Renal deletion of LRRC8/VRAC channels induces proximal tubulopathy

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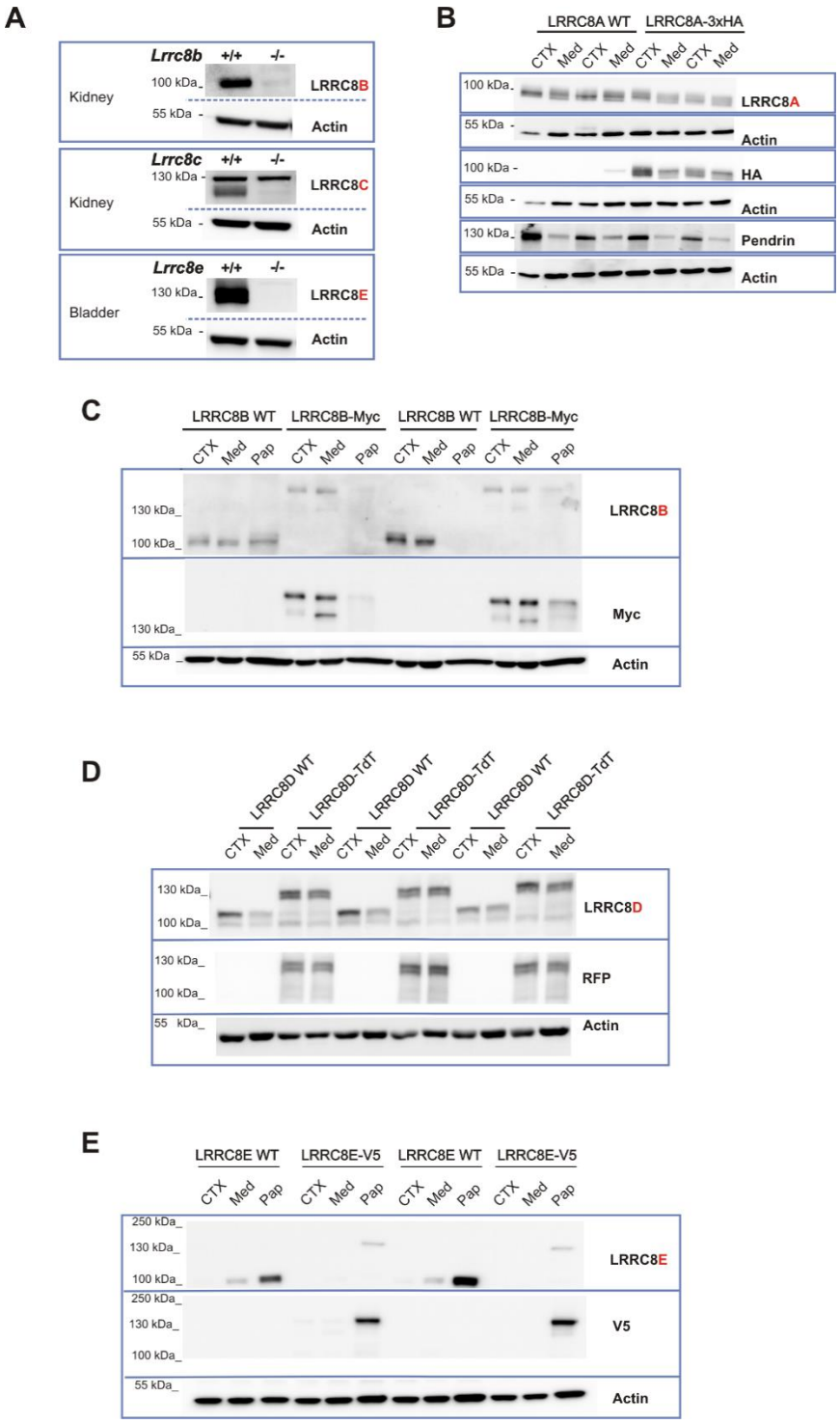
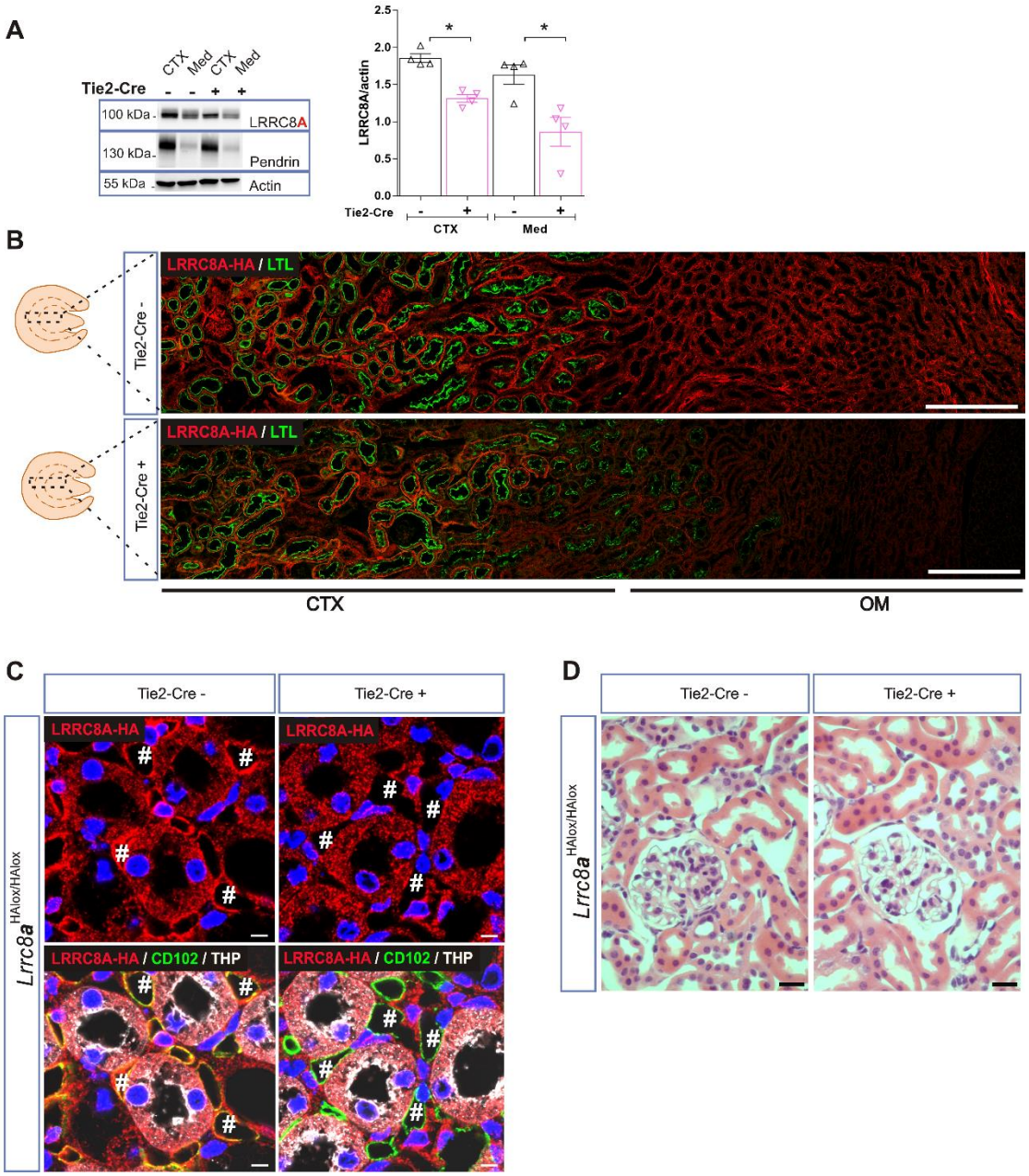
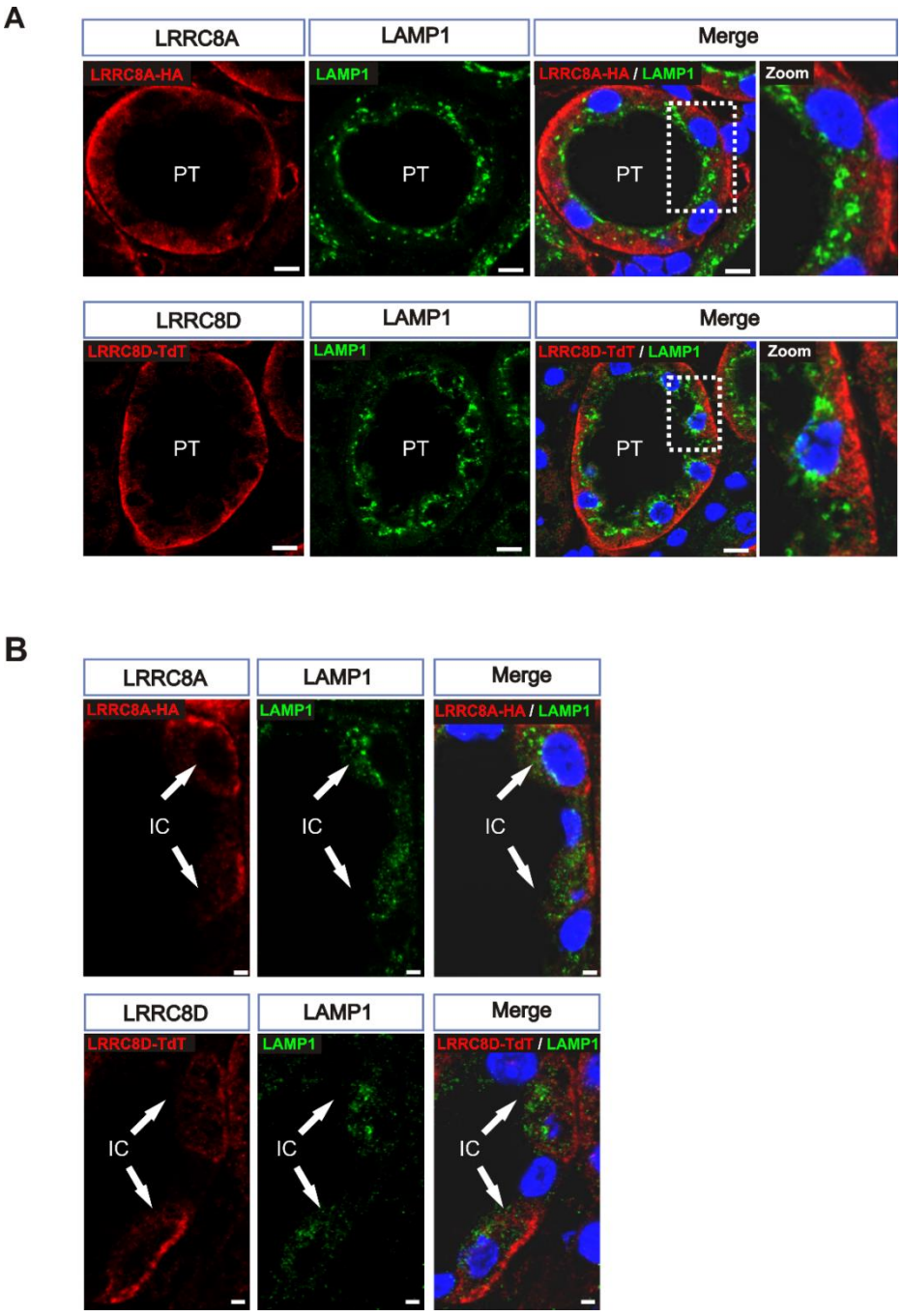
Supplemental Methods



