SUPPLEMENTARY MATERIALS

The Human FSGS-causing *ANLN* R431C mutation induces dysregulated PI3K/AKT/mTOR/Rac1 signaling in podocytes

#Gentzon Hall MD PhD[1,2,3], #Brandon M Lane PhD[1,2], Kamal Khan MS[4], Igor Pediaditakis BS[4], Jianqiu Xiao PhD[4], Guanghong Wu BS[1,3], Liming Wang MD, PhD[3], Maria E Kovalik[1,2,3] Megan Chryst-Stangl MS[1,2], Erica E Davis PhD[1,4], Robert F. Spurney MD[3], *Rasheed A Gbadegesin MBBS, MD[1,2,3]

1. Department of Pediatrics, Duke University School of Medicine

2. Duke Molecular Physiology Institute

3. Department of Medicine, Duke University School of Medicine

4. Center for Human Disease Modeling, Duke University Medical Center

Running Title: *ANLN* R431C causes FSGS through mTOR and Rac1 activation

SUPPLEMENTARY METHODS (SM)

Transient suppression of anln and in vivo complementation assays in zebrafish:

All studies performed in zebrafish were approved by the Duke University Institutional Animal Care and Use Committee (IACUC). We designed a splice blocking MO targeting the donor site of exon 5 of the *anln* ortholog in zebrafish (Ensembl ID: ENSDART00000113574.3, GRCz10; 48% amino acid identity and 62% similarity vs. human; 5´- AGATGACAAGCTCCACTAACCAGGC - 3´; Gene Tools; **Supplementary Figure 1a**). To determine MO efficiency, we injected 3 ng of MO into WT zebrafish embryos (ZDR) at the 1-4 cell stage (1 nl / embryo) and harvested them at 2 days post fertilization (dpf) for RNA extraction. We used Trizol (Invitrogen) to extract total RNA from morphants and uninjected controls following the manufacturer's protocol. We used the QuantiTect Reverse Transcription kit (QIAGEN), to perform RT-PCR on resulting RNA, and amplified the *anln* targeted region. After migrating the amplified product on a 1 % agarose gel, PCR fragments were purified using the QIAquick gel extraction kit (QIAGEN) and cloned using the TOPO-TA cloning kit for sequencing (ThermoFisher). To characterize aberrant splicing, we Sanger sequenced individual colonies on an ABI3730 sequencer using BigDye Terminator chemistry (Applied Biosystems) and analyzed data using Lasergene software (DNASTAR). We assessed the effect of progressive doses of MO (3 ng, 6 ng and 9 ng) and subsequently used 3ng MO for the *in vivo* complementation assays. We cloned the human $ANLN_{WT}$ open reading frame into the pCS2+ vector, and performed site directed mutagenesis as described[1] to introduce the R431C, G618C and R185K variants; all constructs were sequence confirmed. To perform *in vitro* transcription, we linearized plasmids with *Not*I, and

generated capped mRNA using the SP6 mMessage mMachine transcription kit (ThermoFisher). We injected 150 pg mRNA for all embryo injections. Live bright field images were acquired on 4 dpf larvae anesthetized with tricaine using a Nikon AZ100 microscope with a Digital Sight color camera and NIS elements software.

CRISPR/Cas9 genome editing of the anln locus in zebrafish:

We used CHOPCHOP v2[2] to identify an sgRNA targeting the coding region of exon 8 (Ensembl ID: ENSDART00000113574.3, GRCz10; 5´-GAAGGCTTATTATCATGCAGTGG -3´; **Supplementary Figure 1a**). We synthesized sgRNAs using the Gene Art precision gRNA synthesis Kit (Invitrogen) as described.[3] We then injected 100 pg sgRNA in combination with 200 pg Cas9 protein (PNA Bio) in WT zebrafish embryos (ZDR) at the 1-cell stage and harvested them at 2 dpf for genomic DNA extraction using proteinase K (Life Technologies). We PCR-amplified the targeted region and assessed for indels by heteroduplex analysis as described.[3] Briefly, the amplified products from control embryos (n=2) and mutant embryos (n=8) were migrated by polyacrylamide gel electrophoresis (PAGE) using precast gels (Invitrogen) following heat denaturation and slow reannealing (95º C for 5 min, ramp down to 85º C at -1º C/s, and then to -0.1º C/s). The PCR fragments were then visualized using ethidium bromide and a UV lamp. For estimation of mosaicism in individual embryos we then cloned PCR product from F0 mutant embryos (n= 5) and a control embryo (n=1) using the TOPO-TA cloning kit for sequencing (ThermoFisher) per manufacturer's protocol. We sequenced 16-24 colonies from each embryo on an ABI 3730 sequencer

using BigDye Terminator chemistry (Applied Biosystems) and we analyzed the data using Lasergene software (DNASTAR).

