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Aberrant Polycystin-1 Expression Results in Modification of Activator Protein-1 Activity, whereas Wnt Signaling Remains Unaffected*

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Polycystin-1, the polycystic kidney disease 1 gene product, has been implicated in several signaling complexes that are known to regulate essential cellular functions. We investigated the role of polycystin-1 in Wnt signaling and activator protein-1 (AP-1) activation. To this aim, a membrane-targeted construct encoding the conserved C-terminal region of mouse polycystin-1 reported to mediate signal transduction activity was expressed in human embryonic and renal epithelial cells. To ensure specificity and minimal cotransfection effects, we focused our study on the endogenous proteins that actually transduce the signals, β-catenin and T-cell factor/lymphoid-enhancing factor for Wnt signaling and (phosphorylated) c-Jun, ATF2, and c-Fos for AP-1. Our data indicate that the C-terminal region of polycystin-1 activates AP-1 by inducing phosphorylation and expression of at least c-Jun and ATF2, whereas c-Fos was not affected. Under our experimental conditions, polycystin-1 did not modulate Wnt signaling. AP-1 activity was aberrant in human autosomal dominant polycystic kidney disease (ADPKD) renal cystic epithelial cells and in renal epithelial cells expressing transgenic full-length polycystin-1, resulting in decreased Jun-ATF and increased Jun-Fos activity, whereas Wnt signaling remained unaffected. Since our data indicate that aberrant polycystin-1 expression results in altered AP-1 activity, polycystin-1 may be required for adequate AP-1 activity.

Progressive development of fluid-filled cysts in autosomal dominant polycystic kidney disease (ADPKD)[1] results in chronic renal failure. In the majority of patients, the disease can be accounted for by a mutation in the *PKD1* gene (1, 2), whereas a minority suffers from a mutation in the *PKD2* gene (3, 4). The precise function of polycystin-1 and polycystin-2, the proteins encoded by the *PKD1* and *PKD2* gene, respectively, remains to be elucidated. Polycystin-1 is predicted to be a transmembrane protein of ~460 kDa. The large extracellular N terminus contains multiple domains thought to be involved in cell-cell and cell-matrix interactions. The intracellular C terminus of polycystin-1 contains putative phosphorylation sites and a coiled-coil domain that can mediate protein-protein interactions.

Several studies have implicated a role for polycystin-1 in signal transduction. Overexpression of the C-terminal region of polycystin-1 in human embryonic kidney 293T (HEK293T) cells has been shown to activate the Wnt signaling pathway (5) and the activator protein-1 (AP-1) transcription factor complex (6, 7). Furthermore, overexpression of a full-length polycystin-1 construct has been reported to activate the Janus kinase and signal transducer and activator of transcription (JAK-STAT) signaling pathway (8). These signaling pathways are all involved in key cellular processes such as proliferation and differentiation, cell cycle regulation, and cell survival. Since these cellular processes are essential for normal function, the signaling pathways governing them are tightly regulated. We set out to investigate the activation of signaling pathways by polycystin-1. To identify relevant signaling events, a membrane-targeted construct containing the C-terminal domain of polycystin-1, the highly conserved region that has been proven previously to successfully activate luciferase reporters for AP-1 and Wnt signaling (5–7), was expressed in renal cells. To determine the physiological relevance of this approach, renal epithelial cells expressing transgenic full-length polycystin-1 and ADPKD renal cystic epithelial cells were subsequently analyzed. Our study focuses on the Wnt signaling pathway and the AP-1 transcription factor complex.

The canonical Wnt signaling pathway is involved in cell proliferation, differentiation, polarity, migration, and survival (reviewed in Ref. 9). Upon stimulation by Wnt, cytoplasmic free β-catenin is stabilized and subsequently translocated to the nucleus. Binding of β-catenin to T-cell factor/lymphoid-enhancing factor (TCF/LEF) transcription factors then results in

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[1] The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; TCF, T-cell factor; LEF, lymphoid-enhancing factor; AP-1, activator protein-1; MDCK, Madin-Darby canine kidney; NRK-52E, normal rat kidney 52E; HEK, human embryonic kidney; β-cat S33, β-catenin Ser^{33}.

transactivation. Thus, β-catenin plays a dual role in the cell, as a transducer of canonical Wnt signaling and as a key component of cell adhesion, since β-catenin is also an integral part of adhesion junctions. Cellular adhesion and signaling are therefore coupled via β-catenin.

The AP-1 transcription factor complex regulates key cellular responses such as cell proliferation, differentiation, and survival and can be activated by a variety of stimuli such as growth factors and stresses (reviewed in Refs. 10 and 11). The AP-1 complex can be composed of homo- or heterodimers of a variety of transcription factors including Jun, ATF, and Fos family members. The heterogeneity of the AP-1 complex is thought to provide a mechanism to regulate the cellular response. In most cell types, growth factors, serum, and phorbol esters predominantly induce Jun-Fos transcriptional activity, whereas stress-inducing stimuli such as UV-C irradiation and alkylating agents predominantly result in activation of Jun-ATF. Heterogeneity is further illustrated by the fact that Jun-Fos heterodimers bind to the 7-bp consensus sequence TGAGTCA, whereas Jun-ATF heterodimers recognize the 8-bp consensus sequence TGACNTCA.

To ensure specificity, we focused our study on the proteins that actually transduce the signal, β-catenin and TCF/LEF for Wnt signaling and c-Jun, ATF2, and c-Fos for AP-1. We report here the activation of Jun-ATF heterodimers by the membrane-targeted mouse C-terminal polycystin-1 fusion protein construct. Moreover, AP-1 activity was aberrant in human ADPKD renal cystic epithelial cells and in renal epithelial cells expressing transgenic human full-length polycystin-1, resulting in impaired Jun-ATF and increased Jun-Fos activity, whereas Wnt signaling was not affected.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The membrane-targeted mouse C-terminal polycystin-1 fusion protein construct, mPKD1HT, was reported earlier (12) (Fig. 1*A*, *lower panel*). Deleting the insert subsequently generated the empty vector control, pcDNA1.1ΔHindIII-NotI, in short pcDNA1.1. The following constructs have been described previously: TOP-TK and FOP-TK luciferase reporter constructs (13) (Fig. 1*A*, *upper two panels*) in short TOP and FOP; the β-catenin Ser^{33} (β-cat S33) construct containing full-length β-catenin with a mutation at Ser^{33} and its corresponding empty vector control, pcDNA3ZeoΔMCS, in short pcDNA3Zeo (14); the 5×jun2 TATA pGL3, in short 5×jun, 5×collTRE TATA pGL3, in short 5×coll, TATA pGL3 (15, 16) (Fig. 4*A*, *upper panel*), −1600/+740 wt c-*jun* TATA pGL3, and −1600/+740 m1 + 2 c-*jun* TATA pGL3, luciferase reporter constructs (17); the Myc-tagged cdc42 V12 construct, encoding constitutively active Cdc42, and its corresponding empty vector control, pMT2 (18); and the HA-tagged ATF2 construct, HA-ATF2 (19). The p-AP-1-Luc or 7×AP-1 reporter construct (Stratagene, Cedar Creek, TX) was a kind gift from M. Karperien (Leiden University Medical Center, Department of Endocrinology, Leiden, The Netherlands). The *Renilla* luciferase reporter construct, pRL-TK, was purchased from Promega (Leiden, The Netherlands), and pEGFP-N1 was from BD Transduction Laboratories (Erembodegem-Aalst, Belgium). Plasmids were isolated using the Nucleobond® DNA isolation kit from Machery-Nagel GmbH & Co. (Düren, Germany) according to the manufacturer's instructions.

