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RESEARCH ARTICLE

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Klotho regulation by albuminuria is dependent on ATF3 and endoplasmic reticulum stress

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Abstract

Proteinuria is associated with renal function decline and cardiovascular mortality. This association may be attributed in part to alterations of Klotho expression induced by albuminuria, yet the underlying mechanisms are unclear. The presence of albumin decreased Klotho expression in the POD-ATTAC mouse model of proteinuric kidney disease as well as in kidney epithelial cell lines. This downregulation was related to both decreased Klotho transcription and diminished protein half-life, whereas cleavage by ADAM proteases was not modified. The regulation was albumin specific since it was neither observed in the analbuminemic *Col4α3*$^{-/-}$ Alport mice nor induced by exposure of kidney epithelial cells to purified immunoglobulins. Albumin induced features of ER stress in renal tubular cells with ATF3/ATF4 activation. ATF3 and ATF4 induction downregulated Klotho through altered transcription mediated by their binding on the Klotho promoter. Inhibiting ER stress with 4-PBA decreased the effect of albumin on Klotho protein levels without altering mRNA levels, thus mainly abrogating the increased protein degradation. Taken together,

Abbreviations: Alb, albumin; Atf4/3, activating transcription Factor 4/3; C57BL/6J, C57 black 6 mice; CCr, creatinine clearance; CD2AP, CD2 associated protein; Chop/Gadd153, DNA damage-inducible transcript 3 protein; c-Jun, transcription factor AP-1; Col4α3, collagen type IV alpha 3 chain; CQ, chloroquine; CTL, control; db/db, dyslipidemic mouse model of Type II diabetes; DIM, dimerizer-injected; eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; FePi, fractional excretion of phosphate; FKBP, FK506 Binding Protein; FNL-7, HEK-293 cells expressing Firefly and Nano Luciferase in the control of the human Klotho promoter; FVB, albino mouse strain susceptible to Friend leukemia virus B; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Grp78, endoplasmic reticulum chaperone BiP; HEK-293, human embryonic kidney 293 cells; HK-2, human kidney 2 cells; LPS, lipopolysaccharides; MAPK, mitogen-Activated Protein Kinase; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; Nphs2, podocin; POD-ATTAC, podocyte apoptosis through targeted activation of caspase-8; RLU, relative luminescence units; ROS, reactive oxygen species; shRNA, short hairpin RNA; TUN, Tunicamycin; Xbp-1s, X-box-binding protein 1 spliced variant.

albuminuria decreases Klotho expression through increased protein degradation and decreased transcription mediated by ER stress induction. This implies that modulating ER stress may improve proteinuria-induced alterations of Klotho expression, and hence renal and extrarenal complications associated with Klotho loss.

KEYWORDS

albuminuria, ATF3, CKD, ER stress, Klotho

1 | INTRODUCTION

Proteinuria is a major factor associated with progression of kidney fibrosis, loss of renal function, and End-stage renal disease (ESRD).[1-5] In addition, proteinuria is associated with increased cardiovascular (CV) risk and global mortality, independently of renal function, in both high-risk patients and the general population.[1-5] Albuminuria may be a marker of vascular lesions.[6] However, in primary glomerulonephritis, albuminuria is also predictive of CV mortality, despite being independent of vascular lesions.[1] The pathway linking albuminuria with enhanced CV risk is yet elusive.

α-Klotho is a transmembrane glycoprotein expressed in the distal and, to a lesser extent, the proximal convoluted tubules.[7,8] Klotho is an obligate co-receptor of Fibroblast Growth Factor 23 (FGF-23), thus regulating phosphate homeostasis.[8] Apart from the transmembrane form, Klotho has been detected in the urine, blood, and cerebrospinal fluid and may be generated mainly by proteolytic cleavage at the membrane by ADAM metallopeptidase domain proteases, ADAM10 and ADAM17 (soluble form), or through a splicing variant (secreted form),[9-15] the fate of which has been recently debated since this mRNA may be rapidly degraded.[16] Renal Klotho levels decline in experimental models of Chronic Kidney Disease (CKD). This likely contributes directly to hyperphosphatemia, increased vascular calcification, and cardiac hypertrophy, but potentially also has indirect effects via the parallel elevation of FGF-23.[17-20] In humans, measurement of soluble Klotho levels has yielded more contradictory results, likely due to the low reproducibility of available methods.[21]

In a recent study, phosphate handling was shown to be altered by the presence of albuminuria.[22] In albuminuric compared to non-albuminuric CKD patients, plasma phosphate levels were higher at similar levels of renal function despite elevated plasma levels of FGF-23. Similar observations were made in nephrotic children without the alteration of glomerular filtration rate. Experimentally, Klotho protein expression was downregulated in two different murine proteinuric models. Klotho regulation by proteinuria was further confirmed in other models of proteinuria and was shown to be independent of inflammatory cytokines.[23] In humans, albuminuria has been associated with decreased soluble Klotho, alterations of FGF-23 and increased serum phosphate.[22,24-27] Another study confirmed the association of albuminuria to decreased FGF-23 biological activity and a worse prognosis in a CKD cohort.[28] Interestingly, in the Chronic Renal Insufficiency Cohort (CRIC) study, the association of proteinuria with mortality was noticeably lessened by the integration of FGF-23 levels.[29] All these studies underline the essential function of Klotho in regulating phosphate and FGF-23 levels and emphasize the necessity to find new therapeutic avenues to decrease CV mortality and improve renal survival in proteinuric kidney disease.

Endoplasmic reticulum (ER) stress has been shown to be activated in different models of proteinuric kidney disease, either by the primary disease, or by proteinuria itself.[30-34] ER stress induces different cell responses including increased proteasomal and lysosomal degradation, autophagy, or apoptosis. Pharmacological inhibition of ER stress has been achieved with different classes of drugs, including the chemical chaperones 4-PBA and TUDCA that both improve protein-folding capacity.[30] Proteinuric kidney disease is characterized by induction of ER stress that may be improved by 4-PBA treatment.[35-38] Among transcription factors induced by ER stress are ATF4 and ATF3, which may act as activators or repressors depending on the gene of interest and their binding to the promoter as homo- or heterodimers.[39] ATF3 has been shown to be able to bind to the α-Klotho promoter.[40] ATF3 deficiency was reported to be detrimental in ischemia-reperfusion injury[41,42] and cyclosporin A-induced nephrotoxicity.[43]

We here study the mechanism of decreased Klotho expression by albuminuria using an animal model of high-grade albuminuria generated through podocyte apoptosis, and cultured kidney epithelial cells (HEK-293 and HK-2). We show that exposure of renal epithelial cells to serum albumin decreases Klotho expression by altering both the protein half-life and its gene transcription. We demonstrate that ATF3 induction may participate in the decreased Klotho transcription by binding to the Klotho promoter. Finally, we show that ER stress decreases Klotho expression and that inhibiting ER stress reverses Klotho downregulation in kidney epithelial cells exposed to serum albumin.

2 | MATERIALS AND METHODS

2.1 | Animal experimentation

All animal experiments were approved by the Institutional Ethical Committee of Animal Care in Geneva and Cantonal authorities with the authorization number 94-16. All mice had free access to food and water.

2.1.1 | POD-ATTAC mice

POD-ATTAC mice expressing an inactive form of caspase-8 fused with the FKBP protein under the control of the human podocin promoter Nphs2[44] were maintained on a FVB background. Genotyping was performed as described.[45] Podocyte apoptosis was induced by injection of the dimerizer AP20187 (#635058; Takara, Saint-Germain-en-Laye, France). Transgenic male FVB mice aged 8 weeks were injected intraperitoneally with a single dose of 0.5 μg/g body weight of dimerizer[44] or equal volume of vehicle and euthanized after 3 or 7 days. The mice were acclimatized in individual metabolic cages (Techniplast, Buguggiate, Italy) for 2 days, 24-hour urine samples were collected and the animals were sacrificed at the indicated time points. Proteinuria was assessed with a Combur10 Test strip (#11379208; Roche Diagnostics Ltd., Rotkreuz, Switzerland). Mice were anesthetized with an intraperitoneal injection of ketamine (100 μg/g) and xylazine (5 μg/g) (GRAEUB, Bayer Healthcare, Berlin, Germany) before the sacrifice. Blood was collected in Li-heparin microtubes (20.1345, SARSTEDT, Nümbrecht, Germany). Urine and plasma parameters analysis was performed at the Zurich Integrative Rodent Physiology (ZIRP) platform of the University of Zurich using the UniCel DxC 800 analyser (Beckman Coulter, Brea, CA). Following that we used the standard equation to calculate creatinine clearance in 24-hour urine:

$$\text{CCr (mL/min)} = \text{U}\left(\frac{\text{mg}}{\text{mL}}\right) \times \text{V (mL)} \Big/ \text{P}\left(\frac{\text{mg}}{\text{mL}}\right) \times 24 \times 60$$

To calculate the fractional excretion of phosphate (FePi), we used the formula:

$$\text{FePi} = \text{Urine Pi}\left(\frac{\text{mg}}{\text{mL}}\right) \times \text{Plasma Cr}\left(\frac{\text{mg}}{\text{mL}}\right) \Big/ \text{Plasma Pi}\left(\frac{\text{mg}}{\text{mL}}\right) \times \text{Urine Cr}\left(\frac{\text{mg}}{\text{mL}}\right)$$

For ER stress inhibition, 1 mg/g/day of 4-phenylbutyric acid sodium salt (Scandinavian Formulas, Sellersville, PA) or vehicle (water corrected for pH and Na^+ concentration, with HCl and NaCl, respectively) was provided in the drinking water starting on the same day of the dimerizer injection for 7 days. Water consumption was not different among the experimental groups (Normalized values: CTL 1.00 ± 0.03, CTL 4-PBA 0.80 ± 0.12, DIM 0.99 ± 0.15, DIM 4-PBA 0.79 ± 0.08).

