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INF2 Mutations in Charcot–Marie–Tooth Disease with Glomerulopathy

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ABSTRACT

BACKGROUND
Charcot–Marie–Tooth neuropathy has been reported to be associated with renal diseases, mostly focal segmental glomerulosclerosis (FSGS). However, the common mechanisms underlying the neuropathy and FSGS remain unknown. Mutations in INF2 were recently identified in patients with autosomal dominant FSGS. INF2 encodes a formin protein that interacts with the Rho-GTPase CDC42 and myelin and lymphocyte protein (MAL) that are implicated in essential steps of myelination and myelin maintenance. We therefore hypothesized that INF2 may be responsible for cases of Charcot–Marie–Tooth neuropathy associated with FSGS.

METHODS
We performed direct genotyping of INF2 in 16 index patients with Charcot–Marie–Tooth neuropathy and FSGS who did not have a mutation in PMP22 or MPZ, encoding peripheral myelin protein 22 and myelin protein zero, respectively. Histologic and functional studies were also conducted.

RESULTS
We identified nine new heterozygous mutations in 12 of the 16 index patients (75%), all located in exons 2 and 3, encoding the diaphanous-inhibitory domain of INF2. Patients presented with an intermediate form of Charcot–Marie–Tooth neuropathy as well as a glomerulopathy with FSGS on kidney biopsy. Immunohistochemical analysis revealed strong INF2 expression in Schwann-cell cytoplasm and podocytes. Moreover, we demonstrated that INF2 colocalizes and interacts with MAL in Schwann cells. The INF2 mutants perturbed the INF2–MAL–CDC42 pathway, resulting in cytoskeleton disorganization, enhanced INF2 binding to CDC42 and mislocalization of INF2, MAL, and CDC42.

CONCLUSIONS
INF2 mutations appear to cause many cases of FSGS-associated Charcot–Marie–Tooth neuropathy, showing that INF2 is involved in a disease affecting both the kidney glomerulus and the peripheral nervous system. These findings provide new insights into the pathophysiological mechanisms linking formin proteins to podocyte and Schwann-cell function. (Funded by the Agence Nationale de la Recherche and others.)
INF2 is known to promote not only actin polymerization but also filament severing and depolymerization. The interaction of INF2 with other diaphanous-related formins, such as mDia1–DIAPH1 and the Rho-GTPase CDC42, through its DID. In addition, it has been shown to bind (through its C-terminal) the myelin and lymphocyte protein (MAL) in Jurkat T cells and MAL2 in Madin–Darby canine kidney cells and HepG2 hepatocytes to regulate intracellular protein transport. Although very little is known about the role of diaphanous-related formins in the peripheral nervous system, the implication of CDC42 and MAL in essential steps of myelination and myelin maintenance led us to hypothesize that INF2 mutations may be responsible for the association between FSGS and Charcot–Marie–Tooth neuropathy.

### METHODS

#### STUDY PARTICIPANTS

The study was conducted from March 2010 through September 2011. Sixteen index patients (seven with apparent autosomal dominant inheritance and nine with sporadic disease) from 16 unrelated families were included in the study. Twelve were from our French FSGS DNA cohort, including 2 families described previously. We contacted authors of published cases of FSGS and Charcot–Marie–Tooth neuropathy and thereby obtained DNA samples from 4 additional families. Twenty-five members of the patients’ families were also tested. All index patients presented with clinical manifestations of Charcot–Marie–Tooth disease by a neurologist in their primary care centers on the basis of their clinical history, physical examination, and electrophysiological or histologic testing. Mutations in PMP22 or MPZ were ruled out in all patients. Written informed consent was obtained from all study participants or their parents, and the study was approved by the Comité de Protection des Personnes Ile-De-France II.

