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Cadmium targeting transcription factor EB to inhibit autophagy-lysosome function contributes to acute kidney injury

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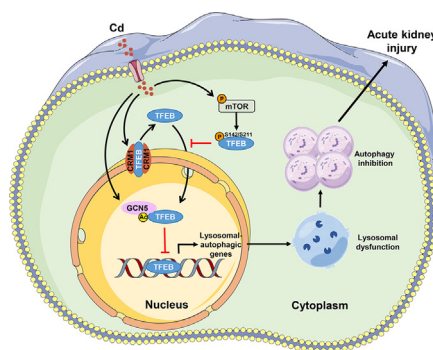
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HIGHLIGHTS

- Inhibited TFEB activity is involved in Cd-induced autophagy and lysosomal dysfunction.
- Cd blocks TFEB nuclear translocation via mTORC1-mediated TFEB phosphorylation.
- Activation of CRM1 facilitates Cd-induced TFEB nuclear export.
- Cd decreases TFEB transcriptional activity via GCN5-mediated TFEB acetylation.
- There is no correlation among CRM1 activation, TFEB phosphorylation and acetylation.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Environmental and occupational exposure to cadmium (Cd) has been shown to cause acute kidney injury (AKI). Previous studies have demonstrated that autophagy inhibition and lysosomal dysfunction are important mechanisms of Cd-induced AKI.

Objectives: Transcription factor EB (TFEB) is a critical transcription regulator that modulates autophagy-lysosome function, but its role in Cd-induced AKI is yet to be elucidated. Thus, *in vivo* and *in vitro* studies were conducted to clarify this issue.

Methods and results: Data firstly showed that reduced TFEB expression and nuclear translocation were evident in Cd-induced AKI models, accompanied by autophagy-lysosome dysfunction. Pharmacological and genetic activation of TFEB improved Cd-induced AKI via alleviating autophagy inhibition and lysosomal dysfunction, whereas *Tfeb* knockdown further aggravated this phenomenon, suggesting the key role of TFEB in Cd-induced AKI by regulating autophagy. Mechanistically, Cd activated mechanistic target of rapamycin complex 1 (mTORC1) to enhance TFEB phosphorylation and thereby inhibiting TFEB nuclear translocation. Cd also activated chromosome region maintenance 1 (CRM1) to promote TFEB nuclear export. Meanwhile, Cd activated general control non-repressed protein 5 (GCN5) to enhance nuclear

Abbreviations: AKI, acute kidney injury; B3, Butyrolactone 3; BUN, urea nitrogen; Cre, creatinine; CRM1, chromosome region maintenance 1 protein; CTSB, Cathepsin B; CTSD, Cathepsin D; GCN5, General control non-repressed protein 5; HE, Hematoxylin-eosin staining; KIM-1, Kidney injury molecule 1; LAMP1, Lysosomal-associated membrane protein 1; LAMP2, Lysosomal-associated membrane protein 2; LC3, Microtubule-associated protein 1 light chain 3; LMB, Leptomycin B; mTORC1, mechanistic target of rapamycin complex 1; NGAL, neutrophil gelatinase associated lipocalin; PTMs, post-translational modifications; TFEB, transcription factor EB.

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TFEB acetylation, resulting in the decreased TFEB transcriptional activity. Moreover, inhibition of CRM1 or GCN5 alleviated Cd-induced AKI by enhancing TFEB activity, respectively.

Conclusion: In summary, these findings reveal that TFEB phosphorylation, nuclear export and acetylation independently suppress TFEB activity to cause Cd-induced AKI via regulating autophagy-lysosome function, suggesting that TFEB activation might be a promising treatment strategy for Cd-induced AKI.

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Introduction

Cadmium (Cd) is a heavy metal pollutant that causes high harm to humans and animals via environmental contamination and occupational exposure [1,2]. Cd is absorbed into the organism causing multi-system and multi-organ damage via various pathways including industrial pollution, cigarette smoke, food and water [3–5]. Cd is highly accumulative in the body, and the kidney is its main accumulation site and a key target organ [6,7]. Emerging evidence has shown that environmental and occupational exposure to Cd can cause acute kidney injury (AKI) [8–10]. It must be stressed that the renal tubular is the main damage site of Cd-caused AKI [8]. It is now clear that AKI relates to complicated and multifactorial pathogenesis, in which autophagy and lysosomal dysfunction play vital roles [9,11–14]. However, the role of autophagic-lysosomal function in Cd-induced AKI remains yet to be fully elucidated.

Autophagy is a lysosome-dependent degradation route that eliminates unacceptable components by forming autophagosomes and autolysosomes, which has gained much attention as a therapeutic strategy for AKI [15–19]. Our previous reports have demonstrated that lysosomal dysfunction-caused autophagy inhibition participates in Cd-induced AKI, in which transcription factor EB (TFEB) plays a key role [20–22]. TFEB is a crucial transcription factor essential for maintenance of autophagy and lysosomal function, which has been confirmed to regulate various renal diseases such as lipopolysaccharide-induced septic AKI, cisplatin-induced AKI and diabetic nephropathy [23–28]. Nevertheless, the underlying mechanism of TFEB in Cd-induced AKI is not yet clear.

TFEB is widely expressed in various cell types *in vivo*, and its intracellular localization and distribution determine its function and activity [29,30]. Activation and intracellular localization of TFEB are majorly regulated through protein–protein interactions and post-translational modifications (PTMs) [31]. Wang et al. demonstrated that activation of mechanistic target of rapamycin (mTOR) leads to TFEB inactivation, thereby disturbing lysosomal function via inhibiting lysosomal biogenesis and degradation activity [32]. Emerging evidence has revealed that Chromosomal region maintenance 1 (CRM1) inhibition can improve autophagy flux by restoring TFEB nuclear localization [33,34]. Moreover, emerging evidence indicates that TFEB acetylation controls its activity [35–37]. For instance, general control non-repressed protein 5 (GCN5)-modulated acetylation restrains TFEB to bind to its target genes via perturbing its dimerization [35,38]. Additionally, inhibition of TFEB activity is associated with autophagic-lysosomal dysfunction-related renal diseases [39,40]. Thus, elucidating the function of TFEB in Cd-triggered autophagic-lysosomal dysfunction will be of great significance in alleviation of AKI.

This study was to dissect the regulatory mechanisms of TFEB in Cd-induced AKI. The results showed that Cd inhibits TFEB nuclear localization via the activation of mTOR and CRM1 signals, and enhances TFEB acetylation to suppress its transcriptional activity by activating acetyltransferase GCN5, leading to autophagy-lysosome dysfunction and resultant AKI. The current study clarified the regulatory effect of TFEB on Cd-induced autophagy-lysosome

dysfunction via its phosphorylation, nuclear export and acetylation modification, indicating that it acts as a new therapeutic target in Cd-induced AKI.

Materials and methods

Reagents

Cadmium Chloride (CdCl₂), DMEM (High Glucose) medium, DAPI (10236276001) and siRNA GCN5 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine[®] RNAiMAX Transfection Reagent (13778150), Lipofectamine[®] 3000 Transfection Reagent (L3000015), siRNA TFEB and siRNA mTORC1 were purchased from Invitrogen (Carlsbad, CA, USA). Lenti-TFEB and Lenti-NC were obtained from GeneChem (Shanghai, China). BCA protein assay kit (W9811) was obtained from TIANGEN (Beijing, China). Nuclear and Cytoplasmic Protein Extraction Kit (P0028) and Chromatin Immunoprecipitation (ChIP) Assay Kit (P2078) were purchased from Beyotime (Shanghai, China). Butyrolactone 3 (B3, GC10226) and TFEB activator 1 (GC39530) were purchased from Glpbio (CA, USA). Leptomycin B (LMB, S7580) was purchased from Selleck (Shanghai, China). The following primary antibodies were used: NGAL (GB111134, Servicebio, Hubei, China), KIM-1 (A2831, ABclonal, Hubei, China), p62/SQSTM1 (P0067, Sigma, MO, USA), LC3 (M186-3, MBL, Tokyo, Japan), TFEB (13372-1-AP, Proteintech, Hubei, China), phospho-TFEB (Ser211) (AF3708, Affinity, Jiangsu, China), phospho-TFEB (Ser142) (AF3845, Affinity, Jiangsu, China), mTOR (2972, CST, MA, USA), phospho-mTOR (Ser2448) (2971, CST, MA, USA), CRM1 (A19625, ABclonal, Hubei, China), GCN5 (66575-1-Ig, Proteintech, Hubei, China), IgG (B900610, Proteintech, Hubei, China), acetylated-lysine (9441, CST, MA, USA), GAPDH (GB12002, Servicebio, Hubei, China), Histone 3 (4499, CST, MA, USA). Secondary antibodies were purchased from CWBIO (Jiangsu, China).

Cell culture and treatment

NRK-52E cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were treated with CdCl₂ dissolved in sterile ultrapure water with reference to the Cd dose in our previous study [9]. NRK-52E cells were treated with 10 μM CdCl₂ for 12 h, and then co-treated with 5 nM LMB for 2 h.

Ethics statement

The animal experiments were reviewed and approved by the Experimental Animal Ethics Committee of Shandong Agricultural University (Approval no. SDAUA-2021-038).

Animal experimental design

Specific pathogen-free Sprague-Dawley (SD) healthy male rats (5 weeks old, weighing 100–110 g) were obtained from Jinan Pengyue Experimental Animal Breeding Co Ltd (Shandong, China).

After a 7-day acclimatation, 30 rats were divided into 5 groups based on a random number table ($n = 6$ per group): (1) Control group: injected intraperitoneally with the same volume solvent (physiological saline or ethanol solution). (2) Cd group: CdCl_2 (1 mg/kg b.w., dissolved in physiological saline) injected intraperitoneally for 7 consecutive days. (3) Cd + C1 group: CdCl_2 injection as in the Cd group, and TFEB activator 1 (5 mg/kg b.w., dissolved in ethanol solution) injected intraperitoneally on days 2–7. (4) Cd + LMB group: CdCl_2 injection as in the Cd group, and LMB (0.2 mg/kg b.w., dissolved in ethanol solution) injected intraperitoneally on days 2–7. (5) Cd + B3 group: CdCl_2 injection as in the Cd group, and B3 (1 mg/kg b.w., dissolved in ethanol solution) injected intraperitoneally on days 2–7. The experiment lasted for 7 days. At the end of the experiment, the rats were killed by cervical dislocation under deep anesthesia after fasting for 12 h. Blood samples and kidneys were dissected and immediately stored at -80°C for further assays.

Serum biochemical analysis

Blood samples were clotted overnight at 4°C , and isolated at 2 000 g for 5 min at 4°C . The serum levels of urea nitrogen (BUN, C013) and creatinine (Cre, C011) were examined harnessing specific kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as per the manufacturer's protocols.

Histopathological observation

Parts of kidney tissues were isolated and fixed using 10% formalin. Fixed tissues were embedded in paraffin, and 5 μm sections were prepared, stained with hematoxylin-eosin (H&E), and observed by light microscopy.

RT-qPCR

Total RNA from rat kidney and NRK-52E cells was collected using RNAiso Plus in accordance to manufacturer's manual. The concentration and purity of RNA were determined based on A260/A280 ratio by the MicroDrop[®] spectrophotometer (BIO-DL Corporation, Shanghai, China). Subsequently, RNA was reversed into cDNA using Transcriptor cDNA Synth. Kit. RT-qPCR reactions (10 μL /reaction) were conducted on a LightCycler 96 (Roch, Switzerland) with SYBR Green I Master. Relative expressions of targeted genes were reckoned by $2^{-\Delta\Delta\text{Ct}}$ method by normalizing to the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers used in this study were stated in Table 1.

Immunofluorescence (IF) staining

NRK-52E cells were cultured on sterile cover glasses located in the 24-well plates. After the corresponding treatments, cells on

each slide were immersed in 4% paraformaldehyde for 6 min and then incubated in PBS containing 2% BSA for 50 min at 25°C . The slides were incubated with primary anti-TFEB dissolving in 2% BSA (at the final dilution ratio of 1:150) at 4°C for 18–20 h and then incubated with Alexa Fluor[®] 488 (1:500 dilution using PBS) away from light for 1 h. Nucleuses were stained with DAPI. Fluorescence in different cells was observed using a confocal microscope (Leica TCS SPE, Germany).

