COQ6 mutations in human patients produce nephrotic syndrome with sensorineural deafness

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Steroid-resistant nephrotic syndrome (SRNS) is a frequent cause of end-stage renal failure. Identification of single-gene causes of SRNS has generated some insights into its pathogenesis; however, additional genes and disease mechanisms remain obscure, and SRNS continues to be treatment refractory. Here we have identified 6 different mutations in coenzyme Q10 biosynthesis monoxygenase 6 (COQ6) in 13 individuals from 7 families by homzygosity mapping. Each mutation was linked to early-onset SRNS with sensorineural deafness. The deleterious effects of these human COQ6 mutations were validated by their lack of complementation in coq6-deficient yeast. Furthermore, knockdown of COQ6 in podocyte cell lines and coq6 in zebrafish embryos caused apoptosis that was partially reversed by coenzyme Q10 treatment. In rats, COQ6 was located in cell processes and the Golgi apparatus of renal glomerular podocytes and in stria vascularis cells of the inner ear, consistent with an oto-renal disease phenotype. These data suggest that coenzyme Q10-related forms of SRNS and hearing loss can be molecularly identified and potentially treated.

Introduction
Nephrotic syndrome (NS), a malfunction of the kidney glomerular filter, leads to proteinuria, hypoalbuminemia, and edema. Steroid-resistant NS (SRNS) represents a frequent cause of end-stage renal failure (ESRF), which requires renal replacement therapy for survival. Identification of single-gene causes of NS (1–7) has generated the first insights (8, 9) into its pathogenesis: (a) that single-gene causes of NS result in SRNS (10, 11) with very few exceptional cases that respond to treatment (3, 12); (b) that SRNS-causing genes are expressed in a specialized cell type, the glomerular podocyte (13); and (c) that the pathohistology ranges from diffuse mesangial sclerosis (DMS) to the childhood-onset phenotype of focal segmental glomerulosclerosis (FSGS). Two-thirds of all SRNS cases with onset in the first year of life (14) and 10%–28% of all childhood cases (15) are caused by single-gene mutations in 1 of only 4 genes, NPHS1 (1), NPHS2 (2), LAMB2 (16), and WTI (17). However, the molecular cause of more than 80% of all cases of SRNS is unknown, and treatment options have yet to be discovered. We therefore performed total genome search for linkage to identify further causative recessive genes.
Results

**COQ6 mutations cause SRNS with sensorineural deafness (SND).** To identify further single-gene causes for SRNS, we performed a genome-wide search for linkage in 14 different consanguineous families with SRNS. Calculating lod under the hypothesis of locus heterogeneity yielded a significant maximum heterogeneity lod score of 4.9 (α = 5%) on human chromosome 14q24.3, covering a region of 6.4 Mb (Figure 1A). Of 7 families homozygous at this locus (SRNS2), families A1072 and F1082 from Turkey revealed haplotype sharing (Figure 1B), restricting the critical genetic region to 2.1 Mb under the hypothesis that an ancestor common to both families introduced the disease allele (i.e., homozygosity by descent). This interval contained 32 positional candidate genes (Figure 1C).

Exon sequencing of COQ6 yielded homozygous mutations in 3 of the 7 families homozygous at the SRNS2 locus (Table 1). In both affected individuals of family A1072, we detected a homozygous A353D substitution (Figure 1G and Table 1) that was also found in family F1082, as predicted from haplotype sharing at SRNS2 (Figure 1B). All 3 affected individuals that were examined in the northern Lebanese family F252 exhibited the homozygous change A353D (Figure 1G and Table 1). This mutation caused by shared by 3 families (A234, A1072, and F252) is predicted to be expressed in mitochondria with a Predotar score of 0.45. Both missense mutations altered amino acid residues that are uniformly conserved from COQ6 orthologs across species, and yeast synthesizes CoQ6. Using in silico analysis, we identified a single heterozygous nonsense mutation, c.1022dup11 (p.R341W), in one allele. We did not detect any further mutations. The 3 protein-truncating mutations in COQ6 that we detected in subjects A988, A1904, and A3331-21 (Figure 1G and Table 1) may be considered null alleles. Similarly, the fully conserved homologous missense mutations G225R and A353D that we observed in 4 families with SRNS and SND may also represent loss-of-function mutations (Figure 1G and Table 1).

To test this hypothesis, we examined all the 6 COQ6 mutations for potential deleterious effects by complementation in yeast. Yeast cells harboring a deletion mutation in the COQ6 gene were unable to grow on yeast extract/peptone/glycerol media (YPG; in which glycerol acts as a nonfermentable carbon source) and were deficient in CoQ6 (Figure 2D and E, and ref. 18). Expression of human WT COQ6 with an aminoterminal yeast mitochondrial leader sequence rescued both YPG and CoQ6 content (Figure 2D and E). Although not as robustly as did yeast harboring the WT COQ6 gene (pSRI-1; Figure 2, D and E). Rescue of growth on YPG was observed with either low-copy number (pQM; Figure 2D) or high-copy number (pRCM; data not shown) expression constructs. Both constructs also rescued the deficiency in CoQ6 content (Figure 2E). In contrast, none of the COQ6 constructs harboring the human mutations (Figure 1G and Table 1) were able to rescue the growth deficiency phenotype or CoQ6 content phenotypes (Figure 2, D and E), thereby demonstrating the deleterious effects of these mutations.

