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RESEARCH ARTICLE

CRB2 depletion induces YAP signaling and disrupts mechanosensing in podocytes

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Abstract

Focal segmental glomerulosclerosis (FSGS) is a histologic lesion caused by a variety of injurious stimuli that lead to dysfunction/loss of glomerular visceral epithelial cells (i.e., podocytes). Pathogenic mutations in crumbs homolog-2 (CRB2), encoding the type 1 transmembrane protein crumbs homolog-2, have been shown to cause early-onset corticosteroid-resistant nephrotic syndrome (SRNS)/FSGS. Here, we identified a two-generation Indian kindred (DUK40595) with biopsy-proven SRNS/FSGS caused by a compound heterozygous mutation in CRB2 comprised of the previously described truncating mutation p.Gly1036_Alafs*43 and a rare 9-bp deletion mutation p.Leu1074_Asp1076del. Because compound heterozygous mutations involving the truncating p.Gly1036_Alafs*43 variant have been associated with reduced CRB2 expression in podocytes and autosomal recessive SRNS/FSGS, we sought to define the pathogenic effects of CRB2 deficiency in podocytes. We show that CRB2 knockdown induces yes-associated protein (YAP) activity and target gene expression in podocytes. It upregulates YAP-mediated mechanosignaling and increases the density of focal adhesion and F-actin. Using elastic resonator interference stress microscopy (ERISM), we demonstrate that CRB2 knockdown also enhances podocyte contractility in a substrate stiffness-dependent manner. The knockdown effect decreases with increasing substrate stiffness, indicating impaired mechanosensing in CRB2 knockdown cells at low substrate stiffness. Although the mechanical activation of CRB2 knockdown cells is associated with increased YAP activity, the enhanced cell contractility is not significantly reduced by the selective YAP inhibitors K-975 and verteporfin, suggesting that multiple pathways may be involved in mechanosignaling downstream of CRB2. Taken together, these studies provide the first evidence that CRB2 deficiency may impair podocyte mechanotransduction via disruption of YAP signaling in podocytes.

NEW & NOTEWORTHY We identified a rare compound heterozygous CRB2 mutation as the cause of familial SRNS/FSGS in a two-generation East Asian kindred. Modeling the effect of the mutation, we show that CRB2 knockdown in podocytes induces YAP transcriptional activity and upregulates YAP-mediated mechanosignaling. Using elastic resonator interference stress microscopy (ERISM), we demonstrate that CRB2 knockdown enhances podocyte contractility in a substrate stiffness-dependent manner. The knockdown effect decreases with increasing substrate stiffness, indicating impaired mechanosensing in CRB2-deficient podocytes.

cell mechanics; CRB2; FSGS; podocytes; YAP signaling

INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is a histologic injury caused by dysfunction or loss of glomerular visceral epithelial cells (i.e., podocytes) (1). In over 70% of patients with FSGS, the most common clinical manifestation is nephrotic syndrome (NS) and nearly 50% of patients with FSGS progress to end-stage kidney disease (ESKD) over a

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decade (2). Notably, the incidence of FSGS has increased more than 10-fold over the past three decades and it is now the most common primary glomerular lesion that causes ESKD in the United States (3, 4).

FSGS can result from a variety of stimuli, contributing to a heterogeneity of the histologic lesion and variability in the clinical presentation and treatment response (1). Regardless of the cause, it is clear that the final common pathway to developing FSGS involves podocyte injury or loss (4–7). To date, mutations in over 60 podocyte-expressed genes have been shown to cause familial steroid-resistant nephrotic syndrome (SRNS)/FSGS (8–10). Mutations in the gene that encodes crumbs homolog-2 (*CRB2*; *FSGS9*) have been shown to cause early-onset SRNS/FSGS (11–20). CRB2 is an essential slit diaphragm (SD) protein that organizes in clusters in the podocyte apical membrane and interacts with nephrin and other SD proteins (Supplemental Fig. S1) (14). CRB2 is one of three members of the crumbs family (CRB1, CRB2, and CRB3) of apical polarity-related type I transmembrane proteins (21). Crumbs proteins are widely expressed in epithelial cells and regulate cell shape, polarity, and intracellular signaling (21). Although the expression of CRB1 is largely restricted to the central nervous system, CRB2 and CRB3 are both expressed throughout the body, including in the kidneys (22, 23). Notably, inherited glomerular disease has only been associated with *CRB2* mutations, and no known human genetic disease has been associated with mutations in *CRB3* (19, 22, 24). CRB2 is widely recognized as a critical upstream regulator of Hippo pathway signaling in epithelial cells, and dysregulated Hippo signaling in podocytes has been implicated in the pathogenesis of glomerular disease (25–27). However, the relationship between CRB2 deficiency and yes-associated protein (YAP)-mediated gene regulation via the Hippo pathway in the pathobiology of podocyte injury has not been explored.

Here, we report the identification of a rare compound heterozygous *CRB2* mutation in a two-generation Indian kindred with biopsy-proven FSGS. Affected family members expressed the previously reported truncating frameshift mutation p.Gly1036AlafsTer43 (11) and a rare, in-frame 9-bp deletion mutation, p.Leu1074_Asp1076del. We hypothesized that these variants would exert a loss-of-function effect and sought to explore the pathogenic mechanisms of CRB2 deficiency in podocytes using an in vitro model of CRB2 knockdown combined with extensive measurements of the mechanical activity of podocytes.

In the present study, we sought to clarify the role of CRB2 as an upstream regulator of YAP mechanosignaling in podocytes. Using siRNA-mediated CRB2 knockdown in conditionally immortalized human podocytes, we demonstrate that YAP target gene and protein expression, focal adhesion density, and mechanosignaling are significantly increased. Using elastic resonator interference stress microscopy (ERISM), a recently developed force microscopy modality for long-term mapping of mechanical activity of cells (28, 29), we demonstrate that contractile force development is significantly increased in CRB2 knockdown podocytes. In addition, CRB2 knockdown podocytes exhibit a higher F-actin density and paxillin expression and phosphorylation while their cell area is significantly decreased. To examine whether CRB2 knockdown has an impact on how podocytes adjust contractile forces to the mechanical properties of the micro-environment (mechanosensing), we performed a detailed characterization of podocyte force development over a range of substrate stiffnesses throughout differentiation. In line with the proposed function of CRB2 being an upstream regulator of YAP activation, we found that the relative effect of CRB2 knockdown on cell contractility decreases with substrate stiffness. Interestingly, treatment of CRB2 knockdown podocytes with the YAP inhibitors verteporfin or K-975 did not reduce podocyte contractile force development relative to controls, suggesting that other pathways than YAP signaling may also contribute to mechanosignaling downstream of CRB2. Taken together, these findings provide the first evidence that CRB2 deficiency may impair podocyte mechanotransduction via disruption of YAP signaling in podocytes.

MATERIALS AND METHODS

IRB

Patient recruitment, consent, and research protocols were approved by Duke Institutional Review Board (IRB). Written informed consent was received from every participant or legal guardian.

Genome Sequencing

Proband blood DNA was subjected to targeted gene capture and sequencing to a mean >80–100 times coverage on the Illumina sequencing platform through MedGenome Labs Pvt. Ltd., Bangalore, India. The sequences obtained were aligned to the human reference genome (GRCh37/hg19) using the Burrows-Wheeler Alignment (BWA) program (30, 31) and were analyzed using Picard and the Genome Analysis Toolkit (GATK) version 3.6 (32, 33) to identify variants relevant to the clinical indication. Gene annotation of the variants was performed using the Variant Effect Predictor program (34) against the Ensembl release 87 human gene model (http://www.ensembl.org/). Clinically relevant mutations were annotated using published variants in literature and the ClinVaar, OMIM, GWAS, HGMD, and SwissVar disease databases (35–39). Common variants were filtered based on allele frequency in 1000 Genome Phase 3, ExAC, EVS, dbSNP147, 1000 Japanese Genome, and regional Indian population database (40–42). Only nonsynonymous and splice-variants found in the exome panel consisting of 8,322 genes were used for clinical interpretation.

CRB2 Primers and Direct Sequencing

CRB2 family PCR.
*CRB2*_Nested2_F: AGATTCTGCCAGGAGTTGCT.
*CRB2*_Nested2_R: AACACAACGCCTGGACATTC.
Chr19:123372844 + 123374338 1,495 bp.

PCR was performed on previously extracted samples from blood using the PrimeStar GXL Polymerase kit, with final concentrations of 1× PrimerStar GXL Buffer, 200 μM each 2′-deoxyribonucleoside 5′-triphosphate, 0.1 μM each primer, 1.25 U/50 mL PrimeStar GXL Polymerase, and 0.5 M betaine. PCR amplification was performed using a thermocycler (GeneAmp 9700) with 30 cycles of 98°C for 10 s, 67°C for 15 s, and 68°C for 15 s using the following primers, *CRB2*_Nested2_F: AGATTCTGCCAGGAGTTGCT and *CRB2*_Nested2_R: AACAC-

AACGCCTGGACATTC. Products were then confirmed via a 1% agarose gel run at 100 V for 30 min and Sanger Sequenced using both forward and reverse primers at GeneWiz. Sequencing files were uploaded to Sequencher, trimmed, and assembled to the reference sequence. Heterozygous bases were called using the internal software, and all mismatches to the reference sequence were visually checked for accuracy using the chromatogram sequences.

Protein Structure Modeling

In silico modeling of the CRB2 p.Leu1074_Asp1076del variant was performed using the I-TASSER protein structure prediction software (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (43, 44). Structural renderings were generated using the PyMOL molecular graphics program.

Lentiviral Vector Production and Transduction of siScr and si*CRB2* Lines

Lentiviral vectors were generated using the transient infection protocol described by Tagliafierro et al. (45). Briefly, 15 μg of vector plasmid, 10 μg of psPAX2 packaging plasmid (Addgene No. 12260 generated in Dr. Didier Trono's laboratory, EPFL, Switzerland), 5 μg of pMD2.G envelope plasmid (Addgene No. 12259, generated in Dr. Trono's laboratory), and 2.5 μg of pRSV-Rev plasmid (Addgene No. 12253, generated in Dr. Trono's laboratory) were transfected into 293 T cells. Vector particles were collected from the filtered conditioned medium at 72 h posttransfection. The particles were purified using the sucrose-gradient method and concentrated >250-fold by ultracentrifugation (2 h at 20,000 rpm). Vector and viral stocks were aliquoted and stored at −80°C. Conditionally immortalized podocytes were plated on collagen I-coated six-well flasks and maintained under growth-permissive conditions until 70% confluence was achieved. Cells were then transduced with 4 μL of siScr and si*CRB2* lentiviruses at a concentration of 1.0×10^5 particles/μL in serum-free media. Cells were incubated overnight before washing with complete media and further incubation for 48 h. At confluence, antibiotic selection was initiated for 5–7 days. Surviving cells were expanded in complete media before transition to growth-restrictive conditions at confluence. After 12 days of differentiation, cells were harvested and assayed for *CRB2* mRNA expression as described in *RNA Extraction and qPCR*.

