Rubicon-deficiency sensitizes mice to mixed lineage kinase domain-like (MLKL)-mediated kidney ischemia-reperfusion injury

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The cytosolic protein rubicon (RUBCN) has been implicated in the removal of necrotic debris and autoimmunity. However, the role of RUBCN in models of acute kidney injury (AKI), a condition that typically involves necrotic kidney tubules, was not investigated. Here, we demonstrate that RUBCN-deficient mice are hypersensitive to renal damage induced by ischemia-reperfusion injury (IRI) and cisplatin-induced AKI. Combined deficiency of RUBCN and mixed lineage kinase domain-like (MLKL) partially reversed the sensitivity in the IRI model suggesting that the absence of RUBCN sensitizes to necroptosis in that model. Necroptosis is known to contribute to TNFα-induced severe inflammatory response syndrome (SIRS), but we detected no statistically significant difference in overall survival following injection of TNFα in RUBCN-deficient mice. We additionally generated RUBCN-deficient mice which lack gasdermin D (GSDMD), the terminal mediator of pyroptosis, but no reversal of the AKI phenotype was observed. Finally, and in contrast to the previous understanding of the role of RUBCN, we did not find a significant autoimmune phenotype in RUBCN-deficient mice, but detected chronic kidney injury (CKD) in aged RUBCN-deficient mice of both sexes. In summary, our data indicate that RUBCN-deficient mice are hypersensitive to kidney injury.

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INTRODUCTION
Phagocytosis of dead or dying cells was studied in detail over the last decade [1, 2]. The protein rubicon (RUBCN) was initially demonstrated as an autophagy regulator associated with the protein Beclin-1 [3, 4], and later demonstrated to be involved in endosomal maturation [5, 6]. It is also known that RUBCN recruits NADPH oxidases to participate in the defence against bacteria [7], such as Listeria monocytogenes [8]. Recently, RUBCN was demonstrated to be involved in a specific cellular uptake of extracellular content referred to as LC3-associated phagocytosis (LAP) [9–12]. This process is thought to be critically important in the removal of necrotic debris and may be critical for tissues to allow regeneration following acute injury while avoiding autoimmunity [13].

Acute kidney injury (AKI) is associated with the loss of nephrons and therefore may fundamentally contribute to the progression of chronic kidney disease [14–16]. As a clinical consequence of progressive nephron loss, end-stage renal disease (ESRD) requires dialysis treatment or renal transplantation. Acute tubular necrosis is a hallmark condition of AKI, and we reasoned that failure to remove necrotic debris following AKI might affect renal injury in murine models of AKI. We and others recently demonstrated that mixed lineage kinase domain-like (MLKL)-mediated necroptosis is involved in acute kidney injury in mouse models [17–20] and in humans [21]. Likewise, other forms of regulated necrosis, such as ferroptosis [22, 23] and gasdermin D (GSDMD)-mediated pyroptosis [24], are potentially involved in AKI.

Here, we demonstrate that RUBCN-deficient mice are hypersensitive to AKI, an effect that is partially reversed on the combined loss of RUBCN and MLKL, but not RUBCN and GSDMD. In addition, we find aged RUBCN-deficient male mice to develop chronic kidney disease and proteinuria.

MATERIAL AND METHODS

Reagents

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rubicon (D9F7) rabbit monoclonal antibody | Cell Signalling | 8465 |
| β-actin antibody    | Cell Signalling | 3700 S |
| Anti-mouse IgG; HRP-linked antibody | Cell Signalling | 7076 S |
| Anti-rabbit IgG; HRP-linked antibody | Cell Signalling | 7074 S |

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Mice

Mice of the C57BL/6 N strain, further referred to as wild-type mice, were purchased from the company Charles River, Germany. Rubicon<sup>−/−</sup> (Rub<sup>−/−</sup>) mice on a C57BL/6 background were described previously [10] and were provided by Douglas R. Green. Mice on a C57Bl/6 background were described previously [10] and were kindly provided by James M. Murphy and Warren S. Alexander (The Walter and Eliza Hall Institute of Medical Research, purchased from the company Charles River, Germany). Mice double-deficient for both rubion/MLKLor rubicon/GSDMD, respectively, were then set up as homozygotic breeder pairs.