2.1.2 | Aminonucleoside-induced nephrotic syndrome (PAN) rats

Paraffin-embedded kidney tissues from male Wistar rats injected with a single intraperitoneal injection of Puromycin aminonucleoside at a dose of 130 μg/g and euthanized after 14 days, were used, as described.[22]

2.1.3 | Alport mouse model

Isolated kidney tubules and kidney cortex were derived from *Col4α3*^{tm1Jhm} (*Col4α3*$^{-/-}$) Alport mice on the C57BL/6J background crossed with albumin-deficient mice[46] and were used for mRNA and protein analysis, respectively. The samples originated from 120 to 180 days old male mice of this previous study. The genotypes used were *Col4α3*$^{+/-}$; *Alb*$^{+/-}$ (control mice), *Col4α3*$^{-/-}$; *Alb*$^{+/-}$ (Alport mice), and *Col4α3*$^{-/-}$; *Alb*$^{-/-}$ (Alport albumin-deficient mice).

2.1.4 | Tunicamycin-injected mice

Whole kidney lysates derived from a previous study were used for protein analysis.[47] More specifically, mice at the C57BL6 background were injected intraperitoneally with two doses of Tunicamycin (3 μg/g/day) to induce ER stress-mediated acute kidney injury and the kidneys were harvested 48 hours later.

2.2 | Renal morphology and immunohistochemistry

Kidney tissue was fixed in 4% paraformaldehyde and embedded in paraffin. 5 μm sections were stained with Sirius red to assess kidney fibrosis or Periodic acid-Schiff (PAS) to assess general renal morphology. Immunohistochemical staining for Klotho, ATF4, and ATF3 was performed. The antigen retrieval technique was microwave-based using either Tris-EGTA (10 mM Tris and 0.5 mM EGTA, pH 9.0) or citrate buffer (0.1 M, pH 6.0). The list of antibodies is summarized in Table 1. All slides were counterstained with hematoxylin for nuclei staining, mounted, and then scanned with the Zeiss Axioscan.Z1 (ZEISS, Oberkochen, Germany) at a 20× magnification.

TABLE 1 Antibodies and kits used for immunohistochemistry

Name	Host and isotype	Manufacturer	Cat. No.	Dilution
Primary antibody				
Klotho	Rat IgG2a	TransGenic	KO603	1:50
ATF4	Rabbit IgG	Cell signaling	#11815	1:250
ATF3	Rabbit IgG	Abcam	ab207434	1:50
Detection system				
Klotho	ImmPRESS HRP Anti-Rat IgG	Vector Laboratories	MP-7404-50	
	EnVision Flex kit	DAKO	K8010	
ATF4, ATF3	CSA II Biotin-Free Catalyzed Amplification System	DAKO	K149711-2	

2.3 | Cell culture and transfection

Human epithelial kidney HEK-293 (ATCC CRL-1573) and HEK-293T (ATCC CRL-3216) and immortalized human proximal tubular HK-2 cells (ATCC CRL-2190) were used. A HEK-293 Klotho reporter cell line (FNL-7), which is a modified version of clone KL5[48] containing 1.8 kb of the proximal promoter in the dual luciferase reporter system, was provided by Carmela R. Abraham (Boston University School of Medicine, Boston, MA).

2.3.1 | Generation of a stable line overexpressing human Klotho

A stable HEK-293 cell line overexpressing the human transmembrane form of α-Klotho was generated after transfection with the overexpressing plasmid containing the full-length Klotho cDNA, kindly provided by Orson W. Moe (UT Southwestern Medical Centre, Dallas, TX).[49] Twenty-four hours after transfection (FuGENE HD Transfection Reagent, E2311; Promega, Madison, WI), 500 μg/mL Geneticin G418 (#10131019; Gibco-Thermofisher, Ecublens, Switzerland) was added continuously for 3 weeks to select the clone with the highest Klotho overexpression.

2.3.2 | Transient overexpression of human transmembrane Klotho and the cleavage mutant KLΔ9

HEK-293 or HK-2 cells were transiently transfected with plasmids expressing the human transmembrane Klotho, either wild-type (KLWT) or the mutant KLΔ9 in which the amino acids 954-962 that are close to the transmembrane region have been deleted leading to almost complete inhibition of cleavage by ADAM proteases.[11] Transfection was performed with the use of FuGENE HD. Twenty-four hours later the cell medium was changed and the cells were kept in culture for an additional of 24 hours.

2.3.3 | Transient overexpression of human ATF4 and human ATF3 transcription factors

HK-2 or FNL-7 cells were transiently transfected with plasmids expressing the human ATF4 (pRK-FLAG-ATF4, #26114; Addgene, Cambridge, MA) and human ATF3 (pRK-FLAG-ATF3, #26115; Addgene) transcription factors with the use of FuGENE HD as described before. As a control, an empty vector was transfected in parallel. All measurements for both cell lines were performed 48 hours post-transfection.

2.3.4 | Production of cell line stably expressing shRNA against human ATF4 and human ATF3

In order to knock down ATF4 and ATF3 expression, shRNA targeting sequences against the human ATF4 and ATF3 transcription factors were designed (ATF4: 5′-CCTAGGTCTCTTAGATGATTA-3′ and ATF3: 5′-ACGAG AAGCAGCATTTGATAT-3′) and subcloned between the restriction sites of AgeI and EcoRI of the pLKO.1 lentiviral vector (#10878, Addgene). As nontargeting (scrambled) shRNA, we used the sequence 5′-CAACAAGATGAAGAGCACCAA-3′ (SHC016; Sigma-Aldrich, St. Louis, MO). In order to produce lentiviral particles, packaging HEK-293T cells were transfected with the pLKO.1 vector expressing the targeting or scrambled shRNA along with the helper plasmids pMD2.G (#12259; Addgene) and psPAX2 (#12260; Addgene) using jetPRIME (#114-15; VWR, Radnor, PA) according to the manufacturer's instructions. The cell medium was changed 4 hours post-transfection. To isolate the lentiviral particles, the cell medium was collected 48 hours after and centrifuged at 1500 rpm for 7 minutes and passed through a

0.45-μM pore size filter to remove cell debris. To generate the stable cell line, HK-2 cells at 30% of confluency were transduced and 48 hours later 2.5 μg/mL of Puromycin (#P9620; Sigma-Aldrich) was added for additional 72 hours for selection.

2.3.5 | Chemical treatments

The two kidney epithelial cell lines were maintained in serum-free medium for 24 hours and subsequently treated for another 24 hours with 10 mg/mL of fatty acid-poor and endotoxin-free bovine serum albumin (#126579; Calbiochem, Sunnyvalle, CA) or human normal immunoglobulins IgG (KIOVIG; Baxalta, Lessines, Belgium) in the absence of serum, to mimic proteinuric conditions. To inhibit protein degradation, 5 μM of the proteasomal inhibitor MG132 (M7449; Sigma-Aldrich) or the lysosomal inhibitor chloroquine was added for 4 hours before the completion of the 24-hour albumin treatment. The ER stress inhibitors Sodium phenylbutyrate (4-PBA, SMLO309; Sigma-Aldrich) and Sodium tauroursodeoxycholate (TUDCA, T0266; Sigma-Aldrich) were used at a concentration of 1 mM for the 24 hours of albumin treatment. As a positive control for ER stress induction, 2 μg/mL of Tunicamycin (#654380; Calbiochem) was used for 2 or 4 hours. For exogenous administration experiments, recombinant human Klotho (5334-KL; R&D Biosystems, Minneapolis, MN) at a final concentration of 1 nM was added in the medium for 24 hours.

2.4 | Protein half-life assay

The protein half-life was assessed as described before.[50] Briefly, the HEK-293 Klotho overexpressing cells were exposed to albumin for 24 hours, and subsequently incubated with 1 mM of cell-permeable biotinylation reagent EZ-Link NHS-SS-Biotin (#21441; Thermofisher) at room temperature for 30 minutes diluted in serum-free medium. After quenching with DPBS (D8537; Sigma-Aldrich), the cells were incubated in full medium at 37°C and the total cell extract was isolated at the indicated time points. The Pierce BCA Protein Assay Kit (#23225; Thermofisher) was used to determine the protein concentration and 50 μg of protein were incubated with equal volume of streptavidin beads (#20349; Thermofisher) overnight at 4°C in lysis buffer containing 0.3% w/v bovine serum albumin (A1391; Applichem, Darmstadt, Germany). The following day, the beads were collected and washed by centrifugation at 2000 g for 2 minutes and the bound protein was eluted by incubation in 2× Laemmli sample buffer at 95°C for 5 minutes and analyzed by western blot. The band intensity was quantified for each time point and was normalized in comparison to the respective time 0, for each condition and for multiple experiments, so we can depict the amount of the remaining labeled fraction over time.

