A novel *AQP2* sequence variant causing aquaporin-2 retention in the cytoplasm and autosomal dominant nephrogenic diabetes insipidus

Supplementary:

Methods [9, S12-S13]

In addition to routine clinical nephrological diagnostics, a standard diagnostic water deprivation test was carried out under constant medical supervision. Blood and urine samples were collected every hour, and the well-being of the proband was monitored along with blood pressure and body weight. Stop criteria were a rise in serum sodium >150 mmol/L or loss of body weight > 3 %; otherwise, intranasal desmopressin 20 µg was administered, and observations continued for 2 h. A genetic analysis was performed. Written informed consent was obtained from the patient for publication.

Expression construct and Cloning

Human *AQP2* in pcDNA3.1+/C-(K) DYK was purchased from Genscript (Leiden, Netherlands, Plasmid Number: OHu109362) and used as a template to introduce the c.799 800; p.Arg267Glyfs*12 mutation that leads to a C-terminal extension of 6 amino acids (AQP2-MUT). AQP2-MUT was generated by site-directed mutagenesis using the QuickChange XL Kit (Agilent Technologies, Santa Clara, USA) with overlapping primers harboring the two base pair deletion that corresponds to positions 799 and 800 in the coding sequence of *AQP2* (forward primer sequence: 5'-CCGCAGAGCCTGCCAGGGTACCAAGGCCGA-3'; reverse primer sequence: 5'- TCGGCCTTGGTACCCTGGCAGGCTCTGCGG-3'). The product was digested with 1 µL Dpn I enzyme and transformed into DH5α competent cells (New England Biolabs, Massachusetts, USA). Clones were selected based on Colony PCR and sequenced using *AQP2* sequencing primers (forward primer: 5'-TAATACGACTCACTATAGGG-3'; reverse primer: 5'-TAGAAGGCACAGTCGAGG-3') by LGC Genomics (Berlin, Germany). For expression of the constructs in Madin-Darby canine kidney (MDCK.1) high resistance and Human embryonic kidney (HEK) 293 cells, the AQP2 wild type (AQP2-WT) and AQP2-MUT sequences were

cloned into the pCMV6-Entry vector using Nhe I and Xho I restriction sites (forward primer: 5'-CTGGCTAGCGTTTAAACTTAAGCTTG-3'; reverse primer AQP2-WT: 5'-CATTCTCGAGTTAGGCCTTGGTACCCCGTGGCAG-3'; reverse primer AQP2-MUT: 5'-CATTCTCGAGTCACAAGCGTCCGTCGGGGCCGT-3'). Correct orientation and sequence of the insert was confirmed by sequencing (LGC Genomics, Berlin, Germany), and protein expression was analyzed by Western blotting.

Cell Culture and Transfection

MDCK.1 cells were grown in DMEM-GlutaMAX (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 5 % (v/v) FCS at 37°C in 5 % CO_2. HEK293 cells were grown in DMEM-GlutaMAX (Thermo Fisher Scientific) supplemented with 10 % (v/v) FCS at 37°C in 10 % CO_2. At approximately 80 % confluency, cells were transfected with 1 µg DNA using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The medium was replaced with fresh cell culture medium 5 h later, and the cells were grown overnight. MDCK.1 cells were additionally treated with 10 µM indomethacin until the next day to lower their basal transcellular osmotic water permeability (Pf) prior to stimulation with forskolin (Fsk)[9, S12, S13].

Immunoblotting

Immunoblotting was essentially carried out as previously described[S13]. MDCK.1 and HEK293 cells were grown in 6-well cell culture plates. Cells were washed once with ice-cold 1x PBS, lysed with lysis buffer (10 mM K_2HPO_4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5 % Triton X-100, 0.2 % sodium deoxycholate; pH 7.4; 1x PhosSTOP EASY, 1x Complete mini EDTA-free (Roche, Basel, Switzerland) and detached using a cell scraper. The cell lysate was passed five times through a syringe and cleared from cell debris by centrifugation. Protein concentrations were determined by Bradford assays, and equal amounts of protein were boiled with Laemmli sample buffer for 5 min at 95°C. AQP2 and Hsp90 (loading control) were detected by Western blotting using specific primary antibodies (anti-AQP2 E-2, Santa Cruz Biotechnology,

