Supplementary data

Index

Methods Supplement

Animal Studies

All experiments were approved by the Austin Health Animal Ethics Committee. Mice were maintained under specific pathogen-free conditions with a 12-hour light/12-hour dark cycle. Animals were given free access to food and water. Homozygote lines of wild type (WT) and ACC 1/2 whole body knockin (KI) mice,[19] on a C57/Bl6 background were used.

The folic acid nephropathy (FAN) model was used to create an experimental model of renal fibrosis, as has been described.[20] Male WT and ACC 1/2 KI mice received intraperitoneal injections of folic acid (240μg/g bodyweight dissolved in 300mM $NaHCO_3$) at 8 to 12 weeks old. On day 14, blood and kidneys were harvested and mice euthanized.

The FAN model was used to assess the effect of metformin on renal fibrosis. Liquid metformin hydrochloride (Focus Pharmaceuticals, London, UK) 0.4mg/mL was added into the drinking water and changed every 48 hours. Mice were monitored and samples were harvested in the same way as described earlier.

Unilateral ureteric obstruction (UUO) was also used to produce a model of TIF as has been described.[3] Mice at 8-10 weeks of age had the left ureter ligated. Sham mice underwent a laparotomy without ureter ligation. Seven days later, blood and kidney samples were collected under anesthesia and the mice euthanized.

Western Blot Analysis

Kidney lysates were prepared and Western blots performed as we have previously described.[21] Quantification of Western Blots was by densitometry with analysis using Image J software.[22] The following antibodies were used; α-Smooth Muscle-FITC (Sigma-Aldrich, St. Louis, USA), sheep anti-Fluorescein-POD (Roche Applied Science, Indianapolis, USA), rabbit anti-GAPDH antibody (Cell Signalling technology, Massachusetts, USA), rabbit anti-phospho-acetyl CoA carboxylase (Cell Signaling Technology, Massachusetts, USA), rabbit anti-acetyl CoA carboxylase antibody (Cell Signaling Technology, Massachusetts, USA), and swine anti-rabbit immunoglobulin HRP-linked (Dako, Glostrup, Denmark).

Histology

Kidneys were sliced in half transversely and fixed in formalin. Kidney halves were paraffin embedded by the Department of Anatomical Pathology, Austin Health, Melbourne. 4μm kidney sections were cut using a rotary microtome (Leica, Germany).

Picro-Sirius Red Staining

Kidney sections were stained using Picro-Sirius Red Stain Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Oil Red O Staining

Kidneys halves were embedded in plastic Tissue-Tek Cryomold moulds (VWR, Pennsylvania, USA) using Tissue-Tek O.C.T compound (VWR, Pennsylvania, USA) and then snap frozen in liquid nitrogen. Samples were processed for Oil Red O staining by the Department of Anatomical Pathology, Austin, Melbourne.

Quantification

The light microscope Leica DMLB2 connected to a high resolution Optronics Microfire camera was used to capture images of kidney sections at 20-fold magnification. Stereo-Investigator 10 (MicroBrightField Bioscience, Vermont, USA) software was used to stitch the image. For each kidney sample, a whole cortical field from a kidney section was analyzed. The area occupied by collagen in Picro-Sirius Red stained sections and lipid droplets in Oil Red O stained sections was measured using Image J software. The values obtained were expressed as a percentage of the whole cortical area.

Triglyceride (TG) Assay

Half kidney tissue from the UUO model was weighed and homogenised in either 500 µl or 1000 µl buffer depending on weight. It was then processed according to the TG assay protocol (Abcam, ab65336). Samples were diluted 1:10 and used 30 µl per well for assay. One set of samples was treated with lipase to digest the TG to glycerol and fatty acids, the other set was left untreated to test for background glycerol levels in the tissue. There were 6 samples per group.

Cell Culture

Primary cultures of renal tubular epithelial cells (TEC) were prepared by sieving whole mouse kidneys. Cells were grown in K1 medium (DMEM/F12 supplemented with 5% FCS, insulin-

transferrin-selenium supplement (ITS), 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 ng/ml prostaglandin, 5 × 10−11 M triiodothyronine, and 5 × 10^{-8}M hydrocortisone) at 37°C with 5% CO_2. To mimic injury, cells were treated with 10mM folic acid in serum free KI media for 24 hours.[23] Cell lysates were prepared for Western blots as per kidney lysates. The experiment to determine the level of ATP in folate stimulated TEC was performed in 96-well plates, using serum-free media. The ability of cells to survive for 242-hours in this media was first determined by microscopy and ATP levels. Cells were stimulated with 10 mM folate, as outlined above, and harvested after 3 and 24 hours. There were 23-30 wells for each condition. ATP was assayed using the Abcam kit ab83355 according to the manufacturer's instructions.

