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Supplementary Materials for

Cyclin G1 and TASCC regulate kidney epithelial cell G_2-M arrest and fibrotic maladaptive repair

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at www.sciencetranslationalmedicine.org/cgi/content/full/11/476/eaav4754/DC1)

Data file S1 (Microsoft Excel format). Primary data.

Materials and Methods

Transfection

LLC-PK1 or HKC-8 cells were plated at 50% confluence and transfected with Lipofectamine 2000 (ThermoFisher) following the manufacturer's instructions. Briefly, plasmid DNA and Lipofectamine 2000 were mixed in serum free DMEM/F12 at a ratio of 1 μg DNA:2 μl Lipofectamine 2000 and incubated for 20 min. The mixture was then added to the cells with full media. In some experiments, pCMV-6xMyc-HsCyclin G1 (kindly provided by Drs. Hiroshi Nojima and Norikazu Yabuta) was co-transfected with YFP at a 5:1 ratio to identify the transfected cells. pmRFP-LC3 was a gift from Tamotsu Yoshimori and purchased from Addgene (plasmid 21074 (*18*)). YFP was purchased as part of the ATCC subcellular localization kit (ATCC).

Super resolution imaging by structured illumination microscopy

Paraffin embedded tissue was sectioned at 4-6 um thickness and mounted on (3-Aminopropyl) trimethoxysilane treated coverslips (mounting the tissue to the coverslip is necessary as super-resolution microscopes have limited working distances). Antigen retrieval was performed by incubating the sections under high temperature (120 ºC) and high pressure in citrate buffer using a pressure cooker. Sections were then blocked with BSA and normal donkey serum, and stained with anti-mTOR and/or anti-LC3 antibodies and corresponding secondary antibodies (Jackson Immunoresearch). Tissues were imaged on a Nikon structured illumination microscope (N-SIM) as a z-stack at 0.12 μm thickness using a 100x TIRF objective. The resulting z-stack was 3D rendered in NIS elements for image analysis.

Western blot

Cells or kidney tissues were lysed, and lysates were prepared as previously described(*50*). Briefly, proteins were separated by molecular weight using tris-glycine SDS-polyacrylamide gels and transferred to Polyvinylidene fluoride (PVDF membranes). Membranes were

incubated with primary antibodies followed by appropriate horseradish peroxidase–conjugated secondary antibodies. Enhanced chemiluminescence (Amersham Biosciences) was used to detect proteins. Supernatant from cultured HK-2 and LLC-PK1 cells was analyzed via western blot to determine CTGF protein levels with a rabbit anti-human antibody. Densitometry was performed using Image J software.

Isolation of primary renal proximal tubular cells

Renal cortical tissues were harvested from wild-type or CG1$^{-/-}$ animals to isolate proximal tubular cells for culture as previously described (*51*). Briefly, cortical tissues were minced on an ice-cold glass plate, digested with collagenase in oxygenated media at 37^{o}C with vigorous shaking, and centrifuged in 32% Percoll, diluted in DMEM/F-12, to purify proximal tubular cells from the suspension. Cell aggregates were then plated on collagen-coated dishes. Cells were maintained in DMEM/F-12 medium supplemented with 5 μg/ml transferrin, 5 μg/ml insulin, 0.05 μM hydrocortisone, and 50 μM vitamin C.

β-galactosidase staining

β-gal staining was performed using the β-gal staining kit from Sigma-Aldrich according to the manufacturer's instructions. Approximately 7 days after isolation of primary proximal tubule cells, they were treated with 10 μg/ml AA for 7 days. The cells were then fixed with the formaldehyde buffer provided with the kit and stained according to the manufacturer's instructions.

Flow cytometry

Flow cytometry was performed using a MACSQuant Analyzer (MiltenyiBiotec) as described previously (*2*). Briefly, LLC-PK1 cells were trypsinized and fixed with 70 % ice cold ethanol and stained with propidium iodine (PI) solution (FxCycle ThermoFisher). For experiments involving YFP co-transfection, cells were fixed in 4% PFA for 1 h and then permeabilized with 70 % ethanol as described above. Cell cycle analysis was performed using FlowJo software.

