Supplementary Materials:

Methods

Animal studies

The *Pkd1*$^{RC/RC}$ (*PKD1* p.R3277C) mice, a murine model of ADPKD, were provided by Dr. Peter C. Harris. Wild-type (WT) C57BL/6J mice were purchased from Jackson Labs. PAPP-A deficient mice were generated by crossing PAPP-A deficient mice, *Pappa*^{tm1Cac} *(1)* and *Pkd1*$^{RC/RC}$ mice. *Pkd1*$^{RC/RC}$ mice were crossed with *Prkar1a*$^{f/f}$*; Pkhd1*-Cre mice to generate ADPKD mice with kidney-specific over-activation of PKA (*2*). Five animals per cage were housed in standard cages in a room maintained at constant temperature and humidity, and 12 h light/dark cycles.

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Mayo Clinic (Protocol no. A47715 and A00003864), and studies were conducted in adherence to the NIH Guide for the Care and Use of Laboratory Animals. For IGF-1 antibody experiment, the animals were injected intraperitoneally with 0.2 mg/kg IGF-1 antibody or IgG control (R&D Systems) per week for 6 weeks. 5 mg/kg FSK or control (5% DMSO) was administered intraperitoneally for FSK experiment.

At the end of the study mice were sacrificed, blood was taken for biochemical analysis, and organs were weighed. Portions of tissue were placed in formalin and processed for histological studies or snap frozen in liquid nitrogen and stored at -80 °C for gene expression analysis.

Real time Polymerase chain reaction

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and cDNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen). Commercially available TaqMan gene expression probes were obtained from Applied Biosystems and quantitative real-time

PCR was performed in duplicates as described previously (*3*). The relative mRNA expression of target genes was calculated using the 2^{-ddCq} method with *Gapdh* as internal reference gene.

Serum IGF-1 levels

Serum was collected from C57BL6 WT and *Pkd1*$^{RC/RC}$ mice and stored at -80º C. The amounts of IGF-1 in the serum were measured using the Ultra-Sensitive Mouse IGF-1 ELISA kit from Crystal Chem, Inc., according to the manufacturer instructions.

cAMP ELISA

cAMP levels were measured in renal tissues of WT and *Pkd1*$^{RC/RC}$ -*Pappa* mutant mice using Direct cAMP ELISA kit from Enzo Life Sciences, Inc. kidneys were homogenized in 0.1M HCl and supernatant was used for assay. Protein was measured using Bradford assay.

Cell culture

RCTE and 9-12 cells were cultured in DMEM:F12 with 10% FBS and 1% penicillin/streptomycin. Cells were treated with 10μM FSK in presence or absence of IL1β (5 ng/ml), IL-2 (2 ng/ml), EGF (10 ng/ml, TGFβ (1 ng/ml), 0.5 μM ionomycin and 0.2 μM verapamil for 16 hour and RNA was isolated. Cells were treated with 10μM FSK in presence or absence of budesonide (150 μM) or decitabine (0.5 μM) for 16 hours followed by RNA isolation. Cells were pre-treated wuth Budesonide (150 μM) or decitabine for 24 hours before adding FSK. The cells were treated with FSK for 16 hours in presence or absence of compound C (10μM) or ERK inhibitor, SCH772984 (2μM) which were added to the media after 1 hour of FSK treatment and RNA was isolated. The recombinant PAPP-A (*4*) was used in pathway studies.

Madin-Darby canine kidney (MDCK) cells were maintained in DMEM/F12 (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin (Invitrogen). Cystogenesis studies with MDCK cells were performed essentially as previously described (*5*). Briefly, cells were suspended in collagen I solution, seeded in

24-well plates at 4,000 cells per well, and incubated at 37 °C for 30-45 min. for collagen to polymerize. Cells were then given medium (DMEM/F12, 1% FBS) containing the cAMP agonist forskolin (FSK, 10 μM) and IGF-1 (50 ng/ml). Cells were cultured for 14 days, with media change every other day. At the end of the study, the number of cysts in each well was counted.

PKA kinase activity assay

RCTE and 9-12 cells were treated with or without FSK for 24 hours and protein was extracted with 1 ml lysis buffer (20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT) and protease inhibitor cocktail). The PKA kinase activity was measured using 2 μg of extracted protein using PKA kinase assay kit following manufacturer's instructions (Enzo Life Sciences, Inc.)

