

# Increased Activity of Activator Protein-1 Transcription Factor Components ATF2, c-Jun, and c-Fos in Human and Mouse Autosomal Dominant Polycystic Kidney Disease

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Autosomal dominant polycystic kidney disease is a common inherited disorder that predominantly manifests with the formation of fluid-filled cysts in both kidneys. The disease can be accounted for by a mutation in either the *PKD1* or the *PKD2* gene. It was demonstrated previously that aberrant expression of the *PKD1* gene product, polycystin-1, results in modification of activator protein-1 (AP-1) transcription factor activity in cultured renal epithelial cells. Here, it is reported that activity of the AP-1 components c-Jun, ATF2, and c-Fos is altered in renal cystic tissue of patients with autosomal dominant polycystic kidney disease and of hypomorphic *Pkd1* mice with polycystic kidney disease. Data were obtained using immunohistochemical and Western blot analysis. Significant upregulation of Thr71- and Thr69/71-phosphorylated ATF2 and Ser73-phosphorylated c-Jun and increased c-Fos were detected in small cysts and (dilated) ducts and tubules surrounded by fibrotic interstitium. The data indicate that various AP-1 components are constitutively activated in polycystic kidney disease and suggest that aberrant AP-1 activity is relevant for cyst formation.

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Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited disorder that manifests predominantly with progressive development of fluid-filled cysts in both kidneys. Cyst formation ultimately 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), and a minority have a mutation in the *PKD2* gene (3–5). Several studies have implicated a role for the *PKD1* gene product, polycystin-1, in signal transduction pathways governing activator protein-1 (AP-1) activity (6–8). Moreover, we have reported that AP-1 activity is aberrant in an immortalized renal cystic epithelial cell line derived from a patient with ADPKD (8). AP-1 transcription factors are composed of multiple homo- or heterodimers that regulate key cellular processes such as cell proliferation, differentiation, and survival (9–11). The heterogeneity of AP-1 is thought to provide a mechanism to regulate a broad spectrum of cellular responses, because AP-1 can be activated by a variety of stimuli, such as growth factors and genotoxic stress. These stimuli affect intracellular signaling pathways, resulting in activation of one or more AP-1 components, including Jun, ATF, and Fos family members. Both the

activity and the expression levels of these transcription factors can be regulated by mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) (12). Phosphorylation of c-Jun at Ser73 results in increased transactivation potential and stability. Transactivation capacity of ATF2 is regulated *via* phosphorylation at Thr71 and Thr69 (13).

To elucidate further the role of AP-1 in ADPKD cystogenesis, we analyzed various AP-1 components in human and mouse ADPKD renal cystic tissue using immunohistochemical and Western blot analysis. Here, we report that Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, and c-Fos are increased in small cysts and (dilated) ducts and tubules surrounded by fibrotic interstitium. Our data indicate that AP-1 activity is upregulated in human and mouse polycystic kidneys, suggesting that renal cyst formation and accompanying interstitial fibrosis may in part result from changes in AP-1 activity.

## Materials and Methods

### Human Tissue Samples of Normal and Cystic Kidneys

Renal tissue from patients with ADPKD and chronic renal failure was fixed in 4% formalin and embedded in paraffin as described (14). Control renal tissue was isolated from donor kidneys intended for transplantation but not suitable for technical reasons (*e.g.*, branching of the renal artery). In total, three human control renal tissue samples (ET10, ET13, and ET14) and six human ADPKD renal cystic tissue samples of patients with known mutations in or positive linkage to *PKD1* were analyzed (Table 1).

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Table 1. Human normal and ADPKD patients analyzed for AP-1 activity<sup>a</sup>

Name	Gender	Age at Isolation	Diagnosis	Gene	Mutation	Exon	Predicted aa Change	Reference
ET10	M	29	Healthy	—	—	—	—	—
ET13	F	47	Healthy	—	—	—	—	—
ET14	F	58	Healthy	—	—	—	—	—
PK4.2 (H91-7975)	M	60	ADPKD	<i>PKD1</i>	C>T at nt 1198	5	R400X	(15)
PK133 (H87-9609B)	M	61	ADPKD	<i>PKD1</i>	G>A at nt 5622	15	Trp1874X	(16)
PK284 (E96-8918A)	F	52	ADPKD	<i>PKD1</i>	G>A at nt 5622	15	Trp1874X	(16)
PK143 (H84-3821)	F	55	ADPKD	<i>PKD1</i>	12251 <sup>+</sup> 12252 insTGTCACC	45	?	—
PK131 (LUMC9.089.237)	M	43	ADPKD	<i>PKD1</i>	?	?	?	—
PK63 (H79-10784)	F	45	ADPKD	<i>PKD1</i>	?	?	?	—

<sup>a</sup>ADPKD, Autosomal dominant polycystic kidney disease; AP-1, activator protein-1.

