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Loss of *Zeb2* in mesenchyme-derived nephrons causes primary glomerulocystic disease

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Abstract

Primary glomerulocystic kidney disease is a special form of renal cystic disorder characterized by Bowman's space dilatation in the absence of tubular cysts. ZEB2 is a SMAD-interacting transcription factor involved in Mowat-Wilson syndrome, a congenital disorder with an increased risk for kidney anomalies. Here we show that deletion of *Zeb2* in mesenchyme-derived nephrons with either *Pax2-cre* or *Six2-cre* causes primary glomerulocystic kidney disease without tubular cysts in mice. Glomerulotubular junction analysis revealed many atubular glomeruli in the kidneys of *Zeb2* knockout mice, which explains the presence of glomerular cysts in the absence of tubular dilatation. Gene expression analysis showed decreased expression of early proximal tubular markers in the kidneys of *Zeb2* knockout mice preceding glomerular cyst formation, suggesting that defects in proximal tubule development during early nephrogenesis contribute to the formation of congenital atubular glomeruli. At the molecular level, *Zeb2* deletion caused aberrant expression of *Pkd1*, *Hnf1β,* and *Glis3*, three genes causing glomerular cysts. Thus, *Zeb2* regulates the morphogenesis of mesenchyme-derived nephrons and is required for proximal tubule development and glomerulotubular junction formation. Our findings also suggest that *ZEB2* might be a novel disease gene in patients with primary glomerular cystic disease.

Keywords

kidney development; obstructive nephropathy; pediatric nephrology; proximal tubule; ADPKD

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DISCLOSURE

All the authors declared no competing interests

INTRODUCTION

Cystic kidney disease is a group of genetically heterogeneous disorders that are characterized mainly by renal tubular dilatation.[1,2] Glomerulocystic kidney disease (GCKD) is a special form of cystic kidney disease that confines cystic dilatation to the Bowman's space.[3–5] GCKD is defined as two- to three-fold dilatation of Bowman's space in more than 5% of identifiable glomeruli in the plane of a kidney section, and the glomerular cysts in primary GCKD are mainly localized to the subcapsular region of the kidney.[4–6] Glomerular cysts in association with tubular cysts can be found in several syndromes including tuberous sclerosis complex, orofaciodigital syndrome 1, and Meckel-Gruber syndrome.[3,7–9] Glomerular cysts can also be found in patients with mutations in the *UMOD* and *HNF1β* genes,[10,11] and in patients of autosomal dominant polycystic kidney disease with *PKD1* mutations, other ciliopathies such as nephronophthisis, multicystic dysplastic kidney or urinary tract obstruction.[3] However, the genetic basis of primary glomerulocystic kidney disease without tubular dilatation remains largely unknown.

ZEB2 is a zinc finger E-box binding homeobox transcription factor that promotes epithelial-mesenchymal transition via a TGF-β/SMAD/ZEB/miR-200 signaling network.[12–14] *ZEB2* mutations in human cause Mowat-Wilson syndrome (MWS: OMIM 235730), a congenital disorder characterized by intellectual disability, craniofacial abnormalities, Hirschsprung's disease, congenital heart defects, and an increased risk for congenital kidney anomalies.[15] However, the pathological features of its kidney defects have not been examined and defined. [15,16] *ZEB2* is also a known target for microRNA miR-200.[17] Recently, it has been shown that upregulation of ZEB2 in *Hnf1b* knockout mice and downregulation of miR-200 in Dicer knockout mice are associated with glomerular and tubular cysts.[18,19] However, no direct link between ZEB2 downregulation and renal cystic disease has been established and the pathological effect of ZEB2 downregulation in early nephrogenesis remains unknown.

Here we report that deletion of *Zeb2* in early mouse nephrogenesis with either *Pax2-cre* or *Six2-cre* resulted in primary glomerulocystic disease without tubular dilatation. We found that loss of *Zeb2* caused renal proximal tubule hypotrophy and reduced glomerulotubular junction integrity and maturation, which contributed to the formation of congenital atubular glomeruli leading to glomerulocystic disease. By gene expression analysis, we found that *Zeb2* nephron specific knockout kidney had aberrant expression of *Pkd1*, *Hnf1β,* and *Glis3*, three genes associated with glomerular cysts. These results suggest that *Zeb2* regulates the morphogenesis of metanephric mesenchyme derived early nephrons and is required for proximal tubule development and normal glomerulotubular junction formation.

RESULTS

Because aberrant expressions of *Zeb2* and miR-200 have been reported in young mice with a cystic kidney phenotype and *ZEB2* loss-of-function mutations are associated with an increased risk for renal anomalies in MWS patients,[15,18,19] we hypothesized that *ZEB2* plays an important role in early kidney development. To test this hypothesis, we analyzed a *Zeb2* floxed conditional knockout (cKO) mouse line[20] as *Zeb2* null mice die at E9.5 before kidney development begins.[21] We first crossed the *Zeb2* floxed homozygotes ($Zeb2^{flox/flox}$)

with a *Pax2-cre*$^{+}$ deleter strain[22] that expresses the *Cre* recombinase in both the metanephric mesenchyme and ureteric bud (Supplemental Figure 1a). After genotyping 96 three-week old weanlings of *Zeb2*$^{flox/+}$;*Pax2-cre*$^{+}$ heterozygous matings, we did not find any *Zeb2*$^{flox/flox}$;*Pax2-cre*$^{+}$ homozygotes (Supplemental Figure 1b). We then analyzed newborn mice and E18.5 embryos from timed-pregnant females. Five dead newborn *Zeb2*$^{flox/flox}$;*Pax2-cre*$^{+}$ homozygotes and 21/55 (38%) E18.5 homozygous embryos were found (Supplemental Figure 1b), suggesting that *Zeb2*$^{flox/flox}$;*Pax2-cre*$^{+}$ homozygotes die at birth.