Conditionally Immortalized Human Podocyte Culture: Conditionally immortalized human podocyte cell lines were generously provided by Dr. Jeffrey Kopp (Chief, Kidney Diseases Branch, NIDDK) and were cultured and harvested as described[4]. For immunoblotting experiments examining apoptosis, cells were exposed to serum starvation for 48 hours at growth restrictive temperatures before the media and cells were collected by cell scraping.

Lentiviral Constructs and Infection: Standard molecular cloning methods were used to replace the ubiquitin-EGFP of FUGW 19 with CMV-turboGFP, CMV-hANLN$_{WT}$-tGFP and CMV-hANLN$_{R431C}$-tGFP. For calcineurin inhibition, the ubiquitin-EGFP of FUGW 19 was replaced with CMV-hVIVIT-tGFP. Lentivirus was made and conditionally immortalized human podocytes were transduced as described[5].

Immunofluorescence: Conditionally immortalized human podocytes were differentiated per established protocols on collagen I–coated coverslips (BD Biosciences) and processed as described[6]. Cells were then exposed to phalloidin alexa-568 (Invitrogen) for one hour at RT followed by two PBS washes and then 4′,6-diamidino-2-phenylindole stain at a concentration of 1:20,000 diluted in PBS. Immunofluorescence imaging was performed using a Carl Zeiss AxioImager and the ZenBlue Bioimaging Software.

F-actin bundling/polymerization assay: HEK293T cells were grown in DMEM +5% FBS in T75 flasks at 37 degrees Celcius until 85% confluency and transfected using Lipofectamine 2000 with plasmids containing CMV-turboGFP, CMV-hANLN$_{WT}$-tGFP, or CMV-hANLN$_{R431C}$-tGFP. 65 ul of Lipofectamine 2000 (Thermo-Fisher) was combined with 1.7 mls of Opti-MEM (Gibco) and allowed to incubate at room temperature for 5 mins before combining with 1.7mls of Opti-MEM containing 26ug of plasmid DNA. After 20 mins of incubation, the mixture was added to the cells in 15 mls of DMEM with 5% FBS. Cells were analyzed using flouresense microscopy between 40- 48hrs to ensure adequate transfection levels and then harvested at 48 hrs and lysed using a syringe in 400 ul of 20mM HEPES, 20mM NaCl buffer. Supernatant was collected after spinning for 3 mins at 14,000 x G at 4 degrees Celsius and analyzed using immunoblotting to ensure equivalent levels of hANLN between samples. Bundling reactions including appropriate positive and negative controls were performed using the Actin Binding Protein Biochem Kit (Cytoskeleton Inc) according to the provided bundling method protocol. 30 ul of experimental lysate was combined with 50 ul of human non-muscle F-actin stock or an F-actin buffer and incubated for 30 mins at room temperature. After spinning at 14,000 x G for one hour, the supernatant was collected and the F-actin pellet was mixed with 30 ul of water and 30 ul of 2X Laemmli reducing-sample buffer and analyzed using immunoblotting with an F-actin mouse monoclonal antibody (1:500; Novus Biologicals).

Immunoblotting: Conditionally immortalized podocytes were harvested, and treated with lysis buffer (Pierce Biotechnology, Rockford, IL) supplemented with

phosphatase/protease inhibitor cocktail, 1:100 dilution (Cell Signaling Technology) and 1:100 PMSF (Sigma-Aldrich) for 15 min on ice. Cells were then spun at 14,000 RPM for 10 mins at 4°C (Eppendorf). Protein Immunoblotting was performed as described[6] using a rabbit monoclonal phospho AKT S473 antibody (1:1000; Cell Signaling Technology), rabbit monoclonal total AKT antibody (1:1000; Cell Signaling Technologies), rabbit polyclonal phospho-mTOR S2481 antibody (1:750; Cell Signaling Technologies), rabbit monoclonal phospho-mTOR S2448 (1:1000; Cell Signaling Technologies), rabbit monoclonal p70-S6K (1:1000; Cell Signaling Technologies), rabbit polyclonal phospho p70-S6K T424/S421 (1:1000; Cell Signaling Technologies), rabbit monoclonal mTOR antibody (1:1000; Cell Signaling Technologies), mouse monoclonal Rac1 antibody (1:500; Cytoskeleton Inc), rabbit polyclonal Anillin antibody (1:1000; Bethyl Laboratories), mouse monoclonal F-actin antibody (Novus Biologicals), rabbit polyclonal Cleaved Caspase 3 antibody (1:1000; Cell Signaling Technologies), rabbit polyclonal Caspase 3 antibody (1:1000; Cell Signaling Technologies), rabbit monoclonal GSK3β (1:1000 Cell Signaling Technologies), rabbit monoclonal phospho-S9 GSK3β (1:1000 Cell Signaling Technologies), mouse monoclonal Stat3 antibody (1:1000 Cell Signaling Technologies), rabbit phospho-S727 antibody (1:1000 Cell Signaling Technologies), mouse monoclonal CHOP antibody (1:500 Cell Signaling Technologies), and mouse monoclonal β-actin antibody (1:3000; Sigma-Aldrich).