**Response to CoQ10 treatment.** Monogenic variants of childhood NS are characterized by a lack of response to therapy (26). Because successful CoQ10 treatment had been described previously in an

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**Available online with this article; doi:10.1172/JCI45693DS1.** Full-length isoforms were expressed in multiple tissues, including the kidney (Supplemental Figure 1, C–F). Using in situ hybridization analysis, we demonstrated Coq6 mRNA expression in the metabolic mesenchyme and forming nephrons of mouse kidney (Supplemental Figure 1, G–L). Isoform a (encoding a 54-kDa protein) is predicted to be expressed in mitochondria with a Predotar score of 0.81 (http://urgi.versailles.inra.fr/predotar) because of a mitochondrial leader peptide, whereas isoform b (encoding a 51-kDa protein), which uses an alternative exon 1, is considered only possibly mitochondrial, with a Predotar score of 0.45. Both isoforms encode a flavin-dependent monoxygenase with 3 flavin adenine dinucleotide–binding motifs (18). The amino acid sequence is highly conserved throughout evolution, with identity to the human COQ6 protein sequence of 66% for *Danio rerio* and 33% for the *E. coli* ortholog *UbiH*. The enzyme coenzyme Q10 monooxygenase 6 (COQ6) is required for biosynthesis of coenzyme Q10 (CoQ10; also referred to as ubiquinone) and is thought to catalyze one or more ring hydroxylation steps. Humans synthesize CoQ10 and yeast synthesizes CoQ6 (where CoQ6 designates a polysoprenyl residue with n isoprene units). CoQ6 operates as a redox carrier in the mitochondrial respiratory chain shuttling electrons from respiratory chain complexes RCCI and RCCII to complex RCCIII (19). CoQ10 also serves as a lipid-soluble antioxidant and has previously been implicated in protection from cell damage by ROS (20).
Figure 1

Positional cloning of COQ6 mutations in individuals with NS and SND. (A) lod score profile across the human genome in affected children from 14 consanguineous kindred with SRNS. Parametric heterogeneity lod (HLOD) scores are plotted against human chromosomal mapping positions, concatenated from p-ter (left) to q-ter (right). (B) Within the SRNS2 locus, haplotypes from 250k SNP analysis are shown for 3 of the 7 families with homozygosity at the SRNS2 locus. Alleles are colored light green (AA), dark green (BB), and red (AB). (C) The 32 genes within the SRNS2 locus; 3 genes preferentially expressed in kidney podocytes are underlined. Mutations were found in COQ6. Transcriptional direction is indicated by < or >. (D) COQ6 extends over 13.2 kb and contains 12 exons (boxes). (E) Exon structure of COQ6 cDNA. Arrows indicate relative positions of mutations (see G). Positions of peptides for antibody generation are shown in yellow. (F) Domain structure of COQ6 protein. Extent of the mono-oxygenase domain is shown in relation to encoding exon position (E). (G) 6 different COQ6 mutations in 7 families with SRNS. Family number and amino acid change (see Table 1) are given above sequence traces. Arrows denote positions of mutations in relation to exons and protein domains. For the 2 missense mutations, G255R and A363D, full conservation across evolution of altered amino acid residues is illustrated.
### Table 1

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Origin</th>
<th>Consang</th>
<th>Exon</th>
<th>Mutation sequence (segregation)</th>
<th>Nucleotide change</th>
<th>Protein sequence change</th>
<th>Protein domains</th>
<th>Histology (at yr of age)</th>
<th>Kidney disease/ treatment (at yr of age)</th>
<th>Age at ESRF (yr)</th>
<th>Age at onset (yr)</th>
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<td>A234-21</td>
<td>Turkey</td>
<td>Y</td>
<td>1058</td>
<td>G1058C&gt;A</td>
<td>9 (het, M, P)</td>
<td>2.5</td>
<td>2</td>
<td>FSGS (2.5)</td>
<td>SND (4 yr)</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>A1072-22</td>
<td>Turkey</td>
<td>Y</td>
<td>1058</td>
<td>G1058C&gt;A</td>
<td>9 (het, M, P)</td>
<td>2.5</td>
<td>2</td>
<td>FSGS (3)</td>
<td>SND (40% of normal)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>A3331-21</td>
<td>Turkey</td>
<td>N</td>
<td>1341</td>
<td>G1341A&gt;G</td>
<td>11 (het, M, P)</td>
<td>3.0</td>
<td>2</td>
<td>CP-R, ACE-I</td>
<td>SND (40% of normal)</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

**Histology** (a yr of a dp)

- FSNS (6.8)
- SM (1.0)
- FSGS (1.2)
- DM (1.1)
- ND (1.1)

**Extrarenal manifestations**

- SRNS, CoQ10
  - SND (10 mo), bilateral nephrolithiasis (5 mo), GR, levamizole
  - FSGS (2.5)
  - SND (4 yr)

**Additional mutations**

- α-CoQ6 mutations only detected in families 1298 (8), 19 and 1494 (89, 90). 

**Notes**

- All mutations were absent from more than 90 healthy control subjects from Central Europe and from 60 healthy control subjects from Turkey. All missense mutations were conserved down to *E. coli*.

