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Supplementary Materials for

Activation of P-TEFb by cAMP-PKA signaling in autosomal dominant polycystic kidney disease

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This PDF file includes:

Supplementary Materials and Methods

Immunohistochemistry (IHC)

The kidney tissues were fixed in 10% formalin overnight and paraffin-embedded. Tissue sections were blocked and incubated with primary antibody against PCNA (Santa Cruz, sc-56). After primary antibody incubation, tissue sections were incubated with goat anti-mouse/rabbit-HRP secondary antibody followed by staining with DAB substrate. IHC sections were imaged using a microscope (BX51, Olympus).

Embryonic kidney culture

Kidneys were dissected from CD1 mice at E13.5, and grown on 0.4 μm transparent Millicell organotypic cell culture inserts (Millipore) in 12-well plates. Embryonic kidneys were maintained in a 37°C incubator with 5% CO2. A maximum of four explants were grown on each membrane. Kidneys were cultured in DMEM/F12 medium supplemented with 2 mM L-glutamine (Sigma, G7513); 10 mM HEPEs (Sigma, H3375); 1/100 insulin-transferrin-selenium (Life, 41400-045); 1/40 penicillin/streptomycin (Life, 15140-122); 25 ng/ml prosta- glandin E1 (Sigma, P5515); 32 pg/ml T3 (Sigma, T5516); 100 μM 8-Br-cAMP (Sigma, B5386). Appropriate amount of each drug or DMSO were added to each well. The medium was changed twice per day. Total area and cyst area measurement were evaluated using the NIH ImageJ software. The cyst index was calculated as the ratio of cyst area to total area.

MDCK I cell cyst growth assay

For cyst generation, 400 MDCK I cells were suspended in 400 µl of MEM containing 10 mM HEPEs; 3.1 mg/mL collagen I (PureCol) and 27 mM NaHCO3. After gel formation by incubation for 90 minutes at 37°C, 1 mL of DMEM/F12 medium supplemented with 10% FBS and 10 µM forskolin (Sigma, F3917) was added to each well. MDCK I cells were cultured up to twelve days in a 37°C incubator with 5% CO2. The medium was changed once per day. Cells were treated with FP from day 4 to day 12. Cells were transfected either with scrambled or CDK9 D167N mutant before cyst generation. Cells were infected with lentivirus carrying either control shRNA or shCDK9 before cyst generation. Micrographs of the same cysts were taken on day 4, 6, 8, 10 and 12 after seeding. For analysis of cyst formation and growth, cyst numbers were counted, and diameters were measured using the NIH ImageJ software. CDK9 shRNA sequences : CDK9 #1 (GCTTCTGAAACACGAGAATGT), CDK9 #2 (GCTGCAAAGGCAGTATATACC), CDK9 #3 (GCTCAACGGCCTCTACTATAT).

Antibodies used for WB

Cyclin T1 (Santa Cruz, sc-10750), CDK9 (Santa Cruz, sc-8338), RNAPII (Santa Cruz, sc-899), GST (Santa Cruz, sc-459), Polycystin-2 (Santa Cruz, sc-28331), EGFP (Santa Cruz, sc-8334), α-Tubulin (Proteintech, 10449-1-AP), MePCE (Protein- tech, 14917-1-AP), LARP7 (Proteintech, 17067-1-AP), RNAPII CTD S2 (Millipore, 04-1571),

RNAPII CTD S5 (Millipore, 04-1572), GAPDH (Millipore, MAB347), p-PKA Substrate (Cell Signaling Technology, 9624), Myc (Cell Signaling Technology, 2278), p-CREB (Cell Signaling Technology, 9198), HEXIM1 (Bethyl, A303-112A), FLAG-HRP (Sigma, F7425), His (Ruiying Biological, RLM3004) and p-HEXIM1(ser158) (Custom made). For p-HEXIM1(ser158) antibody: Rabbit polyclonal antibodies against p-HEXIM1 (ser158) were produced by Jingjie PTM Biolabs (Hangzhou). The peptides used for antibody generation were CKHRRRPpSKKKRHW (phosphorylated) and CKHRRRPSKKKRHW (unphosphorylated).

Primers uesd for RT-PCR

Ngal [GACTTCCGGAGCGATCAGTT (forward) and CTGATCCAGTAGCGACAGCC (reverse)], *p16* [ATGGGTCGCAGGTTCTTGG (forward) and CGTGAACGTTGCCCATCATC (reverse)], *Mcp-1* [CAGGTCCCTGTCATGCTTCT (forward) and CCCATTCCTTCTTGGGGTCA (reverse)], *Il-6* [AGTTGTGCAATGGCAATTCTGA (forward) and GGTACTCCAGAAGACCAGAGGA (reverse)], *Tgfb1* [AGCTGCGCTTGCAGAGATTA (forward) and AGCCCTGTATTCCGTCTCCT (reverse)], *Col1a1* [TTCTCCTGGCAAAGACGGAC (forward) and CTCAAGGTCACGGTCACGAA (reverse)], *7SK snRNA* (Human) [GACATCTGTCACCCCATTGA (forward) and GCGCAGCTACTCGTATACCC (reverse)], *7SK snRNA* (Dog) [GCGACATCTGTCACCCCATT (forward) and

