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Mutations in KIRREL1, a slit diaphragm component, cause steroid-resistant nephrotic syndrome.

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Authors contributions

E.W., A.D., H.H., M.N., N.M., A.J.M., W.T., H.Y.G., C.S., S.S., S.M., R.P.L. and F.H. generated total genome linkage data, performed exome capture with massively parallel sequencing, and performed whole exome evaluation and mutation analysis.

E.W., A.D., H.H., M.N., N.M., A.J.M., W.T., H.Y.G., C.S., C.R., R.B-C., C.B., M.S., S.S., T.H. and F.H. recruited patients and gathered detailed clinical information for the study. C.R., R.B-C. and S.R. provided images, analyzed renal histology and electron microscopy.

A.K.S., E.W., E.A., P.S., S.S., S-H.K., H.H., H.Y.G. carried out the cell experiments. F.H. and D.N. conceived of and directed the project.

A.K.S. and E.W. wrote the paper with help from F.H. and D.N. The manuscript was critically reviewed by all of the authors.

Disclosure

F.H. is a cofounder of Goldfinch-Bio. No other authors have competing financial interests. C.B. is an employee of Bioscientia.

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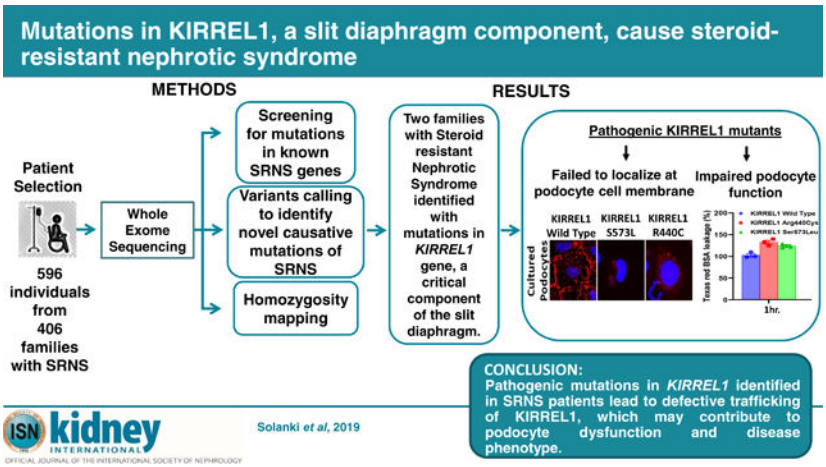
Abstract

Steroid-resistant nephrotic syndrome is a frequent cause of chronic kidney disease almost inevitably progressing to end-stage renal disease. More than 58 monogenic causes of steroid-resistant nephrotic syndrome have been discovered and majority of known steroid-resistant nephrotic syndrome-causing genes are predominantly expressed in glomerular podocytes, placing them at the center of disease pathogenesis. Herein, we describe two unrelated families with steroid-resistant nephrotic syndrome with homozygous mutations in the *KIRREL1* gene. One mutation showed high frequency in the European population (minor allele frequency 0.0011) and this patient achieved complete remission following treatment, but later progressed to chronic kidney disease. We found that mutant *KIRREL1* proteins failed to localize to the podocyte cell membrane, indicating defective trafficking and impaired podocytes function. Thus, the *KIRREL1* gene product has an important role in modulating the integrity of the slit diaphragm and maintaining glomerular filtration function

Graphical Abstract



Keywords

KIRREL1; FSGS; MCD; steroid-resistant nephrotic syndrome (SRNS)

INTRODUCTION

Steroid-resistant nephrotic syndrome (SRNS) represents the second most frequent cause of CKD in the first 3 decades of life[1]. It is characterized by edema, nephrotic-range proteinuria, hyperlipidemia and its histological hallmarks, focal segmental glomerular sclerosis (FSGS). Although there have been advancements in treatment regimens no drug directly targets any of the primary causes of SRNS[2]. Currently, there are more than 58 genes that, if mutated, cause SRNS in a monogenic way[3]. Most of them are relevantly expressed in the glomerular visceral epithelial cell, the podocyte, placing it at the center of the pathogenesis of SRNS[4]. Podocytes are terminally differentiated cells that maintain glomerular filtration barrier. Foot processes of neighboring podocytes form a specialized intercellular junction, known as the slit diaphragm, which is essential for the filtration function of the kidney[5]. The

