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MYO1E Mutations and Childhood Familial Focal Segmental Glomerulosclerosis

Caterina Mele, Biol.Sci.D., Paraskevas Iatropoulos, M.D., Roberta Donadelli, Biol.Sci.D., Andrea Calabria, Eng.D., Ramona Maranta, Biol.Sci.D., Paola Cassis, Ph.D., Simona Buelli, Ph.D., Susanna Tomasoni, Ph.D., Rossella Piras, Chem.Pharm.D., Mira Krendel, Ph.D., Serena Bettoni, Biotech.D., Marina Morigi, Ph.D., Massimo Delledonne, Ph.D., Carmine Pecoraro, M.D., Isabella Abbate, Ph.D., Maria Rosaria Capobianchi, Ph.D., Friedhelm Hildebrandt, M.D., Edgar Otto, M.D., Franz Schaefer, M.D., Fabio Macciardi, M.D., Fatih Ozaltin, M.D., Sevinc Emre, M.D., Tulin Ibsirlioglu, Ph.D., Ariela Benigni, Ph.D., Giuseppe Remuzzi, M.D., and Marina Noris, Ph.D., for the PodoNet Consortium

ABSTRACT

BACKGROUND

Focal segmental glomerulosclerosis is a kidney disease that is manifested as the nephrotic syndrome. It is often resistant to glucocorticoid therapy and progresses to end-stage renal disease in 50 to 70% of patients. Genetic studies have shown that familial focal segmental glomerulosclerosis is a disease of the podocytes, which are major components of the glomerular filtration barrier. However, the molecular cause in over half the cases of primary focal segmental glomerulosclerosis is unknown, and effective treatments have been elusive.

METHODS

We performed whole-genome linkage analysis followed by high-throughput sequencing of the positive-linkage area in a family with autosomal recessive focal segmental glomerulosclerosis (index family) and sequenced a newly discovered gene in 52 unrelated patients with focal segmental glomerulosclerosis. Immunohistochemical studies were performed on human kidney-biopsy specimens and cultured podocytes. Expression studies in vitro were performed to characterize the functional consequences of the mutations identified.

RESULTS

We identified two mutations (A159P and Y695X) in *MYO1E*, which encodes a nonmuscle class I myosin, myosin 1E (Myo1E). The mutations in *MYO1E* segregated with focal segmental glomerulosclerosis in two independent pedigrees (the index family and Family 2). Patients were homozygous for the mutations and did not have a response to glucocorticoid therapy. Electron microscopy showed thickening and disorganization of the glomerular basement membrane. Normal expression of Myo1E was documented in control human kidney-biopsy specimens in vivo and in glomerular podocytes in vitro. Transfection studies revealed abnormal subcellular localization and function of the A159P-Myo1E mutant. The Y695X mutation causes loss of calmodulin binding and of the tail domains of Myo1E.

CONCLUSIONS

MYO1E mutations are associated with childhood-onset, glucocorticoid-resistant focal segmental glomerulosclerosis. Our data provide evidence of a role of Myo1E in podocyte function and the consequent integrity of the glomerular filtration barrier.

From the Mario Negri Institute for Pharmacological Research, Clinical Research Center for Rare Diseases, Aldo e Cele Daccò Ranica (C.M., P.I., R.D., R.M., P.C., R.P., S. Bettoni, G.R., M.N.), Mario Negri Institute for Pharmacological Research, Centro Anna Maria Astori, Science and Technology Park Kilometro Rosso (S. Buelli, S.T., M.M., A.B., G.R.), and the Unit of Nephrology and Dialysis, Ospedali Riuniti di Bergamo (G.R.) — all in Bergamo, Italy; the Department of Medicine, Surgery and Dentistry, Università degli Studi di Milano, Milan (A.C., F.M.); the Center of Functional Genomics, Department of Biotechnologies, University of Verona, Verona (M.D.), the Department of Nephrology and Dialysis, Santobono Hospital, Naples (C.P.), and the Laboratory of Virology, Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani, Rome (I.A., M.R.C.) — all in Italy; the Department of Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY (M.K.); the Department of Pediatrics (F.H., E.O.) and the Howard Hughes Medical Institute (F.H.), University of Michigan, Ann Arbor; the Pediatric Nephrology Division, Center for Pediatrics and Adolescent Medicine, Heidelberg, Germany (F.S.); and the Department of Pediatric Nephrology, Hacettepe University Faculty of Medicine, Ankara (F.O., T.I.), and the Department of Pediatric Nephrology, Istanbul Medical Faculty, Istanbul University, Istanbul (S.E.) — both in Turkey. Address reprint requests to Dr. Remuzzi at the Mario Negri Institute for Pharmacological Research, Centro Anna Maria Astori, Science and Technology Park Kilometro Rosso, Via Stezzano 87, 24126 Bergamo, Italy, or at giuseppe.remuzzi@marionegri.it.

Drs. Mele, Iatropoulos, Remuzzi, and Noris contributed equally to this article.

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Focal segmental glomerulosclerosis is a kidney lesion characterized by glomerulosclerosis that is focal (involving a subgroup of glomeruli) and segmental (involving a portion of the glomerular tuft). As the disease progresses, the pattern of glomerulosclerosis becomes predominantly global. Alterations of the podocytes, resulting in foot-process effacement, constitute the major ultrastructural abnormality. The pathological classification of focal segmental glomerulosclerosis includes collapsing, tip-lesion, cellular, and perihilar variants, as well as a type known as focal segmental glomerulosclerosis not otherwise specified.[1] Although focal segmental glomerulosclerosis may be a primary disorder, secondary cases have been associated with drug toxicity, viral infections, and diseases that affect the renal mass and glomerular hemodynamics.[2] Genetic forms of focal segmental glomerulosclerosis have been reported, some of which affect the kidney exclusively, whereas others are syndromic, with multiorgan involvement.[3-5] The genetic forms are usually glucocorticoid-resistant and often progress to end-stage renal disease.[4]

Patients with focal segmental glomerulosclerosis generally present with proteinuria, with urine protein levels often in the nephrotic range, owing to dysfunction of the glomerular filtration barrier.[6] The disorder accounts for 7 to 20% of cases of the nephrotic syndrome in children[7] and up to 35% of cases in adults,[8,9] and its incidence appears to be increasing.[7,10] An understanding of the glomerular filtration barrier is key to discussions of focal segmental glomerulosclerosis. This barrier is tripartite, consisting of a fenestrated endothelium, the glomerular basement membrane, and podocytes, which are differentiated epithelial cells with interdigitated foot processes interconnected by multiprotein slit diaphragms.[6]

