aPKCλ/ι and aPKCζ Contribute to Podocyte Differentiation and Glomerular Maturation

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[40x633]ABSTRACT

Precise positioning of the highly complex interdigitating podocyte foot processes is critical to form the normal glomerular filtration barrier, but the molecular programs driving this process are unknown. The protein atypical protein kinase C (aPKC)—a component of the Par complex, which localizes to tight junctions and interacts with slit diaphragm proteins—may play a role. Here, we found that the combined deletion of the aPKCλ/ι and aPKCζ isoforms in podocytes associated with incorrectly positioned centrosomes and Golgi apparatus and mislocalized molecules of the slit diaphragm. Furthermore, aPKC-deficient podocytes failed to form the normal network of foot processes, leading to defective glomerular maturation with incomplete capillary formation and mesangiolysis. Our results suggest that aPKC isoforms orchestrate the formation of the podocyte processes essential for normal glomerular development and kidney function. Defective aPKC signaling results in a dramatically simplified glomerular architecture, causing severe proteinuria and perinatal death.


During kidney development, podocytes extend primary processes and foot processes to surround glomerular capillaries.1 Podocyte and glomerular development can be classified into characteristic stages. During the comma-shaped body stage, undifferentiated podocytes are cuboidal epithelial cells with apical sited cell-cell contacts. During the s-shaped body stage, the cell-cell contacts move along the lateral membrane toward the basal sites of podocytes. Subsequently, at the capillary loop stage, podocytes stop cell division, start to express typical podocyte differentiation markers like the slit diaphragm proteins nephrin and Podocin, and extend primary processes and foot processes, which become connected by the slit diaphragm.1–6

There are only few cells known to undergo such dramatic phenotypic changes during development as podocytes. The signaling programs driving these unique complex changes in cell shape, function, and structure are incompletely understood. However, the asymmetric apical membrane expansion and the process formation suggest the involvement of apicobasal polarity complexes. Apicobasal polarity is established by three core polarity complexes, which subdivide the cellular membrane in an apical and a basolateral membrane compartment: the Crumbs complex, consisting of Crumbs, PATJ and PALS1, localizes to the apical membrane; the Par complex,
consisting of Par3, Par6, and atypical protein kinase C (aPKC), localizes to tight junctions; and the Scribble complex, which contains Scribble, Lgl and Dlg, is located at adherens junctions and the basolateral membrane.7 We previously showed that the Par complex translocates together with the cell-cell contacts from apical to basal aspects of the podocyte.8,9 The Par complex interacts with the cytoplasmic tail of the slit diaphragm proteins nephrin and Nep1 and localizes to the insertion site of the slit diaphragm.8,10 Interaction of Par6 with the guanosine-5’-triphosphate (GTP)–bound form of the small GTPases Rac and Cdc42 results in a conformational change of Par6, which facilitates the activation of aPKC.11–14 The two isoforms of aPKC, aPKCα and aPKCζ, are very similar in structure, but are encoded by two different genes.15 Whereas constitutive aPKCζ knockout results in embryonic lethality by embryonic day 9 (E9),16 aPKCζ knockout mice are viable.17 Recently, podocyte-specific conditional knockout mice of aPKCα (aPKCα/α PcKO) were analyzed. Although podocyte-specific deletion of aPKCα results in proteinuria, glomerulosclerosis, and death of knockout mice during adolescence,9,10 knockout podocytes still display regular foot processes connected by slit diaphragms at birth.10 These findings raise the possibility, that aPKCζ might at least partially compensate the loss of aPKCα during podocyte differentiation. To elucidate the full effect of the aPKC complex in development, we generated mice with a podocyte-specific knockout for both aPKC isoforms. This approach revealed that aPKCα is a central regulator of podocyte process formation and glomerular maturation. Furthermore, we demonstrate that basal localization of the centrosome and the Golgi apparatus is a characteristic hallmark of podocyte cell polarity and that the loss of both aPKC isoforms causes an aberrant localization of these organelles.