pathophysiology of nephrotic syndrome is characterized by structural alteration of the cytoskeleton and molecular reorganization of slit diaphragm components leading to foot process effacement[6]. The first monogenic cause of SRNS in humans was discovered in *NPHS1*, an essential and major constituent of the slit diaphragm that is involved in forming glomerular filter via its homophilic or hetherophilic extracellular domain interaction[7]. Subsequently, several other monogenic causes in genes associated with slit diaphragm and actin cytoskeleton were discovered, emphasizing an essential role of this structure[3]. KIRREL1 has been described as a NPHS1-like Ig superfamily cell adhesion molecule[8]. Deletion of *Kirrel* in a mouse model results in podocyte foot processes effacement phenotype causing loss of glomerular filtration function[9]. A direct interaction between NPHS1 and KIRREL1 as well as their co-localization at slit diaphragm were described[10]. KIRREL1 is involved in rearrangement of actin cytoskeleton as a signaling mediator, emphasizing its essential role in organization and functional assembly of slit diaphragm[11]. Evidence suggest that in response to glomerular injury, NPHS1 and KIRREL1 trigger a signaling cascade that initiates downstream signaling and trafficking events, leading to morphological (effacement) and functional (proteinuria) changes in podocytes[12]. It has also been shown that injury to podocytes observed in various glomerular diseases is associated with mislocalization and displacement of KIRREL1 and NPHS1 from the podocyte cell membrane leading to podocyte damage[13]. The signaling events initiated by these proteins in response to injury govern the subsequent morphological and phenotypic changes in podocytes. Collectively, these observations suggest that a specific pathogenic link between SRNS development and alteration in KIRREL1 exists, indicating that similar to NPHS1, mutations in KIRREL1 may have pathological effects leading to renal dysfunction. Human mutations in *KIRREL1* causing SRNS have been not reported to date.

Performing whole exome sequencing and targeted exon sequencing, we here identify mutations in *KIRREL1* gene from two unrelated families with a history of SRNS. Using different *in vitro* functional assays we demonstrate that these mutations result in altered trafficking and mislocalization of KIRREL1 protein as well as affected integrity of slit diaphragm.

RESULTS

Mutations in *KIRREL1* in 2 patients with nephrotic syndrome

To identify novel monogenic causes of SRNS, we performed whole-exome sequencing[14] (WES) and homozygosity mapping[15] in a cohort of 596 individuals from 406 families with SRNS. We first evaluated this data set for mutations in 33 known SRNS causing genes[16]. We discovered two homozygous missense mutations (p.Arg440Cys and p.Ser573Leu) of the *KIRREL1* gene in two individuals of unrelated consanguineous families (A666 and B742) with SRNS. So the frequency of this gene would be (2/406), which is 0.4 % emphasizing its rarity as a monogenetic cause in a highly preselected cohort with SRNS. The mutated alleles causing mutations p.Arg440Cys and p.Ser573Leu appear to be very rare based on the reference data base gnomAD with a minor allele frequency (MAF) of 3.97×10^{-6} and a MAF of 7.87×10^{-4} respectively (Fig.1, Suppl.Fig.1, Suppl.Table.1 and Suppl.Table.2). Notably, the p.Ser573Leu, identified in a child of Italian descent and found mutated at the

homozygous state in one control subject in GnomAD, has an allele frequency of 0.0011 in European populations, which is very frequent. The respective amino acid residues are conserved to *D.rerio* for both mutations (Fig.1B and C, Suppl.Table.1 and Suppl.Figure.2), and mutations are located within a homozygosity peak (Fig.1D and E). Renal histology in both patients was characterized by low-grade mesangial hyper-cellularity (Fig.1F and G). Ultrastructural analysis yielded extensive foot process effacement, increased mesangial matrix and mesangial proliferation (Fig.1H).