The E18.5 *Zeb2*$^{flox/flox}$;*Pax2-cre*$^{+}$ homozygous embryos did not display discernible gross structural defects of the kidney or ureter. However, histological examination of the kidneys from eight E18.5 *Zeb2*$^{flox/flox}$;*Pax2-cre*$^{+}$ homozygous embryos revealed that 100% of the homozygotes had renal cysts, while none of the seven littermate controls (0%) had a cystic phenotype (Table 1, Figure 1). The renal cysts first appeared at E16.5 as no cysts could be detected at E15.5 (Table 1, Figure 1). The renal cysts were apparently from a glomerular origin as 47% of the glomeruli were cystic in the E16.5 *Zeb2* cKO embryos and 30% of the cysts had a visible glomerular tuft (Tables 1 and 2). This phenotype is sufficient for a diagnosis of glomerulocystic kidney disease (GCKD) according to the established criteria.[4]

To determine whether *Zeb2* cKO embryonic kidneys also develop renal tubular cysts, we examined the kidney sections with both proximal tubule specific marker *Lotus tetragonolobus lectin* (LTL) and distal tubules and collecting duct specific marker *Dolichos biflorus agglutinin* (DBA). The cysts were negative for both LTL and DBA staining at E18.5 (Figure 1c and 1d), suggesting that the *Zeb2*$^{flox/flox}$;*Pax2-cre*$^{+}$ homozygous embryos develop primary glomerular cysts. To determine if glomerular cyst formation is due to a deletion of *Zeb2* in the developing nephron, we examined ZEB2 expression during kidney development by analyzing a ZEB2-EGFP reporter mouse that has been reported previously.[23] In addition to the stromal cells, ZEB2 was detected in a subset of cells in which it can be deleted by *Pax2-cre* in the developing nephron (Supplemental Figure 2), including the S-shaped bodies (Supplemental Figure 2e) and the glomeruli (Supplemental Figure 2f). By co-staining, we confirmed that ZEB2 is co-expressed in a subset of cells with PAX2 and JAG1, two markers of developing nephrons (Supplemental Figure 3).

Zeb2$^{flox/flox}$;*Pax2-cre*$^{+}$ homozygous mice died at birth (probably due to high expression of *Pax2-cre* in the nervous system where ZEB2 also plays an important role),[22,23] precluding a longitudinal analysis of glomerulocystic kidney disease progression after birth. A recent study shows that SIX2 regulates ZEB2 expression through miR-200.[24] SIX2 is also a nephron progenitor marker and is expressed in the metanephric mesenchyme that gives rise to all segments of the mature nephron including the parietal and visceral epithelial cells in the glomeruli and the proximal and distal tubular epithelial cells.[25] To further delineate the role of ZEB2 in nephron development and to study the pathological effect of *Zeb2* deletion in mesenchyme-derived nephrons as well as glomerular cystic phenotype in postnatal mature kidney, we crossed *Zeb2*$^{flox/flox}$ mice with *Six2-cre* mice. The *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ homozygous mice survived after birth and were weaned at a Mendelian distribution. Histological examination of the kidneys from nine *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ homozygous embryos revealed that, like the *Zeb2*$^{flox/flox}$;*Pax2-cre*$^{+}$ embryos, all homozygous embryos

had glomerular cysts starting at E16.5 (Figure 2a). At postnatal day 12 (P12), the glomerular cysts in the *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ mice were mainly located in the subcapsular region of the kidney that is consistent with the primary glomerulocystic kidney disease diagnosis (Figure 2b).[4] The cysts were also negative for both LTL and DBA staining (Figure 2c and 2d), confirming their glomerular origin. In comparison, none of the five wild-type littermate controls had a cystic phenotype at P12 (Figure 2b).

To determine the longitudinal effect of glomerulocystic kidney disease on mature kidney, we followed the *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ mice to adulthood. At 7 weeks of age, all *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ mice (n=5, 100%) developed macroscopic renal cysts that were visualized on the surface of the kidney (Figure 3a). Histological analysis revealed the presence of numerous large glomerular cysts in the area of the renal cortex and outer medulla (Figure 3b). Although no significant interstitial fibrosis was observed (Figure 4a), some remaining non-cystic glomeruli in adult *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ mice displayed glomerulosclerosis lesions (Figure 4b). Similar lesions were also observed in few non-cystic glomeruli in P8 *Zeb2* cKO kidneys (Supplemental Figure 4). By 8 weeks of age, all *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ mice (n=5, 100%) developed albuminuria (Figure 4c and 4d) with significantly elevated serum blood urea nitrogen (BUN) levels (Figure 4e). Albuminuria was not directly caused by loss of ZEB2 in podocytes or reduced proximal tubule endocytosis as deletion of *Zeb2* specifically in the podocyte using *Nphs2-cre* did not lead to proteinuria (data not shown), and the expressions of megalin and cubilin were upregulated in *Zeb2* adult cKO kidneys (Supplemental Figure 5), which may increase endocytosis of albumin as previously reported.[26] These data suggest that loss of *Zeb2* in mesenchyme-derived nephrons alone is sufficient to cause glomerulocystic disease in mature kidneys, which leads to secondary glomerulosclerosis, albuminuria and renal failure in adult mice.

One common cause of acquired glomerular cysts is the loss of glomerulotubular junction integrity leading to atubular glomeruli.[27,28] Glomerulotubular integrity can be quantified by examining the connection of the proximal tubule to the Bowman's capsule with the proximal tubule marker LTL.[29] To determine whether the *Zeb2* knockout mice have decreased glomerulotubular integrity, we examined glomeruli from five P12 *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ mice and five littermate controls by serial sections. We found that only 63/604 (10%) glomeruli had a visible LTL positive staining (i.e. glomerulotubular junction) in the *Zeb2* cKO mice as compared to 115/324 (36%) in the wild-type littermate controls (Figure 5), a statistically significant decrease of glomerulotubular integrity, suggesting that *Zeb2* deletion causes formation of atubular glomeruli.

Acquired atubular glomeruli can be caused by atrophy of the proximal tubules in polycystic kidney disease.[30] To determine if reduced glomerulotubular integrity in *Zeb2* cKO is associated with renal proximal tubule atrophy (acquired defect) or hypotrophy (developmental defect), we analyzed the mRNA levels of markers for the proximal tubule (*Hnf1a and Vil1*), the podocyte (*Nphs1* and *Nphs2*), and the collecting duct (*Upk3a*).[31] We found that only the proximal tubule mRNA markers were significantly reduced in the *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ mice compared to the levels in the wild-type littermates at postnatal day 8 (P8) (Figure 6a). To determine if the reduction of proximal tubule mRNA at P8 is caused by an early developmental defect preceding the formation of glomerular cysts in

Zeb2 cKO mice, we analyzed mRNA markers in E14.5 kidneys. Similar to P8, a significant decrease of the proximal tubular markers was detected in the E14.5 *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ kidneys as compared to their wild-type littermates (Figure 6b). Consistent with this finding, the expression of the proximal tubule brush border protein villin 1 (VIL1),[31] was also downregulated in both E15.5 *Zeb2*$^{flox/flox}$;*Pax2-cre*$^{+}$ and E16.5 *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ mutant kidneys (Figure 6c and 6d). Finally, the mean kidney size of the E16.5 *Zeb2* cKO embryos was smaller than that of their wild-type littermate controls (Figure 6e). Taken together, these data suggest that loss of *Zeb2* in the mesenchyme-derived nephrons causes early proximal tubule developmental defects resulting in tubular hypotrophy, reduced glomerulotubular junction integrity, and congenital atubular glomeruli formation.