RNA extraction and real-time quantitative PCR

Total RNA was isolated from snap-frozen kidneys using the TRIzol reagent (Sigma) according to the manufacture's protocol. First-strand cDNA was synthesized using the MML-V reverse transcriptase (Promega). Real-time PCR was performed using the iQ-SYBR Green supermix (BioRad) and the iQ5 Multicolor Real-Time PCR Detection System (BioRad) for mRNA detection. 18S rRNA was used as a housekeeping gene.

The following primer sequences were used:

18SrRNA forward: ATGGCCGTTCTTAGTTGGTG, reverse GAACGCCACTTGTCCCTCTA;

TGF-β forward: GCAACAATTCCTGGCGTTACC, reverse: CGAAAGCCCTGTATTCCGTCT;

CTGF forward: AACAGTGGAGATGCCAGGAG, reverse TAATTTCCCTCCCCGGTTAC;

αSMA forward: AGGGCTGGAGAATTGGATCT, reverse: CCAGCAAAGGTCAGAGAAGG;

Col-4α1 forward: TTCAGATTCCGCAGTGCCCTA, reverse: TTCTCATGCACACTTGGCAGC;

Fibronectin forward: ATGTGGACCCCTCCTGATAGT, reverse: GCCCAGTGATTTCAGCAAAGG.

