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SCF-SKP2 E3 ubiquitin ligase links mTORC1-ER stress-ISR with YAP activation in murine renal cystogenesis

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Running title: SKP2-YAP Promote Renal Cystogenesis

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ABSTRACT

The Hippo pathway nuclear effector Yes-associated protein 1 (YAP) potentiates the progression of polycystic kidney disease (PKD) arising from ciliopathies. The mechanisms underlying the increase in YAP expression and transcriptional activity in PKD remain obscure. We observed that in kidneys from mice with juvenile cystic kidney (jck) ciliopathy, the aberrant hyperactivity of mechanistic target of rapamycin complex 1 (mTORC1) driven by ERK1/2 and PI3K/AKT cascades induced endoplasmic reticulum (ER) proteotoxic stress. To reduce it by reprogramming translation, the protein kinase R-like ER kinase (PERK)-eukaryotic initiation factor 2α (eIF2α) arm of the integrated stress response (ISR) was activated. PERK-mediated phosphorylation of eIF2α drove the selective translation of activating transcription factor 4 (ATF4), potentiating YAP expression. In parallel, YAP underwent K63-linked polyubiquitination by SCF-S-phase kinase-associated protein 2 (SKP2) E3 ubiquitin ligase, a Hippo-independent, nonproteolytic ubiquitination that enhances YAP nuclear trafficking and transcriptional activity in cancer cells. Defective ISR cellular adaptation to ER stress in eIF2α-phosphorylation-deficient jck mice further augmented YAP-mediated transcriptional activity and renal cyst growth. Conversely, pharmacological tuning down of ER stress-ISR activity and SKP2 expression in jck mice by administration of tauroursodeoxycholic acid (TUDCA) or tolvaptan, impeded these processes. Restoring ER homeostasis, and/or interfering with the SKP2-YAP interaction represent novel potential therapeutic avenues for stemming the progression of renal cystogenesis.
**INTRODUCTION**

The primary cilium is a single, nonmotile, sensory organelle present at the cell surface. It senses mechanical and chemical environmental cues and conveys these external signals to the cell's interior (1). Linked fundamentally to the mitotic cell cycle, the primary cilium orchestrates organ development and tissue homeostasis.

In the kidney, the primary cilium in tubular epithelial cells localizes at the apical surface and translates physical events to intracellular signals that modulate cellular functions (2). As genomic and genetic approaches identify novel mutations in genes encoding proteins critical for ciliary function, the number of ciliopathies continues to expand (3). Inherited ciliopathies lead to the development of renal cysts, ultimately causing kidney damage and end-stage kidney disease (ESKD). They encompass autosomal dominant polycystic kidney disease (ADPKD), the most common genetic renal disease and an important cause of ESKD, autosomal recessive polycystic kidney disease (ARPKD), and nephronophthisis (NPH), a genetically heterogeneous group of kidney diseases representing the most common genetic cause of ESKD in children.

*PKD1* and *PKD2* mutated in ADPKD encode for the major ciliary-associated proteins polycystin 1 (PC1) and polycystin 2 (PC2), respectively (4, 5). PC1 is a large transmembrane protein while PC2 is a member of the transient receptor potential (TRP) superfamily. PC2 co-localizes with PC1 and functions to regulate intracellular Ca2+ levels and cell growth (6). In addition, PC2 is found in the same complex as protein kinase R-like endoplasmic reticulum (ER) kinase (PERK; gene name *EIF2AK3*) and eukaryotic translation initiation factor 2α (eIF2α, gene name *EIF2A*) on the ER membrane (7). The PERK-eIF2α arm is a critical component of the integrated stress
response (ISR) and plays a major role in the survival and adaptation of cells to stress by repressing global protein synthesis via PERK-mediated phosphorylation of eIF2α (8, 9).

Loss of PC1 or PC2 leads to hyperproliferation of the tubular epithelial cells, giving rise to cystogenesis and massive kidney enlargement (10). In contrast, decreased proliferation and apoptosis exemplify the NPH group of diseases, a discrepancy ascribed in part to altered Hippo signaling, an evolutionarily conserved pathway that regulates cell proliferation and death, and hence organ size (10). In mammals, it is composed of the Mammalian Ste20-like kinases 1/2 (MST1/2) and Large tumor suppressor 1/2 (LATS1/2) kinase cassette that acts to phosphorylate and inhibit the transcriptional regulator Yes-associated protein 1 (YAP) and its paralog Transcriptional co-activator with PDZ-binding motif (TAZ). MST1/2 phosphorylates LATS1/2, which in turn induces the phosphorylation of the effectors YAP (on Serine 127) and TAZ, targeting them for sequestration by 14-3-3 in the cytoplasm. Alternatively, phosphorylation at other sites leads to YAP/TAZ K48-linked polyubiquitination by SCF-β-TRCP E3 ubiquitin ligase and degradation by the proteasome [reviewed in (11)]. Decreased Hippo signaling results in YAP/TAZ dephosphorylation and translocation to the nucleus, primarily via importin 7 (12), a process crucial for their functionality as transcriptional coactivators. There, YAP/TAZ binds to Transcriptional enhanced associate domain (TEAD) transcription factors and activates YAP/TAZ target gene expression to promote cell proliferation, differentiation, and survival, with one of the major transcriptional target genes being MYC proto-oncogene (MYC) (13, 14). YAP/TAZ nucleocytoplasmic shuttling and MYC, have been widely implicated in the pathogenesis of cystic kidney disease (13-15), as
YAP expression and transcriptional activity increase in \textit{Pkd1}-null mouse kidneys and human ADPKD tissues (14, 16). In this context, it is proposed that ADPKD and ARPKD arise from decreased LATS1/2 phosphorylation and increased YAP/TAZ transcriptional activity, while conversely increased LATS1/2 signaling and reduced cellular proliferation underlie the NPH forms [reviewed in (10)]. Whether in fact, unbalanced Hippo signaling is responsible for these differences remains to be determined (17).

Multiple signaling pathways are implicated in the initiation and progression of ADPKD (14, 18, 19). As a master regulator of cell metabolism, mechanistic target of rapamycin complex 1 (herein mTOR) reprograms the metabolic changes needed for cells to exit the quiescent state and enter the cell cycle. The interplay between mTOR activity and the primary cilium in this process is extensively studied, and inhibition of mTOR activity is a potential therapeutic tactic for the treatment of ADPKD. However, two large-scale randomized clinical trials have failed to confirm the efficacy of this approach (20). Tolvaptan, a selective arginine vasopressin receptor 2 (AVPR2) antagonist that inhibits cAMP production in response to AVP, is presently the only drug approved for the treatment of ADPKD. However, its modest therapeutic efficacy and troublesome side effects (polyuria and rare cases of unexpected liver dysfunction), account for a high drop-out rate (21, 22). In search of more effective therapeutics with fewer adverse events, it becomes paramount to understand how these diverse signaling pathways integrate to initiate and potentiate the cystogenic process.

Here we show that in PKD, the prevailing increase in ER stress that ensues due to proteotoxicity activates the PERK-eIF2-ATF4 arm of the ISR, a branch of the unfolded protein response. The PERK-eIF2-ATF4 arm of the ISR is a key component of the unfolded protein response, which is a cellular system that senses and responds to the accumulation of misfolded proteins in the endoplasmic reticulum (ER). The activation of the PERK-eIF2-ATF4 arm of the ISR results in the phosphorylation of eIF2\(\alpha\), the inhibition of protein translation, and the stabilization of ATF4, which is a key transcription factor that regulates the expression of genes involved in the unfolded protein response. The activation of the PERK-eIF2-ATF4 arm of the ISR is a key event in the development of ADPKD, and its inhibition may provide a potential therapeutic target for the treatment of this disease.
protein response (UPR), raising YAP expression. Increased YAP nuclear transport and YAP/TEAD-mediated transcriptional activity unexpectedly arise from Hippo-independent, non-proteolytic YAP-K63-linked ubiquitination by SCF-S-phase kinase-associated protein 2 (SKP2) E3 ubiquitin ligase complex. Importantly, pharmacological tuning down of ER stress/ISR leads to decreased YAP and SKP2 expression and impaired cyst progression, thereby highlighting the potential clinical utility of targeting this pathway in PKD.
RESULTS

