Reduced Notch Signaling Leads to Renal Cysts and Papillary Microadenomas

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ABSTRACT

The formation of proximal nephron segments requires canonical Notch2 signaling, but other functions of Notch signaling during renal development are incompletely understood. Here, we report that proximal tubules forming with reduced Notch signaling, resulting from delayed conditional inactivation of Notch1 and/or Notch2, are prone to cyst formation and tubular epithelial stratification. Conditional inactivation of the DNA binding factor RBP-J, which mediates Notch signaling, also resulted in multiple congenital cysts arising from the proximal tubule. Moreover, a few stratified foci/microadenomas containing hyperproliferative cells, resembling precursors of papillary renal cell carcinoma, formed in these proximal tubules. Epithelial stratification correlated neither with reduced expression of the transcriptional regulator of ciliary proteins TCF2/HNF1β nor with loss of apical-basal polarity. Instead, Notch signaling helped to restrict the orientation of epithelial mitotic spindles to a plane parallel to the basement membrane during nephron elongation. In the absence of Notch, random spindle orientation may explain the epithelial stratification and cyst formation. Furthermore, post hoc analysis of human class 1 papillary renal cell carcinoma revealed reduced Notch activity in these tumors, resulting from abundant expression of a potent inhibitor of canonical Notch signaling, KyoT3/FHL1B. In summary, these data suggest that canonical Notch signaling maintains the alignment of cell division in the proximal tubules during nephrogenesis and that perturbations in Notch signaling may lead to cystic renal disease and tumorigenesis.


Nephrons are composed of a filtration apparatus (the glomerulus) connected by a segmented tube lined with a single layer of epithelia to the collecting duct. Each tubular segment contains distinct cell types, arranged along the proximal-distal axis,1,2 that engage in vectorial reabsorption of the electrolytes and proteins from glomerular filtrate before its arrival at the duct. The molecules that specify distinct cell fates along the proximal-distal nephron axis and those orchestrating nephron morphogenesis are only beginning to be identified. Recent studies have identified that renal epithelial cells coalesce via a Wnt9b-regulated convergent-extension process during tubule morphogenesis to narrow the tubule diameter,3 which is latter maintained by strict control of division plane orientation parallel to the length of the nephron in proliferating epithelia of elongating tubules.4 Restriction of mitotic spindle poles to a plane parallel to the length of the nephron is regulated by the planar cell polarity pathway and was first shown to be dependent on Pkd1, a basal body protein, and TCF2/HNF1β, a transcriptional regulator of genes encoding ciliary...
proteins, the disruption of which results in cystic kidney disease.\textsuperscript{4,5} Cystic kidney diseases, including autosomal dominant and recessive polycystic kidney disease, Bardet–Biedl syndrome, and nephronophthisis, arise as a consequence of mutations in a large set of genes, most of which have been structurally or functionally linked to cilia or the basal body.\textsuperscript{6–9} It is held that ciliopathies affect the positioning of the centrosome and hence the orientation of the mitotic spindles during cell division, leading to deregulation of tubule diameter and cyst formation.\textsuperscript{10} However, disruption in spindle orientation relative to length of the nephron may not in itself be sufficient to result in cystic kidney disease.\textsuperscript{11} Considering that epithelial stratification in the epidermis may require the mitotic spindles to orient perpendicular to the basement membrane (BM),\textsuperscript{12} it is likely that maintaining the tubular epithelium of the nephron as a monolayer requires that the mitotic spindles be restricted parallel to the BM apart from being restricted parallel to the length of the nephron in proliferating epithelia during tubule elongation (see Supplementary Figure 1E). Whereas many studies have identified regulators that restrict the mitotic spindle poles parallel to the length of the nephron, the identities of regulators restricting the mitotic spindle poles parallel to the BM have not yet been described.

Mammals express four Notch proteins, receptors that mediate short-range communication by interacting with ligands that are presented on the neighboring cell’s surface.\textsuperscript{13} Notch1, Notch2, and genes coding for their ligands Dll1 and Jag1 are all expressed by renal vesicle (RV) cells.\textsuperscript{13,14} After ligand binding, the juxtamembrane negative regulatory region (NRR) unfolds, permitting metalloproteases to cleave Notch. Ectodomain shedding is followed by intramembrane cleavage mediated by γ-secretase, leading to the release of the Notch intracellular domain (NICD). NICD translocates to the nucleus where it associates with the transcription factor RBP-J and the adaptor Mastermind-like to recruit the transcription activation machinery.\textsuperscript{14} Inactivation of Notch2, γ-secretase, or RBP-J in the intermediate mesoderm or in the metanephric mesenchyme before ureteric bud invasion results in complete absence of glomeruli and proximal tubules (PTs), with the RV giving rise to abnormally shaped distal tubules.\textsuperscript{15–17} Notch1 is not able to rescue Notch2 loss and can be removed without consequences from kidneys with functional Notch2. Pharmacologic inhibition of γ-secretase in kidney organ cultures indicated that Notch activity becomes dispensable for nephron morphogenesis after the S-shaped stage.\textsuperscript{17} Finally, when Notch2 deletion is delayed until after nephrogenesis has begun, a sensitized background forms in which the contribution of Notch1 to nephron segmentation is revealed.\textsuperscript{13} These findings demonstrated that canonical Notch signals acting early in the RV specify the proximal nephron fates, and Notch2 contributes the lion share of this signal. However, these experiments do not address whether Notch signaling is required again in nephron differentiation, maintenance, or physiology.

The characterization of mice and humans hints at additional roles for Notch signaling in late stages of kidney development and/or in maintenance of kidney structure and function. Canonical Notch signaling is required for establishing left-right asymmetry,\textsuperscript{18–20} a cilia-dependent process; if Notch activity was to affect cilia formation or function, a phenotype may be observed when Notch signaling is lost during nephron maturation. Mice homozygous for a Notch2 hypomorphic allele develop hypoplastic kidneys that contain aberrantly differentiated glomeruli.\textsuperscript{21} A 2-year-old human infant with Allagille syndrome heterozygous for a Notch2 allele coding for a truncated Notch2 protein developed small kidneys with bilateral cysts.\textsuperscript{22} Renal cyst formation has been associated with several defective cellular processes including increased cell proliferation,\textsuperscript{23} improper convergence of epithelial cells to narrow the tubular diameter,\textsuperscript{3} loss of oriented cell division during the lengthening of nephrons,\textsuperscript{4,24} and loss of cilia-mediated signaling.\textsuperscript{25} Although the cystic kidney phenotype may still reflect defects at the RV to S-shape body (SB) transition, it could also imply that Notch2 has a function after SB formation to prevent cyst formation.