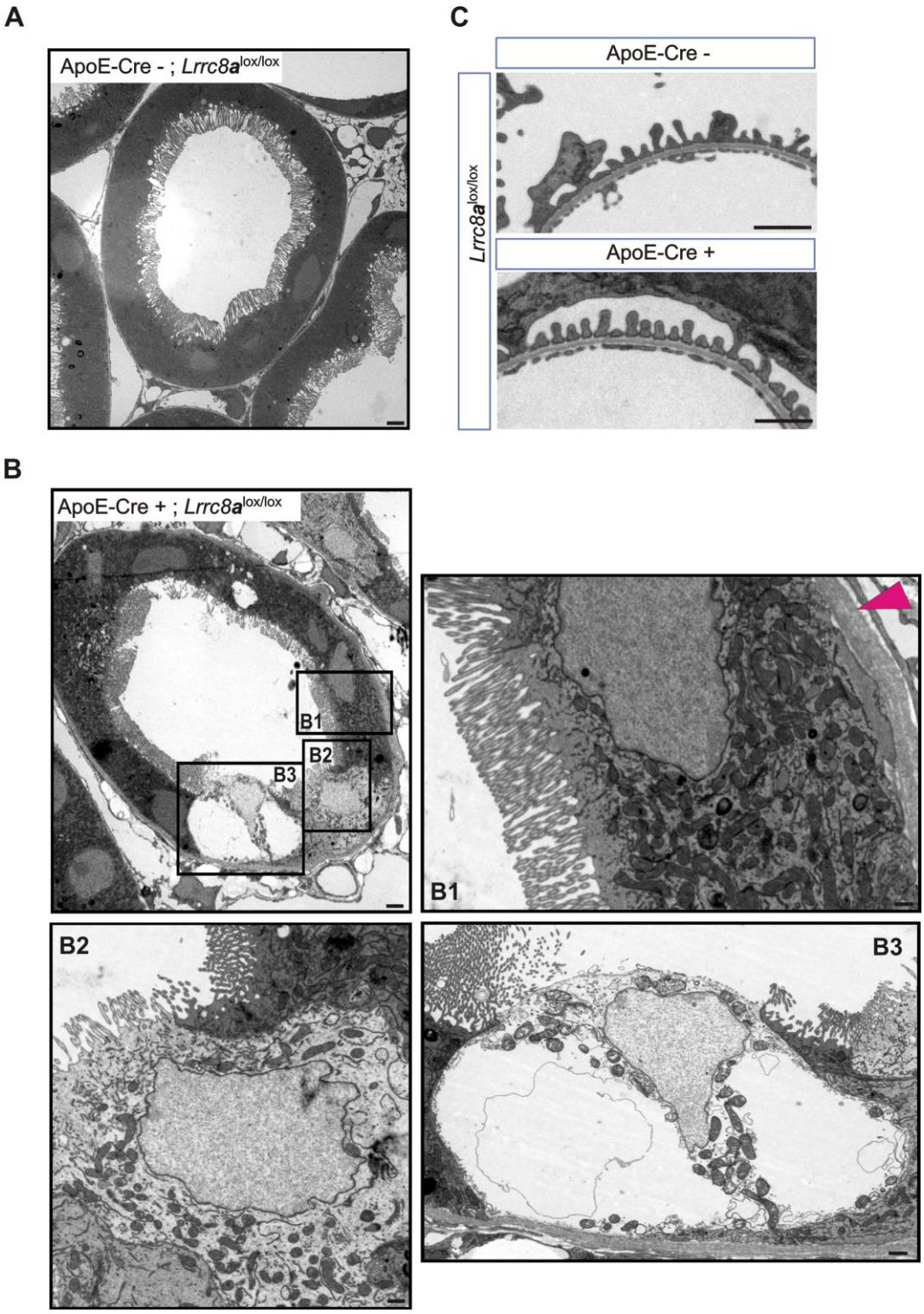
Supplemental Figure 1. Western blot analysis of LRRC8 subunit expression. A. Validation of antibodies against mouse LRRC8B, -C, and –E, using tissues from respective constitutive KO mice as controls; for LRRC8B and -C membrane fraction from whole kidney was used and for LRRC8E from bladder. **B-E**. Comparison of epitope-tagged and WT LRRC8 subunit expression by Western blot using antibodies against native or the epitope-tagged proteins. Membrane fractions isolated from WT or *Lrrc8a*$^{HAlox/HAlox}$ (B), *Lrrc8b*$^{SmFPV5/SmFPV5}$ (C), *Lrrc8d*$^{TdTlox/TdTlox}$ (D), and *Lrrc8e*$^{SmFPV5/SmFPV5}$ mice (E). β-actin, loading control and Pendrin, marker of renal cortex. Images are representative of n ≥ 3 mice per genotype.



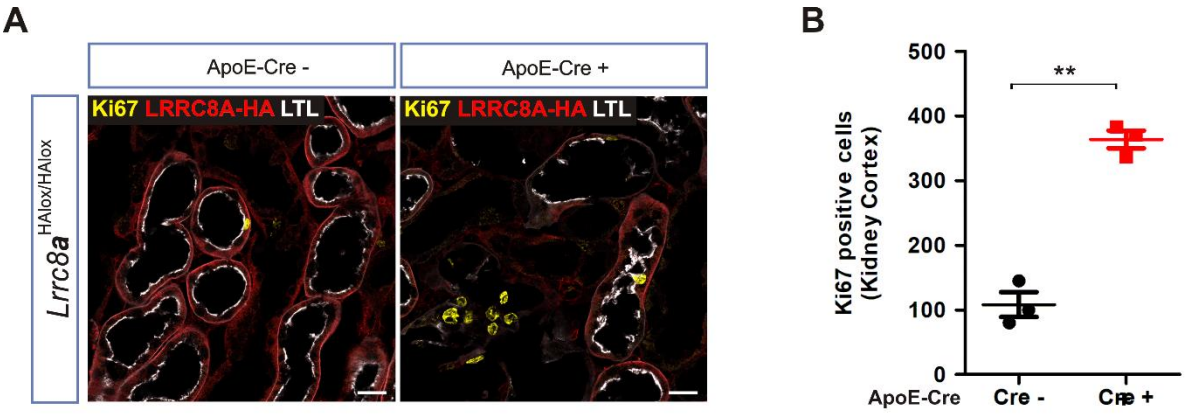
Supplemental Figure 2. In renal medulla LRRC8A is predominantly found in endothelial cells. A. Left, Western blot of LRRC8A in membrane fractions of cortex and medulla from *Lrrc8a*$^{lox/lox}$ mice crossed with endothelial-specific Tie2-Cre mice (+) or controls (-). Pendrin, marker of kidney cortex; β-actin, loading control. Right, Western blot quantification. Bars, mean ± SEM, * p <0.05 (Mann–Whitney U test). n=4 mice/ genotype. **B**. Assessment of Tie2-Cre driven LRRC8A deletion by immunofluorescence. HA labeling (red) of Tie2-Cre;*Lrrc8a*$^{HAlox/HAlox}$ and control kidney sections. PTs identified by LTL labeling (green). Cortex (CTX) and outer medulla (OM) indicated below. Scale bar 200 µm. **C.** LRRC8A-HA staining (red) was reduced in Tie2-Cre;*Lrrc8a*$^{HAlox/Halox}$ mice compared to *Lrrc8a*$^{HAlox/Halox}$ control siblings. Lower panels, overlay of upper images with TAL marker THP (white) and endothelial marker CD102 (green). LRRC8A is specifically deleted in CD102-positive endothelial cells of blood vessels (#). Scale bar 5 µm**. D.** Hematoxylin/eosin staining of kidney cortex shows normal morphology in Tie2-Cre;*Lrrc8a*$^{lox/lox}$ mice. Images are representative of n ≥ 3 mice per genotype. Scale bar 20 µm.



