**Rho GAP myosin IXa is a regulator of kidney tubule function**

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**EPITHELIA** line the cavities and surfaces of the body and separate them from the surrounding environment. The formation of epithelia depends on the development of specific cell-cell adhesion structures between neighboring cells and the establishment of an apicobasal cell polarity. Coordinated distribution of plasma membrane components and dynamic reorganization of the actin cytoskeleton are crucial for the development and maintenance of cell polarity. This, in turn, is regulated by protein signaling networks that include Rho family GTPases and polarity proteins.

Different epithelia define the functional modules of the kidney. Proteins not retained by the glomerulus are reabsorbed by epithelial cells of the proximal tubule. The two main endocytic receptors are megalin and cubilin (34). Endocytosis of proteins in proximal tubules is a very efficient process, as urine is virtually protein free in mammals. However, alterations affecting the glomeruli or the uptake by proximal tubule epithelial cells can result in proteinuria due to system overload (12).

To date, several myosins have been linked to kidney dysfunction. Loss of myosin le causes glomerular lesions that are causative for a glomerular filtration defect resulting in albuminuria (22). Notably, myosin VI-deficient mice also develop albuminuria. They show a defective uptake of urinary proteins into proximal tubular cells (17). Myosin VI is associated with components of clathrin-enriched pits of the intermicrovillar region of the brush border (4). Likewise, myosin VIIb localizes to the distal tips of microvilli in the brush border of renal proximal tubules, where it is supposed to regulate the shuffling of membrane-associated proteins (9).

Until now, class IX myosins have not been associated with kidney dysfunction. In mammals, there are two members expressed: myosin IXα (Myo9α; previously called myr 7) and myosin IXβ (previously called myr 5) (3). Class IX myosins are unique among the myosin superfamily in that they contain a GTPase-activating protein (GAP) domain in their tail region, which allows them to negatively regulate small Rho GTPases. The GAP domain of Myo9α specifically inactivates Rho isoforms A, B, and C (10). Myo9α is expressed most abundantly in the brain and testis and at lower levels in the adrenal gland, kidney, lung, and spleen (10, 16). In the postnatal brain, Myo9α expression correlates with the onset of maturation of ependymal cells that form the epithelial cell layer that separates the brain tissue from the cerebrospinal fluid. Deletion of Myo9α results in the formation of hydrocephalus due to stenosis of the narrowest part of the ventricular system, the Sylvian aqueduct. In the absence of Myo9α, ependymal cells undergo morphological changes as well as alterations in differentiation (2).

In the present study, we investigated the physiological and histological consequences of loss of Myo9α in the kidney. Myo9α-deficient mice developed proteinuria and polyuria and showed signs of hydropnephrosis with dilated renal calyces. Proteinuria was most likely caused by a defect in the endocytic uptake of low-molecular-weight (LMW) proteins in the proximal tubule, as indicated by increased apical accumulation of megalin and its ligand albumin in proximal tubule cells. Loss of Myo9α resulted in a downregulation of the Rho effector murine diaphanous-related formin-1 (mDia1), which might contribute to the defect in endocytosis in the proximal portion of the nephron. We also noted typical secondary effects such as a dedifferentiation of cells and scarring of the tissue.

**MATERIALS AND METHODS**

Mice. Myo9α-deficient (Myo9α<sup>−/−</sup>) mice were generated as previously described (2). Myo9α<sup>+/−</sup> mice were backcrossed into a C57BL/6 background for 7–11 generations. The primers used for genotyping were as follows: F1 (5′-GCAGCTAGGCGTCATGC-3′) and R3 (5′-TGCTACCAGTTGDCATGC-3′) to identify the mutant allele and F3 (5′-GTGGCAGTGTGCAGTG-3′) and R3 to test for wild-type (WT) alleles, respectively.

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All procedures and protocols met guidelines for animal care and experiments in accordance with national and European (86/609/EEC) legislation.

* Urine collection. Urine was collected for 24 h in the presence of a mix of protease inhibitors from Myo9a−/− mice and control animals placed in metabolic cages. The WT and Myo9a−/− mice used were 12–24 wk old. Mice were accustomed to cages for 19–24 h before the collection period. Animals were kept on tap water and standard chow during the collection period. The protein profile in urine from knockout and control mice was examined by nonreducing SDS-PAGE. The urine volume loaded from each mouse was corrected proportionally to the mouse with the highest urinary output. Protein concentration was determined by the Bradford assay. Osmolality was measured using an osmometer and the appropriate calibration solutions (Knauer, Berlin, Germany).

* Antidiuretic hormone measurements. To determine antidiuretic hormone (ADH) levels in urine and blood plasma of WT and Myo9a−/− mice, the Arg8-vasopressin ELISA kit (Enzo Lifesciences) was used. Results were plotted and calculated using SigmaPlot 10.0 (Systat Software, Chicago, IL).

* Immunoblot analysis. Kidneys were snap frozen in liquid nitrogen and homogenized with sucrose lysis buffer (0.32 M sucrose, 15 mM HEPES (pH 7.4), 0.5 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) in a Dounce homogenizer at 4°C. The protein concentration in the homogenates was quantified by the Bradford assay (Bio-Rad Laboratories) using BSA as a standard.

Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). They were incubated with primary antibodies followed by peroxidase-coupled secondary antibodies (1:5,000, Jackson Immunoresearch). The primary antibodies used were as follows: Myo9a [Tü 78, 2 μg/ml (10)], β-actin (AC-15, 1:2,500, Sigma-Aldrich), mDia1 (0.25 μg/ml, BD Bioscience), and megalin (H-245, 1 μg/ml, Santa Cruz Biotechnology). Signals were visualized with SuperSignal West Pico chemiluminescence substrate (Pierce).

* in situ hybridization. Kidneys of adult mice were fixed in 4% paraformaldehyde treated with diethylpyrocarbonate at 4°C overnight, equilibrated in 30% sucrose for cryoprotection, and frozen in OCT compound in isopentane cooled by liquid nitrogen. Frozen sections of 10 μm thickness were prepared in a cryostat (Leica CM 1900) and mounted on Superfrost plus glass slides (Thermo Scientific, Braunschweig, Germany). Subsequent RNA in situ hybridization was performed as previously described (2).

* Histochemistry and immunohistochemistry. Kidneys of 12- to 24-wk-old mice were fixed in Bouin’s fixative (Sigma-Aldrich) for 18–24 h and then cleared and conserved in 70% (vol/vol) ethanol. Tissues were dehydrated, embedded in paraffin, sectioned at 6–10 μm, and stained with hematoxylin and eosin for histological analysis or with antibodies for immunofluorescence.

For cryosections, kidneys were fixed in 4% paraformaldehyde at 4°C overnight, cryoprotected in 30% sucrose in PBS, and frozen in OCT compound in isopentane cooled by liquid nitrogen. Frozen sections of 10 μm thickness were prepared in a cryostat (Leica CM 1900) and mounted on Superfrost plus glass slides.

After deparaffinization and heat antigen retrieval in sodium citrate (0.3% sodium citrate and 0.05% Tween 20, pH 6.0), sections were subsequently permeabilized with 1% Triton X-100 and blocked by incubation in blocking buffer (5% normal goat serum, 1% BSA, 0.05% Tween 20, 0.1% Triton X-100, and 0.75% glycine in PBS) for 1 h at room temperature. Sections were incubated at 4°C overnight with primary antibodies against albumin (1:2,000, DAKO, Glostrup, Denmark), β-catenin and occludin (all 1:500, Zymed, San Francisco, CA), aquaporin (Aqp)1 and Aqp2 (all 4 μg/ml, Alomone Labs, Jerusalem), PCNA (1:1,000, Abcam, Cambridge, MA), Na+/K+-ATPase transporter 2 (NKCC2: 1:2,000, Kerin Mutig, Berlin, Germany), Myo9a [Tü 78, affinity purified (10), 1:300], vimentin (1.5 μg/ml, Abcam), and megalin (LRP2, 2.5 μg/ml, Novus Bioscience, Cambridge, UK). After a wash, an antibody was visualized using horseradish peroxidase-conjugated secondary antibodies (1:500, Jackson Immunoresearch) and 3,3′-diaminobenzidine tetrahydrochloride substrate (Sigma-Aldrich). For indirect immunofluorescence labeling, we used either Cy3-conjugated secondary antibodies (all 1:500, Jackson Immunoresearch) and/or Alexa 488-conjugated secondary antibodies (1:400, Molecular Probes, Eugene, OR). FITC-conjugated Lotus tetragonolobus lectin (LTA) and rhodamine-labeled peanut agglutinin (all 1:1,000, Vector Laboratories, Burlingame, CA) as well as FITC-conjugated phallolidin (1:100, Invitrogen, Karlsruhe, Germany) were used to label proximal tubules, distal tubules, and F-actin, respectively. Nuclei were stained with 4′,6-diamidino-2-phenylindole (4 μg/ml, Sigma-Aldrich). Finally, all sections were mounted in Mowiol. Immunofluorescent staining was imaged using either an Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an ORCA-285 digital camera (Hamamatsu Photonics, Herrsching, Germany) controlled by Wasabi software (version 1.4, Hamamatsu) or a LSM 510 confocal microscope (Carl Zeiss) with LSM Release 4.2 software.

* Tube diameter measurements. Kidney paraffin sections were stained for the proximal tubule marker L. tetragonolobus lectin. Confocal images of 10 random fields in the cortex of Myo9a−/− and Myo9a−/− animals were acquired using a ×40 objective. The inner diameter of the tubules defined by LTA staining was measured using ImageJ (National Institutes of Health, Bethesda, MD).

* Cell culture. Porcine proximal tubule cells (LLC-PK1 cells) were cultured in DMEM containing 0.5 mM l-glutamine, 100,000 U/l penicillin, 100 mg/l streptomycin, and 10% FCS and passaged 2 times/wk. For transfection experiments, cells were either seeded on coverslips or in well plates 24 h before transfection. Transfection with small interfering (si)RNA was carried out with Lipofectamine.

