were measured using EnSpire 2300 Multilabel Reader (PerkinElmer, Waltham, MA). No data points have been excluded. Data were plotted as mean ± s.d. and statistical significance was determined using unpaired t-test calculated using Prism software (GraphPad, La Jolla, CA).

BU (blood area) measurements: BU measurements were performed on the mice serum collected from the eye vein using QuantiCrom Urea Assay Kid (BioAssay Systems, Hayward, CA) based on the manufacturer’s protocol.

LPS and PAN induced proteinuria in rodents: BALB/c mice (8 weeks old, males) were injected intraperitoneally with LPS (200 µg/mouse) twice with a 24 h interval. When indicated in the figure, 24 h after the second injection, mice received single intraperitoneal injection of Bis-T-23 (40 mg/kg) or DMSO (20%). PAN (150 mg/kg body weight) was injected into a tail vein of Sprague-Dawley rats (male 8 weeks old) and urine albumin/creatinine ratio was measured as described above.

Glomerular filtration rate (GFR) and renal plasma flow (RPF) (inulin- and PAH-clearance): Measurements of RPF by PAH clearance and of GFR by inulin clearance were performed 8 days after daily treatment of C57Bl/6N male mice with just sodium citrate buffer (vehicle) on day 1 and 4. Blood glucose level was measured every other day with Glucostat Elite (Bayer, Leverkusen, Bayer). Animals with glucose levels > 16 mmol/l were regarded as hyperglycemic/diabetic. After 16 weeks of diabetes, the animals were subjected to Bi-T-23 treatment for 8 days.

Histological analysis. For histological analysis, the mice were anesthetized with isoflurane and perfused first with saline and then fixation solution (0.15% 1M HEPES buffer, 1.5% glutaraldehyde, 1.5% paraformaldehyde, pH 7.35) via the left ventricle. The kidneys were harvested and 1 × 1 mm cubes were cut from the cortex part of the kidney and fixed overnight in fixation solution. The tissue cubes were post-fixed with osmium in cacodylate buffer and contrasted with 4% uranyl acetate at 4 °C. Finally, the samples were dehydrated in acetone and embedded in EPO/acetone (1:1).

PAF staining: PAF staining was performed on paraffin-embedded tissue sections following a standard protocol. In brief, the kidney tissue sections were first deparaffinized via the following steps: 3 × 5 min Histoclear (Life Science Products, Frederick, MD) 3 × 3 min 100% ethanol, 2 × 2 min 96% ethanol, 1 × 1 min 70% ethanol, briefly in distilled water. The deparaffinized sections were oxidized in 0.5% periodic acid for 10 min, washed 3 × 5 min with distilled water, and incubated in Schiff’s reagent for 20 min. The differentiation was carried out in sulfite water (12 ml 10% Na2SO3, 10 ml 1M HCl, 200 ml distilled water) 3 × for 2 min and a 10 min wash under running tap water. The sections were dehydrated in following steps: 2 × briefly 96%, 3 × 2 min 100% ethanol, 3 × 2 min Histoclear, and covered with Histokitt. The PAS staining was documented using a Leica DMLB microscope with a Leica DFC425C camera (Leica, Wetzlar, Germany).

Generation of DYN1R725A transgenic mice. The construct to generate DYN1R725A transgenic mice was made using the BD Creator cloning system (BD Biosciences, San Jose, CA; Clontech, Mountain View, CA). Briefly, a 2.5-kb fragment containing the full-length cDNA of human DNM1 with a mutation in R725A was isolated from pcDNA-DNM1 (BD Biosciences, San Jose, CA; Clontech, Mountain View, CA). For histological analysis, the mice were anesthetized with isoflurane and perfused first with saline and then fixation solution (0.15% 1M HEPES buffer, 1.5% glutaraldehyde, 1.5% paraformaldehyde, pH 7.35) via the left ventricle. The kidneys were harvested and 1 × 1 mm cubes were cut from the cortex part of the kidney and fixed overnight in fixation solution. The tissue cubes were post-fixed with osmium in cacodylate buffer and contrasted with 4% uranyl acetate at 4 °C. Finally, the samples were dehydrated in acetone and embedded in EPO/acetone (1:1).