All mice have been housed in individually ventilated cages (IVC, Techniplast Deutschland GmbH) under specified pathogen-free conditions (SPF). The mice were weighed and blood samples were taken until the age of 50 weeks. After 1 year the mice were sacrificed for dissection and the organs were removed for subsequent histological analyses.

All work with animals was carried out in accordance with German animal welfare legislation and was notified and approved by the responsible authority, the Landesdirektion Dresden (TVV 57/2017) if not otherwise indicated. As required by German law, all group sizes were pre-calculated with the help of an independent statistician. The calculation basis aimed for detecting a significant difference with an alpha error of 5% and a beta error of 80%.

Long-term assessment of rubicon-deficient mice

Blood collections were done via a puncture of the vena facialis or one of its branches. The samples were kept on ice for 20–30 mins and then centrifuged at 2500 × g for 10 min at 4 °C. Fifty microliters were diluted in 100 μl 0.9% sodium chloride solution for assessment of serum creatinine and urea levels (determined by the Clinical Chemistry and Laboratory Medicine, Universitätssklinikum Carl Gustav Carus, Technische Universität Dresden) or stored for subsequent ELISA assays.

In week 50, the mice were sacrificed and a blood glucose measurement—blood with a glucose monitor was performed. Furthermore, for differential blood counts 200 μl of blood were filled into an EDTA-coated tube and sent to the Clinical Chemistry. Finally, organs were collected (kidneys, spleen, pancreas, caecum, liver, heart, lungs), fixed in 4% neutral-buffered paraformaldehyde solution and transferred to 70% ethanol 24 h later. The left kidney was halved, one half was shock-frozen in a 0.5 ml buffer containing 10% buffered paraformaldehyde for 1 h and then embedded in OCT compound (Sakura Finetek Europe NV, Zoeterwoude, the Netherlands) and snap-frozen in dry ice.

Histological processing

Periodic acid—Schiff reaction (PAS) and tubular damage score evaluation. Histological processing was carried out at the Histology Facility of BIOTEC, Technische Universität Dresden. The PAS staining was carried out using a PAS-staining kit (MORPHISTO GmbH). The samples were incubated in multiwell-plates coated with dsDNA or ANAs and visualized using an antibody coupled with horseradish peroxidase-conjugated (HRP). The hydrogen peroxide 3,3,5,5-tetramethylbenzidine (TMB) substrate was added and the optic density (OD) at 450 nm was quantified with a microelab reader (Tecan Group Ltd.).
Bilateral kidney ischemia and reperfusion injury (IRI)
Bilateral kidney ischemia and reperfusion injury (IRI) was performed as described in detail earlier [23] and a detailed protocol is available [27]. Briefly, 8–12-week-old male mice were rigorously matched for weight. In the experiment, they were handed over by a second person for blinding of the surgeon. After induction of narcosis, they were placed in a supine position and fixed. The abdomen was opened, the caecum and gut were carefully placed to the side. With the use of a surgical microscope (Carl Zeiss, Jena, Germany), sharp forceps were used to pinch retroperitoneal holes directly cranially and caudally in the renal pedicle. Via this access, a 100 g pressure micro serifine (FST 18035-03) was placed on the pedicle to induce ischemia and a timer was started. The same procedure was repeated on the other side with a maximal tolerance of 60 s between both clampings. After exactly 30 min, the vascular clamps were removed. Reperfusion was determined visually for both sides before the gut was returned into the abdominal cavity. The parietal peritoneum and the cutis, respectively, were closed separately. Isoflurane application was stopped immediately thereafter, and 1 mL of prewarmed PBS was administered intraperitoneally to compensate for any possible dehydration during surgery. 0.1 µg/g buprenorphine-HCl was administered every 8 h for analgesia. After a 48 h observation period, blood was collected by retro-orbital puncture and the mice were sacrificed.

Cisplatin-induced acute kidney injury
The model of acute kidney injury by cisplatin injection was described previously [23]. Briefly, 8–12-week-old female mice were rigorously matched for weight, and then received a single dose of 20 mg/kg cisplatin in 0.9% NaCl in a total volume of 400 µl intraperitoneally. The mice were then returned to their cages and monitored frequently. For assessment following cisplatin application, animals were under permanent observation and survival, as determined by the local authorities, was checked every 2 h. In detail, we checked for a combined endpoint of either loss of 20% body weight or the inability to maintain a body temperature higher than 28 ºC or severe movement imbalances or automutilary behavior.

