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SUPPLEMENTAL DATA

Kidney-directed AAV9 Klotho gene delivery improves renal function in a mouse model of chronic kidney disease

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Table S1. Main-cohort: Animal flow

Stage	Sham	5/6Nx or disease groups	Total	Detail
Entered surgery	8	42	50	Eight sham procedures and 42 two-stage 5/6-nephrectomies
Week-4 model assessment	8	42	50	Assessment occurred before randomization and treatment
Excluded before randomization	0	2	2	Both failed serum-creatinine success criterion
Entered longitudinal analysis	8	40	48	Forty eligible modeled mice were randomized to five groups
Humane-endpoint euthanasia	0	3	3	Events occurred at weeks 6, 10, and 13
Completed week 16	8	37	45	Terminal biological *n* summed across groups

The unique model success criterion was a week-4 serum creatinine of at least 50 µmol/L and at least a 50% increase from the presurgical value, with recovery from surgery, body-weight loss below 15%, and no active infection. MODEL_FAIL_TE01 had a week-4 serum creatinine of 46.8 µmol/L, a 41% increase, and MODEL_FAIL_TE02 had 49.1 µmol/L, a 37% increase. Neither of these entered Figure 1 or the longitudinal dataset. Sham animals were not subject to the disease criterion. **Abbreviation**: 5/6Nx, five-sixths nephrectomy.

Table S2. Humane endpoints and missing terminal data

Animal ID	Group	Week	Clinical trigger	Procedure	Data handling
AAV-EV_07	AAV-EV	6	21.4% weight loss with persistent severe inactivity and hunched posture	Cervical dislocation without anesthesia	Earlier observations retained; later and terminal values unavailable
CKD_02	CKD	10	22.1% weight loss with severe dehydration not corrected by supportive care	Cervical dislocation without anesthesia	Earlier observations retained; later and terminal values unavailable
AAV-KL-L_01	AAV-KL-L	13	Body-condition score 1.5 with inability to obtain food and water	Cervical dislocation without anesthesia	Earlier observations retained; later and terminal values unavailable

Humane endpoints included weight loss greater than 20% from week 4, a body condition score of 2 or less, inability to obtain food or water, severe dehydration unresponsive to supportive care, persistent recumbency, or severe inactivity and hunched posture lasting more than 12 hours. Personnel were trained and certified under LBSM2026056. **Abbreviations**: AAV-EV, empty adeno-associated virus serotype 9 vector; AAV-KL-L, low-dose adeno-associated virus serotype 9 vector encoding human Klotho; CKD, chronic kidney disease; ID, identifier.

Table S3. AAV batch release

Attribute	Release criterion	AAV9-KL-TE2401	AAV9-EV-TE2402	Method or product
Cassette identity	Sequence concordant	Pass	Pass	Sanger sequencing of both cassette junctions
Vector-genome titer	At least 1.0×10^{13} vg/mL	2.15×10^{13} vg/mL	2.09×10^{13} vg/mL	Takara Bio AAVpro kit 6233
Genome integrity	Predominant 4.62-kb band	Pass	Pass	Alkaline agarose electrophoresis
Full capsids	At least 40%	48.9%	47.6%	Analytical ultracentrifugation
Protein purity	At least 95%	96.8%	96.1%	SDS-PAGE densitometry
Aggregates	No more than 5%	1.8%	2.0%	Size-exclusion chromatography
Endotoxin	Below 5 EU/mL	0.19 EU/mL	0.22 EU/mL	Thermo Fisher Pierce A39552
Final-vector sterility	No growth	No growth	No growth	USP <71>-compatible aerobic, anaerobic, and fungal culture
Producer-cell mycoplasma	Negative	Negative	Negative	Lonza MycoAlert PLUS LT07-701; producer-cell culture, not purified-vector sterility
AAV9 capsid	Detectable and concordant	Pass	Pass	PROGEN AAV9 ELISA PRAAV9

Abbreviations: AAV, adeno-associated virus; AAV9, adeno-associated virus serotype 9; ELISA, enzyme-linked immunosorbent assay; EU/mL, endotoxin units per milliliter; EV, empty vector; kb, kilobases; KL, Klotho; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; USP, United States Pharmacopeia; vg/mL, vector genomes per milliliter.