Glomerular cysts are reported in several mouse models of renal cystic kidney disease, including *Wwtr1*, *Glis3*, *Ofd1* and *Pkhd1* knockout mice, the *Hnf1β* and *Dicer* cKO mice, and *Pkd1* over expression transgenic mice.[19,31–36] Interestingly, the *Hnf1β* cKO mice using *Six2-cre* develop glomerular cysts due to a drastic reduction in the levels of proximal tubular markers at E14.5, resembling the *Zeb2* cKO phenotype.[31] However, by immunostaining of JAG1, a marker for the renal vesicle and the S-shaped body,[31] we did not observe differences between *Zeb2* cKO and wild-type littermate controls (Supplemental Figure 6). To determine if loss of *Zeb2* affects the expression of these six genes and the microRNA miR-200, we examined mRNA and microRNA levels in the kidney tissues of E14.5 and E18.5 *Zeb2* cKO and wild-type controls. Although there was no significant difference in the expression levels of any of the six genes and miR-200 at E14.5 before glomerular cyst formation (Figure 7a), we detected a decreased expression of *Glis3* and increased expression levels of *Hnf1β* and *Pkd1* in E18.5 *Zeb2* cKO compared to the wild-type littermate controls (Figure 7b). The expression level of *Pkd1* mRNA was the most significantly upregulated in the *Zeb2* cKO kidneys at postnatal day 8 (P8) (Figure 7c). Consistent with the mRNA levels, PKD1 coding protein polycystin-1 (PC1) was also found to be expressed at a higher level in the glomeruli of postnatal day 7 (P7) *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ mice but not at E16.5 and E17.5 when the glomerular cysts are initially observed (Figure 7d). Interestingly, the PC1 expression was upregulated in non-cystic glomeruli but not in the glomeruli with dilated Bowman's space (Figure 7e). These data suggest that loss of *Zeb2* in the kidney leads to upregulation of polycystin-1 expression in non-cystic glomeruli after the initial phase of glomerular cyst formation.

Renal cystogenesis is often associated with increased cell proliferation.[37] To determine if abnormal cell proliferation also plays a role in the formation of glomerular cysts in *Zeb2* cKO mice, we quantified the proliferation of parietal epithelial cells in the Bowman's capsule of *Zeb2* cKO kidneys and wild-type littermate controls using the cell proliferation marker phospho-Histone H3 (pHH3). No significant difference of cell proliferation was observed in *Zeb2* cKO kidneys compared to wild-type littermate controls (Supplemental Figure 7), suggesting that cell proliferation does not play an important role in *Zeb2* cKO glomerulocystic phenotype.

Apoptosis is part of normal kidney development during C-shaped body (CSB) and S-shaped body (SSB) formation.[38,39] To determine if abnormal apoptosis in CSB and SSB may contribute to congenital atubular glomeruli formation in *Zeb2* cKO mice, we performed

TUNEL assays on E15.5 and E16.5 developing kidneys from five *Zeb2* cKO and four littermate controls. Interestingly, 11/34 (32%) CSB/SSB were identified with at least one apoptotic cell in *Zeb2* cKO kidneys, while 25/39 (64%) CSB/SSB were found in wild-type littermate controls (Supplemental Figure 8). These data suggest that loss of *Zeb2* causes aberrant apoptosis in CSB and SSB during nephron development, which may contribute to proximal tubular hypotrophy and abnormal glomerulotubular junction maturation in *Zeb2* cKO mice.

DISCUSSION

In this study, we found that ZEB2 is critical for the mesenchyme-derived nephron development. Conditional deletion of *Zeb2* with *Pax2-cre* and *Six2-cre*, two Cre strains active in the metanephric mesenchyme derived nephron progenitor cells, resulted in the same glomerular cyst phenotype, indicating that ZEB2 regulates the morphogenesis of mesenchyme-derived nephrons and is required for normal nephron development. Decreased levels of proximal tubular markers at both mRNA and protein levels from E14.5 to E16.5 and reduced kidney size at E16.5 in the *Zeb2* cKO mice suggest an early hypotrophy of the proximal tubule, leading to congenital atubular glomeruli formation, accumulation of glomerular filtrate and cystic expansion of the Bowman's space. Although ZEB2 is also expressed in the stromal cells of the developing kidney, stromal cell-specific deletion of *Zeb2* with *Foxd1-cre* did not result in a glomerular cystic phenotype (data not shown), which further supports the specific role of ZEB2 in early developing nephron. Consistent with our findings, haploinsufficiency of *Zeb2* was also reported to be associated with delayed nephrogenesis in a transgenic rat model.[40]

Atubular glomeruli have been reported in polycystic kidney disease, obstructive uropathy, and many other glomerular or tubular diseases in humans and animal models.[27,29,30,41] However, they are thought to be secondary to progressive injury to the proximal tubules inducing degenerative tubular cell changes and atrophy, eventually destroying proximal tubules at the glomerulotubular junctions.[30,42] We found that loss of *Zeb2* is associated with primary congenital atubular glomeruli due to developmental defects and hypotrophy of the proximal tubules and the glomerulotubular junction. Although the link between acquired atubular glomeruli and glomerular cyst formation was proposed two decades ago,[28] our study now demonstrates that primary glomerulocystic disease can also be caused by congenital atubular glomeruli.

ZEB2 is also known as SIP1 (SMAD-interacting protein 1), which interacts with activated SMAD proteins and is part of the TGF-β/SMAD signaling pathway.[43,44] SMADs are highly expressed in developing kidney.[45] Elevated TGF-β/SMAD signaling has also been detected in experimental *Pkd1* mouse models and human patients with polycystic kidney disease.[46] We found abnormal expression of *Glis3* gene in *Zeb2* cKO kidneys at E18.5 compared to their wild type littermates. Transcription factor GLIS3 interacts with TAZ protein (encoded by *Wwtr1* gene), which is also part of the TGF-β/SMAD pathway regulating SMAD shuttling between the cytoplasm and the nucleus.[34,47] Interestingly, both *Glis3* and *Wwtr1* knockout mice develop glomerular cysts as *Zeb2* cKO mice.[34,36] In our study, we also found abnormal expression of *Hnf1β* in the *Zeb2* cKO kidneys at E18.5 compared to the wild-type

littermate controls. *HNF1B* mutations cause glomerulocystic disease in human and *Hnf1β* mesenchyme specific cKO mice also develops glomerular cysts.[11,31] A recent study shows that the expressions of both *Zeb2* and *Pkd1* are upregulated in another *Hnf1β* renal specific knockout mouse model with polycystic kidney disease,[18] suggesting that an optimal and balanced ZEB2 expression in the kidney is required to prevent renal cysts formation.

