Investigation of cellular functions of ADCK4 and its relation to nephrotic syndrome

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Directed by Professor Heon Yung Gee

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ABSTRACT

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Mutations in ADCK4 (aarF domain containing kinase 4, also known as COQ8B) usually manifest as steroid-resistant nephrotic syndrome (SRNS) and sometimes accompany medullary nephrocalcinosis or extrarenal symptoms including seizure. ADCK4 has a helical domain, ABC1 domain, and a kinase-like domain and is similar to yeast Abc1/Coq8 which is required for coenzyme Q_{10} (CoQ_{10}) biosynthesis. ADCK4 localizes to mitochondrial matrix and associates with the inner membranes. Interestingly, patients with ADCK4 mutations exhibit reduced cellular CoQ_{10} contents. However, the role of ADCK4 at the molecular level is unclear. To address this, we knocked out ADCK4 in cultured podocytes and HK-2, a proximal tubule cell line using CRISPR/Cas9. ADCK4 knockout did not affect cell viability in both cell lines. The basal levels of CoQ_{10} levels of podocytes were three-fold higher compared to those of HK-2 cells. The levels of CoQ_{10} were severely decreased in ADCK4 knockout podocytes compared to control podocytes, whereas CoQ_{10} contents in HK-2 cells were not different. As CoQ_{10} is required for electron transfer from complex I and II to complex III of mitochondrial respiratory chain, we measured complex II+III activities. Complex II+III activities were defective only in podocytes, but not in HK-2 cells. In addition, transmission electron microscopy showed that

ADCK4 knockout resulted in disintegrated mitochondria and loss of cristae formation in cultured podocytes, but not in HK-2 cells, suggesting that ADCK4 is indispensable for maintaining mitochondrial function in podocytes. In conclusion, our results showed that podocytes are more vulnerable to loss of ADCK4 than HK-2 cells and this may explain why individuals with ADCK4 mutations mostly manifest only SRNS.

Key words: CoQ_{10}, Steroid Resistant Nephrotic Syndrome, ADCK4

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I. INTRODUCTION

Nephrotic Syndrome is a chronic kidney disease that causes significant proteinuria. Since massive proteins are excreted from the capillaries because of abnormal glomeruli, hypoalbuminemia which means remarkably low albumin level in the blood, occurs subsequently leading to edema by accumulation of fluid in the interstitial tissues. While most children with nephrotic syndrome are sensitive to steroid treatments, approximately 10-20% of patients are not recovered after glucocorticoid therapy and additional immunosuppressive therapy. The cases of those patients are referred to as steroid resistant nephrotic syndrome (SRNS) and most of the patients with SRNS enter into end stage kidney disease (ESKD) requiring dialysis treatment or kidney transplantation.[1]

The visceral glomerular epithelial cell, also called podocyte, is a cell that lines the outer aspect of the glomerular basement membrane forming the filtration barrier which acts as a selective filter and prevents protein leakage. SRNS mostly occurs in association with focal segmental glomerulosclerosis (FSGS) and foot

process effacement.[2] However, the pathogenic mechanisms of glomerular diseases are poorly understood. There are about 45 genes related to SRNS, most of which are related to the structure and function of glomerulus[3]. Mutations of aarF domain containing kinase 4 (ADCK4 or COQ8B) are single-gene cause of SRNS, which were identified in individuals manifesting as isolated adolescent-onset FSGS.[1,2]

Analysis of the ADCK4 amino acid sequence yielded a helical domain, an ABC1 domain and a kinase domain. ADCK4 belongs to ADCK families which have conserved protein kinase motifs corresponding to regions required for ATP binding. As ADCK kinases do not have the classical C-terminal motifs, ADCKs belong to atypical kinases.[4] CABC1 / coq8, yeast homolog of ADCK4, plays a role in stabilizing the phosphorylation state of some components involved in the synthesis of coenzyme Q (Coq3, Coq5, Coq7).[5] Similarly, human ADCK4 proteins also interact with the components of the Coenzyme Q_{10} (CoQ_{10}) biosynthesis pathway (COQ6, COQ7, etc.). In addition, levels of CoQ_{10} in patients with ADCK4 mutations were reduced, and mitochondrial respiratory enzyme activity was found to be lower than in normal controls.[1]

CoQ_{10} or ubiquinone, a lipophilic component located in the inner mitochondrial membrane, Golgi apparatus,[31] and cell membranes, plays a pivotal role in oxidative phosphorylation.[1,2,34] CoQ_{10} shuttles electrons from complexes I and II to complex III.[4] CoQ_{10} also has a critical function in antioxidant defenses by its redox reaction.[7] The CoQ_{10} biosynthesis pathway has been extensively studied in the yeast *Saccharomyces cerevisiae*. It has been found that at least 12 proteins encoded by COQ genes form a complex, simultaneously stabilizing each other, and take part in coenzyme synthesis.[12,13,18,20] On the basis of protein homology, homolog genes have been identified in humans. In humans, at least 15 genes are known to be involved and one of them is ADCK4.[10,17,36] ADCK4 is known to be involved in the synthesis of CoQ_{10} like ADCK3,[6] but the exact cellular functions of ADCK4 are poorly understood. In this study, we investigated ADCK4 functions at the cellular and molecular levels and found that ADCK4 plays a role in the CoQ_{10} biosynthesis

pathway especially in podocytes by interacting with components which have oxidoreductase activities, thereby regulating cellular homeostasis.

II. MATERIALS AND METHODS

1. Cell culture

Human podocytes were a kind gift from Moin Saleem, University of Bristol, Bristol, UK, and cultured as previously described.[33] Human podocytes were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin and 1% insulin transferrin selenium. Human proximal tubule cells (HK-2) and HEK293 were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

2. Molecular cloning

sgRNAs targeting human ADCK4 (sgRNA 1: GCTGCACAATCCGCTCGGCAT, sgRNA 2: GTAAGGTCTGCACAATCCGCT, sgRNA 3: GACCTTATGTACAGTTCGAG,) were cloned into BsmBI-digested lentiCRISPR V2 puro (Addgene plasmid #52961). U6 primer was used for Sanger sequencing to confirm cloned vector. ADCK4 cDNA was cloned into p3XFLAG CMV26 (C-terminal) vector (Sigma Aldrich). BAP cDNA cloned into the p3XFLAG CMV7 was digested by using Kpn1 and EcoR1 restriction enzymes (New England BioLabs, Ipswich, MA) and ligated into a p3XFLAG CMV24 (N-terminal) for stable cell lines. CMV-Forward (5′–ATACTAGTCCACCATGGACTACAAAGACC–3′) and reverse (5′–ATGAATTCCTAGAAGGGCAGCGTGTC–3′) primers were used for Sanger sequencing to confirm cloned vectors.

3. Lentivirus production and transduction

Lenti-X 293T cells were seeded into each 6 well plate dish. Once the cells reached approximately 90% confluence, they were transfected with 1 μg of the expression plasmid, 1 μg of the VSV-G envelope-expressing plasmid pMD2.G, and 1 μg of the 2nd-generation lentiviral packaging plasmid psPAX2 by using 9 μl FuGENE6 (Promega) in OptiMEM. Media were replaced with fresh media 6 hr after transfection. The lentivirus supernatant was collected 48 hr after transfection and

filtered through a 0.20 μM filter. Once podocytes and HK-2 cells were 50% confluent, they were transduced with CRISPR/Cas9 lentivirus targeting ADCK4. Polybrene was used at 4 μg/ml to aid transduction efficiency. Transduced cells were selected using 4 μg/ml puromycin.

