Cyst growth in ADPKD is prevented by pharmacological and genetic inhibition of TMEM16A in vivo

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In autosomal dominant polycystic kidney disease (ADPKD) multiple bilateral renal cysts gradually enlarge, leading to a decline in renal function. Transepithelial chloride secretion through cystic fibrosis transmembrane conductance regulator (CFTR) and TMEM16A (anoctamin 1) are known to drive cyst enlargement. Here we demonstrate that loss of Pkd1 increased expression of TMEM16A and CFTR and Cl− secretion in murine kidneys, with TMEM16A essentially contributing to cyst growth. Uregulated TMEM16A enhanced intracellular Ca2+ signaling and proliferation of Pkd1-deficient renal epithelial cells. In contrast, increase in Ca2+ signaling, cell proliferation and CFTR expression was not observed in Pkd1/Tmem16a double knockout mice. Knockout of Tmem16a or inhibition of TMEM16A in vivo by the FDA-approved drugs niclosamide and benz bromarone, as well as the TMEM16A-specific inhibitor Ani9 largely reduced cyst enlargement and abnormal cyst cell proliferation. The present data establish a therapeutic concept for the treatment of ADPKD.
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dolyctic kidney diseases (PKDs) comprise a number of inherited disorders that lead to bilateral renal cyst development. The most common form, autosomal dominant PKD (ADPKD), affects about 1 in 1000, and accounts for 5–10% of end-stage renal disease. ADPKD is characterized by continuous cyst enlargement over time, leading to compression of adjacent healthy parenchyma. Disease causing mutations in Polycystin-1 (PKD1; ~85% of cases) and Polycystin-2 (PKD2; ~15% of cases) are known, but the complex mechanisms for cyst development and cyst growth are still poorly understood. Cell proliferation and fluid secretion are two essential characteristics of cyst development and enlargement. Epithelial fluid secretion occurs by the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR). CFTR was also shown to cause expansion of renal cysts. However, the Ca\(^{2+}\)-activated Cl\(^{-}\) channel TMEM16A is known to be essential for fluid secretion into renal cysts in vitro. Cyst enlargement is promoted by purinergic signaling and lipid peroxidation, which both activate Tmem16a.

Here, we use in vivo and in vitro models for ADPKD and unmask upregulation of expression of TMEM16A and CFTR in kidneys of Pkd1-knockout mice and Pkd1-knockout MDCK cells. Increased expression of TMEM16A causes enhanced intracellular Ca\(^{2+}\) signaling and induces cyst growth, both being normalized by additional knockdown of Tm16a. The results identify upregulated TMEM16A as an essential player in ADPKD. Two FDA-approved drugs which inhibit TMEM16A, niclosamide and benzbromarone, and the TMEM16A-specific small molecule Ani\(^{12,13}\) strongly suppress the cystic phenotype in vivo. The results demonstrate TMEM16A as a central pharmacological target to suppress cyst growth in ADPKD.

**Results**

TMEM16A supports cyst growth in Pkd1-deficient pMDCK cells. In ADPKD, cysts mainly originate from principal cells of the collecting duct\(^{9,15}\). We have established an in vitro cyst model by means of a subclone of collecting duct MDCK cells which highly resembles principal cells (principal-like (pl)MDCK). We previously demonstrated that blocking of TMEM16A inhibits pMDCK cyst growth in a collagen matrix\(^{8,11}\). This is now investigated by eliminating expression of Pkd1 in two pMDCK cell clones (Pkd1\(^{-/-}\); clones #1 and #2) using CRISPR/Cas9 (Supplementary Fig. 1). Pkd1-knockout induces upregulation of expression of the ion channels TMEM16A, CFTR, and the purinergic receptor P2Y2 (Supplementary Fig. 2). Knockdown of Pkd1 (Pkd1\(^{-/-}\)) causes spontaneous cyst growth, which is much less pronounced in control cells (Pkd1\(^{+/+}\)). Additional stimulation with forskolin further augments cyst size which is more distinct in Pkd1\(^{-/-}\) than in Pkd1\(^{+/+}\) cysts (Fig. 1a, b). Intracellular cAMP levels are enhanced in Pkd1\(^{-/-}\) cells under control condition and are further enhanced after stimulation with forskolin, ATP or inhibitors. Source data are provided as a Source Data file.
forskolin (Fig. 1c). Forskolin, i.e., cAMP does not only increase cyst size, but also induces cyst formation. Pkd1+/+ cells in the absence of forskolin occasionally show tubule-like structures (Fig. 1a, left upper panel; Supplementary Fig. 3a, left upper panel) which are completely absent in Pkd1−/− cells (Fig. 1a, right, upper panel; Supplementary Fig. 3a, right upper panel). Tubular structures are also absent in Pkd1+/+ cells when treated with forskolin. These observations suggest that increase in basal cAMP upon deletion of Pkd1 drives cells into cyst formation.

Cyst growth in the presence of forskolin is attenuated by inhibition of CFTR using CFTRinh172. Inhibition is even more pronounced in the presence of the TMEM16-inhibitor niclosamide. Cyst growth is also inhibited by blocking purinergic receptors with suramin, but, in contrast, is further increased in the presence of ATP (Fig. 1d, e)9,10. The data obtained for clone #1 are reproduced in a second clone #2 (Supplementary Fig. 3). This suggests upregulation of CFTR- and TMEM16A-dependent Cl− secretion in the absence of PKD1, which drives the cystic phenotype.