High levels of polycystin-1 are often detected in the kidney of ADPKD patients.[48–52] Overexpression of polycystin-1 in mice also causes glomerular cysts.[32,53] *Hnf1β* cKO and *Dicer* cKO mice develop renal cysts with increased levels of *Pkd1* and decreased levels of miR-200.[18,19] We found increased expression of *Pkd1* mRNA and polycystin-1 in the glomeruli of *Zeb2* cKO kidneys after the initial phase of glomerular cyst formation. Although miR-200 expression is repressed by ZEB2[54] and *Zeb2* gene is a known target of miR-200[55] in a double-negative feedback loop, we did not observe differential expression of miR-200 in *Zeb2* cKO kidneys. Likewise, no differential expression of *Zeb2* was observed in the *Dicer* cKO mice in which *miR-200* is significantly downregulated.[19] Therefore, *Pkd1* upregulation in the non-cystic glomeruli of *Zeb2* cKO mice is probably secondary to the glomerular stress (e.g. due to hyperfiltration of the non-cystic glomeruli) and not the result of a direct regulation of *Pkd1* gene expression by the ZEB2/miR-200 signaling network. The effects of this increased expression of polycystin-1 in *Zeb2* cKO mice are presently unknown and need further investigation.

In conclusion, by studying animal models of a disease gene causing a human syndrome associated with an increased risk of congenital kidney anomalies, we identified *Zeb2* as a novel gene important in proximal tubule development and glomerulotubular junction formation. Loss of *Zeb2* in mesenchyme-derived nephrons in mice results in reduced glomerulotubular integrity, congenital atubular glomeruli and primary glomerular cystic disease. Future studies are needed to elucidate the molecular pathway of ZEB2 signaling during kidney development and the cell type and mechanism of *Pkd1* overexpression in non-cystic glomeruli, and to determine whether MWS patients also develop glomerular cysts and whether a subset of patients with glomerulocystic kidney disease carry *ZEB2* mutations. As a transcription factor, ZEB2 may also provide a starting point for further identification of new genes important in glomerulotubular junction development and that, when mutated, may cause primary glomerulocystic kidney disease in patients.

MATERIALS AND METHODS

Animals

Zeb2 floxed conditional knockout mouse and ZEB2-EGFP reporter mouse were previously reported.[20, 23] *Pax2-cre*$^{+}$ mice were obtained from the MMRRC (#010569-UNC)[22] and *Six2-cre*$^{+}$ mice were purchased from the Jackson Lab (#009606).[25] All animal studies were approved by the IACUC of Boston University.

Histology

The kidneys of mice at defined ages were dissected and fixed in 4% PFA and processed for paraffin embedding. Serial sections were cut and stained by H&E, Periodic acid–Schiff stain

and Masson Trichrome stain using standard methods. Slides were viewed with an Olympus microscope and photographed using a DP72 digital camera.

Quantification of glomerular cysts and kidney length

The numbers of glomeruli with and without cysts were counted on an H&E-stained median sagittal kidney section from each animal. The glomerular cysts were quantified by counting the total number of glomeruli that are identified by glomerular tuft and by scoring them as cystic when a two- to threefold dilatation of the Bowman's space was observed. Embryonic kidney length was measured using Olympus microscope and cellSens software.

Immunostaining

For immunohistochemistry (IHC), kidney sections were pretreated to quench endogenous peroxidase (3.0% hydrogen peroxide) and endogenous biotin (SP-2001, Vector Labs), and stained with biotinylated LTL (B-1325, Vector Labs) and DBA (B-1035, Vector Labs) following standard IHC protocol. For immunofluorescence staining, mouse kidneys were fixed in 4% PFA followed by incubation in 30% sucrose overnight at 4°C, embedded in OCT compound (Tissue-Tek), and cryosectioned at 10 μm. Frozen sections were permeabilized with 0.1% PBS- Triton X-100 for 10 min and blocked in 5% goat serum for 1 hour. Primary antibodies were incubated overnight at 4°C followed by secondary antibodies incubated at room temperature for 1 hour. Following primary antibodies were used: WT1 (sc-192, Santa Cruz), Polycystin-1 (sc-130554, Santa Cruz), Laminin (L9393, Sigma), GFP (GFP-1020, Aves), Villin-1 (2369, Cell Signaling), JAG1 (sc-8303, Santa Cruz), PAX2 (71-6000, Thermo Fisher), megalin (sc-16478, Santa Cruz), cubilin (sc-20609, Santa Cruz). Tissue sections were mounted in media containing DAPI and imaged by a Zeiss confocal microscope.

Urine and Plasma Analysis

Urine protein excretion was detected by SDS-PAGE followed by Coomassie blue staining and quantified using ImageJ. Urine creatinine was measured using a mouse creatinine assay kit (80350, Crystal Chem). Urine albumin/creatinine ratios were calculated. Serum BUN were measured using the Catalyst Dx Chemistry Analyzer by IDEXX.

Assessment of glomerulotubular integrity

The glomerulotubular integrity was assessed by analyzing the connection between the proximal tubules and the glomeruli on LTL-stained kidney sections as previously described,[29, 30, 56] based on the knowledge that LTL-positive stained epithelial cells constitute part of the Bowman's capsule with normal glomerulotubular junctions but are absent in atubular glomeruli. Briefly, consecutive serial sections of LTL-stained kidneys from *Zeb2* cKO and wild-type controls (5 mice in each group) were used to make positive identification of atubular glomeruli. In order to quantify the glomerulotubular integrity, all glomeruli were counted on a single LTL-stained kidney section from each mouse and were divided into two categories on the basis of presence (positive) or absence (negative) of LTL staining in the Bowman's capsule. Quantification of glomerulotubular integrity was

presented as the percentage of LTL-positive glomeruli in total glomeruli counted in cKO mice and wild-type controls.[29]

Gene expression analysis

Total RNA was extracted from kidneys using miRNeasy Micro kit (217084, Qiagen). cDNA was synthesized using Verso cDNA Synthesis Kit (AB-1453, Life Technologies) and TaqMan MicroRNA RT kit (4366596, Life Technologies). Gene expression was analyzed using 7500FAST real-time PCR machine with TaqMan probes (Life Technologies). Relative gene expression data were analyzed by the delta-delta-Ct method and were normalized to the either *Gapdh* or *Ppp1r3c*.

