Supplemental material Table of Contents

Table S1. List of reagents and resources used in this study.

Reagent or Resource	Source, Catalog number	Dilution IF/IHC/W
Antibodies		
Goat anti-KIM-1 antibody	R&D, Cat#AF1817	1:180 (IF)
Rabbit anti-cleaved caspase-3	Cell signaling, Cat#9661S	1:500 (IF)
Mouse α-Smooth Muscle Actin– FITC	Sigma Aldrich, Cat#F3777	1:200 (IF)
Rabbit Anti-myoglobin antibody	Abcam # ab154292	1:50 (IF)
Mouse anti-megalin antibody	Santa Cruz Cat# sc-74525	1:100 (IHC)
Rabbit anti-megalin antibody	Invitrogen, Cat#PA5-67900	1:1000 (W)
Sheep anti-cubilin antibody	R&D, Cat#AF3700	1:1000 (W)
Mouse anti-myoglobin antibody	Santa Cruz Cat# sc-74525	1:200 (W)
Chemicals, Peptides, and Recombinant Proteins		
Radioimmunoassay (RIPA) buffer	50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2mM EDTA	
FITC-Sinistrin	Fresenius-Kabi Austria	

Antigen unmasking solution (citrate-based)	Vector Laboratories, Cat#H-3300	
DAPI mounting medium	Thermo Fisher Scientific, Cat#P36971	
Paraformaldehyde	Thermo Fisher Scientific, Cat#O4042-500	
Avidin/Biotin blocking kit	Vector Laboratories, Cat#SP-2001	
Peroxidase substrate kit	Vector Laboratories, Cat#SK-4100	
Vectastain ABC Kit	Vector Laboratories, Cat#PK-6100	
Hydrogen peroxide	Thermo Fisher Scientific, Cat#H323	
Methanol	Thermo Fisher Scientific, Cat#A412	
BSA	Sigma-Aldrich, Cat#A7906	
Glycerol	Sigma-Aldrich Cat# G9012	
Protein ladder (standard)	ThermoFisher #26620	
Protein ladder (high molecular weight)	ThermoFisher #LC5699	
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	The Jackson Laboratory, Stock No: 000664	

Supplemental Methods

Perfusion-fixation and histologic preparation

After exsanguination, the left ventricular apex was used to deliver 0.9% sodium chloride solution at a fixed pressure of 135 mmHg until both kidneys and the liver were blanched. The right renal pedicle was clamped, and 4% paraformaldehyde perfused through the left ventricular apex. Both kidneys were then removed; the right kidney (having been perfused only with saline) was immediately placed in liquid nitrogen and stored at -80°C for protein and messenger RNA assays. The left kidney was placed in formalin for 24h, followed by paraffin embedding.

Immunofluorescence, immunohistochemistry, and immunoblotting:

For immunofluorescence, sections were incubated for 2h at room temperature with primary antibodies in 1% bovine serum albumin/phosphate buffered saline, followed by Cy2, Cy3 or Cy5-conjugated secondary antibodies (all 1:500, Thermo Fisher Scientific, Carlsbad CA) for 1h at room temperature, and stained with diaminopyridine (DAPI) in the mounting medium. Immunohistochemistry was performed using the Vectastain ABC kit, (Vector Labs, Burlingame CA) according to manufacturer instructions. Antigens were unmasked in citrate-based unmasking solution (Vector Labs, Burlingame CA), and sections were blocked with 10% normal goat serum for 20 minutes, followed by incubation with primary antibody in 10% normal goat serum for 1h. Then sections were incubated with biotinylated secondary antibody (Vector Labs, 1:200, 30 minutes), Vectastain ABC and DAB substrate, and mounted. For immunoblotting, protein was extracted from renal homogenate in iced radioimmunoassay precipitation buffer and treated with protease inhibitor (Complete, Roche Applied Science, Indianapolis IN). Lysate protein concentration was measured using the bicinchoninic acid assay and 4-12% gradient precast gels (Bis-tris, Invitrogen, Carlsbad, CA) were loaded with 80µg of protein, electrophoresed, and transferred to polyvinyl difluoride membranes. After blocking with 5% milk and incubation in primary anti-myoglobin, megalin, or cubilin and HRP-conjugated secondary antibody (see supplementary table 1), blots were imaged using enhanced chemiluminescence (Thermo Fisher Scientific). For quantification, total protein was optically semiquantified using the Ponceau stain image of the same membrane, and the specific protein quantity expressed as ratio of the specific band density to total Ponceau density.