Genetic studies in familial forms of focal segmental glomerulosclerosis indicate that the disorder is associated with mutations in genes that encode several podocyte proteins.[11-18] However, the molecular cause is unknown in more than 50% of patients with primary, glucocorticoid-resistant focal segmental glomerulosclerosis, and effective treatments are lacking.[3]

We performed whole-genome linkage analysis and high-throughput sequencing in a family with autosomal recessive focal segmental glomerulosclerosis and identified a missense mutation in *MYO1E*, which encodes myosin 1E (Myo1E), a nonmuscle class I myosin. Wild-type Myo1E is expressed mainly at the plasma membrane, but mutant Myo1E localizes to the podocyte cytoplasm. We also sequenced *MYO1E* in a different group of patients and identified another mutation that causes protein truncation and leads to focal segmental glomerulosclerosis. Together, these findings define the important role of Myo1E in maintaining the function of the glomerular filtration barrier in humans.

METHODS

STUDY PARTICIPANTS

The index family was from Italy and included consanguineous, unaffected parents and their three children who had the nephrotic syndrome and lesions typical of focal segmental glomerulosclerosis on kidney biopsy (Fig. 1). We also recruited unrelated patients who had either familial recessive focal segmental glomerulosclerosis (28 patients, including a patient from a family in Turkey [Family 2]), or sporadic, childhood-onset focal segmental glomerulosclerosis (24 patients younger than 13 years of age). Since mutations in the same gene may associate with either focal segmental glomerulosclerosis or diffuse mesangial sclerosis,[16] we included 38 children with the latter disorder (5 with the familial form and 33 with the sporadic form). None of the patients carried mutations in *NPHS1*, *NPHS2*, or *WT1*. DNA samples from 484 healthy white persons (382 Italian and 102 Turkish) were included as controls.

STUDY DESIGN AND ASSESSMENTS

The study was begun on October 1, 2007, and ended on March 31, 2011. Whole-genome linkage analysis was performed in the index family with the use of an array of 1 million single-nucleotide polymorphisms (SNPs). A customized-sequence capture array was used to isolate genomic DNA of the positive-linkage region, which was sequenced on a 454 Genome Sequencer FLX platform (454 Life Sciences). Localization of Myo1E in kidney specimens from controls and patients and in cultured podocytes, as well as its colocalization with other podocyte proteins, was assessed by means of immunofluorescence and immunoperoxidase staining and by means of immunogold electron microscopy. Wild-type and mutant recombinant Myo1E proteins were expressed in cultured podocytes, and their intracellular localization and effects on cytoskeletal and other podocyte proteins and on podocyte motility were evaluated. The effect of

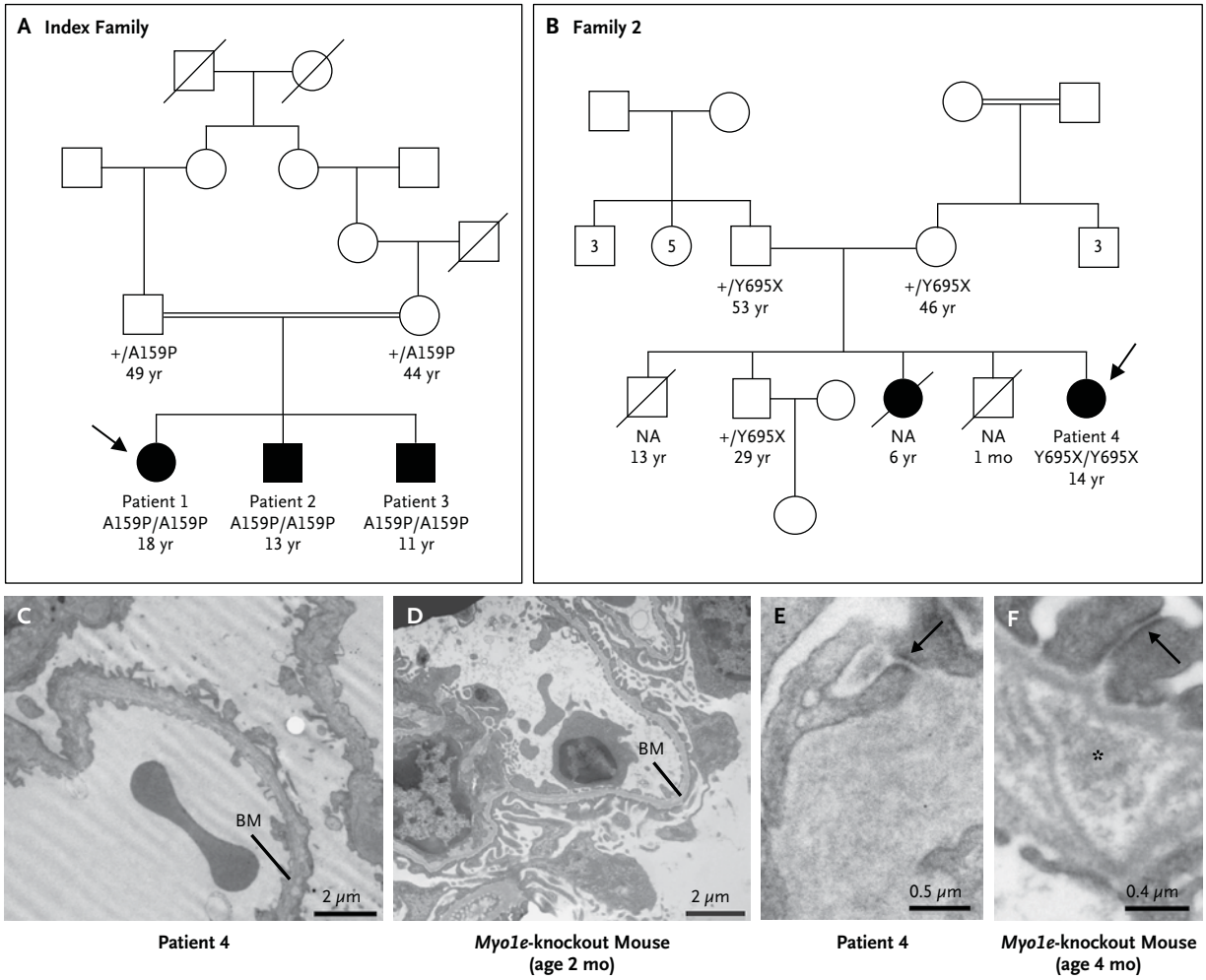


Figure 1. Pedigrees of the Families with Myosin 1E (*MYO1E*) Mutations, and Ultrastructural Changes in the Glomerular Capillary Wall.