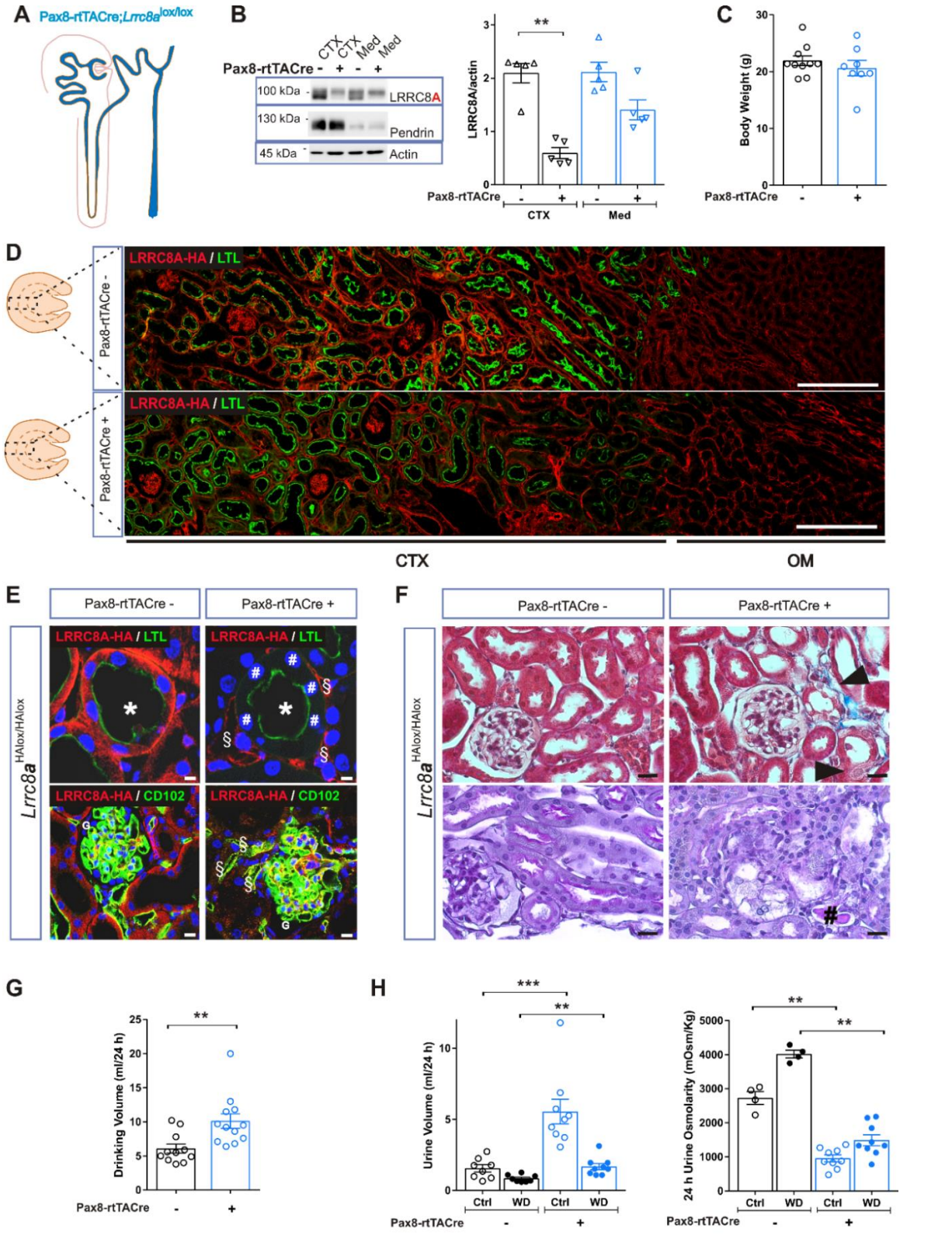
Supplemental Figure 3. LRRC8A and LRRC8D do not colocalize with late endosomal/ lysosomal LAMP1. Immunofluorescence of **A.** Proximal tubules (PT) and **B.** Intercalated Cells (IC) with LAMP1 (green) and in red LRRC8A-HA (upper panel) or LRRC8D-TdTomato (lower panel) in respective homozygous KI mice fails to show any co-localization.



Supplemental Figure 4. Electron microscopy analysis of ApoE-Cre;*Lrrc8a*$^{lox/lox}$ cortex. TEM images of a proximal tubule (PT) from control (**A**) and ApoE-Cre;*Lrrc8a*$^{lox/lox}$ mouse (**B**). Cells with different stages of degeneration are observed side-by side in the same PT segment, from a milder morphological alteration characterized by thickening of the basal membrane (**B1,** arrowhead), diluted cytoplasm (**B2**), and a severe hydropic cell death (**B3**). Normal podocytes foot processes and slit diaphragm is observed in ApoE-Cre;*Lrrc8a*^{loxlox} mice (**C**). n=3 mice/genotype (6 weeks old), scale bar, 2.5 µm in A and B, 1 µm in C and 0.5 µm in the close up pictures (B1, B2, B3).

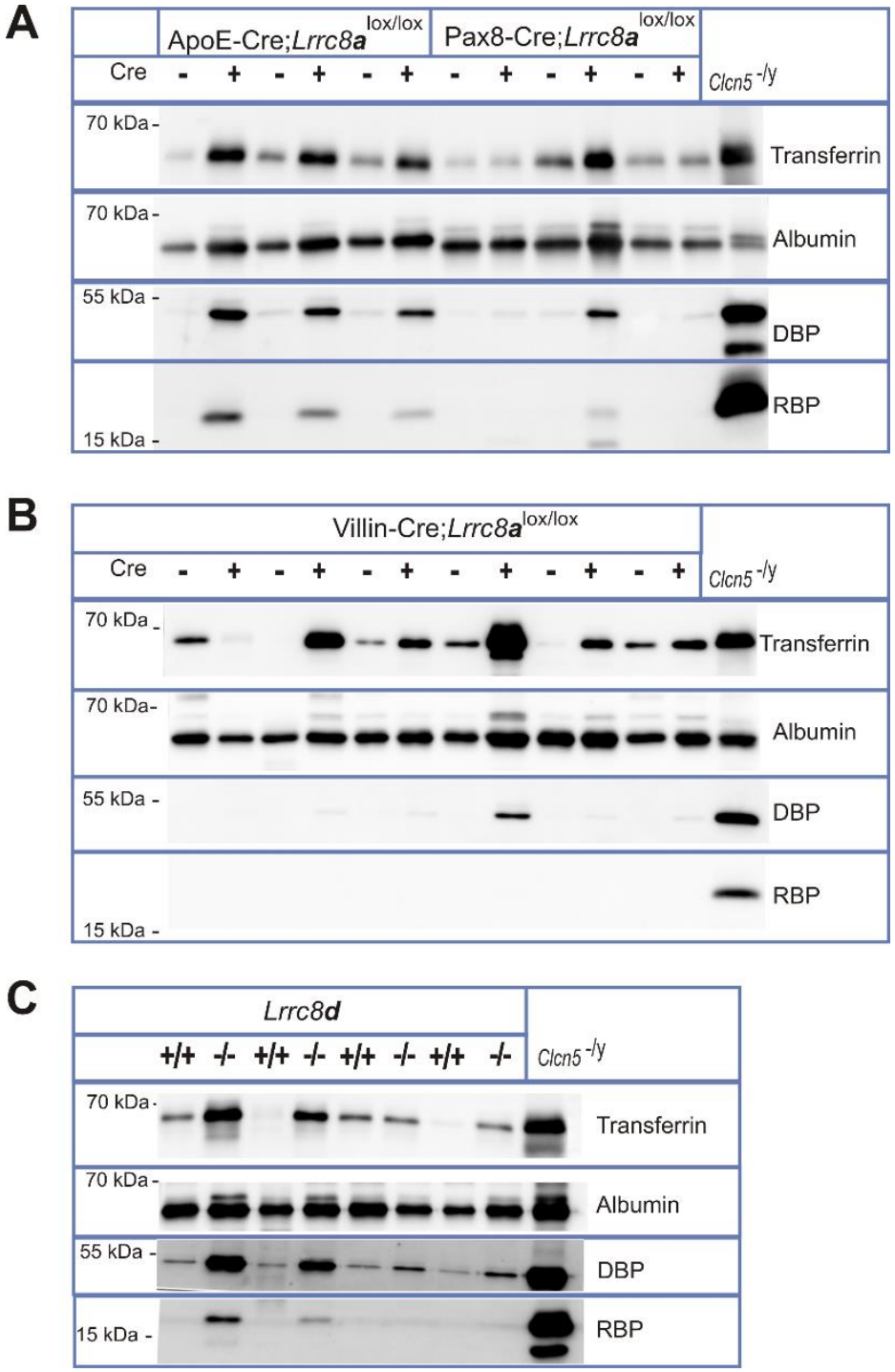


Supplemental Figure 5. Increased expression of proliferation marker Ki67 in ApoE-Cre;*Lrrc8a*$^{HAlox/HAlox}$ mice. A. Representative picture of Ki67 (yellow) in proximal tubule cells (labeled with LTL in white) of Cre + mice. KO tubules are negative for LRRC8A-HA staining (red). **A.** Quantification of Ki67 positive cells in kidney cortex of ApoE-Cre;*Lrrc8a*$^{HAlox/HAlox}$ mice.