4. Cell viability assay

ADCK4-deficient podocytes and HK-2 cells were used for viability assay using Cell Counting Kit-8 (Dong-in bio.). 100 μl cell suspensions (1×10^5/ml) with cultured medium was added to a 96-well plate and incubated for 24 hr at CO_2 incubators. Then, the medium was replaced with phenol-free fresh medium with or without H_2O_2 at 500uM concentrations for 15 hr. Four wells were included in same conditions. 10 μl CCK-8 reagent was added to each well, cells were incubated for 1 hr, and optical density at 450 nm was measured.

5. TEM analysis

The cells were trypsinized and washed twice using PBS. The cells were fixed in 2.5% glutaraldehyde, 1.25% PFA, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C. They were washed in 0.1M phosphate buffer, postfixed with 1% OsO_4 dissolved in 0.1M PBS for 2 hr, dehydrated in ascending gradual series (50 ~ 100%) of ethanol and infiltrated with propylene oxide. Specimens were embedded by Poly/Bed 812 kit (Polysciences). After pure fresh resin embedding and polymerization at 65°C oven (TD-700, DOSAKA, Japan) for 24 hr, sections of about 200~250 nm thickness were initially cut and stained with toluidine blue (sigma, T3260) for light microscope. 70 nm thin section were double stained with 6% uranyl acetate (EMS, 22400 for 20 min) and lead citrate (Fisher, for 10 min) for contrast staining. There sections were cut by LEICA EM UC-7 (Leica Microsystems, Austria) with a diamond knife (Diatome) and transferred on copper and nickel grids. All of the thin sections were observed by transmission electron microscopy (JEM-1011, JEOL, Japan) at the acceleration voltage of 80kV.

6. Immunofluorescence analysis

Cells were grown on 22 mm cover glass and were fixed in 4% PFA for 10 min and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Cells were incubated in blocking buffer containing 10% donkey serum for 1 hr at room temperature. Samples were incubated overnight at 4°C with primary antibodies diluted in blocking buffer. After washes with PBS, samples were incubated with secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) for 1 hr at room temperature, washed, and covered with mounting medium and cover slips. Images were obtained with a LSM 700 microscope (Carl Zeiss).

7. Immunoblotting

Cells were incubated with lysis buffer [150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4), 1% NP-40, and complete proteinase inhibitors], sonicated, and centrifuged at 13,200 rpm for 15 min. The supernatant was collected, and its protein concentration was measured by using Bradford assay. Absorbance at 590 nm was measured by using the SpectraMax microplate reader**.** The protein was mixed with 5x LDS sample buffer (KOMA) and loaded into a 4–12% Glycine-Tris gel (KOMA). The separated protein was then transferred to a nitrocellulose membrane. The blot was blocked with 5% skim milk at room temperature for 1 hr. Primary antibodies were diluted in 5% skim milk and incubated for overnight at 4°C. After washing with TBST, membranes were incubated with secondary antibodies. Protein blots were visualized by using West pico chemiluminescent substrate or West femto maximum sensitivity substrate kit (Pierce). Immunoblotting was quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

8. Immunoprecipitation

HEK293 stably expressing p3XFLAG-ADCK4 or BAP plasmid stable cell lines were lysed in lysis buffer. For 75mg of proteins, 80ul of FLAG M2 agarose beads (Sigma-Aldrich) were added and incubated 48 hr at 4°C orbital shaker. The agarose

beads were washed twice with lysis buffer before lysates were added. After the end of the incubation, centrifuge the tubes, and remove the supernatant from beads. To remove non-specific binding, the beads were washed with lysis buffer 3~4 times. Then, 200 μl of 3xFLAG peptide elution buffer (150ng/μl peptide) was added and samples were incubated overnight at 4 ℃. After first elution, this elution step repeated one more time for 1 hr. 2x SDS buffer was added and samples were incubated at 25 ℃ for 1 hr. After the elution, the elutes were analyzed by immunoblotting, coomassie blue staining, and silver staining.

9. iTRAQ

An isobaric tag for relative and absolute quantification (iTRAQ) proteomic method with 8plex tags (AB Sciex) was used to determine differences in protein expression between control and ADCK4 knockout podocytes. **Digestion.** Each 100 μg samples were precipitated with aceton to eliminate the background for overnight at -20 °C and centrifuged at 6,000 x g for 10 min. Pellets were reconstituted with 20 μl of dissolution buffer (0.5 M triethylammonium bicarbonate), 1 μl of denaturant 2% (vol/vol) SDS, and 2 μl of reducing reagent 50 mM Tris-(2-carboxyethyl) phosphine. The mixtures were vortexed, spun down, and incubated at 60 °C for 1 hr. Free cysteines were blocked by adding 1 μl of 200 mM methyl methanethiosulfonate in isopropropanol and were incubated for 10 min at room temperature. Trypsin (V5111) (Promega) was reconstituted with deionized water at a concentration of 1 mg/mL. 10 μL of trypsin solution were added to each vial, and vials were incubated overnight at 37 °C. **iTRAQ labeling.** The 8plex iTRAQ reagents were allowed to reach room temperature and then were reconstituted with 50 μl of isopropanol. Each labeled reagent was mixed with the corresponding protein digest and incubated at room temperature for 2 hr. Samples were pooled into a new vial and dried in a SpeedVac (Thermo Scientific). After reconstitution with 0.1% formic acid (FA), the digest was desalted on a Waters Oasis HLB Extraction Cartridge and eluted with 60% (vol/vol) acetonitrile (ACN), 0.1% FA. The eluted peptide mixture was dried with a SpeedVac. Strong cation exchange fractionation. The sample was reconstituted with 100 μl

strong cation exchange buffer A (10 mM KH_2PO_4, 20% ACN, pH 2.7) and separated on a PolyLC Polysulfoethyl A column (200 × 2.1 m, 5 μm, 200 Å) with a linear 200 μL/min gradient of 0–70% buffer B (10 mM KH_2PO_4, 20% ACN, 500 mM KCl, pH 2.7) for 45 min on an Agilent 1200 LC device with Chemstation B.02.01 control software (Agilent). Fractions were collected every minute and eventually were pooled into 24 fractions. After drying in a SpeedVac, the fractions were desalted, eluted, and dried as described above. **LC-MS.** The samples were reconstituted with 0.1% FA. Liquid chromatography was performed on an Eksigent nanoLC-Ultra 1D plus system. Peptide digest first was loaded on a Zorbax 300SB-C18 trap (Agilent) at 6 μL/min for 5 mi and then was separated on a PicoFrit analytical column (100 mm long, i.d. 75 μm, tip i.d 10 μm, packed with BetaBasic 5-μm 300-Å particles (New Objective) using a 40-min linear gradient of 5–35% ACN in 0.1% FA at a flow rate of 250 nl/min. Mass analysis was carried out on an LTQ Orbitrap Velos (Thermo Fisher Scientific) with data-dependent analysis mode, where MS1 scanned the full MS mass range from m/z 300–2,000 at 30,000 mass resolution, and six higher-energy collisional dissociation tandem mass spectrometry (HCD MS2) scans were carried out sequentially at a resolution of 7,500 with 45% collision energy, both in the Orbitrap. **Database search and quantitative data analysis.** MS/MS spectra from 24 fractions were searched against the UniProtKB/Swiss-Prot database, taxonomy *H.Sapiense* using our six-processor Mascot (version 2.3; Matrix Science) cluster at the National Institutes of Health, with precursor mass tolerance at 20 ppm, fragment ion mass tolerance at 0.05 Da, trypsin enzyme with two miscleavages, methyl methanethiosulfonate of cysteine and iTRAQ 8plex of lysine and the N terminus as fixed modifications, and deamidation of asparagine and glutamine, oxidation of methionine, and iTRAQ 8plex of tyrosine as variable modifications. The resulting data file was loaded into Scaffold Q+ (version Scaffold_3_00_04; Proteome Software, Inc.) to filter and quantitate peptides and proteins. Peptide identifications were accepted at 80% or higher probability as specified by the Peptide Prophet algorithm and a false-discovery rate (FDR) of less than 1%. Protein identifications were accepted at 90.0% or higher probability and contained at least two identified peptides