Pathologic scoring of kidney injury:

For each experimental replicate (one mouse) 100 periodic-acid Schiff-stained high-power fields were reviewed in a blinded, randomized fashion. Within each high-power field, the severity of three markers (loss of brush border, tubular atrophy, epithelial vacuolization) was assessed. A composite score for each replicate was computed with weighting for the severity of each finding.

Human proximal tubular epithelial cell culture

Human kidney-2 (HK-2) cells were obtained from American Tissue Type Culture and cultured as previously described (19). To test cilastatin inhibition of myoglobin uptake, fluorescein isothyocyanate (FITC)-myoglobin or vehicle were added to wells pretreated for 2h with cilastatin (0.1 mg/mL, 0.3 mM, equivalent to plasma concentration 4 half lives after injection in the animal model) or vehicle (dimethyl sulfoxide 0.1 mg/mL) in triplicate. Cells were washed after 2h, and fluorescence measured and that of treated cells was compared to that of vehicle control. Cell culture experiments were independently repeated 5 times.

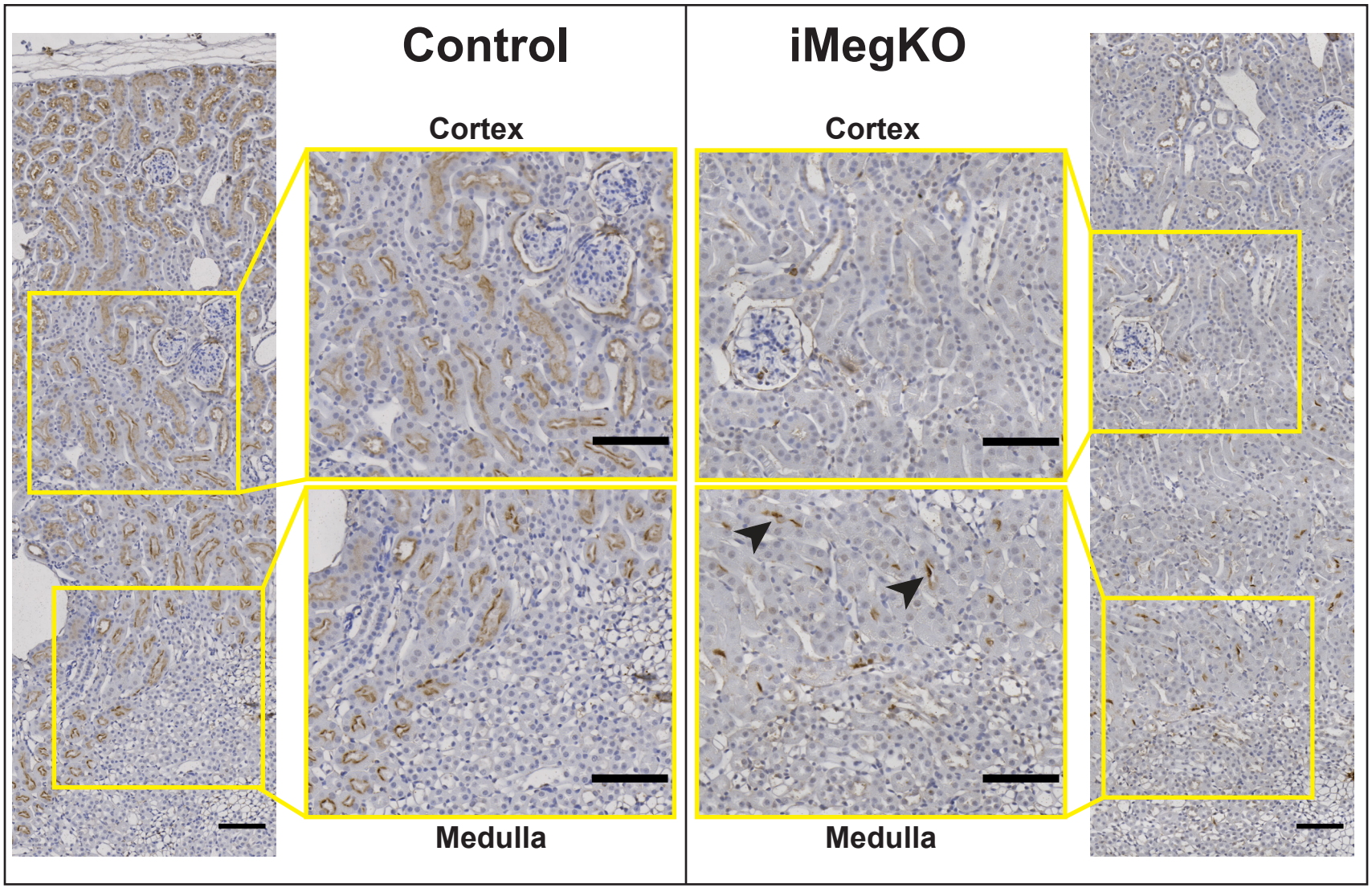
Power analysis for iMegKO cilastatin vs vehicle experiment

A priori data (means and standard deviation) were taken from the wild type cilastatin vs vehicle experiment (depicted in figure 4 in the manuscript) and used to power the experiment testing the effect of cilastatin in iMegKO mice after glycerol injection (results depicted in figure 6 in the manuscript).