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP was conducted with ChIP Assay Kit (Beyotime, P2078) on the basis of the manufacturer's instructions. In brief, cells were fixed proteins to DNA with 1% formaldehyde. DNA-protein complexes were pulled down with anti-TFEB antibody. The specific DNA-protein complexes were reversed and the DNA was purified. The enrichment of particular DNA sequences was detected by downstream qPCR analysis. Primers were stated in the Table 2.

Co-immunoprecipitation (Co-IP)

Co-IP assays were performed using a Dynabeads Protein G Immunoprecipitation kit as recommended by the manufacturer. NRK-52E cells were administrated according to above-mentioned treatments and then lysed in fresh-prepared RIPA buffer containing PMSF. Protein G beads were mixed with 5 μL TFEB or IgG antibody per sample, and rotated for 12 h at 4°C . Then, the t lysates were incubated with beads-antibody complex overnight at 4°C . After washed using cell lysis buffer, the immunoprecipitates were resuspended with $1 \times$ SDS PAGE sample buffer to configure the samples. The targeted proteins were determined using immunoblot assays with anti-acetylated-lysine primary antibodies.

Western blotting analysis

Total protein of kidney tissue and NRK-52E cells were obtained using RIPA lysate containing 1% protease inhibitor cocktail. Nuclear proteins were isolated by Nuclear and Cytoplasmic Protein Extraction Kit. The concentrations of total protein or nuclear protein were examined using the BCA assay. Proteins were separated by SDS-PAGE gels and transferred to PVDF membranes. Then, PVDF membranes were incubated with 5% skim milk at RT and thereafter membranes were treated with primary antibodies (KIM-1, NGAL, p62, LC3, TFEB, p-TFEB, mTOR, p-mTOR, CRM1, GCN5, acetylated-lysine, GAPDH and Histone H3) at 4°C for 18–20 h. The membranes were then incubated with secondary antibodies for 50 min at RT. The specific proteins were visualized on a Chemidoc XRS (Bio-Rad, Marnes-La Coquette, France). Each assay was performed in triplicates with different batches of cells.

Table 1
Primer sequences with their corresponding PCR product size.

Gene	Primer (5'-3')	Product (base pairs)
<i>GAPDH</i>	GACATGCCCTGGAGAAAC AGCCCAGGATGCCCTTAGT	96 bp
<i>LAMP-1</i>	GCCGAGTAGCACTTCAGCAAGG CCAGCAGGCAGGTTCCATTGTC	155 bp
<i>LAMP-2</i>	ATCATTACACCACCGTGCCATC GTAGCCAGCAGACAGGTAGCATTG	122 bp
<i>Cathepsin B</i>	CAGCAGACACCTCCACATTGACTC CGACCATCGCACAGATCAGAGAC	126 bp
<i>Cathepsin D</i>	CTCCTGGCTGCGTCTCTCTC TGACACTGGCTCTTGGTCTAG	168 bp

Table 2
Primers used for ChIP-qPCR analysis.

Gene	Primer (5'-3')	Product (base pairs)
<i>ATG9B</i>	TAGAGTGGTGGTTCCGTGAGAAGG GTTTGGTTGGGTTGAGAGTGTGTG	135 bp
<i>VPS11</i>	CCCTGGCAGTCATAACAAAGCATTG GTGAGAGCCATGTAAGGGAGCATC	85 bp
<i>LAMP-1</i>	ACAGGAGGGACAGGGAGAGT CCATCCATCCCTTGAGAACATTCA	122 bp
<i>Cathepsin D</i>	CACAGATCAGGGAGGGGCGAAG AGAGAATTCTGGATGCGAGTCACG	105 bp
<i>ATP6V1H</i>	CAGGTTTCTGTGGTCTGCTTTTGC TGGAGAGGAATGGGCTCTGTGC	88 bp

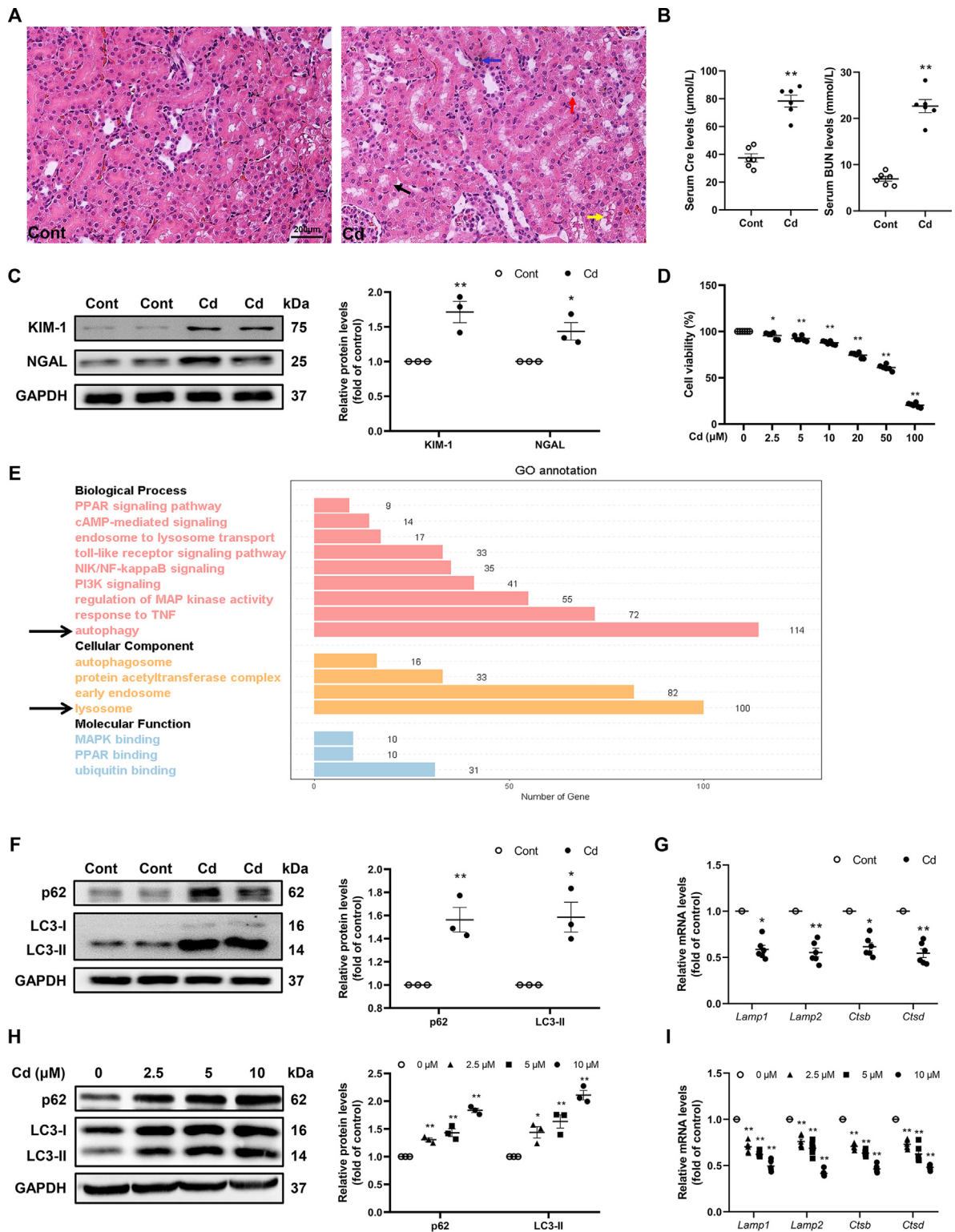


Fig. 1. Autophagy-lysosome dysfunction is involved in Cd-induced AKI. (A) Representative images of HE staining. Blue arrow indicates nuclear pyknosis. Black arrow indicates karyolysis. Red arrow indicates necrotic cells shed in the tubular lumen. Yellow arrow indicates tubular protein deposition. (B) Serum samples were collected to analyze the Cre and BUN levels (mean $\pm$ SEM, $n = 6$). (C) The protein expressions of NGAL and KIM-1 were detected in renal tissues. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (D) Cell viability in NRK-52E cells treated with different doses of Cd for 12 h, detected by CCK-8 test (mean $\pm$ SEM, $n = 6$). (E) Gene ontology pathway enrichment analysis showed an overrepresentation of differentially expressed genes (DEGs) associated with autophagy and lysosome during Cd exposure *in vivo*. (F) Renal tissues were collected to analyze the protein levels of p62 and LC3. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (G) mRNA expression levels of LAMP1, LAMP2, CTSD and CTSB were detected by RT-qPCR method in Cd-exposed rat renal tissues (mean $\pm$ SEM, $n = 6$). (H) NRK-52E cells were treated with a range of Cd doses for 12 h to analyze the protein levels of KIM-1 and NGAL. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (I) Cells were treated with different doses of Cd for 12 h to detect LAMP1, LAMP2, CTSD and CTSB mRNA expression levels by RT-qPCR method (mean $\pm$ SEM, $n = 6$). (J) Cells transfected with RFP-GFP-LC3 plasmid were exposed to 10 μ M Cd for 12 h, then observed under a confocal microscope. Typical confocal images were quantitated by the ratios of autolysosomes and autophagosomes (right image). * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control.

Small interfering RNA (siRNA) treatment

siRNA for TFEB (5-ACAGUCCCAUGGCAUGCUACACAU-3'), mTORC1 (5-CUCUGAAGCAGCUUCAGUACAUAGG-3') and GCN5 (5-GCUACUAUGUGACCCGGAA-3') were transfected into NRK-52E cells at 40% confluency using RNAiMAX Transfection Reagent based on the manufacturer's specifications. After 6 h incubation, NRK-52E cells were incubated with normal medium for 24 h for further experiments.

Transient transfection

Transfection was performed using Lipofectamine® 3000 (Invitrogen, Carlsbad, USA) according to the manufacture's protocol. Briefly, cells were transfected with 1 µg RFP-GFP-LC3 plasmid, then maintained on coverslips in 24-well plates. During incubation for 36 h, transfected cells were treated according to experimental requirements, and then exposed to 10 µM Cd for another 12 h. Coverslips were fetched out and fixed with 4% paraformaldehyde (PFA) in PBS for 8 min, and nuclei were marked with DAPI after washing. Representative images were captured by the confocal microscope (Leica TCS SPE, Germany). The number of puncta per cell was quantified using the "analyze particles" function of Image J under identical threshold conditions.

Lentiviral transfection

NRK-52E cells at a confluence of 40% were transfected with Lenti-TFEB (TFEB-OE) or Lenti-NC (NC-OE) according to the manufacturer's protocols. Cells were cultured for 72 h and then incubated with CdCl₂ for 12 h for the corresponding assays. Immunoblot analysis was employed to evaluate the transfection efficacies.

Transcriptome sequencing

The RNA from the kidneys was used for RNA-Seq. Raw sequencing was trimmed using Cutadapt software (version 1.16) to exclude redundant sequencing. Filtered sequencing reads were mapped to mm9 genome with the default parameter. Differential expression and visualization were yielded by Cufflinks (version v2.2.1) and R package cummeRbund (version v2.22.0).

Statistical analysis

Data in this study were stated as mean ± SEM. Comparisons among different treatments were evaluated by *t* test and one-way analysis of variance (ANOVA). Graphs were generated using GraphPad Prism 8. *p* < 0.05 showed statistical significance.

Results

Autophagic-lysosomal dysfunction is involved in Cd-induced AKI

To investigate possible biological processes and cell signaling pathways involved in Cd-induced renal damage, we established Cd-induced AKI model *in vivo* via intraperitoneal injection. Histopathological examination showed that Cd exposure caused renal tubular epithelial injury, which was characterized by nuclear pyknosis, karyolysis, necrotic cells shed in the tubular lumen and tubular protein deposition (Fig. 1A). Combined with two increased indicators of renal function (serum Cre and BUN levels, Fig. 1B) and two elevated tubular injury marker protein levels in kidney tissues (NGAL and KIM-1, Fig. 1C) in Cd-exposed rats, it can be confirmed that Cd-induced AKI model was successfully established. Meanwhile, *in vitro* model of Cd-induced AKI was established using NRK-52E cells, showing that Cd decreased significantly cell viability with increasing concentrations (Fig. 1D).

