Gitelman-like syndrome caused by pathogenic variants in mtDNA

Viering et al.

Supplementary material

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Supplementary methods

Determination of heteroplasmy

For determination of heteroplasmy levels, DNA was isolated from fibroblasts using the Qiagen QIAamp DNA mini kit (#51306) and/or whole blood by standard clinical procedures. Heteroplasmy percentage of the identified candidate variants was determined by analysis with single molecule Molecular Inversion Probes (smMIPs) in whole blood (for families 1, 2, 4, 6, 8) and in fibroblasts (for families 3, 4, 6, 11). In short, smMIPs were incubated with 200 ng gDNA for 16 hours for hybridization, extension and ligation. This was followed by exonuclease treatment, amplification, purification and sequencing on a NextSeq500 (Illumina) with a 2x150 bp paired-end run. Data-analysis was done with SeqNext (JSI). smMIP sequences are available in Supplementary Table 1. Read depth and variant percentage for individuals 5.II.6 and 6.II.2 is based on exome sequencing data. For family 10 and 12 this is based on long-read sequencing with the Sequel (Pacific Biosciences) and for individual 11.II.1 on the multi-gene panel results. Heteroplasmy measurements in family 13 were performed on DNA isolated from peripheral-blood leukocytes, saliva, urinary epithelial cells and fibroblasts, using labelled amplicons and tetra-primer amplification refractory mutation system PCR as described in (1). A variant was considered homoplasmic if coverage at the variant position was at least 300 and the percentage of reads with the variant was above 99%.

Statistical analysis of genetic data

Fisher's exact test to test the odds of observing a mtDNA variant 6 times (m.591C>T) or 3 times (m.4291T>C) in a cohort of 156 people while the population frequency was 1 in 304,823 or lower, was performed using R version 3.6.2 and the fisher.test() function. Corrected p-values were obtained by a Bonferroni correction for multiple testing, i.e., corrected p-values were calculated by multiplying the obtained p-value by 3 as we screened for three variants.

Fractional magnesium excretion

Fractional magnesium excretion (FEMg) was obtained at the same time as the serum magnesium measurements and was calculated with the following formula:

$FEMg = (magnesium_{urine} * creatinine_{serum})/(creatinine_{urine} * magnesium_{serum})*100.$

Thiazide test

Thiazide tests were performed in two families in conformity with previously described protocols (2, 3). Subjects of family 4 and 12 were allowed a small breakfast without coffee on the morning of the test, and a maximum of two sandwiches during the test. Baseline measurements (weight, blood pressure and first urine sample) were taken at 8 AM (t = 0). Subjects were then instructed to drink 10 mL/kg body weight in 15 minutes. A urine sample was again collected at t = 45 minutes, t = 90 and t = 120. At t =150, blood and urine samples were collected and 50 mg of hydrochlorothiazide was given orally. During this last phase, subjects were instructed to drink 250 mL water per hour until the end of the test. Urine was

collected every 30-60 minutes until *t* = 510. An additional blood sample was collected at *t* = 270 and *t* = 510. At *t* = 510 body weight and blood pressure were measured again, as well as serum electrolytes, before safely ending the test.
A similar protocol was used for the thiazide test in the proband of family 1. This patient was asked to fast from midnight until 11 AM. The subject voided all urine at 7 AM (*t* = 0) and she was instructed to drink 450 mL of water. At *t* = 60 minutes, 50 mg hydrochlorothiazide was given orally. Blood samples were collected at *t* = 60 (baseline) and *t* = 180, while urine samples were collected at *t* = 60 (baseline), *t* = 120, *t* = 180 and *t* = 240. From *t* = 60 until *t* = 240, the patient was asked to drink 200 mL per hour.
Calculation of the fractional chloride excretion (FeCl) was done using the formula $(Cl^-_{urine} * creatinine_{serum})/(creatinine_{urine} * Cl^-_{serum}) *100$. For determining the maximal change in FeCl, we subtracted the average of the FeCl at baseline from the highest FeCl measurement obtained in the six hours after the administration of hydrochlorothiazide.
Values obtained with the thiazide test were compared to reference values determined by Colussi et al.(3) and that have been used before in our center.(2, 4) Colussi et al.(3) reported a diagnostic sensitivity 93% and specificity of 100% for Gitelman syndrome. Later, it was shown to have a lower specificity when it is used to discriminate Gitelman syndrome from Gitelman-like syndromes.(4) Test sensitivity in Gitelman-like syndromes is currently difficult to assess due to the small number of patients and conflicting results,(3-5) but seems to be significantly lower than for Gitelman syndrome.

Fibroblasts
In families 3 and 4, fibroblasts were grown from a skin biopsy. In families 6 and 11, fibroblasts were obtained by nasal brush (Cytobrush Plus, Cooper Surgical, # 176291). After initial culturing of the material at local laboratories, further experiments were performed at the Radboudumc, Nijmegen. Fibroblasts were cultured in Medium 199 (M199) (#P04-07050, Pan Biotech) supplemented with 10% (v/v) fetal calf serum (#10270, Gibco), 100 IU/mL penicillin and 100 µg/mL streptomycin (#15140-122, Gibco) in a humidified atmosphere with 5% (v/v) CO_2 at 37°C. For starting up cultures, 20% (v/v) fetal calf serum was used instead of 10%. All cultures were mycoplasma negative. For all experiments, fibroblasts with passage number between 5 and 20 were used. In addition to one family control (unaffected relative on the paternal line, control 1), two control cell lines of unrelated individuals were included. Both had been shown to have normal mitochondrial function in earlier experiments. One fibroblast line was derived from a skin biopsy done at the Radboudumc, Nijmegen (control 2), the other was obtained commercially (ATCC® PCS-201-012™, lot # 61683453, from a 40-year-old woman, control 3). Cell lines were tested for mycoplasma contamination after arrival and upon defrosting.

OXPHOS activity measurement
Measurements of the activity of the mitochondrial oxidative phosphorylation (OXPHOS) complexes were performed in a clinically certified setting as described previously (6). In short,

mitochondria were isolated from ~10 million cells by mechanical pottering and differential centrifugation. OXPHOS activity of each of the five mitochondrial complexes was determined by the rate of rotenone-sensitive DCIP reduction (complex I), malonate-sensitive DCIP reduction (complex II), cytochrome c reduction (complex III), cytochrome c oxidation (complex IV) and oligomycin-sensitive NADH oxidation (complex V). Additionally, citrate synthase activity and protein concentration were measured. Citrate synthase activity was measured by a colorimetric method (formation of 5-thio-2-nitrobenzoic acid, TNB, from di-5-thio-2-nitrobenzoic acid, DTNB, when acetyl-CoA and oxaloacetate are converted to citrate), protein concentration was measured by the absorption at 600 nm after adding pyrogallol red-molybdate. Obtained values were compared to reference values specific for our center.