Treatment response and renal outcome in both families with *KIRREL1* mutations

Both patients with detected homozygous mutations in *KIRREL1* shared the phenotype of SRNS. There were no extrarenal manifestations or syndromic features in both affected children. Nevertheless, both affected individuals varied remarkably regarding the response to treatment course and the outcome of the renal function.

The patient A666_21 was diagnosed with NS at age of 5 years. He developed SRNS at his initial presentation and was resistant to different immunosuppressive drugs. He has never achieved a complete remission and remained having undulating proteinuria with partial response to treatment. Additional treatment with ACE-I and ARB was not beneficial for the patient. Nevertheless, his renal function remained normal all over the time, so he stopped his clinical visits after 12.5 years of follow up. Interestingly, this patient has a younger sister with reported periorbital edema and proteinuria as well as extended relatives with three children with ESRD and one child with nephrotic syndrome. However, neither the genotype of his sister nor the genotypes from his relatives are known because DNA samples for genetic analysis could not be obtained (Suppl. Table 1).

The patient B742_21 was diagnosed with NS at age of 14 years. He developed SRNS at his initial presentation. Throughout he was started on tacrolimus with weaning off of corticosteroids. He was additionally treated with CCB and ACE-I for his high blood pressure and proteinuria. During the course of treatment with Tacrolimus, CCB and gradual dose increase of ACE-I the patient succeeded a complete remission. However, over 4 years of follow up, he developed chronic kidney disease, which has continued to progress attaining CKD stage 2 at the time of this publication (Suppl.Table.1).

KIRREL1 mutations lead to mislocalization in cultured human podocytes

We have previously demonstrated, that KIRREL1 primarily localizes at the podocyte cell membrane and injury induces its mislocalization[17]. Since *in silico* analysis suggested, that the mutations may increase protein instability, we hypothesized, that this may induce mislocalization of these mutant proteins. To test this hypothesis, we generated cDNA constructs containing patient's mutant alleles with inserted Flag tag at the N-terminus (Fig. 2A). Stably transfected podocyte cell lines expressing these mutant proteins were generated, the expression was confirmed by western blot (Fig.2B). The localization of these mutant proteins and KIRREL1 wild type was analyzed by immunofluorescence microscopy using anti-Flag antibody. In comparison to the KIRREL1 wild type, a mislocalization of both KIRREL1 mutants (Arg440Cys and Ser573Leu) was noted (Fig.2C). The subcellular protein fractionation analysis of these cell lines further confirmed, that in comparison to KIRREL1

wild type protein, the mutant KIRREL1 proteins were present in the membrane fraction at a significant low level.(Suppl.Fig.3A and B). Further, the *KIRREL1* mutations did not affect membrane localization of NPHS1 (Suppl.Fig.4A and B and Suppl.Fig.5A and B).

To narrow down the subcellular localization of mutant KIRREL1 proteins we investigated their co-localization with the markers of early endosomes (Rab5a), late endosomes (Rab7a), Golgi complex (N-acetylgalactosaminyltransferase), and lysosomes (Lamp1). Both KIRREL1 mutants showed increased localization at Golgi apparatus, early endosomes and lysosomes (Suppl.Fig.6A, left panels 1, 2, 3 and 6B). In comparison, KIRREL1 wild type protein was predominantly localized at the cell membrane (Suppl.Fig.3A). No significant differences were noted in the localization of KIRREL wild type and mutant proteins in late endosomes (Suppl.Fig.6A, **left panel 4**). These findings suggest that the *KIRREL1* mutations induce trafficking defects, which allows the retention of mutant proteins in subcellular organelles.

KIRREL1 Mutations induce loss of transepithelial permeability in cultured human podocytes

Since KIRREL1 mutants failed to localize at cell membrane, we hypothesized that this may affect integrity of cell-cell adhesions and transcellular permeability. To assess these changes, we performed a transepithelial permeability assay, where permeability of Texas Red-labeled albumin was measured across the stable podocyte monolayer expressing either KIRREL1 wild type or KIRREL1 mutant proteins. The respective stable podocytes were grown on transwell filters and analyzed for Albumin influx across the podocytes monolayer. In comparison to cells expressing KIRREL1 wild type protein, a significant increase in albumin flux (an indicator of increase in transepithelial permeability) was noted within 1 hour (p <0.01), with a further increase at 4 hours (p< 0.001) (Suppl.Fig.7). Our results conclude that *KIRREL1* mutations adversely affect the podocyte cell junctions and intercellular integrity.