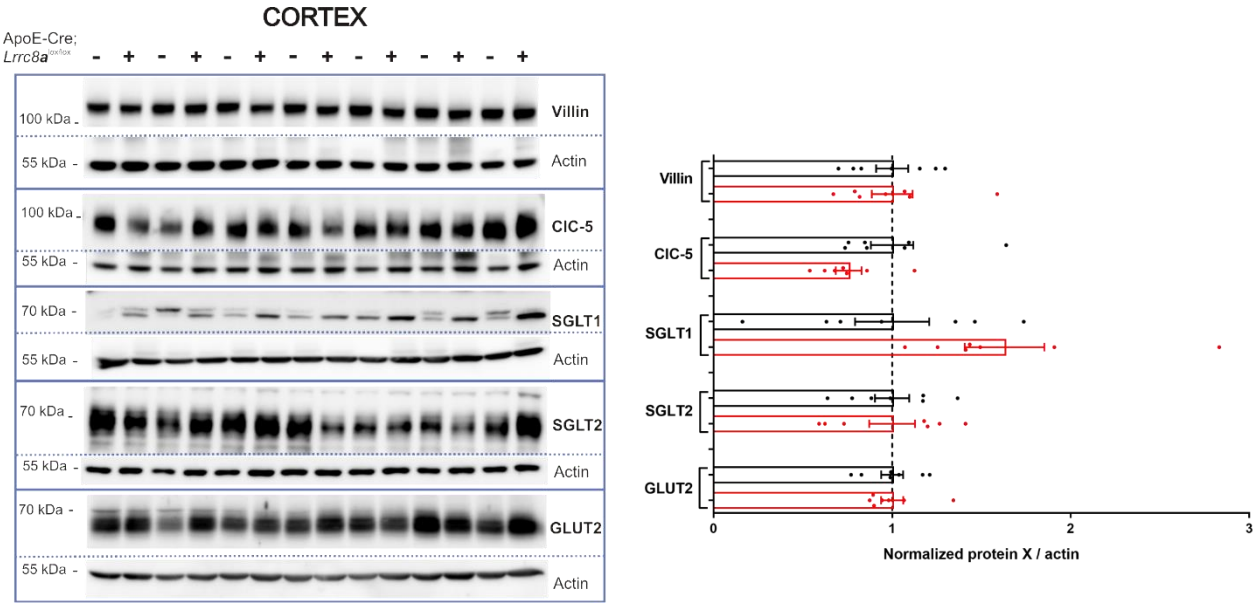


Supplemental Figure 6. Acute deletion of LRRC8A in Pax8-rtTACre;*Lrrc8a*$^{lox/lox}$ mice recapitulates the renal phenotype of ApoE-Cre;*Lrrc8a*$^{lox/lox}$ mice. A. Illustration of doxycycline-induced Cre expression using Pax8-rtTACre mice. Recombinase is expressed in the entire nephron, but not in blood vessels. **B.** Left, representative Western blot of LRRC8A in membrane fractions of renal cortex (CTX) and medulla (Med) from *Lrrc8a*$^{lox/lox}$ control and induced Pax8-rtTACre;*Lrrc8a*$^{lox/lox}$ mice. Pendrin, CTX marker; β-actin, loading control. Right, Pax8-rtTACre driven deletion reduced

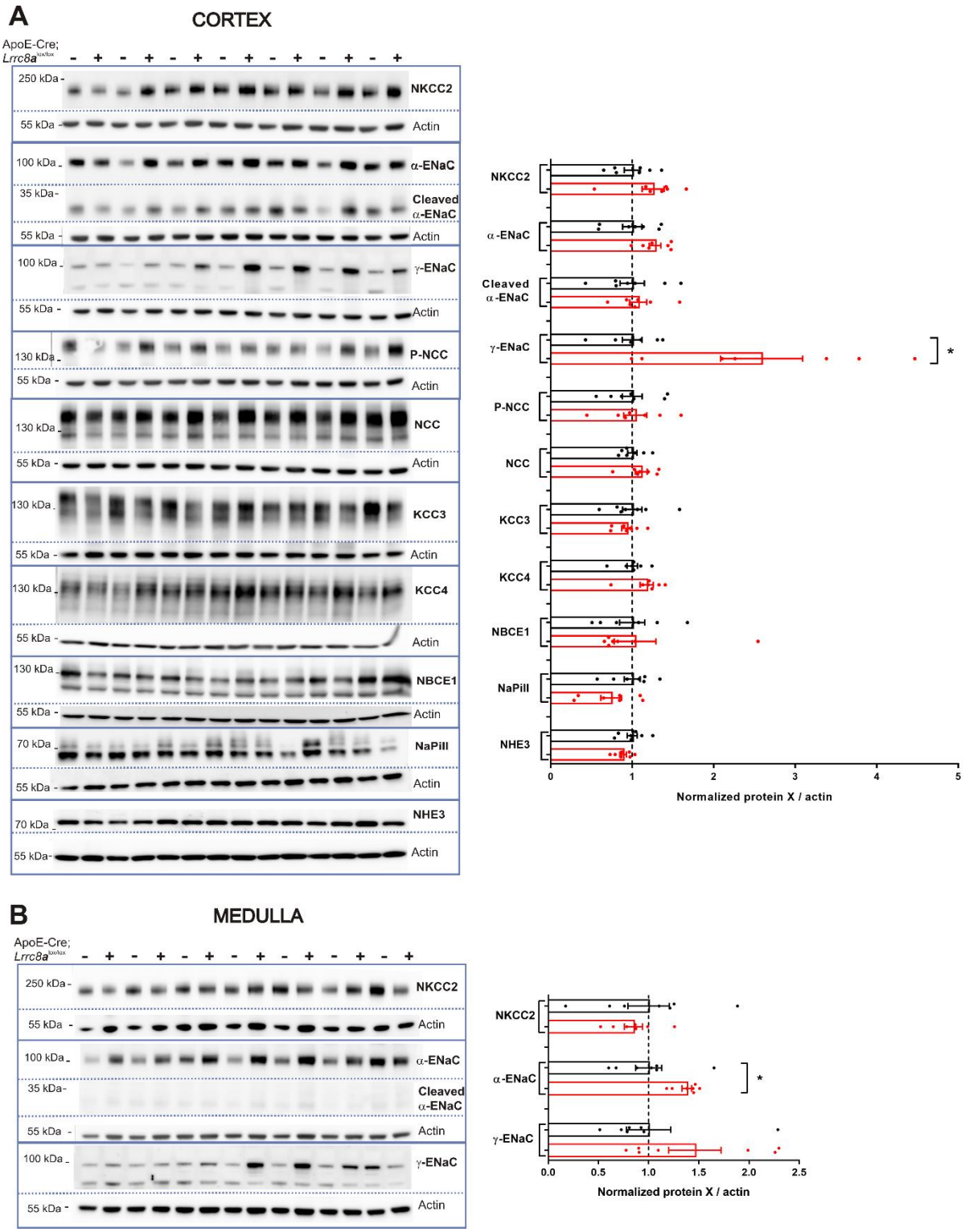
LRRC8A levels in CTX and Med by ~70 % and ~35 %, respectively. **C.** Body weight of doxycycline-treated control and Pax8-rtTACre 11-12 weeks old siblings. **D**. LRRC8A deletion in Pax8-rtTACre animals assessed in induced Pax8-rtTACre;*Lrrc8a*$^{HAlox/HAlox}$ mice using an HA antibody in immunofluorescence (red). LTL used as marker for proximal tubules (green). The region approximately corresponding to cortex and outer medulla (OM) are indicated. Scale bar, 200 µm. **E.** Upper panel, complete deletion (#) of LRRC8A-HA upon Pax8-rtTACre induction in proximal tubules (PT, identified by green apical LTL staining). By contrast, LRRC8A-HA remains in blood vessels (§). Lower panel, no deletion of LRRC8A was observed in endothelial cells labeled with CD102 (green) in glomerulus (G) and vasculature (§). Nuclei are DAPI stained (blue). Scale bar 5 µm. **F.** Upper panel, Masson's trichrome staining of kidney CTX from control and Pax8-rtTACre mice, tubular injury with mild fibrosis (blue) and swollen cells are indicated in induced Pax8-rtTACre mice (arrowheads). Lower panel, PAS (Periodic Acid-Schiff) staining shows hyaline casts (#). Scale bar 20 µm. **G.** Daily water intake, **H.** Volume (left panel) and osmolarity (right panel) in 24 h urine collected in metabolic cages of Pax8-rtTACre and control mice. Urine volume and osmolarity were measured before (Ctrl) and 24h post removal of water from the metabolic cage (WD). Bars, mean ± SEM, ** $p < 0.01$, *** $p < 0.001$ (Mann–Whitney U test). Pictures are representative of at least n=3 mice/ genotype.



