Cathepsin D in podocytes plays an important role in the pathogenesis of proteinuria and chronic kidney disease

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ABSTRACT

A solution for the pathogenesis and treatment of podocyte injury in chronic kidney disease is a very important therapeutical goal. Recent studies have revealed many analogies between podocytes and neurons. These analogies may be the key to elucidation of the pathogenesis of podocyte injury.

Cathepsin D (CD) is a representative aspartic proteinase in lysosomes. Central nervous system neurons in CD-deficient mice have shown a form of lysosomal storage disease with a phenotype resembling neuronal ceroid lipofuscinoses. In the kidney, the role of CD in podocytes has not been fully explored. Herein, we generated podocyte-specific CD knockout mice that developed proteinuria at 5 months and end-stage renal failure by 20-22 months of age. Immunohistochemical analysis of these mice showed apoptotic podocyte death, followed by proteinuria and glomerulosclerosis along with aging. Using electron microscopy, we identified granular osmiophilic deposits (GRODs), autophagosome/autolysosome-like bodies, and fingerprint profiles in podocytes, typical hallmarks of CD-deficient neurons. We also showed that podocyte autophagy was severely inhibited by the cessation of autolysosomal degradation and that the subunit c of mitochondrial ATP synthase had accumulated in the GRODs in CD-deficient podocytes, similarly to CD-deficient neurons.

Furthermore, both podocin and nephrin, two essential components of the slit diaphragm, translocated to Rab7 and Lamp1-positive amphisomes/autolysosomes that accumulate in podocyte cell bodies in CD-deficient mice. The accumulation of podocin and nephrin was most likely due to defective lysosomal activity resulting in foot process effacement. Thus, loss of CD in podocytes causes autophagy impairment triggering the accumulation of toxic subunit c-positive lipofuscins as well as slit diaphragm proteins, followed by apoptotic cell death.

(260 letters)
INTRODUCTION

Chronic kidney disease (CKD) is marked by a progressive loss in renal function over a period of months or years and causes end-stage renal disease. It has become a worldwide problem in public health because the prevalence is growing. Proteinuria is an early sign and a prognostic marker for CKD patients. In addition, proteinuria is an independent risk factor for cardiovascular morbidity and mortality. Numerous studies have demonstrated that reducing proteinuria is associated with an improved renal outcome regardless of the underlying disease process. Glomerular podocytes with their foot processes (FPs) and interposed slit diaphragms (SD) serve as a final filtration barrier to urinary protein loss. The majority of nephrotic syndrome is characterized by FP effacement and/or molecular reorganization of the SD. Defects in podocyte structure, function or number can lead to pathologic lesions, known as glomerulosclerosis. Accordingly, a better understanding of the mechanism of podocyte injury will provide potential therapeutic steps for the management of CKD.

Lysosomes participate in the turnover of cytoplasmic constituents transported via autophagy as well as in the degradation of extracellular materials incorporated via endocytosis or phagocytosis, thus contributing essentially to maintaining cellular homeostasis. More than 20 lysosomal proteinases, including cathepsin L (CL), cathepsin B (CB), and cathepsin D (CD), play principal roles in lysosomal degradation. Most lysosomal proteinases are capable of efficiently cleaving a wide variety of substrates. However, recent investigations have demonstrated that individual lysosomal proteinase is distributed differently in diverse tissues, and is involved in tissue-specific protein degradation.

Recent studies have shown that CL upregulation in podocytes is important for the acceleration of proteinuria, because the induction of a cytoplasmic variant of CL in podocytes precedes FP effacement and proteinuria in mice. In fact, an increased expression of CL in podocytes has been observed in a variety of human proteinuric kidney diseases, including minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous nephropathy, and diabetic nephropathy. Cytosolic CL cleaves the large GTPase dynamin, actin-binding protein synaptopodin,
and SD-associated CD2-associated protein (CD2AP). These events result in a disorganization of the podocyte actin cytoskeleton and in FP effacement. Thus, many glomerular diseases can be regarded as podocyte enzymatic disorders.7-10

CD, a major lysosomal asparatic endopeptidase, which is widely distributed in various mammalian tissues and cells,11 has unique functions. In many cases, the absence of one single cathepsin in mice does not show a fatal course, because it is thought that a functional overlap among multiple cathepsins ensures the proper degree of protein degradation and turnover. However, CD-deficient mice die at approximately postnatal day 26 from a combination of morbidities including intestinal necrosis, thromboembolia and lymphopenia.12 Subsequent studies have indicated that mouse CD-deficiency induces a neuropathology that is strikingly similar to that observed in human neuronal ceroid-lipofuscinosis (or Batten disease). The pathological symptoms include an accumulation of autofluorescent storage material that resembles granular osmiophilic deposits (GRODs) and the subunit c of mitochondrial ATP synthase.13 CD-deficient mice also exhibit seizures and blindness characteristic of terminal-stage neuronal ceroid-lipofuscinosis patients. Furthermore, an accumulation of abundant autophagosomes/autolysosomes has been noted, which indicates that neuron-specific CD-deficiency elicits a strong inhibition of autolysosomal degradation.14 In the kidney, CD is notably present in the glomerulus as well as in the cortical collecting tubule cells.15 It has been suggested that CD-like activity in glomeruli isolated from PAN-treated rats increased significantly compared with control rats.16 However, it is unclear whether CD plays a role in podocytes under pathological conditions.

Podocytes are terminally differentiated cells and process-bearing cells just like neurons; they also express many neuron-specific proteins, such as synaptopodin, nephrin, podocin and NEPH1.17,18 In addition, recent studies have reported that podocytes exert a higher level of autophagy under basal conditions and that autophagy significantly contributes to maintaining the functional integrity of podocytes.19-24 Considering the importance of CD in neuronal autophagy and the resemblance between neurons and podocytes, we hypothesized that it is of particular importance to elucidate the
role of CD in podocytes. In the present study, we investigated the effects of CD-deficiency on podocyte structure and function, using podocyte-specific, CD-deficient mice.
RESULTS

CD deficiency in podocytes resulted in an age-dependent late-onset form of glomerulosclerosis

To determine the potential roles of the CD in podocytes, CD floxed mice were crossed with Podocin-Cre mice to generate podocyte-specific CD knockout (CDpdKO) mice. Immunofluorescence staining confirmed the markedly diminished expression of CD protein in the glomeruli (Fig. 1A). Western blot analysis from isolated glomeruli showed a significant reduction in CD protein in CDpdKO mice compared with that in their control (CDCtrl) littermates (Fig. 1B). Since podocyte protein accounts for ~30–40% of total glomerular protein, the data shown here are consistent with CD-deficiency in podocytes.