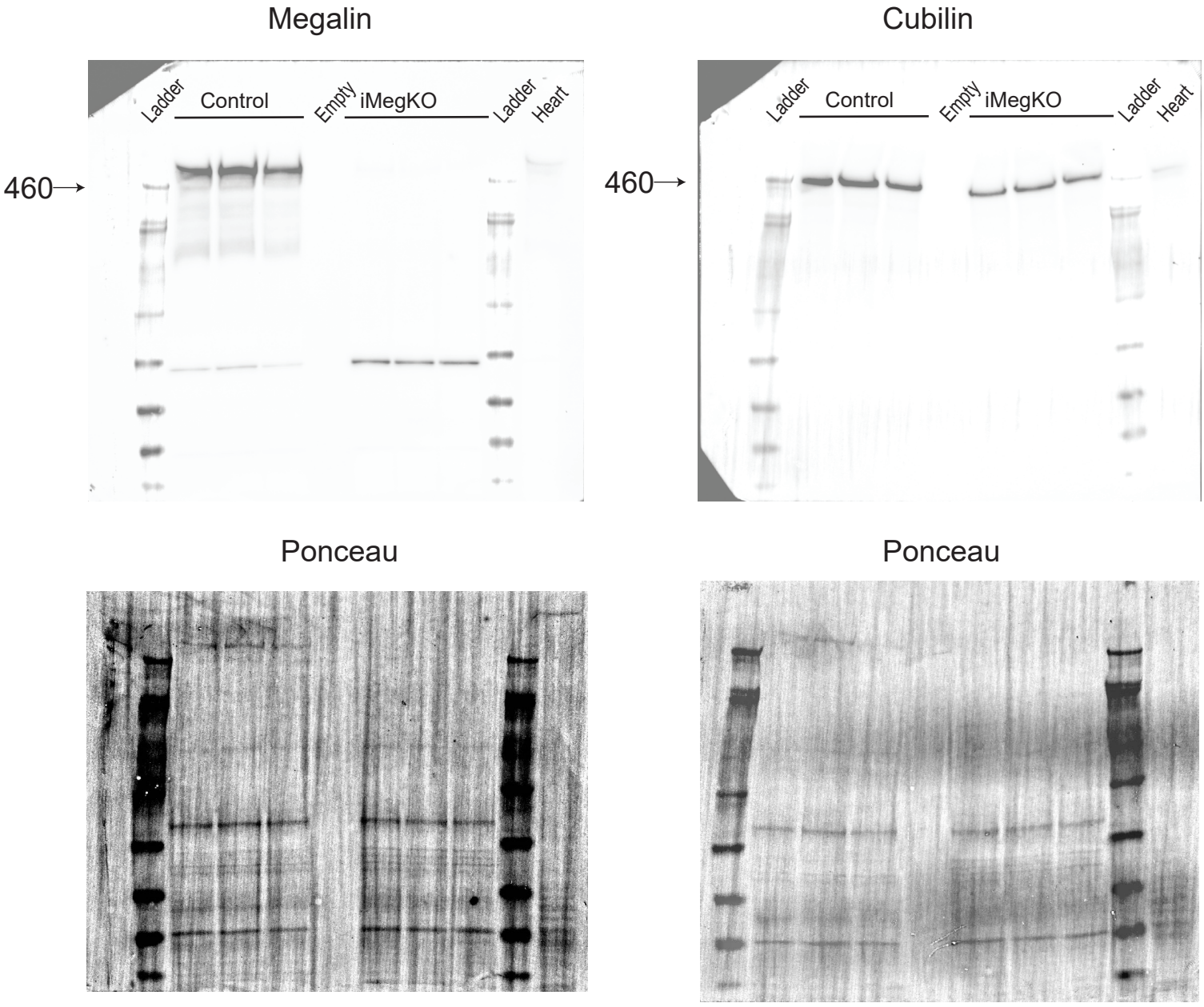
Text below captured from RStudio. Lines in blue beginning with '>' denote user input.

```
> mean1<-67
> mean2<-526
> meandif<-mean2-mean1
> std1<-48.9
> std2<-217.6
> stds<-c(std1,std2)
```