Supplementary Figures

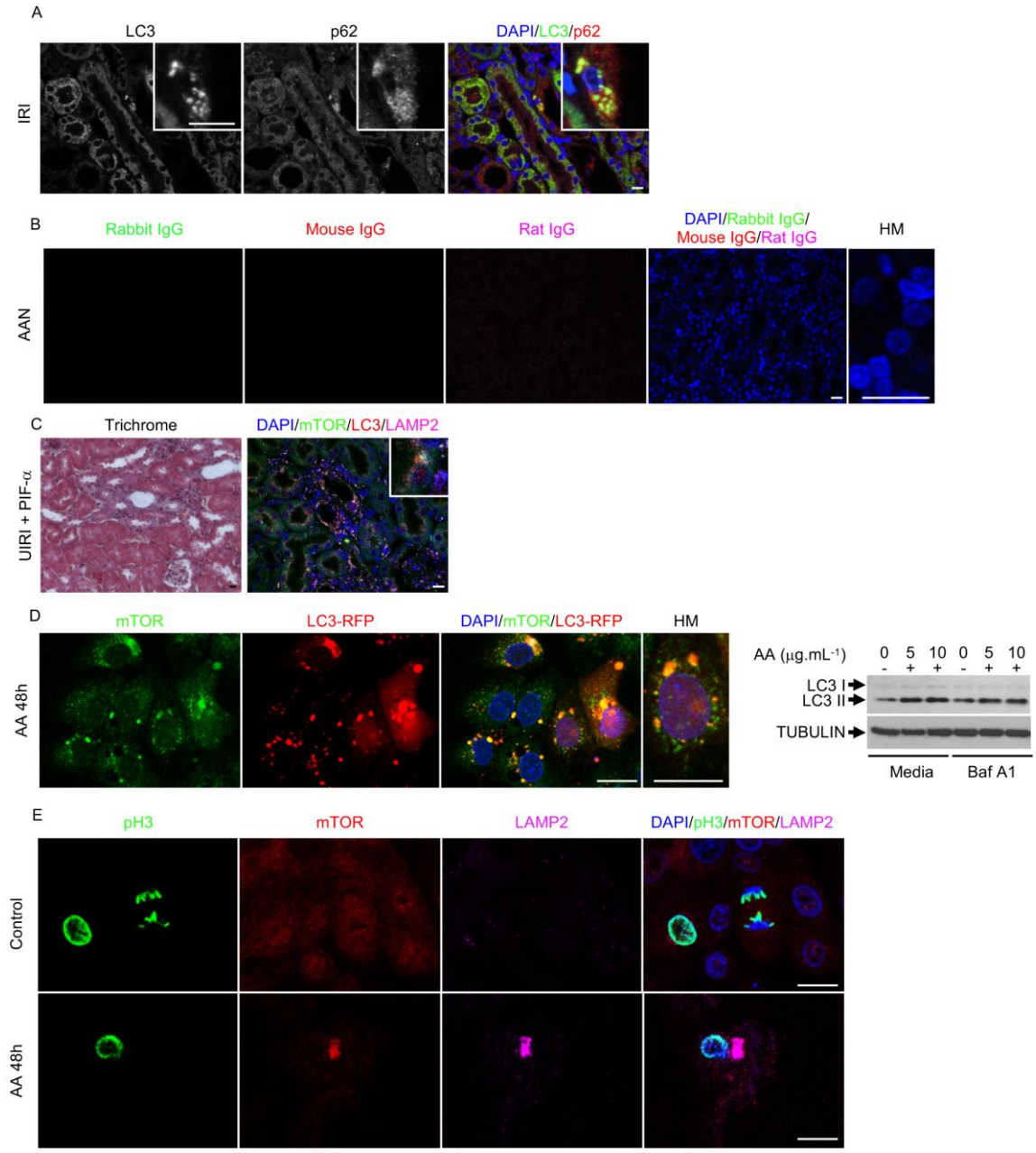
