

Reduced Lon protease 1 expression in podocytes contributes to the pathogenesis of podocytopathy



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Emerging evidence has shown that mitochondrial dysfunction is closely related to the pathogenesis of podocytopathy, but the molecular mechanisms mediating mitochondrial dysfunction in podocytes remain unclear. Lon protease 1 is an important soluble protease localized in the mitochondrial matrix, although its exact role in podocyte injury has yet to be determined. Here we investigated the specific role of this protease in podocyte in glomerular injury and the progression of podocytopathy using podocyte-specific Lon protease 1 knockout mice, murine podocytes in culture and kidney biopsy samples from patients with focal segmental glomerular sclerosis and minimal change disease. Downregulated expression of Lon protease 1 was observed in glomeruli of kidney biopsy samples demonstrating a negative correlation with urinary protein levels and glomerular pathology of patients with focal segmental glomerular sclerosis and minimal change disease. Podocyte-specific deletion of Lon protease 1 caused severe proteinuria, impaired kidney function, severe kidney injury and even mortality in mice. Mechanistically, we found that continuous podocyte Lon protease 1 ablation induced mitochondrial homeostasis imbalance and dysfunction, which then led to podocyte injury and glomerular sclerosis. *In vitro* experiments implicated the kidney protective effect of Lon protease 1, which inhibited mitochondrial dysfunction and podocyte apoptosis. Thus, our findings suggest that the regulation of Lon protease 1 in podocytes may provide a novel therapeutic approach for the podocytopathy.

Kidney International (2021) **99**, 854–869; <https://doi.org/10.1016/j.kint.2020.10.025>

KEYWORDS: glomerular disease; Lonp1; mitochondrial dysfunction; podocyte; podocytopathy

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Received 9 March 2020; revised 26 September 2020; accepted 9 October 2020; published online 10 November 2020

Translational Statement

Mitochondrial dysfunction in podocytes contributes to glomerular disease progression, while the specific therapeutic targets to improve mitochondrial function in podocytes are still absent. Our data from the podocytopathy patients' kidney biopsy tissues demonstrated that the expression of Lon protease 1 (Lonp1) in glomeruli was negatively correlated with the severity of proteinuria and glomerular pathology. Further findings from *in vivo* and *in vitro* experiments suggested an important role of Lonp1 in protecting mitochondria, podocytes, and glomeruli. Taken together, the findings from this study not only provide new insights into the understanding of podocytopathy, but also offer a novel potential strategy for treating podocytopathy by targeting Lonp1.

Glomerular diseases are the main causes of chronic kidney disease (CKD) and renal failure.^{1–3} These diseases pose a challenge to public health and account for significant annual costs worldwide.⁴ Given the current lack of effective therapeutic strategies, there is an urgent need to explore novel targets for the treatment of glomerular diseases.

Podocytopathy serves as a common pathology of glomerular diseases characterized by podocyte injury and proteinuria.⁵ Podocyte injury leads to destruction of the filtration barrier and the development of glomerular diseases.⁶ Studies investigating methods to ameliorate podocyte injury have provided practical approaches for treating glomerular diseases.^{7–9} Podocytes are cells that consume large amounts of energy, in which mitochondria are important for maintaining structure and function.^{10,11} Recently, mitochondrial dysfunction has been implicated in glomerular disease and nephropathy progression.^{12,13} Our previous studies also indicated that mitochondrial dysfunction is an early event in podocyte injury, and that improving mitochondrial function using certain chemicals can ameliorate podocyte injury and proteinuria.^{14,15} However, the mechanism underlying the regulation of mitochondrial dysfunction and cell injury in podocytes has yet to be clarified.

Lon protease 1 (Lonp1), also called mitochondrial Lon protease, is an adenosine triphosphate (ATP)-dependent

protease that maintains mitochondrial metabolism by clearing abnormal proteins, repairing mtDNA, and serving as a chaperone.¹⁶ Recent studies indicate that Lonp1 is essential for maintaining mitochondrial proteostasis and function, and that its deletion or mutation results in the accumulation of insoluble proteins, mitochondrial dysmorphology, and reduced mtDNA expression.^{17–20} Improving mitochondrial function through regulation of Lonp1 is presumably quite important for treating various chronic and developmental diseases, including neurodegeneration, cardiac ischemia, heart failure, and cancer.^{21–24} Until now, research on Lonp1 in renal tissue has been limited to the assessment of changes in its expression at the protein level *in vitro*, which showed decreased expression in injured human renal tubular epithelial cells and embryonic kidney 293T cells.^{25,26} Initially, we analyzed the gene expression of Lonp1 in human renal glomeruli according to an online dataset of human kidney single-cell sequencing (<http://humphreyslab.com/SingleCell/>) and found that Lonp1 was expressed almost in all kinds of kidney cells, with the greatest expression in podocytes. The exact role of Lonp1 in the podocytes and kidneys remains unknown, however.

Previous studies have shown that global deficiency of Lonp1 in mice causes early embryonic lethality.²⁷ In this study, using podocyte-specific Lonp1 knockout (conditional knockout [cKO]) mice and *in vitro* mouse podocyte and human renal biopsy samples, we demonstrated that Lonp1 is downregulated in the glomeruli of patients with focal segmental glomerular sclerosis (FSGS) and minimal change disease (MCD) and shows an inverse association with urine protein levels and glomerular pathology in patients. Evidence from *in vivo* and *in vitro* studies indicates that continuous ablation of Lonp1 in podocytes plays a pathogenic role in mediating podocyte and glomerular injury by inducing an imbalance in and dysfunction of mitochondrial homeostasis. Our findings reveal a novel target for treating glomerular diseases and podocytopathy.

RESULTS

Patients with FSGS and MCD exhibited reduced expression of Lonp1 in podocytes

A costaining of Lonp1 with a podocyte-specific nuclear protein Wilms' tumor 1 gene (*WT1*)²⁸ in the normal human kidney tissue showed the predominant cytoplasmic localization of Lonp1 in podocytes (Figure 1a). MCD and FSGS are the common clinical forms of podocytopathy.²⁹ Histopathological examination of these patients revealed segmental sclerosis in glomeruli and/or features of significant podocyte injury, including podocyte vacuolar degeneration and extensive fusion of the podocyte foot process (Supplementary Figure S1A and C). Furthermore, we detected the expression of Lonp1 using renal biopsy specimens from patients with FSGS and MCD, and the paracarcinoma tissues from patients with renal hamartoma without nephropathy were used as a control. Histological analysis showed significantly reduced Lonp1 expression in the glomeruli of patients with

MCD and FSGS, by 59% and 70.3%, respectively ($P < 0.001$ for both MCD and FSGS; Figure 1b and c), and linear regression analysis indicated a negative correlation between Lonp1 expression and urine protein levels, as well as the degree of glomerular pathology (Figure 1d and e; Supplementary Figure S1B and D), which piqued our interest in exploring the specific role of Lonp1 in glomerular podocytes.

Podocyte-specific loss of Lonp1 impairs renal function and mouse survival

To generate podocyte-specific Lonp1 knockout mice, Lonp1-floxed (Lonp1^{fl/fl}) mice were crossed with podocin-Cre mice to generate podocyte-specific Lonp1 knockout mice (Npsh2-Lonp1 KO, designated Lonp1-cKO). Lonp1^{fl/fl} littermates with no Cre expression served as controls (Supplementary Figure S2A). Podocyte-specific Lonp1 deficiency was confirmed by detecting Lonp1 expression in primary glomerular podocytes derived from cKO and control mice. Primary podocytes were identified by the positive expression of the known podocyte marker podocin (Supplementary Figure S2B). Lonp1 expression in podocytes from cKO mice revealed significantly reduced protein and mRNA levels (Supplementary Figure S2C and D). In contrast, Lonp1 expression in the cortex showed no significant difference between control and cKO mice (Supplementary Figure S2D). These data demonstrate that podocyte-specific Lonp1 knockout mice were successfully established.