### Mouse Tissue Samples of Normal and Cystic Kidneys

Renal cystic tissue was isolated from a mouse model with hypomorphic *Pkd1* alleles as a result of the presence of a neomycin resistance marker in intron 1 (*Pkd1<sup>mt/ml</sup>*) (17). These mice develop polycystic kidney disease comparable to human ADPKD, with the majority of cysts originating from collecting ducts. Healthy, wild-type, age-matched mice were used as controls. Animal experiments were performed with approval of the animal experimentation committee of the Leiden University Medical Center according to the animal experimentation guideline of the Dutch government.

### Antibodies and Reagents for Immunohistochemistry

*Dolichos biflorus* agglutinin (DBA)–horseradish peroxidase (HRP) conjugate was diluted 1:50 for immunohistochemistry (Sigma, Zwijndrecht, The Netherlands), rabbit anti-aquaporin-2 was diluted 1:300 (Calbiochem, Amsterdam, The Netherlands), goat anti-uromodulin was diluted 1:5000 (Organon Teknika–Cappel, Turnhout, Belgium), and rabbit anti-megalin was diluted 1:500 (18,19). Rabbit anti-phospho-Thr71-ATF2 was diluted 1:50, rabbit anti-phospho-Thr69/71-ATF2 was diluted 1:50, rabbit anti-phospho-Ser73-c-Jun was diluted 1:12.5 (Cell Signaling Technology, Beverly, MA), and rabbit anti-c-Fos was diluted 1:3000 (Oncogene Research Products, Nottingham, UK). Mouse anti-polycystin-1, PKS-A, was raised against amino acids 3078 to 4302 of polycystin-1 and was a gift from C. Ward (20). PKS-A was diluted 1:50 for human and 1:20 for mouse tissue sections. Secondary antibodies included rabbit anti-goat–HRP diluted 1:100, rabbit Envision–HRP, and mouse Envision–HRP (DakoCytomation B.V., Heverlee, The Netherlands).

### Immunohistochemical Analysis

Renal tissue sections were stained with nephron segment-specific markers (19). DBA is expressed in collecting duct and, to a lesser extent, in distal tubules. Aquaporin-2 is also expressed in collecting ducts, whereas uromodulin is present in distal tubules and thick ascending limbs of Henle's loop. Megalin is expressed in proximal tubules. Histochemical staining was performed as described (19).

For ATF2, c-Jun, c-Fos, and polycystin-1 tissue sections were stained using the following protocol. Sections were deparaffinized and washed with PBS for 5 min. Antigen retrieval was performed for ATF2, c-Jun, and c-Fos by boiling in 10 mM citrate buffer at pH 6 for 10 min followed by cooling for 20 min. Sections then were washed three times with demiwater. Each wash step was carried out for 5 min. Blocking for endogenous peroxidase activity was performed in 1% H<sub>2</sub>O<sub>2</sub> for 20 min.

Sections were washed three times with demiwater followed by washing with PBS. Sections then were preincubated with 5% normal goat serum in PBS for 1 h. Incubation with primary antibodies for Thr71-phosphorylated ATF2, Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, total c-Fos, and polycystin-1 was done overnight at 4°C. After sections were washed three times with PBS, they were incubated with rabbit Envision–HRP for 30 min, washed three times with PBS, and developed using DAB with 0.7% NiCl<sub>2</sub> for 10 min (black/brown precipitate).

For mouse tissue sections, PKS-A was incubated with mouse Envision–HRP overnight at 4°C. The next day, blocking reagent from the ARK kit was added (Dako). Sections then were incubated with the PKS-A–HRP conjugated antibody for 1 h and developed as described. For Sirius red staining of the interstitial component collagen, sections were incubated in 0.2% phosphormolybdenic acid for 5 min and then in 0.1% Sirius red in picric acid for 15 min. All sections were counterstained with hematoxylin (blue), with the exception of sections stained for ATF2, c-Jun, and c-Fos.