DISCUSSION

In this report, we have identified and characterized two novel recessive mutations in *KIRREL1* gene in two patients with SRNS of 2 unrelated consanguineous families in well conserved amino acid residues indicating the physiological significance of these regions. It was interesting to note that the p.Ser573Leu mutation appeared very frequent in European population with MAF 0.0011 and was found mutated at the homozygous state in one control subject in GnomAD being even slightly more common than the mutation of most common gene (*NPHS2*; p.Arg138Gln)[18]. However it is to be noted that a pathogenic variant is by definition a variant, which contributes mechanistically to disease, but is not necessarily fully penetrant (i.e., may not be sufficient in isolation to cause disease) and thus, there is a possibility of variable expressivity or incomplete penetrance for this variant (MacArthur et al., Nature 2014; 469). Further, as per our knowledge, the gnomAD data base is a reference of healthy individuals; although there is still not a 100% guarantee for it, since those individuals haven’t been screened on a standard procedure, leaving a possibility of including false negative subjects. However, the *in silico* predicting scores, good conservation and the functional data for p.Ser573Leu variant, are conclusive with its functional impact.

Interestingly, although the patient with this mutation achieved complete remission upon treatments with Tacrolimus, CCB and ACE-I, however a later follow up showed progression to CKD. We further, demonstrated that stable expression of KIRREL1 protein containing human mutations in human podocyte cell lines leads to its mislocalization and accumulation in early endosomes, Golgi apparatus, and lysosomes indicating impaired protein trafficking. Additionally, our findings conclude that mislocalization of KIRREL1 alters podocyte cell junctions and leads to impaired intercellular integrity.

It is likely that the differences in the pathophysiology of these mutations are due to the functional differences of these regions. While the extracellular domain of KIRREL1 is involved in interaction with slit membrane protein NPHS1 ensuring structural integrity and filtering function of slit diaphragm[19], the intracellular domain has been shown to be actively involved in signaling events that define actin cytoskeleton organization and modulation[11]. The redistribution of NPHS1 and KIRREL1 from the podocyte cell membrane to the subcellular compartments is strongly associated with the initiation of podocyte dysfunction and eventual loss of glomerular filtration function[20,21]. It was interesting to note that both mutations induced protein mislocalization, where its ability to localize at the podocyte cell membrane was severely diminished indicating affected protein trafficking. It is likely that these mutant proteins are transported to the podocyte cell membrane but due to their increased instability, they are removed and remain trapped in early endosomes which serve as sorting protein recycling station enabling their restoring back to plasma membrane[22]. We have previously shown that mislocalization of KIRREL1 is associated with increased cellular permeability and loss of intercellular junctions[13]. Since these mutant proteins phenocopied that effect, it is likely that this mislocalization may contribute to the disease phenotype. It is difficult to predict which cellular pathway is exactly affected due to these mutations and will need further detailed investigation. Although the follow up studies are required to investigate detailed signaling and trafficking events affected by these mutations. Nevertheless, these novel mutations highlight the biological importance of *KIRREL1* in podocytes homeostasis.

In conclusion, this is the first report describing mutations in human *KIRREL1* that results in SRNS emphasizing its important role in modulating the integrity of slit diaphragm and maintaining glomerular filtration function. The fact that one patient has responded to Tacrolimus, CCB and ACE-I treatment cannot be generalized at this point with regards to other patients with *KIRREL1* mutations that may be discovered in the future, because we feel that it is not justified to draw conclusions on genotype-treatment relationships based on a single case. To study the detailed pathophysiology of these mutations, further studies, especially construction of Knock-in mouse model, will be needed.