Renal epithelial cell proliferation, apoptosis, and fibrosis in jck kidneys

The spontaneously arisen renal cystic mouse model, juvenile cystic kidney (jck), arises from a homozygous G448V substitution in the highly conserved Regulator of Chromosome Condensation 1 (RCC1) domain of the Nephronophthisis 9 (NPHP9)/NEK8 protein (Nek8jck/jck, herein referred to as jck,) (23), a protein critical for cell cycle regulation and ciliary function (24). The jck mutation is transmitted in autosomal recessive mode, and contrary to other forms of NPH9 mutations and more like human ADPKD, cysts in jck kidneys are formed from multiple segments of the nephron leading to a continual decline in renal function and death by 20 weeks of age (25). EGF receptor (EGFR) overexpression and mislocalization, increased cAMP levels, activation of the RAS-RAF-MEK-ERK MAPK pathway, and sexual dimorphism in the progression of the cystic disease, with more aggressive disease in male mice, have also been reported (26). The jck missense mutation leads to YAP expression, YAP nuclear shuttling, and upregulation of YAP target gene transcription (27), findings that parallel those in Pkd1-null mouse kidneys and human ADPKD tissues (16). The molecular mechanisms underlying these observations however remain under active investigation. Therefore, first we set out to further assess the utility of this rodent PKD model for uncovering key molecular mechanisms that partake in these YAP actions and ultimately in the process of renal cystogenesis.

Enlargement due to the formation of multiple cysts distributed throughout the entire organ is the hallmark of jck kidneys, a process discernable even at one month of age, and progressing with advanced age (Supplemental Figure 1A). This feature
recapitulates the ADPKD phenotype and contrasts sharply with other forms of NPH where the kidneys are small. The expression of Aquaporin 2 (AQP2), a member of a family of highly selective transmembrane water channels, is decreased (Supplemental Figure 1B) (28). Normally located at the apical membrane of principal cells in the collecting duct, AQP2 transports water across the cell to regulate urine concentration. Its decreased expression leads to impairment in urinary concentrating ability, a phenomenon also observed in ADPKD and related to the modification of the medullary architecture by the ongoing cystic changes (29).

Ki-67, a marker of G1 to M phase transition, showed significant differences in its expression (Supplemental Figure 1C). While most wild-type renal tubular epithelial cells were Ki-67-negative, reflecting their mainly quiescent G0 status, most cells lining the cysts in jck kidneys remained Ki-67-positive, indicative of these cells not entering G0. Cyclin-dependent kinase 1 (CDK1) and MYC expression were also increased in jck kidney tissue (Supplemental Figure 1D), as in ADPKD, suggestive of the dysregulated cell cycle in these cells and increased cell proliferation.

Expression of molecular markers of renal interstitial damage [Nestin (NES), Vimentin (VIM)] (Supplemental Figure 1E), dedifferentiation [SRY-box transcription factor 2 (SOX2), POU class 5 homeobox 1 (OCT4)] (Supplemental Figure 1F), and epithelial-mesenchymal transition (EMT) [VIM, Actin alpha 2, smooth muscle (αSma), Intercellular adhesion molecule 1 (Icam), Vascular cell adhesion molecule 1 (Vcam), and Vitronectin (Vtn)] (Supplemental Figure 1G) were increased in jck kidneys. Consequently, histological changes notably increased interstitial collagen deposition and tissue fibrosis, were apparent (Supplemental Figure 1H).
TUNEL-positive apoptotic cells were observed among the cyst lining cells but they were more abundant among non-cystic renal tubular epithelial cells (Supplemental Figure 1I). Similar tissue distribution was observed by immunostaining for the pro-apoptotic Bcl-2 family member BCL2 like 11 (BIM) (30). These results differ from previously reported observations, where tubular epithelial cells had few apoptotic cells (26). The discrepancy could be attributed to the age of the animals used in our study, 4-month-old in our case rather than 2-month-old used in (26), at a point in disease progression where cyst enlargement is sufficiently prominent to negatively impact normal kidney architecture (31).

Altogether, these results support the contention that like in ADPKD, increased epithelial cell proliferation is a key hallmark of jck kidney phenotype contributing to cyst development, tubulointerstitial fibrosis, and apoptosis. These observations along with the availability and ease of manipulation of this rodent model, and the slow progression of PKD, validate its suitability for deciphering the molecular mechanisms of renal cystogenesis, with relevance to human PKD pathology.

**Increased intracellular signaling, mTOR activity, and ER stress in jck kidneys**

Next, we sought to validate in jck kidneys, in addition to MYC, the presence of other known mediators of the prevailing hyperproliferative state. In ADPKD, increased cAMP levels and activation of the RAS-RAF-MEK-ERK MAPK and AKT/mTOR pathways mediated in part by AVPR2 signaling and high EGFR activation, have been reported (18). Indeed, levels of phosphorylated extracellular signal-regulated kinase 1/2 (P-ERK1/2) and protein kinase B (P-AKT) were increased in jck kidneys compared to WT (Figure 1A, and B), accompanied by a significant rise in phosphorylated mTOR levels
mTOR positively controls protein synthesis through various downstream effectors, mainly by phosphorylating the eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) and the ribosomal protein S6 kinase B1 (RPS6KB1)/ribosomal protein S6 (RPS6). Indeed, an increase in P-EIF4EBP1 and P-RPS6 levels was observed in jck kidneys, consequent to the increased mTOR activity in the background of the jck mutation (Figure 1E, and F).

Prolonged mTOR activity and excessive rise in protein translation leads to accumulation of misfolded or unfolded proteins giving rise to proteotoxicity, ER stress, and activation of the ISR (9). ISR promotes both cellular repair and survival by reducing the load of unfolded proteins through attenuation of general protein synthesis, a mainly adaptive response initiated by the ER transmembrane receptor, PERK, one of the four arms of the ISR, and a branch of the UPR. PERK is a transmembrane kinase in the ER lumen where it associates with the molecular chaperone heat shock protein family A (Hsp70) member 5 (HSPA5 a.k.a. GRP78, BiP) (Figure 1G). Sensing the protein homeostasis (proteostasis) defect, GRP78 is titrated away from PERK by the unfolded proteins, allowing for the phosphorylation and activation of PERK to take place (Figure 1H, upper panels). Phosphorylated PERK in turn mediates the phosphorylation of eIF2α at serine 52, which blocks general protein translation (Figure 1H, lower panels). Paradoxically, while reducing protein load in the ER (32), P-eIF2α preferentially translates mRNAs encoding several short upstream open reading frames (uORF), such as Activating transcription factor 4 (Atf4) with the protein acting as a master transcription factor of stress-responsive genes to alleviate the stress, restore ER proteostasis, and promote adaptation and cell survival (Figure 1I). In jck kidneys, the expression of GRP78, having multiple
functions in relieving ER stress and maintaining cell viability (33), is increased (Figure 1G), primarily due to the concomitant and prevailing high ATF4 expression that potentiates its transcription.

If homeostasis in the ER cannot be re-established and the damage is irreversible, the IRS switches from the pro-survival to the pro-apoptotic mode (34). ATF4 induces the expression of protein phosphatase 1 regulatory subunit 15A (PPP1R15A, a.k.a. GADD34), a mediator of eIF2α dephosphorylation that allows the restoration of general protein synthesis during prolonged ER stress, eventually leading to cell death. Also, levels of the transcription factor DNA damage-inducible transcript 3 (DDIT3, a.k.a. C/EBP-Homologous protein, CHOP), implicated in ER stress-induced apoptosis, increase (35). Accordingly, CHOP expression is markedly enhanced in jck kidneys but absent from WT, suggesting that the pro-apoptotic mode of the ISR is initiated (Figure 1I).

Taken together, these findings indicate that, in the context of the jck mutation, a state of hyperproliferation of the cyst lining epithelial cells ensues, in part, via increased P-ERK1/2, P-AKT, mTOR activity, recapitulating rather precisely the ADPKD phenotype (26). Under circumstances of relentless mTOR activation and ongoing ER stress, the pro-apoptotic mode of ISR is triggered, ultimately increasing cellular dedifferentiation, collagen deposition, apoptosis, tissue damage, and fibrosis.