RESULTS

The Double aPKCα/α and aPKCζ Knockout in Podocytes Results in Severe Proteinuria and Perinatal Death

To visualize the expression of aPKCα/α and aPKCζ during glomerular development, we performed in situ hybridization analyses of kidneys of newborn mice. Both isoforms were highly expressed in podocytes of developing glomeruli (Figure 1, A and B). Next, aPKCζ–/–,17 aPKCα/αfox/fox18 and NPHS2.Cre+ mice,19 which express Cre under control of a podocyte-specific promoter, were crossed to generate aPKC double mutant mice (NPHS2.Cre+;aPKCα/αfox/fox;αPKCζ–/–). Western blot analysis confirmed the loss of aPKCζ and significant reduction of aPKCα in glomerular lysates of knockout mice (Figure 1, C and D). At postnatal day (P) 0, aPKC double mutant mice already displayed massive proteinuria, whereas podocyte-specific aPKCα/α knockout resulted in significant lower albumin
Severely impaired foot process network formation in aPKC double mutant mice. (A) Whereas scanning electron micrographs of kidneys of control and aPKC\(z\) KO mice show developing foot processes that enwrap the capillaries, podocyte processes of aPKC\(l\) PcKO mice display an irregular pattern. In aPKC double mutant mice, podocyte cell bodies are covered with microvilli and blebs. Podocyte processes cannot be detected. (B) Podocytes of newborn aPKC\(l\) PcKO mice exhibit partially broadened processes and long projections to the glomerular basement membrane. aPKC double knockout podocytes display no elaborated process network (arrows). Furthermore, capillary dilations are observed (asterisk). Scale bars, 10 \(\mu m\) in upper panel in A; 2.5 \(\mu m\) in lower panel in A; 2 \(\mu m\) in upper and middle panels in B; 1 \(\mu m\) in lower panel in B.
Figure 3. Glomerular development arrests at late capillary loop stage in aPKC double mutant mice. Frozen kidney sections of newborn mice of the indicated genotypes (P0) were stained using antibodies against the polarity protein Par3 and the basement membrane marker nidogen and were subjected to confocal laser microscopy. Because glomerular development is asynchronous, kidneys of newborn mice display all glomerular developmental stages (from left to right) as follows: comma-shaped body (I), s-shaped body (II),
excretion, increasing to the level of double mutant mice around P14 (Figure 1E and Supplemental Figure 1). aPKC–/– KO mice showed a mildly elevated albumin/creatinine ratio compared with control mice at P0 and P7. After completion of glomerulogenesis at or beyond P14, no difference was detected between control and aPKC–/– KO mice. Unlike aPKC+/– PcKO mice, double mutant mice showed elevated serum creatinine already at birth (P0), indicating an impaired kidney function (Figure 1F), and a significant reduction in body weight compared with control and aPKC–/– KO mice (Figure 1G). aPKC+/– PcKO mice exhibited developmental retardation from P14 onward, whereas aPKC–/– KO mice showed no difference to control. Around 70% of double mutant mice died during the first week after birth with a median survival of 2.5 days (Figure 1H). The majority of aPKC+/– PcKO died between P14 and P21 with a median survival of 16 days, whereas aPKC–/– KO mice showed no increased lethality compared with control mice.

**Severely Impaired Foot Process Network Formation in aPKC Double Mutant Mice**

Histologic analysis of kidneys of newborn mice demonstrated tubular dilations with protein casts in aPKC double mutant mice (Supplemental Figure 2A). In scanning and transmission electron microscopy of kidneys of newborn mice, podocytes of control and aPKC–/– KO mice displayed foot processes with no detectable abnormalities (Figure 2, A and B). aPKC+/– PcKO mice exhibited podocytes with partially broadened foot processes (Figure 2, A and B). Strikingly, podocytes of aPKC double mutant mice were covered with microvilli and blebs, but no foot processes could be detected (Figure 2A). In line with these findings, a distinct podocyte foot process network was not detected in aPKC double knockout by transmission electron microscopy (Figure 2B).

**Glomerular Development Arrests at the Late Capillary Loop Stage in aPKC Double Mutant Mice**

Because aPKC double mutants showed a disturbed podocyte process network development, individual maturation stages were further analyzed by confocal immunofluorescence microscopy using the junctional marker Par3. In the comma-shaped body stage, Par3 localizes at the apical sited cell-cell contacts of the undifferentiated podocytes. During the s-shaped body stage, Par3 translocates toward the basal membrane together with cell-cell contacts. Simultaneously, endothelial cells migrate into the cleft between immature podocytes and tubule cells. At the capillary loop stage, Par3 localizes at the basolateral sited cell-cell contacts. Subsequently, primary processes and foot processes develop, followed by the formation of slit diaphragms with Par3 localizing to the slit diaphragm insertions (see Figure 3A for schematic illustration).8,10 In early developmental stages, no differences were detectable between individual mutant mice and controls (Figure 3, B–E, I–III), which might be explained by the fact that Cre expression under control of the NPHS2 promoter occurs later during the capillary loop stage.19 Congruently, at the late capillary loop stage, process formation and glomerular infolding were severely impaired in aPKC double mutant mice. Whereas Par3-labeled podocyte processes extended into the glomerulus resulting in a partition of capillaries in control mice (Figure 3BV and Supplemental Figure 3), the infolding process was almost absent in aPKC double mutant mice. Only sporadic and unbranched Par3 positive protrusions were visible in these mice (Figure 3EV and Supplemental Figure 3). In contrast, glomerular development was unaffected in aPKC–/– KO mice (Figure 3CV and Supplemental Figure 3), whereas Par3-positive stained extensions displayed less complex branching in aPKC+/– PcKO mice than in control mice (Figure 3DV and Supplemental Figure 3).