We found that the Lonp1-cKO mice began to die at 3 weeks after birth, and almost all died after 5 weeks (Figure 2a). Blood urea nitrogen (BUN) and serum creatinine levels in cKO mice at age ≥ 4 weeks were significantly increased, indicating severe renal dysfunction in the Lonp1-cKO mice (Figure 2b and c). In addition, the 4-week-old mice developed significant albuminuria, as shown by the urinary albumin:creatinine ratio and semiquantitative measurement of urine protein levels using Coomassie blue staining (Figure 2d and e). Renal hematoxylin and eosin and Masson trichrome staining consistently revealed remarkable pathological glomerular damage, interstitial fibrosis, and features of tubular injury, including tubular atrophy, loss of brush border, and cast formation, along with increased expression levels of tubular injury markers of KIM-1 and NGAL compared with the control mice (Figure 2f and Supplementary Figure S3). However, some other vital organs, such as the lung, liver, and heart, showed no significant pathological changes compared with control mice (Figure 2g). Taken together, these findings indicate a specific and critical role of Lonp1 in podocytes in maintaining normal kidney function *in vivo*.

Podocyte-specific loss of Lonp1 impairs renal function with age

We measured the body weight of the mice over a 4-week period and found progressive weight loss and significantly lower weights in Lonp1-cKO mice compared with control

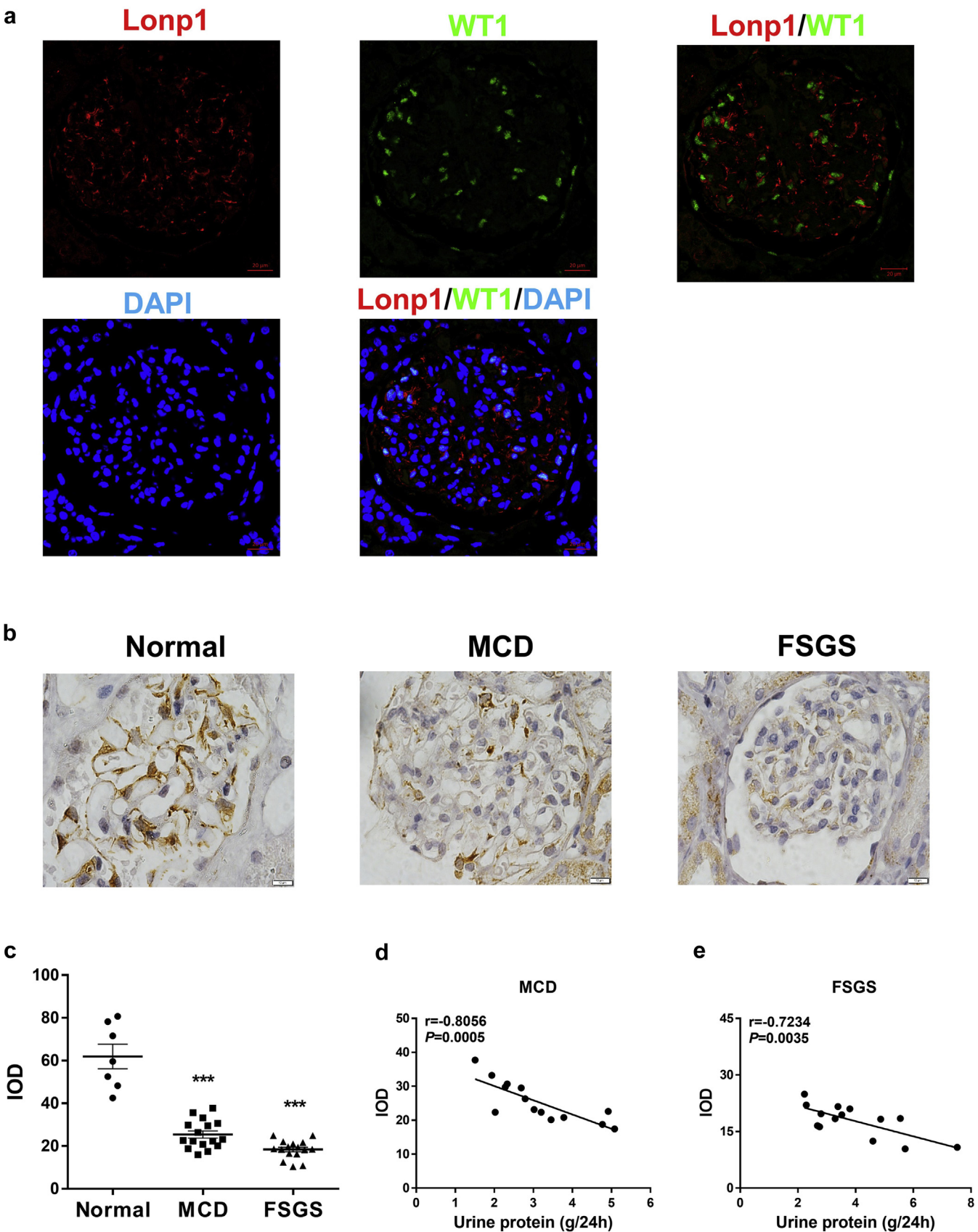


Figure 1 | Glomerular Lon protease 1 (Lonp1) expression is associated with human proteinuria. (a) Localization of Lonp1 predicted by immunofluorescence staining using human normal paracarcinoma kidney tissue. Lonp1 (red), WT1 (green), DAPI (blue), (continued)

mice at 4 weeks of age (Figure 3a). BUN was also measured in plasma samples from mice at 14, 21, 25, and ≥ 28 days. There was no significant difference in BUN levels in cKO mice and controls at 14 and 21 days. However, after 21 days, BUN levels were significantly increased compared with those in the control littermates ($P < 0.05$ for 25 days and $P < 0.001$ for ≥ 28 days; Figure 3b), indicating impaired renal function in the Lonp1-cKO mice. Histologically, as shown in Figure 3c, significantly increased segmental or complete glomerular sclerosis was observed in 3- to 4-week-old mice. These findings suggest that mice with podocytes lacking Lonp1 might sustain progressive renal damage after birth.

Podocyte-specific loss of Lonp1 induces podocyte injury with age

The histological results showed progressive damage in the glomeruli of Lonp1-cKO mice; normal morphological glomeruli were observed in mice age 1–2 weeks, whereas focal glomerular sclerosis was seen at age 3 weeks. In week 4, striking glomerular damage with shrinkage and focal necrosis were observed. Given that podocytes are highly involved in glomerular sclerosis, we next focused on podocytes in the Lonp1-cKO mice. Immunofluorescence analysis showed that for up to 2 weeks after birth, the glomerular podocytes of the cKO mice were indistinguishable from those of control mice, as shown by the expression of glomerular slit diaphragm proteins podocin and synaptopodin, whereas 3- to 4-week-old cKO mice exhibited significantly decreased expression levels of podocin and synaptopodin in the glomeruli (Figure 4a and b), indicating damaged podocytes. Meanwhile, the number of WT1-positive podocytes was also decreased in 3- to 4-week-old mice (Figure 4c). Furthermore, Western blot analysis confirmed reduced expression of another podocyte marker, nephrin, in cKO mice at age 3–4 weeks (Supplementary Figure S4). Moreover, terminal deoxynucleotidyl transferase dUTP nick-end labeling/podocin double-labeled cells were observed in the glomeruli of the Lonp1-cKO mice (Supplementary Figure S5), indicating that Lonp1 depletion induced podocyte apoptosis *in vivo*. Transmission electron microscopy (TEM) analysis consistently revealed extensive degeneration of cell bodies and foot process fusion in podocytes of Lonp1-cKO mice at 3 and 4 weeks after birth (Figure 5e). Therefore, we hypothesized that podocyte-specific loss of Lonp1 induces podocyte injury at an early age in mice and later results in renal dysfunction.

Podocyte-specific loss of Lonp1 causes mitochondrial dysfunction in podocytes with age

Because our previous studies indicated that mitochondrial dysfunction mediates podocyte injury,¹⁵ we examined

mitochondrial function in renal podocytes of Lonp1-cKO mice. Expression of mitochondrial-encoded genes in renal tissues of the cKO mice at approximately 4 weeks of age revealed abnormal changes, and mRNA levels of multiple genes were reduced (Figure 5a). In addition, a reduction in mitochondrial genomic DNA content was confirmed by quantitative polymerase chain reaction, which was significant in 4-week-old mice ($P = 0.16$ at 3 weeks, $P < 0.01$ at 4 weeks; Figure 5b). Mitochondrial ATP production in primary podocytes at 4 weeks was 76.8% lower in the Lonp1-cKO mice compared with the control mice (Figure 5c). The levels of proteins involved in the mitochondrial respiratory chain complex were significantly decreased in primary podocytes from the Lonp1-cKO mice (Figure 5d).