YAP/TAZ expression and nuclear localization in jck kidneys

The PERK-eIF2α-ATF4 arm of the ISR potentiates YAP expression, as ATF4 specifically binds to the YAP promoter following induction of ER stress (36). Therefore, we next sought to determine whether the observed increase in ISR activity in jck kidneys was
accompanied by comparable changes in the expression and transcriptional activity of the Hippo nuclear effectors YAP and TAZ. Levels of T-YAP/TAZ and P-YAP/TAZ were increased in jck kidneys compared to WT (Figure 2A, and B). Moreover, the ratios of P/T-YAP and P/T-TAZ were small (more so for YAP), indicative of increased YAP/TAZ activity, as the phosphorylated forms are targeted for sequestration/degradation. While YAP was marginally detectable by Western immunobLOTS in WT kidneys, as previously reported (37), YAP staining using immunohistochemistry was evident in several WT renal tubular epithelial cells and localized predominantly to the cytoplasm (Figure 2C). Immunostaining for YAP in jck kidney sections was more extensive, localizing almost exclusively to the nuclei of the cells lining the cysts, thereby substantiating its activation. In comparison, TAZ immunostaining in WT sections was both cytoplasmic and nuclear, and this pattern persisted in the epithelial cells lining the tubules and cysts in jck kidneys (Figure 2D).

The increased expression and nuclear localization especially of YAP would then be suggestive of increased transcriptional activity by the Hippo pathway effector. Indeed, the expression of YAP/TAZ target genes Cellular communication network factor 1 (Ccn1, a.k.a. Cyr61) and Cellular communication network factor 2 (Ccn2, a.k.a. Ctgf) was also increased (Figure 2E, F) in jck kidneys compared to WT, consistent with a rise in YAP-TAZ/TEAD transcriptional activity. Collectively, these observations indicate pronounced increases in YAP/TAZ expression and transcriptional activity in jck kidneys.

**Impaired ISR potentiates YAP activity and renal cystogenesis**
Although activation of the mTOR-ER stress-PERK-eIF2α-ATF4 pathway was evident in jck kidneys, its direct role in YAP activity and renal cystogenesis remains speculative. To this end, we next sought to evaluate its contribution by employing the genetic approach. Specifically, the germline knock-in mutation at the eIF2α phosphorylation site [eIF2α(S52A)] (38) was introduced into the jck background and renal cyst development was evaluated. The eIF2α(S52A) mutation decreases the phosphorylation of the α subunit of eIF2 and leads to chronic and unresolved ER stress by further augmenting general protein synthesis. Only mice heterozygous for the S/A knock-in mutation were used, as homozygotes die within 18 hr after birth (38).

Mice of the four genotypes WT, Nek8+/jck;eIF2α+/SA (double heterozygotes), Nek8jck/jck;eIF2α+/+(jck), and jck;eIF2α+/+(jck) were generated (Figure 3A), and renal cyst burden was determined by ultrasonography at three months of age (Figure 3B). Relative kidney volume/body weight ratio increased in the indicated genotype order, with the introduction of the hemizygous SA mutation into the jck background (jck;eIF2α+/SA) fashioning the most impactful positive change on renal cystogenesis. Mice heterozygous for both mutant alleles (Nek8+/jck;eIF2α+/SA) were not different from WT (Figure 3B, and C). The mean individual cyst area was also significantly greater in jck;eIF2α+/SA kidneys compared to jck (Figure 3D). Histologically, multiple microscopic and macroscopic cysts were observed affecting both the cortex and medulla (Figure 3E).

These structural changes were also reflected in pathophysiological outcomes, as the jck;eIF2α+/SA mice exhibited a significant increase in urine output compared to jck,
likely due to defective urine concentrating capacity arising from more aggressive tubular damage (Figure 4A) (39, 40). Phosphorylated mTOR and markers of ISR, ATF4 and CHOP, also increased significantly in \textit{jck;eIF2a}\textsuperscript{+/SA} mice compared to \textit{jck} (Figure 4B). Phosphorylated \textit{eIF2a} antagonizes mTOR activity (41), which may explain in part the higher mTOR phosphorylation in \textit{jck;eIF2a}\textsuperscript{+/SA} than \textit{jck} mice. Moreover, under stress, activated mTOR also stimulates the translation of ATF4 and CHOP (42, 43) and may also contribute to the upregulation of both proteins in \textit{jck;eIF2a}\textsuperscript{+/SA} mice having higher mTOR activity than \textit{jck}.

We then questioned whether potentiation of mTOR-ER stress-eIF2\textit{a}-ATF4 activity in response to the eIF2\textit{a} mutant has affected YAP/TAZ transcriptional activity. While levels of CYR61 protein in \textit{Nek8}\textsuperscript{+/jck};\textit{eIF2a}\textsuperscript{+/SA} samples were comparable to \textit{WT}, they increased in \textit{jck} and became even more pronounced in \textit{jck;eIF2a}\textsuperscript{+/SA} kidneys (Figure 4C). Similarly, MYC expression, while increased in \textit{jck} samples, was further potentiated by the single-allele eIF2\textit{a}(S52A) mutation (Figure 4D). Parallel increases in \textit{Cyr61} and \textit{Myc} mRNA levels were observed as well as for transcripts of several other YAP-target genes including \textit{Ctgf}, \textit{Ankyrin repeat domain 1} (\textit{Ankrd1}), and \textit{Natriuretic peptide B} (\textit{Nppb}), while \textit{Taz} expression, not a YAP target gene, was not significantly altered (Figure 4E-J).

Therefore, the inability of the eIF2\textit{a}\textsuperscript{SA} knock-in mutation to effectively block protein translation arising from the prevailing mTOR activity ultimately potentiates ATF4 and CHOP expression. This in turn, augments YAP transcriptional activity leading to progressive renal cystogenesis and tissue damage.

\textit{SCF-SKP2 E3 ubiquitin ligase regulates YAP activity independent of Hippo}
Although ATF4 augments YAP expression, we next questioned whether other signaling mechanisms could further potentiate YAP/TAZ actions. One obvious possibility is decreased Hippo signaling. We, therefore, sought to determine the status of LATS1/2 activity (phosphorylation) in jck kidneys. Unexpectedly, we observed higher levels of phosphorylated LATS1/2 in jck kidneys than in WT (Figure 5A), supporting neither our contention nor the prevailing paradigm (10). These results, while contrary to our expectations, were nevertheless consistent with our previous report that ATF4 stabilizes LATS1/2 (44).

How could we then reconcile these incongruent findings? Although the Hippo phosphorylation cascade causes cytoplasmic retention and inactivation of YAP, emerging evidence in cancerous cell lines suggests that YAP subcellular localization and hence activity is also regulated in a Hippo-independent manner (45). While polyubiquitination on defined lysine residues, notably on K48 and K29, is related to degradation by the proteasome, other polyubiquitinations (e.g. on K63, K11, K6, and M1) and monoubiquitinations regulate processes such as endocytic trafficking, inflammation, translation, and DNA repair [reviewed in (46)]. In cancer cells, YAP was recently shown to undergo K63-linked polyubiquitination by SCF-SKP2 E3 ubiquitin ligase at K321 and K497 sites. This post-translational modification, in sharp contrast to K48 polyubiquitination, potentiates YAP nuclear translocation and transcriptional activity (45).

We, therefore, examined jck kidney extracts to determine whether YAP was undergoing K63-linked polyubiquitination using an anti-YAP antibody for immunoprecipitation followed by western immunoblotting with an anti-K63-Ub
antibody. Although not a cancerous tissue, *jck* kidney extracts but not *WT* exhibited YAP K63-linked polyubiquitination (Figure 5B, left panel). Similar results were obtained using the anti-K63-Ub antibody for immunoprecipitation and the anti-YAP antibody for immunoblotting (Figure 5B, right panel). In contrast, YAP immunoprecipitation followed by western immunoblotting with an anti-K48-Ub specific antibody revealed decreased K48-linked polyubiquitination of YAP in *jck* kidneys compared to control (Figure 5C). A similar analysis for TAZ failed to demonstrate any differences in K63/K48-linked ubiquitination between *jck* and *WT* kidney extracts (Figure 5D, and E). Subsequent studies therefore focused exclusively on YAP.