Table S4. Main cohort: Groups, interventions, and terminal availability

Group	Week-4 n	Intervention	Schedule	Week-16 or terminal *n*
Sham	8	Sham surgery; no renal-vein treatment	Observation through week 16	8
CKD	8	5/6 nephrectomy; no active treatment	Observation through week 16	7
AAV-EV	8	2×10^{11} vg empty AAV9 in 100 µL by left renal vein	Single dose at week 4	7
AAV-KL-L	8	5×10^{10} vg AAV9-KL in 100 µL by left renal vein	Single dose at week 4	7
AAV-KL-H	8	2×10^{11} vg AAV9-KL in 100 µL by left renal vein	Single dose at week 4	8
Recombinant Klotho	8	R&D Systems 5334-KL soluble ectodomain, 0.02 mg/kg intraperitoneally; buffer exchange with Amicon UFC5010 and sterile filtration with Millex-GV SLGV033RS	Every 48 hours, weeks 4 to 16; final dose 24 hours before blood collection	8

Abbreviations: AAV9, adeno-associated virus serotype 9; AAV9-KL, adeno-associated virus serotype 9 vector encoding human Klotho; AAV-EV, empty adeno-associated virus serotype 9 vector; AAV-KL-H, high-dose adeno-associated virus serotype 9 vector encoding human Klotho; AAV-KL-L, low-dose adeno-associated virus serotype 9 vector encoding human Klotho; CKD, chronic kidney disease; vg, vector genomes.

Table S5. Surgical, analgesic, sampling, and UUO procedures

Procedure	Anesthesia	Analgesia	Timing and endpoint	Key reporting detail
5/6Nx, stage 1	Isoflurane 4% induction and 2% maintenance in O_2 at 1 L/min	Buprenorphine 0.05 mg/kg SC preincision then every 12 h for 48 h; bupivacaine 1 mg/kg at incision	Day 0	Two of three visible left renal-artery branches ligated
5/6Nx, stage 2	Same isoflurane regimen	Same regimen	Day 7	Right nephrectomy
Renal-vein vector delivery	Same isoflurane regimen	Included in week-4 procedural care	Week 4	100 µL over 60 s, followed by 3 min gentle compression
UUO or sham ureteral surgery	Same isoflurane regimen	Same buprenorphine and bupivacaine regimen	Day 0; collection day 10	Two 6-0 silk ligatures placed 5 mm apart on left ureter; sham exposure without ligation; n=6 per group; no study treatment
UUO collagen quantification	Not applicable	Not applicable	Day-10 kidneys	Picrosirius Red, Abcam ab150681; 5-µm sections; 10 nonoverlapping cortical fields per kidney at 200-fold magnification; blinded fixed-threshold ImageJ 1.53t
Humane endpoint	No anesthesia	Not applicable	Weeks 6, 10, and 13	Prompt cervical dislocation by trained personnel
Scheduled terminal collection	Deep isoflurane at 5% in O_2	Not applicable	Week 16	Cardiac puncture followed by bilateral thoracotomy

NSAIDs were omitted from this protocol to avoid confounding renal-function measurements. The planned week-16 procedure is distinct from the cervical dislocation without anesthesia applied to the three humane-endpoint animals. **Abbreviations**: 5/6Nx, five-sixths nephrectomy; SC, subcutaneous; UUO, unilateral ureteral obstruction.

Table S6. TaqMan gene-expression assays

Target	Species	Gene symbol	Assay ID	Role
Klotho	Mouse	Kl	Mm00502002_m1	Endogenous renal Klotho
Klotho	Human	KL	Hs00183100_m1	Human KL exon assay; interpreted as vector-associated only in AAV-KL groups; plasmid-equivalent standard
Collagen type I alpha 1	Mouse	Col1a1	Mm00801666_g1	Fibrosis-associated transcript
Fibronectin 1	Mouse	Fn1	Mm01256744_m1	Fibrosis-associated transcript
Actin alpha 2	Mouse	Acta2	Mm00725412_s1	Myofibroblast-associated transcript
Vimentin	Mouse	Vim	Mm01333430_m1	Mesenchymal-associated transcript
Cadherin 1	Mouse	Cdh1	Mm00486918_m1	Epithelial-associated transcript
Cellular communication network factor 2	Mouse	Ctgf or Ccn2	Mm01192933_g1	Fibrogenic-response transcript
Transforming growth factor	Mouse	Tgfb1	Mm03024053_m1	Ligand transcript