2.5 | Membrane biotinylation

After 24 hours in the presence or absence of albumin, HEK-293 Klotho-overexpressing cells were incubated with 1.6 mM of non-cell-permeable EZ-Link Sulfo-NHS-SS-Biotin (#21331; Thermofisher) for 1 hour at 4°C to label cell surface proteins that were subsequently isolated using streptavidin beads, as described before.[51] The isolated labeled membrane fraction was subjected to western blot analysis where GAPDH was used as a negative control and E-cadherin was used as loading control.

2.6 | Luciferase assay

FNL-7 cells were transiently transfected with plasmids expressing human ATF4 or ATF3 or both and after 48 hours they were trypsinized and subsequently lysed in the Passive Lysis Buffer provided with the Dual-Luciferase Reporter Assay System (#E1910; Promega). The measurement was performed in the SpectraMax Paradigm Microplate Reader (Molecular Devices, San Jose, CA). The fold change in luminescence compared to the empty vector-transfected cells is shown in the graph.

2.7 | Measurements of ADAM enzymatic activity

The kinetic measurement of ADAM10 and ADAM17 (TACE) activities in the mouse kidney cortex were performed with the SensoLyte 520 ADAM10 Activity Assay Kit (AS-72226) and the SensoLyte 520 TACE Activity Assay Kit (AS-72085), respectively (AnaSpec, Fremont, CA). Fold changes of enzyme activity (Relative Fluorescence Units/min) compared to the control samples are shown in the graph.

2.8 | Enzyme-linked immunosorbent assay (ELISA)

Fibroblast Growth Factor 23 (FGF-23) levels were measured in the plasma using the Mouse/Rat FGF-23 (Intact) ELISA Kit (#60-6800) or the Mouse/Rat FGF-23 (C-Term) ELISA Kit (#60-6300), both from Immutopics (San Clemente, CA). The soluble α-Klotho levels were determined using the

Alpha Klotho Human Soluble ELISA Assay Kit (#27998; IBL, Minneapolis, MN).

2.9 | Total RNA extraction and real-time PCR analysis

qRT-PCR was performed with 10 ng cDNA. The set of primers used for every gene of interest are summarized in Table 2. The data were analyzed according to the 2^-ΔΔCT method to obtain the fold change in expression relative to the control samples. The Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) was used as reference gene.

2.10 | Western blot analysis

Antibodies used for western blotting are shown in Table 3. The chemiluminescence was detected by using the SYNGENE imaging system and the Genesys software (SYNGENE, Frederick, MD). The band density was quantified by ImageJ software (NIH, Bethesda, MD) and normalized to the reference gene (β-actin or GAPDH) or the loading control (Coomassie or Ponceau staining). Fold changes compared to the control samples are shown in the graph.

2.11 | Statistical analysis

The results are shown as Mean ± SD and GraphPad Prism (version 7.0; GraphPad Software, San Diego, CA) was used for the statistical analysis. For comparison between two experimental groups, unpaired two-tailed *t* test or multiple *t* test without correction were used to detect differences, while one-way ANOVA (Tukey's multiple comparisons test) or two-way ANOVA (Sidak's multiple comparisons test) were used for three or four experimental groups. Additionally, for the enzymatic activity measurements, linear regression analysis with baseline correction was used to compare the slopes. Finally, for the calculation of protein half-life, we used the non-linear regression model of one-phase decay and the extra sum-of-squares *F* test. In all cases, the confidence intervals were set at 95% ($\alpha = .05$).

3 | RESULTS

3.1 | Klotho is downregulated during the early phase of proteinuric CKD

POD-ATTAC mice injected with a single dose of dimerizer display normal renal function and present high-grade proteinuria from Day 2.[44] The experiments were performed at 7 days,

TABLE 2 List of primers used for Real-time PCR analysis

Gene of interest	Forward primer	Reverse primer
Mouse		
α-Klotho	CCTACGTGTTGGACGACATC	CTCAAACTGATTCGCAGCAT
Secreted α-Klotho	CCAACCCAAATCCCAAAATCC	CCTAACTATAGCCTGTTCCCA
Adam10	TGATGGTGTTCTTGGTCTGC	CTTGCTTTTCTCACATATTCCCC
Adam17	GGGTTTTGCGACATGAATGG	GAAAACCAGAACAGACCCAAC
Rplp0	AATCTCCAGAGGCACCATTG	GTTCAGCATGTTCAGCAGTG
Grp78	AGATGCTTGGCACTATTGCTG	CCAGGTCAAACACAAGGATG
Xbp1s	TGCTGAGTCCGCAGCAGGTG	ACTAGCAGACTCTGGGGAAG
Atf4	GGTTCTCCAGCGACAAGG	GCATCGAAGTCAAACTCTTTCAG
Chop	AACAGAGGTCACACGCACAT	ACCACTCTGTTTCCGTTTCC
Atf3	GAGGATTTTGCTAACCTGACACC	TTGACGGTAACTGACTCCAGC
Human		
α-KLOTHO	CTCAAAGCCCACATACTGGA	CCTTGGGCTCAAACTGATCT
ADAM10	CAAAGTCTGAGAAGTGTCGGG	CTGCACATTGCCCATTAATG
ADAM17	CATTATCAAACCCTTTCCTGCG	CTCGTCCATATGTGAGTCTGTG
RPLP0	CATGTGAAGTCACTGTGC	GCTGCACATCACTCAGGA
GRP78	GTAGCGTATGGTGCTGCTGT	TCATGACACCTCCCACAGTT
XBP1s	TGCTGAGTCCGCAGCAGGTG	GCTGGCAGGCTCTGGGGAAG
ATF4	CCAAGCACTTCAAACCTCATG	ATCCATTTTCTCCAACATCCAATC
CHOP	GACTGAGGAGGAGCCAGAAC	ACCACTCTGTTTCCGTTTCC
ATF3	GCCGAAACAAGAAGAAGGAG	GCTCCTCAATCTGAGCCTTC

TABLE 3 List of antibodies used for western blot analysis

Name	Host and isotype	Manufacturer	Cat. No.	Dilution
Primary				
Klotho	Rat IgG2a	TransGenic	KO603	1:1000
GAPDH	Mouse IgG1	Merck Millipore	MAB374	1:100 000
β-actin	Mouse IgG1	Sigma-Aldrich	A5441	1:10 000
E-cadherin	Mouse IgG2a	BD Transduction	610181	1:2000
ADAM10	Rabbit IgG	Merck Millipore	AB19026	1:500
ADAM17	Rabbit IgG	Merck Millipore	AB19027	1:500
XBP-1s	Rabbit IgG	Cell signaling	#12782	1:500
ATF4	Rabbit IgG	Cell signaling	#11815	1:500
CHOP	Mouse IgG2a	Cell signaling	#2895	1:500
GADD153	Mouse IgG1	Santa Cruz	sc-7351	1:500
ATF3	Rabbit IgG	Abcam	ab207434	1:500
Secondary				
Anti-rat	Goat IgG	Invitrogen	629520	1:2000
Anti-rabbit	Goat IgG	BD Pharmingen	554021	1:2000
Anti-mouse	Goat IgG	BD Pharmingen	554002	1:5000

during a stable proteinuric phase. Both Klotho mRNA and protein expression were decreased, while renal function was preserved and no fibrosis was observed, despite albuminuria (Figure 1A-G). Plasma intact and C-terminal FGF-23 levels were higher in proteinuric animals, whereas no significant modification in fractional excretion of phosphate was observed (Figure 1H-J), indicating some degree of biological resistance to FGF-23. The decrease in Klotho expression was observed already from day 3 (Supplementary Figure 1A-C), suggesting that Klotho downregulation is an early event in response to proteinuria.

3.2 | Klotho half-life decreases in the presence of albumin

To investigate the molecular mechanisms of Klotho downregulation in the presence of albumin, the most abundant plasma protein in the urine of CKD patients, we used a HEK-293 cell line overexpressing the transmembrane form of the human Klotho. When exposed to 10 mg/mL of bovine serum albumin (BSA) for 24 hours, HEK overexpressing cells displayed lower levels of total and membrane Klotho, as well as lower levels of soluble Klotho in the supernatant (Figure 2A-C). We further observed a decreased protein half-life of Klotho (0.58 hours vs 0.32 hours, $P = .0104$), (Figure 2D). This decrease in Klotho protein half-life is likely due to increased proteasomal and lysosomal degradation given the partial reversibility by MG132 and chloroquine, respectively (Figure 2E,F). To our knowledge, there is no distal tubular cell line expressing Klotho at the protein level, so we used HK-2 cells that are of proximal tubular origin[52] and endogenously express Klotho, albeit at lower levels. We confirmed that both mRNA and protein Klotho levels were decreased by albumin exposure (Figure 2G,H).

