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Supplementary data

Methods

Expression profile of *ALG13* gene

Total RNAs from human adult tissues were purchased from Stratagene. Mouse adult tissues were obtained from a healthy animal sacrificed in accordance with the recommendations of the European Commission. All the procedures related to animal treatments were approved by Ethic-Scientific Committee for Animal Experiments and Italian Health Ministry. Murine podocytes were isolated from mouse glomeruli as described in our previous work [9], mouse ES-derived cardiomyocytes were gently provided by Dr. Cristina D'Alessio, mouse macrophage cell lines (RAW-264) derived from ACCT catalog, human podocyte cell line was gently provided by Prof. Saleem [13], HEK-293 (Human Embryonic Kidney 293) cells and HK-2 (human kidney 2) which is a proximal tubular cell (PTC) line derived from normal kidney, were already available in our laboratory. Total RNAs from tissues and cell lines were isolated by using TRIreagent® (Sigma-Aldrich) protocol. 1 μg of total RNA was reverse transcribed with the RevertAid RT Reverse Transcription Kit (Thermo Fisher). qPCR reactions were performed, as described in previous works, in triplicate using gene specific primers and sybr select master mix for CFX (Life Technology) following the manufacturer's directions. qPCR expression values of the specific genes were normalized versus the expression of the *HPRT* and *Gapdh* genes for human and mouse respectively [11, 12]. Primers used are listed in supplementary Table 1.

Immunofluorescence (IF) on brain and kidney mice tissues

IF staining was performed on serial sections of brain and kidney tissues from wild-type female mice at 28 post natal days. Paraffin slides were deparaffinized in xylene and rehydrated in graded alcohols and water at RT. For antigen detection, the slides were immersed in alkaline buffer pH 9 and boiled in microwave vessel for 30 minutes. Then the sections were incubated in a humidified

chamber with blocking buffer (PBS1X/5% serum/0.2% triton X100) for 1 h and with anti-ALG13 is1 (20810-1-AP, Proteintech, 1:50/Goat anti-Rabbit IgG 594 nm Vector, 1:200) or anti-ALG13 is2 (Anti-GLT28D1, ab 172743, abcam, 1:50/Donkey anti-Mouse IgG 488 nm Vector, 1:200) antibodies. The nuclei were stained with DAPI (Thermo Scientific, 1:1000). Positive controls were included for each antibody and negative controls were prepared without the incubation with the primary antibody. Images were captured using DMI6000 LEICA inverted microscope/LAS AF software.

Immunofluorescence on human podocyte cell line

200.000 cells (human podocytes) were seeded in 6-well plates covered with cover slips and were grown at 37°C for twelve days; then were transfected with 10nM of ALG13-is2-siRNA as described. Forty-eight hours after transfection, adherent cells were fixed with 4% paraformaldehyde, incubated overnight with anti-nephrin antibody (1:50) followed by FITC-conjugated secondary antibody (donkey anti-goat IgG-FITC, sc2024), phalloidin and DAPI. Confocal images were acquired using LSM 510 META, Zeiss Microscope.

Cell cultures

Human Embryonic Kidney 293 cells (HEK 293, ATCC CRL-1573) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO), 1% (v/v) glutamine, 1% (v/v) Penicillin Streptomycin, 1% (v/v) Amphotericin B, at 37°C/ 5% CO2.

Conditionally immortalized undifferentiated human podocytes cell line is resistant to hygromycin and neomycin/G418, because of the 2 stably transfected constructs and is able to remain undifferentiated by growing at the permissive temperature of 33°C / 5%CO2 in RPMI-1640 medium, supplemented with 10% fetal bovine serum (GIBCO), 1% (v/v) glutamine, 1% (v/v) Penicillin Streptomycin, 1% (v/v) Amphotericin B, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL

selenium. The differentiation is induced by switching the temperature to 37°C and is completed in 14 days [13]. Tunicamycin treatment was performed as described in reference [14].