### Western Blot Analysis

Tissue samples were homogenized in RIPA buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% DOC, 1% TX-100, and 1% NP-40) supplemented with complete protease inhibitor cocktail (Roche B.V., Almere, The Netherlands), 0.5 μM sodium fluoride, and 0.5 μM sodium vanadate (Sigma) using a mortar and pestle and MagNa Lyzer (Roche). Western blot analysis was performed as described (8). Rabbit anti-phospho-Thr71-ATF2, rabbit anti-phospho-Thr69/71-ATF2, and rabbit anti-phospho-Ser73-c-Jun were diluted 1:500, rabbit anti-c-Fos was diluted 1:1000 (Upstate, Charlottesville, VA), and mouse anti-β-actin was diluted 1:40,000 (MP Biomedicals, Amsterdam, The Netherlands). Primary antibodies were detected using goat anti-rabbit–HRP 1:10,000 (Jackson ImmunoResearch Laboratories, West Grove, PA) or sheep anti-mouse–HRP conjugate 1:10,000 (Amersham Biosciences, Roosendaal, The Netherlands). Proteins were detected using enhanced chemiluminescence (Sigma) on Kodak BioMax Light Film (Kodak, Roosendaal, The Netherlands). Expression was quantified using densitometry and expressed relative to the loading control β-actin. Statistical analysis was performed using the *t* test.

### Imaging

Sections were analyzed using a Leica DM-LB light microscope (Leica, Rijswijk, The Netherlands). Digital images were taken at ×200 magnification using Leica DC500 digital camera and software.

## Results

### Expression of ATF2, c-Jun, c-Fos, and Polycystin-1 in Normal Human Renal Tissue

We analyzed AP-1 activity in three human normal renal tissue samples (Table 1). To distinguish between different nephron segments, we included markers for immunohistochemical analysis. DBA and aquaporin-2 both served as markers for collecting ducts and showed similar expression patterns (Figure 1). Uromodulin was used as a marker for distal tubules and megalin for proximal tubules.

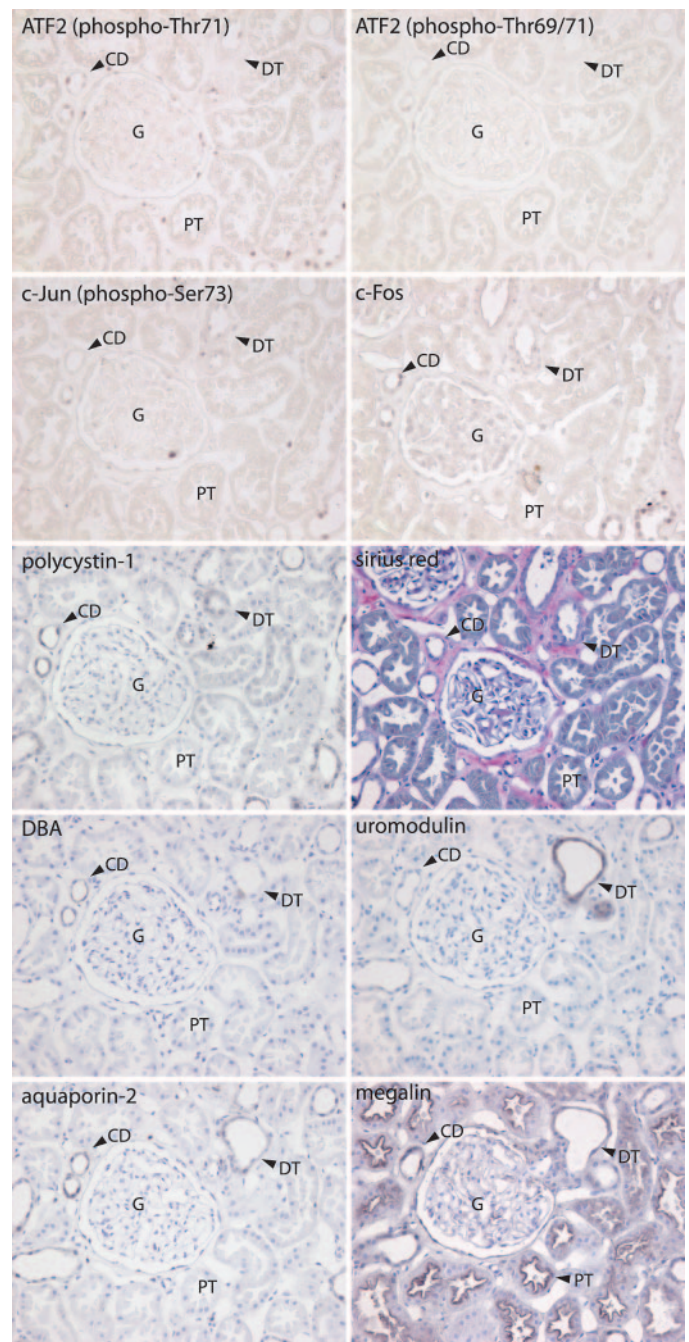
Normal human renal tissue showed coordinate expression of active Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, c-Fos, and polycystin-1 in collecting ducts and distal tubules (Figure 1, Table 2). Expression of polycystin-1 in collecting ducts and distal tubules was reported previously (14,20,21). Expression was also detected in the parietal epithelial cells of Bowman's capsule, in vascular structures, and in the multilayer cubical epithelium of the papillary duct (data not shown). Sirius red staining indicates surrounding interstitium (Figure 1).