Mitochondrial respiration by the Seahorse XFe96 Analyzer

Fibroblasts were trypsinized and counted on a Luna-II automatic cell counter (Logos biosystems, with cell counting slides #L12003). 1.5 *10^4 cells were seeded per well (top and bottom rows were not used), and kept at 37°C, 5% (v/v) CO_2 for 20-24 hours. The Seahorse sensor cartridge was incubated overnight in calibrant buffer (Agilent, #102416-100). Seahorse XF base medium (Agilent #103334-100) was supplemented with L-glutamine (final concentration 2 mM, Life sciences), glucose (10 mM, Sigma) and pyruvate (1 mM, Gibco). Medium pH was set to 7.40 with NaOH (and if needed HCl) at 37°C and filtered (0.2 µm filter). 180 µL of warm medium was added to each well, including top and bottom rows. Plates were then incubated at 37°C, 0% CO_2 for 1-1,5 hour. After sensor calibration, cell plates were inserted in the Seahorse XFe96 analyzer and 16 measurements of OCR and ECAR (extracellular acidification rate) were done with 6-minute intervals: 3 minutes mixing and 3 minutes measuring. Directly after measurement 4 we injected oligomycin A (1 µmol/L final concentration, Sigma), after measurement 7 we injected 3 or 5 µmol/L FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, Sigma), after measurement 10 again FCCP (+1 µmol/L to obtain 4 and 6 µmol/L as final concentrations) and after measurement 13 rotenone 0.5 µmol/L (Sigma) and antimycin A 0.5 µmol/L (Sigma). To reduce inter-plate variation and differences between cell lines, within-plate titration was performed to identify the FCCP concentration at which respiration was maximally uncoupled.

Citrate synthase activity measurements for Seahorse XFe96 experiments

A modified version of the previously described protocol (7) was applied using DTNB (5,5'-dithiobis-2-nitrobenzoic acid; Sigma, #D8130). Seahorse medium was replaced by 20 µL of 0.33% (v/v) Triton X-100 (GE, #17-1315-01) in 10 mM Tris-HCl (pH 7.6), after which plates were stored at -80°C. Plates were subjected to in total 3 freeze-thaw cycles to interrupt all lipid bilayers. 150 µL with 0.17 mM DTNB, 0.035% Triton X-100 solution and 20 µL of 3 mM acetyl-CoA (#10101907001, Sigma) was added to each well. A Tecan Spark spectrophotometer (Tecan, Switzerland) measured background DTNB conversion for 9 minutes at 1-minute intervals (412 nm and 37°C). Subsequently, 10 µL of 10 mmol/L oxaloacetate (Sigma, #O4126) was added to each sample to start the reaction, and the same

measurement cycle was repeated immediately (412 nm and 37°C). Citrate synthase activity was calculated with the following formula:
Citrate synthase activity = ($DTNB_{conversion\ in\ presence\ of\ oxaloacetate}$ - $DTNB_{background\ conversion}$)/ (13.6 * 0.63) * (1/5)
The 13.6 indicates an extinction coefficient of 13.6/mmol/L/cm, the 0.63 the distance that the light travels through the solution in cm and the 1/5 that the reaction volume is $1/5^{th}$ of 1 mL (200 µL). The result is expressed in mU/mL and represents the citrate synthase activity of 1 confluent Seahorse XFe96 well dissolved in 1 mL.

Seahorse AnalyzeR
In short, a text file containing the normalized and background-subtracted OCR values was exported from Wave Desktop Software and analyzed in R, version 3.6.2 for Mac, using Rstudio version 1.1.456. The R-script depends on packages data.table (version 1.12.8) and tidyverse (1.3.0). OCR values < 0 were removed. For each well, the median OCR of each Mito Stress test interval was calculated. Subsequently, for each cell line, the average of all wells on one plate was calculated (*n* = 6-12). For interval 3, the concentration of the uncoupling agent FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, Sigma) resulting in the highest average OCR was selected. Then, all wells that did not respond correctly to the Mito Stress test were removed (i.e., not *interval 3 > interval 1 > interval 2 > interval 4*). Maximum respiration was obtained by subtracting the average OCR of interval 4 from the average OCR of interval 3. Lastly, by expressing the data as a percentage from the average of the three control cell lines on the plate, the effect of the inter-plate effect was reduced.

Preparation of buffers for $^{22}Na^+$ uptake and NCC phosphorylation experiments
Hypotonic-low-chloride buffer was prepared as 70 mmol/L sodium gluconate, 0.5 mmol/L calcium gluconate, 0.5 mmol/L magnesium chloride, 2.5 mmol/L potassium gluconate, 2.5 mmol/L HEPES (i.e., 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and set to pH 7.4 with Tris. Isotonic buffer was prepared as 140 mmol/L sodium chloride, 1 mmol/L calcium chloride, 1 mmol/L magnesium chloride, 5 mmol/L potassium chloride, 5 mmol/L HEPES and also set to pH 7.4 with Tris. Lysis buffer contained the following: 1 mmol/L EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid), 1 mmol/L EDTA (Ethylenediaminetetraacetic acid), 10 mmol/L sodium glycerophosphate, 50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 270 mmol/L sucrose, 150 mmol/L sodium chloride, 1 mmol/L sodium orthovanadate and 50 mmol/L Tris, set to pH 7.5 with hydrochloric acid. 1% (v/v) Triton X-100 and protease inhibitors (pepstatin 1 µg/mL, leupeptin 5 µg/mL, aprotinin 1 µg/mL and PMSF (phenylmethylsulphonyl fluoride) 1 mmol/L) were added fresh.

Effect of complex IV inhibition on NCC-mediated $^{22}Na^+$ uptake
HEK293 cells with passage number 10-20 were seeded on 24-well plates (#734-1604, Costar) coated with poly-L-lysin (#P2636, Sigma), at a density of 8 x 10^4 cells per well. We transfected each well with either 0.5 µg DNA construct containing NCC (pCIneo-NCC-IRES-GFP) or 0.5 µg

construct without NCC (pCIneo-IRES-GFP, hereafter indicated with mock). Transfection was done within 24 hours using lipofectamine (#11668019, Invitrogen; DNA:lipofectamine ratio 1:2) according to the manufacturer's protocol, including supplementation with opti-MEM (#31985-054, Gibco). After two days, transfection success was assessed by visually checking GFP expression under the microscope. Samples were subsequently put on hypotonic-low-chloride or isotonic buffer with or without 100 µmol/L thiazide, and with either 1 mmol/L potassium cyanide (KCN, experimental condition) or as a control 1 mmol/L potassium chloride (KCl). After half an hour incubation, samples were put on isotonic buffer containing both $^{22}Na^+$ and inhibitors of other sodium transporters and channels (i.e., amiloride 100 µmol/L, bumetanide 100 µmol/L and ouabain 1 mmol/L). After half an hour in the $^{22}Na^+$, cells were lysed in 500µL of 0.1% (w/v) SDS (sodium dodecyl sulfate) in PBS (phosphate buffered saline). 3.5 mL of Opti-Fluor (#6013199, Perkin Elmer) was added and after thorough mixing, radioactivity was measured on a liquid scintillation counter (Hidex 600SL). Radioactive buffers were also measured on the counter to exclude differences in $^{22}Na^+$ concentration between the different buffers. Samples were counted for 180 seconds with a coincidence time of 35 nanoseconds. Wavelength measurement window was set at 300-700nm. Ionizer delay was set to 10 seconds, measurement chamber delay to 5 seconds. NCC expression was assessed by immunoblotting, following the same protocol as described below. Culturing and the $^{22}Na^+$ uptake was done in triplicate, the complete experiment was performed four times.