We studied the function of Notch1 during nephron maturation in a sensitized Notch2 background engineered by delaying the inactivation of Notch2 using a BAC Six2-GFP::Cre transgene (described in detail in Surendran et al.\textsuperscript{13}). This transgene is expressed mosaically in the renal epithelial progenitor population,\textsuperscript{13} allowing many progenitor cells to retain the floxed allele. In the induced mesenchyme\textsuperscript{26} and in pretubular aggregates, uniform high levels of Cre expression are detected, leading to complete (>99%) gene inactivation before RV formation.\textsuperscript{13} Because Notch2 expression begins as Cre expression becomes uniform (Supplementary Figure S1), Six2-GFP::Cre\textsuperscript{26};Notch2\textsuperscript{f/f} mice inactivate their Notch2 locus coincident with or after Notch2 mRNA synthesis has begun; the remaining amounts of Notch2 mRNA and protein synthesized before recombination of both Notch2 alleles, together with Notch1, supports normal morphogenesis of over 60% of RV in Six2-GFP::Cre\textsuperscript{26};Notch2\textsuperscript{f/f} kidneys. Although some nephron loss—and visibly smaller kidneys—is detected in 30% of newborn mice, viability, fertility, and fecundity are not affected.\textsuperscript{13} These mice provided a suitable model in which to determine whether there are additional Notch functions during nephrogenesis.

We find that Notch signaling is part of a molecular program that regulates proliferation and restricts the orientation of epithelial cell division relative to the BM during PT morphogenesis. Combined relaxation in these processes leads to epithelial stratification, hyperproliferation, and cyst formation. Consequently, even a partial reduction in Notch signaling results in stratified epithelia and formation of papillary microadenomas. Mice with reduced Notch signaling may model a precursor state of human type 1 papillary renal cell carcinoma (PRCC). Human type 1 PRCC is characterized by elevated levels of Kyoto/FHL1, a splice variant of which is known to act as an inhibitor of the canonical Notch signaling pathway.\textsuperscript{27–29} Indeed, we documented increased expression and nuclear localization of Kyoto/FHL1B in human PRCC samples. Post hoc analysis established that at least one Notch target (Hey1) was reduced, consistent with a role for the canonical Notch signaling pathway in the suppression of type 1 PRCC.
RESULTS

Notch1 and Notch2 Regulate PT Morphogenesis

We previously observed that canonical Notch2 signaling was required for proximal nephron development during a narrow developmental window before the conversion of RVs to SB. 15 Use of the Six2-GFP::Cre transgene, which is expressed mosaically in renal epithelial progenitors and at uniformly high levels coincident with Notch2 expression in the induced mesenchyme (Supplementary Figure S1), allowed for inactivation of all Notch2 floxed alleles in all cells of the SB without a negative effect on survival. The likely perdurance of Notch2 protein synthesized before genetic inactivation, in conjunction with Notch1 signals, supported proximal nephron segment formation. This permitted examination of kidney physiology and morphology in adult mice with renal Notch2 deficiency.

Whereas no cysts were ever observed in the 15 analyzed littermate controls (Figure 1A), 3- to 5-week-old Six2-GFP::Cre N1/2f/f mice revealed a few large renal cysts (Figure 1B). Cysts were evident (albeit at a lower frequency) already in postnatal day 1 (P1) Six2-GFP::Cre N1/2f/f kidneys. We next tested whether loss of Notch1 compromised morphogenesis. We observed that 31% of Six2-GFP::Cre N1/2f/f mice also developed microscopic cysts at P1, and at 3 weeks 38% of these mice had large macroscopic renal cysts (Figure 1C and Table 1). Thus, Notch1 and Notch2 contribute to a similar extent and are both required for the establishment and/or maintenance of the tubular diameter. If Notch1 and Notch2 functioned redundantly to suppress cyst formation, then combined inactivation should result in increased penetrance of the cyst phenotype and in more cysts per individual. We generated Six2-GFP::Cre N1/2f/f; N2/2f/f mice; all P1 pups of this genotype displayed severely impaired nephrogenesis and each contained multiple proximal tubular cysts at birth (Figure 1H). The emergence of a phenotype in single receptor mutants and the increase in penetrance and incidence of cysts per kidney implies that the Notch1 and Notch2 receptors have partially overlapping functions in ensuring normal tubule elongation or morphogenesis.

A role for Notch signaling in patterning the cell fates of the collecting ducts has been recently demonstrated, with hydrenephrosis as a late consequence. The Six2-GFP::Cre activity does not target the duct, avoiding this confounding effect. In both Six2-GFP::Cre N1/2f/f and Six2-GFP::Cre N1/2f/f; N2/2f/f mice, cysts were restricted to the cortex and corticomedullary junctions where proximal nephron segments are found (Figure 1B through E). To conclusively identify the nephron segments from which cysts originated, we stained kidney sections with a collecting duct/principal cell marker aquaporin2 (AQP2), a distal tube/loop of Henle marker chloride channel Kb (CLCKb), a parietal cell marker (Claudin 1), and a PT marker, Lotus teragonolobus lectin (LTL). None of the epithelia lining the cysts expressed AQP2, CLCKb, or Claudin1 (Figure 1, E and F; Supplementary Figure S2), whereas the apical surface of epithelia lining all of the smaller cysts stained with LTL (Figure 1G). However, most epithelial cells lining the larger cysts do not show cross reactivity to any of these markers. These observations confirm that the cysts originated from PTs; cells lining the larger cysts may have lost their mature characteristics as the cysts enlarge.