#sc-515770; anti-AQP2 N-20, Santa Cruz Biotechnology, # sc-9880; anti-Hsp90, Enzo Life Sciences, #SPA-830), HRP-coupled secondary antibodies (Jackson Immuno Research, Ely, UK), and chemiluminescent HRP substrate (Merck Millipore, Burlington, Massachusetts, USA). Protein bands were semi-quantitatively analyzed by densitometry.

Immunofluorescence microscopy

AQP2 was detected as described[9, S13, S14]. MDCK.1 cells were grown on glass coverslips (12 mm diameter) placed in 6-well cell culture plates. Cells were left untreated or treated with 10 µM Fsk for 30 min. Cells were washed once with ice-cold 1x PBS, fixed for 15 min in fixation buffer (2.5 % (w/v) PFA, 100 mM sodium cacodylate, 100 mM sucrose; pH 7.6) at room temperature, and permeabilized for 5 min in 0.1 % Triton X-100 in 1x PBS at room temperature. Unspecific antibody binding was prevented by blocking buffer (0.3 % (v/v) fish skin gelatine in 1x PBS) at 37°C for 45 min. For incubation with primary antibodies, coverslips were transferred into wet chambers and covered with blocking buffer containing mouse anti-AQP2 (E-2; Santa Cruz Biotechnology, #sc-515770; 1:300) and rabbit anti-ERGIC-53/p58 (Sigma-Aldrich, #E1031; 1:300) antibody. After a 45 min incubation at 37°C, the cells were washed three times with 1x PBS and incubated for 45 min at 37°C with fluorophore-coupled secondary antibodies (Cy3 goat anti-mouse IgG, #115-165-003, Alexa Fluor 647 donkey anti-rabbit IgG, #711-605-152; Jackson Immuno Research, Ely, UK; 1:300) and DAPI (1:100) in wet chambers as described above. Residual antibody was removed by washing three times with 1x PBS before mounting the coverslips on microscope slides using Immu-Mount (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Samples were stored in the dark at 4°C overnight to ensure curing before imaging with a confocal laser scanning microscope (LSM) 710 (Carl Zeiss, Jena, Germany) using a Plan-Apochromat 63x/1.40 oil objective. Omissions of primary or secondary antibodies in the controls revealed no labeling.

Supplemental Figure 1: (A) Scheme illustrating arginine vasopressin (AVP)-mediated water reabsorption in

renal collecting duct principal cells. AVP binds to the V2 receptor located in the basolateral plasma membrane and stimulates the phosphorylation of aquaporin-2 (AQP2) by protein kinase A (PKA), which initiates trafficking to and insertion of AQP2 into the apical plasma membrane facilitating water reabsorption in the collecting duct. (B) Autosomal dominant mutations of *AQP2* yield mutant AQP2 which is misrouted to other cellular compartments by 4 different mechanisms, retention in Golgi, misrouting to the basolateral membrane, attenuated phosphorylation of C-terminal residues, or as we report here retention in the ERGIC, the ER-Golgi intermediate compartment. (C) The presented mutation causes cellular retention of mutant AQP2 between ER and Golgi in the ERGIC

Supplemental Figure 2: A schematic presentation of the AQP2 protein. The C terminus is highlighted showing sequence variations leading to autosomal dominant nephrogenic diabetes insipidus. The presented novel mutation is shown in pink.

Supplemental Figure 3: Vector Map of AQP2 wild-type. The AQP2-WT sequence was sub-cloned from pcDNA3.1 vector into the pcMV6-Entry backbone. Vector features and restrictions sites are shown. The AQP2-WT sequence is highlighted in light blue.

Supplemental Figure 4: Vector Map of AQP2 mutant. The AQP2-MUT sequence was sub-cloned from pcDNA3.1 vector into pcMV6-Entry backbone. Vector features and restrictions sites are shown. The AQP2-MUT sequence is highlighted in light blue.