TNFα-induced systemic inflammatory response syndrome
The model of TNFα-induced shock has been described in detail previously [28]. In our experiments, female 8–12-week-old mice were rigorously weight-matched before mice receiving injections of either vehicle or 25 ng murine TNFα in 200 µL PBS via the tail vein. Under binding to the genotype, animals were under permanent observation and survival, as defined by the local authorities, was checked every 2–4 h. In detail, we checked for a combined endpoint of either loss of 20% body weight or the inability to maintain a body temperature higher than 28 ºC or severe movement imbalances or automutilary behavior.

Analysis of serum creatinine and urea
Blood collections were done via a retro-orbital puncture. The samples were rapidly transferred to the Institute for clinical chemistry and laboratory medicine (Universitätsklinikum Carl Gustav Carus, Technische Universität Dresden) for standardized and completely blinded analysis of serum creatinine and urea levels.

Isolation of murine kidney tubules
Isolation of tubules was performed according to a recently published protocol [23]. The kidneys of 8-week-old male mice were collected and transferred to a petri dish with 2 ml PBS. In the dish the kidneys were decapsulated. The kidney was sliced and those were transferred into a tube containing 1 ml of collagenase solution and incubated for 5 min at 37 ºC and 850 rpm. This was repeated two more times and those supernatants were transferred into a new tube and the tubules were washed twice with ice-cold incubation solution. The sorting solution was added, and the tubules were distributed on a 24-well plate containing 1 ml of DMEM/F12, Thermo Fischer, supplemented with 0.01 mg/ml recombinant human insulin, 5.5 µg/ml human transferrin, 0.005 µg/ml sodium selenite (Na2SeO3) (ITS) without linoleic acid, Sigma–Aldrich), 50 nM hydrocortisone, 100 µM penicillin, and 100 µg/ml streptomycin (Pen/Strep, Thermo Fisher). Per timepoint (0 h, 0.5 h, 1 h, 2 h, 4 h) and per mouse one well was plated. After the respective timepoints 200 µl of cell suspension was collected and stored on ice for LDH-release assay.

Lactate dehydrogenase (LDH)-release assay
The assay was performed using the CytoTox96 Assay Kit (Promega Corporation) according to the manufacturer’s instructions. For each sample 200 µl of supernatant was taken, kept on ice and then 50 µl of lysis solution (0.8% Triton X-100) was added to the well and incubated for 45 min at 37 ºC to determine the maximum LDH release. Of this, 50 µl of the supernatant was plated in triplicates on a 96-well plate. Then, the CytoTox 96® Reagent was added and incubated at room temperature for 30 min in the dark. The stop solution was added and the optical density at 490 nm was immediately determined with a plate reader.

Cell lines and cell culture
Murine NIH-3T3 cells were purchased from the American Type Culture Collection. NIH-3T3 cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Thermo Fisher) supplemented with 10% (v/v) FBS (Thermo Fisher), 100 U/ml penicillin, and 100 µg/ml streptomycin (Pen/Strep, Thermo Fisher). The cells were cultured in a humidified 5% CO2 atmosphere.

siRNA-mediated knockdown of RUBCN
NIH-3T3 cells were plated in a petri dish in 15 ml antibiotic-free medium. After 24 h 180 pmol RNAi and 24 µl Lipofectamine® (Thermo Fisher) were each mixed in 1.5 ml Opti-MEM® Medium (Thermo Fisher) without serum, combined and incubated at room temperature for 20 min before dropping the mixture on the cells. The following day, cells were harvested, plated into six-well plates and cell death assays were performed as described below. Knockdown efficacy was determined on protein level using Western Blot.