Short Methods:

Study Approval and participants

Approval for human subject research was obtained from the University of Michigan and the Boston Children's Hospital Institutional Review Boards. Patients were enrolled between 1998 and 2016. We performed whole exome sequencing in 596 individuals from 406 families. Patients were recruited in collaboratingon with centers worldwide and were

ethnically diverse. Individuals of consanguineous kindred were preferentially sent for whole exome sequencing. All participants or their guardians provided written informed consent. Following informed consent, we obtained pedigree information, clinical data, and blood samples from individuals with proteinuria or nephrotic syndrome (NS) (onset < 25 years of age). Clinical data and blood samples were obtained from individuals with NS or their guardians. Clinical data were obtained using an established questionnaire (http://www.renalgenes.org). The diagnosis of NS was made by (pediatric) nephrologists, on the basis of standardized clinical and renal histologic criteria. Renal biopsy samples were evaluated by renal pathologists. Treatment response to immunosuppressive therapy in NS was defined as follows:

1. Complete response: remission of proteinuria, with a urine protein to creatinine ratio of <0.2 mg/mg, normalization of albumin.
2. Partial response: decrease in proteinuria by 50%.
3. Resistance: persistent, fixed proteinuria.

In cases with complete response, the absence of proteinuria has been confirmed in laboratory values of negative proteinuria either in 24-hours urine specimen or in the first morning urine specimen.

Whole exome sequencing

Whole exome sequencing was performed on genomic DNA isolated from blood lymphocytes or saliva and subjected to library preparation and exome capture using Agilent SureSelect™ human exome capture arrays (Life technologies™) followed by next-generation sequencing on a HiSeq Illumina sequencing platform. Sequence reads were mapped to the human reference genome (NCBI build 37/hg19) using CLC Genomics Workbench™ (version 6.5.2; CLC bio, Aarhus, Denmark). Subsequent bioinformatics analysis excluded all variants with minor allele frequencies greater than 1% in the dbSNP database (version 142). Non-coding variants outside canonical splice side regions as well as synonymous variants were excluded, and variants were annotated using the ExAC database. Variants that were reported more than 10 times in the homozygous state in the ExAC database were excluded as likely non-pathogenic polymorphisms. Remaining variants were ranked based on established criteria[3,23,24] and were evaluated for potential pathogenicity under consideration of phenotypic as well as pedigree information.