Supplemental Table 1: Baseline blood cell counts and serum parameters of the AQP2-R267G proband

References

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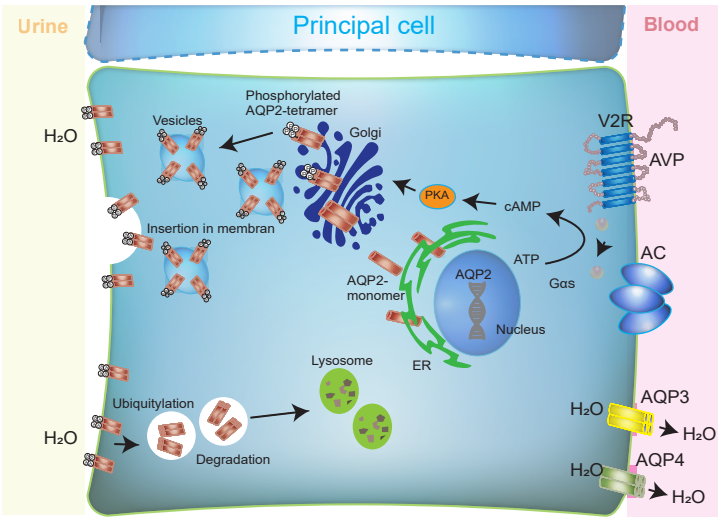
Supplemental Table 1: Baseline blood cell counts and serum parameters of the AQP2-R267G proband

Erytrocytes, vol.fr.,B.	0.40-0.50	0.48
Erytrocytesvol. Middel [MCV fL]	82-98	89
Ferritin, P (µg/L)	15-300	24
Hemoglobin, B (mmol/L)	8.3-10.5	10
Leukocytes, B (10E9/L)	3.5-8.8	7.24
Reticulocytes, B (10E9/L)	36-113	64
Trombocytes, B (10E9/L)	145-350	216
Neutrophils (10E9/L)	1.50-7.50	4.06
Albumin, P (g/L)	36-50	48
Calcium-ion (pH=7.4), P (mmol/L)	1.18-1.32	1.28
Phosphate, P (mmol/L)	0.71-1.53	1.10
Potassium, P (mmol/L)	3.5-4.4	3.8
Urea,P (mmol/L)	3.2-8.1	3.7
Creatinine,P (µmol/L)	60-105	73
eGFR ml/min per 1.73m2 (mL/min)	> 60	109
Magnesium,P (mmol/L)	0.71-0.94	0.85
Sodium,P (mmol/L)	137-145	141
Urat,P (mmol/L)	0.23-0.48	0.32
Bicarbonate (mmol/L)	22-26	24.1
Alanine transaminase [ALAT] (U/L)	10-70	27
Alkaline phosphatase,P (U/L)	35-105	65
Bilirubin,P (µmol/L)	5-25	13
gamma-Glutamyltransferase (U/L)	10-80	20
Lactate dehydrogenase,P (U/L)	105-205	185
Cortisol,P (nmol/L)	200-700	290
C-reaktive protein [CRP],P (mg/L)	< 6.0	1.7

Supplemental Figure 1

A



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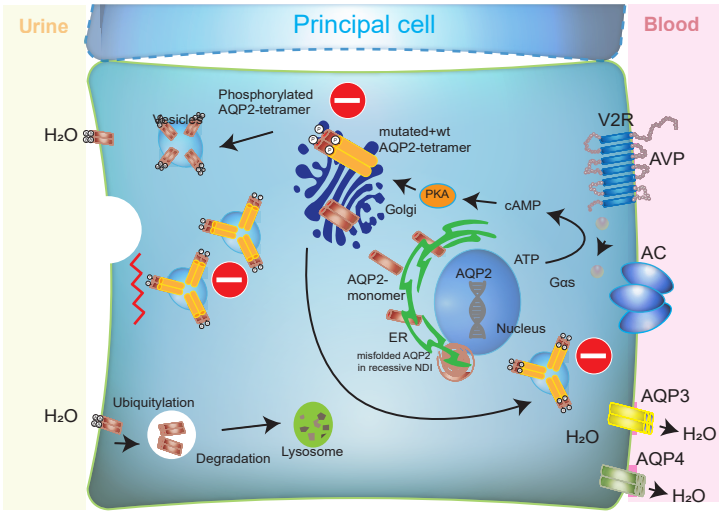