Cell Culture—Cells were maintained in Dulbecco's modified Eagle's medium/F-12 with 100 units/ml penicillin and streptomycin, 1 mM sodium pyruvate, 0.1 mM HEPES, 2 mM glutaMAX™-I, and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cell culture reagents were purchased from Invitrogen B.V. (Breda, The Netherlands) and disposables from Greiner Bio-One B.V. (Alphen a/d, Rijn, The Netherlands). Madin-Darby canine kidney (MDCK) and normal rat kidney 52E (NRK-52E) cells were obtained from ATCC (number CCL-34 and CRL-1571, respectively). Human embryonic kidney cells, HEK293 and HEK293T, generously provided by J. Dorsmann (Department of Molecular Cell Biology, Leiden, The Netherlands), HCT116 (ATCC number CCL-247), and SW480 (ATCC number CCL-228) were maintained in Dulbecco's modified Eagle's medium with 4500 mg/ml glucose and supplements as stated above. The following cell lines have been described previously: M7 and M8 (20), RCTEC, PKD9–7WT, and PKD10–7WT (21). Osmotic shock was induced by the addition of 250 μM NaCl to culture medium for 15 min at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, after which cells were lysed as described below (see "Western Blot"). UV-C irradiation was performed by removing culture medium, washing cells twice with phosphate-buffered saline, exposing cells to 40 J/m^2 UV-C, and subsequently culturing for 6–8 h. Cells were incubated with 100 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) for 2 or 24 h.

Luciferase Reporter Assays—Cells were cultured in 6-well plates and co-transfected with 250 ng of TOP or FOP; 100 ng of 5×jun, TATA pGL3, 5×coll, 7×AP-1, −1600/+740 wt c-*jun* TATA pGL3, or −1600/+740 m1 + 2 c-*jun* TATA pGL3; 5 ng of pRL-TK; and 1000 ng of mPKD1HT, 250 ng of β-cat S33, 500 ng of cdc42 V12, or the corresponding empty vectors. Total DNA amount was standardized using pKNUN. All samples were performed in triplicate unless stated otherwise. HEK293 and HEK293T cells were transfected with 6 μl, M7 and M8 with 10 μl, RCTEC, PKD9–7WT, and PKD10–7WT with 3 μl, and NRK-52E with 8 μl of FuGene™ 6 (Roche Applied Science), and MDCK cells were transfected with 6 μl of Transfast™ (Promega) per 1 μg of DNA as described by the manufacturers. Cells were maintained under serum-free conditions from the moment of transfection, unless stated otherwise. Firefly and *Renilla* luciferase activities were measured 1–2 days post-transfection using the Dual-Luciferase® reporter assay from Promega according to the manufacturer's instructions. Samples that were subsequently used for Western blotting experiments were prepared as described below. Statistical analysis was performed using the paired *t* test.

Western Blot—Cells were lysed in passive lysis buffer (Promega) with 1 mM phenylmethylsulfonyl fluoride (Roche Applied Science), 100 μg/ml trypsin inhibitor, 0.5 μM sodium fluoride, and 0.5 μM sodium vanadate (Sigma). Western blotting was performed as described (15). Primary antibodies used include mouse-anti-β-catenin (BD Transduction Laboratories), diluted 1:1000; mouse anti-human IgG (Fcγ fragment-specific), diluted 1:1000 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA); rabbit anti-P73-c-Jun, diluted 1:1000 (Cell Signaling Technology, Beverly, MA); rabbit anti-c-Jun, diluted 1:1000 (H79; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-phospho-ATF2, diluted 1:1000 (Thr^{71}; Cell Signaling Technology); rabbit anti-ATF2, diluted 1:1000 (C19; Santa Cruz Biotechnology); rabbit anti-c-Fos, diluted 1:1000 (06-431, Upstate, Charlottesville, VA); and rabbit anti-MSH2, diluted 1:15,000 (22). Primary antibodies were detected using sheep anti-mouse horseradish peroxidase conjugate, diluted 1:10,000 (Amersham Biosciences) or goat anti-rabbit-horseradish peroxidase, diluted 1:10,000 (Jackson ImmunoResearch Laboratories). Proteins were detected using enhanced chemiluminescence (Sigma) or the Supersignal® WestPico chemiluminescent substrate (Perbio Science, Etten-Leur, The Netherlands).

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described (23). Briefly, cells were fixed with methanol/acetone (1:2) or 2% paraformaldehyde and 0.2% Triton X-100, blocked in 5% nonfat dry milk/phosphate-buffered saline, and incubated with primary and secondary antibodies. Mouse monoclonal anti-β-catenin, diluted 1:500, and mouse anti-human IgG (Fcγ fragment-specific), diluted 1:100, were detected with sheep anti-rabbit Alexa594 conjugate, diluted 1:2000 (Molecular Probes, Leiden, The Netherlands), goat anti-mouse Alexa594 1:1000 (Molecular Probes), or sheep-anti-mouse fluorescein isothiocyanate, diluted 1:200 (Sigma). Coverslips were embedded in gelvatol (5 g of polyvinylalcohol in 30% glycerol and 100 mg/ml DABCO) with 1 μg/ml 4′,6-diamidino-2-phenylindole·2HCl as a nuclear marker. Fluorescence was obtained using a Leica DMRBE microscope type 301–371.011 (Leica, Rijswijk, The Netherlands). Images were digitally stored using IP Lab Spectrum version 3.1 software.

Immunohistochemistry—Human renal tissue sections from healthy individuals and from patients diagnosed with ADPKD were immunostained for β-catenin as described (24). The Envision+ kit (DakoCytomation B.V., Heverlee, Belgium) was used as a secondary reagent. Staining was developed using DAB (brown precipitate). Slides were counterstained with hematoxylin.

RESULTS

Membrane-targeted Mouse C-terminal Polycystin-1 Does Not Activate Wnt Signaling in HEK293 and MDCK Cells—Wnt activation is reflected by transactivation of luciferase in the TOP *versus* the control FOP reporter construct (Fig. 1*A*). Since the luciferase reporter construct contains three binding sites for Wnt-specific TCF/LEF transcription factors only, activation requires intact components of the signaling cascade in the cells