Fluorescent immunostaining suggested co-localization of YAP and K63-Ub in the nuclei of epithelial cells lining the cysts in *jck* kidneys (Figure 5F), and that was further validated using laser scanning confocal fluorescence microscopy (Figure 5G). The Pearson’s correlation coefficient (*r*) for YAP/K63-ubiquitin co-localization was 0.939, highly supportive of the proximity association for the two proteins (Figure 5H).

To follow up on these rather unexpected observations, we employed an antibody against K63-linkage-specific polyubiquitin and demonstrated prominent immunostaining in cells lining the renal cysts in *jck* mice but significantly less so in *WT* tubular epithelial cells (Figure 5I). Since *SKP2* is reported to be a direct YAP target gene (47), we then examined *SKP2* expression in *jck* and *WT* renal tissue extracts. *SKP2* was undetectable in immunoblots of *WT* kidney extracts but highly expressed in *jck* (Figure 5J). *SKP2* also mediates K48-linked polyubiquitination and degradation of the tumor suppressor cyclin-dependent kinase inhibitor 1B (CDKN1B, herein p27).
that regulates G₀ to S phase transition (48). Consistent with our expectations, p27 expression was present in *WT* kidneys but was undetectable in *jck* given the prevailing high SKP2 levels.

In summary, these observations support the contention that in the setting of ER stress-ISR-ATF4 activation, increased SKP2 expression ensues. One outcome is YAP’s nuclear relocation and transcriptional activity via K63-linked YAP polyubiquitination. In parallel, SKP2-mediated K48-linked polyubiquitination targets p27 for proteolysis, thereby contributing to the dysregulation of the cell cycle and the observed hyperproliferative state of the cyst lining cells.

**Deciliation increases ER stress and YAP activity**

Primary cilia are sensors on the cell membrane that sense surrounding mechanical and chemical signals. YAP is now recognized as a mediator of mechanical cues provided by the cellular microenvironment (49). The observed activation of YAP in human ADPKD and mouse *Pkd1* kidney cysts (14), and now in *jck* kidneys, raises the possibility that sensing external signals is linked to YAP regulation. To this end, we employed Madin–Darby canine kidney II (MDCKII) cells that express a primary cilium (50) and first examined the role of laminar flow shear stress on YAP expression and transcriptional activity. Cells were plated in tissue culture plates and at the corresponding density in parallel-plate flow chambers (PPFCs) with either static culture medium fluid or experiencing unidirectional flow for 12 hours at 3 dynes/cm² laminar shear stress, maintained constant by the presence of an in-line dampener. The expression of YAP, its target gene protein MYC, and other cell proliferation markers was then assessed.
In the absence of flow, YAP levels in cells grown in the chamber were comparable to those in cells grown in tissue culture plates (Figure 6A). However, exposure to constant laminar flow markedly diminished YAP levels. In parallel, levels of YAP target gene effector protein MYC declined, as well as those of cellular proliferation markers CDK1 and Proliferating cell nuclear antigen (PCNA) following exposure to fluid shear stress. These changes were partly mitigated when laminar flow shear stress was not constant, i.e., in the absence of a dampener.

The above-described changes may arise from the bending of the primary cilium due to flow (50). Alternatively, pressure on the cell surface by fluid flow may underlay these alterations (51). To further examine a link between loss of ciliary function and YAP activation, we assessed the molecular alterations that follow the deciliation of MDCKII cells using chloral hydrate (2). Increasing concentrations of chloral hydrate potentiated levels of P-ERK1/2, P-AKT, P-RPS6, and CDK1 (Figure 6B). In parallel, levels of ATF4 and CHOP also increased following deciliation, while the ratio of P/T-YAP diminished, consistent with activation of YAP. Interestingly, chloral hydrate treatment also potentiated SKP2 expression in MDCKII cells.

Next, the effect of deciliation on YAP subcellular re-localization in MDCKII cells was examined using immunofluorescence. In untreated cells, YAP immunostaining localized mainly in the cytoplasm but following treatment with chloral hydrate, it relocated almost exclusively to the nucleus (Figure 6C). Similar observations were made using mouse embryonic fibroblasts (MEFs) prepared from WT embryos (Figure 6D). In contrast, in MEFs derived from jck embryos, YAP immunofluorescence localized mainly in the nucleus in the absence of chloral hydrate treatment.
Collectively, deciliation with chloral hydrate activates cellular signaling pathways that promote ERK1/2 and AKT phosphorylation, mTOR activation, and potentiation of the ISR effectors ATF4 and CHOP, leading to YAP dephosphorylation, SKP2 expression, and YAP nuclear translocation.

**YAP inhibition impairs cystogenesis in an in vitro model**

Given the aforementioned observations, we next asked whether attenuation of ER stress impairs cyst formation. To evaluate potential therapeutics targeting ER stress entails the availability of an in vitro model of cystogenesis to carry out the initial drug screening. Numerous in vitro models have been developed, each with its advantages and limitations (52). Here, we employed the MDCKII two-dimensional (2D) cyst-like model with forskolin stimulation (53, 54) and first assessed its utility as a screening tool.

Following stimulation of adenylate cyclase for 14 days with forskolin (10 μM) to increase intracellular levels of cyclic AMP, numerous multicellular 3D cystic-like structures appeared (Supplemental Figure 2A). As shown by immunofluorescent staining, strong YAP expression was detected in the cells lining these cyst-like structures. Co-treatment with verteporfin (1 μM), a potent YAP-TAZ/TEAD inhibitor blocking the transcription of target genes downstream of YAP (55), completely abolished the formation of these cystic structures (Supplementary Figure 2B, and C), and greatly reduced the expression of YAP/TAZ target genes *Ctgf* and *Cyr61* (Supplementary Figure 2D). In the presence of verteporfin, levels of P-ERK1/2, P-mTOR, and P-RPS6 decreased over time whereas T-ERK1/2, T-mTOR, and T-RPS6
were maintained. In parallel, YAP levels were also reduced significantly following veporfin treatment (Supplemental Figure 2E).

Altogether, these findings indicate that in this in vitro cystogenesis model, inhibition of YAP actions impacts negatively on parameters initiating and promoting the hyperproliferative state and cyst-forming capacity of forskolin treatment.

**Alleviation of ER stress impedes YAP activity in vitro**

Next, we proceeded to assess whether restoration of ER stress will also impact negatively on cyst formation following forskolin treatment. The naturally occurring hydrophilic bile acid derivative tauroursodeoxycholic acid (TUDCA) is a taurine conjugate of ursodeoxycholic acid (UDCA), a Food and Drug Administration (FDA)-approved drug for the treatment of primary biliary cholangitis. Several functions are ascribed to the action of TUDCA, primarily the alleviation of ER stress (56, 57). We, therefore, assessed the impact of TUDCA treatment on forskolin-induced cystic structure formation by cultured MDCKII cells.

Indeed, TUDCA, in a concentration-dependent fashion, significantly reduced the number of cyst-like foci that developed in the presence of forskolin (Supplemental Figure 3A, and B). The addition of TUDCA to the culture medium also decreased levels of P-mTOR and P-RPS6 (Supplemental Figure 3C), likely a consequence of ER stress attenuation by TUDCA. Treatment of MDCKII cells for 24 hours with TUDCA progressively increased P-YAP levels (Supplemental Figure 3D). Accordingly, transcript levels of YAP target genes *Ctgf* and *Cyr61* declined significantly (Supplemental Figure 3E).
Following treatment of MDCKII cells with forskolin, YAP immunofluorescence localized primarily in the nucleus. The concurrent addition of TUDCA however, shifted its subcellular location, as YAP-associated green fluorescence became exclusively cytoplasmic (Supplemental Figure 3F).

We then questioned whether TUDCA will also alter YAP subcellular localization in response to AVP, a powerful potentiator of cystogenesis in vivo (58) via cAMP and the only approved therapeutic target for patients with ADPKD. Following treatment of MDCKII cells with AVP (10 nM), YAP immunofluorescence once again became almost exclusively nuclear. Yet, the concurrent addition of TUDCA re-localized YAP to the cytoplasm (Supplemental Figure 3G). Interestingly, treatment of MDCKII cells with AVP also increased SKP2 expression (Supplemental Figure 3H).