**aPKC Double Mutant Mice Exhibit a Disturbed Glomerular Maturation**

Glomerular maturation continues in mice during the first postnatal days with ongoing capillary formation and glomerular volume increase. To monitor this process, we analyzed glomeruli at P4 of surviving aPKC double mutant mice. Histologic analysis of kidneys at P4 revealed severe impairment in glomerular architecture of aPKC double mutant mice with capillary dilation, mesangiolysis, and mesangial hemorrhagia (Figure 4A). In transmission electron microscopy, aPKC double knockout podocytes displayed an undifferentiated morphologic appearance. Most podocytes lacked any processes and their cell bodies directly contacted the glomerular basement membrane (Figure 4B). The missing envwrapping of the glomerular capillaries by foot processes and basement membrane infolding was associated with capillary dilations and an impaired separation of the capillaries from the mesangium, accompanied with severe mesangiolysis. In contrast, control and aPKC–/– KO mice exhibited foot processes connected by slit diaphragms (Figure 4B), whereas broadened foot processes with narrowed filtration slits were detected in aPKC+/– PcKO mice (Figure 4B).
Immunofluorescence staining of P4 kidney sections for the mesangial cell marker desmin, the endothelial marker CD31, and the podocyte marker podocin confirmed capillary dilation and the loss of podocyte projections in aPKC double mutant mice, ultimately resulting in the formation of capillary aneurysms (Figure 5, A and B) and in ballooned glomeruli, which almost completely lacked any capillary partition.

Figure 4. aPKC double mutant mice display severely disturbed glomerular maturation. (A) Periodic acid–Schiff staining of P4 kidney sections of mice of the genotypes as indicated. Arrows mark mesangiolysis and mesangial hemorrhagia in aPKC double mutant mice. Asterisks mark tubular dilations with protein casts. (B) Ultrastructural analysis of P4 kidney sections reveals a regular foot process network in control and aPKCζ KO mice. In aPKCζ/PcKO mice, broadened foot processes with narrowed filtration slits (arrows) can be observed. In aPKC double mutant mice, podocytes (arrowheads) exhibit an undifferentiated appearance, sitting directly on the glomerular basement membrane. Asterisks indicate dilated capillaries, dotted line indicates the glomerular basement membrane, and white arrowhead indicates mesangiolysis. Scale bars, 20 μm in A; 2 μm in B.
Aberrant Expression of Slit Diaphragm Proteins and Incorrect Positioning of Centrosomes and Golgi Apparatus in aPKC Double Knockout Podocytes

We next analyzed the expression pattern of Par3, the slit diaphragm proteins nephrin and podocin, and the primary process marker detyrosinated α-tubulin by confocal laser microscopy. Under physiologic conditions, slit diaphragm proteins distribute in a linear pattern along the capillary wall. In contrast, a granular and cytoplasmic distribution of Par3, nephrin, and podocin was detected in aPKC double knockout podocytes (Figure 6, A–C). In addition, nephrin staining seemed to be reduced (Figure 6B) and aPKC double knockout