Metanephros organ culture

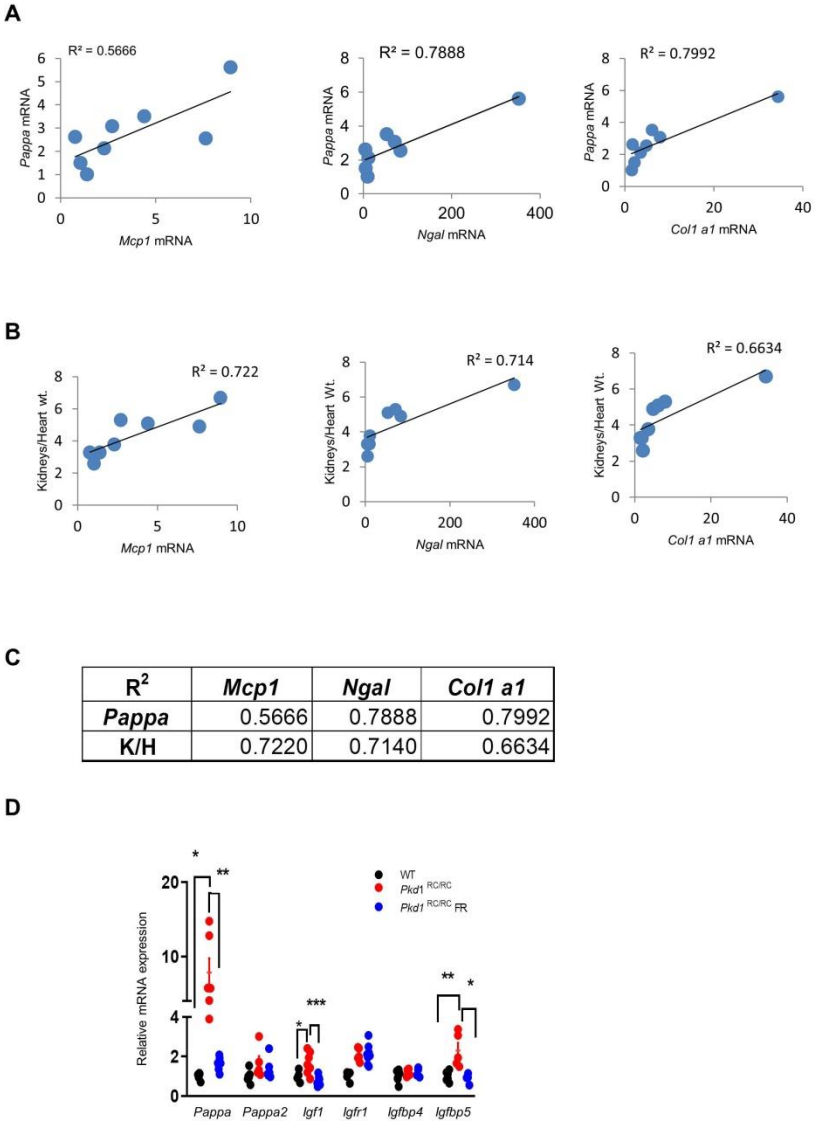
Metanephros organ culture was performed as previously described(*6*) (*7*) except the addition of insulin to the media. Embryos were aseptically isolated from C57BL/6J or *Pkd1*$^{RC/RC}$ mice at embryonic day 12 – 13 (E12 - 13). Metanephroi from embryos were collected and placed onto a transparent Falcon 0.4 μm cell culture insert floating on a defined serum-free medium. The metanephroi were incubated for 0-14 days in 12-well Falcon tissue culture plates (Fisher Scientific, CA) at 37 °C in a humidified incubator (5% CO_2). Defined medium was DMEM/F12 supplemented with 5 μ /ml transferrin, 2.8 nM selenium, 25 ng/ml prostaglandin E1, 6 ng/ml triiodothyronine (T3) and 1% penicillin and streptomycin. IGF-1 was added to the medium for C57BL/6J. Growth medium was changed every other day. Cysts were generated by treating with forskolin (FSK) and the development of cysts was observed daily under microscope.

Western blotting

Western blot analysis on kidney tissues and cultured cells was performed as described earlier (*3*). Antbodies against LC3 (4108), p62 (#5114) cleaved caspase 3 (#9664), pERK(#4370), ERK(#4695), pAkt (#4060), Akt (#4691), pS6 (#4858) and S6 (#2217) were used and purchased from Cell signaling. Membranes were stripped and probed with tubulin or GAPDH antibody to control for equal gel loading and transfer. Films were scanned and densitometry was performed using ImageJ.

Statistical analysis

Data are expressed as means ± SEM. Comparisons were made by unpaired Student's *t-test* and analysis of variance (ANOVA). Nonparametric tests were used wherever required by data distribution. Significance was set at $P < 0.05$.