NCC phosphorylation experiments

Culturing and transfection was done the same as for the $^{22}Na^+$ uptake experiments, with the following changes. Cells were seeded in 6-well plates at a density of 4 x 10^5 cells per well. Transfection was performed with 2 µg NCC or mock construct. Two days after transfection, samples were put on hypotonic-low-chloride or isotonic buffer with or without 100 µmol/L thiazide, and with either 1 mmol/L potassium cyanide (KCN, experimental condition) or as a control 1 mmol/L potassium chloride (KCl). Cells were lysed in 200 µL ice-cold lysis buffer. Culturing was done in duplicate; the complete experiment was performed three times.

NKCC2 phosphorylation experiments

HEK293 cells with passage number 10-14 were seeded on 10cm petri-dishes (Cellstar, #664160) coated with poly-L-lysin (#P2636, Sigma), at a density of 4 x 10^6 cells per dish. We transfected each well with either 14 µg DNA construct containing human NKCC2 isoform A (pCIneo-NKCC2-HA-IRES-GFP) or 14 µg construct without NKCC2 (pCIneo-IRES-GFP, hereafter indicated with mock). Transfection was done within 24 hours at 50-80% confluency using lipofectamine (#11668019, Invitrogen; DNA:lipofectamine ratio 1:2) according to the manufacturer's protocol, including supplementation with opti-MEM (#31985-054, Gibco). Samples were subsequently put on hypotonic-low-chloride or isotonic buffer with either 1 mmol/L potassium cyanide (KCN, experimental condition) or as a control 1 mmol/L potassium chloride (KCl) for 30 minutes. Cells were lysed in 1 mL ice-cold lysis buffer. Culturing was done in duplicate; the complete experiment was performed three times.

Measuring protein concentration

Protein concentration was measured by adding 200 µL of Bradford reagent (Sigma-Aldrich, #B6916-500ML) to 5 µL of diluted sample, measuring light absorbance at 595 nm with a Benchmark Plus microplate reader (Bio-Rad), and calculating protein concentration by comparing the absorbance to a standard curve of known protein concentrations (diluted albumin standard, ThermoFisher Scientific, #23209).

Immunoprecipitation (NKCC2 only)

Pierce anti-HA beads (Thermo Scientific, #26181) were washed 4 times with PBS, spinning the beads down at 1,600 rpm in between washes. 4.5 mg of the protein sample was added to 40 µL of 1:1 beads:PBS solution and incubated on a rotor overnight. Beads were washed 3 times with ice-cold lysis buffer, supernatant was removed and the beads were incubated with 30µL 2x Laemmli-dithiothreitol (DTT) buffer for 30 minutes at 37°C. The supernatant was subsequently used for SDS-PAGE immunoblotting.

Immunoblotting NCC

Equal amounts of protein were supplemented with Laemmli buffer (final concentration 1x) and dithiothreitol (final concentration 100 mmol/L). 25 µg of protein of each sample was subjected to SDS-PAGE (8% 40:1 bisacrylamide gel) and transferred to polyvinylidene membranes by blotting at 100 volts for 2 hours on ice. Membranes underwent blocking in 5% (w/v) non-fat dry milk dissolved in 1x Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 60 minutes at room temperature. Incubation of the membranes with primary antibody was done overnight at 4°C: rabbit anti-NCC (1:2000, Millipore, #AB3553), rabbit anti-pT58-NCC (NCC phosphorylated at human position p.Thr60, 1:2000, kind gift from Robert Fenton(8)). Specificity of antibodies was confirmed by the inclusion of a negative control in all experiments (i.e., HEK293 cells transfected with a mock construct instead of NCC construct, Supplementary Figure 3). Membranes were subsequently washed three times with TBS-T and incubated in secondary antibody for 1 hour at room temperature (peroxidase anti-rabbit-IgG, 1:10 000, Sigma Aldrich, #A4914). Three more washes with TBS-T were performed, after which proteins were visualized with chemiluminescent reagent (SuperSignal West femto/pico, Thermo Fisher Scientific, # 34095/#34078) on an ImageQuant LAS 4000 (Supplementary Figure 2). Densitometric analysis of band intensities was done using ImageJ Software (version 1.51).

Immunoblotting NKCC2

1 µL of PNGase F (NEB, P0704) was added to the Laemmli-DTT samples and incubated for 30 more minutes at 37°C. 10 µL of sample was subjected to SDS-PAGE (7% 40:1 bisacrylamide gel). Transfer to a membrane and blocking was performed the same as for NCC. Incubation of the membranes with primary antibody was done overnight at 4°C: sheep anti-NKCC2 (1 µg/mL, bought from MRC Dundee (contact: James Hastie), sheep anti-pS91-NKCC2 (NKCC2

phosphorylated at human position p.Ser91, 1 µg/mL + 10 µg/mL non-phospho-peptide, bought from MRC Dundee (contact: James Hastie). Specificity of antibodies was confirmed by the inclusion of a negative control in all experiments (i.e., HEK293 cells transfected with a mock construct). Membranes were subsequently washed three times with TBS-T and incubated in secondary antibody for 1 hour at room temperature (peroxidase anti-sheep-IgG, 1:10 000, Jackson ImmunoResearch, #213-032-177). Subsequent washing and imaging was the same as for NCC.

