Supplemental Methods

Mutation Calling

Mutation calling was performed in line with proposed guidelines[1], and the following criteria were employed as previously described[2, 3]: Only rare variants were included, with mean allele frequency <1% in dbSNP147, and with only 0-1 homozygotes in the adult genome database gnomAD. Furthermore, variants were non-synonymous and/or located within splice sites. Subsequently, variant severity was stratified based on protein impact (truncating frameshift or nonsense mutations, essential or extended splice-site mutations, and missense mutations). Splice-site mutations were assessed by *in silico* tools MaxEnt and NNSPLICE splice-site mutation prediction scores[4, 5] as well as conservation across human splice-sites as described previously[6]. Missense mutations were assessed based on SIFT, MutationTaster, PolyPhen 2.0 prediction, REVEL and CADD scores[7-11] and evolutionary conservation evaluated based on manually derived multiple sequence alignments.

Plasmids, siRNAs, cell culture, transfection, and transduction

Human full-length *ARHGEF6* cDNA was subcloned following PCR from human full-length cDNA (RC208074, Origene). Human *ILK, PARVA, PARVB* cDNA was obtained from Origene (RC218650, RC205086, RC220460). Truncation constructs were generated either by PCR or mutagenesis was performed by using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). The splice site variant (c.2135+4A>G) was predicted to disrupt the SRp40 binding site, thereby potentially leading to skipping of exon 20 or (partial) retention of intron 20[12, 13]. We therefore used conservative modeling by including exon 20 in the cDNA construct (p.Lys712*). The following expression vectors were used: pRK5-N-Myc, pCDNA6.2-N-GFP.

Overexpression experiments were performed in HEK293T or IMCD3 cells (ATCC biological resource center), as well as immortalized human podocytes (gift from Moin Saleem, University of Bristol, Bristol, UK). Podocytes were cultured as previously described[14]. HEK293T and IMCD3 cell were maintained in DMEM or DMEM/F12 supplemented with 10% fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin. Plasmids were transfected using Lipofectamine 2000. COS7 cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% serum (Sigma-Aldrich, Merck, Darmstadt, Germany) and penicillin-streptomycin (100 U/ml and 100 mg/ml, respectively) (Sigma-Aldrich) at 37°C and 5% CO_2. For integrin internalization assays, cells were plated on 6-well plates to 50-70% confluency. Cells were transiently transfected with 2 µg GFP-tagged ARHGEF6 expression constructs using 2.5 µl jetOPTIMUS reagent in 200 µl buffer W in DMEM with 10% serum. MDCK-II cells (obtained from Keith Mostov, UCSF) were maintained (37°C, 5% CO_2, humidified atmosphere) in modified Eagle's Medium (MEM) with L-glutamine (Gibco), supplemented with 10% fetal bovine serum (Sigma-Aldrich), penicillin (100 U/ml) and streptomycin (100 µg/ml; both PAA). All cell lines were routinely tested negative for mycoplasma contamination (MycoAlert, Lonza).

ARHGEF6 expression constructs in pEntry-GFP were cloned into pLenti6.2v5 (Clontech) using LR Gateway clonase. Lentiviral particles containing pLenti6.2v5-eGFP or pLenti6.2v5-ARHGEF6-eGFP (Clontech), respectively, were produced in HEK293FT cells (ThermoFisher; Cat: R70007) by transfecting these constructs (Lipofectamine 2000) along with a Lentiviral Packaging Mix (ViraPower, ThermoFisher Scientific). Lentivirus-containing supernatants were collected at 48 hours after transfection and filtered through a 0.45 µm filter.

MDCK cells plated at 50% confluency were transduced 6 hours after attachment with the lentiviral particles in antibiotic-free medium in the presence of 4 µg/ml polybrene (Sigma-Aldrich). The virus-containing medium was replaced after 18 h with antibiotic-free medium, and cells were cultured for another 24 hours, followed by blasticidin selection (4 µg/ml) and cell sorting for GFP-expressing cells (FACS Aria SORP, Becton Dickinson). After selection and sorting, cells were maintained in growth medium with blasticidin until freezing cell stocks. Subsequent subculturing and experiments were performed in normal growth medium in the absence of blasticidin.