Finally, the selective AVPR2 antagonist tolvaptan, which blocks cAMP production in response to AVP, presently the only drug approved for the treatment of ADPKD (21), increased YAP phosphorylation in a dose-dependent manner in MDCKII cells (Supplemental Figure 3I).

In summary, these in vitro observations underscore the contention that restoration of ER homeostasis mitigates the formation of cyst-like structures in response to forskolin, in part by promoting molecular avenues that potentiate YAP phosphorylation and its cytoplasmic localization.

**Alleviating ER stress reduces renal cyst growth in jck mice**

Based on the aforementioned in vitro observations, we next questioned whether TUDCA would be equally impactful as tolvaptan in vivo in slowing renal cyst
progression in PKD kidneys (59). We tested and compared in a 2-month trial the efficacy of these compounds individually using the jck murine model of PKD.

Starting at 1 month of age and for 2 months, jck mice were treated with either TUDCA or tolvaptan introduced into the regular chow. Before euthanasia, ultrasonography of the kidneys was performed (Figure 7A) and measurements of kidney volume relative to body weight were obtained (Figure 7B). Both TUDCA- and tolvaptan-treated mice demonstrated a 40-50% reduction in the calculated ratio which was associated with a 65-75% decrease in the average cyst area (Figure 7C), and with the gross anatomic comparison of the ex-vivo size of the procured kidneys (Figure 7D). Measurements of serum urea nitrogen levels demonstrated significant renal function preservation following treatment with TUDCA and tolvaptan, compared to regular chow (Figure 7E). On the other hand, urine osmolality in the three jck groups was significantly decreased due to the urine-concentrating defect attributed to peripheral resistance to circulating AVP (60). Treatment with tolvaptan however trended toward a greater decrease, likely due to its additional inhibitory effects on AVP signaling (Figure 7F) (21).

Histological assessment of kidney tissues further corroborated the ultrasonographic findings (Figure 8A). Indeed, the cyst number and size were significantly reduced in response to either treatment. Most of the amelioration was evident in the medulla, although a modest improvement was also noted in the renal cortex of treated mice. A reduction in collagen deposition and fibrosis was evident in the pericystic microenvironment in tissue sections of treated animals following staining with Sirius red and Masson’s trichrome, respectively. YAP immunostaining was drastically altered by TUDCA and tolvaptan treatment, as staining in cyst lining epithelial cells was
significantly diminished. In parallel, treatment was TUDCA or tolvaptan impacted profoundly on SKP2 expression whose immunostaining was greatly reduced. Notably, in \textit{jck} sections SKP2 cytoplasmic staining was more extensive, underscoring its pivotal role in promoting AKT activation (Figure 8B) (61).

To further decipher the mechanism by which these two disparate compounds impact positively on the PKD phenotype, we investigated their effect on the PERK-eIF2α arm of the ISR. Levels of P-eIF2α decreased following treatment with either drug, underscoring once more the central role of this pathway in renal cystogenesis (Figure 8C, and D). As anticipated, both drugs were effective in decreasing YAP expression in \textit{jck} kidneys, as levels of both T-YAP and P-YAP decreased following treatment (Figure 8E). Consequently, YAP activity was offset, as the level of \textit{Ctgf} transcripts normalized with either treatment (Figure 8F). In parallel, while increased SKP2 levels were observed in \textit{jck} kidneys and were associated with the near-complete absence of p27 and higher PCNA and MYC expression consistent with the existing hyperproliferative state, treatment with either TUDCA or tolvaptan profoundly impacted these parameters. SKP2 expression normalized, thereby corroborating the immunohistochemistry findings (Figure 8G). Consequent to the decrease in SKP2, levels of p27 and PCNA reverted to control, while MYC expression decreased following either of the treatments.

Overall, these results demonstrate a major amelioration of the PKD phenotype ensues TUDCA administration, similar to that seen by tolvaptan. Both compounds impact profoundly on ISR activity, ultimately decreasing YAP/SKP2 expression and YAP transcriptional actions.
SKP2 inhibition restores p27 expression and impairs YAP activity

The selective SKP2 inhibitor (SKPin) C1 is a small-molecule inhibitor of SKP2-mediated p27 degradation. It fits into a molecular surface pocket at the SKP2 interface with the adaptor protein CDC28 protein kinase regulatory subunit 1B (CKS1B), which is indispensable for the interaction and recognition of p27 by SKP2 and blocks p27 K48-linked ubiquitination (62).

One week following a single intraperitoneal administration of SKPin C1 to jck mice (63), p27 expression increased in whole kidney lysates (Figure 8H). In parallel, levels of P-YAP and T-YAP decreased, consistent with degradation (Figure 8I). These findings implicate SKP2 as a central contributor to cell cycle dysregulation and YAP actions that potentiate the hyperproliferative and inflammatory/fibrotic consequences associated with renal cystogenesis (15).

SKP2 expression increases following Pkd1 deletion

Numerous rodent models have been developed for the study of ADPKD (52), yet as of today no model perfectly recapitulates the human disease. It is proposed, therefore, that any observation or ADPKD treatment strategy should be tested in at least two different animal models, of which one should be based on a Pkd mutation (64). Hence, we set out to determine whether increased SKP2 expression is observed in a tamoxifen-inducible, kidney epithelium-specific Pkd1-deletion mouse model (65).

Here, inactivation of Pkd1 before postnatal day 13 results in severely cystic kidneys within 3 weeks (Early Onset, EO), whereas inactivation at day 14 and later results in cysts only after 5 months (Late Onset, LO).
We first examined YAP expression in kidneys from Cre- and Cre+ EO (induction P10-P11, termination P20) and LO (induction P27, termination P160) mice by immunohistochemistry (Figure 9A). In the Cre- kidney, YAP immunostaining was prominent in some tubular epithelial cells, albeit exclusively cytoplasmic in its subcellular distribution. In contrast, in the Cre+ EO kidney, tubular epithelial cells displayed cytoplasmic and nuclear staining, while nearly all cyst-lining epithelial cells stained positive for nuclear YAP. In the Cre+ LO sample, the majority of the tubular epithelial cells and most cyst lining cells expressed YAP that localized predominantly to the nucleus. These findings support the contention that in the absence of PC1, cystogenesis associates with YAP nuclear localization (65).

Next, we examined the phosphorylation status of mTOR and YAP and SKP2 expression in kidney tissue extracts from Cre- and Cre+ EO mice (Figure 9B). Levels of P-mTOR increased in the absence of PC1 compared to the control, as were those of T-YAP. Upregulation of YAP transcriptional activity was further substantiated by the rise in CYR61 expression in Cre+ EO kidney extracts. Lastly, immunoblots for SKP2 confirmed its expression in whole-kidney extracts from Cre+ EO kidney but not in extracts from Cre- controls. Collectively, these observations support the applicability of our findings in jck kidneys to the ADPKD model, as it closely recapitulates the molecular processes in the latter (Figure 9C).
DISCUSSION

The lack of effective treatment options for PKD calls for a fundamental re-examination of the molecular pathways that underpin the onset and progression of renal cystogenesis in this highly heterogeneous group of ciliopathies. Our findings have uncovered several unexpected outcomes with important clinical implications.