### Expression of ATF2, c-Jun, c-Fos, and Polycystin-1 in Human ADPKD Renal Cystic Tissue

AP-1 activity was determined in six human renal cystic tissue samples (Table 1). Small cysts and (dilated) ducts and tubules surrounded by fibrotic interstitium showed increased AP-1 activity. The level of Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, and c-Fos was elevated (Figure 2, Table 2). It is interesting that polycystin-1 was coordinately detected and increased in these regions. Constitutive activity of AP-1 components may reflect increased stress as a result of progressive development of cysts and interstitial fibrosis. The expanded and fibrotic interstitium surrounding the cysts, as detected by Sirius red staining, also displayed nuclear expression of phosphorylated ATF2, c-Jun, and c-Fos.

In large cysts, levels of ATF2, c-Jun, c-Fos, and polycystin-1 were variable and generally lower than regions with small cysts. These large cysts mainly expressed markers for collecting ducts (data not shown). Some cyst-lining epithelial cells stained for multiple nephron segment markers, indicating that these cysts originated from multiple nephron segments or that these cyst-lining cells may have undergone de-differentiation and therefore lost nephron segment-specific markers.

To confirm immunohistochemical data, we performed Western blot analysis on total renal tissue lysates (Figure 3A), because regions with small cysts, (dilated) ducts and tubules, and surrounding fibrotic interstitium contribute to the majority of total renal tissue compared with large fluid-filled cysts, which are relatively low in cell content. Expression levels were subsequently quantified using densitometry and expressed relative to the loading control  $\beta$ -actin (Figure 3B). Overall, total cystic tissue lysates also showed significant increases in Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, and c-Fos. Levels of phosphorylated ATF2 and c-Jun in cystic kidneys were significantly increased when compared with total ATF2 and c-Jun protein levels (Figure 3B). These data indicate that indeed activity (ratio phosphorylated to total protein level)



**Figure 1.** Immunohistochemical staining of normal human renal tissue showing a glomerulus surrounded by ducts and tubules. Serial tissue sections obtained from three independent healthy kidneys were sequentially stained for Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, c-Fos, and polycystin-1 (black/brown). Results were correlated to collecting duct (*Dolichos biflorus* agglutinin [DBA] and aquaporin-2), distal tubule (uromodulin), proximal tubule (megalin; black/brown), and interstitial markers (Sirius red; pink/red). All sections, with the exception of sections stained for ATF2, c-Jun, and c-Fos, were counterstained with hematoxylin (blue). Representative images are shown of human renal tissue sample ET10. G, glomerulus; CD, collecting duct; DT, distal tubules; PT, proximal tubules.

**Table 2.** Summary of expression levels of Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, and c-Fos in renal control tissue, small cysts and (dilated) ducts and tubules, and large cysts<sup>a</sup>

	CD	DT	PT
<b>Normal renal tissue</b>			
ATF2 (phospho-Thr71)	+	++	–
ATF2 (phospho-Thr69/71)	+	+	–
c-Jun (phospho-Ser73)	+	++	–
c-Fos	+	+	–
<b>Small cysts/dilated tubules</b>			
ATF2 (phospho-Thr71)	+++	+++	+++
ATF2 (phospho-Thr69/71)	++	++	++
c-Jun (phospho-Ser73)	+++	+++	+++
c-Fos	+++	+++	++
<b>Large cysts</b>			
ATF2 (phospho-Thr71)	++		
ATF2 (phospho-Thr69/71)	+		
c-Jun (phospho-Ser73)	–/++		
c-Fos	–/++		

<sup>a</sup>Expression levels are indicated as – representing no expression and +++ representing highest expression. CD, collecting duct; DT, distal tubules; PT, proximal tubules.