Given that cystic kidney disease can progress to end-stage renal failure, we next determined whether Notch signaling is required for normal renal function by monitoring blood urea nitrogen (BUN) and creatinine levels. Whereas serum creatinine levels...
were similar in 1-year-old wild-type and Six2-GFP::Cre8; N1f/f littermates (0.3875 ± 0.083 mg/dl, n = 8, versus 0.3 ± 0.141 mg/dl, n = 4), all Notch1-deficient mice displayed increased BUN (42.9 ± 8.5 mg/dl, n = 7) compared with wild-type littermates (26 ± 1.1 mg/dl, n = 5 controls; Figure 1I; P = 0.0015) by 7 weeks of age. BUN remained elevated at 20 weeks in Six2-GFP::Cre8;N1f/f mice (43.5 ± 5.9 mg/dl, n = 10) when compared with wild-type littermates (23.7 ± 3.8 mg/dl, P = 5.3 × 10^{-8} in n = 10 controls; Figure 1I). In contrast, only 47% of Six2-GFP::Cre8;N2f/f mice (7 weeks and older, n = 19) had increased (>30mg/dl) BUN levels (Figure 1I). Thus, the cystic kidney disease in mice lacking Notch1 or Notch2 in renal epithelia does not progress to renal failure by 1 year of age. These observations reveal that Notch1 and 2 contribute equally in preventing cyst formation within Pts, and that even partial inactivation of renal Notch signaling impairs renal tubule morphogenesis.

### Canonical Notch Signaling Pathway Components Are Required for Suppression of PT Cysts

To determine if the adhesive property of Notch was involved in suppression of PT cysts, or whether loss of canonical Notch signals mediated this pathology, we examined Six2-GFP::Cre8;RBP-Jf/f mice. In such mice adhesion will remain, but canonical signaling will be eliminated. Six2-GFP::Cre8;RBP-Jf/f mice died within 2 days after birth and contained kidneys with fewer glomeruli and Pts than Six2-GFP::Cre8;N1f/f; N2f/f mice, consistent with severely impaired nephron segmentation.3 To Pt cysts were observed in only 20% of Six2-GFP::Cre8;RBP-Jf/f mice at P1 (Table 1), and very few cysts were detected per kidney. This result could reflect the stochastic nature of cyst formation in a very small pool of Pt cells or confirm a requirement for adhesion supplied by the Notch proteins present in these kidneys. The Notch1+/− allele (N1+/−)32,33 encodes for a full-length Notch1 protein with a single amino acid substitution in its transmembrane domain that drastically decreases the half-life of NICD1,34,35 rendering this allele inactive in canonical signaling. If Notch contributed adhesion but not signaling, N1+/− proteins should support development of normal Pt diameter. Adding this signaling-deficient but adhesion-competent allele to the Six2-GFP::Cre8;N2f/f mice increased the penetrance of the Pt cyst phenotype from 23% to 73% (Figure 2, A and H, and Table 1), consistent with a requirement for signaling, not adhesion, in the suppression of renal cysts. To examine the possibility that the low frequency of cyst formation in Six2-GFP::Cre8;RBP-Jf/f mice reflected the combined effect of low numbers of Pts, of which only a small fraction are perturbed, we inactivated RBP-J with the RARb2-Cre line. RARb2-Cre8 is less efficient than Six2-GFP::Cre8 since RARb2-Cre8; RBP-Jf/f mice survive for 2 weeks after birth (in rare instances, 4 weeks) because of improved nephron formation rates. Consistent with less efficient RBP-J inactivation in the progenitor pool, we detected more RBP-J protein in neural-cell-adhesion-molecule-positive, mesenchymal cap condensate cells surrounding the ureteric bud in RARb2-Cre8; RBP-Jf/f kidneys at P1 compared with the Six2-GFP::Cre8;RBP-Jf/f kidneys (yellow arrowheads in Figure 2D versus Figure 2C). RBP-J protein was not detected in Pts (Figure 2F), indicating that complete inactivation of the floxed alleles was achieved before expression of LTL. Importantly, the increase in Pts coincided with an increased penetrance (100%) and incidence of cysts at P1 in RARb2-Cre8; RBP-Jf/f kidneys (Figure 2, B and H; Table 1), suggesting dependence on signaling and not adhesion. In the few RARb2-Cre8; RBP-Jf/f mice (n = 3) that survived to weaning, the cysts had enlarged to occupy most of the kidney (Figure 2G; note that RARb2-Cre did not delete RBP-J in the duct but did remove it in the stroma, Figure 2D). Combined, our results indicate a function for canonical Notch signaling (RBP-J-dependent) in the suppression of Pt cysts, most likely through the activation of unknown transcriptional targets. The frequency of cyst formation within kindreds correlated with the increased loss of canonical Notch signaling (Figure 2H).

### Increased PT Epithelial Cell Proliferation and Epithelial Stratification Preceded Cyst Formation in Notch-Signaling-Deficient Pts

To understand the cellular basis of cyst formation, we analyzed Notch-signaling-pathway-deficient kidneys at various time points by light and electron microscopy. Whereas simple cuboidal epithelia with prominent apical brush borders form the normal Pt (apical periodic acid–Schiff staining, Figure 3A; red arrow in Figure 3B), Pt cysts contain cells with a flattened

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**Table 1. The penetrance of the cystic phenotype correlates with the extent of reduction in canonical Notch signaling**

<table>
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<th>Genes</th>
<th>+/+</th>
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<td>Percent of pups with cyst at birth (# of pups)</td>
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<td>3 to 5 weeks</td>
<td>63% (8)</td>
<td>38% (8)</td>
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<td>NS</td>
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<td>40 to 52 weeks</td>
<td>60% (10)</td>
<td>50% (18)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
<td>100% (3)</td>
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NS, no survivors; ND, not determined; +, wild-type allele; f, floxed allele.

*The frequency of cyst formation is presented as a percentage, and the number of mice analyzed per time point for each genotyping is indicated within parentheses. No cysts were seen in the wild-type littermates. All floxed alleles were inactivated using the Six2-Cre line. Wild-type littermates and those of the genotypes Six2-Cre;N2+/−, N2+/+, N2f/f, and N1f/f had no cyst at birth (0 of 13), after 3 to 5 weeks (0 of 15), or after 42 to 52 weeks (0 of 20)."
and elongated appearance and without brush borders (black arrows in Figure 3B’). The focal nature of improper differentiation or dedifferentiation (loss or lack of brush borders) was confirmed by electron microscopy (EM) (Figure 3E, inset). Such cells are not seen in any wild-type kidneys or in Notch-signaling-deficient PTs outside of the cysts (Supplementary Figure S3). In addition, many vacuolated cells are seen in some cysts (Figure 3F); again, no such cells were seen outside of the cysts.