Molecular Cloning of Luciferase Reporter Lines

The 8× GTIIC-luciferase plasmid was obtained from Addgene (No. 34615; gift of Dr. Stefano Piccolo). The following primers were used to amplify the 8× GTIIC-luciferase region: forward- CCGACTCGAGAATTACACGGC; reverse-CTATCGATATCTACCGAGCTCTTACGC. The XhoI and EcoRV restriction sites were added at the 5′ and 3′ ends, respectively. The fragment was cloned into a standard self-inactivating lentivirus (SIN-LTR) lentiviral vector cassette. Plasmid No. 1773 (Addgene, gift of Dr. Bob Weinberg) was then amplified using the following primers: forward-TGGACTCGAGTCGACCCTGT and reverse-GATCCCGCTCGAGATCTACTCTATTCC. The fragment carrying SV40- promoter hygromycin resistant gene flanked by XhoI restriction sites was cloned into an intermediate vector to create the final pBK909 plasmid used for cell transfection. All vectors were validated by direct Sanger sequencing and by digestion. The final plasmid was amplified and prepared for transfection as described by Brown et al. (46). Once siScr and si*CRB2* lines were established, cells were plated on collagen I-coated six-well flasks and maintained under growth-permissive conditions until 70% confluence was achieved. Cells were then transduced with 4 μL of the 8× GTIIC lentivirus at a concentration of 1.0×10^5 particles/μL in serum-free media. Cells were then incubated overnight before washing with complete media and further incubation for 48 h. At confluence, antibiotic selection was initiated for 5–7 days. Surviving cells were expanded in complete media.

Cell Culture

Human conditionally immortalized podocytes contained human telomerase reverse transcriptase (hTERT). The thermosensitive SV40 large T antigen was kindly provided by J. Kopp (Bethesda, MD). Both siScr and si*CRB2* podocytes were established through lentiviral transduction with scrambled and *CRB2* siRNA (Applied Biological Materials), respectively. Podocytes were incubated on collagen-I-coated flasks (Corning, NY) under permissive temperature (33°C) and 5% CO_2 and in RPMI 1640 (R8758, Gibco) supplemented with 10% of fetal bovine serum (FBS) (A5209402, Gibco), 1% of penicillin-streptomycin (15140122, Gibco), and 1% of insulin-transferrin-selenium (ITS) (354351, Corning). Podocytes were split at a confluency of ∼90%. Both cell lines were seeded onto an ERISM substrate before performing the thermoswitch to the differentiation conditions at 37°C, 5% CO_2 for 12 days. Culture medium was refreshed every 2 to 3 days. Podocytes were exposed to the YAP-transcriptional inhibitors verteporfin (SML0534, Sigma-Aldrich) and K-975 (HY-138565, MedChemExpress) and to the mammalian target of rapamycin (mTOR) inhibitor rapamycin (HY-10219, MedChemExpress) for 24 h using the concentrations indicated in Fig. 7. All groups of the inhibitor study were treated with 0.2% (vol/vol) of DMSO.

RNA Extraction and qPCR

Cells were grown to 95% confluency in a T75 collagen-coated flask, rinsed with 1× PBS, and incubated for 5 min at room temperature in 5 mL of trypsin. Media was added to each sample to neutralize the trypsin, and all flask contents were transferred to a 15-mL conical tube. The conicals were centrifuged at 1,500 rpm for 5 min with two washes of 1× PBS. RNA was extracted using the Qiagen RNeasy miniprep kit according to the manufacturer's instructions. RNA eluates were quantified using Nanodrop and normalized to 500 ng/μL. cDNA was generated using the BioRad iScript cDNA synthesis kit in 2 μg of RNA input in a 40 μL reaction volume according to the manufacturer's instructions. TaqMan Gene Expression Assays (Invitrogen) were used in conjunction with TaqMan Fast Advanced Master Mix (Invitrogen) using 0.5 μL of Assay mix, 5 μL of Master Mix, 3.5 μL of nuclease-free water, and 1 μL of cDNA per reaction. The assays were run on a ViiA7 with manufacturer's cycling parameters. All samples were run in triplicate along with no-template controls. β-Actin was used as the internal control gene, and the relative quantification was calculated using the ViiA7 software.

Immunohistochemistry

The immunofluorescence staining of human kidney biopsy specimen was performed, as previously described (47). Briefly, 4-μm sections of paraffin-embedded human kidney tissues were deparaffinized with xylene, followed by hydration using a gradient of ethanol concentrations at room temperature. Antigen epitope retrieval was performed by incubating the kidney sections in 10 mM sodium citrate buffer with 0.05% Tween 20 (pH = 6.0) for 10 min. Subsequently, the sections were blocked with 3% BSA in 1× PBS buffer for 1 h at room temperature. The sections were then incubated overnight at 4°C with CRB2 rabbit polyclonal antibody (1:100, VWR, AP5724B, RRID: AB_10820013) and nephrin guinea pig polyclonal antibody (1:200, GP-N2, PROGEN Biotechnik, RRID: AB_2904121). Following the incubation, the slides were washed three times with 1× PBS and incubated with Alexa Fluor 488 goat anti-rabbit antibody (1:200, Invitrogen, A11034, RRID: AB_2576217) and Alexa Fluor 594 goat anti-guinea pig antibody (1:200, Invitrogen, A11076, RRID: AB_2534120) at room temperature for 1 h in the dark. After three additional washes with 1× PBS, the sections were mounted with DAPI Slowfade (Invitrogen, S36938). Images were acquired using an Andor CSU-WDi spinning disk confocal microscope equipped with a Nikon Ti-E CFI Plan Apochromat Lambda ×60 oil immersion objective.

Immunocytochemistry

Podocytes were seeded either in Petri dishes (80416, Ibidi) for nephrin and synaptopodin, on glass coverslips for focal adhesion kinase (FAK)1, CRB2, and transient receptor potential cation channel subfamily C member 6 (TRPC6), or on ERISM substrates for paxillin immunocytochemistry. The latter was combined with ERISM measurements; for this, a coordinate array of 5 cells/chamber were recorded before taking a full-range ERISM scan of the array to yield the reference local thickness of the field of view and further fast one-minimum ERISM scans (48) were performed before the fixation.

Cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 15 min (Petri dishes and ERISM substrates) or 20 min (glass coverslips) and were washed three times in 1× PBS with 5 min of incubation. Except for membrane staining, cells were permeabilized using 0.1% or 0.2% Triton X-100 in 1× PBST (1× PBS + 0.2% Tween 20) for 5 min, after which cells were blocked with 1% or 2% BSA for 30 min and then rinsed with 1× PBST. After washing the cells for 5 min twice, they were incubated with the following primary antibodies at 4°C overnight: nephrin rabbit polyclonal antibody (1:200, Thermo Fisher Scientific, PA5-20330, RRID:AB_11153994), synaptopodin mouse monoclonal antibody (1:200, PROGEN Biotechnik, 61094, RRID:AB_1543006), FAK1 mouse monoclonal antibody (1:100, Millipore Sigma, 05-537, RRID:AB_2173817), CRB2 rabbit polyclonal antibody (1:100, BIOSS, BS-14046R, Ref. 49), TRPC6 mouse monoclonal antibody (1:100, Abcam, 105845, RRID:AB_10901396, Ref. 50), and at room temperature for 1 h: paxillin rabbit monoclonal antibody (1:1,200, Cell Signaling Technologies, MA, 50195) in 1% BSA. After washing three to five times with 1× PBS or 1× PBST, cells were incubated in the following secondary antibodies for 1 h at room temperature in dark: Alexa Fluor Plus 647 donkey anti-mouse (1:800, Thermo Fisher Scientific, A32787, RRID:AB_2762830), Cy5 donkey anti-rabbit (1:400, Jackson ImmunoResearch, 711-175-152, RRID:AB_2340607), Alexa Fluor 555/488 donkey anti-mouse and donkey anti-rabbit (1:1,000, Thermo Fisher Scientific, A-31570, RRID: AB_2536180 and A-21206, RRID:AB_2535792), TRITC-phalloidin (1:500, Sigma Aldrich, FAK100), Alexa Fluor 488/555-phalloidin (1:1,000, Thermo Fisher Scientific, A12379, RRID: AB_2535792 and A30106), and DAPI (1:800 or 1:2,000). The stained cells were washed three to five times in 1× PBS or 1× PBST. Cells on glass coverslips were placed on ProLongTM Gold Antifade Mountant (Thermo Fisher Scientific, P36930); the other samples were imaged in 1× PBS.

For nephrin/or synaptopodin/DAPI staining in Petri dishes, cells were imaged under a Leica Stellaris 8 confocal microscope at Humboldt Centre for Nano- and Biophotonics; for phalloidin/CRB2/TRPC6 and FAK1/DAPI on glass coverslips a Leica SP8-confocal microscope at the Duke Light Microscopy core facility; and for phalloidin/paxillin/DAPI on ERISM substrates a Nikon Eclipse Ti2 epi fluorescence microscope.

Quantification of Immunofluorescence Images

Fluorescence intensity was quantified via the ImageJ software. For the intensity of actin and paxillin, a 50 pixel of rolling ball radius background was subtracted. The actin intensity was normalized to the used exposure time (1–2 s for the control and 1 s for CRB2 knockdown podocytes).

The calculation of the actin orientation order parameter was adapted from Ref. (51). The quantification of counts and mean size of paxillin stains was adapted from Ref. (52). In brief, background subtraction was performed as described earlier. The local contrast of the images was enhanced by the contrast-limited adaptive histogram equalization (CLAHE) plug-in using ('block size' = 19, 'histogram bins' = 256, 'maximum slope' = 6, 'no mask and fast'). The background was minimized by the "Exponential" command. After auto-adjusting brightness and contrast, the Mexican Hat Filter plug-in was used with radius = 3.5. Next, a threshold of 0–255 was applied on the images with the "Default" method and with selecting "Calculate threshold for each image." Finally, the "Analyze Particles" command was executed with a "size (μm^2)" parameter of 0.01–15.

Western Blot Analysis

Following treatment, mature immortalized murine podocyte cultures were washed once with ice-cold PBS. Cells were then harvested in loading buffer (Cell Signaling Technologies; Boston, MA) supplemented with 1 μM Calyculin A (EMD Chemicals; Billerica, MA) and protease inhibitor cocktail, 1:400 dilution (Sigma). Whole cell extracts were passed through a 1-mL syringe (BD Biosciences; San Jose, CA) 10 times. Cell lysates were then subjected to SDS-polyacrylamide gel electrophoresis using NuPAGE 10% Bis-Tris pre-cast gels (Invitrogen), followed by transfer to 0.2-μM pore size PVDF membrane (Millipore; Billerica, MA). Membranes were blocked with 1 h incubation in 5% milk in PBS. Protein immunoblotting was performed using phospho-paxillin (Y118) rabbit polyclonal antibody (1:1,000, Cell Signaling Technologies, 2541, RRID:AB_2174466), phospho-FAK (Y397) rabbit monoclonal (D20B1) antibody (1:1,000, Cell Signaling Technologies, 8556, RRID:AB_10891442), and total FAK rabbit monoclonal

antibody (1:1,000, Cell Signaling Technologies, 71433, RRID: AB_2799801). Membranes were washed once with PBS and incubated for 1 h with horse radish peroxidase-conjugated goat anti-rabbit polyclonal secondary antibody at 1:10,000 dilution (Invitrogen, 108-035-003, RRID:AB_2337489). Immuno-labeled proteins were detected using a chemiluminescence detection system (Pierce Biotechnology; Rockford, IL) on Kodak Biomax film (VWR Scientific; Radnor, PA).