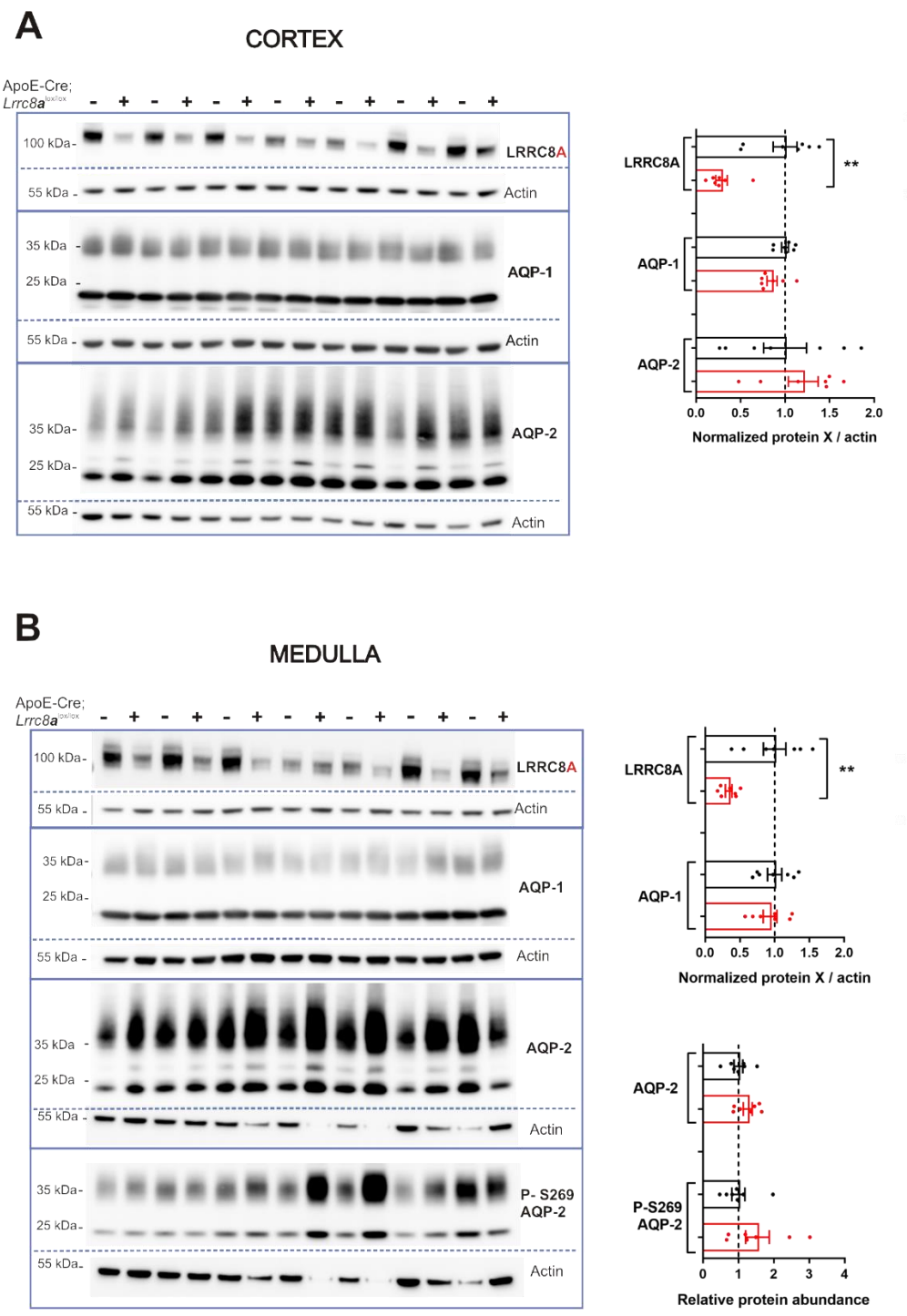
Supplemental Figure 7. Mild proteinuria in ApoE-Cre, Pax8-rtTACre;*Lrrc8a*$^{lox/lox}$ mice. Immunoblot on urine samples using antibodies against transferrin, albumin, vitamin D-binding protein (DBP), and retinol-binding protein (RBP) shows mild proteinuria in ApoE-Cre;*Lrrc8a*$^{lox/lox}$ and Pax8-rtTACre;*Lrrc8a*$^{lox/lox}$ mice **(A)** that is absent from Cre recombinase (-) control siblings. Variable proteinuria is detected in Villin-Cre;*Lrrc8a*$^{lox/lox}$ (**B**) and *Lrrc8d*$^{-/-}$ mice (**C**). Samples were normalized by creatinine concentration detected in 24 h urine. *Clcn5* KO (-/y) urine was used as positive control. ApoE-Cre;*Lrrc8a*$^{lox/lox}$ (n=3 mice/ group), Pax8-rtTACre;*Lrrc8a*$^{lox/lox}$ (n=4 cre + and n=3 cre - mice), Villin-Cre;*Lrrc8a*HA$^{lox/HAlox}$ (n=6 mice/ group), *Lrrc8d*$^{-/-}$ (n=3 mice/ genotype).



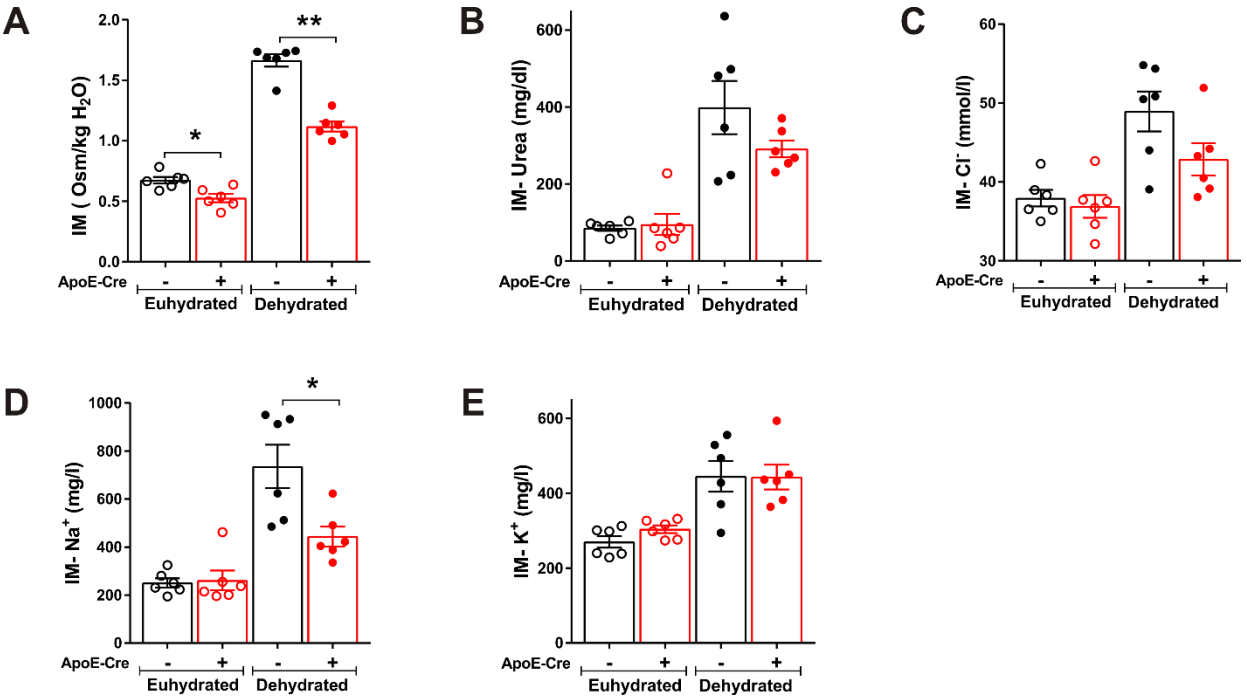
Supplemental Figure 8. Normal protein abundance of SGLT2, SGLT1 and GLUT2 in ApoE-Cre*;Lrrc8a*$^{lox/lox}$ mice. Left, abundance of SGLT2 and GLUT2 transporters in membrane fractions in cortex isolated from *Lrrc8a*$^{lox/lox}$ mice expressing the ApoE-Cre recombinase (+) compared to control mice ApoE-Cre recombinase (-). Villin and ClC-5 were also assessed as housekeepers showing similar protein abundance in both genotypes. β-actin, loading control. Right, Western blot quantification. Bars, mean ± SEM, n=7 mice/ genotype.



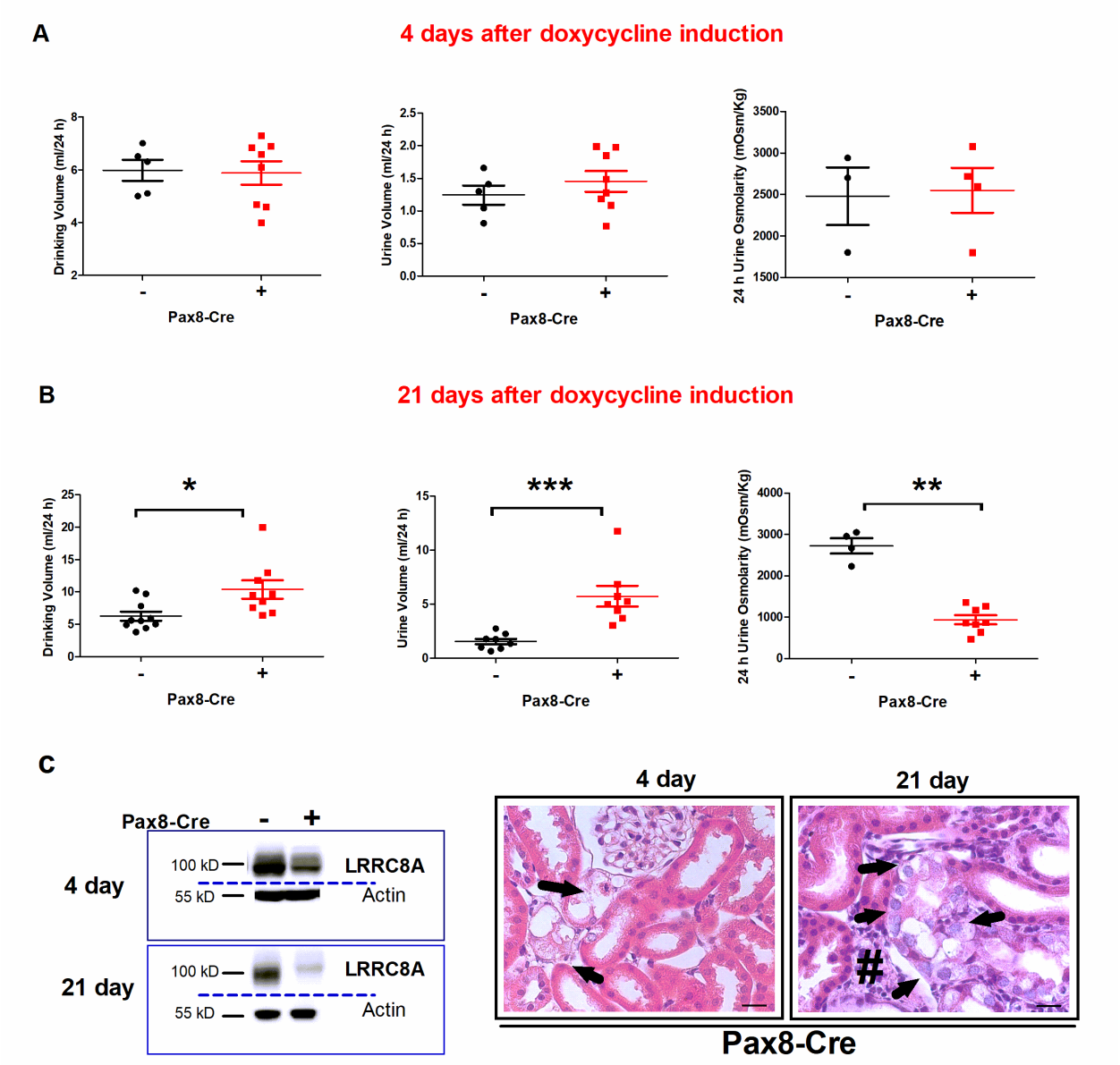
Supplemental Figure 9. Protein abundance of selected ion transporters and channels in renal cortex and medulla of ApoE-Cre;*Lrrc8a*$^{lox/lox}$ mice. Western blot analyses and quantification of membrane protein levels in kidney cortex (**A**) and medulla (**B**) from ApoE-Cre;*Lrrc8a*$^{lox/lox}$ (+) and control *Lrrc8a*$^{lox/lox}$ (-) mice. β-actin, loading control. Bars, mean ± SEM, p <0.05 * (Mann–Whitney U test), n=7 mice/ genotype.