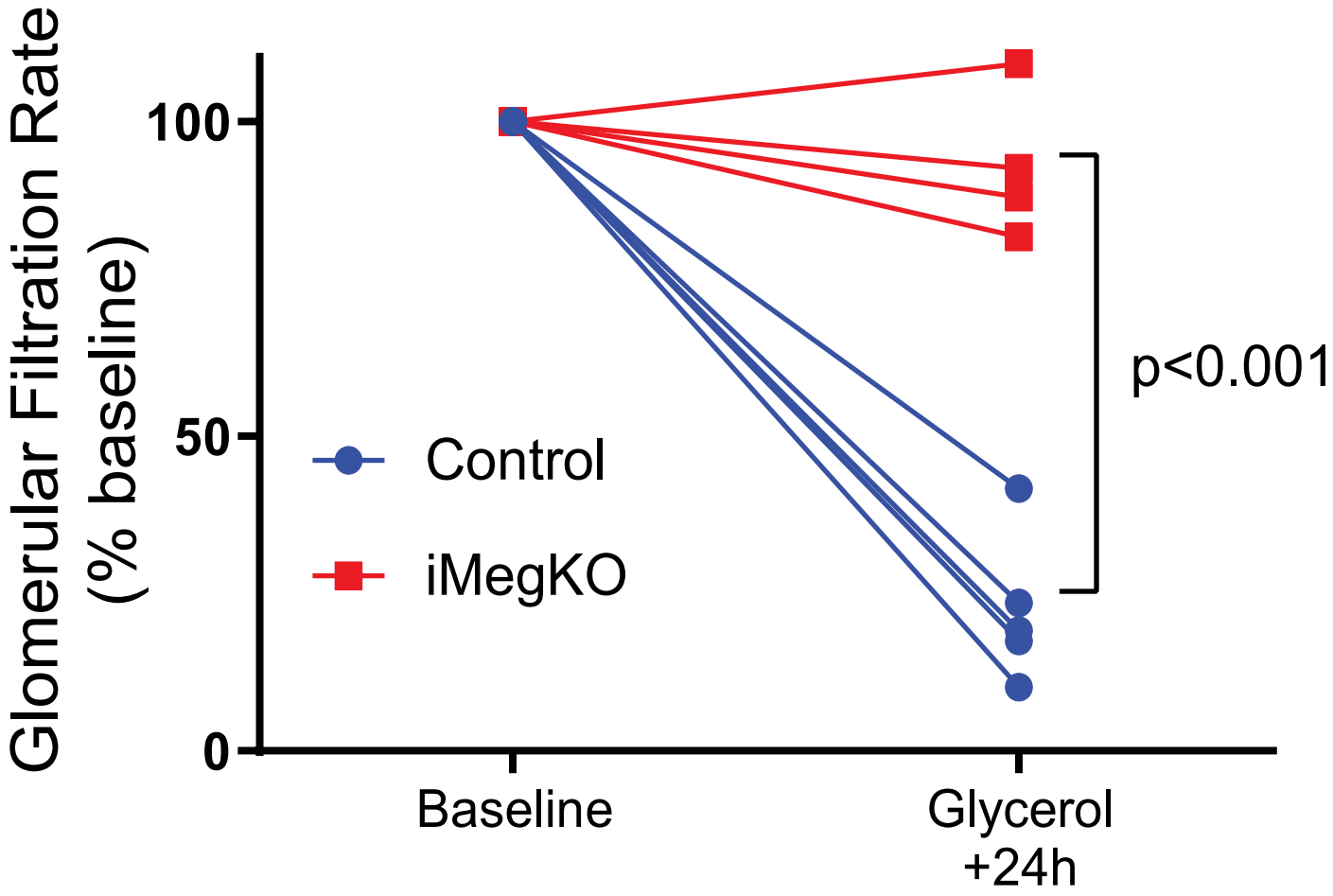
```
> sstds<-stds^2
> pooled_std<-sqrt(mean(sstds))
> dee<-meandif/pooled_std
> pwr::pwr.t.test(d=dee, power=0.8, type="two.sample")

     Two-sample t test power calculation

              n = 3.180799
              d = 2.897838
      sig.level = 0.05
          power = 0.8
    alternative = two.sided

NOTE: n is number in *each* group
```