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Table 1. Physiological parameters measured in renal clearance experiments of Myo9a+/+ and Myo9a−/− mice aged 12–24 wk

<table>
<thead>
<tr>
<th>Physiological Parameter</th>
<th>Genotype</th>
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<tbody>
<tr>
<td></td>
<td>Myo9a+/+ mice</td>
</tr>
<tr>
<td></td>
<td>Water access</td>
</tr>
<tr>
<td></td>
<td>Water access</td>
</tr>
<tr>
<td>Osmolality, mosmol/kg H2O</td>
<td>3.034 ± 1.456 (n = 3)</td>
</tr>
<tr>
<td>Na+, mmol/l/24 h</td>
<td>125.92 ± 28.11 (n = 3)</td>
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<tr>
<td>K+, mmol/l/24 h</td>
<td>94.28 ± 136.88 (n = 3)</td>
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<tr>
<td>Creatinine, μg/24 h</td>
<td>311.85 ± 105.03 (n = 3)</td>
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<tr>
<td>Urea, mg/24 h</td>
<td>71.75 ± 17.8 (n = 3)</td>
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<tr>
<td>Glucose, μg/24 h</td>
<td>602.95 ± 315.49 (n = 12)</td>
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Values are means ± SD; n, number of animals. Myo9a+/+ mice, mice with wild-type myosin IXA (Myo9a); Myo9a−/− mice, Myo9a-deficient mice. *P = 0.01; †P = 0.04; ‡P = 0.05.
RNAiMax (Life Technologies, Darmstadt, Germany) according to the manufacturer’s instructions. Stealth siRNA sequences were from Invitrogen. The Myo9a siRNA sequences used were as follows: siRNA 1, 5'-AUGGCAAGCAUACUUUGACUUUG-3'; siRNA 2, 5'-UCUACUGCAAUAGUAGAAGUUUC-3'; and siRNA 3, 5'-UGAAGGUUGACACUGCCACGGC-3'. Cells were harvested for Western blot analysis or processed for immunofluorescence staining 72 h after transfection. For Western blot analysis, cells were scraped off with a rubber policeman, centrifuged, and then resuspended in cell lysis buffer [50 mM Tris·HCl (pH 7.4), 100 mM NaCl, 2 mM MgCl₂, 1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml Pefabloc]. Protein concentrations of the lysates were determined by a Bradford assay using BSA as a standard.

**Endocytosis of albumin.** For albumin uptake experiments, cells were washed and starved in Ringer solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂, 0.8 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, and 10 mM HEPES, pH 7.4) 2 h before the experiment. Cells were incubated with 10 μg/ml FITC-conjugated albumin (Sigma-Aldrich) for 15, 30, and 60 min at 37°C. For binding assays, cells were incubated at 4°C for 15 or 60 min, respectively. Before the cells were lysed in detergent containing solution (0.1% Triton X-100 in 20 mM MOPS, pH 7.4), unbound albumin was removed in both cases by washing the cells eight times with ice-cold Ringer solution. Cell-associated fluorescence was measured using a FluoroMax 3 spectrofluorometer (Horiba, Kyoto, Japan) at an excitation wavelength of 495 nm and an emission wavelength of 519 nm. Autofluorescence of cell lysates was measured and subtracted. The protein concentration of the lysates was measured by the Bradford assay, and results were plotted as nanogram FITC-albumin per milligram of total protein. For fluorescence imaging, extracellular FITC-albumin was removed after uptake at 37°C by an acid wash step (0.2 M acetic acid and 0.2 M NaCl, pH 2.5). Cells were fixed with 4% paraformaldehyde at room temperature for 20 min and mounted in Mowiol.

**Immunofluorescence of cells.** Cells grown on coverslips were fixed with methanol at -20°C for 20 min. Unspecific binding sites were blocked by incubation with 5% normal goat serum in PBS. Primary antibodies were diluted in blocking solution and incubated for 1 h. Secondary antibodies were diluted in blocking solution and added for no longer than 1 h in a humid chamber in the dark. If necessary, nuclei were stained with 4′,6-diamidino-2-phenylindole. Coverslips were mounted with Mowiol and stored at 4°C in the dark.

**Statistical analysis.** Statistical analysis was performed using Microsoft Excel 2010 (Redmond, WA). P values indicating statistical significance were calculated using an unpaired Student’s t-test. P values of <0.05 were considered to be statistically significant. Values are means ± SD.

**RESULTS**

Kidney physiology is impaired in Myo9a⁻/⁻ mice. To determine whether loss of Myo9a in mice affects renal function, urine was collected for 24 h from mice with unrestricted access to water or under water deprivation. While the water intake of WT and Myo9a⁻/⁻ littersmates was comparable (7.26 ± 5.4 ml/24 h in WT mice vs. 8.53 ± 2.45 ml/24 h in Myo9a⁻/⁻ mice; Table 1), Myo9a⁻/⁻ mice excreted three times more urine (137.85 ± 61.08 μl·g⁻¹ body wt⁻¹·24 h⁻¹ in Myo9a⁻/⁻ mice vs. 53.48 ± 37.28 μl·g⁻¹ body wt⁻¹·24 h⁻¹ in WT mice). Myo9a⁻/⁻ mice also excreted more urine when they were kept under water deprivation for 24 h (52.4 ± 23.17 μl·g⁻¹ body wt⁻¹·24 h⁻¹ in Myo9a⁻/⁻ mice vs. 18.49 ± 16.52 μl·g⁻¹ body wt⁻¹·24 h⁻¹ in WT mice). However, similar to WT littersmates,
Myo9a<sup>−/−</sup> mice were able to concentrate their urine by approximately threefold under water deprivation (Table 1 and Fig. 1A). While concentrations of proteins in urine from Myo9a<sup>−/−</sup> animals were comparable with those of WT littermates, the total amount of protein excreted within 24 h was increased threefold in both Myo9a<sup>−/−</sup> mice both with and without access to drinking water (136.38 ± 17.84 μl·g body wt<sup>−1·24 h<sup>−1</sup></sup> in Myo9a<sup>−/−</sup> mice vs. 39.65 ± 8.21 μl·g body wt<sup>−1·24 h<sup>−1</sup></sup> in WT mice with water access and 28.37 ± 8.39 μl·g body wt<sup>−1·24 h<sup>−1</sup></sup> in Myo9a<sup>−/−</sup> mice vs. 11.05 ± 4.18 μl·g body wt<sup>−1·24 h<sup>−1</sup></sup> in WT mice without water; Fig. 1B). Analysis of urine samples collected from Myo9a<sup>−/−</sup> mice showed high levels of proteinuria. SDS-PAGE of urine from WT and Myo9a<sup>−/−</sup> mice revealed extensive excretion of LMW proteins in Myo9a<sup>−/−</sup> mutants. The majority of proteins excreted had a size of <25 kDa (Fig. 1C). This result suggests a defect in proximal tubule protein endocytosis that is either due to a decreased absorption of filtered LMW proteins or an increased excretion of protein by damaged tubules. Urine samples of Myo9a<sup>−/−</sup> mice also showed small amounts of proteins above the molecular mass of BSA.