beta 1				
SMAD family member 7	Mouse	Smad7	Mm00484742_m1	Inhibitory-Smad transcript; mRNA only
Glyceraldehyde-3-phosphate dehydrogenase	Mouse	Gapdh	Mm99999915_g1	Reference gene

RNA was extracted using the QIAGEN RNeasy Mini Kit (catalog no. 74104) with the RNase-Free DNase Set (catalog no. 79254). cDNA was synthesized using the Applied Biosystems High-Capacity Kit (catalog no. 4368814). PCR was performed using TaqMan Gene Expression Master Mix (catalog no. 4369016) on a QuantStudio 6 Flex system. Human KL was calibrated to a plasmid standard, and the lower limit of quantification was 5,000 plasmid-equivalent copies/µg total RNA. **Abbreviations**: AAV-KL, adeno-associated virus serotype 9 vector encoding human Klotho; *Acta2*, actin alpha 2, smooth muscle, aorta; *Ccn2*, cellular communication network factor 2; *Cdh1*, cadherin 1; cDNA, complementary deoxyribonucleic acid; *Col1a1*, collagen type I alpha 1 chain; *Ctgf*, connective tissue growth factor; DNase, deoxyribonuclease; *Fn1*, fibronectin 1; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; ID, identifier; *KL*, human Klotho gene; *Kl*, mouse Klotho gene; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; RNA, ribonucleic acid; RNase, ribonuclease; *Smad7*, mothers against decapentaplegic homolog 7; *Tgfb1*, transforming growth factor beta 1; *Vim*, vimentin.

Table S7. Western blot and immunohistochemistry antibodies

Target	Supplier and catalog	Host	Application and dilution	Secondary	Lot ID
Klotho	R&D Systems AF1819	Goat	WB 0.2 µg/mL	Jackson ImmunoResearch 705-035-147, 1:10,000	TE-AB-2404
Klotho	Abcam ab203576	Rabbit	IHC-P 1:200, in-house optimized	Agilent Dako EnVision+ K4003 and DAB+ K3468	TE-AB-2412
PAI-1 or Serpin E1	R&D Systems AF3828	Goat	WB 0.25 µg/mL under reducing conditions; approximately 45 to 50 kDa	Jackson ImmunoResearch 705-035-147, 1:10,000	TE-AB-2414
Phospho-Smad3 Ser423/425	Cell Signaling Technology 9520	Rabbit	WB 1:1,000	Cell Signaling Technology 7074, 1:3,000	TE-AB-2405
Smad3	Cell Signaling Technology 9523	Rabbit	WB 1:1,000	Cell Signaling Technology 7074, 1:3,000	TE-AB-2406
Phospho-Smad2 Ser465/467	Cell Signaling Technology 3108	Rabbit	WB 1:1,000	Cell Signaling Technology 7074, 1:3,000	TE-AB-2407
Smad2	Cell Signaling Technology 5339	Rabbit	WB 1:1,000	Cell Signaling Technology 7074, 1:3,000	TE-AB-2408
TGF-β receptor I	Abcam ab235578	Rabbit	WB 1:1,000	Cell Signaling Technology 7074, 1:3,000	TE-AB-2409
Type IV collagen	Abcam ab6586	Rabbit	WB 1:1,000	Cell Signaling Technology 7074, 1:3,000	TE-AB-2410
Beta-actin	Cell Signaling Technology 4970	Rabbit	WB 1:1,000	Cell Signaling Technology 7074, 1:3,000	TE-AB-2411
GAPDH	Cell Signaling Technology 5174	Rabbit	WB 1:1,000	Cell Signaling Technology 7074, 1:3,000	TE-AB-2413

Smad7 was measured only by qRT-PCR; no Smad7 protein antibody or protein results are included. **Abbreviations**: DAB, 3,3′-diaminobenzidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC-P, immunohistochemistry on paraffin-embedded tissue sections; PAI-1, plasminogen activator inhibitor 1; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Ser, serine; Serpin E1, serpin family E member 1; Smad2, mothers against decapentaplegic homolog 2; Smad3, mothers against decapentaplegic homolog 3; Smad7, mothers against decapentaplegic homolog 7; TGF-β, transforming growth factor beta; WB, western blotting.