Ciliary dysfunction is a recognized cause of aberrant intracellular signaling (RAS-RAF-MEK-ERK, PI3K-AKT, and mTOR activation) (66). We now show that deciliation using chloral hydrate has similar effects on these molecular cascades, consequently inducing ER stress-ISR-ATF4 and SKP2 expression in kidney tubular epithelial cells while promoting increased YAP levels and nuclear translocation. Ciliogenesis and cell cycle are linked intimately to each other, and our observations suggest that perhaps cilium-mediated mechanosensation of extracellular signals (for example, fluid shear stress arising from glomerular filtrate flowing on the surface of renal tubular cells) may be pivotal for halting renal epithelial cell proliferation. PC1, localizing largely in the primary cilium as well as apical membranes, is proposed to act as a sensory molecule for fluid shear stress that transmits the signal from the extracellular fluid environment to PC2. The latter negatively regulates cell proliferation by enhancing PERK-dependent eIF2α phosphorylation (7, 67). Both molecules then provide baseline tonic inhibition of a signal effector that potentiates cyst growth when unchecked. We propose that one such major effector is YAP. In the absence of a functional sensor, aberrant intracellular signaling ensues and YAP expression and its nuclear actions rise, ultimately potentiating cyst formation (68). Further studies however will be required to add support or refute this supposition.
What underlies the increase in ER stress observed in PKD kidneys? In the setting of aberrant and uncontrolled EGFR and AVPR2 signaling amongst others (69), high mTOR kinase activity prevails. The phosphorylation of EIF24EBP and RPS6K/RPS6 impacts positively on protein synthesis, as the former impedes translational inhibition while the latter activates translation (70). The profound and relentless rise in protein translation has consequences, as it leads to proteotoxic ER stress. To reduce it by reprogramming translation, activation of the eIF2α arm of the ISR ensues. General translation is shut off and preferential translation of mRNAs such as the transcription factor ATF4 and its target gene products is initiated to restore ER homeostasis. In addition to this adaptive response, ATF4 translation is also activated by growth signals that stimulate mTOR independent of the ISR and eIF2α phosphorylation (42, 71). The contribution of this pathway to the observed high ATF4 levels however remains to be determined.

It is in this background setting that we employed two experimental approaches to establish the pivotal role of ER stress-ISR pathway on YAP action and ultimately on renal cystogenesis. The first was a genetic approach that introduced a heterozygous S/A knock-in mutation at the eIF2α phosphorylation site [eIF2α(S52A)] (38) into the jck background. The inability to adequately shut down general protein synthesis in this animal model, further augmented ER stress, YAP expression and activity, and renal cyst progression. The second was a pharmacological approach employing the administration to jck mice of either TUDCA which resolves ER stress and restores proteostasis or tolvaptan, an AVP R2 antagonist and the only approved therapy for ADPKD. While the two drugs affect seemingly disparate signaling pathways, they both converged to downregulate the eIF2α-ATF4 arm of the ISR, and decrease YAP.
expression and transcriptional activity. As such, our studies provide preclinical proof of principle that targeting ER stress is a compelling, previously unrecognized therapeutic strategy for PKD.

Interestingly, in PKD kidney not only are YAP levels increased but so is YAP transcriptional activity, all while the Hippo phosphorylation cascade is amplified and targeting YAP for cytoplasmic sequestration and degradation. Here, in the PKD kidney, a noncancerous tissue, we report that YAP is subject to a Hippo-independent, nonproteolytic K63-linked polyubiquitination by the SCF-SKP2 E3 ligase complex, described in cancer cells. This complex is known to serve primarily as a central component of cell cycle progression at the G1 phase by K48-linked ubiquitination and degradation of its primary substrate, p27 (72, 73). Apart from p27, SKP2 also initiates K48-linked ubiquitination and proteasomal-mediated destruction of FOXO1, CARM1, p21, p57, and other mediators of cell cycle regulation and diverse cellular functions (74). In addition to K48 ubiquitination of its proteolytic substrates, in cancerous cells, the SCF-SKP2 E3 ligase complex also mediates K63-linked ubiquitination that modulates the function of its target substrates (74). Prominent in this group are AKT and YAP (45, 75). Via this process, the ligase complex promotes the YAP-TEAD interaction, which in turn retains YAP in the nucleus (45), enhances its transcriptional activity (45), and contributes to the relentless hyperproliferation of cancerous cells. Our findings now indicate that PKD cyst epithelial cells also utilize this process to increase YAP transcriptional activity and thereby potentiate cyst growth and the progression of tubulointerstitial fibrosis by Myc (13) and Ctgf (15) overexpression, respectively.
Several potential mechanisms could explain the increased levels of SKP2 in jck kidneys. First, the PI3K/AKT pathway, which is activated in this setting, increases Skp2 transcription while promoting SKP2 protein stability via phosphorylation (73). Upstream regulation of mTORC2 by the PI3K/AKT axis also contributes to SKP2 stability, as the mTORC2 kinase directly phosphorylates SKP2 and prevents its degradation (76). Finally, SKP2 was identified recently as a YAP target gene (77), a pivotal observation that requires further consideration. Our demonstration that SKP2 levels are also increased in Pkd1-null kidneys points to the wider applicability of our findings in other forms of ciliopathy-related kidney cyst disease, rather than being viewed as a select consequence of the jck mutation.

It is of interest here to assess the commonalities between PKD and cancer which are provocative (78). While PKD does not predispose to cancer development (79), the extensive similarities between PKD and solid tumors are intriguing, so that PKD is often referred to as “neoplasia in disguise” (80). The similarities, underscored primarily by sustained proliferative signaling, are reinforced further by our observations of SKP2-mediated YAP-K63 ubiquitination. Owing to its vital role in cell cycle regulation, SKP2 plays a critical role in human cancers where its overexpression is associated with poor survival and adverse therapeutic outcomes (74). In normal cells, SKP2 localizes mainly in the nucleus, while during cancer progression, SKP2 translocates to the cytoplasm, an observation that strikingly parallels its localization (primarily cytoplasmic) in jck kidney tubular epithelial cells. The phosphorylation of SKP2 by AKT is the molecular switch that critically controls the formation, localization, and function of the SKP2-SCF complex (61).
Based on the aforementioned discussion, the development of strategies interfering with a number of these signaling pathways involved in the pathogenesis of PKD is envisioned. Having now underscored the importance of the mTOR-ER stress-ISR pathway in the onset and progression of PKD, consideration should be given to using compounds such as TUDCA, either alone or in combination with EGFR or SRC inhibitors, or rapalogs [reviewed in (81)], for the treatment of PKD (82, 83). A growing number of pre-clinical and clinical studies highlight the potential benefit of this naturally occurring bile acid in a variety of ER stress-driven pathologies (84).

Alternatively, SKPins, small molecule inhibitors of SKP2-mediated p27 degradation (62) with potent anti-tumor activities in vitro and pre-clinical mouse models (63) are now highlighted in our studies as a potential therapeutic approach for the treatment of PKD. The mechanism by which restoration of p27 expression by SKPin C1 impacts YAP phosphorylation remains to be determined. Studies aiming to address whether the inhibitor also interferes with SKP2-YAP interaction and/or YAP K63-linked polyubiquitination are ongoing. Nevertheless, based on our findings, the development of small molecules that interfere with SKP2 action in general or specifically with SKP2-YAP interaction would serve as potential therapeutic approaches for PKD. In the future, it may be of interest to also investigate the therapeutic efficacy of these compounds in a combinatorial fashion.

While rodent models based on the engineering of Pkd1 and Pkd2 are an obvious choice to study the pathophysiological mechanisms of ADPKD, these models present several limitations hindering their utility (52). The Nek8 jck mutation is transmitted in autosomal recessive mode, yet it recapitulates closely the ADPKD phenotype (26). Our analysis of these mice here, in addition to previous reports (26, 37, 85),
further validates their utility as a suitable rodent model for deciphering the molecular mechanisms of renal cystogenesis, and for testing new therapies. These mice therefore could be viewed as a suitable murine PKD model for dissecting these mechanisms and a valuable preclinical tool for evaluating the efficacy and safety of novel therapeutics (85).

In conclusion, our findings provide the necessary backdrop for a previously unrecognized molecular mechanistic framework in PKD kidneys that could be targeted pharmacologically. Restoring ER homeostasis, and/or interfering with the SKP2-YAP interaction represent innovative therapeutic approaches that aim to stem the progression of cyst growth in ciliopathy-related cystic kidney disease.
METHODS

*Mice and genotyping.* jck mice in the C57/BL6J background (stock no. 002561; The Jackson Laboratory) were housed in a temperature-controlled animal facility [21°C, 60 ± 5% humidity], with a 12:12-h light-dark cycle, and were allowed free access to autoclaved standard rodent chow (2018 Rodent Laboratory Chow; Harlan, Indianapolis, IN) and drinking water (tap water). Genotyping was performed according to the protocol provided by the supplier using tail genomic DNA. Only males were analyzed, as jck mice show sexual dimorphism in disease progression, with more aggressive disease in male mice. For preparation of *WT* and *jck* MEFs, embryos of the corresponding genotype were identified at E13.5 using yolk sac genomic DNA and MEFs were isolated, as previously described (86).