Renal tubule-specific knockout of Pkd1 upregulates expression of TMEM16A and drives cyst growth. We use KspCreER<sup>T2</sup>; Pkd1<sup>lox/lox</sup> mice to obtain tamoxifen inducible tubule-specific Pkd1 knockout<sup>16,17</sup>. Tamoxifen is applied for 3 days starting at postnatal day 20. Kidneys isolated 10 weeks after deletion of Pkd1 demonstrates clear knockdown of Pkd1-expression as shown by Western blotting and when compared to non-induced mice serving as controls (Pkd1+/+) (Fig. 2a, b). In contrast, expression of TMEM16A is upregulated in Pkd1−/− kidneys (Fig. 2c, d). Knockdown of Pkd1 induces PKD with multiple renal cysts and enlarges kidneys. The calculated cystic index (c.f. "Methods") is strongly enhanced (Fig. 2e, f). In contrast, additional knockout of Tmem16a in Pkd1−/− mice (KspCreER<sup>T2</sup>;Pkd1<sup>lox/lox</sup>:T16a<sup>lox/lox</sup>), hereafter termed Pkd1−/−/T16a−/− (Fig. 2a−d), results in a significantly attenuated cystic phenotype (Fig. 2e, f). The results demonstrate an essential role of TMEM16A for proliferation of cysts, electrolyte secretion into the cysts, or both<sup>8,18</sup> (Fig. 2e, f).

Knockdown of Pkd1 causes upregulation of tubular expression of CFTR and TMEM16A. Induction of cyst formation by downregulation of Pkd1 (Pkd1−/−) is paralleled by upregulation of TMEM16A expression (Fig. 2c). Immunolabelling of TMEM16A and CFTR indicate upregulation of both ion channels in the cyst epithelium of Pkd1−/− kidneys, where both ion channels are strongly colocalized (Fig. 3a−c, Supplementary Figs. 4 and 5a). In contrast to cystic kidneys, TMEM16A is much less abundant in normal kidneys, where it is found to be expressed in apical membranes and in the primary cilium<sup>19</sup> (Supplementary Fig. 5b, c). Remarkably, enhanced expression of CFTR in cystic kidneys is entirely reversible upon additional knockout of Tmem16a in Pkd1−/−/T16a−/− animals (Fig. 3a−d and Supplementary Fig. 4). These results confirm the relationship between CFTR and TMEM16A detected earlier.

**Fig. 2 Tubule-specific deletion of TMEM16A reduces cyst progression in an ADPKD mouse model.** a−d Western blotting from whole kidney lysates detecting tubule-specific deletion of Pkd1 (450 kDa) (P = 0.03) and increase in TMEM16A expression (glycosylated 130 kDa and non-glycosylated forms<sup>34</sup>) (P = 0.003) (10 weeks after induction at postnatal days 20−22 using tamoxifen. Mice co-deleted for Pkd1 and Tmem16a (Pkd1−/−/T16a−/−) showed reduced Pkd1 expression (P = 0.022) and TMEM16A expression (P = 0.0004) (n = 3 animals each). e, f Tubule-specific knockout of Pkd1 (Pkd1−/−) induced polycystic kidney disease (P < 0.0001, n = 11 animals), which was largely reduced in mice with an additional knockout of Tmem16a (Pkd1−/−/T16a−/−) (P = 0.0001, n = 11 animals). Non-induced Pkd1<sup>fl/fl</sup> mice served as control (Pkd1+/+) (n = 8 animals). Corresponding cystic indices (defined as cortical cyst area normalized to whole cortex area) Mean and error bars indicating ±SEM. #Unpaired two-sided t test comparing Pkd1+/+ with Pkd1−/−, §unpaired two-sided t test comparing Pkd1−/− with Pkd1−/−/T16a−/−. Source data are provided as a Source Data file.
Fig. 3 Expression of CFTR and cell proliferation in Pkd1−/− kidneys depends on TMEM16A. a Enhanced tubular epithelial expression of CFTR and TMEM16A in kidneys from Pkd1−/− mice that was abolished in kidneys from Pkd1−/−/T16a−/− double-knockout mice. Both, CFTR and TMEM16A were colocalized in the apical membrane of the cyst epithelium. (blue; DAPI). b–d Expression of TMEM16A (**P < 0.0001) and CFTR (**P < 0.0001) were enhanced (n = 25 independent images over 5 animals) in Pkd1−/− mice. Significant colocalization of TMEM16A and CFTR was detected (**P = 0.0006) using colocalization finder algorithm (ImageJ, V.1.49 by Laumonerie and Mutterer). Expression of CFTR (**P = 0.0006) and TMEM16A (**P = 0.0006), as well as colocalization (**P = 0.0001) were lower in Pkd1−/−/T16a−/− mice. e, f ki-67 staining was enhanced in tubular epithelial cells from Pkd1+/+ (§P < 0.0001) animals but was lower in cells from Pkd1−/−/T16a−/− kidneys (§P < 0.0001) (n = 25 independent images from 5 animals). Mean and error bars indicating ±SEM. #one-way ANOVA and Tukey's post-hoc test comparing Pkd1+/+ with Pkd1−/− and Pkd1−/− with Pkd1−/−/T16a−/−, respectively. Source data are provided as a Source Data file.
demonstrating the requirement of TMEM16A for proper expression and function of CFTR in mouse and human airway and intestinal epithelial tissues. Earlier studies showed a strong correlation between expression of TMEM16A and cell proliferation (reviewed in Refs. 18,21). This is also observed in the present study using the proliferation marker ki-67. Proliferation is enhanced in Pkd1+/− mice, but is lowered to almost normal levels in Pkd1−/−/T16a−/− double-knockout kidneys (Fig. 3e, f).

