SI Materials and Methods

Reagents and antibodies

Recombinant human TNF-α (#210-TA), recombinant mouse TNF-α (#410-MT) and Z-VAD-FMK (#FMK001) were purchased from R&D Systems. Birinapant (#TL32711) was from Chemietek. Necrostain-1 (#N9037), cycloheximide (#C7698), digitonin (#D141), erastin (#E7781), deferoxamine mesylate (#D9533), ferrostatin-1 (#SML0583) and calcium oxalate hydrate (#289841) were purchased from Sigma-Aldrich. Necrostatin-1s was purchased from BioVision (#2263). Sodium oxalate was from Santa Cruz Biotechnology (#sc-203396).

The primary antibody antibodies used for Western blotting and immunofluorescence are as follows: anti- RIP3 (ab62344, Abcam; #2283, ProSci), anti-MLKL (AP14272b, Abgent), anti-phospho-MLKL (Thr357) (ABC234, Millipore), anti-megalin (sc-16478, Santa Cruz Biotechnology), anti-caspase-3 (#9662, Cell Signaling), anti-caspase-8 (#4790, Cell Signaling), anti-LC3 (NB100-2331, Novus), anti-p62 (ab56416, Abcam), anti-p-Smad 1/5/8 (#9511, Cell Signaling), anti-Smad1 (#9743, Cell Signaling), anti-HMGB1 (ab79823, Abcam), anti-Ngal (AF1857, R&D Systems), anti-Flag (F1804, Sigma-Aldrich), anti-Na^+/K^+-ATPase α1 (#3010, Cell Signaling), anti-Na^+/K^+-ATPase β1 (sc-21713, Santa Cruz), anti-GAPDH (sc-25778, Santa Cruz Biotechnology) and anti-β-actin (A1978, Sigma-Aldrich). The previously validated rabbit anti-RGMb antibody (63) and rat anti-RGMb antibody clone 307.1H6 (43) were used for immunofluorescence and Western blotting respectively.

Generation of renal tubule specific Rgmb knockout mice

Floxed Rgmb mice on C57BL/6 background have been described (46). Excision of the loxP-flanked region in kidney tubular epithelial cells to establish conditional kidney knockout mice (cKO) was obtained by interbreeding with Ksp-Cre mice on the C57BL/6 background.

Ischemic reperfusion injury (IRI)

Bilateral IRI was performed as previously described with minor modifications (64). Briefly, male Rgmb cKO ($Rgmb^{f/f}$; Ksp-cre) mice and wild-type littermates ($Rgmb^{f/f}$, $Rgmb^{f/wt}$ or $Rgmb^{wt/wt}$; Ksp-cre) at 8 weeks of age were anesthetized with ketamine (100 mg/kg) and xylazne (10 mg/kg). The hair on both sides of the back was removed, and the skin in the surgical area was wiped with 70% alcohol swab. The mouse was placed on a heat plate at a temperature of 36.5-37°C. Right and left flank incisions were made. The right kidney was first pushed out from the right flank incision, and the tissue around the renal pedicle was removed to expose the right renal pedicle for clamping. Then the left kidney was pushed out from the left flank incision to expose the left renal pedicle. The renal pedicles were clamped for 40 min using a micro aneurysm clip (160-863, George Tiemann & Co). After removal of clamps, the kidneys were subjected to reperfusion as indicated by the change of color from dark purple to red. The kidneys were returned to the abdomen cavity. Temgesic was used for post-surgery analgesia. The mice were sacrificed at 24 hours after ischemia. Kidney, urine and serum samples were collected for further analysis. Mice with sham operation were used as control.

For necrostatin-1 injection, necrostatin-1 was dissolved in DMSO and diluted in PBS before it was injected (i.p.) into mice at 1.65 mg/kg body weight. 30 min after injection, mice were subjected to renal pedicle clamping. For GSK'963 injection, GSK'963 or its inactive enantiomer GSK'962 (Glixx Laboratories) was dissolved in DMSO at 5% final DMSO concentration, and then 40% Cavitron ((2-Hydroxypropyl)-β-cyclodextrin average Mw~1,380, Sigma-Aldrich) in sterile saline was added (6% final concentration). GSK'963 or GSK'962 was injected (i.p.) 30 min before and 8 h after the ischemic surgery at the same dose of 2.5 mg/kg body weight.

All animal studies were approved by The Chinese University of Hong Kong Animal Experimentation Ethics Committee. Experiments were conducted in accordance with The Chinese University of Hong Kong animal care regulations.

Oxalate nephropathy

Oxalate crystal kidney injury was performed as previously described (37). Male Rgmb cKO and WT littermates at 8 weeks of age were injected (i.p.) with a single dose of sodium oxalate at 100 mg/kg body weight, and provided with drinking water containing 3% sodium oxalate. Serum, urine and kidney samples were harvested after 24 h.

Serum creatinine and BUN assays

Serum creatinine levels were measured using the Creatinine Liquicolor® kit (#0430-120, Stanbio Lab). Briefly, 3 µl of serum samples, water (as blank) and standards were added into a 96 well plate in duplicates. 135 µl of Reagent 1 (R1) was immediately added into each well, followed by incubation at 37°C for 5 mins. Absorbance A1 was measured at 550 nm. 45 µl of Reagent 2 (R2) then was added into each well, and incubated at 37°C for 5 mins. Absorbance A2 was measured at 550 nm. Serum creatinine concentrations were calculated according to the manufacturer's instructions.

Serum BUN levels were measured using the Stanbio Enzymatic Urea Nitrogen kit (#2050-450). Briefly, 1µl serum samples and standards were added into a 96 well plate in duplicates. 100 µl Enzyme Reagent was immediately added into each well, followed by incubation at room temperature for 10 min.100 µl Color Reagent 2 was then added into each well, and incubated at room temperature for 10 min. Absorbance was measured at 600 nm. Serum BUN concentrations were calculated using the formula provided by the manufacturer.

Histology and Immunofluorescence

Paraffin kidney sections were used for periodic acid-Schiff staining. The degree of tubular damage including tubular dilation, tubular atrophy, and cast formation was scored by three investigators without knowing the genotypes.

Cryostat kidney sections were used for immunofluorescent staining to examine RGMb, megalin, RIP3, and MLKL cellular localization. Sections were treated with 1% SDS for 4 minutes, and then were incubated with anti-RGMb, anti-megalin, anti-RIP3, anti-MLKL or anti-CD31 antibodies, followed by Alexa Fluor® 546/488-labeled secondary antibodies. The sections were mounted with a mounting medium (#H-1200, VECTOR) containing DAPI. Images were captured with our confocal microscope (Olympus FV1000).

To quantify MLKL fluorescence intensity, regions corresponding to the MLKL-associated fluorescence in the apical poles of the proximal tubular cells was selected using the freeform as the drawing/selection tool to measure Area, Integrated density and Mean gray value in ImageJ software. The background was determined for each image taken by measuring the pixel intensity in several locations of the 5–10 darkest cells. Corrected mean signal was calculated {corrected mean signal= [integrated density- (selected area x mean fluorescence of background readings)]/area}. 10 fields per section and three sections per kidney were used to calculate the average corrected mean signal for each animal.

TUNEL

TUNEL assay was performed on paraffin kidney sections using ApopTag Plus Fluorescein In Situ Apoptosis Detection kit (Chemicon International, S7111). 10 fields per section and three sections per kidney were examined for each animal to determine the apoptotic tubular epithelial cells per field in the outer medulla using ImageJ.

Cell culture and transfection

Human colon carcinoma HT-29 cells were originally obtained from ATCC (#HTB-38). The cells were cultured in Dulbecco's Modified Eagle Medium (#12800-017, Gibco) supplemented with 10% Fetal Bovine Serum (#10270, Invitrogen). Mouse proximal tubular TKPTS cells were published previously (65) and were a generous gift from Dr. Rafia Al-Lamki (University of Cambridge). TKPTS cells were maintained in DMEM / F12 (1:1) (#11330-032, Invitrogen) supplemented with 7% FBS and 1% SITZ liquid medium (#S4920, Sigma-Aldrich). Human proximal tubular HK-2 cells (#CRL-2190, ATCC) were cultured in DMEM / F12 (1:1) medium supplemented with 10% FBS. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cells were seeded in 6, 12 or 96 well plates and transfected with Rgmb or 3xFlag-Rgmb expression construct using Lipofectamine™ 2000 (#11668-019, Invitrogen). Transfected cells were incubated in completed medium for 1 or 2 days prior to experiments.

Induction of necroptosis and ferroptosis

Necroptosis in TKPTS cells was induced by combined treatment with TNF-α, cycloheximide (CHX) and the pan-caspase inhibitor Z-VAD-FMK (zVAD) (TCZ: 100 ng/ml mTNF-α, 2 μg/ml CHX and 25 μM zVAD) for the indicated durations before cell viability was measured. Necroptosis in HT-29 cells was induced by TNF-α, the smac mimetic Birinapant (S) and zVAD (TSZ: 30 ng/ml hTNF-α, 50 nM Birinapant and 20 μM zVAD) as indicated. Necroptosis was inhibited by co-treatment of TCZ or TSZ with the RIP1 kinase inhibitor necrostain-1 (50 μM). Necroptosis in HK-2 cells were induced by 1 mg/ml CaOx crystals for 24 h. CaOx was sonicated for 15 cycles of 60 seconds (#JGB15015954A, Branson SLPE Energy Sonifier) before using.

Ferroptosis in HK-2 cells was incubated by ersatin as indicated. Ferroptosis was defined by co-treatment of erastin with100 μM desferoxamine (DFO) or 2 μM ferrostatin-1 (Fer-1) for 24 h before cell viability was measured.

Cell viability

MTT assay was performed as previously described (45). Briefly, cells in 96-well microtiter plates were incubated with 20 µl/well MTT solution at the concentration of 5 mg/ml for 4 hours. The formazan crystals were dissolved in 100 µl DMSO. Absorbance was measured at 570 nm in a microplate reader (Thermo Scientific Multiskan Go Microplate Spectrophotometer).

ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay kit (#G7571, Promega). Cells were seeded in 96-well Flat Clear Bottom White Polystyrene TC-Treated Microplates (#3610, Corning). Luminescence was measured in a microplate reader (Molecular Devices SpectraMax I3 Multimode Reader).

Isolation of membrane proteins

Cytosolic and crude membrane proteins from HT-29 cells and kidney tissues were performed as previously described (24). Briefly, HT-29 cells were washed three times with ice-cold PBS before the cells were harvested and permeabilized in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.025% digitonin, protease inhibitor mixture (#P8304, Sigma-Aldrich) and phosphatase inhibitor mixture (#78428, Thermo Fisher). The cortical and outer medullary region was dissected and homogenized in the same buffer. Cytosolic and crude membrane fractions were separated by centrifugation at 3,000 g for 5 min. The supernatants were taken as cytosolic proteins. The pellets were washed 3 times with PBS and then solubilized in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Trition-100 and protease and phosphatase inhibitors for 30 min on ice before centrifugation at 10,000 g for 15 min to collect membrane proteins.

Western blotting

A total of 20-40 μg of protein was separated by SDS-PAGE under reducing or non-reducing conditions, and subjected to Western blot analysis using primary antibodies as indicated. Specific bands were scanned and quantified by densitometry.

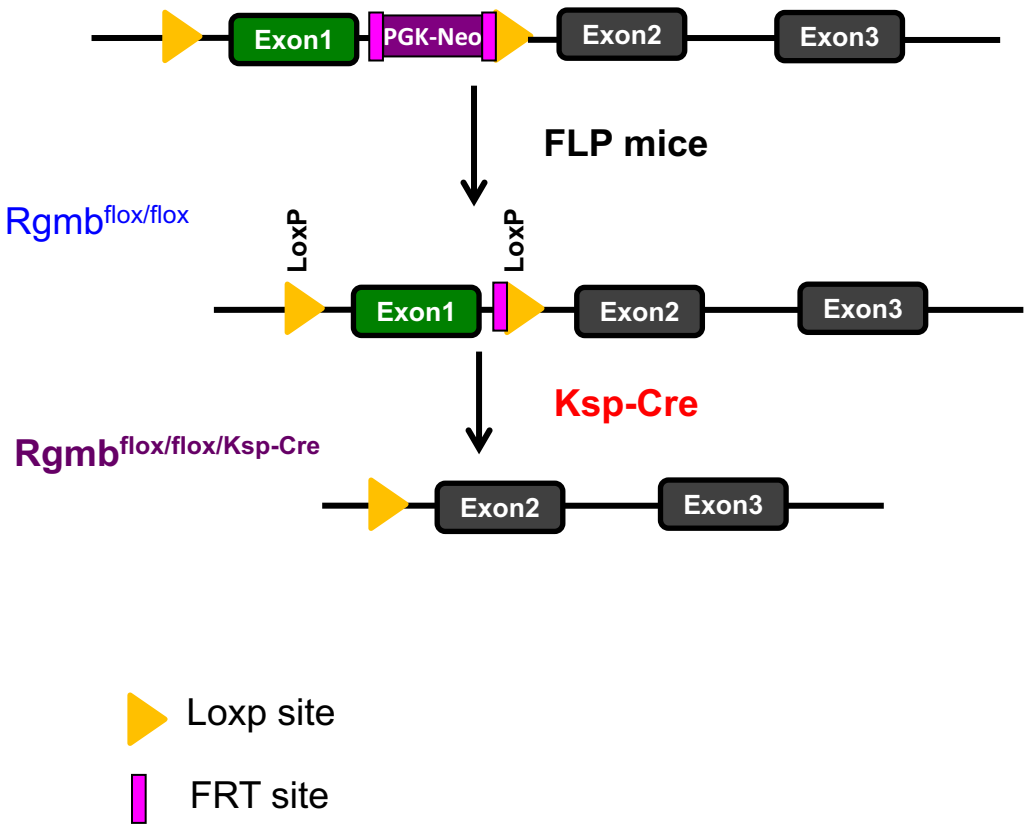
RNA isolation and real-time PCR analysis

Total RNA was extracted using illustra RNAspin Mini (#25-0500-71, GE Healthcare) according to the manufacture's instruction. Reverse transcription was performed using PrimeScript RT reagent kit (TAKARA). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on ABI Prism 7900 Sequence Detection System (PE Biosystems).

Statistical analysis

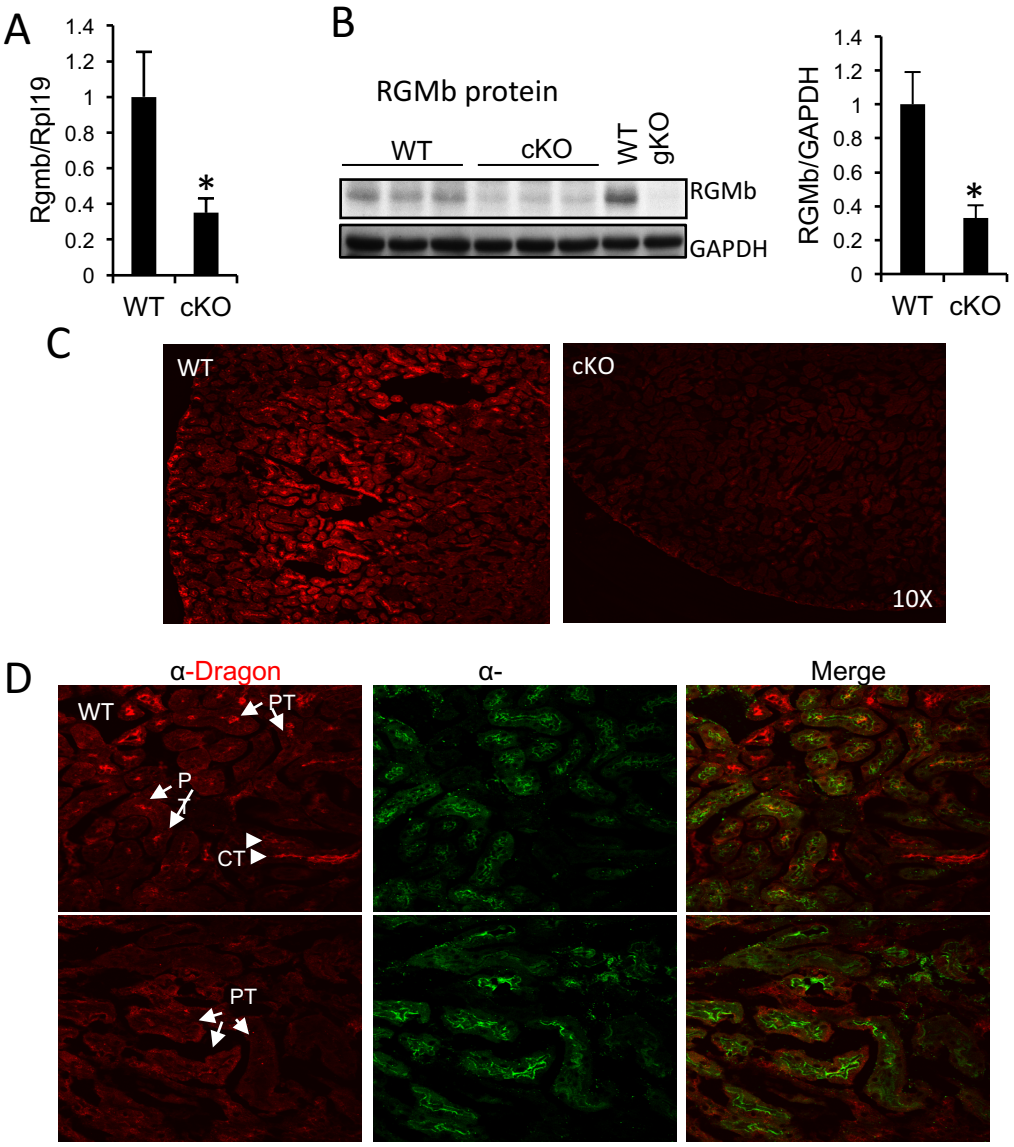
All data are represented as mean ± S.E.M of independent replicates. Student's *t*-test was applied for statistical analysis. A *p*-value of less than or equal to 0.05 was considered statistically significant.

Supplemental Figure 1



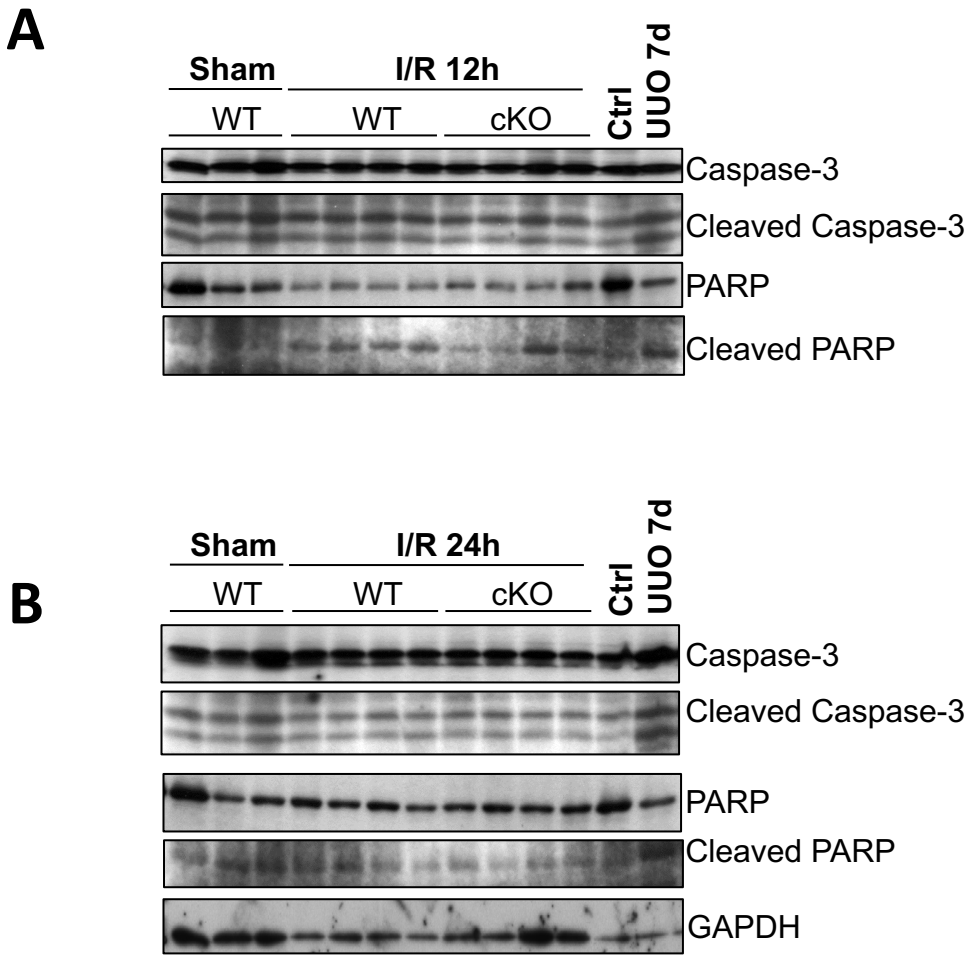
Supplemental Figure 1. Scheme of conditional knockout mice with tubule-specific deletion of Rgmb. The targeting vector contains Loxp-sites flanking Exon I of the Rgmb gene and a neomycin gene flanked by FRT-sites. The FRT-flanked neomycin gene is removed by crossbreeding floxed Rgmb mice with FLP mice. Excision of the loxP-flanked region in kidney tubular epithelial cells to establish conditional kidney knockout mice (cKO) is achieved by interbreeding with Ksp-Cre mice.