Transfection and knock-down experiments

HEK-293 cells. The day before transfection, 180.000 cells were seeded in 6-well plates and were grown to 70–90% confluence. The standard co-transfection mix was prepared for triplicate samples by adding 2 μg of the nephrin expression vector (OCAB17 B05 pDEYFP_N1GEN) and 3 unique 27mer siRNA duplexes (10 nM) specifically targeting the short isoform 2 of ALG13 gene (DsiRNA TriFECTa Kit, RNC.RNAI.N001013951.12, IDT) in 100 μl of OPTimem (Gibco); 12 μl of IBAfect reagent (IBA) were added separately in 100 μl of OPTimem. The two solutions were mixed, incubated at room temperature for 15 min, and then added to each well. The final volume of the medium plus the transfection mixture was 2 ml. As control, cells co-transfected with nephrin expression vector and 10 nM of Trilencer-27 Universal Scrambled Negative Control siRNA Duplex (IDT) and cells transfected only with nephrin expression vector were used. Forty-eight hours after transfection, RNA and proteins were extracted.

Human Podocyte cells. Conditionally immortalized undifferentiated human podocytes cell line was grown as described in supplementary methods and as previously reported [13]. The differentiation is induced by switching the temperature to 37°C and is completed in 14 days [13]. Tunicamycin treatment was performed at day 12 of differentiation (5 μg/ml) for 20 h as described in reference [14].

200.000 cells were seeded in 6-well plates and were grown at 37°C for twelve days, then were transfected as described before without the nephrin expression vector. Cells were transfected with: 10nM ALG13-is2 siRNA gene, 10 nM of Universal Scrambled Negative Control, 2 μg of the ALG13-is2141T, 2 μg of the ALG13-is2141L expression vectors (the Alg13 cDNA clone (NM_018466) was synthesized at GeneCust Europe, this clone was used as template for PCR

reaction to generate the full length cDNAs fragment to introduce in pcDNA3.1D/V5-His-Topo vector (Invitrogen). The T141L (ALG13) mutagenesis was carried out by the Quick Change II XL site-directed mutagenesis technique according to the manufacturer's instructions (Stratagene). The whole coding sequences of wild-type and the mutant plasmids were sequenced to confirm the mutagenesis and to exclude undesired mutations) [9].The standard co-transfection mix was prepared for triplicate samples by adding 2 μg of the ALG13-is2141T , 2 μg of the ALG13-is2141L expression vectors and 10nM ALG13-is2 siRNA gene. Forty-eight hours after transfection, RNA and proteins were extracted from cell lysates and medium was collected.

Immunoprecipitation

Immunoprecipitation (IP) was performed by means of the immunomatrix protein A/G PLUS-Agarose (sc2003 SantaCruz Biotechnology). Briefly, pre-clearing was performed by mixing 20 μl of protein A/G PLUS-Agarose together with 500 μg of conditioned medium proteins at 4 °C for 4 h. After centrifugation cell lysate were incubated with 10 μl (10 μg) of anti-nephrin antibody (N20/sc-19000, 1:200, Santa Cruz) at 4 °C for 1 h and then 20 μl of protein A/G PLUS-Agarose were added and incubated at 4 °C overnight. Pellets were washed 4 times with 1ml RIPA buffer and then resuspended in 100 μl of 1x electrophoresis sample buffer.

Glycosylation analysis: Immunoprecipitated samples from podocyte cells treated with ALG13-is2 siRNA, tunicamycin and untreated cells were separated onto 8% polyacrylamide gels and then proteins were transferred to nitrocellulose sheets which were blocked with 0.2% non-fat milk in PBS and incubated with biotinylated concanavalin A (Vector Laboratories) for 1 h at final concentration of 10 μg/ml. After several washing with 0.1% Tween/PBS, blots were incubated for 1 h with streptavidin horseradish peroxidase-conjugated (Vector Laboratories) diluted 1:3000 in PBS. Labeled proteins were developed using the chemiluminescent protein detection system (BioRad).