Medullary epithelial cells isolated from Pkd1+/+, Pkd1−/−, and Pkd1−/−/T16a−/− mice are grown on permeable supports to analyze Ca2+ dependent ion transport by TMEM16A. Transepithelial voltage deflections are induced by purinergic stimulation with UTP, and both basal and UTP-activated short-circuit currents are enhanced in renal epithelium lacking expression of Pkd1 (Fig. 4a–c). In contrast, renal epithelium from mice with double knockout of Pkd1 and Tm6sa demonstrate normal ion transport properties, indicating the role of TMEM16A for enhanced ion transport in ADPKD (Fig. 4a–c and Supplementary Fig. 6a–c). Enhanced Ca2+ dependent Cl− secretion in Pkd1−/−/T16a−/− cells is also well detected in whole cell patch-clamp recordings as well as iodide quenching experiments (Supplementary Fig. 6d–g). Importantly, Cl− secretion activated by increase in intracellular cAMP (IBMX and forskolin; IF) is also enhanced in Pkd1−/−/T16a−/− cells, but not in epithelial cells from Pkd1−/−/T16a−/−-double knockout animals (Supplementary Fig. 6h, i).

Enhanced ion secretion in Pkd1−/− animals is due to upregulated Ca2+ signaling. Enhanced intracellular Ca2+ signaling is observed in cells expressing Tm6sa.22–24 We therefore compare intracellular Ca2+ signals in primary medullary epithelial cells isolated from Pkd1+/− animals, Pkd1−/−/T16a−/− double knockout and Pkd1−/+ animals. Basal Ca2+ levels and ATP-induced Ca2+ rise are both enhanced in Pkd1−/− cells when compared to double knockout and wt cells (Fig. 4d–f). ATP-induced Ca2+ increase is strongly inhibited by the TMEM16A-inhibitor benzbromarone13, supporting the role of TMEM16A for intracellular Ca2+ signaling (Fig. 4g, h). We employ a Ca2+ store release protocol by removing extracellular Ca2+ and emptying the endoplasmic reticulum (ER) Ca2+ store using the ER Ca2+ pump inhibitor cyclopiazonic acid (CPA). We find that deletion of Pkd1 enhances Ca2+ release and largely enhances store-operated Ca2+ entry (SOCE) after re-adding extracellular Ca2+ (Fig. 4i, j). Thus, Ca2+ release and SOCE are coupled and are augmented by deletion of Pkd1, probably due to upregulation of Tm6sa.23,25–27. SOCE is potently blocked by the inhibitors of Orai channels and transient receptor potential (Trp) channels, YM58483 and SK&F96365, respectively, suggesting a contribution of both channels to enhanced SOCE in Pkd1−/− cells (Fig. 5a–c). Analysis of expression of renal epithelial Trp channels suggests upregulation of Trpm6, which, however, appears unlikely to be in charge of enhanced Ca2+ entry as Trpm6 is not inhibited by SK&F9636528 (Fig. 5e, f). ER tethering by Tm6sa is therefore likely to be cause for enhanced Ca2+ signaling22,23.

FDA-approved inhibitors of Tm6sa and Ani9 attenuate renal cyst growth in vivo. The present data indicate an essential contribution of Tm6sa to the development of PKD. Recent studies identified two FDA-approved drugs, niclosamide and benzbromarone, as potent inhibitors of Tm6sa and benzamorpana, in vitro. We therefore examined if treatment with niclosamide and benzamorpana in vivo will inhibit enlargement of renal cysts in Pkd1−/− animals. To that end, Pkd1−/− animals are maintained at normal diet or are fed a diet supplemented with 0.2% niclosamide, which is well tolerated. Animals are sacrificed 10 weeks later and kidneys are analyzed. The animals are monitored daily. No side effects are observed, but in contrast, normalization of weight gain is observed. Cyst development is strongly attenuated in niclosamide-treated mice when compared to animals on standard diet (Fig. 6a, b). Proliferative activity is analyzed by staining medullary epithelial cells with the proliferation marker Ki-67. Knockdown of Pkd1 largely enhances proliferation, which is significantly reduced by treatment with niclosamide (Fig. 6c, d).