Supplemental Figure 2



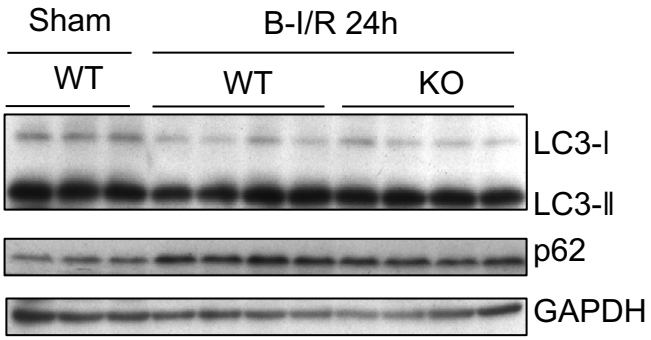
Supplemental Figure 2. RGMb mRNA and protein expression are ablated in Rgmb cKO mice. (A and B) Rgmb mRNA and protein levels in the kidneys of WT and Rgmb cKO mice. Kidneys collected from male WT and Rgmb cKO mice at 2 months of age were analyzed for Rgmb mRNA levels by real-time PCR (A), and for RGMb protein by Western blotting (B). Kidney samples from WT and global Rgmb KO (gKO) mice at 10 days of age were used to identify the RGMb specific band (37 kDa) (B, left panel). RGMb proteins were quantified by densitometry (B, right panel). (C) Cellular localization of RGMb in WT and Rgmb cKO kidneys. Photographs at low magnification show expression of RGMb in various tubules in WT kidney tissue, which was efficiently depleted in cKO kidney tissue. (D) Expression of RGMb in proximal tubules was efficiently depleted in cKO mice. Double staining for RGMb and megalin was performed in WT and Rgmb cKO kidneys. RGMb was expressed in the apical membrane of proximal tubules, and this expression was much reduced in the Rgmb cKO mice. Rpl19 was used as the internal control for real-time PCR. GAPDH was used as the loading control for Western blotting. n =3 for panel A. * $P< 0.05$

Supplemental Figure 3



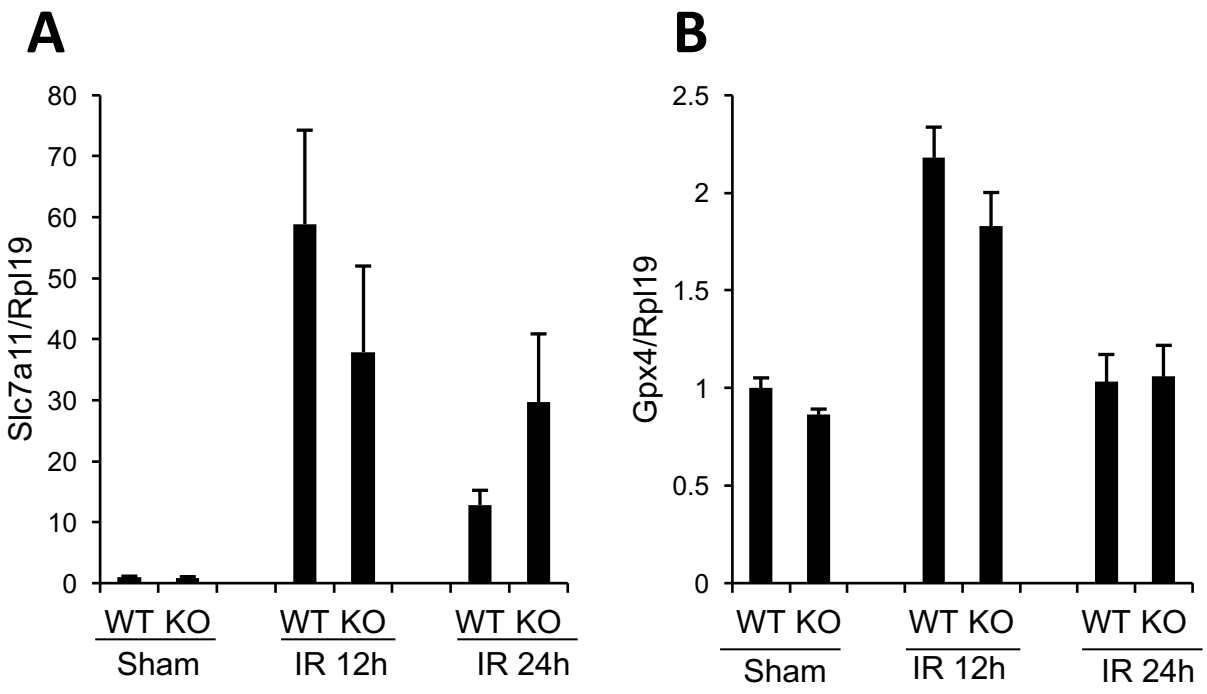
Supplemental Figure 3. Cleaved caspase-3 and cleaved PARP were not increased by ischemia/reperfusion in WT and Rgmb cKO kidneys. Male WT and Rgmb cKO mice at 2 months of age underwent 40 min of bilateral renal pedicle clipping. Sham-operated mice were used as control. Mice were sacrificed 12 h (A) or 24 h (B) after reperfusion. Kidney lysates were subjected to Western blotting for full-length and cleaved caspase-3, and full-length and cleaved PARP. Kidneys from sham operation and unilateral ureteral obstruction (UUO) for 7 days were used as positive controls to show increased cleaved caspase-3 and PARP by UUO.

Supplemental Figure 4



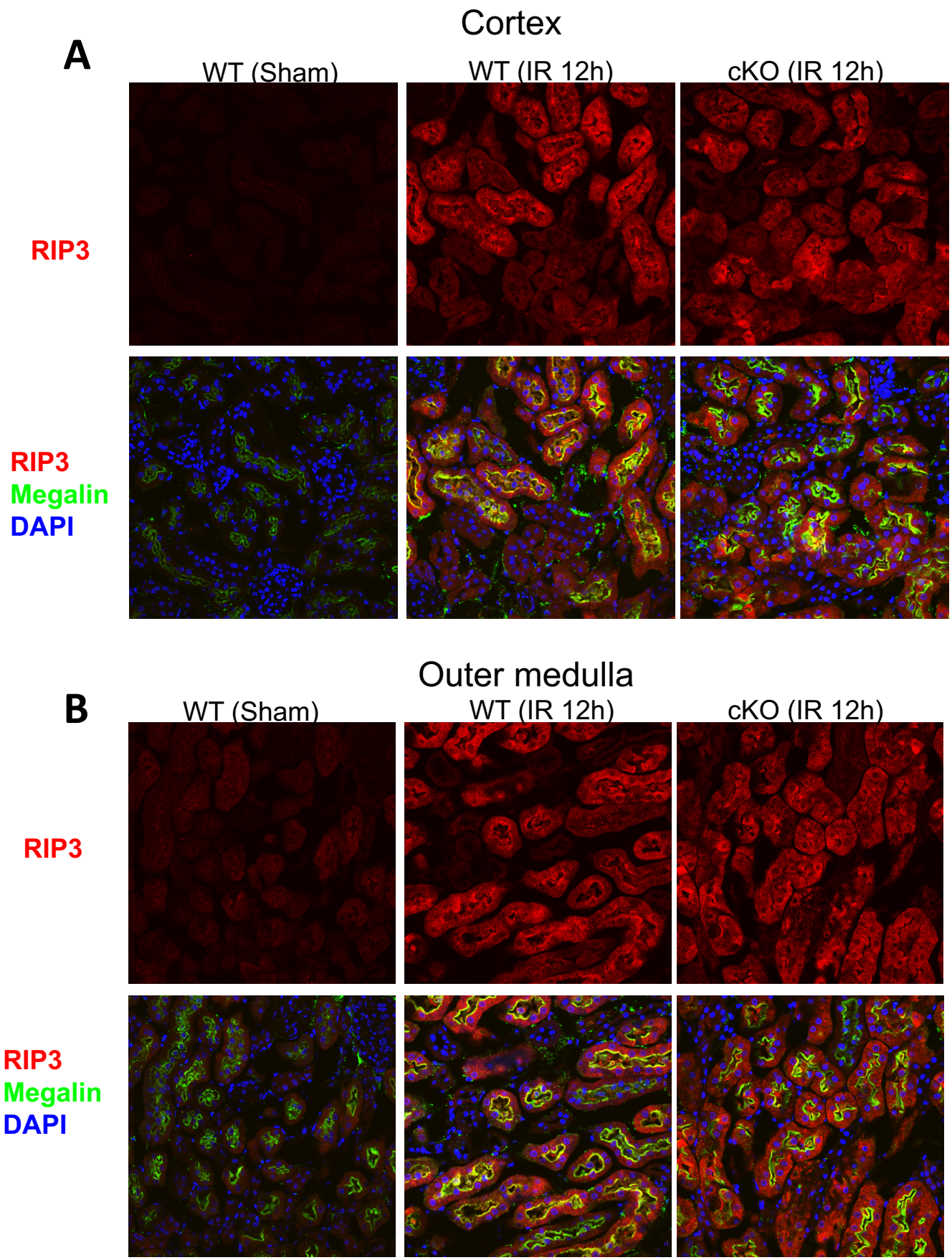
Supplemental Figure 4. Expression of the autophagy markers LC3-I, LC3-II and p62 in WT and Rgmb cKO kidneys after ischemia/reperfusion (IR). Male WT and Rgmb cKO mice at 2 months of age underwent 40 min of bilateral renal pedicle clipping. Sham-operated mice were used as control. Mice were sacrificed 24 h after reperfusion. Kidney lysates were subjected to Western blotting for LC3-I, LC3-II and p62. GAPDH was used as the loading control.