We noticed that almost all randomly sampled Notch-signaling-deficient cysts (29 of 30 seen in four mice) had at least one area of densely packed cells containing stratified epithelia (inset in Figure 1D; black arrowhead in Figure 3C). Some appeared as polyp-like structures within the larger cysts (black arrowhead in Figure 3D). The cells within these polyp-like structures have nuclei that are rounded and appear to have small and clear cytoplasm. Epithelial stratification was never detected in wild-type controls (Supplementary Figure S3, A, D, and G). To determine whether stratification also could be detected before cyst formation, we performed ultrastructural analysis of P1 kidneys and compared the anatomy of PTs from Six2-GFP::CreF;RBP-Jf/f and RARb2-CreF;RBP-Jf/f with wild-type littermate PTs. Wild-type PTs (Supplementary Figure S3, A, D, and G) and normal but Notch-deficient PTs (Supplementary Figure S3, B) contained polarized cuboidal cells with basal contacts to the BM and apical surfaces exposed to the tubular lumen. In contrast, some precystic Notch-deficient PTs contain foci of epithelial cells that have lost contact with the BM, whereas others had apical surfaces covered entirely by their neighbors (arrowheads in Figure 3, G and H; asterisk in Supplementary Figure S3, C, E, F, and H through J). These foci were seen before enlargement of tubular diameter (Figure 3, G and H; Supplementary Figure S3, C, E, and F) and remained identifiable within most cysts (Figure 3D; asterisks in Supplementary Figure S3, H through J).

Stratification could be a result of hyperplasia. To ask if an increase in cell division occurred in the absence of Notch signaling, we determined the number of mitotic cells before cyst formation. To ensure we analyzed animals that will develop cysts, we confined the analysis to Six2-GFP::CreF;N1+/−;N2f/f mice and their
Before overt enlargement of tubular diameter, epithelial stratification results in the formation of polyp-like structures.5 Although TCF2/HNF1β is downregulated in some cyst-lining epithelia in Notch-deficient kidneys (box 1, Figure 4C), TCF2/HNF1β was expressed in all normal-diameter PTs (box 2, Figure 4C) and in some cyst-lining epithelia (box 3, Figure 4C). TCF2/HNF1β is also present in some stratified, undifferentiated epithelia that lack LTL reactivity (box 4, Figure 4C). Thus, reduced TCF2/HNF1β expression does not correlate with Notch-deficiency-dependent epithelial stratification and cyst formation.

**Notch Signaling Is Required in Epithelial Cells to Restrict Mitotic Spindle Orientation Parallel to the BM, Which May Be How Notch Signaling Prevents Epithelial Stratification**

Maintaining the diameter of renal tubules requires first convergent-extension movement8 followed by alignment of the mitotic spindles parallel to the long axis of the nephron (x) and control littermates at P1. We analyzed only morphologically normal PTs for the presence of phospho-ser10-histone H3 (pS10H3). Although many mitotic cells are present in the nephrogenic zone of all kidneys (Figure 3I), there is an approximately 2.5-fold increase in the number of mitotic cells within morphologically normal PTs of Six2-GFP::Creαβ;N1+/f/N2f/f kidneys at P1 when compared with that of wild-type littermate kidneys (Figure 3, I through J; P = 0.0064). Thus, increased proliferation precedes cyst formation and could be detected in otherwise normal PTs. A similar trend was also seen in other genotypes (data not shown).
spindle orientation relative to the BM in a cultured renal epithelial cell line. For this we measured the orientation of the mitotic spindles in MDCK cells relative to the plated surface. Previous studies have reported that the metaphase spindles of MDCK cells are almost always oriented parallel (within 10°) to the plated surface, resulting in a division plane perpendicular to the plate and maintenance of a monolayer. In control MDCK cells treated with DMSO, the angle between the plane of the spindles/spindle poles and the plate surface was <10° in 94% of cells in late metaphase and early anaphase (n = 50; Figure 5, A and C). In contrast, inhibition of the Notch signaling pathway using the γ-secretase inhibitor (GSI) DAPT reduced the fraction of cells dividing parallel to the BM to 58% (n = 50). Whereas none of the control cells had a mitotic angle >25°, 8% of GSI-treated cells had a mitotic angle of >25° (Figure 5, B and C).

To confirm in vivo that Notch deficiency resulted in altered mitotic spindle orientation within PTs, we analyzed P1 kidneys of Six2-GFP::CreRb2-Cregr;RBP-Jf/f mice and their littermates. pS10H3 staining was used to identify anaphase and telophase chromosomes and determine the plane in which the two spindle poles are positioned (this plane is perpendicular to the final division plane; see schematic in Figure 5D). Whereas the angles between the mitotic spindle plane and the apical surface or the BM were always <30° (and hence approximately parallel to the BM; n = 16) in control littermate kidneys, 47% of the PT epithelial cell divisions in Six2-GFP::CreRb2-Cregr;RBP-Jf/f kidneys had a mitotic angle >60° (n = 17) and 23% were approximately perpendicular (>80°) to the BM. This will result in a division plane parallel to the BM and stratification (Figure 5, D and E).

The occurrence of mitotic spindle planes perpendicular to the BM before cyst formation in Notch-deficient PT epithelia indicates that Notch1 and Notch2 are components of a molecular program that restrict mitotic spindles to a plane parallel to the BM. Randomized spindle orientation coupled with a mild increase in proliferation rates in cells lacking Notch signaling could account for stochastic stratification; stenosis; and, secondarily, the increased diameter and cyst formation in Notch-signaling-deficient kidneys.