To elucidate the possible mechanism of Cd-induced AKI, we analyzed RNA-seq data of rat kidney tissues between control and Cd groups. Data in Fig. 1E showed that differentially expressed genes were mainly enriched in the pathways associated with autophagy and lysosome. Next, autophagy marker protein levels and lysosomal-related genes expression levels were determined in rats and cell lines. As shown in Fig. 1F and Fig. 1H, Cd exposure significantly upregulated the expression of p62 and LC3-II. Simultaneously, the expression of *LAMP1*, *LAMP2*, *CTSB* and *CTSD* was markedly decreased by Cd treatment (Fig. 1G and Fig. 1I). Moreover, RFP-GFP-LC3 plasmid transfection was used to perform autophagic flux assay. As shown in Fig. 1J, yellow puncta are increased with a concomitant decrease in red puncta in Cd exposed cells, suggesting that autophagosome maturation into autolysosomes is blocked, i.e., inhibition of autophagic flux. Thus, our result clearly suggest that autophagy suppression and lysosomal dysfunction occurs in Cd-induced AKI.

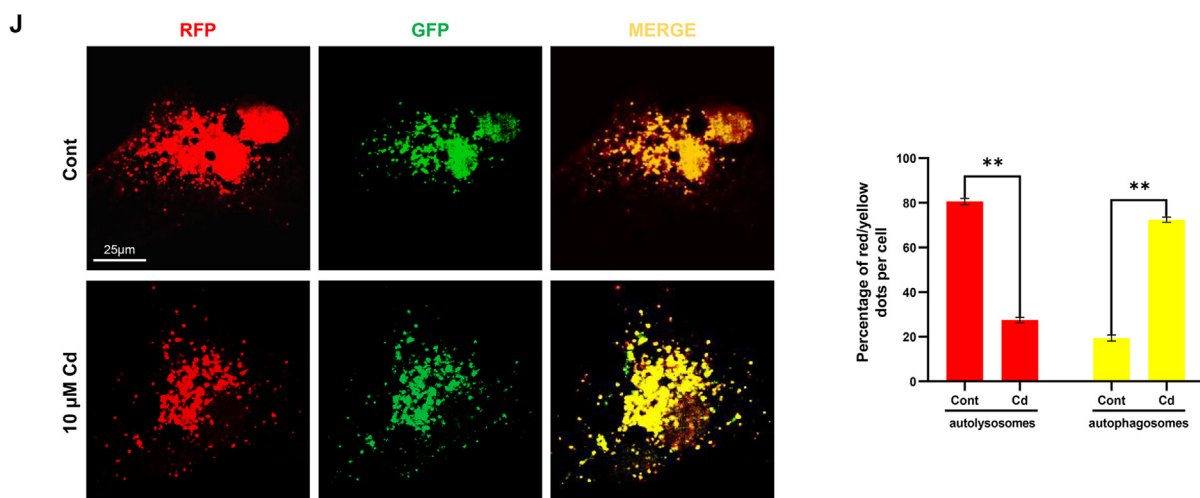


Fig. 1 (continued)

TFEB dysregulation is revealed in Cd-induced AKI

To predict the potential targeted genes involving in autophagy inhibition and lysosomal dysfunction, 26 differentially expressed genes co-expressed in the autophagy and lysosomal pathways were identified by transcriptomic analysis (Fig. 2A). Strikingly, the differentially expressed gene TFEB expression was markedly inhibited by Cd exposure (Fig. 2B). Next, TFEB protein levels were assessed to verify the RNA-seq data. Consistently, Cd exposure significantly reduced the TFEB protein levels in kidney tissues (Fig. 2C) and NRK-52E cells (Fig. 2D). Moreover, inhibited TFEB nuclear translocation in kidney tissues was revealed in Cd-induced AKI rats (Fig. 2E). Immunoblot and immunofluorescence analyses demonstrated that TFEB was inhibited to translocate into nuclear in Cd-treated NRK-52E cells (Fig. 2F and 2G). Given

these results, we speculate that TFEB dysregulation plays an important role in Cd-induced AKI through modulating autophagy and lysosome function.

To further elucidate the effect of TFEB in Cd-induced AKI, rats were administered TFEB activator 1 (C1). Levels of TFEB were significantly upregulated by C1 treatment (Fig. 3C). Histopathological examination showed that C1 alleviated Cd-induced kidney injury (Fig. 3A). Meanwhile, C1 significantly decreased the expression of KIM-1 and NGAL in the kidney and significantly reduced the serum levels of Cre and BUN (Fig. 3B and 3C). These data indicate that TFEB attenuates Cd-induced kidney injury in rats. To further verify the action mechanisms of TFEB in Cd-induced kidney injury, NRK-52E cells with overexpressing or silencing TFEB were generated. Fig. 3E and 3F showed that TFEB overexpression and knockdown models were successfully established. CCK-8 assay revealed that

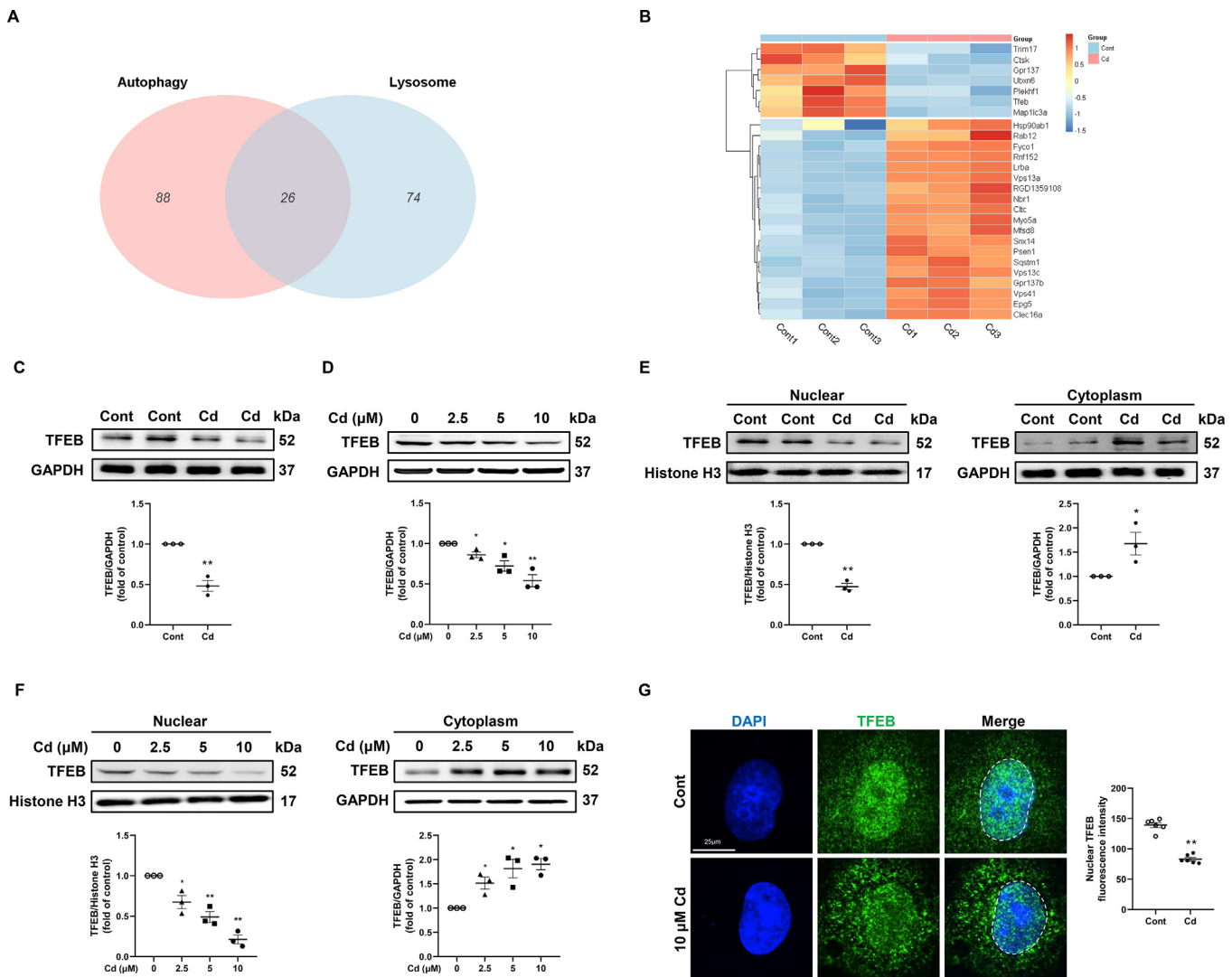


Fig. 2. Cd inhibits TFEB nuclear translocation (A) Venn diagram of DEG between the control and Cd-exposed group. (B) Heat map of DEGs of autophagy and lysosomal pathways in the control and Cd-exposed groups. (C) Renal tissues were collected to analyze the protein levels of TFEB. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (D) NRK-52E cells were treated with a range of Cd doses for 12 h to analyze the protein levels of TFEB. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (E) Renal tissues were collected to analyze the nuclear and cytoplasmic protein levels of TFEB. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (F) NRK-52E cells were treated with a range of Cd doses for 12 h to analyze the nuclear and cytoplasmic protein levels of TFEB. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (G) Detection of TFEB nuclear translocation in NRK-52E cells by IF staining method. Nuclear TFEB fluorescence intensity quantification in right panels. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control.