with FDR less than 1%. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Peptides were quantitated using the centroided reporter ion peak intensity, with a minimum of 5% of the highest peak in the spectrum. The isobaric tagged samples were normalized by comparison with the median protein ratios for the reference channel. Quantitative protein values were derived only from uniquely assigned peptides. Protein quantitative ratios were calculated as the median of all peptide ratios. SDs were calculated as the interquartile range around the median. Quantitative ratios were $\log_2$ normalized for final quantitative testing

10. Statistical analysis

Data are presented as the means ± standard error. Statistical analysis was performed with unpaired t-test using the GraphPad Prism software package (version 7.0). $P<0.05$ was considered statistically significant.

III. RESULTS

1. Loss of ADCK4 causes mitochondrial defects in podocytes.

To investigate the function of ADCK4, we generated stable cell lines with human cultured podocytes and proximal tubule cells. Each cell was targeted to deletion of the ADCK4 gene locus (Fig. 1A) with a CRISPR/Cas9 containing targeting sgRNA. Disruption of coding exon 6 yielded ADCK4 frameshift alleles which confirmed by Sanger sequencing (Fig. 1B-C), and the absence of ADCK4 was proved by western blot of cell lysates (Fig. 1D-E). The generated ADCK4 knockout (KO) cell lines showed no difference in cell viability assay, revealing that loss of ADCK4 does not have impact on cell survival (Fig. 1F-G).

Since levels of coenzyme Q_{10} (CoQ_{10}) are decreased in cells derived from patients with ADCK4 mutations[1], we next examined this using established KO cells and found that the total CoQ_{10} was reduced in cultured podocytes, but not in HK-2 cells (Fig. 2A). The basal levels of coenzyme Q_{10} (CoQ_{10}) levels of podocytes were three-fold higher compared to those of HK-2 cells. The levels of CoQ_{10} were severely decreased in ADCK4 KO podocytes compared to control podocytes, whereas CoQ_{10} contents in HK-2 cells were not different. The activity of respiratory chain complex II+III is dependent on CoQ_{10} content in the mitochondria,[8] as CoQ_{10} shuttles electrons from complex I and II to complex III of respiratory chain. Therefore, we measured complex II and II+III activities, and found that even though complex II activities were impaired in both cell lines, complex II+III activities were defective only in, but not in HK-2 cells. Taken together, these data showed that loss of ADCK4 causes defects consistent with CoQ_{10} deficiency.

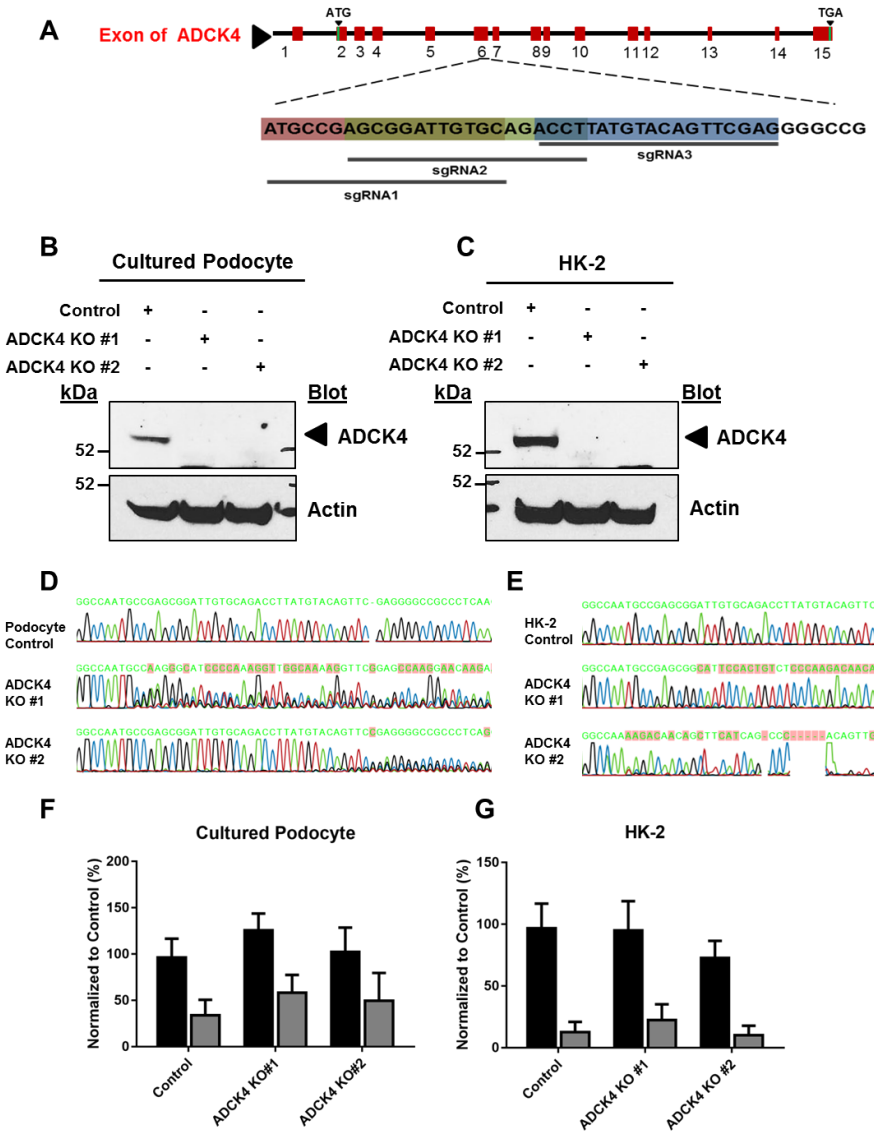


Figure 1. Generation of ADCK4 knockout cells using CRISPR/Cas9. A. Three sgRNAs targeting colored, green-colored, and blue-colored sequences, respectively. **B-C.** Western blots of ADCK4 expression in the CRISPR/Cas9-edited podocytes (B) and HK-2 cells originated from proximal tubule (C). **D-E.** Sanger sequencing of the exon 6 of ADCK4 confirmed genome editing in KO cell lines. **F-G.** Cell viability was measured using cell count kit-8 (CCK) assay. H_2O_2 was treated at 500μM for 15 hr. Cell viability was not different between KO and control cells in both podocytes (F) and HK-2 cells (G). Data are representative of at least three independent experiments.