- hom, homozygous in affected individual; het, heterozygous in affected individual; M, heterozygous mutation identified in mother; P, heterozygous mutation identified in father.

- phosphamide resistant; GR, growth retardation; CoQ10, treatment with CoQ10.

- Treatment-sensitive case (renal replacement therapy not listed).

- Exogenous Coq6 localizes to mitochondria. To study the subcellular localization of COQ6, we generated GFP-labeled clones of human full-length isoforms a, b, and c (Supplemental Figure 1, A and B). We also generated anti-COQ6 antibodies directed against the synthetic peptides, whose positions in relation to exons and protein domains are shown in Figure 1E. Following affinity purification, antibody α–CoQ6-TPEP2 detected a major band at the expected size of 50.8 kDa for full-length human COQ6 (Supplemental Figure 2). Because human full-length COQ6 isoform contains a putative mitochondrial leader sequence, we predicted that exogenous expression would result in mitochondrial localization. Indeed, after transient exogenous transfection into Cos-7 or HeLa cells or into murine podocyte cell lines, exogenous COQ6 isoform co-localized quantitatively in mitochondria with mitochondrial markers cytochrome c and cytochrome c oxidase (COXIV; Figure 3, A and B).

- Surprisingly, whereas α–COQ6-TPEP2 clearly detected exogenously expressed COQ isoform a, thereby confirming specicity of the antibody, it detected an additional endogenous signal that appeared to be localized in Golgi apparatus (Figure 3C). In fact, this endogenous signal detected by α–COQ6-TPEP2 was located in Golgi, as confirmed by double labeling with the Golgi marker Golgin 97 (Figure 3D). Interestingly, α–COQ6-TPEP2 did not reveal any endogenous COQ6 expression in mitochondria (Figure 3, D and E). Furthermore, exogenously individual with COQ2 mutations (12), we administered CoQ10 in 2 children whose parents consented. Treatment was administered orally, giving 1 Softgel CoQ10 capsule (50 mg; GNC Preventive Nutrition) twice per day. Individual A234-26 presented with proteinuria without edema at 2 months of age, when the COQ6 mutation was detected in his sister (A234-26) and cousin (A234-21). Urinary protein/creatinine ratio was 40 mg/mg initially (normal, <0.2 mg/mg). Treatment was commenced at 2 months of age, giving CoQ10 orally at 15 mg/kg/d divided in 3 doses over 2 months together with enalapril (1.25 mg/d orally). CoQ10 treatment was then increased to 30 mg/kg/d, and urine protein/creatinine ratio decreased to 8 mg/mg within 2 months. Proteinuria decreased further to 5.8 mg/mg and remained stable at 4.8 mg/mg during the last follow-up at 15 months of age. Renal function was normal throughout. Bilateral severe SND and severe growth retardation were noted at 1 months of age. After treatment with 50 mg CoQ10 orally twice per day, SND substantially improved in A234-26. Individual A1072-22 manifested with NS at age 2.5 years. CoQ10 treatment was started at 5.5 years of age, when the subject was in partial remission from CsA treatment, which was discontinued at 5.8 years. At the beginning of CoQ10 treatment, 24-hour protein excretion was 7 mg/mg (117 mg/d); 2 months into treatment, it decreased to 3.7 mg/mg (76 mg/d), and remission was maintained at the end of the study period. Hearing was not improved after CoQ10 treatment. When CoQ10 treatment was inadvertently interrupted, proteinuria reappeared at a level of 57 mg/mg (1,100 mg/d). Following reinstitution of CoQ10 treatment, proteinuria decreased again to 9 mg/mg (188 mg/d).

- Exogenous Coq6 localizes to mitochondria. To study the subcellular localization of COQ6, we generated GFP-labeled clones of human full-length isoforms a, b, and c (Supplemental Figure 1, A and B). We also generated anti-COQ6 antibodies directed against the synthetic peptides, whose positions in relation to exons and protein domains are shown in Figure 1E. Following affinity purification, antibody α–CoQ6-TPEP2 detected a major band at the expected size of 50.8 kDa for full-length human COQ6 (Supplemental Figure 2). Because human full-length COQ6 isoform contains a putative mitochondrial leader sequence, we predicted that exogenous expression would result in mitochondrial localization. Indeed, after transient exogenous transfection into Cos-7 or HeLa cells or into murine podocyte cell lines, exogenous COQ6 isoform co-localized quantitatively in mitochondria with mitochondrial markers cytochrome c and cytochrome c oxidase (COXIV; Figure 3, A and B). Surprisingly, whereas α–COQ6-TPEP2 clearly detected exogenously expressed COQ isoform a, thereby confirming specificity of the antibody, it detected an additional endogenous signal that appeared to be localized in Golgi apparatus (Figure 3C). In fact, this endogenous signal detected by α–COQ6-TPEP2 was located in Golgi, as confirmed by double labeling with the Golgi marker Golgin 97 (Figure 3D). Interestingly, α–COQ6-TPEP2 did not reveal any endogenous COQ6 expression in mitochondria (Figure 3, D and E). Furthermore, exogenously
expressed full-length COQ6 isoform a spared Golgi expression, as demonstrated with Giantin as a Golgi marker (Figure 3F). Likewise, in podocyte cell lines, the cell type central to the disease mechanism of SRNS, α–COQ6-TPEP2 detected endogenous COQ6 in Golgi, but not in mitochondria (Figure 3G). The endogenous COQ6 expression pattern was confirmed with the antibody α–COQ6–925-1 (Supplemental Figure 3, A and B), which was derived from a different peptide (Figure 1E).