Figure 5. aPKC double mutant mice develop capillary aneurysms during glomerular maturation. (A) Frozen kidney sections of mice of the indicated genotypes at P4 are stained using antibodies against the mesangial marker desmin and the podocyte marker podocin and are subjected to confocal laser microscopy. Arrows indicate podocyte extensions to the mesangium, which regress during glomerular maturation in aPKC double mutant mice. (B) Frozen kidney sections of mice at P4 were stained using antibodies against the endothelial marker CD31 and the podocyte marker podocin. Arrows mark CD31-positive stained capillaries. In aPKC double mutant mice, massive aneurysms can be observed (asterisks). Scale bars, 5 μm.
Figure 6. Impaired expression of slit diaphragm proteins in aPKC double mutant mice. (A–C) Staining of frozen kidney sections at P4 for the slit diaphragm proteins Par3, nephrin, podocin, and the basement membrane marker nidogen. Arrows mark expression of the
podocytes nearly completely lacked detyrosinated α-tubulin (Figure 6D), indicating a compromised microtubular network and primary process formation. To test whether growth hormones like vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), which have a well documented role in glomerular maturation, are affected in aPKC double knockout mice, we performed in situ hybridization and immunofluorescence staining analysis for vascular endothelial growth factor A (VEGFA) and platelet-derived growth factor B (PDGFB) as well as their respective receptors. Supplemental Figure 4 indicates indeed a reduced glomerular expression of VEGFA and PDGFB during late glomerular maturation at P4 in aPKC double knockout mice, which might at least partially contribute to the observed aneurysm formation and mesangiolysis.

In neurons, localization of the centrosome and the Golgi apparatus precedes and determines the site of neuronal polarization and process outgrowth.\(^{20}\) In the comma-shaped body stage of the glomerulus, the centrosome and Golgi apparatus localize at the apical cell pole of podocytes (Figure 7, Supplemental Figures 5 and 6). Strikingly, unlike in tubular cells, where pericentrin (centросome marker) and giantin (Golgi apparatus marker) signals remain in an apical position, in podocytes these markers translocate to the basal compartment during transition from the s-shaped body stage and the capillary loop stage to the glomerulus (Figure 7, Supplemental Figures 5 and 6). Although the centrosome and the Golgi apparatus localized to the basal side of the nucleus in podocytes of control mice, these subcellular structures were not restricted to this localization in aPKC double mutant mice (Figure 8, Supplemental Figures 7 and 8).

**aPKC Is Required for Glomerular Maintenance**

To investigate whether aPKC is required not only for development but also for maintenance of the glomerular filtration barrier, aPKC\(^{ζ}\)/\(^{ζ}\)×flo TC×flo TC mice were crossed with NPHS2,rtTA:tetO.Cre mice to generate doxycycline-inducible podocyte-specific aPKC\(^{ζ}\) knockout mice. Administration of doxycycline to the drinking water between 5 and 7 weeks of age resulted in loss of glomerular aPKC\(^{ζ}\)/ expression, proteinuria, and glomerulosclerosis between 2 and 4 weeks after birth (Supplemental Figures 2B and 9).\(^{9,10}\) whereas these mice initially showed fully established foot processes connected by slit diaphragms at birth.\(^{10}\) We speculated that the second aPKC isoform, aPKC\(^{ζ}\), might partially compensate for the loss of aPKC\(^{ζ}\), resulting in the only mild developmental phenotype. To study the full effect of aPKC for podocyte differentiation and glomerular development, double mutant aPKC mice were generated.

**Podocyte Process Formation Requires aPKC Function**

We demonstrate that aPKC is required for podocyte differentiation and process network formation. The fundamental importance of aPKC for podocyte function is underlined by the fact that both isoforms are highly enriched in podocytes. Indeed, loss of aPKC\(^{ζ}\) appears to be partially compensated by aPKC\(^{ζ}\), suggesting that both isoforms can act synergistically. In agreement, aPKC\(^{ζ}\) knockout itself displays a slightly increased and transient albuminuria postnatally. In contrast, expression of only one aPKC isoform or different localization of the both isoforms have been reported in other tissues.\(^{21,22}\) Recently, it was demonstrated that aPKC\(^{ζ}\) and aPKC\(^{ζ}\) are dispensable for certain cell types because the double knockout of aPKC\(^{ζ}\) and aPKC\(^{ζ}\) in primitive and adult hematopoietic stem cells did not cause any obvious phenotype.\(^{23}\) In podocytes, aPKC seems not only to be important for podocyte maintenance and foot process assembly but also for the initial steps of process formation. To date, most published genetic and acquired injury models were reported to result in foot process effacement, but did not focus on the exact mechanism of podocyte process formation. For example, knockouts of other slit diaphragm proteins result in effaced, irregularly configured, or misguided foot processes, but not in such a severe failure of process formation. Mice lacking the slit diaphragm protein podocin reveal reduced number of foot processes with narrowed filtrations slits and lacking slit diaphragms.\(^{24}\) Knockout of nephrin results in irregular shaped foot processes without slit diaphragms.\(^{25}\) Nephr1 deficiency causes misguidance and effacement of foot processes.\(^{26}\) The effect of aPKC for podocyte differentiation might be even underestimated in our conditional mouse model, because NPHS2.Cre-driven deletion of aPKC\(^{ζ}\) does not occur before the capillary loop
Podocytes exhibit astonishing similarities with neurons. Both cell types are postmitotic and develop long microtubule-based protrusions cobbled with short actin-based foot processes (podocytes) or dendritic spines (neurons). Interestingly, in primary cultured neurons, the Par complex localizes to the tip of an immature neurite to specify the axon and establishes neuronal polarity by the regulation of the neuronal microtubule machinery. Disrupting the function of the Par complex prevents neurites to differentiate into either axons or dendrites. At later steps of neuronal differentiation, the Par complex is required for the morphogenesis of the actin-based dendritic spines. It is conceivable to speculate that aPKC-mediated regulation of the microtubule and/or actin machinery in podocytes, like in neurons, contributes to cell process formation. Furthermore, the site of axon outgrowth in neurons is defined not only by the Par complex but also by the localization of the centrosome and the Golgi apparatus. Interestingly, we could discern that the centrosome and the Golgi apparatus translocate from the apical cell pole to the basal site of the nucleus during normal podocyte development. Correct basal centrosome positioning appears to fail in aPKC-deficient podocytes, indicating that aPKC might be required to instruct podocyte polarity via the positioning of the centrosome and the Golgi apparatus. It was consistently shown that in migrating astrocytes aPKC is needed for the centrosome and Golgi positioning toward the leading edge, which was controlled by Cdc42. Recently, a podocyte-specific Cdc42 knockout has been shown to cause severe nephrotic syndrome and postnatal lethality in mice, indicating that Cdc42 and aPKC could indeed act in the same pathway in podocytes.