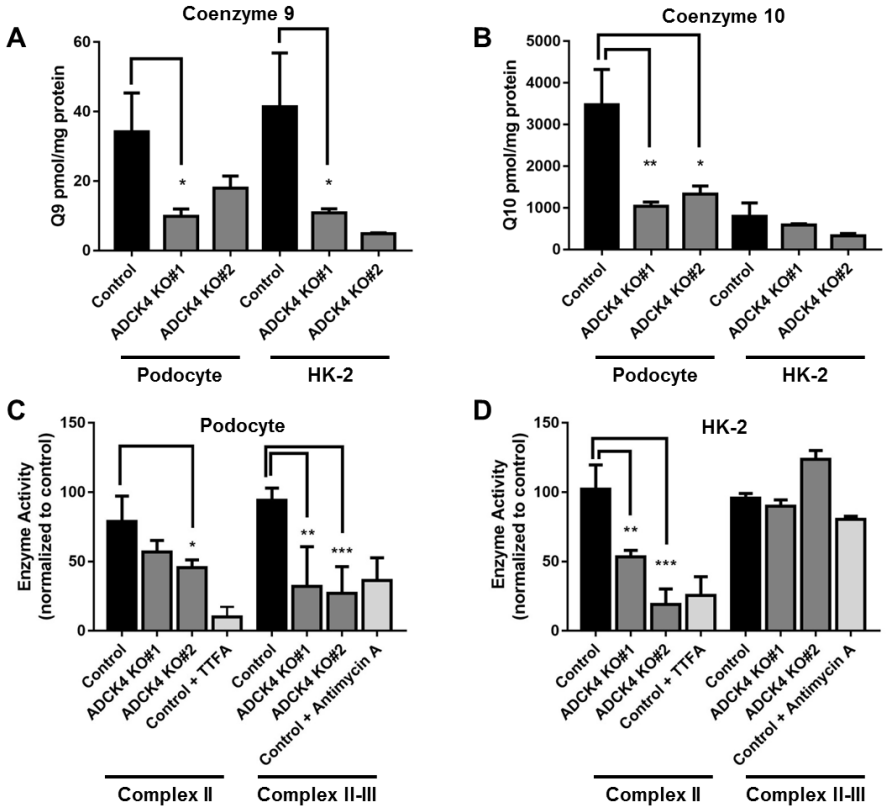


Figure 2. Coenzyme Q is deficient in ADCK4 knockout (KO) podocytes. A-B. Coenzyme Q contents of cultured podocytes and HK-2 cells. CoQ_{10} levels are severely deficient in ADCK4 KO podocytes. **C-D.** Respiratory chain complex II and succinate-cytochrome c reductase (complex II-III) enzyme activities were measured in podocytes (C) and HK-2 cells (D). Complex II-III activities were decreased only in podocytes, whereas complex II activities were affected in both cell lines. *$P < 0.05$; **$P < 0.005$; ***$P<0.001$.

2. Disrupted mitochondrial morphology and inactivation of mitochondrial membrane potential was observed in ADCK4 knockout (KO) podocytes.

As seen in Figure 2A-D, the loss of ADCK4 demonstrated defects in CoQ_{10} biosynthesis and respiratory enzyme activities. Notably, patients with ADCK4 mutations showed mitochondrial abnormalities in podocytes and proximal tubules.[9] These abnormal proliferation of polymorphous mitochondria in the cytoplasm of podocytes is one of the characteristic ultrastructural findings of CoQ_{10}-related diseases.[9,10,11] Therefore, we next examined ultrastructure of mitochondria of ADCK4 KO cells by transmission electron microscopy (TEM). Formation of cristae was wrecked and shapes of mitochondria were disintegrated in ADCK4 KO podocytes (Fig. 3A–C'), whereas ADCK4-deficient HK-2 cells showed normal features of mitochondria (Fig. 3D-F").

To gain functional insights into the observed mitochondrial morphological alterations, we assessed the mitochondrial membrane potential by loading the cells with the JC-10 dye, which concentrated in the mitochondrial matrix based on membrane polarization. JC-10 formed red fluorescent aggregates in normal cells, while monomeric form of JC-10 diffused and fluoresced green in apoptotic cells with low mitochondrial potential.[37] Mitochondrial membrane potential analysis disclosed the presence of inactive mitochondria in podocytes, but not in HK-2 cells (Fig. 3G-H). Therefore, podocytes are more susceptible for loss of ADCK4 than HK-2 cells and loss of ADCK4 induced mitochondrial defects.

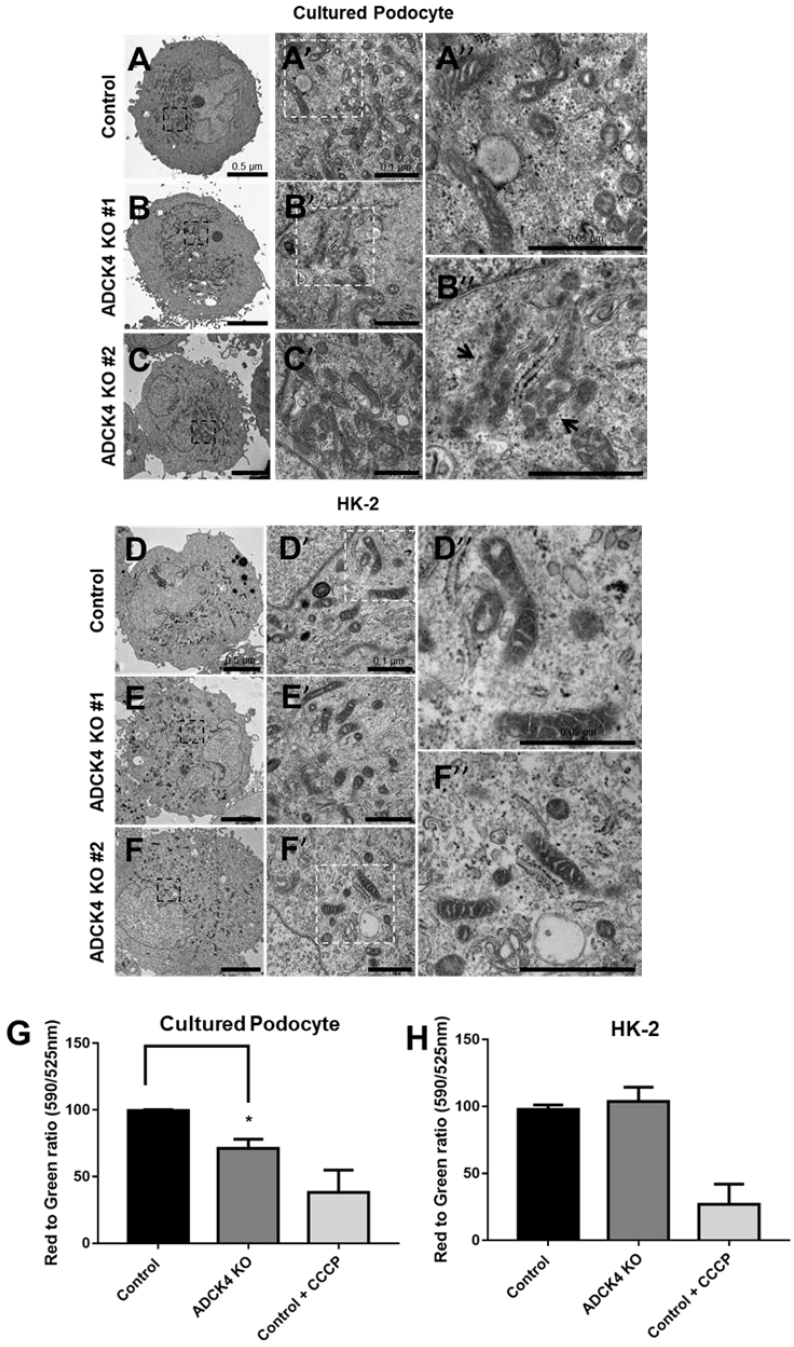


Figure 3. Transmission electron microscopy (TEM) images of ADCK4 knockout (KO) cell lines. A-C'. TEM of podocytes showing mitochondrial morphology. Black and white-boxed areas are enlarged. Mitochondria in ADCK4-deficient