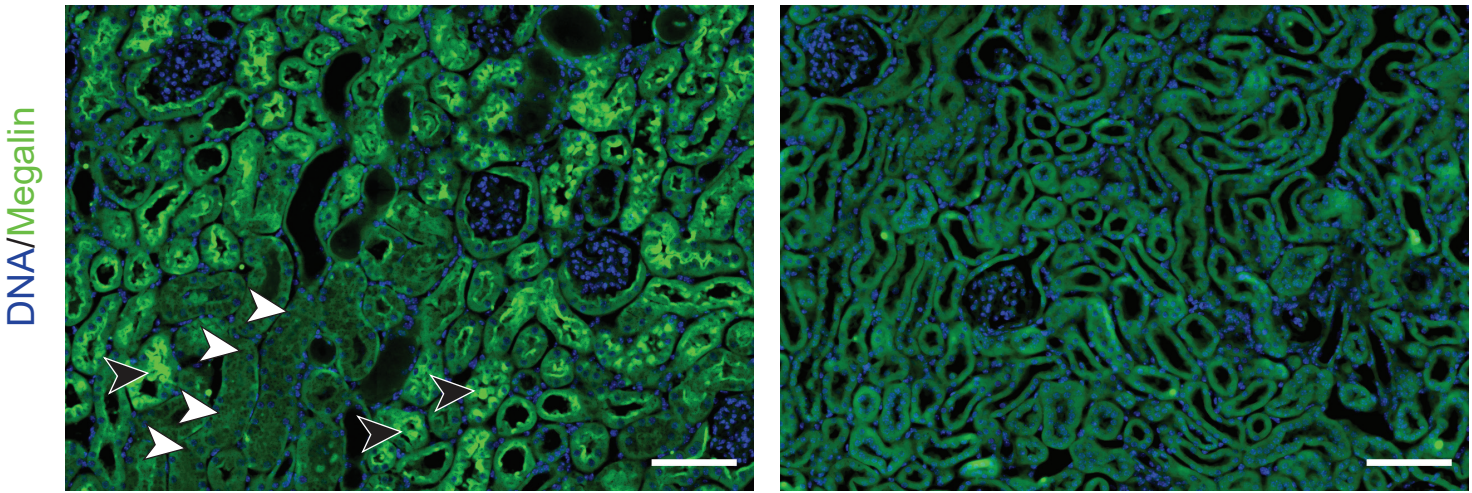
Supplemental Figure 1: Immunohistochemistry performed using antibody directed at megalin in kidneys of control and iMegKO mice after induction of cre recombinase. Control mice demonstrate robust expression of megalin along the brush border of tubular epithelial cells in the cortex and medulla (i.e. in S1, S2, and S3 segments of the proximal tubule). After induction of cre recombinase. iMegKO mice demonstrate absence of megalin from tubular brush border in the cortex with small areas of expression in tubular epithelial cells of the outer medulla (arrowheads). Scale bars are 100 μm.



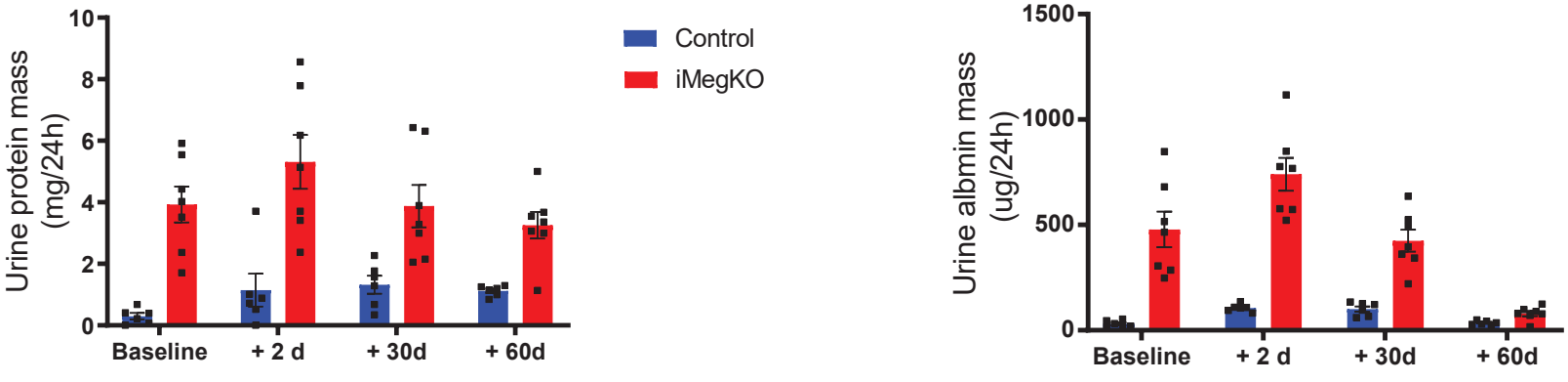
Supplemental Figure 2: iMegKO mice do not express megalin in the kidney. Full-length kidney lysate immunoblots directed at megalin (left) and cubilin (right) with respective Ponceau stains. Induction of cre recombinase after tamoxifen injection results in the deletion of megalin, without change in the abundance of cubilin.