Table S8. Biochemical assays and immunoassays

Analyte	Matrix	Supplier	Catalog	Lot ID	QC
Creatinine	Serum and urine	BioAssay Systems	DICT-500	TE-KIT-2412	Duplicate CV at most 15%; dilution linearity 90% to 110%
Urea or BUN	Serum	BioAssay Systems	DIUR-100	TE-KIT-2413	Duplicate CV at most 15%
Total protein	Urine	Thermo Fisher Scientific	23225	TE-KIT-2414	Seven-point standard curve
Albumin	Urine	Abcam	ab108792	TE-KIT-2415	Duplicate CV at most 15%
Human soluble alpha-Klotho	Mouse serum	IBL International	27998	TE-KIT-2416	Human-reactive; cross-matrix blank controls, parallelism, and spike recovery; cannot resolve vector from injected protein
Mouse soluble alpha-Klotho	Mouse serum	R&D Systems	DY1819-05 with DY008C	TE-KIT-2417	DuoSet with ancillary reagents; serum matrix qualified by

					parallelism and spike recovery 92% to 108%
Mouse soluble alpha-Klotho	Mouse urine	IBL International	27601	TE-KIT-2426	Urine-validated kit
Intact FGF23	Serum	QuidelOrtho	60-6800	TE-KIT-2418	Four-parameter logistic curve
IL-6	Serum	R&D Systems	M6000B	TE-KIT-2419	Four-parameter logistic curve
TNF-alpha	Serum	R&D Systems	MTA00B	TE-KIT-2420	Four-parameter logistic curve
CCL2 or MCP-1	Serum	R&D Systems	MJE00B	TE-KIT-2421	Four-parameter logistic curve
Total TGF-β1	Serum	R&D Systems	DB100C	TE-KIT-2422	1 N HCl activation for 10 min at room temperature; equal-volume 1.2 N NaOH in 0.5 M HEPES neutralization
Phosphate	Serum	BioAssay Systems	DIPI-500	TE-KIT-2424	Duplicate CV at most 15%
Calcium	Serum	BioAssay Systems	DICA-500	TE-KIT-2425	Duplicate CV at most 15%

Abbreviations: BUN, blood urea nitrogen; CCL2, C-C motif chemokine ligand 2; CV, coefficient of variation; FGF23, fibroblast growth factor 23; HCl, hydrochloric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ID, identifier; IL-6, interleukin 6; M, MCP-1, monocyte chemoattractant protein 1; NaOH, sodium hydroxide; QC, quality control; TGF-β1, transforming growth factor beta 1; TNF-α, tumor necrosis factor alpha.

Table S9. Prespecified longitudinal statistical model

Element	Specification
Primary endpoint	Serum creatinine at week 16
Primary contrast	AAV-KL-H versus AAV-EV at week 16
Analysis visits	Postbaseline weeks 8, 12, and 16; week 4 entered as baseline
Fixed effects	Group, categorical visit, group-by-visit interaction, baseline serum creatinine, and baseline-by-visit interaction
Repeated subject	Animal ID
Analysis population	Five randomized 5/6-nephrectomy groups; Sham displayed descriptively and excluded from treatment contrasts
Estimation	Restricted maximum likelihood with R 4.5.3 and nlme 3.1-168 generalized least squares
Within-animal covariance	Fully unstructured correlation with corSymm and visit-specific residual variances with varIdent
Inference	Model-based coefficient covariance and residual t distribution with 96 residual degrees of freedom
Prespecified week-16 contrasts	Primary: AAV-KL-H versus AAV-EV. Supportive: AAV-KL-H versus untreated CKD
Multiplicity	None. One confirmatory primary contrast; the supportive contrast reported separately
Sample-size planning	Two-sided alpha 0.05, 80% power, difference 18 µmol/L, unadjusted SD 12 µmol/L, baseline correlation 0.50, implied residual SD 10.4 µmol/L; t-test approximation 6.35 per group, rounded to seven, with eight assigned for attrition
Missing data	All observed postbaseline values used; no post-euthanasia imputation; likelihood inference assumes missing at random conditional on model variables and prior outcomes
Sensitivity analysis	Adverse-rank analysis placing humane-endpoint animals below observed week-16 animals