Supplemental Figure 5



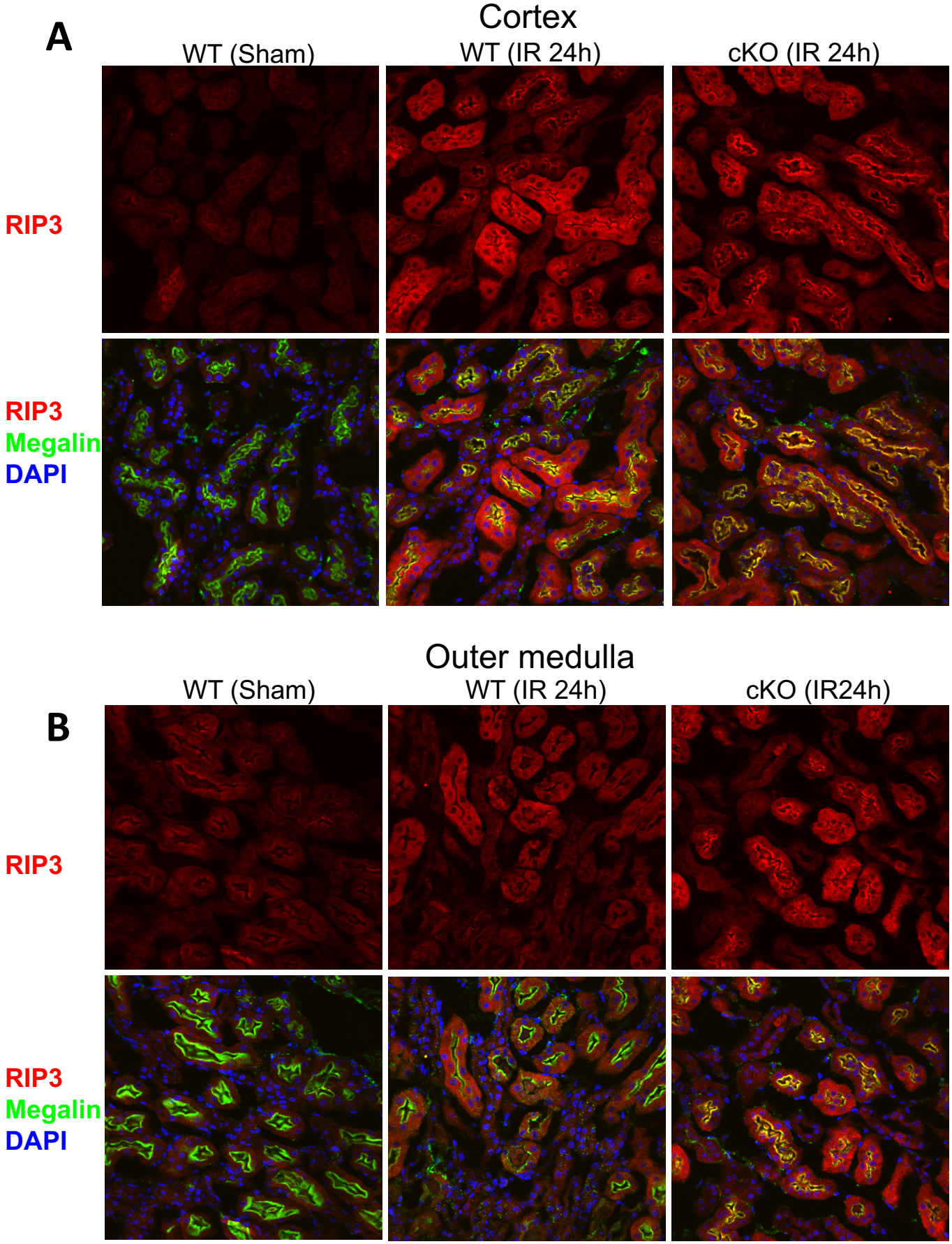
Supplemental Figure 5. Changes in expression of the ferroptosis regulators Slc7a11 and Gpx4 in the kidneys of WT and Rgmb cKO mice after ischemia/reperfusion injury (IRI). Male WT and Rgmb cKO mice at 2 months of age underwent 40 min of bilateral renal pedicle clipping. Mice were sacrificed 12 h and 24 h after reperfusion. Kidneys were analyzed for mRNA levels of Slc7a11 and Gpx4 by real-time PCR. Rpl19 was used as the internal control.

Supplemental Figure 6

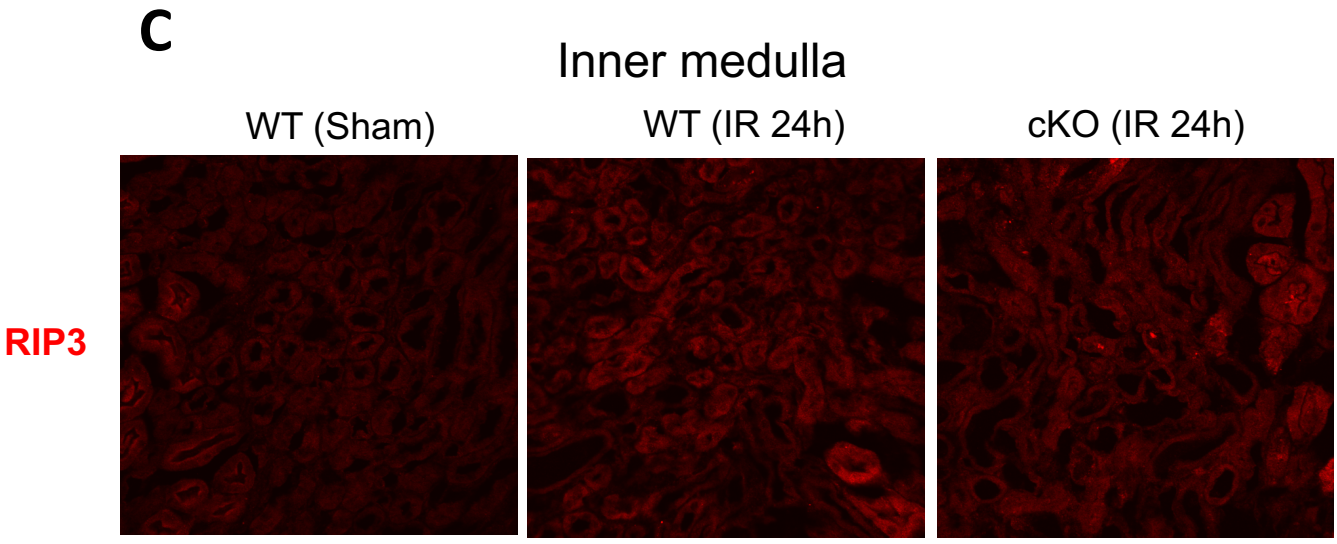


Supplemental Figure 6. Cellular localization of RIP3 in WT and Rgmb cKO kidneys 12 h after ischemia/reperfusion (IR). Immunofluorescence was performed on sections from sham-operated kidneys and kidneys of WT and Rgmb cKO mice 12 h after ischemia (40 min) and reperfusion for RIP3 (red). Co-staining with megalin (green) was included to identify proximal tubules. The cortex (A) and outer medulla (B) are presented. No staining for RIP3 was found in the inner medulla and papilla. The RIP3 antibody used: ab62344, Abcam.