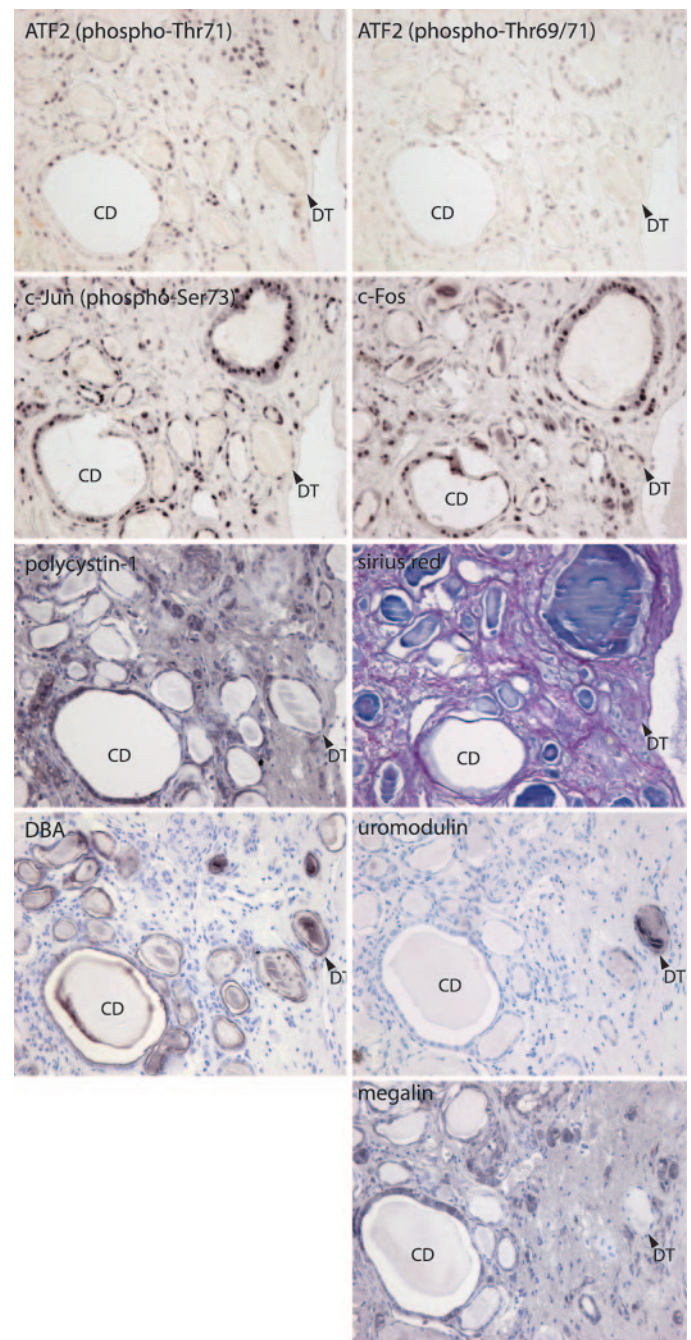
rather than expression (total protein level) of ATF2 and c-Jun is increased in renal cystic tissue. Noteworthy, both control and ADPKD cystic kidneys displayed some variations in AP-1 activity. This may be due to differences between individuals. The expression level of c-Jun in tissue sample PK131 was below the detection threshold and could not be detected in the Western blot depicted in Figure 3A. However, when this tissue sample was overloaded, increased Ser73-phosphorylated c-Jun was detected (data not shown).

In summary, AP-1 activity was increased in small cysts and (dilated) ducts and tubules surrounded by fibrotic interstitium. These cells displayed significant elevation of nuclear Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, and c-Fos.

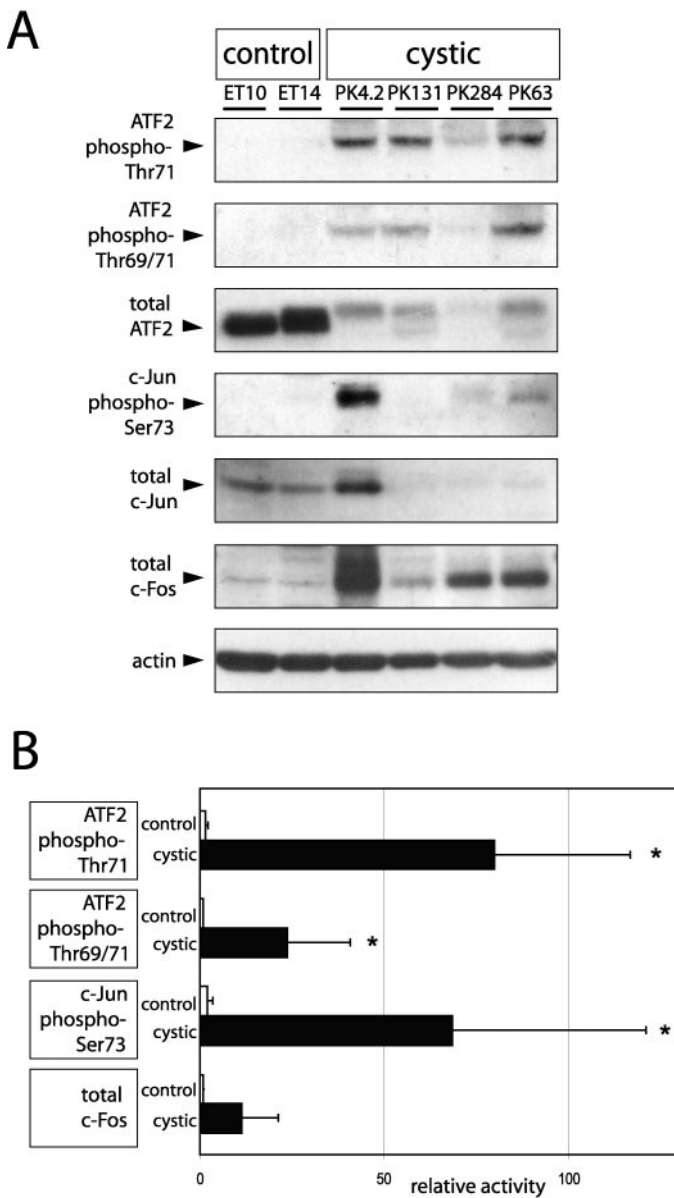
*Expression of ATF2, c-Jun, and c-Fos in Normal and Polycystic Kidney Tissue of the Mouse*