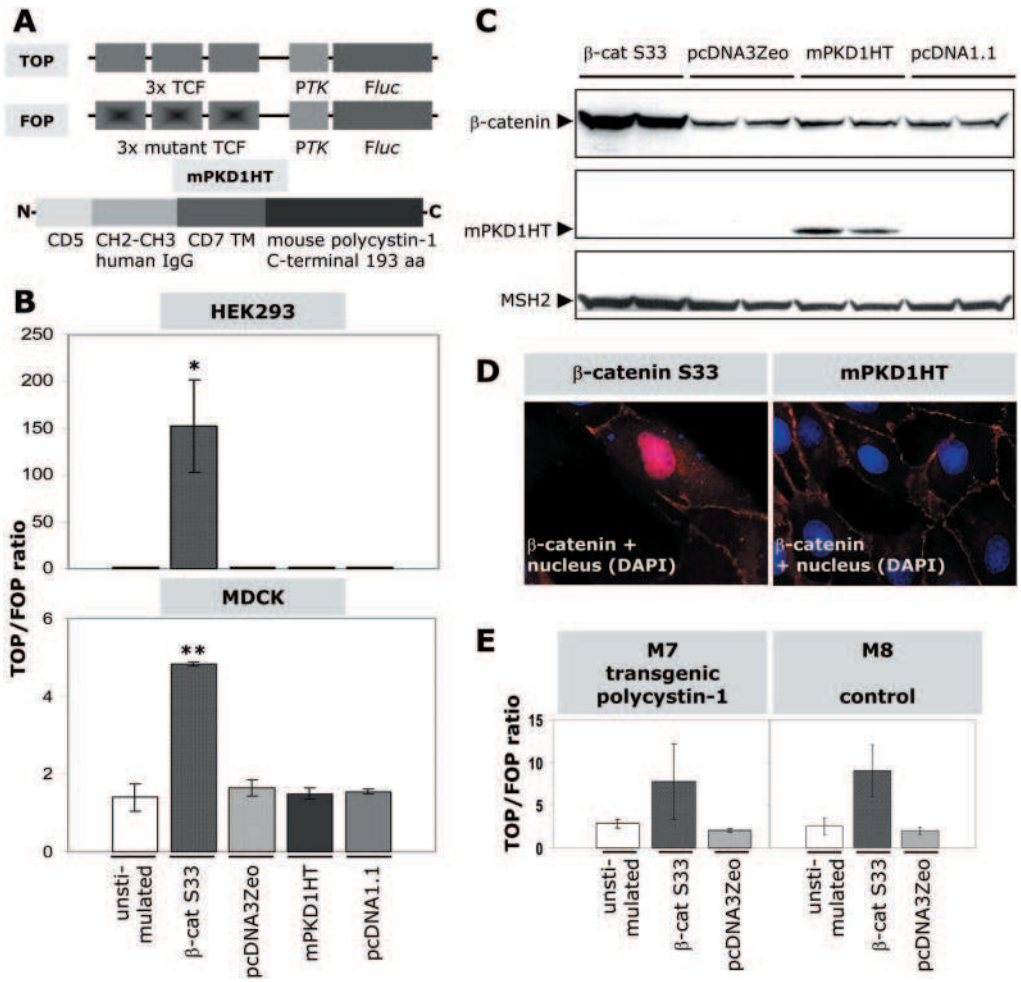

FIG. 1. **Membrane-targeted mouse C-terminal polycystin-1 does not activate Wnt signaling.** *A*, schematic representation of the *TOP* and *FOP* luciferase reporters for Wnt signaling (*upper two panels*). The TOP construct contains three Wnt-specific binding sites for TCF/LEF transcription factors (*3×TCF*) and the firefly luciferase reporter (*Fluc*) under control of a minimal promoter from herpes simplex virus thymidine kinase (*PTK*). The FOP construct is identical to the TOP construct, with the exception that the three TCF binding sites are mutated and therefore inactive. The mPKD1HT construct (*lower panel*) contains the C-terminal 193 amino acids (amino acids 4101–4293) of mouse polycystin-1 fused to the CD5 signal sequence, CH2-CH3 sequences of human IgG, and the CD7 transmembrane domain, in the pcDNA1.1 vector backbone. *B*, TOP/FOP luciferase reporter assay in HEK293 (*upper panel*) and MDCK cells (*lower panel*). Cells were transfected with plasmid constructs (TOP or FOP and pRL-TK reporters and either mPKD1HT or β-cat S33), cultured under serum-free conditions, and assayed for luciferase activity 1–2 days post-transfection. As a positive control for induction of Wnt signaling, the constitutive active β-catenin Ser^{33} construct, β-cat S33, was included. The pcDNA3Zeo and pcDNA1.1 vectors were included as empty vector controls for the β-cat S33 and the mPKD1HT construct, respectively. Data are shown of 2–4 independent triplicate experiments as the mean ± S.D. of the ratio between the TOP and FOP reporters. Statistical significant measurements are indicated. *, $p < 0.05$; **, $p < 0.005$. *C*, Western blot analysis of β-catenin in HEK293 total cell lysates. Cells were transfected with β-cat S33, mPKD1HT, or the empty vector controls, pcDNA3Zeo and pcDNA1.1; assayed for luciferase activity; and assayed subsequently for β-catenin protein level using Western blot. β-Catenin was detected with mouse-anti-β-catenin (*upper panel*). To detect the mPKD1HT construct, the same blot was incubated with mouse anti-human-IgG (*middle panel*). As a loading control, rabbit anti-MSH2 was included (*lower panel*). Representative data are shown. *D*, immunofluorescence microscopy for β-catenin in MDCK cells. Cells were transfected with β-cat S33 (*left panel*) or with the mPKD1HT construct (*right panel*). β-Catenin was detected using mouse-anti-β-catenin and goat anti-mouse Alexa594 (*red*). Transfected cells were identified by co-transfection with an enhanced green fluorescent protein construct (pEGFP-N1; not shown). Cell nuclei were visualized using 4′,6-diamidino-2-phenylindole·2HCl (*DAPI*) (*blue*). Representative images are shown. *E*, TOP/FOP luciferase reporter assay in mouse renal tubular epithelial cells expressing full-length human polycystin-1 (M7, *left panel*) and the control cell line (M8, *right panel*). Cells were transfected with plasmid constructs (TOP or FOP and pRL-TK reporters and β-cat S33 or pcDNA3Zeo) and assayed for luciferase activity 2 days post-transfection. Data are shown of two independent triplicate experiments as the mean ± S.D. of the ratio between the TOP and FOP reporter. The β-cat S33 construct was included as a positive control for induction of Wnt signaling.

tested. HEK293 and canine renal tubular epithelial (MDCK) cells transfected with the constitutive active β-catenin Ser^{33} mutant, β-cat S33, showed significant Wnt activation, as reflected by the markedly increased TOP/FOP ratio compared with unstimulated and empty vector control (Fig. 1*B*). Therefore, the cells tested were indeed capable of generating an adequate cellular response upon induction of Wnt signaling. However, no significant Wnt activation was detected after transfection with the membrane-targeted mouse C-terminal polycystin-1 construct, mPKD1HT, in both HEK293 (Fig. 1*A*) and MDCK cells (Fig. 1*B*). To exclude the possibility that induction of Wnt signaling by mPKD1HT was below the measuring threshold of the luciferase reporter assay, we tested for the hallmarks of Wnt activation, cytoplasmic accumulation, and nuclear translocation of β-catenin, using Western blotting and immunofluorescent staining for β-catenin (Fig. 1, *C* and *D*). Significant accumulation and nuclear translocation of β-catenin were detected only in cells transfected with the β-catenin Ser^{33} mutant construct. The mPKD1HT construct was correctly expressed in transfected cells (Fig. 1*C*, *middle panel*) and was correctly targeted to the plasma membrane, although a significant amount was also present in the cytoplasm as detected using immunofluorescence microscopy (data not shown). We and other groups have previously reported the expression of

endogenous polycystin-1 in the plasma membrane (23, 25, 26). The housekeeping protein MSH2 was incorporated as a loading control (Fig. 1*C*, *lower panel*). In mPKD1HT-transfected cells, β-catenin was exclusively detected associated to the plasma membrane as a component of adherens junctions (Fig. 1*D*, *right panel*). Cells expressing β-cat S33 exhibited both the plasma membrane-associated and nuclear localization (Fig. 1*D*, *left panel*).

We conclude that under the defined experimental conditions, the membrane-targeted mouse C-terminal polycystin-1 construct does not induce Wnt signaling in HEK293 and MDCK cells. Furthermore, in M7 cells, mouse SV40 large T-immortalized renal tubular epithelial cells expressing transgenic human full-length polycystin-1, Wnt activation was detected but did not differ from M8 control cells (Fig. 1*E*). Both cell lines were capable of responding adequately to Wnt induction by β-cat S33. Thus, expression of polycystin-1 did not directly activate Wnt signaling.

Membrane-targeted Mouse C-terminal Polycystin-1 Does Not Augment β-Catenin Ser^{33}-induced Wnt Activation—HEK293 and MDCK cells co-transfected with mPKD1HT and β-cat S33 did not show a significant difference in Wnt activation as compared with co-transfection of β-cat S33 with pDNA1.1, the vector backbone of mPKD1HT (Fig. 2*A*). Moreover, in the colon epithelial carcinoma cell lines, HCT116 (Fig. 2*B*) and SW480 (data not shown), which exhibit constitutively active Wnt signaling due to mutations in the β-catenin and *APC* gene, respectively, mPKD1HT did not have an effect on canonical Wnt signaling as detected using the TOP/FOP assay. Transfection of β-catenin Ser^{33} in HCT116 and SW480 did induce Wnt signaling above the activation level in the unstimulated status.

In conclusion, the membrane-targeted mouse C-terminal polycystin-1 construct did not augment β-catenin Ser^{33}-induced Wnt activation.

Wnt Signaling Is Not Affected in ADPKD Renal Cystic Epithelium—Since overexpression of polycystin-1 did not result in activation or augmentation of Wnt signaling, we investigated Wnt signaling in the human renal ADPKD cystic epithelial cell lines, PKD9–7WT and PKD10–7WT. PKD9–7WT and PKD10–7WT as well as the control cell line, RCTEC, did not differ in Wnt activation in the unstimulated state as detected by the TOP/FOP reporter assay, whereas cells did exhibit an adequate cellular response upon activation of Wnt signaling by β-cat S33 (Fig. 3*A*, 2-fold induction by β-cat S33 compared with the empty vector in all cells). Immunofluorescent staining for β-catenin revealed only the expected plasma membrane-associated localization of β-catenin (Fig. 3*B*). Furthermore, immunohistochemical staining of renal cystic tissues of four ADPKD patients with mutations in PKD1 did not show distinct cytoplasmic accumulation or nuclear translocation of β-catenin (Fig. 3*C*, ADPKD patient H84–3821 shown in the *right panel*).