In summary, this study uncovers the fundamental role of aPKC for podocyte orientation, polarity, and process development.

**Podocyte Process Formation and Polarity Establishment Contributes to Glomerular Maturation**

Very little has been known on the contribution of podocytes to the development of the overall glomerular structure and capillary formation. This is partially due to the fact that, as mentioned above, most known genetic podocyte models still exhibit podocytes with an extending process network. Therefore, the aPKC double mutant mice offered a unique opportunity to study the influence on the glomerular development by podocytes that arrest at their development during the late capillary loop stage. In general, podocyte process formation...
starts at the capillary loop stage. Together with mesangial cells, extending processes and glomerular basement membrane appear to separate glomerular capillaries to form the immature glomerulus.

During perinatal glomerular maturation, arborization of surface infolding increases and further capillary lumina are formed, which are enwrapped by basement membranes and podocyte foot processes (Figure 10A). Because podocytes stop cell divisions at the capillary loop stage, the increase of glomerular surface area is associated with an increase of extensions of every single podocyte to cover the new shaped capillaries.

Here we demonstrate that the failure of aPKC double knockout podocytes to extend their process network results in a dramatic phenotype with a reduced number of capillaries.
and massive capillary aneurysm formation of the remaining capillaries (Figure 10B). Subsequently, microruptures of the aneurysmatic capillaries seem to occur, leading to a spillover of erythrocytes and plasma components into the mesangium causing the extensive mesangiolysis seen in \textit{aPKC}\textsubscript{mutant} mice. These observations indicate that podocyte processes trigger the glomerular basement infolding and the shaping of the glomerular capillaries. Due to the polarization and differentiation defects, impaired secretion of soluble factors, such as VEGF\textsuperscript{5}, might contribute to the capillary phenotype (Supplemental Figure 4) because VEGFA expression is reduced in \textit{aPKC}\textsubscript{deficient} podocytes. However, complete absence of VEGFA in the glomerulus causes a different phenotype with rather small or missing capillary loops and diminished numbers of endothelial cells that always lack fenestrations.\textsuperscript{33} However, glomerular aneurysm formation and ballooning were described in a few other mouse models. For example, failure of mesangial cells to invade the glomerulus in mice lacking either PDGFB or PDGF receptor \(\beta\) results in massively enlarged glomeruli without a capillary tuft but a single capillary lumen.\textsuperscript{34} In addition, the genetic disruption of the connection between mesangial cells and the glomerular basement membrane leads to a lack of capillary convolution.\textsuperscript{35}

Our model highlights the role of podocytes for glomerular development, indicating that the interplay of both the mesangial cells (from inside the glomerulus) and the podocyte process network (from the outer side) regulate the formation of the capillary tuft.

In summary, both \textit{aPKC} isoforms are molecular guideposts to orchestrate fundamental podocyte polarity programs, podocyte development, and podocyte maintenance. In addition, our data illustrate that an extending foot process network is required for capillary formation and glomerular maturation.