Luciferase Reporter Assay

Cells were plated in triplicate at 2×10^5 cells/mL in 2 mL on six-well collagen-coated plates. The plates were incubated at 33°C and 5% CO_2 until 95% confluency was reached. Protein lysates were obtained by removing the media and rinsing cells twice with 1 mL of 1× PBS. Cells were then incubated in 200 μL of 1× passive lysis buffer (PLB, Promega E1500) on a 150 rpm orbital shaker for 45 min. Lysates were collected in a microcentrifuge tube and centrifuged at 800 rpm for 5 min at 4°C. The supernatant was transferred to a clean microcentrifuge tube and stored on ice. Quantification of the whole protein lysates was performed using the BioRad protein assay (Cat. No. 5000001) using 0.5 mg/mL to 0.05 mg/mL of BSA standards and the microtiter plate protocol. Each standard sample was run in triplicate with 10 μL of each pipetted into 200 μL of diluted 1× dye reagent. The plate was incubated for 50 min at room temperature, and then the absorbance at 595 nm was read using a microplate reader (M200Pro, Tecan). The absorbance of the samples was plotted against the standard curve with an R^2 value of >0.98 to measure the concentration of each protein sample. Each sample was diluted to 0.35 mg/mL to perform the luciferase assay (Promega E1500). Luciferase was thawed in a room temperature water bath, and the luciferase assay buffer was added to the lyophilized luciferase assay substrate. Each sample was plated in triplicate, along with no template controls containing only 1× PLB in a 3990 Costar Plate. Normalized protein lysate (20 μL) was added to the plate, and 100 μL of diluted luciferase assay reagent was added immediately before loading samples into the microplate reader to measure luminescence (integration time, 10 s).

ELISA

Immunoassays were used to quantify concentrations of connective tissue growth factor (CTGF) (Abcam, ab261851), thrombospondin-1, endothelin-1, Cyr61/CCN1 (R&D Systems; DTSP10, DET100, DCYR10, respectively), and transforming growth factor-beta 1, 2 and 3 (TGF-β1, -2, and -3) (MesoScale Discovery, Rockville, MD, K15241K), in cell culture supernatants. Due to all samples having concentrations below the lowest limit of detection for the assay, TGF-β3 was excluded from the analysis. CTGF required a 10-fold dilution of supernatant and had a mean intra-assay coefficient of variation (CV) of 1.6%. Thrombospondin (diluted 2-fold), endothelin-1 (diluted 10-fold), and Cyr61 (undiluted) had mean intra-assay CVs of 1.3, 2.1, and 3.1%, respectively. All samples required acid treatment for the TGF-β assay. Ten microliters of 1 M HCl was added to 50 μL of the sample and incubated for 10 min after which 7 μL of 1.2 M NaOH was added before vortexing the sample and loading onto the plate. All assays were performed according to the manufacturer's instructions.

Elastic Resonator Interference Stress Microscopy

ERISM substrates were fabricated as described earlier (28). In brief, a bottom mirror, consisting of a 0.5-nm thick Cr adhesive layer, a 10-nm thick Au mirror layer, and a 50-nm thick Ta_2O_5 protective layer, was deposited on clean glass coverslips (No.5) by electron beam evaporation, thermal evaporation, and sputtering, respectively. The siloxane-based elastomer (GEL8100, NuSil) was prepared in a 1:1 mass ratio and spin-coated onto the bottom mirror. The top surface of the elastomer was treated with 2.5%–15% power of oxygen plasma (max. power, 600 W; duration, 10 s) to reduce its surface energy and improve the quality of the 15-nm thick Au mirror deposited on top.

A set of four silicon chambers (surface area each, 0.75 × 0.75 cm^2; 81201, Ibidi) were applied on the substrate. The chip surface was coated with 8 $\mu g/cm^2$ of type I collagen (Rat Tail, A10483-01, Gibco) for 1 h at room temperature. Chambers were washed three times with 1× PBS and three times with fresh cell medium (RPMI 1640/10% FBS/1% penicillin-streptomycin/1% ITS), and then incubated at 33°C, 5% CO_2 for 30 min. Trypsinized podocytes were seeded at a density of 1,000 cells/chamber. Podocytes were incubated at 33°C, 5% CO_2 for 24 h before transfer to 37°C, 5% CO_2 for the 12-day differentiation. For the maintenance of cell culture on ERISM substrates, the cell medium was exchanged daily by washing each chamber three times with prewarmed fresh cell medium.

For ERISM measurements, the podocyte cultures were placed in a microscope on-stage incubator (Okolab) and maintained at 37°C, 5% CO_2. The incubator was mounted on a modified inverted fluorescence microscope (Nikon Ti2). Monochromatic probe light with wavelengths ranging from 550 to 750 nm was provided by a halogen lamp (ASBN Series, Oceanhood Taiwan Opto-electronics) filtered by a 1/8 m monochromator (CM110, Spectral Products). The probe light was coupled to the microscope and projected onto the substrate with either a ×20/0.45 numerical aperture (NA) or ×40/0.60 NA objective (Nikon S Plan Fluor) depending on the required field of view and magnification. Reflection and phase contrast images of the sample were detected by a scientific complementary metal oxide semiconductor (sCMOS) camera (0.05-s exposure time; Zyla, Andor Technology) and a standard CMOS camera (iCube C-NS4133BU, NET GmbH), respectively. The local resonance wavelengths of the sensor for each cell were measured by recording wide-field reflectance images for a series of illumination wavelengths. The wavelength selection and image capturing were controlled by an in-house developed software (LabView, NI, Austin, TX) written by Phillip Liehm. Images of control and CRB2 knockdown cells were collected under the same conditions.

Reflectance images were converted into local displacement maps by comparing the local resonance wavelengths against the predictions of an optical model of the ERISM substrate for different elastomer thicknesses, as described previously (28, 48). The Fourier-filtered ERISM displacement maps were obtained by targeting spatial frequencies in the range of 0.016–1.007 μm^{-1} in the obtained displacement maps using the fast Fourier transform plugin of the ImageJ software.

Statistical Analysis

Data groups for both cell lines were compared using two-sample or paired-sample Student's *t* test (equal variance not assumed).

RESULTS

Family Clinical Characteristics

Family DUK40595 is a two-generation kindred from India with two affected offspring from a nonconsanguineous marriage (Fig. 1*A*). A summary of all relevant clinical information for the family is provided in Table 1. The proband (DUK40595-1) and her affected male sibling (DUK40595-100) developed SRNS in their first decade of life (Table 1). The parents are healthy with no evidence of kidney disease. The female sibling was the only member of the family to undergo kidney biopsy that was diagnostic for SRNS/FSGS (Table 1). Subsequent clinical genetic testing of the proband revealed a compound heterozygous mutation of *CRB2* comprised of the previously reported 16-bp duplication p.Gly1036_Alafs*43 mutation (11) and a rare in-frame, 9-bp deletion mutation [c.3220_3228del (p.Leu1074_Asp1076del); minor allele frequency—0.00004]. The duplication results in a frameshift and premature truncation of the protein 43 amino acids downstream of codon 1,036 within the third laminin G domain (p.Gly1036Ala*43; ENST00000373631) (Fig. 1*B*). In addition, a second rare, heterozygous 9-bp deletion in exon 10 of *CRB2* (chr9:126136027_126136035del; Depth—167×) was identified. The 9-bp deletion results in the loss of three highly conserved amino acid residues in the 12th EGF-binding domain (Fig. 1*B*). The Asp-Leu-Phe residues are conserved to zebrafish (Fig. 1*C*), and their deletion is predicted to disrupt the secondary structure of the 12th EGF-binding domain (Fig. 1, *B* and *D*).

Direct sequencing of all family members confirmed the compound heterozygous *CRB2* mutation in the proband and her male sibling. The father (DUK40595-1001) was found to be heterozygous for the p.Gly1036-Alafs*43 mutation and the mother (DUK40595-1000) was heterozygous for the p.Leu1074_Asp1076del mutation (Table 1 and Fig. 1). The male sibling eventually progressed to ESKD at age 17 and received a kidney transplant without recurrence of disease in his allograft.

CRB2 Knockdown Alters Podocyte Spreading Area and Maturity Marker Expression

Prior studies of the p.Gly1036Alafs*43 mutation have shown that it functionally eliminates the CRB2 transmembrane domain and markedly reduces glomerular CRB2 expression (Supplemental Fig. S2) (11). To evaluate the effects of CRB2 deficiency on podocyte mechanosignaling, we generated conditionally immortalized human podocytes stably expressing *CRB2* siRNA (si*CRB2*) and compared them with controls expressing a scrambled RNA segment of equivalent length (siScr) (53). Both cell lines contained human telomerase reverse transcriptase (hTERT) and a temperature-sensitive SV40 transgene. The podocytes were initially cultured under growth-permissive conditions and then differentiated over the course of 12 days under growth-restrictive conditions. In differentiated podocytes, we achieved an ∼80% reduction in *CRB2* mRNA expression in the si*CRB2* line relative to controls (siScr), as shown in Fig. 2*A*.

Figure 2*B* shows phase contrast images of siScr and si*CRB2* podocytes under growth-permissive and growth-restrictive conditions. The quantification of cell area in Fig. 2*C* indicates that both cell lines increased their spreading area during differentiation. However, the spreading area was approximately half as much for the CRB2 knockdown podocytes compared with the siScr cells (siScr: mean cell spreading area: proliferating—3,032 μm^2, differentiated—14,638 μm^2; si*CRB2*: mean cell area: proliferating—2,862 μm^2, differentiated—6,397 μm^2).

We further assessed differentiated podocytes for expression and localization of the slit diaphragm protein nephrin and the foot process protein synaptopodin looking at both proliferating and differentiated podocytes (Fig. 2*B*). Confocal imaging of podocytes revealed a marked increase in both nephrin and synaptopodin expression in differentiated cells for both lines. However, nephrin staining appeared to be reduced in si*CRB2* podocytes relative to controls, consistent with prior observations (14, 20).

Taken together, CRB2 knockdown reduces the podocyte spreading area in differentiated cells and alters the expression of nephrin.

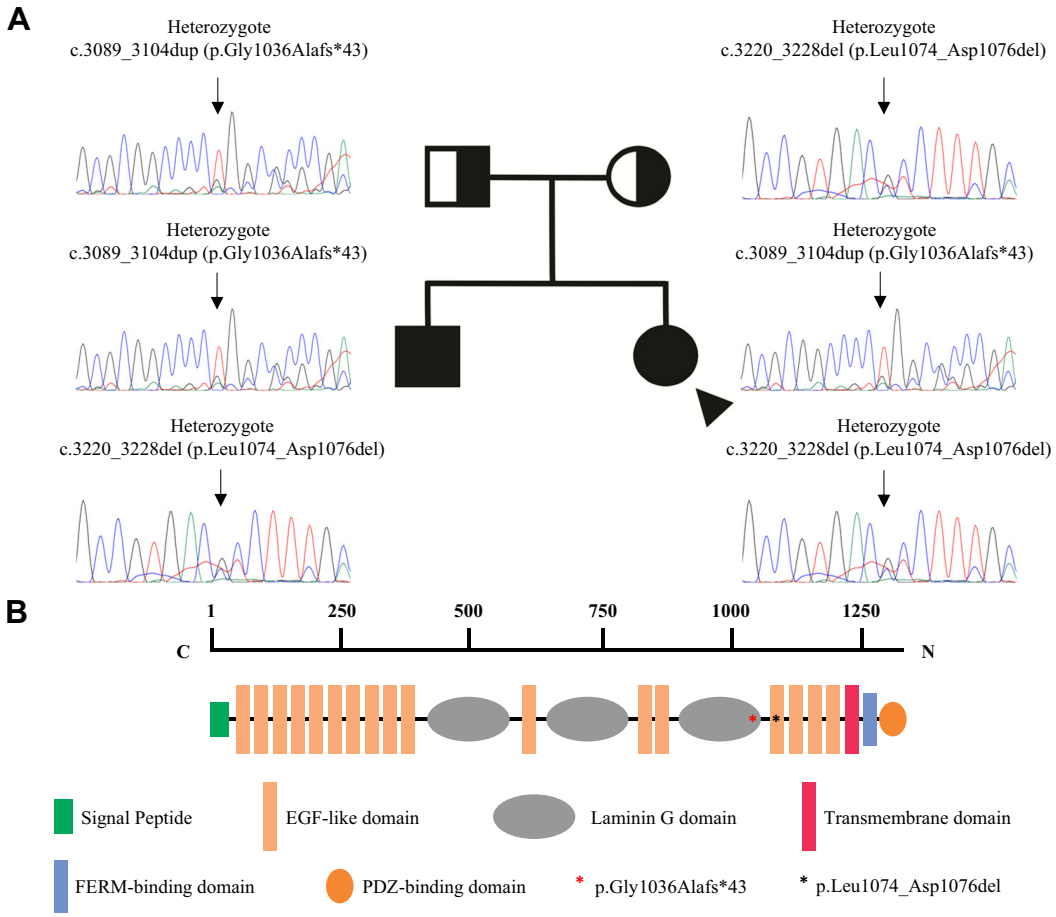
CRB2 Knockdown Induces YAP Target Gene and Protein Expression in Immortalized Podocytes