**Figure 4.** The mechanism driving epithelial stratification and cyst formation is not loss of apical-basal polarity or reduced TCF2/HNF1β expression. (A) Na+/K+-ATPase is expressed in the basolateral membrane of PT epithelia in P1 wild-type (RBP-Jf/f) littermates. (B) RBP-J deficient PT epithelia lining the cysts retain basolateral expression of Na+/K+-ATPase, although some regions (marked by asterisks) show a reduction in Na+/K+-ATPase expression. Inset in B shows a magnified view of the cyst-lining epithelium in which the arrowhead points to a stratified epithelium that retains polarized expression of Na+/K+-ATPase. (C) Kidneys from P1 Rarb2-Cregr;RBP-Jf/f mice were stained for TCF2/HNF1β (red). Although TCF2/HNF1β is downregulated in some cyst-lining epithelia in Notch-deficient kidneys (box 1), TCF2/HNF1β was expressed in all normal diameter PTs (box 2) and in some cyst-lining epithelia (box 3) containing stratified epithelia (arrowhead in box 4). (D) E-cadherin expression is restricted to the basolateral membrane, with a strong punctate staining at the adherence junctions of PT cells that express megalin in the apical membrane of wild-type kidneys. The inset shows magnified PTs with an asterisk in the lumen of a PT composed of epithelia that express megalin in the apical membrane of wild-type kidneys. The inset shows magnified PTs with an asterisk in the lumen of a PT composed of epithelia that express megalin in the apical membrane of wild-type kidneys. The inset shows magnified PTs with an asterisk in the lumen of a PT composed of epithelia that express megalin in the apical membrane of wild-type kidneys. The inset shows magnified PTs with an asterisk in the lumen of a PT composed of epithelia that express megalin in the apical membrane of wild-type kidneys. The inset shows magnified PTs with an asterisk in the lumen of a PT composed of epithelia that express megalin in the apical membrane of wild-type kidneys. The inset shows magnified PTs with an asterisk in the lumen of a PT composed of epithelia that express megalin in the apical membrane of wild-type kidneys.

Maintaining the tubule as an epithelial monolayer requires alignment of epithelial mitotic spindles parallel to the BM along a third axis (z; Supplemental Figure S1E). The occurrence of epithelial stratification and renal cysts in Six2-GFP::CreRb2-Cregr;N2f/f mice within the first 5 weeks of age may indicate that the mechanism maintaining cell divisions parallel to the BM and/or parallel to the length of the nephron is compromised in the absence of Notch. We posited that the mechanism underlying cyst formation in Notch mutants reflects a combination of relaxed control over mitotic spindle orientation in at least one axis coupled with enhanced proliferation.

Because the convolution of the postnatal PT makes it difficult to accurately measure orientation of cell division along the length of the PT, we focused first on determining whether the Notch signaling pathway contributes to regulation of mitotic spindle orientation relative to the BM in a cultured renal epithelial cell line. For this we measured the orientation of the mitotic spindles in MDCK cells relative to the plated surface. Previous studies have reported that the metaphase spindles of MDCK cells are almost always oriented parallel (within 10°) to the plated surface, resulting in a division plane perpendicular to the plate and maintenance of a monolayer. In control MDCK cells treated with DMSO, the angle between the plane of the spindles/spindle poles and the plate surface was <10° in 94% of cells in late metaphase and early anaphase (n = 50; Figure 5, A and C). In contrast, inhibition of the Notch signaling pathway using the γ-secretase inhibitor (GSI) DAPT reduced the fraction of cells dividing parallel to the BM to 58% (n = 50). Whereas none of the control cells had a mitotic angle >25°, 8% of GSI-treated cells had a mitotic angle of >25° (Figure 5, B and C).

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**Notch-Deficient Cyst-Lining Epithelia Have Reduced Expression of p21, Detected before Forming Papillary Microadenomas Containing Proliferating Cells**

Next, we examined the connection of Notch with the cell cycle in the PT. The loss of Notch signaling could be sufficient to trigger proliferation; alternatively, Notch signaling may only be required to promote exit from the cell cycle during...
nephron maturation. If loss of Notch signaling could trigger proliferation, and this was sufficient to drive cyst formation, we would expect a progressive increase in the number of cysts as the mice age. To begin to differentiate between these possibilities, we compared 5- and 52-week-old Six2-GFP::Cre;N1f/f kidneys to determine if more renal cysts formed with time. The penetration and incidence of renal cysts in 5- and 52-week-old Notch2-deficient kidneys were indistinguishable, suggesting that all cysts formed early in life and that new cysts were not added with age. Similar results were obtained in Six2-GFP::Cre;N1f/f kidneys (Table 1). These observations are consistent with Notch signaling contributing to the exit from the cell cycle at the end of nephron lengthening rather than acting in the maintenance of a steady state in mature renal PT epithelia.

The 21-kD protein p21<sup>wAF1/Cip1</sup> (p21) is a cyclin-dependent kinase inhibitor and a direct target of Notch1 in keratinocytes<sup>38</sup>; its down-regulation in human autosomal dominant polycystic kidney disease<sup>39</sup> and in murine polycystic kidneys is caused by mutations in <i>pkd1</i>.<sup>23</sup> For these reasons we examined the expression of p21 in Notch-signaling-deficient and control littermate kidneys. PT epithelia contained p21 in RBP-J<sup>f/f</sup> and noncystic PTs of RARb2-Cre<sup>e8</sup>;RBP-J<sup>f/f</sup> mice (Figure 6A and data not shown). In contrast, cyst-lining epithelial cells showed little or no nuclear p21 staining (Figure 6B, arrowheads). We confirmed that global reduction in p21 expression is secondary to cyst formation and not a direct consequence of Notch loss. Nonetheless, reduced p21 could facilitate hyperplasia in the cyst-lining epithelia of Notch-signaling-deficient kidneys using quantitative reverse transcription PCR (<i>n</i> = 4 per group, <i>P</i> = 0.12). In contrast, the PT precursor marker cadherin6<sup>19</sup> was reduced approximately 3-fold in the same sample (<i>P</i> < 0.045, Figure 6C). These observations are consistent with a model in which loss of p21 expression is secondary to cyst formation and not a direct consequence of Notch loss. Nonetheless, reduced p21 could facilitate hyperplasia in the cyst-lining epithelia of Notch-signaling-deficient kidneys.