Thus, in established ADPKD cystic epithelium, Wnt signaling was not significantly affected.

Membrane-targeted Mouse C-terminal Polycystin-1 Activates AP-1 via Jun-ATF2 in HEK293 and NRK-52E Cells—The AP-1 transcription factor complex can be activated by a variety of stimuli such as growth factors and stresses that can induce both Jun-Fos and Jun-ATF activity. We tested activation of AP-1 using distinct luciferase reporter constructs (Fig. 4*A*, *upper panel*). The 5×coll and 7×AP-1 reporters are activated by Jun-Fos heterodimers and can be strongly induced by TPA (19, 27). The 5×jun reporter construct mainly monitors Jun-ATF activity and is hardly enhanced by TPA. In HEK293 cells, TPA specifically activated the 5×coll and 7×AP-1 luciferase reporters but not the 5×jun reporter in cells cultured under serum-free conditions (Fig. 4*A*). In contrast, the constitutively

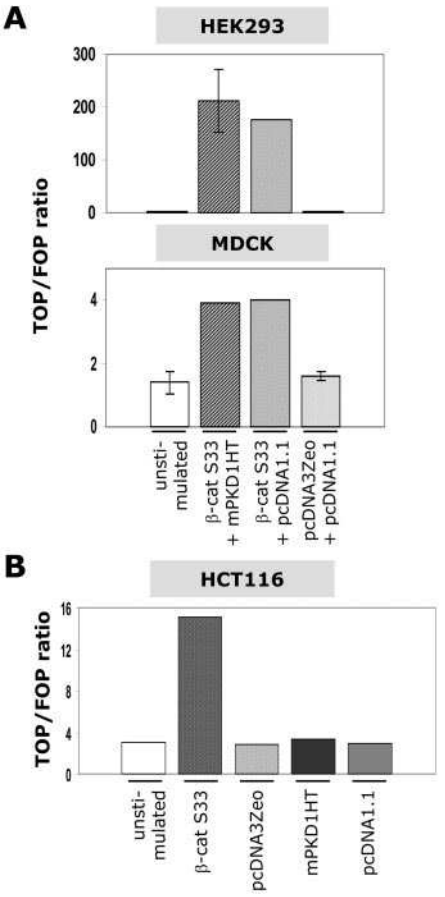

FIG. 2. **Membrane-targeted mouse C-terminal polycystin-1 does not augment β-cat S33-induced Wnt activation.** *A*, TOP/FOP luciferase reporter assay to detect synergism between mPKD1HT and β-cat S33 in HEK293 (*upper panel*) and MDCK cells (*lower panel*). Cells were transfected with plasmid constructs (TOP or FOP and pRL-TK reporters, constitutive active β-cat S33 and/or the mouse C-terminal polycystin-1 construct, mPKD1HT, or the corresponding empty vector controls, pcDNA3Zeo and pcDNA1.1), cultured under serum-free conditions, and assayed for luciferase activity 1–2 days post-transfection. Data are shown of 1–2 triplicate experiments as the (mean ± S.D. of the) ratio between the TOP and FOP reporter. *B*, TOP/FOP luciferase reporter activity assay in a human colon carcinoma epithelial cell line with constitutive active Wnt signaling, HCT116. Cells were transfected with plasmid constructs (TOP or FOP and pRL-TK reporters, mPKD1HT, β-cat S33 or the empty vector control, pcDNA1.1 or pcDNA3Zeo) and assayed for luciferase activity 1 day post-transfection. Data are shown of 1–2 independent triplicate experiments as the (mean ± S.D. of the) ratio between the TOP and FOP reporter. The β-cat S33 construct was included as a positive control.

active Rho GTPase, cdc42 V12, strongly induced the 5×jun reporter. Therefore, activation of Jun-ATF and Jun-Fos heterodimers can indeed be distinguished in this cell type using these reporters. The membrane-targeted mouse C-terminal polycystin-1 construct specifically induced the 5×jun reporter, whereas activation of the 5×coll and 7×AP-1 reporters was not detectable. Similar results were obtained in HEK293 (Fig. 4*A*), HEK293T cells (data not shown), and the renal epithelial cell line, NRK-52E (Fig. 4*B*). The effect of the mPKD1HT construct on activation of the 5×jun reporter in NRK-52E cells was similar to the activation observed with the known inducers of Jun-ATF2 activity, cdc42 V12 and UV-C. Moreover, the effect of the mPKD1HT construct on the 5×jun reporter was dosage-dependent (Fig. 4*C*).

Thus, under the defined experimental conditions membrane-targeted mouse C-terminal polycystin-1 enhanced Jun-ATF rather than Jun-Fos activity.

Membrane-targeted Mouse C-terminal Polycystin-1 Induces Phosphorylation and Expression of c-Jun and Increases

