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Blockade of Cysteine Cathepsin Caused Lysosome Dysfunction and Aggravated Adenine-induced Nephropathy by Inducing Tubular Cell Senescence

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Running title: Cysteine cathepsin inhibition exacerbates AN

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Abstract

Lysosomal cysteine cathepsins are a group of endopeptidases which exert critical, context-dependent roles in chronic kidney disease (CKD) with underlying mechanisms incompletely clarified. In this study, we investigated the consequences of pan-cysteine cathepsin inhibition using the pharmacological inhibitor E-64 in C57BL/6 mice with adenine-induced nephropathy (AN), followed by proteomic analysis to resolve underlying mechanisms. Our results showed an overall reduction of cysteine cathepsin activity in the kidneys of AN mice, which was further impaired upon E-64 treatment and accompanied by exacerbated renal structural and functional deterioration. Notably, proteomic analysis revealed significant enrichment of lysosomal proteins and lysosomal degradation pathways in AN kidneys following E-64 treatment. This was associated with accumulation of dysmorphic lysosomes with compromised substrate degrading ability. Importantly, E-64-induced lysosomal dysfunction provoked renal tubular cell senescence, as evidenced by elevated expression of cell cycle suppressor genes *p19* and *p21*, increased senescence-associated β -galactosidase activity, and a blunted adaptive proliferation of tubular cells. Collectively, pan-cysteine cathepsin inhibition induces lysosomal dysfunction and cellular senescence in renal tubular cells, resulting in aggravated severity of adenine-induced nephropathy. Maintaining the homeostasis of cysteine cathepsin activity is crucial for preventing renal tubular cell injury in the treatment of CKD.

Key words: chronic kidney disease; cysteine cathepsin; lysosomal dysfunction; senescence; E-64; adenine nephropathy

Introduction

Chronic kidney disease (CKD) is defined as a disease condition with the presence of structural or functional abnormalities in kidney for more than three months ¹. This disease represents a substantial burden on public health worldwide, with a global prevalence estimated at 14.6% ². In the context of CKD, detrimental factors such as

61 uremic toxins, oxidative stress, and inflammatory mediators can lead to pathological
62 alterations in renal parenchymal cells ³. Although these cells may undergo adaptive
63 proliferation in an attempt to compensate for declining renal function, a subset of
64 them exhibits classic hallmarks of cellular senescence ^{3,4}. These features include cell
65 cycle arrest, upregulation of cyclin-dependent kinase inhibitor (CDKI) genes (e.g.,
66 *p16*, *p21*), enhanced activity of senescence-associated β -galactosidase (SA- β -Gal),
67 and the development of a senescence-associated secretory phenotype (SASP). In turn,
68 renal cell senescence exacerbates the progression of CKD and contributes to
69 associated cardiovascular complications ³. Consequently, a thorough understanding of
70 the molecular mechanisms underlying renal cell senescence is essential for the
71 development of therapeutic strategies aimed at attenuating CKD pathogenesis.

72 Due to its involvement in extensive blood filtration and nutrient reabsorption, the
73 kidney is characterized by a high metabolic rate ⁵. Consequently, renal cells,
74 particularly renal tubular epithelial cells, are subject to considerable metabolic stress
75 and must therefore possess a robust capacity for the clearance of damaged organelles
76 and macromolecular waste to sustain intracellular homeostasis ⁶. Through specialized
77 intracellular sorting mechanisms, such as autophagy, damaged organelles and
78 metabolic waste macromolecules are delivered to lysosomes-the primary sites of
79 waste degradation and nutrient recycling ⁷. Lysosomes are abundantly distributed
80 within renal tubular cells and their dysfunction can impair renal cell function or even
81 its survival under hazardous stress, thereby contributing to the pathogenesis of CKD ⁸.
82 Genetic mutations in the lysosome-associated genes *GLA* and *CTNS* lead to
83 intra-lysosomal accumulation of undegraded products, namely glycosphingolipids and
84 cystine, and directly underlie the inherited disorders Fabry disease and cystinosis,
85 both of which show characteristic CKD manifestations in humans ⁹. In non-inherited
86 situations, it is reported that structurally dysmorphic lysosomes are significantly more
87 abundant in the tubular cells of patients with hypertensive CKD and those with CKD
88 of uncertain etiology ¹⁰. In diabetic kidney disease, advanced glycation end products
89 (AGEs) induce lysosomal dysfunction in proximal tubular cells, promoting
90 exocytosis-mediated excretion of Lrp2 (Megalin), reducing the re-absorptive capacity

of tubular cells, and exacerbating proteinuria ¹¹. In cystinosis resulting from *CTNS* mutations, intra-lysosomal cystine accumulation causes tubular cell apoptosis ⁹. Despite these achievements, it should be noted that the mechanisms by which lysosomal dysfunction contributes to tubular cell injury are heterogeneous and etiologically dependent. Thus, a thorough clarification of these mechanisms is crucial for improving future CKD management.

Cathepsins constitute a family of endopeptidases that play essential roles in the degradation, maturation, and functional modification of substrate proteins. Predominantly localized to endosomal and lysosomal compartments, these proteases are involved in diverse physiological and pathological processes, such as cellular protein turnover, antigen processing and presentation, tumor metastasis, and viral infection ^{12, 13}. Based on the amino acid residues involved in catalytic activity, cathepsins are classified into three groups: cysteine cathepsins, serine cathepsins, and aspartic cathepsins ¹². Among these, cysteine cathepsins represent the largest subgroup, consisting of cathepsins B, C, F, H, K, L, O, S, W, and Z. Many of these cysteine cathepsins exhibit detectable expression in the kidney, with cathepsins B and L being robustly expressed in renal proximal tubules ^{14, 15}. Current evidence suggests that cysteine cathepsins play distinct and context-dependent roles in CKD. For instance, cathepsins B and L have been reported to protect against, whereas cathepsins S and K exacerbate, renal fibrosis induced in mice by unilateral ureteral obstruction (UUO) ¹⁴. Conversely, cathepsins B and/or L facilitate glomerular cell injury and proteinuria in glomerular diseases, including streptozotocin (STZ)-induced experimental DKD and nephrotoxic serum-induced glomerulonephritis ^{16, 17}. However, in consistence with the case in UUO mice, cathepsin S also contributes to the pathogenesis of DKD-related glomerular injury in db/db mice subjected to uninephrectomy and mice with immune glomerulonephritis ^{18, 19}. Nevertheless, pharmacological inhibition of pan-cysteine cathepsins using the irreversible inhibitor E-64 attenuates proteinuria in rats subjected to anti-glomerular basement membrane antibody-induced glomerulonephritis ²⁰. Conversely, inhibition of cysteine cathepsins with E-64 has no effect on either renal pathology or function in Dahl salt-sensitive

rats, a model of hypertensive nephropathy²¹. Therefore, the precise roles of cysteine cathepsins in CKD warrant further investigation in a context-dependent manner.

In this study, we investigated the effects of pan-cysteine cathepsin inhibition using E-64 on renal structure and function in a mouse CKD model with adenine-induced nephropathy (AN). Our findings demonstrate that inhibition of cysteine cathepsins induces lysosomal dysfunction, leading to cellular senescence and impaired proliferation of tubular cells, and ultimately exacerbates the severity of CKD in the AN model.