*Generation of jck;eIF2α+/SA mice.* eIF2α+/SA female mice in the C57BL/6J background were crossed with Nek8<sup>jck/jck</sup> (jck) males to obtain Nek8<sup>+/jck</sup>;eIF2α<sup>+/SA</sup> offspring. Male and female Nek8<sup>+/jck</sup>;eIF2α<sup>+/SA</sup> offspring were then mated to obtain jck;eIF2α<sup>+/SA</sup> male mice. The primers for the eIF2α<sup>SA</sup> mutation selection were: Forward: CACACACCCATTCCATGATAGTAAAAT and Reverse: CAATGTTGTAGACCCTGACAA-TGAAGG, as previously reported (38).

*Antibodies.* Antibodies used for immunohistochemistry and western immunoblotting:

- ATF4 (10835-1-AP), SKP2 (15010-1-AP) from Proteintech; CHOP (2895), P-YAP (13008 Ser127), YAP (14074), P27 (3686), MYC (9402), P-RPS6 (4858 Ser235/236), T-RPS6 (2217), P-mTORC1 (5536T Ser2448), T-mTORC1 (2983T), P-ERK1/2 (9101 Thr202/Tyr204), T-eIF2α (2103), P-AKT (9271), T-AKT (4691), EIF4FBP1 (2855), K48-Ub (8081), P-PERK (3179 Thr980), PERK (3192) from Cell Signaling.
Technologies; CYR61 (E-AB-14920), CTGF (E-AB_12339), from Elabscience Biotechnology Inc.; CDK1 (SC-54), T-ERK1/2 (SC-94) from Santa Cruz Biotechnology; α-tubulin (T5168), K63-Ub (05-1308) from Sigma-Aldrich; PCNA (MS-106-P1ABX), P-LATS1/2 (PA5-64591 Ser809/Ser872) from Thermo Fisher Scientific; Aquaporin2 (E-AB-30540), Oct4 (NB100-2379) from Novus Biologicals; Sox2 (ab97959), Vimentin (ab92547), Ki67 (Clone SP6), P-eIF2α(P51) (ab32157), BIM (ab32158), TAZ (ab224239) from Abcam; GRP78 (SPC-180) from StressMarq Biosciences; Nestin-1 (rat-401s) from DSHBU Iowa).

Peptides and chemicals. Forskolin (BioShop Canada) was used at 10 µM concentration. AVP was purchased from Sigma Aldrich and used at 10 nM concentration. SKP2 inhibitor (SKPin C1, S8652) was purchased from Selleckchem and was administered as a single IP injection (20 mg/kg) to 1-month-old jck mice. Animals were euthanized one week later and kidneys were procured for analysis.

Laser scanning confocal fluorescence microscopy. Paraffin tissue sections were deparaffinized, hydrated, and permeabilized using PBST (PBS containing 0.5% Triton X-100). Normal horse serum (Vector Laboratories) was used for blocking. Fluorescent labeling was done with anti-YAP-Alexa Fluor 594 (Santa Cruz Biotechnology, sc-271134) and anti-ubiquitin (linkage-specific K63)-Alexa Fluor 488 [EPR8590-448] (Abcam, ab192539) antibodies at a concentration of 1 µg/ml for both. Images were acquired using an LSM 800 Laser Scanning Confocal Microscope (Carl Zeiss) under 20X magnification. Pearson’s correlation coefficient was calculated with image analysis software (Volocity, USA, Version 6.5.1).
Treatment of jck mice with TUDCA and tolvaptan. TUDCA and tolvaptan were purchased from Best of Chemicals Sciences (USA). To verify structure and purity, NMR and LC-MS analysis was performed at the Drug Discovery Platform, Research Institute of the McGill University Health Center (>98% pure). For chow preparation, tolvaptan was first suspended in 0.5% hydroxypropyl cellulose (0.5%) followed by sonication for 20 min to obtain an emulsified form because of its very low water solubility and then added (0.1% wt/wt) to grounded chow powder (Teklad, Envigo). Following homogeneous mixing, the chow was cut to appropriate size pieces and thoroughly dried before administration. TUDCA was dissolved in water, added to grounded chow powder (0.2% wt/wt), and processed similarly. Male jck mice, starting at 1 month of age, were fed with either regular chow, tolvaptan-, or TUDCA-containing chow for 4 consecutive days followed by regular chow for the remaining 3 days of the week for 2 months. This treatment schedule avoided the attrition of animals observed when drug-containing chow was administered daily. At the end of two months (3-month-old), ultrasonography was performed, and serum and kidneys were procured for analysis.

Statistics. All data are presented as mean ± SEM. A Student’s unpaired t-test was used for the comparison of two groups. One-way ANOVA, followed by a Tukey-Kramer multiple-comparisons test was used for comparisons of more than two groups. Statistical differences with $P<0.05$ were considered significant.

Study approval. All animal procedures were reviewed and approved by the McGill University Institutional Animal Care Committee (protocols JGH-8121 to ACK and JGH-
The mice were maintained in accordance with university guidelines for the care and use of laboratory animals.
**AUTHOR CONTRIBUTIONS**

DKP, ACK, and MLL conceived the study. DKP, XB, YZ, and NAS conducted the experiments and acquired the data. DKP, ACK, and MLL analyzed the data. AEK contributed to work with eIF2αS52A mice, and provided reagents and invaluable scientific input. DKP and ACK wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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**CONFLICT OF INTEREST**

The authors declare that no conflict of interest exists.
REFERENCES


Figure 1
Figure 1. Aberrant mTOR activity, ER stress, and ISR in jck kidneys.

Representative expression of (A) P-ERK1/2, T-ERK1/2, and (B) P-AKT, T-AKT in jck kidneys relative to WT. (C) P-mTOR and T-mTOR levels in WT and jck kidneys. (D) P-mTOR expression using immunohistochemistry. Bars correspond to 200 μm. (E) Levels of P-mTOR downstream target proteins EIF4EBP1, and (F) RPS6. (G) Increased GRP78 expression, consistent with increased ER stress in jck kidneys. (H) Activation of the PERK arm of the ISR (P-PERK) and phosphorylation of eIF2α in jck. (I) Increased ATF4 and CHOP expression in jck kidneys compared to WT.
Figure 2
Figure 2. Increased YAP/TAZ expression in jck kidneys. (A) P-YAP and T-YAP expression in WT and jck kidneys. (B) P-TAZ and T-TAZ expression by western blot in WT and jck kidneys. (C) YAP and (D) TAZ immunohistochemistry in WT and jck kidney sections. Bars correspond to 50 μm. (E) YAP/TAZ target gene expression (Cyr61 and Ctgf) in jck kidneys compared to WT by quantitative real-time PCR, and (F) by immunohistochemistry. Data represent mean ± SEM. Differences between groups were analyzed for statistical significance by Student’s unpaired t-test; *P<0.05, **P<0.01.
**Figure 3**

A. The genetic interactions and resulting phenotypes of different genotypes:
- **Aa** = jck mutant allele
- **Bb** = eIF2α WT allele
- **a** = jck mutant allele
- **b** = eIF2α SA mutant allele

**AA/BB**
Nek8^{+/-};eIF2α^{+/+} (jck)

**X**

**Aa/Bb** (50% frequency)
Nek8^{+/-};eIF2α^{+/+} (jck)

**aa/Bb** (12.5% frequency)
Nek8^{+/-};eIF2α^{+/+} (jck)

B. Ultrasound images showing kidney morphology:
- **WT**
- **Nek8^{+/-};eIF2α^{+/+} (jck)**
- **jck**
- **jck;eIF2α^{+/+}**