### Figure 5

Notch signaling is required in epithelial cells to restrict mitotic spindle orientation parallel to the BM. (A, B) The restriction of the mitotic spindle orientation in MDCK cells requires the Notch signaling mediator, γ-secretase. Staining for γ-tubulin (red), β-tubulin (green), and Hoechst (blue) are shown for representative cells. Images of z-stack sections taken 0.5 μm apart of (A) DMSO- or (B) DAPT-treated MDCK cells were used to measure the mitotic angle. (C) The histogram tabulates the distribution of the mitotic angles in DMSO (<i>n</i> = 50) and DAPT (<i>n</i> = 50) late metaphase or early anaphase cells, with the x-axis showing increments of 5°. F-test was performed to test the null hypothesis that the variation in the spindle angles between the DMSO-treated versus DAPT-treated cells is the same. Because <i>P</i> < 0.0001, we reject the null hypothesis; the variation in spindle angle is significantly different between cells treated with DAPT versus DMSO. (D) Schematic of dividing cells at the bottom of the panel illustrates that the mitotic spindle plane (MSP; red line) is perpendicular to the cell division plane. Hence, a cell that has an MSP perpendicular to the BM (tan cell, blue lines) will result in a cell division plane parallel to the BM and in epithelial stratification, whereas a cell that has a MSP parallel to the BM (gray) will give rise to daughter cells that remain attached to the BM and within the epithelial monolayer. Above, representative images of pSer10H3- and LTL-stained Six2-GFP::Cre<sup>e8</sup>;N1<sup>f/f</sup>;N2<sup>f/f</sup> and wild-type control littermate P1 kidneys. Kidney sections from five mice per genotype were analyzed, and a total of 33 mitotic angles were measured. The left panels depict division planes of normal PT epithelial cells: the MSP is parallel to BM and/or apical surface. The asterisks mark the lumen of the nephrons. (E) A histogram tabulating the distribution of the mitotic angles in wild-type (<i>n</i> = 16) and Six2-GFP::Cre<sup>e8</sup>;N1<sup>f/f</sup>;N2<sup>f/f</sup> (Notch-deficient; <i>n</i> = 17) anaphase and telophase cells, with the x-axis showing increments of 30°. The Mann–Whitney U test was performed to determine that the median mitotic angles were significantly different (<i>P</i> = 0.038). All scale bars are 10 μm.
Ki-67 served in 3 of 6 Six2-GFP::Cre\textsuperscript{tg}/H11001 microadenomas still contained proliferating cells in perivascular regions (Supplementary Figure S4, A and B), were quiescent and proliferating cells were located in the micropolyps containing stratified epithelia in the cysts of 5-week-old mice (D, arrowhead), which are Ki-67 positive (G, arrowhead). By 52 weeks of age, these mice contained epithelial outgrowths into the lumen of the cysts, termed microadenomas (E and F, which is a higher magnification of the boxed area in E). The epithelial cells within the microadenomas are positive for (H) Ki-67 and (I) pS10H3, an indication that they have failed to exit the cell cycle. All scale bars are 100 μm.

multiple layers of relatively undifferentiated, proliferating cells (Figure 6, D and G). At 52 weeks, the renal cyst-lining stratified epithelium had frequently assembled into finger-like projections that protruded into the lumen, termed papillary microadenomas (Figure 6, E and F). In stark contrast to 52-week-old N2\textsuperscript{ff} littermates, in which most renal epithelium were quiescent and proliferating cells were located in the perivascular regions (Supplementary Figure S4, A and B), these microadenomas still contained proliferating cells (Ki67\textsuperscript{+}; Figure 6H; pS10H3\textsuperscript{+}; Figure 6I). This was observed in 3 of 6 Six2-GFP::Cre\textsuperscript{E8}; N2\textsuperscript{ff} mice with cystic kidneys (50%) and in 2 of 9 Six2-GFP::Cre\textsuperscript{E8}; N1\textsuperscript{ff} cystic kidneys (22%). To determine whether these adenomas progressed to tumors we analyzed the kidneys of five 18-month-old Six2-GFP::Cre\textsuperscript{E8}; N2\textsuperscript{ff} or Six2-GFP::Cre\textsuperscript{E8}; N1\textsuperscript{ff} mice. None had tumors (data not shown).

Renal papillary adenomas are considered to be precursors to PRCCs in humans,\textsuperscript{40} which prompted us to examine the literature describing molecular features of human PRCCs. Strikingly, the second most highly elevated transcript in human molecular class 1 PRCCs is \textit{four and a half LIM domains 1} (\textit{FHL1}),\textsuperscript{27} which codes for a protein thought to inhibit tumor formation.\textsuperscript{41} The splice forms \textit{FHL1B} and \textit{FHL1C} (also known as \textit{KyoT3} and \textit{KyoT2}, respectively) are potent inhibitors of canonical Notch signaling.\textsuperscript{28,29} Because only these two splice variants produce nuclear, Notch-inhibitory FHL1 proteins, we examined the subcellular localization of FHL1B/KyoT3 in papillary growths of six human PRCC samples. We first stained for cytokeratin7 (krt7), which is upregulated in molecular class 1 PRCCs and not in molecular class 2 PRCCs.\textsuperscript{27} In adjacent sections we determined that KyoT3 was expressed and it localized to the nucleus in most cells of four krt7-positive class 1 PRCCs but not in two PRCC samples with weak or no krt7 expression or in normal kidney samples (Figure 7). To seek evidence of reduced Notch signaling activity, we reanalyzed the array data deposited by Yang \textit{et al.}\textsuperscript{27} in the GEO database (GSE2748; http://www.ncbi.nlm.nih.gov/geo/gds/gds_browse.cgi?gds = 1344) by clustering the PRCC molecular class 1 (krt7, FHL1 positive) with the histologic type 1 and 2A and performing a two tailed \textit{t} test analysis against the PRCC molecular class 2 and histologic type 2B. We scanned the list of altered transcripts (at \textit{P} < 0.05) for known Notch targets and identified Hey1. Aligning the FHL1 and Hey1 profiles for the 34 human PRCC samples clearly demonstrates a striking correlation between elevated FHL1 and reduced Hey1 expression (Supplementary Figure S4), consistent with reduced Notch signaling in human class 1 PRCCs. This \textit{post hoc} analysis offers an obvious mechanistic explanation for the emergence of class 1 PRCCs in humans: Similar to the microadenoma formation we observe in Notch-deficient PTs, human type 1 PRCCs may arise because of reduced Notch signaling, which, in turn, results in renal epithelial hyperproliferation and failure of renal epithelia to exit the cell cycle. However, we did not observe expression of krt7 in Notch-deficient mouse kidneys with microadenomas (arrowheads, Supplementary Figure S4D); krt7...
DISCUSSION

During PT Morphogenesis the Canonical Notch Signaling Coordinates the Maintenance of Epithelia in a Monolayer with Epithelial Proliferation

Epithelial characteristics such as cell-extracellular matrix adhesion, cell-cell adhesion, and apical-basal polarity are acquired early in the developing nephron and need to be maintained during tubule elongation and morphogenesis for the development of functional nephrons. Our studies reveal that the canonical Notch signaling pathway is required for maintaining the alignment of a division plane perpendicular to the BM in the renal PTs during morphogenesis. This new function of Notch signaling in renal tubule morphogenesis is required to develop a consistent PT diameter and to maintain a polarized epithelial monolayer during elongation, a process involving epithelial cell proliferation. In the absence of Notch1 or Notch2, PT epithelia exit the cell cycle less frequently. Because most Notch-signaling-deficient epithelial cells lining the cysts remain in a monolayer, we conclude that a partially randomized spindle orientation relative to the BM coupled with increased numbers of mitotic events in the absence of Notch signaling allows some cells to stratify. Subsequently, hyperproliferation will be sustained, leading to enlarged tubular diameter. Alternatively, stratified foci will form that could result in the partial obstruction of tubular segments where the stratification occurs, which would then trigger stochastic enlargement of the tubular diameter.