TTGGAGGTTCTAGCAGGGGA(reverse)]

fig. S1

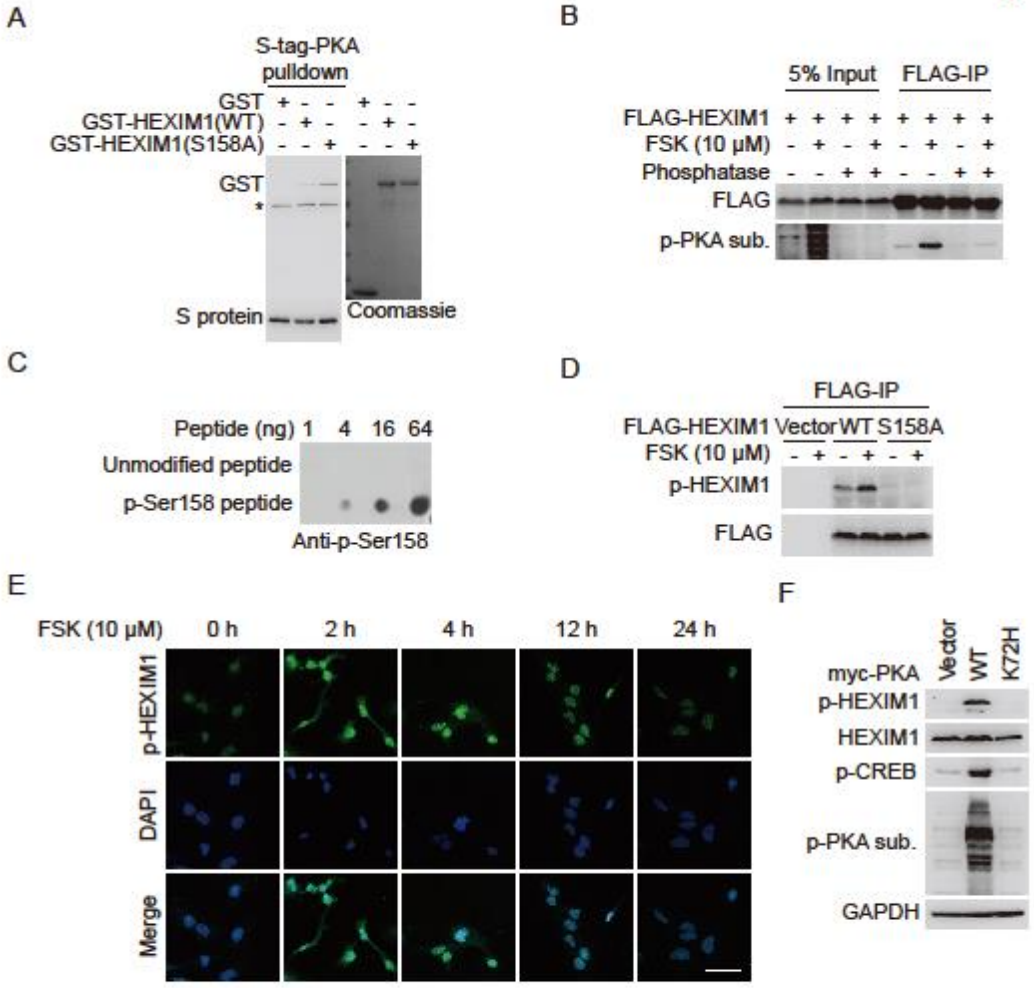

A
S-tag-PKA
pulldown
GST + - - + - -
GST-HEXIM1(WT) - + - - + -
GST-HEXIM1(S158A) - - + - - +
GST
*
S protein
Coomassie
B
5% Input
FLAG-IP
FLAG-HEXIM1 + + + + + + + +
FSK (10 μM) - + - + - + - +
Phosphatase - - + + - - + +
FLAG
p-PKA sub.
C
Peptide (ng) 1 4 16 64
Unmodified peptide
p-Ser158 peptide
Anti-p-Ser158
D
FLAG-IP
FLAG-HEXIM1 Vector WT S158A
FSK (10 μM) - + - + - +
p-HEXIM1
FLAG
E
FSK (10 μM) 0 h 2 h 4 h 12 h 24 h
p-HEXIM1
DAPI
Merge
F
myc-PKA Vector WT K72H
p-HEXIM1
HEXIM1
p-CREB
p-PKA sub.
GAPDH