Endogenous COQ6, COQ7, and COQ9 colocalize to cell processes and Golgi in rat podocytes. Monogenic forms of SRNS are caused by dysfunction of the glomerular podocyte, a terminally differentiated cell critical for the filtering function of the kidney glomerulus. (13, 26). We therefore examined expression of the COQ6 protein in kidney. COQ6 was seen almost exclusively in glomeruli, rather than in tubules (Figure 4A). As predicted, within glomeruli, COQ6 was expressed specifically in podocytes, as marked by WT1 labeling (Figure 4A). Within podocytes, COQ6 was absent from mitochondria (labeled with COXIV; Figure 4A); conversely, it was expressed within cellular processes and within Golgi apparatus (Figure 4, B and C), which confirmed the results obtained in cell lines (Figure 3). Because nonmitochondrial localization and function have been previously described for CoQ6 (27), we hypothesized that nonmitochondrial expression would also apply to COQ7 and COQ9, which are known as mitochondrial proteins but share a multienzyme complex together with COQ6 for the biosynthesis of CoQ10 and CoQ6 (21). Indeed, upon immunofluorescence, both COQ6 and COQ7 fully colocalized to cellular processes and Golgi of rat glomerular podocytes (Figure 4, B and C), as did COQ7 and COQ9 (Supplemental Figure 3C). We demonstrated specificity of this signal for α-COQ6-TPEP2 by showing the absence of the podocyte signal after preabsorption with the cognate peptide TPEP2, in contrast to its presence after preabsorption with the noncognate peptide TPEP1 (Supplemental Figure 3D). In addition, COQ6 colocalized to podocyte cellular processes with podocin (Figure 4E), another gene product that, if mutated, causes a Mendelian form of SRNS (2). These findings demonstrate that COQ6, COQ7, and COQ9 are all 3 expressed in cellular processes and Golgi apparatus of podocytes rather than in mitochondria.
We then investigated expression of COQ6 in the inner ear, because hearing loss is the other phenotype in this oto-renal syndrome. Immunofluorescence studies using α–COQ6-TPEP2 revealed COQ6 expression in the spiral ganglion as well as in cells of stria vascularis and spiral ligament (Figure 4, F and G), which are involved in maintaining the high potassium concentration in the cochlear duct necessary for sound transduction of the hair cells of Corti organ.

Coq6 knockdown in cultured podocytes induces apoptosis that is reversed by CoQ10 treatment. In order to study COQ6 function in glomerular podocytes, the cell type involved in the disease phenotype, we performed knockdown of Coq6 expression in vitro using a vector-based siRNA approach (28). Murine podocytes (29) were stably transfected with COQ6 siRNA constructs, and clones were analyzed by RT-PCR for Coq6 expression levels. We compared clones transfected using a scrambled siRNA oligonucleotide with non-transfected WT podocytes. The 3 clones that showed the highest degree of Coq6 downregulation (clones 1, 2, and 5) were selected for functional studies (Supplemental Figure 1M). First, we analyzed the growth behavior of Coq6 knockdown clones when kept in an undifferentiated and proliferative state (Figure 5A). When we seeded a defined number of cells and counted them after 24, 48, and 72 hours of cultivation, we found that the growth rate of the 3 Coq6 knockdown clones was strongly decreased, while the scrambled siRNA clone C proliferated at the same rate as WT cells (Figure 5A). This difference reached statistical significance after 24 hours for clones 2 and 5 (P < 0.05) and after 72 hours for clone 1 (P < 0.005; Figure 5A). As the scrambled siRNA clone C did not
show any difference in growth behavior compared with WT podocytes, the scrambled clone was used as negative control for further experiments. To analyze whether the diminished overall growth rate in podocytes after Coq6 knockdown is associated with activation of the intrinsic apoptotic pathway, we next determined the inner mitochondrial membrane potential (\(\Delta\Psi_m\)) in these podocyte clones by using the fluorescent dye tetramethylrhodamine ethylester (TMRE). Compared with control cells, all 3 knockdown clones showed a substantial drop in TMRE fluorescence (data not shown), indicative of an increased percentage of podocytes with depolarized \(\Delta\Psi_m\).