Abbreviations: AAV-EV, empty adeno-associated virus serotype 9 vector; AAV-KL-H, high-dose adeno-associated virus serotype 9 vector encoding human Klotho; CKD, chronic kidney disease; ID, identifier; SD, standard deviation.

Table S10. Biological sample availability by visit

Group	Week 4	Week 8	Week 12	Week 16	Terminal tissue	RNA sequencing
Sham	8	8	8	8	8	3
CKD	8	8	7	7	7	3
AAV-EV	8	7	7	7	7	0
AAV-KL-L	8	8	8	7	7	0
AAV-KL-H	8	8	8	8	8	3
Recombinant Klotho	8	8	8	8	8	0

Technical duplicates were averaged within each animal and did not increase biological *n*. Missing values after humane-endpoint euthanasia are structural and are not imputed. **Abbreviations**: AAV-EV, empty adeno-associated virus serotype 9 vector; AAV-KL-H, high-dose adeno-associated virus serotype 9 vector encoding human Klotho; AAV-KL-L, low-dose adeno-associated virus serotype 9 vector encoding human Klotho; CKD, chronic kidney disease; RNA, ribonucleic acid.

Table S11. RNA-sequencing workflow and quality summary

Component	Detail
Biological samples	Renal cortex from Sham, CKD, and AAV-KL-H, n=3 mice per group
RNA acceptance	RIN at least 8.0
Library preparation	NEBNext Poly(A) mRNA Magnetic Isolation Module E7490L and Ultra II Directional RNA Library Prep Kit E7760L
Sequencing	Illumina NovaSeq 6000 S4, paired-end 150 bp
Depth	Mean 46.8 million read pairs per library, range 42.1 to 52.6 million
Base quality	Mean Q30 93.2%
Quality and trimming	FastQC 0.11.9 and Trim Galore 0.6.7
Alignment	STAR 2.7.10a to GRCm38
Counting and annotation	featureCounts in Subread 2.0.3 with GENCODE mouse release M25
Differential-expression calculation	Python 3.12.14, NumPy 2.3.5, and pandas 2.2.3; median-ratio normalization, Wald-style normal approximation, and Benjamini-Hochberg adjustment
Significance rule	Adjusted *p* below 0.05 and absolute log2 fold change above log2 of 1.5
Feature universe	15,000 genes after filtering
Summary	417 CKD-versus-Sham DEGs; 159 AAV-KL-H-versus-CKD DEGs; 123 significant genes changed in the opposite direction
Heatmap	Predefined 40-gene renal-response panel labeled with standard Mus musculus gene symbols

Raw RNA-sequencing data are deposited under accession OMIX017482. Complete underlying study data are available from the corresponding author upon reasonable request, subject to institutional and ethical requirements. **Abbreviations**: AAV-KL-H, high-dose adeno-associated virus serotype 9 vector encoding human Klotho; bp, base pairs; CKD, chronic kidney disease; DEGs, differentially expressed genes; GRCm38, Genome Reference Consortium mouse genome build 38; mRNA, messenger

ribonucleic acid; NumPy, Numerical Python; poly(A), polyadenylic acid; Q30, percentage of bases with a Phred quality score ≥30; RIN, RNA integrity number; RNA, ribonucleic acid; STAR, Spliced Transcripts Alignment to a Reference.