**CONCISE METHODS**

**Mice**

\textit{aPKC}\textsubscript{\(\alpha\)}-\textit{floxed} mice (\textit{aPKC}\textsubscript{\(\alpha\)\textsubscript{\textit{floxed}}}\textit{; NPHS2}\textit{rtTA}\textit{;tetO.Cre}) and \textit{aPKC}\textsubscript{\(\xi\)} knockout mice (\textit{aPKC}\textsubscript{\(\xi\)}\textsuperscript{\textit{mutant}}) were previously described.\textsuperscript{17,18} \textit{NPHS2.Cre}\textsuperscript{\(\dagger\)} mice,
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which express Cre under control of a podocyte-specific promoter, were kindly provided by Lawrence Holzman (Renal, Electrolyte, and Hypertension Division, University of Pennsylvania School of Medicine, Philadelphia, PA).19 aPKC\textsubscript{fl/fl} mice (aPKC\textsubscript{fl/fl} and NPHS2.Cre\textsuperscript{+/-}) were crossed to generate aPKC\textsubscript{double mutant} mice (NPHS2.Cre\textsuperscript{+/-};aPKC\textsuperscript{fl/fl}), podocyte-specific aPKC\textsubscript{cre} single knockout littermates (aPKC\textsubscript{cre};aPKC\textsubscript{fl/fl}), and NPHS2.Cre negative aPKC\textsubscript{KCN} knockout littermates (aPKC\textsubscript{KCN/-};NPHS2.Cre\textsuperscript{+/-}) were crossed with aPKC\textsubscript{PcKO} littermates (aPKC\textsubscript{PcKO}/aPKC\textsubscript{PcKO} or aPKC\textsubscript{PcKO/aPKC\textsuperscript{PcKO}+/+}). To generate doxycycline-inducible podocyte-specific aPKC\textsubscript{cre}-knockout mice (aPKC\textsubscript{cre}/aPKC\textsubscript{cre};NPHS2.PcKO/tetO.Cre), aPKC\textsubscript{cre}-floxed mice (aPKC\textsubscript{cre}/aPKC\textsubscript{cre}) were crossed with NPHS2.PcKO/tetO.Cre mice; tetO.Cre negative littermates served as the control. For the induction of aPKC\textsubscript{cre} deletion, mice received doxycycline hydrochloride (Sigma-Aldrich) via drinking water (2 mg/ml with 5% sucrose, protected from light) between 5 and 7 weeks of age. All animal studies were approved by the Committee on Research Animal Care, Regierungspräsidium Freiburg.

Urine and Serum Analyses
Urinary albumin and urinary creatinine were measured using a flurometric albumin kit (Progen) and an enzymatic colorimetric creatinine kit (Labor+Technik) following the manufacturers’ instructions. Proteinuria was expressed as milligram of albumin per milligram of creatinine. For Coomassie gel analysis, BSA standard (1, 5, 10, and 20 μg) and 1 μl of urine of mice of the indicated genotypes were separated by 10% SDS-PAGE. The gel was stained by Coomassie blue.

Morphologic Analyses
Kidneys were fixed in 4% paraformaldehyde or glutaraldehyde, embedded in paraffin or Epon, and further processed for periodic acid–Schiff staining or electron microscopy, respectively. For scanning electron microscopy, glutaraldehyde fixed kidney samples were dehydrated by sequential incubation in 70% ethanol, 80% ethanol, 90% ethanol, and 100% ethanol, followed by incubation in 50:50 ethanol/Hexamethyldisilazane. After incubation in 100% Hexamethyldisilazane, the solvent was allowed to evaporate. Samples were coated with Gold (Zeiss Semco Nanolab7, Polaron Cool Sputter Coater E 5100, Balzer Cpd 020) and imaged using a Leo 1450 VP scanning electron microscope.

Immunofluorescence Staining of Kidney Sections
Kidneys were frozen in optimal cutting temperature compound and sectioned at 6 μm (Leica Kryostat). The sections were fixed with 4% paraformaldehyde, blocked in PBS containing 5% BSA, and incubated for 1 hour with primary antibodies as indicated. After washing the sections with PBS for several times, fluorophore-conjugated secondary antibodies (Invitrogen) were applied for 30 minutes. Images were taken using a Zeiss laser scan microscope equipped with a ×63 water immersion objective.