3.3 | Klotho downregulation is specific to albuminuria in glomerular disease

To determine the specificity of the effects of albumin on the regulation of Klotho, we used another model of glomerular disease, the *Col4α3*$^{-/-}$ Alport mice, in the presence or absence of circulating serum albumin.[46] In this mouse model of Alport syndrome, Klotho mRNA and protein expression were decreased in isolated kidney tubules and whole kidney cortex, respectively (Figure 3A,B). In contrast, in the albumin-deficient Alport mice, Klotho expression was not downregulated, implying specific effects of albumin that are not caused by the primary renal disease itself. The albumin-specific effects were validated in vitro where the incubation of HEK-293 Klotho overexpressing cells or HK-2 cells with 10 mg/mL of human immunoglobulins IgG (KIOVIG) for 24 hours had no effect on Klotho expression (Figure 3C-E).

3.4 | Klotho cleavage by ADAM proteases is not altered by proteinuria

Next, we examined how the levels of the alternatively spliced isoform were affected. We observed a comparable downregulation in the expression of this secreted isoform related to the main transcript in the 7 days POD-ATTAC model (Figure 1E). Regarding proteolytic cleavage of Klotho at the membrane, ADAM17 expression did not change,

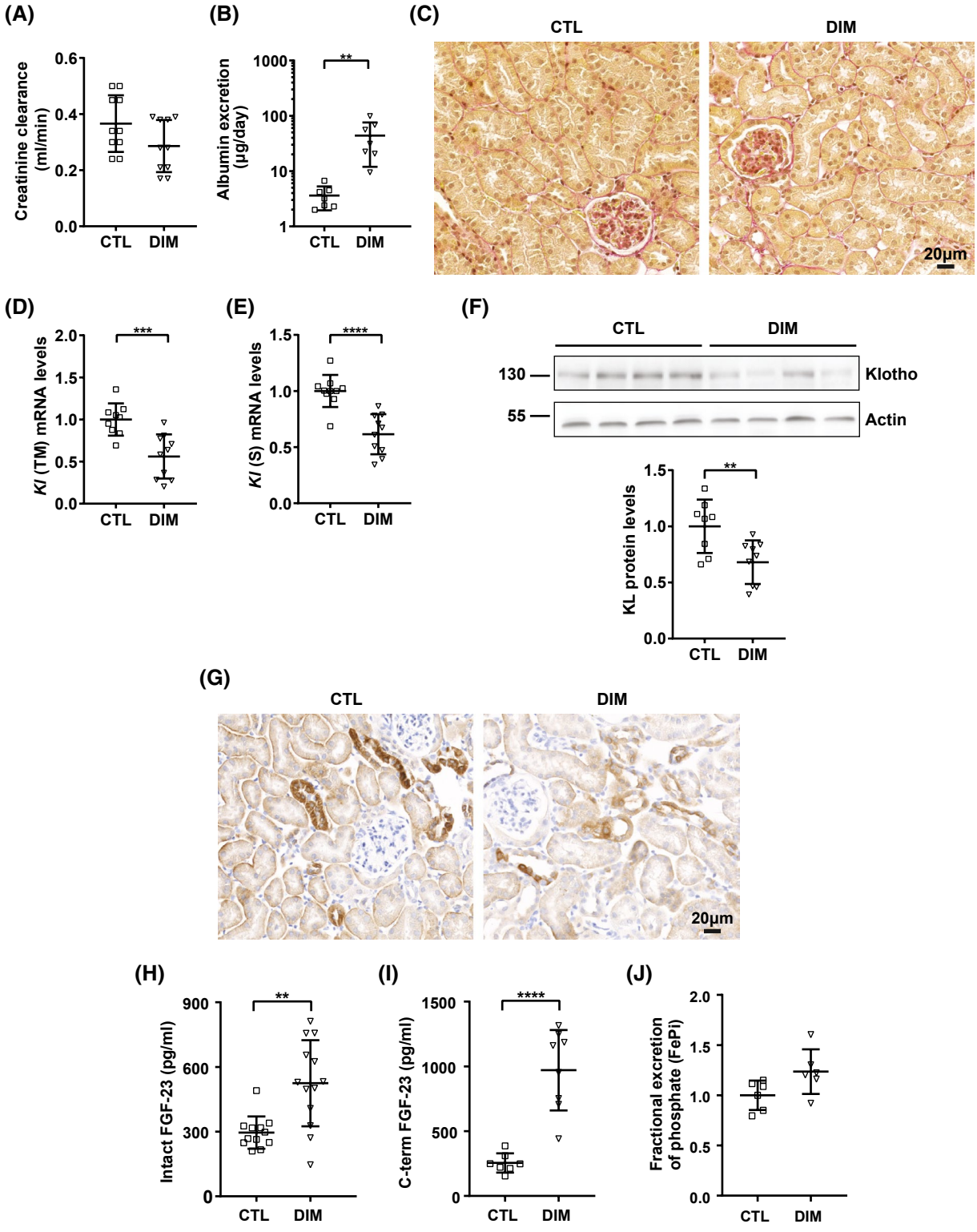

FIGURE 1 Klotho is downregulated in proteinuric CKD. Creatinine clearance ($N = 10$), (A), albumin excretion ($N = 7$), (B) and representative Sirius red staining (C) of kidney sections of control (CTL) and proteinuric (DIM) mice 7 days upon injection with vehicle or dimerizer (0.5 µg/g). Relative mRNA levels of transmembrane—TM ($N = 9$), (D) and secreted—S Klotho ($N = 10$), (E) in the kidney cortex 7 days upon injection. Western Blot analysis of renal cortex expression of Klotho and quantification ($N = 8$), (F). Representative immunohistochemical staining of Klotho in kidney sections of control and proteinuric mice (G). Levels of intact Fibroblast Growth Factor 23 (FGF-23) in the plasma of control ($N = 12$) and proteinuric ($N = 13$) mice as measured by ELISA (H). Levels of C-terminal Fibroblast Growth Factor 23 (FGF-23) in the plasma of control ($N = 7$) and proteinuric ($N = 8$) mice as measured by ELISA (I). Normalized fractional excretion of phosphate in control and proteinuric mice ($N = 6$), (J). The mRNA levels are normalized to RPLP0 gene expression and the protein levels to β-actin. Mean ± SD, unpaired two-tailed *t* test $^*P < .05$