Results

Blockade of cysteine cathepsin activity aggravated adenine-induced nephropathy

To investigate the role of cysteine cathepsin in CKD, we established the adenine-induced nephropathy (AN) in C57BL/6 mice and concurrently administered E-64, a broad-spectrum, irreversible cysteine cathepsin inhibitor²², *via* intraperitoneal injection (Fig. 1A). Adenine-containing diet led to a progressive decline in body weight, which was not significantly altered by E-64 treatment at doses of 25 and 50 mg/kg/day, respectively (Fig. 1B). Compared with healthy controls, AN mice exhibited markedly reduced cysteine cathepsin activity in kidney lysates. E-64 administration further suppressed cathepsin activity in AN mice, confirming effective inhibition of renal cysteine cathepsins (Fig. 1C).

AN mice showed elevated serum creatinine and urea levels relative to controls, and these parameters were further increased following E-64 treatment (Fig. 1D and E). Macroscopically, kidneys from AN mice showed a pale appearance with a rough surface, features that were more pronounced in E-64-treated AN mice (Fig. 1F). HE staining of AN kidney sections revealed substantial structural disruption, characterized by tubular dilation, tubular epithelial cell degeneration, and interstitial expansion. Sirius Red staining showed increased interstitial deposition of collagen fibers. These structural abnormalities were exacerbated by E-64 treatment (Fig. 1F–H). Furthermore, Western blot and immunohistochemical analyses confirmed upregulation of fibrotic markers including Colla1, fibronectin, and α -SMA in AN

kidneys. Expression of these fibrotic proteins was further elevated after E-64 administration (Fig. 1I–N).

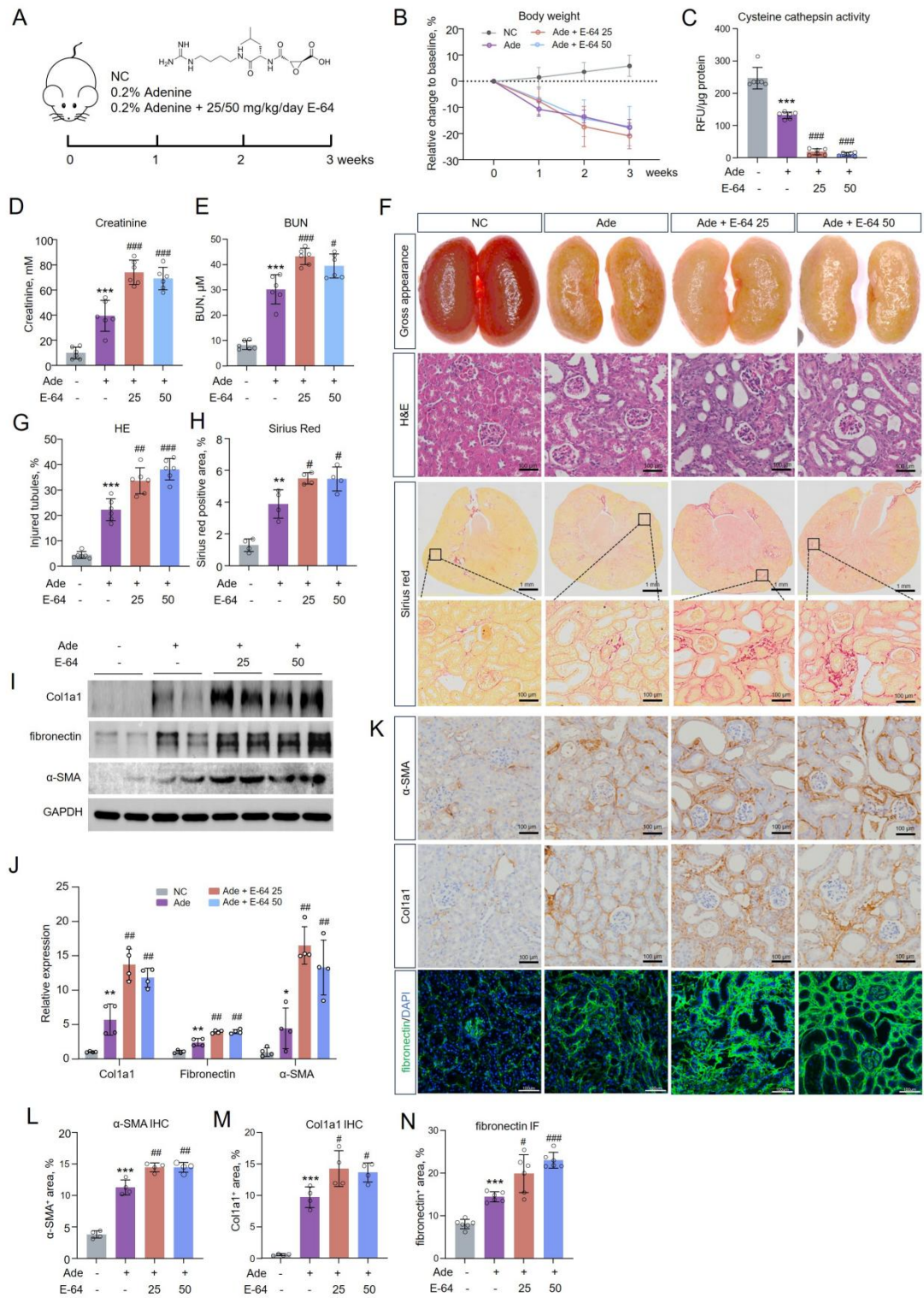


Figure 1. E-64 treatment exacerbates renal functional impairment and fibrotic lesions in AN mice. A. Experimental design of E-64 intervention in AN mice. The chemical structure of E-64 is

shown in the top panel. B. Weekly body weight of mice in each group. C. Cysteine cathepsin activity normalized by protein concentration in kidney lysate. D and E. Serum creatinine and blood urea nitrogen (BUN) levels in mice. F. Representative macroscopic images of kidneys and corresponding pathological staining (HE and Sirius Red) from each group after 3 weeks of treatment. G. Quantification of the percentage of injured renal tubules based on HE staining. H. Quantification of collagen fiber deposition based on Sirius Red staining. I and J. Western blot analysis showing expression levels of fibrosis-related proteins (α -SMA, fibronectin, and Colla1) in renal tissues and corresponding quantitative results. K-N. Immunohistochemical and immunofluorescence analysis of the expression and distribution of fibrotic marker proteins (α -SMA, fibronectin, and Colla1) in kidney sections, together with quantitative assessments. Each dot indicates datum derived from individual animal. WB, western blot. IHC, immunohistochemistry. IF, immunofluorescence. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ Vs. NC. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ Vs. Ade.

We further employed RT-PCR to analyze the expression of mature renal tubular marker genes closely associated with kidney function—*Aqp1*, *Lrp2*, *Cdh16*, *Slc41a1*, *NkAa1*, and *Slc12a3*. The results revealed a significant downregulation of these genes in AN kidney. Notably, E-64 treatment further reduced the expression levels of *Aqp1* and *Lrp2* (Fig. 2A). Immunohistochemical analysis further confirmed that E-64 treatment further suppressed the protein expression of *Aqp1* and *Lrp2* in AN kidney (Fig. 2B-D). Collectively, these data indicate that inhibition of cysteine cathepsin exacerbates renal structural and functional impairment in AN mice.

