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Altered Hippo signaling in polycystic kidney disease

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Abstract

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is characterized by progressive deterioration of renal function and formation of cysts, and is an important cause of end stage renal disease. Previously we showed that tubular epithelial injury accelerates cyst formation in inducible *Pkd1*-deletion mice. In these mice expression of the Planar Cell Polarity (PCP) component Four-jointed (*Fjx1*) is decreased during epithelial repair, while in control mice *Fjx1*-expression is increased and may be required during tissue regeneration. In cystic kidneys, however, *Fjx1* expression is also increased.

Besides a PCP component, Four-jointed is also implicated in the Hippo-signaling pathway. This pathway is involved in organ size control by regulating proliferation and apoptosis. The role of Hippo signaling, together with the opposing expression pattern of *Fjx1* during epithelial repair and at cystic stages, triggered us to investigate the activity of the Hippo pathway during these processes. Therefore, we examined its final effector molecule, the transcriptional co-activator Yes-associated protein (YAP) and observed that during tissue repair, YAP expression was not different between *Pkd1*-deletion mice and controls, i.e. during tissue regeneration YAP expression was increased and predominantly localized in the cytoplasm but normalized after tissue repair. At a later stage however, in cystic epithelia and epithelia of dilated tubules, strong nuclear YAP accumulation was observed, accompanied by up-regulation of the YAP transcriptional targets *Birc-3*, *Ctgf*, *InhbA* and *Fjx1*. Altered activity of the Hippo pathway was confirmed in renal tissues from human ADPKD and ARPKD patients, as well as in cystic renal tumors. Our data strengthen the concept that during epithelial repair Four-jointed is involved in PCP signaling, while in cystic kidneys it is related to Hippo-signaling and cyst growth.

Introduction

ADPKD is a systemic disease with cystogenesis as its major hallmark. Extra-renal manifestations are hypertension, cerebral aneurysms, and cysts in liver and pancreas.[1] In young adults only a few cysts can be detected ultrasonographically, whereas at middle age renal function declines and almost all normal renal parenchyma has been replaced by thousands of cysts and fibrotic tissue.
In most patients, ADPKD is caused by either a mutation in the *PKD1* gene, encoding the protein polycystin-1 (PC1), or a mutation in the *PKD2* gene, encoding polycystin-2 (PC2).[2-4] It is known that, in kidneys, the primary defect occurs in the epithelium and that a balanced expression level of these proteins is critical to maintain renal epithelial architecture. In renal epithelial cells deregulated *PKD1/2*-gene expression either by gene dose reduction, or by complete gene inactivation through somatic mutations, initiates cystogenesis.[5-7] Both PC1 and PC2 are expressed in several cellular compartments and form different multimeric protein complexes, in which they modulate several signaling pathways that in concert, control essential cellular functions such as proliferation, apoptosis, cell adhesion and differentiation.[8, 9] Disruption of any of these processes can lead to cyst formation, as demonstrated by a variety of mouse models.[10-14] Previously we generated an inducible *Pkd1*-deletion mouse model in which renal epithelial specific *Pkd1* deletion can be induced by the administration of tamoxifen. Recently we showed that tubular epithelial injury accelerates cyst formation in the adult induced *Pkd1*-deletion mice. This was not the result of uncontrolled cell proliferation, but probably due to altered integrity of the newly formed cells, rendering them more susceptible for cyst formation.[15] In that study we also showed that aberrant canonical Wnt and planar cell polarity (PCP) signaling as well are early changes after *Pkd1* deletion. During the repair phase of tubular epithelial injury, when PCP signaling is needed, renal mRNA expression of the PCP-pathway component Four-jointed (Fjx1), was increased in controls, but not in kidneys of the *Pkd1*-deletion mice. Here, the expression of *Fjx1* decreased after *Pkd1*-gene disruption and persisted at relatively low levels at pre-cystic stages. However, later on, in cystic kidneys *Fjx1* expression dramatically increased.
It has previously been shown that *Fjx1* is not only involved in PCP signaling but is also a regulator of the Hippo pathway, via a complex and currently not completely understood mechanism.[16] The Hippo pathway has extensively been studied in *Drosophila*, where it is involved in controlling cell proliferation and apoptosis.[17-19] In addition, an important role for the Hippo pathway has been suggested in the regulation of organ and tissue size control. Hippo pathway mutants show dramatic alterations in e.g. wing size.[17-20] The pathway is named after the core pathway component, the kinase Hippo. Via several phosphorylation steps eventually, the non-DNA binding transcriptional co-activator Yorkie (Yki) is phosphorylated and retained in the cytoplasm. This prevents Yki target genes from being transcribed. The core components of the *Drosophila* Hippo pathway are well conserved and also function as tumor suppressors in mammals in addition to regulating growth in terms of organ size control.[21]

The abovementioned functions of the Hippo pathway, and the increased expression of *Fjx1* in cystic kidneys, led us to hypothesize that altered Hippo signaling might also be involved in cyst growth. Therefore we studied the nuclear localization of the final effector molecule of the Hippo pathway, the Yki mammalian ortholog YAP, as well as expression of its down-stream target genes. We show that cysts display strong nuclear staining for YAP, while it is absent in pre-cystic tubules. We also show that the expression of a number of suspected mammalian YAP targets is up-regulated in cystic kidneys of *Pkd1*-mutant mouse models. During tissue-repair and at pre-cystic stages, however, Hippo-signaling was not differentially activated in *Pkd1*-mutants although *Fjx1* expression was reduced. In addition, we provide evidence that activation of the Hippo pathway is altered during cyst growth in human ADPKD and autosomal recessive polycystic kidney disease, ARPKD, as well as in cyst growth in renal cancer. Our data suggest a role for aberrant Hippo signaling as a mechanism for cyst growth in polycystic kidney disease.

Materials and Methods

Mice

The inducible conditional *Pkd1*-deletion (cKO) mice (tam-KspCad-CreERT2;*Pkd1*$^{del2\text{-}11/lox2\text{-}11}$) and tamoxifen treatment have been described previously. [22, 23] For the injury experiments, cKO mice with gene disruption at adult age (3 months) and controls were used. Renal tubular epithelial injury was induced using 15mg/kg 1,2-dichlorovinyl-cysteine (DCVC) i.p.[15] *Cre;Pkd1*$^{del2\text{-}11,lox}$ mice were treated with DCVC (cKO+DCVC) or vehicle (cKO-DCVC); as controls, *Pkd1*$^{del2\text{-}11,wt}$ and *Pkd1*$^{del2\text{-}11,lox}$ mice were treated with DCVC (control+DCVC).