#### GENETIC, HISTOLOGIC, AND FUNCTIONAL STUDIES

INF2 exons 2, 3, and 4 were sequenced for all participants, and in the absence of mutations, the remaining exons were sequenced (as previously described). Localization of INF2, MAL, and MAL2 in normal human kidney and peripheral-nerve specimens and in cultured Schwann cells was assessed by means of immunoperoxidase and immunofluorescence staining. The interaction of INF2 with MAL in Schwann cells was demonstrated by

### CHAUCOT–MARIE–TOOTH DISEASE REFERS to a heterogeneous group of inherited chronic peripheral motor and sensory neuropathies. Affected persons typically present with progressive distal-muscle weakness and atrophy, reduced tendon reflexes, and foot and hand deformities. Three Charcot–Marie–Tooth disease subtypes have been distinguished by means of electrophysiological and neuropathological studies — a glial myelinopathy (type 1) characterized by slow motor-nerve conduction velocities and demyelinating neuropathy, an axonal form (type 2) associated with normal or subnormal nerve conduction velocities and axonal degeneration, and an intermediate form with demyelinating and axonal features in which patients from the same family may have either subnormal or reduced nerve conduction velocities. At least 40 different genes or loci have been associated with this disease (as has been reviewed by Lupski and colleagues). Autosomal dominant Charcot–Marie–Tooth type 1 is the most prevalent form, with mutations in the peripheral myelin protein 22 gene (PMP22) and the myelin protein zero gene (MPZ) underlying most cases.

An increased prevalence of nephropathies, particularly focal segmental glomerulosclerosis (FSGS), has been documented in patients with Charcot–Marie–Tooth neuropathy, but the pathophysiological mechanism linking these two clinical entities is unknown. FSGS is a histologic pattern of renal damage that is associated with a spectrum of primary and secondary glomerular diseases, including isolated proteinuria and glucocorticoid-resistant nephrotic syndrome. In the past few years, the identification of genes involved in hereditary glomerulopathies has expanded knowledge about the crucial role of the podocyte, a glomerular epithelial cell with interdigitating foot processes, as well as its actin cytoskeleton, in the function of the glomerular filtration barrier.

INF2 mutations account for 12 to 17% of autosomal dominant cases of FSGS. The gene encodes a member of the diaphanous-related formin family, which is involved in remodeling the actin and microtubule cytoskeletons. INF2 possesses functional domains characteristic of other diaphanous-related formins: an N-terminal diaphanous-inhibitory domain (DID), the formin homology domains FH1 and FH2, and a C-terminal diaphanous-autoregulatory domain (DAD). However, INF2 has a unique ability to promote not only actin polymerization but also filament severing and depolymerization.

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pull-down assay. The effects of INF2 mutant expression on interaction with active CDC42 and IQGAP1 were evaluated by coimmunoprecipitation, and the effects of INF2 mutant expression on intracellular localization of MAL and CDC42 and on the actin cytoskeleton were evaluated by immunofluorescence. Two INF2 mutants associated with FSGS and Charcot–Marie–Tooth neuropathy and of mutants associated with FSGS alone. A detailed description of the methods used is provided in the Supplementary Appendix, available with the full text of the article at NEJM.org.

RESULTS

INF2 MUTATIONS

Heterozygous INF2 mutations were detected in 12 of the 16 index patients (75%). Nine different mutations were identified: eight missense mutations and one in-frame deletion of three amino acids. All were new mutations located in exons 2 and 3, which encode the DID domain (Fig. 1A, and Table 1S in the Supplementary Appendix), and all caused nonconservative changes in highly conserved amino acids. Scores from PolyPhen-2 software analysis (http://genetics.bwh.harvard.edu/pph2) to predict the functional effects of missense INF2 variants ranged from 0.993 to 1, predicting that INF2 variants were probably damaging. No INF2 variants were present in any of the 670 control chromosomes assayed or referenced in the National Heart, Lung, and Blood Institute's
Exome Sequencing Project server. Mutations segregated with the disease in each familial case, although intrafamilial variability was noted (Fig. 1S in the Supplementary Appendix). A de novo mutation was confirmed in all three sporadic cases for which DNA was available from both parents of the patient. Most mutations identified in patients exhibiting FSGS and Charcot–Marie–Tooth neuropathy were localized in the 3’ end of exon 2 and in exon 3, in which no mutation has been identified to date (to our knowledge). The INF2 variants were clustered between nucleotides 300 and 500, whereas most isolated FSGS mutants were located downstream of nucleotide 500. To make functional predictions, we mapped mutants associated with FSGS alone8,10 and those associated with FSGS and Charcot–Marie–Tooth neuropathy onto a human INF2 DID in silico model (Fig. 1B); although all involved DID residues, mutations in the two groups of patients were distinctly localized, the latter being located mostly in the second and third DID armadillo repeats and the former mostly in the fourth armadillo repeat (Fig. 1B).