Morphological damage in mitochondria was visualized by TEM. As shown in Figure 5e, there was striking evidence of mitochondrial damage, including mitochondrial disarray, degeneration and fragmentation, in the cKO mice at age 3 and 4 weeks. Notably, at age 2 weeks, just before significant morphological abnormalities of podocytes were observed in the cKO mice, impaired mitochondria were found in podocytes (Figure 5e). These results suggest that continuous depletion of Lonp1 in podocytes induced mitochondrial dysfunction, which resulted in podocyte injury.

Lonp1 silencing induces podocyte apoptosis and mitochondrial dysfunction *in vitro*

To confirm this phenomenon *in vivo*, a mouse podocyte cell line and Lonp1 RNA interference were used to knock down Lonp1 expression in podocytes. The effects of the RNA interference vectors (shLonp1-01 and shLonp1-02) were examined after podocytes were transfected for 48 hours (Figure 6a). As shown in Figure 6b, the percentage of annexin V-positive cells was higher in Lonp1-knockdown podocytes than in negative controls, indicating that Lonp1 silencing induced podocyte apoptosis.

Next, several independent parameters were used to evaluate mitochondrial function *in vitro*, including the production of total reactive oxygen species (ROS) and mitochondrial superoxide (indicating mitochondrial ROS [mtROS]), the mitochondrial membrane potential, the mtDNA copy number, and oxygen consumption rate (OCR). As shown in Figure 6c and d, total ROS and mtROS production were significantly increased in the Lonp1-knockdown podocytes. In addition, tetramethylrhodamine, methyl ester fluorescence, which indicates the mitochondrial membrane potential level, was significantly decreased ($>30\%$ reduction; Figure 6e). Similarly, Lonp1 knockdown resulted in reductions in the

Figure 1 | (continued) and merged image. Bbar = 20 μm . (b) Representative images of Lonp1 expression in glomeruli. Bar = 10 μm . (c) Scatterplot showing the average integrated optical density values of Lonp1 in paracarcinoma kidney ($n = 7$) and kidney tissue biopsy samples from patients with minimal change disease (MCD) ($n = 16$) and patients with focal segmental glomerular sclerosis (FSGS) ($n = 16$). (d,e) Correlation between 24-hour urine protein level and glomerular Lonp1 expression in kidneys from patients with MCD (d; $n = 14$) and patients with FSGS (e; $n = 14$). Data are presented as mean \pm SEM. *** $P < 0.001$ compared with the normal group. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

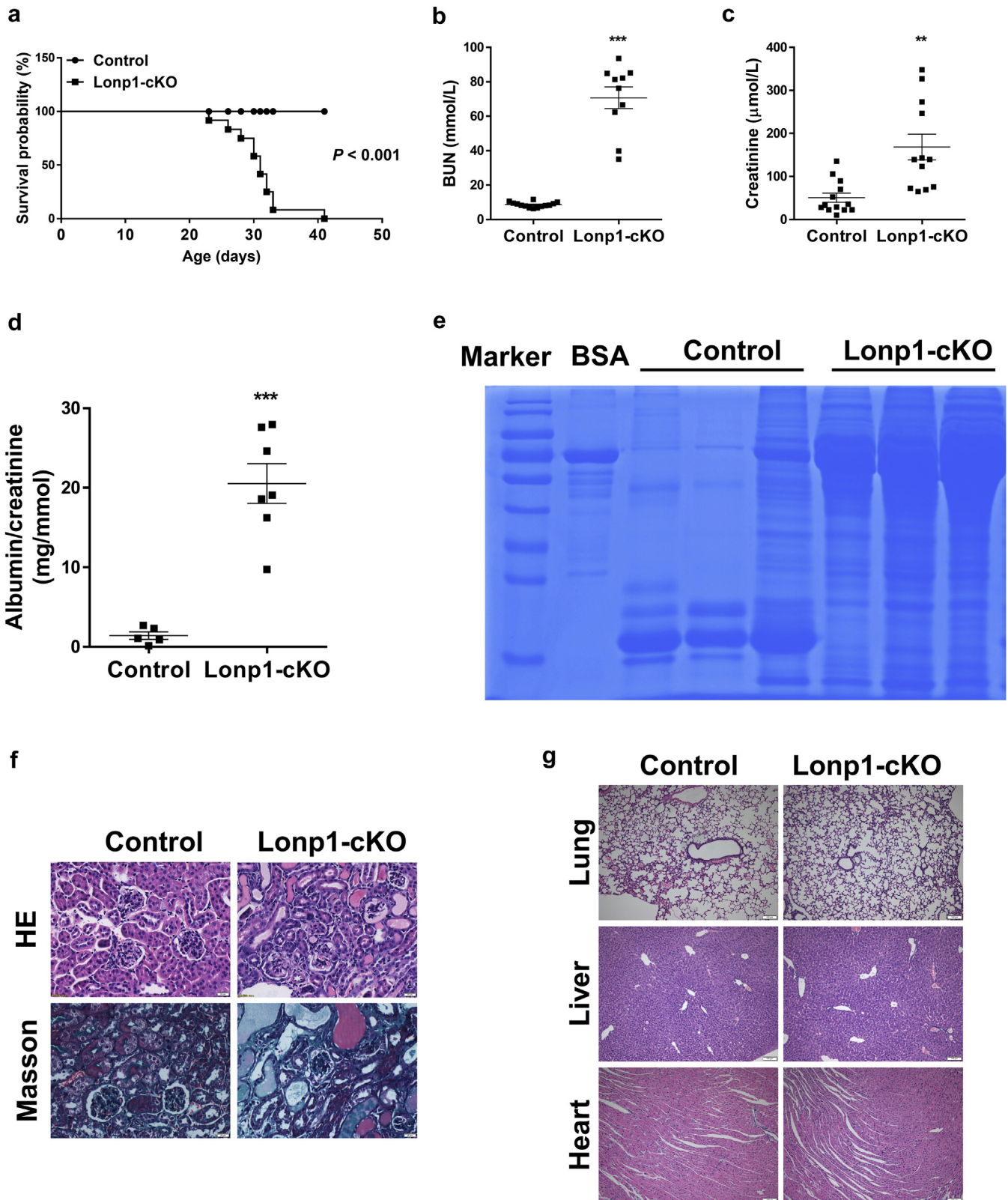


Figure 2 | Podocyte-specific Lon protease 1 (Lonp1) knockout causes albuminuria, impaired renal function, and reduced survival in mice. (a) The survival rates of control and podocyte-specific Lonp1 knockout (cKO) mice. Both control and cKO mice were collected at approximately age 4 weeks, and the serum, urine, kidney, lung, liver and heart tissues were used for further study. (b) Blood urea nitrogen (BUN) levels ($n = 10-14$). (c) Serum creatinine levels ($n = 12-13$). (d) Albumin:creatinine ratios in random urine samples ($n = 5-7$). Data are presented as mean \pm SEM. ** $P < 0.01$; *** $P < 0.001$ compared with control. (e) Coomassie blue staining of representative urine samples ($n = 3$) by sodium dodecyl sulfate polyacrylamide gel electrophoresis. (f) Representative hematoxylin and eosin (H&E)- and Masson (continued)