Supplemental Figure 7

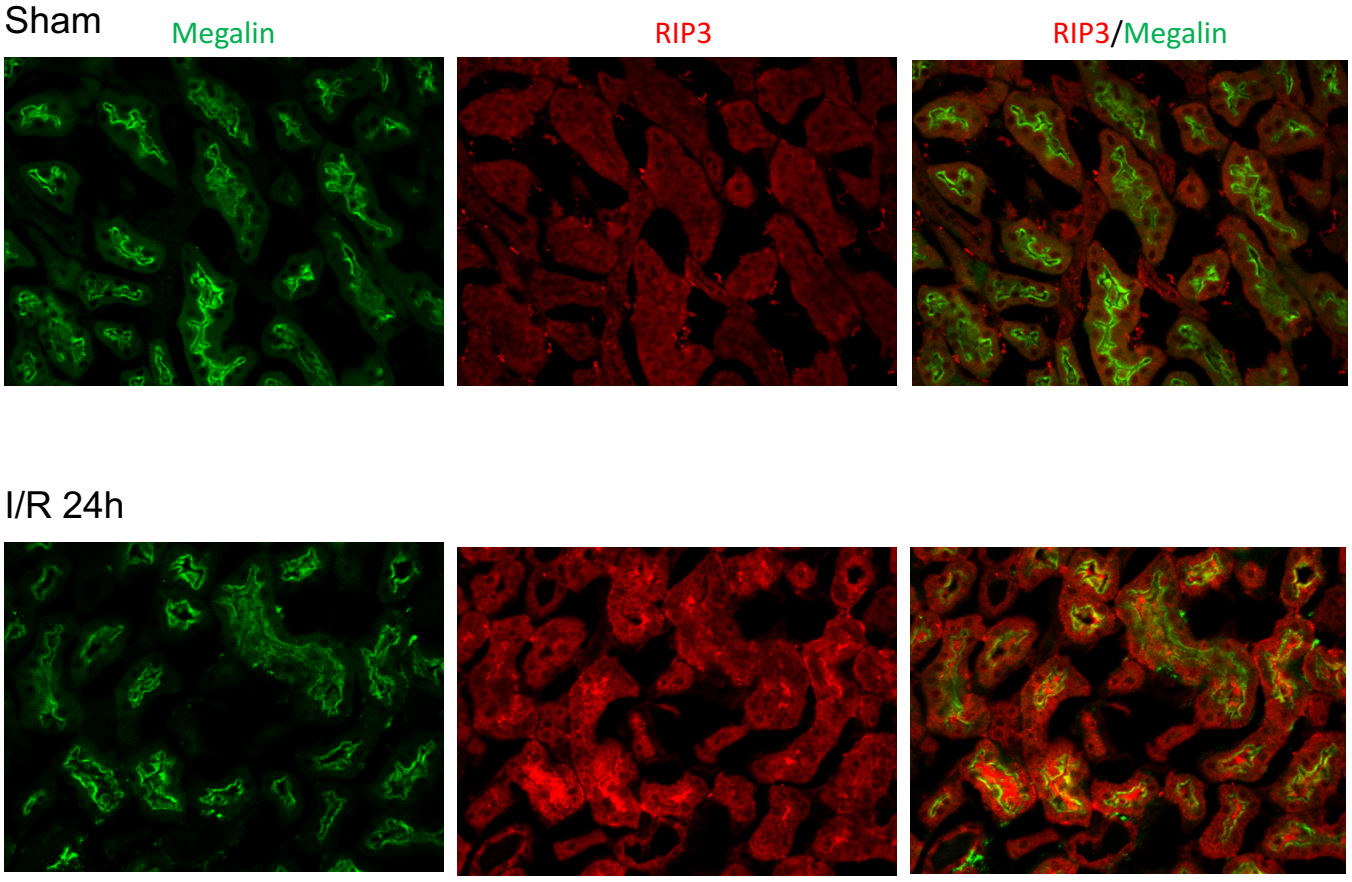


Supplemental Figure 7 (continued)



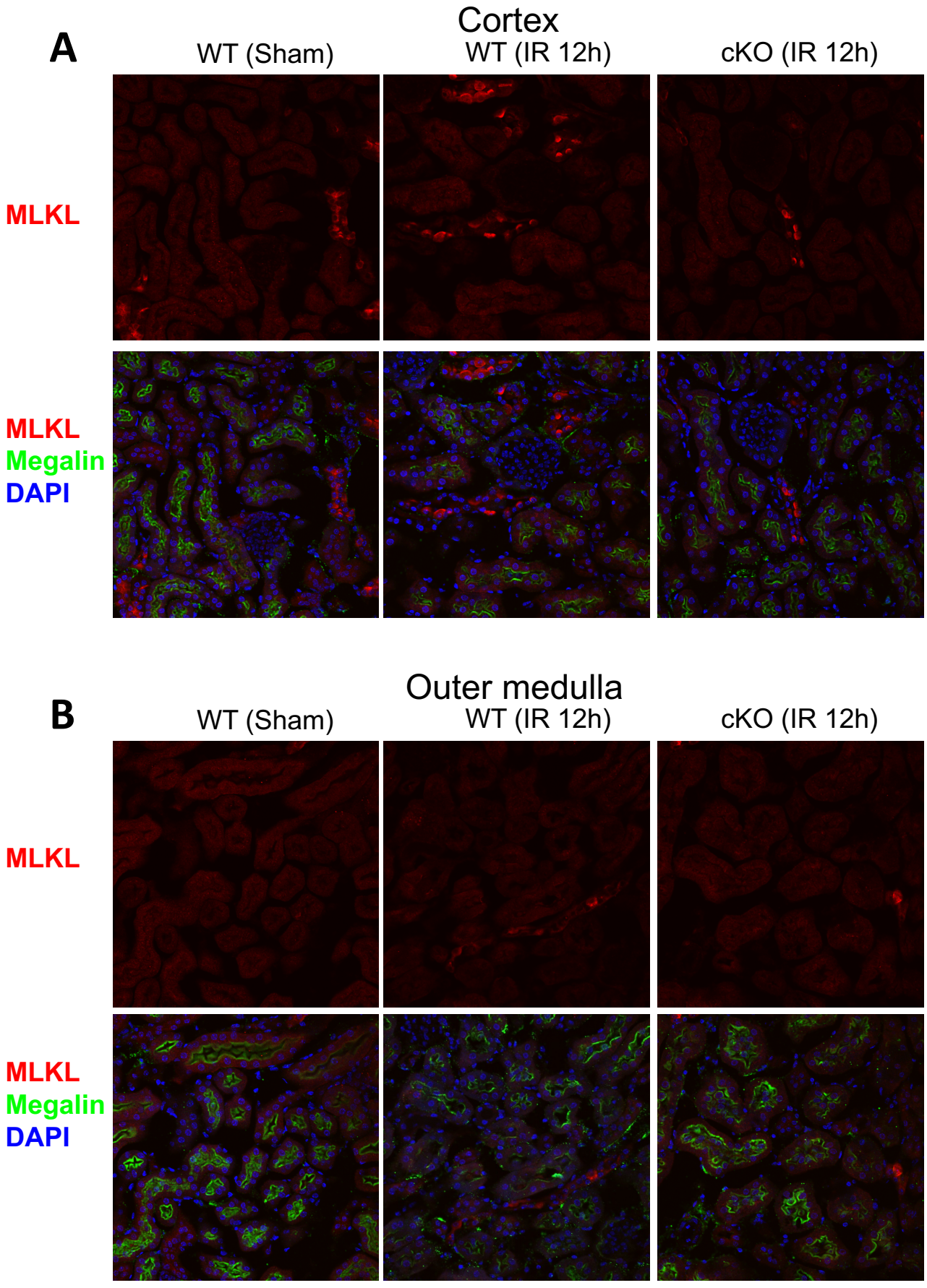
Supplemental Figure 7. Cellular localization of RIP3 in WT and Rgmb cKO kidneys 24 h after ischemia/reperfusion (IR). Immunofluorescence was performed on sections from sham-operated kidneys and kidneys of WT and Rgmb cKO mice 24 h after ischemia (40 min) and reperfusion for RIP3 (red). Co-staining with megalin (green) was included to identify proximal tubules. The cortex (A), outer medulla (B) and inner medulla (C) are presented. The RIP3 antibody used: ab62344, Abcam.

Supplemental Figure 8



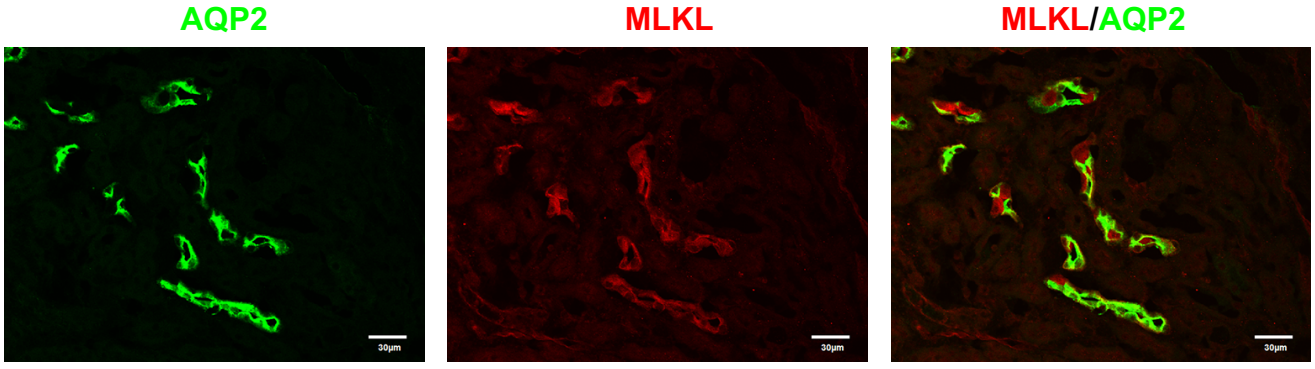
Supplemental Figure 8. Immunostaining of RIP3 in WT kidneys 24 h after ischemia/reperfusion (IR) using a different RIP3 antibody. Immunofluorescence was performed on sections from sham-operated kidneys and kidneys of WT mice 24 h after ischemia (40 min) and reperfusion for RIP3 (red). Co-staining with megalin (green) was included to identify proximal tubules. The RIp3 antibody used: #2283, ProSci.