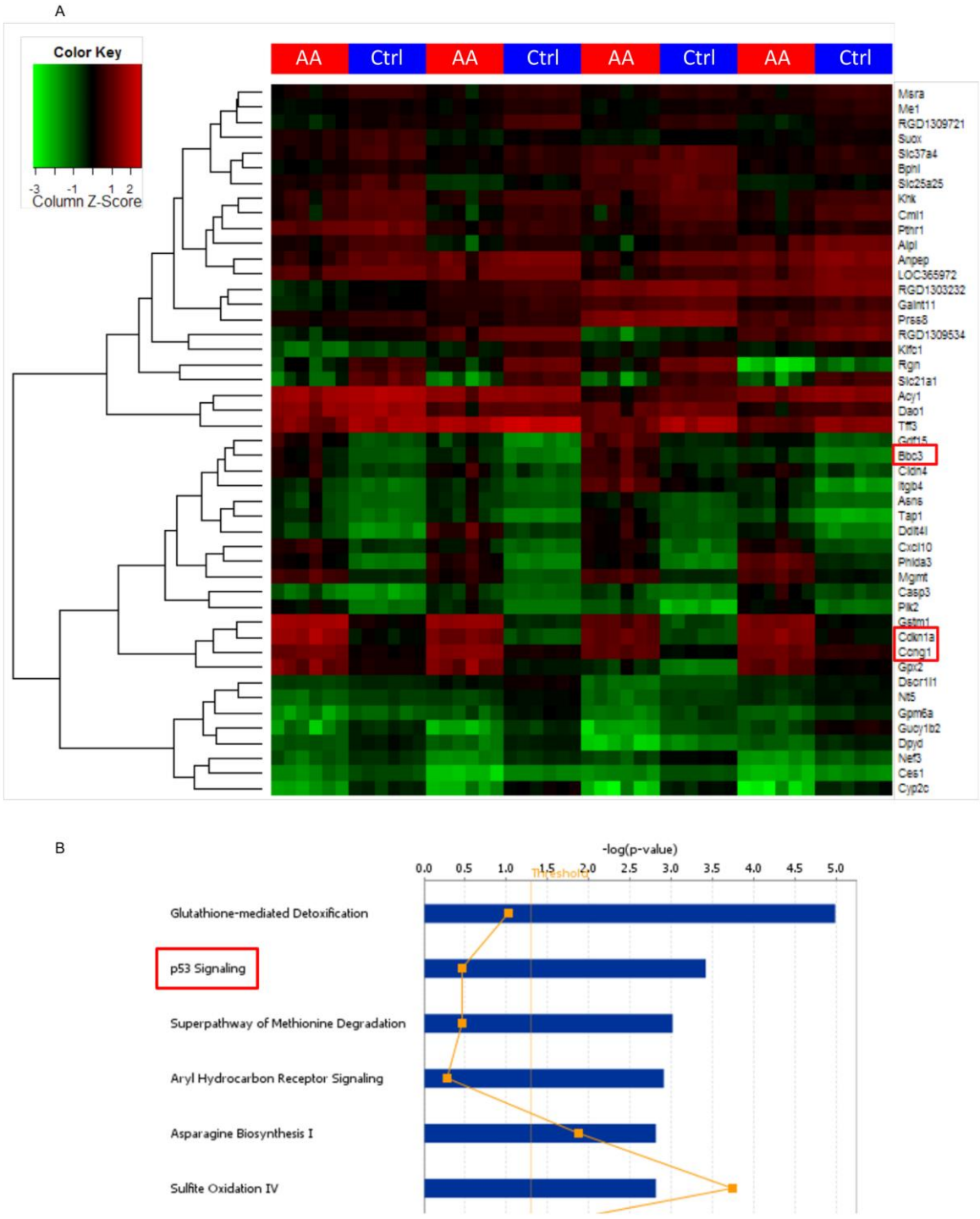