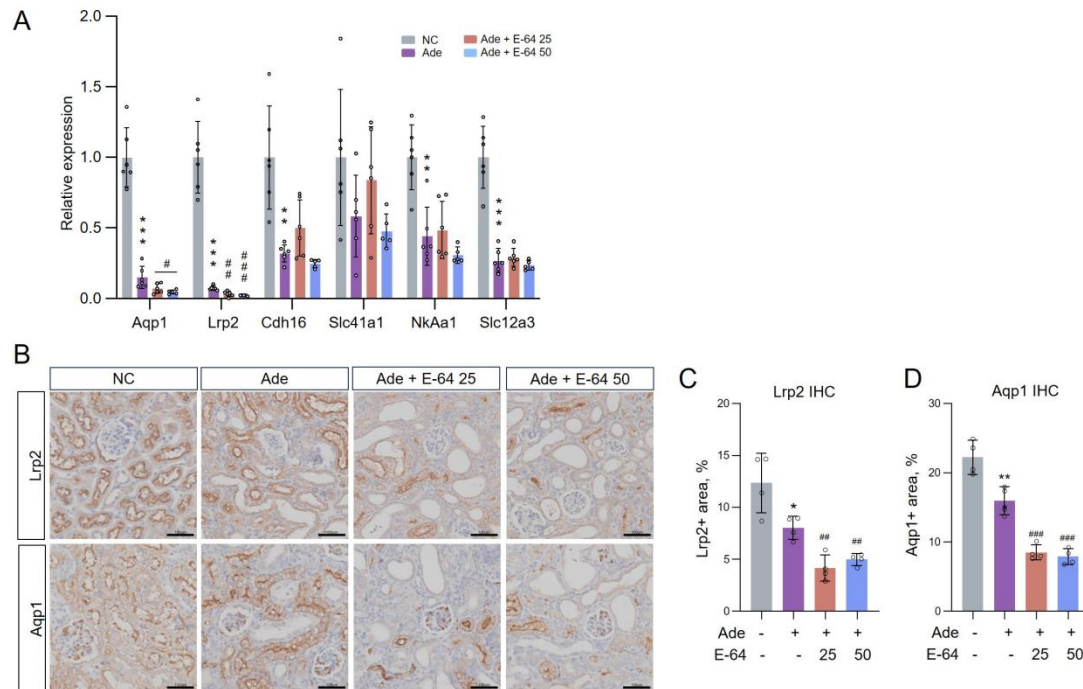


Figure 2. E-64 intervention inhibits the expression of renal tubular marker genes in AN kidney. A. RT-PCR analysis of mRNA expression levels of *Aqp1*, *Lrp2*, *Cdh16*, *Slc41a1*, *NkAa1*, and *Slc12a3* in renal tissue. B–D. Immunohistochemical analysis of *Lrp2* and *Aqp1* expression in renal tissue, followed by their quantitative analyses, respectively. IHC, immunohistochemistry. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ Vs. NC. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ Vs. Ade.

Proteomic analysis revealed that inhibition of cysteine cathepsin activity impaired lysosomal metabolic pathways

To investigate the mechanism by which inhibition of cysteine cathepsin exacerbated renal injury in AN, we performed DIA quantitative proteomic analysis on renal tissues from AN mice treated with or without E-64 (50 mg/kg/day). Principal component analysis (PCA) showed characteristic clustering of the samples from different treatment groups, indicating significant differences in protein expression between the two groups (Fig. 3A). As revealed by volcano plot and heatmap analysis, a total of 8405 proteins were identified, among which 347 proteins showed significantly increased abundance, whereas 157 proteins demonstrated decreased abundance in renal tissues of E-64-treated AN mouse (Supplementary Table 3, Fig. 3B and C). Notably, KEGG pathway enrichment analysis revealed that upregulated proteins were

notably enriched in lysosome and lysosomal substrate degradation-related pathways, including lysosome, glycosaminoglycan degradation, sphingolipid metabolism, antigen processing and presentation, and phagosome (Fig. 3D). Among the differentially expressed proteins, 52 upregulated proteins were localized to the lysosome (Fig. 3E). Since E-64 is a cysteine cathepsin inhibitor, we focused on the expression levels of cathepsins among the upregulated proteins and found that not only cysteine cathepsins (B, C, F, H, K, L, O, S, W, Z) were increased at the protein level, but also aspartic cathepsin (D) and serine cathepsin (A) were significantly elevated (Fig. 3F, proteases are classified based on <https://enzyme.expasy.org/enzyme-bynome.html>).



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Blockade of cysteine cathepsin activity caused ectopic lysosome accumulation while with compromised lysosomal degradation capability

Given that proteomic analysis indicated abnormal expression of numerous lysosomal proteins upon E-64 treatment, we subsequently examined the expression of the lysosomal marker protein Lamp1 using immunohistochemistry. The results revealed increased Lamp1 expression in renal tissues of AN mice, while E-64 treatment further upregulated Lamp1 expression (Fig. 4A and 4B). These findings were corroborated by western blot analysis (Fig. 4C and 4D). Given that elevated Lamp1 expression may reflect changes in lysosomal abundance, we performed transmission electron microscopy to assess the ultrastructure of renal tubular cells. The results demonstrated an increased number of lysosomes (red arrows) in renal tubular cells of AN mice compared to normal controls. However, E-64 treatment induced further lysosomal accumulation within tubular cells (Fig. 4E).