The Notch-deficient renal epithelia form papillary microadenomas, which we hypothesize to be a precursor state for PRCCs. In support of this hypothesis, human type 1 PRCC is characterized by elevated levels of KyoT/FHL1, a splice variant of which can act as an inhibitor of the canonical Notch signaling pathway. Indeed, we observed increased expression of KyoT3/FHL1B in human PRCC samples, and post hoc analysis identified an inverse correlation between Notch target genes and KyoT3 expression. In further support of this hypothesis, a recent report determined that Notch1 and Notch4 were expressed in normal tissue but their expression was greatly reduced in human renal cell carcinoma tissues. Thus, it appears that the canonical Notch signaling pathway may actively suppress intracystic papillary microadenoma formation in mice and renal cell carcinoma in humans. Further studies will be required to determine whether Hey1 or any other genes regulated by the Notch pathway in normal renal epithelia act as tumor suppressors in the renal epithelia.

Additional Somatic Mutations within the Notch-Deficient Stratified Epithelial Cells May Be Necessary for Progression to Renal Carcinoma

Some Notch-deficient PT epithelia do not fully differentiate, but instead remain in a proliferating phase to form microad-
enomas. Because we observed that the frequency of microdenoma formation is lower than that observed for epithelial stratification and cyst formation, it is possible that microdenoma formation reflects the acquisition of additional somatic mutations in Notch-deficient stratified epithelial cells within polyps. Mutations in any number of genes, in combination with Notch deficiency, may suffice to drive stratified epithelial cells to transform into renal carcinomas. In this context, it is interesting to note the similarities between kidneys with reduced Notch signaling activity and kidneys with tuberous sclerosis complex (tsc) gene mutations. One commonality is adenoma formation within renal cysts that may progress to renal cell carcinoma. Another similarity is the loss of control over mitotic spindle orientation in the renal epithelia lacking tsc1, tsc2, or Notch signaling capacity. These similarities raise the speculative possibility that if some Notch-deficient renal epithelia acquire mutations that partially inactivate the functions of tsc gene products this may transform the stratified epithelia into adenomas, which could possibly further progress to renal cell carcinoma.

Whereas Too Little Notch Signaling Results in Abnormal Renal Physiology, Too Much Notch Signaling Can Result in Renal Pathology

Delayed inactivation of Notch1 or Notch2 in all of the nephron segments but the collecting ducts leads to volume contraction as indicated by elevated BUN without an increase in serum creatinine. An even more severe compromise in renal function is observed when loss of Notch signaling occurs in the collecting duct. In contrast, constitutive activation of Notch signaling is observed when loss of Notch signaling occurs in the collecting duct. In this context, it is interesting to note the similarities between kidneys with reduced Notch signaling activity and kidneys with tuberous sclerosis complex (tsc) gene mutations. One commonality is adenoma formation within renal cysts that may progress to renal cell carcinoma. Another similarity is the loss of control over mitotic spindle orientation in the renal epithelia lacking tsc1, tsc2, or Notch signaling capacity. These similarities raise the speculative possibility that if some Notch-deficient renal epithelia acquire mutations that partially inactivate the functions of tsc gene products this may transform the stratified epithelia into adenomas, which could possibly further progress to renal cell carcinoma.

Histology and Immunohistochemistry

For all immunohistochemistry, kidneys were fixed overnight in Bouin’s fixative at 4°C (except 4% paraformaldehyde was used for detection of RBP-J), washed, and stored in 70% ethanol before embedding in paraffin and sectioning at 5- to 7-μm thickness. Before immunostaining, the sections were deparaffinized, boiled for 20 minutes in Trilogy (Cell Marque) or antigen unmasking solution (Vector Labs H-3300 for detecting RBP-J), and cooled at room temperature (RT) for 20 minutes. Detection of RBP-J required quenching of endogenous hydrogen peroxidase activity with 3% hydrogen peroxide treatment for 10 minutes at RT. Sections were then blocked in PBS containing 1% BSA, 0.2% powdered skim milk, and 0.3% Triton X-100 for 30 minutes at RT before incubation in primary antibody overnight in a humidified chamber at 4°C. For anti-RBP-J staining (Cosmo Bio Co., SIM-2ZRBP2, 1:100), the sections were additionally blocked with avidin and then biotin (Vector Labs SP-2001) before incubation with secondary antibody conjugated with biotin (1:500), then incubated with avidin and then biotin (Vector Labs SP-2001) before incubation with primary antibody. Tissue sections were then incubated with secondary antibody conjugated with biotin (1:500), then developed using the ABC Vectastain Kit (Vector Labs, PK-6100) followed by tyramide amplification (PerkinElmer, NEL744001KT).

Additional primary antibodies and lectins utilized in the study include p21 (1:200; Santa Cruz SC-6246), neural cell adhesion molecule (1:200; Sigma), E-cadherin (1:400; BD Transduction Labs C20820), β-catenin (1:200, BD Transduction Labs), WT-1 (1:100; Santa Cruz Sc-192), CLCK (1:100; Alomone labs ACL-004), AQP2 (1:500; gift from Dr. Mark Knepper), Na+/K+-ATPase α-1 (1:500; Upstate 05-369), megalin (1:1000; gift from Marilyn Farquhar), Claudin 1 (1:100, Invitrogen), TCF2/HNF1β (1:400; Santa Cruz SC-22840 and gift from Dr. Marco Pontoglio), acetylated-tubulin (1:500; Sigma T6793), FITC-conjugated LTL (1:200; Vector Labs), Ki-67 (1:200; Novocastra NCL-Ki67p), and pS10H3 (1:500; Upstate 05 to 817). Secondary antibodies conjugated with Cy3 (Jackson ImmunoResearch) or Alexa488 (Molecular Probes) were utilized to visualize the binding pattern of primary antibodies.