To investigate whether CRB2 knockdown alters YAP activity in differentiated podocytes, we performed transcriptional enhanced associate domains (TEAD) luciferase reporter assays using the well-validated 8xGTIIC-luciferase reporter (54). We detected that TEAD reporter activation was significantly increased in si*CRB2* podocytes relative to control (Fig. 3*A*). In addition, we performed Western blot analyses to evaluate YAP phosphorylation at serine 127 (Ser127). Phosphorylation of YAP at Ser127 promotes its degradation or cytoplasmic sequestration through binding with 14-3-3 family proteins. Cytosolic retention of the cotranscriptional regulator renders it inactive and prevents target gene activation (55). Conversely, when YAP is not phosphorylated at serine 127, it correlates with increased nuclear translocation and transcriptional activity. In Fig. 3, *B* and *C*, we show that transcriptionally active nonphosphorylated YAP (np-YAP) is significantly upregulated in the si*CRB2* line relative to controls, which correlates with a marked enhancement of thrombospondin-1 (THBS-1) expression (Fig. 3*D*), a YAP target gene known to drive mechanosignaling via direct stimulation of the integrin receptor (56, 57). Finally, to more broadly assess the effects of CRB2 deficiency on YAP mechanosignaling target gene expression, we performed ELISA assays and demonstrate significantly increased release of the YAP target genes transforming growth factor-β1 (TGF-β1), transforming growth factor-β2 (TGF-β2), THBS-1, connective tissue growth factor/CCN family member 2 (CTGF/CCN2), cysteine-rich angiogenic inducer 61/CCN family member 1 (Cyr61/CCN1), and endothelin-1 (EN1) from si*CRB2* podocytes relative to controls (Fig. 3*E*).

Taken together, these data suggest that CRB2 knockdown induces YAP dephosphorylation/activation and mechanotransduction gene expression in podocytes.

CRB2 Knockdown Enhances Podocyte Contractile Force Development and Contractility In Vitro

YAP is a key regulator of cellular mechanotransduction in multiple cell types (54, 58). Prior studies have shown that the YAP target genes *CTGF/CCN2*, *Cyr61/CCN1*, and *THBS-1* are all direct activators of mechanosignaling through integrin receptors (59–62). Downstream effectors of integrin receptor signaling, such as focal adhesion kinase (FAK) and the adaptor protein paxillin, populate focal adhesions (FAs) and transmit outside-to-inside signals to direct various aspects of cellular motility and attachment (63, 64). Since CRB2 knockdown induces YAP activity and the expression of YAP mechanotransduction target genes, we next explored the



C

p.Leu1074_Asp1076del	D	L	F
Human	D	L	F
Rhesus	*	*	*
Mouse	D	L	F
Dog	D	L	F
Elephant	D	L	F
Chicken	D	L	F
X_tropicalis	D	L	F
Zebrafish	D	L	F

(*) No data available for Rhesus

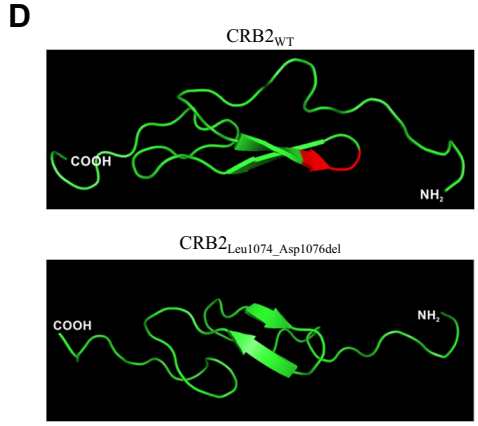


Figure 1. Pedigree of family DUK40595 and identification of CRB2 variant. *A*: family DUK40595 is a two-generation Indian kindred. The proband (arrow head) has biopsy-proven focal segmental glomerulosclerosis (FSGS), and her younger male sibling has proteinuric nephropathy likely caused by the FSGS lesion. The cause of FSGS in both affected siblings was found to be a compound heterozygous mutation of crumbs homolog-2 (*CRB2*; *FSGS-9*). The pathogenic mutation is comprised of a previously described truncating frameshift mutation (p.Gly1036_Alafs*43) and a rare 9-bp deletion mutation (p.Leu1074_Asp1076del). *B*: schematic of CRB2 protein functional domains. Family DUK40595 pathogenic mutations identified in the 3rd Laminin G domain [p.Gly1036Alafs*43, (* in orange)] and the 12th EGF-like domain [p.Leu1074_Asp1076del, (* in black)]. The 9-bp, in-frame deletion results in the loss of three highly conserved amino acids (Asp-Leu-Phe) (*C*), which significantly distorts the structure of the EGF-like domain (*D*).

Table 1. *Family DUK40595 clinical characteristics*

Family and Individual	Gender	Genotype	Amino Acid Change	Proteinuria, g/24 h	Age at Diagnosis	Diagnosis	Transplant/ Recurrence
DUK40595-1000	Female	c.3220_3228del	p.Leu1074_Asp1076del	None	NA	No disease	No/NA
DUK40595-1001	Male	c.3089_3104dup	p.Gly1036Alafs*43	None	NA	No disease	No/NA
DUK40595-1	Female	c.3089_3104dup/ c.3220_3228del	p.Gly1036Alafs*43/ p.Leu1074_Asp1076del	Yes; >3.5	16	FSGS	No/NA
DUK40595-100	Male	c.3089_3104dup/ c.3220_3228del	p.Gly1036Alafs*43/ p.Leu1074_Asp1076del	Yes; >3.5	15	FSGS	Yes/NA

FSGS, focal segmental glomerulosclerosis; NA, not applicable.

effect of CRB2 knockdown on podocyte contractile force development using ERISM.

ERISM detects cell-induced deformations of an elastic, optical micro-cavity by detecting and analyzing the interference of monochromatic probe light across a field of view captured by a modified inverted microscope (28, 32). We have previously used ERISM to observe the near-complete loss of immortalized human podocyte forces in a puromycin amino nucleoside (PAN) injury model (29).

To characterize the effects of CRB2 knockdown on podocyte force development, isolated siScr and si*CRB2* podocytes were seeded on collagen-I-coated ERISM substrates with an apparent stiffness ranging from 11 to 142 kPa, and ERISM measurements for $n \geq 14$ cells were performed on four or five nonconsecutive days during the 12 days of podocyte differentiation. Figure 4, *A* and *B* shows representative phase-contrast and ERISM displacement images of both cell lines when using a substrate with an apparent mechanical stiffness of 74 kPa. Contractile forces generated inside the cell result in an expansion of the elastomer at the position of FAs (indicated by bright areas at the cell periphery) (29). At the same time, cell contraction also leads to a compression of the elastomer underneath the cell body (indicated by the dark regions). These vertical deformation patterns are a response of the deformable substrate to predominantly horizontally directed cellular forces (28). (For a more-detailed discussion of force transmission in podocytes, see Fig. 6 and Ref. 29.)

The magnitude of the exerted contractile podocyte forces was quantified by calculating the total volume of downward sensor deformation underneath the cell soma, referred to as the indented volume (*IV*) in the following. Figure 4, *C–F* shows the *IV* for si*CRB2* and siScr podocytes during the differentiation period when cultured on substrates with different mechanical stiffnesses and demonstrate that cell contractility generally increases during differentiation. From *day 4* of the differentiation onward, the contractility of si*CRB2* podocytes proved significantly higher than that of siScr controls, regardless of substrate stiffness. These findings suggest that CRB2 knockdown significantly enhances podocyte contractility during differentiation.

Next, we calculated the relative change in *IV* during differentiation, i.e., between *day 12* and *day 1* of the differentiation, for all tested substrates (Fig. 4*G*), and found that it increases with substrate stiffness for both cell lines. To evaluate, if the mechanosensing capability of CRB2 knockdown cells is changed, we normalized the relative change in *IV* during differentiation of si*CRB2* podocytes to the value of siScr controls ('relative knockdown effect' in Fig. 4*G*). Strikingly, the relative effect of CRB2 knockdown is largest for the softest substrate and decreases with increasing substrate stiffness. This indicates that the contractility of CRB2 knockdown cells remains high on soft substrates compared with control cells, which implies that mechanosensing might be impaired in CRB2 knockdown cells, rendering them unable to reduce contractility on highly compliant substrates. This finding is in line with the suggested role of CRB2 as an upstream regulator of YAP activity.

Collectively, the data from this series of experiments show that CRB2 knockdown increases force generation in human podocytes. Since this effect is the highest on soft substrates, these changes may be more relevant early on in disease to initiate a cascade of podocyte functional impairment. Taken together, these findings suggest that CRB2 may function as an upstream regulator of YAP-induced mechanoactivation.

CRB2 Knockdown Enhances Filamentous Actin and Mechanosensitive Protein Expression in Podocytes

Because CRB2 knockdown increases podocyte force development, we wanted to determine whether this enhancement in force is associated with an increase in the expression of mechanosensitive proteins. To evaluate this, we performed immunocytochemistry in si*CRB2* and siScr podocytes fixed on ERISM substrates with an apparent stiffness of 74 kPa. First, we explored the expression and localization of F-actin and the FA adaptor protein paxillin (Fig. 5*A*). Although actin stress fibers are visible in both cell lines, quantifying the mean fluorescent intensity of F-actin shows a significant increase of F-actin in differentiated si*CRB2* podocytes as compared with the siScr control group (Fig. 5*C*). This finding is in contrast to results from an earlier study that found a reduction in F-actin formation for *CRB2* knockout podocytes (15). In addition, to test whether the CRB2 knockdown leads to a rearrangement of the actin cytoskeleton, we calculated the orientation order parameter *S* of actin fibers (Fig. 5*A*; $S = 0$ for isotropic, nonpolarized; $S = 1$ for perfectly linearly polarized actin cytoskeleton) (51). The orientation order of si*CRB2* podocytes displayed a slight increase compared with siScr controls, yet with no statistical significance (Fig. 5*D*).