podocytes showed abnormal fission and disrupted cristae in panel B" (black arrows). **D-G'.** TEM of HK-2 cells indicates tubular mitochondrial morphology and normal shape of cristae. Scale bars, 0.5μm in A-C and D-F, 0.1μm in A'-C' and D'-F', 0.05μm in A", B", D", and F". **G-H.** Effect of ADCK4 KO on mitochondrial membrane potential (MMP). MMP were measured in JC-10 stained podocytes and HK-2 cells and quantified using Fluorometric assay. Data showed the presence of inactive mitochondria in ADCK4 KO podocytes, but not in HK-2 cells.

3. ADCK4 knockout (KO) results in cytoskeletal changes in cultured podocytes, but not in HK-2 cells.

Previous study showed that knockdown ADCK4 by siRNAs in podocytes resulted on reduced migration.[1] We next observed cytoskeleton of ADCK4 KO cells using phalloidin staining and COXIV (mitochondrial marker) antibodies. In case of podocytes, ADCK4 deficient causes reduced phalloidin intensity compared to control cells (Fig. 4A). However, HK-2 cells did not show any differences between control and ADCK4 KO (Fig. 4B). Therefore, ADCK4 may have a role in the maintenance of cytoskeleton structure.

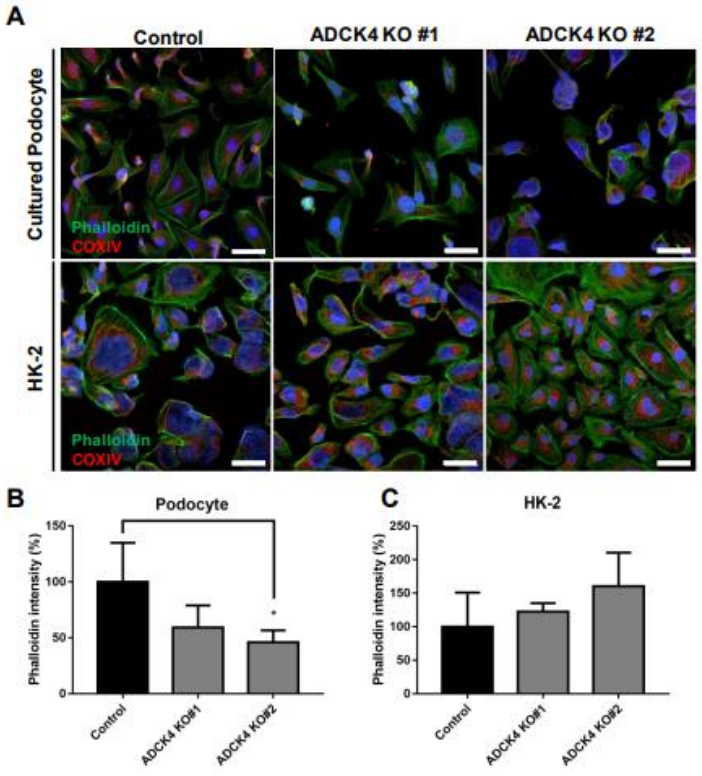


Figure 4. ADCK4 knockout (KO) results in cytoskeletal changes in cultured podocytes, but not in HK-2 cells. A. Cells were stained with phalloidin (green) and COXIV, a mitochondrial marker (red). Scale bar, 50μm. **B.** Quantification of phalloidin intensity in control and ADCK4 KO cells.

4. Sensitivity of ADCK4 knockout (KO) cells to polyunsaturated fatty acids.

The reduced form of CoQ (QH_2) may play a role as a potent lipid-soluble antioxidant scavenging free radicals and preventing lipid peroxidative damage.[12,13] We treated cells with arachidonic acid (AA), a polyunsaturated fatty acid, which is prone to autoxidation and breakdown into toxic products.[12] Then, we examined MAPK pathway, which is involved various in cell signalings such as cellular inflammation and cell cycle, in order to see whether ADCK4 deficiency causes hypersensitivity to the oxidative damages of polyunsaturated fatty acids. As a result of polyunsaturated fatty acid treatment, there were marked elevations of MAPK signaling including p-p38, p-ERK1/2, p-JNK in ADCK4 KO podocytes (Fig. 5B-C). However, loss of ADCK4 already induced activation of p-ERK1/2 signals before treatment of arachidonic acid (Fig. 5B). However, loss of ADCK4 did not induce apoptosis whether arachidonic acids are treated or not (Fig. 5A). We next observed ultrastructure of mitochondria of ADCK4 KO cells by transmission electron microscopy (TEM) after AA treatment. As a result, AA-treated ADCK4 KO podocytes showed swollen mitochondria exhibited shortened cristae and fewer inner mitochondrial membranes (Fig. 5D-E'). These results indicate that ADCK4 KO confers susceptibility to cellular stress, such as autoxidation products of polyunsaturated fatty acids.