Fig. S1. PKA phosphorylates HEXIM1 at serine-158. (**A**) S-tag pulldown assays examining the interactions between S-tag-PKA and GST-HEXIM1 (WT and S158A). Asterisk indicates the non-specific bands. (**B**) FLAG-IP assays analyzing the phosphorylation status of FLAG-HEXIM1 in 293T cells treated with the indicated agents. (**C**) Dot blot analysis of p-HEXIM1. (**D**) FLAG-IP assays examining the phosphorylation status of FLAG-HEXIM1 (WT and S158A) using the anti-p-HEXIM1 antibody. (**E**) Immunofluorescence analysis of p-HEXIM1 in 293T cells treated with FSK. (**F**) Western blot analysis of p-HEXIM1 and p-CREB in 293T cells transfected with vector or myc-PKA (WT and K72H). Scale bar: 50 μm.

fig. S2

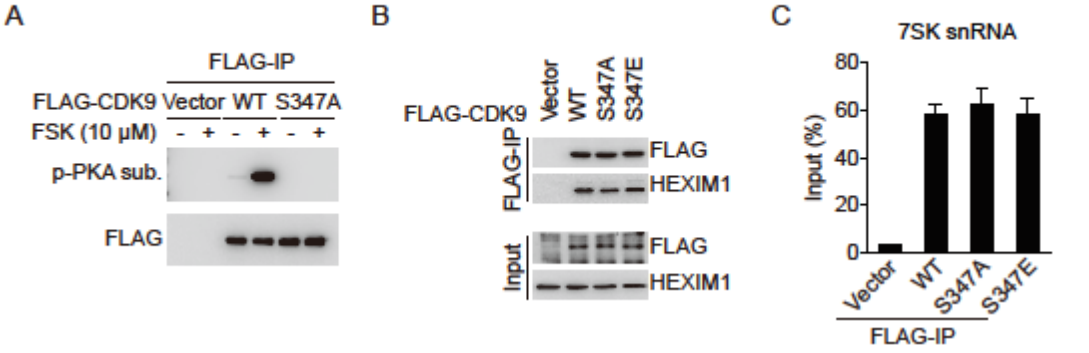

A
FLAG-IP
FLAG-CDK9 Vector WT S347A
FSK (10 μM) - + - + - +
p-PKA sub.
FLAG
B
FLAG-CDK9 Vector WT S347A S347E
FLAG-IP
FLAG
HEXIM1
Input
FLAG
HEXIM1
C
7SK snRNA
Input (%)
80
60
40
20
0
Vector
WT
S347A
S347E
FLAG-IP

Fig. S2. Phosphorylation of CDK9 at serine-347 by PKA does not affect P-TEFb/HEXIM1/7SK snRNP complex assembly. (**A**) FLAG-IP assays analyzing the phosphorylation status of FLAG-CDK9 (WT and S347A) in 293T cells treated with FSK. (**B**) Co-IP assays examining the interactions between FLAG-CDK9 (WT, S347A and S347E) and endogenous HEXIM1 in 293T cells. (**C**) RNA-IP assays examining the associations between the indicated FLAG-CDK9 and 7SK snRNA. Data represent as mean ± SEM of three replicates.