The resultant CDpdKO mice were born at the expected Mendelian frequency with no gross renal anomalies noted. Kaplan-Meier survival curves for a total of 39 months showed that the CDpdKO mice had a significantly lower survival ratio than their CDCtrl littermates (p=0.0002) (Fig. 1C). With regard to body weight, there were no significant differences between the CDpdKO mice and their CDCtrl littermates (data not shown). But at around 24 months, the CDpdKO mice became thin (Fig. 1D), and showed atrophic kidneys with a rough surface and a slightly yellowish appearance (Fig. 1E). CDpdKO mice developed significantly higher levels of albuminuria when compared with their CDCtrl littermates starting at around 5 months of age (Fig. 2A). The levels of albuminuria were increased in the CDpdKO mice with aging. Blood biochemical analyses on 22-24-month-old mice demonstrated that, compared with their CDCtrl littermates, the CDpdKO mice had significantly higher serum urea nitrogen (UN) levels (21.07±1.33 versus 48.30±8.58 mg/dl; p<0.001; n=7 per group), and higher serum creatinine levels (0.13±0.01 versus 0.36±0.07 mg/dl; p<0.001; n=7 per group) (Fig. 2, B and C). These results might indicate that the CDpdKO mice were suffering from CKD and consequently had a shorter life expectancy compared with their CDCtrl littermates.

We next examined the kidney histology. For as long as 3 months of age, the CDpdKO mice were indistinguishable from their CDCtrl littermates via analysis by kidney histology and albuminuria (Figs. 2A and 3A). The number of sclerotic glomeruli in the CDpdKO mice was significantly greater than that...
in their CD<sup>Crl</sup> littermates at 12 months (Fig. 3, B and D). At 20 months of age, many sclerotic
glomeruli and severe tubulointerstitial lesions with cystic dilation of tubules were detected in the
CD<sup>pdKO</sup> mice (Fig. 3, C and D). The percentage ratio of sclerotic glomeruli at 12 months was
1.25±0.27 in the CD<sup>Crl</sup> littermates and 3.89±0.67 in the CD<sup>pdKO</sup> mice (p<0.01, n=3), and at 20
months it was 5.27±0.91 in the CD<sup>Crl</sup> littermates and 42.9±12.17 in the CD<sup>pdKO</sup> mice (p<0.01, n=3).
These results strongly suggest that CD deficiency in podocytes led to an age-dependent, late-onset
form of glomerulosclerosis.

Electron microscopy analysis of CD-deficient podocytes revealed characteristic morphological
changes

As the glomerular ultrastructure was analyzed via electron microscopy, the newborn
CD<sup>pdKO</sup> mice were indistinguishable from their CD<sup>Crl</sup> littermates (data not shown). In podocytes of
3-month-old CD<sup>pdKO</sup> mice that did not exhibit albuminuria (Fig. 2A), the obvious histological
phenotype was not detected (Fig. 3A). However, significant changes, including an accumulation of
electron-dense materials, lesions resembling GRODs (Fig. 4, inset in A bottom; asterisk and Fig.4D),
and autophagosome/autolysosome-like bodies were identified (Fig. 4, inset in A bottom; arrowhead).
These granular structures, which varied in size, content, and electron density, were further increased
in CD<sup>pdKO</sup> mice with aging. At 8 months, the CD<sup>pdKO</sup> mouse podocytes were completely filled with
GRODs (Fig. 4B); furthermore, autophagosome/autolysosome-like bodies were observed containing
portions of the cytoplasm and encircled with multilayered membranes (Fig. 4E). In addition, this
portion of GRODs contained fingerprint profiles with tightly and concentrically arrayed compact
membranes, as in the structures observed in juvenile neuronal ceroid-lipofuscinosis (Fig. 4F). These
characteristic morphological changes were strikingly similar to those seen in CD-deficient mouse
neurons.  

By 12 months of age, when the glomerulosclerosis could be partially recognized by kidney
histology (Fig. 3), the FPs of CD<sup>pdKO</sup> mouse podocytes had effaced along the glomerular basement
membrane (GBM) to form a continuous band of cytoplasm (Fig. 4C bottom; large arrow). A dense band of actin filaments (Fig. 4, inset in C bottom; small arrows) were seen along the effaced FPs running parallel to the GBM. These morphological changes in the structure of podocyte FPs were characterized as a development of nephrotic syndrome. Moreover, irregularly shaped nuclei also appeared, with a small and dispersed heterochromatin and an unclear nucleolus, similar to the nuclei in dying CNS neurons (Fig. 4C bottom; arrowhead). Scanning electron microscopy analysis showed prominent microvillous transformations, which were characterized as severe proteinuria (Fig. 4G bottom; arrow).

Electron micrographs of irregularly shaped nuclei show an association with apoptotic podocyte death in aging CDpdKO mice

Many studies have shown that a reduction in the number of podocytes correlates with proteinuria and leads to glomerulosclerosis. To count the number of podocytes in the glomeruli, WT1 staining, a nuclear marker for podocytes, was performed at 3, 12 and 20 months (Fig. 5A). The number of WT1-positive cells was significantly decreased in CDpdKO mice compared with their CDCtrl littermates. The number of WT1-positive cells in the CDCtrl littermates and in the CDpdKO mice were 17.49±0.21 versus 14.94±0.26 per glomerulus at 3 months, 15.33±0.47 versus 12.14±0.15 per glomerulus at 12 months, and 14.90±0.16 versus 6.54±0.22 per glomerulus at 20 months, respectively (n=3; p< 0.01) (Fig. 5B). These observations imply either an increase in podocyte cell death or a detachment from the GBM in CDpdKO mice.

It is well known that podocyte injury causes either podocyte apoptosis or detachment. First, we assessed the podocyte apoptosis by analyzing cleaved caspase-3, which is classified as an effector caspase. We counted cleaved caspase-3 dots in the glomeruli at 3, 12 and 20 months (Fig. 5, E and F). At 3 months, cleaved caspase-3 dots were not detected in either the CDpdKO mice or in their CDCtrl littermates. Quantification of the number of cleaved caspase-3 positive cells in 12 and 20 month-old mice showed a significantly higher rate in the CDpdKO mice than in their CDCtrl littermates (CDCtrl
littermates versus CD\textsuperscript{pdKO} mice: 0.45±0.20 versus 22.82±3.38% at 12 months, 2.67±0.86 versus 10.72±2.11% at 20 months, respectively; n=3; \(p<0.01\)). Next, we assessed the podocyte apoptosis by analyzing cleaved caspase-8, which is classified as an initiator caspase. Quantification of the number of cleaved caspase-8 positive dots in 12 month-old mice showed a significantly higher rate in CD\textsuperscript{pdKO} mice than in their CD\textsuperscript{Ctrl} littermates (CD\textsuperscript{Ctrl} littermates versus CD\textsuperscript{pdKO} mice: 0.94±0.27 versus 19.44±2.29%, respectively; n=4; \(p<0.001\)) (Fig. 5, C and D). Finally, we performed TUNEL staining at 12 and 20 months (Fig. 5, G and H). As shown in Fig. 5H, the quantification of TUNEL-positive cells in 20-month old mice revealed a significantly higher rate in CD\textsuperscript{pdKO} mice than in their CD\textsuperscript{Ctrl} littermates (CD\textsuperscript{Ctrl} littermates versus CD\textsuperscript{pdKO} mice: 1.18±0.38 versus 3.06±0.73% at 12 months, 1.19±0.08 versus 12.09±3.16% at 20 months, respectively; n=3; \(p<0.01\)). These findings indicate that podocyte cell death in the CD\textsuperscript{pdKO} mice, which is characterized by typical apoptotic features, such as positive staining for cleaved caspase-3, cleaved caspase-8, and TUNEL staining, was increased with aging longer than 12 months.