Software and websites used for genetic analyses

- OMIM: https://omim.org/. Identifiers of syndromes named in the text: Gitelman syndrome/biallelic pathogenic variants in *SLC12A3* (263800), biallelic pathogenic variants in *CLCNKB* (607364), monoallelic pathogenic variant in HNF1B (137920), monoallelic pathogenic variant in *FXYD2* (154020), biallelic pathogenic variant in *KCNJ10* (612780), monoallelic pathogenic variant in ATP1A1 (618314), biallelic pathogenic variants in EGF (611718), HUPRA syndrome (613845), Kearns-Sayre syndrome (530000), congenital myopathy and gastro-intestinal pseudo-obstruction (613662), hypercholesterolemia with hypertension (500005), MELAS (540000), MT-TI associated cardiomyopathy (*590045), Leigh syndrome (256000), nonsyndromic hearing loss (500008).
- MITOMAP: A Human Mitochondrial Genome Database. http://www.mitomap.org, 2021. A database of mitochondrial variation based on GenBank. Accessed on June 28, 2021. The database at that moment contained 51 836 full sequences from GenBank. As a caveat, sequences from GenBank might not be of equal quality and are considered enriched for patients with mitochondrial disease.
- HelixMTdb: https://www.helix.com/pages/research. A population-based database of mitochondrial variation -accessed on June 28, 2021- contained sequences from 196,554 unrelated individuals.
- Gnomad: https://gnomad.broadinstitute.org/. Accessed June 28, 2021, version 3.1.1. A population-based database of mitochondrial variation -accessed on June 28, 2021- contained sequences from 56,434 unrelated individuals.
- MitoTIP: accessible via https://www.mitomap.org/mitomaster/index_snvs.cgi, accessed on June 28, 2021. Predicts pathogenicity of mitochondrial tRNA variants based on variant frequency in GenBank, annotations of pathogenicity from MITOMAP, conservation across species, the position of the variant within the tRNA and the type of nucleotide change (transition/transversion).
- PON-mt-tRNA: http://structure.bmc.lu.se/PON-mt-tRNA/. Accessed on June 28, 2021. Machine-learning based predictor of pathogenicity of mitochondrial tRNA variants. This algorithm is not influenced as much by GenBank records or literature reports on the variant.
- Clustal O: https://www.ebi.ac.uk/Tools/msa/clustalo/ for aligning the sequence of *MT-TI* and *MT-TF* of the 10 selected species. Default settings were used, and small manual alterations were done to improve alignment. Used accession numbers of the mitochondrial genome, from Homo sapiens to Drosophila melanogaster in the same

order as figure 2: NC_012920.1, NC_001643, NC_001644, NC_002082, NC_005089, NC_001665.2, NC_006853.1, NC_040970.1, NC_002081, NC_024511.2.

- rtools: http://rtools.cbrc.jp/cgi-bin/index.cgi for modelling of tRNA secondary structure with CentroidHomFold. Default settings were used (inference engines: *McCaskill(BL)* and *CONTRAlign*, base pair weight: *2^3*, E-value for homology search against Rfam: *0.01*, number of homologous sequences: *30*). Accessed 5 September 2020.
- R version 3.6.2 for Mac, using Rstudio version 1.1.456.
    - The code written for analysis of Seahorse XFe96 data has been made publicly: https://github.com/DaanViering/Seahorse-analyzeR. Version 1.2 of date 26-03-2021 was used.
    - Code for Fisher's exact test:
      > x <- data.frame(c(6,156), c(1,304824))
      > fisher.test(x)
- MtHap: For mitochondrial haplogroup determination. https://dna.jameslick.com/mthap/
- ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/. Variant classifications were submitted to ClinVar in July 2021, accession numbers: SCV001745859, SCV001739513, SCV001745860, SCV001745835

Supplementary table 1: primer and smMIP sequences

Nucleotide sequences of the primers and single molecule Molecular Inversion probes (smMIPs) used. Start and end indicate positions in the mitochondrial genome.

Primer/smMIP	Sequence	Start	End
MT-TF sequencing primer forward	ACCCTAACACCAGCCTAACCA	368	388
MT-TF sequencing primer reverse	GCTTGTTCCTTTTGATCGTGGTG	734	756
MT-TI sequencing primer forward	GTCACCAAGACCCTACTTCTAACC	4084	4107
MT-TI sequencing primer reverse	GCTGTGATGAGTGTGCCTGC	4512	4531
MT-TF smMIP 1	GGTTTGGTCCTAGCCTTTCNNNNNNNNCTTCAGC TTCCCGATATCCGACGGTAGTGTACACCGCTGCTA ACCCCATAC	542	651
MT-TF smMIP 2	AGGATGGGCGGGGGTTGTATTGNNNNNNNNCT TCAGCTTCCCGATATCCGACGGTAGTGTGATGTG AGCCCGTCTAAA	508	617
MT-TF smMIP 3	AAGCTACATAAACTGTGGNNNNNNNNCTTCAGC TTCCCGATATCCGACGGTAGTGTAACTCACTGGA ACGGGGATGCT	590	699
MT-TI smMIP 1	AATCCAAAATTCTCCGTGCCNNNNNNNNCTTCAG CTTCCCGATATCCGACGGTAGTGTATTACAATCTC CAGCATTCC	4251	4360
MT-TI smMIP 2	GGTAGGAAGTTTTTTCATAGGANNNNNNNNCTT CAGCTTCCCGATATCCGACGGTAGTGTGTTTAAG CTCCTATTATT	4191	4300
MT-TI smMIP 3	CATATTTCTTAGGTTTGANNNNNNNNCTTCAGCT TCCCGATATCCGACGGTAGTGTCTTTAGGATGGG GTGTGATAGG	4272	4381

Supplementary Table 2: Age-specific reference for urinary calcium:creatinine ratio
Age-specific reference for urinary calcium:creatinine ratio. m, month; y, year.

Age group	Normal calcium/creatinine ratio (mmol/mmol)
1 m - 1 y	0.09 - 2.2
1 - 2 y	0.07 - 1.5
2 - 3 y	0.06 - 1.4
3 - 5 y	0.05 - 1.1
5 - 7 y	0.04 - 0.8
7 - 17 y	0.04 - 0.7
Adults	0.16-0.7

Supplementary Table 3: Full clinical data
All available relevant clinical data on patients included in this study. CKD, chronic kidney disease as defined by KDIGO; eGFR, estimated glomerular filtration rate; RBP, retinol-binding protein. Please find this table as a supplementary Excel attachment.

Supplementary Table 4: Additional variant information

Variant information obtained from different databases. For PON-mt-tRNA the cut-off for likely pathogenic is > 0.5, for MITOTIP, the cut-off is > 12.66. Higher scores denote a higher probability of pathogenicity. The sensitivity and specificity of MITOTIP are slightly higher than those of PON-mt-tRNA. The numbers written in brackets in the column headers represent the number of individuals included in each population database. Position indicates position of the variant on the mitochondrial genome. Ref, reference allele; Alt, alternative allele; VUS, variant of uncertain significance.