To evaluate the potential role of INF2 in isolated Charcot–Marie–Tooth disease, we performed mutational analysis of INF2 exons 2, 3, and 4 in an additional group of 50 patients who presented with Charcot–Marie–Tooth disease without a known renal phenotype, nerve conduction velocities in the intermediate range (25 to 45 m per second), and limited hand function, muscle wasting, and abolition of deep tendon reflexes in the lower and upper limbs. Four patients also had mild or moderate sensorineural hearing loss. In addition, magnetic resonance imaging of the brain, performed in 2 patients (15 and 48 years old), showed central nervous system anomalies characterized by white-matter hyperintensity and ventricular dilation, which were more severe in the older patient (Fig. 2B). Median-nerve conduction velocities were in the range of intermediate Charcot–Marie–Tooth neuropathy (23 to 45 m per second). Patient K.3 had almost normal median-nerve conduction velocities (45 m per second), whereas her maternal aunt had reduced velocities (30 m per second; data not shown). The six available sural-nerve biopsy specimens all showed a pattern of lesions with a combination of axonal and demyelinating changes, characterized by a marked decrease in myelinated fibers (Fig. 2C), as compared with that in age-matched controls, and numerous multilayered “onion bulbs” (Fig. 2D). Together, these data suggest an intermediate Charcot–Marie–Tooth phenotype in patients with INF2 mutations.

**INF2 Expression in Podocytes and Schwann Cells**

In peripheral nerves, we detected robust INF2 staining in Schwann cells and lighter staining in some axons (Fig. 3A, and Fig. 2S in the Supplementary Appendix). In the kidney, we confirmed that INF2 expression occurs predominantly in podocytes. We detected weak staining in the proximal and distal tubules and found no INF2 in vessels. In kidney-tissue and sural-nerve sections from the patients, INF2 staining persisted, but the severity of the lesions precluded our drawing any conclusion with respect to putative overexpression.

**INF2 and MAL in Schwann Cells**

MAL interacts with INF215 and is a major component of myelin.18 We therefore hypothesized that mutant INF2 proteins could alter the INF2–MAL pathway in Schwann cells. We demonstrated the presence of INF2, together with MAL, in normal human peripheral-nerve serial sections and the endogenous colocalization of INF2 and MAL in mouse Schwann cells (Fig. 3A and 3B). Moreover, glutathione S-transferase–pull-down experiments revealed an interaction between the INF2 C-terminal and endogenous MAL in Schwann cells (Fig. 3C). We also confirmed INF2 and MAL2 localization in human podocytes, and the absence of MAL in the glomeruli (Fig. 3A).
### Table 1. Neurologic and Renal Phenotype of the 12 Index Patients with *INF2* Mutations.

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<th>Index Patient</th>
<th>Age at Proteinuria Onset</th>
<th>Time of Biopsy</th>
<th>Age at ESRD Onset</th>
<th>Age at Onset of Peripheral-Nerve Dysfunction</th>
<th>Muscle Weakness</th>
<th>Sural-Nerve Histologic Characteristics</th>
<th>Sensorineural Hearing Loss</th>
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<td>Age yr</td>
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* To convert the values for creatinine to micromoles per liter, multiply by 88.4. ESP denotes external sciatic popliteal, ESRD end-stage renal disease, MRI magnetic resonance imaging, and ND not done.
† This family has been previously described by Lemieux and Neemeh.21
We then investigated the effects of INF2 mutants on MAL localization (Fig. 3D). In contrast to the perinuclear localization of the wild-type form of INF2,9,25 INF2 mutants in patients with FSGS and Charcot–Marie–Tooth disease were diffusely localized throughout the cytoplasm, similar to the FSGS mutants studied here and by others.9 We also observed that MAL had a perinuclear localization when transfected alone or with the wild-type form of INF2, whereas its distribution was diffuse throughout the cell with INF2 mutants, and that the two proteins were colocalized (Fig. 3C). Costaining of endogenous protein disulfide isomerase (PDI) confirmed the predominant localization of wild-type INF2 to the endoplasmic reticulum.25 In cells expressing INF2 mutants, the mislocalization of INF2 coincided with a diffuse pattern of PDI staining, which could reflect cytoskeleton disorganization (Fig. 3S in the Supplementary Appendix).