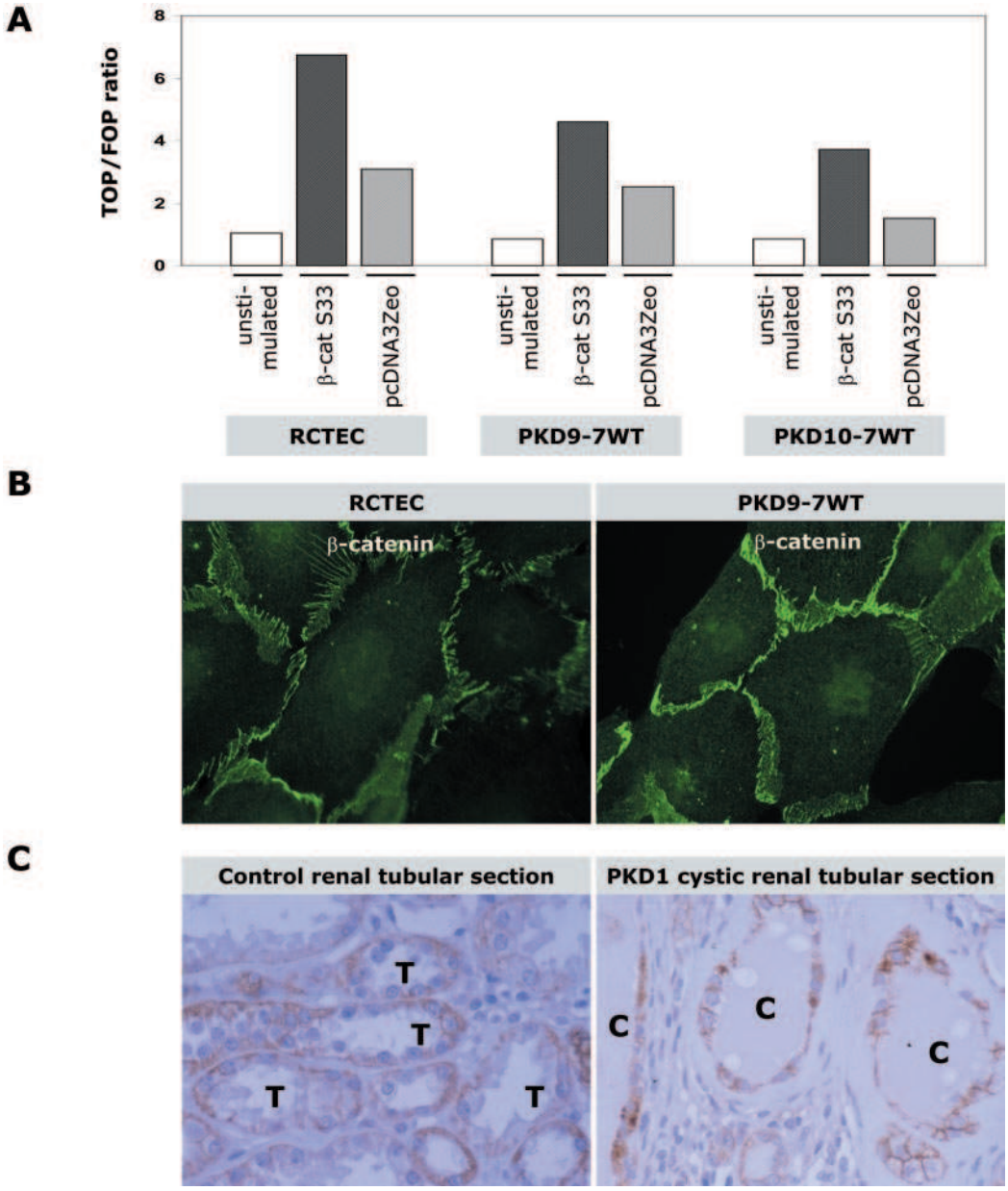

FIG. 3. **Wnt signaling is not activated in ADPKD renal cystic cells.** *A*, TOP/FOP luciferase reporter activity assay in control (RCTEC, *left panel*) and human ADPKD renal cystic epithelium (PKD9–7WT and PKD10–7 WT; *middle* and *right panels*). Cells were transfected with plasmid constructs (1000 ng of TOP or FOP and 25 ng of pRL-TK reporters, 500 ng of constitutive active β-cat S33, or the empty vector control, pcDNA3Zeo) and assayed for luciferase activity 1 day post-transfection. Data are shown of a triplicate experiment as the ratio between the TOP and FOP reporter. *B*, immunofluorescence microscopy for β-catenin in RCTEC (*left*) and PKD9–7WT cells (*right*). Cells were fixed and immunostained for β-catenin (*green*). Cell nuclei were visualized using 4′,6-diamidino-2-phenylindole·2HCl (*data not shown*). Representative images are shown. *C*, immunostaining for β-catenin in human renal sections derived from control (*left panel*) and ADPKD cystic tissue (*right panel*). The ADPKD cystic tissue shown is derived from a patient who has a mutation in exon 45 of the *PKD1* gene (12251Î2252insTGTCACC). Tissue sections were stained for β-catenin (*brown*) and counterstained with hematoxylin (*blue*). *T*, tubule lumen; *C*, cystic tubule lumen. Images were taken at ×400 magnification.

Phosphorylation of ATF2—In conjunction with the activation of the Jun-ATF dependent 5×jun luciferase reporter, expression of mPKD1HT increased both total and Ser^{73} phosphorylation of endogenous c-Jun in HEK293 (Fig. 5*A*, *left panel*) and HEK293T cells (data not shown). Densitometry analysis of Western blots indicated that the effect of the mPKD1HT construct occurred predominantly by induction of Ser^{73} phosphorylation (12-fold increase compared with the empty vector control) and to a lesser extent by increasing total protein level of c-Jun (1.6-fold; data not shown). Cells treated with the known inducers of c-Jun activity, osmotic shock, and cdc42 V12, showed similar enhancement.

In contrast, the total protein level of endogenous c-Fos in mPKD1HT-transfected cells did not differ from control, whereas cells treated with TPA did show a distinct increase in c-Fos level (Fig. 5*B*). Thus, expression of mPKD1HT induced phosphorylation and increased total protein level of c-Jun, whereas c-Fos protein level was unaffected.

Co-expression of HA-tagged ATF2 with the mPKD1HT construct in HEK293 cells increased Thr^{71} phosphorylation of ATF2 compared with the empty vector control, pcDNA1.1, although the increase was less intense than the effect of osmotic shock and cdc42 V12 expression (Fig. 5*C*, *left panel*). Data were confirmed by assaying for endogenous ATF2 in cells transfected with mPKD1HT using Western blotting (Fig. 5*C*, *right panel*).