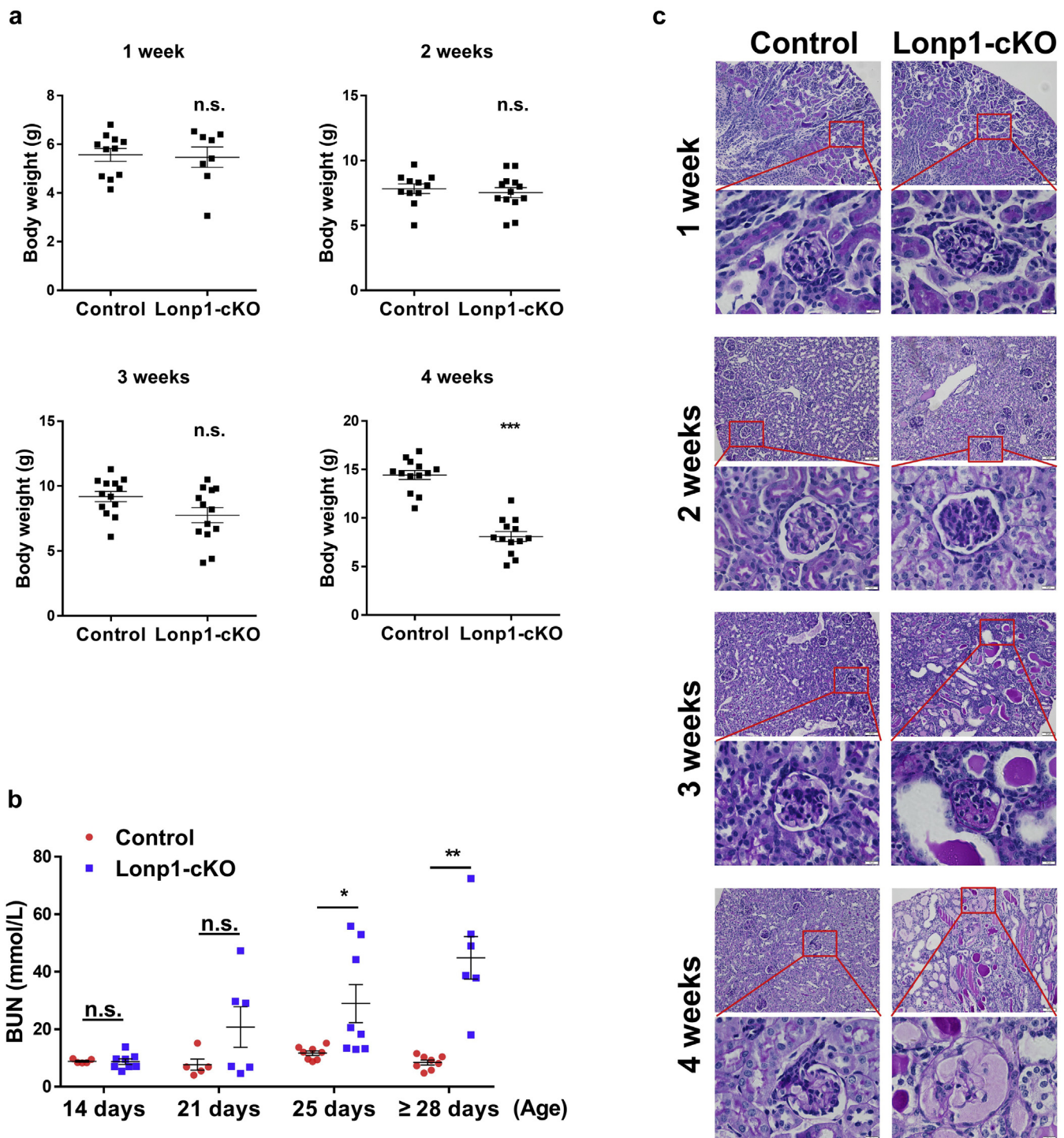


Figure 3 | Podocyte-specific Lon protease 1 (Lonp1) knockout impairs renal function with age. (a) Body weight of mice at the indicated ages ($n = 8-13$). (b) Blood urea nitrogen (BUN) levels in mice at the indicated ages ($n = 5-8$). Data are presented as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with controls. (c) Representative periodic acid Schiff-stained renal sections showing the development of renal injury (bar = 50 μ m) and glomerular enlargement with lesions (bar = 10 μ m). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 2 | (continued) trichrome-stained sections showing renal pathological damage and fibrosis. Bar = 20 μ m. (g) Representative H&E-stained sections of lung, liver, and heart tissue from control and cKO mice. Bar = 100 μ m. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

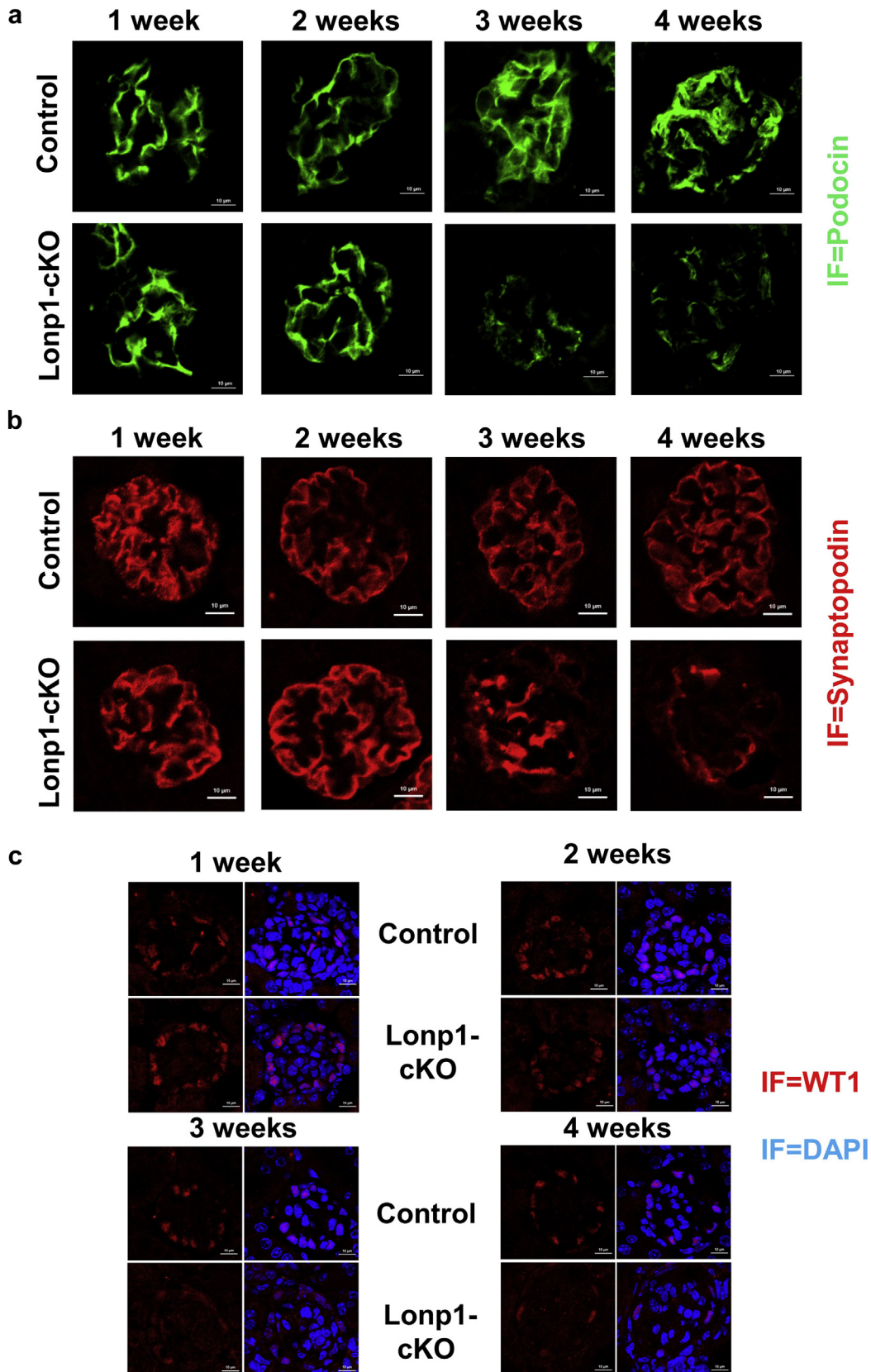


Figure 4 | Podocyte-specific Lon protease 1 (Lonp1) knockout induces podocyte injury with age. (a,b) Representative immunofluorescence images of podocin (a, green) or synaptopodin (b, red) staining in renal sections from control and podocyte-specific Lonp1 knockout (cKO) mice at the indicated ages (labeled). (c) Representative images of immunofluorescence staining of WT1 (left, red) and DAPI (blue, shown in the right merged image) in renal sections from control and cKO mice at the indicated ages. Bar = 10 μ m. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