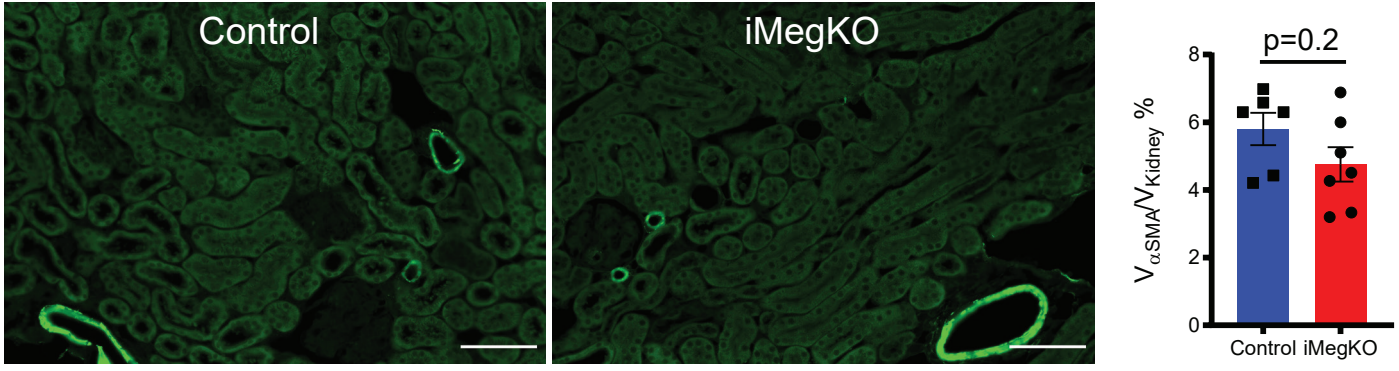
Supplemental Figure 3: Glomerular filtration rate (GFR) expressed as percent of baseline, measured before and 24h after glycerol injection. While control mice developed severe impairment of renal function (no control GFR was >50% of baseline and most were <30% of baseline), iMegKO mice demonstrated stable GFR.