Image and statistical analyses

Images were acquired and digitized by Image Scanner using LabScan software version 6.0 (GE Healthcare). Optical density was quantified and normalized by the Quantity One Imaging system (BioRad) or TotalLab software version 1.0 (Nonlinear Dynamics). Student's t-test was used to compare the expression of each gene in two different tissues; ANOVA test for multiple comparisons with Bonferroni's corrections was used to analyze multiple data sets.

Protein extraction and Western blot analysis

For Western blotting, cells were solubilized in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 4 mM EDTA, 10 mM Na4PO7, 2 mM Na3VO4, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 mg/mL aprotinin, 1 mM leupeptin) for 60 min. at 4°C. Cell lysates were clarified at 5,000g for 15 min. Conditioned medium proteins (normalized versus the number of cells) were precipitated over night by using pre-cooled acetone. Proteins were recovered after centrifugation at 1500 g at 4° C. Solubilized proteins were separated by SDS-PAGE and transferred onto Immobilon-P membranes (0.45 μm pore size; Millipore, Bedford, MA). The membrane was probed with anti-nephrin antibody (N20/sc-19000, 1:200, Santa Cruz), anti-ALG13-is2 antibody (antiGLT28D1, 1:200 ABCAM), anti-GRP78/BiP antibody (1:1000, Sigma) and anti-β-actin antibody (sc-47778, 1:3000 Santa Cruz Biotechnology) followed by HPR-conjugated secondary antibodies. Densitometric analysis was performed using Scion Image (Ver.4.0.2;Scion Corporation, USA). Total and cytoplasmic signals were normalized against β-actin; conditioned medium signals were normalized with Ponceau Solution (Sigma).

Supplementary Table 1. The complete list of gene specific primers used for qPCR

Gene	Primer Forward	Primer Reverse	Size	MT
ATF6	5'- AGGCTGGATGAAGATTGGGA-3'	5'-CTGGAGAAAGTGGCTGAGGT-3'	223 bp	60°C
BIP	5'-TTCTTGCCGTTCAAGGTGGT-3'	5'-CTTGGCGTTGGGCATCATTA-3'	208bp	60°C
CALR	5'-TGATAACTTTGGCGTGCTGG-3'	5'-CTCCTCCTCTTTGCGTTTCT-3'	217bp	60°C
CD2AP	5'CGAGTTGGAGAAATCATCAG-3'	5'ATTCCCATGCCTTTCCCGTT-3'	171bp	60°C
CHOP	5'-TTAAGTCTAAGGCACTGAGCG-3'	5'-GTCTGATGCCTGTTTTTGTAG-3'	221bp	60°C
HPRT	5'-TGGCGTCGTGATTAGTGATG-3'	5'-TCCAGCAGGTCAGCAAAGAA-3'	222bp	60°C
NPHS1	5'-AGTAATGCCTTGGGGGACAG-3'	5'-ACAGGAGGCATTGGAGAGGA-3'	195bp	60°C
NPHS2	5'-ACCTTCGTCTCCAAACTCTG-3'	5'-TGAGGGATCGATGTGCTAGG-3'	198bp	60°C
XBP1	5'-GCTGAGTCCGCAGCAGGTGG-3'	5'-GAACTGGGTCCTTCTGGGTA-3'	330bp	60°C
ALG13-is1	5'-TGGAGACTCTGGAAAAAGGA-3'	5'-AGGAGCAGAAGCAATGGACT-3'	170bp	55°C
ALG13-is2	5'-ATCTTGTTATTAGTCACGCA-3'	5'-GTAATCCAACAACTTTATCC-3'	267bp	55°C
Alg13-is1M	5'-ATTTCCGAGCAGTTGTTTCA-3'	5'-ACTTGAGTTGGTGGCTTTCC-3'	242bp	55°C
Alg13-is2M	5'-AGTCTGGAGAAAGGCAAACC-3'	5'-AATCCAACAACTTTATCCAA-3'	224bp	55°C
Gapdh-M	5'-TGGAGAAACCTGCCAACTAT-3'	5'- CATACCAGGAAATGAGCTTG-3'	198bp	55°C

All primer sequences are specific to human genes except sequences of Alg13-is1M, Alg13-is2 and Gapdh-M which are murine.