Supplemental Figure 9



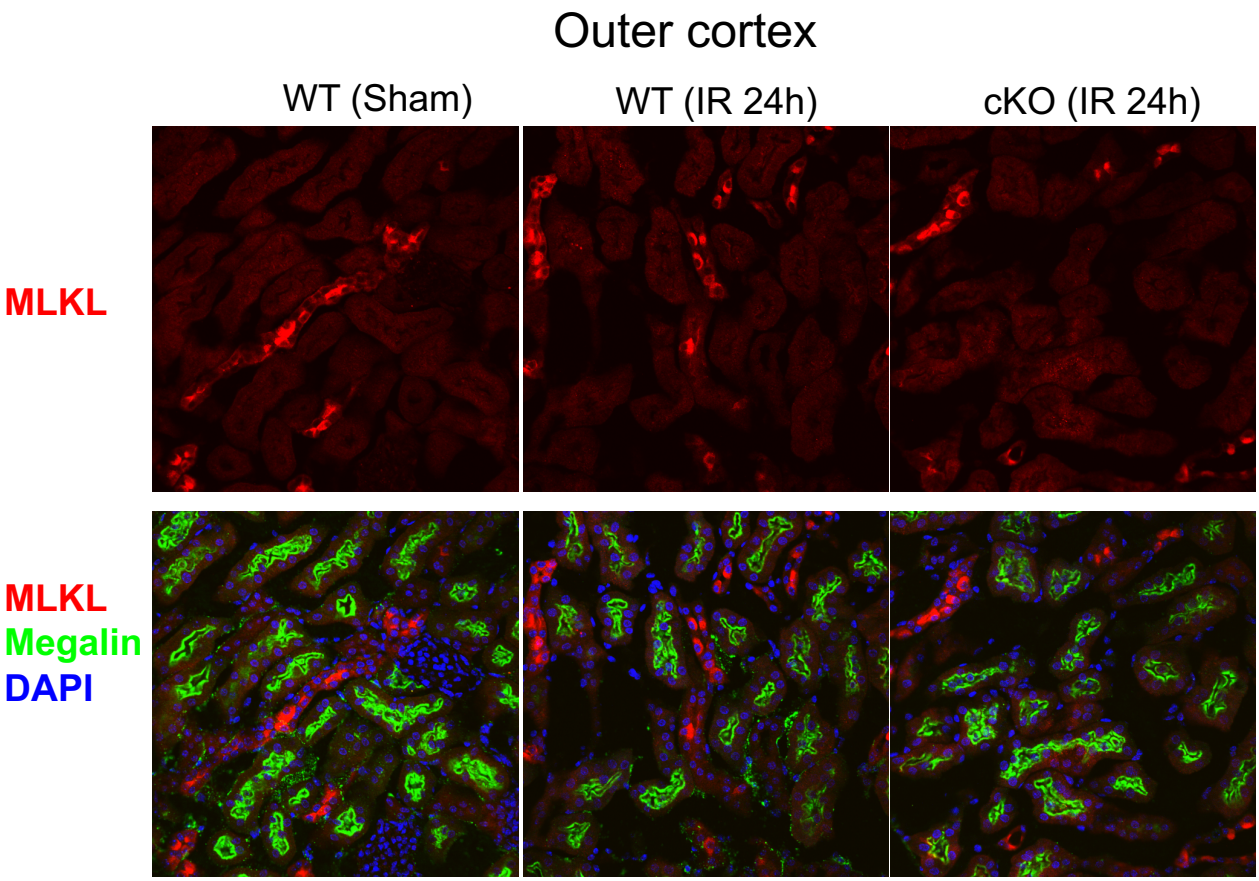
Supplemental Figure 9 (continued)

C



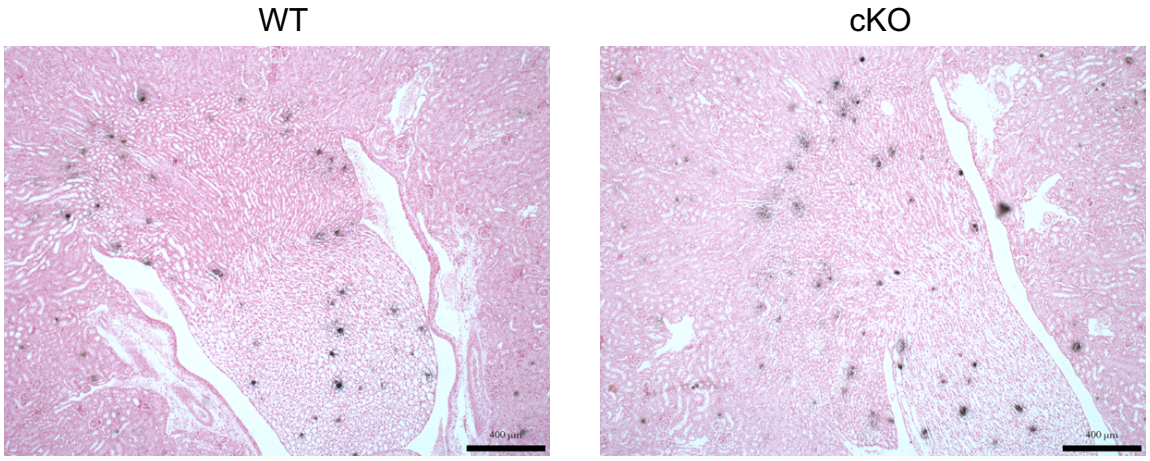
Supplemental Figure 9. Cellular localization of MLKL in WT and Rgmb cKO kidneys 12 h after ischemia/reperfusion (IR). Immunofluorescence was performed on sections from sham-operated kidneys and kidneys of WT and Rgmb cKO mice 12 h after ischemia (40 min) and reperfusion for MLKL (red). Co-staining with megalin (green) was included to identify proximal tubules. The cortex (A) and outer medulla (B) are presented. Co-localization of MLKL (red) and AQP2 (green) was also performed (C). MLKL was only found in collecting ducts, and no staining for MLKL was observed in proximal tubules.

Supplemental Figure 10



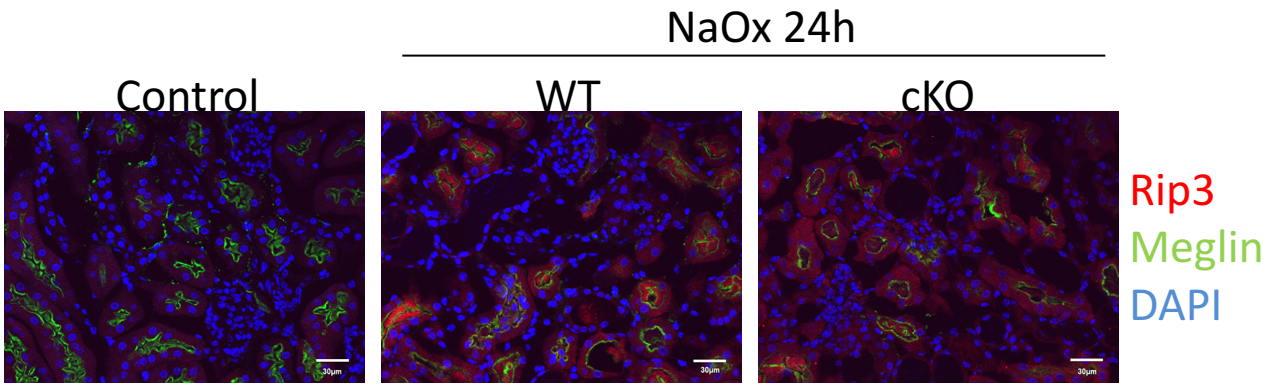
Supplemental Figure 10. Cellular localization of MLKL in outer cortex in WT and Rgmb cKO kidneys 24 h after ischemia/reperfusion (IR). Immunofluorescence was performed on sections from sham-operated kidneys and kidneys of WT and Rgmb cKO mice 24 h after ischemia (40 min) and reperfusion for MLKL (red). Co-staining with megalin (green) was included to identify proximal tubules. MLKL staining was only found in collecting ducts.

Supplemental Figure 11



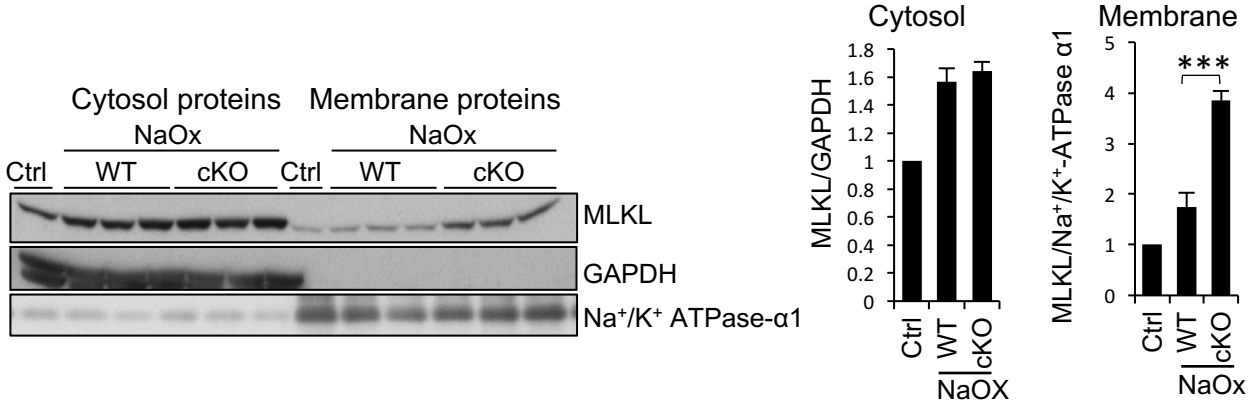
Supplemental Figure 11. Pizzolatto staining of WT and Rgmb cKO kidneys injected with NaOx. Male WT and Rgmb cKO mice at 2 months of age were injected with a single dose of NaOx (100 mg/kg body weight). Mice were sacrificed 24 h after injection. Kidney sections were used for Pizzolatto staining.