In the PN40 series of cKO mice, the *Pkd1* gene was disrupted by tamoxifen administration at 3 consecutive days around day 40. Animals were sacrificed at 1, 2, 3 and 4 months upon tamoxifen treatment. Tissues were used for DNA- and mRNA-isolation or formalin-fixed for immunohistochemistry. Recombination efficiency was determined by eMLPA analysis according to Leonhard *et al.*[24]

The hypomorphic mouse model was described earlier by our group. [6] Mice were on a C57BL/6J genetic background. Control and cystic kidneys were isolated from 3-4 week old mice.

The local ethical animal experimental committee of the Leiden University Medical Center (LUMC) and the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture approved the experiments performed.

Human Tissue Samples of Normal, Cystic Kidneys and Renal Tumors

Renal tissues from ADPKD patients with end stage renal failure and controls were fixed in 40mM phosphate buffered 4% formaldehyde at room temperature for at least 24 hours, dehydrated and paraffin embedded. Control tissues were isolated from donor kidneys intended for

transplantation, but found not suitable for technical reasons. In addition normal appearing areas in renal tissue from renal tumor patients were evaluated. In total 4 control samples, 5 ADPKD kidneys (4 with known mutations in, or positive linkage to, *PKD1*), 2 fetal ARPKD kidneys and 5 patients with renal cancer were analyzed. Two were diagnosed with clear cell renal cell carcinoma (CCRCC), 2 with papillary renal cell carcinoma (PRCC) and 1 with renal mixed epithelial stromal tumor (MEST).[25] All human tissue samples were obtained following procedures approved by the LUMC medical ethical committee (institutional review board).

Immunohistochemistry

Formalin-fixed, paraffin-embedded kidney sections (4 µm) were stained with rabbit-anti-YAP antibodies (#4912, Cell Signaling, Danvers, MA, USA). Segment marker staining was performed on sequential sections using rabbit polyclonal anti-megalin (Pathology LUMC, Leiden, the Netherlands[26]), goat polyclonal anti-Tamm-Horsfall protein (uromodulin, Organon Teknika-Cappel, Turnhout, Belgium), and rabbit polyclonal anti-aquaporin-2 (Calbiochem, Amsterdam, The Netherlands. rabbit envision HRP (Dako, Glostrup, Denmark) or rabbit-anti-goat HRP (Dako) were used as secondary antibodies. Immune reactions were revealed using diaminobenzidine and counterstained with hematoxylin. See supporting information for a more detailed protocol.

Quantification of nuclear YAP in cysts

The percentage of tubules and cysts displaying ≥50% and 25-50% YAP-positive nuclei was calculated in injury-induced cKO and control mice at 5 and 10 weeks after DCVC treatment. Therefore YAP-positive and negative nuclei in tubules and cysts in 3-4 random, non-overlapping fields in the renal cortex of each mouse were counted. The tubular diameter and percentage of YAP nuclei was determined of 10 randomly selected tubules and/or cysts in each field.

Nuclei with strong or apparent nuclear staining were considered positive. The average tubular diameter in control mice was set to 1. The relative tubular diameter (RTD) was calculated for all tubules were divided into 4 groups; tubule (T) with a relative tubular diameter <1.25; dilated tubule (DT), 1.25≤ RTD <1.75; small cysts or cyst (C), 1.75≤RTD<3.5; large cyst RTD ≥ 3.5.

mRNA expression analysis

Kidneys were snap frozen in liquid nitrogen upon isolation and stored at -80°C until further processing. Total RNA was isolated from renal tissue homogenates using TRI-Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturers' protocol. cDNA was synthesized with Superscript III (Invitrogen, Paisley, UK). Quantitative gene expression analysis of *Birc-3*, *Ctgf*, *InhbA* and *Yap* was performed on a Light Cycler 480 (Roche Applied Science, Penzberg, Germany) using 2x Sybr-Green master (ROX) (Roche Applied Science) according to manufacturers' protocol. Data were analyzed according to the Pfaffl-method, taking PCR efficiencies into account.[27] The median expression in control+DCVC at 2 wks after DCVC treatment or wild types in case of the

hypomorphic mouse model was set to 1 and expression was normalized to *Hprt* expression. Expression of *Fjx1* was determined as previously described. [15] The detailed protocol can be found in the supporting information.

Statistical analysis

Statistical comparisons between groups concerning the expression of YAP targets were performed using one-way ANOVA with Bonferroni post-testing for cKO mice and controls. For B6 *Pkd1*^{nl,nl} mice and controls, an unpaired t-test or an unpaired Welch's corrected t-test in case of unequal variance was used. P-values <0.05 were considered significant.

Results

YAP expression alters during tissue-repair and cyst growth

We previously showed that adult conditional *Pkd1*-deletion mice (cKO) develop cystic kidneys, upon treatment with the nephrotoxicant 1,2-dichlorovinyl-cysteine (DCVC) while heterozygous *Pkd1*$^{del/+}$-mice or vehicle treated mice do not.[15] Because of the biphasic differential expression pattern of *Fjx1* in this model we propose a role for disturbed Hippo signaling in cyst formation. To validate this hypothesis, YAP expression was analyzed in renal sections of mice sacrificed at 1, 2, 5 and 10 weeks after DCVC-induced injury.

One week after DCVC treatment, epithelial repair is in its final phase. At this stage, increased cytoplasmic YAP was observed in those parts of the proximal tubules the inner cortex, that were most severely damaged by the DCVC, in both cKO as well as controls treated with DCVC, compared with vehicle treated cKOs (fig. 1A-C). The kidney cortex of vehicle treated cKOs only shows nuclear and cytoplasmic staining of YAP in collecting ducts and distal tubules (fig. S1 A-D). Collecting ducts in the medulla also show nuclear staining of YAP.