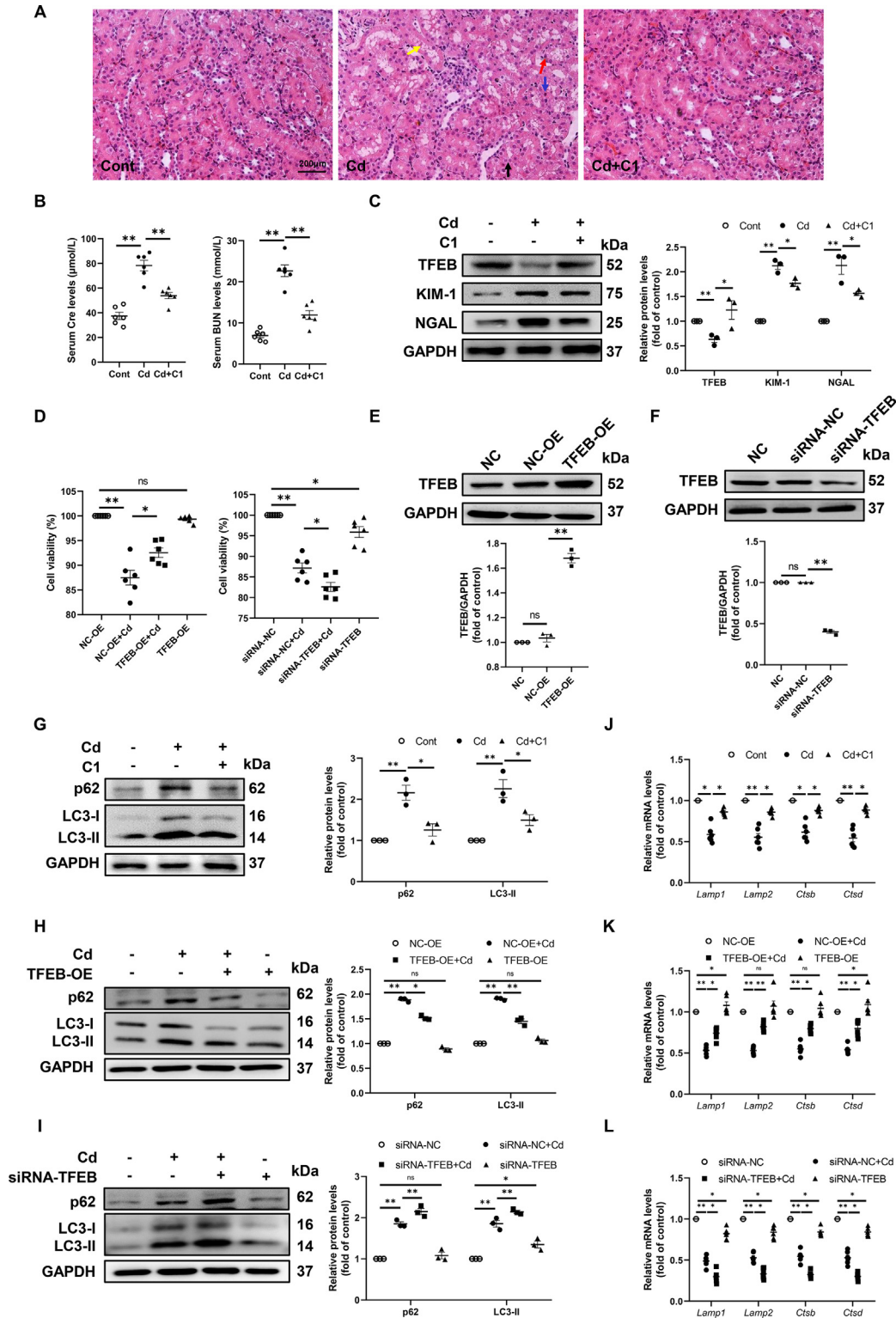


Fig. 3. TFEB-dependent autophagic-lysosomal dysfunction contributes to Cd-induced nephrotoxicity. (A) Representative images of HE staining. Blue arrow indicates nuclear pyknosis. Black arrow indicates karyolysis. Red arrow indicates necrotic cells shed in the tubular lumen. Yellow arrow indicates tubular protein deposition. (B) Serum samples were collected to analyze the Cre and BUN levels (mean $\pm$ SEM, $n = 6$). (C) Renal tissues were collected to analyze the protein levels of TFEB, KIM-1 and NGAL. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (D) After TFEB overexpression or knockdown, NRK-52E cells were treated with 10 μM Cd for 12 h to detect cell viabilities (mean $\pm$ SEM, $n = 6$). (E) NRK-52E cells were transfected with Lenti-TFEB (TFEB-OE) and Lenti-NC (NC-OE). Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (F) NRK-52E cells were transfected with control siRNA (siRNA-NC) and TFEB siRNA (siRNA-TFEB). Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (G) Renal tissues were collected to analyze the protein levels of p62 and LC3. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (H, I) After TFEB overexpression or knockdown, NRK-52E cells were treated with 10 μM Cd for 12 h to analyze the protein levels of p62 and LC3. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (J) mRNA expression levels of *LAMP1*, *LAMP2*, *CTSB* and *CTSD* in Cd-exposed rat renal tissues (mean $\pm$ SEM, $n = 6$). (K, L) mRNA expression levels of *LAMP1*, *LAMP2*, *CTSB* and *CTSD* in Cd-exposed NRK-52E cells (mean $\pm$ SEM, $n = 6$). (M) After TFEB overexpression, cells transfected with RFP-GFP-LC3 plasmid were exposed to 10 μM Cd for 12 h, then observed under a confocal microscope. Typical confocal images were quantitated by the ratios of autolysosomes and autophagosomes (right image). * $p < 0.05$; ** $p < 0.01$.

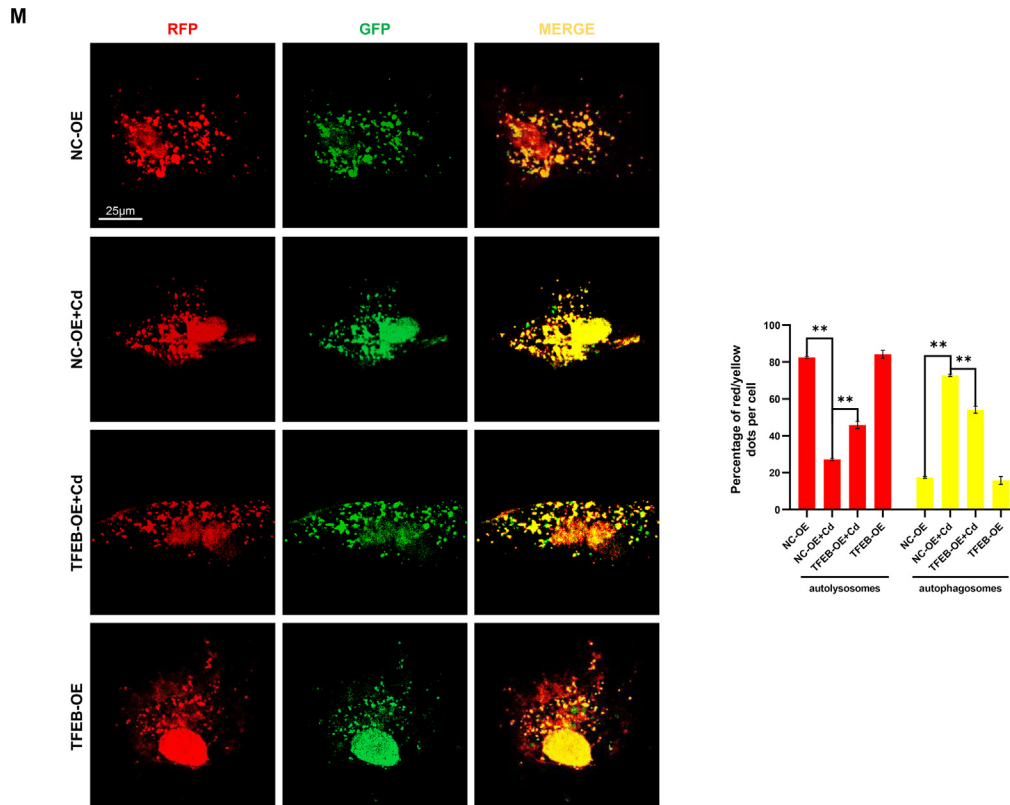


Fig. 3 (continued)

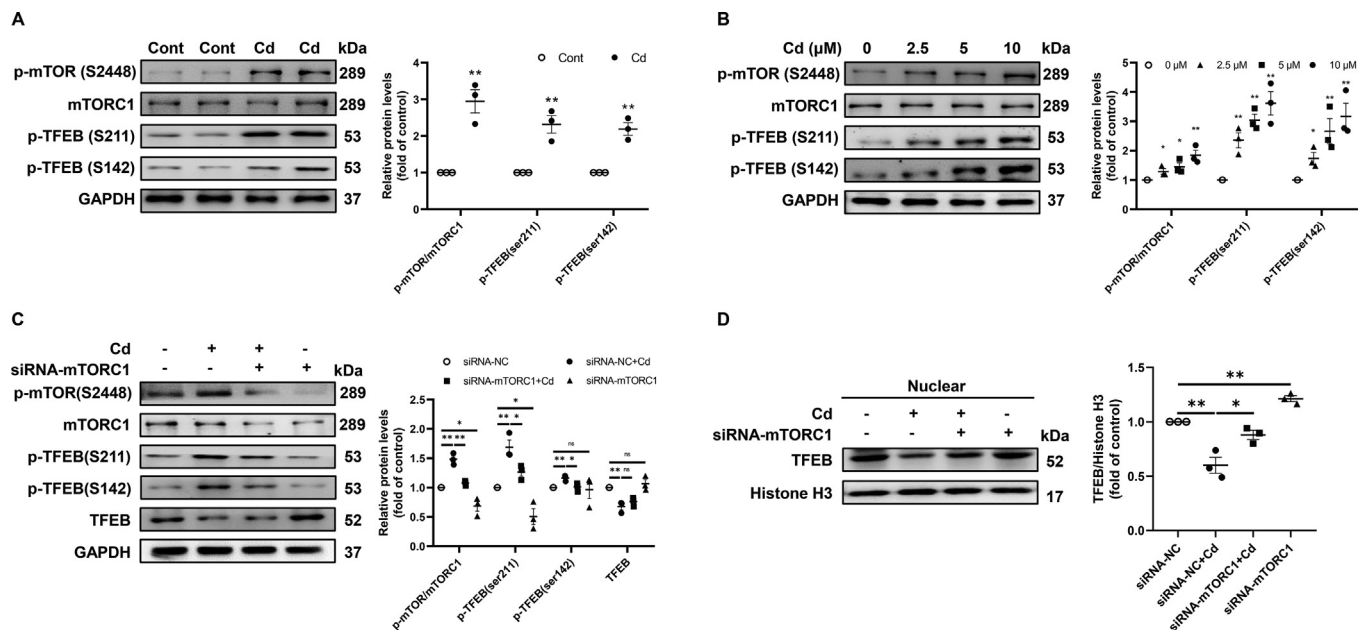


Fig. 4. Mtorc1 activation results in tfef phosphorylation and consequently inhibited tfef nuclear translocation during Cd exposure. (A) Renal tissues were collected to analyze the protein levels of mTOR, p-mTOR (S2448), p-TFEB (S211) and p-TFEB (S142). Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (B) NRK-52E cells were treated with a range of Cd doses for 12 h to analyze the protein levels of mTOR, p-mTOR (S2448), p-TFEB (S211) and p-TFEB (S142). Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (C) NRK-52E cells were transfected with control siRNA (siRNA-NC) and mTORC1 siRNA (siRNA-mTORC1). After mTORC1 knockdown, NRK-52E cells were treated with 10 μ M Cd for 12 h to analyze the protein levels of mTOR, p-mTOR (S2448), TFEB, p-TFEB (S211) and p-TFEB (S142). Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (D) After mTORC1 knockdown, NRK-52E cells were treated with 10 μ M Cd for 12 h to analyze the nuclear protein levels of TFEB. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. * $p < 0.05$; ** $p < 0.01$.

TFEB overexpression enhanced cell viability, while TFEB knock-down further decreased cell viability (Fig. 3D). These data support that Cd exposure induces AKI by inhibiting TFEB activity.

Tfeb-dependent autophagic-lysosomal dysfunction contributes to Cd-induced AKI

To further decipher if the beneficial action of TFEB activation against Cd-induced kidney injury is responsible for maintaining autophagy and lysosomal homeostasis, relevant indicators were examined *in vivo* and *in vitro* models. As shown in Fig. 3G and 3H, increased expression of p62 and LC3-II in Cd-exposed cells and tissues was notably reversed by activation of TFEB, indicating the amelioration of Cd-induced autophagy inhibition. However, these two protein levels were further increased in si-Tfeb NRK-52E cells exposed to Cd (Fig. 3I). Furthermore, activation of TFEB evidently enhanced the expression levels of mRNA of lysosome-related genes (*LAMP1*, *LAMP2*, *CTSB* and *CTSD*), while TFEB knock-down further suppressed the expression of lysosomal genes (Fig. 3J, 3K and 3L). Data in Fig. 3M showed that Cd-induced changes of autophagosomes (increased yellow dots) and autolysosomes (decreased red dots) were significantly reversed by TFEB overexpression treatment, furthermore suggesting that TFEB restored Cd-blocked autophagosome-lysosome fusion. The above results revealed that Cd exposure induced inhibition of TFEB activity and autophagic-lysosomal dysfunction, ultimately contributing to cell death or kidney injury. Thus, these data suggest that defective TFEB is the basis of Cd-caused autophagic-lysosomal dysfunction and resultant AKI.

mTORC1 activation results in TFEB phosphorylation and consequently inhibited TFEB nuclear translocation in Cd-induced AKI

Given the important roles of TFEB in Cd-induced nephrotoxicity, it is essential to delve into the mechanisms by which Cd regulates TFEB activity. To investigate whether mTORC1 is responsible for Cd-inhibited TFEB activity, the expression levels of mTORC1 and p-mTOR (S2448) were examined *in vivo* and *in vitro* after Cd exposure. mTORC1 protein expression levels were not affected by Cd exposure, but p-mTOR (S2448) expression levels were significantly increased by Cd exposure (Fig. 4A and 4B). It is reported that mTORC1 can modulate TFEB activities through Ser211 and