Position	**Ref**	**Alt**	**Gene**	**HelixMTdb** (196,554)	**gnomAD** (56,434)	**GenBank Seqs (MITOMAP)** (51,836)	**MITOTIP**	**PON-mt-tRNA**
591	C	T	*MT-TF*	Homoplasmic: 0 Heteroplasmic: 3	Homoplasmic: 0 Heteroplasmic: 1	0	3.2	0.11
616	T	C	*MT-TF*	Homoplasmic: 0 Heteroplasmic: 6	0	1	17.5	0.18
4291	T	C	*MT-TI*	0	0	0	10.0	0.34
643	A	G	*MT-TF*	Homoplasmic: 3 Heteroplasmic:1	0	0	11.27	0.59

Supplementary Table 5: Criteria for pathogenicity in each of the variants

Criteria are applied as proposed by Wong et al.(9) Position indicates position of the variant on the mitochondrial genome. References in this table.(1, 9-12) Ref, reference allele; Alt, alternative allele; VUS, variant of uncertain significance.

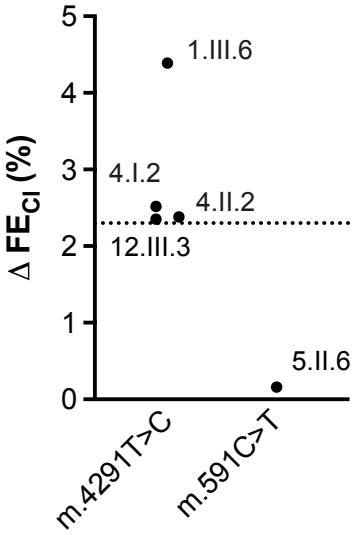
Position	Ref	Alt	Gene	Criteria for pathogenicity added based on this study	Criteria for pathogenicity previously met	References	Verdict
591	C	T	*MT-TF*	PS4, PM9, PM10, PP6, PP7, BP4	N.A.	None	**Pathogenic**
616	T	C	*MT-TF*	-	PS5, PM7, PM9, PM10	Connor et al. 2017, Wong et al. 2020, Riedhammer et al. 2020, Lorenz et al. 2020	**Pathogenic**
4291	T	C	*MT-TI*	PS4, PM9, PM10, PP6	PP7, PS5	Wilson et al. 2004, Roshan et al. 2012	**Pathogenic**
643	A	G	*MT-TF*	PM9, PM10	BS4, PP7	Wong et al. 2020	**VUS**

Supplementary Table 6: Number of phenylalanine and isoleucine residues in the mitochondrial genes

Number of phenylalanine and isoleucine residues in the mitochondrial genes, and their relative abundance in all mitochondrial genes.

Gene	Subunit	# of phenylalanine residues	# of isoleucine residues	# total residues	Ratio phenylalanine residues/total residues (%)	Ratio isoleucine residues/total residues (%)
ND1	I	16	22	318	5.0	6.9
ND2	I	15	31	347	4.3	8.9
ND3	I	8	9	115	7.0	7.8
ND4	I	20	41	459	4.4	8.9
ND4L	I	3	7	98	3.1	7.1
ND5	I	38	54	603	6.3	9.0
ND6	I	8	12	174	4.6	6.9
CYB	III	24	38	380	6.3	10.0
COX1	IV	41	38	513	8.0	7.4
COX2	IV	10	22	227	4.4	9.7
COX3	IV	23	14	261	8.8	5.4
ATP6	V	9	29	229	3.9	12.7
ATP8	V	1	3	68	1.5	4.4