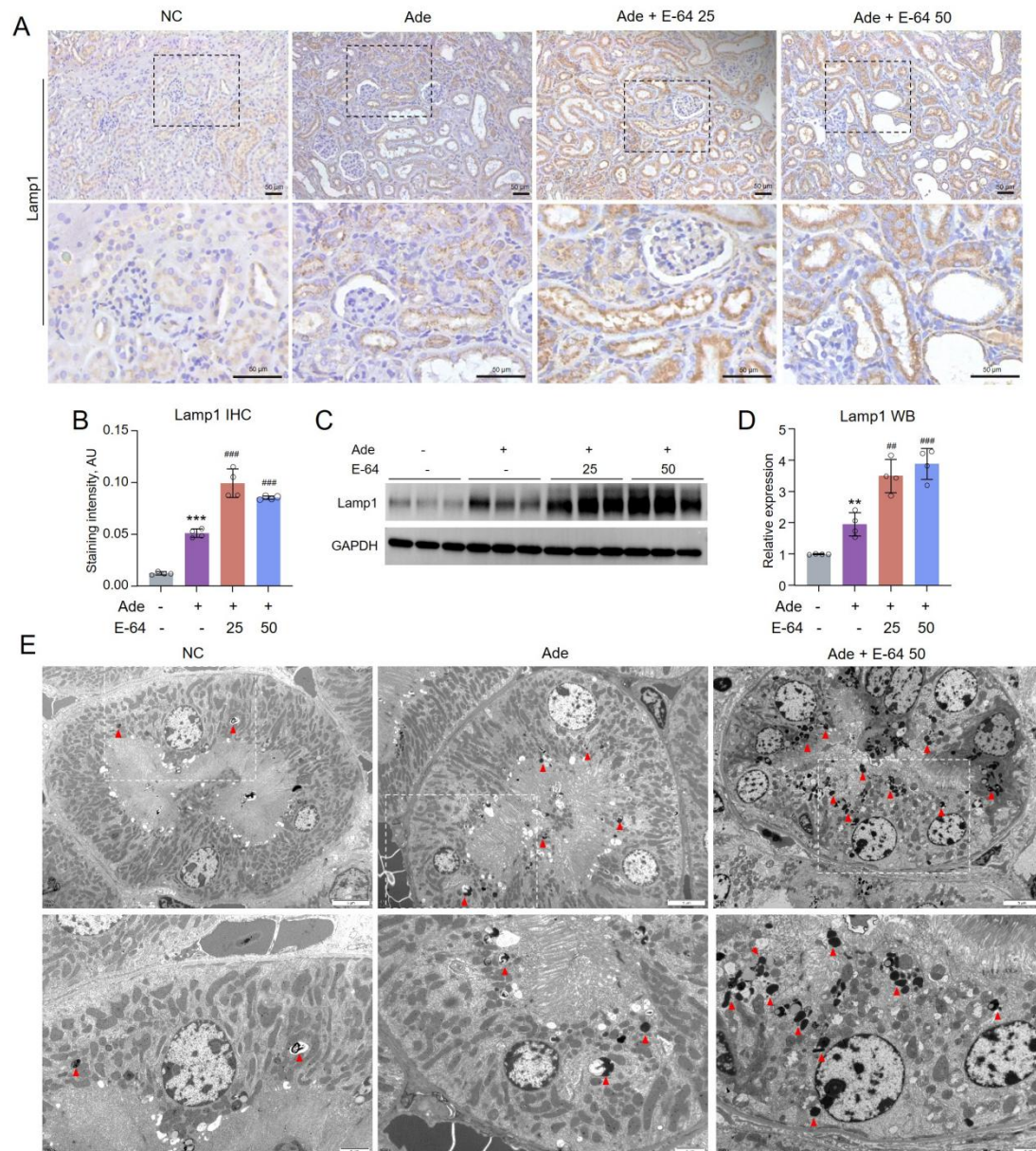


Figure 4. Inhibition of cysteine cathepsin activity induces aberrant lysosomal accumulation in renal tubular cells of AN mice. A and B. Immunohistochemical analysis of Lamp1 expression in kidney tissues of mice with indicated treatments and its quantitative analysis. C and D. Western blot analysis of Lamp1 protein expression in kidney tissues and its quantitative analysis. E. Transmission electron microscopy analysis to reveal the subcellular ultrastructure of renal tubular cells, with lysosomes indicated by red arrows. WB, western blot. IHC, immunohistochemistry. ** $p < 0.01$ and *** $p < 0.001$ Vs. NC. ## $p < 0.01$ and ### $p < 0.001$ Vs. Ade.

Inhibition of cysteine cathepsin activity induced aberrant lysosomal accumulation and functional impairment in cultured renal tubular cells

We further investigated the effects of cysteine cathepsin inhibition on lysosomes in cultured renal tubular epithelial cells (NRK52E). NRK52E cells were treated with or without 100 μ M E-64 and 1 mM adenine for three days. Enzymatic activity assays demonstrated that while adenine administration inhibited cellular cysteine cathepsin activity (consistent with the *in vivo* findings in Fig. 1C), E-64 treatment more potently suppressed enzyme activity (Fig. 5A). Next, lysosomal status was assessed using the lysosome-specific tracer dye LysoTracker. Adenine treatment increased LysoTracker staining, whereas E-64 treatment further enhanced LysoTracker staining intensity regardless of the presence or absence of adenine stimulation (Fig. 5B). These results were confirmed by flow cytometry quantification (Fig. 5C and D), suggesting that E-64 induced an increase in lysosomal number within NRK52E cells. Notably, transmission electron microscopy observation not only revealed an increased number of lysosomes upon E-64 treatment but also demonstrated that these lysosomes exhibited abnormal morphology characterized by increased intralysosomal content and swelling appearance (Fig. 5E).

We next assessed lysosomal proteolytic activity using the DQ-BSA-Green, a reagent consisting of BSA conjugated with a high-density fluorescent dye. Due to fluorescence self-quenching, intact DQ-BSA-Green is non-fluorescent. Upon degradation by lysosomal enzymes, the fluorescent dye is released, generating a detectable fluorescent signal. Therefore, the intensity of DQ-BSA-Green fluorescence reflects lysosomal substrate degradation activity²³. Both microscopy observation and flow cytometry analysis revealed that, compared with the blank-treated cells, E-64 treatment reduced lysosomal degradation activity. Adenine treatment significantly enhanced lysosomal degradation activity. However, E-64 markedly suppressed the adenine-induced lysosomal degradation activity (Fig. 5F-H). Taken together with the *in vivo* data presented in Figure 4, these findings indicate that although E-64 treatment increases lysosomal number, these lysosomes exhibit aberrant morphology and compromised function, presenting a pathological manifestation resembling human lysosomal storage diseases (LSDs)²⁴.