We then analyzed activity of caspase-9 (Figure 5B), the major caspase downstream of mitochondria-mediated apoptosis (30). Using fluorometric immunosorbent enzyme assay (FIENA), we detected a more than 2-fold increase of caspase-9 activity in all 3 knockdown clones compared with control podocytes (\(P < 0.005\); Figure 5B). Moreover, caspase-3, the final downstream executioner caspase of different apoptotic signaling pathways (31), showed significant elevation of activity for all 3 siRNA clones (\(P < 0.05\); Figure 5C), consistent with the effect seen in zebrafish (see below). These data suggest that the observed decline in podocyte growth after Coq6 knockdown is caused, at least in part, by increased apoptosis. Since mitochondria-induced apoptosis is associated with depolarization of the inner mitochondrial membrane (32) as well as caspase-9 activation (30), it is likely that lack of growth upon Coq6 knockdown is caused by activation of the intrinsic apoptotic pathway. Because some of the SRNS patients in our study that carry COQ6 mutations responded partially to CoQ10 treatment, we then analyzed whether CoQ10 has a beneficial effect on the phenotype caused by knockdown
of Coq6 expression in podocytes in vitro. When culturing podocyte clones 1, 2, and 5 in the presence of CoQ_{10} at a concentration of 50 μM for 48 hours, we observed a decrease in caspase-9 and caspase-3 activities (Figure 5, B and C). Compared with clones cultured in the absence of CoQ_{10}, this decrease was statistically significant ($P < 0.05$), whereas we did not detect any changes in caspase activities of negative control clones (Figure 5, B and C).

To examine COQ6 loss of function in a vertebrate organism rather than cell lines, we performed morpholino oligonucleotide (MO) knockdown of coq6 in zebrafish. We demonstrate that coq6-MO4 MOs directed against zebrafish coq6 intron 7 splice donor blocked proper splicing of coq6 mRNA (see Supplemental Figure 1N). Negative controls were injected with 0.1 mM coq6 mismatch MO. Note the gray appearance of zebrafish heads upon differential interference contrast (DIC) microscopy as a sign of increased cell death. (E and F) Zebrafish dorsal head and lateral trunk views. Embryos were injected as indicated with 0.1 mM coq6-MO4 splice targeting MO (E), MO targeting the AUG translation start site (dcoq6 MO1; F), coq6 mismatch (mm) MO negative controls, or left uninjected (WT). Cells were stained by an antibody against cleaved caspase-3, a specific marker for apoptotic cells. Lens (asterisk), yolk sac (ys), and cloaca (arrow) are indicated. Scale bars: 1 mm (D); 100 μm (E and F).
of COQ6 mutations is remarkably similar to that of mutations in COQ2 (23) and PDSS2 in humans (34) and Pds2 in mice (24), all of which respond to CoQ10 treatment (12, 35). Patients with defects in genes required for CoQ biosynthesis — PDSS2, COQ2, and COQ9 — exhibit renal disease, and in some cases there is a dramatic response to CoQ10 therapy, but this is quite variable (36).

**Discussion**

In this study, we report what we believe to be a novel cause of SRNS that appears to respond to oral CoQ10 supplementation. CoQ10, an essential component of the mitochondrial electron transport chain and one of the most potent lipophilic antioxidants (37), is also required for pyrimidine nucleoside biosynthesis and has been implicated in the inhibition of apoptosis by its prevention of inner mitochondrial membrane collapse (38). In multiple studies, the deleterious effect of CoQ10 deficiency to mitochondria has been shown. CoQ10 deficiency can lead to the opening of the mitochondrial permeability transition pore (MPTP) directly, but an increased amount of ROS caused by CoQ10 deficiency can also induce the MPTP by opening of nonspecific high-conductance permeability transition pores in the mitochondrial inner membrane (39). Experiments in HEK293 cells recently showed that CoQ10 inhibits Bax-induced mitochondrial dysfunction and protects mitochondria from permeability transition pore opening (9). Here, we confirmed that COQ6 mutations that cause CoQ10 deficiency led to the upregulation of proapoptotic factors. Interestingly, CsA inhibits the MPTP through interaction with cyclophilin D, an essential component of the MPTP (40). We showed that incubation of COQ6 knockdown podocytes with CsA had a mild rescue effect and decreased activity of caspase-3. Patient A1072-22, who had been treated with CsA in the past, also showed partial remission of proteinuria after treatment with CsA.

Mutations in 5 other CoQ10 biosynthesis enzymes leading to CoQ10 deficiency have been recently implicated in other monogenic mitochondrialopathies, generally characterized by central nervous system signs and myopathy (22, 34, 41–43); 1 patient with mutations in PDSS2 and 5 patients with mutations in COQ2 also presented with SRNS (22, 34, 43). So far, the exact pathogenic mechanism has remained unclear. Interestingly, 1 patient with mutations in COQ2 was successfully treated with oral CoQ10 supplementation, showing progressive recovery of renal function and a reduced level of proteinuria until 5 years after completion of treatment (22). The podocyte-specific phenotype caused by PDSS2 mutations leading to primary CoQ10 deficiency was demonstrated in the Pbs2 knockout mouse (24). Conditional knockouts targeted to renal tubular epithelium, monocytes, or hepatocytes did not show disease manifestation. It remains unclear why the podocyte in particular is affected by PDSS2-dependent CoQ10 deficiency.