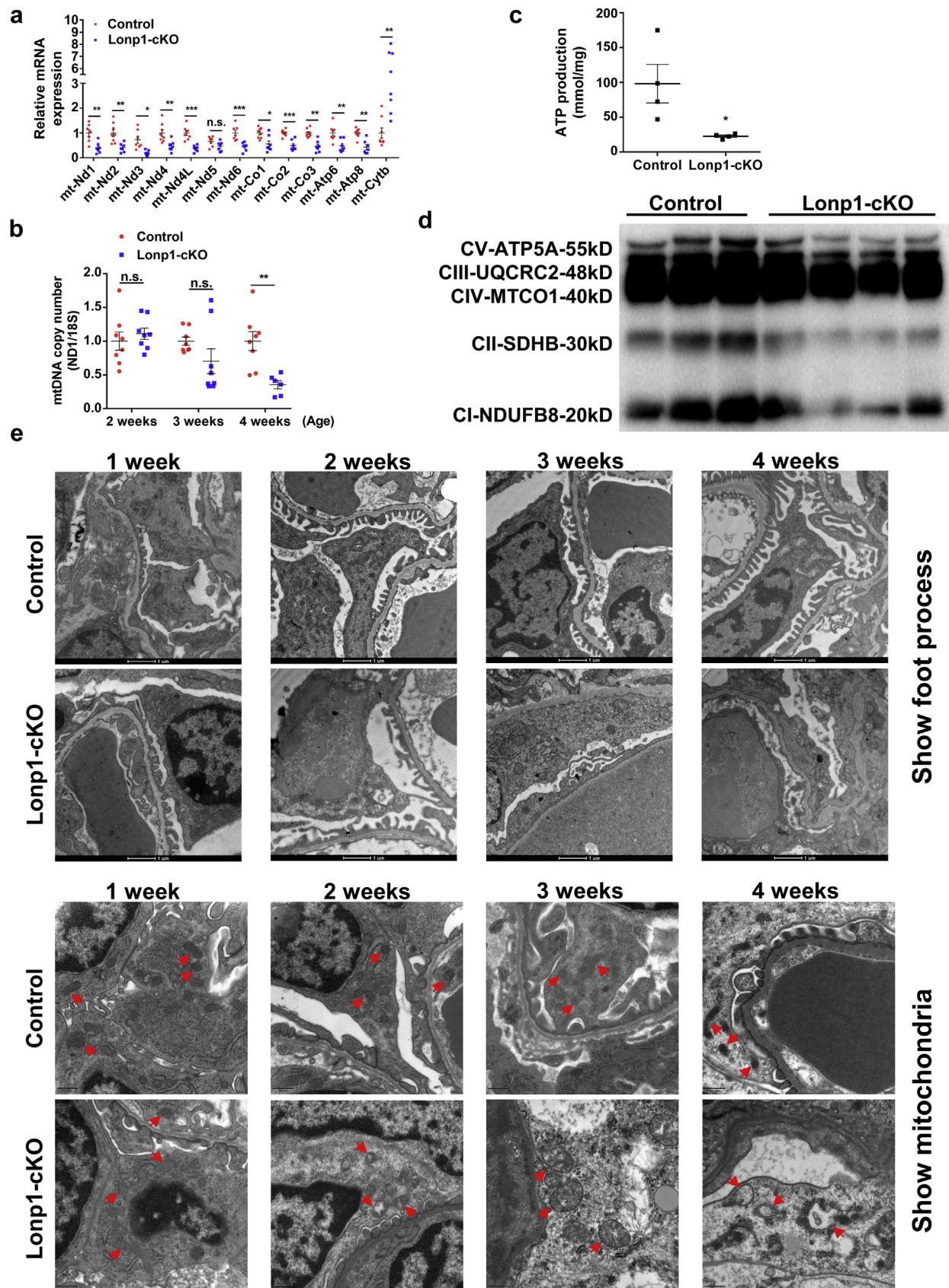


Figure 5 | Podocyte-specific Lon protease 1 (Lonp1) knockout induces mitochondrial dysfunction in podocytes. (a) Expression of genes encoded by the mitochondrial genome in renal tissues from 4-week-old mice ($n = 7-8$). (b) Renal mitochondrial DNA (mtDNA) (continued)

mtDNA copy number and OCR (Figure 6f–h). These data showed notable mitochondrial dysfunction in Lonp1-knockdown podocytes.

We next performed a proteomic study using mitochondria from Lonp1-knockdown podocytes and a negative control. Mass spectrometry analysis identified the proteins from mitochondria and grouped them according to their cellular location and biological functions. As shown in Figure 7a, mitochondrial proteins that were classified into various groups according to biological function, including ATP production, the respiratory chain, and mitochondrial transport, were downregulated. Because podocytes began to undergo apoptosis when transfected with sh-Lonp1 for 48 hours, another proteomic study was performed using mitochondria from podocytes transfected with sh-Lonp1 for 24 hours. Gene Ontology (GO) enrichment analysis revealed that the most significantly changed cell functions were involved mainly in the oxidation reduction process, metabolic process, and regulation of ATP synthesis (Figure 7b). These data suggest an imbalance of mitochondrial homeostasis in Lonp1-deficient podocytes.

Overexpression of Lonp1 protects against mitochondrial dysfunction and podocyte apoptosis

Puromycin aminonucleoside (PAN) is an analog of puromycin commonly used to induce podocyte injury and nephropathy models.^{30,31} Here we explored the effect of overexpressing Lonp1 in PAN-treated podocytes. First, a Lonp1-overexpressing vector with a Flag tag was established, and the effect of transfection was detected Western blot analysis with a Flag antibody (Figure 8a). Lonp1 overexpression ameliorated PAN-induced podocyte apoptosis, as the apoptotic cell numbers and the expression of an apoptotic marker, cleaved caspase-3, were both reduced in Lonp1-overexpressing podocytes after PAN treatment (Figure 8b and c).

Furthermore, the reductions in the mitochondrial membrane potential, mtDNA copy number, and OCR and the upregulation of total ROS and mtROS induced by PAN were significantly alleviated by Lonp1 overexpression (Figure 8d–h). Finally, we also found that the beneficial effect of prednisolone, a potent glucocorticosteroid used in the clinical treatment of FSGS, MCD, and other types of nephrotic syndromes,³² against PAN-induced podocyte injury was possibly associated with enhanced Lonp1 activity and mitochondrial protection (Supplementary Figure S6). These results demonstrate that Lonp1 might serve as a target for protecting against mitochondrial dysfunction and podocyte apoptosis.

DISCUSSION

Here we report the downregulation of Lonp1 expression in glomeruli in renal biopsy specimens from patients with FSGS and MCD and the negative correlation between Lonp1 expression and urine protein levels in patients. We provide *in vivo* evidence showing that Lonp1 deletion in podocytes triggered severe podocyte injury, resulting in proteinuria, glomerular sclerosis, and interstitial fibrosis. Our *in vitro* data demonstrate the critical function of Lonp1 in mitochondria and podocytes, as Lonp1 knockdown in podocytes caused mitochondrial dysfunction and podocyte apoptosis, whereas Lonp1 overexpression repaired similar injuries induced by PAN. Our findings indicate that Lonp1 in podocytes is a potential therapeutic target for the amelioration of podocyte and glomerular injury.

Globally, glomerular diseases have been considered the main cause of CKD.² According to clinical data from China, 61.7%–68.6% of renal biopsy specimens obtained from hospitalized patients resulted in a diagnosis of primary glomerulonephritis.³³ In the USA and UK, glomerular diseases are the third-leading cause of end-stage renal disease.^{34,35} Podocytopathy is the most common type of glomerular disease. A primary injury to podocytes can cause MCD and FSGS.³⁶ Podocytes are essential components of the filtration barrier and have a vital role in maintaining the integrity of the glomerular filtration barrier. As highly specialized, terminally differentiated visceral epithelial cells, podocytes largely do not proliferate and are lost once they are damaged, resulting in destruction of the filtration barrier and the occurrence of proteinuria.^{6,37} Recent studies have demonstrated that podocyte injury plays a fundamental role in the development of glomerular disease, and strategies to ameliorate podocyte injury have proven as effective as therapy for proteinuria in experimental animals.^{38–40} Clinically, however, no specific drugs for the treatment of podocyte injury have been identified to date, and novel treatment targets for protecting podocytes are still urgently needed.