Two weeks after injury, tissue repair is completed and also the increase in cytoplasmic YAP, observed during epithelial repair, is no longer present, showing no differences compared to non-injured renal tissue (fig. 1D). The same expression pattern was also observed at 5 weeks after injury. However, 10 weeks after injury, when kidneys of DCVC treated cKOs show many cysts of proximal tubular origin[15] (fig. S1 F), strong nuclear accumulation of YAP is observed in cystic tubular epithelial cells (fig. 1E and fig. S1 E). In contrast, nuclear accumulation of YAP is only weak or absent in proximal tubules of vehicle treated cKO (fig. 1F) or DCVC treated controls (fig. S1 A-D), which show no cysts (controls) or only mildly dilated tubules (vehicle treated cKO's).

mRNA expression analysis in cystic kidneys of injury-induced cKO animals 10 weeks after injury, as well as 3 week old *Pkd1*^{nl,nl} mice, did not show significant differences in *Yap* expression levels compared with controls (data not shown).

Nuclear YAP is absent in pre-cystic kidneys but accumulates in cystic epithelia of different *Pkd1*-mutant mouse models

Next we examined the YAP expression in several *Pkd1*-mutant mouse models. cKO mice, in which the *Pkd1* gene was deleted from the renal epithelial cells at approximately 40 days of age, develop polycystic kidneys within 4 months. At 3 months dilated tubules and the first cysts derived from proximal tubules are observed. 4 months after the gene deletion cysts derived from all nephron segments are presents but the largest cyst are of proximal tubular origin. In these mice nuclear YAP is virtually absent in proximal tubular epithelial cells at pre-cystic stages while distal tubules and collecting ducts show nuclear and cytoplasmic YAP (fig. 1G), similar to wild-type animals. However, in cystic epithelia, strong nuclear localization of YAP was observed, although also a minority of the cysts was negative for nuclear YAP (fig. 1H).

Another model, the *Pkd1*^{nl,nl} mice, have reduced levels of *Pkd1* in all cells and show massive cystic kidneys 3 weeks after birth. The majority of these cysts originate from distal tubules or collecting ducts.[6] Also in cystic kidneys of these mice, nuclear YAP is present in cyst-lining epithelia of cysts derived from all segments including proximal tubules, although not in all cysts (fig. 1J-L). Age-matched wild type mice (fig. 1I), show the same phenotype as control kidneys shown in fig. 1F and S1A.

Cystic epithelia in human renal ADPKD, ARPKD and tumor tissues also show nuclear YAP

To extend the observation made in mouse models to the pathology in patients, we stained renal tissues from ADPKD patients and control kidneys for YAP. In a substantial number of cysts, strong nuclear YAP accumulation was observed in the cyst-lining cells (fig. 1M), although cysts without nuclear YAP were present as well. Nuclear YAP was also seen in fibroblasts in fibrotic tissue in cystic ADPKD renal tissue (not shown). No nuclear YAP was present in proximal tubular epithelia of human renal control tissues, while distal tubules and collecting ducts do show some nuclear YAP expression (fig. S2).

Since nuclear translocation of YAP might be a more common mechanism in cyst formation, we also examined cystic kidneys from autosomal recessive polycystic kidney disease (ARPKD) patients, as well as cysts associated with different types of renal tumors.

Similar to renal cysts in ADPKD, cystic epithelia in ARPKD renal tissues also show prominent nuclear localization of YAP (fig. 1N). However, very large cysts with extremely flattened epithelia do not show nuclear YAP accumulation.

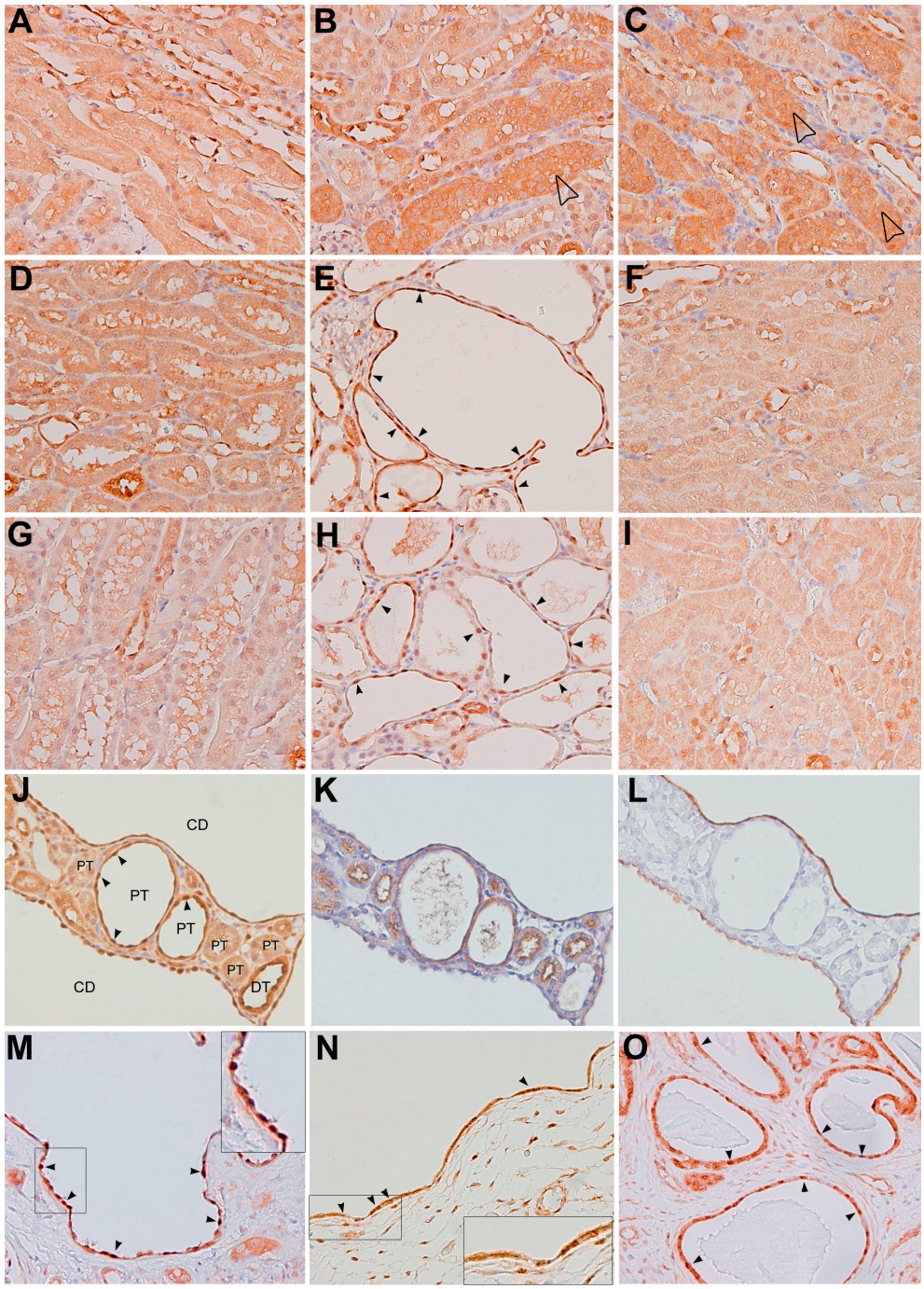
Furthermore, in cysts associated with CCRCC, PRCC and renal MEST, nuclear YAP was observed as well (fig. 1O and S1E-F). As in ADPKD renal tissue, nuclear YAP is also observed in fibroblasts in fibrotic tissue in ARPKD kidneys (fig. 1N) and renal tumors (not shown).

