Inverted Formin 2 Regulates Actin Dynamics by Antagonizing Rho/Diaphanous-related Formin Signaling

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ABSTRACT

Mutations in inverted formin 2 INF2 are a common cause of familial FSGS. INF2 interacts with diaphanous-related formins (mDia) and antagonizes mDia-mediated actin polymerization in response to active Rho signaling, suggesting that dysregulation of these pathways may mediate the development of INF2-related FSGS. However, the precise mechanisms by which INF2 regulates actin-dependent podocyte behavior remain largely unknown. Here, we investigated the possible role of INF2 in both lamellipodia-associated actin dynamics and actin-dependent slit diaphragm (SD) protein trafficking by manipulating the expression of INF2 and the activity of Rho/mDia signaling in cultured podocytes. Activation of mDia in the absence of INF2 led to defective formation of lamellipodia and abnormal SD trafficking. Effects of mutations disrupting the INF2-mDia interaction suggested the specificity of the mDia-antagonizing effect of INF2 in maintaining the lamellipodium. Furthermore, we found that SD trafficking requires INF2 interaction with lipid raft components. In summary, INF2 regulates lamellipodial actin dynamics and the trafficking of slit diaphragm proteins by opposing Rho/mDia-mediated actin polymerization. Thus, in podocytes, INF2 appears to be an important modulator of actin-dependent behaviors that are under the control of Rho/mDia signaling.


Podocytes are terminally differentiated epithelial cells with interdigitating processes that wrap around and support the capillaries of the kidney’s glomeruli. The terminal portions of these actin-rich extensions, known as foot processes (FPs), are bridged by cell-cell junctions called slit diaphragms (SD), a complex of proteins anchored at adjacent FPs.1,2 FPs are polarized cytoplasmic processes with an apical-basal junction demarcated by the SD complex.3 The SD protein complex participates in regulating the morphology and filter function of podocytes through crosstalk with actin remodeling pathways.4,5

Ultrastructural studies have shown that the FPs contain a central actin bundle surrounded by a cortical actin network. This cortical actin is essential for maintaining the morphology and function of podocytes.6 Through direct connections with the plasma membrane of FPs, cortical actin serves as a scaffold for SD proteins and their communication with actin filaments through signaling pathways and actin-binding proteins.6,7 Actin reorganization and SD protein translocation accompany the foot process effacement and loss of the filtration barrier seen in proteinuric diseases.8

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Members of the Rho family of small GTPases play a central role in controlling the actin architecture of cells. Perturbations in the activity of several of the Rho family GTPases can disrupt the morphology of podocytes and lead to proteinuria. Constitutive activation of RhoA in podocytes in mice causes proteinuric kidney disease and FSGS, with FP effacement, prominent intracellular stress fibers, and altered distribution of SD proteins nephrin and podocin. HIV nephropathy and nephrotoxicity studies have also confirmed that factors altering Rho activity lead to FP effacement and SD dysfunction. Conversely, treatments that antagonize overactivated Rho may be able to restore proper actin dynamics, FP morphology, and the targeting of SD proteins, although expression of a dominant negative RhoA in podocytes also leads to glomerular disease. These and other studies point to the significance of fine-tuning RhoGTPase activity for preserving the actin-dependent phenotype of podocytes. Nevertheless, the precise understanding of the regulation of Rho-mediated actin dynamics required for maintaining the podocyte phenotype remains rudimentary.

Members of the family of the diaphanous subfamily of mammalian formins (or mDia) are major downstream effectors of Rho signaling. The mDias contain an N-terminal GTase-binding domain (GBD) that overlaps a diaphanous inhibitory domain (DID), two regions affecting actin dynamics, the formin homology 1 and 2 (FH1 and FH2) domains, and a C-terminal diaphanous autoregulatory domain (DAD). mDia’s actin polymerizing activity is normally silenced by an intra-molecular DID/DAD interaction (termed autoinhibition). GTP-bound RhoA binding to the GBD disrupts the DID/DAD interaction, allowing mDia-mediated actin nucleation and elongation through the FH1 and FH2 domains.

Previously, we identified a direct interaction between mDia and inverted formin 2 (INF2) using a yeast two-hybrid screen. INF2, like other formin family members, contains an N-terminal DID, a C-terminal DAD, and FH domains. Mutations in the INF2-DID cause FSGS that typically develops in adolescence or adulthood, often leading to overt kidney failure. Some patients with INF2 mutations display both FSGS and the demyelinating condition Charcot-Marie-Tooth disease. In an earlier study, we showed that INF2-DID interacts with and antagonizes members of the mDia formin subfamily, major downstream effectors of Rho. Therefore, we examined whether the effect of INF2 depletion on cortical actin retraction might result from loss of inhibition of Rho-mediated mDia activity. We coexpressed dominant negative forms of either mDia1 or mDia2 (DN-mDia1, encoded by pEGFP-mDia1-DN3 (Hind III)) in cells with INF2 knockdown and found that DN-RhoA or DN-mDia1 could partially restore the normal cortical actin meshwork. We found that treatment with a potent Rho inhibitor, permeable C3 transferase, could also antagonize the retraction of lamellipodia induced by INF2 knockdown (Figure 1). Therefore, we propose that the altered actin architecture in INF2-deficient cells is mediated, at least in part, by loss of inhibition of Rho-mediated actin polymerization and filament elongation in the absence of sufficient INF2 to antagonize the action of mDia.

