SUPPLEMENTAL MATERIAL

Supplemental Methods:

Immunofluorescence Staining

For double immunofluorescence stainings of MANF with Kim-1, PDIA3 and CHOP respectively, kidneys were fixed in 4% paraformaldehyde (PFA) at 4 °C overnight, then incubated in 15% sucrose/PBS at 4 °C for 2 hours and 30% sucrose/PBS at 4°C overnight. OCT-embedded tissues were cryosectioned into 8 μm sections and mounted on Superfrost slides. Sections were blocked in 1% BSA for 30 minutes and incubated with primary antibodies specific for MANF (Abnova), Kim-1 (R&D) and PDIA3 (Abnova). For co-staining of MANF and CHOP, the CHOP antigen was retrieved by BD Retrievagen A working solution (BD Pharmingen) for 10 minutes at 89 °C before sections were incubated with 1% BSA. Secondary antibodies were Alexa 488-conjugated anti-rabbit antibody for MANF and Alexa 594-conjugated anti-goat antibody for Kim-1, anti-mouse IgG1 antibody for PDIA3, and anti-mouse IgG2b antibody for CHOP. Slides were analyzed under a fluorescence microscope (Nikon).

Renal Bilateral Ischemia/Reperfusion Mouse Model

The animal experiments conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Washington University Animal Studies Committee. C57Bl/6 mice (10 males at age of 9 weeks) were subjected to bilateral renal ischemia for 30 minutes. Briefly, mice were anesthetized with isoflurane, their body temperature was maintained at 37 °C on a heating pad, and monitored with a rectal probe throughout surgery. Ischemia was induced by nontraumatic clamping of bilateral renal vascular pedicles for 30

minutes, during which time the kidneys were kept warm and moist. The clamps were then removed and the kidneys were observed for return of blood flow. Sham-operated C57Bl/6 mice (4 males at age of 9 weeks) underwent an identical procedure without vascular pedicle clamping. 6 hours after reperfusion, urines were collected from metabolic cages. 9 hours after reperfusion, sera were obtained, and kidneys were harvested. One kidney was snap frozen in dry ice and stored at -70°C until further processing for protein and RNA. The other kidney was fixed in 4% PFA (pH 7.4) at 4 °C, then processed for paraffin blocks or transferred to 15% and 30% sucrose/PBS for OCT-embedded cryosections.

Serum Creatinine Measurement

Serum creatinine was measured by using a QuantiChrom^{TM} creatinine assay kit (DICT-500) (BioAssay Systems, Hayward, CA).

Peptide Competition Assay

In the peptide blocking assay, the immunoblotting experiment with the anti-MANF antibody (Abnova) was run in duplicate; one with the antibody that was pre-incubated with its immunogen peptide (a generous gift from Abnova) and another with the same antibody without pre-incubation with the blocking peptide. 1 µg/ml of primary antibody was incubated with a 200-fold molar excess of peptide for 30 minutes at 37°C prior to immunoblotting.

Immunoprecipitation/LC-MS/MS

Around 250 µl urine (normalized to the same amount of urine creatinine for different genotypes) pooled from three *Lamb2*$^{-/-}$; NEPH-C321R-LAMB2 mice and their *Lamb2*$^{+/-}$ littermates at P26, were diluted in PBS to 650 µl final volume respectively. The diluted urines were incubated with a goat anti-MANF antibody (Santa Cruz, Santa Cruz, CA) overnight at 4 °C and immune-complexes were precipitated using protein G agarose (Sigma, St. Louis, MO). After washing with PBS three times, the immunoprecipitated proteins were eluted by boiling in 2× sodium dodecyl sulfate (SDS)-sample buffer for 10 minutes.