In conclusion, membrane-targeted mouse C-terminal poly-

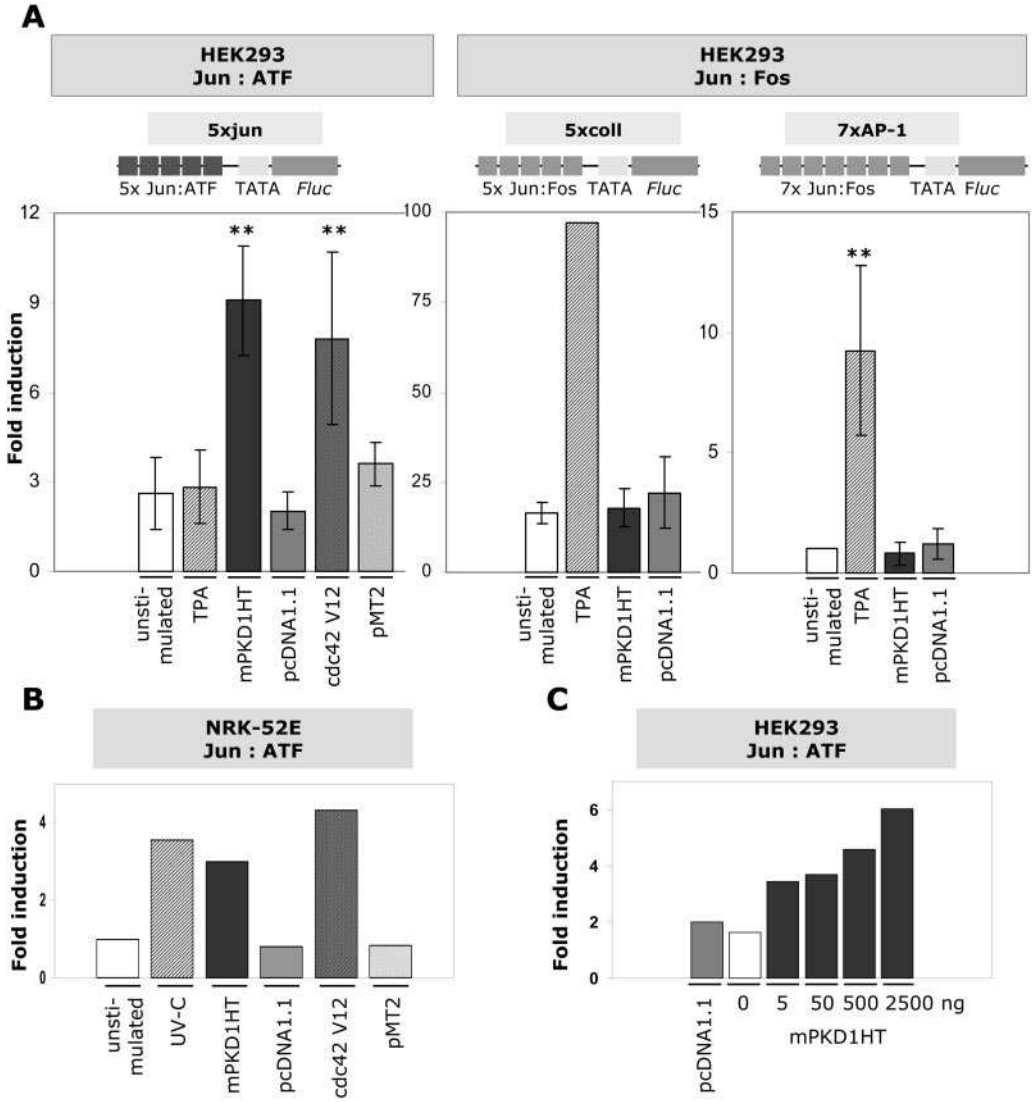

FIG. 4. **Membrane-targeted mouse C-terminal polycystin-1 activates the 5×jun luciferase reporter.** *A*, AP-1 luciferase reporter activity assays in HEK293 cells. The *upper panel* shows the schematic representation of the 5×jun reporter construct containing five Jun-ATF binding sites (*5×Jun:ATF*), the 5×coll reporter containing five Jun-Fos binding sites (*5×Jun:Fos*), and the *7×AP-1* reporter containing seven Jun-Fos binding sites (*7×Jun:Fos*), a TATA box (*TATA*), and the firefly luciferase reporter (*Fluc*). Cells were transfected with plasmid constructs (5×jun, 5×coll, 7×AP-1, or TATA pGL3 and pRL-TK reporters, and the mouse C-terminal polycystin-1 construct, mPKD1HT, or the empty vector control, pcDNA1.1), cultured under serum-free conditions, and assayed for luciferase activity 2 days post-transfection. TPA was included as a negative control for the 5×jun reporter and as a positive control for the 5×coll and 7×AP-1 to indicate that Jun-ATF and Jun-Fos activation can be distinguished using these reporters. As a positive control for induction of the 5×jun reporter, constitutive active cdc42 V12, with the corresponding empty vector control, pMT2, was included. Data are shown of a minimum of two independent triplicate experiments as the mean ± S.D. of the fold induction between the 5×jun (n = 6–10 independent experiments; *left panel*), the 5×coll (n = 5, except for TPA (n = 1); *middle panel*), or the 7×AP-1 (n = 9; *right panel*) reporter and the TATA pGL3 (control) reporter. Statistical significant measurements are indicated. **, $p < 0.005$. *B*, 5×jun luciferase reporter assay in NRK-52E cells. Cells were transfected with plasmid constructs (250 ng of 5×jun or TATA pGL3 and 5 ng of pRL-TK reporters, 2000 ng of mPKD1HT or pcDNA1.1, and 1000 ng of cdc42 V12 or pMT2), cultured under serum-free conditions, and assayed for luciferase activity 1 day post-transfection. As positive controls for activation of the 5×jun reporter, cells irradiated with 40 J/m^2 UV-C (*UV-C*) and cells transfected with cdc42 V12 (*cdc42 V12*) were included. Data are shown of a triplicate experiment as the fold induction between the 5×jun and the TATA pGL3 reporter. *C*, 5×jun luciferase reporter assay in HEK293 cells transfected with a dosage range of mPKD1HT (5–2500 ng). Cells were transfected with plasmid constructs (5×jun or TATA pGL3 and pRL-TK reporters and mPKD1HT or pcDNA1.1), cultured under serum-free conditions, and assayed for luciferase activity 2 days post-transfection. Data are shown of a triplicate experiment as the fold induction between the 5×jun and the TATA pGL3 reporter.

cystin-1 induced phosphorylation and activation of c-Jun and ATF2, whereas the c-Fos protein level remained unaffected.

AP-1 Activity Is Aberrant in Human ADPKD Renal Cystic Epithelial Cells and in Renal Epithelial Cells Expressing Transgenic Full-length Polycystin-1—To determine the physiological relevance of the data obtained using our membrane-targeted mouse C-terminal polycystin-1 construct, we investigated AP-1 activity of the human renal cystic epithelial cell line, PKD9–7WT, which is derived from an ADPKD patient. PKD9–7WT cells exhibited significantly less 5×jun reporter activity than the control cell line, RCTEC (Fig. 6*A*, *left panel*). Similarly, data obtained using the −1600/+740 c-*jun* TATA pGL3 luciferase reporter revealed that transcription of c-*jun* itself was also decreased in PKD9–7WT cells (Fig. 6*C*). Upon treatment with UV-C irradiation, RCTEC and PKD9–7WT did exhibit increased 5×jun reporter activity, indicating that both cell lines were capable of generating an adequate cellular response to induce Jun-ATF activity (data not shown). In accordance with the impaired 5×jun reporter activity, expression of total and active Ser^{73}-phosphorylated c-Jun was decreased in

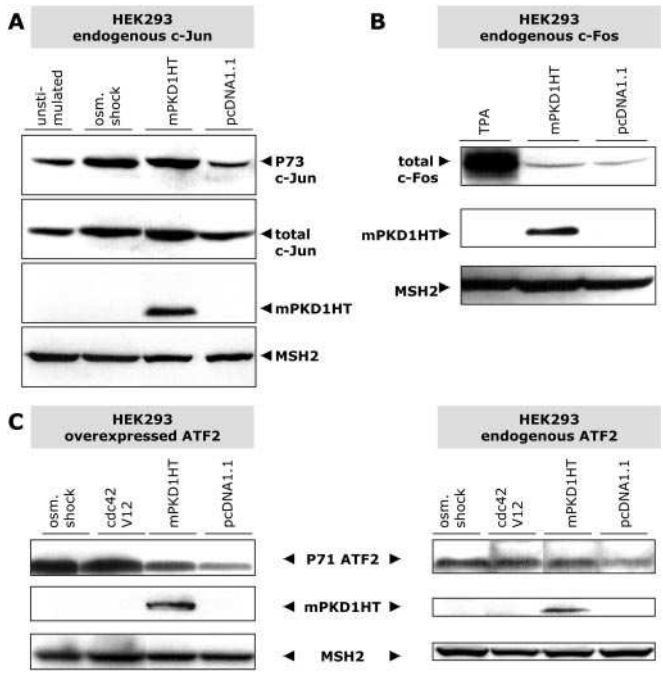

FIG. 5. **Membrane-targeted mouse C-terminal polycystin-1 increases phosphorylation and expression of c-Jun and phosphorylation of ATF2.** *A*, HEK293 cells were transfected with the mouse C-terminal polycystin-1 construct, mPKD1HT, or the corresponding empty vector control, pcDNA1.1, cultured under serum-free conditions, and assayed for endogenous c-Jun on a Western blot. Expression levels of Ser^{73}-phosphorylated c-Jun (*P73-c-Jun*), total c-Jun (*total c-Jun*), the mPKD1HT construct (*mPKD1HT*), and the loading control MSH2 (*MSH2*) were analyzed. As a positive control for induction of (phosphorylation of) c-Jun, cells treated with osmotic shock (*osm. shock*) were included. Representative data are shown. *B*, HEK293 cells were transfected with mPKD1HT or pcDNA1.1, cultured under serum-free conditions, assayed for luciferase activity, and subsequently assayed for endogenous c-Fos using Western blotting. Total endogenous c-Fos (*total c-Fos*), the mPKD1HT construct (*mPKD1HT*), and the loading control MSH2 (*MSH2*) were analyzed. As a positive control for induction of c-Fos, cells treated with TPA (*TPA*) were included. Representative data are shown. *C*, HEK293 cells were co-transfected with HA-ATF2 and cdc42 V12, mPKD1HT, or pcDNA1.1 (*left panel*) or transfected with cdc42 V12, mPKD1HT, or pcDNA1.1 alone (*right panel*); cultured under serum-free conditions; lysed 1 day post-transfection; and assayed for ATF2 on Western blot. Thr^{71}-phosphorylated ATF2 (*P71-ATF2*), the mPKD1HT construct (*mPKD1HT*), and the loading control MSH2 (*MSH2*) were analyzed. As positive controls for induction of phosphorylation of ATF2, cells treated with osmotic shock (*osm. shock*) and cells transfected with cdc42 V12 were included.

PKD9–7WT compared with RCTEC cells (Fig. 6*A*, *middle panel*). In contrast, expression level of total and Thr^{71}-phosphorylated ATF2 was increased in PKD9–7WT cells (Fig. 6*B*, *right panel*). This increased expression of ATF2 may reflect enhanced activity of more upstream extracellular signal-regulated kinases (ERK) (19). Furthermore, increased activity of the Jun-Fos-dependent AP-1 reporters, 5×coll (Fig. 6*B*, *left panel*) and 7×AP-1 (data not shown) in PKD9–7WT cells coincided with an increased total expression level of c-Fos (Fig. 6*B*, *right panel*).

Upon transfection of PKD9–7WT cells with the membrane-targeted mouse C-terminal polycystin-1 construct, mPKD1HT, 5×jun reporter activity was restored to levels above the reporter activity of RCTEC control cells (Fig. 6*D*). These data indicate that the Jun-ATF-activating properties of polycystin-1 can be mimicked by expression of this C-terminal region of polycystin-1.

In conclusion, AP-1 activity is aberrant in PKD9–7WT cells, resulting in impaired Jun-ATF activity and increased Jun-Fos activity. Moreover, data indicate that expression of c-Jun is regulated at the level of both gene transcription and post-transcriptional modifications, suggesting that c-Jun is the limiting factor for impaired Jun-ATF activity in PKD9–7WT cells.