**RESULTS**

**INF2 Maintains Podocyte Cortical Actin on Lamellipodial Membrane by Opposing Rho/mDia Activity**

Previously, we showed that INF2 antagonizes the effect of Rho/mDia on actin polymerization. FSGS-causing INF2 mutations lead to an ultrastructural phenotype characterized by abnormally thick actin filament bundles. We used small interfering RNA (siRNA) to knock down the endogenous expression of INF2 in cultured podocytes. As shown in Figure 1, knockdown of INF2 to approximately 30% of normal, as indicated by immunoblotting (with an siRNA transfection efficiency of >98%), led to a loss of the cortical actin network (as labeled by cortactin) with more prominent actin stress fibers and parallel actin bundles compared with control cells. These INF2-deficient cells also exhibited elongated morphology. We quantified the fraction of cells containing lamellipodia by measuring the relative area of cortical actin meshwork and the relative length of plasma membrane enriched with cortactin -25 (Figure 1), a protein that is highly associated with the branched lamellipodial actin network. We observed a statistically significant decrease in the relative size of the lamellipodia in cells with INF2 depletion.

In an earlier study, we showed that INF2-DID interacts with and antagonizes members of the mDia formin subfamily, major downstream effectors of Rho. Therefore, we examined whether the effect of INF2 depletion on cortical actin retraction might result from loss of inhibition of Rho-mediated mDia activity. We coexpressed dominant negative forms of either RhoA (DN-RhoA, encoded by pEGFP (Enhanced Green Fluorescent Protein)-RhoA T19N mutant) or mDia1 (DN-mDia1, encoded by pEGFP-mDia1-DN3 (Hind III)) in cells with INF2 knockdown and found that DN-RhoA or DN-mDia1 could partially restore the normal cortical actin meshwork. We found that treatment with a potent Rho inhibitor, permeable C3 transferase, could also antagonize the retraction of lamellipodia induced by INF2 knockdown (Figure 1). Therefore, we propose that the altered actin architecture in INF2-deficient cells is mediated, at least in part, by loss of inhibition of Rho-mediated actin polymerization and filament elongation in the absence of sufficient INF2 to antagonize the action of mDia.

**INF2 Counteracts mDia in Maintaining Cortical Actin Dynamics Independent of Rho**

The dynamic balance between stress fiber formation and cortical actin is thought to be coordinated by Rho/Rac/Cdc42 GTase activity. To exclude the possibility that direct changes in Rho/Rac/Cdc42 GTase activity after INF2 depletion might underlie the altered actin dynamics, we compared Rho/Rac/Cdc42 activity in cells with and without INF2 knockdown by pulldown assays. We observed no changes in Rho, Rac, or Cdc42 activity (Figure 2A). This suggests that INF2 depletion induced actin reorganization independent of
direct activation of Rho but occurs through disinhibition of mDia. By immunofluorescence staining, we observed peripheral membrane enrichment of mDia, but not RhoA, in INF2 knockdown cells. We also observed a shift in mDia, but not Rho, from cytosolic to membrane fractions of cell lysates upon INF2 knockdown (Figure 2C). In INF2 knockdown cells, the size of the actin cortex decreased in concert with plasma membrane enrichment of mDia and a more fusiform cell morphology with prominent parallel actin bundles (Figure 2B). Thus, INF2 depletion disinhibits mDia activity downstream of RhoA.

**INF2 Preserves Lamellipodial Trafficking of SD Proteins by Opposing Rho/mDia Activity**

The cortical actin that is maintained through the active polymerization and depolymerization dynamics of actin provides a platform for cellular events, such as vesicle trafficking and membrane protein turnover.29,30 Loss of cortical actin impairs the trafficking of endosomes and newly synthesized membrane receptor proteins.31 We found that absence of INF2 leads to a decrease in cortical actin and lamellipodia formation. We therefore wished to also determine the role of INF2 in coordinating cortical actin remodeling and membrane trafficking of SD proteins. As shown in Figure 3A, podocin resides along the lamellipodial membrane distal to the cortical mesh, together with areas of cortactin enrichment. However, with INF2 knockdown, the plasma membrane distribution of podocin was strongly reduced. We also compared the distribution of SD protein nephrin in podocytes with and without INF2 knockdown. Nephrin is normally enriched at the plasma membrane adjacent to the cortical actin meshwork in control cells, whereas INF2 knockdown cells showed a decreased cortical actin mesh and a defect in the membrane trafficking nephrin, which appeared to be trapped in vesicular structures (Figure 3B).