Table S12. Cross-document consistency and package acceptance checklist

Requirement	Implementation	Package evidence
Animal-level Figure 1 data	Forty-eight IDs with weeks 4, 8, 12, and 16, status, and three humane-endpoint events	Figure 1
Primary longitudinal model	One group-by-visit nlme MMRM with baseline and baseline-by-visit terms	Table S9
Human-Klotho attribution	Last-dose interval and human-KL-specific qPCR reported	Figure 2, Figure S1, Tables S4, S6, and S8
Reagent detail	Manufacturers, catalogs, and lot identifiers	Tables S3, S6, S7, and S8
UUO role	Independent untreated model-establishment control, n=6 per group, day 10	Figure 7 and Table S5
Smad7 scope	mRNA only; no protein panel or claim	Figure 3, Figure 4 legend, and Tables S6 and S7
RNA heatmap	Forty standard Mus musculus gene symbols and complete source table	Figure 6f
Figure legibility	Figures 1 to 8 redrawn at journal width with Arial 8 to 12 pt; Figure 8 uses plain connecting lines without arrows	Figures folder
No arrow symbols	Plain text, punctuation, and connecting lines only	Automated text and figure scan
Data availability	Raw data deposited under OMIX017482	Data availability statement

Abbreviations: ID, identifier; KL, human Klotho gene; MMRM, mixed model for repeated measures; mRNA, messenger ribonucleic acid; pt, typographic points; qPCR,

quantitative polymerase chain reaction; RNA, ribonucleic acid; Smad7, mothers against decapentaplegic homolog 7; UUO, unilateral ureteral obstruction.