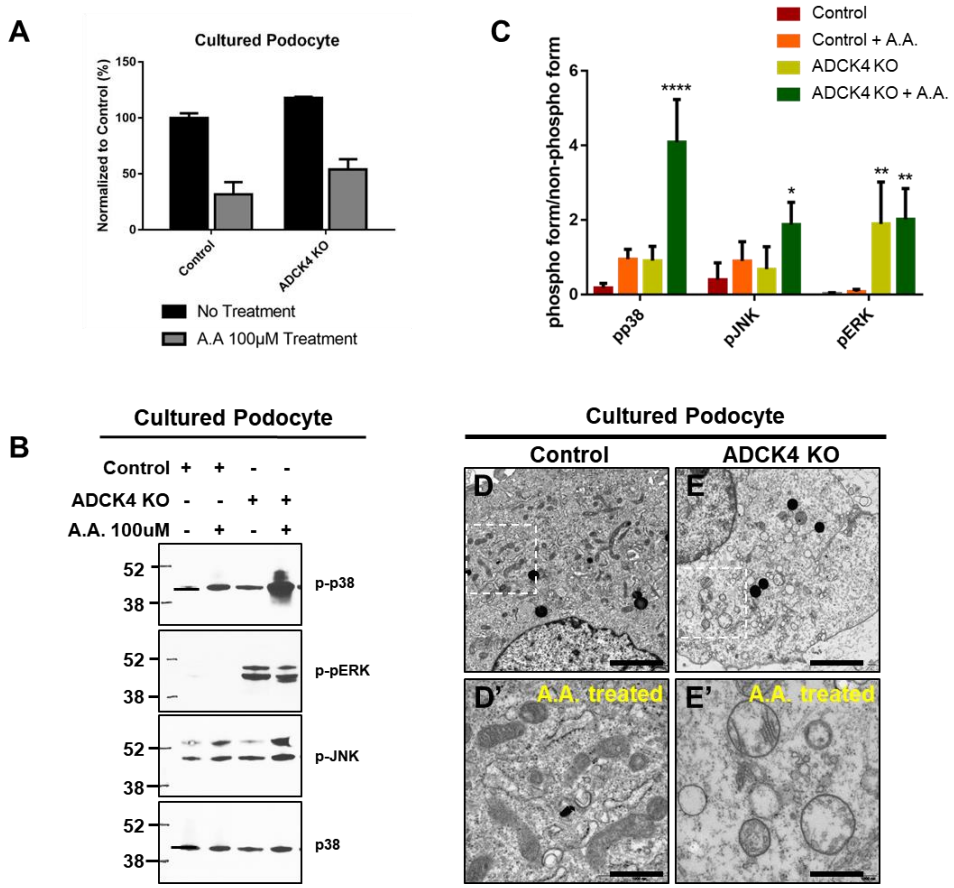


Figure 5. ADCK4 knockout (KO) results in the activation of MAPK pathway in podocytes and sensitivity to polyunsaturated fatty acids. **A.** Cell viability was decreased by 100 μM AA treatment for 4 hr, however, there was no difference between control and ADCK4 KO podocytes. Cell viability was measured using CCK assay. **B.** Cells were treated with 100μM arachidonic acid (AA), a polyunsaturated fatty acid. Representative immunoblots analyses of phosphorylated p-p38, pERK1/2, p-JNK in podocytes. Unphosphorylated p38 was used as a loading control. p-p38 and p-pERK1/2 were increased by ADCK4 KO and p-p38 was further induced by arachidonic acid. **C.** Bar graphs represent band intensities of the blot shown in panel A, and data represent the mean ± standard deviation of more than three independent experiments. Band intensities were normalized to p38. **D-E'.** TEM images of podocytes showing mitochondrial morphology after AA treatment in control (D-D') and ADCK4 KO (E-E'). White-boxed areas are enlarged. Scale bars, 0.2μm in D-E, 0.1μm in D'-E'.

5. Proteomic analysis in ADCK4 KO cells using iTRAQ

Toward understanding the molecular basis for the CoQ_{10} deficiency of ADCK4 KO cells, we quantified protein abundance changes by MS-based proteomics using isobaric tag for relative and absolute quantification (iTRAQ)[27] in podocytes in the absence and presence of AA treatment. Proteomic characterization of control and ADCK4 KO podocytes identified more than about 2500 proteins, and 421 (16%) proteins were mitochondrial proteins. Using Gene Ontology (GO), using the DAVID functional annotation tool (david.abcc.ncifcrf.gov), the differentially expressed proteins in control and ADCK4 KO cells were divided into three categories of GO annotations: biological process, cellular component, molecular function. The results indicate that proteins related to cellular defense response were up-regulated in ADCK4 KO podocytes compared to control cells. In an injury situation using arachidonic acid treatment, coenzyme metabolism-related proteins and intermediate filament related proteins were down-regulated, and DNA regulation proteins were up-regulated in ADCK4 KO podocytes (Fig. 6A). Gene ontology terms of up- and down-regulated proteins were also consistent with proteomic survey, especially metabolic process related proteins were down-regulated upon AA treatment (Fig. 6B-E). These results demonstrate that ADCK4 is involved not only in coenzyme biosynthesis proteins, but also cellular metabolism related-proteins in podocytes, thereby maintaining CoQ_{10} biosynthesis complex.

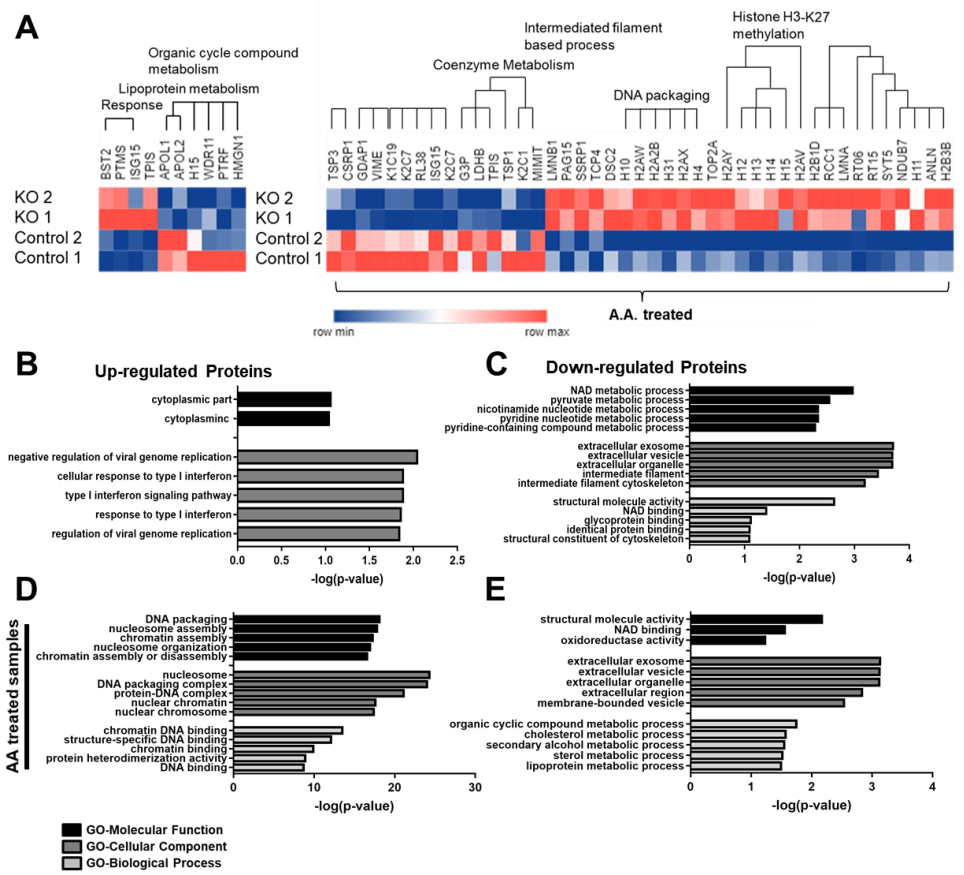
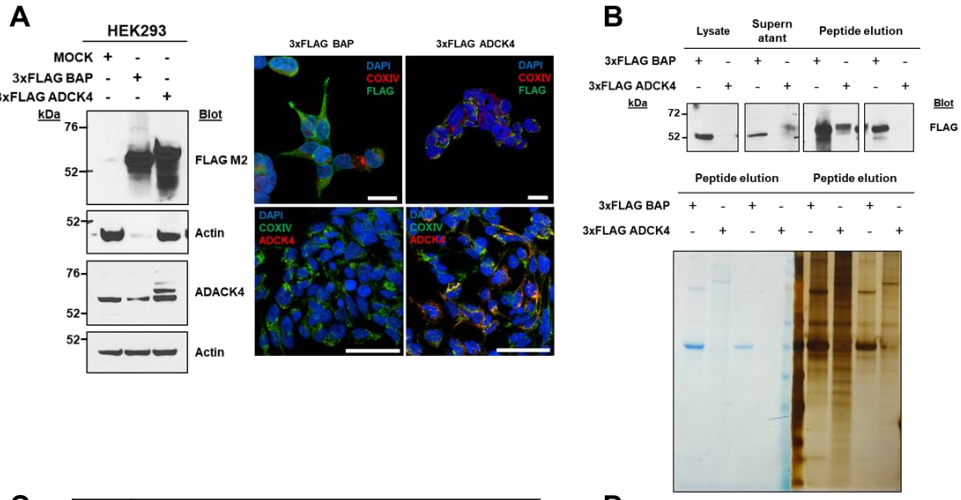


Figure 6. Identification of differential protein expression in ADCK4 KO podocytes. A. Relative abundance of 10 proteins which were differentially expressed (>1.5 fold) between control and ADCK4 KO podocytes (left). Heat map showing 42 differentially expressed proteins upon AA treatment (right). **B-C.** Gene ontology (GO) terms of the top 5 up-regulated and down-regulated proteins in ADCK4 KO podocytes compared to control cells. **D-E.** GO terms of proteins showing changes upon AA treatment**.** GO representing Molecular Function, Cellular Component, and Biological Process were separately analyzed for enrichment. Five most significantly enriched GO terms in three categories are plotted against –log(p-value). p-value < 0.05.