Staining of sequential sections with nephron segment markers, revealed that in ADPKD, ARPKD and renal tumor tissue most cysts already lost their marker. However, in both ADPKD and ARPKD, cysts, originating from distal tubules, collecting ducts, as well as proximal tubules (fig. S3A-D),

displaying nuclear YAP, are observed. CCRCC and PRCC are derived from proximal tubular epithelia while cysts in renal MEST originate from proximal tubular epithelia, distal tubule and loop of Henle (thin segment).[28-30]

In summary, robust nuclear accumulation of YAP is present in cystic epithelia derived from different segments of the nephron in various *Pkd1*-mutant mouse models, but not in pre-cystic and in control tissues. Accumulation of nuclear YAP was also observed in human ADPKD and ARPKD cystic epithelia, as well as in cysts associated with renal tumors, indicating that altered Hippo signaling is a common feature in cyst growth.

Figure 1: YAP expression in renal tissues. A-C shows YAP expression in inducible *Pkd1-deletion* mice (cKO) 1 week after DCVC treatment; (A) cKO–DCVC, (B) cKO+DCVC and (C) control+DCVC. D shows cKO+DCVC 2 weeks after DCVC treatment, E and F display respectively cKO+DCVC and cKO-DCVC 10 weeks after DCVC treatment. G and H show (G) non-cystic tubules and (H) cystic tubules in an inducible *Pkd1*-deletion mouse 3 months after gene disruption (at 40 days). I shows staining for YAP in 3 week old $Pkd1^{wt,wt}$ and J-L in $Pkd1^{nl,nl}$ mice. K and L stained for (I,J) YAP (K) megalin and (L) aquaporin 2. PT; proximal tubule as judged by anti-megalin staining, DT; distal tubule as judged by anti-Tamm Horsfall protein staining (not shown), CD; collecting duct as judged by anti-aquaporin 2 staining.
M, N and O show human renal tissue of (M) ADPKD, (N) ARPKD and (O) mixed epithelial and stromal renal tumor. Open arrowheads indicate increased cytoplasmic staining of YAP; closed arrowheads indicate examples of nuclear YAP staining. Original magnification 20x.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Dilated tubules and cysts show increased numbers of nuclei with nuclear YAP

To confirm the findings observed with immunohistochemistry described above, nuclear staining of YAP was quantified in the injury-induced cKO mice at 5 and 10 weeks after nephrotoxic injury, as this model develops cysts predominantly from proximal tubular origin. The percentage of tubules, dilated tubules cysts and large cysts containing >50% and 25-50% of YAP positive nuclei was calculated. Tubules and cysts derived from distal tubules or collecting duct, as judged by the cytoplasmic YAP staining, were excluded from the analysis.

Table 1 shows that 70% of the cysts from kidneys at 10 weeks after injury show ≥50% YAP-positive nuclei where only 10% of the tubules in time-matched controls show ≥50% YAP- positive nuclei. The percentage of large cysts with ≥50% YAP-positive nuclei is lower than in cysts (i.e. 58%).

In addition, the analysis revealed that in dilated tubules and tubules in cystic kidneys, the number of YAP positive nuclei is already increased (60% and 65% respectively) compared to tubules in control kidneys. However, it must be noted that these tubules, although showing a normal tubular diameter, show aberrant morphology, including enlargement of the tubular lumen and disappearance of brush border. Tubules in pre-cystic, injury-induced kidneys do not show this marked increase in YAP positive nuclei.

In summary, in injury-induced cKO mice morphologic aberrant tubules, dilated tubules and cysts show increased percentages of YAP positive nuclei.

Table 1: Quantification of nuclear YAP in tubules and cysts of injury-induced cKO mice

Group	Time after DCVC	Type	n	% of tubules and cysts with	
				≥50% positive nuclei	≥25< 50% positive nuclei
control + DCVC (n=2)	5 weeks	T	46	0	2
		DT	1	0	0
cKO + DCVC (n=3)	5 weeks	T	41	2	10
		DT	37	14	8
		C	2	0	100
Control +DCVC (n=3)	10 weeks	T	68	10	12
		DT	3	33	33
cKO +DCVC (n=3)	10 weeks	T	34	65	27
		DT	55	60	45
		C	30	70	13
		LC	12	58	17

T; tubule, <1.25 times normal tubular diameter, DT; dilated tubule, ≥1.25 < 1.75 times normal tubular diameter, C;cyst, ≥1.75 < 2.5 times normal tubular diameter, LC; large cyst ≥2.5 times normal tubular diameter.

Up-regulation of transcriptional targets of YAP

YAP is a transcriptional co-activator which can drive the expression of target genes. A number of Yki target genes have been identified in *Drosophila*. In mammals however, the targets of YAP are barely known. We investigated the expression of several "suspected" YAP targets that were chosen according to homology with *Drosophila* Yki target genes, or because of previous reports. We analyzed the expression of baculoviral IAP repeat-containing-3 (*Birc-3;* also known as *cIAP-1* and *cIAP-2*), inhibin beta-A (*InhbA)* and connective tissue growth factor *(Ctgf)* in the injury-induced adult *Pkd1*-deletion mice and the hypomorphic *Pkd1*^{nl,nl} mice. *Birc-3* is the homologue of *dIAP1,* a well established Yki target. *Ctgf* and *InhbA* have been reported as YAP targets in mice.[31, 32] In cKOs, renal expression of YAP targets was not increased 2 weeks after injury (fig. 2A-C). Even more, *Birc-3* expression is lower in vehicle treated cKOs relative to DCVC treated controls (fig. 2A).