Immunoblotting, immunoprecipitation, and immunofluorescence staining

Immunoblotting, immunoprecipitation, and immunofluorescence staining were performed as described previously[15]. Briefly, for immunoprecipitation experiments, HEK293T cells were lysed and pre-cleared using rec-Protein A-Sepharose 4B Conjugate (Life Technologies) overnight. Equal amounts of protein were then incubated with EZview™ Red Anti-c-Myc Affinity Gel (Sigma-Aldrich). Coimmunoprecipitation experiments were performed in two to three independent experiments. Immunoblotting was performed using mouse anti-c-Myc (sc-40, Santa Cruz Biotechnology), goat anti-GFP (sc-9996, Santa Cruz Biotechnology), HRP-labeled secondary antibodies were purchased from Santa Cruz Biotechnology. The following antibodies were used for clone characterization: rabbit anti-Arhgef6 (HPA003578, Sigma; ab91562, Abcam; 4573S, Cell Signaling), mouse anti-Arhgef6

(H00009459-B01P, Novus), rabbit anti-Parva (4026S, Cell Signaling), rabbit anti-Parvb (PA5-42805, Life Technologies), mouse anti-Ilk (NBP2-37448, Novus), Mouse HRP-linked anti-beta actin (ab20272, Abcam). For cell immunofluorescence, cells were seeded on coverslips and grown at a permissive temperature. For overexpression studies, cells were transiently transfected using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Experiments were performed 24–48 hours after transfection. Cells were fixed for 10 minutes using 4% paraformaldehyde (PFA) and permeabilized as above. Staining was performed similarly to tissue sections. The same primary antibodies were used as described above. Donkey anti-mouse, anti-guinea pig, and anti-rabbit Alexa 488– and Alexa 594–conjugated secondary antibodies (ab150105, ab150108, ab150185, ab150073, ab150076) and DAPI staining reagents were used. Confocal laser scanning microscopy was performed using the Leica SP5X system with an upright DM6000 microscope and images were processed with the Leica AF software.

Antibody-based integrin internalization assay

Cells were kept under serum-deprived conditions (0.1% serum) for 2-3 h and detached with StemProAccutase (Thermo Fisher Scientific). Cells were incubated on ice and cell surface □1 integrin was stained with Alexa Fluor 647-conjugated antibody clone TS2/16 (sc-53711 AF647, Santa Cruz Biotechnology, Inc.; detects the active conformation of □1 integrin) or isotype control mouse IgG1, κ (14-4714-82, Invitrogen) diluted 1:100 in FACS-buffer (PBS containing 0.5% BSA and 2 mM EDTA) for 60 minutes at 4°C and rotation. After 1 h cells were washed twice with FACS-buffer. To start integrin internalization, cells were incubated in pre-warmed media (0.1% serum/DMEM) at 37°C for 15 min. After internalization, cells were immediately transferred on ice and cooled down. As a control, antibody treated cells were left on ice (0 min internalization). The remaining cell surface bound antibody was removed by acidic wash buffer (0.2 M acetic acid, 0.5 M NaCl, pH 2.3) in three washing steps (3 min each), followed by neutralization with 0.3% BSA/DMEM. For measurement of the total amount of cell surface integrin stained by the □1 integrin antibody, a parallel cell culture was left on ice without acid wash. Fluorescent intensities of total cell surface and internalized integrin β1 were measured by using FACS Canto II (BD Biosciences). Only GFP-positive cells (20,000 cells per condition) were included in the analysis.

3D MDCK spheroid culture and analysis

Cysts were processed for immunofluorescence staining as follows: cysts were rinsed twice in PBS and then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Samples were blocked in 5% normal donkey serum in PBS/TX-100 for 30 min at room temperature. Primary antibodies (mouse anti-gp135 1:500, obtained from George Ojakian (SUNY, Downstate Medical Center, Brooklyn, NY), rabbit polyclonal anti-laminin (1:250; L9393, Sigma-Aldrich) were added to cyst samples and incubated overnight at 4°C. After extensive washing with PBS/TX-100, the samples were incubated with secondary antibody (Alexa Fluor 568, AlexaFluor 647) and/or Alexa Fluor-568-conjugated phalloidin (Thermo Fisher) to stain F-actin at a 1:250 dilution overnight at 4°C. Cysts were photographed on a Zeiss 900 LSM inverted confocal microscope and W Plan-Apochromat 40x/1.0 DIC objective. Images were adjusted for brightness and contrast with Fiji v 1.53.

Mouse breeding, maintenance and CAKUT phenotype evaluation

Mice were housed under pathogen-free conditions with a light period from 7:00 AM to 7:00 PM and had *ad libitum* access to water and rodent chow. *Arhgef6*$^{-/-}$ and *Arhgef6*$^{-/Y}$ mice have been previously described[16] and were backcrossed co C57B6 for more than 15 generations. In brief, a targeting vector inserting a neomycin cassette in exon 1 of *Arhgef6* was designed to delete exons 1 and 2 as well as intron 1 and part of intron 2. The vector was electroporated into R1 ES cells, positive clones selected by G418, screened by multiplex PCR and confirmed by southern blotting. Positive ES cell clones were then microinjected into C57BL/6 blastocysts. Resulting male chimeras were backcrossed onto C57BL/6 mice to generate an N1 population. *Arhgef6*$^{-/+}$ and *Arhgef6*$^{-/Y}$ mice were then incrossed. Genotyping was performed by multiplex PCR using the following primers:
#1: GTTCAAATCCCCATTGCATCATAGTCTG,
#2: GTCTTTAACCGCTGTGCTTCTTTTTGGATA,
#3: GATATGGGTCTGTAAACTGTTGCTGCTAAT and
#4: GTTGGCGCTACCGGTGGATGTGG.