Theoretically, podocytes consume large amounts of energy because of their numerous complex foot processes. Experimental evidence has shown the presence of numerous mitochondria in the podocyte cell body and even in the narrow peripheral foot processes.⁴¹ We and others have suggested that mitochondria play an essential role in maintaining the normal structure and function of podocytes and are key targets for preventing or treating experimental podocyte injury *in vivo* and *in vitro*.^{15,42–46}

Figure 5 | (continued) was assessed by quantitative polymerase chain reaction, and the number of mitochondrial genome copies (detected by the ND1 primer) were normalized to 18S ribosomal RNA ($n = 6–8$). (c) Measurement of adenosine triphosphate levels in primary podocytes from control and podocyte-specific Lonp1 knockout (cKO) mice ($n = 4$). Data are presented as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with controls. (d) Mitochondrial OXPHOS proteins (CV-ATP5A, CIII-UQCRC2, CIV-MTCO1, CII-SDHB, and CI-NDUFB8) in primary podocytes from control and cKO mice. Equal loading of total proteins on the gel for each sample. (e) Transmission electron microscopy analysis of kidney tissues from control and cKO mice at the indicated ages. The representative images show foot process (bar = 1 μ m) and podocyte mitochondria (red arrows) (bar = 500 nm). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

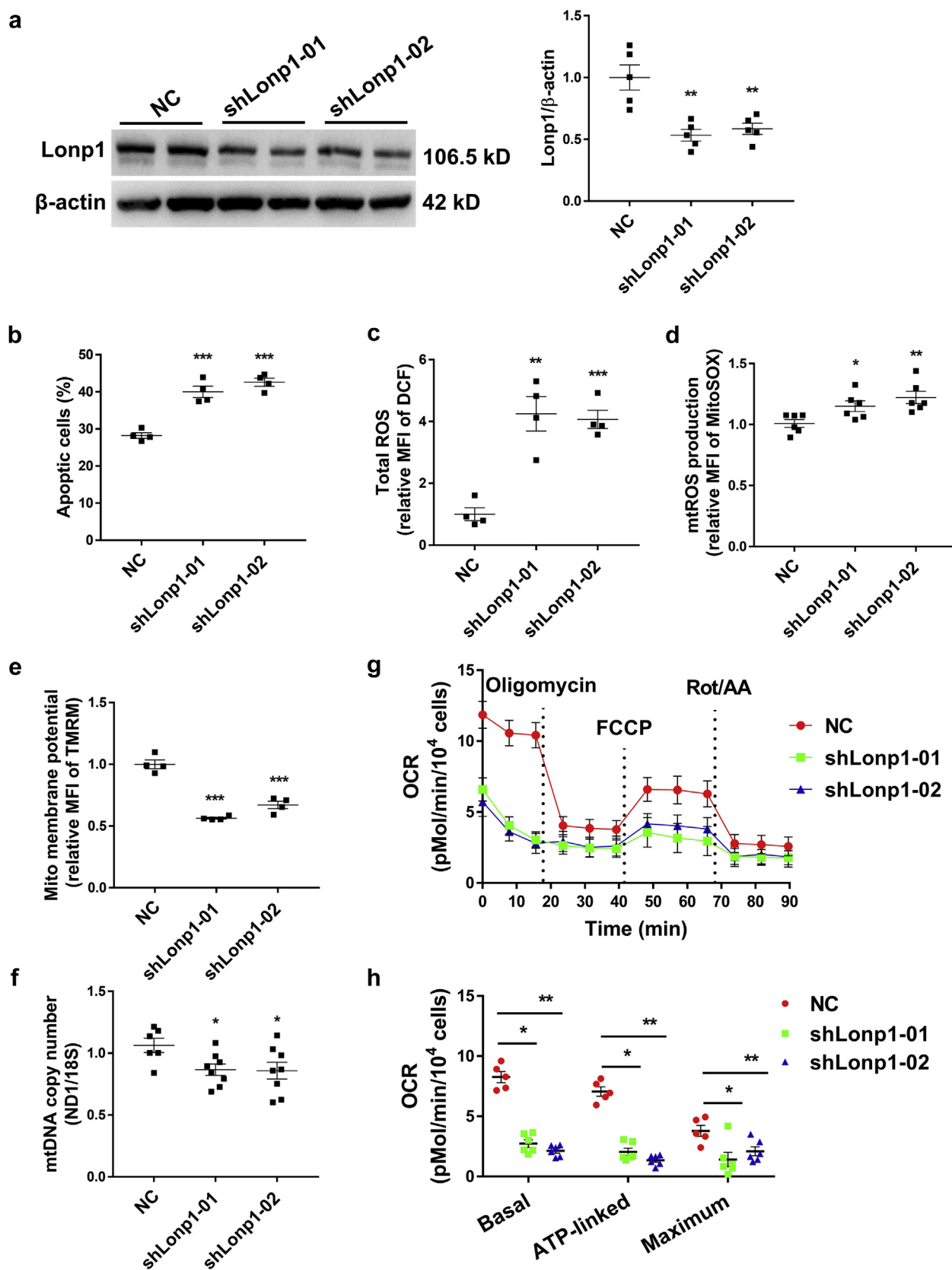


Figure 6 | Lon protease 1 (Lonp1) knockdown induces podocyte injury *in vitro*. Mouse podocytes were transfected with negative control vector (NC) and 2 kinds of shRNA vectors for Lonp1 (shLonp1-01 and -02) for 48 hours. (a) Representative Western blots ($n = 2$ for each group shown) of Lonp1 and β -actin and a scatterplot showing the densitometry results for Lonp1 ($n = 5$). (b) Apoptotic cells were (continued)

Lonp1 is reported to be abundantly expressed in metabolically active organs, such as the adrenal glands, kidneys, fat, and thyroid.⁴⁷ It is an essential enzyme that maintains mitochondrial metabolism and serves as a protein chaperone and a major regulator involved in mtDNA replication. Several studies have suggested that Lonp1 mutation and/or dysregulation could result in abnormal mitochondrial morphology, decreased respiratory capacity, and disruption of the mitochondrial redox balance, all of which are involved in the pathological mechanisms underlying CODAS syndrome and heart disease.^{23,24} In this study, using biopsied kidney tissues from patients with FSGS and MCD and paracarcinoma kidney tissue from patients with renal carcinoma, we first reported the localization of Lonp1 in podocytes and the prominent downregulation of Lonp1 in the glomeruli of patients with podocytopathy. Furthermore, a negative correlation between Lonp1 expression and urine protein levels and glomerular pathology was observed in patients with podocytopathy, suggesting the key role of Lonp1 in podocyte injury. Based on our results from a clinical study, we hypothesized that decreased Lonp1 expression in renal glomeruli might mediate podocytopathy and glomerular diseases.

We generated podocyte-specific Lonp1-cKO mice to explore the role of Lonp1 in podocyte injury. We found that these cKO mice were born with a normal phenotype, indicating that deletion of Lonp1 in podocytes did not affect normal development. However, abnormal mitochondria were observed in podocytes of cKO mice at 2 weeks after birth, and significant podocyte injury occurred in mice age ≥ 3 weeks. Consistently, visible focal glomerular sclerosis and increased BUN levels were found in 3-week-old mice. Severe renal dysfunction developed at 4 weeks after birth, as evidenced by worsening podocyte injury, complete sclerosis in glomeruli, significantly elevated levels of BUN and serum creatinine, proteinuria, and interstitial fibrosis. These data suggest the important role of Lonp1 in podocyte function.

Furthermore, tubular injury was also observed in the cKO mice at age 4 weeks, as evidenced by the impaired histopathology and increased expression levels of tubular injury markers KIM-1 and NGAL. Given that these 4-week-old cKO mice also developed severe podocyte injury and massive proteinuria, the tubular injury might be caused by damaged podocyte and the overloaded urinary protein. In fact, the interaction between intrinsic renal cells such as podocytes and tubular cells has been identified as a common pathway in the

kidney disease.^{48–51} For example, podocyte injury and apoptosis could lead to damage of the glomerular filtration barrier, resulting in proteinuria and subsequent tubular injury.⁵² In addition, other mediators, such as inflammatory factors and exosomes produced from damaged podocytes, could also induce tubulointerstitial inflammation and fibrosis.³⁶ Our findings demonstrate the specific contribution of Lonp1 to podocyte function and show that continuous Lonp1 ablation in podocytes could progressively cause glomerular damage and tubular injury.

We further explored the regulation of mitochondrial function in podocytes by Lonp1. Our *in vivo* studies emphasize the importance of Lonp1 in the maintenance of mitochondrial function, as significant mitochondrial damage, including mtDNA copy number depletion, abnormal changes in mitochondrial-encoded genes, reductions in ATP production and respiratory chain complex expression, and abnormal morphology, were observed in the podocytes of Lonp1-cKO mice. The *in vitro* data also demonstrate that downregulation of Lonp1 alone in cultured mouse podocytes directly induced mitochondrial dysfunction by increasing total ROS and mtROS and decreasing the mitochondrial membrane potential, OCR, and mtDNA copy number. Furthermore, knockdown of Lonp1 ultimately induced podocyte apoptosis. Accordingly, similar damage in podocytes induced by PAN was significantly alleviated by Lonp1 overexpression, indicating the renoprotective effects of Lonp1. Recently, several specific compounds directly or indirectly targeting Lonp1 have shown improvements in mitochondrial function. For example, SRT1720, a small-molecule activator of sirt1, has been reported to attenuate high-glucose-induced neurotoxicity by Lonp1 induction *in vitro*.⁵³ Inhibition of Lonp1 proteolytic activity by CDDO-Me or Obtusilactone A could greatly induce cytotoxicity and suppress cell proliferation in cancer cells.^{22,54–56} Moreover, we found that the beneficial effect of a glucocorticoid steroid prednisolone against PAN-induced podocyte injury was possibly associated with the enhanced Lonp1 activity and mitochondrial protection. The successful development of medications targeting Lonp1 and our data indicate that decreased Lonp1 expression in podocytes might be a mediator of mitochondrial dysfunction and cell apoptosis and thus may serve as a novel target for treatment of podocytopathy.