Thr71-phosphorylated ATF2 and Ser73-phosphorylated c-Jun expression in mouse control renal tissue was comparable to the pattern observed in human renal control tissue (Figure 4). However, Ser73-phosphorylated c-Jun was also detected in the cytoplasm of collecting duct epithelial cells. Cytoplasmic localization of c-Jun has been demonstrated previously (22–25). Expression of Thr69/71-phosphorylated ATF2 and c-Fos was not detected by immunohistochemistry in normal renal tissue.

Renal cystic tissue from *Pkd1<sup>nl/nl</sup>* mice that had polycystic kidney disease showed similar alterations in AP-1 activity as observed in human ADPKD renal cystic tissues. Upregulation of nuclear Thr71-phosphorylated ATF2 and Ser73-phosphorylated c-Jun in particular was a feature in kidneys with relatively small cysts (Figure 5). Increases in Thr69/71-phosphorylated

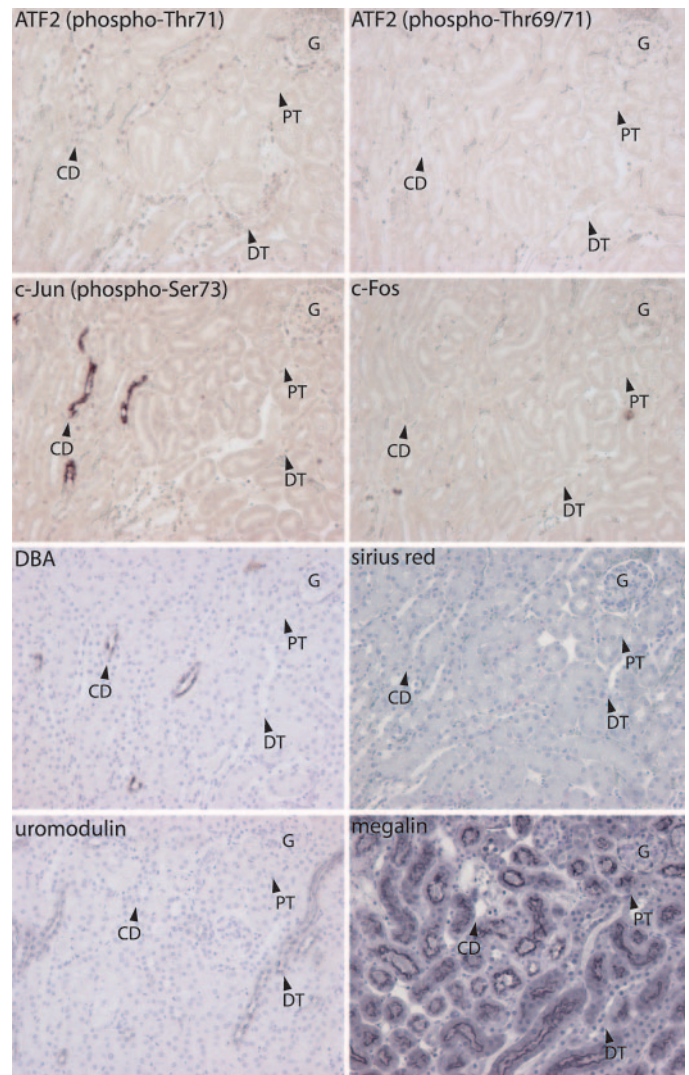


**Figure 2.** Immunohistochemical staining of human autosomal dominant polycystic kidney disease (ADPKD) renal cystic tissue showing several small cysts and (dilated) ducts and tubules surrounded by fibrotic interstitium. Serial renal tissue sections from six patients with ADPKD and chronic renal failure were sequentially stained for Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, c-Fos, and polycystin-1 (black/brown). Results were correlated to collecting duct (DBA), distal tubule (uromodulin), proximal tubule (megalin; black/brown), and interstitial markers (Sirius red; pink/red). All sections, with exception of sections stained for ATF2, c-Jun, and c-Fos, were counterstained with hematoxylin (blue). Representative images are shown of ADPKD patient PK4.2.