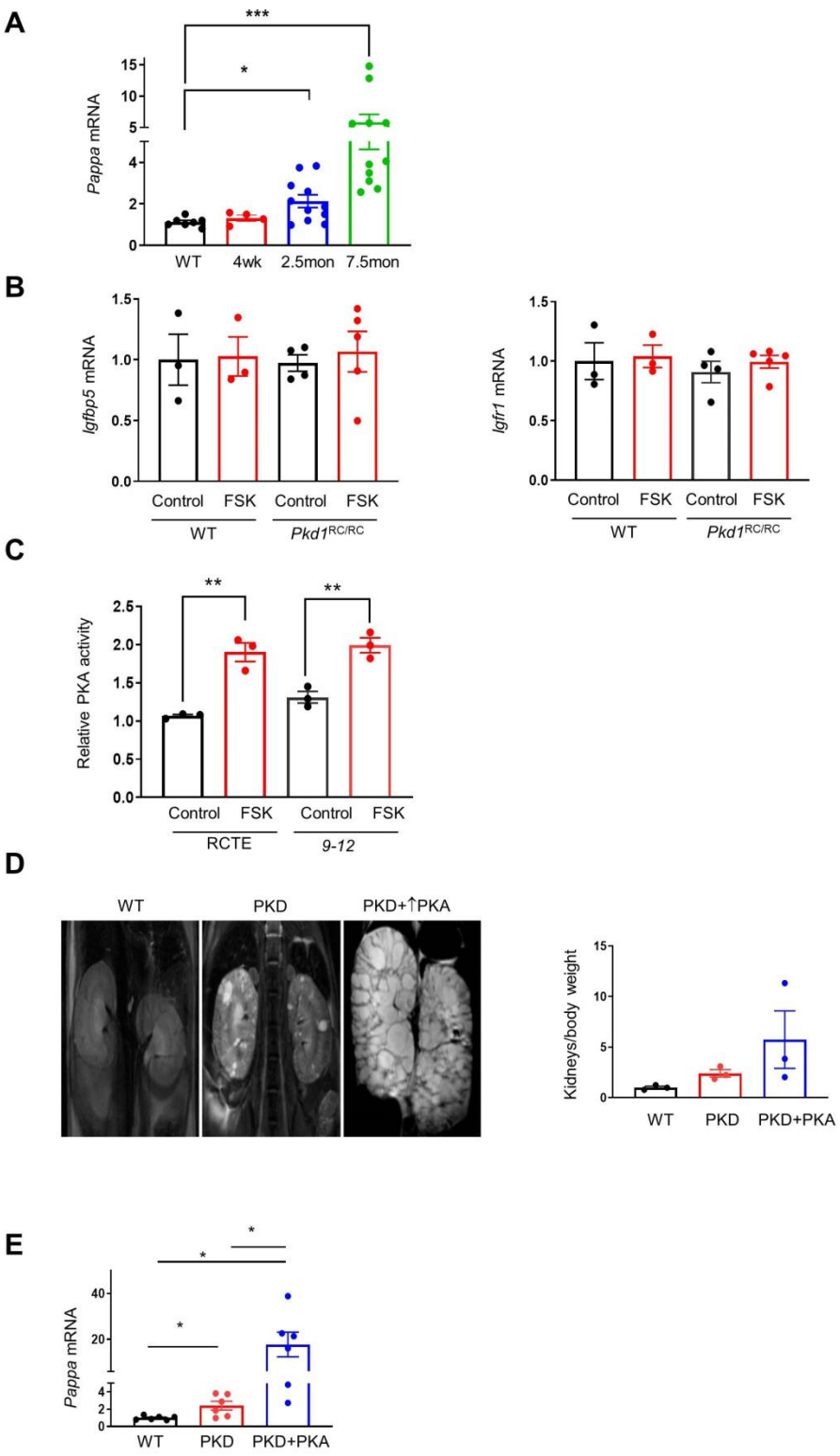
C

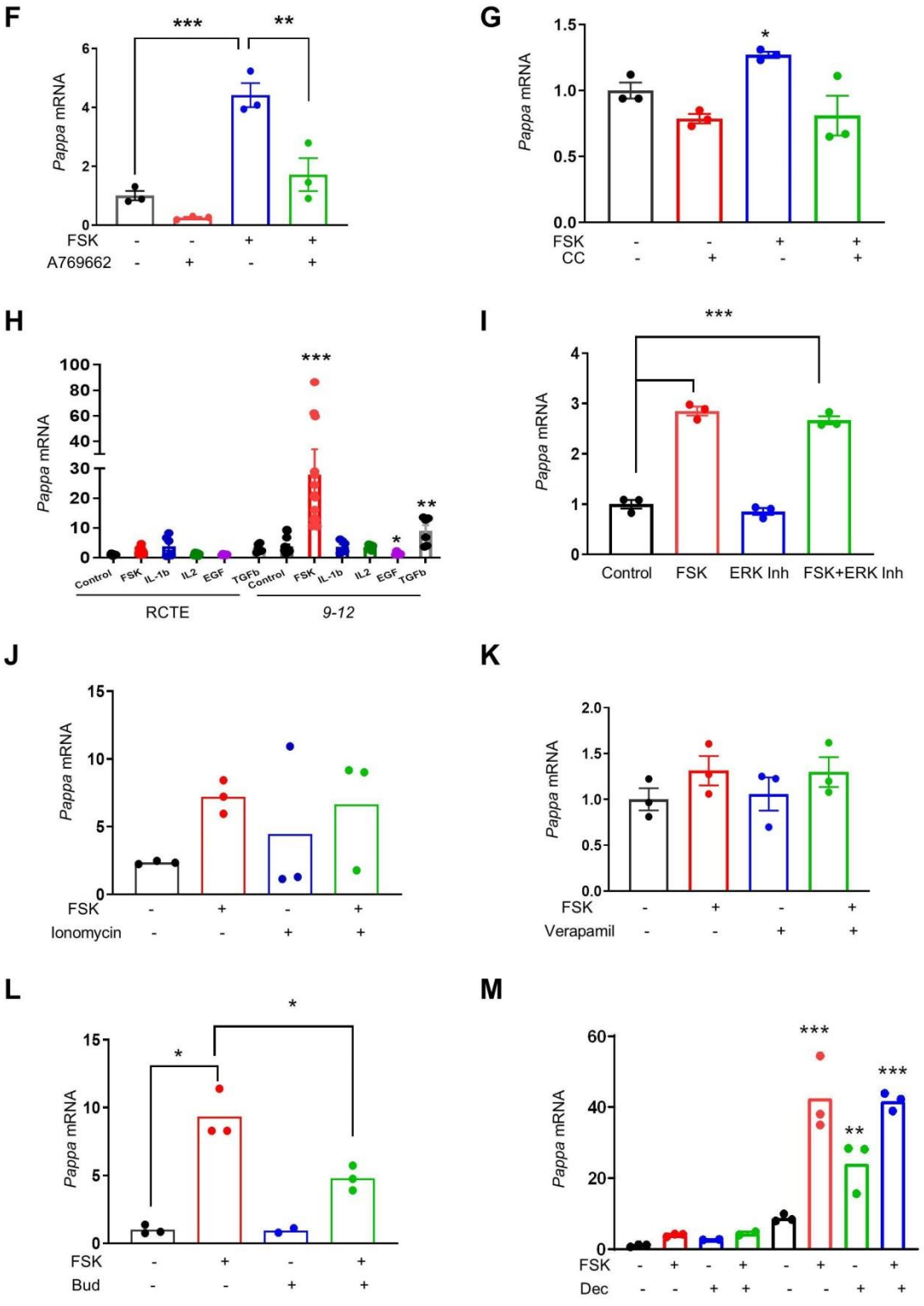
R^2	*Mcp1*	*Ngal*	*Col1 a1*
Pappa	0.5666	0.7888	0.7992
K/H	0.7220	0.7140	0.6634

D

Fig. S1. Food restriction ameliorated the IGF-1 pathway components in ADPKD. (A-B) Graphs showing correlation from *Pkd1*$^{RC/RC}$ mice (n=8, 3-7.5 months old) between **(A)** *Pappa* and *Mcp1*, *Ngal* and *Col1a1* mRNA expression levels, **(B)** kidneys to heart ratio and *Mcp1*, *Ngal* and *Col1a1* mRNA expression levels. **(C)** Table showing the R^2 values for figures **(A)** and **(B). (D)** Relative mRNA expression of IGF-1 pathway components in kidneys of 7.5 month old WT and *Pkd1*$^{RC/RC}$ mice fed a standard diet ad libitum (AL) or 40% food restriction (FR) for 6 months. PCR data are expressed relative to *Gapdh*, (n=4-8). A *p*

value of < 0.05 was considered significant. *P< 0.05, **P< 0.01, ***P< 0.001 by two-tailed Student's t-test (One-tailed for WT vs *Pkd1*$^{RC/RC}$ AL).