Cell culture and generation of podocytes overexpressing KIRREL1 and mutants

The immortalized human podocyte cell line was obtained from Dr. Moin Saleem[25] and the cells were cultured in RPMI 1640-based medium supplemented with 10% FBS (Invitrogen), 2 g/liter of sodium bicarbonate ($NaHCO_3$), insulin-transferrin-selenium supplement (Sigma-Aldrich), and 200 U/ml of penicillin and streptomycin (Invitrogen). Retroviruses overexpressing KIRREL1 and its mutants were generated by the transfection of the respective plasmids, cloned into the pBABE vector into Phoenix cells according to the manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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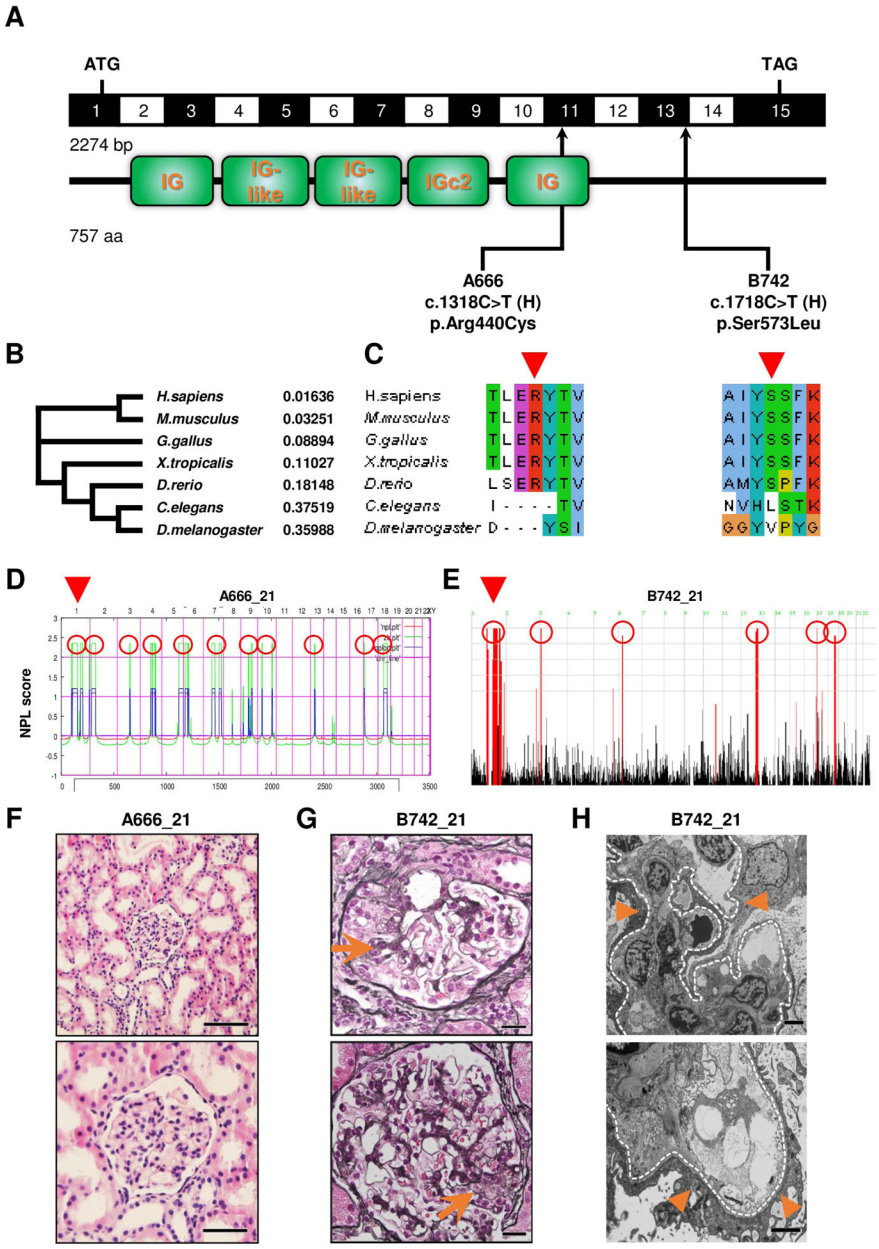

Figure 1. Homozygosity mapping and whole exome sequencing identify recessive mutations of *KIRREL1* in 2 families with steroid-resistant nephrotic syndrome

(**A**) *KIRREL1* (NM_018240.6/NP_060710.3). Exon structure of human cDNA with the corresponding protein including its functional domains obtained from SMART. Positions of start codons and of stop codon are indicated. Arrows indicate positions of pathogenic mutations detected in families with SRNS. H, homozygous. (**B**) Phylogram of KIRREL1 protein was generated using multiple sequence alignment in Clustal omega. Numbers are representing the percent identity graph data comparing evolutionary relationship among different organisms to human reference for KIRREL1 protein (0: 100% identical; 1: 0% identical). (**C**) Evolutionary conservation of amino acid residues that are altered in patients with SRNS. Altered amino acid residues of KIRREL1 (p.Arg440Cys, p.Ser573Leu). (**D**)

Homozygosity mapping identifies regions of homozygosity with recessive candidate loci (red circles) in patient A666_21. Nonparametric LOD (NPL) scores and SNP positions (Affymetrix 250K StyI array) are plotted on human chromosomes concatenated from p-ter (left) to q-ter (right). Genetic distance is given in centimorgans (cM). Whole exome sequencing identifies a homozygous mutation of *KIRREL1* (p.Arg440Cys) that is positioned within the maximum NPL peak on chromosome 1 (arrowhead). (**E**) Homozygosity mapping identifies regions of homozygosity with recessive candidate loci (red circles) in patient B742_21. Nonparametric LOD (log of the odds ratio) (NPL) score profile is plotted across the human genome. Within the maximum NPL peak on chromosome 1 (arrowhead), we identified a homozygous mutation in *KIRREL1* (p.Ser573Leu). (**F**) Renal histology of individual A666_21 with *KIRREL1* mutation. Presence of only minor findings of mesangial hypercellularity is consistent with minimal change disease on light microscopy (H&E staining). (Scale bars: upper panel 200 μm and lower panel 100 μm). (**G**) Renal histology of individual B742_21 with *KIRREL1* mutation shows focal segmental glomerular sclerosis (arrows) on light microscopy (MPAS staining). (Scale bars: upper and lower panels 20 μm). (**H**) Renal histology of B742_21 with *KIRREL1* mutation showing podocyte foot process effacement (arrowheads) on transmission electron microscopy (TEM). Glomerular basement membrane is highlighted by a dotted line. (Scale bars: upper and lower panels 2 μm).