In Situ Hybridization
Whole mRNA extracts from P1 mouse kidney served as a template for RT-PCR and subsequent cloning of fragments of the coding sequence and 3’-untranslated region. The following primers were used: mPKC\textsubscript{a} (5’-GGAGCTCAAGAGAGGAGGA-3’), mPKC\textsubscript{b} (5’-GAGCTCAGAGAGGAGGA-3’), mPKC\textsubscript{c} (5’-ACTAGCCAGCTGCTCAAGA-3’), mPKC\textsubscript{d} (5’-GCCAAATATGACGAGGTA-3’), and mVEGFA (5’-GAAAGGAAGATGAGGAAG-3’). PCR products were cloned into pbScript II KS (-), linearized, and transcribed with T3 and T7 RNA polymerases (Promega) to generate sense and antisense digoxigenin-labeled probes (digoxigenin RNA labeling mix; Roche Applied Science). Kidneys were fixed overnight at 4°C in 4% paraformaldehyde, embedded in paraffin, and sectioned at 8 μm. For mRNA detection, slides were treated with proteinase K, refixed with 4% paraformaldehyde, acetylated by using acetic anhydride (0.25% acetic anhydride in 0.1 M triethanolamine; Sigma-T-1377) and hybridized at 68°C in hybridization buffer (50% formamide, 50 μg/ml yeast RNA, 1% SDS, 50 μg/ml heparin, 0.1% probe). Stringency washes were performed with wash I (50% formamide, 5× SSC pH 4.5, 1% SDS) and wash II (50% formamide, 2× SSC). For detection, slides were incubated with alkaline phosphatase-conjugated antidigoxigenin antibody (Roche Applied Science) 1:3000 at 4°C overnight followed by BM purple staining (Roche Applied Science). Digital photographs were captured on an Axioplan2 microscope (Zeiss).

Isolation and Characterization of Mouse Glomeruli
Glomeruli were isolated using Dynabead perfusion and were glass-glass homogenized in lysis buffer (containing 20 mM 3-{[3-cholamidopropyl]dimethylammonio}-1-propanesulfonate and 1% Triton X-100).35 After centrifugation (15,000×g, 15 minutes, 4°C)
protein concentration was determined by Dc Protein-Assay (Bio-Rad). Equal amounts of protein were separated on SDS-page.

Antibodies
Antibodies were obtained from Millipore (anti-Par3 rabbit pAb, 07–330; anti-nidogen rat mAb, MAB1946; anti-WT1 mouse mAb, 05–73; anti-detyrosinated tubulin rabbit pAb, AB3201), Progen (anti-nephrin guinea pig pAb, GP-N2), Sigma (anti-α-tubulin mouse mAb, T6199; anti-β-Actin mouse mAb, A1978; anti-podocin rabbit pAb, P0372; anti-PDGFβ rabbit pAb, HPA01972), Abcam (anti-WT1 rabbit pAb, ab15249; anti-giantin rabbit pAb, ab24586; anti-pericentrin rabbit pAb, ab4448), BD Biosciences (anti-CD31 rat mAb, 550274; anti-pericentrin mouse mAb, 611814), Invitrogen (anti-ZO-1 mouse mAb, 339100), Dako (anti-desmin mouse mAb, M0760), and Cell Signaling (anti-PKCγ rabbit mAb, 9368; anti-aPKCα/β rabbit mAb, 2998; anti-PDGFRβ rabbit mAb, 3169; anti-VEGFR2 rabbit mAb, 2479). Polyclonal antibody specific for aPKCa/α was raised in rabbit by injection of aPKCa/α amino acid 184–234 and was described previously.22 Nuclear staining reagent (To-Pro-3, T3605) and fluorescein-conjugated secondary antibodies were obtained from Invitrogen.

Statistical Analyses
Data were expressed as the mean ± SEM. Statistical comparisons were performed using two-tailed t test if not stated otherwise. Differences with \( P<0.05 \) were considered significant.

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DISCLOSURES
None.

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Supplemental Figure 1: Double knockout of $aPKC_{\lambda/\lambda}$ and $aPKC_{\zeta}$ in podocytes results in severe albuminuria.

Follow up analysis of urinary albumin (n = 7-48 each group; * P < 0.05, ** P < 0.01, *** P < 0.001, compared to $aPKC_{\lambda/\lambda}$ PcKO; +, compared to $aPKC_{\zeta}$ KO accordingly; #, compared to control accordingly). Urinary albumin concentration at P0: control 60.2 mg/l, $aPKC_{\zeta}$ KO 96.3 mg/l, $aPKC_{\lambda/\lambda}$ PcKO 959.9 mg/l, $aPKC$ double mutant 9,351.2 mg/l.
Supplemental Figure 2: Histological analysis at P0 and P16.