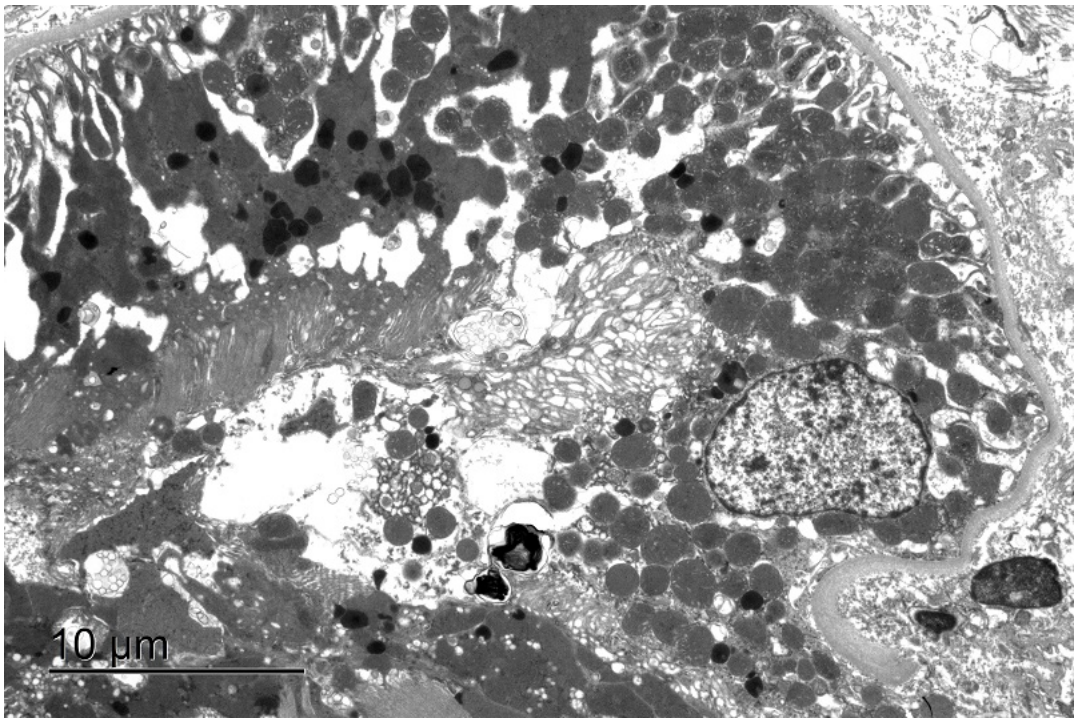
Supplementary Figure 1: Thiazide test



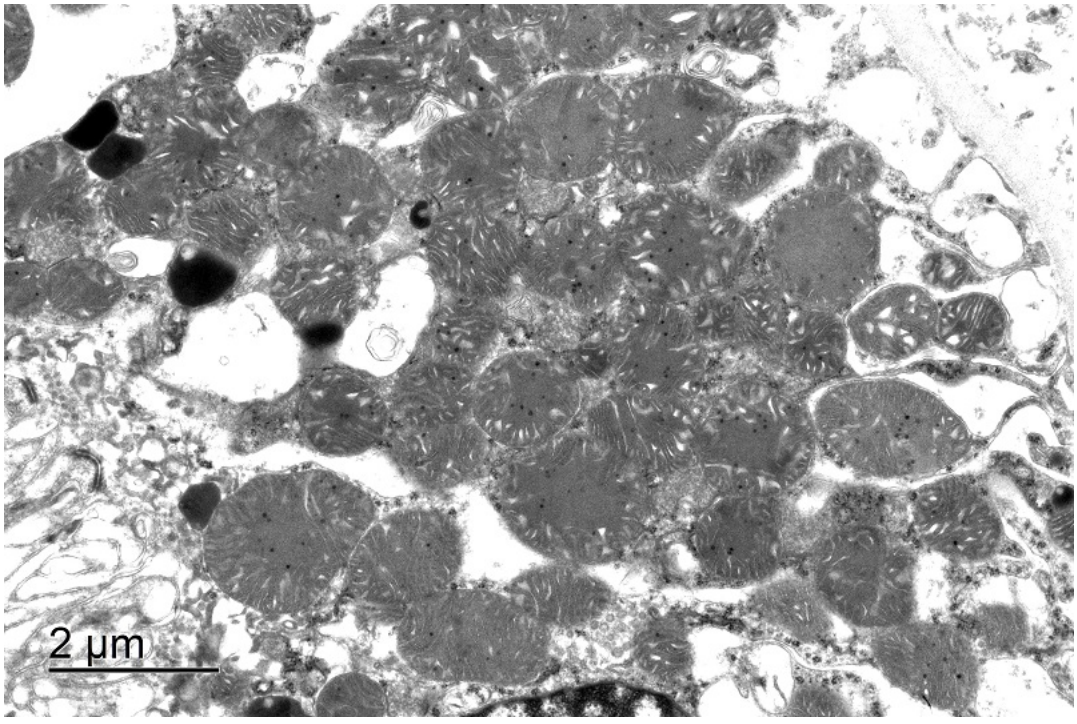
*Maximal change in the fractional excretion of chloride (**Δ** FE_{Cl}) after administration of a single dose of 50 mg hydrochlorothiazide orally. The followed protocols were slightly different per patient as described in the Supplementary Methods. The dotted line represents the cut-off value proposed by Colussi et al. (3) for Gitelman syndrome. Patients with Gitelman syndrome typically have a **Δ** FE_{Cl} values below this cut-off, i.e., below 2.3%.*

Supplementary Figure 2: Electron microscopy of proximal tubular cells

A

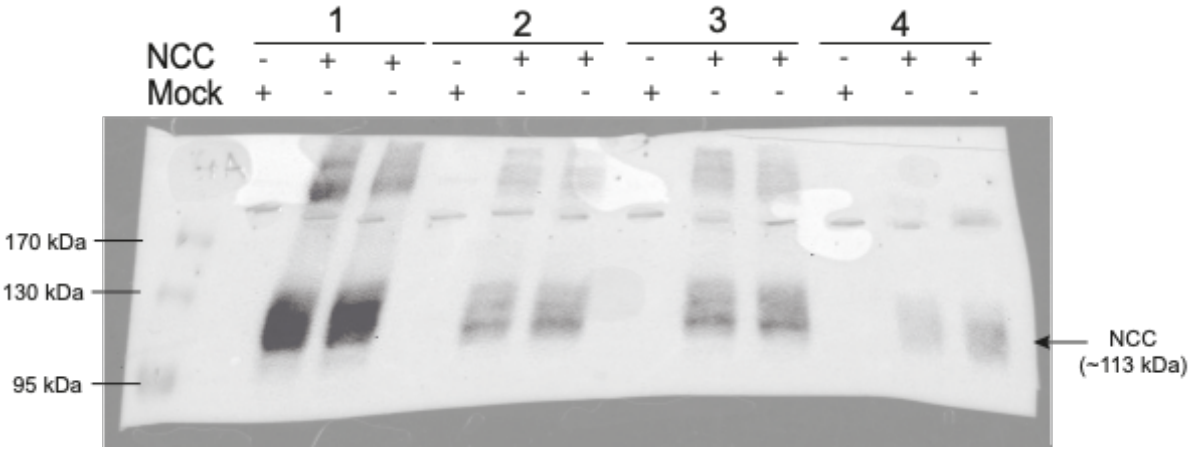


B



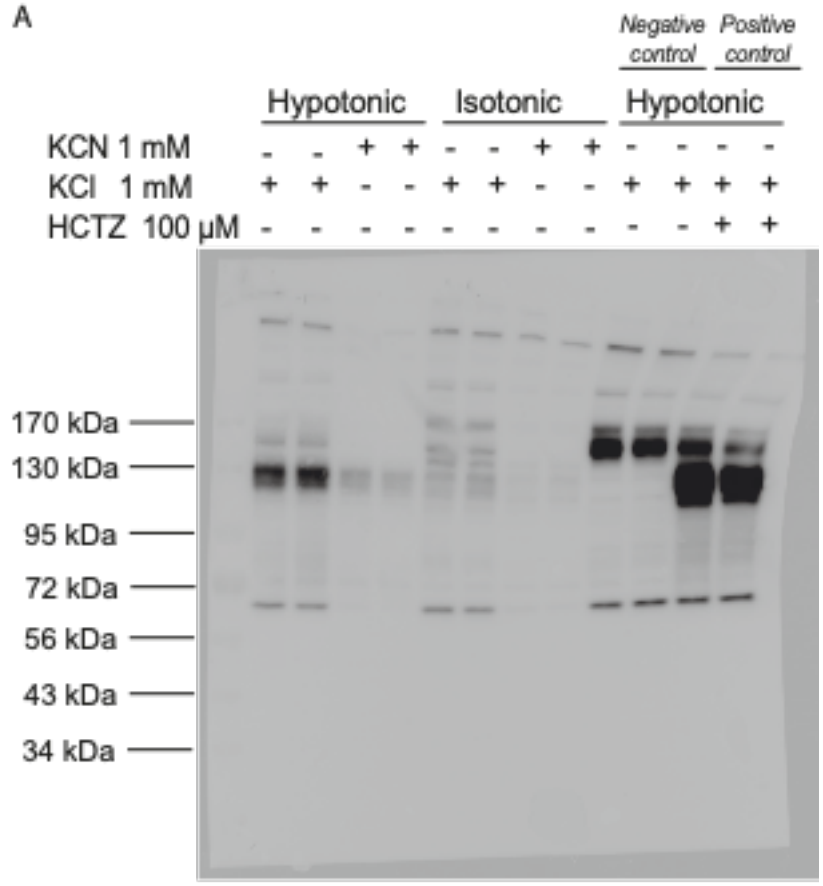
Examination of proximal tubular cells in the kidney biopsy of patient 10.II.3. (A) Note the apical brush border that is specific for the proximal tubule. Mitochondria look enlarged and abnormal in shape. (B) A close-up of part of panel A shows that most mitochondria exhibit widened cristae structures and sometimes partial absence of cristae. This contrasts with the distal tubular cells shown in Figure 4 D-E; there, cristae were not visible at all in most mitochondria.