Rac1 and RhoA Activity Assays: GTP-bound Rac1 and RhoA were analyzed using PAK and RBD bead assays (Cytoskeleton Inc). Conditionally immortalized podocytes were grown to 80% confluence, washed twice in ice cold PBS and lysed on ice for 15 minutes

with 600 ul of ice cold lysis buffer supplemented with phosphatase inhibitor. Lysate was collected using a cell scraper and spun at 10,000 x G at 4 degrees Celsius for 3 mins and 30 ul of lysate was set apart to analyze for total Rac1 or RhoA expression. The remaining lysate was applied to 15mg of PAK or RBD bound beads for 1 hour in rotation at 4 degrees Celsius before 2 washes with 500ul Wash Buffer with centrifugation at 5000 X G at 4 degrees. 25ul of 2X SDS buffer was applied to the washed beads before boiling for 2 mins and running them on a gel for immunoblotting with Rac1 or RhoA monoclonal antibodies (1:500; Cytoskeleton Inc).

Targeted Inhibition: For inhibition experiments, stock inhibitors were made in DMSO and diluted 1:1000 in media to reach final working concentrations of 100nM Wortmannin (Calbiotech), 1.5uM MK-2206 (Selleckchem), 5uM KU-0063794 (Selleckchem), 100nM Rapamycin (Selleckchem), 80uM NSC-23766 (Tocris), 1µM FK-506 (Tacrolimus).

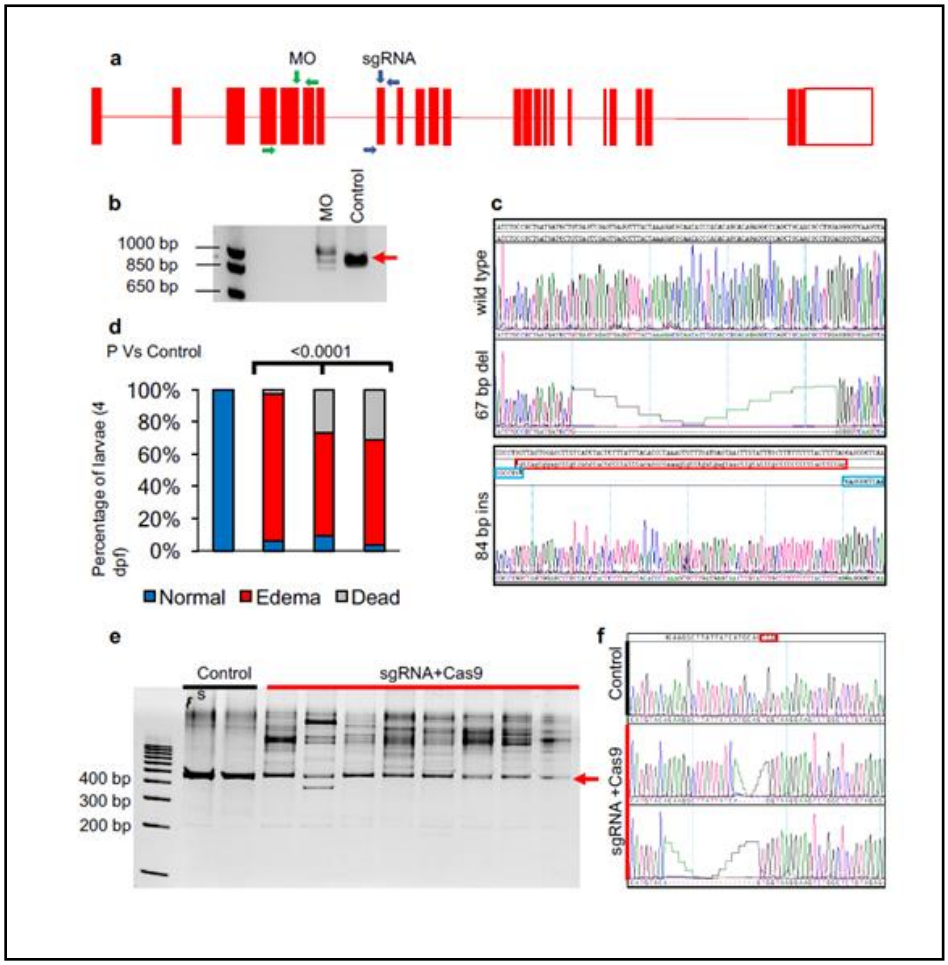
Migration Assay: Stably transfected immortalized podocytes expressing *tGFP, $ANLN_{WT}$* or *$ANLN_{R431C}$* were grown to confluence on collagen coated 6 well plates. Cell monolayers were washed and scratch wounds were applied using a 1000-*µ*l pipet tip before removing media and applying new media. Podocytes were imaged using an EVOS microscope at time 0 immediately after wound creation. Cells were then returned to growth restrictive conditions for 24 hours before final imaging of wound healing. 2 mls of media containing inhibitors or DMSO controls were added to each well (6 wells per inhibitor for each cell line repeated in triplicate) 1 hr prior to scratch wound and then fresh inhibitor media was applied post scratch wound.