The eluate from each genotype was divided into two aliquots (15 µl and 60 µl) and run on duplicate SDS-polyacrylamide gels. The gel loaded with small aliquots of eluates was immunoblotted with the rabbit anti-MANF antibody (Abnova) to verify the ~ 30 kDa band after immunoprecipitation. The other gel was loaded with two times 30 µl of eluates and stained with Sypro-Ruby. The ~ 30 kDa bands were sliced in 1 mm horizontal slices for in-gel digestion with Glu-C protease (Sigma, St. Louis, MO) as previously described. [1] The desalted peptides were analyzed by LC-MS/MS using a high-resolution quadrupole-Orbitrap mass spectrometer (Q Exactive, ThermoFisher, St. Louis, MO). [2] The LC (Easy nLC) was interfaced to the Q Exactive with a nanoelectrospray source (ThermoFisher). The peptides were separated on a C_{18} PepMap® column (75 µM x 50 cm) with a 2 µM particle size (ThermoFisher) using a linear gradient of 2 to 40% of Solvent B (0.1% formic acid in acetonitrile) for 78 minutes followed by a linear increase to 95% B in 1 minutes. The flow rate was 500 nL/min for peptide loading and reduced to 300 nL/min for gradient peptide elution. MS data acquisition was performed in data-dependent mode using survey scans (375-1500 Th) that were acquired at 70,000 resolution to a target value of 1 x 10^6. Dynamic exclusion was enabled for a duration of 20 seconds and the ten most intense ions were sequentially isolated (1.2 Th) for high-energy collision-induced dissociation. The

normalized energy was set to 27% and the MS/MS ion selection threshold was set to 1 x 10^5 counts. The injection time was set at 60 milliseconds with a resolution of 17,500. The interface conditions were as follows: spray voltage of 2 kV; capillary temperature 275 ºC; no auxiliary or sheath gas. The raw data were processed using Mascot Distiller (Version 2.5.1.0) and the processed MS/MS spectra were used to search the UniProt database (downloaded 10/08/2013, 43296 entries) with taxonomy of Mus Musculus selected. The results were filtered to an FDR of 1% using Scaffold version 3.3.1 (Proteome Software, Portland, USA).

Supplemental Figure Legends:

Supplemental Figure 1: Upregulation of MANF precedes Kim-1 induction in renal tubular cells in the TM-induced AKI mouse model. Double immunofluorescence staining for MANF (green, a, e and i) and Kim-1 (red, b, f and j) on PFA-fixed, sucrose-cryoprotected kidney sections from mice treated with vehicle or TM (1 mg/kg) in the indicated time points. Nuclei (d, h and l) were counterstained with Hoechst 33342 (blue). The expression of MANF in renal tubules was increased 24 hours post-injection whereas significant upregulation of Kim-1 was observed on day 5 post-injection. Scale bars, 40 µm.

Supplemental Figure 2: MANF co-localizes with an ER stress marker PDIA3 in ER-stressed renal tubular cells and MANF-positive tubular cells exhibit nuclear CHOP staining 24 hours following TM injection. PFA-fixed frozen kidney sections from TM untreated- and treated- mice were examined by dual immunofluorescence staining of the indicated antibodies at 24 hours and 5 days post-injection. (A) Co-localization of MANF (green,

e, g and h) with PDIA3 (red, f, g and h) in renal tubules 24 hours after 1 mg/kg TM injection. (B) The immunostaining of MANF (green, e, g and h) and the nuclear staining of CHOP (red, f, g and h), a transcriptional regulator induced by ER stress, were substantially upregulated 24 hours following TM injection and subsided 5 days post-injection. In addition, the same tubular cells were stained positive for both MANF and CHOP at 24 hours following TM injection. Scale bars, 40 μm.