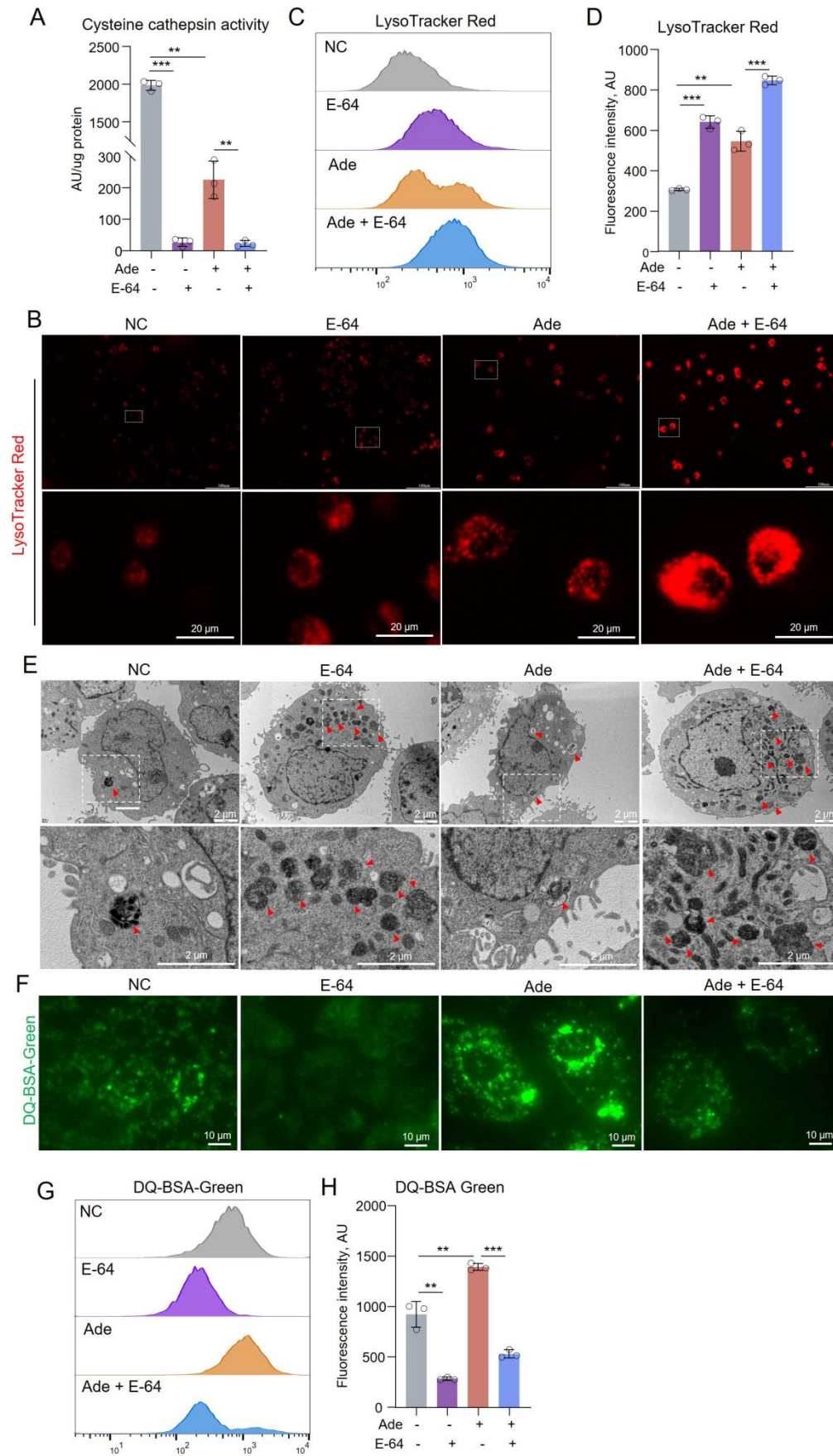


Figure 5. Inhibition of cysteine cathepsins causes lysosomal accumulation and functional

impairment in NRK52E cells. A. Quantification of cysteine cathepsin activity in NRK52E cells subjected to the indicated treatments. B. Representative images of LysoTracker Red staining for lysosomal labeling in NRK52E cells. C and D. Flow cytometric analysis of LysoTracker Red fluorescence intensity and corresponding quantitative summary. E. Transmission electron microscopy images depicting subcellular ultrastructure. Red arrowheads indicate lysosomes. F. Fluorescence microscopy images showing lysosomal degradation activity assessed using the DQ-BSA-Green substrate. G and H. Flow cytometric analysis of DQ-BSA-Green-derived fluorescence and corresponding quantification. Each dot represents an independent cell culture. ** $p < 0.01$ and *** $p < 0.001$.

Inhibition of cysteine cathepsin activity induced senescence and impaired proliferation in renal tubular cells

Lysosomal dysfunction has been reported to induce cellular senescence^{25,26}, a pivotal process contributing to renal cell injury in the pathogenesis of CKD^{8,9}. Therefore, we investigated whether cysteine cathepsin deficiency could influence cellular senescence in CKD environment. RT-PCR analysis of cyclin-dependent kinase inhibitor (CDKI) family genes revealed that all CDKI members, including *p15*, *p16*, *p18*, *p19*, *p21*, *p27*, and *p57*, were upregulated in kidney tissues from AN mice. Among these, treatment with E-64 further promoted the expression of *p19* and *p21* (Fig. 6A). Given the more pronounced changes in *p21* expression, we checked p21 protein levels by western blot, which confirmed its upregulation in the kidneys of AN mice following E-64 treatment (Fig. 6B, C). Moreover, E-64-treated AN mice exhibited enhanced SA- β -Gal staining in kidney tissues, especially in the high-dose group (50 mg/kg) (Fig. 6D, E).

In NRK52E cells, adenine treatment reduced p21 expression and decreased SA- β -Gal staining compared with control cells. However, E-64 supplementation upregulated p21 protein levels and increased the proportion of SA- β -Gal-positive cells, regardless of the presence or absence of Ade (Fig. 6F-H).

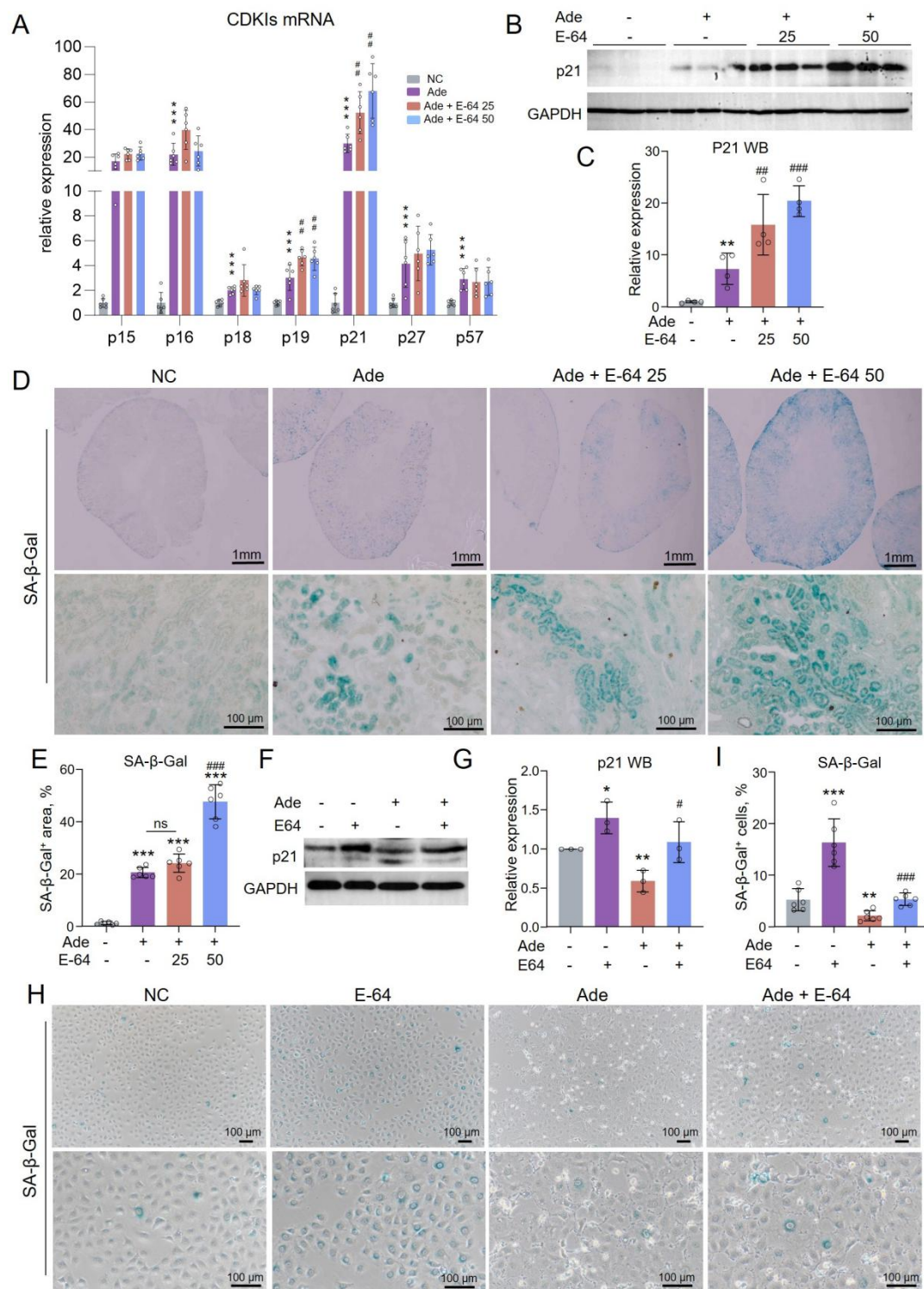


Figure 6. Cysteine cathepsin inhibition induces renal tubular cell senescence. A. RT-PCR analysis of mRNA expression levels of CDKI family members. B and C. Western blot analysis of p21 protein expression in kidney tissue and corresponding quantification. D and E. Representative images and quantification of SA-β-Gal staining in kidney tissue. F and G. Western blot analysis of p21 expression in NRK52E cells with indicated treatments and corresponding quantification. H

and I. Representative images and quantification of SA- β -Gal staining in NRK52E cells under indicated treatment conditions. Each data point represents an independent animal or cell culture replicate. ns, no significance. WB, western blot. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ Vs. healthy mice or blank-treated NRK52E cells. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ Vs. AN mice or Ade-treated NRK52E cells.