Proliferation Assay: Stably transfected immortalized podocytes expressing *tGFP,* $ANLN_{WT}$ or $ANLN_{R431C}$ were grown to 80% confluence as described[4] and then transferred to collagen coated 96 well plates at 5,000 cells per well in 100ul of media and grown at growth permissive temperatures. 12 replicate wells were used for each sample per time-point. 10 ul of a cell counting colorimetric assay (Dojindo Molecular Technologies, Inc. Cell Counting Kit-8, Rockville, MD, USA) colorimetric assay) was added to each sample according to manufacturer protocol at time-points of 0 hrs, 3hrs, 24hrs, and 48hrs before reading on a Tecan Infinite 200 PRO microplate reader (Switzerland). For the 48-time point reading, fresh inhibitor or control media as re-applied to the cells after 24hrs. All experiments were repeated in triplicate.

Apoptosis Assay: Stably transfected immortalized podocytes expressing *tGFP,* $ANLN_{WT}$ or $ANLN_{R431C}$ were grown at growth permissive temperatures as described previously[4] to 80% confluency. 200,000 cells were plated on 6-well plates in triplicate for each sample and allowed to grow to 80% confluency. The cells were then given serum free media and moved to growth restrictive temperatures for 48hrs before harvesting. Annexin V and 7-AAD staining (BD Annexin V Apoptosis Detection Kit) was applied to the cells according to established protocols before flow cytometry analysis (BD FACSCalibur). The apoptosis assay was individually repeated 4 times for each cell line.

Statistical Analyses: Zebrafish larval batches were compared using χ^2 tests (GraphPad software). All *in vitro* data are represented as the mean±SEM. Group differences were

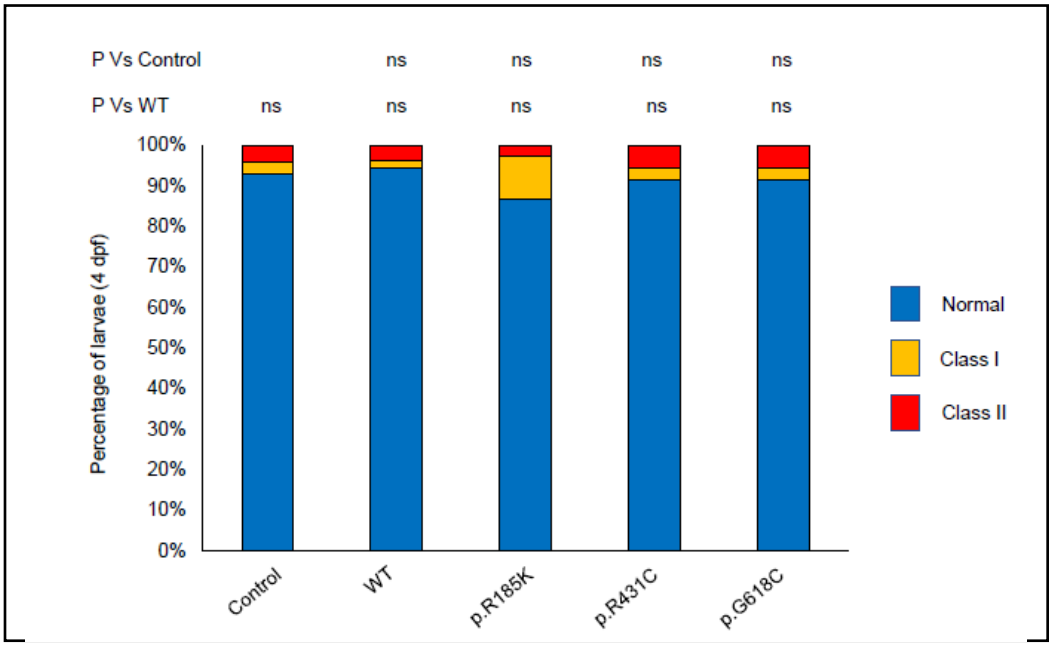
assessed by the *t* test with unequal variances. One-way analysis of variance (ANOVA) followed by a Tukey HSD post hoc test was used to determine the differences between means where there are three or more groups. Statistical significance was established at $P<0.05$.