In both cell lines, paxillin showed a pronounced dotted/punctuate expression pattern along the cell periphery (Fig. 5*A*), consistent with its position within the FAs of cells (65, 66). No difference in the distribution of paxillin was observed between the cell lines. However, Western blotting of phosphorylated paxillin (p-paxillin) and focal adhesion kinase (p-FAK, Fig. 5*B*), and the immunolabeling of FAK (Supplemental Fig. S3) suggest the presence of enhanced paxillin and FAK activity in CRB2-deficient cells. Assuming

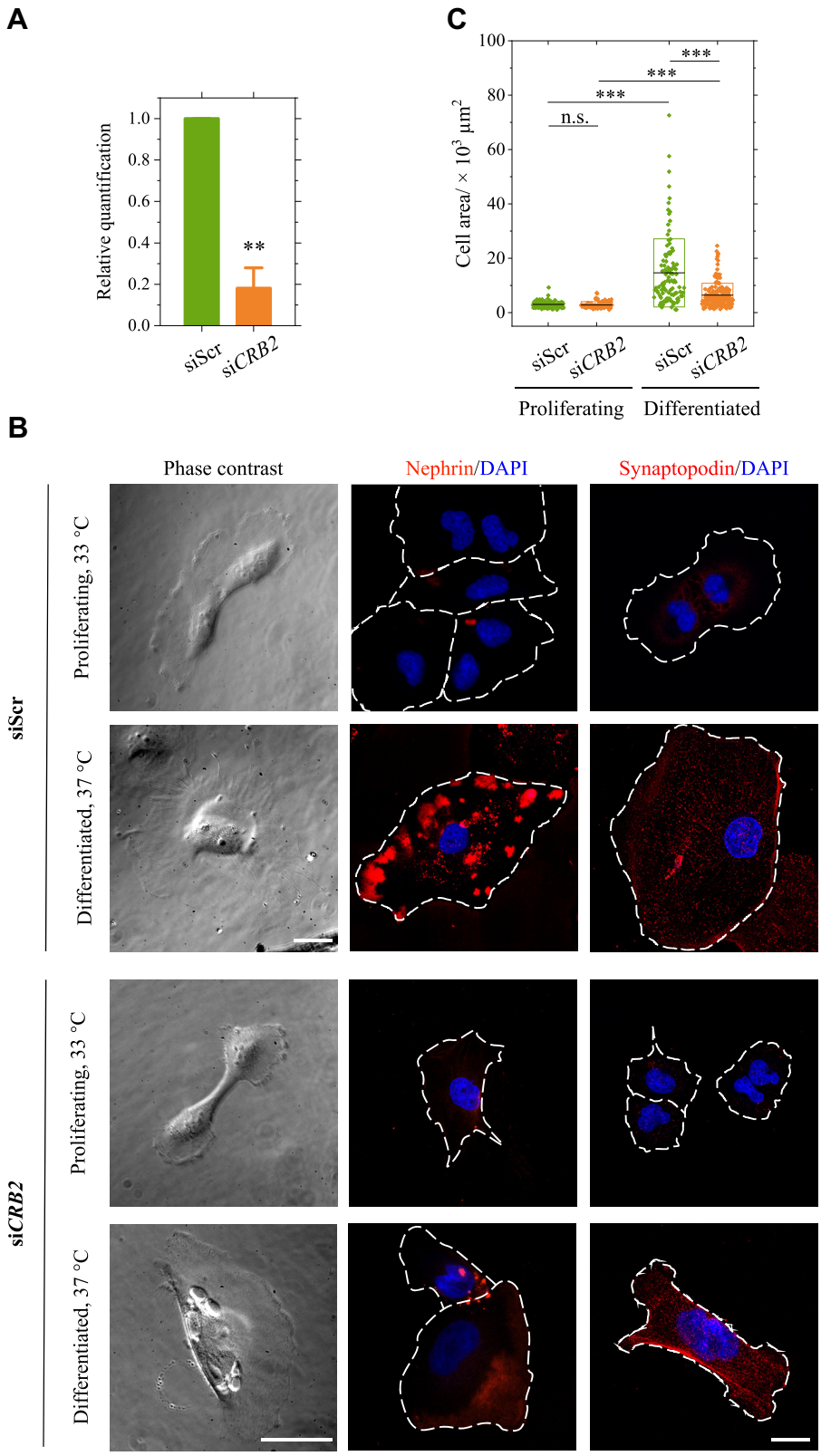


Figure 2. Immortalized podocytes express differentiation markers and increase in cell area upon maturation. *A*: plot of relative quantification of crumbs homolog-2 (*CRB2*) gene expression in scrambled siRNA (siScr) and CRB2-knockdown (si*CRB2*) podocytes from three quantitative polymer chain reaction (qPCR) replicates. Mean values (boxes) and standard deviation (error bar). Groups were compared using a paired-sample *t* test with nonequal variance, $n = 3$, $P = 0.00487$. $^{**}P \leq 0.005$. *B*: phase contrast and max projection of confocal immunofluorescence images of nephrin (red), synaptopodin (red), and nuclei (detected by DAPI, blue) for proliferating (at 33°C) and 12-day-differentiated (at 37°C) siScr and si*CRB2* podocytes. Cell outlines are indicated by white-dashed lines in the immunofluorescence images. Scale bars, 50 µm. *C*: comparison of cell area between proliferating and 12-day-differentiated podocytes (dots), showing mean (lines) and ±1 SD (boxes) for the siScr and si*CRB2* cells. $n \geq 80$. Groups were compared using two-tailed Student *t* tests with nonequal variance. $P = 0.38742$, 2.28571e-16, 1.08013e-18, 8.78202e-10, respectively. n.s., $P > 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

that each paxillin dot in the immunofluorescence images corresponds to a FA, we quantified the number of FAs per cell; on average 150 and 85 FAs were found in siScr and si*CRB2* podocytes, respectively (Fig. 5*F*). The average size of individual FAs, ~1.7 µm^2 (Fig. 5*G*), was independent of cell type and consistent with earlier measurements of FA size (66, 67). However, due to the smaller cell area of knockdown cells, the ratio of total FA area to cell area was significantly higher in si*CRB2* podocytes relative to siScr controls (Fig. 5*H*). Since FAs grow in response to increased cell contractility (68), these data suggest that CRB2

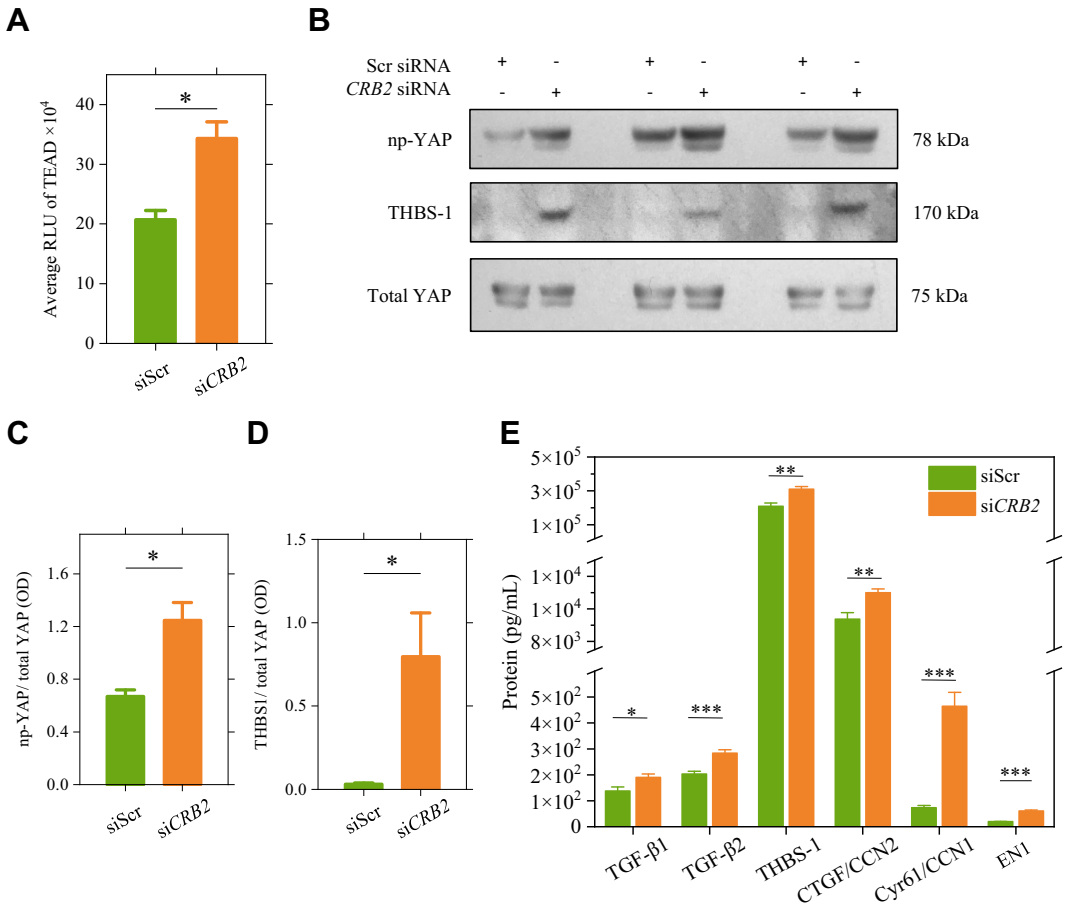


Figure 3. Crumbs homolog-2 (*CRB2*) knockdown increases the expression of yes-associated protein (YAP) target genes and proteins in immortalized podocytes. *A*: luciferase reporter assay of average expression of transcriptional-enhanced associate domain (TEAD) in scrambled siRNA (siScr) and *CRB2*-knockdown (si*CRB2*) podocytes. $n = 3$, $P = 0.02233$. *B*: Western blots of nonphosphorylated YAP (np-YAP), thrombospondin-1 (THBS-1), and total YAP expression in siScr and si*CRB2* podocytes. *C* and *D*: relative expression level of np-YAP and THBS-1 compared with total YAP for both cell lines using data obtained from *B*. $n = 3$, $P = 0.03856$ and 0.09999, respectively. *E*: enzyme-linked immunoassay (ELISA) analysis of expression of YAP-target genes for both cell lines. CCN1/2, cellular communication network factor 1 or 2; CTGF, connective tissue growth factor; Cyr61, cysteine-rich angiogenic inducer 61; EN1, endothelin 1; TGF, transforming growth factor. $n = 6$, $P = 0.02635$, 8.32864*e*-4, 0.00255, 0.00711, and 3.86608*e*-5, respectively. Mean values (boxes) and ±SE (error bars). Groups in *A*, *C*, *D*, and *E* were compared using two-tailed Student *t* tests with nonequal variance, n.s.: $P > 0.05$, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

knockdown enhances the formation of F-actin fibers, FAK and paxillin activation, FA density, and contractility in podocytes, probably downstream of YAP target gene expression.

Figure 5*A* shows that paxillin localizes at the termini of actin fibers in both cell lines. To investigate how contractile forces are transmitted to the substrate at the FA sites in podocytes, we used Fourier filtering of the ERISM displacement maps. This procedure filters out any broad substrate deformations and thus provides a more detailed view of subtle and localized deformations (Fig. 6*C* and Supplemental Fig. S4). The Fourier-filtered displacement maps show a series of clearly distinguishable push/pull features (shown in blue/red) at the periphery of the cell. This twisting behavior is consistent with earlier observations of the torque being applied by FAs (28). Areas of high paxillin expression are located at the center of these features, confirming that they are associated with FAs transferring traction forces horizontally to the substrate. This twisting is more pronounced in the si*CRB2* cell than the siScr cell, which is in line with the higher overall contractility of the latter (Fig. 4) (29, 69).