For determination of cyst frequency, at least ten sections were examined per kidney that were stained with hematoxylin and eosin, periodic acid–Schiff, or FITC-conjugated LTL (see Table 1 for the number of animals analyzed per genotype). For quantification of pS10H3-positive proximal tubular epithelia, three midsections of kidneys from five (N1+/−;N2+/−) and six (2-GFP::Cre+/−; N1+/−;N2+/−) mice per genotype were analyzed. Fields (10×) covering the entire section were photographed and the number of pS10H3- and LTL-positive

CONCISE METHODS

Mice

All experiments involving mice were approved by the Washington University Institutional Animal Care and Use Committee. The Six2-GFP::Cre+/− transgenic mice were bred with mice with floxed alleles of RBP-J (RBP-J flox/flox), Notch1 (N1+/−), Notch2 (N2+/−), both N1f/f;N2f/f, or mice with a null allele and a floxed allele of Notch1 (N1Δf/f). Compound heterozygotes were bred with mice homozygous for the floxed allele of the desired gene. The Rarb2-Cre+/− transgenic mice were bred with RBP-J flox/flox mice. RBP-J−/− mice. All mice used in this study were maintained on mixed backgrounds. Mice and embryos were genotyped following the universal PCR genotyping protocol (primer sequences are available upon request), and their age was estimated based on the assumption that the day on which a vaginal plug was observed was embryonic day 0.5. Blood was collected by cardiac puncture at the time of tissue harvest and analysis of serum BUN and creatinine levels was done by the O’Brien Center renal chemistry core and presented as milligrams per deciliter.
cells per LTL-positive tubule cross section was determined for each kidney. This was then multiplied by 1000 to calculate the number of mitotic proximal tubular epithelial cells per 1000 proximal tubular cross sections for each kidney and the results are presented as the average ± SD for each genotype.

**Human PRCC**

PRCC samples were obtained through the O’Brien center IRB07-0430. A total of six samples were analyzed for cytokeratin7 and FHL1B expression. Cytokeratin7 was detected using anti-CK7 (Ventana, 760-2224) and anti-FHL1B was a gift from Dr. Mary Waye.

**Measurement of Mitotic Spindle Orientation**

MDCK cells were grown in Eagle’s minimum essential medium (ATTC #20-2003) containing 10% heat-inactivated FBS at 37°C with 5% carbon dioxide. The mitotic spindle orientation (the angle between the plane containing the spindle poles and the plane of fibronectin-coated surface) was in essence measured and calculated as described by Toyoshima and Nishida. In brief, for measurement of mitotic spindle orientation in late metaphase and early anaphase, the cells were plated on fibronectin-coated coverslips (BD Biosciences, 354088) and cell cycle synchronized by double-thymidine block. The cells were released from the block by washing them in fresh media and incubating them for an additional 5 hours. For inhibition of Notch signaling, 10 μm of DAPT was added to media during the second thymidine block and replenished after the release from the block. The cells were washed in PBS and fixed in 4% paraformaldehyde for 5 minutes at 37°C before incubation with cold methanol at −20°C for 20 minutes. The cells were blocked with 3% BSA in PBS for 1 hour before incubation with anti-γ-tubulin conjugated with cy3 (Sigma) and β-tubulin conjugated with FITC (Sigma). The DNA was stained with Hoechst before acquiring 0.5-μm thick z-stack images of cells in late metaphase or early anaphase. The linear (shortest, x) distance between the two spindle poles and the vertical distance (y) were measured from the z-stack images. Then the trigonometric function \( \sin^{-1}(y/x) \) was used to calculate the spindle angle relative to the plated surface.

For mitotic angle measurements in vivo, kidney sections were stained with pS10H3 and LTL. Images (10×) were utilized to locate PT epithelia that were pS10H3 positive. Higher magnification images were taken to identify anaphase and telophase PT epithelia in which the apical and/or BM orientation was unambiguous. A line was drawn parallel to the plane of the apical surface and BM. A second line was drawn parallel to the separating pS10H3-stained chromosomes in the plane in which the two mitotic spindles were located. The angle formed between these two lines was determined to be the mitotic angle. The Mann–Whitney U test was performed to determine whether the mitotic angles were significantly different.

**Quantitative PCR**

Total RNA was isolated from P1 mouse kidneys using the RNase Mini-Kit (Qiagen) and reverse transcribed using random hexamers and the SuperScript RT II Kit (Invitrogen). The cDNA was amplified with SYBR Green mix (Applied Biosystems), and gene-specific exon-exon junction-spanning primers, using the ABI7700 sequence detection system (Applied Biosystems). Primer sequences are available upon request. Dissociation curves were analyzed to ensure that one PCR product was amplified. Standard curves were made using cDNA reverse transcribed from wild-type P1 kidneys. The samples were measured in triplicate and gene expression levels were normalized with hypoxanthine phosphoribosyltransferase 1. One unit of expression was set as the average, normalized level of expression in wild-type kidneys.

**Statistical Analysis**

All results are presented as mean ± SD. In the graphs, the height of the bar represents the mean and the error bars represent SD. Two-tailed, unpaired t tests were performed to compare mice with Notch-deficient kidneys with their wild-type littermates. The resulting \( P \) values are mentioned in the text and figure legends.

**EM**

The EM work was done with the assistance of Howard Wynder in the EM core of the Department of Developmental Biology, Jeanette Cunningham in the O’Brien EM facility, and Marilyn Levy in the EM core of the Department of Cell Biology and Physiology. For EM, P1 kidneys were fixed in 2.5% glutaraldehyde-buffered in 0.1 M sodium cacodylate, rinsed in buffer, treated with 1.25% osmium tetroxide, rinsed, dehydrated, stained in uranyl acetate, and then embedded in epoxy plastic (Polybed 812) before thin sections were cut on a Reichert-Jung Ultra-Cut. Sections were poststained in 4% uranyl acetate and lead citrate and viewed on a Zeiss 902 electron microscope. Photographs were recorded with Kodak EM film and then scanned. Kidneys from two mice per genotype were analyzed.

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**DISCLOSURES**

None.

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