Polyuria observed in Myo9a<sup>−/−</sup> mice is not caused by a dysfunction of the pituitary gland. A factor critical to the regulation of fluid balance is arginine vasopressin, also known as ADH. ADH is produced in the hypothalamus of the brain and is stored in and released from the posterior pituitary gland.
(28). Because Myo9a is expressed in various regions of the brain, it was important to rule out a dysfunction in ADH release in Myo9a−/− animals that could interfere with kidney function. As expected, ADH levels were low in urine samples when mice had free access to drinking water, and values for WT and Myo9a−/− samples were not significantly different (292.29 ± 152.61 vs. 115.71 ± 43.67 pg·ml−1·24 h−1). When animals had no access to drinking water for 24 h, levels of urinary ADH increased to 1,969.58 ± 1,287.95 pg·ml−1·24 h−1 in WT mice and 2,145.95 ± 928.98 pg·ml−1·24 h−1 in Myo9a−/− mice, respectively (Fig. 2A). Blood plasma levels of ADH were nearly identical in WT and Myo9a−/− samples (190.58 ± 105.46 pg·ml−1·24 h−1 in WT mice vs. 190.3 ± 106.7 pg·ml−1·24 h−1 in Myo9a−/− mice; Fig. 2B). We thus conclude that ADH regulation is unaffected in Myo9a−/− mutant mice.

Polyuria observed in Myo9a−/− mice is not caused by a dysfunction of the distal parts of the nephron. Dysfunction of channels or transporter molecules specific to distal tubule cells is a frequent cause of polyuria. To further search for a cause for the observed decreased urinary osmolality in Myo9a−/− mice (Table 1), we investigated the distribution of water channel Aqp2. In principal cells of WT and Myo9a−/− collecting ducts, Aqp2 was found enriched at the apical membrane, whereas a smaller fraction was found in the cytosol. Costaining with β-catenin showed a clear separation of apical and basolateral membranes in both genotypes; thus, cell polarization was maintained in both genotypes.
unaffected (Fig. 3A). When primary collecting duct cells were isolated and cultured, no obvious differences between Myo9a<sup>−/−</sup> and WT cells were observed. Cells from both genotypes formed properly polarized epithelial sheets without any detectable defect (data not shown). Based on these results, we conclude that a defect in collecting duct cells is not likely to be the origin for the kidney defect observed in Myo9a<sup>−/−</sup> animals. Since Aqp1, the main water channel in the proximal tubule, and NKCC2 in the thick ascending limb of the loop of Henle have previously been ascribed a role in urine concentration, the strong polyuria and decreased osmolality may be attributed to dysfunction of either of those channels. When we checked for localization and expression levels of Aqp1 and NKCC2, we did not observe any differences between WT and Myo9a<sup>−/−</sup> kidneys (Fig. 3, B and C, and data not shown).

**Myo9a<sup>−/−</sup> mice show signs of hydronephrosis.** Differentiation of renal epithelial cells is completed around 3 wk after birth, as indicated by the loss of the late proliferation marker PCNA and the appearance of epithelial and apical differentiation markers, such as E-cadherin and megalin (27). In immature Myo9a<sup>−/−</sup> kidneys, we did not observe differences in the tissue architecture. However, inspection of fully differentiated kidney tissue from 28-day-old (postnatal day 28) Myo9a<sup>−/−</sup> mice revealed some drastic morphological alterations. Myo9a<sup>−/−</sup> mice showed thinning of the renal parenchyma and dilation of the renal pelvis calyces (Fig. 4).

**Myo9a<sup>−/−</sup> kidneys reveal dedifferentiation and scarring.** Renal tubule cells divide at a low rate balancing the loss of tubular epithelial cells into the urine. Certain events, e.g., ischemia, that induce apoptosis or necrosis can dramatically alter the cell division rate (5). In agreement with these findings, adult WT kidneys exhibited almost no PCNA-positive cells, as revealed by immunostaining. In contrast, increased numbers of PCNA-positive nuclei were noted in Myo9a<sup>−/−</sup> kidney cortices (Fig. 5). Virtually all PCNA-positive nuclei were located in the distal part of the nephron, which stained positive for the marker peanut agglutinin. Additional PCNA-positive cells were located in the clusters of interstitial cells in between tubules (Fig. 5A). Colabeling of PCNA with the proximal tubule marker LTA confirmed the absence of PCNA-positive nuclei in proximal tubule cells (Fig. 5B).