(A, B) PAS staining of mouse kidney sections of the genotypes as indicated. Asterisks mark tubular dilations with protein casts in kidneys of newborn aPKC double mutant mice (P0) and in kidneys of aPKCλ/λ PcKO mice at P16. Arrows mark sclerosed glomeruli. Scale bars: 20 μm.
**Supplemental Figure 3: Impaired glomerular infolding in aPKC double mutant mice.**

Frozen kidney sections of newborn mice of the indicated genotypes (P0) were stained for the polarity protein Par3 and the basement membrane marker Nidogen. Arrows indicate surface invagination and enwrapping of the capillaries by the Par3 positive stained podocyte process network. In aPKC double mutant mice, surface infolding is severely impaired and Par3 displays a granular staining pattern. Scale bars: 5 μm.
Supplemental Figure 4: Glomerular expression of VEGFA, PDGFB and their respective receptors.

(A) In situ hybridization studies of kidneys of control and aPKC double mutant mice at P4. During capillary loop stage and in glomeruli during early maturation no significant differences in VEGFA expression could be detected (upper panel), while during late maturation glomeruli of aPKC double mutant mice displayed reduced VEGFA expression (arrows, lower panel). (B) Frozen kidney sections of control and aPKC double mutant mice at P4 were stained for VEGFR2 and the podocyte marker Nephrin. Asterisk marks aneurysm. (C, D) Reduced PDGFB and PDGFRβ expression during late glomerular maturation at P4. Arrowhead marks mesangiolysis. Scale bars: 20 μm in A, 5 μm in B, C and D.
Supplemental Figure 5: Translocation of centrosome and Golgi-apparatus during podocyte differentiation in the newborn rat.

Frozen kidney sections of newborn wildtype rat were stained using antibodies against the junction marker ZO-1 and (A) the centrosome marker protein Pericentrin (arrows) or (B) the Golgi-apparatus marker Giantin (arrows) respectively (developmental stages in A: top, comma-shaped body stage; middle, late s-shaped body stage; bottom, immature glomerulus; developmental stages in B: top, comma-shaped body stage; middle, late s-shaped body stage/early capillary loop stage; bottom, immature glomerulus). Scale bars: 5 μm.
Supplemental Figure 6: Translocation of centrosome and Golgi-apparatus during podocyte differentiation in the newborn mouse.

(A) Frozen kidney sections of newborn wildtype mice were stained using antibodies against the centrosome marker protein Pericentrin (arrows) and the polarity protein Par3 and were subjected to confocal laser microscopy (top, comma-shaped body stage; middle, s-shaped body stage; bottom, capillary loop stage). (B) Frozen kidney sections of newborn wildtype mice were stained using antibodies against the Golgi-apparatus marker protein Giantin (arrows) and the podocyte marker protein WT1 (top, comma-shaped body stage; middle, late s-shaped body stage/early capillary loop stage; bottom, late capillary loop stage). Scale bars: 5 μm.
Supplemental Figure 7: Aberrant positioning of centrosomes in αPKC double knockout podocytes.

(A, B) Frozen kidney sections of control and αPKC double mutant mice at P4 were stained for the centrosome marker Pericentrin and the podocyte marker Podocin. While in control mice centrosomes (arrows) are restricted to the basal pole of the cytoplasm, in αPKC double knockout podocytes aberrant localization (arrows) can be detected. Scale bars: 5 μm.
Supplemental Figure 8: Aberrant positioning of Golgi-apparatus in aPKC double knockout podocytes.

(A, B) Frozen kidney sections of control and aPKC double mutant mice at P4 were stained for the Golgi-apparatus marker Giantin and the podocyte marker Nephrin. While in control mice Golgi-apparatus (arrows) are restricted to the basal pole of the cytoplasm, in aPKC double knockout podocytes aberrant localization (arrows) can be detected. Scale bars: 5 μm.
Supplemental Figure 9: Ultrastructural analysis of glomeruli at P16.

(A) Scanning electron micrographs show differentiated podocytes at P16. While podocytes of control and aPKCζ KO mice have regular primary processes and foot processes, the surface of aPKCζ/ζ knockout podocytes is covered with microvilli, and no regular foot process network is detectable. (B) Transmission electron microscopy revealed foot process effacement (arrows) and podocyte microvilli and blebs (arrowhead) in aPKCζ/ζ PcKO mice. Scale bars: 10 μm in A upper panel, 2.5 μm in A lower panel, 2 μm in B.