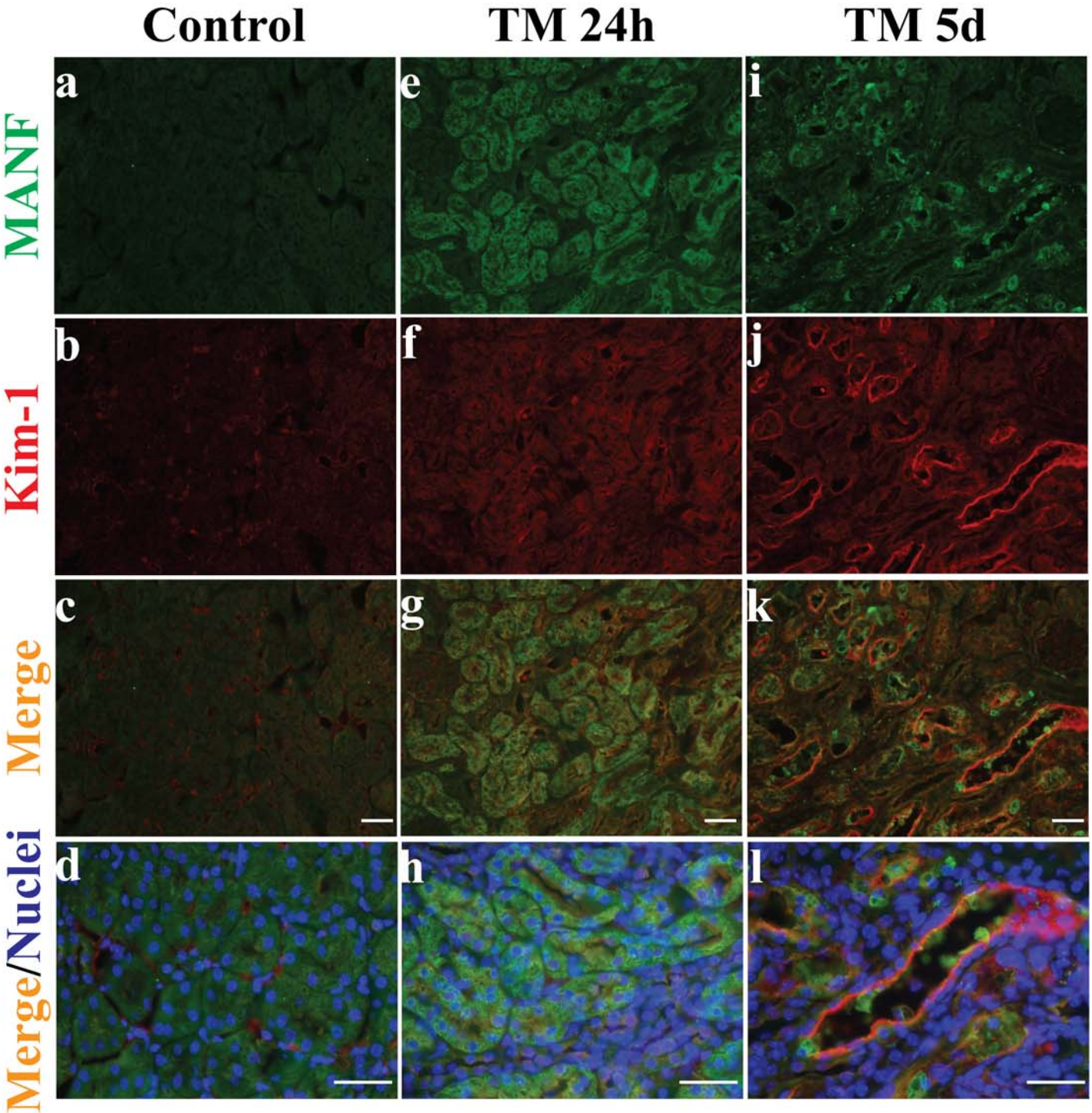
Supplemental Figure 3: MANF is induced and excreted in urine in a mouse model of AKI caused by bilateral ischemia/reperfusion. (A) H&E and PAS staining of paraffin sections from sham-operated (a-b, n=4) and post-ischemic (c-d, n=5) kidneys at 9 hours of reflow. Note acute tubular necrosis (arrows in c and d) in the corticomedullary junction area of kidneys undergoing I/R insult. Scale bars: 40 μm. (B) Serum Cr levels for the indicated groups. Mean ± SD; P=0.15 by t test. (C) Quantitative RT-PCR showed relative transcript levels of BiP, CHOP and MANF in kidneys from mice undergoing sham operation (n=4) or I/R (n=5) after 9 hours of reperfusion. Absolute levels were normalized first to those of GAPDH and then to the levels in the sham-operated kidneys. Mean ± SD; *, $P < 0.05$ and **, $P< 0.001$ by t test. (D) Representative immunoblotting results from sham-operated and I/R-challenged kidneys for levels of CHOP, MANF and β-actin at the indicated time point. (E) Double immunofluorescence staining for MANF (green, a and d) and LTL (red, b and e) on paraffin kidney sections from mice with sham operation or I/R injury. Scale bars, 40 μm. (F) Representative WB analysis of MANF in crude urine specimens collected from sham-operated and I/R-injured mice 6 hours after ischemic AKI. The urinary MANF excretion was normalized to urine Cr excretion such that the urine volume applied to the gel reflected the amount of urine containing 2 μg of Cr.