Histological analysis of Myo9a<sup>−/−</sup> kidneys at 12 wk of age revealed the development of fibrosis adjacent to proximal tubules. Whereas WT kidneys showed a normal tubular structure with occasional interstitial cells, Myo9a<sup>−/−</sup> kidneys exhibited large clusters of interstitial cells that expressed the mesenchymal marker vimentin (Fig. 6). In WT kidneys, vimentin antibody labeled, as expected, the glomeruli and blood vessels (38). Additionally, a subset of Myo9a<sup>−/−</sup> kidneys showed defects that resembled focal glomerulosclerosis that might have developed in response to an antecedent tubular or interstitial injury, explaining the presence of larger-molecular-weight proteins in the urine of Myo9a<sup>−/−</sup> animals. We considered this to represent a secondary effect, especially since Myo9a is absent from glomerular cells.

**Myo9a localizes to the luminal side of proximal tubule epithelial cells.** To determine how loss of Myo9a might affect renal function, we investigated the cellular expression of Myo9a in the kidney. In situ hybridization on tissue sections of 3-mo-old WT mice revealed that Myo9a mRNA was predominantly expressed in the cortex of the kidney and was absent from the medulla. Myo9a mRNA was present in epithelial cells of the proximal tubule but was absent from the glomeruli and medullary rays. Furthermore, Myo9a was absent from the blood vessels (Fig. 7A). We found that Myo9a protein was expressed in WT but not Myo9a<sup>−/−</sup> mouse kidney homogenates (Fig. 7B). When tissue sections were stained for Myo9a protein, the signal was concentrated at the luminal side of epithelial cells of the proximal tubules, where it overlapped with apical actin filaments (Fig. 7C). This signal was absent in Myo9a<sup>−/−</sup> proximal tubules, demonstrating the specificity of the antibody staining. Fluorescent signals between the tubules were due to unspecific binding by secondary antibodies (arrowheads in Fig. 7, C and C').

**Myo9a<sup>−/−</sup> mice show dilation of proximal tubules, but epithelial cell polarity is not altered.** In Myo9a<sup>−/−</sup> kidneys, tubules within the superficial cortex and midcortex were frequently dilated, as observed with hematoxylin and eosin staining (Fig. 8, A–D). This dilation concerns proximal tubules, as revealed by staining with LTA, which specifically binds to the apical membrane of proximal tubule cells (Fig. 8, E and F). Notably, the phenotype varied between different Myo9a<sup>−/−</sup> kidneys examined. Some animals showed dilation of calyces and proximal tubules, whereas others showed only dilation of proximal tubules and some did not show any dilation of renal proximal tubules. To assess this phenotype, kidney sections were subjected to LTA staining, and inner diameters of labeled tubules in the cortex were measured. Figure 8G shows that the frequency distribution of proximal tubule diameters was altered in Myo9a<sup>−/−</sup> kidneys. In WT cortices, no diameters of >30–35 μm were measured, whereas in Myo9a<sup>−/−</sup> mice, tubule diameters ranged up to 50 μm. Concomitantly, the fraction of tubules with diameters of 5–10 μm, which constituted the majority in WT kidneys,
was reduced to almost one-third in Myo9a−/− kidneys. The mean diameter in Myo9a−/− kidneys was nearly twice that of WT kidneys (18.1 ± 7.5 vs. 10.6 ± 6.8, n = 5 Myo9a−/− kidneys and 5 WT kidneys, P < 0.01). Notably, physiological defects in the kidneys were also observed in animals without detectable alterations in morphology, suggesting that dilation of tubules and calyces are secondary effects due to the loss of the motor protein Myo9a.

A crucial prerequisite for epithelia to function properly is their apical-basal polarity, for which adherens and, especially, tight junctions are essential. Therefore, sections were stained for the adherens junction marker β-catenin and the tight junction marker occludin. Proximal tubules were identified by only low levels of occludin and diffuse staining of β-catenin at the plasma membrane. While the tubules were dilated and cells appeared flattened, polarity was unchanged. In addition, distal...
tubules that form a tighter barrier than proximal tubules did not reveal any loss or improper localization of occludin and β-catenin in Myo9a−/− mice (Fig. 9).

Albumin and its receptor megalin accumulate at the apical cell surface in proximal tubules of Myo9a−/− mice. The endocytic receptor megalin is most abundantly expressed in proximal tubules of the nephron. It is located at the apical cell membrane, along the endocytic and recycling pathways, and, to some extent, in lysosomes. Since megalin knockout mice exhibit LMW proteinuria (24) and megalin mosaic knockout mice yielded no indications of a nonmegalin receptor-mediated uptake of albumin (12), we wondered if loss of Myo9a would also influence megalin localization or function. Megalin was enriched at the apical plasma membrane of proximal tubules in WT and Myo9a−/− kidneys (Fig. 10). However, the apical megalin signal was significantly increased in Myo9a−/− kidneys compared with WT kidneys, and only a faint diffuse staining could be observed in the cytosol (Fig. 10, A–D). Compared with WT littermates, proximal tubules in Myo9a−/− mice showed an increased staining intensity for megalin at the apical brush border of epithelial cells even in nondilated tubules (Fig. 10, E and F). The observed staining appeared to be specific for megalin, since it was not due to the secondary antibody but rather to the primary antibody directed against megalin (Fig. 10, G and H). To quantify the effect of Myo9a deletion on megalin distribution, the ratio of intracellular to apical cell membrane fluorescence intensities was determined. A significant decrease in this ratio was observed in Myo9a−/− proximal tubule cells, indicating an enrichment of megalin at the apical plasma membrane (Fig. 10I). No change in total megalin levels was observed using Western blot analysis (Fig. 10J).