Western blotting
Cells were lysed in ice-cold 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5 mM EDTA supplemented with PhosSTOP® (Merck), Complete® (Merck), and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice. Insoluble material was removed by centrifugation (14,000 × g, 30 min, 4 ºC). Protein concentration was determined using a commercial BCA assay kit according to the manufacturer’s instructions (Thermo Fisher). Equal amounts of protein (25 µg per lane) were resolved on a 4–15% gradient SDS/PAGE gel and transferred to a PVDF membrane (Bio-RAD). After blocking for 1 h at RT, primary antibody incubation (dilution of 1:1000 if not otherwise indicated) was performed at 4 ºC overnight. Secondary antibodies (anti-mouse, HRP-linked antibody, anti-rabbit, HRP-linked antibody, Cell Signaling) were applied at concentrations of 1: 5000. Proteins were then visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

Cell death assays
To induce necroptosis in the NIH-3T3 cell line after siRNA-mediated knockdown, cells were seeded in six-well plates and were stimulated with 20 ng/ml human TNFα (Biolegend), and 20 µM zVAD-fmk (Selleckchem). Ten micromolar Necrostatin-1s (Nec-1s, Merck KGaA) served as protection control. After the indicated timepoints, cells were collected and prepared for flow cytometry.
Cells were harvested and the pellets were washed twice in PBS and stained with 5 $\mu$l of 7-AAD (BD Biosciences) and 5 $\mu$l of annexin-V-FITC (BD Biosciences) added to 100 $\mu$l annexin-V binding buffer (BD Biosciences). After 15 min, cells were recorded on the LSRII with the FACS Diva 6.1.1 software (BD Biosciences) and subsequently analyzed with the FlowJo v10 software (Tree Star). The flow cytometry procedure was supported by the Flow Cytometry Core Facility of the CMCB Technology Platform at TU Dresden.

**Flow cytometry**
Cells were harvested and the pellets were washed twice in PBS and stained with 5 $\mu$l of 7-AAD (BD Biosciences) and 5 $\mu$l of annexin-V-FITC (BD Biosciences) added to 100 $\mu$l annexin-V binding buffer (BD Biosciences). After 15 min, cells were recorded on the LSRII with the FACS Diva 6.1.1 software (BD Biosciences) and subsequently analyzed with the FlowJo v10 software (Tree Star). The flow cytometry procedure was supported by the Flow Cytometry Core Facility of the CMCB Technology Platform at TU Dresden.

**Statistical analysis**
Statistical analyses were performed with Prism 8 (GraphPad software, San Diego, CA, USA). Two cohort comparisons of discrete values were analyzed by a one-sided Student’s $t$-test. If more than two groups were involved, a one-way ANOVA was performed with correction by Tukey post-hoc test. In both such datasets, the bars represent the mean ± standard deviation if not otherwise indicated. For analyses of survival data, a log-rank test was performed. Data were considered significant when $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, or $^{****}p \leq 0.0001$.

**RESULTS**
**RUBCN-deficient mice are hypersensitive to kidney ischemia-reperfusion injury**

It was previously suggested that RUBCN-deficient mice might exhibit glomerular injury after the age of 20 weeks [29]. However, because necrotic debris occurs within hours in acute kidney injury (AKI)-models [30], we hypothesized that RUBCN might also be
involved in the control therein. As demonstrated in Fig. 1A, RUBCN-deficient mice showed higher serum levels of the AKI marker creatinine. Along similar lines, we observed higher levels of serum urea (Fig. 1B), indicating AKI-induced uremia. Histologically, kidney cortical sections were stained for periodic acid Schiff (PAS). More tubular necrosis (Fig. 1C) and a higher tubular damage score (Fig. 1D) were detected. We previously developed a model for the assessment of tubular necrosis in freshly isolated kidney tubules [23]. In that assay, spontaneous LDH release remained unchanged in tubules isolated from RUBCN-deficient mice (Fig. S1). Another preclinical model of renal damage is cisplatin-induced AKI (CP-AKI) in which we typically investigated two timepoints in independent experiments of 48 h and 72 h following cisplatin injection. We detected a trend toward higher levels of serum creatinine (Fig. S2A) and serum urea (Fig. S2B) in the RUBCN-deficient mice, but due to high standard deviations, no statistical significance was reached despite 13 vs. 10 mice per group. However, in an entirely separate approach, we investigated the overall survival of

Fig. 2 The hypersensitization of RUBCN-deficient mice is partially reversed on a combined RUBCN/MLKL-double-deficient background. Indicated 8–12-weeks-old male mice underwent bilateral kidney IRI as demonstrated in Fig. 1. 48 h following the onset of reperfusion, serum creatinine (A) and serum urea (B) concentrations were measured and mice were sacrificed. Kidneys were PAS-stained and two representative magnifications are demonstrated in C and quantified using the tubular damage score D. Statistical analysis was performed using one-way ANOVA analysis (p-values are indicated for each experiment).
13 wild-type and 24 RubcnKO mice following cisplatin injection and detected a significantly shorter time to death in the RUBCN-deficient mice (Fig. S2C, \( p = 0.006 \)). In conclusion, these data indicate that RUBCN-deficient mice are sensitive to at least two different mouse models of acute kidney injury, and that this effect cannot be directly attributed to kidney tubules.