Pedigrees of the index family (Panel A) and of Family 2 (Panel B) are shown. The arrows point to the probands, and the plus signs indicate the wild-type alleles. A159P and Y695X indicate the c.475G→C and c.2085T→G mutations in the transcript of *MYO1E*, corresponding to the amino acid changes Ala159Pro and Tyr695*, respectively. The parents in Family 2 are from the same village. Patient 4 in Family 2 was homozygous in all single-nucleotide polymorphisms in *MYO1E* (Table 5 in the Supplementary Appendix), indicating consanguinity. Ages at last observation (or at death) are shown, when available. Two deaths occurred in Family 2: one member died at 13 years of age as a result of meningitis, and the other died suddenly at 1 month of age. Squares indicate male family members, circles female family members, slashes deceased persons, solid symbols persons with focal segmental glomerulosclerosis, and double horizontal bars consanguinity. Numbers within circles and squares indicate the sibling number. NA indicates that DNA was not available. Panels C through F show ultrastructural changes in the glomerular capillary wall in Patient 4 with the homozygous Y695X *MYO1E* mutation in Family 2 (Panels C and E) and in *Myo1e*-knockout mice (at 2 months of age in Panel D and at 4 months of age in Panel F). (The white dot in Panel C is an artifact in the ultrathin specimen.) In both the patient and the mice, the glomerular basement membrane (BM) is thickened and disorganized, with multiple electron-lucent areas and multilamination (Panels C and D). Intramembranous granular material is present in lucent areas between the laminae (asterisk in Panel F), and the foot processes are effaced. Intercellular contacts show focal areas of increased plasma-membrane density (arrows in Panels E and F). Podocytes have undergone microvillous transformation.

Myo1E deficiency on podocyte motility was assessed by knocking down *MYO1E* with small interfering RNA (siRNA). For a detailed description of the assessment methods, see Figure 1 and Tables 1 and 2 in the Supplementary Appendix, available with the full text of this article at NEJM.org. The study was approved by the ethics committee of Azienda Sanitaria Locale, Bergamo, Italy. Participants or their parents or guardians gave written informed consent.

Table 1. Clinical Characteristics of Patients with *MYO1E* Mutations.*

Patient No.	Age at Diagnosis	Age at Onset of ESRD	Treatment	Finding on Renal Biopsy	First Observation			Last Observation		
					Age	Urinary Protein	Serum Creatinine	Age	Urinary Protein	Serum Creatinine
	yr	*yr*			*yr*	*g/24 hr*	*mg/dl*	*yr*	*g/24 hr*	*mg/dl*
Index family										
Patient 1	9	13	Glucocorticoids (NR), cyclosporine (NR), ACE inhibitor (NR)	Advanced FSGS	9	3.00	0.6	18	0.05†	1.1†
Patient 2	4	—	Cyclosporine (PR), ACE inhibitor (PR)	FSGS	4	1.56	0.4	13	0.53	0.7
Patient 3	2	—	Glucocorticoids (NR), cyclosporine (PR), ACE inhibitor (PR)	FSGS	2	3.40	0.4	11	0.59	0.7
Family 2										
Patient 4	1	—	Glucocorticoids (NR), cyclosporine (PR), ACE inhibitor (PR)	FSGS	1	+++‡	0.3	14.5	2.8	0.3

* To convert the values for creatinine to micromoles per liter, multiply by 88.4. ACE denotes angiotensin-converting enzyme, ESRD end-stage renal disease, FSGS focal segmental glomerulosclerosis, NR no response, and PR partial remission. The older sister of Patient 4 in Family 2 died before these studies were done and is not represented in the table (no DNA was available).
† These data were obtained after kidney transplantation.
‡ Dipstick was used for this evaluation; the three plus signs denote a strongly positive result.

RESULTS

CLINICAL HISTORY AND HISTOLOGIC CHARACTERISTICS OF THE PATIENTS

The Index Family

The proband (Patient 1) presented with nephrotic-range proteinuria, microhematuria, hypoalbuminemia, and edema at 9 years of age (Table 1 and Fig. 1A). Thirty percent of the glomeruli had segmental sclerohyalinosis with capsular crescents and flocculocapsular adhesion, and 30% had global sclerohyalinosis and collapse. The remaining glomeruli had focal mesangial hyperplasia, as well as tubular atrophy with tubulitis and moderate interstitial inflammatory infiltrates. Diffuse glomerular parietal deposits of IgM, C3, C4, and C1q and peritubular deposits of IgA, IgG, and C3 were present. Electron microscopy was not performed. The patient was treated with prednisone, cyclosporine, and an angiotensin-converting–enzyme (ACE) inhibitor, with no reduction in urine protein levels. After 1 year of therapy, prednisone and cyclosporine were discontinued. Renal function progressively deteriorated, and end-stage renal disease developed when she was 13 years of age. At the age of 17 years, she received a cadaveric kidney transplant. One year after transplantation, graft function was normal, with no proteinuria.

After focal segmental glomerulosclerosis was diagnosed in the proband, her two siblings (Patients 2 and 3) were found to have proteinuria and milder renal injury, with 10 to 30% of glomeruli affected by segmental or diffuse sclerohyalinosis with segmental flocculocapsular adhesion and mesangial hyperplasia. Parietal and mesangial focal deposition of IgM, C3, and C1q and peritubular staining for IgG, C3, and C4 were noted in biopsy specimens from both siblings. The histologic findings for all three siblings are compatible with focal segmental glomerulosclerosis not otherwise specified.[1] Although the proband did not have a response to treatment with glucocorticoids and cyclosporine, in both her siblings, cyclosporine therapy reduced proteinuria but did not eliminate it (Table 1).