A
***
*
Pappa mRNA
15
10
5
4
2
0
WT
4wk
2.5mon
7.5mon
B
Igfbp5 mRNA
1.5
1.0
0.5
0.0
Control
FSK
Control
FSK
WT
Pkd1RC/RC
Igfr1 mRNA
1.5
1.0
0.5
0.0
Control
FSK
Control
FSK
WT
Pkd1RC/RC
C
**
**
Relative PKA activity
2.5
2.0
1.5
1.0
0.5
0.0
Control
FSK
Control
FSK
RCTE
9-12
D
WT
PKD
PKD+↑PKA
Kidneys/body weight
15
10
5
0
WT
PKD
PKD+PKA
E
*
*
*
Pappa mRNA
40
20
4
2
0
WT
PKD
PKD+PKA



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101 **Fig. S2. cAMP pathway induces PAPP-A expression in ADPKD.** (**A**) Time course of

102 *Pappa* expression in *Pkd1*$^{RC/RC}$ mice compared to WT at different time points (n=4-11). **(B)**

103 mRNA expression levels of *Igfbp5* and *Igf-1R* in kidney tissues of *Pkd1*$^{RC/RC}$ (n=4-5) and

WT (n=3) mice treated with vehicle (5% DMSO) or 5 mg/kg FSK for 24 hours. **(C)** Relative PKA activity in RCTE and 9-12 cells following FSK treatment for 24hrs compared to control. **(D)** Overactivation of PKA exacerbates cystic disease in ADPKD mice. *Pkd1*$^{RC/RC}$ mice were crossed with *Prkar1a*$^{f/f}$*; Pkhd1*-Cre mice to generate ADPKD mice with kidney-specific overactivation of PKA (PKD + ↑PKA). Representative MR Images of WT and PKD kidneys at 3 months old compared to PKD + ↑PKA at 5 weeks old and graph of Kidneys/body weight. **(E)** *Pappa* mRNA expression in kidneys of 3 month old WT and *Pkd1*$^{RC/RC}$ and 5 weeks old PKD + ↑PKA mice. **(F)** *Pappa* mRNA expression levels in 9-12 cells treated with/without an AMPK activator, A769662 for 6 hours followed by 10 μM FSK treatment (with/without) for 16 hours. **(G)** mRNA expression levels of *Pappa* in RCTE cells treated with 10 μM FSK in presence or absence of an AMPK inhibitor, compound C for 16 hours. **(H)** mRNA expression levels of *Pappa* in RCTE and 9-12 cells treated with FSK (10 μM), IL1β (5 ng/ml), IL-2 (2 ng/ml), EGF (10 ng/ml) or TGFβ (1 ng/ml) for 16 hours. **(I)** 9-12 cells treated with 10 μM FSK in presence or absence of an ERK inhibitor, SCH772984 (2μM) for 16 hours. **(J)** 9-12 cells treated with 10 μM FSK in presence or absence of 0.5 μM ionomycin and **(K)** RCTE cells treated with 10 μM FSK in presence or absence of 0.2 μM verapamil for 16 hours. **(L)** 9-12 cells were treated with 10 μM FSK in presence or absence of budesonide (150 μM) or **(M)** RCTE and 9-12 cells treated with 10 μM FSK in presence or absence of decitabine (0.5 μM) for 16 hours. Cells were pre-treated with budesonide or decitabine for 24 hours before FSK treatment. Data are mean ± SEM. A p value of < 0.05 was considered significant. *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$ by two-tailed Student's t-test or Mann Whitney test or one way ANOVA and then Tukey's post-hoc test.