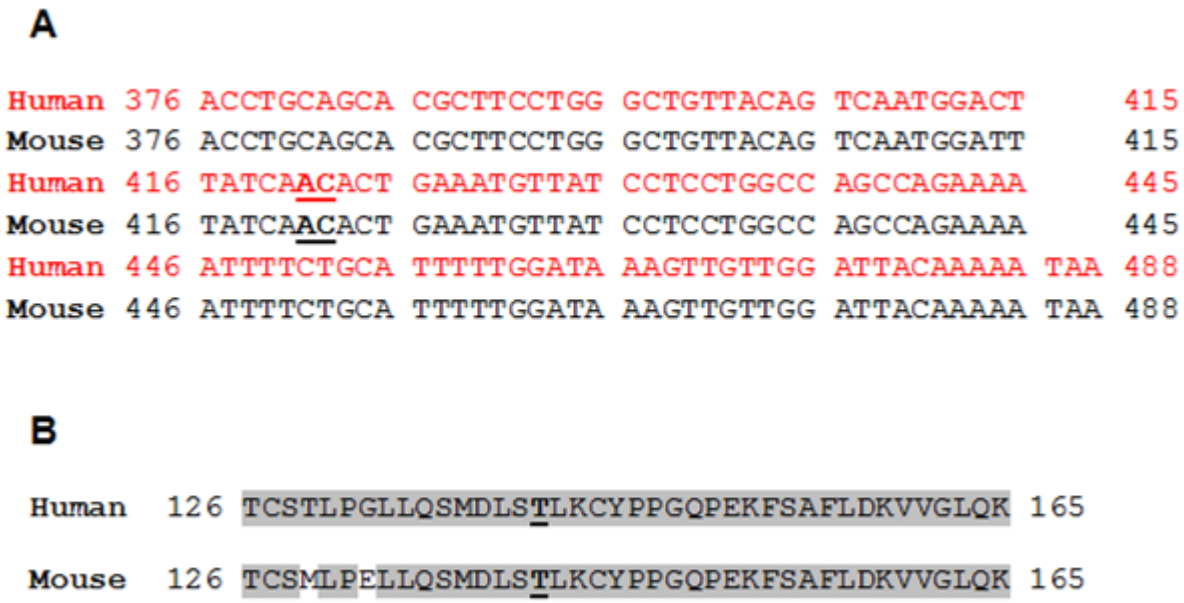
Legends to supplementary figures

Figure 1. Human-Mouse alignment. A, Nucleotide and **B**, amino acid comparison of the C-terminal tail of the human and mouse ALG13-isoform2.