Family 2

The proband in Family 2 (Patient 4) presented with edema, nephrotic-range proteinuria, hematuria, and hypoalbuminemia at 1 year of age (Table 1 and Fig. 1B). When she was 4 years of age, renal biopsy showed segmental or global sclerosis of

20% of glomeruli (Fig. 2 in the Supplementary Appendix) and focal tubular dilatation and atrophy with interstitial fibrosis. Focal glomerular IgG, IgA, and C3 staining was noted. All these features are compatible with focal segmental glomerulosclerosis not otherwise specified. Staining with the use of two antibodies against the C-terminal of Myo1E was negative for Myo1E; glomerular synaptopodin staining was greatly reduced, whereas podocin staining was almost normal (Fig. 3 in the Supplementary Appendix). Electron-microscopical examination showed foot-process effacement and microvillous transformation of podocytes, focal thickening and disorganization of the glomerular basement membrane with loss of identifiable layers (Fig. 1C and 1E), and focal expansion of the mesangial matrix. These changes are almost identical to those seen in *Myo1e*-deficient mice[19] (Fig. 1D and 1F, and Fig. 4 in the Supplementary Appendix). Other abnormalities included multilamination in the basement membrane of Bowman's capsule, focal mesangial interposition in the glomerular basement membrane, and electron-dense areas in the mesangium.

From 1.5 to 13 years of age, the patient received glucocorticoid therapy alone or in combination with cyclosporine. Proteinuria reached a nadir with cyclosporine (Fig. 5 in the Supplementary Appendix), but complete remission was not achieved. She is currently taking ramipril and losartan and has mild edema, proteinuria (Table 1), and microhematuria. Renal function and blood pressure are normal.

Her older sister had presented with microhematuria and mild proteinuria at 3 months of age. Renal biopsy showed segmental glomerular hyalinization and thickening of the glomerular basement membrane associated with mesangial proliferation. Immunofluorescence studies and electron microscopy were not carried out. She received glucocorticoid therapy (without cyclosporine or ACE inhibitors). Renal function progressively deteriorated to end-stage renal disease, and she died at 6 years of age. No relevant extrarenal manifestations were reported in affected members of either the index family or Family 2, which would rule out a syndromic form of focal segmental glomerulosclerosis with multiorgan involvement.

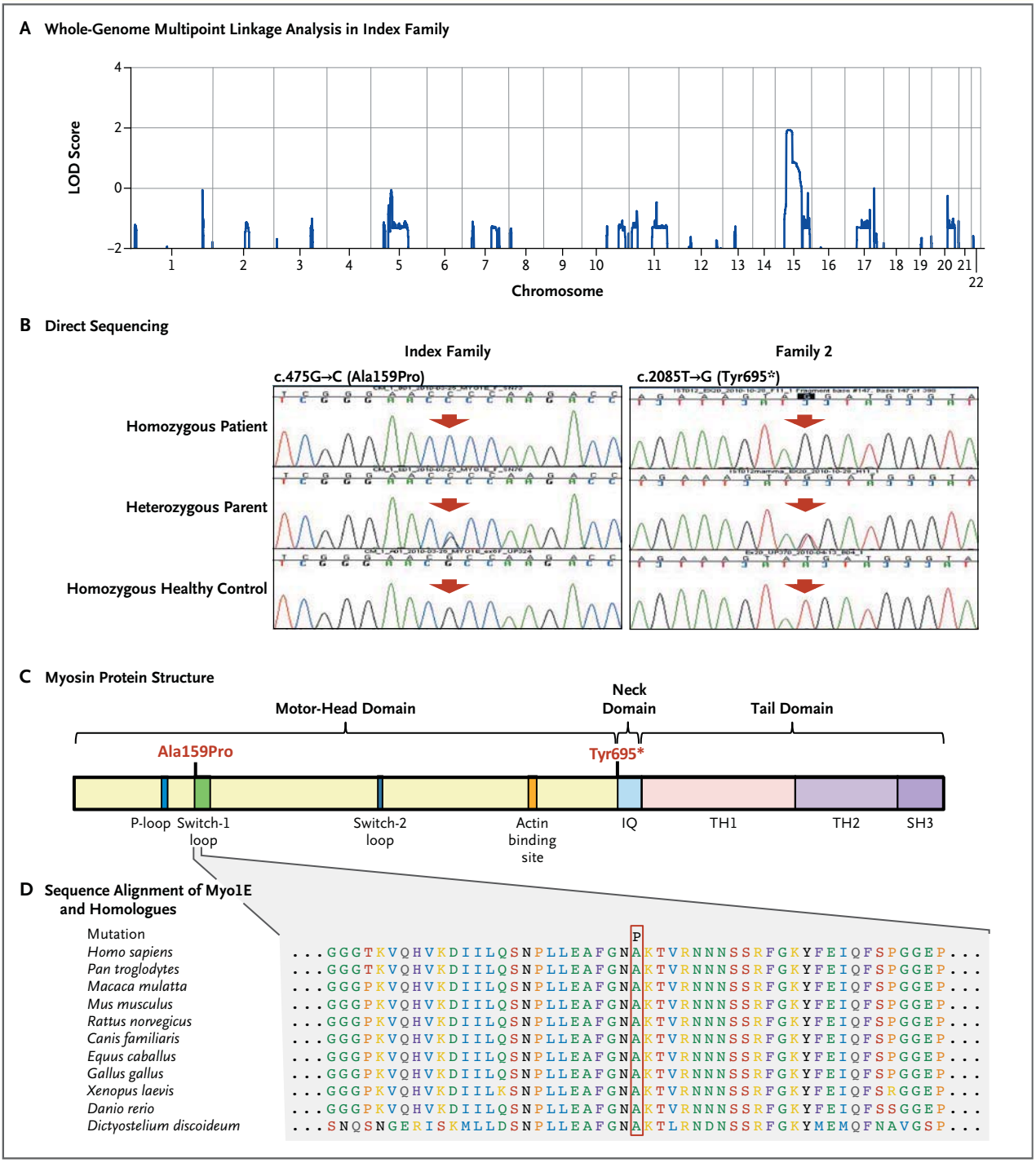
GENETIC AND FUNCTIONAL STUDIES

Whole-genome multipoint linkage analysis of the three siblings and the father in the index family showed a single positive peak on chromosome 15q21 (logarithm of the odds [LOD] $\text{score}_{\text{max}}=1.9$; z $\text{score}_{\text{max}}=2.7$) (Fig. 2A). We also genotyped the mother for nine rare SNPs in the linkage peak, with rs7181069 reaching the highest LOD score, at 2.7 (z $\text{score}_{\text{max}}=7.4$), and defined a new putative locus for focal segmental glomerulosclerosis. The linkage area spanned 16 megabases between rs12900916 and rs2278545. The expected length of the identity-by-descent (IBD) region around the disease locus is a function of sibling inbreeding (inbreeding coefficient $F=0.00195$), predicting an IBD region of about 16 centimorgans.[20]