To clarify the mechanism of Lonp1 in podocytes involved in regulating mitochondrial function, we determined proteomic expression in mitochondria in Lonp1-knockdown

Figure 6 | (continued) detected using double staining with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI), and a quantitative analysis of apoptotic cells (FITC-annexin V-stained; $n = 4$) was performed. (c) Quantification of the mean fluorescence of DCF (dichlorodihydrofluorescein diacetate; indicating total reactive oxygen species [ROS]; $n = 4$). (d) Quantification of the mean fluorescence of mitoSOX (indicating mitochondrial ROS [mtROS]; $n = 6$) using flow cytometry. (e) Quantification of the mean tetramethylrhodamine, methyl ester (TMRM) fluorescence using flow cytometry showing the mitochondrial membrane potential ($n = 4$). (f) Quantitative polymerase chain reaction analysis of the mitochondrial DNA copy number ($n = 6–8$). (g,h) Cellular respiration assays ($n = 5–6$). (g) Mitochondrial respiration shown as oxygen consumption rate (OCR). (h) Quantification of other respiratory parameters (basal OCR, ATP-linked OCR, and maximum OCR). FCCP, p-trifluoromethoxyphenylhydrazone; Rot, rotenone; AA, antimycin A. Data are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$ compared with the NC group.

a

Functional categories	Fold (sh-Lonp1/NC)	Biological process associated
Mitochondrial metabolism & energy		
Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial (ETFDH)	0.756	Electron transfer and ATP production
Acyl-coenzyme A synthetase, mitochondrial (ACSM1)	0.827	Involved in respiratory chain
Glyoxylate reductase/hydroxypyruvate reductase (Grhpr)	0.770	Convert glyoxylate into the less reactive glycolate
Propionyl-Coenzyme A carboxylase alpha polypeptide (Pcca)	0.743	Catalyse the carboxylation reaction of propionyl CoA
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial (Ndufa10)	0.781	Subunit of Complex I of the respiratory chain
Sarcosine dehydrogenase, mitochondrial (SARDH)	0.810	Catalyze the demethylation of sarcosine to make glycine
mtDNA & mitochondrial stability		
Succinate--CoA ligase [GDP-forming] subunit beta, mitochondrial (SUCLG2)	0.655	Maintain mtDNA content and respiratory chain complexes activity
Mitochondrial carrier		
Sideroflexin-1 (Sfxn1)	0.824	Transport for iron into or out of the mitochondria
Unclassified		
RAB32 member RAS oncogene family (Rab32)	0.811	Acts as an A-kinase anchoring protein by binding to the type II regulatory subunit of protein kinase A and anchoring it to the mitochondrion. Also involved in synchronization of mitochondrial fission
Translation initiation factor IF-2, mitochondria (MTIF2)	0.811	Catalyzing the binding of initiation tRNA to mitochondrial 28S ribosomes, initiate of protein synthesis.

b

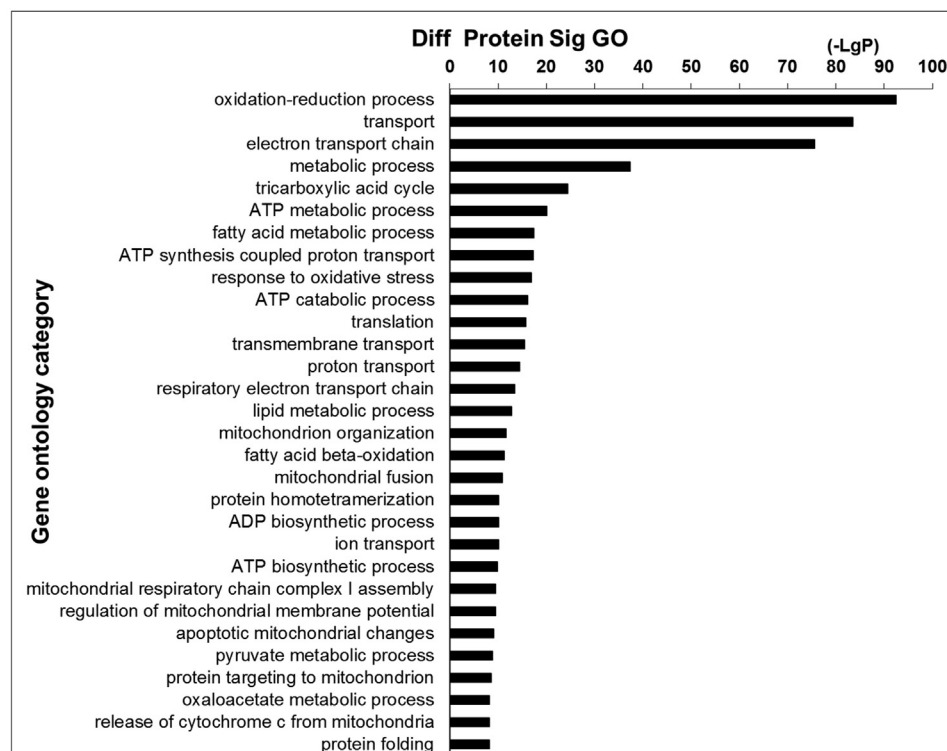


Figure 7 | Proteomic analysis of the mitochondria-enriched fraction from Lon protease 1 (Lonp1)-knockdown podocytes. Mouse podocytes were transfected with the negative control vector (NC) or shRNA vector for Lonp1 (shLonp1-01) for 48 hours (a) or 24 hours (b), after which mitochondria were extracted for proteomic analysis with mass spectrometry. (a) Functional categories of the changed Lonp1-associated mitochondrial proteins. (b) Functional analyses were generated via Gene Ontology.