Fig. S1. TASCC formation in vitro and in vivo. (A) Co-immunostaining of LC3 and p62 in IRI injured mouse kidney tissue. (B) Kidneys were stained with rabbit, mouse and rat isotype-matched IgGs 21 days after exposure to AA to control for secondary Ab non-specific staining. (C) Morphology (trichrome) of kidneys and co-immunostaining between mTOR, LC3 and LAMP2 in mice with UIRI and treated with pifithrin-α (PIF-α), 21 days after kidney injury.

(D) Co-immunostaining of mTOR and *LC3-RFP* in HK-2 cells 48h after AA treatment (5μg/ml) (*n* = 3 independent experiments) (left panels). Representative western blot of LC3 accumulation in HK-2 cells treated with AA in the presence or absence of bafilomycin A1 (100nM). (*n* = 3 independent experiments) (right panels). (E) Lower magnification of Figure 2i: Co-immunostaining of pH3 (pH3), mTOR and LAMP2 in HK-2 cells 48h after AA treatment (*n* = 3 independent experiments). Scale bar: 10 μm.



Top p53 regulated genes: *CDKN1A* (p21), *BBC3* (PUMA) and *Ccng1* (cyclin G1)

Fig. S2. Microarray analysis reveals link between p53 signaling, cyclin G1 and fibrosis. (A) Unsupervised hierarchical clustering of mRNA expression patterns in kidneys from rats exposed to either vehicle (Ctrl) or aristolochic acid (AA) for 12 weeks (10 mg/kg 5 times per week by oral gavage) (*19*). Red boxes highlight some upregulated genes. p53 pathway genes

most highly upregulated in this study were *CDKN1A* (p21), *BBC3* (PUMA) and *Ccng1* (cyclin G1). (B) Ingenuity Pathway Analysis was performed on the data in A to identify the pathways most likely to be involved in kidney fibrosis. The top results were graphically ranked based on the *P* value. Glutathione-mediated detoxification is known to be upregulated by AA and is necessary for the processing of AA into a biologically active form and was therefore excluded as a pathway to evaluate further in tubulointerstitial kidney fibrosis.