The 112 genes in this region were screened by means of genomic locus capture followed by high-throughput sequencing in the proband. There were 2141 homozygous mismatches, of which 3 were putative functional variants not reported in SNP databases (2 splice variants and 1 nonsynonymous change). Only the coding variant was predicted to be damaging on the basis of bioinformatic analysis (Table 3 in the Supplementary Appendix). There is a G→C change in exon 6 of *MYO1E* (c.475G→C) replacing alanine-159 of Myo1E with proline (p.A159P). Direct sequencing showed that the three affected siblings were homozygous for the mutation and the unaffected parents were heterozygous for the mutation, confirming that the mutation segregates with focal segmental glomerulosclerosis (Fig. 2B). The mutation was not found in 764 normal chromosomes from Italian subjects.

Myo1E is a membrane-associated class I myosin with a motor-head domain that binds ATP and F-actin, a calmodulin-binding neck domain and a tail domain (Fig. 2C).[21-23] The amino acid A159 is highly conserved in myosins from protozoa to humans and lies in the switch-1 loop of the motor-head domain (Fig. 2C and 2D).[24] Our structural tridimensional model shows that the switch-1 region is located in the ATP-binding pocket close to the actin-binding domain (Fig. 6 in the Supplementary Appendix).

MYO1E was sequenced in the other patients, and a homozygous nonsense mutation (c.2085T→G) was identified in a girl in a second family (Family 2), who had familial focal segmental glomerulosclerosis (Patient 4). This mutation causes protein interruption at tyrosine 695 (p.Y695X), at the start of the calmodulin-binding domain (Fig. 2B and 2C, and Fig. 6 in the Supplementary Appendix). Her parents and her unaffected brother were



heterozygous for this mutation (Fig. 1B), which was not found in 968 normal human chromosomes from 382 Italian and 102 Turkish subjects. The other non–disease-causing *MYO1E* variants found in the patient cohort are shown in Table 4 in the Supplementary Appendix.

Myo1e has been localized in podocytes in mice, and *Myo1e*-knockout mice have proteinuria, podocyte injury, and chronic renal disease.[19] Immunohistochemical studies in control human kidney-biopsy specimens showed that Myo1E is predominantly expressed in the glomerulus (Fig. 3A, and Fig. 7 in the Supplementary Appendix). Myo1E partially colocalized with synaptopodin,

Figure 2 (facing page). Identification of *MYO1E* Mutations in Focal Segmental Glomerulosclerosis.

Panel A shows a parametric, multipoint logarithm-of-the-odds (LOD) score profile across the human genome, as calculated in the index family. There is a single positive peak on human chromosome 15. Panel B shows sequencing electropherograms of the index family (left) and Family 2 (right). The affected child in the index family has a homozygous G→C substitution (arrow), and the unaffected father is heterozygous for the mutation. The affected child in Family 2 has a homozygous T→G substitution (arrow), and the unaffected mother is heterozygous for the mutation. The two lowermost electropherograms are from healthy controls. Panel C shows the functional domains in the myosin 1E protein (Myo1E). Myo1E contains a motor-head domain (including a phosphate-binding loop [P-loop] and the switch-1 and switch-2 loops), which binds ATP and F-actin (actin binding site); a light-chain–binding neck domain (IQ), which binds calmodulin; and a tail domain, which contains the lipid-binding tail homology 1 (TH1) domain, the proline-rich tail homology 2 (TH2) domain, and the Src homology 3 (SH3) domain. The Ala159Pro change is within the switch-1 loop, and Y695X causes a truncation at the beginning of the neck domain. Panel D shows the sequence alignment of the Myo1E protein and its homologues among various species. The red box indicates the location of the highly conserved Ala159 amino acid that is changed to Pro (P) in the index family. (Sequences are from the National Center for Biotechnology Information.)

a podocyte-specific protein essential for the integrity of the podocyte cytoskeleton,[25] but not with the endothelial marker VE-cadherin (Fig. 3B and 3C), indicating that human Myo1E is mainly expressed in podocytes, as is the case in mice. Myo1E expression in human podocytes was confirmed by Western blot analysis (Fig. 8 in the Supplementary Appendix). In cultured human podocytes, Myo1E localized close to the cytoplasmic membrane, with enrichment at the lamellipodia tips (Fig. 3D, 3E, and 3F). In costaining experiments, Myo1E localized at the tips of F-actin bundles (Fig. 3D, 3E, and 3F) and partially colocalized with CD2-associated protein (CD2AP) (Fig. 3G). Immunogold labeling for Myo1E in control human glomeruli revealed gold particles almost exclusively on the cytoplasmic side of the podocyte plasma membrane (Fig. 3H and 3I).

Human podocytes transfected with green fluorescent protein (GFP)–tagged wild-type Myo1E or E753K, I531M, D465N, and F307L (control SNPs) showed predominant localization at the plasma membrane, whereas podocytes transfected with GFP-tagged mutant A159P Myo1E showed diffuse cytoplasmic localization, which at a higher plasmid dose acquired a punctate pattern (Fig. 4A through 4F, and Fig. 9 and 10 in the Supplementary Appendix).

Similar findings were observed in mouse podocytes and HEK293 cells (Fig. 11 and 12 in the Supplementary Appendix). A Western blot analysis of wild-type and mutant A159P Myo1E expression in human and mouse podocytes is shown in Figure 13 in the Supplementary Appendix. Total-internal-reflection fluorescence microscopy of live mouse podocytes showed enhanced green fluorescent protein (EGFP)–tagged wild-type Myo1E in a bright, discrete, punctate pattern on lamellipodia and filopodia and in endocytic invaginations on the bottom surface of plasma membrane (Fig. 14 in the Supplementary Appendix, and video, available at NEJM.org), whereas the EGFP-tagged mutant A159P Myo1E was mainly cytosolic (Fig. 14 in the Supplementary Appendix).