Cell proliferation and apoptosis analyses

Proliferative cells were identified using an anti-pHH3 antibody (9701, Cell Signaling). To quantify cell proliferation, all the glomeruli were counted on a single kidney section from each of the 8 mice and were divided into two categories on the basis of presence (positive) or absence (negative) of pHH3 staining in the Bowman's capsule. Quantification is presented as the percentage of pHH3-positive glomeruli out of total glomeruli counted. Apoptosis was analyzed as previously described using the TUNEL assay.[57] Apoptotic cells were detected using Apoptag Peroxidase *In Situ* Apoptosis Detection Kit (S7100, Millipore). To quantify apoptosis in the CSB and SSB, we analyzed all the CSB/SSB in one kidney section from 9 different embryos, and counted them as either "positive" when at least one apoptotic cell was present or "negative" when no apoptotic cell was visualized. The immunofluorescent TUNEL staining was performed using the ApopTag Red *In Situ* Apoptosis Detection Kit (S7165, Millipore).

Statistical Analyses

Data are given as mean and standard deviation (SD). A minimum of three mice were used for each analysis, unless stated otherwise. Statistical analysis was performed by using the Student t-test or the chi-square test, and significance was determined at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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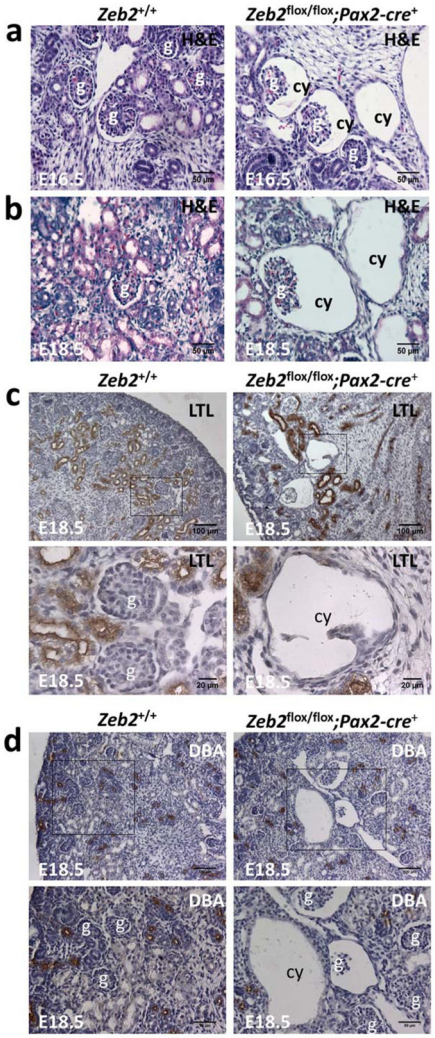

Figure 1. Deletion of *Zeb2* with *Pax2-cre* leads to embryonic glomerulocystic kidney disease without tubular dilatation

Congenital glomerular cysts were observed in the kidney of E16.5 (**a**) and E18.5 (**b**) *Zeb2*$^{flox/flox}$;*Pax2-cre*$^{+}$ homozygous embryos but not in the *Zeb2*$^{+/+}$ littermate controls (H&E staining, 200x magnification). The cysts in the *Zeb* $^{flox/flox}$;*Pax2-cre*$^{+}$ kidneys at E18.5 are negative for the proximal tubules marker *Lotus Tetragonolobus Lectin* (LTL) (**c**), and the collecting duct and distal tubules marker *Dolichos Biflorus Agglutinin* (DBA) (**d**). Upper panels - 100x magnification; lower panels - enlarged images of boxed regions from upper panels (n≥3 for each group). Scale bars are shown. Abbreviations: g-glomerulus; cy-cyst.

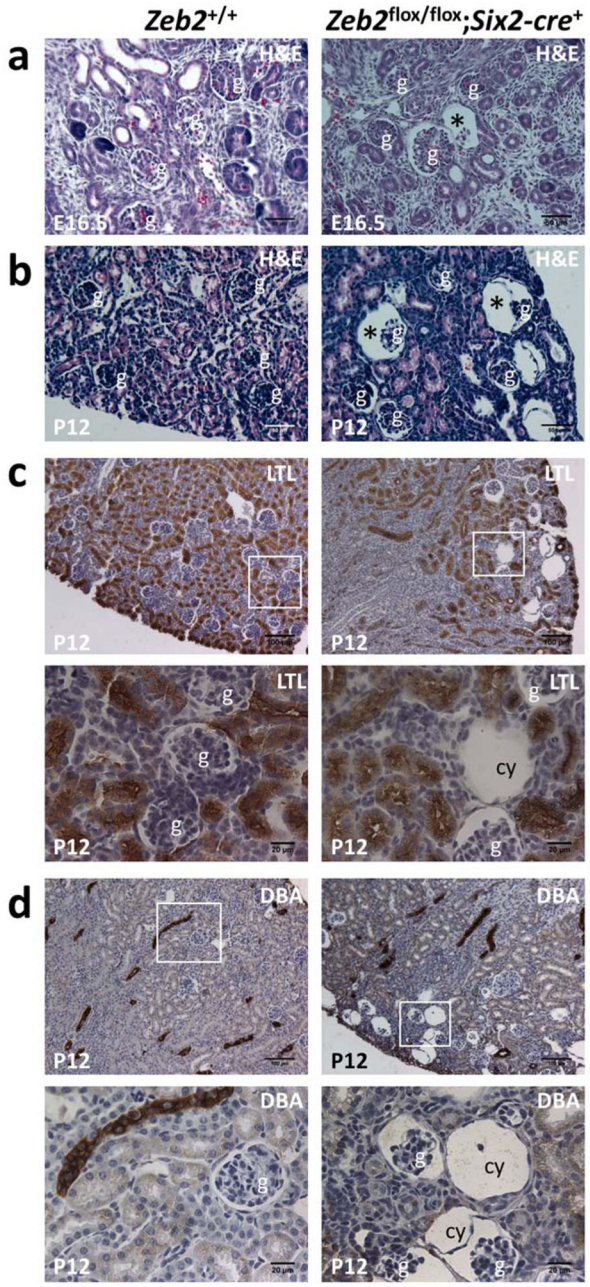

Figure 2. Mesenchyme-specific deletion of *Zeb2* using *Six2-cre* also leads to glomerulocystic kidney disease without tubular dilatation