We next examined the impact of cysteine cathepsin inhibition on tubular cell proliferation. Immunostaining for the proliferating cell marker Ki67 revealed increased cell proliferation in kidney tissues of AN mice, keeping in line with an adaptive reparative response to injury⁴, which was significantly attenuated by E-64 treatment (Fig. 7A, B). Consistent with these *in vivo* findings, adenine treatment alone enhanced proliferation (Ki67-positive cells) in NRK52E cells *in vitro*, whereas E-64 supplementation suppressed proliferation both in the presence and absence of Ade (Fig. 7C, D). Collectively, these results indicate that cysteine cathepsin deficiency leads to suppressed proliferation of renal tubular cells.

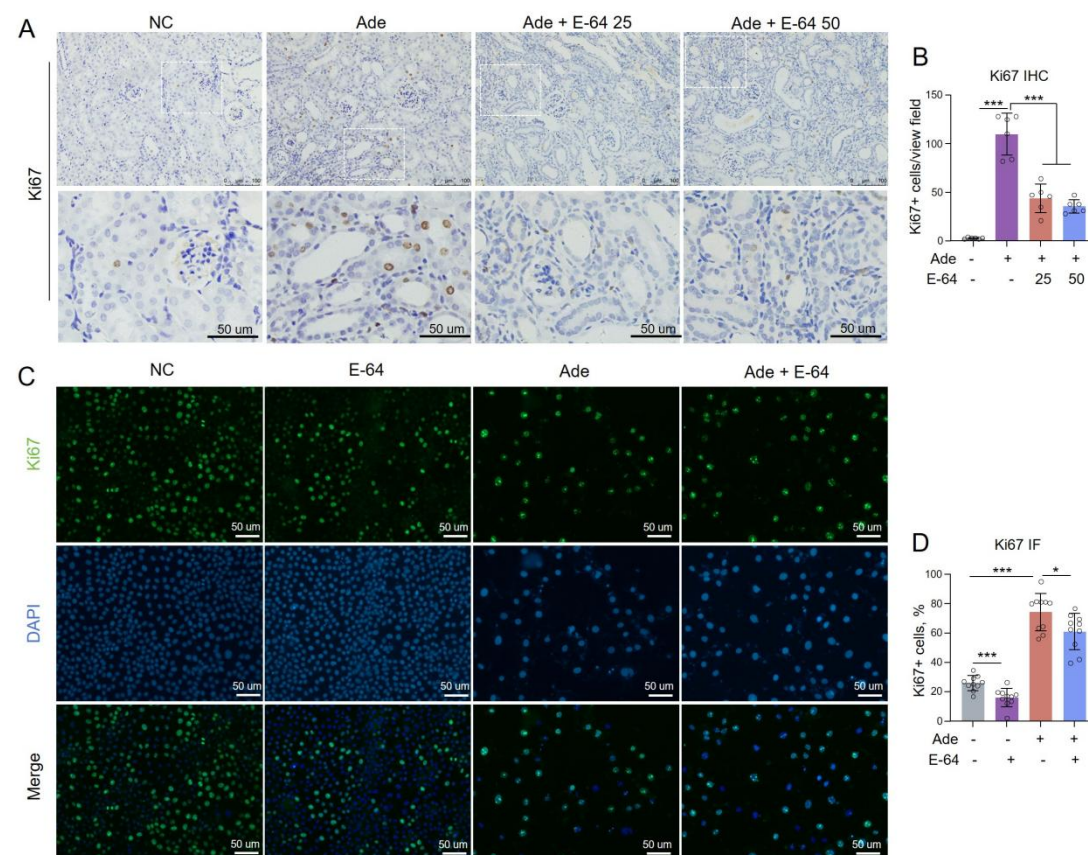


Figure 7. Cysteine cathepsin inhibition impairs renal tubular cell proliferation. A and B.

Representative images and quantification of immunohistochemical staining for Ki67 in kidney tissue. C and D. Representative images and quantification of immunofluorescence staining for Ki67 in NRK52E cells under the indicated treatment conditions. Each data point represents an independent animal or cell culture replicate. IHC, immunohistochemistry. IF, immunofluorescence. * $p < 0.05$ and *** $p < 0.001$.

Discussion

CKD is a heterogeneous disorder whose underlying pathological mechanisms remain incompletely elucidated. Reported evidence indicates that cysteine cathepsins exert paradoxical, context-dependent functions in distinct subtypes of CKD^{14, 16, 20, 21}. In the current study, we examined the impact of pan-cysteine cathepsin activity inhibition in AN mouse, a well-established CKD characterized by tubule-centered injury, tubulointerstitial fibrosis, and progressive decline in renal function²⁷. Our results demonstrated that adenine-induced nephropathy exhibited reduced cysteine cathepsin activity in renal tissue. Vigorous inhibition of pan-cysteine cathepsin activity using E-64 significantly exacerbated tubulointerstitial fibrosis and further impaired renal function in AN mice, as assessed by blood biochemistry, histopathological examination, and analysis of fibrotic protein expression (Fig. 1). Moreover, cysteine cathepsin inhibition led to increased loss of the proximal tubular cell maturation markers Aqp1 and Lrp2, indicating more severe degeneration of proximal tubular epithelia (Fig. 2). Collectively, these *in vivo* findings indicate that despite the distinct or even opposing roles of individual cysteine cathepsins, pan-cysteine cathepsin inhibition is overall detrimental in the context of adenine-induced renal injury. Previous studies have reported that cathepsins B and L, the primary cysteine cathepsins localized in lysosomes, exert renoprotective effects in tubular cells of UUO mice^{12, 14}. Therefore, it is possible that the renoprotective functions of cathepsins B and L may outweigh the pro-injury effects of other cysteine cathepsins, such as cathepsins S and K¹⁴, ultimately leading to aggravated renal injury in E-64-treated AN mice in current study.