Subunit c of mitochondrial ATP synthase accumulated in CD\textsuperscript{pdKO} mice podocytes

The CD\textsuperscript{pdKO} mouse podocytes exhibited characteristic morphological changes like GRODs and autophagosome/autolysosome-like bodies that are common in CD-deficient mouse neurons. Biochemical investigations have revealed that subunit c of mitochondrial ATP synthase is a major storage protein in the GRODs of CD-deficient mouse neurons\textsuperscript{13} and that, in particular, an accumulation of autophagosomes/autolysosomes is involved along with the GRODs.\textsuperscript{14} To assess the autophagy-lysosome pathways in the podocytes of CD\textsuperscript{pdKO} mice, we performed immunohistochemical analysis. Lysosomal CB, lysosomal-associated membrane protein 1 (Lamp1), microtubule-associated protein 1 light chain 3 (LC3), p62, and ubiquitin were all markedly accumulated in the podocytes of CD\textsuperscript{pdKO} mice (Fig. 6). These data were further supported by western blots of glomerular lysates, which showed that Lamp1, polyubiquitin, and LC3-II had accumulated in CD\textsuperscript{pdKO} mouse glomeruli (Suppl. Fig. 1). Quantification of the number of p62-positive cells in 10-12
month-old mice showed a significantly higher rate in CD\textsuperscript{pdKO} mice than in their CD\textsuperscript{Ctrl} littermates (Fig. 6E). Strikingly, enlarged Lamp1 dots were colocalized with LC3 dots, indicating that autolysosomes accumulate in podocytes (Fig. 6B). These data are consistent with the data published previously on CD-deficient neurons\textsuperscript{14, 30}.

Interestingly, we confirmed that these CD-deficient podocytes emitted autofluorescence, particularly after 8 months of age (data not shown). Since these data are consistent with those due to the lysosome-like bodies containing ceroid lipofuscin\textsuperscript{13}, we further sought to determine whether subunit c of mitochondrial ATP synthase is present in these lysosomal structures. No immunoreactivity for subunit c was detected in any of the kidney sections obtained from the CD\textsuperscript{Ctrl} littermates. However, dotted immunoreactivity for subunit c was detected in CD\textsuperscript{pdKO} mouse podocytes (Fig. 7A).

To identify the subcellular localization of subunit c in podocytes from CD\textsuperscript{pdKO} mice and their CD\textsuperscript{Ctrl} littermates, immunoelectron microscopy using the cryothin section immunogold method was applied to the tissues. In the podocytes of the CD\textsuperscript{Ctrl} littermates, subunit c was detectable by immunogold labeling in the mitochondrial inner membrane (Fig. 7, B and C top, arrowheads). In podocytes from CD\textsuperscript{pdKO} mice, the labeling for subunit c was associated with both the inner membrane of intact mitochondria and the membrane-bound compartments with dense materials (GRODs) (Fig. 7, B and C bottom, arrowhead, arrows). These findings indicate that subunit c of mitochondrial ATP synthase is a major storage protein of GRODs in CD\textsuperscript{pdKO} mouse podocytes.

\textit{SD proteins, podocin and nephrin that accumulate in the podocyte cell bodies of CD\textsuperscript{pdKO} mice}

The majority of nephrotic syndrome is characterized by FP effacement resulting from the molecular reorganization of the SD\textsuperscript{2}. We assessed the expression of podocin and nephrin, two essential proteins that help maintain SD integrity in CD\textsuperscript{pdKO} mice. Immunofluorescence staining clearly showed the localization of podocin and nephrin in the GBM in CD\textsuperscript{Ctrl} littermates. By contrast, podocin and nephrin were found to be significantly distributed in the podocyte cell bodies in CD\textsuperscript{pdKO}
mice (Fig. 6 and Suppl. Fig 2A). Notably, the enlarged cytoplasmic staining of podocin and nephrin in podocyte cell bodies was found to be well colocalized with enlarged Lamp1 staining (Fig. 8A and Suppl. Fig. 3A).

Endocytosis and degradation of SD proteins play an important role in the maintenance of SD function. Neither the CDpdKO mouse podocytes nor the podocytes of their CDCtrl littermates displayed significant colocalization of podocin when tested for with an early endosomal marker Rab5 (Suppl. Fig. 2B). By contrast, a late endosomal marker for Rab7 were increased in CDpdKO mouse podocytes compared with CDCtrl littermate podocytes (Fig. 8B and, Suppl. Figs. 2C and 3B). Furthermore, both podocin and nephrin were considerably colocalized with Rab7 and Lamp1 in CDpdKO mice compared with their CDCtrl littermates. These data indicated that podocin and nephrin were mainly localized in the late endosomes and lysosomes of CDpdKO mouse podocyte cell bodies.

To further identify the subcellular localization of podocin in CDpdKO mouse podocytes, immunoelectron microscopy using paraffin-embedded tissue sections was performed (Fig. 8C). As clearly shown, podocin was localized in the GRODs accumulating in the podocyte cell bodies of CDpdKO mouse glomeruli. This finding indicates that cytoplasmic podocin is localized together with other storage proteins on the GRODs in CDpdKO mouse podocytes (Fig. 8C).

**Podocyte CD expression may be related to the progression of human glomerular diseases**

To examine the expression of CD in the podocytes of human glomerular diseases with nephrotic syndrome by immunohistochemical staining, we stained the CD in human kidney biopsy specimens of minor glomerular abnormalities, MCD and FSGS. The expression of CD was significantly increased in the MCD samples (Fig. 9B) compared with that in the FSGS samples (Fig. 9C) (MCD versus FSGS: 27.34±3.72 versus 13.25±1.77%, respectively; *p* < 0.01). These results indicated that the degree of podocyte injury is related to the expression levels of CD in the glomeruli.
In addition to the physiological role in lysosomal degradation, recent reports have linked CD to apoptosis\textsuperscript{32} as well as to several other diseases such as Alzheimer’s disease,\textsuperscript{33} atherosclerosis,\textsuperscript{34} cancer,\textsuperscript{35} and neuronal ceroid-lipofuscinosis.\textsuperscript{36} In the kidney, CD is expressed not only in podocytes but also in tubular cells. Previous studies showed the expression of CD is increased in the renal tubules of nephrotic rats and mice to decompose endocytosed proteins.\textsuperscript{16, 37} However, the association between CD and CKD is not yet fully understood. The present study provides the first evidence that the deletion of CD in podocytes impairs autophagy through an accumulation of toxic subunit c-positive lipofuscins and delocalization of SD proteins leading to podocyte apoptotic cell death.