In contrast, *Birc-3* and *InhbA* show significant up-regulation only at cystic stages of injury-induced cKOs compared to DCVC treated controls and vehicle treated cKOs (fig. 2A). Expression of these genes is also markedly increased in cystic kidneys of 3-4 weeks old *Pkd1*^{nl,nl} mice, as well as *Ctgf* which is not increased in cystic kidneys of DCVC treated cKOs (fig. 2C-F).

Besides regulating the Hippo pathway, *Fjx1* is also thought to be a transcriptional target of YAP.[18, 33, 34] We previously reported increased expression of *Fjx1* in cystic kidneys of DCVC treated cKO mice.[15] Also cystic kidneys of *Pkd1* ^{nl,nl} mice show up-regulation of *Fjx1* (data not shown).

In summary, three or four out of four "suspected" transcriptional targets of YAP were up-regulated in cystic kidneys of injury-induced cKO mice or hypomorphic *Pkd1* mutant mice, respectively. Thereby further supporting *Birc-3*, *InhbA* and *Fjx1* as targets of YAP, but also indicating a more complex regulation of *Ctgf* expression.

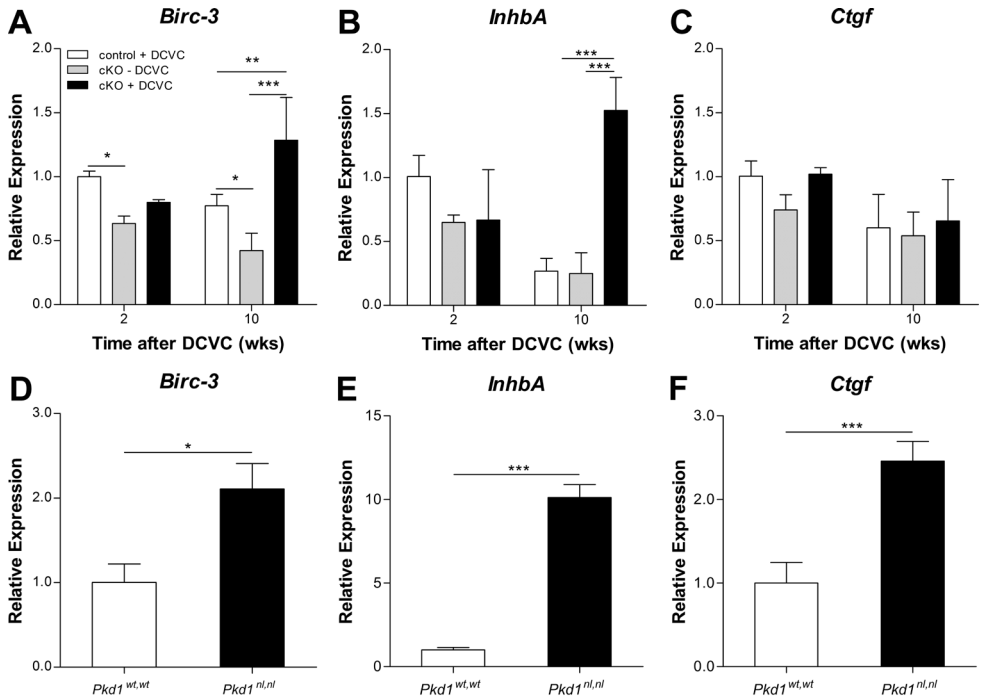

Figure 2: Relative expression of YAP targets in *Pkd1*-mutant mouse models. Expression of *Birc-3*, *InhbA* and *Ctgf* (A through C respectively) in controls treated with DCVC (white bars), and cKO animals treated with vehicle (grey bars) and with DCVC (black bars). Expression levels of controls+DCVC at 2 wks after injury have been set to 1. (D-F) Expression of the same genes in 3-4 weeks old $Pkd1^{nl,nl}$ mice and wild types; expression in wild types has been set to 1. * P< 0.05, **P<0.01, *** P<0.001

Discussion

The Hippo signaling pathway is emerging as an important pathway not only in organ size control but also in cancer. The product of the neurofibromatosis type II tumor suppressor gene (*NF2*), Merlin, is a regulator of the Hippo pathway. In addition, increased YAP expression and nuclear accumulation were shown in multiple types of human cancer. [35-38]

Here, we provide for the first time evidence for altered activation of the Hippo signaling pathway in cyst growth in ADPKD and other cystic diseases. In mouse models for ADPKD nuclear localization of YAP is only observed in cystic epithelia and dilated tubules but not in pre-cystic tubules. Nuclear YAP was detected in cysts derived from all segments. Although normal distal tubules and collecting ducts already show nuclear YAP, it is very likely that the Hippo pathway is also affected in cysts derived from collecting ducts and distal tubules, as $Pkd1^{nl,nl}$ cystic kidneys also show up-regulation of YAP targets.

Increased amounts of YAP-positive nuclei are observed in morphological aberrant tubules, dilated tubules and cysts but not in normal looking pre-cystic tubules. This suggests that aberrant Hippo signaling is not an initiating event in cyst formation but accompanies cyst growth.

Our findings, showing altered Hippo signaling during cyst growth, are in line with previous studies reporting renal cyst formation caused by disturbed Hippo signaling, e.g. deregulation of YAP in Zebrafish results in pro-nephric cyst formation. [39, 40] In addition, mice lacking TAZ (Wwtr1), the YAP ortholog, show renal cystic disease. [40-42] In one of the studies however, increased protein levels of polycystin-2 were reported as a result of TAZ deficiency. [40]

The Hippo pathway is also reported to be involved in the control of cell size. [43] Therefore, altered Hippo signaling may explain the observed hypertrophy of *Pkd1* knock-out cells, in addition to loss of control of the mTOR pathway. [44]

Together with Four-jointed, the PCP proteins Fat and Dachsous not only regulate PCP, but also regulate the Hippo pathway through a complex and not completely understood mechanism. *Fat4* knockout mice develop cystic kidneys and it has been postulated that disturbed PCP is the underlying defect.[13] However, its effect on the Hippo signaling pathway and YAP/TAZ translocation has not been addressed yet. It also remains to be investigated whether in our model increased expression of *Fjx1* in cystic kidneys is causing altered Hippo signaling or is the result of YAP driven transcription.