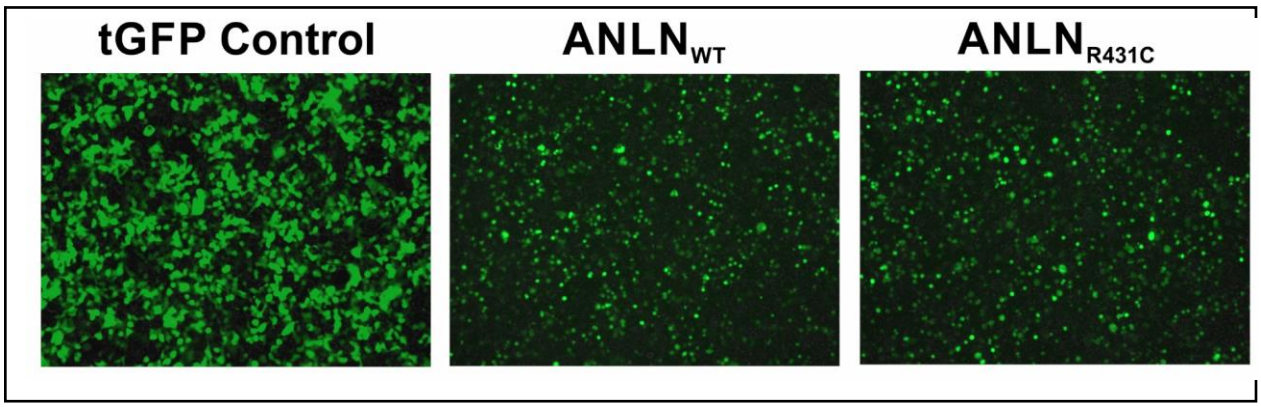
Supplementary Figure 1: Validation of transient suppression and CRISPR/Cas9 genome editing reagents targeting *anln* in zebrafish.

(**a**) Schematic of zebrafish *anln* locus (Ensembl ID: ENSDART00000113574.3, GRCz10). Red boxes, coding exons; white boxes, untranslated regions. Vertical green arrow indicates position of MO, horizontal green arrows correspond to primers flanking MO targeted region for RT-PCR shown in (b). Vertical blue arrow indicates position of sgRNA, horizontal blue arrows correspond to primers flanking sgRNA targeting region for heteroduplex analysis shown in (e). (**b**) Agarose gel image shows RT-PCR products amplified around the *anln* target region in uninjected control embryos and *anln* morphants. Red arrow indicates wild type *anln* product; upper band, middle band and lower band in MO indicate 84 bp insertion, wild-type and 67 bp deletion, respectively. (**c**) Chromatograms indicate aberrant splicing events in *anln* morphants. Top, wild type;

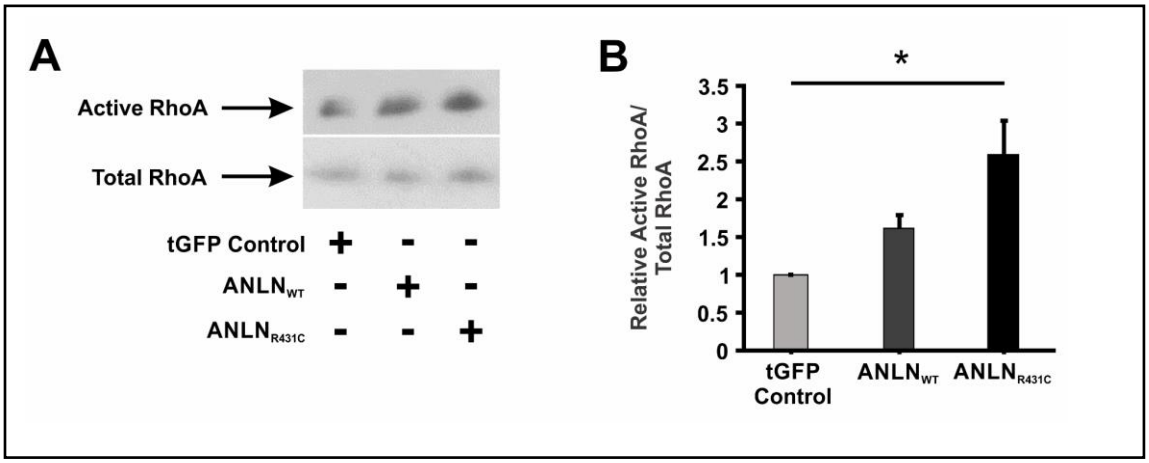
center, partial excision of exon 5 (67 bp); bottom, inclusion of intron 5-6 (84 bp). (**d**) *anln* e5i5 MO dose curve. Zebrafish embryos at the 1-4 cell stage were injected with progressively increasing doses of MO (3 ng, 6 ng and 9 ng); larvae were scored qualitatively based on edema phenotype and mortality at 4 dpf; see Figure 1b for representative images. Statistical calculations were performed using a χ^2 test; ns, not significant (n=63-81 per batch, repeated). (**e**) Heteroduplex analysis of uninjected controls and *anln* F0 mosaic mutants. DNA was extracted at 2 dpf and the targeted region was PCR-amplified, denatured and reannealed slowly, then migrated on a 20% polyacrylamide gel. Red arrow indicates the WT *anln* product. (**f**) Representative chromatograms of PCR products from uninjected control embryos and *anln* F0 mutants indicating insertion deletion events in F0 mutants; sgRNA target, top; protospacer adjacent motif (PAM), red box.