C. Statistical analysis of kidney volume/body weight:
- Significant differences indicated by asterisks:
  - *******

D. Individual cyst area (mm²):
- Significant differences indicated by asterisks:
  - ********

E. Histological sections of kidneys:
- **WT**
- **jck**
- **jck;eIF2α^{+/+}**
**Figure 3.** ER stress potentiates cystogenesis in *jck* mice. (A) Mice heterozygous for the SA knock-in mutation at the eIF2α phosphorylation site [eIF2α(S52A)] were crossed into the *jck* background. Mice of the four genotypes (*WT, Nek8+/jck·eIF2α+/SA, Nek8jck/jck·eIF2α+/+, and Nek8jck/jck·eIF2α+/SA*) were obtained, as shown schematically. Expected percentages of offspring with the indicated genotypes are shown. (B) Ultrasonographic examination of renal cysts at 3 months of age (top panels). Shown are representative images of kidneys generated by 3D reconstruction (red images, lower panels) used to determine relative kidney volume. (C) Kidney volume relative to body weight measurements. (D) Individual cyst area measurements (mm²) from kidneys of the corresponding genotypes, as determined from ultrasonographic 2D images (3 mice from each group were used). (E) H&E-stained kidney sections from mice of the indicated genotypes. Data represent mean ± SEM. In panel C, differences between the groups were analyzed for statistical significance by one-way ANOVA followed by a Tukey-Kramer multiple-comparisons test. In panel D, a Student’s unpaired *t*-test was used for the comparison of the two groups. ***P<0.001, ****P<0.0001.
Figure 4
Figure 4. YAP transcriptional activity associates with renal cystogenesis (A) Urine output (μl of urine over 4 hours) by mice of the indicated genotypes. (B) Representative expression levels of P-mTOR and T-mTOR, ATF4, and CHOP in the kidneys of mice of the indicated genotypes (n=4 for each genotype) (C) CYR61, and (D) MYC protein levels in kidneys from the corresponding genotypes, as indicators of YAP target gene transcriptional activity (n=2 and 4, respectively, for each of the indicated genotypes). YAP target genes (E) Cyr61, (F) Myc, (G) Ctgf, (H) Ankrd1, and (I) Nppb mRNA expression by quantitative real-time PCR relative to β-Actin in kidney extracts from mice of the indicated genotypes (n=4 for each genotype). Data are representative of two independent experiments. (J) Taz mRNA expression in the kidneys of the indicated genotypes. Data represent mean ± SEM. Differences between the groups were analyzed for statistical significance by one-way ANOVA followed by a Tukey-Kramer multiple-comparisons test. *P<0.05, **P<0.01, ***P<0.001. ns, not significant.
Figure 5
**Figure 5. YAP K63-linked polyubiquitination and nuclear colocalization.**

(A) P-LATS1/2 expression in WT and jck kidney extracts. (B) Total YAP in kidney samples immunoprecipitated using a YAP antibody followed by immunoblotting with a K63-linked polyubiquitin-specific antibody (left panel). Total K63-Ub in kidney samples was immunoprecipitated using a K63-linked polyubiquitin-specific antibody followed by immunoblotting with a YAP-specific antibody (right panel). (C) Total YAP in kidney samples was immunoprecipitated using a YAP antibody followed by immunoblotting with a K48-linked polyubiquitin-specific antibody. (D) Total TAZ was immunoprecipitated using a TAZ antibody followed by immunoblotting with a K63-linked polyubiquitin-specific antibody. (E) Total TAZ in kidney samples was immunoprecipitated using an antibody against TAZ followed by immunoblotting with a K48-linked polyubiquitin-specific antibody. Results from two different kidney samples from each of the two genotypes are shown. (F) Representative immunofluorescence micrographs of jck kidney sections, showing K63-Ub and YAP co-localization in the nucleus. Upper panel: Immunostaining with Ub-K63 fluorescein (FITC)-specific antibody (green); middle panel: immunostaining with a YAP-specific rhodamine antibody (red); bottom panel: merging of K63-Ub-FITC with YAP-rhodamine. (G and H) Laser scanning confocal fluorescence microscopy of jck kidney sections using DAPI, anti-YAP Alexa Fluor 594, and anti-K63-Ub Alexa Fluor 488. The Pearson correlation coefficient \( r \), a measure of the strength of the linear relationship between two variables, YAP and K63-Ub on the left panel \( (r = 0.939) \), and DAPI and K63-Ub on the right panel \( (r = 0.895) \), are indicated. (I)
Immunohistochemical localization of K63-linkage specific ubiquitinated proteins in kidney sections. (J) SKP2 and p27 expression in WT and jck kidneys.
Figure 6
Figure 6. Deciliation induces ER stress-ATF4, SKP2 expression, and YAP nuclear localization. (A) YAP, CDK1, PCNA, and MYC expression in MDCKII cells cultured either in a tissue culture (TC) dish or in a parallel-plate flow chamber (PPFC) unexposed or exposed to steady unidirectional laminar fluid shear stress (3 dyn/cm²) in the presence or absence of the dampener. (B) P/T-ERK1/2, P/T-AKT, P/T-RPS6, CDK1, ATF4, CHOP, P/T-YAP, and SKP2 expression following treatment of MDCKII cells with increasing concentrations of chloral hydrate to induce deciliation. (C) YAP immunofluorescence microscopy of MDCKII cells without and with chloral hydrate treatment (4 mM). DAPI fluorescence demarcates the nuclei. (D) YAP immunofluorescence in WT MEFs before and following chloral hydrate treatment (left panels). YAP immunofluorescence in jck MEFs (right panels).
Figure 7
Figure 7. TUDCA and tolvaptan alleviate ER stress and slow renal cyst growth in jck kidneys. (A) In vivo ultrasonography of kidneys from WT and jck mice fed regular chow or chow containing TUDCA or tolvaptan. Yellow arrows indicate cysts. Shown in red are representative images of kidneys generated by 3D reconstruction used to determine relative kidney volume. (B) Kidney volume/body wt ratio for WT, jck, and jck treatment groups. (C) Average cyst area in mm² in control and two treatment group kidneys. Cyst content was assessed from 2D images at the level where the renal artery can be identified. (D) Representative gross morphology of kidneys (arrows) procured from each group at 3 months of age. (E) Serum urea nitrogen levels. (F) Urine osmolality following a 4-hour fast with only access to water. Number of animals used for each experiment are indicated by the symbols shown on the respective graph column. Data represent mean ± SEM. Differences between groups were analyzed for statistical significance by one-way ANOVA, followed by a Tukey-Kramer multiple-comparisons test; *P<0.05, **P<0.01, ***P<0.001; ns, not significant.
**Figure 8.** SKP2 plays a central role in renal cystogenesis. (A) Representative H&E, Sirius Red (dark red indicating collagen), and Trichrome staining (blue indicating fibrosis), YAP, and SKP2 immunostaining in kidney sections from control and treatment groups. (B) Higher magnification of SKP2 immunostaining in WT and jck kidneys demonstrating its differential subcellular distribution. Bars indicate 50 μm. (C) Representative western immunoblotting for P/T-eIF2α. (D) Graphic representation of the quantified P/T-eIF2α ratio derived from western blots using four kidney samples for each group. (E) Representative western blot for P/T-YAP expression in kidneys from control and treatment groups. (F) Graphic representation of Ctgf expression using quantitative real-time PCR in the four kidney samples examined for each group using β-Actin as internal control. (G) Representative western blot analysis for SKP2, p27, PCNA, and MYC in kidneys from each of the groups. (H) p27, and (I) P/T-YAP levels as determined by western blot in two representative kidneys from jck mice, treated with vehicle or the SKP2 inhibitor SKPin C1. Number of animals per treatment group used for each experiment is represented by the number of points shown on the respective graphs. Data represent mean ± SEM. Differences between groups were analyzed for statistical significance by one-way ANOVA, followed by a Tukey-Kramer multiple-comparisons test; *P<0.05, **P<0.01, ***P<0.001.
Figure 9
Figure 9. YAP and SKP2 expression in kidney following Pkd1 deletion.  
(A) YAP immunostaining in kidney sections from Cre- (control) and Cre+ EO and LO Pkd1cond;Pax8;Tet-On- mice. Bars shown are 100 μm. (B) P-mTOR, T-mTOR, YAP, CYR61, and SKP2 expression in kidney tissue extracts from Cre- and Cre+ EO mice. (C) Schematic representation of the mechanistic pathways proposed to contribute to YAP-mediated renal cystogenesis and its progression. Lines represent direct/indirect activation (arrowhead) or inactivation (blunt end). The various drugs used for this study are indicated in red. Additional details are found in the body of the manuscript.