A video showing microscopy of Myo1E in infected mouse podocytes is available at NEJM.org

In contrast to wild-type Myo1E, the A159P mutant did not stain with an anti-Myo1E antibody, suggesting conformational changes in the mutant protein (Fig. 9 in the Supplementary Appendix). GFP-tagged wild-type Myo1E substantially costained with F-actin at the podocyte plasma membrane, whereas the A159P mutant did not colocalize with F-actin (Fig. 4G and 4H). Wild-type Myo1E partially colocalized with CD2AP at the cell surface, whereas A159P Myo1E and CD2AP costaining was mainly cytoplasmic and often punctate (Fig. 4I and Fig. 4J). Wild-type Myo1E overexpression increased human podocyte motility (Fig. 4K). In contrast, A159P Myo1E had no effect on podocyte motility. Conversely, Myo1E knockdown in human podocytes (Fig. 4K) and Myo1E knockout in mouse podocytes impaired migration (unpublished data). A159P Myo1E transfection did not modify alpha-actinin-4, podocin, or nephrin expression in podocytes (Fig. 15 and 16 in the Supplementary Appendix).

DISCUSSION

This study identifies two mutations in *MYO1E*, which encodes myosin 1E (a nonmuscle class I myosin). These mutations are closely associated with autosomal recessive focal segmental glomerulosclerosis, and several findings support their potential causative role in the development of this disorder: first, the segregation of the mutations with the disease; second, the absence of the mutations in a large number of controls; third, the mutations occurring in important functional do-

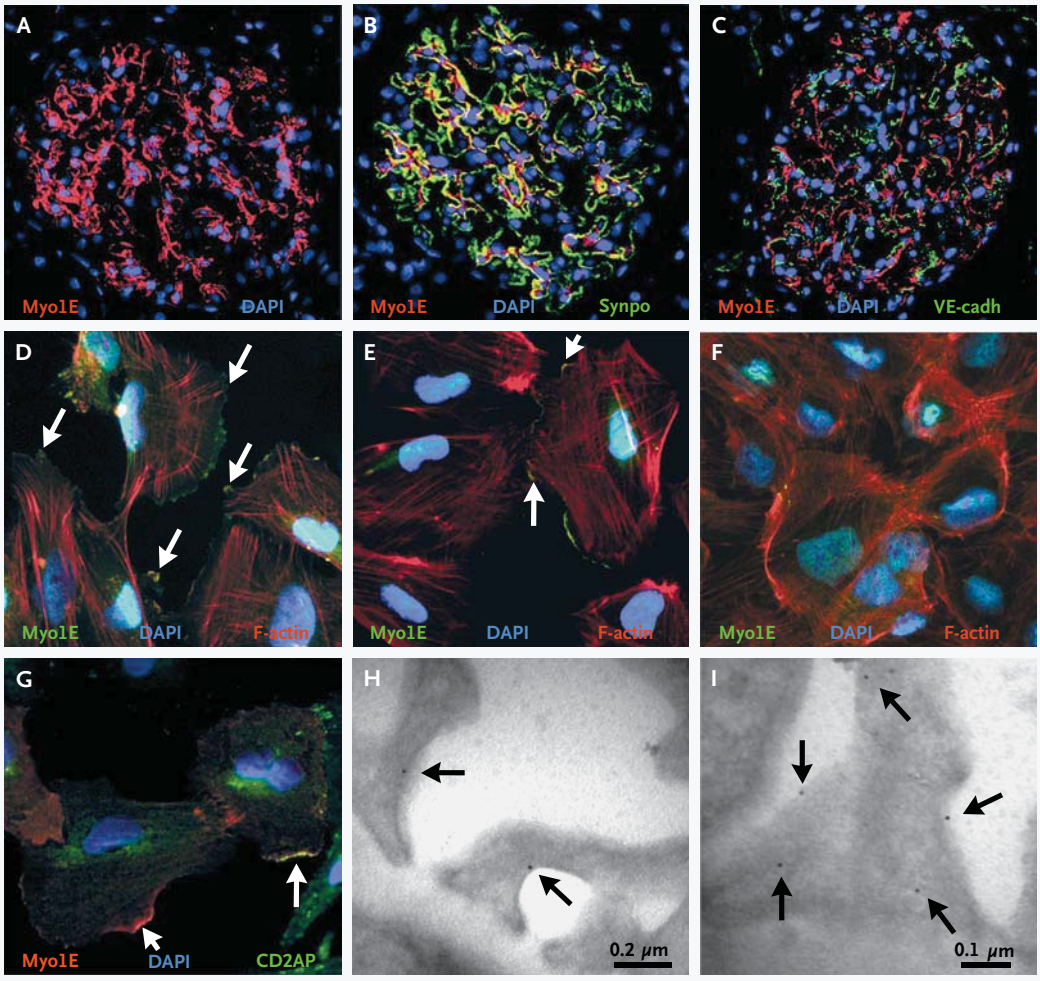


Figure 3. Endogenous Myosin 1E (Myo1E) Expression.

Panels A, B, and C show immunofluorescence staining of normal human kidney tissue with 4′,6-diamidine-2-phenylidole dihydrochloride (DAPI) (blue staining of cell nuclei in all three panels, as well as Panels D, E, F, and G) and antibodies against Myo1E (red in all three panels) and the podocyte marker synaptopodin (Synpo) (green in Panel B) or the endothelial-cell marker VE-cadherin (VE-cadh) (green in Panel C). Myo1E staining can be seen in the glomerulus. The yellow areas in Panel B indicate the merging of Myo1E and synaptopodin staining in the podocytes. Panels D, E, and F show staining of endogenous Myo1E in cultured undifferentiated podocytes (Panel D), terminally differentiated nonconfluent podocytes (14-day culture at 37°C) (Panel E), and confluent human podocytes (Panel F). The Myo1E staining localizes predominantly along the plasma membrane, mainly on cell lamellipodia, both in undifferentiated and in fully differentiated podocytes (green in Panels D, E, and F), with fainter staining in confluent podocytes. The arrows in Panels D and E show areas of colocalization (yellow) of Myo1E and the F-actin tips (rhodamine–phalloidin stain, red). Panel G shows CD2-associated protein (CD2AP) (green) in cultured differentiated podocytes; the arrows show partial costaining (yellow) of endogenous Myo1E (red). Panels H and I show immunogold labeling of Myo1E in podocytes from control human glomeruli. Gold particles (arrows) localize to the cytoplasmic side of the podocyte plasma membrane.

mains; fourth, the specific expression of Myo1E in human kidney podocytes and cultured podocytes; fifth, the finding that the A159P mutation greatly alters Myo1E subcellular localization, its interaction with the podocyte cytoskeleton, and its ability to promote podocyte migration; and finally, the similarity of the ultrastructural glomerular lesions in the patient with the Y695X mutation and *Myo1e*-deficient mice.