Supplementary Figure 2: Overexpression of *ANLN* mRNA in zebrafish.
Zebrafish larvae injected with 150 pg *ANLN* mRNA were scored live for edema phenotypes based on objective criteria (see Figure 1b). Statistical calculations were performed using a χ^2 test; ns, not significant; n=53-75 per batch, repeated.

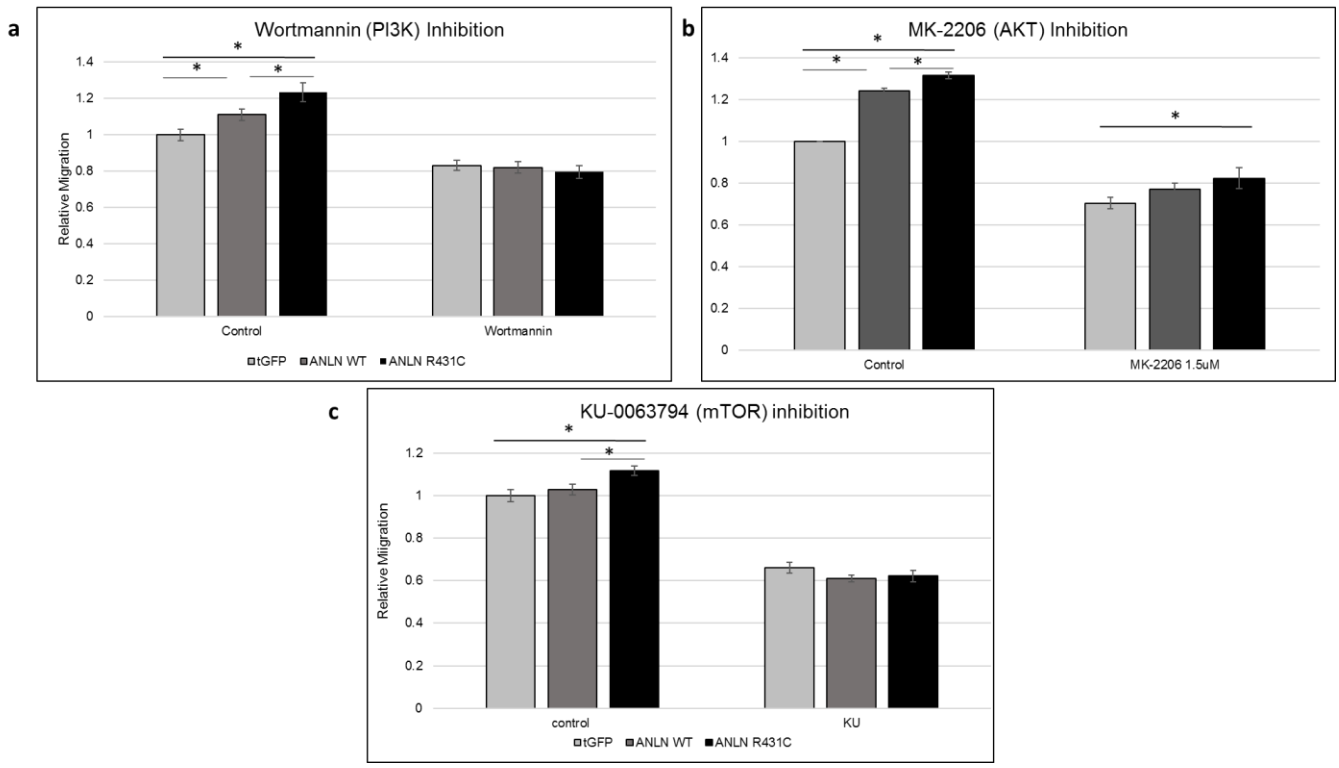


Supplementary Figure 3: Transient transfection of HEK 293 cell lines showed similar expression of ANLN between *tGFP-ANLN$_{WT}$* and *tGFP-ANLN$_{R431C}$* cell lines.