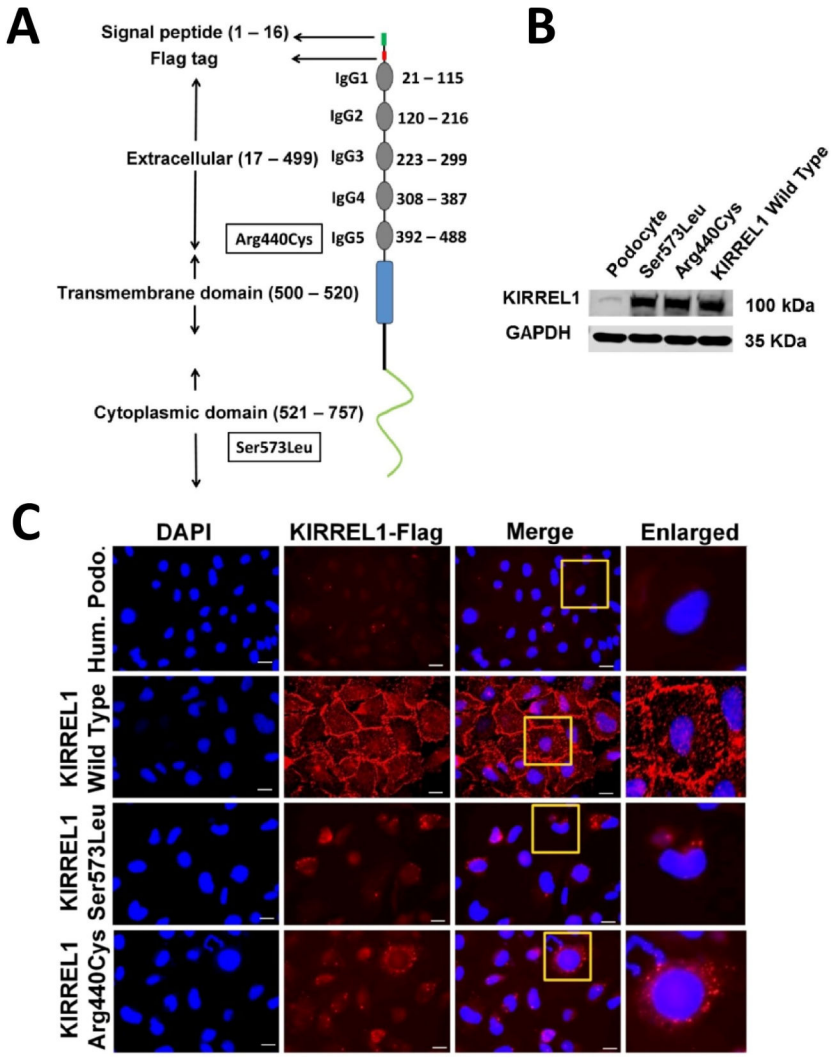

Figure 2. KIRREL1 mutants mislocalize in human podocytes.

(**A**) Schematic domain distribution, position of Flag tag and mutations in the KIRREL1 protein. (**B**) Western blot analysis showing the expression of the wild type control and KIRREL1 mutants plasmid constructs as indicated. (**C**) Unpermeabilized wild type human podocytes, human podocytes stably expressing flag tagged wild type KIRREL1 and flag tagged KIRREL1 mutants p.Ser573Leu and p.Arg440Cys were stained with Flag antibody, that labeled Flag-KIRREL1 extracellularly. Their membrane localization was evaluated by immunofluorescence microscopy using a confocal microscope. KIRREL1 mutants p.Ser573Leu and p.Arg440Cys show mislocalization compared to wild type control in cultured human podocytes cell lines. (Scale bars: 25 μm).