The discovery of genetic abnormalities in proteins that bind or regulate the podocyte cytoskeleton[13,14,18] in focal segmental glomerulosclerosis

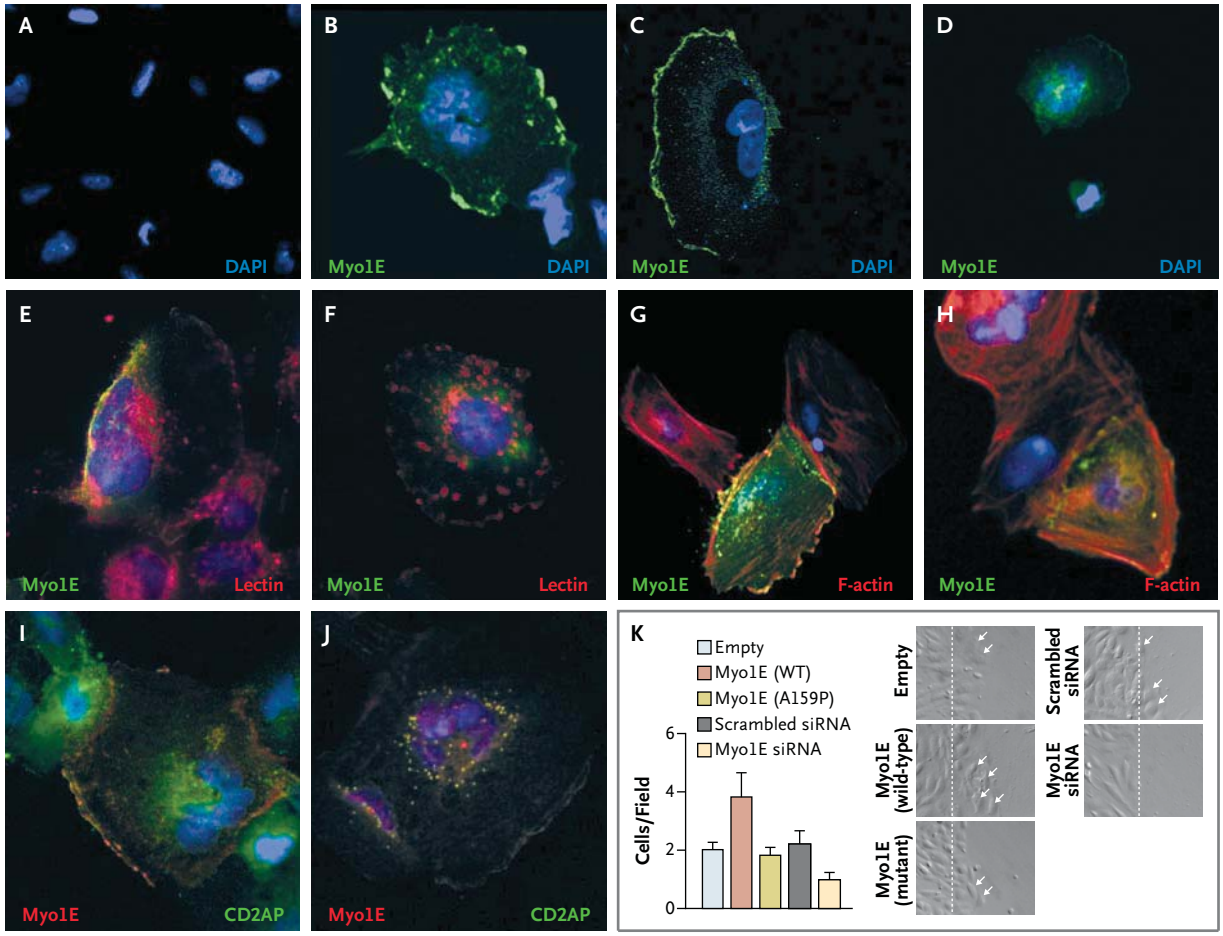


Figure 4. Expression of Recombinant Myo1E in Cultured Human Podocytes.

Shown in Panels A through J are undifferentiated human podocytes transfected with green fluorescent protein (GFP)–tagged wild-type Myo1E, E753K Myo1E (control SNP, rs8024923), or mutant A159P Myo1E (green, 1 μg plasmid) and analyzed by means of confocal microscopy; the nuclei were stained with 4′,6-diamidine-2-phenylidole dihydrochloride (DAPI) (blue). Panel A shows untransfected human podocytes. Panels B, C, and D show human podocytes transfected with wild-type Myo1E, control E753K Myo1E, and mutant A159P Myo1E, respectively. Wild-type and control E753K Myo1E localize along the plasma membrane, while the A159P mutant shows a cytoplasmic distribution. Transfection efficiency was about 30% for the GFP-tagged wild-type, E753K, and A159P constructs (determined by calculating the ratio of the number of green cells to the number of blue nuclei, multiplied by 100, in 10 to 15 high-power fields). Panels E and F show staining of podocyte plasma membranes with rhodamine-labeled lectin (red). GFP-tagged wild-type Myo1E partially colocalizes with lectin (Panel E, yellow), whereas the A159P mutant does not (Panel F, green). The mislocalization of the mutant protein is not attributable to instability or degradation, since the sizes and amounts of the wild-type and A159P proteins expressed in transfected podocytes were the same on Western blotting (see Fig. 13A and 13B in the Supplementary Appendix). Panels G and H show staining of F-actin filaments (red) in transfected human podocytes. GFP-tagged wild-type Myo1E (Panel G) partially colocalizes with F-actin (yellow), whereas the A159P mutant does not (Panel H, green). Panels I and J show staining with an anti–CD2-associated protein (CD2AP) antibody (green). Myelocytomatosis virus–associated sequence (MYC)–tagged wild-type Myo1E (Panel I, red) partially colocalizes with CD2AP at the plasma membrane (yellow), whereas in cells transfected with the MYC-tagged A159P mutant (Panel J), MYC-tagged Myo1E, and CD2AP abnormally colocalize in the cytoplasm (yellow). Panel K shows the results of a scrape-wound migration assay of cultured human podocytes. Transfection with GFP-tagged wild-type Myo1E (WT) increased podocyte migration, as compared with the migration of podocytes transfected with an empty GFP-tagged vector, whereas the GFP-tagged A159P Myo1E had no effect on migration. Knockdown of endogenous Myo1E by small interfering RNA (siRNA) reduced podocyte migration, as compared with the migration of podocytes transfected with universal scrambled siRNA. The numbers of podocytes per field that migrated into the wound track 6 hours after scraping of the podocyte layer are shown (one field=71,500 μm^2), representing the mean (±SE) value for 3 wells, with 5 fields counted per well. Images of representative fields are shown. Dashed lines indicate the left margin of the scrape wound; arrows show migrating cells. $P<0.05$ for the comparison of wild-type with the empty vector and with A159P (by one-way analysis of variance) and for the comparison of scrambled siRNA and Myo1E siRNA (by Student's t-test).