Interestingly, the presentation of primary CoQ10 deficiency caused by genetic mutations is very heterogeneous. It seems that the mutations in PDSS2 and COQ2 are partial loss-of-function mutations. Therefore, the phenotypes (ATP synthesis, ROS production) may depend on the content of Q in the cell, which is determined by the severity of the mutation. Mutations in PDSS2, for example, cause reduced ATP synthesis in cultured fibroblasts, but no increase in ROS production, whereas mutations in COQ2 cause no difference in ATP synthesis in cultured fibroblasts, but increase ROS production (36). One could speculate that different cell types and/or tissues react differently to ROS and ATP synthesis defects according to their antioxidant defense mechanisms or the level of respiratory activity, explaining the wide spectrum of clinical features. Based on the surprising Golgi localization of COQ6, COQ7, and COQ9, we speculate that the antioxidant function of Q/QH2 in certain cells, such as podocytes, may depend on targeted synthesis of CoQ in the Golgi for delivery to the plasma membrane, which in the podocyte constitutes an enormous surface area that must contain vulnerable lipid components. Whereas most other forms of monogenic childhood NS are characterized by a lack of response to therapy (10, 16, 17, 44), the identification by mutation analysis of individuals with NS caused by COQ10 biosynthesis defects, as described here, is important because those individuals may respond to treatment with CoQ10.

**Methods**

**Subjects**

We obtained blood, tissue samples, and pedigrees following informed consent from individuals with NS and/or from their parents. Human subject research was approved by the University of Michigan Institutional Review Board. The diagnosis of NS was made by a pediatric nephrologist based on either chronic or recurrent high-grade proteinuria (>40 mg/m²/h) or persistent low-grade proteinuria (>4 mg/m²/h) (45). Steroid-sensitive NS and SRNS were defined according to standard criteria (45, 46). Renal biopsies were evaluated by a reference renal pathologist. Age at onset of ESRF was defined as age at first renal replacement therapy, i.e., dialysis or renal transplantation. Clinical data were obtained using a standardized questionnaire (www.renalgenes.org).

**Genetic mapping and exon sequencing**

We performed a genome-wide search for linkage by homozygosity mapping using the 250k Affymetrix SNP microarray (47). Data were evaluated by calculating nonparametric lod scores and scoring for homozygosity (Z) across the whole genome in order to identify regions of homozygosity. The GENEHUNTER-MODSCORE program (48) was used to calculate multipoint lod scores assuming recessive inheritance with complete penetrance, a disease allele frequency of 0.001, and the marker allele frequencies for individuals of mixed European descent specified by Affymetrix. Parametric and nonparametric lod scores were plotted over genetic distance across the entire human genome using GnuPLOT software (http://www.gnuplot.info/). See Supplemental Table 2 for exon sequencing primers.

**COQ6 mRNA expression**

The probe for human Northern blot analysis was obtained by digesting the COQ6 isoform a coding region cloned into pCRITOPO with EcoRI and was radiolabeled with α-32P dCTP (Amersham Biosciences) using the Random Primers DNA Labeling System kit (Invitrogen) and successively purified with Quick-Spin columns (Roche) according to the manufacturer’s protocol. Radiolabeled probes were hybridized to a commercial preblotted membrane (FirstChoice Human Blot 1 membrane; Ambion) containing 2 μg/lane poly(A)− RNA from 10 human tissues. Prehybridization and hybridization were both performed in a 50% formamide buffer at 42°C, with a working concentration of 1.5 × 10⁶ cpm and 0.1 mg salmon sperm DNA per milliliter. The excess probe was removed by washing at 65°C for 15 minutes using a 0.1% SDS, 0.1x sodium chloride–sodium citrate buffer solution. Radioactivity was detected with a Storm PhosphorImager (Molecular Dynamics) after overnight exposure. Selective amplification of COQ6 isoform b was carried out using primers in exon 1b and in exon 4. Simultaneous amplification of isoform a and isoform b was carried out using primers on exon 2 and on exon 4. PCR conditions were 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds,
was verified by direct sequencing. HeLa cells stably expressing mitochondrial COQ6 were transferred in 80% glycerol prior to documentation.

**Generation of plasmids**

Yeast COQ6 was amplified from genomic DNA extracted from a WT BY4741 strain and cloned in pCM189 vector. Site-specific mutagenesis was performed using the QuickChange kit (Stratagene) according to the manufacturer’s protocol. To generate constructs that expressed the gene under the control of the endogenous yeast coq6 promoter, the different pCM189–yeast COQ6 constructs were digested with SacI and MslI (to eliminate the CYC1 promoter and control elements); religated with a fragment encompassing the yeast COQ6 promoter, the 5’ end of the gene, and the MslI site; amplified from genomic DNA; and cut accordingly. Plasmid maps were described previously (49). Yeast growth and transformation were performed as described below.

**COQ6-GFP expression studies**

The complete coding region of full-length human COQ6 isoform α, lacking termination codon but including the Kozak sequence, was PCR amplified from pCRITTOPO-COQ6 and subcloned into the HindIII and BamHI sites of pEGFP-N1 (BD Biosciences – Clontech). The correctness of the construct was verified by direct sequencing. HeLa cells stably expressing mitochondrial targeting red fluorescent protein (50) were grown on coverslips in complete DMEM (Sigma-Aldrich) containing 10% fetal bovine serum until 70% confluent, then transfected with the purified plasmid using Effectene Transfection Reagent (Qiagen) according to the manufacturer’s instructions. Cells were visualized after 48 hours using a Nikon Video Confocal microscope.

**In situ hybridization analysis in mice**

Whole-mount in situ hybridization was performed following a standard procedure with digoxigenin-labeled antisense riboprobes (51). Stained specimens were prepared as described previously (52).