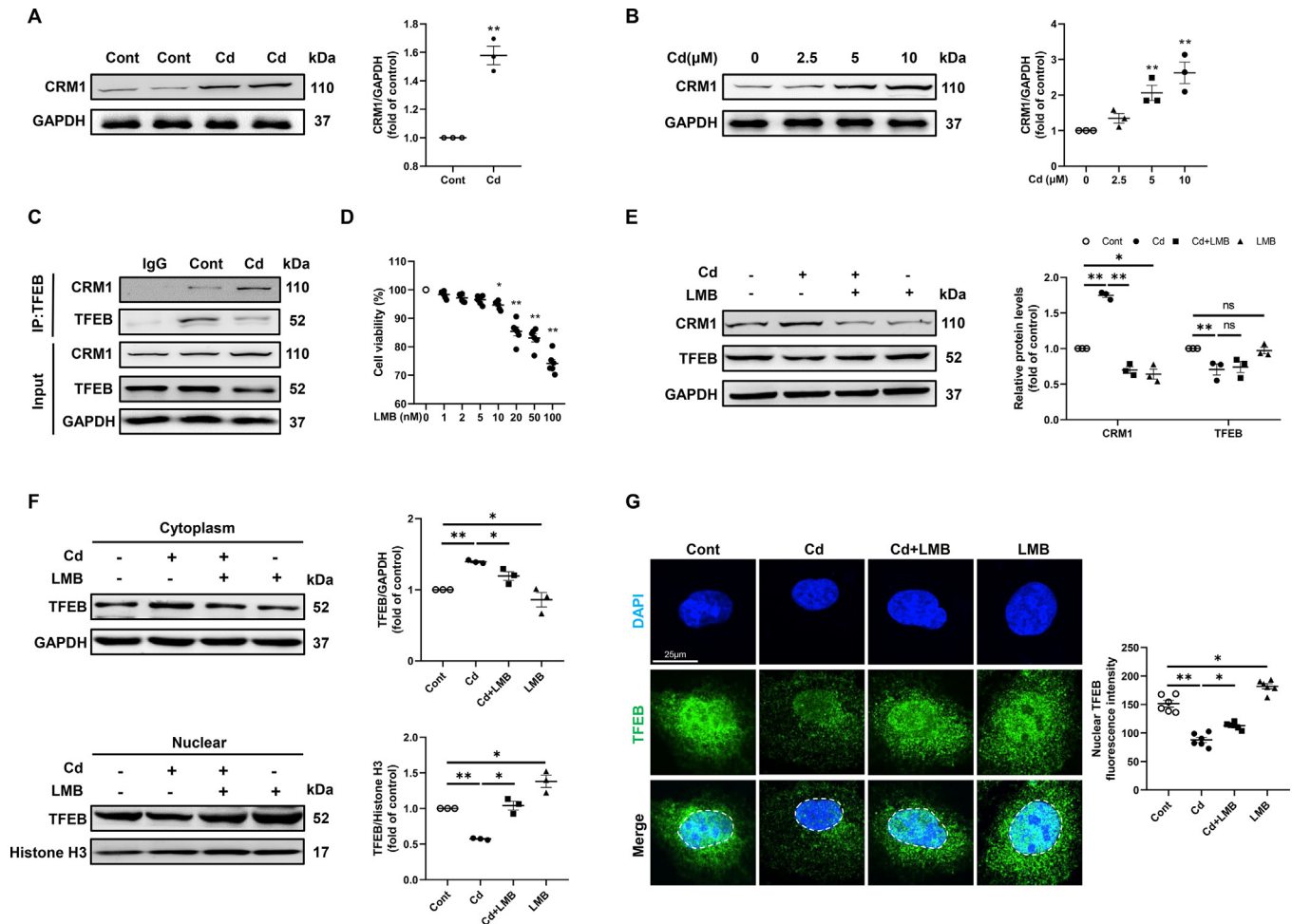


Fig. 5. Cd activates CRM1 to promote TFEB nuclear output. (A) Renal tissues were collected to analyze the protein levels of CRM1. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (B) NRK-52E cells were treated with a range of Cd doses for 12 h to analyze the protein levels of CRM1. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (C) Co-precipitation of CRM1 with TFEB. TFEB was immunoprecipitated from NRK-52E cells using anti-TFEB, and the precipitates were analyzed using anti-CRM1. (D) NRK-52E cells were treated with different doses of LMB for 2 h to detect cell viabilities (mean $\pm$ SEM, $n = 6$). (E) NRK-52E cells were treated with 10 μ M Cd and/or 5 nM LMB to analyze the protein levels of CRM1 and TFEB. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (F) NRK-52E cells were treated with 10 μ M Cd and/or 5 nM LMB to analyze the nucleus and cytoplasm protein levels of TFEB. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (G) TFEB nuclear translocation in NRK-52E cells was detected by IF staining. Nuclear TFEB fluorescence intensity quantification in right panels. * $p < 0.05$; ** $p < 0.01$.

Ser142 phosphorylation[26]. Next, the phosphorylation levels of S211 and S142 sites of TFEB were examined, respectively. Cd exposure evidently increased the expression levels of p-TFEB (S211) and p-TFEB (S142) *in vivo* and *in vitro* (Fig. 4A and 4B). To evaluate the effect of mTORC1 on TFEB phosphorylation, mTORC1 was knocked down using siRNA transfection *in vitro*. Our results showed that mTORC1 deletion partially dampened Cd-induced TFEB phosphorylation (Fig. 4C). mTORC1 silencing attenuated Cd-suppressed TFEB nuclear translocation, which was mitigated by inhibition of phosphorylation but not total protein (Fig. 4C and 4D). The above results suggest that Cd exposure activates mTORC1 for phosphorylation modification of TFEB, contributing to inhibition of TFEB activity and nuclear translocation.

Activation of CRM1 promotes nuclear TFEB export during Cd exposure

TFEB activity was determined by translocation from cytoplasm into nucleus. So, TFEB shuttling inside or outside the nucleus is a critical event. Aforementioned analyses showed that Cd exposure reduced the total protein levels of TFEB and concomitantly elevated the cytoplasmic TFEB, highlighting nuclear transport receptor protein may participate in regulating the activity of TFEB. Recent reports have revealed that CRM1 as a nuclear transporter can regulate the nuclear output of TFEB [33]. To investigate whether CRM1 is involved in the TFEB activity inhibited by Cd, the expression changes of CRM1 were examined under the condition of Cd exposure *in vivo* and *in vitro*. The expression level of CRM1 was markedly

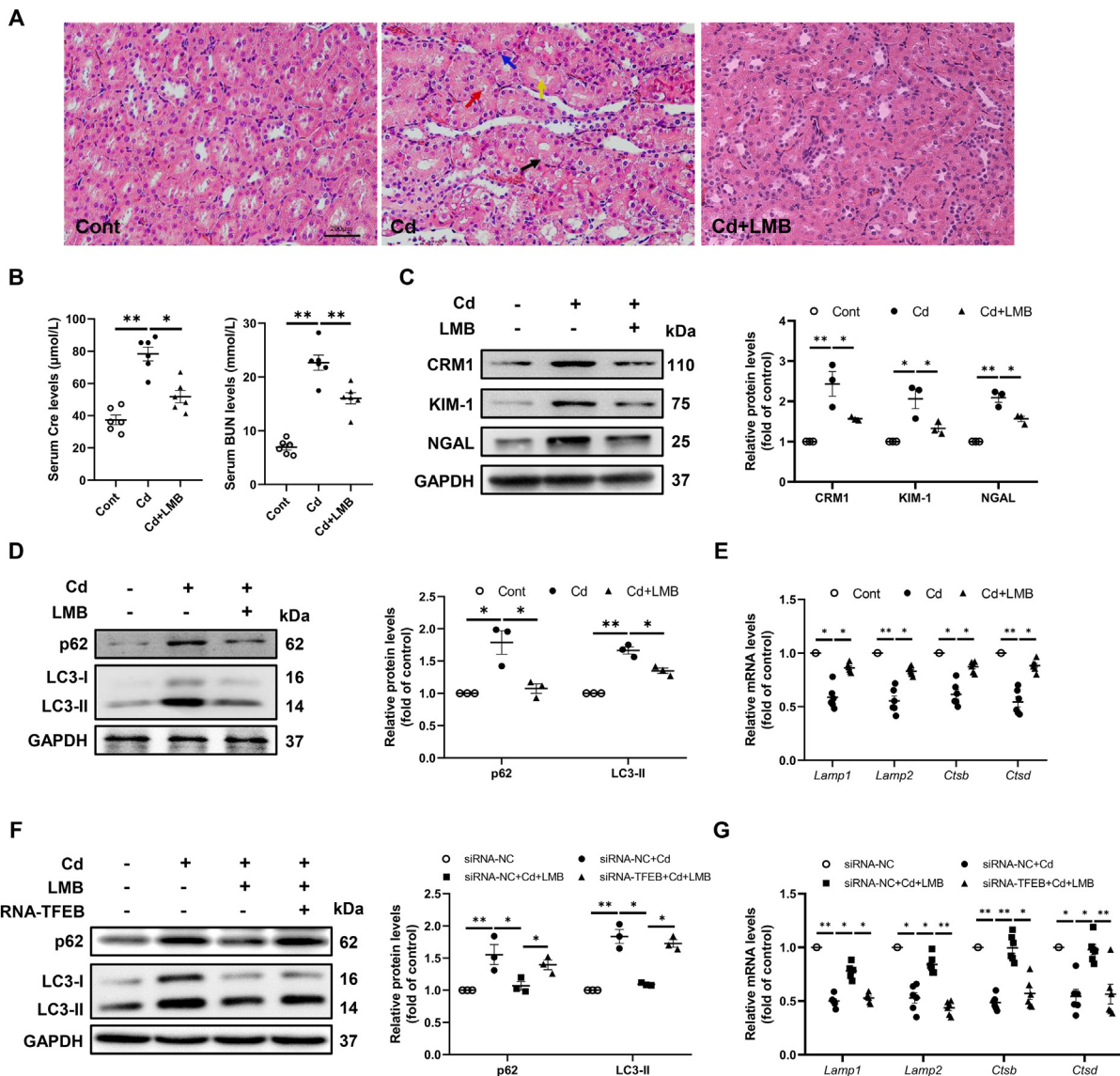


Fig. 6. CRM1 inhibitor LMB alleviates Cd-induced AKI by improving autophagy and lysosomal function. (A) Representative images of HE staining. Blue arrow indicates nuclear pyknosis. Black arrow indicates erythrocytes. Red arrow indicates necrotic cells shed in the tubular lumen. Yellow arrow indicates tubular protein deposition. (B) Serum samples were collected to analyze the Cre and BUN levels (mean ± SEM, n = 6). (C) Renal tissues were collected to analyze the protein levels of CRM1, KIM-1 and NGAL. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean ± SEM, n = 3) were shown in right panels. (D) Renal tissues were collected to analyze the protein levels of p62 and LC3. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean ± SEM, n = 3) were shown in right panels. (E) mRNA expression levels of *LAMP1*, *LAMP2*, *CTSD* and *CTSB* in Cd-exposed rat renal tissues (mean ± SEM, n = 6). (F) After TFEB knockdown, NRK-52E cells were treated with 10 μM Cd and/or 5 nM LMB to analyze the protein levels of p62 and LC3. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean ± SEM, n = 3) were shown in right panels. (G) mRNA expression levels of *LAMP1*, *LAMP2*, *CTSD* and *CTSB* in Cd-exposed NRK-52E cells (mean ± SEM, n = 6). (H) After TFEB knockdown, cells transfected with RFP-GFP-LC3 plasmid were exposed to 10 μM Cd and/or 5 nM LMB, then observed under a confocal microscope. Typical confocal images were quantitated by the ratios of autolysosomes and autophagosomes (right image). *p < 0.05; **p < 0.01.