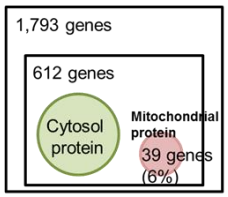
6. ADCK4 interactors have various functions in mitochondria.

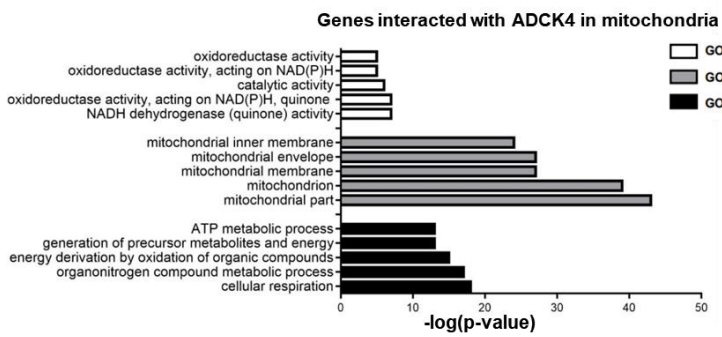
To identify interactors of ADCK4, we generated BAP and ADCK4 stable cell lines. As a control, cells expressing BAP were used and each cell line was confirmed by immunoblot and immunofluorescence (Fig. 7A). ADCK4-interacting proteins were purified by immunoprecipitations and elutes were resolved by SDS-PAGE for immunoblot, coomassie blue staining, and silver staining (Fig. 7B). The eluted proteins were subject to for LC-MS/MS analysis. As a result, 1,793 proteins were detected and 612 proteins were exclusively interacted with ADCK4. Among them, 39 proteins were mitochondrial proteins (Fig. 7D). To address the analysis consequences of ADCK4 association, we performed GO (Gene ontology) analysis. Although ADCK4 is also present in cytoplasm, we focused on mitochondrial proteins because ADCK4 is enriched in mitochondria. Gene ontology analysis showed that proteins involved in oxidoreductase activity, catalytic activity, and NADH dehydrogenase activity were significantly associated with ADCK4 in mitochondria (Fig. 7E). Those results are consistent with protein list of ADCK4 interactors listed in order of highest correlations (Fig. 7C). As we generated iTRAQ data, we looked into abundance changes of ADCK4 interactors. In ADCK4 KO cells, protein associated with cytokine production pathway were down-regulated compared to control cells with statistical significance. Meanwhile, genes associated with cellular response to stress were up-regulated in ADCK4 KO cells. When AA was treated, genes linked to actin skeleton organization were down-regulated in ADCK4 KO cells compared to control cells (Fig. 7F). These results are consistent with differential protein expression in ADCK4 KO podocytes (Fig. 6A-E)



C

STRBP	Spermatid perinuclear RNA-binding protein
ATP5L	ATP synthase subunit g, mitochondrial
CX6A1	Cytochrome c oxidase subunit 6A1, mitochondrial
AGK	Acylglycerol kinase, mitochondrial
SUCB1	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial
SUCB2	Succinate--CoA ligase [GDP-forming] subunit beta, mitochondrial
ABCB7	ATP-binding cassette sub-family B member 7, mitochondrial
PYC	Pyruvate carboxylase, mitochondrial
SLIRP	SRA stem-loop-interacting RNA-binding protein, mitochondrial





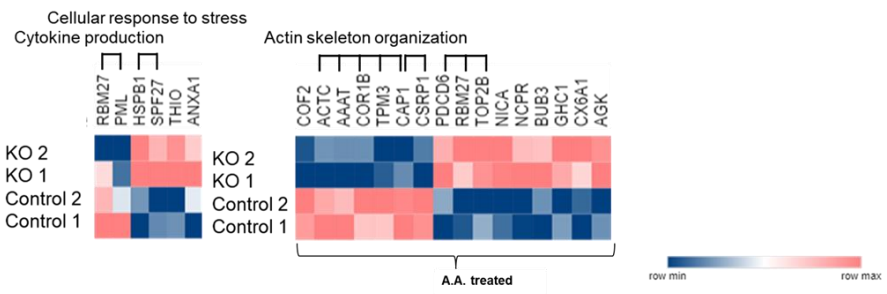


Figure 7. ADCK4 interactors were identified by LC/MS-MS and analyzed Gene Ontology. A. HEK293 cells were transfected with 3xFLAG-tagged ADCK4 and BAP vector and confirmed by immunoblot analysis. HEK293 stably expressing 3xFlag-tagged ADCK4, BAP vectors were confirmed by immunofluorescence using COXIV (red), FLAG (green) antibodies. **B.** Cell lysates were immunoprecipitated with FLAG M2 antibody conjugated agarose beads and eluted by competing with 3xFLAG peptides. Each step was confirmed by immunoblot. The elutes were resolved by SDS-PAGE and prepared for coomassie blue staining and silver staining. **C-E.** Gene ontology analysis of mitochondrial proteins which interact with ADCK4. 612 proteins exclusively interacted with ADCK4 were detected in LC-MS/MS analysis. And 39 of 612 (6%) proteins were mitochondrial proteins (D). List of ADCK4-interacting mitochondrial proteins (C). GO terms of ADCK4 interactors (E). GO representing Molecular Function (MF), Cellular Component (CC), and Biological Process (BP) were separately analyzed for interactions. Five most significantly associated GO terms in three categories are plotted against –log (p-value). t-test, p-value < 0.05. **F.** Relative abundance of 22 proteins which were differentially expressed (>1.5 fold) between control and ADCK4 KO podocytes. Heat map showing 22 differentially expressed proteins upon AA treatment.