Arhgef6$^{-/+}$ and *Arhgef6*$^{-/Y}$ mice were bred to achieve 1:1 ratios of potentially affected and healthy animals, both for female and male mice, following an X-linked recessive disease hypothesis. Litters were separated from their parents at day 4 postnatally (P4), euthanized, dissected and subjected to

macroscopic evaluation. The macroscopic evaluation criteria for CAKUT were defined as: suspected duplex kidney, numeric abnormality, hydronephrosis or hydroureter, suspected hypoplasia, and suspected dysplasia based on side differences in color or opacity. If a CAKUT phenotype was suspected based on these criteria, kidneys and urinary tract were dissected and processed for paraffin embedding and histological staining (Masson's Trichrome). All specimens were then reevaluated according to the following microscopic criteria: duplex kidney, hydronephrosis or hydroureter, hypodysplasia as evidenced by differences in size, cystic structures, very heterogeneous parenchyma, or loss of zonal compartmentalization. To achieve objective evaluation, genotyping was only performed after all phenotypic evaluation was completed.

Generation of Xenopus models

For whole-embryo CRISPR/Cas9 knockout, two-cell *X. tropicalis* embryos were injected bilaterally with 1500 pg of *arhgef6* gRNA (version 1, 2 or 3) or *slc45a2* gRNA together with 4500 pg of Cas9 protein (PNA-Bio). For targeted CRISPR/Cas9 knockout, four-cell *X. tropicalis* embryos were injected unilaterally in the left ventral cell with 900 pg of *arhgef6* gRNA (version 1, 2 or 3) or *tyrosinase* gRNA (version 1 or 2) together with 2700 pg of Cas9 protein. For Morpholino knockdown, four-cell *X. tropicalis* embryos were injected in the left ventral blastomere with 4.8 ng of *arhgef6* translation-blocking morpholino (5'-GCTGTGTAAATTGGCTTCGTACAG) or standard control morpholino (5'-CCTCTTACCTCA GTTACAATTTATA-3') (GeneTools, LLC.).

Fluorescence imaging of Xenopus embryos

Whole-mount immunofluorescence was performed as follows. Stage 38 embryos were fixed overnight at 4°C in 1x MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde), dehydrated to 100% MeOH and incubated at -20°C overnight. Next, embryos were incubated in bleaching buffer (10% H_2O_2, 23% H_2O, 67% MeOH) overnight at room temperature under bright light until completely bleached. Embryos were rehydrated to 1x TBST and incubated in blocking buffer (155 mM NaCl, 10 mM Tris-HCl pH 7.5, 10% Fetal Bovine Serum (FBS), 5% DMSO) for two hours. Staining was performed with LE-Lectin-DyLight488 (1:100, ThermoScientific, L32470) diluted in blocking buffer overnight at 4°C. After washes in 1x TBST, embryos were transferred to 1x TBS and fluorescence stereomicroscopy was performed with a MZ10 F (Leica) taking pictures of both kidneys at each side of the embryo. Resulting images were processed using an U-Net deep learning pipeline[17] to calculate either, the log2 fold change between kidney size when compared to the average kidney size in non-injected embryos, or the log2 fold change in size between the left kidney (manipulated) and the right kidney (unmanipulated) within a single *X. tropicalis* embryo.

Target sites and oligos for CRISPR/Cas9

	Genomic target site	Oligo for IVT
Arhgef6 gRNA1	GTCAGAACATGAACG GCCATAGG	GAATtaatacgactcactataGGCAGAACATGAACGGCCATgttttag agctagaaATAG
Arhgef6 gRNA2	TGGGACATTTATGGA GAATCAGG	GAATtaatacgactcactataGGGGACATTTATGGAGAATCgttttaga gctagaaATAG
Arhgef6 gRNA3	GTGGATGTCAGCTCA TTGCTTGG	GAATtaatacgactcactataGGGGATGTCAGCTCATTGCTgttttaga gctagaaATAG
Tyrosinase gRNA1	GGGGGTTCTGCTCCG ATCGTGG	GAATTAATACGACTCACTATAGGGGGTTCTGCTCCGATCG GTTTTAGAGCTAGAAATAG
Tyrosinase gRNA2	GGGCCCCTGTTCAGA AATCCTGG	GAATTAATACGACTCACTATAGGGCCCCTGTTCAGAAATC CGTTTTAGAGCTAGAAATAG

Slc45a2 gRNA	CTCCCAGGAGATCTACGGGCCGG	Gcagctaatacgactcactatagctcccaggagatctacgggcgttttagagctagaaatagcaag

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