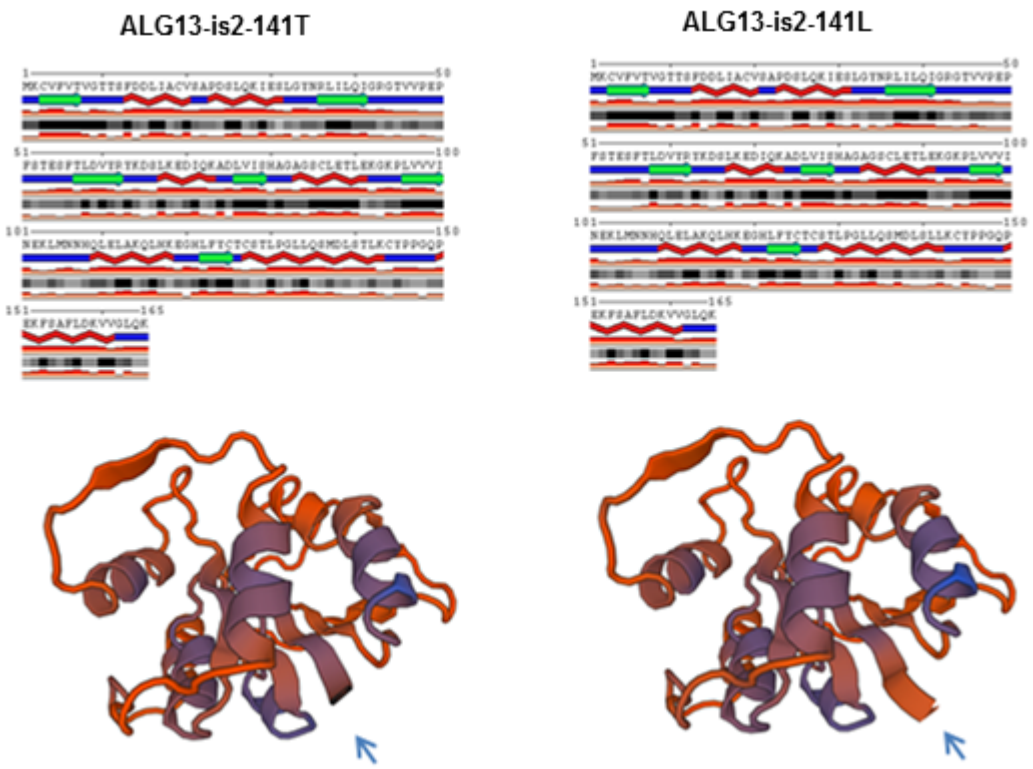
Figure 2. Secondary and tertiary structure prediction. Bioinformatics analysis of the ALG13-is2 wt (141T) and mutant (141L) proteins. No trans-membrane domain is reported. Differences in the C-terminal alpha helix region, of the mutated (141L) protein, are indicated with arrows.

Figure 3. Expression of the two Alg13 isoforms in brain and kidney mice tissues. 10 µm thick paraffin sections were stained using anti-ALG13 is1 (20810-1-AP, Proteintech, 1:50/Goat anti-Rabbit IgG 594 nm Vector, 1:200) and anti-ALG13 is2 (Anti-GLT28D1, ab 172743, abcam, 1:50/Donkey anti-Mouse IgG 488 nm Vector, 1:200) antibodies. The nuclei were stained with DAPI (Thermo Scientific, 1:1000). Images (20X) were captured using DMI6000 LEICA inverted microscope/LAS AF software. **A**, The long isoform 1 is highly expressed in brain specifically in cortex and in the areas CA1 and CA3 of the dorsal hippocampus and dentate gyrus (DG3), while the short isoform 2 was undetectable (data not shown). **B**, In kidney both antibodies, anti-ALG13 is1 and anti-ALG13 is2 (Anti-GLT28D) intensily stain tubular cells, however, a slightly more intense signal in glomerulous and podocyte cells (white arrows) was observed for the short isoform 2.

Figure 4. Localization of Nephrin protein in ALG13-is2 knock-down podocyte cells. Nephrin protein was stained using anti-Nephrin antibody (Santa Cruz)/Fitc secondary green antibody (Santa Cruz). F-actin fibres were stained with rhodamin phalloidin and nuclei were stained with DAPI. The localization of the Nephrin protein is indicated by green spots. The down-regulation of the short isoform 2 of ALG13 gene produces a disorganization of the actin fibers and a reduced expression of the Nephrin on cellular membrane. NT: not transfected; siRNA: podocyte cells transfected with 10nM of ALG13-is2 siRNA (DsiRNA TriFECTa Kit (IDT)); SRC: podocyte cells transfected with 10nM of scrambled molecule (IDT).