We next tested whether megalin that accumulated at the apical membrane of proximal tubules in Myo9a−/− mice was...
saturated with its ligand albumin. Therefore, endogenous albumin was visualized by antibody staining. In kidney sections from WT and Myo9a−/− mice, albumin was detected in blood vessels and some glomeruli (arrowheads in Fig. 11). In Myo9a−/− sections, albumin was additionally found at the luminal side of proximal tubule cells (Fig. 11). This is in line with the noted increase in cell surface localization of its receptor megalin at the apical luminal side of proximal tubule cells.

**Downregulation of Myo9a in LLC-PK1 cells alters morphology and increases cell surface binding of albumin.** To analyze the role of Myo9a in endocytosis of epithelial cells further, we downregulated Myo9a by siRNA in cultured LLC-PK1 proximal tubule cells, which endogenously express Myo9a. Of the three siRNA sequences tested, two efficiently downregulated Myo9a protein levels by >90% (Fig. 12A). In LLC-PK1 cells, Myo9a was located along the apical F-actin belt that is linked to adherens junctions. This circumferential staining was completely absent in cells transfected with Myo9a siRNA (arrows in Fig. 12B). Myo9a further localized to F-actin arcs in the lamellae of migrating proximal tubule cells (arrowheads in Fig. 12B). The spotted cytosolic signal was unaffected in knockdown cells and, therefore, is most likely due to unspecific binding of the antibody. Upon knockdown of Myo9a, LLC-PK1 cells underwent distinct morphological changes. Myo9a-depleted cells failed to spread and detached from the surface. In the remaining adherent cell patches, cells grew on top of each other, forming a multilayer. However, adherens junctions were maintained properly at the apical side, as indicated by the localization of β-catenin (Fig. 12B). Lamellipodia were absent in these cells. Such alterations never occurred in cells treated with control siRNA or siRNA 2, which did not downregulate Myo9a. To check for potential alterations of endocytosis in cells with downregulated Myo9a, we determined the amount of albumin binding to the apical membrane and the uptake into LLC-PK1 cells. When the binding of albumin to the cell surface was analyzed by blocking endocytosis at 4°C, Myo9a-deficient cells showed a 1.58-fold increase compared with control cells. Despite this increase in surface binding, the amount of FITC-albumin in Myo9a knockdown cells after uptake at 37°C was not increased compared with control cells. Thus, Myo9a-deficient proximal tubule cells internalize albumin less efficiently than control cells (Fig. 12, C–E). This is in line with the observation of increased levels of apical megalin and albumin in proximal tubules of Myo9a−/− kidneys, indicating that uptake of proteins into Myo9a-deficient cells is impaired.

**Myo9a regulates protein levels of the Rho effector mDia1.** By means of its Rho GAP domain, Myo9a can act as a negative regulator of Rho signaling. We have previously shown that

Fig. 10. Increased accumulation of megalin at the apical plasma membrane of Myo9a−/− proximal tubules. Paraffin sections were immunolabeled with megalin antibody (LRP2) and analyzed by confocal microscopy. A: megalin was only expressed in proximal tubules and predominantly localized to the base of the brush border in WT animals. B: loss of Myo9a was associated with an increase in brush border labeling for megalin and little to no signal in the cytosol. Scale bars = 20 μm. C and D: magnifications of individual tubules from the boxed regions in A and B. E and F: nondilated proximal tubules in Myo9a−/− kidneys showed increased apical megalin staining. G and H: negative controls of the same samples confirmed the specificity of the signal. Scale bars = 20 μm. I: the ratio of intracellular to apical membrane fluorescence signal intensities of megalin was calculated. In Myo9a−/− proximal tubule cells, megalin was enriched at the apical membrane compared with WT cells, as determined by a decrease in the ratio. n = 100 cells from 3 independent samples from each genotype. *P ≤ 0.05. J: expression of megalin was not altered in Myo9a−/− kidneys, as determined by Western blot analysis.
knockdown of Myo9a results in upregulation of the Rho signaling pathway (2). To shed light on the signaling pathway(s) Myo9a is regulating in the kidney, we analyzed the expression of proteins operating downstream of Rho activation. Myo9a-deficient kidneys showed a decrease in the amount of the Rho effector mDia1 (Fig. 13A). Concurrently with the knockdown of Myo9a in LLC-PK1 cells, a decrease was observed in mDia1 protein levels (Fig. 13B). Semiquantitative determination of mDia1 levels in cell homogenates by Western blot analysis indicated that the amount of mDia1 in Myo9a cells transfected with siRNA 1 was reduced by 65% of that in control cells and in cells transfected with siRNA 3 by 50%. However, Myo9a did not seem to interact with mDia1, as we could not detect an interaction of Myo9a with mDia1 by coimmunoprecipitation experiments (data not shown). The GAP domain of Myo9a specifically inactivates RhoA, RhoB, and RhoC in vitro (10). Therefore, elimination of the Rho GAP Myo9a may result in an upregulation of Rho activity and that, in turn, might contribute to the downregulation of mDia1 protein levels and the observed alterations in cell morphology and endocytosis. Whether that is indeed the case will be subject to future studies.