**Immunofluorescence**

Rat kidneys were perfusion-fixed with paraformaldehyde/lensine/periodate and processed as previously described (3). For absorption experiments, the immunopurified α–COQ6-TPEP2, COQ7, and COQ9 antibodies were preabsorbed with an equal weight of cognate or noncognate peptide overnight at 4°C. The following antibodies were used: WT1 mouse monoclonal antibody (catalog no. sc-7385; Santa Cruz Biotechnology Inc.); PLCγ1 goat polyclonal antibody (catalog no. sc-28404; Santa Cruz Biotechnology Inc.); α–COQ6-TPEP2 immunopurified rabbit antipeptide antibody (against peptide sequence N-DKDNLDDMGYIVEND-C; anti-rat Glepp1 mouse monoclonal antibody (1B4; ref. 53), CoQ7 goat polyclonal antibody (catalog no. sc-66353; Santa Cruz Biotechnology Inc.); CoQ9 rabbit polyclonal antibody (catalog no. 14874-1-AP; Protein Tech); TGN38 monoclonal mouse antibody (catalog no. 610898; BD Biosciences); and GM130 mouse monoclonal antibody (catalog no. 610823; BD Biosciences).

**Yeast studies**

Yeast growth. The S. cerevisiae coq6-null mutants W303ΔG63 (Mat α ade2-1 his 3-1,15 leu2-3,112 trpl-1 ura3-1 ΔCOQ6-HIS3; ref. 18) and W303ΔCOQ8 (Mat α ade2-1 his 3-1,15 leu2-3,112 trpl-1 ura3-1 ΔAABC1/COQ6-HIS3; ref. 54) were used in this study. Yeast growth media were prepared as described previously (55): YPD, containing 1% yeast extract, 2% peptone, and 2% dextrose; YPGal, containing 1% yeast extract, 2% peptone, 2% galactose, and 0.1% dextrose; YPG, containing 1% yeast extract, 2% peptone, and 3% glycerol; and SD-Ura, containing 0.18% nitrogen base without amino acids, 2% dextrose, 0.14% NaH2PO4, 0.5% (NH4)2SO4, and a complete amino acid supplement (56), minus uracil. Solid plate media contained 2% agar.

**Yeast transformation.** Yeast was subjected to transformation as described previously (57), with either a low–copy number (pQM; ref. 58) or a high–copy number (pRCM; ref. 59) yeast expression plasmid. These vectors were used to prepare plasmids containing the human COQ6 open reading frame with an in-frame aminoterminal yeast mitochondrial leader sequence (pQM_hCOQ6-MLS and pRCM_hCOQ6-MLS). Each of the following mutations in human COQ6 were introduced by site-directed mutagenesis (Stratagene): R162X, W188X, G255R, A353D, W447X, and Q461X/Q78. See Supplemental Table 1 for a complete list of plasmids used in yeast transformation experiments.

**Lipid extraction and analysis.** Yeast cells were grown in YPGal and harvest- ed during log phase growth (1.0 OD600nm). Lipid extracts were prepared from yeast whole cells as described previously (60). Lipids were separated by reverse-phase high-performance liquid chromatography with a Phenyl-hexyl column (Phenomenex 5-μm, 100 × 4.6 mm). The column was equili- brated with a mobile phase consisting of 95.5 methanol/2-propanol with 2.5 mM ammonium formate as solvent A and a flow rate of 650 μl/min. Upon sample injection, the percentage of solvent B (2-propanol with 2.5 mM ammonium formate) was increased linearly from 0% to 5% over 6 minutes, and the flow rate was also increased linearly to 800 μl/min. The flow rate and mobile phase were linearly changed back to initial conditions by 8 minutes. Q was quantified with an Applied Biosystems-MDS Sciex 4000 Q Trap (hybrid triple-quad linear ion trap analyzer with autosampler and a Turbo-V source equipped with ESI and APCI sources) as described previ- ously (35). The samples were analyzed in multiple reaction monitoring (MRM) mode; MRM transitions were as follows: m/z 591.6/197.1 for Q6; m/z 610.6/197.1 for Q8; with ammonium adduct; m/z 455.6/197.1 for Qα (Sigma-Aldrich), used as internal standard.

**Podocyte expression studies**

Preparation of CoQα solution for use in cell culture. Pure CoQα in powder form (Kaneka Corp.) was dissolved in 1 ml of molecular biology–grade isopropanol at a concentration of 10 mM by heating it briefly in a 95°C water bath. Thereafter, the CoQα solution was diluted 1:10 in podocyte culture media to a final stock concentration of 1 mM and reheated for an additional 5 minutes at 95°C with gentle shaking. This CoQα stock solution was stored at 4°C, protected from light for up to 4 weeks, and diluted immediately before use at 1:20 in cell culture medium to a final working concentration of 50 μM.

**Podocyte culture.** Immortalized murine podocytes were cultured as described previously (29). For treatment studies of undifferentiated podocytes, cells were incubated in the presence of 50 μM CoQ0, 1 μM dexamethasone (Sigma-Aldrich), or 1 μM CsA (Sigma-Aldrich) for 48 hours. For analysis of differentiated podocytes, undifferentiated podocytes were cultured under nonpermissive conditions in the presence of 1 μM dexamethasone for 14 days. Cultivation was then continued in the absence of dexamethasone for an additional 48 hours. For determination of cell number and growth curve analysis, cells were seeded in 24-well plates at 20,000 cells/well. After 24, 48, or 72 hours, cells were trypsinized and counted using a hemocytometer. Experiments were done 4 times with at least 3 internal replicates.