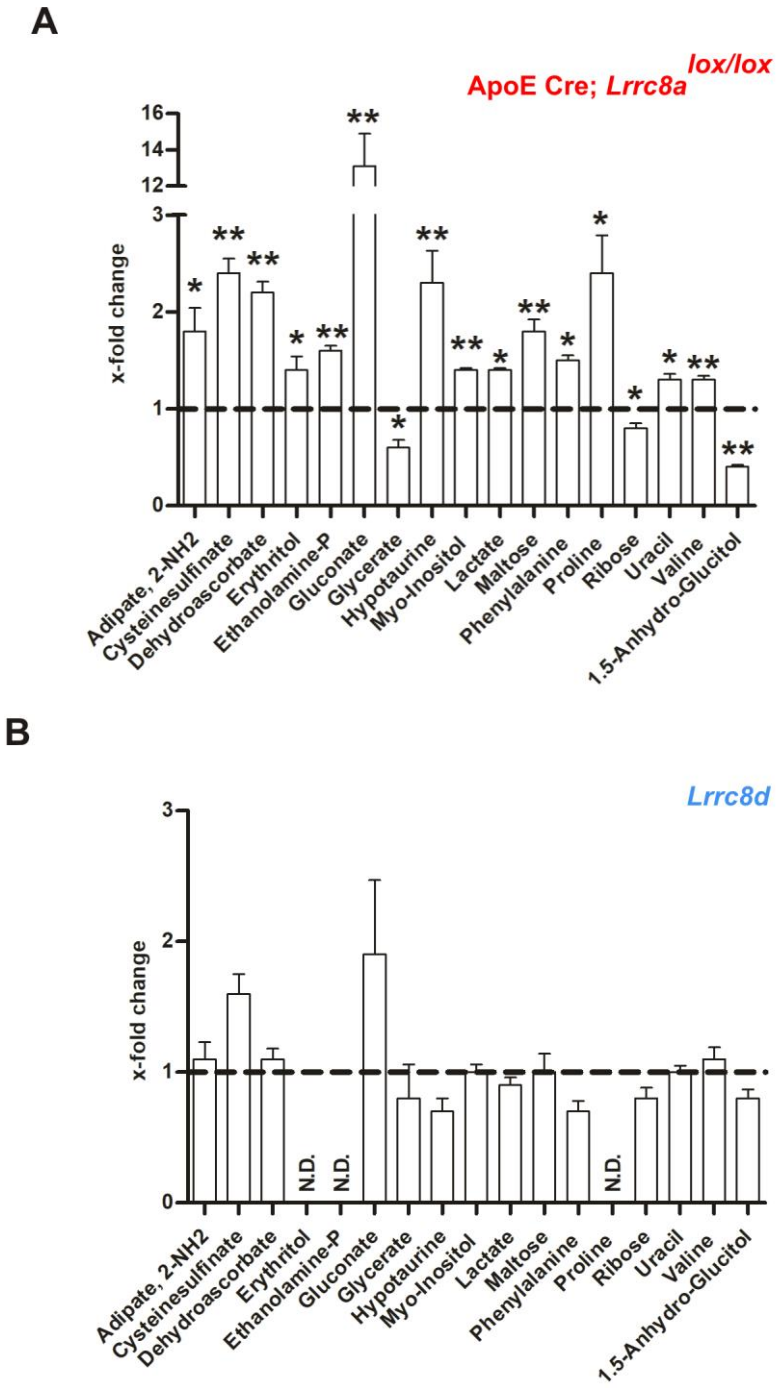
Supplemental Figure 10. Unchanged expression of aquaporins 1 and 2 in ApoE-Cre;*Lrrc8a*$^{lox/lox}$ kidney. Western blotting analyses and quantification of AQP1 and AQP2 in kidney cortex (**A**) and medulla (**B**) of membrane fractions isolated from ApoE-Cre;*Lrrc8a*$^{lox/lox}$ (+) and control *Lrrc8a*$^{lox/lox}$ (-) mice. Phosphorylated P-S269 AQP-2 was also assessed in kidney medulla. LRRC8A expression is shown as control of reduction of LRRC8A protein in ApoE-Cre;*Lrrc8a*$^{lox/lox}$ (+) mice. β-actin, loading control. Protein bands were quantified and normalized to actin, except AQP-2 and Phosphorylated AQP-2 in the medulla, where actin was not detectable in 3 samples. Bars, mean ± SEM, p <0.01 ** (Mann–Whitney U test), n=7 mice/ genotype.



Supplemental Figure 11. Effect of ApoE-Cre driven LRRC8A disruption on interstitial inner medulla osmolarity and concentrations of selected osmolytes, under both water *ad libitum* and 24h water restriction. A. Tissue osmolarity from inner medulla plus papilla (IM) excised from ApoE-Cre;*Lrrc8a*$^{lox/lox}$ mice (+) and control *Lrrc8a*$^{lox/lox}$ euhydrated or water-deprived. Each data point is from IM pooled from 3 mice. **B-E.** Chloride, sodium, potassium and urea concentrations in same samples. Error bars, mean ± SEM, *p<0.05, ** p<0.01 (Mann–Whitney U test).



Supplemental Figure 12. Progression of kidney injury after acute *Lrrc8a* deletion. Water intake and urine excretion was assessed in inducible Pax8-rtTACre;*Lrrc8a*$^{lox/lox}$ mice. Two different times of doxycycline-induction (and thus *Lrrc8a* deletion) were evaluated. **A.** 4 days of induction with doxycycline or **B**. One week after 14 days of induction with doxycycline (in total 21 days). Data obtained in metabolic cages with mice having free access to water. Results in B are identical to supplemental figure 6 and are show here again for comparison. **C**. Right, LRRC8A protein reduction at day 4 or 21 and, left panel, kidney morphological alterations became prominent at day 21, including tubular injury (arrows) and immune cells infiltration (#). Morphological alterations are correlated with the observed polyuria and polydipsia at the same time point (B). Error bars, mean ± SEM, *p<0.05, ** p<0.01, *** p<0.001 (Mann–Whitney U test).



Supplemental Figure 13. Metabolomics analysis reveals differences in the abundance of several metabolites in ApoE-Cre;*Lrrc8a*$^{lox/lox}$ but not in *Lrrc8d*$^{-/-}$ kidney cortices. Differential abundance of metabolites in ApoE-Cre;*Lrrc8a*$^{lox/lox}$ **(A)** or *Lrrc8d*$^{-/-}$ **(B)** kidney cortices normalized to their respective control siblings (represented as 1 in the graphs). Tissues were acutely dissected for analysis of several metabolites. N.D., not determined. Error bars, fold of change ± SEM, *p<0.05, ** p<0.01 (Mann–Whitney U test used to compare control vs. Cre + mice), n=6 male mice/ group with ages between 9-11 weeks.

Supplemental Table 1. Serum creatinine and GFR of control, ApoE-Cre, Pax8-rtTACre, Ksp-Cre and Villin-Cre animals bred to *Lrrc8a*$^{lox/lox}$ mice.

	ApoE-Cre;*Lrrc8a*$^{lox/lox}$		Pax8-Cre;*Lrrc8a*$^{lox/lox}$		Ksp-Cre;*Lrrc8A*$^{lox/lox}$		Villin-Cre;*Lrrc8a*$^{HAlox/HAlox}$	
	Control	**Cre +**	**Control**	**Cre +**	**Control**	**Cre +**	**Control**	**Cre +**
Creatinine (mg/dL)	0.080 ± 0.004 (6)	**0.108 ± 0.009 (6)***	0.105 ± 0.003 (4)	**0.148 ± 0.01 (4)***	N.D.	N.D.	N.D.	N.D.
GFR (µl/min)	269± 15 (8)	**206 ± 12 (8)****	266 ± 14 (8)	**200 ± 18(7)***	321 ± 16 (7)	306 ± 26(7)	247 ± 2 (8)	238 ± 28 (7)

Data: Mean ± SEM (N). ** p < 0.01; * p < 0.05. N.D., not determined.

Supplemental Table 2. Urinary pH and excretion rates of control and ApoE-Cre, Pax8-rtTACre, Ksp-Cre and Villin-Cre animals bred to *Lrrc8a*$^{lox/lox}$ mice, access to water *ad libitum*.