DISCUSSION

In the present study, we demonstrate the importance of the Rho GAP Myo9a for normal kidney function and morphology. Myo9a was expressed predominantly in proximal tubules of the renal cortex, where it localized to the apical junctional F-actin ring, suggesting a role in epithelial function of the proximal tubule. In agreement with this notion, the proximal portion of the nephron was frequently dilated, whereas distal parts appeared normal. Additionally, we frequently observed a dilated renal pelvis and atrophied renal papillary parenchyma in kidneys of Myo9a−/− mice. These kidney defects resembling hydronephrosis developed during the first weeks of life and were not congenital. They might be explained by a complete or partial ureteral obstruction (20, 31) that increases hydrostatic pressure. However, the tubular dilation in the proximal region of Myo9a−/− nephrons is unlikely to be caused by intratubular obstruction, because in chronic ureteral obstruction tubular dilation is greater in the distal nephron than in the proximal part (7). Because Myo9a localized to the luminal side of proximal tubule cells and dilation was restricted to the proximal tubules in Myo9a−/− kidneys, this might be a direct effect of the loss of Myo9a function in that segment.

Absence of Myo9a resulted in an elevated excretion of water and LMW proteins. A common cause for LMW proteinuria is decreased tubular protein reabsorption (17). This indicates that Myo9a might influence the endocytic uptake of urinary proteins. The main receptor responsible for endocytic uptake of urinary proteins in the proximal tubule is megalin/cubilin. In contrast to several mouse models with LMW proteinuria in which megalin or cubulin expression is decreased (11, 30, 32), megalin expression in Myo9a−/− kidneys was comparable to that in WT kidneys. Furthermore, megalin was correctly localized subapically in proximal tubules of Myo9a−/− kidneys. This suggests that Myo9a is not required for targeting the megalin receptor to the apical plasma membrane. However, an increased apical immunoreactivity was observed for megalin in proximal tubules of Myo9a−/− kidneys compared with WT kidneys, indicating an altered trafficking of the receptor. Endogenous albumin, the most abundant plasma and urinary protein and a ligand of the megalin receptor, accumulated at the apical membrane of the proximal tubule, supporting this notion. In Myo9a-depleted LLC-PK1 proximal tubule cells, the binding of albumin to the cell surface was also increased, again pointing to a higher number of receptors at the cell surface. Nevertheless, these cells did not internalize more albumin

Fig. 11. Impaired uptake of endogenous albumin in Myo9a−/− kidneys. Paraffin-embedded kidney sections obtained from Myo9a−/− and WT (Myo9a+/+) littersmates were stained using an anti-albumin antibody. Whereas albumin could only be detected in blood vessels and glomeruli (arrowheads) of Myo9a+/+ kidneys (A and A'), an additional accumulation of albumin was observed at the luminal side of proximal tubule epithelia in Myo9a−/− nephrons (B and B'). A negative control without primary antibody confirmed the specificity of the signal (C and C'). Scale bars = 20 μm.
Fig. 12. Knockdown of Myo9a disrupts the epithelial architecture and increases the binding of albumin to the apical surface in a proximal tubule cell line. A: LLC-PK1 cells were transfected with three different small interfering (si)RNA sequences against Myo9a and a control sequence for 72 h before homogenates were subjected to immunoblot analysis using an affinity-purified anti-Myo9a antibody. β-Actin served as a loading control. Myo9a siRNA 3 yielded the most efficient knockdown, with only 6.9% of the initial amount of Myo9a remaining. B: in proximal tubule epithelial cells, Myo9a localized in a circumferential pattern along the apical part of the basolateral membrane (arrows) and to the F-actin arcs in the lamellae of marginal cells (arrowheads). LLC-PK1 cells transfected with a siRNA sequence directed against Myo9a (siRNA 3) and control cells were stained for Myo9a with an affinity-purified rabbit anti-Myo9a antibody. The circumferential staining was absent in siRNA-transfected cells. Myo9a-deficient LLC-PK1 cells grew in islets, on top of each other, and lacked lamellipodia. Adherens junctions, as identified by β-catenin staining, remained intact in Myo9a-deficient cells. Images shown are maximum projections and single apical planes, respectively. Scale bars = 20 μm. C–E: endocytosis of albumin was impaired in Myo9a-deficient LLC-PK1 cells. Control and siRNA-transfected cells were exposed to 10 mg/l FITC-BSA and incubated at either 4 or 37°C for the indicated times. C: quantification of total amounts of FITC-albumin revealed increased surface binding in Myo9a-deficient LLC-PK1 cells, whereas uptake was comparable between WT, control siRNA-transfected, and Myo9a siRNA-transfected cells. D: Myo9a-deficient LLC-PK1 cells took up reduced amounts of surface-bound albumin. Albumin binding and endocytosis of control and Myo9a siRNA-transfected LLC-PK1 cells are shown. E: Myo9a-depleted cells showed increased binding of FITC-albumin to their apical surface at 4°C. Uptake of albumin into cells at 37°C was comparable between control and Myo9a siRNA-transfected cells. Maximum projections are shown. Scale bars = 20 μm.
compared with control cells, indicating that uptake of protein into the cells is slowed. Loss of Myo9a, a negative regulator of Rho activity, leads to increased Rho activity in epithelial cells (2). The increased Rho activity could lower endocytic internalization in Myo9a−/− proximal tubules since Rho activity is able to regulate internalization of membrane vesicles from the plasma membrane (23). Increased Rho activity might result in increased tension at the membrane. An increase in membrane tension and inhibition of actin dynamics are known to stall coated pits at a late stage of assembly (6) and could thus decrease the internalization of megalin. However, the role of Rho in endocytosis remains controversial and probably depends on the particular endocytic pathway or cell type examined. While in nonpolarized cells constitutively active RhoA inhibits clathrin-mediated endocytosis (23), it stimulates it in Madin-Darby canine kidney cells (25). Deletion of the Rho GAP oligophrenin 1 resulted in upregulation of the Rho-Rho kinase (ROCK) signaling pathway and reduced clathrin-mediated endocytosis in fibroblasts and astroglial cells (19). The overexpression of the *Drosophila* guanine nucleotide exchange factor RhoGEF2 reduced the turnover of E-cadherin in the embryonic epidermis (37). Rho can activate a number of different downstream effectors, such as mDia1, endophilin, PKN1/PRK-1, and PKN2, which have been implicated in endosomal trafficking (15, 18, 26, 33). Therefore, Rho over-activation in Rho GAP Myo9a-deficient cells might interfere with normal endocytosis through the stimulation of a downstream signaling pathway.