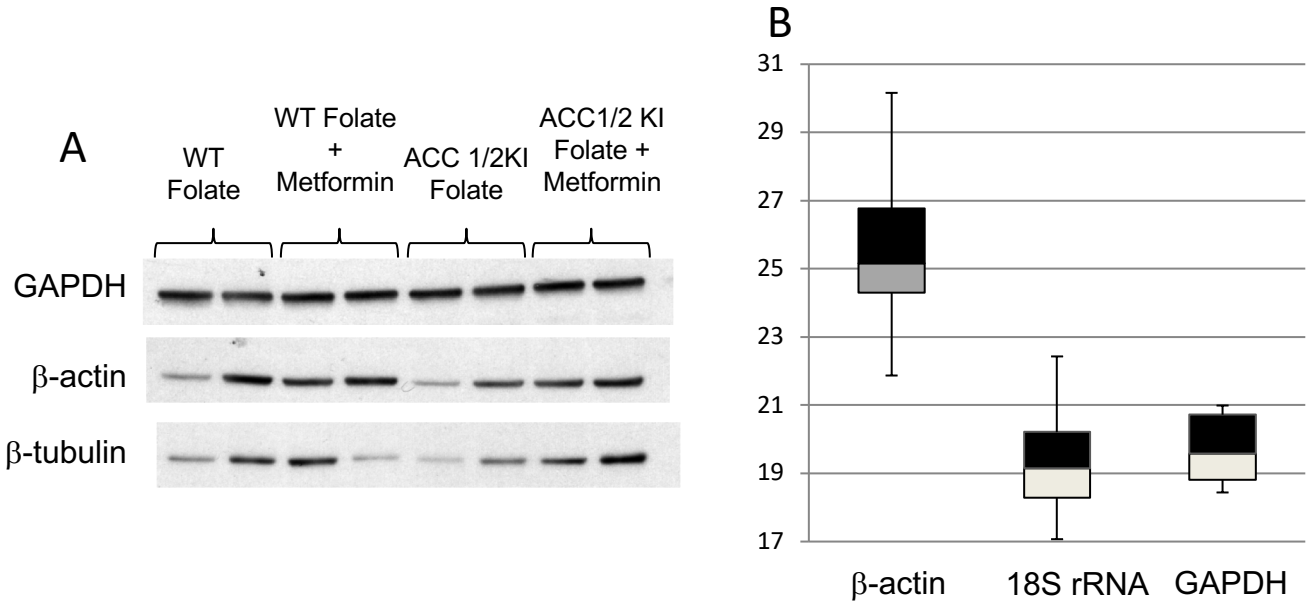
Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was purified from whole mouse kidney samples and reverse transcribed as previously described.[21] Real-time PCR was performed on a Stratagen MX3000 real-time PCR system with Solis Biodyne EvaGreen master mix. The sequence for the primers used are listed in Supplementary Table 1. The Pfaffl method was used to calculate relative expression.[24] Data was expressed as fold expression relative to littermate WT controls. Primer sequences are shown in Supplementary Table 1.

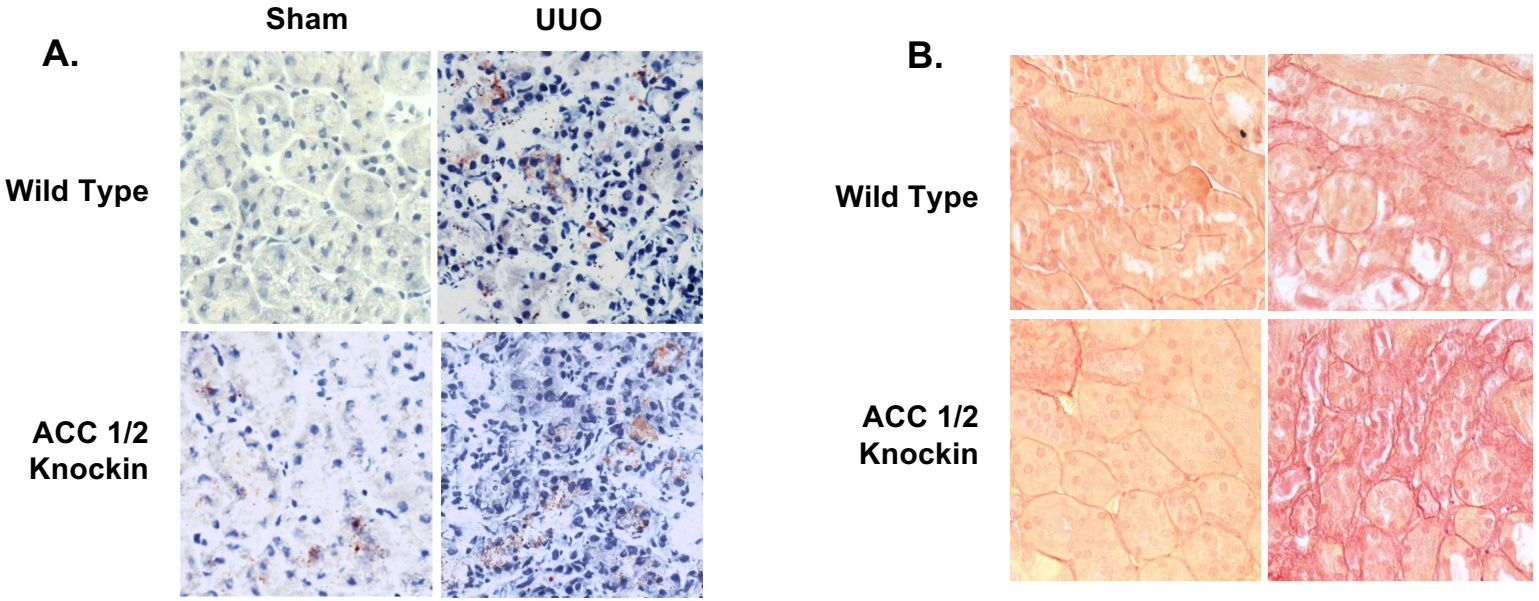
Supplementary Table 1. Sequences of oligonucleotide primers used for qRT-PCR.