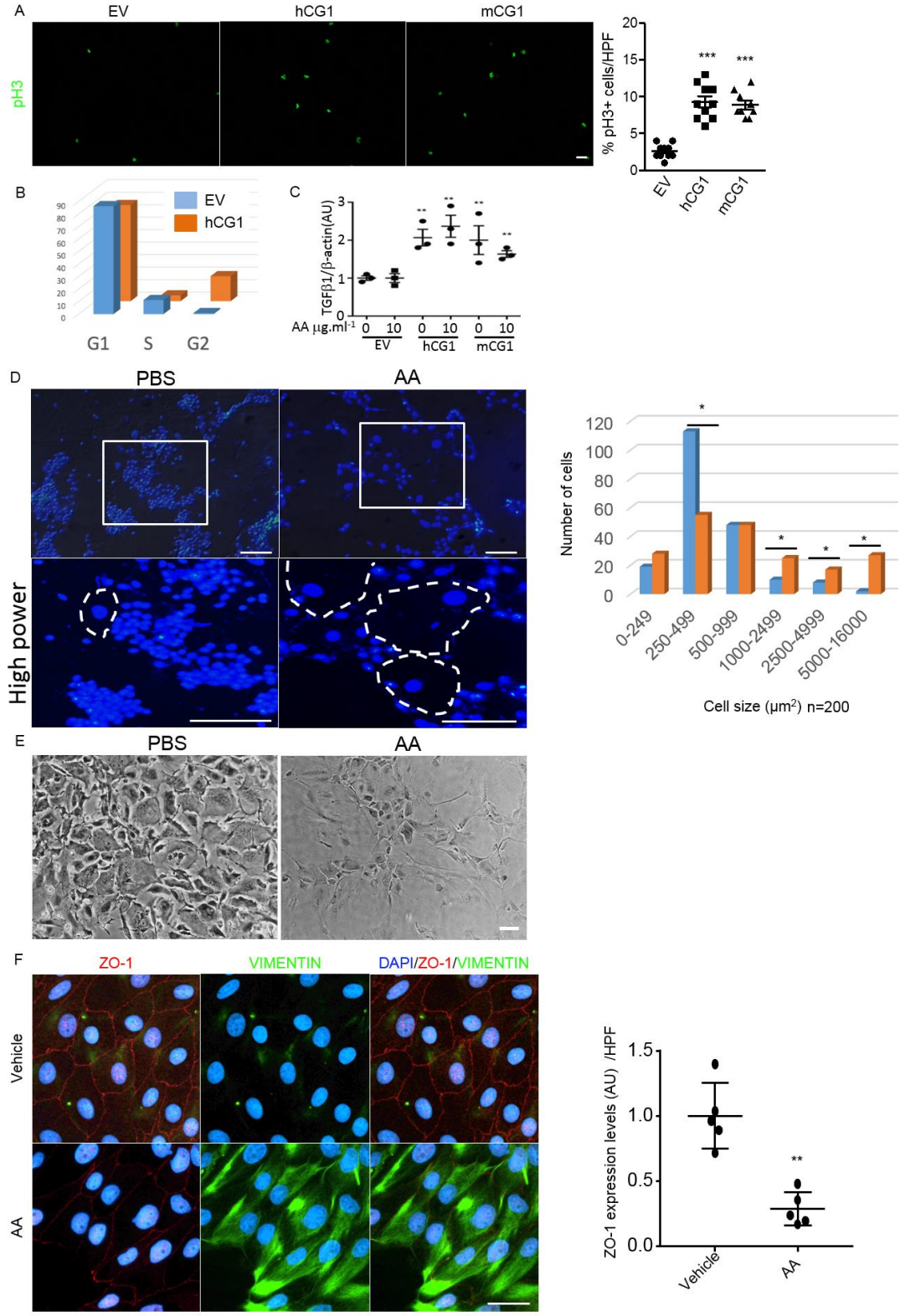


Fig. S3. AA treatment induced cell damage, up-regulation of vimentin, and decreased expression of ZO-1. (A) Representative images and quantification of pH3 staining in cells transfected with human (h) or mouse (m) CG1. *** $P = 0.001$. Scale bar: 10 μm. (B) Cell cycle