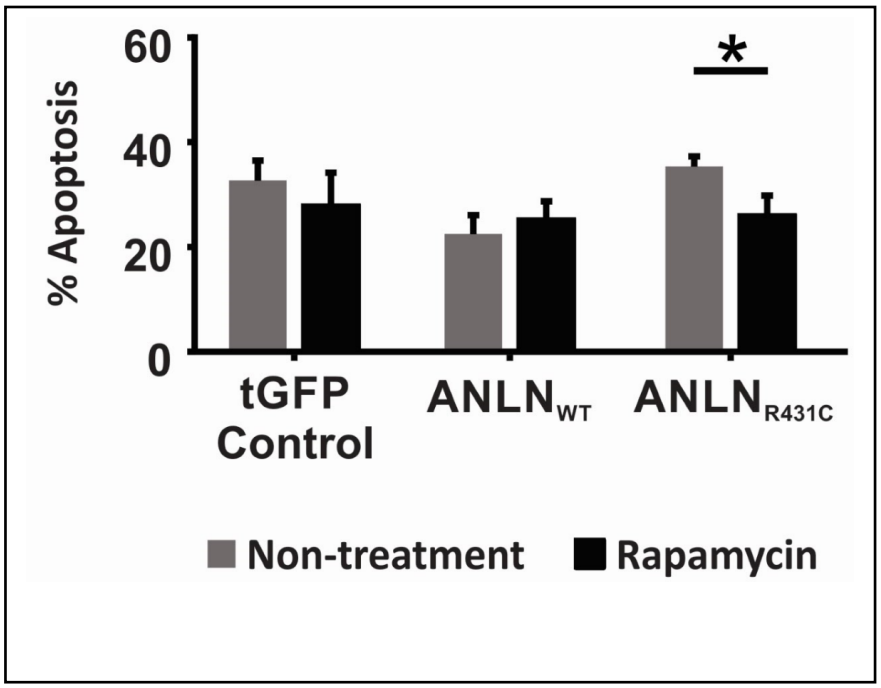


Supplementary Figure 4: Overexpression of *ANLN$_{R431C}$* *does not* alter podocyte RhoA Activity *in vitro:*

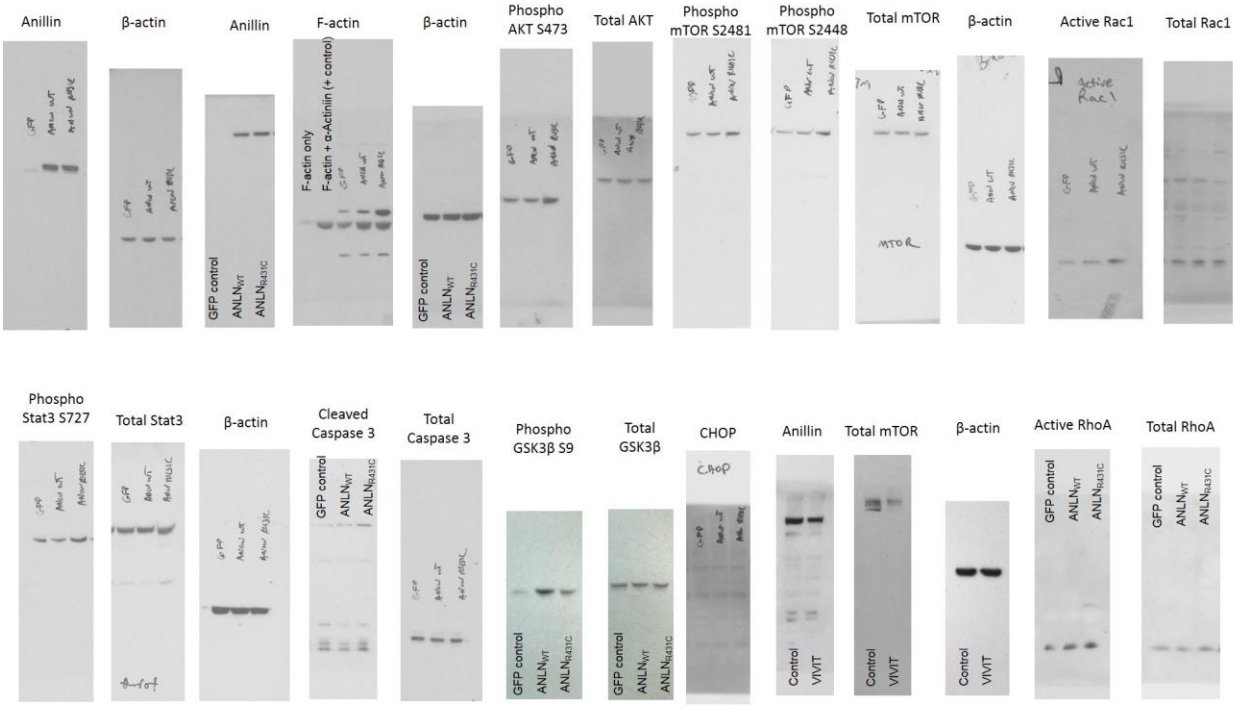
Conditionally immortalized podocyte cell lines were examined for RhoA activity using an RBD bead pulldown assay. **(A)** Representative immunoblots for GTP bound RhoA and total RhoA in control, *ANLN$_{WT}$* and *ANLN$_{R431C}$* cells. **(B)** Quantification of 3 independent assay results revealed no significant difference between *ANLN$_{WT}$* and *ANLN$_{R431C}$* cells (p=0.107), but there was an increase in both cells lines when compared to control (p=0.025 and 0.022 respectively)