Supplemental Figure 4: Confirmation of the upper bands in urine as containing MANF. (A) Urinary MANF excretion within 24 hours of TM injection was assessed by WB using an independent anti-MANF antibody (Proteintech) recognizing different epitopes. (B) Crude urine samples from three pairs of *Lamb2*$^{-/-}$; NEPH-C321R-LAMB2 mice and their *Lamb2*$^{+/-}$ littermates at P19-26 were analyzed by the same Proteintech antibody for MANF excretion. The urinary MANF excretion was normalized to urine Cr excretion such that the urine volume applied to the gel reflected the amount of urine containing 3 µg of Cr for (A) and 2 µg for (B). (C) A peptide competition test for the anti-MANF antibody (Abnova) was performed using urines from vehicle-treated and TM (1 mg/kg)-treated mice with or without the immunizing peptide. A recombinant mouse MANF protein with a carboxy-terminal polyhistidine tag dissolved in PBS was included as a positive control in the inhibition test. (D) The peptide competition test was performed using urines from one pair of *Lamb2*$^{+/-}$ and *Lamb2*$^{-/-}$; NEPH-C321R-LAMB2 littermates at P23 with or without the immunizing peptide.

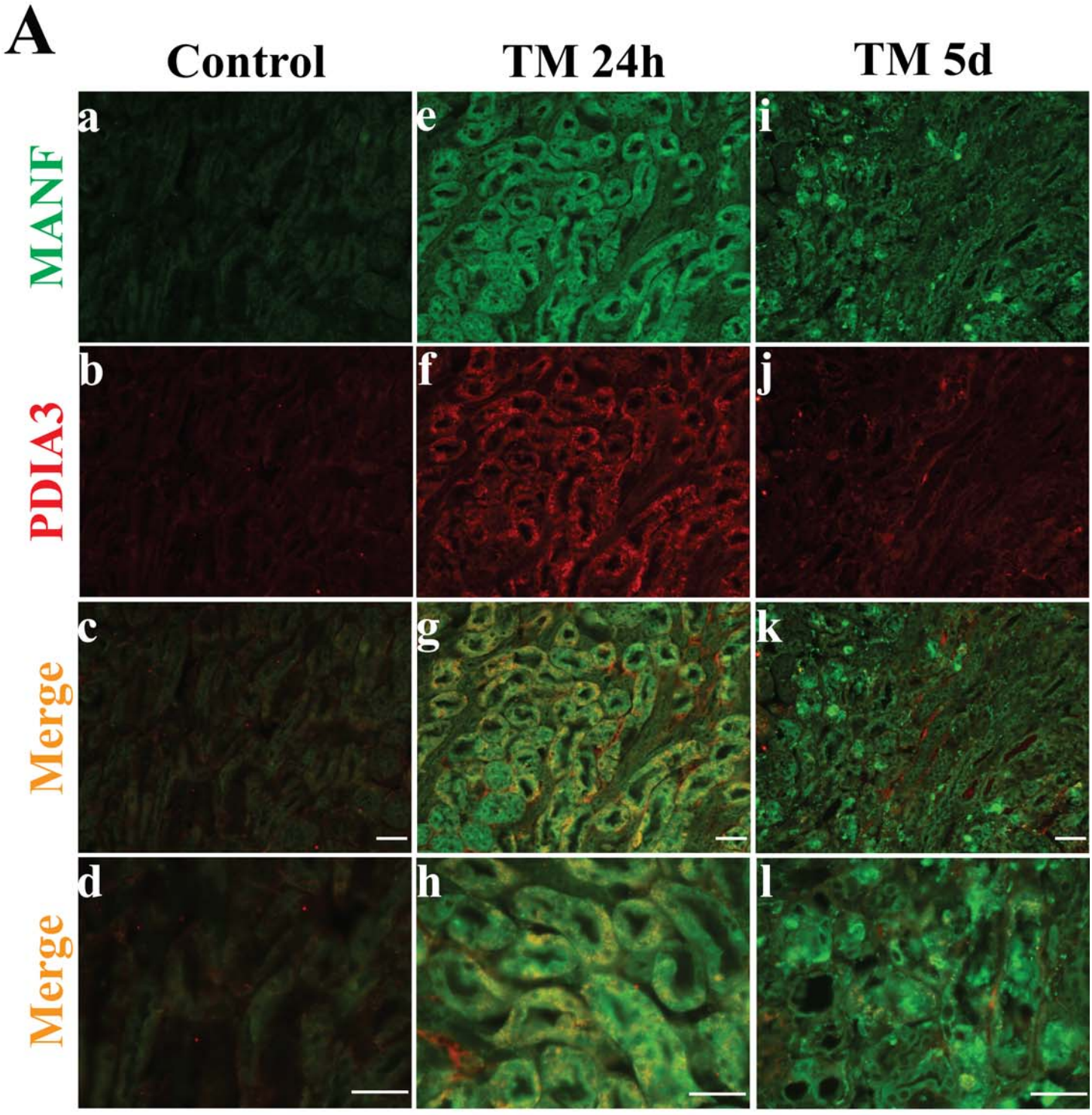
Supplemental Figure 5: Coupled immunoprecipitation and LC-MS/MS are conducted to identify MANF in the ~30 kDa band from the urine of Tg-C321R mice. (A) Urines from WT and Tg-C321R mice at P26 (3 mice/group respectively) were immunoprecipitated with a goat anti-MANF antibody and then probed with the rabbit anti-MANF antibody (Abnova). The arrow points to the ~30 kDa band in the urine of Tg-C321R mice. (B) Urine precipitates from the groups mentioned in (A) were separated on SDS-PAGE and then stained with Sypro-Ruby. The protein bands of interest indicated by red boxes were excised for in-gel Glu-C digestion and subsequent LC-MS/MS analysis. (C) LC-MS/MS identified the peptide as QDDAATKIINE, which shares 100% homology only with mouse MANF.