Nevertheless, our results further strengthen the concept that during epithelial repair Four-jointed is involved in PCP signaling while in cystic kidneys it is related to cyst growth. Decreased expression of *Fjx1* is a direct effect of *Pkd1* inactivation while increased *Fjx1* and YAP target expression can be regarded as an effect of altered signaling during cyst growth, an indirect effect of *Pkd1* inactivation.

Although both under and over-expression of YAP as well as TAZ deficiency, can result in cyst formation, in renal cystic disease it seems to be associated with an increase in nuclear YAP as shown in the different mouse models for ADPKD. This is confirmed by the results in patients with ADPKD and ARPKD, demonstrating nuclear localization of YAP in epithelia in the majority of cysts. This slightly heterogeneous pattern, also observed for a variety of signal transduction components, may be explained by the fact that cystic epithelial cells show bursts in proliferation. [23, 45, 46]

In this study we showed that several postulated targets of YAP are up-regulated in cystic kidneys, which is in agreement with nuclear localization of YAP in cystic epithelia. *Ctgf,* however, is up-regulated in cystic kidneys of hypomorphic *Pkd1* mutant mice which show first signs of fibrosis[47], while cystic kidneys of injury-induced cKO mice show no differential *Ctgf* expression. These data suggest that regulation of *Ctgf* expression is complex.

Transcriptional regulation by YAP is complex; nuclear translocation of YAP can differentially affect transcriptional regulation, depending on cellular context. For example, YAP mostly inhibits apoptosis whereas the YAP-p73 complex shows pro-apoptotic activity. [48] Furthermore, emerging

evidence shows that the availability of transcription factors that can bind to the co-activator YAP/Yki is dependent on tissue, cell type and probably cellular context. [21, 49] Interestingly, YAP/TAZ are transcriptional co-activators for a variety of transcription factors, e.g. TEAD, Glis3, SMAD, ErbB4-CTF, p73, Runx2, many of them implicated in polycystic kidney disease. [17, 21, 50-52] Also modulation of canonical Wnt-signaling, one of the signaling cascades altered in cystic epithelia, by Hippo signaling has been reported recently. [15, 50, 53, 54] By binding Dishevelled, cytoplasmic TAZ inhibits canonical Wnt signaling, while increased nuclear accumulation of TAZ results in increased nuclear β-catenin accumulation and induction of Wnt-target gene expression.[54]

Further research, to gain insight in the binding partners of YAP in different cell types and contexts, is required.

Increased nuclear YAP is not only observed in cystic epithelia in ADPKD, ARPKD but also cystic epithelia in different renal tumors. In these tumors, cystic epithelial expression of nuclear YAP is independent of the VHL status of the tumor. We also observed nuclear YAP in liver cysts derived from bile ducts of hypomorphic *Pkd1* mutant mice (not shown). Therefore, it is tempting to speculate that altered Hippo signaling is a general feature in cystogenesis.

Polycystic kidneys were elegantly called "neoplasia in disguise". [55] Indeed many pathways involved in neoplastic growth are also involved in cyst formation. It is not surprising that a number of drugs developed for the treatment of cancer have shown to be effective in attenuating cyst growth in PKD-mouse models. [56-60] Since altered Hippo signaling plays a role in tumorigenesis, the search for inhibitors of this pathway for cancer treatment can be beneficial for the hunt in the much needed treatment for polycystic kidney disease. [61] The critical role for TAZ/YAP in integrating and modulating signals from different morphogen signaling pathways, including Wnt-, Notch- and TGF-β-signaling further supports the idea that targeting the Hippo pathway could be effective in inhibiting cyst expansion and slowing down progression towards end-stage renal failure in PKD. [47, 53, 54] An interesting future experiment will be to explore whether *in vivo* absence of YAP during cyst growth, indeed, will attenuate cyst expansion.

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Author contributions statement

HH conceived and carried out experiments, analyzed and interpreted data, and generated figures. WNL carried out and analyzed experiments. AMvdW and SJK carried out experiments. HH, EH DJMP and MHB were involved in study design and data interpretation. HH, EH and DJMP were involved in writing the manuscript. All authors had final approval of the submitted manuscript.