In our study, we demonstrated that aging CD\textsuperscript{pdKO} mice developed age-dependent, late-onset glomerulosclerosis, which explains the shorter life expectancy of CD\textsuperscript{pdKO} mice (Fig. 1). The entire process of disorder, which finally leads to glomerulosclerosis, proceeded in a stepwise manner. First, CD\textsuperscript{pdKO} mice exhibited spontaneous proteinuria as early as 5 months after birth, which gradually increased until 20 months (Fig. 2). Then, the mice manifested remarkable podocyte loss when they grew older—between 12 and 20 months of age (Fig. 5). An increase in the number of cleaved caspase-3 positive cells as well as TUNEL positive cells strongly suggested that apoptosis had partially contributed to the podocyte loss. In addition, an increase in the number of cleaved caspase-8 positive cells indicated a contribution of the extrinsic apoptotic signaling pathway in CD-deficient podocytes, because caspase-8 activation functions as an upstream activator of caspase-3.\textsuperscript{38} The increase in podocyte loss in the aging CD\textsuperscript{pdKO} mice in turn evoked the marked impairment of glomerular filtration typically shown by high levels of serum UN and serum creatinine (Fig. 2), which eventually leads to obvious glomerulosclerosis, including the FP effacement and the detachment of podocytes from the GBM (Fig. 3-5).

In view of the gradual increase in proteinuria (5-20 months) as well as no obvious latent postnatal phenotype at 5 months, the accumulation of detrimental effects directly caused by
CD-deficiency seemed to play an important role. When considering the direct effect of CD-deficiency, it should be noted that CD-deficient podocytes share many common characteristics with CD-deficient neurons. In electron microscopic analysis, accumulations of GROD-like electron dense materials, fingerprint profiles, and autophagosome/autolysosome-like bodies (Fig. 4) also are often reported as most characteristic features in the neuronal tissues of CD-deficient mice. In addition, the subunit c of mitochondrial ATP synthase had accumulated in the GRODs in CDpdKO mouse podocytes (Fig. 6 and 7). This characteristic is reminiscent of the neuronal ceroid-lipofuscinosis that is caused by CD-deficiency in neuronal cells, as reported by Koike et al. and other groups. Thus, the loss of CD evoked disorders that are common to both neurons and podocytes, confirming that the two cell systems resemble each other even under pathological conditions in addition to normal physiological conditions, as reported previously.

It should be emphasized that these characteristics are closely connected to the autophagic inhibition of CDpdKO mouse podocytes. Both immunohistochemical and western blot analyses clearly showed that CB, Lamp1, LC3-II, p62, and ubiquitin were markedly accumulated in the podocytes of CDpdKO mice (Fig. 6 and suppl. Fig. 1). The accumulation of p62 is a hallmark of autophagy impairment. Furthermore, enlarged Lamp1 dots that were colocalized with LC3-dots were noted, indicating abundant autolysosome accumulation (Fig. 6). Using western blotting, we confirmed that S6-kinase and ribosomal S6 were dephosphorylated in the isolated glomeruli of both CDpdKO and CDctrl mice (data not shown), indicating mTOR inactivation. Taken together, these characteristics clearly demonstrated that podocyte autophagy was severely inhibited during autolysosomal degradation due to the absence of CD.

Unexpectedly, we found that podocin and nephrin were abnormally distributed in the podocyte cell bodies and colocalized with Rab7 and Lamp1 in CDpdKO mice (Fig. 8, Suppl. Figs. 2 and 3). As Rab7 is a late endosomal marker and Lamp1 is also an endosomal/lysosomal marker, our finding strongly suggests that the two SD proteins must shift their localization to remain on accumulated
autolysosomes. In SD, podocin and nephrin play an essential role in maintaining glomerular filtration barrier. In this role their homeostatic secretion to SD and the subsequent endocytic trafficking is important.\textsuperscript{43} In CD\textsuperscript{pdKO} mouse podocytes, autophagosome formation proceeded normally, but autolysosomal turnover was severely hindered. This resulted in more late endosomes and lysomes that were recruited to form autolysosomes in a compensatory manner. As a result, endocytosed nephrin and podocin becomes confined in Rab7 and/or Lamp1-positive autolysosomes. The disproportionate endosomal/lysosomal localization of podocin and nephrin must affect the integrity of SD, which directly connects to the vulnerability of the SD.

In recent reports, podocyte-specific \textit{Atg5} (autophagy-related 5) knockout mice developed significantly higher levels of proteinuria compared with their control littermates by 8-12 months of age, and the level of proteinuria was increased with aging. Furthermore, 20-24 month-old podocyte-specific \textit{Atg5}-knockout mice displayed significantly increased glomerulosclerosis compared with their control littermates.\textsuperscript{19} Thus, autophagy impairment in podocytes, whether it is elicited directly by the loss of an autophagy-essential gene (\textit{Atg5}) or indirectly by the loss of lysosomal CD, compromises the normal ability of podocytes to sustain a glomerular filtration barrier. It is curious that CD\textsuperscript{pdKO} mice developed proteinuria and glomerulosclerosis at an earlier age compared with podocyte-specific \textit{Atg5} knockout mice (CD\textsuperscript{pdKO} mice versus podocyte-specific \textit{Atg5} knockout mice: 5 months versus 8-12 months for the onset of proteinuria, 12 months versus 20-24 months for the onset of glomerulosclerosis). The reasons for this difference may have been the presence of toxic subunit c-positive lipofuscins, podocin and nephrin accumulating in the podocytes of the CD\textsuperscript{pdKO} mice.

To determine if a compensatory increase in podocyte CL might explain the development of podocyte injury in the CD\textsuperscript{pdKO} mice,\textsuperscript{7,8} we examined CL expression by immunofluorescence (Suppl. Fig. 4A). Although there was a suggestion of increased podocyte staining for CL, the expression of synaptopodin and CD2AP, which are considered the substrates for CL, displayed no significant
differences between CD^{pdKO} mice and CD^{Ctrl} littermates (Suppl. Fig 4). These findings suggest that the effect of CD deficiency is independent of CL, however this question needs further study.

Immunohistochemical staining of human kidney biopsy specimens indicated that the expression of CD was significantly increased in MCD compared with that in FSGS (Fig. 9).

Podocyte injury is more severe in FSGS, which leads to irreversible damage in many cases compared with MCD. Studies have found that podocytes from MCD patients had higher levels of beclin 1-mediated autophagic activity than those from FSGS patients, suggesting that maintaining a relatively high level of autophagic activity may prevent the progression of podocyte injury in MCD.\textsuperscript{44} High CD expression in MCD patients may be due to a high level of autophagic activity. The detailed mechanisms underlying these observations will require further study.