fig. S3

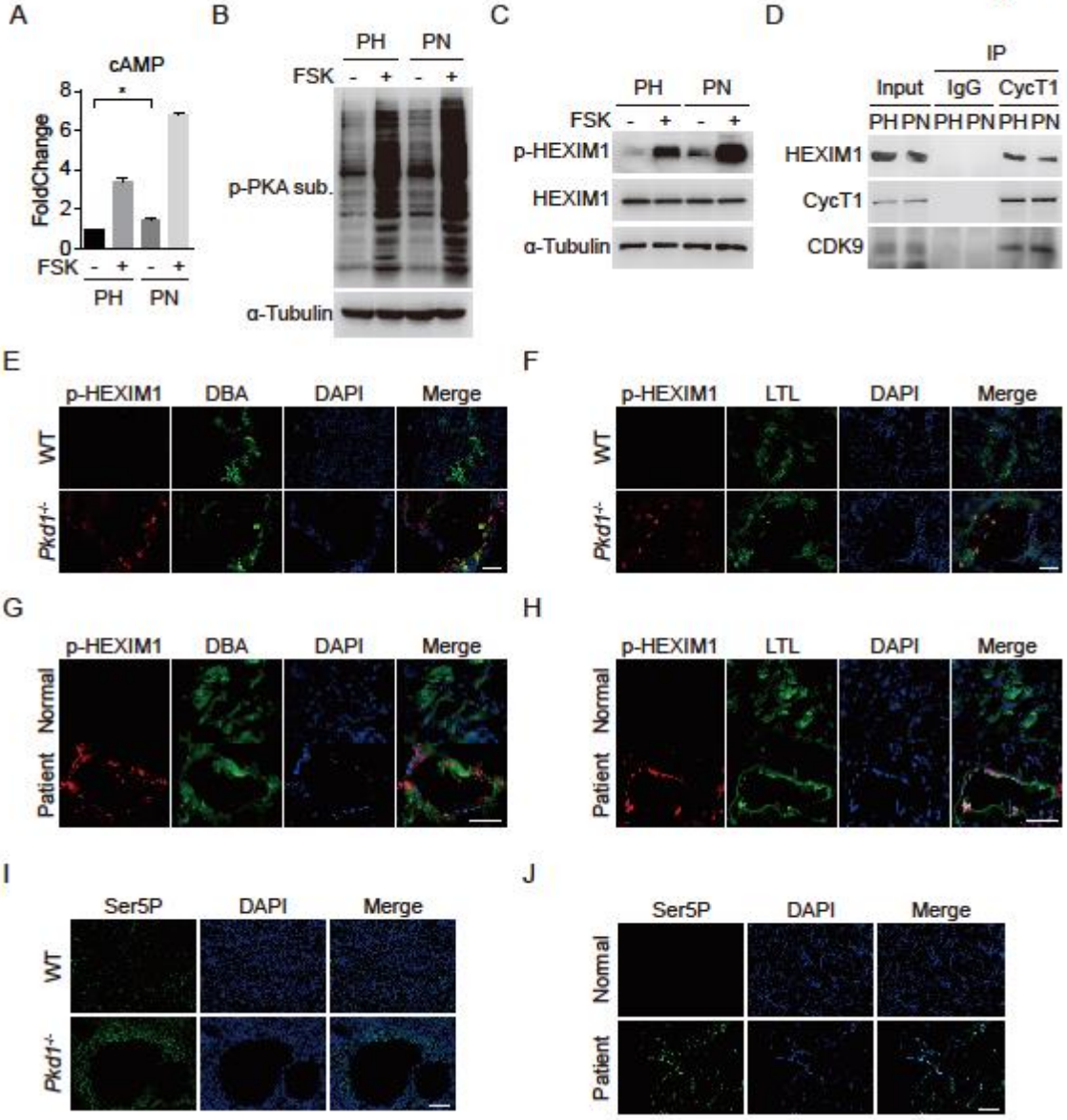
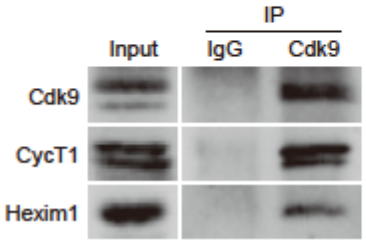

A
cAMP
FoldChange
FSK
PH
PN
B
PH
PN
FSK
p-PKA sub.
α-Tubulin
C
PH
PN
FSK
p-HEXIM1
HEXIM1
α-Tubulin
D
IP
Input
IgG
CycT1
PH PN PH PN PH PN
HEXIM1
CycT1
CDK9
E
p-HEXIM1
DBA
DAPI
Merge
WT
Pkd1-/-
F
p-HEXIM1
LTL
DAPI
Merge
WT
Pkd1-/-
G
p-HEXIM1
DBA
DAPI
Merge
Normal
Patient
H
p-HEXIM1
LTL
DAPI
Merge
Normal
Patient
I
Ser5P
DAPI
Merge
WT
Pkd1-/-
J
Ser5P
DAPI
Merge
Normal
Patient

Fig. S3. The phosphorylation level of HEXIM1 is increased in cystic epithelial cells. (**A**) cAMP levels detection in WT and cystic cells with or without FSK treatment. (**B** and **C**) Western blot analysis of PKA activity (**B**) and p-HEXIM1 (**C**) in WT and cystic cells with or without FSK treatment. (**D**) Co-IP assays examining the interactions between P-TEFb and HEXIM1 in WT and cystic cells. (**E** and **F**) Immunofluorescence analysis of p-HEXIM1 levels in the DBA-positive (**E**) or LTL-positive (**F**) kidney tissues from WT and *Pkd1*$^{-/-}$ mice. (**G** and **H**) Immunofluorescence analysis of p-HEXIM1 levels in the DBA-positive (**G**) or LTL-positive (**H**) tissues from human kidneys. (**I**)Immunofluorescence analysis of Pol II Ser5P levels in mice kidneys. (**J**)Immunofluorescence analysis of Pol II Ser5P levels in human kidneys. Scale bars: 50 μm.

fig. S4

A


IP
Input
IgG
Cdk9
Cdk9
CycT1
Hexim1

B

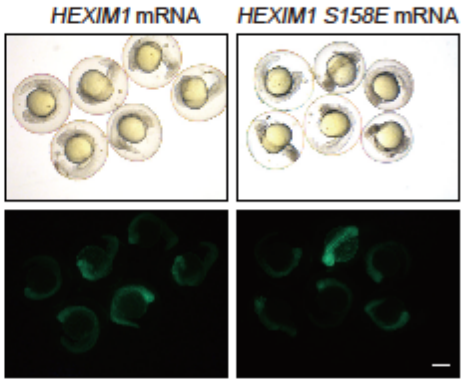

HEXIM1 mRNA
HEXIM1 S158E mRNA

Fig. S4. The interaction between P-TEFb and Hexim1 is conserved in zebrafish. (**A**) Co-IP assays examining the interactions between zebrafish P-TEFb and Hexim1. (**B**) Live cell images showing EGFP-HEXIM1 expression in zebrafish embryos injected with human EGFP-HEXIM1 mRNA (WT and S158E). Scale bar: 300 μm.