Force Development in CRB2 Knockdown Podocytes Is Not Significantly Altered by YAP Inhibitors or Rapamycin

So far, we have established that CRB2 knockdown leads to upregulation of YAP activity and YAP target gene expression, and to an increase in cell forces and an impairment of mechanosensing at low substrate stiffnesses. Next, we asked whether the observed mechanical phenotype of CRB2 knockdown cells is lost when the transcriptional activity of YAP is inhibited. To this end, we treated differentiated podocytes of both the siScr and si*CRB2* lines with either K-975 (100 nM) or verteporfin (72–359 nM; equivalent to 50–250 ng/mL). Both reagents inhibit the interaction between YAP and TEAD (70). However, no significant reduction in cellular forces was observed after treating the cells with either of the two drugs for 24 h; there was also no difference in response between the two cell lines (Fig. 7, *B* and *C*). Higher concentrations of verteporfin (1.5 and 5 μM, equivalent to 1.0 and 3.5 μg/mL) did not result in a reduction of the contractility of CBR2 knockdown cells either (Supplemental Fig. S5).

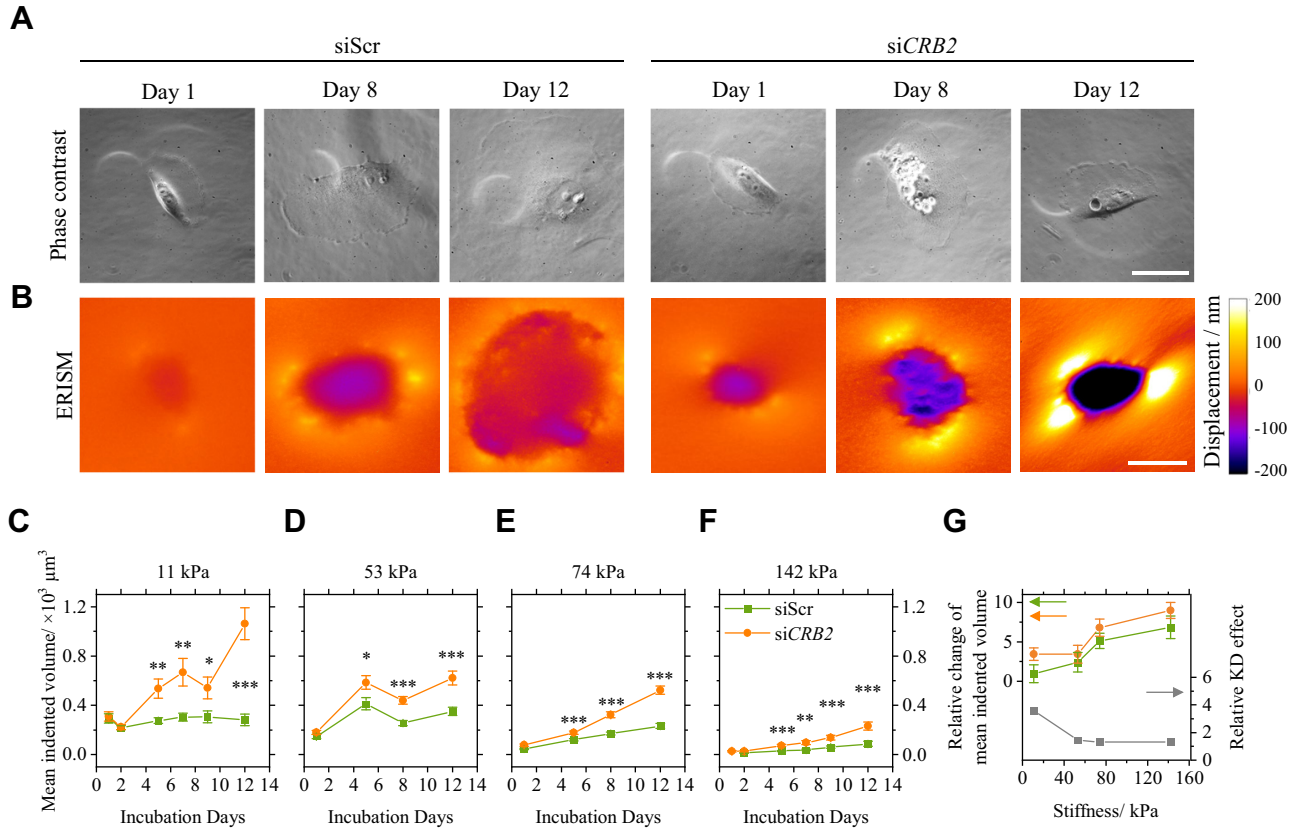


Figure 4. Cell contractility of crumbs homolog-2 (*CRB2*) siRNA (si*CRB2*) podocytes increases throughout differentiation. Phase-contrast images (*A*) and elastic resonator interference stress microscopy (ERISM) displacement maps (*B*) of representative control (scrambled siRNA, siScr) and *CRB2* knockdown podocytes on ERISM substrates with an apparent stiffness of 74 kPa on *days 1*, *8*, and *12* of the differentiation at 37°C. Scale bars, 50 µm. *C*–*F*: evolution of mean indented sensor volume (symbols) and ±SE (error bars) of siScr (green) and si*CRB2* (orange) podocytes during differentiation, measured on ERISM substrates with an apparent stiffness of 11, 53, 74, and 142 kPa, respectively. $n \geq 14$ for each group. $P = 0.00293$, 0.00419, 0.0263, and 4.63094e-6 in *C*; $P = 0.02098$, 2.39707e-6, and 5.94428e-5 in *D*; $P = 7.1584e\text{-}4$, 2.18473e-7, and 2.00674e-11 in *E*; $P = 5.71389e\text{-}5$, 0.00144, 8.07563e-4, and 5.58459e-4 in *F*. *G*: relative change of mean indented volume between *day 12* and *day 1* of differentiation (symbols) and ±SD (error bars) of siScr (green) and si*CRB2* (orange) podocytes. Relative knockdown (KD) effect, i.e., the ratio of relative change, si*CRB2*/siScr cell line, for ERISM substrate of different stiffness (gray symbols, right *y*-axis). Groups were compared using two-tailed Student *t* tests with nonequal variance, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

We also investigated the effect of the mTOR inhibitor rapamycin on the contractility of matured si*CRB2* and siScr podocytes as rapamycin might enhance the autophagy-mediated degradation of YAP (71, 72). Again, no effect on the contractility of either cell line was found after 24 h of treatment (Fig. 7*D*).

The findings of the experiments with YAP-inhibitors suggest that the increase in contractility of CRB2 knockdown cells may not be exclusively a result of YAP activation, but that other pathways may also play a role or that cells can activate compensation pathways if YAP is inhibited via drugs.

DISCUSSION

In this study, we report the identification of a rare compound heterozygous mutation in Crumbs homolog 2 (*CRB2*; *FSGS9*) in an Indian kindred with early-onset FSGS/SRNS. To model the effects of the truncating compound heterozygous mutation in Family DUK40595, we established stably expressing scrambled (siScr) and *CRB2* (si*CRB2*) siRNA lines to determine whether CRB2 functions upstream of YAP regulate podocyte mechanotransduction signaling. We acknowledge the use of siRNA-mediated knockdown as opposed to CRISPR Cas9-mediated gene deletion for these studies as a weakness, and will make the effort to generate these lines for future studies. We showed that CRB2 knockdown significantly enhanced YAP/TEAD reporter activity, YAP target gene expression, and mechano-transductory signaling. CRB2 knockdown also significantly reduces podocyte cell area, upregulates F-actin and mechanosensitive protein expression, and increases focal adhesion (FA) density. In addition, we used ERISM at various substrate stiffnesses to measure the effects of CRB2 knockdown on podocyte force development during differentiation. siScr and si*CRB2* podocytes demonstrated contractile forces transmitted at FA sites that were primarily distributed along the cell periphery. We found that CRB2 knockdown increases podocyte contractility in a substrate stiffness-dependent manner. The knockdown effect decreased with increasing substrate stiffness, indicating impaired mechanosensing in CRB2 knockdown cells at low substrate stiffness. Although we acknowledge that our model does not fully recapitulate the phenotype observed in DUK40595, we believe that the studies provide valuable new insights into the possible mechanisms of CRB2-associated podocytopathy that can inform future