Many lysosomal disorders cause neurodegenerative problems, but very few lysosomal defects are known to cause renal disease. Lysosomal dysfunction in Fabry’s disease impairs podocyte function by disrupting podocyte FPs, but the mechanism by which lysosomes accumulate glycosphingolipids in podocytes and trigger renal failure is not yet fully understood.\textsuperscript{45} Mutations in the CD gene cause a congenital neuronal ceroid-lipofuscinosis classified as CLN10 in humans. CLN10 manifests severe symptoms such as early blindness and psychomotor disability.\textsuperscript{36, 46} Due to the ephemerality of patients with fatal neurodegeneration, whether or not the infants of these patients suffer renal failure has not been investigated.

In conclusion, the present study indicates that CD plays a principal role in autophagic degradation and contributes to maintaining podocyte homeostasis. The results, together with a previous report by Hartleben \textit{et al.} \textsuperscript{47} underscores the importance of autophagy in podocyte homeostasis and health. The regulation of SD proteins through the membrane trafficking system is also considered to contribute greatly to the maintenance of podocyte FPs. The clinical relevance of CD in human podocyte injury requires further investigation in future studies.
CONCISE METHODS

Generation of CD\textsuperscript{\textit{dKO}} Mice

CD-floxed mice (CD\textsuperscript{\textit{floxed/floxed}}) were crossed with Podocin-Cre mice to generate podocyte-specific CD knockout mice CD\textsuperscript{\textit{floxed/floxed};Podocin-Cre\textsuperscript{+}} (CD\textsuperscript{\textit{dKO}}), CD\textsuperscript{\textit{floxed/WT};Podocin-Cre\textsuperscript{+}} and CD\textsuperscript{\textit{floxed/floxed};Podocin-Cre\textsuperscript{+}} littermates served as control animals (CD\textsuperscript{\textit{Ctrl}}) in the present study. These animals showed no pathological phenotypes when examined by histological, immunohistochemical, and biochemical methods. CD-floxed mice are described elsewhere. Podocin-Cre mice have been previously reported\textsuperscript{48} All mice were kept in specific pathogen-free facilities. All animal experiments were guided and approved by the Committee for Animal Experiments of Juntendo University, Tokyo, Japan.

Biochemical measurements

Body weight and urinary albumin to creatinine ratios (ACR) were measured in the mice once a month. Urinary albumin and creatinine levels were measured by immunoassay (DCA 2000 system; Siemens Healthcare, Erlangen, Germany)\textsuperscript{49} Blood samples were obtained from anesthetized mice before death, and then centrifuged at 5,000 rpm for 30 minutes at 4 °C to obtain serum. Serum urea nitrogen (UN) levels were measured by ultraviolet-visible spectroscopy, and creatinine levels were determined using enzymatic methods.

Antibodies

Commercially available antibodies were obtained from the following sources: polyclonal goat anti-mouse CD antibody (R&D systems, Inc.) and polyclonal goat anti-mouse CL antibody (R&D systems, Inc.) for immunofluorescence study; polyclonal rabbit anti-human CD antibody (Cell Signaling Technology, Inc.); polyclonal rabbit anti-WT-1 antibody (Santa-Cruz Biotechnology, Inc.); polyclonal rabbit anti-cleaved caspase 3 (Asp175) antibody (Cell Signaling Technology, Inc.); polyclonal rabbit anti-cleaved caspase 8 (Asp387) antibody (Cell Signaling Technology, Inc.);
monoclonal rat anti-Lamp1 antibody (1D4B: Santa-Cruz Biotechnology, Inc.) for immunofluorescence study; monoclonal rat anti-mouse Lamp1 antibody (1D4B: BD Pharmigen.) for western blotting; polyclonal rabbit anti-Rab5 antibody (Cell Signaling Technology, Inc.);
monoclonal rabbit anti-Rab7 antibody (Cell Signaling Technology, Inc.) for western blotting;
monoclonal mouse anti-synaptopodin antibody (Progen Biotechnik GmbH); polyclonal guinea pig anti-nephrin antibody (Progen Biotechnik GmbH); monoclonal mouse anti-β-Actin antibody (Sigma-Aldrich); polyclonal rabbit anti-LC3 antibody (abcam); polyclonal rabbit anti-SQSTM1/A170/P62 antibody (Wako Pure Chemical Industries, Ltd.); polyclonal rabbit anti-ubiquitin antibody (Dako); and monoclonal mouse anti-multi ubiquitin antibody (FK2: Medical & Biological Laboratories CO., LTD.). Rabbit polyclonal antibodies against podocin and synaptopodin were prepared as previously described. The antibodies against CD, CL and LC3 for western blotting, CB, and Rab7 for immunofluorescence study were kindly donated by Prof. Kominami (Juntendo University), and the antibodies against subunit c of mitochondrial ATP synthase were kindly donated by Dr. Ezaki (Fukushima University).

**Histologic Analysis and Immunohistochemistry**

Kidneys were fixed via the cardiac ventricular perfusion of 4% paraformaldehyde and 20% sucrose in PBS, and embedded in paraffin. The 3-μm-thick kidney sections were stained either with a periodic acid–Schiff (PAS) method or with primary antibodies followed by the respective secondary antibodies. Nuclei were stained with Mayer's Hematoxylin Solution in immunohistochemical staining. All histological and immunohistochemical specimens were observed via a light microscope (Olympus BX41; Olympus, Japan).

To detect podocyte apoptotic cells, the TUNEL assay was performed using the Apoptag Plus Peroxidase *in situ* Apoptosis Detection Kit (Chemicon International Inc., Temecula, Calif., USA). The kit was used according to the manufacturer’s instructions.

The results of the immunofluorescence study are shown in Figs. 5 and 6, and in supplemental
Fig. 4, the fixed kidneys were frozen in optimal cutting temperature compound. Frozen sections (3μm) were incubated with primary antibodies and developed with secondary antibodies. Nuclei were stained with DAPI (4’, 6-diamidine-2-phenylindole). For immunofluorescence study in Figs. 1, 8 and supplemental Figs 2 and 3, paraffin-embedded tissue sections (3μm) were deparaffinized and hydrated according to standard procedures. Sections were subjected to antigen retrieval by boiling in citrate buffer (10 mM, pH 6.0) for 5 min, and treated with 0.3% H2O2 in methanol for 10 min. They were then stained in the same manner as mentioned above.

Immunofluorescence specimens were analyzed using a confocal fluorescence microscope (Leica TCS SP8; Leica Microsystems, Germany).