Analysis of M7 cells, mouse SV40 large T-immortalized renal epithelial cells expressing transgenic human full-length polycystin-1, revealed that Jun-ATF-dependent 5×jun reporter activity was also significantly impaired in these cells compared with the control cell line, M8 (Fig. 7*A*, *left panel*). Moreover, Ser^{73} phosphorylation of c-Jun was decreased in M7 cells (data not shown). Expression of the membrane-targeted mouse C-terminal polycystin-1 construct mPKD1HT, in M7 cells restored the impaired 5×jun reporter activity (Fig. 7*A*, *right panel*). Reporter activity of the 5×coll construct was increased in M7 compared with M8 control cells (Fig. 7*B*). Since M7 cells showed similar impaired Jun-ATF and increased Jun-Fos-mediated AP-1 activation as PKD9–7WT cells, overexpression of full-length polycystin-1 may result in a defect in AP-1 activity as well. M7 cells were isolated from a transgenic mouse model that expresses functional full-length polycystin-1 (20, 28, 29). Intriguingly, transgenic mice developed mild polycystic kidney disease, indicating that expression levels of polycystin-1 are important for normal renal function, since both too low and too high expression of polycystin-1 results in polycystic kidney disease (28). In accordance, our data indicate that in both polycystic kidney cells (PKD9–7WT) and in cells expressing transgenic full-length polycystin-1 (M7) AP-1 activation is aberrant, thereby implicating a role for polycystin-1 in regulating AP-1 activity.

DISCUSSION

The goal of our study was to gain a better understanding of the complex role of polycystin-1 in Wnt signaling and AP-1 activation. For this, we expressed a membrane-targeted mouse C-terminal polycystin-1 construct, mPKD1HT, in cell lines that have no known defect in polycystin-1 or polycystin-2, in order to identify relevant signaling events. To determine the physiological relevance of overexpressing this membrane-targeted mouse C-terminal polycystin-1 construct, human ADPKD renal cystic epithelial cells and renal epithelial cells expressing transgenic human full-length polycystin-1 were subsequently analyzed.

Intriguingly, under defined experimental conditions, we observed preferential Jun-ATF-dependent AP-1 activation by the membrane-targeted mouse C-terminal polycystin-1 construct. The membrane-targeted mouse C-terminal polycystin-1 fusion protein construct did not activate or augment canonical Wnt signaling as detected using the TOP/FOP luciferase reporter assay, Western blotting, and immunostaining for β-catenin (Figs. 1 and 2). Since this was observed in human embryonic kidney, HEK293 and HEK293T, and the more relevant renal epithelial MDCK cells, cell type-specific effects are less likely. Kim *et al.* (5) have previously reported that a membrane-targeted human C-terminal polycystin-1 construct activated a Siamois promoter-based luciferase reporter assay for Wnt signaling and stabilized β-catenin. The discrepancy in data may be attributed to differences in luciferase reporter constructs or in mouse and human polycystin-1. However, mouse and human sequences of polycystin-1 are highly conserved (79% identity between human and mouse) (30). Moreover, we show that Wnt signaling did not significantly differ in a mouse renal epithelial cell line expressing transgenic human full-length polycystin-1 (M7; Fig. 1*E*). Although highly specific, bare TCF binding sites used in our TOP/FOP assays may only be functional within the appropriate environment requiring additional regulatory elements for induction by the mouse C-terminal polycystin-1 construct. Conversely, the Siamois promoter fragment may contain additional regulatory elements that render it activated via a variety of routes and not exclusively by Wnt signaling. In

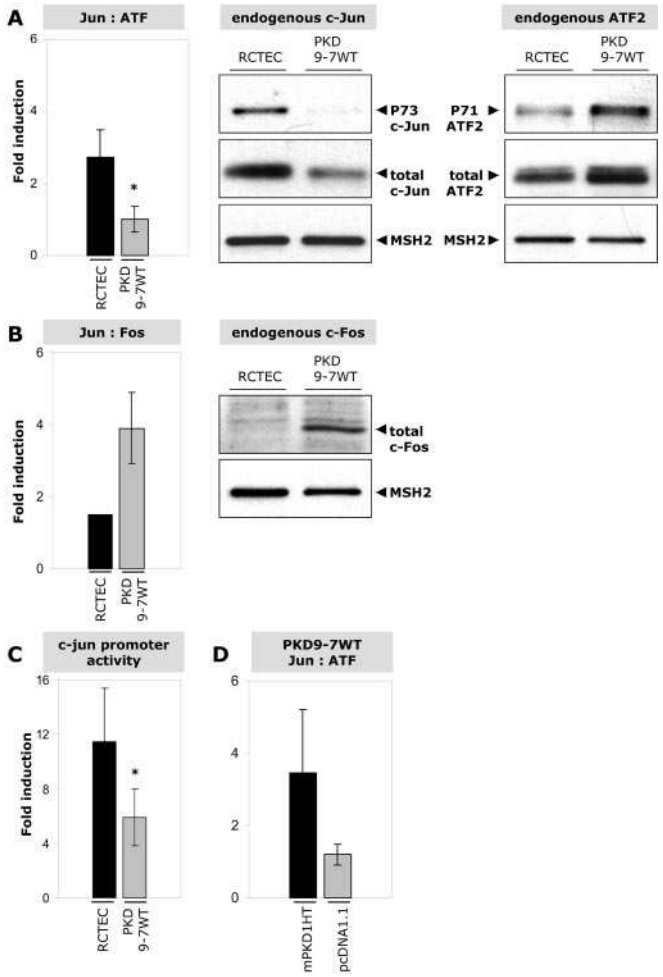

FIG. 6. **AP-1 activity is aberrant in human ADPKD renal cystic epithelial cells.** *A*, 5×jun luciferase reporter assay in the human ADPKD renal cystic epithelial cell line, PKD9–7WT, compared with the control cell line, RCTEC (*left panel*). Cells were transfected with plasmid constructs (1000 ng of 5×jun or TATA pGL3 reporter), cultured under serum-free conditions, and assayed for luciferase activity 2–3 days post-transfection. Data are shown of 2–4 independent triplicate experiments as the mean ± S.D. of the fold induction between the 5×jun and the TATA pGL3 (control) reporter. Statistically significant measurements are indicated. *, $p < 0.05$. RCTEC and PKD9–7WT cells were subsequently analyzed for Ser^{73}-phosphorylated endogenous c-Jun (*P73-c-Jun*), total c-Jun (*total c-Jun*), Thr^{71}-phosphorylated ATF2 (*P71-ATF2*), total ATF2 (*total ATF2*), and the loading control MSH2 (*MSH2*) using Western blotting (*middle* and *right panel*). Representative data are shown. *B*, 5×coll luciferase reporter assay in PKD9–7WT and RCTEC cells (*left panel*). Cells were transfected with plasmid constructs (1000 ng of 5×coll or TATA pGL3 reporter), cultured under serum-free conditions, and assayed for luciferase activity 2–3 days post-transfection. Data are shown of two independent duplicate experiments as the mean ± S.D. of the fold induction between the 5×coll and the TATA pGL3 (control) reporter. RCTEC and PKD9–7WT cells were subsequently analyzed for total protein level of endogenous c-Fos (*total c-Fos*) and the loading control MSH2 (*MSH2*) using Western blotting (*right panel*). Representative data are shown. *C*, −1600/+740 c-*jun* TATA pGL3 luciferase reporter assay in PKD9–7WT and RCTEC cells. Cells were transfected with plasmid constructs (1000 ng of ×1600/+740 wt c-*jun* TATA pGL3 or −1600/+740 m1 + 2 c-*jun* TATA pGL3 reporter), cultured under serum-free conditions, and assayed for luciferase activity 1–2 days post-transfection. Data are shown of three independent duplicate experiments as the mean ± S.D. of the fold induction between the −1600/+740 wt c-*jun* TATA pGL3 and the mutant −1600/+740 m1 + 2 c-*jun* TATA pGL3 reporter. Statistically significant measurements are indicated. *, $p < 0.05$. *D*, 5×jun luciferase reporter assay in PKD9–7WT cells. Cells were transfected with plasmid constructs (1000 ng of 5×jun or TATA pGL3 reporter and 1000 ng of mPKD1HT or pcDNA1.1), cultured under serum-free conditions, and assayed for luciferase activity 1–2 days post-transfection. Data are shown of two independent duplicate experiments as the mean ± S.D. of the fold induction between the 5×jun and the TATA pGL3 (control) reporter.