**COQ6 RNAi**

For the targeted downregulation of protein expression in podocytes, we used the pSuper RNAi System (OligoEngine) (28) following the manufactur- er’s instructions and as done previously (61). Briefly, we cloned a 60-nt oligo including a 19-nt COQ6 target sequence and a hairpin (5′-TTCAGA-
GA-3') into a modified pSuper vector, which also encoded for a zeocin selection marker (61). After transfection of undifferentiated podocytes using FuGene 6 (Roche) followed by zeocin selection at 500 μg/ml for 7–10 days, appearing podocyte clones were covered by cloning chambers, trypsinized, and expanded separately from each other in culture medium containing 100 μg/ml of zeocin. In total, 5 different COQ6 targeting sequences were used (clone 1, AACCAGCAGCTTCCATAT; clone 2, AATGACGTCATCATGATTG; clone 3, CTGTGGCAGATCGAGTGAA; clone 4, AACTGTTGATGGTGGCTGA; clone 5, AGGATCTTGAGGCTCATA). As well as 1 scrambled control sequence (5′-CCCGACTCCGGCCTCTGCG-3′). Stable transfected clones were expanded and used for RNA isolation and functional studies.

**RNA isolation and RT-PCR**

RNA was isolated from 2 × 10⁶ undifferentiated podocytes using the RNeasy kit (Qiagen) following the manufacturer’s protocol. Prior to RT-PCR, total RNA samples were digested with DNase I (Roche) and RNA was transcribed into cDNA using Superscript II (Invitrogen). For RT-PCR, 100 ng cDNA, AmpliTaq Gold DNA polymerase (Applied Biosystems), and was transcribed into cDNA using Superscript II (Invitrogen). For RT-PCR, 100 ng cDNA, AmpliTaq Gold DNA polymerase (Applied Biosystems), and sequence-specific, intron-spanning primers were used (COQ6 forward, CCAGAAGCCGTAGGTACGAC; COQ6 reverse, CCAACAGCTTTGCTCTTCTG). PCR amplification was performed using a PerkinElmer 9600 Thermal Cycler with the following conditions: 5 minutes at 95°C, 30 seconds at 53°C, and 30 seconds at 72°C) were run on a GeneAmp PCR System 9700 (Applied Biosystems). PCR reactions were analyzed on a 2% agarose gel, and ethidium bromide signals were captured with a FluorMax 3 imaging system (Molecular Devices).

**Accession numbers**

Accession numbers of COQ6 orthologs were NP_872282 for Homo sapiens, NP_001038869 for D. rerio, and ZP_00723222 E. coli. Accession numbers of COQ6 orthologs were NP_872282 for Homo sapiens, NP_001038869 for D. rerio, and ZP_00723222 E. coli.

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Address correspondence to: Friedhelm Hildebrandt, Howard Hughes Medical Institute, Departments of Pediatrics and Human Genetics, University of Michigan Health System, 8220 MSRB III, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109-5646, USA. Phone: 734.615.7285; Fax: 734.615.1386; E-mail: fhilde@umich.edu.

**Statistics**

To evaluate caspase-3 and -9 activity in cell lines, Pearson χ² test was used with a critical P value of 0.05 (Figure 5).

**Databases**

Genetic mapping was performed using http://genome.ucsc.edu (May 2004 freeze).

**Zebrfish MO-mediated knockdown**

MOs were obtained from Gene Tools. The nucleotide sequences of the translation-blocking (coq6-MO1) and splice-blocking (coq6-MO4) MOs against D. rerio coq6 were 5'-GCTTAGCTTTAGCCAGAGACAGCAT-3' and 5'-ACACATAAGGGTCAGTCCGGGAA-3', respectively. The 5-bp mismatch controls for the MOs were as follows: coq6-MO1mm, 5'-GGTACCTTTACCCAGACACACAT-3' and coq6-MO4mm, 5'-AACAAGTGTCACCTAGCGGAG-3'. Fertilized eggs were microinjected with the specified amount of MO dissolved in 0.1M KCl. For immunohistochemical labeling, embryos were fixed with 4% PFA in PBS (pH 7.4) and stained with antibody against activated caspase-3 (1:200; BD Biosciences) following standard protocols (63).

**Zebrafish MO-mediated knockdown**

MOs were obtained from Gene Tools. The nucleotide sequences of the translation-blocking (coq6-MO1) and splice-blocking (coq6-MO4) MOs against D. rerio coq6 were 5'-GCTTAGCTTTAGCCAGAGACAGCAT-3' and 5'-ACACATAAGGGTCAGTCCGGGAA-3', respectively. The 5-bp mismatch controls for the MOs were as follows: coq6-MO1mm, 5'-GGTACCTTTACCCAGACACACAT-3' and coq6-MO4mm, 5'-AACAAGTGTCACCTAGCGGAG-3'. Fertilized eggs were microinjected with the specified amount of MO dissolved in 0.1M KCl. For immunohistochemical labeling, embryos were fixed with 4% PFA in PBS (pH 7.4) and stained with antibody against activated caspase-3 (1:200; BD Biosciences) following standard protocols (63).