**Figure 3.** Western blot analysis of human renal tissue samples of normal and cystic kidneys. Total tissue lysates from two normal human control subjects (ET10 and ET14) and four patients with ADPKD (PK4.2, PK131, PK284, and PK63) were analyzed for ATF2, c-Jun, and c-Fos activity.  $\beta$ -Actin was included as a loading control. (A) Western blots of Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, c-Fos, and  $\beta$ -actin. Representative results are shown of two independent experiments. (B) Densitometry analysis of Western blot experiments. Expression was correlated to the loading control  $\beta$ -actin, and expression level of ET10 was set as 1. For ATF2 and c-Jun, the ratio of phosphorylated to total ATF2 and c-Jun protein level was determined to indicate relative activity. Results are displayed as the mean  $\pm$  SD of two independent experiments. \* $P \leq 0.05$ .

ATF2 and c-Fos were not as clear in mouse cystic tissue samples as in human samples. This may be due to slight species-specific differences between human and mouse. It is interesting that older mice that had more severe, progressive polycystic kidney

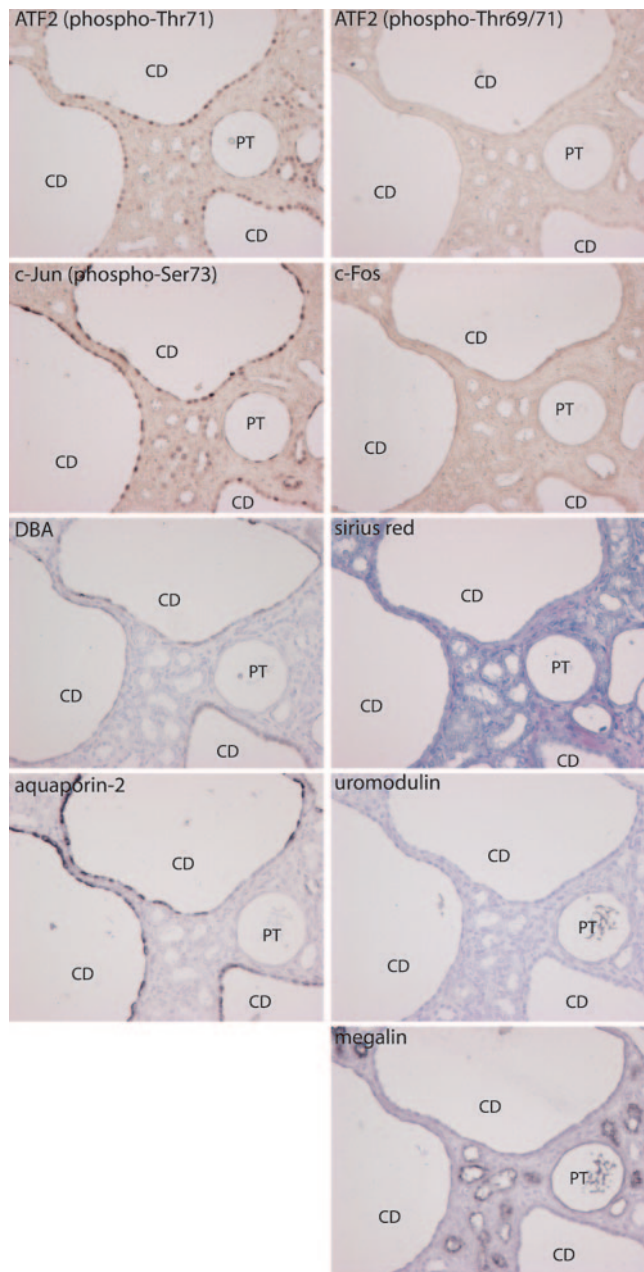


**Figure 4.** Immunohistochemical staining of normal mouse renal tissue. Serial tissue sections were sequentially stained for Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, and c-Fos (black/brown). Results were correlated to collecting duct (DBA), distal tubule (uromodulin), proximal tubule (megalin; black/brown), and interstitial markers (Sirius red; pink/red). All sections, with exception of sections stained for ATF2, c-Jun, and c-Fos, were counterstained with hematoxylin (blue). Representative images are shown of a 4-wk-old wild-type mouse.

disease showed more variable expression of ATF2, c-Jun, and c-Fos, comparable to the large cysts in human samples (data not shown). In summary, AP-1 is increased in both human (Figures 2 and 3) and mouse renal cystic tissue samples analyzed (Figure 5).