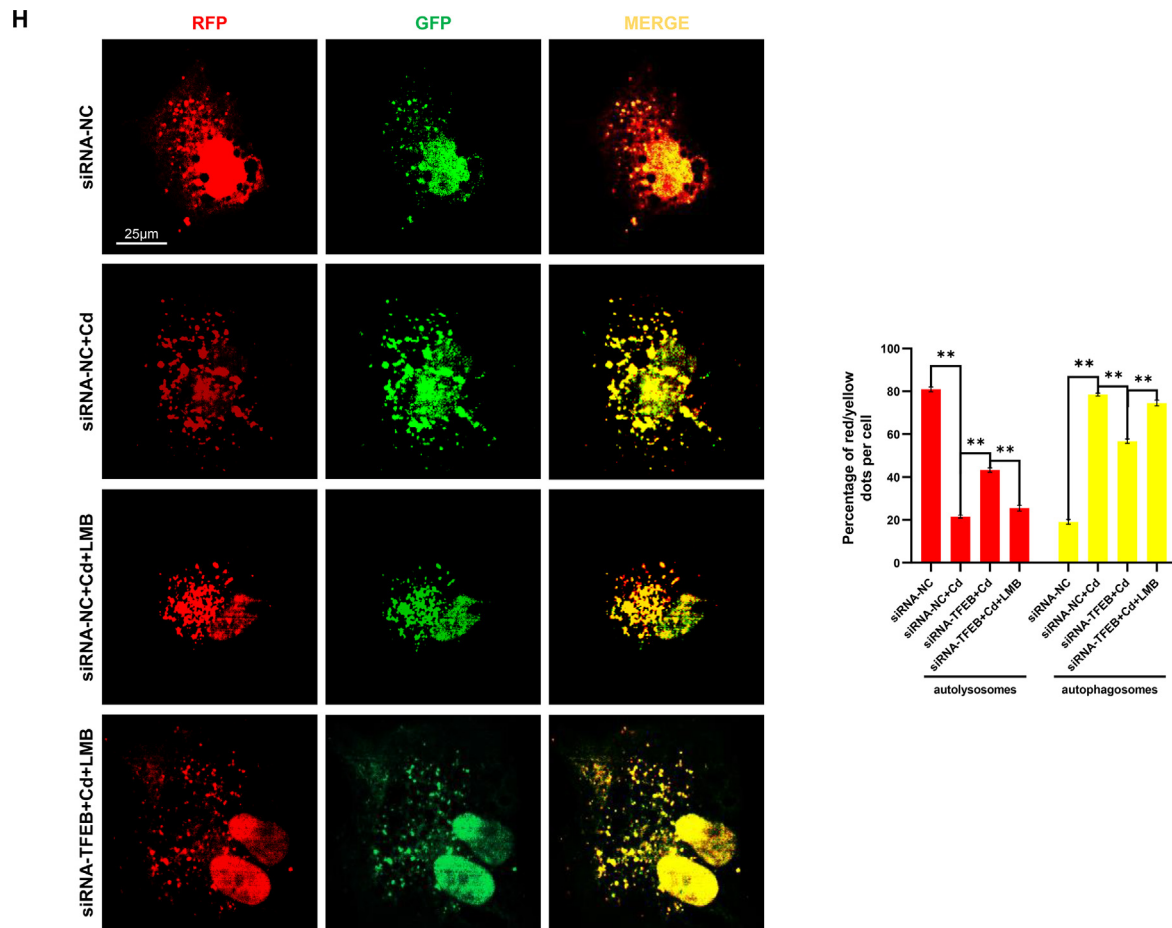


Fig. 6 (continued)

increased in kidney tissues and NRK-52E cells after Cd exposure (Fig. 5A and 5B). To discern whether CRM1 interfered TFEB activity, the interaction between CRM1 and TFEB was detected on NRK-52E cells by IP experiment, and the interaction was strengthened after Cd exposure, which probably indicated that CRM1 plays a regulatory role in AKI through interacting with TFEB (Fig. 5C).

To further explore how CRM1 regulates TFEB, CRM1 inhibitor LMB (5 nM for 2 h) was employed for relevant tests in NRK-52E cells (Fig. 5D). The immunoblot results revealed that the total protein level of TFEB in Cd group is not evidently altered by LMB addition (Fig. 5E). However, the nuclear levels of TFEB in Cd and LMB co-treatment group were significantly higher than those in Cd-treated alone group, and the cytoplasm expression levels of TFEB were remarkably lower (Fig. 5F). Interestingly, in comparison with the control group, the nuclear levels of TFEB in the LMB group also increased evidently, and the cytoplasm expression levels of TFEB decreased noticeably (Fig. 5F), suggesting that LMB suppressed the nuclear export of TFEB. The translocation of TFEB was detected by immunofluorescence method to further verify this phenomenon (Fig. 5G). The above results demonstrate that Cd-induced CRM1 activation promotes the interaction between TFEB and CRM1, leading to the enhancement of TFEB nuclear export. Moreover, inhibited CRM1 could not affect total protein of TFEB but inhibit the nuclear export of TFEB.

Activation of CRM1 aggravates Cd-induced AKI via the suppression of autophagic-lysosomal function

To investigate whether nuclear export of TFEB is involved in Cd-induced AKI, LMB was added to detect indicators related to renal injury. H&E staining demonstrated that treatment with LMB rein-

stated the normal renal tubules except slightly necrosis (Fig. 6A). In addition, LMB treatment overtly reduced the expression of KIM-1 and NGAL, and also decreased Cd-elevated contents of Cre and BUN (Fig. 6B and 6C). The data showed that LMB treatment alleviated AKI induced by Cd exposure. To investigate whether the nuclear output of TFEB affects the autophagic and lysosome function in Cd-induced AKI, autophagy and lysosome-related indicators were evaluated. As shown in Fig. 6D and 6E, Cd-elevated p62 and LC3-II levels were dramatically declined by LMB treatment in rat kidney, while the mRNA levels of Cd-decreased lysosome-related genes were significantly increased by LMB addition. These findings suggested that activation of CRM1 can relieve Cd-induced autophagy inhibition and lysosomal dysfunction. *In vitro* and *in vivo* results were consistent with the fact that LMB treatment alleviated the renal autophagy inhibition and lysosome dysfunction in NRK-52E cells, while this alleviation was inhibited after TFEB knockdown (Fig. 6F and 6G). Cd-induced increase in yellow puncta and decrease in red dots were significantly attenuated by co-treatment with LMB. In addition, yellow puncta increased and red dots decreased after TFEB knockdown (Fig. 6H). These results confirm that Cd exposure activated CRM1 and promoted the nuclear output of TFEB, resulting in autophagy inhibition and lysosome dysfunction, and ultimately leading to AKI. In contrast, inhibiting CRM1 reduced nucleus export of TFEB and promoted autophagy and lysosomal function, which in turn alleviated Cd-induced AKI.

Cd-activated GCN5 acetylates TFEB to inhibit its transcriptional activity

So far, our data suggest that TFEB activation and lysosomal biogenesis are regulated by mTORC1-mediated phosphorylation

and CRM1-mediated nuclear export. Such results inspired us to delve in other types of PTMs in regulating TFEB activity. TFEB was reported to undergo acetylation modifications in the nucleus, such as the acetyltransferase GCN5, which may affect the TFEB acetylation to control TFEB to bind to chromatin and thus regulate target gene transcription. Western blot analysis revealed the expression level of GCN5 *in vivo* and *in vitro* increased significantly after Cd exposure (Fig. 7A and 7B). Furthermore, IP analysis showed an interaction between GCN5 and TFEB, which was strengthened after Cd exposure (Fig. 7C). Our result suggested that TFEB could be acetylated under normal conditions, and increase the acetylation level of TFEB was involved in Cd-treated NRK-52E cells (Fig. 7D). To in-depth explore whether and how GCN5 affects the activity of TFEB, GCN5 knockdown was conducted in NRK-52E cells (Fig. 7E). Data in Fig. 7F showed that Cd-increased TFEB acetylation was alleviated by GCN5 knockdown, suggesting that GCN5 mediates acetylation of TFEB during Cd exposure.

This study also found that GCN5 knockdown did not alter TFEB abundance in the cytoplasm and nucleus (Fig. 7G and 7H). The

above results suggest that GCN5, mainly located in the nucleus, has no effect on TFEB nuclear translocation and may inhibit the function of TFEB in the nucleus by acetylation. To ascertain the transcriptional activity of TFEB, the binding ability of TFEB and its target gene (*ATG9B*, *VPS11*, *LAMP1*, *CTSD*, *ATP6V1H*) promoters was tested by ChIP assay. As shown in Fig. 7I, the binding ability of TFEB to promoters of its target genes could be inhibited after Cd exposure, and GCN5 knockdown could evidently improve the promoter activity of the target gene suppressed by Cd. Together, the findings indicate that Cd-induced GCN5 activation facilitates TFEB acetylation, not affecting the translocation of TFEB, but rather reducing the binding capacity of TFEB to its target genes.

GCN5 inhibition alleviates Cd-induced AKI by improving autophagic-lysosomal function

The above experimental results suggest that Cd-activated GCN5 can directly acetylate TFEB, so it is necessary to investigate whether acetylation of TFEB plays a role in AKI. Rats were

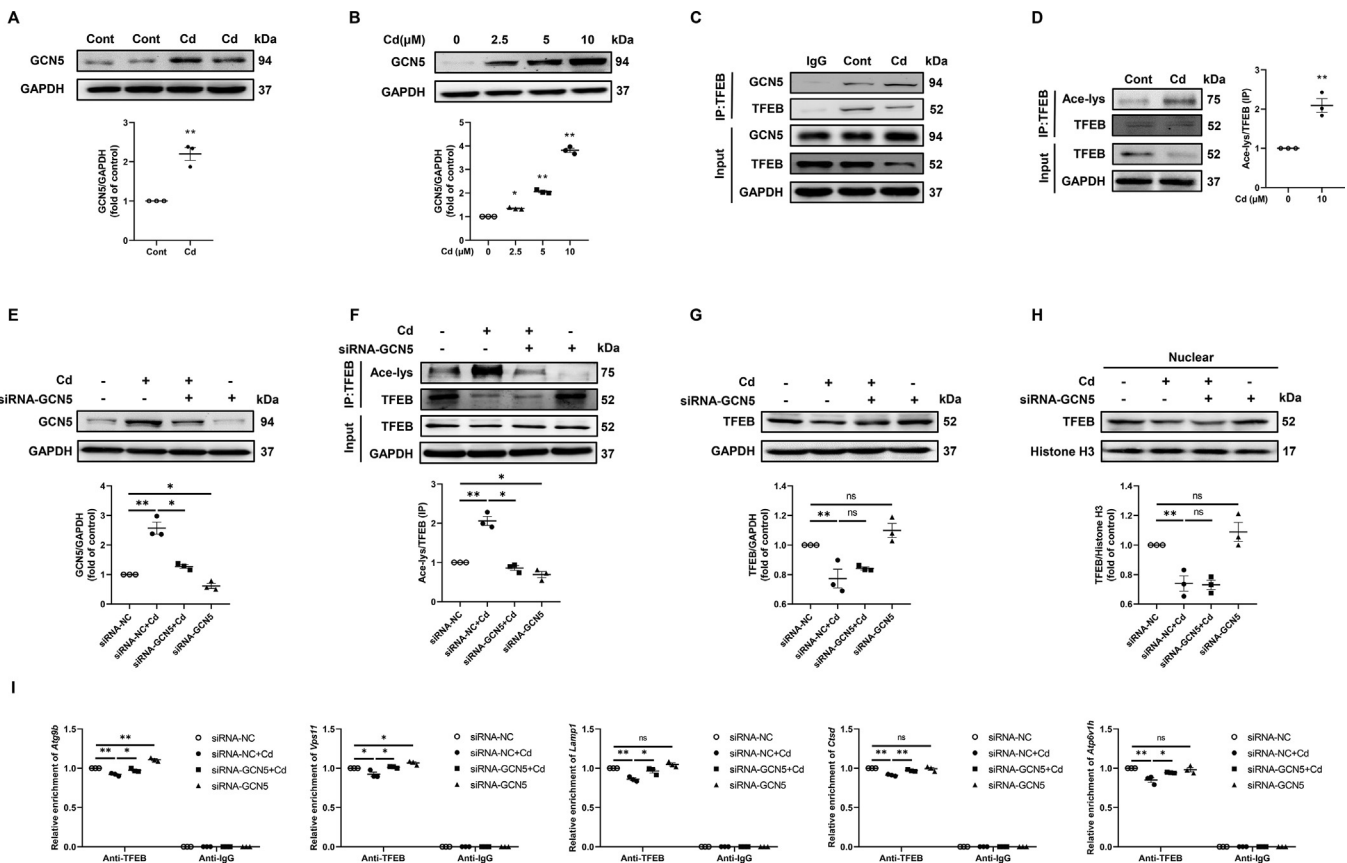


Fig. 7. GCN5 upregulation induced by Cd exposure is responsible for TFEB acetylation and resultant inhibition of TFEB transcriptional activity. (A) Renal tissues were collected to analyze the protein levels of GCN5. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (B) NRK-52E cells were treated with a range of Cd doses for 12 h to analyze the protein levels of GCN5. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (C) Co-precipitation of GCN5 with TFEB. TFEB was immunoprecipitated from NRK-52E cells using anti-TFEB, and the precipitates were analyzed using anti-GCN5. (D) NRK-52E cells were treated with 10 μ M Cd for 12 h to analyze the acetylation levels of TFEB. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (E) NRK-52E cells were transfected with control siRNA (siRNA-NC) and GCN5 siRNA (siRNA-GCN5). After GCN5 knockdown, NRK-52E cells were treated with 10 μ M Cd for 12 h to analyze the protein levels of GCN5. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (F) After GCN5 knockdown, NRK-52E cells were treated with 10 μ M Cd for 12 h to analyze the acetylation levels of TFEB. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (G) After GCN5 knockdown, NRK-52E cells were treated with 10 μ M Cd for 12 h to analyze the protein levels of TFEB. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (H) After GCN5 knockdown, NRK-52E cells were treated with 10 μ M Cd for 12 h to analyze the nucleus protein levels of TFEB. Representative immunoblot images were shown in upper panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in lower panels. (I) NRK-52E cells were treated with 10 μ M Cd to analyze the binding capacity of TFEB and IgG with autophagy and lysosomal gene promoters using ChIP assay (mean $\pm$ SEM, $n = 3$). * $p < 0.05$; ** $p < 0.01$.