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Supplemental figures

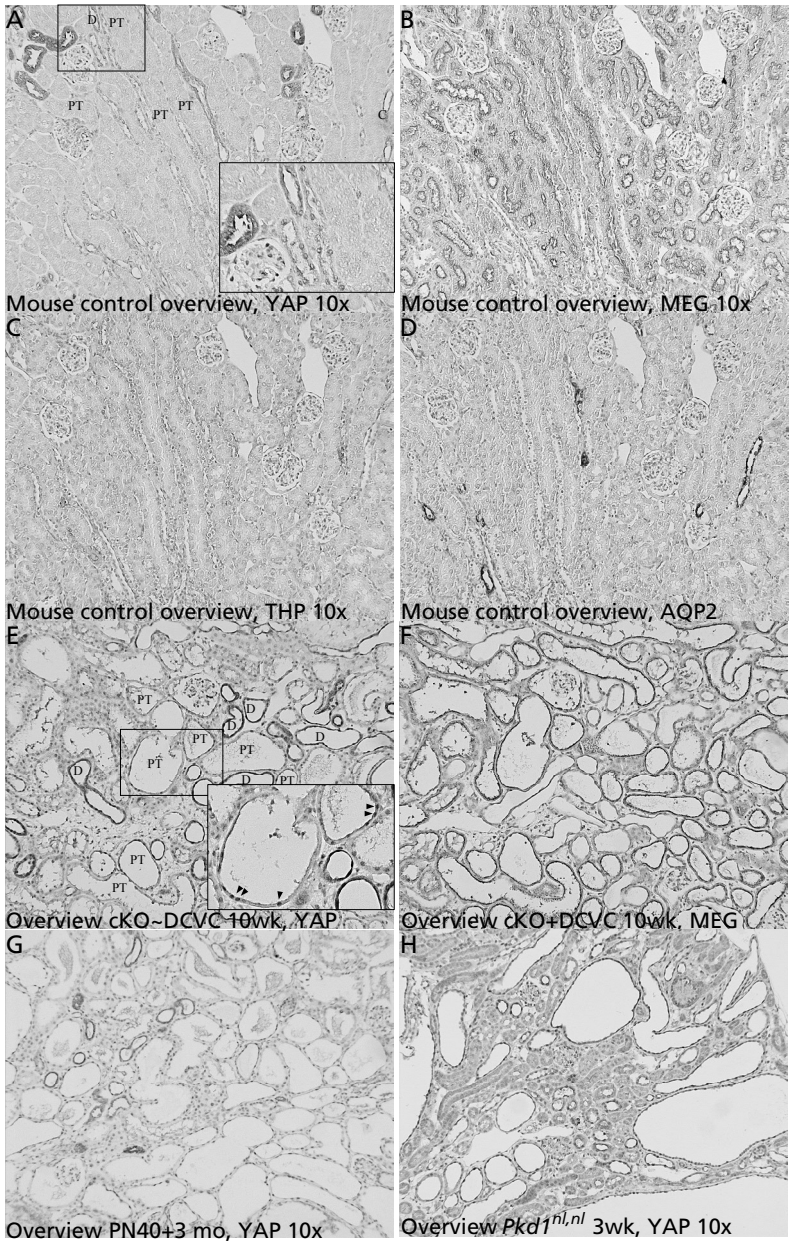

Figure S1: Overview of mouse renal tissues. A-D show control+ DCVC mice 10 weeks after DCVC treatment stained for; (A) YAP, (B) megalin, (C) Tamm-Horsfall protein and (D) aquaporin 2. E and F display an cKO+DCVC 10 weeks after DCVC treatment stained for (E) YAP and (F) megalin. G shows *Pkd1*-deletion mice 3 months after gene disruption at 40 days, stained for YAP, H shows 3 week old *Pkd1* ^{nl,nl} mice, stained for YAP.

PT; proximal tubule as judged by anti-megalin staining, DT; distal tubule as judged by anti-Tamm Horsfall protein staining, CD; collecting duct as judged by anti-aquaporin 2 staining. MEG; megalin, THP; Tamm-Horsfall protein, AQP2; aquaporin 2. Arrowheads show examples of nuclear staining.

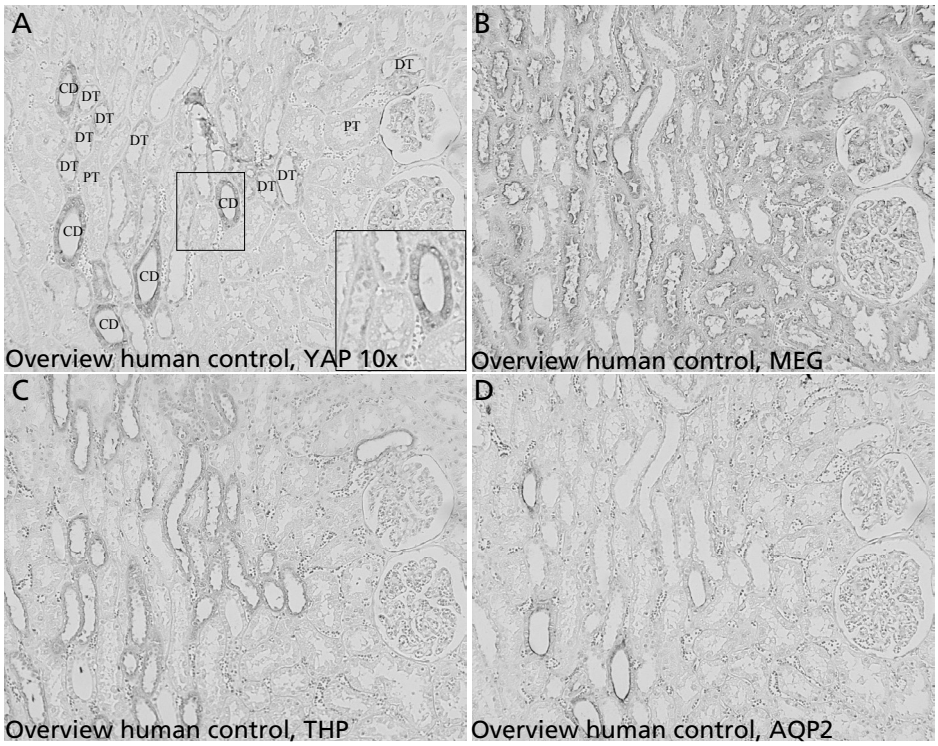

Figure S2: Overview of human renal control tissues. Normal renal tissue; area without irregularities obtained from patient with renal mixed epithelial and stromal tumor, stained for; (A) YAP, (B) megalin and (C) Tamm-Horsfall protein and (D) aquaporin 2. MEG; megalin, THP; Tamm-Horsfall protein, AQP2; aquaporin 2. PT; proximal tubule as judged by anti-megalin staining, DT; distal tubule as judged by anti-Tamm Horsfall protein staining, CD; collecting duct as judged by anti-aquaporin 2 staining.