**Transmission Electron Microscopy**

Kidney samples (approximately 2 mm³) were immersed in 2.5% glutaraldehyde with 0.1 M phosphate buffer (pH 7.4) (PB) for 2 hours. Next, the samples were postfixied in 2% OsO4 with 0.1 M PB for 2 hours. Tissues were dehydrated in ethanol and embedded in epoxy resin (Epok 812; Okenshoji, Japan). Ultrathin sections (1μm) were cut with an ultramicrotome (Leica Ultracut UC7; Leica Microsystems, Austria) and mounted on a copper grid. The sections were stained in uranyl acetate for 30 minutes and in lead citrate for 4 minutes at room temperature. Grids were viewed with a transmission electron microscope (HT7700; Hitachi, Tokyo, Japan) at 100.0kV.

**Scanning Electron Microscopy**

Kidney samples (approximately 2 mm³) were immersed in 2.5% glutaraldehyde with 0.1 M PB (pH 7.4) for 2 hours. Next, the samples were fixed in 2% OsO4 with 0.1 M PB for 2 hours. Tissues were dehydrated in ethanol and freeze-dried with tert-butyl alcohol in a freeze dryer (ES-2030; Hitachi, Tokyo, Japan). After drying, the samples were coated with OsO4 and were visualized with a scanning electron microscope (S-4800; Hitachi) at an accelerating voltage of 3.0 kV.
Immunoelectron microscopy

Immunoelectron microscopy for subunit c using ultrathin cryosections was performed as previously described. Briefly, mice were deeply anesthetized with pentobarbital (25 mg/kg i.p.) and fixed by cardiac perfusion with 4% paraformaldehyde buffered with 0.1 M PB (pH 7.4). Kidneys were quickly removed from the mice, cut into small pieces, further immersed in the same fixative at 4 °C for 2 hours, washed thoroughly with 7.5% sucrose in 0.1 M PB (pH 7.4), embedded in 12% gelatin, and immersed in 2.3 M sucrose in 0.1 M PB overnight at 4 °C. The samples were then placed on a specimen holder (Leica microsystems) and quickly plunged into liquid nitrogen. Ultrathin sections were cut with a microtome using a cryo-attachment (Ultracut UC7/FC7; Leica microsystems) and mounted on Formvar carbon-coated nickel grids. The sections were rinsed with PBS, treated with 1% bovine serum albumin (BSA) in PBS, and incubated overnight with anti-subunit c (10 mg/ml) in PBS, then for 1 hour with anti-goat IgG conjugated with 10 nm colloidal gold particles (1:40; British Biocell International, UK). After the immunoreactions, the sections were embedded in 2% methyl cellulose containing 0.4% uranyl acetate and observed with a Hitachi H-7100 electron microscope. For control experiments, deparaffinized and ultrathin sections were incubated with nonimmunized rabbit serum diluted 1:1,000, followed by respective second antibodies.

Immunoelectron microscopy for podocin was performed in the following way. The deparaffinized section that was immunostained with anti-podocin antibody, as described above, was immersed in 1% OsO4/0.1M PB for 1 hr. After being washed with distilled water, the sections were dehydrated with a graded series of ethanol and embedded in Epon 812. Ultrathin gold sections were processed with a diamond knife, transferred to copper grids (50 mesh) that were coated with Formvar membrane, and observed with a JEM1230 (JEOL, Tokyo, Japan) transmission electron microscope.

Western Blotting

Our glomerulus isolation technique was a modification of a method reported previously.
Isolated glomeruli were lysed in 0.5% CHAPS buffer. Samples were resolved on SDS–polyacrylamide gels. Proteins from the gels were transferred to membranes and then the membranes were blocked with 5% non-fat milk and incubated with the appropriate primary antibodies.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, S Diego, CA). Data are represented as the mean ± s.e.m. Comparisons between groups were analyzed via a Mann-Whitney U test. Differences at $p < 0.05$ were considered significant.

**Human kidney specimens**

Human kidney specimens were collected from kidney biopsies that were performed at the Juntendo University Hospital, Tokyo, Japan. This study was conducted according to the Declaration of Helsinki and approved by the Institutional Review Board of Juntendo University Hospital. Informed consent was obtained from all patients. We selected the cases that developed nephrotic syndrome and that were proven to be minimal change disease (MCD) (n=6) and focal segmental glomerulosclerosis (FSGS) (n=6). As control human samples, we used biopsy samples from the patients with minor glomerular abnormalities (n=5). The clinical data of the patients are presented in supplemental Table 1 (Suppl. Table 1).

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded human kidney specimens with the use of standard techniques as mentioned above. CD positive dots were evaluated using Image J software by blind analysis.

**Acknowledgments**

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Medicine, Tokyo, Japan, for their excellent technical assistance. We also thank the staff of the Laboratory of Molecular and Biochemical Research, Research Support Center, Juntendo University Graduate School of Medicine, Tokyo, Japan, for excellent technical assistance.

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Figure 1. (A and B) Effective deletion of CD protein due to podocin-Cre recombinase confirmed by immunofluorescence staining (A) and western blotting analysis of isolated glomerular homogenates (B) in 3-month-old mice.

(C) Results from a 39-month follow-up of CD<sup>pdKO</sup> mice for survival of data. Kaplan-Meier survival curves show CD<sup>pdKO</sup> mice had a significantly lower survival ratio than their CD<sup>Ctrl</sup> littermates. A Hazard Ratio (Mantel-Haenszel test) is 7.279. \( P=0.0002 \) by Mantel-Cox log-rank test. Median survival time: CD<sup>Ctrl</sup> littermates 30.5 months, CD<sup>pdKO</sup> 25 months.

(D and E) At 24 months, CD KO mice became thin (D), and showed atrophic kidneys with a rough surface and a slightly yellowish appearance (E).

Figure 2. CD deficiency results in age-dependent late-onset glomerulosclerosis.

(A) Results from a 26-month follow-up of CD<sup>pdKO</sup> mice for urinary albumin/creatinine ratios. CD<sup>pdKO</sup> mice developed significantly higher levels of albuminuria when compared with CD<sup>Ctrl</sup>
littermates starting at around the 5th month. The level of albuminuria was increased in aging
CD$^{pdKO}$ mice.

(B and C) Blood chemistry analysis on 22-24 month-old mice demonstrated that, compared with
CD$^{Ctrl}$ littermates, CD$^{pdKO}$ mice had significantly higher serum UN levels, and higher serum
creatinine levels, indicating that the CD$^{pdKO}$ mice had a kidney insufficiency.

The data are the mean ± s.e.m. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by 2-tailed
Mann-Whitney U test.

Figure 3. Podocyte-specific loss of CD causing severe glomerulosclerosis and renal fibrosis.

(A-C) Periodic acid-Schiff (PAS) staining images of kidney sections showed CD$^{pdKO}$ mice had
normal renal histologic findings at 3 months of age (A), but developed severe kidney lesions (focal
sclerosis) by 12 months of age, including sclerotic glomeruli, severe tubulointerstitial lesions with
cystic dilation of tubules, tubular atrophy, and interstitial fibrosis (B and C).