administered GCN5 inhibitor B3 to detect indicators related to renal injury. The data revealed that all these Cd-induced kidney injury indices were reversed by B3 treatment (Fig. 8A, 8B and 8C). These findings suggested that inhibiting GCN5 could alleviate Cd-induced AKI. To explore if acetylation of TFEB regulated Cd-induced AKI involved autophagy and lysosomal dysfunction, inhibition of GCN5 was performed *in vivo* and *in vitro*. As shown in Fig. 8D-G, inhibition of GCN5 decreased the expression of p62 and LC3-II and increased the expression of lysosome-related genes. Elevation of yellow dots and reduction of red dots in Cd-exposed cells were significantly reversed by GCN5 knockdown (Fig. 8H), further confirming the alleviation of Cd-induced autophagy inhibition. The above results showed that GCN5 by targeting TFEB acetylation contributes to autophagic-lysosomal dysfunction and consequent AKI during Cd exposure.

mTORC1, CRM1 and GCN5 do not interfere with each other in Cd-induced AKI

Given the verified correlation of nuclear export, phosphorylation, and acetylation, it is essential to understand whether the nuclear export of TFEB and the 2 types of PTMs of TFEB are functionally cross-talked [31,41,42]. Whether there is a potential relationship between mTORC1-mediated phosphorylation, CRM1-mediated nuclear export and GCN5-mediated acetylation was further explored. First, whether mTORC1 inhibits the TFEB activity by CRM1 and GCN5 was explored. Our data suggested that mTORC1 knockdown has no effect on CRM1 and GCN5 expression (Fig. 9A). IP assay showed no effect on the interaction of TFEB with GCN5 or CRM1 after mTORC1 knockdown compared to the Cd-treated group (Fig. 9B). Therefore, TFEB phosphorylation does not directly affect its acetylation. Next, whether CRM1 represses TFEB

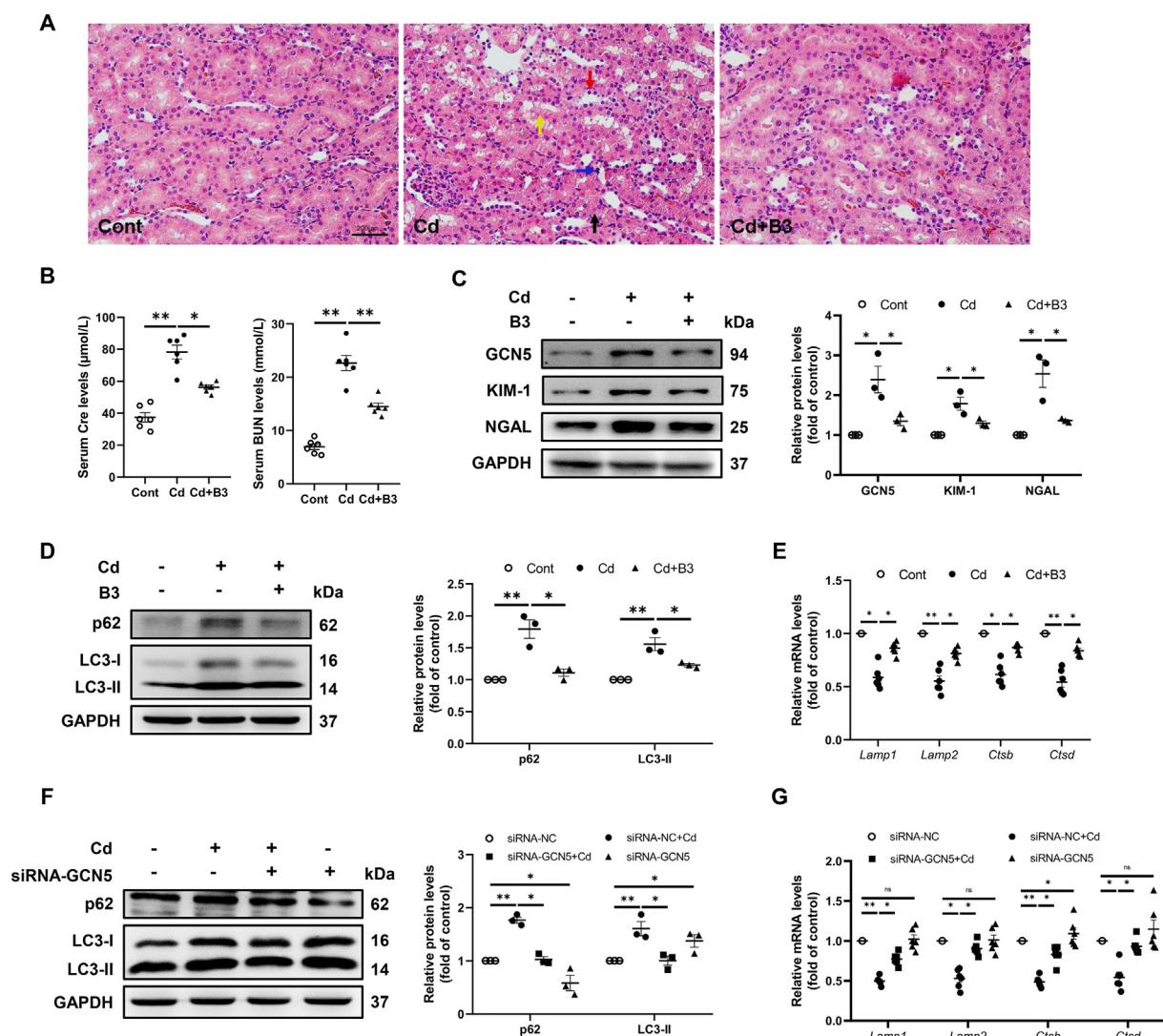


Fig. 8. GCN5 inhibition alleviates Cd-induced AKI by improving autophagy-lysosome function. (A) Representative images of HE staining. Blue arrow indicates nuclear pyknosis. Black arrow indicates karyolysis. Red arrow indicates necrotic cells shed in the tubular lumen. Yellow arrow indicates tubular protein deposition. (B) Serum samples were collected to analyze the Cre and BUN levels (mean $\pm$ SEM, $n = 6$). (C) Renal tissues were collected to analyze the protein levels of GCN5, KIM-1 and NGAL. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (D) Renal tissues were collected to analyze the protein levels of p62 and LC3. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (E) mRNA expression levels of *LAMP1*, *LAMP2*, *CTSD* and *CTSB* in Cd-exposed rat renal tissues (mean $\pm$ SEM, $n = 6$). (F) After GCN5 knockdown, NRK-52E cells were treated with 10 μ M Cd to analyze the protein levels of p62 and LC3. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, $n = 3$) were shown in right panels. (G) mRNA expression levels of *LAMP1*, *LAMP2*, *CTSD* and *CTSB* in Cd-exposed NRK-52E cells (mean $\pm$ SEM, $n = 6$). (H) After GCN5 knockdown, cells transfected with RFP-GFP-LC3 plasmid were exposed to 10 μ M Cd for 12 h, then observed under a confocal microscope. Typical confocal images were quantitated by the ratios of autolysosomes and autophagosomes (right image). * $p < 0.05$; ** $p < 0.01$.

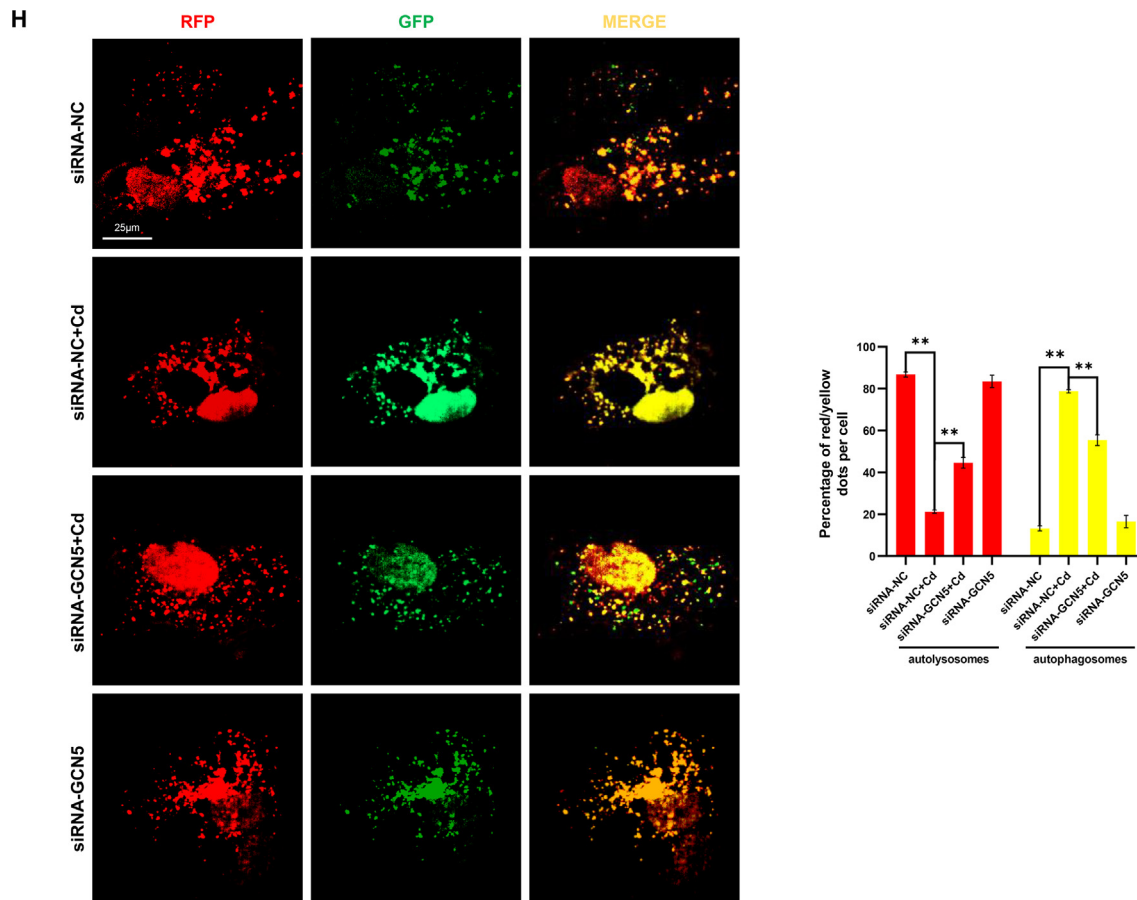


Fig. 8 (continued)

activity via mTORC1-mediated phosphorylation and GCN5 was explored. These findings indicated that LMB addition did not influence p-mTOR and p-TFEB and did not affect the expression of GCN5 (Fig. 9C). No changes were observed in the immunoprecipitation of mTORC1 or GCN5 with TFEB after addition of LMB compared to the Cd-treated group (Fig. 9D). Finally, whether GCN5 affects TFEB phosphorylation was explored. As shown in Fig. 9E, GCN5 knock-down had no effect on p-mTOR and p-TFEB, and also did not affect CRM1 expression. Moreover, results of IP assay showed that GCN5 knockout did not affect the interaction between mTORC1 or CRM1 and TFEB compared to the Cd group (Fig. 9F). Taken together, these findings demonstrate that TFEB phosphorylation, nuclear export and acetylation independently suppress TFEB activity to cause Cd-induced AKI via regulating autophagy-lysosome function.