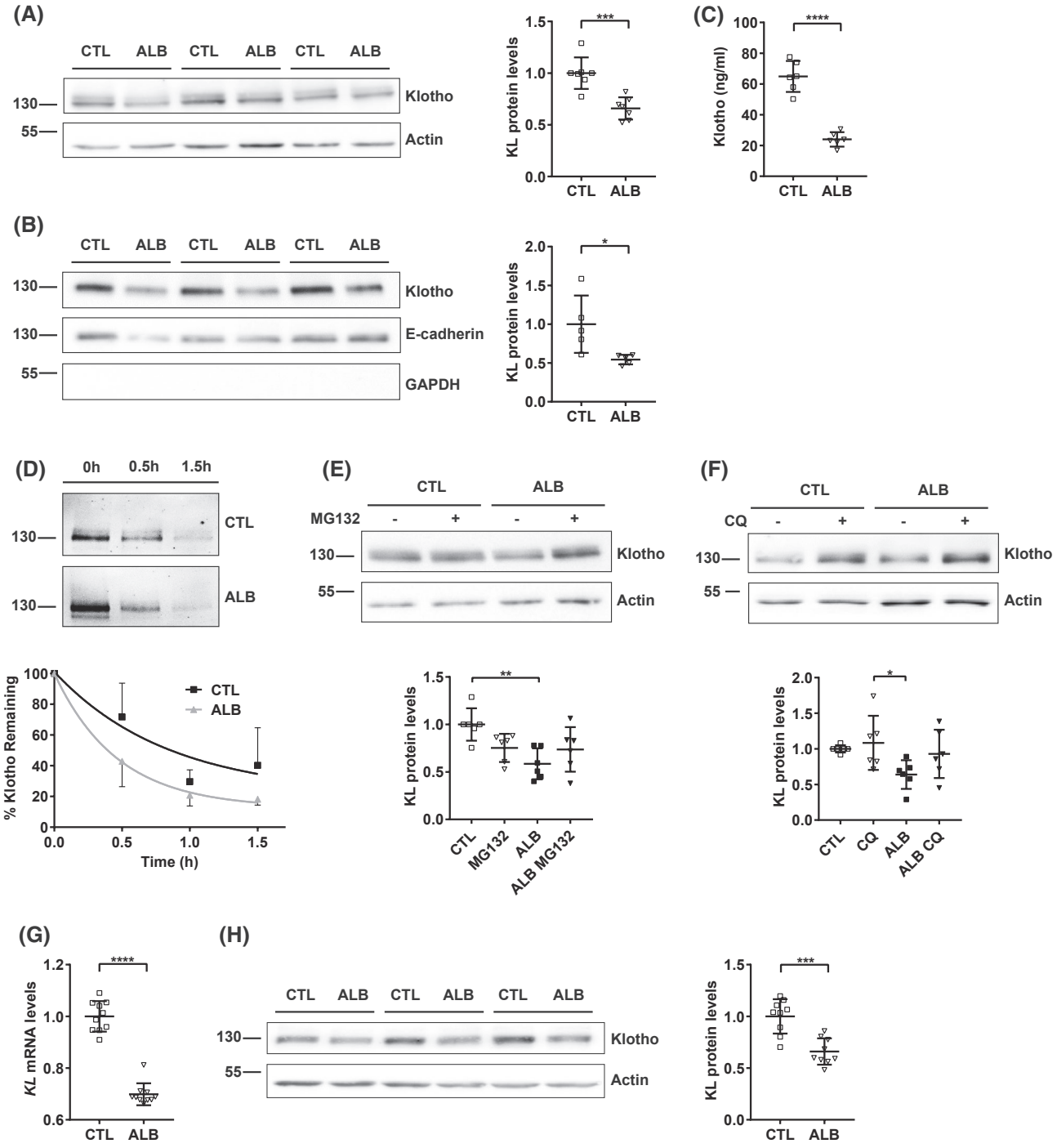

FIGURE 2 Albumin treatment reduces Klotho mRNA and protein expression and increases Klotho protein degradation in vitro. HEK-293 Klotho overexpressing cells were exposed to 10 mg/mL bovine serum albumin (BSA) for 24 hours and western blot analysis was performed with total cell lysates ($N = 7$), (A) or cell surface-biotinylated lysates pulled down with streptavidin beads ($N = 5$), (B). The Klotho protein levels were also measured in the cell supernatants by ELISA ($N = 6$), (C). In order to investigate for changes in protein half-life, the HEK Klotho overexpressing cells were exposed to BSA and then biotinylated for 30 minutes and after the biotinylation was quenched, the cell lysates were pulled down with streptavidin beads at different time points and subjected to western blot analysis. The graph represents the percentage of Klotho remaining over time ($N = 6$). The half-life values as computed by the non-linear regression model of one-phase decay were 0.58 hours for the control and 0.32 hours for the albumin-treated cells, and they were significantly different ($P = .0104$), (D). The cells were incubated with 5 μM of the proteasomal inhibitor MG132 ($N = 6$), (E) or the lysosomal inhibitor chloroquine ($N = 6$), (F) for 4 hours and the Klotho protein levels were measured by western blot analysis, in the presence or absence of albumin. Human kidney proximal HK-2 cells were also exposed to BSA for 24 hours and the Klotho mRNA and protein levels were assessed by qPCR ($N = 10$), (G), and western blot ($N = 9$), (H) respectively. The mRNA levels are normalized to RPLP0 gene expression and the protein levels to β-actin or E-cadherin. Mean ± SD, unpaired two-tailed *t* test or one-way ANOVA or extra sums of squares *F* test $^*P < .05$

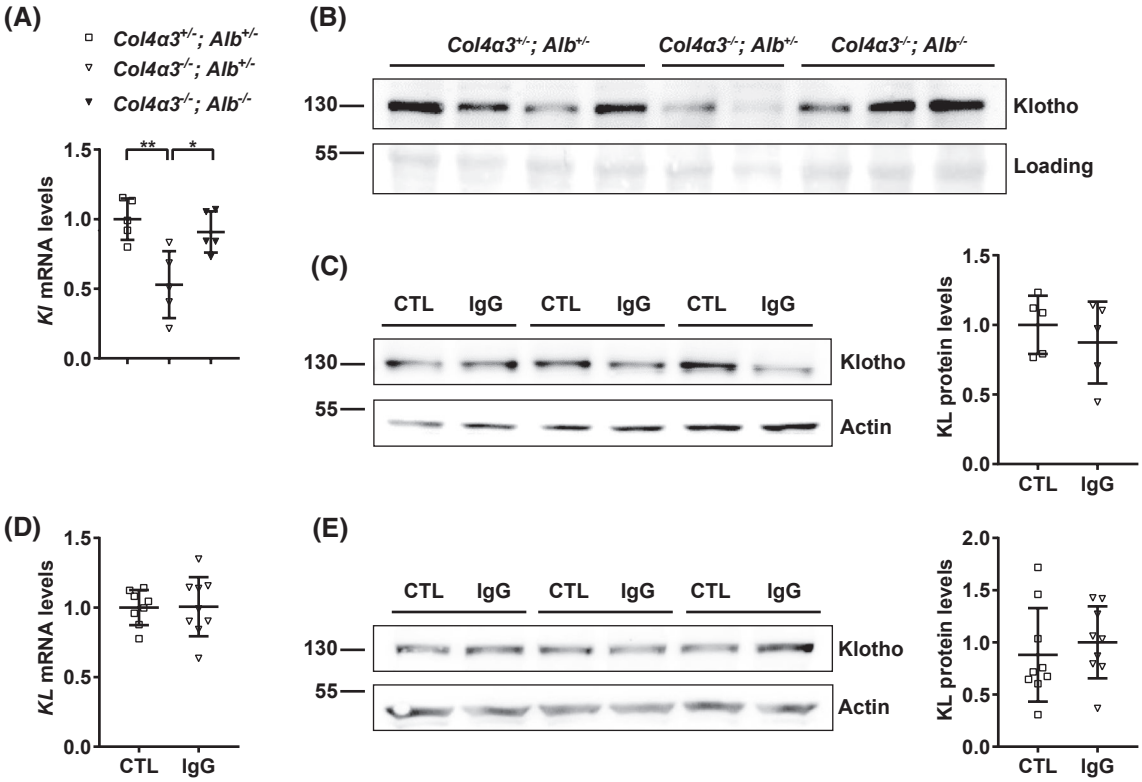

FIGURE 3 Klotho downregulation is specific to albuminuria. Relative mRNA levels of Klotho ($N = 5$) in isolated tubules of normal ($Col4\alpha3^{+/-}; Alb^{+/-}$) and Alport mice, wild type ($Col4\alpha3^{-/-}; Alb^{+/-}$) or albumin deficient ($Col4\alpha3^{-/-}; Alb^{-/-}$), (A). Western Blot analysis of renal cortex expression of Klotho in the three experimental groups (B). Klotho protein levels in the HEK-293 overexpressing cells treated with 10 mg/mL of human immunoglobulins (IgG) for 24 hours ($N = 5$), (C). Klotho mRNA and protein levels in HK-2 cells treated with IgG as measured by qPCR ($N = 8$), (D) and western blot ($N = 9$), (E) respectively. The mRNA levels are normalized to RPLP0 gene expression and the protein levels to β-actin. Mean ± SD, one Way ANOVA or unpaired two-tailed *t* test $^*P < .05$

while ADAM10 protein levels, but not mRNA, increased (Figure 4A-D). However, ADAMs proteolytic activity was unchanged in the kidney of POD-ATTAC mice (Figure 4I). ADAMs mRNA and protein levels were unaltered in cultured renal cells exposed to albumin (Figure 4E-H). Moreover, the reduction of Klotho protein levels by albumin exposure was still observed when the cleavage-resistant Klotho mutant KLΔ9 was overexpressed in HEK-293 and HK-2 cells compared to the wild-type form (Supplementary Figure 2A,B). In addition, Klotho levels were decreased to the same extent intracellularly and in the supernatant (Figure 2C), arguing against an alteration of cleavage in vitro.

3.5 | Albuminuria induces features of ER stress

Proteinuria has been associated with ER stress in kidney disease.[30] Exposure of cultured renal cells to albumin in vitro led to upregulation of typical ER stress genes (Figure 5A-D). In animals, the complete ER stress pattern was not observed, but an early and persistent activation of ATF4 and ATF3 transcription factors was detected by qPCR and increased nuclear staining in the kidney sections of 3 days and 7 days after induction of proteinuria in POD-ATTAC mice (Figure 5E,F and Supplementary Figure 1D,E). The induction of ATF3 was detected in both proximal and distal tubules agreeing with a global downregulation of Klotho (Supplementary Figure 3). The upregulation of ATF4 and ATF3 in parallel with reduction in Klotho levels was also observed in another proteinuric model, the Puromycin Aminonucleoside-induced Nephrotic syndrome (PAN) rats (Supplementary Figure 4A). The absence of ER stress in the albumin-deficient collagen 4α3 knockout mice and in cultured cells incubated in the presence of purified immunoglobulins (Supplementary Figure 4B-D) further demonstrates the major role of albumin in this process.