IV. DISCUSSION

A biosynthetic complex for producing CoQ was revealed in yeast and mammals. This complex likely contains proteins, lipids, and polar small molecules, but its precise composition, structure, and activities remain largely unknown.[17] Primary CoQ deficiencies are known to be caused by mutations in CoQ biosynthesis proteins (e.g. COQ2, COQ5, ADCK3, ADCK4, PDSS1, PDSS2, etc.) These diseases variably disrupt multiple organ system – organs with high demand for mitochondrial oxidative phosphorylation.[17] For example, mutations in ADCK3, the ortholog of ADCK4, disrupt brain function with cerebellar ataxia[6,32], while mutations in ADCK4 disrupt kidney function with SRNS.[1,2,8,9,23] Although ADCK3 and ADCK4 have highly similar domains of an ancient atypical kinase family and appear to results from gene duplication in vertebrates,[6] ADCK3 and ADCK4 are independently required for synthesis of CoQ_{10}, maintaining distinct functions.[1,21,22]

Coq8p, the yeast homolog of ADCK4, is required for CoQ synthesis complex integrity, and Coq8p overexpression has ability to rescue complex Q stability in various mutant yeast strains that would otherwise be complex Q deficient.[18,19] However, how Coq8p stabilizes complex Q is unknown. Initially, Coq8p was hypothesized to be a protein kinase because phosphorylation of Coq3p, Coq5p, Coq7p is altered in coq8 null mutant yeast.[20] However, whether this effect is caused directly by the absence of Coq8p is unclear.[21]

In this study, ADCK4 KO cell lines were generated using cultured podocytes and proximal tubule cells (HK-2) to investigate cellular functions of ADCK4. Although loss of ADCK4 did not effect on cell survival in both cell lines, only podocytes demonstrated disrupted CoQ_{10} biosynthesis pathway and severely reduced CoQ_{10} contents. In addition, respiratory chain enzyme activity in complex II+III and mitochondrial membrane potential were more reduced in podocytes compared to those of HK-2 cells. TEM analysis also revealed that ADCK4-deficient podocytes had mitochondrial cytopathy which showed disintegrated mitochondria and loss of cristae formation while mitochondria of HK-2 were maintained normally. These data

have shown that loss of ADCK4 causes defects consistent with CoQ_{10} deficiency in podocytes.[8,9,10,17]

The phalloidin intensity of ADCK4 KO cells were analyzed and results showed that cytoskeleton was defected only in podocytes, but not in HK-2 cells. This observation could suggest that mitochondrial dynamics may also play important roles in maintaining podocyte's shape and function because podocytes, like neurons[25], would require a proper and high distribution of energy to their foot process. Also, as the foot process has rich microfilaments, these interactions might also participate in podocyte homeostasis.[26,27] Moreover, these cytoskeletal defects in ADCK4 KO podocytes were also consistent with iTRAQ data which described that cytoskeleton related proteins were down-regulated in ADCK4 KO podocytes.

The CoQ_{10} is well known for its antioxidants activities protecting cells from oxidative stress.[31] In this study, we treated arachidonic acid, one of the polyunsaturated fatty acid, to the ADCK4 KO podocytes as lipid peroxidation stress and revealed that loss of ADCK4 induced MAPK pathway signaling while it does not impact on cell viability. The MAPK signaling pathway is essential in regulating many cellular processes including inflammation, cell stress response, and cell proliferation. The reduced form of CoQ_{10} may scavenge lipid peroxyl radicals and function as an antioxidant and prevent initiation of lipid peroxidation as it has been reported to eliminate perferryl radicals.[16,17] In this regard, ADCK4 KO induced hypersensitivity to lipid peroxidation stress.

Recent studies in ADCK3 revealed that ADCK3 was shown to lack canonical protein kinase activity in trans, and to instead bind lipid CoQ_{10} intermediates and have ATPase activity.[22,23,29] GO analysis also revealed that interactors of ADCK4 especially in mitochondria are significantly associated with oxidoreductase activity which could be related to antioxidant features of CoQ_{10}. Furthermore, Proteomic analysis identified ATP binding protein as ADCK4 interactor, suggesting ATPase activity of ADCK4 like ADCK3. Yet, precise role of ADCK4 is not clear, further studies are required for whether ADCK4 has ATPase activities or kinase activity against an undiscovered substrate.

In conclusion, our study indicates that ADCK4 in podocytes associated with proteins that are involved in coenzyme metabolism, ATP metabolism, maintaining cytoskeleton structures. These results gave insights into functions of ADCK4 involved in CoQ biosynthesis pathway and pathogenesis of nephrotic syndrome.

V. CONCLUSION

In this study, I demonstrated that the loss of ADCK4 induce CoQ10 deficiency, mitochondrial defects, and cytoskeletal changes in cultured podocytes. Podocytes are more susceptible to loss of ADCK4 than HK-2 cells, which are originated from renal proximal tubules.

Our results provide insights into the molecular functions of ADCK4. ADCK4 is involved not only in CoQ10 biosynthesis by retaining oxidoreductase activity in mitochondria, but also in maintenance of well-structured cytoskeleton outside of mitochondria. In addition, proteomic analysis on ADCK4 interactors suggests that ADCK4 may have an ATPase activity which is essential in CoQ10 biosynthesis pathway.

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ABSTRACT (in Korean)

ADCK4 돌연변이에 의한 신증후군 발생 기전

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소변으로 과량의 단백질이 빠져나가는 단백뇨를 증상으로 갖는 신증후군(nephrotic syndrome)은 신장 사구체의 이상으로 모세혈관으로부터 과량의 단백질이 빠져나가기 때문에 혈액 내 알부민 수치가 현저하게 적은 hypoalbuminemia 가 생기고 그로 인해 혈액 내 수분이 빠져나가 부종이 생기게 된다. 신증후군을 앓고 있는 소아의 10-20%는 steroid 치료제나 추가적인 면역억제 치료에도 회복되지 않고 말기신질환으로 진행 되는 steroid-resistant NS(SRNS)이다.

SRNS 와 관련된 45여개의 유전자들이 발견되었는데 그 중 하나가 ADCK4로, CoQ_{10} 생합성에 관여한다고 알려져 있다. ADCK4는 세포질에도 존재하지만 CoQ_{10} 합성이 일어나는 mitochondria 안에 주로 위치한다. Kinase-like domain 을 가지고 있지만 전형적인 kinase motif 와 다른 motif 을 가지고 있어서 atypical kinase 라고도 한다. 그러나 ADCK4가 실제로 kinase 기능을 갖고 있는지 여부는 불분명하며 CoQ10 합성과정에서 구체적인 역할은 알려진 바가 없다. 따라서, human

cultured podocytes와 tubule cells (HK-2) 를 이용해 ADCK4의 기능과 병리기전을 분자적 수준에서 규명해 보고자 한다.

CRISPR/Cas9 을 이용하여 ADCK4 KO cell lines을 제작하였고, cell viability 를 측정한 결과 ADCK4가 podocytes 와 HK-2 모두에서 cell survival에 영향을 주지 않았다. 그러나, CoQ_{10} 의 양은 ADCK4가 KO된 podocytes 에서 심각하게 감소되었고, CoQ_{10}이 미토콘드리아 전자전달계에서 complex Ⅰ과 Ⅱ에서 complex Ⅲ로 전자를 전달하는 역할을 하기 때문에, respiratory enzyme activity 를 측정한 결과 complex Ⅱ+Ⅲ에서 enzyme activity가 감소되어 있었다. 반면, HK-2 는 ADCK4가 사라져도 CoQ_{10}과 complex Ⅱ+Ⅲ enzyme activity 모두 영향을 받지 않았다. 투과전자현미경을 이용하여 ADCK4가 KO된 두 세포들에서 미토콘드리아의 morphology 를 관찰한 결과, podocytes 에서는 미토콘드리아가 조각이 나고, cristae가 잘 형성되지 않았던 반면 HK-2의 미토콘드리아는 정상적인 모양을 유지했다.

위 결과를 토대로 ADCK4가 HK-2 보다 podocytes에서 미토콘드리아 기능을 유지하는데 필요하며, ADCK4의 결함이 podocytes에 더 심각한 영향을 끼친다는 것을 확인 할 수 있다.

핵심되는 말: 신증후군, SRNS, ADCK4, Coenzyme Q_{10}