fig. S5

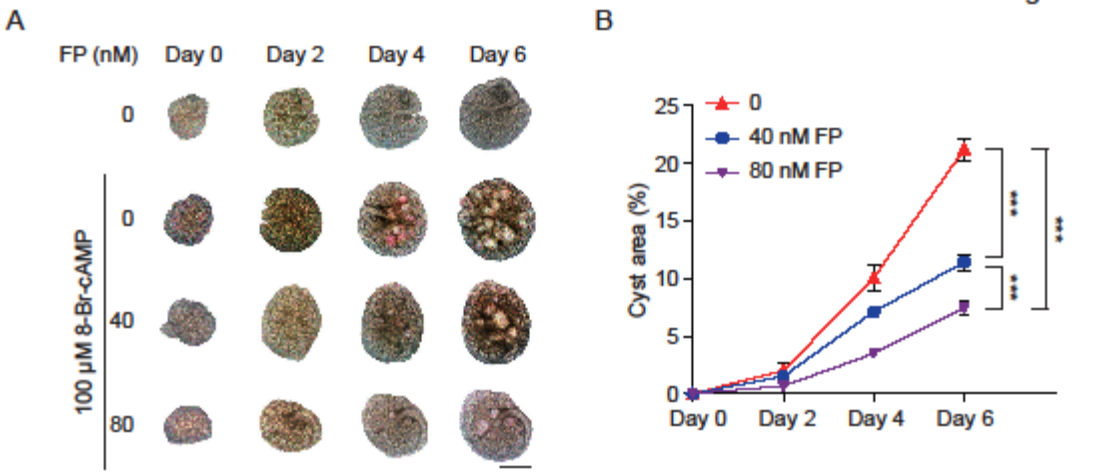

A
FP (nM)
Day 0
Day 2
Day 4
Day 6
0
100 µM 8-Br-cAMP
0
40
80
B
0
40 nM FP
80 nM FP
Cyst area (%)
25
20
15
10
5
0
Day 0
Day 2
Day 4
Day 6
***
***
***

Fig. S5. FP inhibits cystogenesis in the embryonic kidney model. (**A**) Representative images of mouse embryonic kidneys (E13.5) treated with the indicated doses of Flavopiridol. (**B**) Quantification of the percentage of cyst area relative to total kidney area ($n \geq 3$). Scale bar: 1 mm. Two-way ANOVA with Tukey's post-hoc test was used for statistical analysis.