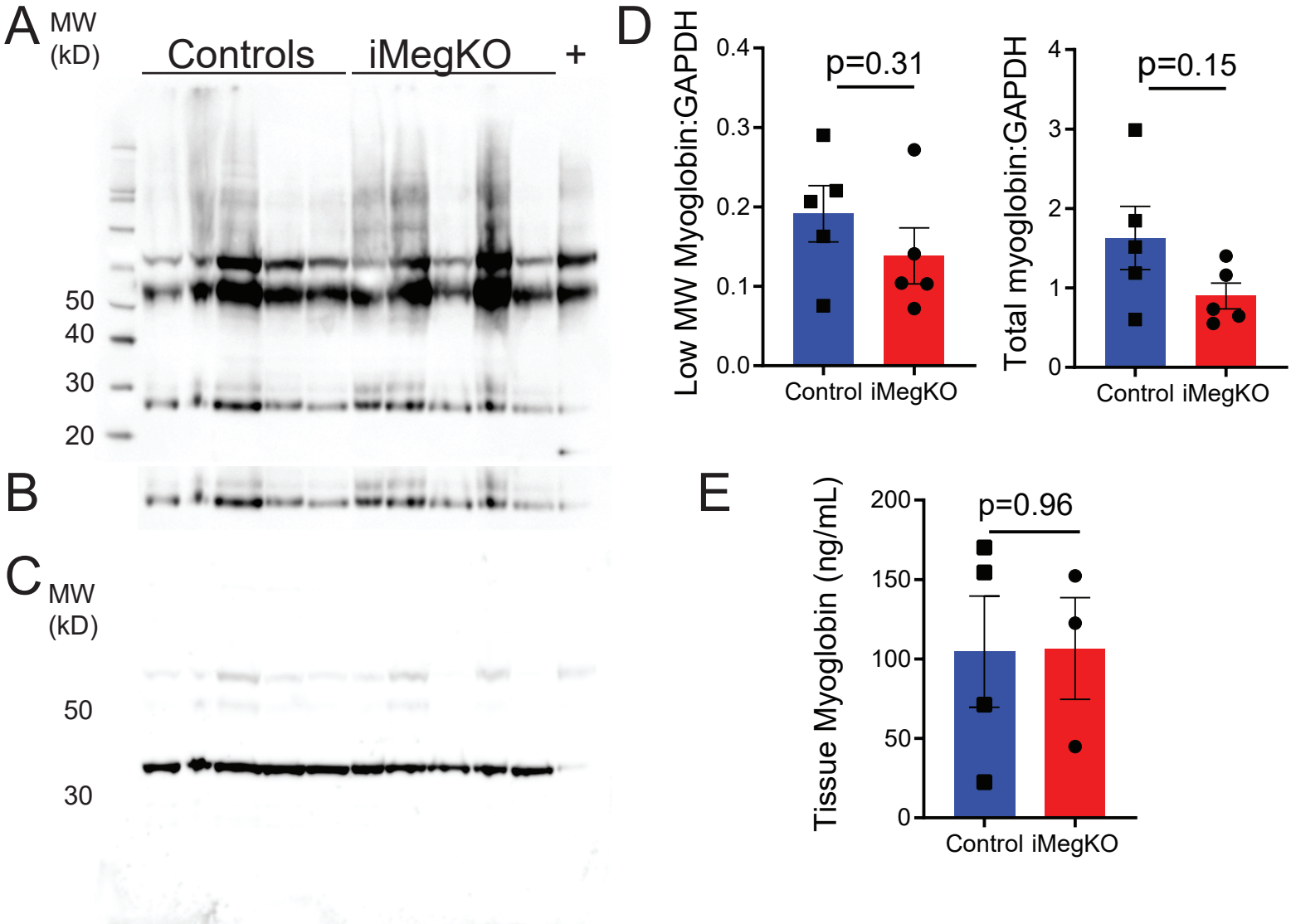
Supplemental Figure 4: Immunofluorescence with antibody directed at megalin 24 hours after glycerol injection in a control mouse (left) and one with inducible, proximal tubule-specific megalin deletion (iMegKO, right). 24h after AKI, megalin is distributed at the brush border of tubular epithelial cells in most tubules. However, tubules which exhibit cell swelling and lumenal effacement consistent with injury also demonstrate absent or reduced megalin expression (white arrowheads). In some tubular lumens, megalin-positive material can be observed (black arrowheads). In iMegKO mouse, megalin is absent. Scale bars are 100 μm.



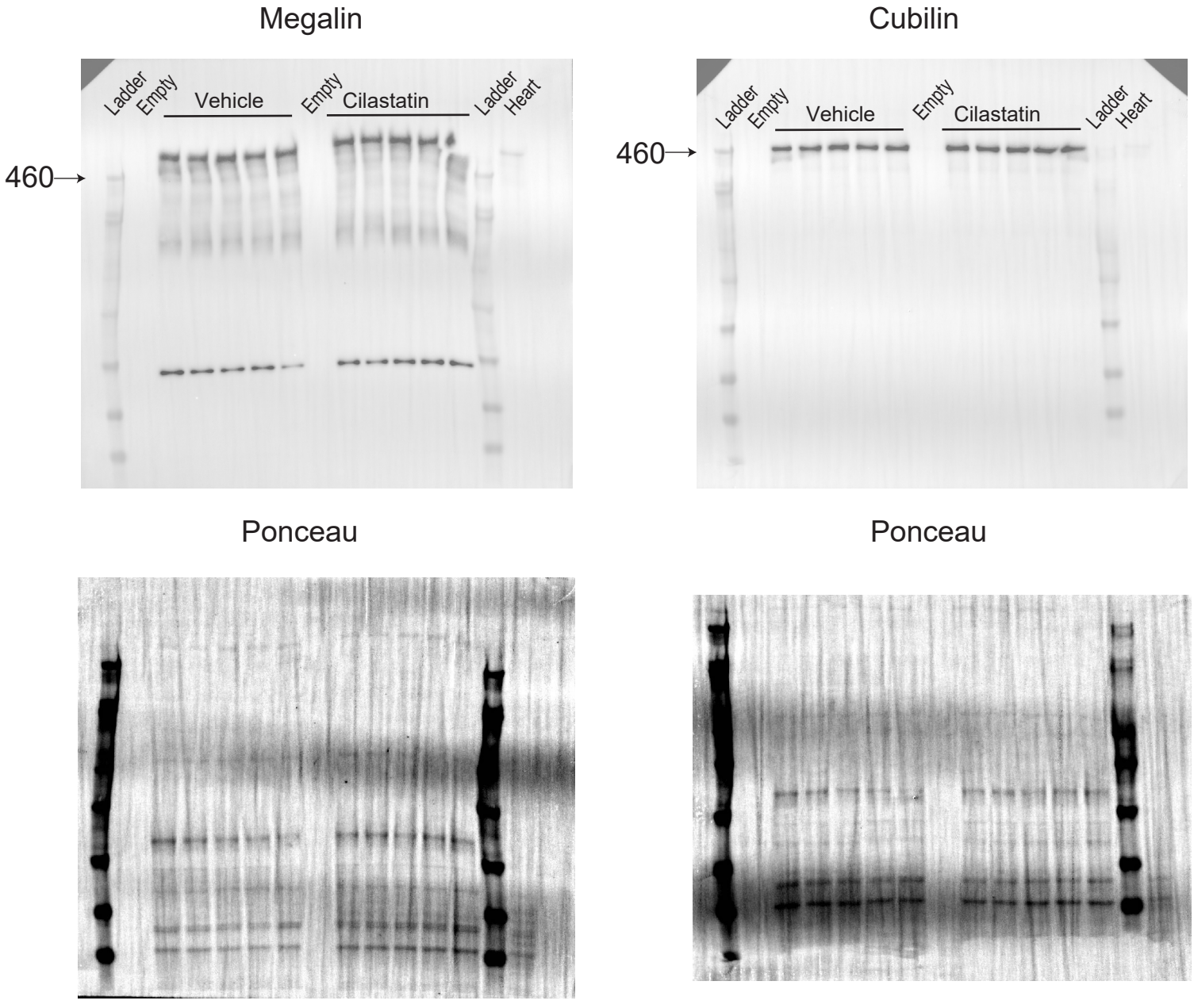
Supplemental Figure 5: Quantitation of urine protein and albumin in control and iMegKO mice over 60 days following glycerol injection (relative change from baseline is presented in figure 3). iMegKO mice demonstrate increased urine protein and albumin all time points when compared to control mice. Both strains of mice demonstrate increased proteinuria and albuminuria after glycerol injection