Supplemental Figure 12



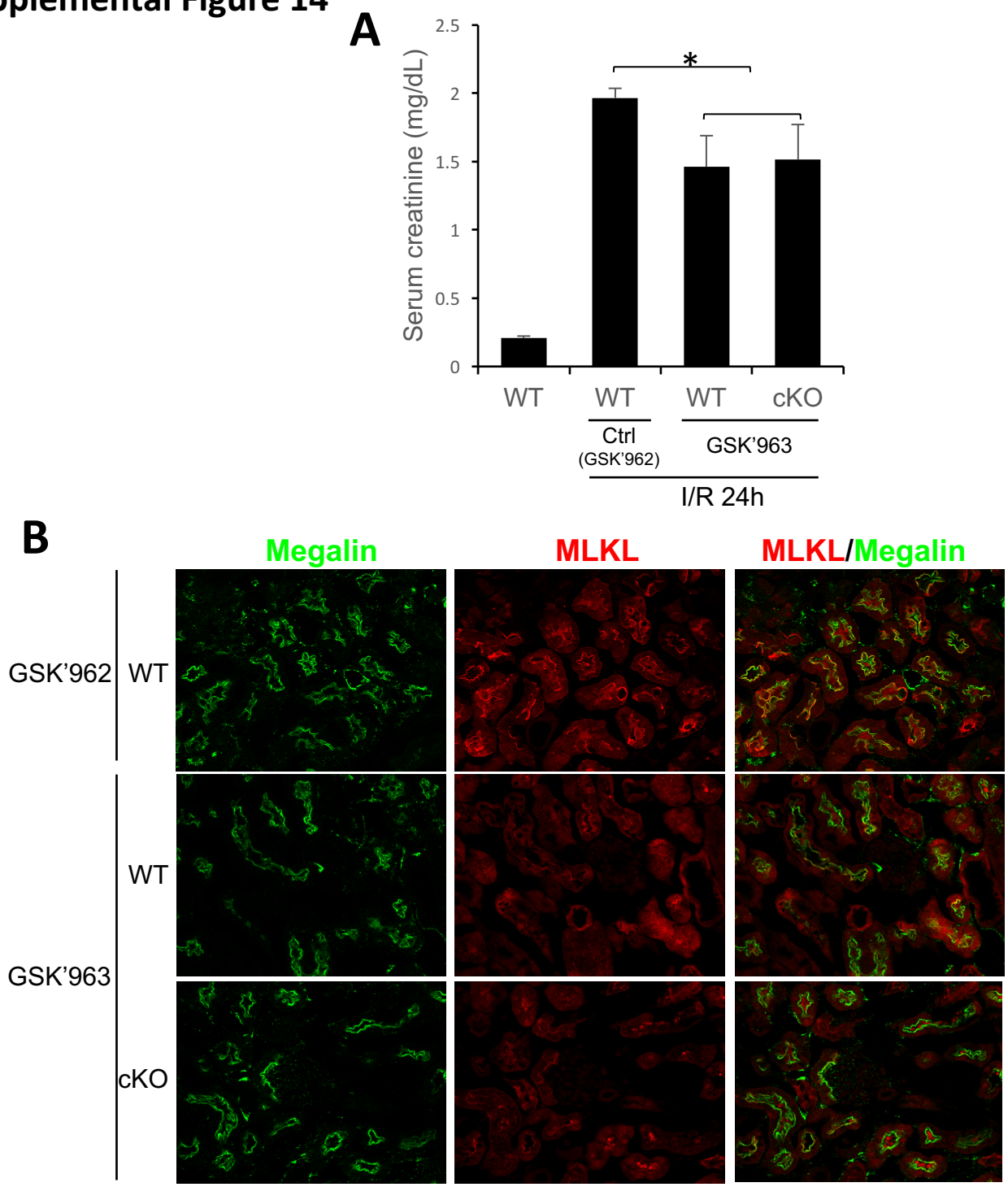
Supplemental Figure 12. Cellular localization of RIP3 in WT and Rgmb cKO kidneys 24 h NaOx injection. Immunofluorescence was performed on sections from control kidneys and kidneys of WT and Rgmb cKO mice 24 h after a single injection of NaOx (100 mg/kg body weight) for RIP3 (red). Co-staining with megalin (green) was included to identify proximal tubules. The cortical regions are presented.

Supplemental Figure 13



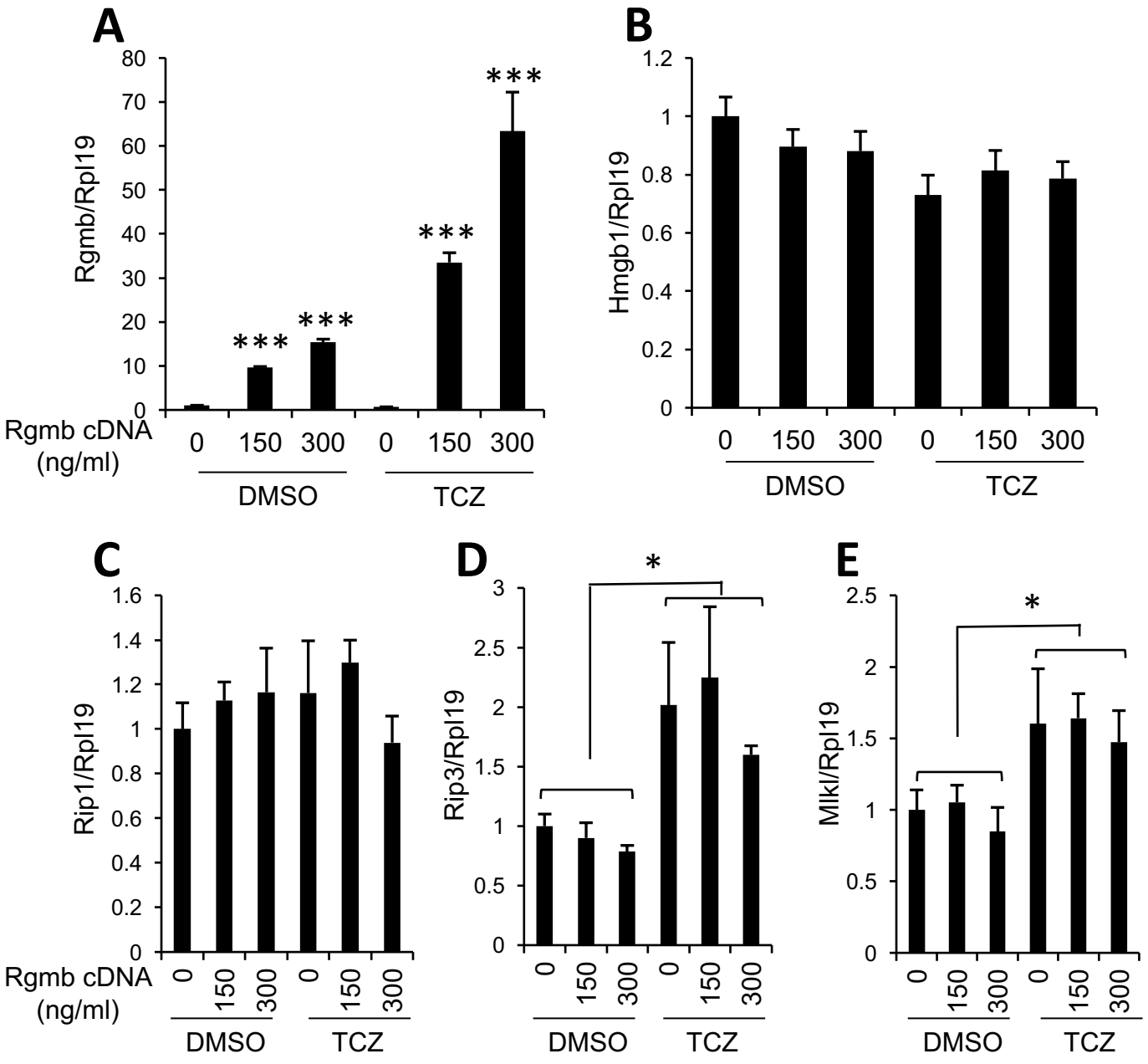
Supplemental Figure 13. MLKL levels in cytosolic and membrane fractions from the kidneys of WT and Rgmb cKO mice. Membrane proteins and cytosolic proteins were isolated from control kidneys or kidneys of WT and Rgmb cKO mice 24 h after NaOx injection, and subjected to Western blotting for MLKL (left panels). MLKL relative to α1-subunit of Na^+/K^+ ATPase in the membrane fractions and MLKL relative to GAPDH in cytosolic fractions were quantified by densitometry. GAPDH was used as the loading control for cytosolic samples, and α1-subunit of Na^+/K^+ ATPase was used as the control for membrane proteins. *** $P < 0.01$.

Supplemental Figure 14



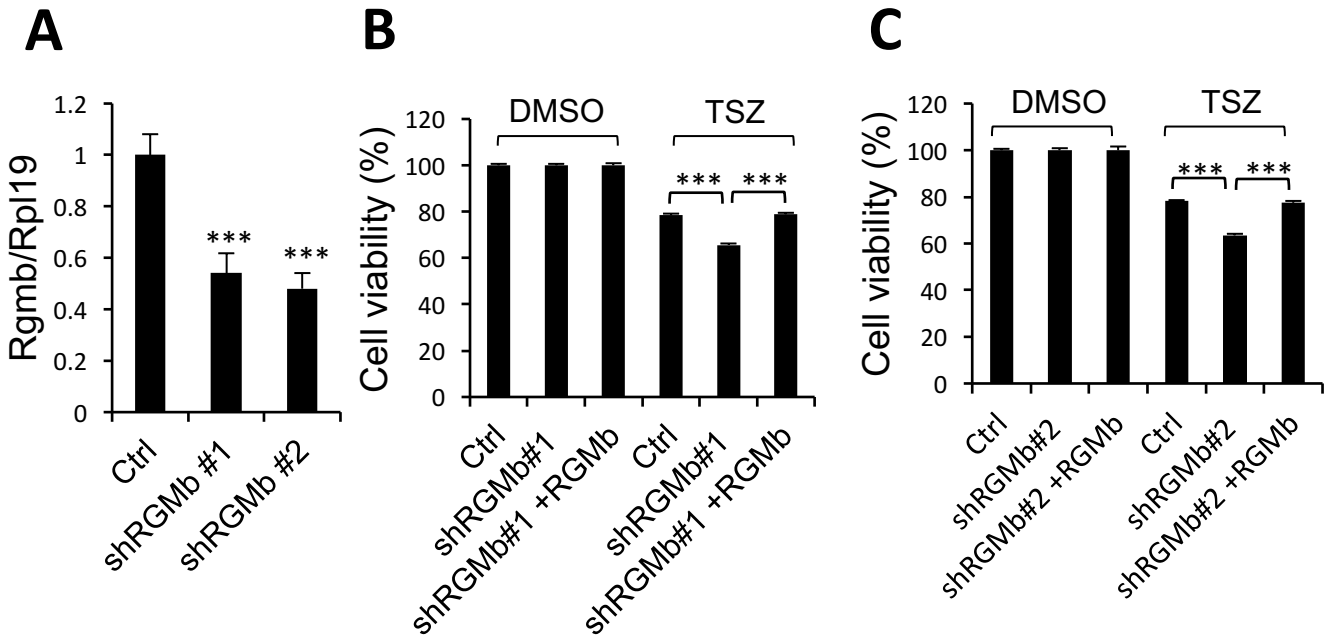
Supplemental Figure 14. Effects of the necroptosis inhibitor GSK'963 on renal function and MLKL localization in WT and Rgmb cKO mice 24 h after ischemia/reperfusion (IR). WT and Rgmb cKO mice udnerwent bilateral ischemia for 40 mins, followed by reperfusion for 24 h. GSK'963 or its inactive anantiomer GSK'962 was injected (i.p.) 30 min before and 8 h after the surgery at the same dose of 2.5 mg/kg body weight. (A) Serum creatinine levels were measured. * $P< 0.05$. n = 4/9/9/8. (B) Frozen kidney sections were used for immunostaining for MLKL (Red). Co-staining with megalin (green) was included to identify proximal tubules. Representative images are presented. 4 mice were used for each group.