An even more pronounced inhibition of cyst growth is observed in animals treated with benzamorpana. Daily intraperitoneal injection of benzamorpana for 8 weeks significantly reduces renal cyst size when compared to sham treated animals (Fig. 7a, b). In line with these data, pathological weight gain caused through polycystic kidneys is completely abolished by treatment with benzamorpana (Fig. 7c). Proliferative activity is analyzed using Ki-67. Knockdown of Pkd1 largely enhances proliferation, which is almost completely eliminated by treatment with benzamorpana (Fig. 7d, e). Finally, we treat Pkd1−/− animals with Ani9, a recently discovered highly specific inhibitor of Tm6sa.29,30 Ani9 likewise potently suppresses cyst formation in vivo (Fig. 8). Taken together, Tm6sa is a crucial protein and clinically relevant pharmacological target in ADPKD. The clinical relevance is further supported by re-analyzing an original data set from a global gene profiling study31, using the transcriptome analysis Console. When inspecting the probe for Tm6sa, a significant increase in Tm6sa-mRNA expression is found in the cystic tissue when compared to normal samples, and levels of expression correlated with cyst size (Fig. 9). These data compare well with a previous study that indicated an upregulation of Tm6sa in kidneys from ADPKD patients.8 We therefore propose inhibitors of Tm6sa, such as niclosamide or benzamorpana, as therapeutics that potentially suppress cyst growth in patients with ADPKD.

Discussion

The present data demonstrate the crucial role of Tm6sa for the growth of renal cysts in a mouse model for ADPKD and in Pkd1-deficient pMDCK cells in vitro. Tm6sa was shown previously to be expressed at low levels in renal epithelial cells of normal human and mouse kidney, and was suggested to be upregulated in polycystic kidneys.8,29 The present data now provide evidence that Tm6sa supports cyst growth by at least three mechanisms: (1) increasing intracellular Ca2+ signaling, (2) supporting Cl− secretion, and (3) enhancing cell proliferation.

The essential role of CFTR for cAMP-regulated chloride secretion in intestine, airways, exocrine glands and numerous other epithelial organs is undisputed.3 Traditionally, Ca2+ activated transport by Tm6sa is regarded as a separate and independent mechanism for transepithelial chloride secretion. Recent studies however suggest that both pathways overlap and cannot be separated easily. Thus CFTR-dependent secretion is also activated through Ca2+ dependent stimulation.32–34. Knockout of Tm6sa in ciliated airway epithelial cells, intestinal epithelial cells and cell lines abrogated CAMP/CFTR-dependent chloride secretion.30 In differentiated epithelial cells plasma membrane expression and activation of CFTR strongly depends on the presence of Tm6sa.7 Plasma membrane tethering of the ER by Tm6sa and increase of compartmentalized Ca2+ signals close to the plasma membrane in conjunction with well described Ca2+ dependent modulations of CFTR function provides a mechanism for Tm6sa-dependent cyst formation.33,35. Niclosamide, benzamorpana, and Ani9 attenuate compartmentalized Ca2+ signaling in renal epithelial cells and other cell types.30,31

The present report demonstrates enhanced Ca2+ signals in Pkd1−/− cells that correlate with enhanced expression of
TMEM16A (Figs. 4 and 5). Increase in purinergic Ca\(^{2+}\) signaling and increase in Ca\(^{2+}\) dependent chloride secretion upon knock-out of Pkd1 was observed in medullary (Figs. 4, 5 and Supplementary Fig. 6) as well as cortical tubular epithelial cells (Supplementary Figs. 7 and 8). Notably, deletion of Pkd1 results in upregulation of hypoxia-inducible factor (HIF)-1\(\alpha\), which worsens cyst formation\(^{17}\). HIF1\(\alpha\) augments expression of P2Y2R. Thus, expression of both P2Y2-receptor and TMEM16A are augmented in ADPKD\(^{10}\). Also, in the present study we demonstrate that in the absence of Pkd1 (Pkd1\(^{-/-}\)), expression of P2Y2 is augmented (Supplementary Fig. 2).

Enhanced Ca\(^{2+}\) mediated signaling in Pkd1\(^{-/-}\) cells is in conflict with previous studies, showing attenuated global Ca\(^{2+}\) levels in renal epithelial cells lacking expression of PKD1\(^{36}\). While these differences currently remain unexplained, the present results, showing enhanced basal intracellular Ca\(^{2+}\) concentrations...
in Pkd1−/− cells, correspond to the enhanced basal short-circuit currents, basal Cl− currents, and the basal halide permeability observed in Pkd1−/− cells (Fig. 4, Supplementary Figs. 6 and 7). The present results are essentially identical to results previously reported for mouse M1 Pkd1−/− cells.

ER tethering by TMEM16A is of particular relevance for submembrane Ca2+ signaling, i.e., Cl− store release and SOCE22,23. Concepcion et al. showed recently that store-operated Ca2+ entry regulates TMEM16A38. In fact TMEM16A-activity is controlled by intracellular Ca2+ as well as plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP2)30,39,40. A large number of studies demonstrate the crucial role of TMEM16A for cell proliferation and cancer development (reviewed in refs. 18,21). From this line, it was shown that knockdown of TMEM16A during kidney development affects ciliogenesis and reduces the number of renal glomeruli, thus causing albuminuria41,42.