In both Myo9a-depleted proximal tubule cells in vitro and Myo9a−/− kidney homogenates, the levels of the Rho effector mDia1 were decreased. This downregulation in Myo9a-deficient epithelial cells may contribute to a defect in endocytosis of LMW proteins in the proximal tubule. In *Drosophila*, depletion of mDia resulted in a reduced uptake of dextran and interfered with clathrin-mediated endocytosis (26). Activated RhoB recruits mDia1 to endosomes. It promotes the assembly of F-actin on the vesicle membrane and connects F-actin dynamics with endocytic membrane trafficking (15). Alternatively, the reduction of mDia1 levels might affect the organization of E-cadherin-mediated cell-cell junctions, as previously reported for human MCF7 epithelial cells (8). Although we did not observe any obvious changes in adherens junctions of epithelial cells in proximal tubules of Myo9a−/− mice, the epithelial organization and morphology of LLC-PK1 cells that had Myo9a downregulated was altered. The downregulation of a single Rho effector upon the loss of the Rho GAP Myo9a points to the intriguing possibility that Myo9a might regulate signaling downstream of Rho differentially.

Exposure of renal epithelial cells to excessive amounts of protein leads to the production of a variety of inflammatory mediators that induce peritubular inflammation, tubular damage, and fibrosis (1, 35). A primary endocytic defect may cause changes in membrane composition at the plasma membrane, endosomes, or the Golgi complex and could thus result in altered renal physiology and finally an elevated stress response (17). Therefore, the observed accumulation of interstitial cells and fibrosis may be secondary effects of the loss of Myo9a. Several studies have revealed that tubular cells challenged with protein overload show an upregulation of inflammatory mediators, such as monocyte chemoattractant protein-1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES), and fractalkine (14, 36, 39). The gradients of chemotactic cytokines guide the infiltration of inflammatory monocytes/macrophages and T cells to the injured sites. These cells, in turn, stimulate mesangial cells, fibroblasts, and tubular epithelial cells to undergo phenotypic transitions and to produce a large amount of extracellular matrix components. Continuous deposition of the extracellular matrix results in fibrous scars and distorts the fine architecture of kidney tissues, leading to the collapse of renal parenchyma and further contributes to the loss of kidney function (22). Downregulation of Myo9a expression has been shown to result in increased NF-κB activity (2), and this might contribute to the inflammatory responses and renal scarring that impair kidney function in Myo9a−/− mice. Inhibition of the Rho effector protein ROCK with Y-27632 prevents fibrosis in a model of unilateral ureteral obstruction (29), showing that Rho signal transduction is able to regulate cell migration and proliferation in progressive fibrosis. If Rho or ROCK inhibition alone could restore renal function in Myo9a−/− mice or whether other factors, such as downregulation of mDia1, contribute to the observed phenotype remain to be investigated. Treatment of hydrocephalic Myo9a-null mice with Y-27632 successfully attenuated enlargement of lateral ventricles (2). The increase in proliferating PCNA-positive cells in the distal tubule is most likely a response of intact tubular cells to compensate the loss of cells, as it occurs after an ischemic injury (5). Additionally, urinary albumin itself can also induce cell proliferation through phosphatidylinositol 3-kinase- and ERK-dependent pathways (13).

In conclusion, the present study shows that Myo9a regulates renal morphology and physiology. The expression of Myo9a is restricted to the circumferential actin belt of renal proximal tubule cells. The data provided in the present study suggest that this unconventional motor plays a role in protein reabsorption in this segment of the nephron possibly via the Rho effector mDia1. Further studies will be necessary to elucidate its precise role in endocytosis and kidney function.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: S.T., M.A., G.C., and M.B. interpreted results of experiments; S.T., M.A., G.C., B.E., and M.B. prepared manuscripts; S.T., M.A., G.C., B.E., and M.B. approved final version of manuscript.

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