fig. S6

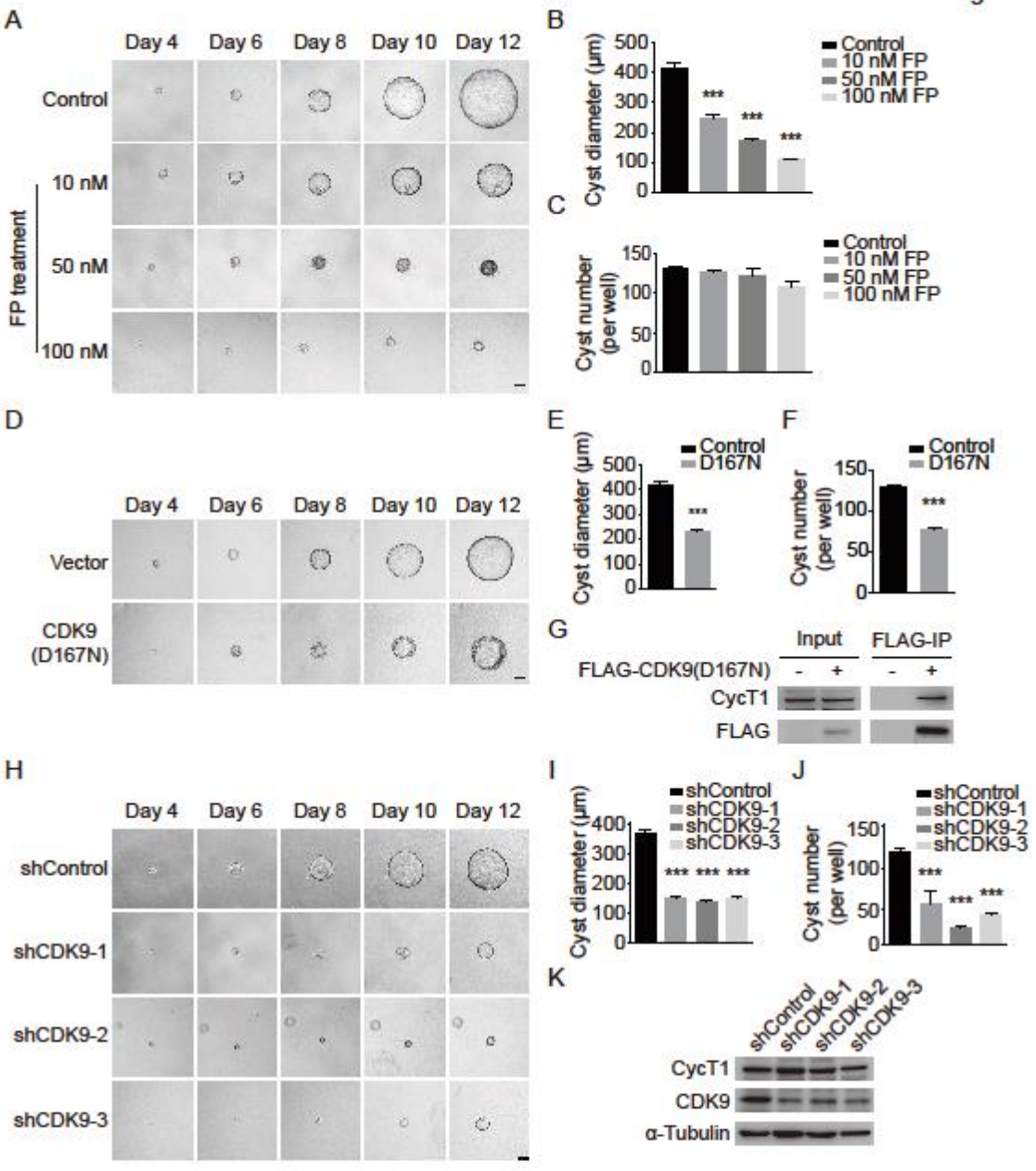

A
Day 4
Day 6
Day 8
Day 10
Day 12
Control
10 nM
50 nM
100 nM
FP treatment
B
Cyst diameter (μm)
500
400
300
200
100
0
***
***
***
Control
10 nM FP
50 nM FP
100 nM FP
C
Cyst number (per well)
150
100
50
0
Control
10 nM FP
50 nM FP
100 nM FP
D
Day 4
Day 6
Day 8
Day 10
Day 12
Vector
CDK9 (D167N)
E
Cyst diameter (μm)
500
400
300
200
100
0
***
Control
D167N
F
Cyst number (per well)
150
100
50
0
***
Control
D167N
G
Input
FLAG-IP
FLAG-CDK9(D167N)
- + - +
CycT1
FLAG
H
Day 4
Day 6
Day 8
Day 10
Day 12
shControl
shCDK9-1
shCDK9-2
shCDK9-3
I
Cyst diameter (μm)
400
300
200
100
0
*** *** ***
shControl
shCDK9-1
shCDK9-2
shCDK9-3
J
Cyst number (per well)
150
100
50
0
***
***
***
shControl
shCDK9-1
shCDK9-2
shCDK9-3
K
shControl
shCDK9-1
shCDK9-2
shCDK9-3
CycT1
CDK9
α-Tubulin

Fig. S6. Inhibition of CDK9 attenuates cyst development in the MDCK cyst model. (**A**) Representative images of MDCK cysts treated with the indicated doses of flavopiridol. (**B** and **C**) Cyst diameter (**B**) and cyst numbers (**C**) of the indicated groups were measured on day 12. (**D**) Representative images of MDCK cysts transfect with vector or human CDK9 (D167N). (**E** and **F**) Cyst diameter (**E**) and cyst numbers (**F**) of the indicated groups were measured on day 12. (**G**) Co-immunoprecipitation (Co-IP) assays examining the interactions between FLAG-CDK9 D167N and endogenous canine CycT1 in MDCK cells transfected with FLAG-CDK9 D167N. (**H**) Representative images of MDCK cells infected with lentiviruses carrying control or CDK9 shRNAs. (**I** and **J**) Cyst diameter (**I**) and cyst numbers (**J**) of the indicated groups were measured on day 12. (**K**) Immunoblotting analysis of CDK9 knockdown efficiencies in MDCK cells. Data represent as mean ± SEM. ***$p < 0.001$. Scale bars: 100 μm. Two-tailed unpaired Student's t test was used for statistical analysis.