The present data strongly suggest TMEM16A as a therapeutic target in ADPKD. Two well established FDA-approved drugs and potent inhibitors of TMEM16A, nicosamide and benz bromarone, strongly suppressed cyst growth in vivo (Figs. 5 and 6). Although both substances potently inhibit TMEM16A12,13, they are not specific to TMEM16A. Benz bromarone is primarily known as an ursolic compound and nicosamide is used for the treatment of tapeworm infestations. Nevertheless, the present results suggest that the observed effects upon treatment with nicosamide, benz bromarone are due to inhibition of TMEM16A, which is further supported by the Ani9-mediated suppression of ADPKD. It has been reported earlier that degradation of TMEM16A is facilitated in the presence of the small molecule TMEM16A-inhibitor CaCCinhAO143,47. Interestingly, we also found a remarkable reduction of TMEM16A-expression when cells were grown in the presence of nicosamide, Ani9, or benz bromarone (Supplementary Fig. 9). Thus, TMEM16A-inhibitors not only block Cl− currents but also inhibit expression of TMEM16A upon long term treatment. Thus, genetic deletion of Tmem16a and inhibition by small molecules ultimately reduces the abundance of TMEM16A in the plasma membrane. This will lower ER-tethering and will attenuate local submembrane Ca2+ signaling, with the functional consequences outlined here. We therefore propose inhibition of Tmem16-mediated intracellular signaling as a therapeutic principle in ADPKD.

**Methods**

**Animals.** Animal experiments were approved by the local institutional review board and all animal experiments complied with the with the United Kingdom Animals Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments. Experiments were approved by the local Ethics Committee of the Government of Unterfranken/Wuerzburg (AZ; 55.2-2532-2-328). Mice with a floxed PKD1 allele were generously provided by Prof. Dr. Dorien J.M. Peters (Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands)44. Animals were housed on a 12:12 h light-dark cycle under constant temperature (24 ± 1 °C) in standard cages. They were fed a standard diet with free access to tap water. Generation of mice with a tamoxifen inducible, kidney epithelium-specific Pkd1 deletion were described recently17. Mice carrying loxP-flanked conditional alleles of Pkd1 were crossed with Ksp-Cre mice in a C57BL/6 background (KspCreERT2;lox;lox, abbreviated as Pkd1 lox/lox). Mice carrying floxed alleles (Tmem16afl/−) were crossed to generate KspCreERT2;Pkd1 fl/lox;Tmem16afl/fl double-knockout mice (abbreviated as Pkd1−/−; Tmem16a−/−). Primers for genotyping are listed in Table 1. We used C57BL/6 male and double-knockout males in the age of 8–10 weeks in the experiments.

**Animal treatment.** Conditional knockout was induced in Pkd1−/− (n = 12) and Pkd1−/−; Tmem16a−/− (n = 12 mice by administering tamoxifen (2 mg/kg body weight) dissolved in 5% ethanol and 95% neutral oil, daily at postnatal days PN 20–22. Non-induced Pkd1−/− (n = 9) served as controls. Induced Pkd1−/− mice were fed with maintenance diet 1320 (Altromin, Lage, Germany) (n = 3) or with Altromin supplemented with 0.2% nicosamide (n = 3). All animals were sacrificed 10 weeks after induction with tamoxifen and kidneys were analyzed. Alternatively, Pkd1−/− mice were maintained for 30 days and subsequently treated with benz bromarone (1 mg/kg/day) dissolved in corn oil or corn oil/DMSO (50 µl) by intraperitoneal injection for 30 days (n = 5). Mice were monitored twice a day by trained staff.

**Renal medullary and cortical primary cells.** Mice were killed by cervical dislocation after exposure to CO2. Kidneys were removed and kept in ice-cold DMEM/F12 medium (Thermo Fisher Scientific, Darmstadt, Germany). The renal capsule was removed under germ-free conditions. Cortex and medulla were separated and chopped into smaller pieces of tissue using a sharp razor blade (Heinz Herzen, Hamburg, Germany). Tissues were incubated in Hanks balanced salt solution/DMEM/F12 (Life Technologies/Gibco®, Karlsruhe, Germany) containing 1 mg/ml collagenase type 2 (Worthington, Lakewood, USA) for 20 min at 37 °C. The digested tissue was passed through a 100 µm cell strainer (Merck KGaA, Darmstadt, Germany), transferred to a 50 ml falcon tube (Corning®), and washed with ice-cold PBS. After centrifugation at 5100 rpm for 4 min/4 °C, cells were resuspended. After resuspension, the cortical cell pellet was centrifuged at 7500 rpm for 30 min at 4 °C through a 45% Percoll (Ge Healthcare, Munich, Germany) 55% 2X PBS-Glucose gradient. After washing with ice-cold PBS, cortical preparations were maintained at 37 °C in 5% CO2 in DMEM/F12 supplemented with 1% FBS, 1% Pen/Strep, 1 L-Glutamine (200 µM), 1% ITS (100×), 50 µM hydrocortisone, 5 µM triiodothyronine, and 5 µM Epidermal Growth Factor (Sigma Taufkirchen, Germany). After 24 h, primary cells grew out from isolated tubules.