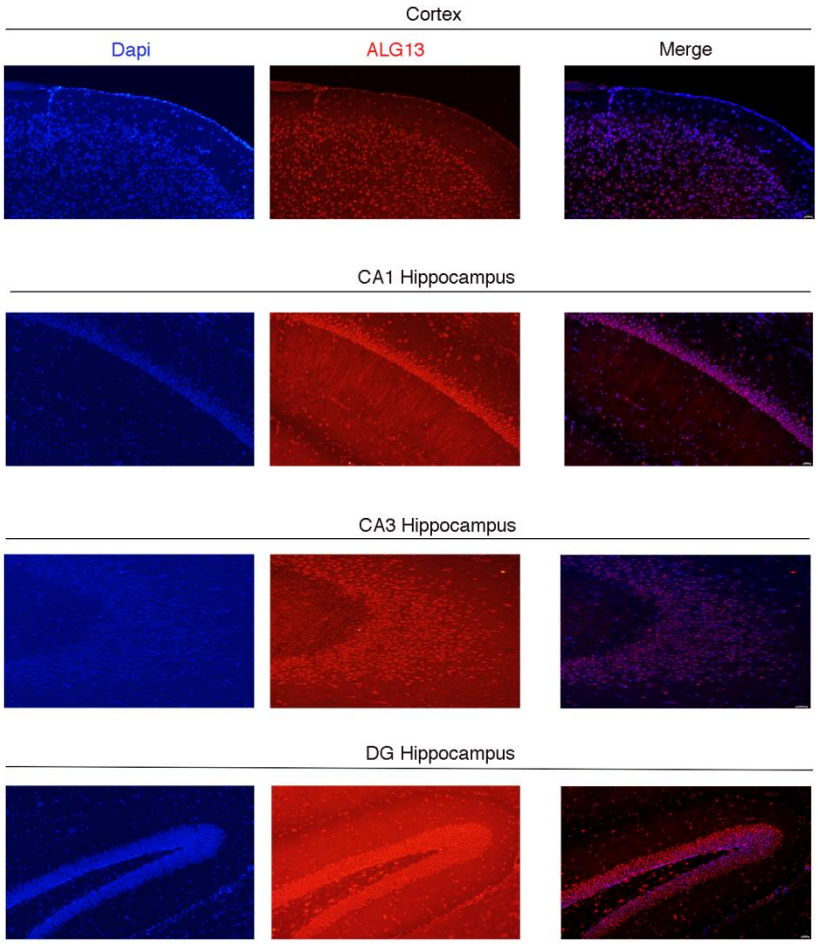


Supplementary Figure 1. Human-Mouse alignment.



Supplementary Figure 2. Secondary and tertiary structure prediction.