References:

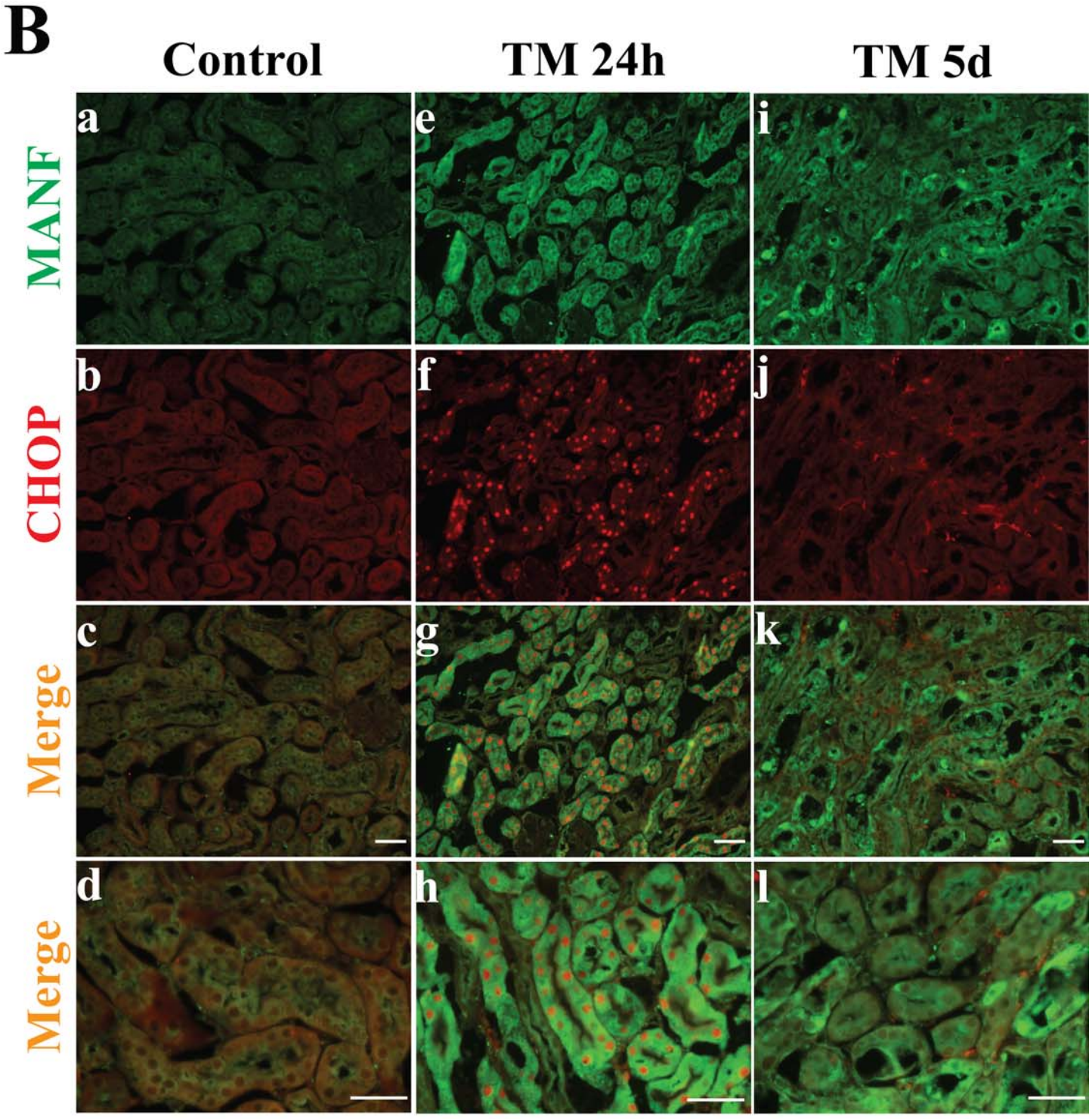
1. Havlis J, Thomas H, Sebela M, Shevchenko A: Fast-response proteomics by accelerated in-gel digestion of proteins. *Anal Chem* 75: 1300-1306, 2003
2. Kelstrup CD, Young C, Lavallee R, Nielsen ML, Olsen JV: Optimized fast and sensitive acquisition methods for shotgun proteomics on a quadrupole orbitrap mass spectrometer. *J Proteome Res* 11: 3487-3497, 2012