	ApoE-Cre;*Lrrc8a*$^{lox/lox}$		Pax8-rtTACre;*Lrrc8a*$^{lox/lox}$		Ksp-Cre;*Lrrc8A*$^{lox/lox}$		Villin-Cre;*Lrrc8a*$^{HAlox/HAlox}$	
Urine	**Cre -**	**Cre +**	**Cre -**	**Cre +**	**Cre -**	**Cre +**	**Cre -**	**Cre +**
pH	6.26 ± 0.13 (9)	**5.57 ± 0.10** (10) **	5.80 ± 0.13 (10)	5.49 ± 0.06 (9)	6.46 ± 0.12 (10)	6.13 ± 0.12 (8)	6.23 ± 0.06 (10)	**5.87 ± 0.07** (10) **
Na^+ (µmol/day)	193.5 ± 14.1 (10)	242.5 ± 23.5 (10)	135.3 ± 26.3 (6)	**270.6 ± 13.9** (9) **	233.9 ± 20 (10)	239.7 ± 20.7 (10)	218.2 ± 26.6 (10)	295 ± 35.9 (10)
K^+ (µmol/day)	420.4 ± 42.3 (10)	579.9 ± 70.6 (10)	585.9 ± 108.6 (6)	706.2± 55.1 (9)	579.2 ± 59.3 (10)	634.9 ± 70.9 (10)	639.9 ± 82.1 (10)	787.5 ± 92.6 (10)
Cl^- (µmol/day)	291.3 ± 30.9 (10)	370.4 ± 46.5 (10)	378.3 ± 78.8 (6)	439.8 ± 36.4 (9)	384.3 ± 42.7 (10)	386.7 ± 43.1 (10)	402.9 ± 51.8 (10)	524.8 ± 60 (10)
Pi (µmol/day)	73.8 ± 8.0 (10)	96.5 ± 10.0 (10)	96.2 ± 25.4 (6)	110.6 ± 12.9 (9)	67.8 ± 7.7 (10)	**98.2 ± 4.8**** (10)	92.9 ± 5.9 (10)	107.1 ± 13 (10)
Urea (µmol/day)	1768 ± 138 (10)	2317 ± 260 (10)	2529 ± 427 (6)	2991 ± 209 (9)	2464 ± 211 (10)	2618 ± 243 (10)	2675 ± 330 (10)	3133 ± 370 (10)
Uric Acid(µmol/day)	1.1 ± 0.1 (10)	1.1 ± 0.2 (10)	0.9 ± 0.1 (6)	0.6 ± 0.1 (9)	1.2 ± 0.1 (10)	1.1 ± 0,1 (10)	1.1 ± 0.1 (10)	1.0 ± 0.1 (10)
Glucose (µmol/day)	3.1 ± 0.3 (10)	**49 ± 11.7** (10) ***	4.2 ± 0.8 (6)	**43.4 ± 12.4 (9) ***	3.4 ± 0.3 (10)	3.6 ± 0.4 (10)	7.6 ± 1.7 (10)	**18.6 ± 2.6** (10) **
Creatinine (µmol/day)	2.5 ± 0.1 (10)	2.50 ± 0.3 (10)	1.8 ± 0.3 (6)	2.2 ± 0.2 (9)	2.9 ± 0.2 (10)	3.2 ± 0.2 (10)	2.5 ± 0.3 (10)	2.7 ± 0.4 (10)
Ammonia (µmol/day)	28.71 ± 3.6 (9)	37.23 ± 4.7 (8)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Data: Mean ± SEM (N). *** p < 0.001;** p < 0.01; * p < 0.05. N.D., not determined.

Supplemental Table 3. Blood and serum biochemical parameters after 24 h water deprivation of control, ApoE-Cre, Pax8-rtTACre, Ksp-Cre and Villin-Cre animals bred to *Lrrc8a*$^{lox/lox}$ mice.

	ApoE-Cre;*Lrrc8a*$^{lox/lox}$		Pax8-rtTACre;*Lrrc8a*$^{lox/lox}$		Ksp-Cre;*Lrrc8A*$^{lox/lox}$		Villin-Cre;*Lrrc8a*$^{HAlox/HAlox}$	
Blood	**Control**	**Cre +**	**Control**	**Cre +**	**Control**	**Cre +**	**Control**	**Cre +**
Hematocrite (%PCV)	48.8 ± 0.7 (10)	50.0 ± 1 (7)	48.2 ± 0,4 (10)	47,7 ± 1,1 (6)	48,0 ± 0,8 (10)	48.9 ± 0.7 (10)	47.5 ± 0.8 (8)	47,1 ± 1,0 (7)
Na^+ (mM)	167.7 ± 1 (10)	**177.7 ± 0.8 (7)*****	168.7 ± 1,3 (10)	**174 ± 1.9 (7)***	168.1 ± 2 (10)	166.8 ± 1.4 (10)	165.0 ± 2.3 (8)	169.1 ± 1.8 (8)
K^+ (mM)	3.5 ± 0.1 (10)	3.6 ± 0.1 (7)	3.5 ± 0.1 (10)	3.2 ± 0.1 (6)	3.8 ± 0.1 (10)	3.5 ± 0.1 (10)	3.4 ± 0.1 (8)	3.5 ± 0.1 (7)
Cl^- (mM)	127.3± 0.8 (10)	**>140 (7)*****	128.3± 1 (10)	**135 ± 2.2 (6)***	128.4 ± 1.9 (10)	128.3 ± 1.1 (10)	126.5± 1.5 (8)	128.6 ± 1.5 (7)
pH	7.12 ± 0.02 (10)	**6.99 ± 0.03 (9)****	7.15 ± 0.02 (10)	7.06 ± 0.04 (7)	7.15 ± 0.02 (9)	7.13 ± 0.01 (10)	7.14 ± 0.03 (8)	7.13 ± 0.02 (7)
HCO_3^-(mM)	22.3 ± 1.4 (10)	**17.4 ± 0.6 (9)****	23.4 ± 0.4 (10)	**20.2 ± 1.2** (7)*	23.8 ± 0.7 (9)	**21.8 ± 0.5 (10)***	21.1 ± 1.3 (8)	21.1 ± 1.0 (7)
pCO_2 (mm Hg)	67.9 ± 3.9 (10)	71.5 ± 3.9 (9)	68.0 ± 2.7 (10)	71.6 ± 5.4 (7)	68.3 ± 2.5 (9)	65± 1.6 (10)	62.9 ± 4.9 (8)	63.5 ± 2.7 (7)
Urea (mM)	18.54 ± 1 (10)	18.75 ± 1 (6)	18.3 ± 1 (10)	17.1 ± 2 (6)	15.9 ± 0.8 (10)	14.3 ± 0.9 (10)	14 ± 0.8 (8)	17.2 ± 1,7 (7)
Glucose (mg/dl)	141 ± 9 (10)	112 ± 14 (9)	189 ± 15 (10)	133 ± 20 (7)	145 ± 13 (10)	160 ± 14 (10)	166 ± 18 (10)	144 ± 12 (8)
Calculated Osmolarity (mosm/kg)	362 ± 7.4 (10)	**384 ± 11.4 (9)*****	364 ± 2.6 (10)	372 ± 4.4 (10)	360 ± 4.1 (10)	357 ± 2.7 (10)	353 ± 4.7 (8)	363 ± 3.7 (8)

Data: Mean ± SEM (N). *** $p < 0.001$;** $p < 0.01$; * $p < 0.05$.

Supplemental Table 4. Urine parameters in *Lrrc8b*, - *Lrrc8*c, and *Lrrc8*e KO and control littermates, with access to water *ad libitum.*

	Lrrc8b		Lrrc8c		Lrrc8e	
Urine	**WT (n=3)**	**KO (n=3)**	**WT (n=3)**	**KO (n=3)**	**WT (n=4)**	**KO (n=4)**
pH	6.09 ± 0.2	5.91 ± 0.4	5.92 ± 0.2	5.45 ± 0.1	6.25 ± 0.2	6.20 ± 0.2
Na^+ (mM) / Crea (mM)	74.4 ± 9	92.8 ± 13	66.5 ± 4	60.4 ± 12	65.0 ± 14	74.8 ± 19
K^+ (mM) / Crea (mM)	166.9 ± 34	181.2 ± 50	92.4 ± 13	99.6 ± 30	120.2 ± 31	122.3 ± 17
Cl^- (mM) / Crea (mM)	99.6 ± 25	131.2 ± 27	66.3 ± 14	91.5 ± 23	72.3 ± 19	89.2 ± 19
Pi (mM) / Crea (mM)	19.8 ± 7	30.9 ± 16	18.5 ± 3	26.3 ± 6	20.8 ± 5	24.4 ± 5
Urea (mM) / Crea (mM)	567 ± 77	626 ± 109	321 ± 33	335 ± 38	479 ± 111	417 ± 48
Uric Acid (mM) / Crea (mM)	0.38 ± 0.02	0.49 ± 0.01	0.36 ± 0.1	0.40 ± 0.1	0.34 ± 0.03	0.39 ± 0.02
Glucose (mM) / Crea (mM)	0.92 ± 0.1	1.29 ± 0.2	0.63 ± 0.1	0.79 ± 0.2	0.81 ± 0.1	0.96 ± 0.1
Creatinine mM	1.45 ± 0.2	1.73 ± 0.5	1.36 ± 0.3	1.98 ± 0.3	2.50 ± 0.9	2.32 ± 0.3

Data: Mean ± SEM, No statistical differences were found.

Supplemental Table 5. Blood parameters of *Lrrc8b*, *Lrrc8*c, and *Lrrc8*e KO and control littermates, with access *to* water *ad libitum*.