**Combined deficiency of RUBCN and MLKL partially overcomes the sensitization of RUBCN-deficient mice in kidney IRI**

Several signalling pathways of regulated necrosis have been implicated in the pathogenesis of AKI [22]. However, we considered it unlikely that ferroptosis is the driver of the sensitization of RubcnKO mice in AKI because renal tubules, in which ferroptosis predominates [23], were not hypersensitive. We therefore decided to investigate MLKL-mediated necroptosis and GSDMD-mediated pyroptosis as hypothetical candidate cell death pathways involved. MLKL-deficient mice, in our hands, were protected from kidney IRI [31]. Indeed, combined deficiency of RUBCN and MLKL partially reversed the significant increase in serum levels of creatinine (Fig. 2A) and urea (Fig. 2B). PAS staining of the kidney cortex is demonstrated in Fig. 2C and blinded assessment revealed less tubular damage in the Rubcn/MlklDKO mice compared to RubcnKO (Fig. 2D). This result suggested a role for necroptosis in the sensitization of RUBCN-deficient mice to AKI.

To assess a potential role of RUBCN in classical necroptosis in general, we pre-treated NIH-3T3 cells with siRNAs against Rubcn for 48 h (Fig. S3A) and stimulated them for an additional 8 h with TNFα and the pan-caspase inhibitor zVAD-fmk to induce necroptosis. As a protection control, we added the specific RIPK1 inhibitor necrostatin-1s (Nec-1s) [32]. As demonstrated in Fig. S3B, RUBCN knockdown did not affect necroptosis in cell culture. To test the potential role of RUBCN in regulating necroptosis in vivo, we intravenously injected female wild-type and RubcnKO mice with recombinant TNFα to induce the well-established necroptosis model of severe inflammatory response syndrome (SIRS) [33]. As demonstrated in Fig. S4, no statistically significant difference in overall survival was detected in the RUBCN-deficient mice. In conclusion, these data strongly suggest that RUBCN does not directly regulate necroptosis, but that a non-cell-autonomous effect mediated by necroptosis might explain the partial reversal of the AKI-sensitivity of the RUBCN-deficient mice.

To next test a potential role of pyroptosis, we crossed RubcnKO mice to GsdmdKO mice (see methods for details). Combined deficiency of RUBCN and GSDMD does not affect the sensitization of RUBCN-deficient mice in kidney IRI as assessed by serum concentrations of creatinine (Fig. S5A) or urea (Fig. S5B) and did not change the morphological appearance of tubular necrosis in histology (Fig. S5C, D).
Fig. 4  RUBCN-deficient female mice display splenic atrophy but no signs of autoimmunity at 50 weeks of age. A Male and female RUBCN-deficient mice fail to gain weight over their first 50 weeks of life compared to wild-type littermates (also see Fig. S6 for details). B In contrast to male mice, female mice show splenic atrophy at an age of 50 weeks as estimated by splenic weight. C Male and female wild-type mice and RUBCN-deficient littermates produce indistinguishable levels of autoantibodies against double-stranded DNA at 36 weeks and 50 weeks of age. Statistical analysis was performed one-way ANOVA analysis (* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \)).
Male RUBCN-deficient mice develop proteinuria and chronic kidney disease at 50 weeks of age while female RUBCN-deficient mice exhibit splenic atrophy