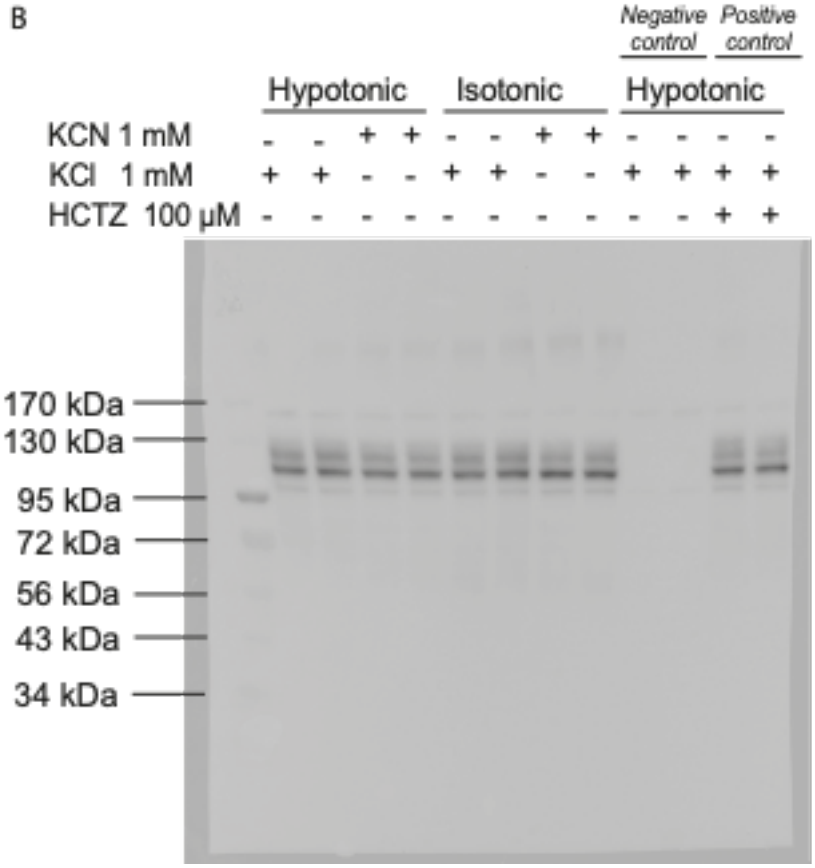
Supplementary Figure 3: Control immunoblots $^{22}Na^{+}$ uptake experiments



*Control immunoblots of the $^{22}Na^{+}$-uptake experiments in Figure 6. Numbers at the top indicate experiment number (*n *= 4*). *The legend indicates which HEK293 samples were transfected with NCC and which with mock. Size of NCC is 113 kDa, size of NCC dimers is 226 kDa. Primary antibody: rabbit anti-NCC (1:2000, Millipore, #AB3553); secondary antibody: peroxidase anti-rabbit-IgG (1:10 000, Sigma Aldrich, #A4914). The bands above at the top likely represent NCC dimers.*

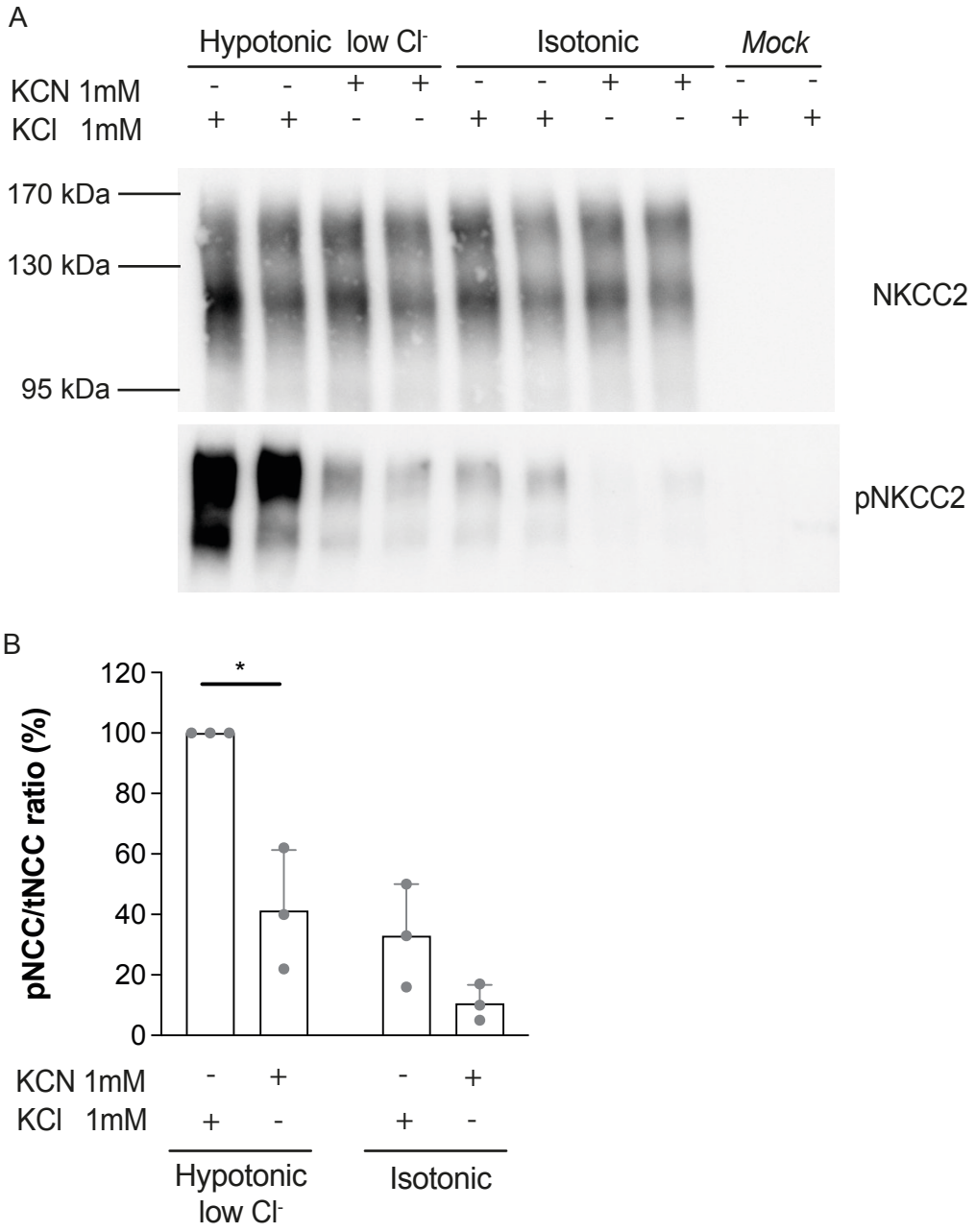
Supplementary Figure 4: Full immunoblot NCC phosphorylation experiments