3.6 | ATF3 and ATF4 decrease Klotho expression and ATF3 mediates part of the transcriptional effect of albumin on Klotho

In addition to protein regulation, Klotho mRNA was also decreased in proteinuric animals. A recent study demonstrated that this downregulation is independent of typical inflammatory cytokines and rather dependent on DNA methylation.[23]

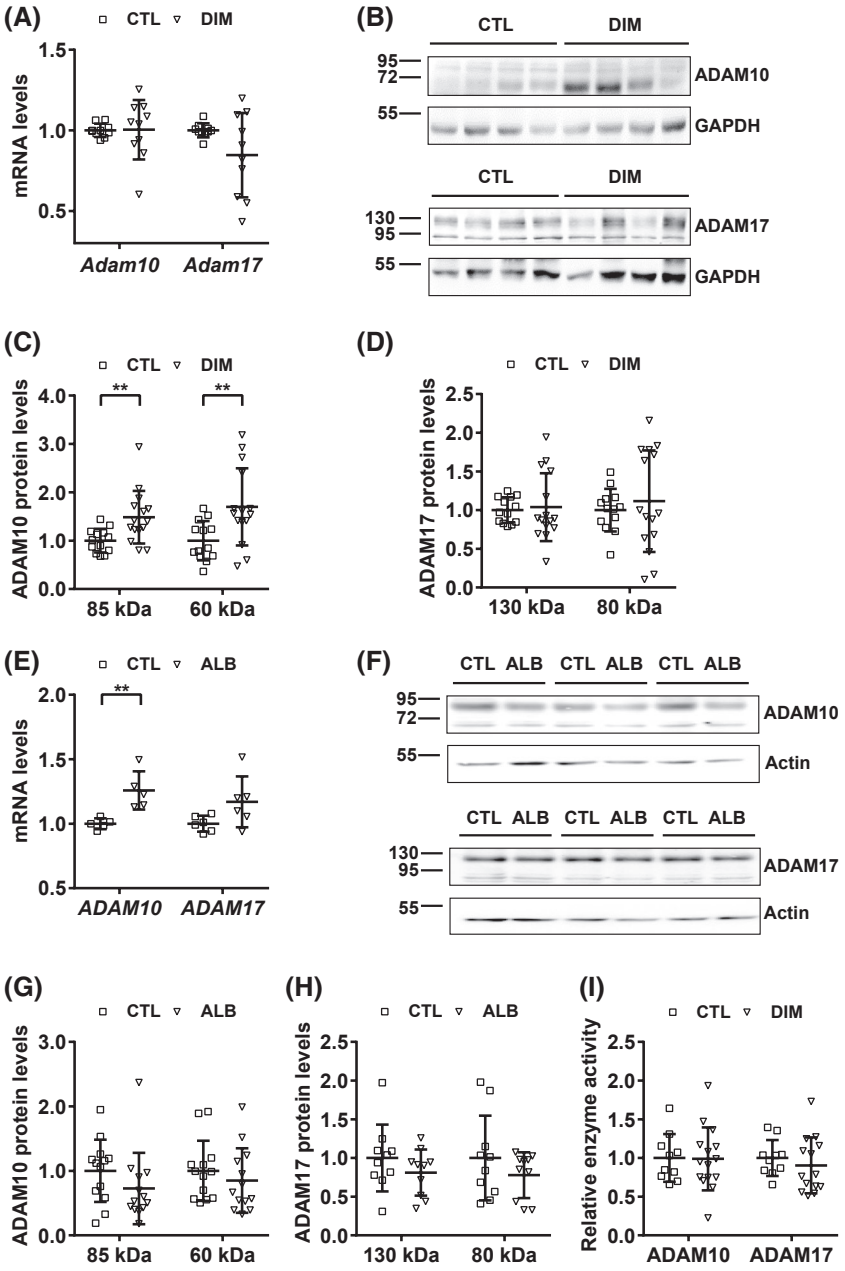

FIGURE 4 Klotho cleavage is most likely not modified by albuminuria. Relative mRNA levels and protein levels of ADAM10 and ADAM17 proteases as measured by qPCR ($N = 10$), (A) and western blot ($N = 13/15$), (B-D), respectively, in the renal cortex of control and proteinuric mice. The expression of ADAM10 and ADAM17 proteases was measured also in vitro, in the HEK-293 Klotho overexpressing cells in the presence or absence of BSA, by qPCR ($N = 6$), (E) and western blot ($N = 13/10$), (F-H). The enzymatic activities of the renal cortical lysates of control ($N = 10$) and proteinuric ($N = 14$) mice were monitored by measuring the cleavage kinetics of a fluorescence FRET substrate (I). The mRNA levels are normalized to RPLP0 gene expression and the protein levels to GAPDH or β-actin. Mean ± SD, multiple *t* test without correction $^*P < .05$

Recent evidence has pointed to the fact that ATF3 binds to the Klotho promoter.[40] Similar to this previous study, we used the Genomatix-MatInspector software[53] and additionally detected putative ATF4 binding motifs in the mouse and human Klotho promoter. Therefore, we tested whether the ATF3 and/or ATF4 upregulation we observed could play a role in Klotho downregulation by albuminuria. Overexpression of ATF4 or ATF3 in the HK-2 cell model led to decreased Klotho mRNA and protein expression, confirming the involvement of these factors in the observed regulation (Figure 6A,B). Using a Klotho reporter cell line,[48] we confirmed that both ATF4 and ATF3 overexpression results in Klotho downregulation via decreased promoter activity changes, and furthermore in a non-additive manner (Figure 6C). As a reverse strategy, we generated HK-2 cells with stable ATF4 and ATF3 knockdown and repeated the albumin exposure experiment (Figure 6D-F). Silencing of both factors increased Klotho transcription at baseline. However, the effect of albumin on Klotho mRNA levels was partially reversed only by ATF3 silencing. At the protein level, silencing of both factors increased Klotho, but did not fully prevent the downregulation by albumin, likely owing to post-transcriptional effects of albumin.

3.7 | ER stress decreases Klotho expression and its reversal by 4-PBA rescues Klotho expression in vivo and in vitro

Next, we investigated whether inhibiting ER stress pharmacologically could reverse Klotho downregulation. In

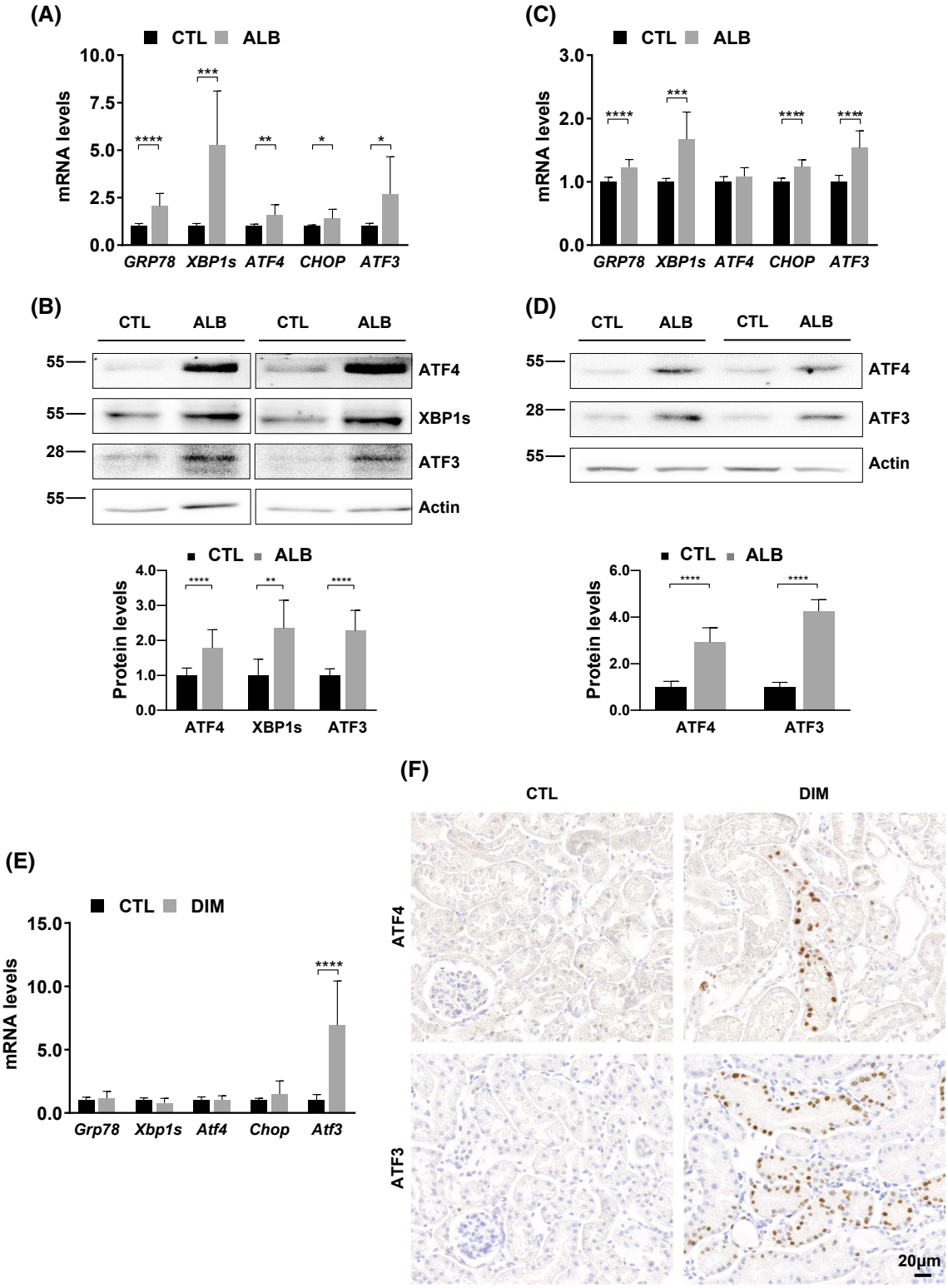

FIGURE 5 Albumin induces ER stress in vitro and in vivo. Relative mRNA and protein levels of ER stress response genes as measured by qPCR ($N = 10$), (A) and western blot ($N = 8$), (B), respectively, in HEK-293 overexpressing cells exposed to 10 mg/mL BSA for 24 hours. Relative mRNA and protein levels of ER stress response genes as measured by qPCR ($N = 10$), (C) and western blot ($N = 8$), (D), respectively, in the HK-2 cells exposed to 10 mg/mL BSA for 24 hours. Relative mRNA levels of ER stress response genes in the cortex of control ($N = 13$) and proteinuric mice ($N = 16$) 7 days post-injection (E). Representative immunohistochemical staining of ATF4 and ATF3 transcription factors in kidney sections (F). The mRNA levels are normalized to RPLP0 gene expression and the protein levels to β-actin. Mean ± SD, multiple *t* test without correction
$^*P < .05$

cultured renal cells, exposure to Tunicamycin, a known ER stress inducer, led to downregulation of Klotho protein and mRNA levels (Supplementary Figure 5A,B). Furthermore, in vivo treatment with Tunicamycin resulted in decrease of Klotho protein in whole kidney lysates (Supplementary Figure 5C). Treatment with Tauroursodeoxycholic acid