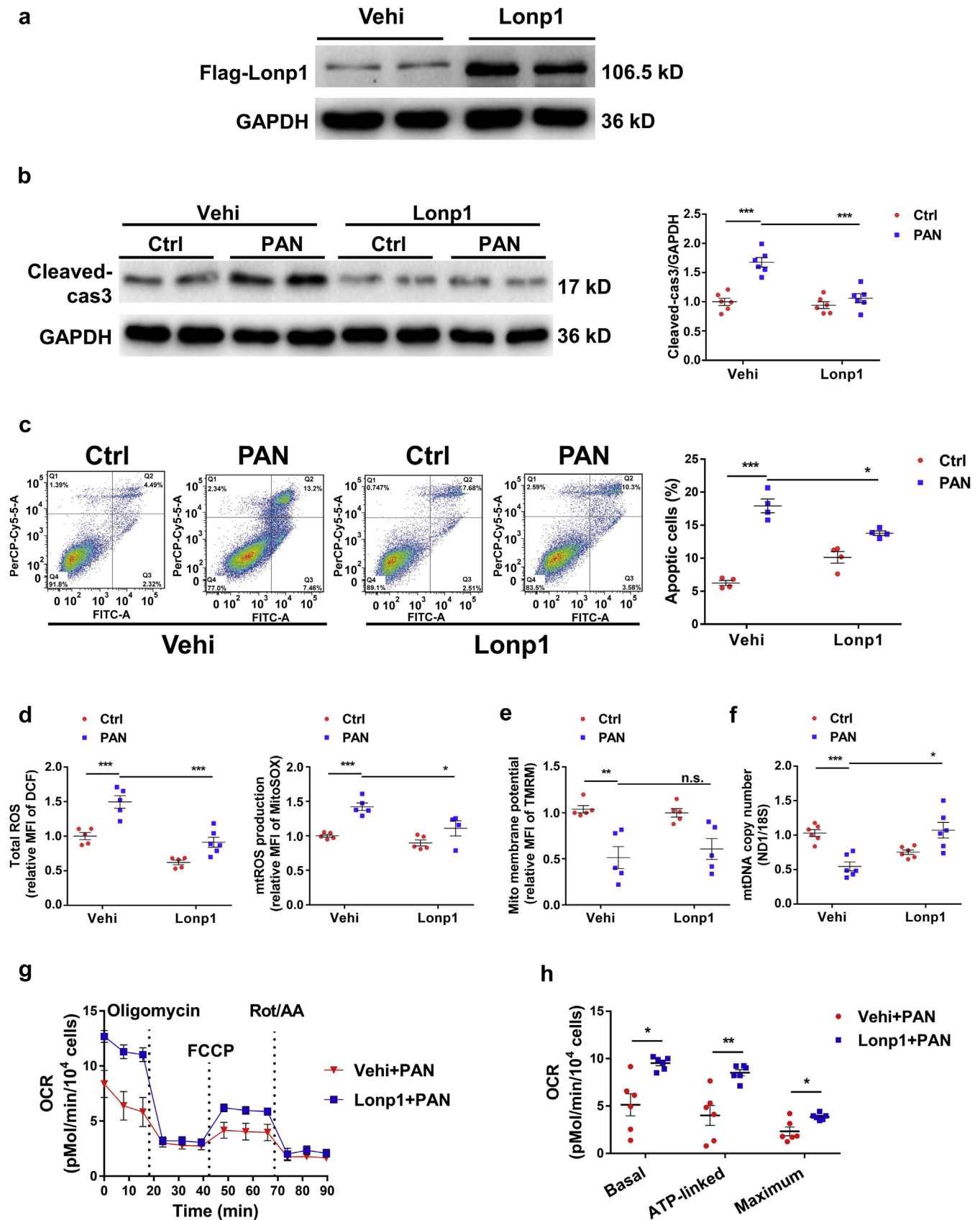


Figure 8 | Overexpression of Lon protease 1 (Lonp1) protects against podocyte apoptosis and mitochondrial dysfunction. Mouse podocytes were transfected with vehicle control (Vehi) or Lonp1-Flag plasmid (Lonp1) for 36 hours, and then cells were treated (continued)

podocytes using mass spectrometry and performed functional analysis. The proteins involved in ATP production, the respiratory chain, and mitochondrial transport were down-regulated, indicating the impaired biological functions of mitochondria in Lonp1-deficient podocytes. GO enrichment analysis performed using mitochondria from podocytes with early Lonp1 deficiency indicated that this caused an imbalance in mitochondrial homeostasis, as proteins involved in the oxidation-reduction process, metabolic processes, and regulation of ATP synthesis were significantly changed. As a proteolytic enzyme, Lonp1 maintains mitochondrial homeostasis by degrading specific substrates.⁵⁷ For example, aconitase, an essential enzyme involved in mitochondrial citrate metabolism, has been identified as a substrate of Lonp1, and its abundance in renal cortical tissue likely contributes to hypocitraturia and hypercitraturia.^{58–60} It is plausible that some substrates that accumulate owing to the deficiency of Lonp1 may mediate processes involved in the subsequent injury to podocytes and the kidneys. In addition, Lonp1 may have other roles independent of its proteolytic activity, and further research is needed to clarify the underlying mechanism.

Some limitations of our study should be noted. First, we did not perform Lonp1-overexpression studies *in vivo*, which would provide more evidence of the function of Lonp1 in renal pathological conditions. In addition, because basal Lonp1 expression is essential for normal mitochondrial function, especially in podocytes, why Lonp1 is reduced in the renal glomeruli of CKD patients remains to be determined. Researchers have indicated several factors that could regulate Lonp1 expression or activity, such as ERK1/2, SIRT1, and Grp75,^{26,53,61} and it is theoretically possible that some of these factors could influence Lonp1 in podocyte injury and podocytopathy.

In conclusion, this study provides new insights into the action of Lonp1 in the glomerular podocytes. Lonp1 plays important roles in mitochondrial function and maintenance of mitochondrial homeostasis in podocytes, which are critical for glomerular and renal function. Our findings identify Lonp1 as a promising therapeutic target for the treatment of podocyte injury and podocytopathy.

METHODS

See [Supplementary Methods](#) for more detailed information.

Patients and study approval

Renal biopsy specimens were obtained from patients with MCD and FSGS who were undergoing diagnostic evaluation at Nanjing Children's Hospital. Patient information is provided in [Supplementary Table S2](#). The agreement describing the use of human specimens was approved by the Human Subjects Committee of Nanjing Medical University. Informed consent was obtained from all participants.

Animals

Podocyte-specific Lonp1 knockout mice (Nehs2^{cre}Lonp1^{fl/fl}, designated Lonp1-cKO) were generated from Nehs2-cre and Lonp1^{fl/fl} mice and maintained in the animal core facility. All animal experiments were performed in accordance with the ARRIVE guidelines approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Serum, random urine, and tissue samples were collected at the indicated times for further study.

Cell culture and treatment

Conditionally immortalized mouse podocytes (obtained from Ding Jie, Peking University) were cultured as described previously.⁶² The primary podocytes were isolated from the mice at approximately 14 days after birth, because the observed podocytes in the cKO mice at this age had normal morphology.

Mass spectrometry analysis

We used 2-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF technology to identify the differentially expressed proteins in mitochondria after preparation and purification of the mitochondrial protein samples. Data were analyzed using the UniProt database as described previously.^{63,64}

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism (GraphPad Software, San Diego, CA). In the figures, the error bars show \pm SEM. A 2-tailed *t* test and one-way ANOVA followed by Bonferroni's comparison test were used for intergroup comparisons. Statistical comparisons between the survival curves were performed using the log-rank (Mantel–Cox) test. A *P* value <0.05 was considered significant.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We thank Prof. Bin Lu for providing the Lonp1-floxed mice and Prof. Ding Jie for providing the mouse podocyte cell line. This work was supported by grants from the National Key Research

Figure 8 | (continued) with puromycin aminonucleoside (PAN; 100 μ g/ml) for another 24 hours. **(a,b)** Representative Western blots ($n = 2$ for each group shown) for Flag-Lonp1, Cleaved-cas3, and GAPDH. Scatterplot shows densitometry results for Cleaved-cas3 ($n = 6$). **(c)** Representative flow cytometry images showing apoptotic cells detected as before. Scatterplot shows the quantitative analysis of apoptotic cells (fluorescein isothiocyanate [FITC]-annexin V-stained; $n = 4$). **(d)** Quantification of the mean fluorescence of DCF (dichlorodihydrofluorescein diacetate; indicating total reactive oxygen species [ROS]; $n = 5–6$) and mitoSOX (indicating mitochondrial ROS [mtROS]; $n = 4–5$) by flow cytometry ($n = 4–6$). **(e)** Quantification of mean tetramethylrhodamine, methyl ester (TMRM) fluorescence using flow cytometry shows the mitochondrial membrane potential ($n = 5$). **(f)** Quantitative polymerase chain reaction analysis of the mitochondrial DNA copy number ($n = 6$). **(g,h)** Cellular respiration assays ($n = 6$). **(g)** Mitochondrial respiration shown as oxygen consumption rate (OCR). **(h)** Quantification of other respiratory parameters (basal OCR, ATP-linked OCR, and maximum OCR). FCCP, p-trifluoromethoxyphenylhydrazone; Rot, rotenone; AA, antimycin A. Data are presented as mean \pm SEM. **P* < 0.05 ; ***P* < 0.01 ; ****P* < 0.001 compared with the indicated group.

and Development Program of China (2016YFC0906103) and National Natural Science Foundation of China (81970581, 81830020, 91742205, 81625004, and 81530023).

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods

Figure S1. Correlation analysis between Lonp1 expression in glomeruli and glomerular pathology.

Figure S2. Identification of Lonp1-cKO mice.

Figure S3. The expression levels of KIM-1 and NGAL were increased in the kidneys of Lonp1-cKO mice.

Figure S4. The expression level of nephrin was decreased in the kidneys of Lonp1-cKO mice with age.

Figure S5. Podocyte apoptosis occurred in the kidneys of Lonp1-cKO mice.

Figure S6. Prednisolone protected against PAN-induced podocyte injury.

Table S1. Genotyping primer sequences.

Table S2. Demographic and clinical data of patients with MCD and FSGS.

Table S3. Primer sequences for quantitative polymerase chain reaction.

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