Another significant finding of the present investigation is the identification of

lysosomal dysfunction in tubular epithelial cells following cysteine cathepsin inhibition. Although prior studies have reported the detrimental effects of deficiencies in individual cysteine cathepsins on renal tubular cells¹⁴, the underlying mechanisms have remained incompletely understood. In the current study, utilizing DIA proteomics, organelle-labeling dyes, and electron microscopy, we noted that pan-cysteine cathepsin inhibition induced pronounced intracellular accumulation and structural abnormalities of lysosomes both *in vivo* and *in vitro*. Importantly, these lysosomes were functionally impaired, as evidenced by compromised clearance of the waste substrate DQ-BSA-Green in affected cells (Fig. 3-5). This dysfunctional lysosomal accumulation recapitulates features characteristic of human LSDs, which arise from mutations in genes encoding lysosome-associated proteins²⁴. Indeed, mutations in genes encoding serine cathepsin A, aspartic cathepsin D, and cysteine cathepsin F have been associated with specific LSDs affecting the brain, eye, ear, skin, skeletal muscle, and other tissues²⁴. Notably, the kidney is known to be affected in galactosialidosis, an LSD can be caused by mutations in *CSTA* (the gene encoding cathepsin A)²⁸. Although cysteine cathepsins execute essential roles in the degradation of macromolecular waste and several family members exert renoprotective functions through various mechanisms, our findings reveal for the first time that cysteine cathepsin inhibition can induce LSD-like accumulation of dysfunctional lysosomes in renal tubular cells, a pathogenic process that may account for the exacerbation of renal injury observed in AN mice.

Lysosomal dysfunction is known to disrupt multiple cellular processes, including the induction of premature senescence, a phenomenon documented in osteoarthritis, Krabbe disease, and Hutchinson-Gilford progeria syndrome^{25, 26, 29, 30}. Cell cycle arrest is a pivotal feature of senescent Cells, usually accompanied by elevated activity of SA- β -Gal³¹. In the present study, renal tubular cells of AN mice exhibited an increased number of lysosomes, reduced cysteine cathepsin activity, and notable senescence (Fig. 4 and 6), findings that are consistent with previous observations in CKD patients and in corresponding animal models⁸⁻¹⁰. However, lysosomal dysfunction induced by cysteine cathepsin inhibition further exacerbated tubular cell

senescence, as evidenced by elevated expression levels of cell cycle inhibitor genes *p19* and *p21*, as well as more pronounced SA- β -Gal staining in the kidneys of E-64-treated AN mice, particularly in the high-dose E-64 treatment group (Fig. 7). In addition, adaptive proliferation of tubular cells was also blunted in E-64-treated AN mice (Fig. 8). The pro-senescence and proliferation-suppressing effects of cysteine cathepsin inhibition were further validated in cultured NRK52E cells (Fig. 6F-H and 7C-D). Collectively, these findings demonstrate that cysteine cathepsin inhibition contributes to adenine-induced nephropathy by inducing lysosomal dysfunction and subsequent cellular senescence in renal tubular cells.

One intriguing observation is that adenine treatment stimulated the lysosomal clearance ability of NRK52E cells in the condition without E-64 as tested by the DQ-BSA-Green degradation assay, despite the cysteine cathepsin activity was suppressed (Fig. 5A, G, and H). It is possible that adenine as a stress factor primes the waste clearance capacity of lysosome although the intra-lysosomal activity of hydrolytic enzyme, including cysteine cathepsin, is partially compromised.

Conclusions

Collectively, the findings of the present study demonstrate that adenine-induced nephropathy is characterized by suppressed cysteine cathepsin activity. Pharmacological inhibition of pcysteine cathepsin leads to the accumulation of dysfunctional lysosomes and promotes cellular senescence in renal tubular cells, thereby exacerbating renal injury in the murine model of in adenine-induced CKD. Restoring the homeostasis of lysosomal cysteine cathepsin may therefore benefit the treatment of CKD subgroups characterized by tubular-centered injury.

Methods

Animal experiment

This study employed male C57BL/6 mice, aged six to eight weeks, obtained from GemPharmatech Co., Ltd. (Chengdu, China). The animals were maintained in a specific pathogen-free facility with *ad libitum* access to water and food. Following a

one-week acclimatization period, the mice were randomly assigned to four experimental groups (n = 6 per group): NC, Ade, Ade + E-64 25, and Ade + E-64 50. Mice in the NC group received a standard diet, whereas the Ade group was provided with a diet containing 0.2% adenine. Animals in both Ade + E-64 groups were maintained on the adenine-supplemented diet and administered daily intraperitoneal injections of E-64 (Beyotime, Cat# SF4135, China) at doses of 25 or 50 mg/kg/day. Mice in the NC and Ade groups received equivalent volumes of vehicle (saline) *via* intraperitoneal injection as a control. After three weeks of treatment, euthanasia was performed on all animals *via* intraperitoneal injection of pentobarbital sodium at a dose of 1 g/kg. Subsequently, blood samples and kidney tissues were harvested for further evaluation. Approval for all experimental procedures involving animals was obtained from the Animal Ethics Committee of Southwest Medical University (Approval No. 20220309-016), and the experiments were conducted following relevant ethical standards.

Histology

Formaldehyde-fixed kidney tissues were embedded in paraffin and cut into 4- μ m-thick sections. Hematoxylin and eosin (HE) staining as well as Sirius Red staining were performed as previously described³².

Biochemical analysis

Measurements of serum creatinine and urea levels were performed with an automated blood biochemistry analyzer (cobas® c702 module, Roche Diagnostics, Switzerland).

Western blot

Kidney tissues and cultured cells were lysed in IP buffer containing 1 mM PMSF for protein isolation, after which protein concentrations were measured using the Coomassie Brilliant Blue assay. Western blot and relative expression quantification of indicated proteins were performed as previously described³³. Antibodies used for western blots are detailed in Supplementary Table 1.

Immunostaining

For immunohistochemical analysis, kidney sections were first deparaffinized in xylene and rehydrated in graded ethanol solutions. Antigen retrieval was carried out in 10 mM citrate buffer (pH 6.0), and endogenous peroxidase activity was blocked with 3% H₂O₂. The sections were then incubated with 5% bovine serum albumin (BSA) for blocking, followed by overnight incubation at 4 °C with the specified primary antibodies. Primary antibody binding was detected using a two-step universal horseradish peroxidase (HRP)-conjugated anti-mouse/rabbit IgG polymer detection kit (ZSGB-Bio, Cat# PV-9000, China). Signal visualization was achieved with diaminobenzidine (DAB), and nuclei were counterstained with hematoxylin. For tissue immunofluorescence, OCT-embedded fresh kidney was sectioned at 4 µm in thickness, followed by fixation in 4% paraformaldehyde and rehydration into phosphate buffered saline (PBS, pH 7.4). For cellular immunofluorescence, post indicated treatments, NRK52E cells grown on a circle glass coverslip were fixed using 4% paraformaldehyde, followed by washing with PBS. Subsequently, the tissue section or cells on the glass coverslip were subjected to permeabilization using 0.25% Triton X-100 and blocking in 5% BSA. Primary antibody incubation was conducted at 4 °C overnight. Following rinses with PBS, the samples were incubated with the fluorescent secondary antibody for one hour at ambient temperature. Nuclear visualization was achieved using 4',6-diamidino-2-phenylindole (DAPI).

Immunohistochemical micrographs were acquired using a light microscope (Leica ICC50W, Germany), whereas immunofluorescence images were captured employing a fluorescence microscope (EVOS FL Auto, Thermo Fisher Scientific, USA). Subsequent quantitative evaluations were conducted utilizing ImageJ software.

RT-PCR

Total RNA was isolated using Trizol reagent, and first-strand complementary DNA (cDNA) was reverse-transcribed with the MightyScript First Strand cDNA Synthesis Master Mix (Sangon Biotech, Cat# B639251, China). Subsequently, quantitative

real-time PCR (RT-PCR) was carried out using the SuperStar Universal SYBR Master Mix (CWBio, Cat# CW3360, China) on the LightCycler[®] 480 System (Roche Diagnostics, Switzerland). The relative expression levels of the target genes were determined using the $2^{-\Delta\Delta Ct}$ algorithm, and gene expression values were normalized to the endogenous reference gene *GAPDH*.