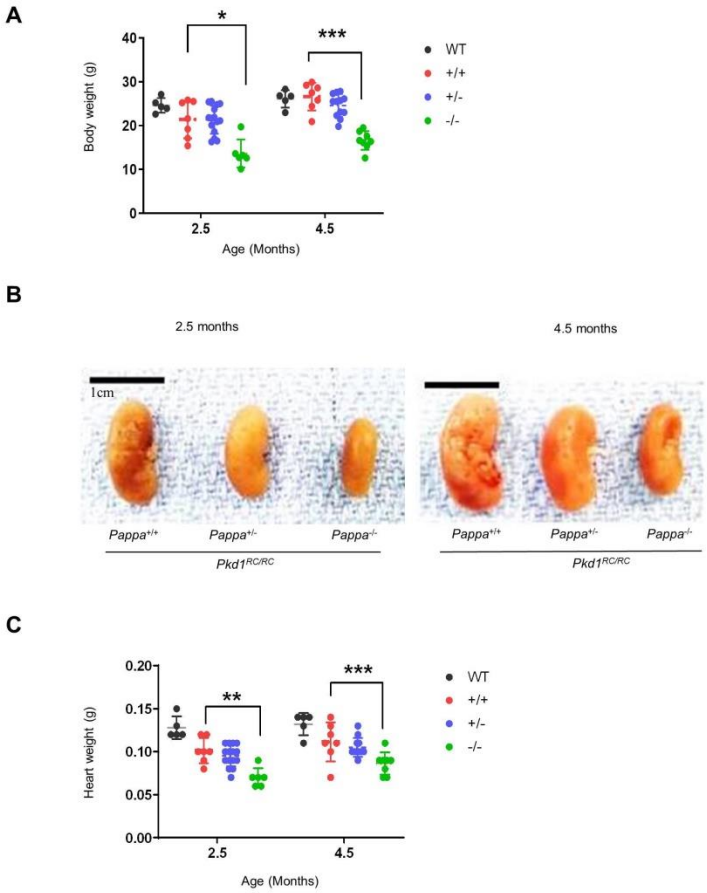


Fig. S3. PAPP-A deficient mice show reduced body size, heart weight and normal kidneys at different ages. (A) Body weight of WT and *Pkd1*$^{RC/RC}$- *Pappa* mutants at different time points. **(B)** Representative gross kidney images from 2.5 and 4.5 month old *Pkd1*$^{RC/RC}$ mice that are *Pappa*$^{+/+}$, *Pappa*$^{+/-}$, or *Pappa*$^{-/-}$. **(C)** Heart weight of WT and *Pkd1*$^{RC/RC}$- *Pappa* mutants at different ages (n=5-15). Data are mean ± SEM. A *p* value of < 0.05 was considered significant. **P*< 0.05, ***P*< 0.01, ****P*< 0.001 by One-way ANOVA followed by Tukey's post-hoc test.