analysis in LLC-PK1 cells 48 hours after transfection with either the EV or hCG1 (n = 3 independent experiments). HM: High magnification. (C) Quantification of TGF-β1 induction in HK-2 cells transfected with EV, hCG1, or mCG1 after 48 hours of vehicle or AA treatment (5 μg/ml) (n = 3 independent experiments). Data are means ± SEM. ANOVA-Tukey's post hoc. ** $P < 0.01$. (D) Upper: Representative images of DAPI stained LLC-PK1 cells treated with/without AA (5 μg/ml). Boxes signify area chosen for high magnification. Dashed lines outline individual cells. Upper right: Quantification of LLC-PK1 cells treated with either vehicle (blue bars) or AA (orange bars) for 48 (5 μg/ml). Scale bar = 50 μm. n = 200 cells. * $P < 0.05$. (E) Phase contrast images of primary PTCs treated with/without AA . Scale bar = 100 μm. (F) Immunofluorescence images of AA-induced changes in vimentin and ZO-1 expression in LLC-PK1 cells. Scale bar: 50 μm. ZO-1 expression was quantified using Nikon NIS software. ** $P < 0.01$.

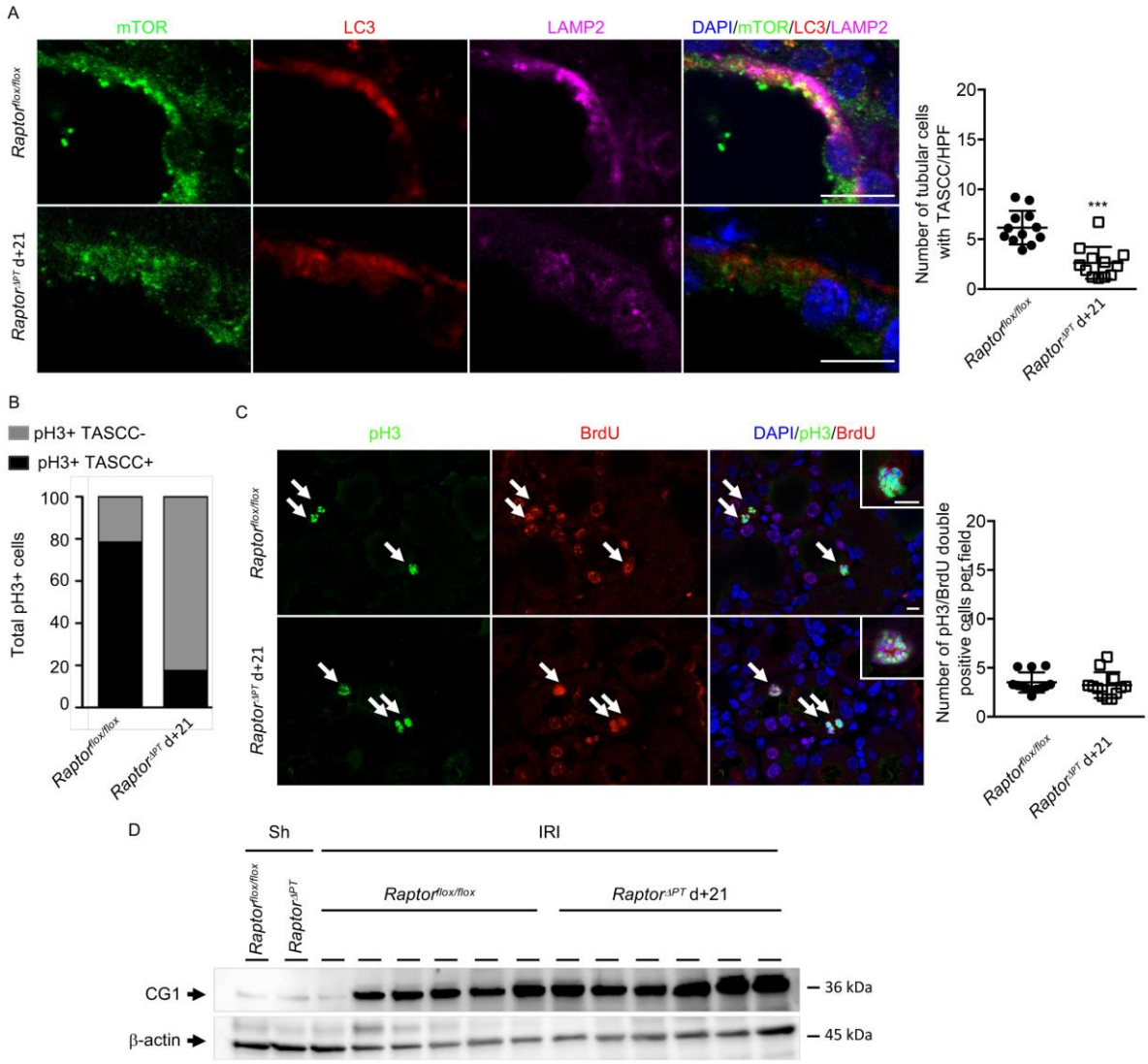


Fig. S4. Characterization of the *Raptor*$^{\Delta PT}$ mice. (A) Co-immunostaining of mTOR, LC3 and LAMP2 in *Raptor*$^{flox/flox}$ and *Raptor*$^{\Delta PT}$ d+21 mice 42 days after IRI (left panel) (n = 12 mice per group). Right panel: Quantification of TASCC formation per field in *Raptor*$^{flox/flox}$ and *Raptor*$^{\Delta PT}$ d+21 mice 42 days after IRI (right panel). *** $P < 0.001$ by t-test comparing *Raptor*$^{flox/flox}$ *vs* *Raptor*$^{\Delta PT}$ d+21. (B) Percentage of pH3^{+} cells with TASCC formation in *Raptor*$^{flox/flox}$ and *Raptor*$^{\Delta PT}$ mice 42 days after IRI (n = 12 mice per group). (C) Co-immunostaining and quantification of pH3 and BrdU in *Raptor*$^{flox/flox}$ and *Raptor*$^{\Delta PT}$ d+21 mice 42 days after IRI (n = 12 per group). Arrows indicate pH3 and BrdU positive nuclei. Insert: High powered image of one of the double positive nuclei. Scale bar: 10 µm. (D) Western blot

of cyclin G1 (CG1) in kidneys of sham operated (Sh) and after IRI in *Raptor*$^{flox/flox}$ and *Raptor*$^{\Delta PT}$ d+21 mice. Note: one *Raptor*$^{flox/flox}$ mouse in the IRI group (lane 3) did not have severe IRI as measured by BUN and developed very little fibrosis. Data are means ± SEM. Scale bar = 10 μm.