Data-independent acquisition (DIA) quantitative proteomics

Renal tissues from Ade and Ade + E-64 50 group were subjected to DIA quantitative proteomic analysis (n = 4 for each group), which was performed by APTBIO Co. Ltd. In brief, the renal tissues were pulverized in liquid nitrogen and subsequently lysed in SDT lysis buffer consisting of 4% SDS and 100 mM Tris-HCl (pH 7.6). The lysate was sonicated, and boiled for 15 min. After centrifugation, protein concentration of the supernatant was quantified using the BCA protein assay method. The quality of the extracted protein was assessed using SDS-PAGE gel separation followed by staining with Coomassie Brilliant Blue R-250. Subsequently, dithiothreitol was added to achieve a final concentration of 40 mM, and the mixture was incubated at 37 °C with agitation at 600 rpm for 1.5 h to reduce disulfide bonds. The reduced bonds were then blocked by treatment with 20 mM iodoacetamide under dark conditions for 30 min. Then, the mixture was transferred to a ultrafilter (molecular weight cutoff 10 kDa) and washed successively with three times of 100 μ L of UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.0) and two times of 100 μ L of NH_4HCO_3 buffer. Next, trypsin was added at 1:50 (wt/wt) to digest at 37°C overnight. Peptides were collected by centrifugation and desalted using the Empore[™] SPE Cartridges (Cat# 186001830BA, Waters), followed by vacuum evaporation and reconstitution in 40 μ L of 0.1% (v/v) formic acid. For DIA proteomics, iRT (indexed retention time) calibration peptides were added to the sample as internal standards. Peptides were subjected to chromatographic separation (Bruker NanoElute, USA) and mass spectrum analysis (Bruker timsTOF HT, USA) in the DIA mode.

Protein expression levels were presented as the intensity of MS signals. For protein with no detected signals in all samples from one group, a value of 1/10 of the mean

expression intensity among groups was assigned to enable downstream statistical analysis. Differentially expressed protein was defined when fold change is ≥ 1.5 or ≤ 0.67 with $P < 0.05$ (Student's t test) among two groups. Principal component analysis (PCA), volcano plots, heatmaps, and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted using R software (version 4.5.1). Specifically, PCA was performed utilizing the prcomp function, volcano plots were generated with the ggplot2 package (version 4.0.1), and heatmaps were produced using the pheatmap package (version 1.0.13). KEGG pathway enrichment analysis was carried out employing the clusterProfiler package (version 4.18.2). Protein subcellular localization was predicted using CELLO (<https://cello.life.nctu.edu.tw/>).

Cell culture and treatment

Rat renal tubular epithelial NRK52E cells were maintained in DMEM supplemented with 4.5 g/L glucose and 10% fetal bovine serum (FBS, Gibco, Cat# 16000-044, United States). For treatment, cells were inoculated onto 24- or 6-well plates. Upon reaching 50% confluence, cells were treated with 100 μ M E-64 with or without 1 mM adenine for 3 days, after which downstream assays were performed as indicated.

Quantification of cysteine cathepsin activity

Lysates of kidney tissues and NRK52E cells were prepared in IP buffer without PMSF. Cysteine cathepsin activity was measured using a fluorometric assay in a reaction buffer composed of 50 mM sodium acetate (pH 5.5), 100 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, and 20 μ M of the cysteine cathepsin-specific fluorogenic substrate Z-FR-AMC (MCE, Cat# HY-P1759, USA). After incubation at 37°C for 30 min, fluorescence intensity was quantified with a microplate reader (Flash SuperMax 3000FL, China) using excitation at 380 nm and emission at 440 nm. The results were normalized to the protein concentration of each sample and are presented as relative fluorescence units per microgram of protein

Lysosome labelling with LysoTrakcer Red

After indicated treatments, NRK32E cells were incubated with 100 nM LysoTracker Red (MCE, Cat# HY-D1300, USA) for 30 min. Then, the culture medium was replaced with PBS. The cells were observed and photographed under a fluorescent microscope (EVOS, Invitrogen, USA). The fluorescence intensity was quantitatively determined by flow cytometry (BD Bioscience, FACS Canto II, USA).

DQ-BSA-Green assay

After indicated treatments, DQ-BSA-Green (ShareBio, Cat# D-12050SB, China) was added to a final concentration of 10 μ g/mL to the NRK32E cells followed by incubation for additional 6 h. Then, fluorescent microscopy and flow cytometry-mediated quantitative analysis were conducted as described above.

Transmission electron microscopy

Fresh kidney slices were pre-fixed using 2.5% glutaraldehyde for over 24 h, followed by secondary fixation in 1% osmium tetroxide. The tissues were then dehydrated using a graded series of acetone and embedded in Epon-812 resin. Ultrathin sections were subsequently cut, placed onto copper grids, and successively stained with uranyl acetate for 10-15 min and lead citrate for 1-2 min. Finally, the sections were visualized under a transmission electron microscope (JEOL, JEM-1400FLASH, Japan).

Senescence-associated β -Galactosidase Staining

Senescence-associated β -galactosidase (SA- β -Gal) staining on kidney sections and cultured cells was performed using a commercially available kit (Solarbio, Cat# G1580, China) following the provided instructions.

Statistics

One-way analysis of variance (ANOVA) was used to examine all quantitative data, which are expressed as mean $\pm$ standard deviation. The least significant difference test was then used to compare groups. GraphPad Prism (version 10.1.2) was used to create

statistical graphs. Statistical significance was defined as a P-value of less than 0.5.

Data availability statement

The DIA proteomics data has been provided as Supplementary Table 3. Additional raw data associated with this study are available upon reasonable request.

Author contributions

Lu Wang: Investigation, Formal analysis. **Xu Yan:** Investigation. **Ke-Liang Yang:** Investigation, software. **Ruo-Xin Zhang:** Investigation. **Cong Wei:** Funding acquisition. **Chen Chen:** Investigation. **En-Yao He:** Investigation, Funding acquisition. **Jian-Chun Li:** Resources. **Rui-Zhi Tan:** Resources. **Li Wang:** Supervision, Funding acquisition. **Hong-Lian Wang:** Funding acquisition, Conceptualization, Writing– review & editing, Project administration.

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Conflicts of interest

The authors declare no conflicts of interest.

Abbreviations

AGEs: advanced glycation end products; AN: adenine-induced nephropathy; ANOVA: one-way analysis of variance; BSA: bovine serum albumin; CDKI: cyclin-dependent kinase inhibitor; cDNA: complementary DNA; CKD: chronic kidney disease; DAB: diaminobenzidine; DAPI: 4',6-diamidino-2-phenylindole; DIA: Data-independent

acquisition; FBS: fetal bovine serum; HE: hematoxylin and eosin; HRP: horseradish
peroxidase; KEGG: Kyoto Encyclopedia of Genes and Genomes; LSDs: lysosomal
storage diseases; PBS: phosphate buffered saline; PCA: Principal component analysis;
RT-PCR: real-time PCR; SA- β -Gal: senescence-associated β -galactosidase; STZ:
streptozotocin; UUO: unilateral ureteral obstruction.

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