Collagen IV staining: Paraffin-embedded kidney tissue sections were deparaffinized as described in PAF staining and pretreated by incubation with 1 mg/ml trypsin (Sigma-Aldrich, St. Louis, MO) at 37 °C for 30 min. To block the sections were incubated with 10% donkey serum (Jackson Immunoresearch, Suffolk, England) and diluted in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) for 30 min at RT. The sections were incubated with 1:50 diluted Collagen IV antibody (Southern Biotech, Birmingham, AL) at 4 °C overnight. On the second day the unbound primary antibodies were washed away by rinsing 3 × 5 min with TBS and the sections were incubated with anti-goat Alexa Fluor 555 (Invitrogen, Carlsbad, CA) for 1 h at room temperature. The sections were then washed 3 × 5 min with TBS and mounted with Activaplumbum medium (Polysciences Inc., Warrenington, PA). The staining was analyzed under a Leica DMLB microscope with a Leica DFC425C camera.

Toluidine blue staining: One-micron-thick sections were cut using glass knives and a Sorval MT-1 (Dupont, Wilmington, DE) ultramicrotome and floated on water droplets on glass slides. The slides were dried in a humidity chamber on a warm hot plate. Toluidine blue stain (0.5% toluidine blue in aqueous 0.5% sodium borate) was pipetted over the sections and placed onto the hot plate until a slight goid rim could be seen around the stain droplet. The sections were rinsed in a stream of distilled water, dried, placed on cover slips, and examined by light microscopy. Representative sections were stained and the blocks were trimmed accordingly. Thin sections were cut using a diamond knife and an LKB 2088 ultramicrotome (LKB, Stockholm, Sweden) and then placed on copper grids. Sections were stained with lead citrate and examined in a FEI Morgagni

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Transmission and scanning electron microscopy. For scanning electron microscopy, renal tissues were dissected and sliced thinly into 2 mm pieces. The tissues were fixed in 4% PFA overnight, washed 3× in PBS, dehydrated, critically point dried using the 850 Critical Point Dryer (EMS) and sputter coated in gold on the 108 Auto Sputter Coater (Cressington, Watford, England). For transmission electron microscopy, renal tissues were collected and dissected into 2-3 mm pieces. The tissues were fixed in 4% PFA O/N, washed 3× in 0.1M cacodylate buffer and post fixed in 1% OsO4 for 1 h. Tissues were once again washed, dehydrated and embedded in Epon812. Ultrathin kidney sections (70 nm) obtained on the EM UC7 Ultramicrotome (Leica) were mounted onto Formvar coated Ni slot grids (EMS). Grids were stained for 15 min in 5% uranyl acetate followed by 0.1% lead citrate for 5 min. Scanning electron microscopy and transmission electron microscopy were done on the same scope (Sigma HDVP Electron Microscope). Electron microscopy micrographs were obtained using the Sigma HDVP Electron Microscope (Zeiss).

Foot process longitudinal length measurements: Scanning electron microscopy images were used to measure longitudinal length from the beginning of each final branch point to the end of a foot process. Each measurement was analyzed using Image J. ‘Foot process length’ from each animal was averaged from a total of 100 measured processes. Briefly, 15–18 individual foot processes were traced from each image taken from 5–6 different glomeruli of each genotype by a naive observer. Data were plotted as mean ± s.d. and statistical significance was determined using unpaired t-test calculated using Prism software (GraphPad, La Jolla, CA).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc.). Results are depicted as median ± s.d. For a two-group comparison, a Student’s t-test was applied if the pretest for normality (D’Agostino-Pearson normality test) was not rejected at the 0.05 significance level. P values less than 0.05 were considered significant. No statistical method was used to predetermine sample size. Survival was determined using log-rank (Mantel-Cox) test from GraphPad Prism software package. Survival P value tested the null hypothesis that the survival curves are identical in the two populations and is not rejected if P value equals 1.0 minus half the two-tail P value. P value less then 0.05 is considered statistically significant.

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