(**a**) Dilated glomerular Bowman's space (asterisk) was observed in the *Zeb2*$^{flox/flox}$*;Six2-cre*$^{+}$ embryonic kidneys at E16.5 but not in littermate controls (H&E staining, 200x magnification). (**b**) Glomerular cysts (asterisk) in the subcapsular region of the kidney cortex at P12 in the *Zeb2*$^{flox/flox}$*;Six2-cre*$^{+}$ kidneys but not in the littermate controls (H&E staining, 200x magnification). (**c**) IHC staining shows the cyst (cy) in a P12 *Zeb2*$^{flox/flox}$*;Six2-cre*$^{+}$ kidney is negative for the proximal tubules marker *Lotus Tetragonolobus Lectin* (LTL). Upper panels - 100x magnification; lower panels - high magnification of boxed regions in the upper panels (n≥3 for each group). (**d**) IHC staining shows the cysts (cy) in a P12

Zeb2$^{flox/flox}$;*Six2-cre*$^{+}$ kidney is negative for the collecting duct and distal tubules marker *Dolichos Biflorus Agglutinin* (DBA). Upper panels - 100x magnification; lower panels - high magnification of boxed regions in the upper panels (n≥3 for each group). Abbreviations: g-glomerulus; cy-cyst.

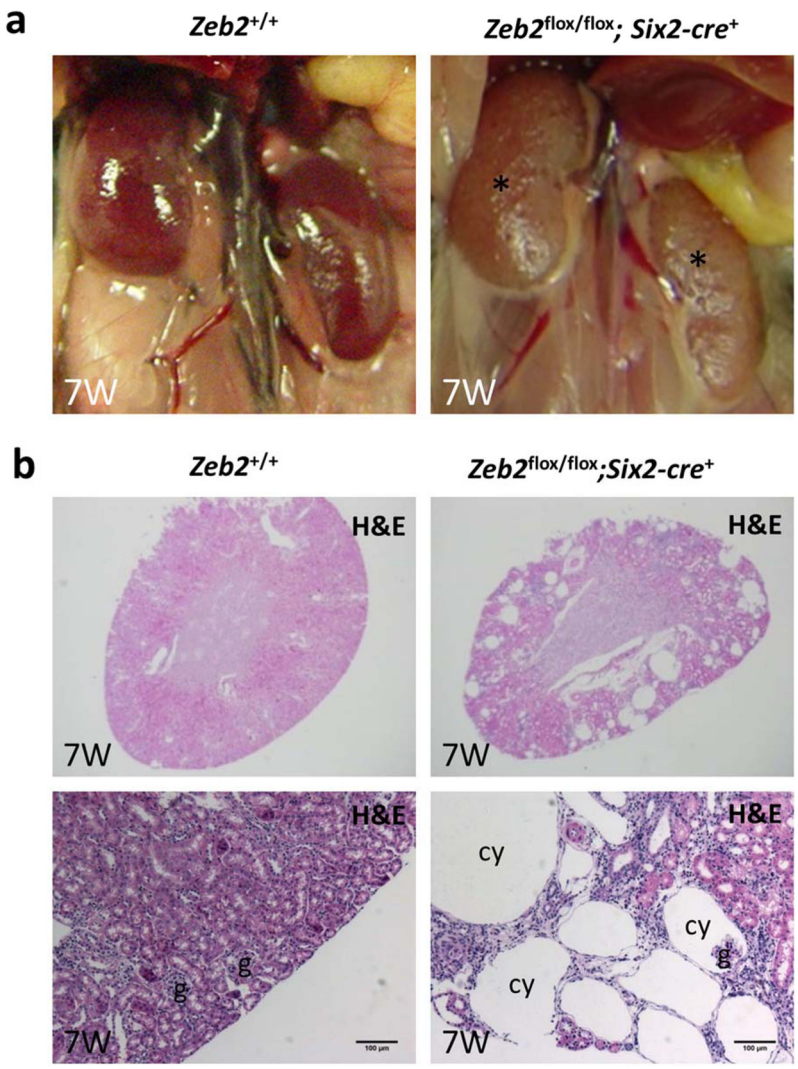

Figure 3. Mesenchyme-specific deletion of *Zeb2* with *Six2-cre* causes glomerulocystic kidney disease in adult mice

(**a**) Macroscopic images of normal kidneys from a 7 weeks old control mouse (left panel) and pale and cystic kidneys (marked by asterisk) from a *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ homozygous mouse (right panel); n=5 in each group. (**b**) Histological images of kidneys from 7 weeks old control *Zeb2*$^{+/+}$ mice and *Zeb2*$^{flox/flox}$;*Six2-cre*$^{+}$ cKO mice show large cysts in the renal cortex and outer medulla region in the cKO mice (n=3 in each group). Upper panels - 25x magnification; lower panels - 100x magnification with scale bars at 100 μm; g-glomerulus; cy-cyst.