A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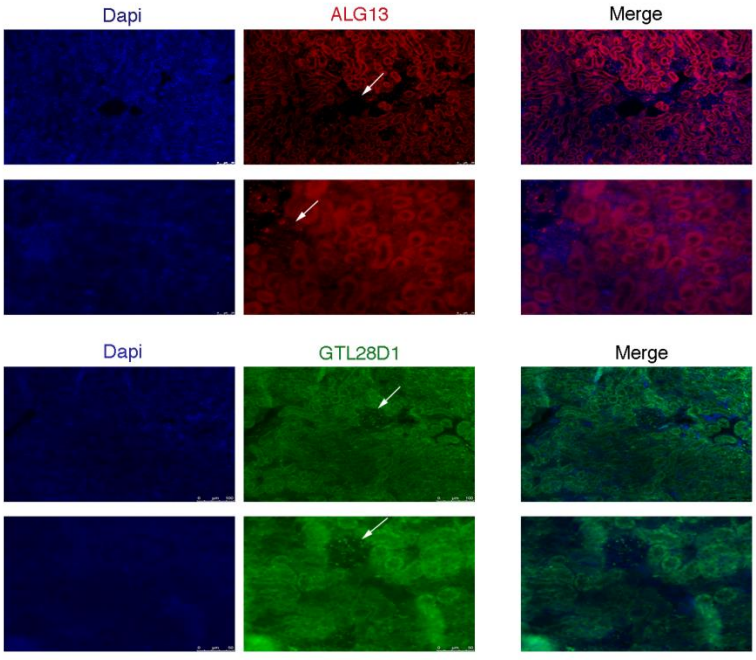
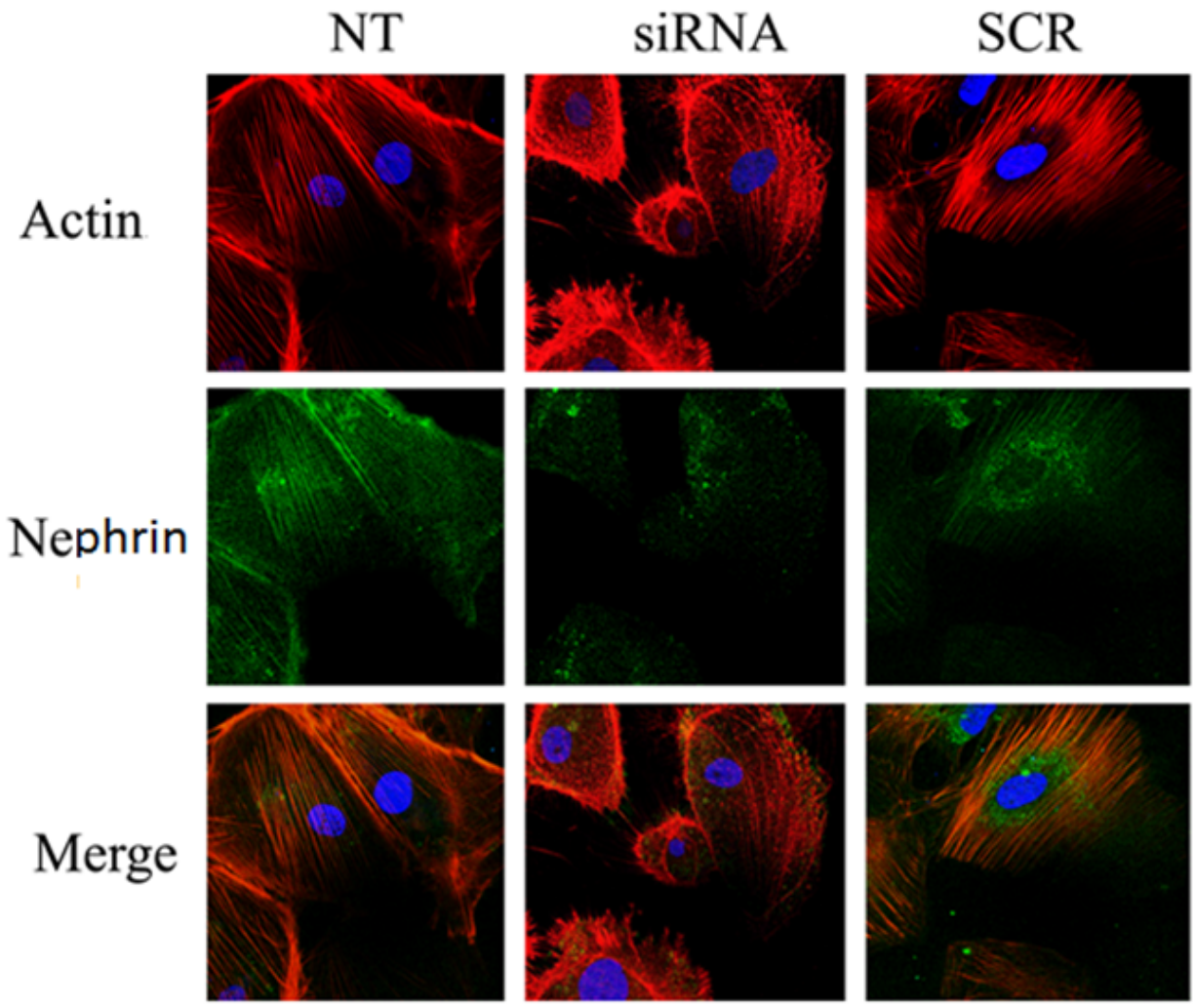
Dapi
ALG13
Merge
CA1 Hippocampus
CA3 Hippocampus
DG Hippocampus

B


Dapi
ALG13
Merge
Dapi
GTL28D1
Merge

Supplementary Figure 3. Expression of the two Alg13 isoforms in brain and kidney mice tissues.



Supplementary Figure 4. Localization of Nephrin protein in ALG13-is2 knock-down podocyte cells.