Discussion

TFEB is proven as a master modulator of lysosomal biogenesis and autophagy-related gene transcription. Recent studies have confirmed autophagy-lysosome dysfunction in Cd-induced kidney injury [9], which prompted us to explore if there is a correlation between TFEB function and Cd-induced AKI via regulating autophagy-lysosome pathway. To this aim, this study was conducted to testify this hypothesis, predominantly focused on the effect of TFEB localization and its transcriptional activity on Cd-induced autophagy-lysosome dysfunction and resultant AKI. As far as we know, this study was the first to systematically dissect the regulatory effect of TFEB on Cd-induced AKI, providing solid evidence of its potential therapeutic target for AKI.

Considerable evidence has shown that autophagy inhibition of renal tubular cells due to lysosomal dysfunction is a key event of Cd-induced AKI [9,43–45]. Consistently, transcriptome analysis of rat renal tissues revealed that the different expressed genes mainly occurred in autophagy and lysosomal signaling pathways between control and Cd groups in this study, down-regulated TFEB was significantly enriched in these two pathways. Notably, TFEB is related to ischemia-reperfusion and cisplatin-induced AKI. Our data demonstrated that upregulated expression genes were majorly enriched into biological processes such as apoptotic process, cellular response to DNA damage stimulus, skeletal muscle contraction, aging, negative regulation of microtubule depolymerization, protein ubiquitination, and skeletal muscle tissue development [27,46,47], but its role in Cd-induced AKI is yet to be elucidated. Data confirmed that Cd-induced AKI is accompanied by autophagy-lysosome dysfunction and inhibited TFEB activity. Given the critical effect of TFEB on autophagy-lysosome function, we hypothesized that TFEB dysregulation may be act as a driver in Cd-induced AKI. As expected, Cd-induced cellular damage and concurrent autophagy-lysosome dysfunction are alleviated by pharmacological or genetic activation of TFEB, which are exacerbated by TFEB silencing (Fig. 3). Our findings highlight the regulatory role of TFEB in Cd-induced AKI via inhibiting autophagy-lysosome function, thus restoration of impaired TFEB activity can be a novel treatment approach for Cd-induced nephrotoxicity.

In physiological condition, TFEB can regulate lysosomal biogenesis and autophagy function [48,49], which attracted us to investigate the reason of inhibited TFEB activity in Cd-induced AKI. TFEB activity is closely regulated by PTMs and protein–protein interac-

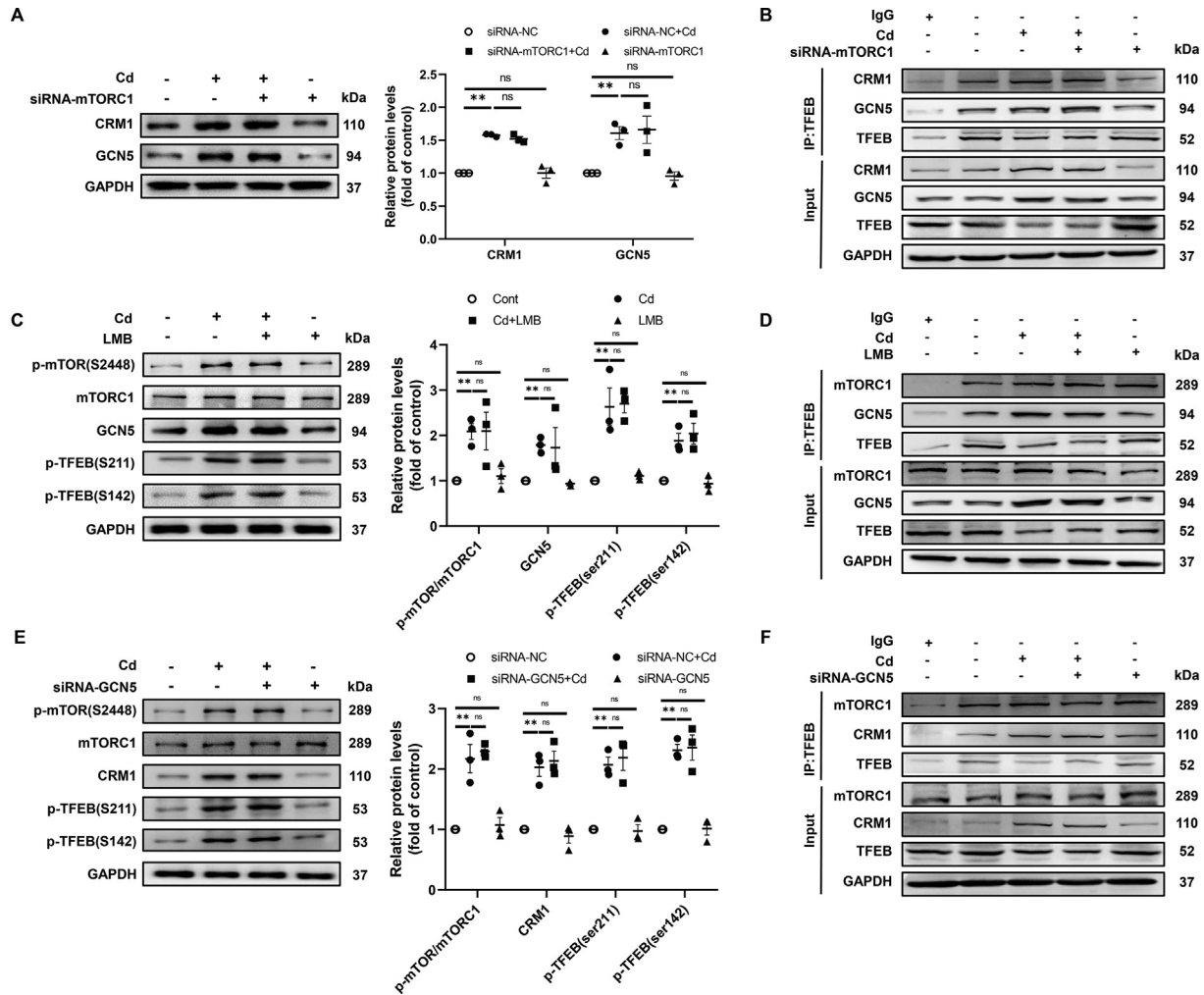


Fig. 9. Mutual non-interaction among CRM1 activation, TFEB phosphorylation and acetylation. (A) After mTORC1 knockdown, NRK-52E cells were treated with 10 μ M Cd to analyze the protein levels of CRM1 and GCN5. (B) Co-precipitation of CRM1 or GCN5 with TFEB. TFEB was immunoprecipitated from NRK-52E cells using anti-TFEB, and the precipitates were analyzed using anti-CRM1 or anti-GCN5. (C) NRK-52E cells were treated with 10 μ M Cd and/or 5 nM LMB to analyze the protein levels of mTOR, p-mTOR (S2448), GCN5, p-TFEB (S211) and p-TFEB (S142). (D) Co-precipitation of mTORC1 or GCN5 with TFEB. TFEB was immunoprecipitated from NRK-52E cells using anti-TFEB, and the precipitates were analyzed using anti-mTORC1 or anti-GCN5. (E) After GCN5 knockdown, NRK-52E cells were treated with 10 μ M Cd to analyze the protein levels of mTOR, p-mTOR (S2448), CRM1, p-TFEB (S211) and p-TFEB (S142). (F) Co-precipitation of mTORC1 or CRM1 with TFEB. TFEB was immunoprecipitated from NRK-52E cells using anti-TFEB, and the precipitates were analyzed using anti-mTORC1 or anti-CRM1. Representative immunoblot images were shown in left panels and corresponding densitometric levels (mean $\pm$ SEM, n = 3) were shown in right panels. *p < 0.05; **p < 0.01.

tions [23]. Phosphorylation is a well-documented PTM that modulates TFEB distribution in the cytoplasm and nucleus [50–52]. Moreover, mTORC1 is confirmed as a key negative modulator to regulate TFEB phosphorylation and its nuclear localization via directly phosphorylation of TFEB at sites of Ser142 and Ser211, mutation of which causes constitutive TFEB nuclear translocation and activation of its target genes, demonstrating that such phosphorylation determines TFEB distribution inside or outside the nucleus [53,54]. Consistently, data in this study has revealed that Cd-induced mTORC1 activation results in TFEB phosphorylation at sites 211 and 142, leading to inhibited TFEB nuclear translocation. Moreover, our previous findings have demonstrated that mTORC1 activation results in autophagy suppression through its recruitment to lysosomes and consequently causes the blockage of lysosomal degradation in Cd-exposed rat proximal tubular cells [22]. Given the above mentioned, it can be seen that restoration of the inhibited TFEB activity by mTORC1 inhibitor may be a promising strategy to alleviate Cd-induced AKI.

Notably, TFEB is a nuclear-cytoplasmic shuttle protein, which depends on nuclear export protein to control TFEB shuttling.

CRM1 is a confirmed nuclear export protein that promotes TFEB across the nuclear membrane to the cytoplasm [55,56]. Also, previous research has shown that CRM1-driven TFEB nuclear export requires mTOR-dependent TFEB phosphorylation [51]. In the present study, Cd-induced CRM1 activation promoted the interaction between TFEB and CRM1, leading to the enhancement of TFEB nuclear export (Fig. 5). Meanwhile, studies have revealed that inhibiting CRM1 by LMB causes rapid transport of TFEB to the nucleus where it enhances autophagy-lysosome function [51,57]. Consistently, our findings have shown that CRM1-mediated TFEB nuclear export contributes to autophagy-lysosome dysfunction in Cd-induced AKI, in which CRM1 interacts with TFEB to play the regulatory role (Fig. 6). Contrary to the idea from Napolitano et al. [51], CRM1-mediated TFEB nuclear export has no concern with mTOR-mediated TFEB phosphorylation, and they play its role in Cd-inhibited TFEB activity independently (Fig. 9).

Furthermore, TFEB acetylation is reported to be the predominant PTM to regulate TFEB transcriptional activity [38,42,58]. It's been shown that GCN5 is a specific acetyltransferase of TFEB. TFEB acetylation by GCN5 inhibited its activity and consequent

autophagy-lysosome dysfunction [38]. Given the importance of GCN5 in regulating TFEB activity and autophagy-lysosome function, we envisioned that GCN5 may play a key role in Cd-inhibited TFEB activity and resultant AKI. As expected, Cd-induced GCN5 activation promotes TFEB acetylation to inhibit its transcriptional activity via augmented affinity of GCN5 with its substrate TFEB (Fig. 7). Previous research has also shown that deacetylation of TFEB in microglia promotes lysosomal biogenesis by enhancing TFEB target gene expression levels [59]. Consistently, our data has shown that inhibition of GCN5 results in deacetylation of TFEB and enhanced TFEB binding capacity for its target genes, thereby alleviating Cd-induced autophagy-lysosome dysfunction and resultant AKI (Figs. 7 and 8). Meanwhile, our findings provide solid evidence that GCN5-modulated acetylation only blocks TFEB activity but not affect its subcellular localization during Cd exposure (Fig. 7). However, Wang et al., suggests that there is a potential regulatory effect of mTORC1 on GCN5, which may coordinate with mTORC1-controlled TFEB nuclear translocation [38]. Contrarily, with immunoprecipitation analysis, it has been shown that Cd-induced GCN5 activation is independent of mTORC1-mediated TFEB phosphorylation or CRM1 activation (Fig. 9). The evidence provided by this study suggests that the acetylation by GCN5 serves as an additional step for controlling the activity of TFEB after it enters the nucleus.

Taken together, the underlying mechanism of inhibited TFEB activity in Cd-induced AKI is illuminated. Cd-inhibited TFEB activity depends on the combined effects of mTORC1-mediated TFEB phosphorylation, CRM1-mediated TFEB nuclear export and GCN5-mediated TFEB acetylation, but TFEB phosphorylation, nuclear export and acetylation independently suppress TFEB activity to cause Cd-induced AKI via regulating autophagy-lysosome function. These results provide the underlying molecular mechanism of Cd-induced kidney damage, suggesting that TFEB is a promising treatment target for Cd-induced AKI.

Acknowledgments

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