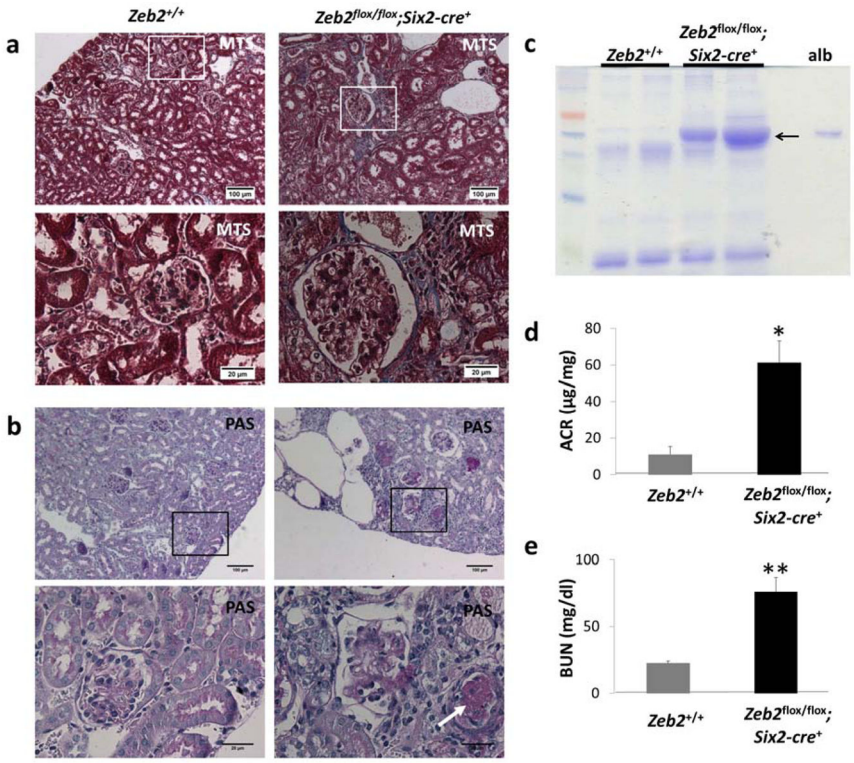

Figure 4. Mesenchyme-specific deletion of *Zeb2* with *Six2-cre* causes glomerulosclerosis, albuminuria and renal failure in adult mice

(**a**) Masson Trichrome staining (MTS) shows minimal fibrosis (blue) in the kidney of a 7 weeks old $Zeb2^{flox/flox};Six2\text{-}cre^{+}$ mouse. Upper panels - 100x magnification; lower panels - high magnification of boxed regions in the upper panels (n=3 for each group). (**b**) Periodic acid Schiff (PAS) staining shows glomerulosclerosis (arrow) in non-cystic glomeruli of a 7 weeks old $Zeb2^{flox/flox};Six2\text{-}cre^{+}$ mouse kidney. Upper panels - 100x magnification; lower panels - high magnification of boxed regions in the upper panels (n=3 for each group). (**c**) Representative SDS-page gel with Coomassie blue staining shows albuminuria (arrow) in two $Zeb2^{flox/flox};Six2\text{-}cre^{+}$ 5 weeks old mice but not in two littermate controls (n=5 mice in each group); alb - albumin. (**d**) Increased albumin-to-creatinine ratio (ACR) in 5 weeks old $Zeb2^{flox/flox};Six2\text{-}cre^{+}$ mice compared to $Zeb2^{+/+}$ littermate controls (n=4 mice in each group, *p < 0.05) (**e**) Significantly elevated BUN in 5 weeks old $Zeb2^{flox/flox};Six2\text{-}cre^{+}$ mice compared to $Zeb2^{+/+}$ littermate controls (n=3 mice in each group, **p-value< 0.001). Data are represented as means +/− standard deviation.

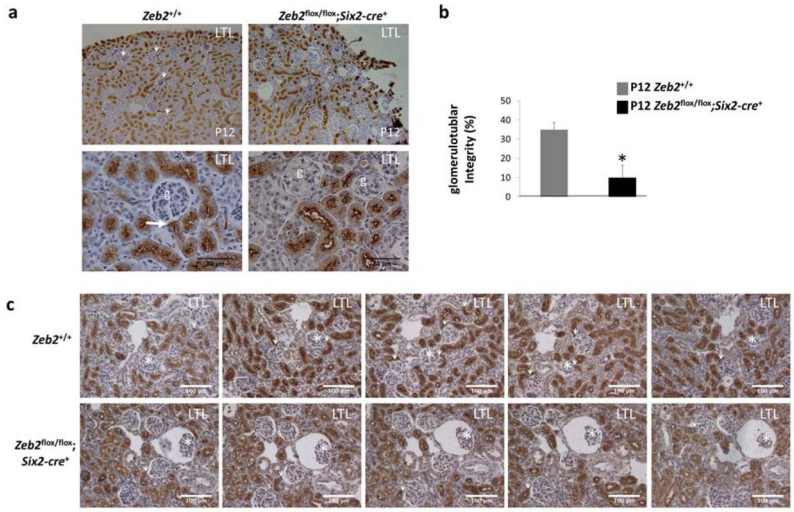

Figure 5. Congenital atubular glomeruli in *Zeb2* mesenchyme-specific knockout mice

(**a**) LTL staining shows many glomerular Bowman's capsule and proximal tubule connections (arrows) in P12 *Zeb2*$^{+/+}$ wild-type control mice but very few connections in *Zeb2*$^{flox/flox}$*;Six2-cre*$^{+}$ mutant mice. Upper panels - 100x magnification; lower panels - 400x magnification; g -glomerulus. (n=5 in each group); Scale bars are shown. (**b**) Percentage of LTL-positive glomeruli out of total glomeruli counted in sagittal kidney sections from five *Zeb2* cKO and five *Zeb2*$^{+/+}$ littermate controls (n=604 glomeruli in *Zeb2* cKO and n=324 glomeruli in wild-type were counted, *$p < 10^{-3}$). (**c**) Representative serial sections of P12 *Zeb2*$^{+/+}$ wild-type kidneys (upper panels) and *Zeb2*$^{flox/flox}$*;Six2-cre*$^{+}$ cKO kidneys (lower panels) show the detection of glomerulotubular junctions (arrows) in the wild-type glomeruli but absence in many *Zeb2* cKO glomeruli (* indicating the same glomerulus on serial sections).

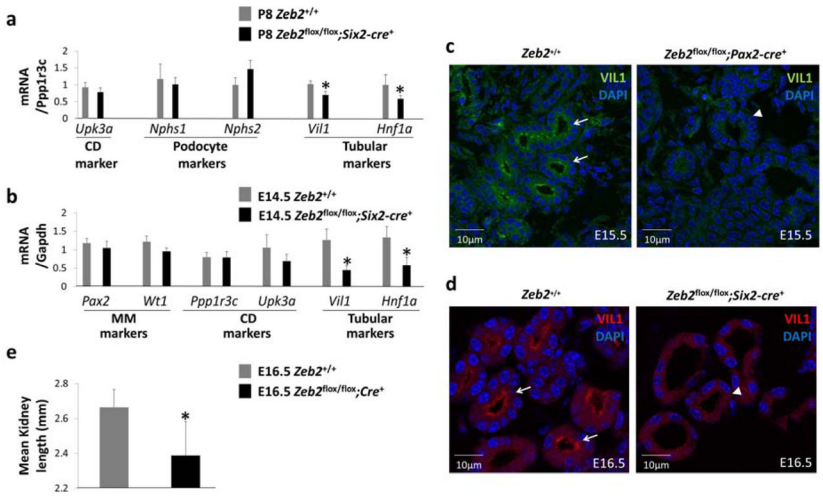

Figure 6. Decreased early proximal tubular marker expression in *Zeb2* mesenchyme-specific knockout mice