fig. S7

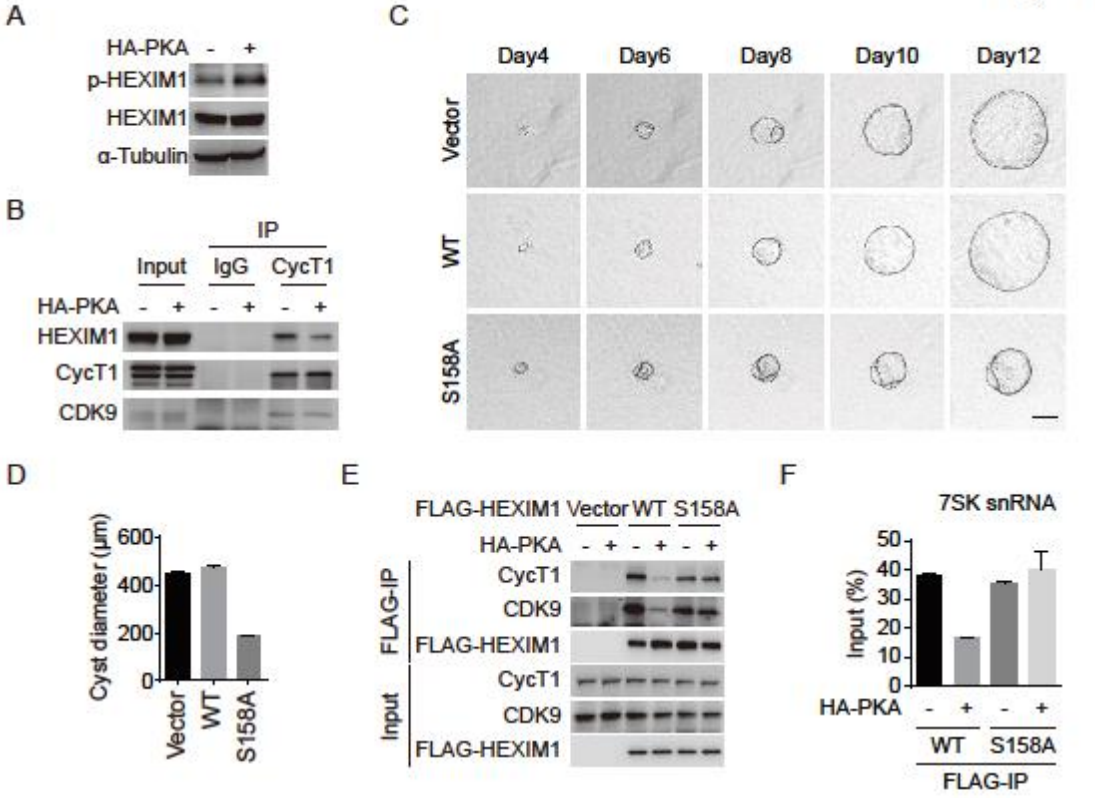

A
HA-PKA - +
p-HEXIM1
HEXIM1
α-Tubulin
B
IP
Input
IgG
CycT1
HA-PKA - + - + - +
HEXIM1
CycT1
CDK9
C
Day4
Day6
Day8
Day10
Day12
Vector
WT
S158A
D
Cyst diameter (μm)
600
400
200
0
Vector
WT
S158A
E
FLAG-HEXIM1 Vector WT S158A
HA-PKA - + - + - +
FLAG-IP
CycT1
CDK9
FLAG-HEXIM1
Input
CycT1
CDK9
FLAG-HEXIM1
F
7SK snRNA
Input (%)
50
40
30
20
10
0
HA-PKA - + - +
WT
S158A
FLAG-IP

Fig. S7. HEXIM1 phosphorylation and P-TEFb/HEXIM1/7SK snRNP complex assembly in the MDCK cyst model. (**A**) Western blot analysis of p-HEXIM1 in MDCK transfected with or without PKA. (**B**) Co-IP assays examining the interactions between P-TEFb and HEXIM1 in MDCK trascfected with or without PKA. (**C**) Representative images of MDCK cysts transfect with vector or human HEXIM1 (WT or S158A). (**D**) Cyst diameter of the indicated groups were measured on day 12. (**E**) Co-IP analysis of the interactions between the indicated FLAG-HEXIM1 and P-TEFb in MDCK cells transfected with or without PKA. (**F**) RNA-IP assays examining the associations between the indicated FLAG-HEXIM1 and 7SK snRNA in MDCK cells transefected with or without PKA. Scale bar: 100 μm.