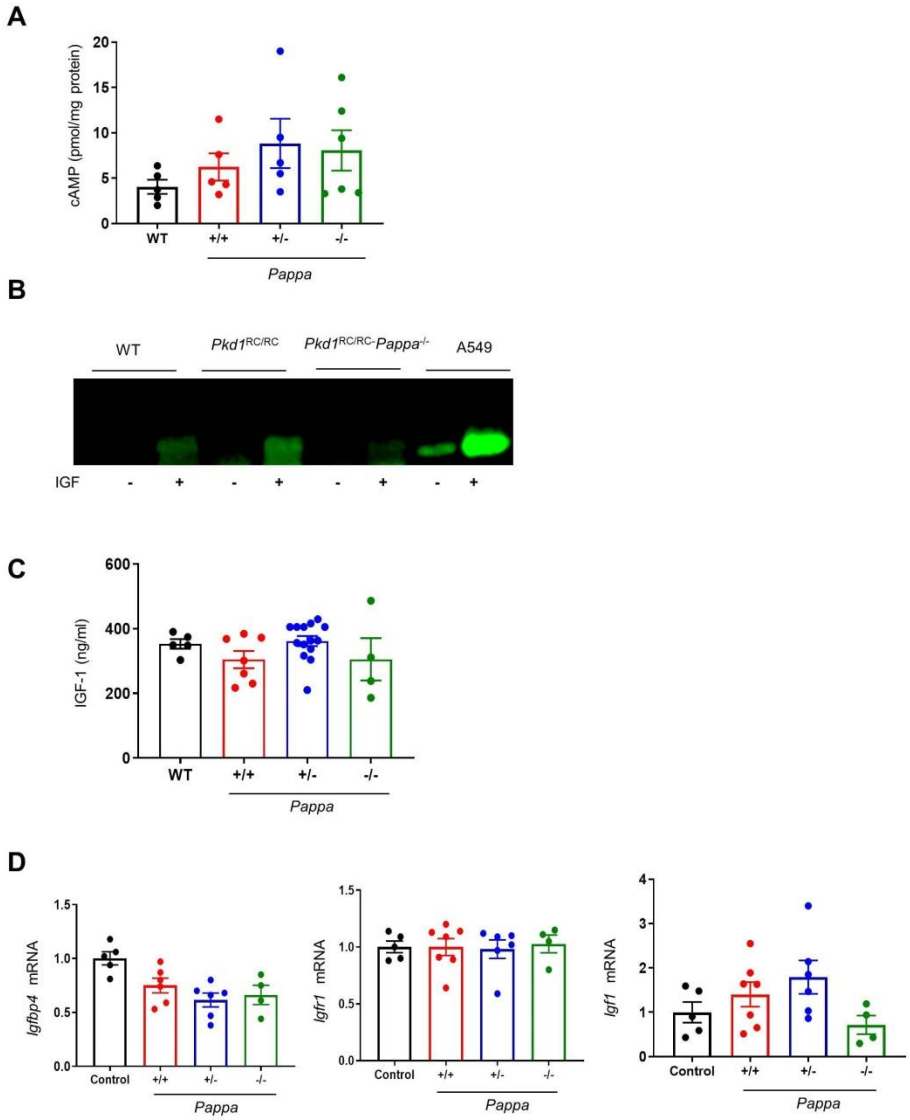
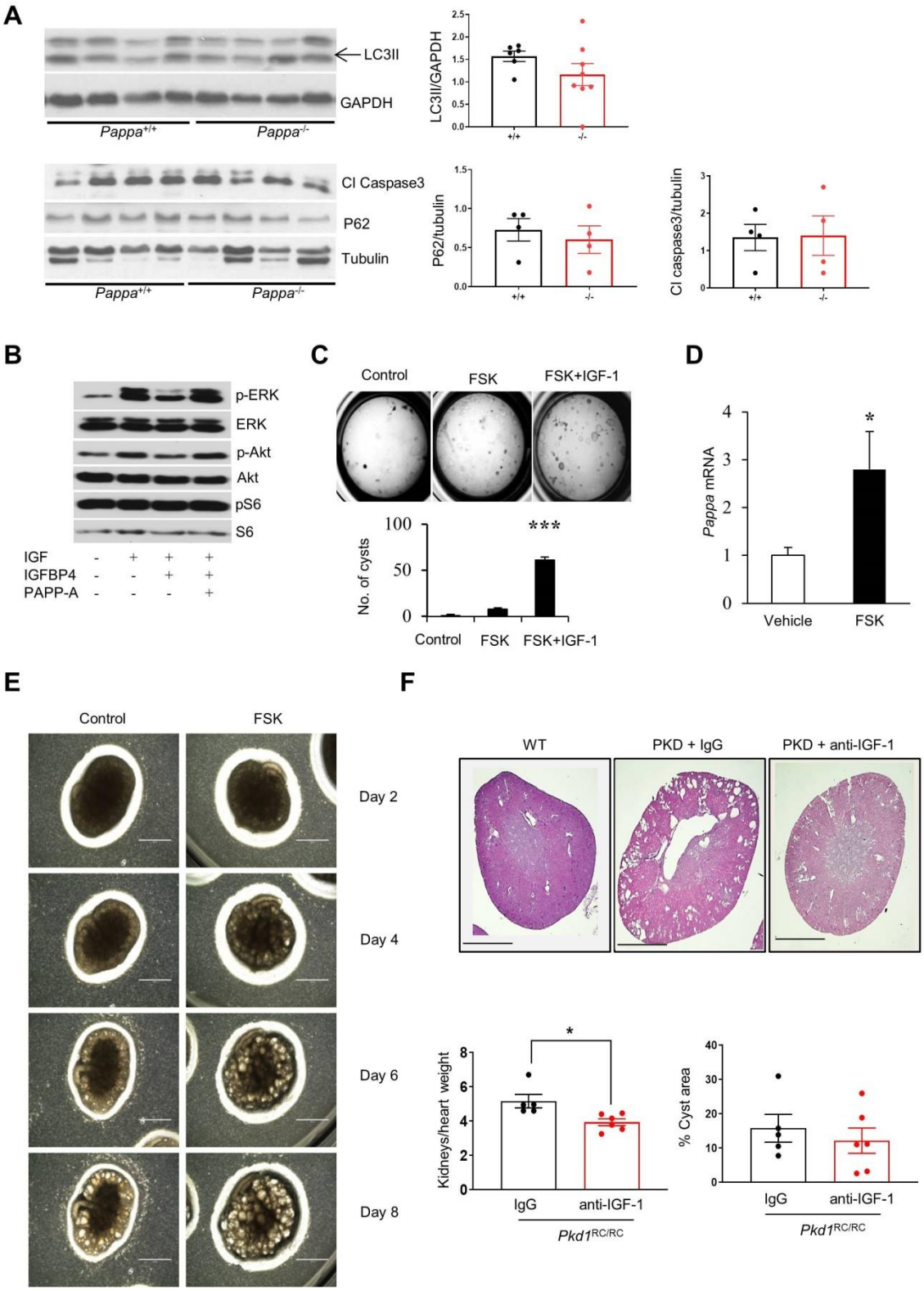