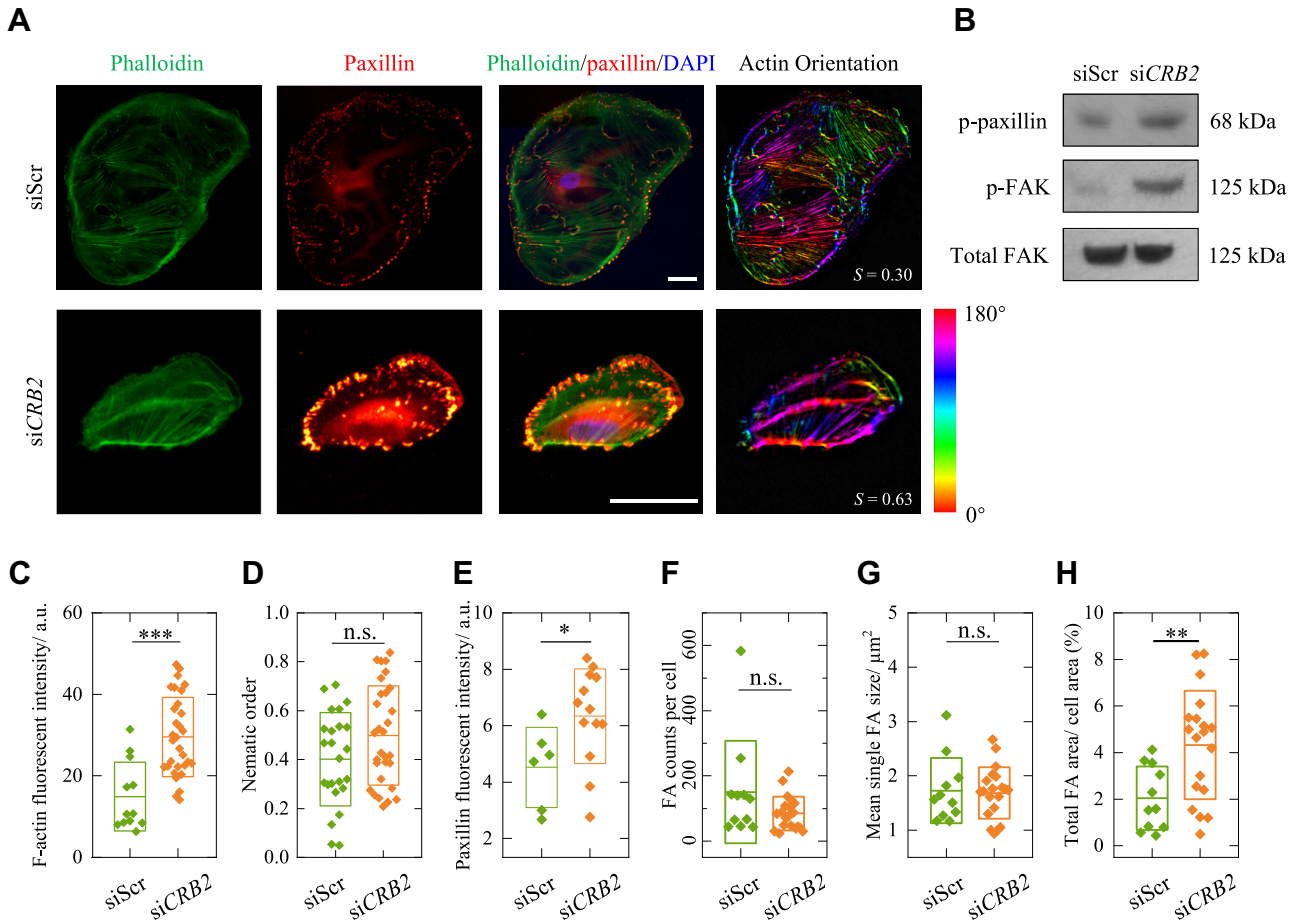


Figure 5. Crumbs homolog-2 (*CRB2*) knockdown enhances filamentous actin and mechanosensitive protein expression in immortalized podocytes. *A*: representative immunofluorescence images of F-actin (labeled by TRITC-phalloidin, green), paxillin (red), and nuclei (labeled by DAPI, blue) for 12-day-differentiated control (scrambled siRNA, siScr) and CRB2 knockdown (si*CRB2*) podocytes. Scale bars, 50 μm. *B*: Western blot of phosphorylated paxillin, focal adhesion kinase (p-paxillin, p-FAK), and total FAK in siScr and si*CRB2* podocytes. Since the experiment was not repeated, we did not perform a quantitative analysis. Mean fluorescence intensity of F-actin ($P = 6.2319e\text{-}5$) (*C*) and paxillin ($P = 0.0321$) (*E*) per cell for both cell lines. *D*: actin cytoskeleton order parameter (nematic order, *S*) for both cell lines. $S = 0$ for isotropic, nonpolarized, and $S = 1$ for perfectly linearly polarized actin cytoskeleton. Numbers of focal adhesion (FA) clusters per cell (quantified via the counts of paxillin dots in immunofluorescence images) (*F*), mean size of individual FAs (*G*), and ratio of total FA area across a cell to its size for differentiated siScr and si*CRB2* podocytes (*H*), $P = 0.00211$. Shown are the values for individual cells (symbols), the mean (central lines), and ±SD (boxes). At least six cells were measured per group. Groups were compared using two-tailed Student *t* tests with nonequal variance, n.s., no significance, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. F-actin, filamentous actin; YAP, yes-associated protein.

studies of podocyte injury and potential therapies in whole animal models.

Together, these results suggest a role of CRB2 as upstream activator of YAP activity. In siScr cells, we hypothesize that YAP is deactivated by phosphorylation through CRB2-MST1/2-LATS-mediated Hippo pathway signaling (Fig. 8*A*). This post-translational modification prevents YAP from entering the nucleus and can lead to its cytoplasmic sequestration with 14-3-3 protein or degradation (Fig. 8*A*). In contrast, CRB2 knockdown may promote the downregulation of Hippo pathway signaling in podocytes, leading to an inappropriate upregulation of YAP activity and target gene expression (Fig. 8*B*). Inappropriate upregulation of gene targets such as *THBS-1* could then lead to hyperactivation of integrin signaling, FAK activation, and FA growth (73) (Fig. 8*B*). Increased YAP activity is also known to activate TGF-β/Ras homolog family member A (RhoA)/Rho-associated protein kinase (ROCK) signaling and myosin II activity (74). Both activation pathways could promote an increase in cell contractility (75, 76) (Fig. 8*B*). Mechanical compression of the nucleus as a result of the increased cell contractility has been shown to directly lead to further nuclear translocation of YAP (77). Moreover, RhoA (via actomyosin) and FAK activity are also known to inhibit LATS1/2, which can lead to a further activation of YAP (78).

Interestingly, we did not find evidence that the increased cell contractility of CRB2 knockdown cells was significantly reduced when cells were treated with the YAP inhibitors K-975 and verteporfin (70, 79). This might suggest that the increase in contractility of CRB2 knockdown cells is not only a direct result of YAP activation, but that other pathways also play a role. A possible alternative activation path is that the association of the protease activated receptor (PAR) complex to PALS1 in the crumbs complex is disrupted upon CRB2 knockdown (80–82) (Fig. 8). This might lead to reduced activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) downstream of the PAR complex, resulting in the observed

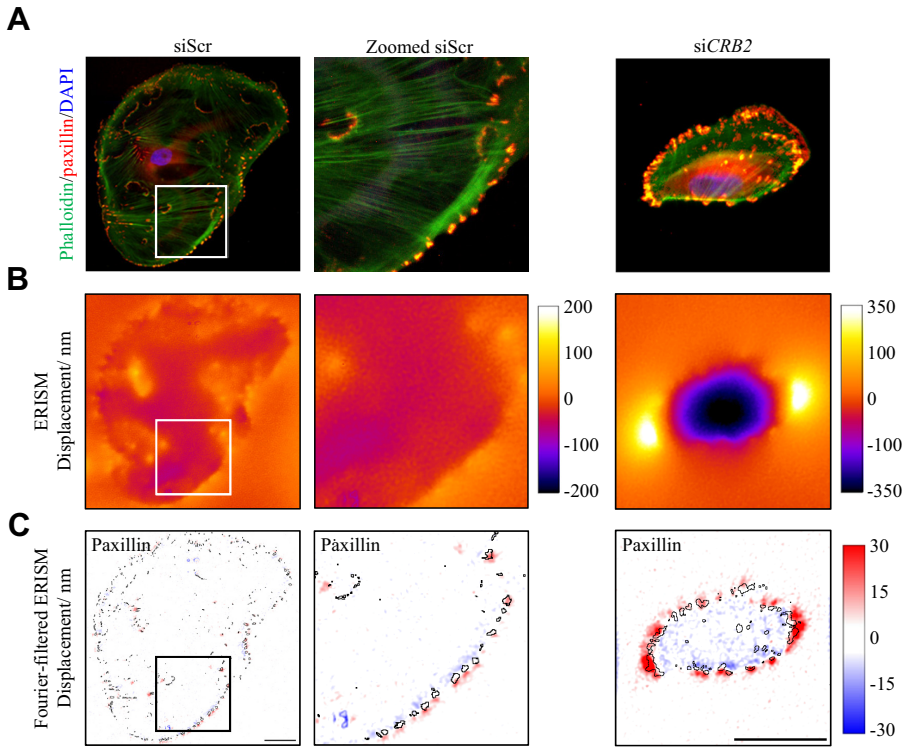


Figure 6. Podocyte contractile forces are colocalized with the paxillin expression focal adhesions (FAs) at the cell periphery. *A*: composite immunofluorescence images of the representative cells shown in Fig. 5. F-actin (labeled by TRITC-phalloidin, green), paxillin (red), and nuclei (labeled by DAPI, blue) of a differentiated control podocyte (*left*), a zoom-in on the area marked by the white square (*middle*), and same staining for a differentiated CRB2 knockdown (si*CRB2*) podocyte (*right*). Corresponding elastic resonator interference stress microscopy (ERISM) displacement (*B*) and Fourier-filtered ERISM maps, with black lines denoting the outlines of paxillin-rich areas (*C*). Shown are scrambled siRNA (siScr) representative of 10 cells and si*CRB2* of 21 cells. Scale bars, 50 μm. F-actin, filamentous actin.

reduction in cell area (83), and to increased RhoA activity and thus the observed increase in cell contractility (67, 84).

It is important to point out that K-975 has not been applied in podocytes before. Based on prior work by Kaneda et al. (70), we selected a treatment dose of 100 nM, which was sufficient to reduce YAP target gene expression in multiple mesothelioma cell lines. Yet, in in vitro studies, dosing ranges up to 10 μM were evaluated with similar effects on gene expression (70). Similarly, with verteporfin, prior studies of the molecule in immortalized podocytes saw effective inhibition of YAP activity and gene expression with doses used as high as 5 μg/mL (85). However, in our study, doses higher than 1.0 μg/mL (1.5 μM) significantly reduced cell viability for both cell lines. With respect to rapamycin, the persistent activation of YAP in si*CRB2* podocytes may have limited the possibility of autophagic degradation in the absence of regular nuclear-to-cytoplasmic shuttling.

Significant progress has been made over the past decade in our understanding of disease-causing *CRB2* mutations in SRNS/FSGS. The first description by Ebarasi et al. (11) clearly established *CRB2* mutations as a cause of autosomal recessive podocyte injury and impaired glomerular integrity. Moreover, using a *crb2*-deficient zebrafish model, they demonstrated that crb2 loss impaired apical basal polarity of podocytes, disrupted foot process morphology and arborization, altered trafficking of SD proteins such as nephrin, podocalyxin, and zonula occludens-1 (ZO-1), disrupted slit diaphragm formation, and compromised glomerular filtration barrier integrity (11). Since then, several studies have confirmed and expanded upon these findings documenting ~30 human disease-causing *CRB2* mutations (15, 19, 20). Nonetheless, insight into the pathogenic mechanisms of CRB2-mediated podocyte injury remains limited. A recent report by Yang et al. (20) suggested that novel pathogenic *CRB2* mutations reduce sphingosine-1-phosphate receptor 1 (S1PR1) expression/phosphorylation and that S1PR1 deficiency disrupts SD protein expression. This mechanism is plausible as S1PR1 signaling has been shown to be cytoprotective against podocyte injury in diabetic nephropathy, and disruption of this protection mechanism could predispose podocytes to dysfunction and loss (86, 87). Prior studies by Möller-Kerutt et al. (14, 18) showed that CRB2 is an essential SD protein that organizes in adjacent clusters with nephrin and that pathogenic mutations in *CRB2* may promote defective posttranslational modification and inappropriate retention in the endoplasmic reticulum (ER) leading to podocyte injury or loss by ER stress. In addition, Hamano et al. (88) demonstrated that CRB2 associates with mTORC1 in developing podocytes and may influence mTORC1-mediated management of cellular energy resources. The changes in YAP activity in CRB2 knockdown podocytes presented by the data may be linked to Hippo pathway signaling. Prior studies of Hippo signaling in podocytes have shown that YAP activity is cytoprotective and that nuclear exclusion of the transcriptional co-regulator drives podocyte injury (25–27, 89, 90). Because YAP is recognized as a universal mechanotransducer (91), we focused on understanding the potential contribution of CRB2 to regulation of YAP-mediated mechanosignaling in podocytes. A prior study by Rinschen et al. (92) showed that puromycin aminonucleoside (PAN)-induced glomerular injury in rats increased YAP activity and target gene expression (i.e., Cyr61/CCN1, CTGF/CCN2, and Ankrd1) and that inhibition of Hippo signaling with verteporfin reduced YAP activation and proteinuria. In addition, they showed that podocyte YAP activity was influenced by substrate rigidity and F-actin (92). Although the Rinschen study did not address the role of CRB2, our findings related to YAP activation and mechanosignaling in podocytes were consistent.