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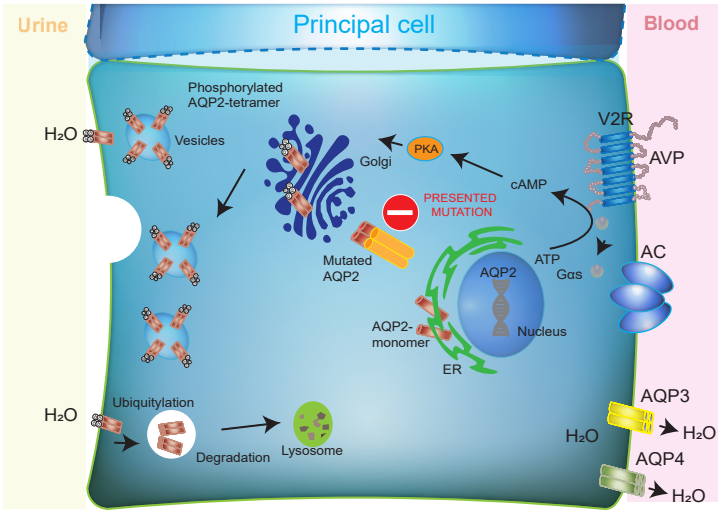

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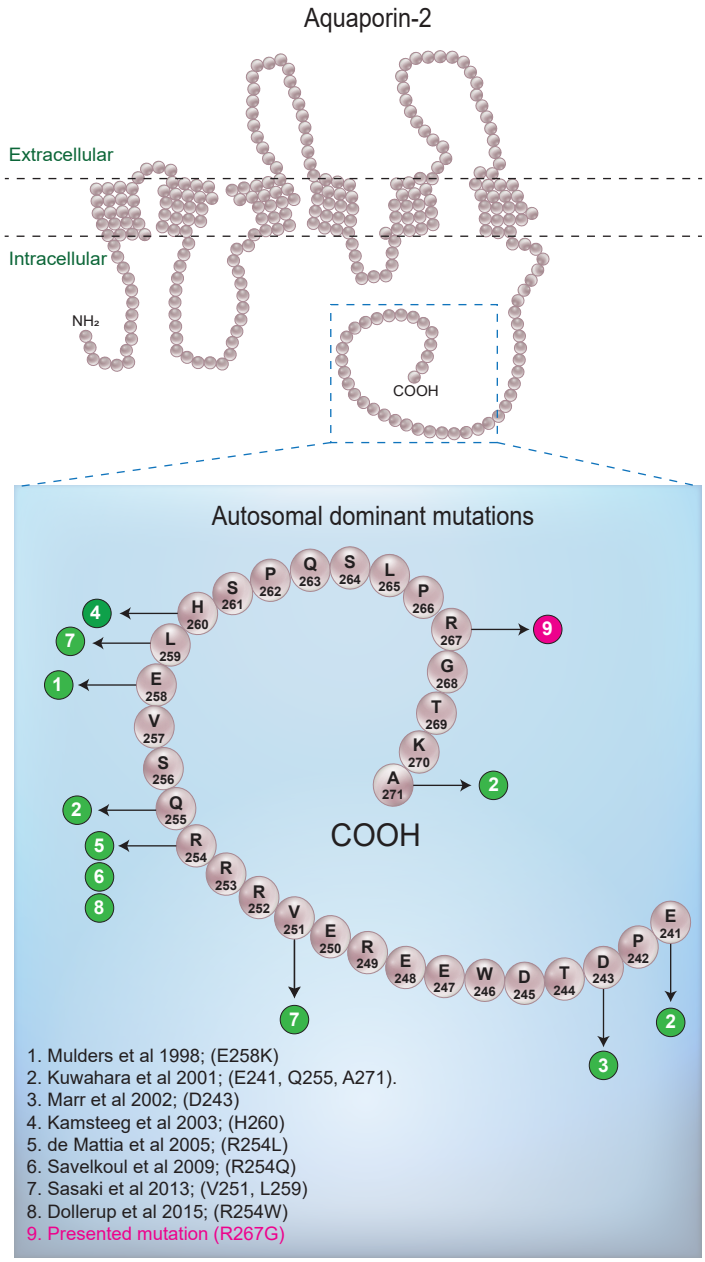
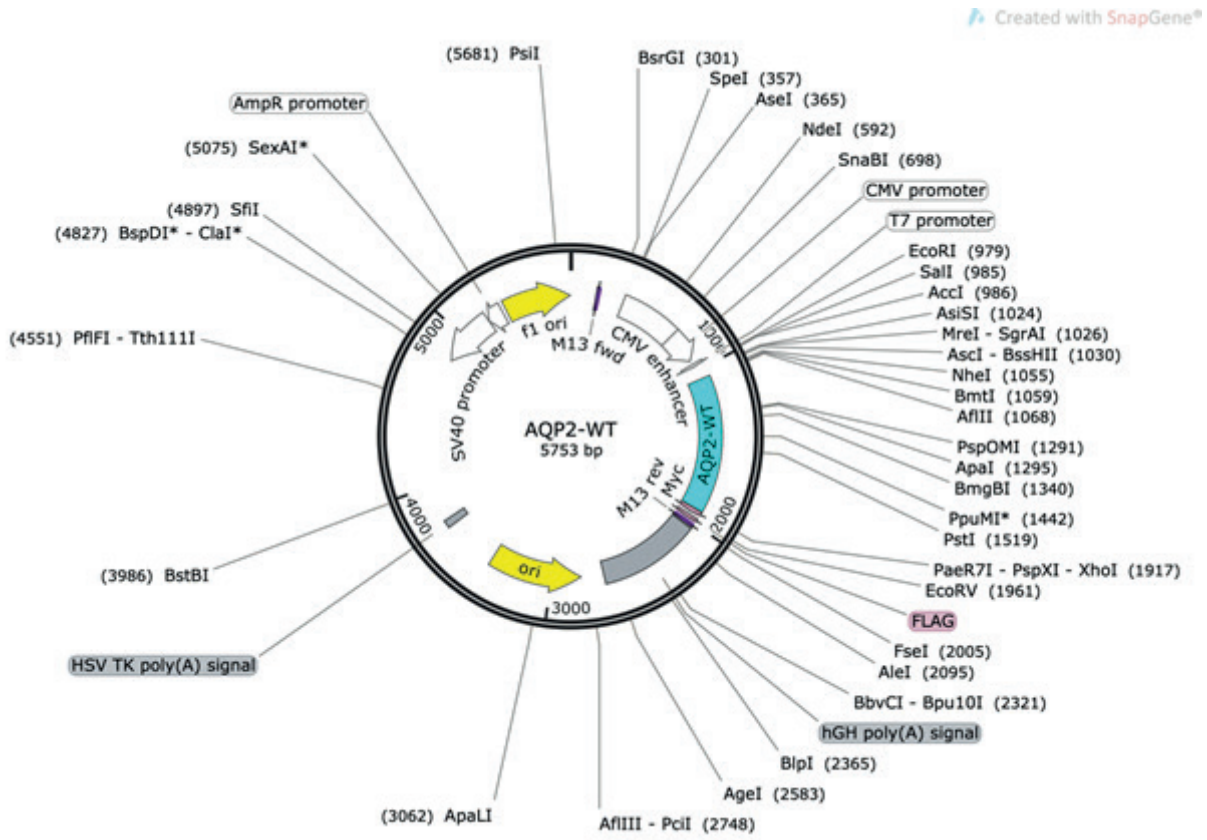


Supplemental Figure 3



Supplemental Figure 4