Fig. S4. cAMP and IGF pathway components in *Pkd1* $^{RC/RC}$-*Pappa* mutant mice. (A) cAMP levels in kidney tissues of WT and *Pkd1*$^{RC/RC}$- *Pappa* mutants (n=5-6). **(B)** Proteolytic assay of PAPPA mediated IGFBP4 using kidney membrane fractions from WT, *Pkd1*$^{RC/RC}$ and *Pkd1*$^{RC/RC}$- *Pappa*$^{-/-}$ mice at 6 months age. Membrane fractions were incubated for 72 h at 37 °C with IGFBP-4 without (−) or with (+) pre-complexing to IGF. N-terminal cleaved band is shown strongly in *Pkd1*$^{RC/RC}$ compared to WT mice when IGF is pre-incubated but nearly absent in *Pkd1*$^{RC/RC}$- *Pappa*$^{-/-}$ mice. A549 cell line is used as positive control. **(C)** Plasma

IGF-1 levels and **(D)** renal mRNA expression levels of *Igfbp4, Igfr1 and Igf1* in WT and *Pkd1*$^{RC/RC}$- *Pappa* mutants mice. Data are mean ± SEM.


A
LC3II
GAPDH
Pappa+/+
Pappa-/-
LC3II/GAPDH
Cl Caspase3
P62
Tubulin
P62/tubulin
Cl caspase3/tubulin
B
p-ERK
ERK
p-Akt
Akt
pS6
S6
IGF
IGFBP4
PAPP-A
C
Control
FSK
FSK+IGF-1
No. of cysts
***
D
Pappa mRNA
*
Vehicle
FSK
E
Control
FSK
Day 2
Day 4
Day 6
Day 8
F
WT
PKD + IgG
PKD + anti-IGF-1
Kidneys/heart weight
% Cyst area
IgG
anti-IGF-1
Pkd1RC/RC

150

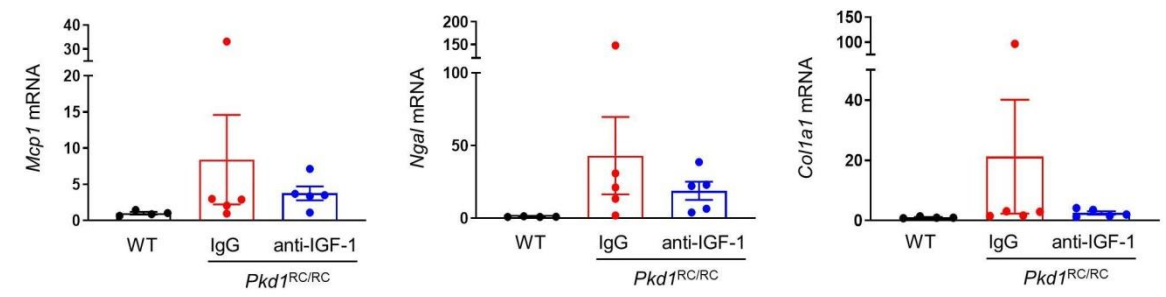


Fig. S5. Role of PAPP-A-IGF-1 pathway in pathogenesis of ADPKD in experimental models. (A) Western blot analysis of LC3, cleaved caspase 3 (Cl Caspase3) and p62 in kidneys of 2.5 month old *Pkd1*$^{RC/RC}$;-*Pappa*$^{+/+}$ and *Pkd1*$^{RC/RC}$-*Pappa*$^{-/-}$ mice. Graphs show quantitative analysis of the specific bands by densitometry. **(B)** Western blot analysis of IGF-1 and MAP kinase pathways in 9-12, human PKD cells in presence of IGF-1, IGFBP4 or IGFBP+PAPP-A. **(C)** three dimensional MDCK cystogenic assay in the presence of FSK alone or FSK + IGF1 (top) and quantification of MDCK cysts (bottom) IGF-1. **(D)** *Pappa* mRNA expression in metanephros treated with FSK or vehicle. (**E)** Photomicrographs showing the cystic growth in a metanephric model of cystogenesis in *Pkd1* $^{RC/RC}$ mice at Day 13.5 embryonic kidneys that were stimulated with FSK (10 µM) or control in absence of any growth hormone. Scale bar, 1 mm. **(F)** *Pkd1* $^{RC/RC}$ mice were treated with IGF-1 neutralizing antibody (0.2 mg/kg, n=6) or control IgG (n-5) weekly by I.P. injection for 6 weeks starting at 4 months old age. Representative H&E sections of kidneys and graphs of kidneys/body weight and cystic area in IgG (n=5) and anti-IGF-1 (n=6) treated *Pkd1*$^{RC/RC}$ mice. **(G)** mRNA levels of *Mcp1, Ngal and Col1a1* in IgG and anti-IGF1 (n=5 for each group) treated *Pkd1*$^{RC/RC}$ mice compared to WT mice (n=4). Data are mean ± SEM. A *p* value of < 0.05 was considered significant. **P*< 0.05, ****P*< 0.001 compared to control by two-tailed Student's t-test.

References

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