	Lrrc8b		Lrrc8c		Lrrc8e	
Blood	**WT (n=3)**	**KO (n=3)**	**WT (n=3)**	**KO (n=3)**	**WT (n=4)**	**KO (n=4)**
Hematocrite (%PCV)	35.7 ± 2.33	40 ± 1.5	38.7 ± 0.88	41 ± 0.6	38.7 ± 0.96	38.3 ± 1
Na^+ (mM)	151 ± 0.58	150.7 ± 1.9	149 ± 0.88	149.7 ± 0.7	151 ± 1.09	149.5 ± 1.2
K^+ (mM)	3.6 ± 0.19	3.4 ± 0.2	3.8 ± 0.15	3.3 ± 0.1	3.8 ± 0.21	3.7 ± 0.1
Cl^- (mM)	117.0 ± 1	119.3 ± 0.9	116.7 ± 0.33	116 ± 0.6	119.5 ± 0.65	119.3 ± 0.9
pH	7.23 ± 0.01	7.19 ± 0.03	7.17 ± 0.01	7.19 ± 0.02	7.13 ± 0.02	7.17 ± 0.04
HCO_3^- (mM)	22.7 ± 0.89	20.7 ± 1.1	19.5 ± 0.26	21.3 ± 1.3	18.2 ± 1.37	20.2 ± 1.4
pCO_2 (mm Hg)	53.7 ± 2.17	54.2 ± 2.5	54 ± 1.7	55.7 ± 2.5	56.9 ± 4.42	55.6 ± 2.6
Urea (mM)	9.7 ± 0.75	9.5 ± 1.3	6.6 ± 0.1	8 ± 0.6	8.9 ± 1.05	8.2 ± 0.6
Glucose (mg/dl)	207.7 ± 19.4	211.7 ± 18.4	246 ± 7.51	182.3 ± 5	208.3 ± 26.5	225.8 ± 5.5

Data: Mean ± SEM, No statistical differences were found.

Supplemental Table 6. Urinary pH and excretion rates of control and *Lrrc8d* KO mice, access to water *ad libitum*..

	Lrrc8d	
Urine	**+/+**	**-/-**
pH	6.45 ± 0.11 (9)	6.27 ± 0.07 (10)
Na^+ (µmol/day)	199.5 ± 19.4 (10)	257.5 ± 25.8 (10)
K^+ (µmol/day)	562.3 ± 45.6 (10)	699.6 ± 52.2 (10)
Cl^- (µmol/day)	372.0 ± 28.2 (10)	445.5 ± 36.8 (10)
Pi (µmol/day)	83.6 ± 8.5 (10)	102.9 ± 7.7 (10)
Urea(µmol/day)	2356.4 ± 130.2 (10)	2741.5 ± 194.5 (10)
Uric Acid (µmol/day)	1.6 ± 0.4 (10)	1.1 ± 0.1 (10)
Glucose (µmol/day)	3.2 ± 0.3 (10)	**20.1 ± 4.9** (10) *******
Creatinine (µmol/day)	2.3 ± 0.3 (10)	2.9 ± 0.2 (10)

Data: Mean ± SEM (N). *** $p < 0.001$.

Supplemental Table 7. Blood parameters after 24h water deprivation of control and *Lrrc8d* KO mice. .

	Lrrc8d	
Blood	**+/+**	**-/-**
Hematocrite (%PCV)	48.2 ± 1.7 (10)	50.8 ± 1 (9)
Na^+ (mM)	164.4 ± 1.6 (10)	**169.2 ± 1.2 (10)***
K^+ (mM)	4.1 ± 0.35 (10)	3.7 ± 0.2 (10)
Cl^- (mM)	123.5± 1.4 (10)	**127 ± 1.1 (10)***
pH	7.11 ± 0.01 (8)	**7.07 ± 0.02 (10)***
HCO_3^-(mM)	23.7 ± 1.1 (8)	22.1 ± 0.8 (10)
pCO_2 (mm Hg)	73.7 ± 2.3 (8)	77.3 ± 3.7 (10)
Urea (mM)	16.7 ± 1.5 (9)	17.3 ± 1.9 (10)
Glucose (mg/dl)	128.5 ± 13.6 (10)	120.6 ± 13.1 (10)
Calculated Osmolarity (mosm/kg)	356 ± 5.3 (10)	357 ± 6.34 (10)

Data: Mean ± SEM (N). * $p < 0.05$.

Supplemental Table 8: Additional antibodies used throughout the study..

Antibody	Supplier	Application
Guinea pig Barttin	Jentsch lab	IF
Sheep THP	RD system (AF5175)	IF
Mouse β-actin	Sigma (A2228)	WB
Guinea pig Pendrin	Carsten A. Wagner lab	WB and IF
Rabbit ICAM1	Proteintech (10020-1-AP)	IF
Rat CD102	BD Pharmingen (553325)	IF
Guinea pig AE1	Carsten A. Wagner lab	IF
Rabbit NKCC2	Sebastian Bachman lab	WB
Rabbit NCC	Millipore (AB3553)	WB
Rabbit pThr-NCC	PhosphoSolutions (p1311-53)	WB
Rabbit KCC3	Jentsch lab	WB
Rabbit KCC4	Jentsch lab	WB
Guinea pig KCC4	Jentsch lab	IF
Rabbit NBCE1	BioScience (LBB14890)	WB
Rabbit NaPiII	Heini Murer Lab	WB
Rabbit NHE3	Chemicon (AB3085)	WB
α-ENaC	Johannes Loffing Lab	WB
γ-ENaC	Johannes Loffing Lab	WB
Mouse Villin	Proteintech/Acris (66096-Ig)	WB
Rabbit ClC-5	Jentsch lab	WB
Rabbit SGLT1	Biozol (LS-C332376)	WB
Rabbit SGLT2	Novusbio (NBP1-92384)	WB
Rabbit GLUT2	Bernard Thorens Lab	WB
Rabbit AQP1	Millipore (AB2219)	WB
Rabbit AQP2	Thermo Fisher (PA5-38004)	WB
Rabbit pS269 AQP2	Robert Fenton Lab	WB
Goat Transferrin	Immunovision (EJ2-0027)	WB
Goat Albumin	Biotrend (2006130+2)	WB
Rabbit DBP	Biogenesis (9580-2710)	WB
Rabbit RBP	Bill Blaner Lab	WB
Rabbit Ki67	Abcam (Ab15580)	IF
Mouse α-Tubulin	Sigma (T6199)	WB

IF=Immunofluorescence, WB= Western blot

SUPPLEMENTAL METHODS

Transmission EM: Deeply anesthetized mice were perfused retrogradely via the aorta with rinsing fluid (0.1M Na-cacodylate buffer added with 3% hydroxyethyl starch) followed by fixation fluid (3% paraformaldehyde in 0.1M Na-cacodylate buffer plus 3% hydroxyethyl starch). Kidneys were collected in post-fixed in 1.5% paraformaldehyde/1.5% glutaraldehyde in 0.1M cacodylate buffer overnight. After washing in cacodylate buffer, tissue was osmificated in 1% osmium tetroxide and 1.5% potassium cyanoferrat (III) in water followed by washing in 1% aqueous uranyl acetate. After dehydration in methanol gradients, tissue was infiltrated by epoxy resin with the help of propylene oxide and embedded in pure epoxy resin. Following polymerization, tissue was trimmed and sectioned. Ultrathin sections were imaged at a Zeiss EM 906 transmission electron microscope equipped with a 2K digital camera (TRS Tröndle, Moorenweis).

Measurement of tissue osmolarity, urea, and electrolyte concentration: Kidney IM/papillas from 3 mice were dissected and pooled and the weight was recorded. The tissue was dried under vacuum overnight. The lyophilized tissue was weighed and then boiled in 200 µl of ultrapure H_20 for 1h and centrifuged for 10 min at 13000g. The supernatant was used to measure osmolarity, urea, and electrolyte concentration. Tissue osmolarity was calculated as: (osmolality supernatant x volume water added) / (weight of wet tissue - weight of dry tissue). Na^+ and K^+ concentrations were determined by ICP-MS, the analysis of Cl^- was by potentiometry (ion-selective electrode, Roche Diagnostics) and urea by a kinetic UV test (Urease-GLDH-method, Roche Diagnostics). Analyses were performed by Synevo Central Labs (Germany).

Metabolomics: 50 mg of kidney cortex were transferred in a vial containing Zilicia Beads and 200 µl of MCW (Chloroform/MeOH/Water, 2/5/1, v/v/v including cinnamic acid 2µg/ml). Samples were homogenized 2x 6500 rpm for 20 s. Samples were transferred in new vials and the remaining pellets with the beads were incubated with 800 µl MCW and rigorously vortexed. The supernatant was transferred in the sample collection vial, 500 µl of water were added to the samples and the vials were shaken for 1 hour at 4°C at 1400 rpm, 260µl of the upper polar phase were transferred into a new vial and dried under vacuum using a speed vac. All solvents were of highest purity (LC-MS grade). Samples were derivatised for GC-MS analysis and measured on a PegasusIV (LEGO) as described[1]. Standards for compound identification were used as described[2] The retention index for 1,5-Anhydro-glucitol was additionally validated. MetMax software[3] was used to create the data matrix for further statistical analyses.

Gluthatione/GSSG determination: Collected whole kidneys were weighed and immediately homogenized in ice-cold 3% sulfosalycilic acid, 10 mM EDTA. After centrifugation, deproteinized supernatants were diluted in 0.1 M potassium phosphate buffer, neutralized with triethanolamine (TEA) to determine total glutathione (GSH + GSSG). GSSG was determined by incubating the deproteinized supernatant with 2-Vynilpiridine and TEA for 1h. Both total glutathione and GSSG were determined following the Tietze enzymatic recycling assay.

Ki67 quantification: Pictures from the whole kidney cortex were taken, and Ki67 positive cells were manually counted.

REFERENCES SUPPLEMENTAL MATERIAL

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