(D) Quantification of sclerotic glomeruli in CD$^{Ctrl}$ and CD$^{pdKO}$ mice at 12 and 20 months showed a
significantly higher rate in CD$^{pdKO}$ mice than in their CD$^{Ctrl}$ littermates. The sclerotic glomeruli per
section were assessed via a total number count. At least 240 glomeruli were randomly selected for
the determination of glomerulosclerosis. The data are the mean ± s.e.m. **$p<0.01$ via a 2-tailed
Mann-Whitney U test.

Figure 4.

(A) Up to 3 months after birth, CD$^{pdKO}$ mice were indistinguishable from WT mice as analyzed by
proteinuria levels and kidney histology. But electron microscopy analysis showed significant changes
including granular osmiophilic deposits (GRODs, asterisk) and autophagosome/autolysosome-like
bodies (arrowhead) in the podocytes of 3-month-old CD$^{pdKO}$ mice (inset in A bottom).
(B) At 8 months of age, electron micrographs in CD<sup>pdKO</sup> mice showed that podocytes were filled with GRODs.

(C) At 12 months of age, electron micrographs in CD<sup>pdKO</sup> mice showed FP effacement (large arrow) containing a dense band of actin filaments running parallel to the GBM (inset in C, small arrows). Irregularly shaped nuclei (arrowhead) also appeared.

(D, E and F) Representative high magnified images of GRODs (D), autophagosome/autolysosome-like bodies with multilayered membranes (E) and a finger print profile (F) from CD<sup>pdKO</sup> mice podocytes obtained at 8 months of age are shown. To show the fingerprint pattern clearly, a boxed area in F is enlarged in the inset.

(G) Scanning electron microscopy analysis also showed microvillous transformations (arrow).

Figure 5. Irregularly shaped nuclei in electron micrographs were associated with apoptotic podocyte death in aging CD<sup>pdKO</sup> mice.

(A) Immunofluorescence staining showed that WT1 positive dots (green) in glomeruli from CD<sup>pdKO</sup> mice were decreased at 15 months of age.

(B) WT1-positive cells in CD<sup>pdKO</sup> mice were significantly decreased when compared with their CD<sup>Ctrl</sup> littermates at 3, 12 and 20 months of age. Analysis was performed on 50-60 glomeruli of each mouse.

(C) Immunohistochemical staining with anti-cleaved caspase-8 showed cleaved caspase-8 positive podocytes of 12-month-old CD<sup>pdKO</sup> mice.

(D) Quantification of the number of cleaved caspase-8 positive dots in 12 month-old mice showed a significantly higher rate in CD<sup>pdKO</sup> mice than in their CD<sup>Ctrl</sup> littermates. At least 350 glomeruli of each mouse were analyzed.
(E) Immunofluorescence staining showed that cleaved caspase-3 positive dots (green) in the glomeruli from CD\textsuperscript{pdKO} mice were increased at 12 months of age. (red: synaptopodin, green: cleaved caspase3, DAPI)

(F) Quantification of the number of cleaved caspase-3 dots in 12 and 20 month-old mice showed a significantly higher percent ratio in CD\textsuperscript{pdKO} mice than in their CD\textsuperscript{Ctrl} littermates. At least 200 glomeruli of each mouse were analyzed.

(G) Tunel staining showed that Tunel positive dots (arrow) in the glomeruli from CD\textsuperscript{pdKO} mice were increased at 20 months of age.

(H) Quantification of the number of Tunel positive dots in 20 month-old mice showed a significantly higher rate in CD\textsuperscript{pdKO} mice than in their CD\textsuperscript{Ctrl} littermates. At least 200 glomeruli of each mouse were analyzed.

The data are the mean ± s.e.m. **p<0.01, ***p<0.001 by 2-tailed Mann-Whitney U test.

Figure 6. Cathepsin B (CB), lysosomal-associated membrane protein 1 (Lamp1), microtubule-associated protein 1 light chain 3 (LC3), ubiquitin and p62 accumulated in CD\textsuperscript{pdKO} mice podocytes.

(A and B) Immunofluorescence staining showed that lysosomal CB (A), Lamp1 and LC3 (B) were increased in 10-month-old CD\textsuperscript{pdKO} mice podocytes. Most dots were enlarged to form aggregates in the podocytes. LC3 dots were significantly colocalized with Lamp1 (B merged).

(C and D) Immunohistochemical staining with anti-P62 and anti-ubiquitin showed P62-positive and ubiquitin-positive podocytes of 10-month-old CD\textsuperscript{pdKO} mice.

(E) Quantification of the number of p62 dots in 10-12 month-old mice showed a significantly higher rate in CD\textsuperscript{pdKO} mice than in their CD\textsuperscript{Ctrl} littermates. At least 150 glomeruli of each mouse were analyzed.
analyzed. The data are the mean ± s.e.m. ***p < 0.001 via a 2-tailed Mann-Whitney U test (CD<sup>Ctrl</sup> littermates versus CD<sup>pdKO</sup> mice: 3.23 ± 0.75 versus 67.83 ± 3.26%, respectively; n=4).

**Figure 7.** Subunit c of mitochondrial ATP synthase is accumulated in lysosomal structures of podocytes as in CD-deficient neurons.

(A) Immunohistochemical staining showed subunit c of mitochondrial ATP synthase accumulated in 10-month-old CD<sup>pdKO</sup> mouse podocytes.

(B and C) Immunoelectron microscopy indicated that, in podocytes from CD<sup>pdKO</sup> mice, the labeling for subunit C was associated with membrane-bound structures containing GRODs. Immunocytochemical staining of subunit c of mitochondrial ATP synthase in podocytes from CD<sup>pdKO</sup> mouse and their CD<sup>Ctrl</sup> littermate at 10 months, using the cryothin section immunogold method. Subunit c. Gold particles label only the mitochondrial inner membrane in the control littermates (arrowheads), whereas they are associated with both the inner membrane of intact mitochondria (arrowhead) and the membrane-bound compartments with dense materials (GRODs) (arrows) in the CD<sup>pdKO</sup> mice (FPs; Foot Processes).

**Figure 8.** The accumulation of podocin in 10-month-old CD<sup>pdKO</sup> mouse podocyte cell bodies was particularly colocalized with Lamp1 and Rab7.

(A and B) Immunofluorescence staining showed the localization of podocin in the GBM in CD<sup>Ctrl</sup> littermates. By contrast, podocin was localized in both the GBM and podocyte cell bodies in CD<sup>pdKO</sup> mice. The late endosomal marker Rab7 was increased in CD<sup>pdKO</sup> mouse podocytes compared with CD<sup>Ctrl</sup> littermate podocytes. Podocin localized in podocyte cell bodies was particularly colocalized with Lamp1 (A) and Rab7 (B) in CD<sup>pdKO</sup> mice.
(C) For immunohistochemical staining, we detected a strong accumulation of podocin in CD\textsuperscript{pdKO} mouse podocyte cell bodies. Immuno-electron microscopy using paraffin-embedded tissue sections indicated that the granular structures (GRODs) that accumulate in the podocyte cell bodies of CD\textsuperscript{pdKO} mouse glomeruli are autophagosomes/autolysosomes containing degraded podocin (GBM; Glomerular Basement Membrane).