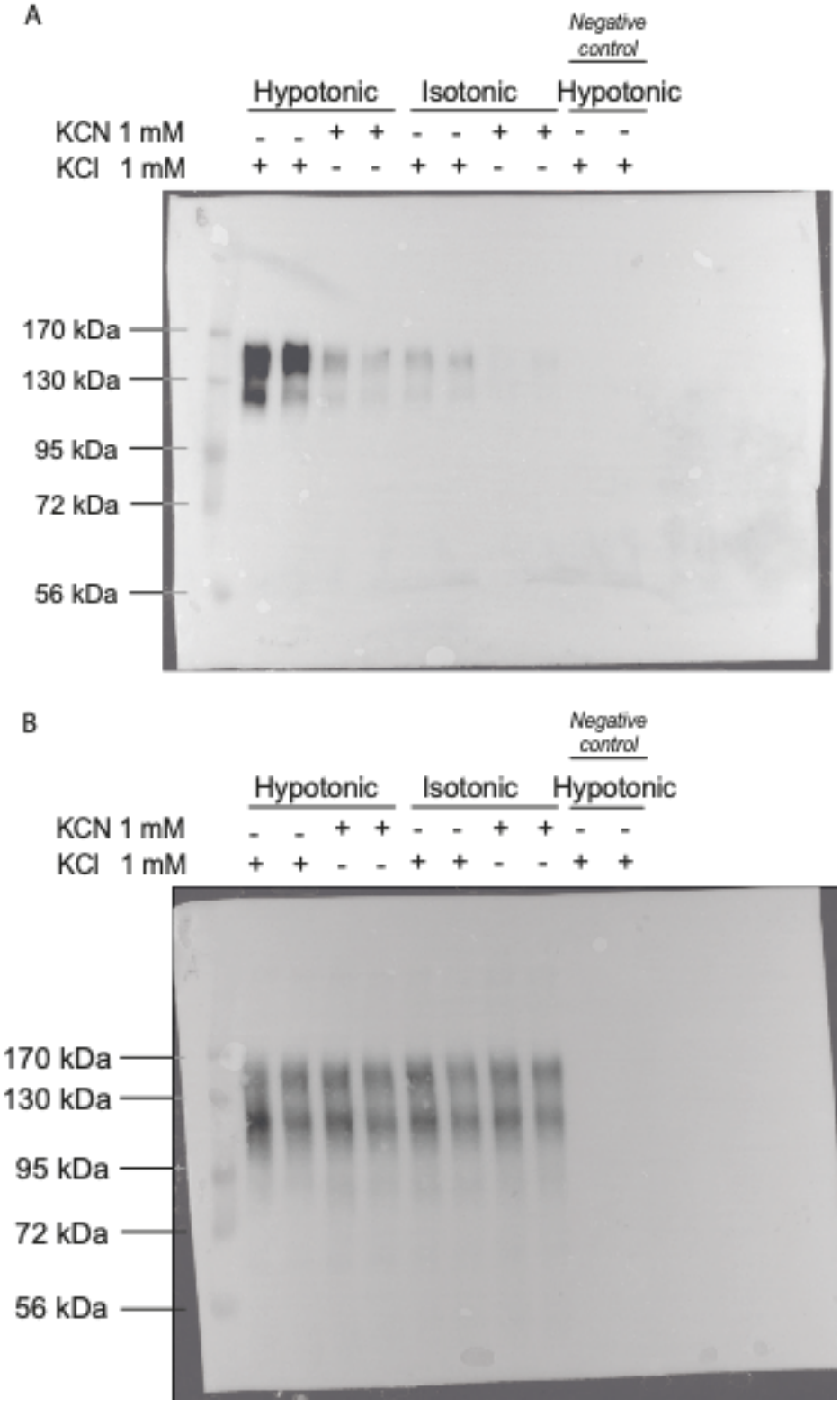
*Full immunoblot of a representative experiment on which Figure 6 and its conclusions are based. Every experimental condition was cultured and blotted in duplicate; every experiment was executed three times. Size of NCC is 113 kDa. Negative control denotes the condition transfected with a mock expression vector instead of NCC expression vector. (**A**) Primary antibody: rabbit anti-pT58-NCC (NCC phosphorylated at human position p.Thr60, 1:2000, kind gift from Robert Fenton(8)); secondary antibody: peroxidase anti-rabbit-IgG (1:10 000, Sigma Aldrich, #A4914). Some non-specific bands are visible, as can be seen in the mock condition. Some bands might represent pNKCC1 (~160-200 kDa), which is a close homologue of NCC and has a phosphorylation site at a similar position. (**B**) Primary antibody: rabbit anti-NCC (1:2000, Millipore, #AB3553); secondary antibody: peroxidase anti-rabbit-IgG (1:10 000, Sigma Aldrich, #A4914). The bands at the top likely represent NCC dimers.*

Supplementary Figure 5: NKCC2 phosphorylation with complex IV inhibition



*(**A**) Representative immunoblots showing NKCC2 and phosphorylated NKCC2 after a 30-minute incubation in hypotonic-low-chloride or isotonic buffer, with either KCN or KCl treatment. The mock condition has been incubated in hypotonic-low-chloride buffer as well. (**B**) Densitometry analysis of band intensity, pNCC/tNCC ratio (*n *= 3 of duplicates in each experiment). Significance was assessed with unpaired t-tests and corrected for multiple testing. KCN, potassium cyanide; KCl, potassium chloride; NKCC2, Na^+-K^+-Cl^--cotransporter; pNKCC2, NKCC2 isoform A phosphorylated at Ser91. *, p < 0.05.*

Supplementary Figure 6: Full immunoblot NKCC2 phosphorylation experiments



*Full immunoblot of a representative experiment on which Supplementary Figure 5 is based. Every experimental condition was cultured and blotted in duplicate; every experiment was executed three times. Size of NKCC2 isoform A is 121 kDa. Negative control denotes the condition transfected with a mock expression vector instead of NKCC2 expression vector. (**A**) Primary antibody: sheep anti-pS91-NKCC2 (NKCC2 phosphorylated at human position p.Ser91,*

*1 µg/mL + 10 µg/mL non-phospho-peptide); secondary antibody: peroxidase anti-sheep-IgG (1:10 000, Jackson ImmunoResearch, #213-032-177). The upper band might represent residual glycosylated NKCC2, despite treatment with PNGase F. (**B**) Primary antibody: sheep anti-NKCC2 (1 µg/mL); secondary antibody: peroxidase anti-sheep-IgG (1:10 000, Jackson ImmunoResearch, #213-032-177). The upper band might represent residual glycosylated NKCC2, despite treatment with PNGase F.*

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