(**a**) At postnatal day 8 (P8), TaqMan assays show decreased mRNA expression levels of proximal tubular markers (*Vil1* and *Hnf1a*) but not podocyte markers (*Nphs1* and *Nphs2*) in $Zeb2^{flox/flox}$;*Six2-cre*$^{+}$ kidneys when normalized to a collecting duct marker *Ppp1r3c*. Collecting duct marker *Upk3a* was used as a control (n= 3 in each group, mean relative quantification adjusted to *Ppp1r3c*, *p < 0.05). (**b**) At E14.5, TaqMan assays show decreased mRNA expression levels of proximal tubular mRNA markers (*Vil1* and *Hnf1a*) in the cKO kidneys compared to wild-type kidneys. There are no differences for the metanephric mesenchyme markers (*Pax2* and *Wt1*) and the collecting duct markers (*Ppp1r3c* and *Upk3a*) in cKO and control kidneys (mean relative quantification adjusted to *Gapdh*, n=3 in each group, *p <0.05). (**c**) Immunofluorescent staining shows decreased expression of VIL1 (villin-1) protein in E15.5 $Zeb2^{flox/flox}$;*Pax2-cre*$^{+}$ cKO proximal tubules (arrowhead) as compared to the proximal tubules in wild-type littermates (arrows). (**d**) Decreased expression of VIL1 (villin-1) protein in E16.5 $Zeb2^{flox/flox}$;*Six2-cre*$^{+}$ cKO proximal tubules (arrowhead) as compared to the proximal tubules in wild-type littermates (arrows). (**e**) The *Zeb2* cKO embryos have smaller kidney length at E16.5 as compared to wild-type controls (n=7 for cKO kidneys and n=6 for wild-type kidneys, *p $<10^{-3}$).

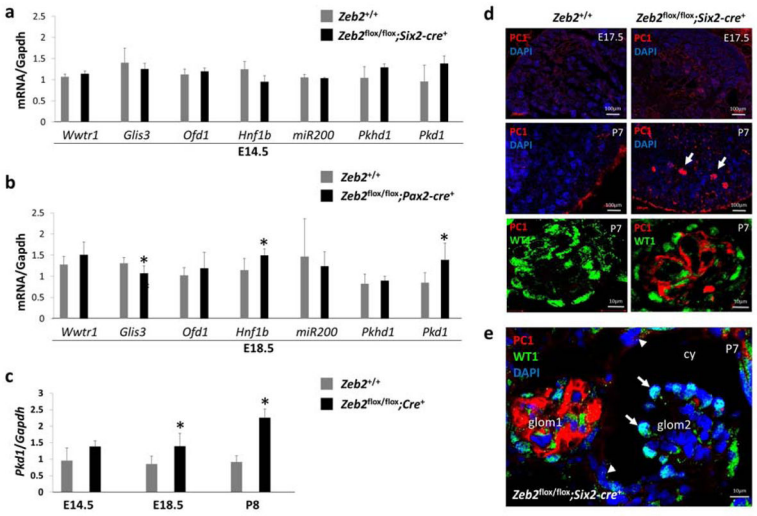

Figure 7. Abnormal expression of known glomerulocystic disease genes in *Zeb2* conditional knockout kidneys

(**a**) TaqMan assays show no differences of the mRNA levels for the 6 genes and miRNA miR200 between E14.5 *Zeb2* cKO kidneys and wild-type controls (mean relative quantification adjusted to *Gapdh*, n≥3). (**b**) TaqMan assays show significant upregulation of mRNA levels of *Pkd1* and *Hnf1β* and downregulation of *Glis3* mRNA in E18.5 *Zeb2* cKO kidneys compared to wild-type controls (mean relative quantification adjusted to *Gapdh*, n=5, *p <0.05) (**c**) Significant upregulation of *Pkd1* mRNA at E18.5 and P8 but not E14.5 in *Zeb2* cKO kidneys compared to wild-type controls (mean relative quantification adjusted to *Gapdh*, n=3, *p <0.05). (**d**) Immunofluorescence staining show increased levels of polycystin 1 (PC1) protein in the glomeruli (arrows) of $Zeb2^{flox/flox};Six2\text{-}cre^{+}$ kidney at P7 (middle and lower panels), but not at E17.5 (upper panel). PC1 staining in the glomeruli was confirmed by co-expression of WT1, a glomerular podocyte marker (lower panel); Scale bars are shown. (**e**) Triple immunofluorescence staining with PC1, WT1 and DAPI in *Zeb2* cKO kidney shows that increased level of polycystin 1 (PC1) protein was detected only in non-cystic glomeruli (glom 1) but not in an adjacent glomerulus (glom 2) with significantly enlarged Bowman's space (cy). The podocytes (arrows) and parietal epithelial cells (arrowheads) are visible.

Table 1

Cystic phenotype observed in H&E stained kidney samples of *Zeb2*$^{flox/flox}$*;Pax2-cre*$^{+}$ and wild-type littermates between E16.5 and E18.5

	E16.5		E17.5		E18.5	
	Zeb2$^{+/+}$	***Zeb2***$^{flox/flox}$***; Pax2-cre***$^{+}$	***Zeb2***$^{+/+}$	***Zeb2***$^{flox/flox}$***; Pax2-cre***$^{+}$	***Zeb2***$^{+/+}$	***Zeb2***$^{flox/flox}$***; Pax2-cre***$^{+}$
Embryos with kidney cysts (more than 1 cyst)	0/2 (0%)	3/3 (100%)	0/3 (0%)	3/3 (100%)	0/7 (0%)	8/8 (100%)
Glomerular Cysts/Number of glomeruli	1/48 2%, (n=4)00	26/55 47%, (n=5)	0/40 0%, (n=3)	18/59 30.5%, (n=4)	1/363 0.2%, (n=13)	61/391 15.6%, (n=15)

n= number of kidneys analyzed

Table 2

Percentage of cysts with glomerular tufts on H&E stained kidney samples between E16.5 and E18.5.

	Cysts with Glomerular tufts/total cysts (n= number of kidneys analyzed) ***Zeb2*$^{flox/flox}$*;Pax2-cre*$^{+}$
E16.5	26/85 (30%, n=5)
E17.5	18/46 (39%, n=4)
E18.5	61/226 (27%, n=15)

n= number of kidneys analyzed