**Figure 9.** Kidney sections from FSGS showed a reduction in the CD expression in podocytes.

(A-D) Staining of CD in human kidney biopsy specimens. The number of CD-positive cells per glomerulus (D) are indicated as the means±s.e.m. of five minor glomerular abnormalities, six MCNS and six FSGS samples. The expression of CD was significantly decreased in FSGS (C) as compared with that in MCD (B). **p<0.01, 2-tailed Mann-Whitney U test.

**Supplemental Figure 1.**

(A) The expression of Lamp1, poly ubiquitin, and LC3 were examined by western blotting of isolated glomerular homogenates in 3-month-old mice. Note that a much higher levels of Lamp1, poly-ubiquitylated polypeptides, and the autophagosome marker, LC3-II were detected in CD\textsuperscript{pdKO} mouse glomeruli.

(B, C and D) Quantitative analysis of western blotting images of Lamp1 (B), LC3- II /LC3-I ratio (C) and LC3 (D) from 3 isolated glomerular homogenates. The levels of Lamp1, LC3- II /LC3-I ratio and LC3- II significantly increased in CD\textsuperscript{pdKO} mice. The data are the mean ± s.e.m. *p<0.05, **p<0.01 by 2-tailed Student’s t-test.

**Supplemental Figure 2.**
Immunofluorescent staining of SD proteins podocin and nephrin was performed on kidney samples from 10-month-old CD<sup>Ctrl</sup> littermates and CD<sup>pdKO</sup> mice. Podocin and nephrin localized to the GBM and podocyte cell bodies in CD<sup>pdKO</sup> mice. And nephrin localized in podocyte cell bodies was particularly colocalized with podocin.

(B) Podocin localized in podocyte cell bodies was hardly colocalized with the early endosomal marker Rab5 in both CD<sup>Ctrl</sup> littermates and CD<sup>pdKO</sup> mice.

(C) Western blotting analysis of Rab5 and Rab7 in isolated glomerular homogenates in 3-month-old mice. Both CD<sup>pdKO</sup> mouse podocytes and CD<sup>Ctrl</sup> littermate podocytes expressed similar levels of Rab5, while Rab7 were increased in CD<sup>pdKO</sup> mouse podocytes compared with CD<sup>Ctrl</sup> littermate podocytes as well as in immunofluorescent staining.

Supplemental Figure 3. Nephrin, as well as podocin, accumulated in 10-month-old CD<sup>pdKO</sup> mouse podocyte cell bodies, and both were particularly colocalized with Lamp1 (A) and Rab7 (B).

Supplemental Figure 4.

(A) Immunofluorescence staining showed that CL was increased in 10-month-old CD<sup>pdKO</sup> mouse podocytes.

(A and B) Expression of synaptopodin (A) and CD2AP (B) which were substrates for CL displayed no significant differences between CD<sup>pdKO</sup> mice and CD<sup>Ctrl</sup> littermates.

Supplemental Table 1. Clinical data from glomerular disease patients. The data are expressed as the mean ± s.e.m.
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Figure 1

A. Podocin

CD<sup>Ctrl</sup> CD<sup>pdKO</sup>

24 months

B. Kidney lysate

CD<sup>Ctrl</sup> CD<sup>pdKO</sup>

Glomerular lysate

C. Percent survival

n=14 CD<sup>Ctrl</sup>

n=13 CD<sup>pdKO</sup>

D. 24 months

CD<sup>Ctrl</sup> CD<sup>pdKO</sup>

E. 24 months

CD<sup>Ctrl</sup> CD<sup>pdKO</sup>
Figure 2

A

Urinary albumin/creatinine ratio (mg/g)

B

Serum UN (mg/dl)

C

Serum CRE (mg/dl)

12 months n=5, 22-24 months n=7
Figure 3

A 3 months  B 12 months  C 20 months

D: Sclerotic glomeruli/total glomeruli (%)

CD Ctrl  CD pdKO  CD Ctrl  CD pdKO
12 months  22-24 months n=3

* *
Figure 5

A  WT1
B  Cleaved caspase8
C  Cleaved caspase3
D  TUNEL

B, F and H n=3
D n=4

WT1 positive dots/gomerulus

Cleaved caspase8 positive dots/total glomeruli (%)

Cleaved caspase3 positive dots/total glomeruli (%)

TUNEL positive dots/total glomeruli (%)
Figure 6

**A** Synaptopodin | CB | Merged+DAPI
---|---|---
CD<sub>Ctrl</sub> |  |  |
CD<sub>pdKO</sub> |  |  |

**B** LC3 | Lamp1 | Merged+DAPI
---|---|---
CD<sub>Ctrl</sub> |  |  |
CD<sub>pdKO</sub> |  |  |

**C** P62

**D** Ubiquitin

**E**

![Bar graph showing P62 positive glomeruli percentage](image)

P62 positive glomeruli / total glomeruli (%)

CD<sub>Ctrl</sub> | CD<sub>pdKO</sub>
---|---
10-12 months | 10-12 months

n=4

***
Figure 7

A  Subunit C

CDCtrl

CDpdKO

B  Subunit C

CDCtrl

FPs

C

FPs

Subunit C

Subunit C
Figure 8

A

Podocin | Lamp1 | Merged

CDctrl

CDpdKO

20μm

Podocin Rab7 Merged

CDctrl

CDpdKO

20μm

High magnification

B

Podocin | Rab7 | Merged | High magnification

CDctrl

CDpdKO

20μm

GRODs

C

CDctrl | CDpdKO | CDpdKO

Podocin

GBM

GRODs

2μm
Figure 9

A

Minor abnormalities

B

MCD

C

FSGS

D

CD dots/total nuclei (%)

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**p < 0.01
Suppl. Figure 1

A. Western blots showing Lamp1, Poly UB, LC3-I, LC3-II, and β-Actin in CD Ctrl (control) and CD pdKO (papillar deficiens knockout) cells.

B. Graph showing the ratio of Lamp1/GAPDH in CD Ctrl and CD pdKO cells. The ratio is significantly higher in CD pdKO cells compared to CD Ctrl cells.

C. Graph showing the ratio of LC3-II/LC3-I in CD Ctrl and CD pdKO cells. The ratio is significantly higher in CD pdKO cells compared to CD Ctrl cells.

D. Graph showing the ratio of LC3 to β-actin in CD Ctrl and CD pdKO cells for LC3-I and LC3-II. The ratio for LC3-II is significantly higher in CD pdKO cells compared to CD Ctrl cells.
Suppl. Figure 2

A

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Suppl. Figure 3

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Scale bars: 20μm/5μm
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