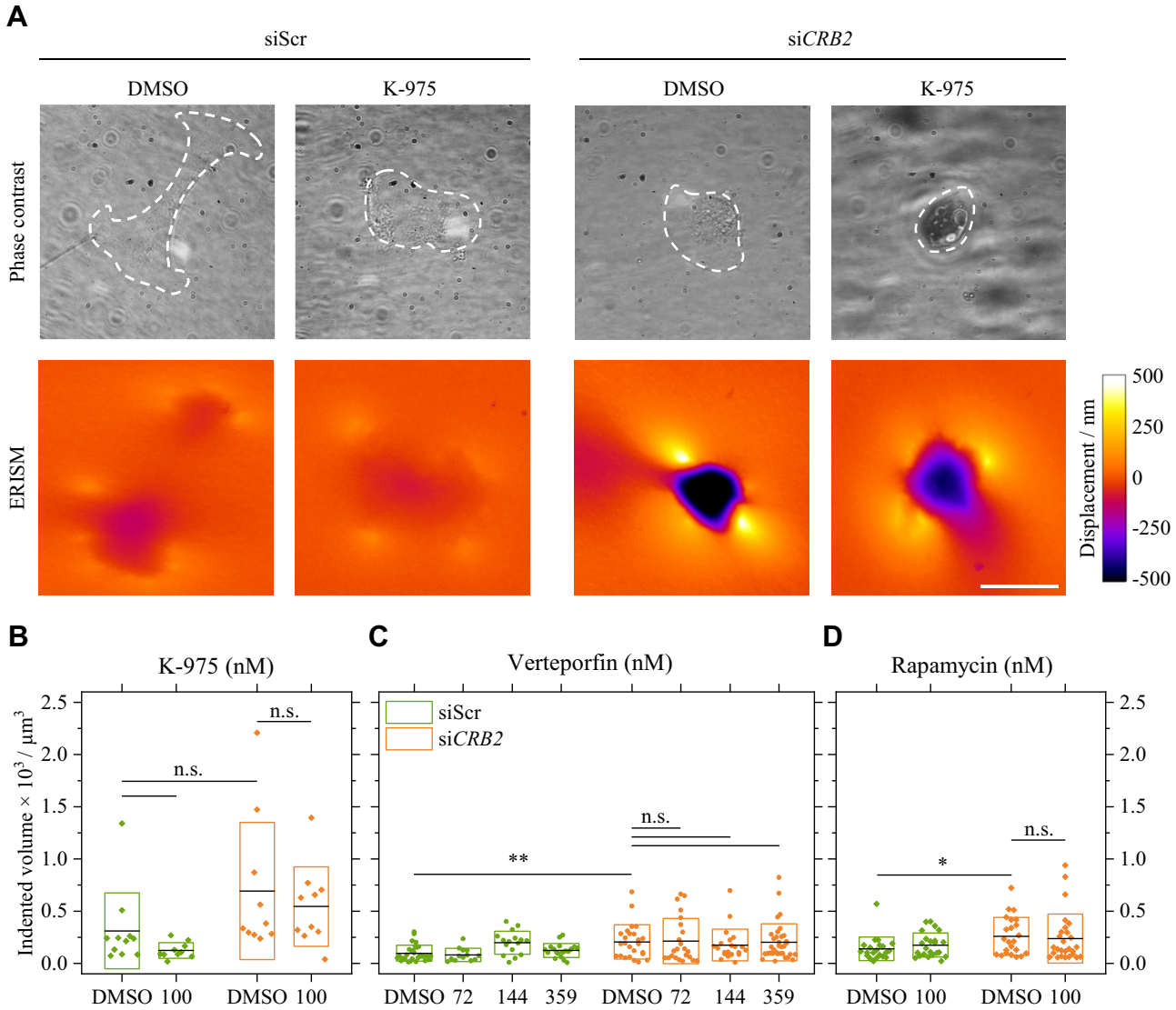


Figure 7. Force development in crumbs homolog-2 (*CRB2*) knockdown podocytes is not significantly altered by yes-associated protein (YAP) or mammalian target of rapamycin (mTOR) inhibitors. *A*: phase contrast images and elastic resonator interference stress microscopy (ERISM) displacement maps of representative differentiated control (siScr) and CRB2 knockdown (si*CRB2*) podocytes on an ERISM substrate with an apparent stiffness of 53 kPa. Cell outlines are indicated by white-dashed lines. Groups were treated without (DMSO) or with 100 nM of K-975 for 24 h. *B–D*: mean indented volume (symbols), means (central lines) and ±SD (boxes) of single podocytes after 24 h of treatment with 100 nM of K-975 (*B*), verteporfin with various of concentrations ($P = 0.00436$) (*C*), and 100 nM of rapamycin (*D*) for siScr and CRB2 knockdown (si*CRB2*) podocytes ($P = 0.01212$). All groups were treated with 0.2% of DMSO. $n \geq 10$. Scale bar, 50 µm. Groups were compared using two-tailed Student *t* tests with nonequal variance, n.s., no significance, $*P \leq 0.05$, $**P \leq 0.01$.

Although the majority of these results suggests a link between CRB2 and YAP activity, the data do not prove whether this link is made through Hippo pathway signaling, e.g., via the activation of MST1/2 and LATS1/2 (Fig. 8*A*). It has been reported in the literature that YAP also directly associates with the crumbs complex (Fig. 8*A*). This association could potentially promote cytoplasmic retention/degradation of YAP without the involvement of the Hippo pathway (93). Indeed, YAP signaling is an essential mediator of a growing number of essential podocyte functions and further insight into the breadth of its involvement in podocyte physiology is warranted.

In conclusion, we identified a novel human *CRB2* gene variant contributing to familial FSGS in combination with a known variant. CRB2 knockdown in human urinary podocytes resulted in increased TEAD gene expression, enhanced YAP and YAP-target protein levels, and higher cell contractility. In addition, CRB2 knockdown reduces the ability of podocytes to adapt their contractile force to the stiffness of their substrate (mechanotransduction). We also noted elevated F-actin, paxillin, and FAK protein expression induced in CRB2-deficient podocytes. These results suggest that CRB2 is an upstream regulator of YAP-mediated mechanotransduction signaling in podocytes, even though other pathways may also play a role in mechanosignaling downstream of CRB2. Although our studies do not demonstrate a direct functional relationship between CRB2 and YAP, these findings provide crucial mechanical insights for understanding *CRB2*-associated podocytopathy.

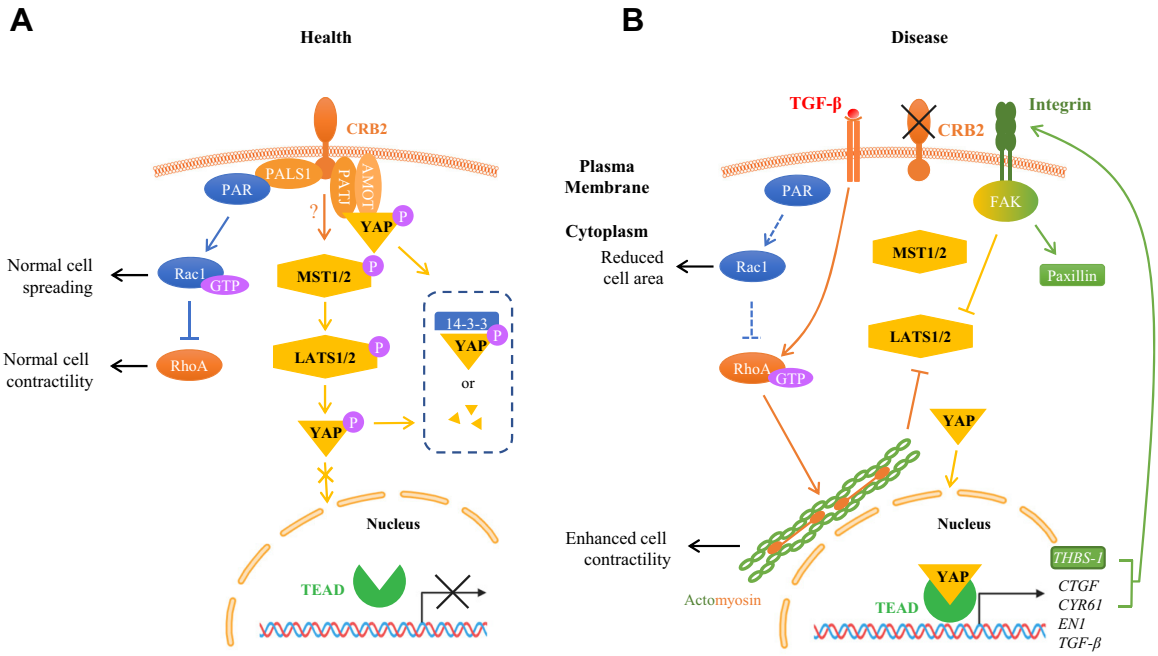


Figure 8. Proposed effect of crumbs homolog-2 (CRB2) deficiency on podocyte mechanotransduction. Schematic diagram of the possible signaling pathways involved upon CRB2 knockdown. *A*: in healthy podocytes, the crumbs complex associates with the protease-activated receptor (PAR) complex, thereby facilitating normal cell spreading and contractility. Either the interaction with crumbs complex or the activation of mammalian sterile 20-like kinase 1/2 (MST1/2) and large tumor suppressor kinase 1/2 (LATS1/2) signaling of the Hippo pathway may promote yes-associated protein (YAP) phosphorylation (deactivation), leading to retention or degradation of cytoplasmic YAP. *B*: in *CRB2* mutant-related nephropathy, the activity of ras-related C3 botulinum toxin substrate 1 (Rac1) might be suppressed by the disassociation of the PAR complex from the crumbs complex. Together with the activated transforming growth factor beta (TGF-β) signaling, this may lead to a higher activity of Ras homolog family member A (RhoA)/Rho-associated protein kinase (ROCK)/Myosin II signaling, thereby resulting in a smaller cell spreading area and an enhanced cell contractility. In the absence of CRB2, upregulated RhoA- and focal adhesion kinase (FAK)-mediated LATS1/2 suppression may also result in nuclear translocation of YAP and upregulation of YAP target genes such as thrombospondin 1 (*THBS-1*), connective tissue growth factor (*CTGF*), cysteine-rich angiogenic inducer 61 (*CYR61*), endothelin-1 (*EN1*) and *TGF-β*, etc. FAK signaling is activated by THBS-1, leading to the upregulation of paxillin. Question marks, assumptions. Solid lines, normal signaling. Dashed lines, reduced signaling. AMOT, angiomotin; GTP, guanine triphosphate; P, phosphate; TEAD, transcriptional enhanced associate domain.

ETHICAL APPROVALS

Human subject studies were approved by Duke University Institutional Review Board, Duke University, USA. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from all participants, authorizing publication of any potentially identifiable images or data included in this article.

DATA AVAILABILITY

The research data sets supporting this publication can be accessed via the PURE repository of the University of St Andrews at https://doi.org/10.17630/e71a5370-2576-441f-aee3-d21ac094b44b.

SUPPLEMENTAL MATERIAL

Supplemental Figs. S1–S5: https://doi.org/10.5281/zenodo.14935108.

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DISCLOSURES

N.M.K. and M.C.G. are inventors on patent US20170322193A1, which describes the ERISM method. G.H. is a consultant for Travere Therapeutics, Otsuka Pharmaceuticals, and Health Monitor Network. G.H. is also a speaker and writer for Otsuka Pharmaceuticals, Inside Edge Consulting, and Health Monitor Network. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

G.H. and M.C.G. conceived and designed research; Y.S., S.K.S., S.N.D., M.E.K., B.S., S.S., and X.T. performed experiments; Y.S.,

N.M.K., R.R., C.J.S., and S.I. analyzed data; Y.S., N.M.K., G.H., and M.C.G. interpreted results of experiments; Y.S. prepared figures; Y.S. and N.M.K. drafted manuscript; Y.S., N.M.K., S.K.S., S.N.D., M.E.K., B.S., S.S., R.R., C.J.S., X.T., S.I., G.H., and M.C.G. edited and revised manuscript; Y.S., N.M.K., G.H., and M.C.G. approved final version of manuscript.

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