**Principal-like MDCK cell cyst model and PKD1-knockout cells.** Principal-like MDCK cells were grown at 37 °C, 21% O2 and 5% CO2 in modified MEM containing Earls balanced salt solution supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS, 30 U/ml penicillin, and 50 µg/ml streptomycin. In vitro cyst assays were performed as described previously39. In brief cells were resuspended as a single-cell suspension in type I collagen and transferred to 24-well plates (3 wells per condition). Forskolin (FSK; 10 µM), ATP (10 µM), suramin (Sur; 100 µM), nicosamide ethanalamine (Niclo; 1 µM) and CFT/Ri172 (CFTRI; 10 µM) were added to the medium at day 0, and medium was changed every 2 days. All substances were purchased from Sigma (Taufkirchen, Germany). After 5 days, images were taken from four random visual fields per well using Zeiss Primo Vert microscope and Zeiss Axioscam 105 camera (Zeiss Microscopy GmbH, Jena, Germany). Diameters of all captured spherical cysts were analyzed using ImageJ and spherical cyst volumes were calculated according to 4/3πr3. A subclone of MDCK cells was used that resembles principal cells of the collecting duct as described earlier39. For genome editing pSPCas9(BB)-2A-puro vectors (PX459) V2.0 (Addgene, Watertown, MA, USA) were used to generate a PKD1−/− cell line. The guide RNA was designed according to the algorithms.
Fig. 5 Inhibition of enhanced store-operated Ca\(^{2+}\) entry (SOCE) by TRP and Orai channel inhibitors. a–d Original recordings and summaries showing inhibition of upregulated ER Ca\(^{2+}\) store release (Store) and store-operated Ca\(^{2+}\) entry (SOCE) in primary renal epithelial cells from Pkd1\(^{−/−}\) mice in the presence of 5 \(\mu\)M Orai channel inhibitor YM58483 (#\(P < 0.0001\) and #\(P < 0.0001\), respectively) and 5 \(\mu\)M TRP-channel inhibitor SK&F96365 (#\(P < 0.0001\) and #\(P < 0.0001\), respectively) (\(n = 73\) cells from 5 independent cultures of 4 animals each). e, f Semiquantitative RT-PCR analysis of Orai1 and TRP-channel (Trpc1, Trpc6, Trpm6, Trpv4, Trpv5) expression in Pkd1\(^{+/+}\) and Pkd1\(^{−/−}\) renal epithelial cells. In Pkd1\(^{−/−}\) cells mRNA for Pkd1 (\(\#P = 0.03\)) and Trpc6 (\(\#P = 0.03\)) were reduced, while mRNA for Trpm1 was enhanced (\(\#P = 0.03\)) (\(n = 5\) reactions from 3 animals each). Mean and error bars indicating ±SEM. #\(P < 0.0001\).

AMP measurements. MDCK cells were grown in a pre-coated assay capture plate (cAMP-Screen Direct System P/N 4412186; Applied Biosystems, Foster City, CA, USA) in serum-free medium. Cells were exposed to serum-free control medium or medium supplemented with 10 \(\mu\)M forskolin. cAMP measurements were performed by the use of the GloMax-Multi Detection System (Promega) according to the manufacturer’s protocol.

Western blotting. Protein was isolated from MDCK cells using a sample buffer containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 0.1% SDS and 1% protease inhibitor mixture (Roche, cOmplete, EDTA-free, Mannheim, Germany) and 1% Triton X-100. Proteins were separated using an SDS polyacrylamide gel (10% for P2Y2R and 8% for TMEM16A) or NuPAGE.
Fig. 6 Niclosamide inhibits polycystic kidney disease in a mouse model for ADPKD. **a**, **b** Representative images of kidneys from non-induced (Pkd1+/+) (n = 4 animals) and induced (Pkd1−/−) mice, untreated (n = 5 animals) or treated with niclosamide (n = 8 animals). Scale bar 5000 µm. Tubule-specific deletion of Pkd1 (Pkd1−/−) was induced at postnatal days 20–22. Thereafter, mice were either treated with standard diet or diet supplemented with 0.2% niclosamide (Niclo) for 10 weeks. Pkd1+/+ mice fed with standard diet served as control. Cyst formation in Pkd1−/− kidneys (§P = 0.003) was strongly inhibited by niclosamide treatment (§P = 0.005). **c**, **d** Proliferating cells identified in Pkd1+/+ and Pkd1−/− kidneys by the proliferation marker ki-67 (red). Proliferation was enhanced in Pkd1−/− kidneys (§P < 0.0001), but was significantly inhibited by niclosamide (§P < 0.0001). Number of ki-67 positive cells was normalized to the tissue area (n = 25 independent images from 5 animals each). Mean and error bars indicating ±SEM. #§One-way ANOVA and Tukey’s post-hoc test. Source data are provided as a Source Data file.

**Immunohistochemistry and antibodies.** Two-micron thick transverse kidney sections were stained. For co-staining of CFTR and TMEM16A, anti-CFTR (rabbit; 1:100; Alomone Labs; 1:500), anti-TMEM16A DOG-1 Polyclonal (Thermo Fisher; 1:1000) or anti-P2Y2R A20 (1:500, Santa Cruz Biotechnology) overnight. Proteins were visualized using horseradish peroxidase-conjugated secondary antibody and ECL detection. Beta-Actin was used as a loading control. Full uncropped blots are shown in Supplementary Figs. 10 and 11.