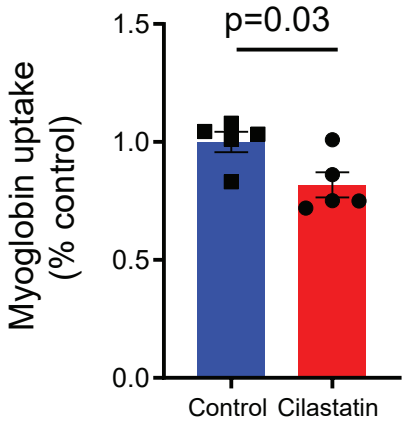
Supplemental figure 6: Representative renal sections from control and iMegKO mice 60 days after glycerol injection, stained with fluorescent antibody to α-smooth muscle actin (αSMA). Interstitial α SMA is not observed; vascular smooth muscle in vessel walls serves as postitive control. Quantification, shown at right, demonstrates no difference between strains.



Supplemental Figure 7: Uncropped immunoblot performed on renal homgenate from control and iMegKO mice 24 hours after glycerol injection. A. Visualization of blot after incubation with anti-myoglobin antibody demonstrates multiple bands, consistent with multimers of myoglobin. The positive control is dilute mouse cardiac lysate. B. Cropped, contrast-enhanced image of the low molecular-weight bands from image A as seen in figure 6. C. Visualization of blot after incubation with anti-GAPDH antibody. D. Quantification of the ratio of all myoglobin-positive bands ("total myoglobin") to GAPDH and that of the low molecular-weight myoglobin-positive band to GAPDH. There was no difference between groups in the quantity of myoglobin retained in the kidney. Results are representative of >4 repeated experiments. E. Confirmatory quantitative ELISA performed on the same renal homogenates demonstrates nearly-identical renal tissue concentration of myoglobin.



Supplemental Figure 8: Full blots for megalin and cubilin obtained from renal homogenate of wild-type mice treated with vehicle or cilastatin. 200 mg/kg cilastatin was injected retroorbitally and kidneys harvested 24h later. Presented are full-length kidney lysate immunoblots directed at megalin (left) and cubilin (right) with respective Ponceau stains for total protein (below). Quantification is in figure 6.



Supplemental Figure 9: Results of cell culture experiment testing uptake of FITC-myoglobin in human kidney cells (HK2 cell line). Cells were pretreated for 2h with vehicle or cilastatin; then FITC myoglobin was added to confluent cells for 2h, and rinsed off. Total fluorescence was measured; cilastatin-treated cells demonstrated reduced fluorescence, indicating that *in vitro*, cilastatin interferes with FITC-myoglobin uptake by proximal tubular epithelial cells.