The detection of RubcnKO mice being sensitive to AKI raised the question of the spontaneous development of chronic kidney injury in these strains. To this end, we set up a one year follow up experiment with at least 10 mice per group of wild-type mice, RUBCN-deficient mice, Rubcn/MikKO mice, and Rubcn-GsdmdKO mice. This approach was independently followed up for both sexes. At 50 weeks of age, mice were sacrificed and serum creatinine, serum urea and urine protein-to-creatinine ratios were determined as demonstrated in Fig. 3. While both male and female RUBCN-deficient mice developed signs of chronic kidney injury (significant increases in serum urea levels), only male on a RUBCN-deficient background additionally presented significant proteinuria. This observation correlated with the failure to gain normal body weight in the RubcnKO and the Rubcn-GsdmdKO mice (Fig. S6A). Interestingly, and somewhat pheno-copying the partial reversal of the IRI phenotype, the failure to thrive of Rubcn/MikKO mice did not reach statistical significance (Fig. S6A). Chronic kidney disease on RUBCN-deficient background did not correlate with renal anemia or erythrocytopenia in the assessment of a standard blood count (Fig. S6B). In this blood test, we additionally observed an increased number of lymphocytes, exclusively in the female mice (Fig. S6B), while serum glucose levels remained unchanged (Fig. S6C). The female lymphocytosis, unexpectedly, correlated with splenic atrophy in all RUBCN-deficient conditions (Fig. 4B), while spleen weight was indistinguishable upon all male groups investigated. Finally, and unlike previously demonstrated, we did not detect a statistically significant increase in double-stranded DNA autoantibodies in the RUBCN-deficient male or female mice at 36 or 50 weeks of age (Fig. 4C). These data indicate that the sole explanation of autoimmunity as a conclusive cause of the failure to gain weight in the RubcnKO mice is not correct. In summary, we identify RUBCN-deficient mice as a potential model for the investigation of chronic kidney injury.

DISCUSSION

We provide evidence for RUBCN-deficient mice to be sensitive to kidney ischemia-reperfusion injury (IRI). We further demonstrate that pyroptosis-deficiency does not reverse this sensitization, while MLKL-deficiency does. As the knockdown of Rubcn does not affect a standard necroptosis assay, we conclude a non-cell-autonomous involvement of MLKL. Indeed, it was recently demonstrated that metabolites released from dying cells function as tissue messengers [34], and similar mechanisms are potentially involved in models of AKI. However, we previously demonstrated that MLKL-deficient mice are partially but significantly protected in the kidney IRI model and discussed a direct effect of necroptosis in kidney tubules [31]. It is beyond this investigation to identify the detailed mechanism of how RUBCN-deficiency triggers MLKL-mediated necroptosis in this setting, but it is important to realize that pyroptosis does not appear to be involved. It was recently demonstrated that deletion of rubicon from one specific compartment, the renal tubular epithelial cells, did not affect the outcome of kidney ischemia-reperfusion injury [35]. Despite different mouse strains used in that study compared to the one presented here, we interpret these data to be in keeping with our findings. As clear as the data on AKI in RUBCN-deficient and Rubcn/MikKO mice are, several open questions remain unanswered. In contrast to previous models, we did not detect signs for the development of autoimmunity in RubcnKO mice [29]. While we do see a failure to develop normal body weight in the RUBCN-deficient and the combined RUBCN/GSDMD-deficient male and female mice, we do not see elevated levels of antineutrophilic antibodies (ANAs) or autoantibodies against double-stranded DNA. Importantly, we detected lower spleen weight in RubcnKO female mice compared to control female mice, while no differences were observed in the males. Why RUBCN-deficient mice fail to gain normal body weight in comparison to littermates, therefore, remains an open question.

Another limitation of our study is the lack of explanation of the progressing chronic kidney disease in these mice. In particular, we observed higher urea levels in the RUBCN-deficient male and female mice, alongside an increase in urine protein-to-creatinine-ratio (proteinuria) exclusively in the male mice. We did not observe any typical signs of glomerulonephritis.

In conclusion, we present evidence for RubcnKO mice to be significantly sensitive to kidney IRI, an effect that is partially reversed on a combined MLKL-deficient background. In addition, we identify the RUBCN-deficient mice as mildly affected by chronic kidney disease over the first year of life, but we did not detect the development of a severe autoimmune phenotype.

DATA AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

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WT, SL, FM, CA, AvM, and LE performed experiments. SRB and DRG provided critical reagents. AL designed the experiments and wrote the paper.

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Competing interests

The authors declare no competing interests.

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