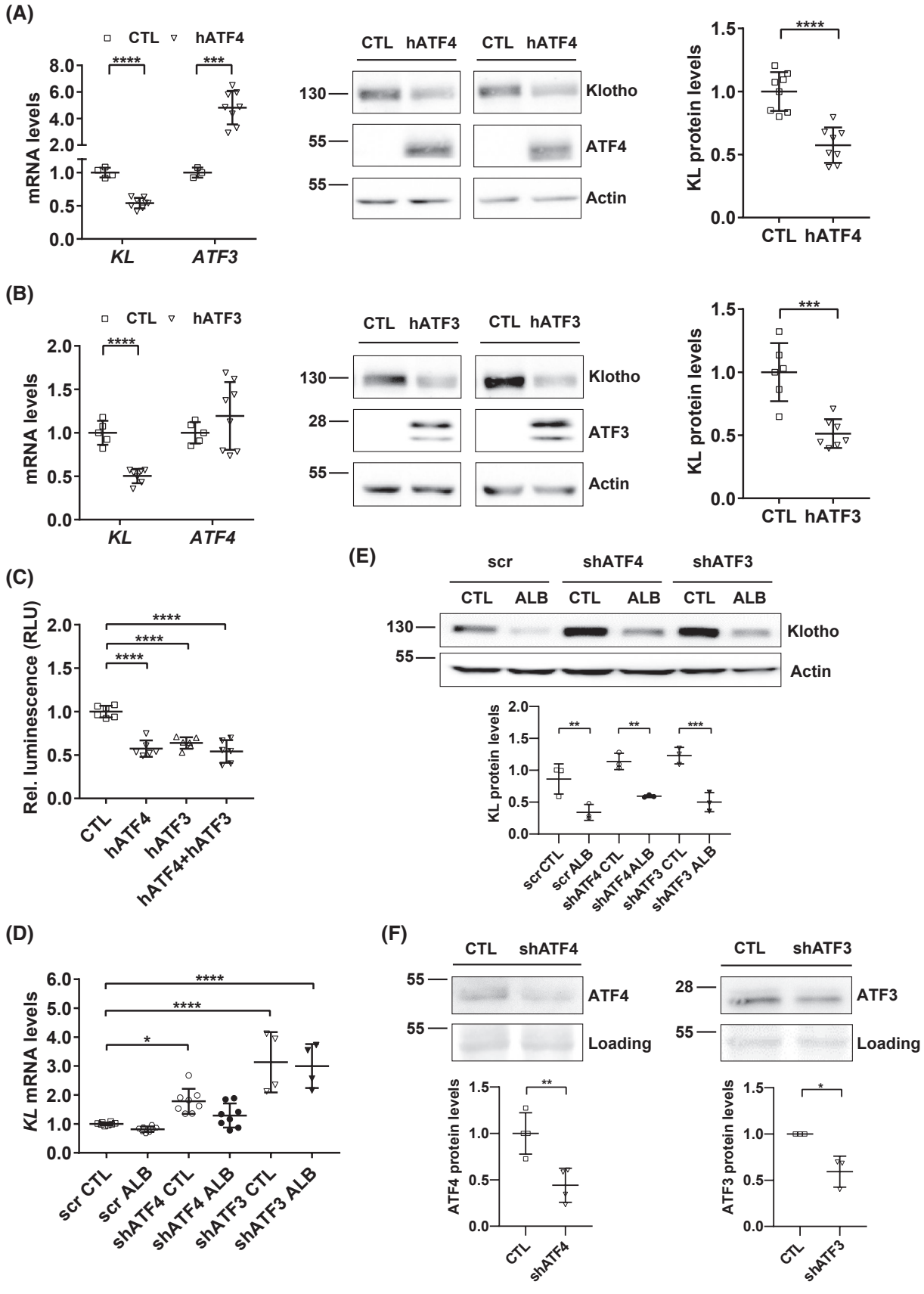

FIGURE 6 ATF4 and ATF3 regulate Klotho expression. Relative mRNA and protein levels of Klotho, ATF4 and ATF3 as measured by qPCR and western blot, respectively, in HK-2 cells transiently overexpressing the human ATF4 transcription factor (two experiments), (A) or the human ATF3 transcription factor (three experiments), (B). Relative luminescence in the FNL-7 cell line expressing the human Klotho promoter driving firefly luciferase expression in control and cells overexpressing human ATF4, ATF3, or both factors ($N = 6$), (C). The HK-2 cells were also transduced with shRNA lentiviral vectors targeting ATF4 or ATF3 and the relative mRNA levels of Klotho as well as the protein levels were assessed by qPCR ($N = 8/8/4$), (D) or western blot analysis ($N = 3$), (E), respectively, in the presence or absence of albumin in comparison to the scrambled shRNA vector. The silencing efficiency was verified by western blot analysis ($N = 4/3$), (F). The mRNA levels are normalized to RPLP0 gene expression and the protein levels to β-actin or Ponceau staining. Mean ± SD, unpaired two-tailed *t* test or multiple *t* test without correction or one-way ANOVA $*P < .05$

(TUDCA) or 4-Phenylbutyric acid (4-PBA) rescued Klotho protein downregulation in the presence of albumin in the HEK-293 cells overexpressing Klotho (Figure 7D). In vivo, 4-PBA effectively reduced the expression of markers of ER stress in the 7-days POD-ATTAC model of proteinuric disease. Additionally, it increased Klotho protein levels, however, without restoring the reduction in mRNA levels (Figure 7A-C). Addition of recombinant human Klotho at the medium did not protect from the albumin-induced upregulation of ER stress response genes and did not affect endogenous Klotho mRNA or protein levels (Supplementary Figure 2C-E).

4 | DISCUSSION

In this study, we demonstrate that Klotho downregulation occurs largely before renal function loss and fibrosis in proteinuric CKD. We show that albuminuria alone is sufficient to specifically downregulate Klotho at the mRNA and protein level in vivo and in vitro. This regulation is related in part to the activation of the ER stress pathway. ATF3 downregulates Klotho mRNA expression and may act as a repressor under conditions of albuminuria. Furthermore, ER stress increases proteasomal and lysosomal Klotho degradation, and these effects can be reversed by ER stress inhibition in vitro and in vivo.

We confirm that Klotho is downregulated under conditions of isolated proteinuria with normal renal function, as it has been observed in different models of renal disease.[22,23] We show that this downregulation was independent of fibrosis and resulted in elevated FGF-23 levels. This confirms that CKD is a Klotho deficiency state, even at early stages of the disease, and indicates causality between proteinuria and decreased Klotho expression. In adults and children, serum Klotho levels have been associated with eGFR,[54,55] but have also been shown to be dependent on the level of proteinuria.[24,25]

We then provide evidence that Klotho loss is specific to albumin, since it was not observed in analbuminemic collagen 4α3 knockout mice, a model of Alport syndrome characterized by proteinuria, increased FGF-23 plasma levels and decreased Klotho expression.[56,57] Whether the rescue of Klotho loss is related to fewer kidney lesions rather than the absence of albumin can be debated since the samples originated from a previous study where the analbuminemic Alport mice had less renal tubular injury, although renal function was still altered at late stages.[46] Together with our in vitro data, we suggest a specific role of albumin on Klotho regulation, which may directly participate to milder lesions given its effect on fibrosis progression. Early micropuncture studies have demonstrated that protein reabsorption along the nephron is extremely effective and the filtrate is practically devoid of protein.[58,59] Filtered albumin is mostly internalized in the proximal tubules[59,60] but the participation of the distal nephron has also been demonstrated,[61] and seems to be important especially in proteinuric conditions where the proximal clearance mechanism might get saturated. In accordance with this hypothesis, in our conditions, Klotho was downregulated in both segments with parallel increase in ATF3 nuclear staining in proximal and distal tubules.