Supplementary Figure 5: Inhibition of components of the PI3K/AKT/mTOR eliminates the increased migration induced by *ANLN$_{R431C}$* mutation. (a) Inhibition of PI3K signaling through treatment with 100nM Wortmannin (p=0.599). **(b)** Inhibition of AKT signaling through treatment with 1.5 uM MK-2206 (p=0.46). **(c)** Inhibition of both mTOR complexes through use of 5uM KU-0063794 (p=0.76). *p<0.05



Supplementary Figure 6: Rapamycin ameliorates serum starvation induced apoptosis in *ANLN$_{R431C}$* podocytes. FACS analysis of Annexin V stained, serum starved tGFP, *ANLN$_{WT}$* and *ANLN$_{R431C}$* expressing podocytes treated with Rapamycin or vehicle control. Analysis revealed a significant decrease in apoptosis in Rapamycin treated *ANLN$_{R431C}$* expressing podocytes when compared to tGFP controls and *ANLN$_{WT}$* expressing podocytes.



Supplementary Figure 7: Uncropped original Western Blot images

Shown in order of appearance in publication. From left to right, Top Row: Anillin (~130 kDa), β-actin (~45 kDa), Anillin (~130 kDa), F-actin (~37 kDa + 50 kDa), β-actin (~45 kDa), Phospho AKT S473 (~60 kDa), Total AKT (~60 kDa), Phospho mTOR S2481 (~250 kDa), Phospho mTOR S2448 (~250 kDa), Total mTOR (~250 kDa), β-actin (~45 kDa), Active Rac1 (~20 kDa), Total Rac1 (~20 kDa). Bottom Row: Phospho Stat3 S727 (~85 kDa), Total Stat3 (~85 kDa), β-actin (~45 kDa), Cleaved Capase 3 (~19 kDA and 17 kDa), Total Caspase 3 (~35 kDa), Phospho GSK3β S9 (~50 kDa), Total GSK3β S9 (~50 kDa), CHOP (~28 kDa), Anillin (~130 kDa), Total mTOR (~250 kDa), β-actin (~45 kDa), Active RhoA (~20 kDa), Total RhoA (~20 kDa)

References

1. Niederriter AR, Davis EE, Golzio C, Oh EC, Tsai I-C, Katsanis N: In vivo modeling of the morbid human genome using Danio rerio. *J. Vis. Exp. JoVE* e50338, 2013

2. Labun K, Montague TG, Gagnon JA, Thyme SB, Valen E: CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Res.* 44: W272-276, 2016

3. Küry S, Besnard T, Ebstein F, Khan TN, Gambin T, Douglas J, et al: De Novo Disruption of the Proteasome Regulatory Subunit PSMD12 Causes a Syndromic Neurodevelopmental Disorder. *Am. J. Hum. Genet.* 100: 689, 2017

4. Hall G, Gbadegesin RA, Lavin P, Wu G, Liu Y, Oh EC, et al: A novel missense mutation of Wilms' Tumor 1 causes autosomal dominant FSGS. *J. Am. Soc. Nephrol. JASN* 26: 831–843, 2015

5. Gbadegesin RA, Hall G, Adeyemo A, Hanke N, Tossidou I, Burchette J, et al: Mutations in the gene that encodes the F-actin binding protein anillin cause FSGS. *J. Am. Soc. Nephrol. JASN* 25: 1991–2002, 2014

6. Hall G, Lane B, Chryst-Ladd M, Wu G, Lin J-J, Qin X, et al: Dysregulation of WT1 (-KTS) is Associated with the Kidney-Specific Effects of the LMX1B R246Q Mutation. *Sci. Rep.* 7: 39933, 2017