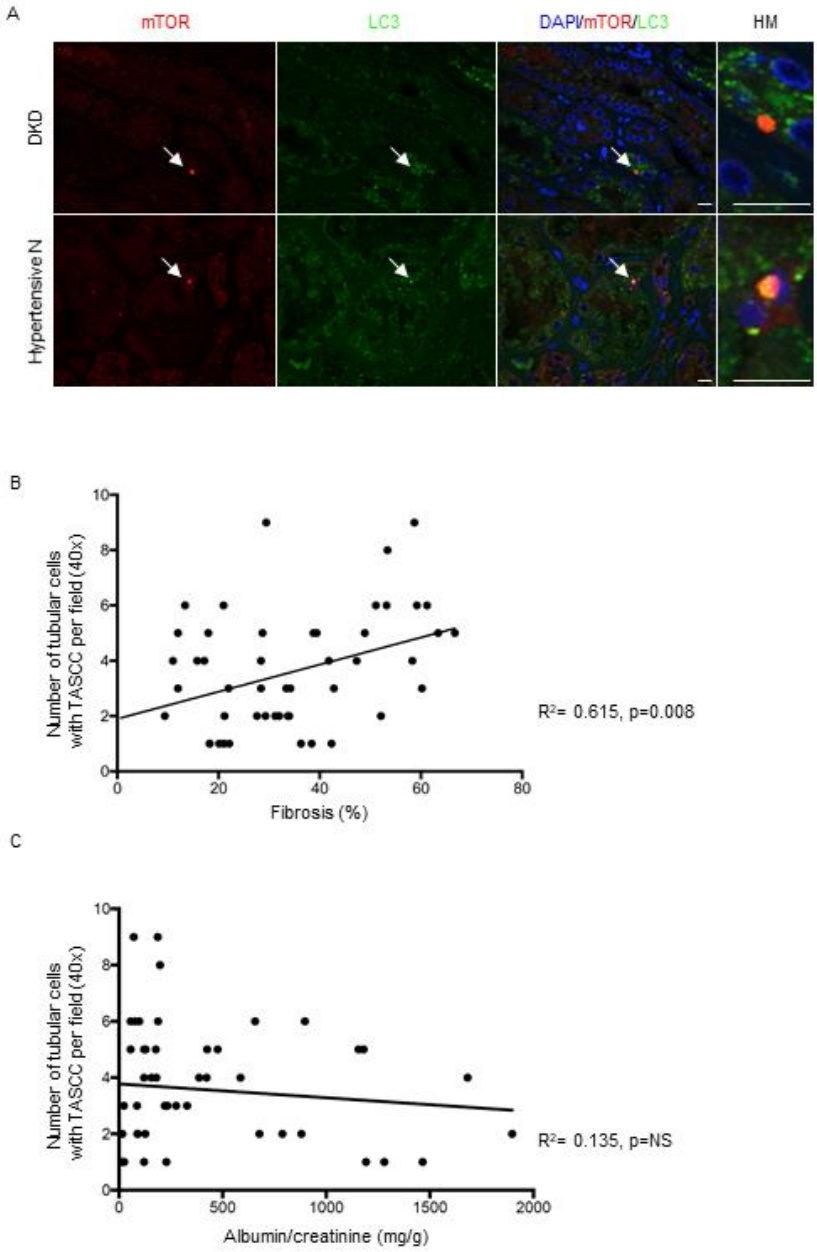


Fig. S5. TASCC and human kidney diseases. (A) Coimmunostaining of mTOR and LC3 in several human kidney biopsies with fibrosis (Type 2 diabetic kidney disease (DKD), $n = 10$ and hypertensive nephropathy, $n = 10$). HM: High magnification. Scale bar: 10 µm. (B) Correlation between TASCC$^+$ tubular cells per field (40X) and fibrosis; and (C) TASCC$^+$ tubular cells per field (40X) and albumin/creatinine (mg/g).

Table S1. Demographic and clinical characteristics of patients from whom kidney tissue was obtained.

Characteristics	Age (years)	Males (%)	eGFR (ml/min/1.73m^2)	A/C (mg/g)	Fibrosis (%)
Controls (*n* = 5)	52±4	80	82±6.3	20±5	5.2±3.3
Type 2 Diabetic Kidney Disease (*n* = 10)	58.5±2.5	40	32.1±8.1	980±110	34.3±11.1
Acute kidney injury (*n* = 10)	49.8±3.4	50	24.3±7.5	221±89	22.4±9.2
Chronic interstitial nephritis (*n* = 10)	57.9±4.1	30	38.2±10.7	198±45	42.7±14.4
Aristolochic acid nephropathy (*n* = 10)	47.3±3.3	60	40.1±7.8	425±96	47.9±14.2
Hypertensive nephropathy (*n* = 10)	56.2±4.1	60	37.4±10.1	330±105	24.8±13.1

eGFR: estimated Glomerular Filtration Rate; A/C: Albumin/Creatinine ratio in the urine. Data are +/- SD.