Figure 2. Features on Pathological Analysis and Neuroimaging in Selected Patients with Focal Segmental Glomerulosclerosis (FSGS) and Charcot–Marie–Tooth Neuropathy.

Panel A shows the results of trichrome staining of kidney sections from a normal control and Patient J.3, who has typical lesions of FSGS not otherwise specified. Panel B shows the results of magnetic resonance imaging (MRI) of the brain (fluid-attenuated inversion recovery [FLAIR] sequences) of a normal control and two patients: one 15 years of age (Patient C.1) and the other 48 years of age (Patient A.3). Arrows point to bilateral symmetric hyperintensities visible in the internal capsule and the periventricular white matter. Panel C shows sural-nerve semithin sections revealing a marked loss of myelinated fibers in the patients (arrows). The severity of the lesions appears to increase with the patient’s age (from left to right, 15, 30, and 48 years); all myelinated fibers were absent in the oldest patient (Patient A.3). Panel D shows electron micrographs of sural-nerve sections revealing multilayered “onion bulbs” (in Patient I.1) and supernumerary elongated extensions from the cytoplasm of many nonmyelinating Schwann cells (in Patient H.1, arrows), occasionally including collagen fibers (in Patient H.1, arrowheads). In the oldest patient (Patient A.3), the endoneurium was completely replaced by numerous whirls of nonmyelinating Schwann-cell cytoplasm.
INF2 MUTANTS AND ACTIN CYTOSKELETON REGULATION

Cells expressing mutant INF2 exhibited less cortical actin and a reduced number of long actin stress fibers than those expressing wild-type INF2, and a disorganized microtubule network (Fig. 4S, 5S, and 6S in the Supplementary Appendix). Similar features were observed with the K/A–3L/A–INF2 mutant that lacks both the polymerization and depolymerization activities of wild-type INF2,14 although with this mutant the decrease in the content of long actin filaments was less severe.

We therefore investigated whether the mutations in INF2 proteins affect their binding to CDC42, an actin-regulating Rho-GTPase known to interact, in its GTP-loaded active state, with the INF2 DID.14 An enhanced interaction was observed between the INF2 mutants and a constitutively active form of CDC42 (CDC42-Q61L) as compared with the wild-type INF2 protein (Fig. 4A and 4B). Moreover, INF2 mutants affected the subcellular localization of CDC42-Q61L, with the fraction of active CDC42 at the plasma membrane being lost in a large proportion of mutant cells as compared with cells expressing wild-type INF2 (Fig. 4C), but did not significantly perturb CDC42 activity (Fig. 7S in the Supplementary Appendix), a result consistent with INF2 being a downstream target of CDC42.14,15 We also demonstrated the interaction of INF2 with IQGAP1, a CDC42 effector known to interact with mDia1.26,27 INF2 mutants still interacted with IQGAP1 and altered the endogenous IQGAP1 subcellular distribution (Fig. 8S in the Supplementary Appendix), as they did for MAL and CDC42.

DISCUSSION

We have demonstrated that, in addition to leading to isolated FSGS, INF2 mutations are a major cause of Charcot–Marie–Tooth disease associated with FSGS, accounting for approximately 75% of all cases. These results shed new light on the genetic basis of the dual neurologic and renal phenotype first described by Lemieux and Neemeh in 1967.21

Since the initial description, several cases of renal involvement (mostly glomerular disorders with FSGS lesions) have been reported in association with Charcot–Marie–Tooth disease.6 Plaisier and colleagues28 demonstrated the presence of myelin protein zero (MPZ) in podocytes and an increased urinary albumin excretion in Mpz knock-out mice, indicating a potential role of myelin components in glomerular permselectivity. No PMP22 or MPZ mutation has been reported in patients with FSGS and Charcot–Marie–Tooth neuropathy. In contrast, three quarters of the patients in the present study had INF2 mutations. None of these mutations were present in patients with Charcot–Marie–Tooth disease only, suggesting that INF2 is not involved in cases of the disease without an apparent renal phenotype. Nevertheless, because of the individual and intrafamilial phenotypic variability we observed, physicians should be alert for proteinuria in all patients who have Charcot–Marie–Tooth disease. Similarly, pes cavus was the only clinical sign of Charcot–Marie–Tooth disease in some members of patients’ families; therefore, a careful clinical neurologic evaluation should be considered for patients with FSGS.