## Discussion

Cyst formation in ADPKD ultimately leads to chronic renal failure. Patients with ADPKD and chronic renal failure represent 8 to 10% of all patients who require renal dialysis and transplantation (26). Therefore, further insight into the mechanism of ADPKD cyst formation is required. Several studies have linked MAPK signaling to cyst formation. Upregulation of



**Figure 5.** Immunohistochemical staining of mouse *Pkd1<sup>nl/nl</sup>* renal cystic tissue showing several relatively small cysts with (dilated) ducts and tubules and surrounding interstitium. Serial tissue sections were sequentially stained for Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, c-Fos, and polycystin-1 (black/brown). Results were correlated to collecting duct (DBA and aquaporin-2), distal tubule (uromodulin), proximal tubule (megalin; black/brown), and interstitial markers (Sirius red; pink/red). All sections, with exception of sections stained for ATF2, c-Jun, and c-Fos, were counterstained with hematoxylin (blue). Representative images are shown of a 4-wk-old mouse.

ERK has been reported previously in the Han:SPRD rat model for ADPKD and the *bcl-2<sup>-/-</sup>* mouse model for cystic kidney disease (27,28). Increased activity of JNK was reported recently in small cysts in another mouse model for polycystic kidney

disease (29). In addition, MAPK signaling has been demonstrated to play a major role in the uretic bud during kidney development (30,31). AP-1 is a major downstream target of MAPK signaling. Transactivation potential and expression of ATF2, c-Jun, and c-Fos can be regulated by ERK, p38, and JNK family members. We and others previously reported that polycystin-1 can modulate AP-1 activity (6–8). To investigate further the cellular defects in ADPKD cyst formation *in vivo*, we analyzed AP-1 activity in renal cystic tissue isolated from human ADPKD patients and in renal cystic tissue from *Pkd1<sup>nl/nl</sup>* mice with polycystic kidney disease. It is interesting that upregulation of Thr71- and Thr69/71-phosphorylated ATF2, Ser73-phosphorylated c-Jun, and c-Fos protein was detected in small cysts and (dilated) ducts and tubules, representing initial stages of cyst formation (Figure 2). Increased c-Fos mRNA was reported previously in two animal models for polycystic kidney disease (32,33). Because cellular stress induces phosphorylation and activation of AP-1, our data indicate that these cells may be in an increased state of cellular stress as a result of progressive development of cysts and interstitial fibrosis. Our data therefore suggest that AP-1 may play a role in ADPKD by promoting cyst formation and accompanying interstitial fibrosis. On the basis of the observation that AP-1 activity was increased in small cysts, it can be speculated that constitutive AP-1 activity may play a role during early stages of cyst formation. However, to further study this hypothesis, cyst formation should be studied at earlier stages under more controlled laboratory conditions, for instance using conditional knockout mouse models for *Pkd1* and *Pkd2*.

The immortalized renal cystic epithelial cells that were analyzed in our previous report may represent a subset of end-stage cystic cells from large cysts displaying decreased level of Ser73-phosphorylated c-Jun and increased c-Fos in addition to an increase in Thr71-phosphorylated ATF2 (8). Furthermore, cultured renal cystic cells may differ from renal cystic epithelium because these cells are surrounded by an abundant expanded fibrotic interstitium *in vivo*. The fibrotic interstitium may contribute to aberrant AP-1 activity of renal cystic epithelium *via* extracellular matrix-to-epithelium signaling.

It is interesting that polycystin-1 was coordinately expressed and upregulated with the AP-1 components in small cysts and (dilated) ducts and tubules. These data are in line with previous reports that increased polycystin-1 expression results in polycystic kidney disease (8,34). Renal epithelial cells expressing transgenic *PKD1* show similar defects in AP-1 transcriptional activity as human ADPKD renal cystic epithelial cells (8), and transgenic *Pkd1* mice develop polycystic kidney disease (34). Increased Thr71-phosphorylation of ATF2, observed in both human and mouse ADPKD, may reflect constitutive ERK activity (13). Upregulation of Ser73-phosphorylated c-Jun may reflect constitutive JNK activity.

We show here for the first time, to our knowledge, that AP-1 activity is increased *in vivo* in human and mouse polycystic kidney disease. Further experiments to investigate whether polycystins modulate AP-1 activity directly or *via* known AP-1 regulators, such as MAPK, will provide further insight into the

function of polycystins and the mechanism of cyst formation in ADPKD.

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