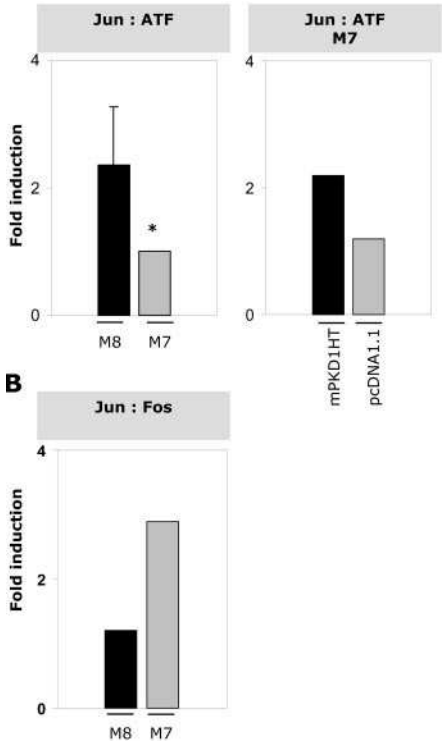

FIG. 7. **AP-1 activity is aberrant in renal epithelial cells expressing transgenic human full-length polycystin-1.** *A*, 5×jun luciferase reporter assay in renal epithelial cells expressing transgenic human full-length polycystin-1 (M7) compared with control cells (M8). Cells were transfected with plasmid constructs (500 ng of 5×jun, TATA pGL3 and 50 ng of pRL-TK reporters, 1000 ng of mPKD1HT or pcDNA1.1), cultured under serum-free conditions, and assayed for luciferase activity 1–2 days post-transfection. Data are shown of 2–5 independent triplicate experiments as the mean ± S.D. of the fold induction between the 5×jun and the TATA pGL3 (control) reporter. Statistically significant measurements are indicated. *, $p < 0.05$. *B*, 5×coll luciferase reporter assay in M7 and M8 cells. Cells were transfected with plasmid constructs (500 ng of 5×coll or TATA pGL3 and 50 ng of pRL-TK reporters), cultured under serum-free conditions, and assayed for luciferase activity 1 day post-transfection. Data are shown of a triplicate experiment as the fold induction between the 5×coll and the TATA pGL3 (control) reporter.

addition, we cannot exclude the possibility that the effect of polycystin-1 on Wnt signaling is too subtle to be detected using existing techniques. This sensitivity threshold is an inherent effect of any experimental design. To date, the TOP/FOP reporter assay and immunodetection of nuclear β-catenin remain the most specific methods to detect Wnt activation.

If the C-terminal polycystin-1 construct can indeed activate Wnt signaling, ADPKD cystic cells should show aberrant Wnt signaling. Our data indicate that canonical Wnt signaling is not significantly aberrant in cells and tissue sections derived from human ADPKD renal cystic epithelium (Fig. 3). In accordance, Kugoh *et al.* (31) have reported that in TCS2-deficient cells lacking plasma membrane-localized polycystin-1, β-catenin localization and function is not affected. Recently, a polycystin-1 knockout mouse model has been described in which total β-catenin protein level was decreased in heart and kidney tissue (32). Administration of pioglitazone rescued cardiac and renal abnormalities and subsequently elevated β-catenin levels to control values, indicating that polycystin-1 function and β-catenin are linked. Functional assays using (cells derived from) this mouse model to determine whether β-catenin function is indeed affected, would provide more insight. In addition, transgenic mice expressing mutant β-catenin develop cysts in the kidneys (33). These mice are deficient in binding to α-catenin, a crucial component linking adhesion junctions to the cytoskeleton, and ex-

hibit constitutively active Wnt signaling. Therefore, aberrant β-catenin function results in cystogenesis. However, whether the primary defect in polycystin-1 in ADPKD affects β-catenin-mediated Wnt activation and thus cystogenesis remains to be elucidated. Cyst development in the kidney has been reported previously in a variety of mouse models, suggesting that several routes can lead to cyst formation (34–37). We postulate that Wnt signaling may not be a major factor in established ADPKD renal cystic epithelium, although it may yet be a factor in earlier stages of ADPKD cystogenesis as the actual trigger that sets off or augments cystogenesis.

The membrane-targeted mouse C-terminal polycystin-1 construct did activate AP-1 and more specifically Jun-ATF heterodimer activity in HEK293 and renal epithelial NRK-52E cells (Fig. 4). To our knowledge, we report here for the first time that expression of the C-terminal region of polycystin-1 induces phosphorylation and activation of endogenous c-Jun (Fig. 5). Our data suggest that the C-terminal polycystin-1 construct also increases phosphorylation of ATF2. The protein level of c-Fos was not affected by the mouse C-terminal polycystin-1 construct, and activation of Jun-Fos dependent luciferase reporters was not detected under our experimental conditions. Parnell *et al.* (7) have recently reported activation of a Jun-Fos-specific luciferase reporter using a construct containing the C-terminal 222 amino acids of mouse polycystin-1. We propose that polycystin-1 is capable of inducing at least c-Jun and ATF2 activity and that the gene transcriptional effect of this activation is tightly regulated and depends on variables such as cellular context and experimental conditions. The C-terminal 29-amino acid difference between our sequence and the construct used by Parnell *et al.* (7) may contain a regulatory domain determining Jun-ATF or Jun-Fos activation. Divergent mechanisms of AP-1 activation have been reported to be a major regulatory mechanism to determine the cellular response upon a certain stimulus (reviewed in Refs. 10 and 11).

Analysis of human ADPKD renal cystic epithelial cells (PKD9–7WT) and a renal tubular epithelial cell line expressing transgenic human full-length polycystin-1 (M7) subsequently revealed that AP-1 activity was aberrant in both cell lines (Figs. 6 and 7). Total and Ser^{73}-phosphorylated levels of c-Jun were decreased in PKD9–7WT cells and coincided with a decrease in Jun-ATF-dependent reporter activity. Total protein level of ATF2 was strikingly increased, possibly due to upstream activation of ERK (19). Total protein level of c-Fos was increased also and was reflected by an increase in Jun-Fos-dependent reporter activity.

In conclusion, we hypothesize that polycystin-1 may affect the upstream activation of c-Jun and therefore modulate AP-1 activity, since ADPKD renal cystic epithelial cells as well as renal epithelial cells expressing transgenic human full-length polycystin-1 show aberrant AP-1 activity. Our data indicate that polycystin-1 primary exerts its effect on transcription and post-transcriptional modifications of c-Jun and that regulation of AP-1 activity may be a physiological function of polycystin-1. Expression of the membrane-targeted C-terminal polycystin-1 construct restored the impaired Jun-ATF activation level of PKD9–7WT cells. Thus, the Jun-ATF activating property of polycystin-1 lies in this C-terminal region, and expression of our polycystin-1 construct provides an adequate tool to study this signaling event. To determine whether aberrant AP-1 activity plays a significant role in ADPKD cystogenesis in general, additional cells from ADPKD patients should be analyzed.

Recent studies have shed some light on the complex interaction between signaling pathways. c-Jun was reported to be essential for regulation of Dickkopf-1 expression, a known inhibitor of canonical Wnt signaling, thereby establishing a direct link between AP-1 and Wnt signaling (38). Moreover, c-Jun and LEF-1 transcription factors have been reported to act cooperatively in regulating the matrix metalloprotease matrilysin promoter (39). The interplay between seemingly different signaling pathways may contribute to fine tune the cellular response upon a certain stimulus. Our data suggest that polycystin-1 primarily exerts its effect on c-Jun and to modulate AP-1 activity. The possible effect on Wnt signaling may occur via AP-1. This then results in a feedback loop regulating polycystin-1 expression, since the promoter region of polycystin-1 has been reported to contain putative AP-1 and TCF/LEF sites (40).

Our data indicate that polycystin-1 regulates AP-1 activity and that AP-1 plays a relevant role in ADPKD cystogenesis.

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