We further show that the decreased renal Klotho protein expression is most likely unrelated to increased cleavage, since we did not observe any activation of ADAM proteases in the kidney of proteinuric mice, modifications of Klotho cleavage in cultured cells exposed to albumin or differential regulation at the protein level when a cleavage resistant isoform was overexpressed. Although we cannot fully exclude that in vivo Klotho cleavage is enhanced independently of ADAM enzymes activity, the observed downregulation appears to be related mainly to a decrease in Klotho transcription and an increase in its proteolytic degradation. The decrease in Klotho transcription in kidney disease has been attributed to regulation by NF-κB or Angiotensin II or more recently to modifications of Klotho promoter acetylation[23] or methylation.[62-64] We observed an induction of ATF3 by proteinuria in different models, both in proximal and distal tubular segments. Recently, it was demonstrated by chromatin immunoprecipitation that ATF3 binds to the Klotho promoter as a heterodimer with c-Jun.[40] In our study, ATF3 and ATF4 overexpression inhibited Klotho transcription and decreased protein expression both in a cell line expressing Klotho endogenously and in a human Klotho promoter reporter assay. Silencing of ATF3 and ATF4 increased Klotho expression at baseline, but only ATF3 appears to be involved in the albumin-induced Klotho mRNA

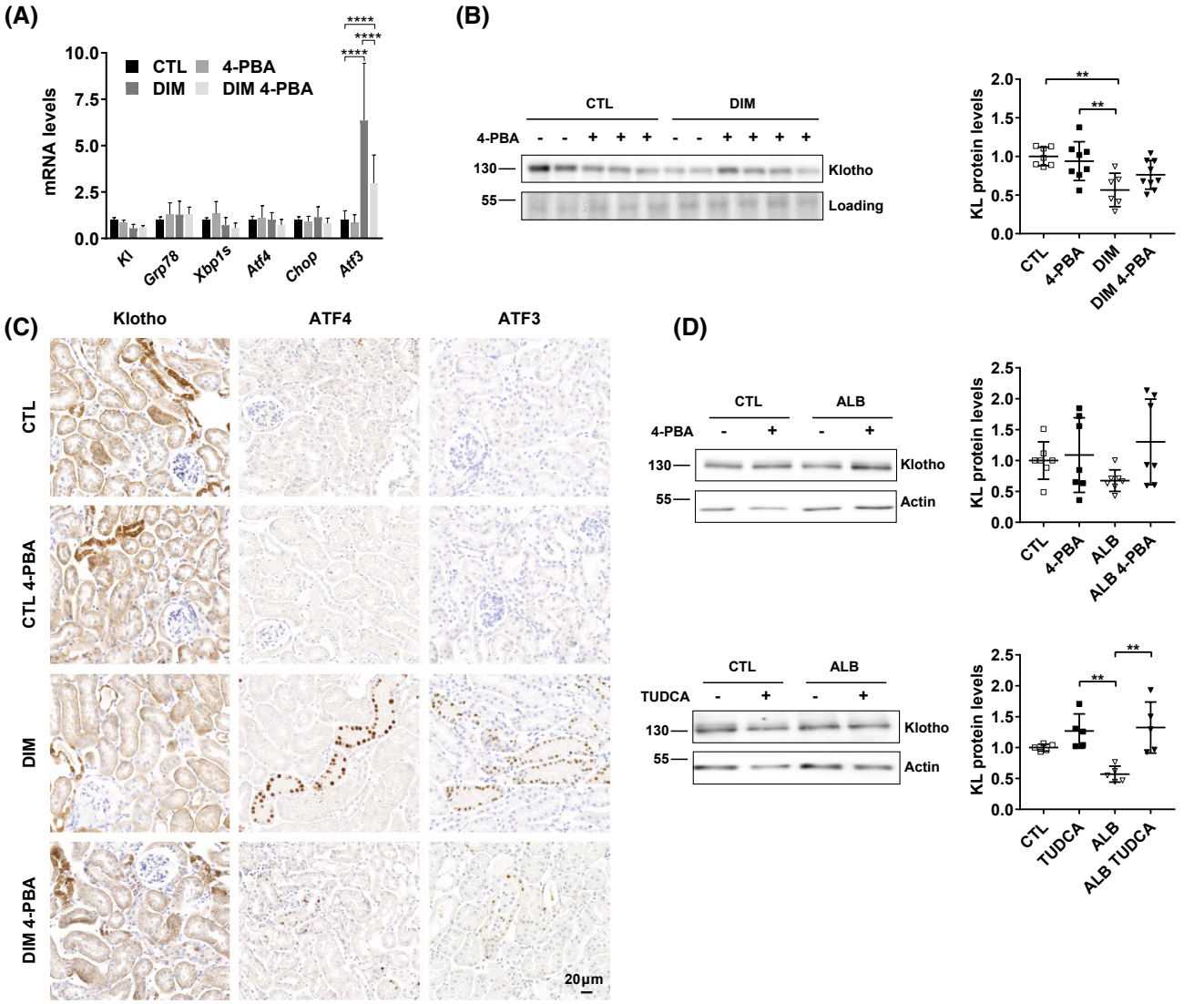

FIGURE 7 ER stress inhibition reverses Klotho downregulation. Relative mRNA levels ($N = 13$), (A) and western blot analysis ($N = 7/8/6/9$), (B) of Klotho and ER stress response genes in the cortex of control and proteinuric mice treated with vehicle or 4-PBA (1 mg/g/day in drinking water for 7 days). Representative immunohistochemical staining of Klotho, ATF4 and ATF3 in kidney sections of control and proteinuric mice, treated with 4-PBA or vehicle (C). In order to inhibit ER stress in vitro, the HEK-293 overexpressing cells were treated with 1 mM 4-PBA ($N = 7$) or 1 mM TUDCA ($N = 5$), (D) for 24 hours in the presence or absence of albumin. The Klotho protein levels were measured by western blot analysis. The mRNA levels are normalized to RPLP0 gene expression and the protein levels to Coomassie staining or β-actin. Mean ± SD, one-way or two-way ANOVA $*P < .05$

suppression. At the protein levels, silencing of ATF4 and ATF3 did not fully reverse the effect of albumin. Therefore, it seems that the post-transcriptional regulation of Klotho by albumin is a crucial determinant of the protein levels. Due to the different observations at the mRNA level, it is possible that ATF3 alone is sufficient to exert its repressive effect, whereas modulating ATF4 expression may have off-target effects since ATF4 is upstream in the signaling pathway. ATF3 has been described to be induced in ischemia reperfusion, a model where Klotho is noticeably downregulated.[65] In this model, ATF3 plays a protective role, due to the diminishing of NF-κB binding capacity.[42] In contrast, the induction of ATF3 has been mentioned in human glomerulopathies and in the LPS or db/db mouse model where it aggravates podocyte injury.[66] The effects of ATF3 induction may thus be disease and cell dependent, so the specific role of ATF3 in chronic proteinuric kidney disease merits further investigation.

It has been known since long time that Klotho administration in vivo or overexpression in vitro can ameliorate ER stress-induced apoptosis,[67,68] even though in our experimental settings, supplementation in vitro did not protect from the albumin effects. We are showing for the first time an inverse relationship where chemically induced ER stress by Tunicamycin suppresses Klotho expression in vitro and in vivo. ER stress induces modifications of protein processing

together with induction of both lysosomal and proteasomal degradation.[30] In our proteinuric model, albumin mediates the reduction of Klotho protein half-life and the increase in ER stress likely contributes to this enhanced protein degradation. This observation is supported by the fact that the ER stress inhibitor 4-PBA could increase Klotho protein levels in vivo and in vitro, independently of gene transcription. Despite decreasing ATF3 expression, 4-PBA did not reverse Klotho transcription inhibition, likely because of the residual ATF3 expression, or modulation by other factors that inhibit Klotho transcription. Albumin has been shown to be an ER stress inducer but the underlying mechanisms have not been extensively studied. Others have shown that albumin may provoke inflammasome activation and calcium mobilization in tubular cells[31,34,35] while other mechanisms such as reduced expression of the adaptor protein CD2AP, ROS production and activation of the MAPK/caspase-12 pathway have been reported to be involved in podocyte apoptosis.[69-71] Of interest, 4-PBA has been described to decrease fibrosis in chronic proteinuric disease.[35] This may be related to a direct effect on ER stress but may also potentially rely on an early rescue of Klotho expression, in turn modulating CKD progression. 4-PBA may thus offer an interesting therapeutic option for proteinuric CKD to ameliorate the renal and extrarenal complications through enhancing Klotho expression.

Altogether, we demonstrate that proteinuria alters both Klotho transcription and protein half-life, resulting in decreased renal Klotho expression. This may contribute to explaining the higher levels of FGF-23 in proteinuric patients and propose a link between albuminuria and increased CV complications. We further show that ATF3 and ATF4 both decrease Klotho transcription, which may also participate in Klotho downregulation during kidney injury. Finally, we demonstrate that pharmacological inhibition of ER stress significantly rescues Klotho expression at the post-transcriptional level in proteinuric kidney disease. Restoration of Klotho expression may be an interesting therapeutic perspective and supplementation with 4-PBA could thus help maintain Klotho expression in proteinuria, in order to improve both cardiac and renal disease evolution in CKD.[19,72]

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

S. de Seigneux conceived the original idea of the project; S. de Seigneux and JH Miner designed the experiments; V. Delitsikou, G. Jarad, F. Ino, C. Chen, and CX Santos performed the experiments; V. Delitsikou, S. de Seigneux, and RD Rajaram analyzed data; PE Scherer, JM Rutkowski, CR Abraham, JH Miner, and AM Shah provided cell lines or mouse models for the study; V. Delitsikou, RD Rajaram, E. Feraille, and S. de Seigneux wrote and revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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