**Immunohistochemistry and fluorescent signals.** Ten random photographs were taken from the cortex of each kidney at a magnification of X200. Immunofluorescence (TMEM16A and CFTR) was analyzed as described previously. Briefly, fluorescent...
Fig. 7 Benz bromarone inhibits polycystic kidney disease in a mouse model for ADPKD. a Representative images of kidneys from non-induced (Pkd1+/+) and induced (Pkd1−−) mice (n = 5 animals each), untreated (daily i.p. injection of corn oil) or treated with benz bromarone in corn oil (BBR, 1 mg/kg/day, i.p.) for 30 days. Scale bars: 1000 μm. b Cystic index (%) 8 weeks after induction of Pkd1+/+ and Pkd1−− mice, untreated or treated with benz bromarone. Cyst growth was induced in Pkd1−− mice (P < 0.0001), but was inhibited by benz bromarone (P < 0.0001) (n = 10 kidneys analyzed from n = 5 animals each). c Time course of body weight for untreated and benz bromarone-treated Pkd1+/+ and Pkd1−− mice. Body weight gain was enhanced in Pkd1−− mice (P < 0.05), but was inhibited by benz bromarone (P < 0.05) (n = 8 kidneys analyzed from n = 5 animals each). d, e Proliferating cells identified in Pkd1+/+ and Pkd1−− kidneys by the proliferation marker ki-67 (red). Proliferation was upregulated in Pkd1−− kidneys (P < 0.0001), but was inhibited by benz bromarone (P < 0.0001) (25 independent images from n = 5 animals each). Scale bar 50 μm. Number of ki-67 positive cells was normalized to the tissue area. Mean and error bars indicating ±SEM. ***One-way ANOVA and Tukey’s post-hoc test comparing Pkd1+/+ with Pkd1−− and Pkd1−− with Pkd1−−/T16a−−, respectively. Source data are provided as a Source Data file.

Morphological analyses. Photographs from hematoxylin and eosin–stained kidney sections were taken at a magnification of ×25 and stitched to obtain single photographs of the whole transverse kidney sections using a Leica DM6000B microscope and a Leica DFC 450C camera. The whole kidney cortex was defined as region of interest using ImageJ and Cinteq 13HD creative pen display (Wacom, Düsseldorf, Germany). Next, we used an algorithm that separates normal tubule space from cystic area by defining diameters of noncystic tubules < 50 mm. The whole cortex cyst area was divided by the whole cortex area and defined as the cystic index.

signals were turned into 8-bit images after subtracting background (ImageJ) and a predefined threshold was used for all images to capture signals. Colocalization was visualized in white by use of ImageJ and an algorithm (http://rsb.info.nih.gov/ij/plugins/colocalization-finder.html, Laumonerie and Mutterer, Institut de Biologie Moléculaire des Plantes, Strasbourg, France). For quantification of ki-67, the color deconvolution algorithm (ImageJ) was applied to dissect the different signals, followed by binarization and particle analysis to obtain the ratio of the number of positive cells and cortex area (normalized to mm² cortex tissue).
Fig. 8 Ani9 inhibits polycystic kidney disease in a mouse model for ADPKD. a Representative images of kidneys from induced (Pkd1−/−) mice, untreated (daily i.p. injection of corn oil) (n = 8 kidneys analyzed from n = 4 animals) or treated with Ani9 in corn oil (Ani9, 0.5 mg/kg/day, i.p.) for 30 days (n = 8 kidneys analyzed from n = 4 animals). Scale bars 1000 µm. b Cystic index (%) 8 weeks after induction of Pkd1−/− and Pkd1+/− mice, untreated or treated with Ani9. Ani9 reduced cyst growth in Pkd1−/− mice (\(P = 0.001\)). §Unpaired two-sided t test. c, d Proliferating cells identified in Pkd1+/− and Pkd1−/− kidneys by the proliferation marker ki-67 (red). Scale bars 50 µm. Proliferation was upregulated in Pkd1−/− mice (\(P < 0.0001\)), but was largely inhibited by Ani9 (\(P = 0.021\)). One-way ANOVA and Tukey's post-hoc test. Number of ki-67 positive cells was normalized to the tissue area. Mean and error bars indicating ±SEM. Source data are provided as a Source Data file.

Ussing chamber. Medullary or cortical primary cells were grown as polarized monolayers on permeable supports (Millipore). Filters were mounted into a perfused micro Ussing chamber and the luminal and basolateral surfaces of the epithelium were perfused continuously with ringer solution (in mM: NaCl (145), KH2PO4 (0.4), K2HPO4 (1.6), Glucose (5), MgCl2 (1) Ca-gluconate (1.3)) at a rate of 6 ml/min (chamber volume 2 ml). All experiments were carried out at 37 °C under open-circuit conditions. Transepithelial resistance (Rte) were recorded continuously. Values for Vte were referred to the basolateral side of the epithelium. Rte was determined by applying short (1 s) current pulses (\(\Delta J = 0.5 \mu A\)) and the corresponding changes in transepithelial voltage (Vte) were recorded continuously. Values for Vte were referred to the basolateral side of the epithelium. Rte was calculated according to Ohm's law (Rte = ΔVte/ΔJ). The equivalent short-circuit current (Isc) was calculated according to Ohm's law from Vte and Rte (Isc = Vte/Rte).

YFP-quenching assay. For YFP-quenching assays, primary renal cells were infected with lentiviral vectors to express halide-sensitive YFP152L, as previously described49. Cells were isolated from 4 different mice and for each mouse 40 cells were measured. Quenching of the intracellular fluorescence generated by the iodide sensitive Enhanced Yellow Fluorescent Protein (EYFP-152L) was used to measure anion conductance. YFP-152L fluorescence was excited at 500 nm using a polychromatic illumination system for microscopic fluorescence measurement (Visitron Systems, Puchheim, Germany) and the emitted light measured at 535 ± 15 nm with a Coolsnap HQ CCD camera (Roper Scientific). Quenching of YFP-152L fluorescence by I− influx was induced by replacing 5 mM extracellular Cl− with I−.