Although INF2 mutations have been shown to be the major cause of autosomal dominant isolated FSGS, accounting for 12 to 17% of all cases9,10 the prevalence of INF2 mutations in association with FSGS and Charcot–Marie–Tooth disease is much higher (75%). Several lines of genetic and functional evidence indicate that these variants are pathogenic mutations. Given the high prevalence of INF2 mutations, the detection of various distinct INF2 mutations, and the absence of mutations in PMP22 and MPZ, the occurrence of a mutation in another gene underlying Charcot–Marie–Tooth disease in patients with INF2-related FSGS is unlikely. Moreover, it is improbable that the frequent de novo mutations we detected would occur in two distinct genes in the same patient. Furthermore, all of the nine INF2 mutations we identified are new. Although the nine mutations encode DID residues, as do mutations associated with isolated FSGS, they had a distinct localization, corresponding mostly to the 3′ end of exon 2 as well as in exon 3, in which no isolated FSGS mutation has been identified.

We further explored the functional effects of some INF2 mutations. We postulated that the mechanisms linking INF2 to the development of Charcot–Marie–Tooth disease involved perturbation of cytoskeletal networks and thus intracellular transport of myelin components. Indeed, INF2 has been shown to regulate specialized routes of protein targeting to the plasma membrane in various types of cells in association with CDC42 and MAL or MAL2.14,15 This targeting involves vesicular carriers that associate with actin filaments and re-
quires both the actin polymerization and depolymerization properties of INF2. 14,15 In addition, CDC42 and MAL are fundamental players in peripheral myelination. 17,18,29 Here we show that INF2 is also expressed in Schwann cells and to a lesser extent in neurons. We also demonstrate the endogenous colocalization of INF2 and MAL and their in vivo interaction in Schwann cells, thereby providing a clear rationale for the role of INF2 mutations in Charcot–Marie–Tooth disease. In addition, we show that INF2 mutations disrupt the INF2–MAL–CDC42 pathway. The reduction in cortical actin and stress fibers in cells expressing INF2 DID mutants was even more severe than in cells expressing the K/A–3L/A–INF2 mutant lacking both the polymerization and depolymerization properties of INF2. This suggests that INF2 DID mutants might not only alter these two functions but also have additional effects on INF2 partners. Indeed, we demonstrated an enhanced interaction between the INF2 DID mutants and CDC42 and a reduced fraction of active CDC42 at the plasma membrane. Together, our results suggest that the mislocalization of the INF2–MAL–CDC42 complex in the cytoplasm, as well as the defects in the polymerization and depolymerization activities of INF2 required for actin dynamics, could disrupt protein targeting to the plasma membrane and therefore also disrupt proper myelin formation and maintenance.

The implication of the Rho-GTPase CDC42 pathway in the effect of the INF2 mutants is reminiscent of mutations in two genes involved in dominant intermediate Charcot–Marie–Tooth disease: DNM2, which encodes the GTPase protein dynamin 2, 30 and ARHGEF10, which encodes a guanine exchange factor that activates Rho-GTPases. 31

Finally, sensorineural hearing loss was present in 4 of the 12 families (33%) with an INF2 mutation, which is a prevalence significantly higher than the approximately 5% prevalence reported among patients with Charcot–Marie–Tooth disease. 32 Mutations in DIAPH1, which encodes mDia1, have been associated with autosomal dominant sensorineural progressive hearing loss. 33 The biologic role of mDia1 in hearing is likely to include regulation of actin polymerization in hair cells of the inner ear. Sun and colleagues 13 recently demonstrated that the INF2 DID interacts with the mDia1 DAD. Thus, similar mechanisms are likely to be involved in deafness related to either DIAPH1 or INF2 mutations.

The reasons why INF2 mutations do not always lead to a neurologic phenotype still need to be clarified. One clue, however, is that the mutations underlying FSGS alone or in combination with Charcot–Marie–Tooth neuropathy are clustered in different parts of the DID. The latter mutations are located between two putative DID-binding pockets, suggesting that they could affect DID function more severely than mutations related to FSGS alone, by simultaneously disrupting the interaction of INF2 with multiple proteins, some of which could be specific myelin proteins. This is consistent with the renal phenotype that is more severe in patients with FSGS and Charcot–Marie–Tooth disease than in patients with FSGS (median age at

### Figure 3 (facing page). Colocalization and Interaction of Wild-Type and Mutant INF2 with Myelin and Lymphocyte Protein (MAL).