highlights the central role of the actin cytoskeleton in podocyte structure and function.[26] The podocyte cytoskeleton is connected to the glomerular basement membrane through $\alpha 3\beta 1$ integrins and a trimolecular nonmuscle myosin–actin–utrophin complex attached to α-dystroglycans and β-dystroglycans.[5,26,27] Foot-process effacement, the hallmark of podocyte injury, is often accompanied by the disappearance of these aligned filaments.[28,29] The cytoskeleton filaments of human podocyte foot processes contain nonmuscle myosins IIa and IIb.[30] Our study documents that Myo1E is specifically expressed in human podocytes and is associated with the plasma membrane. The observed enrichment of Myo1E in podocyte processes in conjunction with F-actin filament tips implicates this protein as a key component of the foot-process cytoskeleton.[27] Myo1E is important for podocyte structure and spreading on the extracellular matrix, as documented by the observation that *Myo1e*-deficient mice have defects in podocyte organization, with foot-process effacement.[19] The phenotype of *Myo1e*-deficient mice is characterized by proteinuria, hemoglobinuria, glomerulosclerosis, and impaired renal function[19] — features that are also present in patients with *MYO1E* mutations. Glomeruli of both *Myo1e*-deficient mice[19] and the patient with the Y695X mutation have peculiar ultrastructural features, with thickening and disorganization of the glomerular basement membrane, suggesting that Myo1E plays a role in regulating the crosstalk between podocytes and their matrix.

Nonmuscle myosin activity generates tension, and the interaction among actin, myosins, and alpha-actinin-4 probably allows the foot processes to generate the contractile forces that help the glomerular capillaries to resist the high intraluminal hydrostatic pressure and to change their morphologic structure actively, modifying the permeability of the glomerular filtration barrier.[26,30,31] Mutations in *MYH9*, encoding the nonmuscle myosin IIa heavy chain, cause autosomal dominant disorders with macrothrombocytopenia, leukocyte inclusions, and in some cases, glomerulopathy.[32] Multiple *MYH9* SNPs have been associated with idiopathic and human immunodeficiency virus–related focal segmental glomerulosclerosis and with hypertensive end-stage renal disease in blacks.[33] However, a follow-up study indicated that the strongest association no longer clustered in *MYH9* but rather in the neighboring *APOL1*.[34]

The A159P missense mutation we identified impairs the ability of Myo1E to promote podocyte motility, which is consistent with 159P localization within the switch-1 loop in the motor-head domain of Myo1E. The switch-1 loop is highly conserved among myosins and, together with the phosphate-binding loop and the switch-2 loop, forms the ATP-binding domain of myosins.[24] In both human and mouse podocytes, the A159P Myo1E mutant (in contrast with wild-type Myo1E) was mislocalized to the cytoplasm, indicating an important role for the switch-1 loop in the proper subcellular distribution of Myo1E. Mutations in the switch-1 loop of another nonmuscle myosin (*MYO7A*) cause type 1B Usher's syndrome (deafness and retinitis pigmentosa),[35] confirming the important role of the switch-1 loop in nonmuscle myosin function.

The second mutation causes the formation of a truncated protein that lacks the calmodulin domain, which is important for calcium-mediated regulation of actin binding and ATP hydrolysis,[36] and that lacks the tail domain, which probably results in a nonfunctional protein, mimicking the condition of *Myo1e*-deficient mice. Indeed, the glomerular ultrastructural lesions in Patient 4 and in *Myo1e*-deficient mice were quite similar. Although Myo1E is expressed in several tissues, neither the patients with *MYO1E* mutations nor *Myo1e*-deficient mice had any extrarenal involvement, indicating that Myo1E is not required for the normal functioning of extrarenal tissues and organs.

The patients with *MYO1E* mutations and focal segmental glomerulosclerosis had glucocorticoid resistance, although three of them had a partial response to cyclosporine therapy. Cyclosporine reduces proteinuria in up to 70% of patients with glucocorticoid-resistant focal segmental glomerulosclerosis, but relapse occurs in 40 to 60%.[37,38] Recently, the antiproteinuric effect of cyclosporine in glucocorticoid-resistant focal segmental glomerulosclerosis has been attributed to the drug's direct stabilizing effects on the podocyte cytoskeleton rather than to its immunosuppressive actions.[39,40] Whether such mechanisms may have limited the deleterious consequences of *MYO1E* mutations remains to be clarified. End-stage renal disease developed in one affected member in each family, whereas the other affected family members had milder courses, possibly attributable to earlier diagnosis (in the index family) or to effective antiproteinuric treatment (in Family 2).

Focal segmental glomerulosclerosis is genetically heterogeneous, with mutations in several

genes,[11-18] each of which accounts for a small percentage of cases. *MYO1E* mutations do not appear to be a common cause of focal segmental glomerulosclerosis, since we have found only two independent *MYO1E* mutations among 29 unrelated patients with familial focal segmental glomerulosclerosis and none among those with sporadic cases. Both mutations were homozygous, suggesting that Myo1E-associated focal segmental glomerulosclerosis might be restricted to families with some degree of consanguinity. In conclusion, these studies show that *MYO1E* mutations have a role in focal segmental glomerulosclerosis and suggest the importance of Myo1E in podocyte homeostasis and the consequent integrity of the glomerular filtration barrier.

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