Cells were grown on coverslips and mounted in a thermostatically controlled imaging chamber maintained at 37 °C. Cells were continuously perfused at 8 ml/min with Ringer solution and exposed to I− concentration of 5 mM by replacing same amount of NaCl with equimolar NaI. Background fluorescence was subtracted, while auto-fluorescence was negligible. Changes in fluorescence induced by I− are expressed as initial rates of maximal fluorescence increase (\(ΔF/Δt\)). For quantitative analysis, cells with low or excessively high fluorescence were discarded.

Measurement of intracellular Ca2+. ([Ca2+]i). Renal primary cells were loaded with 2 µM Fura-2/AM and 0.02% Pluronic F-127 (Invitrogen, Darmstadt, Germany) in ringer solution (mmol/l: NaCl 145, KH2PO4 0.4, K2HPO4 1.6, Glucose 5, MgCl2 1) Ca-gluconate (1.3)) at a rate of 6 ml/min (chamber volume 2 ml). All experiments were carried out at 37 °C using an inverted microscope (Axiovert S100, Zeiss, Germany) and a high speed polychromator system (VisiChrome, Puchheim, Germany). Fura-2 was excited at 340/380 nm, and emission was recorded between 470 and 550 nm using a CoolSnap camera (CoolSnap HQ, Visitron). [Ca2+]i was calculated from the 340/380 nm fluorescence ratio after background subtraction. The formula used to calculate [Ca2+]i was \([Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times (S_{f2}/S_{i2})\), where R is the observed fluorescence ratio. The values R_{max} and R_{min} (maximum and minimum ratios) and the constant S_{f2}/S_{i2} (fluorescence of free and Ca2+-bound Fura-2 at 380 nm) were calculated using 1 µmol/l ionomycin (Calbiochem), 5 µmol/l nigericin, 10 µmol/l monensin (Sigma), and 5 mM/EGTA to equilibrate intracellular and extracellular Ca2+ in intact Fura-2 loaded cells. The dissociation constant for the Fura-2/Ca2+ complex was taken as 224 nmol/l.

Patch clamping. Patch-clamp experiments were performed in the fast whole-cell configuration. Patch pipettes had an input resistance of 2–4 MΩ, when filled with a cytotoxic-like pipette filling solution46, containing (mM) KCl 30, K-gluconate 95,
Inspection of the probe covering TMEM16A performed using the Transcriptome Analysis Console (Thermo Fisher). The increase in TMEM16A mRNA expression was found in the cystic tissue when compared to normal samples. Increase in TMEM16A expression correlated with cyst size. Mean and error bars indicating ±SEM. Source data are provided as a Source Data file.

**Table 1 Primers used for genotyping.**

| Primer          | Gene | Sequence 5’-3’          |
|-----------------|------|-------------------------|
| mPKD1loxPF      | Pkd1 | ACCCTTCCTGAGCCTCCAC     |
| mPKD1loxPR      | Pkd1 | CCACAGGGAAAGCATCATA     |
| F427 Va         | Ksp  | CATTTCTCCACACTGAATTGA   |
| F427 Vb         | Ksp  | ACAGATGGTGGTTTGTTGCTG   |
| inv             | Ksp  | AACTGTCCTCCCTTGATCACC   |
| 16αCKOf         | Tmem16a | GGCTCTATCAATGTCGTTCC   |
| 16αCKOr1        | Tmem16a | CCTCAAGTCCCTCAAGTCCAGTC |

**Table 2 Primers used for generation of the MDCK-PKD1−/− cell lines.**

| Guide primers | Exon 1 fw | 5’-CACCCTGCTCCGGGCATTGGAGCTT |
|               | Exon 1 rev | 5’-AAACAAACGTCCAAATGCCTCCAGC |
| Cloning primers | Exon 1 cloning fw | 5’-GCATGGTACCACGAAAGGGAATGGGCGCA |
|                | Exon 1 cloning rev | 5’-GCTAGCTACGGCGTTGGAATGACCCAC |
| Off-target primers | Chrom 34 fw | 5’-ACGTTAGCGCGGCAAGTCCAA |
|                 | Chrom 34 rev | 5’-CTGCTGCTTCACACTCCCAAG |
|                 | Chrom 3 rev  | 5’-AATTGGCTGGTGCTAGAAC |

**Materials and statistical analysis.** All compounds used were of highest available grade of purity. Data are reported as mean ± SEM. Student’s t test for unpaired samples and ANOVA were used for statistical analysis. P < 0.05 was accepted as significant difference. Data are expressed as mean ± SEM. Differences among groups were analyzed using one-way ANOVA, followed by a Bonferroni test for multiple comparisons. An unpaired t test was applied to compare the differences between two groups. Wilcoxon signed-rank test for columns statistics was used for relative values. P < 0.05 was considered statistically significant.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** All relevant data are available from the authors. A source data file has been included that contains all raw data underlying all reported averages in graphs and charts, as well as all uncropped versions of any gels or blots presented in the figures.

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Author contributions
I.C., A.K., R.S., J.K.S., K.S., and B.B. performed experiments, I.C., A.K., R.S., B.B., and K.K. analyzed data and wrote the paper. K.K. analyzed data and wrote the paper.

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