fig. S8

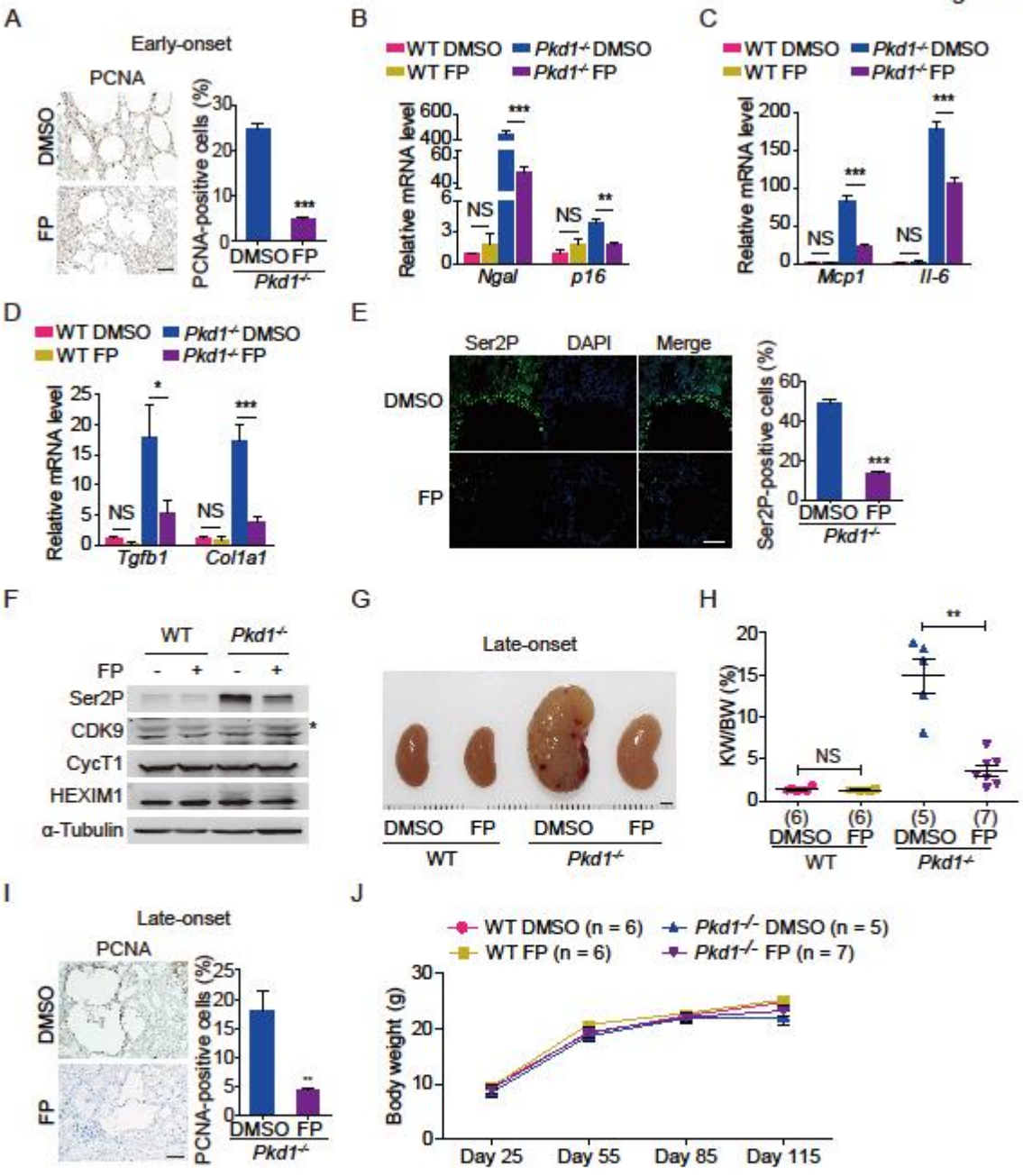

A
Early-onset
PCNA
DMSO
FP
PCNA-positive cells (%)
DMSO FP
Pkd1-/-
B
WT DMSO
Pkd1-/- DMSO
WT FP
Pkd1-/- FP
Relative mRNA level
NS
***
**
Ngal
p16
C
Mcp1
Il-6
D
Tgfb1
Col1a1
E
Ser2P
DAPI
Merge
Ser2P-positive cells (%)
F
WT
Pkd1-/-
FP
Ser2P
CDK9
CycT1
HEXIM1
α-Tubulin
G
Late-onset
H
KW/BW (%)
(6)
(5)
(7)
I
J
WT DMSO (n = 6)
Pkd1-/- DMSO (n = 5)
WT FP (n = 6)
Pkd1-/- FP (n = 7)
Body weight (g)
Day 25
Day 55
Day 85
Day 115

Fig. S8. Effect of FP on cystogensis in the early- and late-onset ADPKD mouse models. (**A**) Immunostaining of PCNA in P29 mice from the indicated groups. (**B**-**D**) Quantitative RT-PCR analysis of relative mRNA levels of injury markers (**B**), inflammatory markers (**C**) and fibrotic markers (**D**) in kidneys from the indicated groups. (**E**) Immunofluorescence analysis of Ser2P Pol II expression in kidneys from *Pkd1*$^{-/-}$ mice treated with DMSO or FP. (**F**) Western blot analysis of P-TEFb, Ser2P Pol II and HEXIM1 expression in kidneys from the indicated groups. (**G**) Representative images of P115 kidneys from the indicated groups. (**H**) Ratios of kidney-weight to body-weight in the indicated groups of P115 mice. (**I**) Immunostaining of PCNA in P115 kidneys from *Pkd1*$^{-/-}$ mice treated with DMSO or FP. (**J**) Growth curve of mouse body weight in late-onset ADPKD model. *, non-specific bands. Scale bars: 100 μm. Data represent the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Two-tailed unpaired Student's t test was used for statistical analysis. (Photo credit: Zhiheng Liu, Tianjin Medical University).