Gene Name	Forward Primer	Reverse Primer
α-SMA	CAGGCATGGATGGCATCAATCAC	ACTCTAGCTGTGAAGTCAGTGTCG
Collagen 1A1	TGACTGGAAGAGCGGAGAGT	GTTCGGGCTGATGTACCAG
Collagen 3A1	ACAGCTGGTGAACCTGGAAG	ACCAGGAGATCCATCTCGAC
Fibronectin	CGAGGTGACAGAGACCACAA	CTGGAGTCAAGCCAGACACA

Cpt1a	GGTCTTCTCGGGTCGAAAGC	TCCTCCCACCAGTCACTCAC
ACox	CTTGGATGGTAGTCCGGAGA	TGGCTTCGAGTGAGGAAGTT
GAPDH	AAGGTCATCCCAGAGCTGAA	CTGCTTCACCACCTTCTTGA
F4/80	TCCTGCTGTGTCGTGCTGTTC	GCCGTCTGGTTGTCAGTCTTGTC
18S	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC
MCP-1	CAAGAAGGAATGGGTCCAGA	GTGCTGAAGACCTTAGGGCA
MAC2	GCTTATCCTGGCTCAACTG	TTCACTGTGGCCCATGATTGT
IL-1β	CAGGCAGGCAGTATCACTCA	TGTCCTCATCCTGGAAGGTC
TNF-α	AGGCTGCCCCGACTACGT	GACTTTCTCCTGGTATGAGATAGC AAA
ICAM-1	TTCACACTGAATGCCAGCTC	GTCTGCTGAGACCCCTCTTG
VCAM-1	GTGGTGCTGTGACAATGACC	ACGTCAGAACAACCGAATCC
PGC1α	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC
Citrate synthase	CAAGCAGCAACATGGGAAGA	GTCAGGATCAAGAACCGAAGTCT
Cox4	TACTTCGGTGTGCCTTCGA	TGACATGGGCCACATCAG



Supplementary Fig. 1: (A) Kidney lysates were prepared from WT mice treated with folate ± metformin and ACC1/2 KI mice treated with folate ± metformin. Protein concentration was measured using the BioRad Protein Assay Dye Reagent and an equivalent concentration of protein was loaded into each well. Gels were resolved, transferred to PVDF membranes and the membranes incubated with GAPDH, developed then stripped and incubated with β-actin, developed and then stripped and β-tubulin to compare the band intensities. GAPDH showed the most uniform band intensities, reflecting the equivalent amount of protein loaded.
(B) Box plot representation of RT-qPCR threshold (Ct) values of RT-PCR data for housekeeping genes β-actin, 18S ribosomal RNA (rRNA) and GAPDH. N=5-6 each of WT control mice, WT mice receiving folate, ACC1/2 KI control mice, and ACC1/2 KI mice receiving folate Injection. Boxes denote median values with upper and lower quartiles, and whiskers minimum and maximum outliers. Data from mice used in Fig. 3.



Supplementary Fig. 2: Representative Images of (A) Oil Red O and (B) Picro-Sirius Red stained kidney sections of both WT and ACC 1/2 KI mice with UUO or following a sham operation.