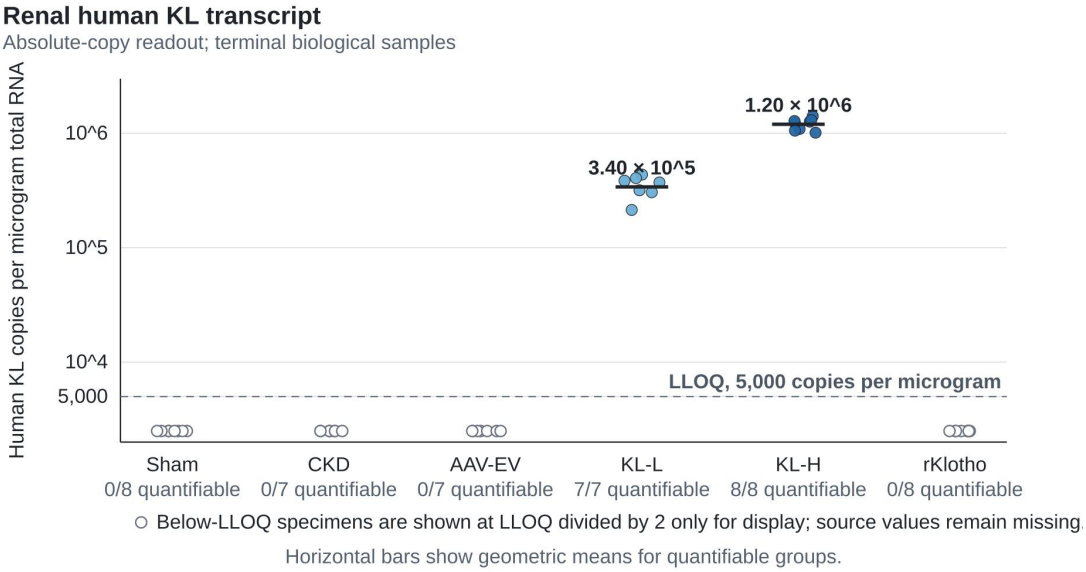


Figure S1. Human KL transcript quantification in the renal cortex at week 16. Human *KL* expression was measured by qPCR using assay Hs00183100_m1 and a plasmid calibration standard. The assay targets a human *KL* exon sequence and does not span a vector junction. Results are expressed as plasmid-equivalent copies per microgram of total RNA on a logarithmic scale. Each point represents one animal; horizontal bars indicate geometric means for groups with quantifiable expression. Human *KL* was quantifiable in all KL-L samples (7/7; geometric mean, 3.40×10^5 copies/µg total RNA) and KL-H samples (8/8; geometric mean, 1.20×10^6 copies/µg total RNA). All samples from Sham (*n* = 8), CKD (n = 7), AAV-EV (*n* = 7), and rKlotho (*n* = 8) were below the LLOQ of 5,000 plasmid-equivalent copies/µg total RNA, indicated by the dashed line. These samples are shown as open circles at half the LLOQ solely to display sample counts; their source values remain missing, and the plotted positions do not represent measured concentrations. **Abbreviations**: AAV-EV, empty adeno-associated virus serotype 9 vector; CKD, chronic kidney disease; KL, human Klotho gene; KL-H, high-dose adeno-associated virus serotype 9 vector encoding human Klotho; KL-L, low-dose adeno-associated virus serotype 9 vector encoding human Klotho; LLOQ, lower limit of quantification; n, number of animals; qPCR, quantitative polymerase chain reaction; rKlotho, recombinant human Klotho; RNA, ribonucleic acid.

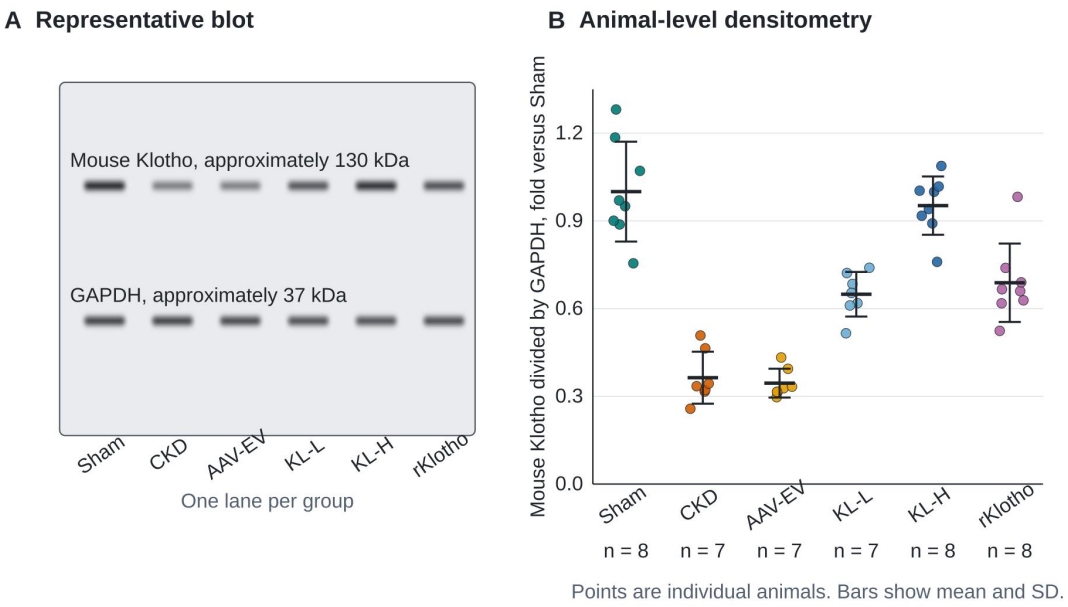


Figure S2. Renal mouse Klotho protein expression at week 16. (A) Representative immunoblots showing one lane per group for mouse Klotho (approximately 130 kDa) and GAPDH (approximately 37 kDa), used as the loading control. **(B)** Animal-level quantification of Klotho band intensity normalized to GAPDH and expressed relative to the Sham mean. Each point represents one animal; horizontal bars indicate means, and whiskers indicate ± SD. Biological sample sizes were n = 8, 7, 7, 7, 8, and 8 for Sham, CKD, AAV-EV, KL-L, KL-H, and rKlotho, respectively. **Abbreviations**: AAV-EV, empty adeno-associated virus serotype 9 vector; CKD, chronic kidney disease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kDa, kilodaltons; KL-H, high-dose adeno-associated virus serotype 9 vector encoding human Klotho; KL-L, low-dose adeno-associated virus serotype 9 vector encoding human Klotho; rKlotho, recombinant human Klotho; SD, standard deviation.