Panel A (immunoperoxidase stain) shows MAL, INF2, and MAL2 proteins in normal human kidney specimens and peripheral-nerve biopsy specimens. The serial sections shown suggest that INF2 colocalizes with MAL2 in podocytes and MAL in Schwann cells. Panel B shows immunostaining of endogenous INF2 (green) and MAL (red) in MSC-80 mouse Schwann cells (with the merged image also shown). 23 The scatter plot represents red and green pixel fluorescence intensities of the corresponding immunostaining. Both proteins were colocalized (for eight samples analyzed: median Pearson’s coefficient, 0.909; range, 0.625 to 0.978). 24 Panel C shows results of an INF2–MAL pull-down assay performed on the detergent-insoluble (I) membrane fraction of S16 rat Schwann cells separated from the soluble fraction (S) and the Ponceau red staining of the glutathione S-transferase proteins used at the bottom. In Schwann cells, endogenous MAL bound the C-terminal of INF2. Panel D shows the colabeling of wild-type or mutant FLAG-tagged INF2 (red) with myelocytomatosis virus–associated sequence (MYC)–tagged MAL (green) transiently expressed in HeLa cells, with the corresponding scatter plot of red and green pixel fluorescence intensities on the right. In contrast to the perinuclear localization of wild-type INF2, the two INF2 mutants (with amino acid change L165P or R106P) in patients with focal segmental glomerulosclerosis (FSGS) and Charcot–Marie–Tooth (CMT) neuropathy were spread throughout the cytoplasm. As observed with endogenous proteins, wild-type INF2 and MAL recombinant proteins were colocalized (see merged images). For INF2 mutants, the colocalization persisted, leading to a broader cytoplasmic distribution of MAL.
INF2 Mutations in Charcot-Marie-Tooth Disease

**Figure A**

Kidney

Peripheral Nerve

MAL

INF2

MAL2

**Figure B**

MSC-80 Cells

**Figure C**

Input

GST

GST-INF2 C-terminal

**Figure D**

HeLa Cells

INF2 Wild Type

INF2 L165P

FSGS-CMT

INF2 R106P
Relative Interaction of FLAG-INF2 and HA-CDC42–Q61L

Cells with Membrane Localization of CDC42–Q61L (%)

<table>
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<th>Wild Type</th>
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<th>R106P</th>
<th>R177H</th>
<th>R218Q</th>
<th>Y193H</th>
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<tr>
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<td>P = 0.04</td>
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<td>Wild Type</td>
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Not Transfected

Total Lysate

IP HA

B

Cells with Membrane Localization of CDC42–Q61L (%)

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<tr>
<th></th>
<th>Wild Type</th>
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<th>R177H</th>
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</table>

Not Transfected

Total Lysate

IP HA

B

Cells with Membrane Localization of CDC42–Q61L (%)

<table>
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<th>Wild Type</th>
<th>L165P</th>
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MIN INF2 as a crucial molecular entity in the occurrence of FSGS and Charcot–Marie–Tooth neuropathy provides additional insight into the role of similar cellular machinery in podocytes and Schwann cells, even though these two highly specialized cell types have distinct functions.

Supported by grants from the Association pour l’Utilisation du Rein Artificiel (to Dr. Antignac), Association Française contre les Myopathies (ANR-08-GENOPAT-017-01, to Dr. Antignac), Agence Nationale de la Recherche (PodoNet project number ANR-07-E-RARE-011-01 in the ERA-Net Consortium [JTC2007], to Dr. Antignac, and ANR-06-MAR-024-01, to Dr. Leguern), Fondation pour la Recherche Médicale (project number DMP 2010-11-20-386, to Dr. Antignac, and doctoral funding, to Dr. Boyer), Association des Malades du Syndrome Néphrotique (to Dr. Mollet), Fonds de la Recherche en Santé du Québec (Fellowship Training Award to Dr. Benoit), and Ministério de Ciencia e Innovación (BFU2009-07886 and CONSOLIDER COAT CSD2009-00016, to Dr. Alonso).

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APPENDIX

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