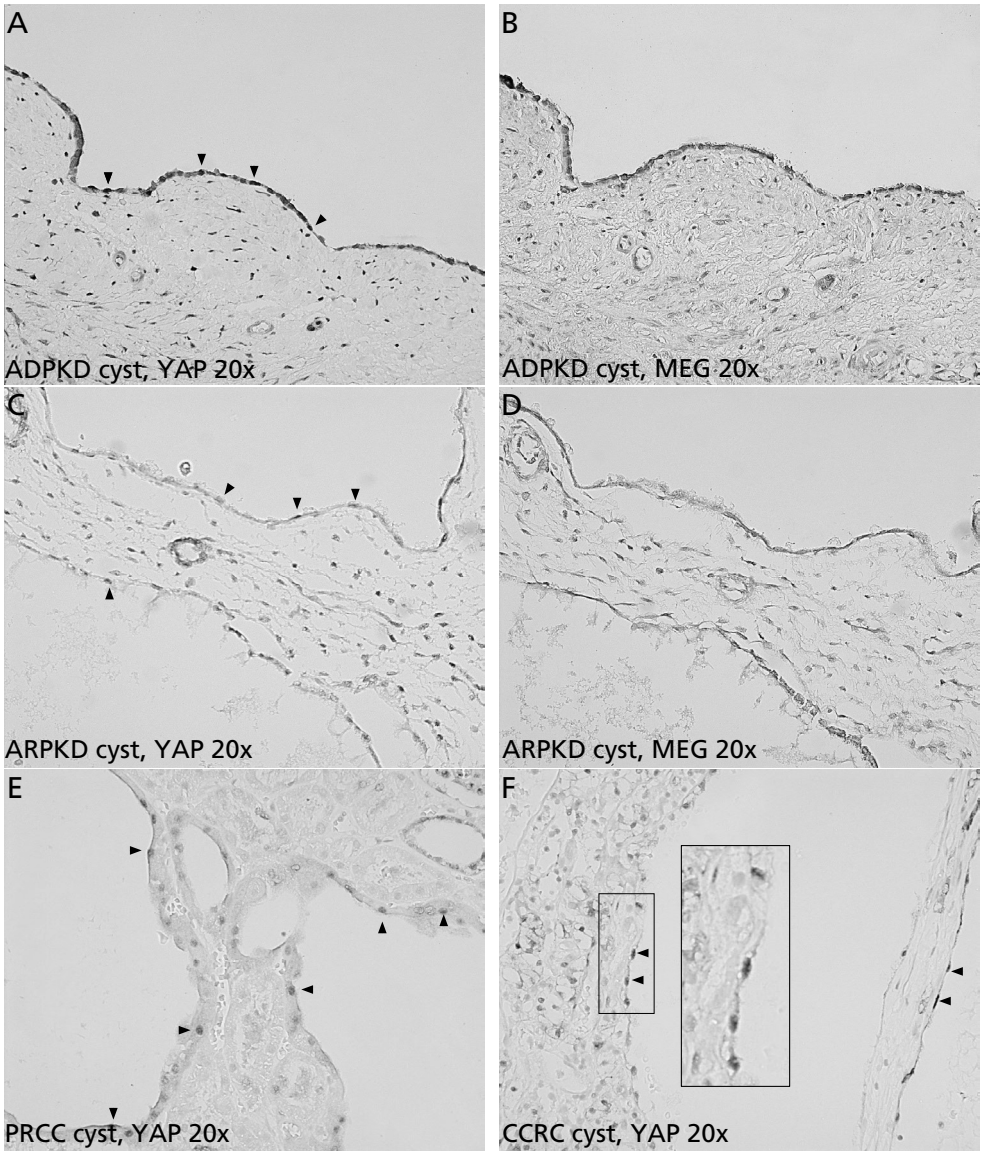

Figure S3: Cysts in human renal tissues. A and B show a cyst in ADPKD renal tissue sequentially stained for (A) YAP and (B) megalin. C and D show a cyst in ARPKD renal tissue sequentially stained for (C) YAP and (D) megalin. E and F show a cyst stained for YAP in a (E) papillary renal cell carcinoma (PRCC) and a (F) clear cell renal cell carcinoma (CCRCC) respectively. MEG; megalin, THP; Tamm-Horsfall protein, AQP2; aquaporin 2. Arrowheads show examples of nuclear staining.

Supplemental Information

mRNA expression analysis

Kidneys were snap frozen in liquid nitrogen upon isolation and stored at -80°C until further processing. Renal tissues were homogenized in PBS containing 1% 2-mercapto-ethanol using a MagNa Lyser Instrument (Roche). Total RNA was isolated from renal tissue homogenates using TRI-Reagent (Sigma-Aldrich) according to the manufacturers' protocol. cDNA was synthesized with Superscript III (Invitrogen). Quantitative gene expression analysis of *Birc-3*, *Ctgf*, *InhbA* and *Yap* was performed on a Light Cycler 480 (Roche Applied Science) using 2x Sybr-Green master (ROX) (Roche Applied Science) according to manufacturers' protocol. Primer sequences are given in table S1. *Hprt* was used as a housekeeping gene. Data were analyzed according to the Pfaffl-method, taking PCR efficiencies into account (Pfaffl,M.W. *Nucleic Acids Res.* **29**, e45 (2001). Expression was calculated relative to the median of control+DCVC at 2 wks after DCVC treatment or wild types in case of the hypomorphic

mouse model, and normalized to *Hprt* expression. Expression of *Fjx1* was determined as previously described (H.Happé *et.al. Hum. Mol. Genet.* **18**, 2532-2542 (2009).

Table S1: Primer sequences for real-time PCR

Gene	Accession	Primer sequence	
Birc-3	NM_007464.3	Fw	agagaggagcagatggagca
		Rv	tttgttcttccggattagtgc
Ctgf	NM_010217.2	Fw	ctgcagactggagaagcaga
		Rv	gatgcacttttgccccttctt
InhbA	NM_008380.1	Fw	tgcagacatgctgtggatct
		Rv	cttaactgtgagccagcaagc
Yap	NM_009534.2	Fw	ttccgatccctttcttaacagt
		Rv	gagggatgctgtagctgctc

Immunohistochemistry

Formalin-fixed, paraffin-embedded kidney sections (4 µm) were used. For YAP staining sections subjected to heat-mediated antigen retrieval procedure (10 mM citrate buffer, pH 6.0). After blocking of endogenous peroxidase activity for 20 min in 0.1% H_2O_2 in water, sections were pre- incubated for 1 hour with 5% normal goat serum. Subsequently sections were incubated over night with rabbit-anti-YAP (#4912, Cell Signaling) diluted 1:50 in 1% BSA in PBS. Segment marker staining was performed on sequential sections of injury-induced cKO and control mice at 10 weeks, of 3 weeks old *Pkd1*^{nl,nl} and wild type mice, and on human renal ADPKD, ARPKD and tumor tissues. To this end rabbit polyclonal anti-megalin (1:500 1h at room temperature, Pathology LUMC, Leiden, the Netherlands (Christensen, E.I. *et al. Eur. J. Cell Biol.* **66**, 349-364 (1995)), goat polyclonal anti-Tamm Horsfall protein (uromodulin, 1:500 1h at room temperature,

Organon Teknika-Cappel, Turnhout, Belgium), and rabbit polyclonal anti-aquaporin-2 (1:4000 o/n at 4°C, Calbiochem, Amsterdam, The Netherlands) diluted in 1% BSA in PBS were used, after to blocking of endogenous peroxidase activity.

Following incubation with rabbit envision HRP (Dako) or rabbit-anti-goat HRP (1:300 Dako), immune reactions were revealed using diaminobenzidine as a chromogen (DAB or Liquid DAB+ substrate chromogen system, DAKO) and counterstained with hematoxylin, dehydrated and mounted.