Supplemental Figure 1



Supplemental Figure 2A



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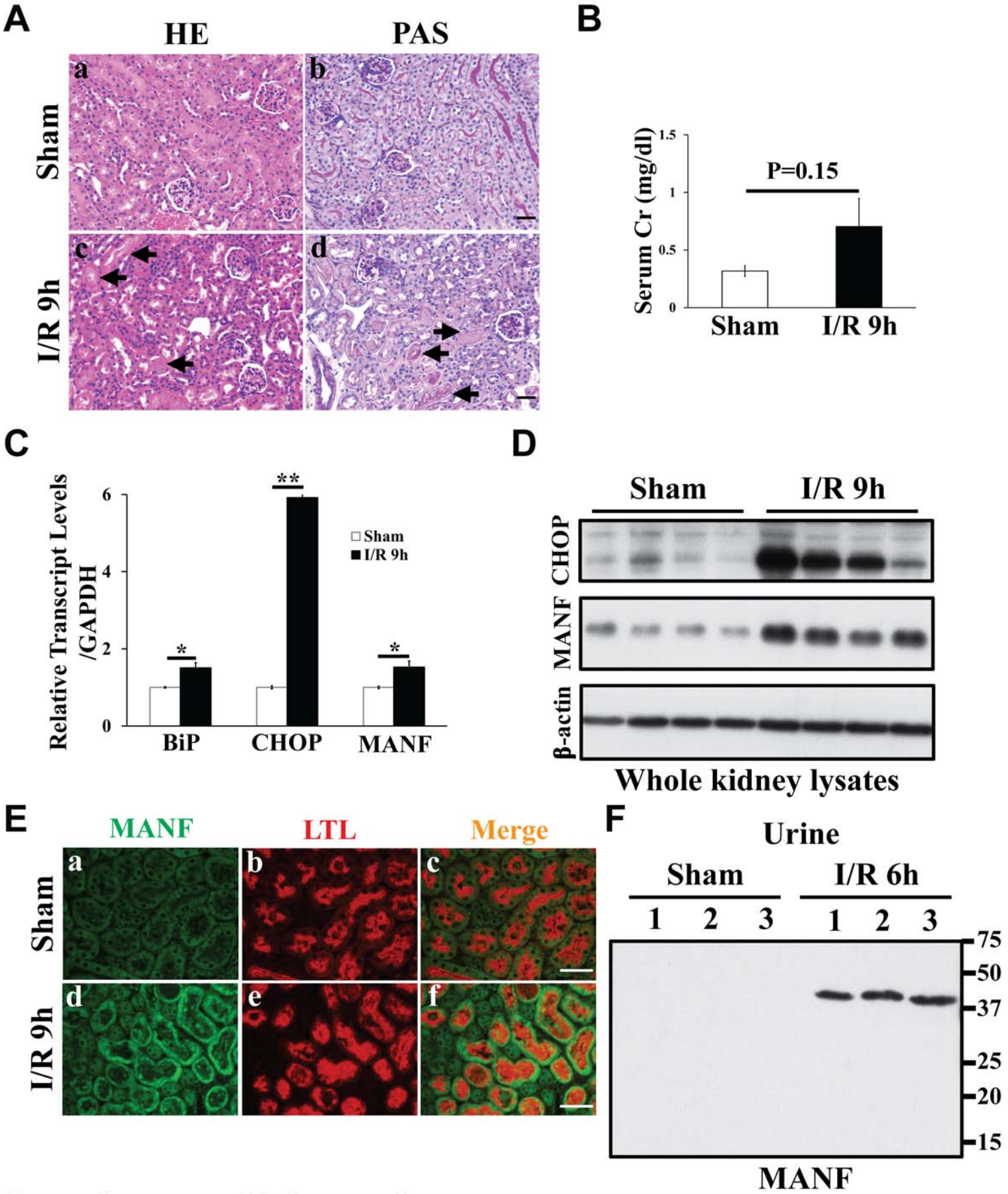
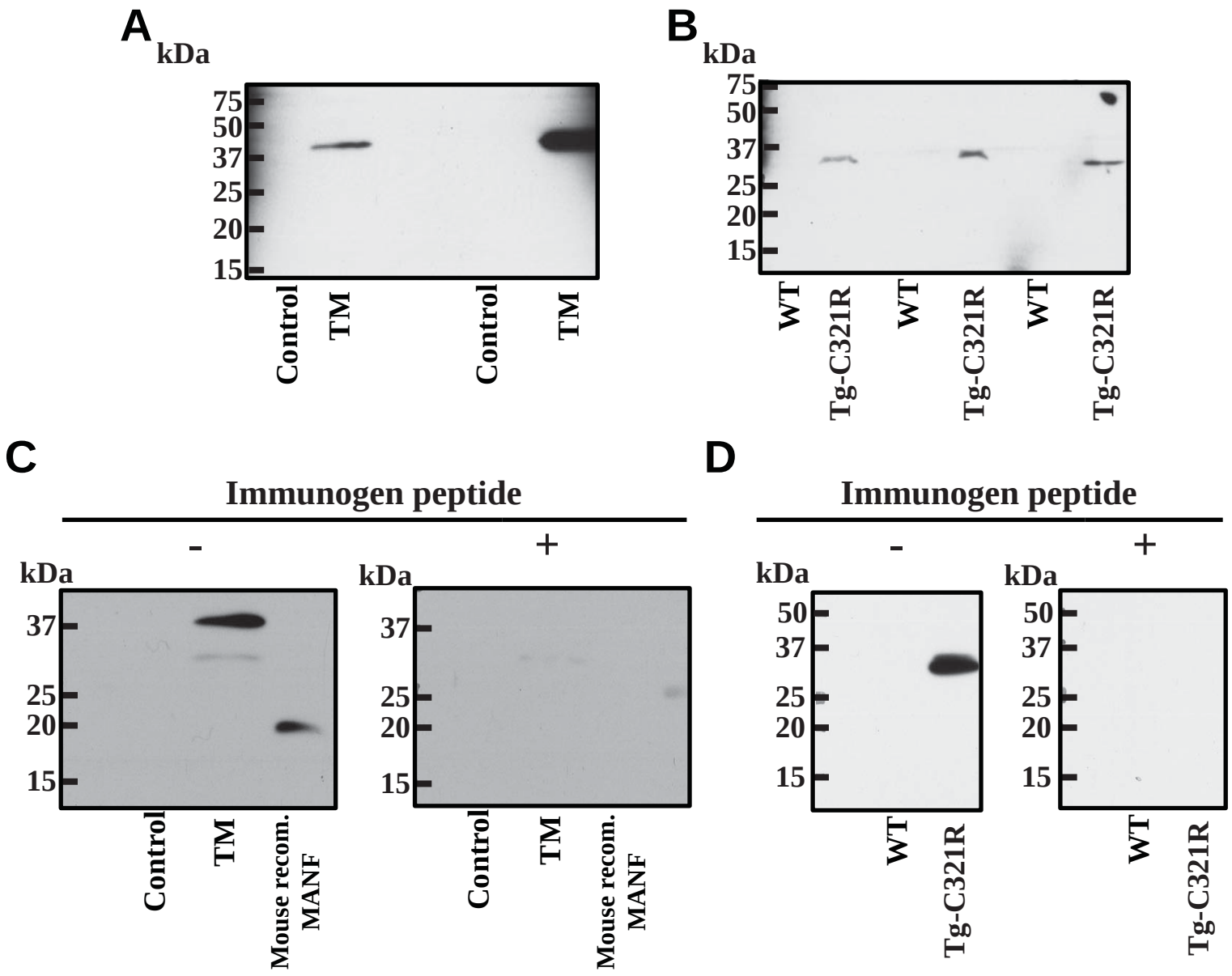
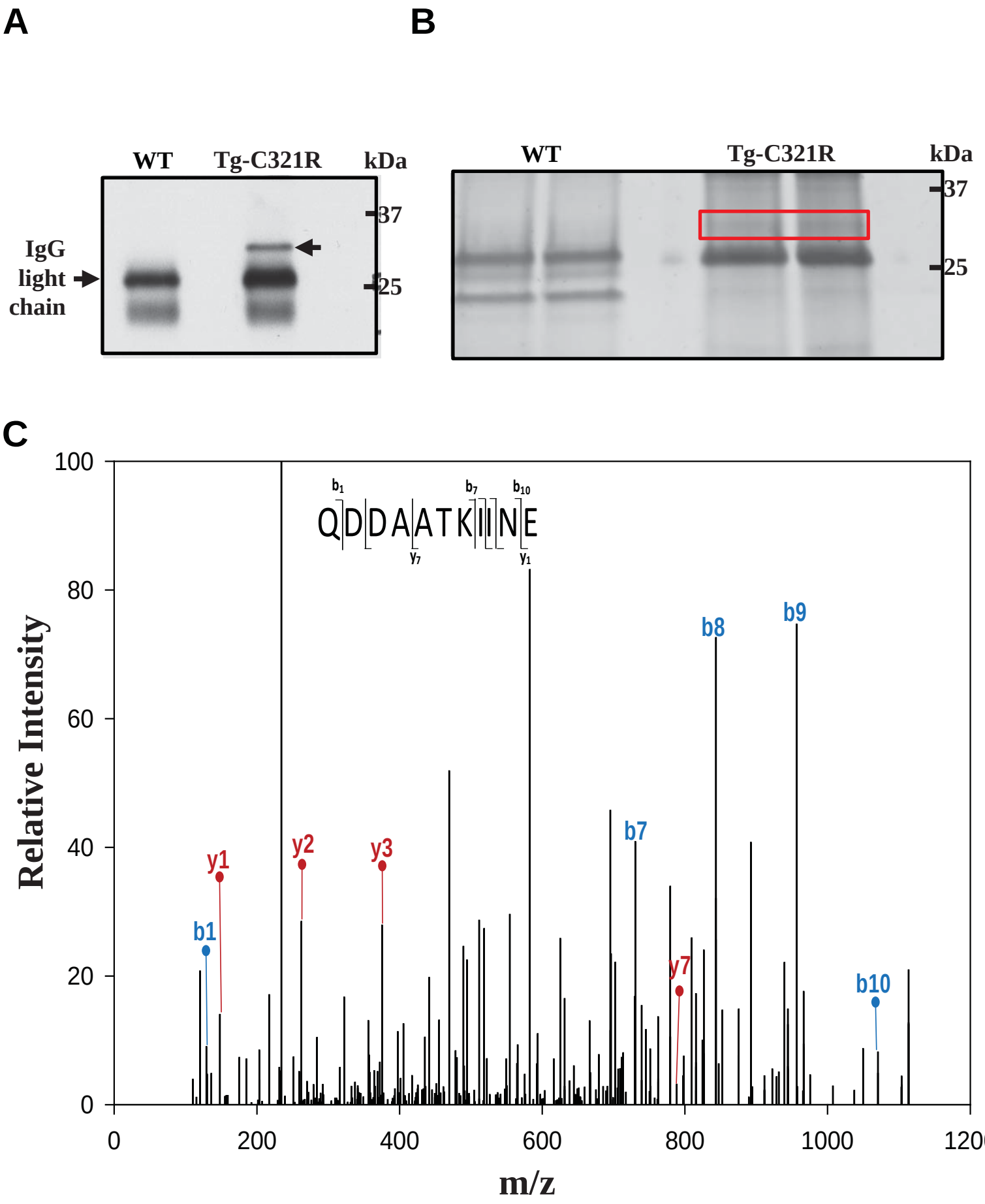


Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5

Supplemental Table 1. Mass error determination for fragment ions for the MANF peptide, QDDAATKIINE[1]

Fragment ion	Observed (*m/z*)	Calculated (*m/z*)	ppm
y1	148.06	148.06	2
y2	262.11	262.10	18
y3	375.20	375.19	20
y7	788.43	788.45	25
b7	730.33	730.34	7
b8	843.41	843.42	11
b9	956.49	956.51	19
b10	1070.52	1070.55	23

[1] $[M+H]^{+1}$ = 1216.59 +/- 1.5 ppm