Supplemental Figure 15



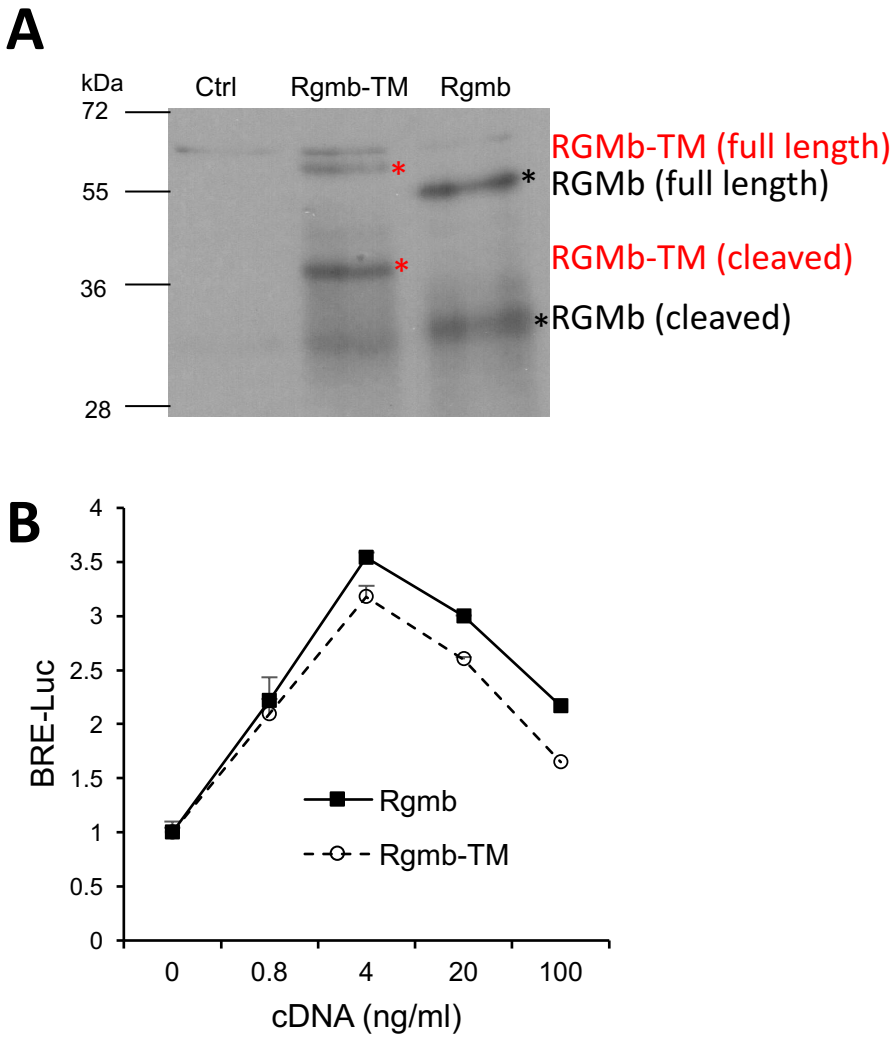
Supplemental Figure 15. Rgmb1 does not regulate Hmgb1, Rip1, Rip3 and Mlkl expression during necroptosis in TKPTS cells. Cells were transfected with increasing amounts of Rgmb cDNA. 48 h after transfection, cells were treated with DMSO (Ctrl) or TCZ (100 ng/ml TNF-α, 2 μg/ml CHX, 25 μM zVAD-fmk) for 24 h. Cells were then collected to analyze Rgmb (A), Hmgb1 (B), Rip1 (C), Rip3 (D) and Mlkl (E) mRNA expression by real-time PCR. Rpl19 was used as the internal control. n = 3. *$P < 0.05$, ***$P < 0.001$.

Supplemental Figure 16



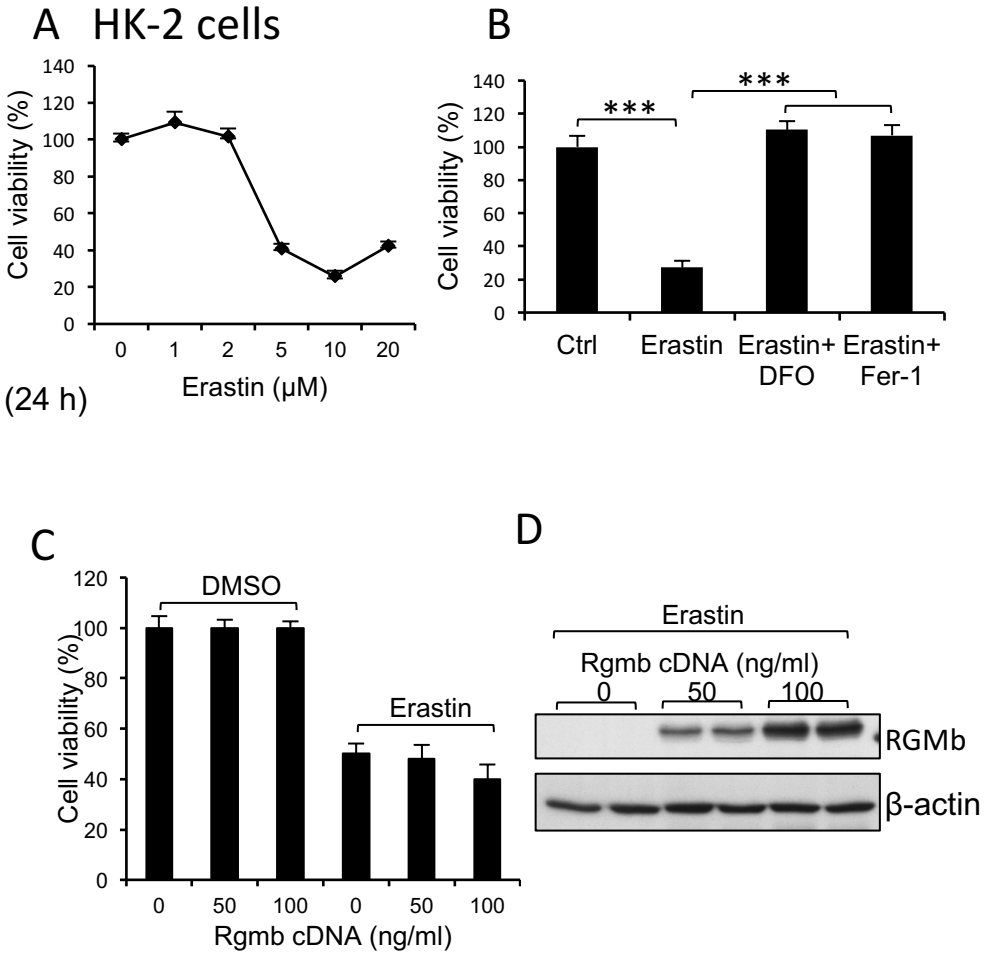
Supplemental Figure 16. Inhibition of Rgmb1 promotes necroptosis in HT-29 cells. (**A**) Knockdown efficacy of two Rgmb shRNA sequences. HT-29 cells were transfected with or without Rgmb shRNA plasmids #1 and #2 (250 ng/ml). 48 h after transfection, cells were collected to analyze Rgmb mRNA expression by real-time PCR. Rpl19 was used as the internal control. (**B and C**) Inhibition of Rgmb1 promotes necroptosis in HT-29 cells. Cells were transfected with or without Rgmb shRNA #1 (B) or Rgmb shRNA #2 (C) in the presence or absence of Rgmb cDNA (300 ng/ml). 48 h after transfection, cells were treated with DMSO or TSZ (30 ng/ml TNF-α, 50 nM Smac mimetic Birinapant , 20 μM zVAD-fmk) for 8 h before the cells were subjected to MTT assay. n= 6.

Supplemental Figure 17



Supplemental Figure 17. Rgm-TM stimulates BMP signaling. (A) Protein expression of the Rgmb-TM plasmid. HT-29 cells were transfected with empty vector, Rgmb-TM or Rgmb plasmid. Cell lysates were used for Western blotting analysis using the Dragon antibody. (B) Effects of Rgmb-TM overexpression on BMP signaling. Cells were transfected with a BMP-responsive firefly luciferase reporter (BRE-Luc) and a control Renilla luciferase vector (pRL-TK), with increasing amounts of Rgmb-TM or Rgmb plasmids. Cell lysates were analyzed for luciferase activity. Relative luciferase activity was calculated as the ratio of firefly to Renilla luciferase activity, to control for transfection efficiency, and is expressed as a multiple of the activity of unstimulated cells transfected with reporter alone (control). n = 3.

Supplemental Figure 18



Supplemental Figure 18. RGMb does not regulate ferroptosis. (A) Erastin dose-dependently induces cell death in HK-2 cells. Cells were incubated with increasing amounts of erastin for 24 h before cell viability were analyzed by MTT assay. (B) Erastin induces ferroptosis in HK-2 cells. Cells were incubated with 5 μM ersatin in the presence or absence of 100 μM desferoxamine (DFO) or 2 μM ferrostatin-1 (Fer-1) for 24 h before cell viability was measured. (C and D) RGMb overexpression has no effect on ferroptosis. HK2 cells were transfected with increasing amounts of Rgmb cDNA. 48 h after transfection, cells were treated with or without 10 μM erastin for 24 h before MTT assay was performed to determine cell viability, and Western blotting was performed to examine RGMb protein expression in cells treated with ersatin. α-actin was used as the loading control. n = 10 for panels A, B and C.