In transgenic mouse experiments, Wang et al. found that active RhoA-induced proteinuria and FSGS were associated with reorganization of the podocyte actin cytoskeleton, FP retraction, and redistribution of SD proteins from a linear to a discontinuous pattern.11 Therefore, to determine whether altered membrane trafficking of SD proteins after INF2 knockdown was also mediated by Rho/mDia signaling, we studied the distribution of podocin and nephrin in the presence of DN-RhoA or DN-mDia1. As shown in Figure 3C, in INF2 knockdown cells, coexpression of DN-RhoA or DN-mDia1 restored the cortical actin meshwork as well as
**A** INF2 knockdown does not influence Rho activity

<table>
<thead>
<tr>
<th>Lysate</th>
<th>Con INF2 KD</th>
<th>INF2</th>
<th>β-actin</th>
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**Rho**

Lysate

INF2

Rhotekin pulldown

**Rac1**

Lysate

INF2

Pak-PBD pulldown

**CDC42**

Lysate

INF2

Pak-PBD pulldown

<table>
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<th>INF2 expression</th>
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<th>Rac1 activity</th>
<th>CDC42 activity</th>
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<td>INF2/β-actin</td>
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<td>GTP/Rac/Total rac1</td>
<td>GTP/CDC42/Total CDC42</td>
</tr>
<tr>
<td>Con</td>
<td>INF2 KD</td>
<td>p&lt;0.05 (n=5)</td>
<td>p&lt;0.05 (n=5)</td>
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**B** INF2 knockdown leads to membrane enrichment of mDia

**C** Translocation of protein mDia from cytosol to membrane fractions

![Figure 2](image)

**Figure 2.** INF2 counteracts mDia in maintaining cortical actin dynamics independent of Rho. (A) Rho/Rac/CDC42 activity in podocytes (undifferentiated) with or without INF2 KD was measured by pulldown assay. Expression of INF2 in cells was illustrated by immunoblotting and normalized to the protein level of β-actin. Cell lysates were incubated with Rhotekin or Pak-PBD–conjugated agarose, and the precipitates were subjected to immunoblotting using anti-Rho for Rhotekin pulldown or anti-Rac/CDC42 for Pak-PBD pulldown. The fraction of active (GTP bound, pulldown) Rho/Rac/CDC42 was compared as a percentage of the total Rho/rac/CDC42 level. (B) Podocytes with or without INF2 KD were costained with anti-Rho/anti-mDia and phalloidin. The changes in peripheral membrane recruitment of Rho/mDia (green) in association with actin filaments (red) were compared. Cortical actin area is circled by yellow lines. Local aggregation of mDia and retraction of lamellipodia in cells with INF2 depletion was highlighted by arrows. (C) Rho and mDia in the cytosolic and membrane fractions of cells were detected by Western blotting, and the membrane/cytosolic (M/C) fractions of Rho and mDia were compared in cells with or without INF2 knockdown. HSP 70 and Na+/K+ ATPase were used as markers for cytosolic and membrane fractions, respectively.
Figure 3. INF2 preserves peripheral membrane trafficking of SD proteins by opposing Rho/mDia activity. Cultured podocytes (un-differentiated) were transfected with INF2-targeting siRNA duplex (INF2 KD) or universal negative control RNA duplex (control). Forty-eight hours later, the cells were cotransfected with plasmids encoding HA-podocin or nephrin. (A) HA (newly synthesized podocin, green) was costained with cortactin (red), and the lamellipodial distribution of podocin was compared in cells with or without INF2 KD. (B) The distribution of newly synthesized nephrin (red) and the association with actin filaments (blue) were compared in cells with or without INF2 KD. (C and D) Cells with INF2 KD were cotransfected with DN RhoA or DN mDia1 or were treated with y27632, a Rock inhibitor. (C) The distribution of podocin and the association with F-actin are illustrated by immunofluorescent stain and phalloidin stain. (D) Surface biotinylation was used to measure the surface membrane trafficking of nephrin. The cytosolic (C) and surface (S) nephrin was measured by Western blotting, and the membrane trafficking of nephrin was quantified as the fraction of biotinylated nephrin (S/S+C)%. HSP 70 and Na+/K+ ATPase were used as controls for unbiotinylated and surface-biotinylated proteins, respectively.

membrane trafficking of podocin. Surface biotinylation experiments confirmed the ability of DN-RhoA or DN-mDia1 to restore the plasma membrane localization of nephrin lost by INF2 knockdown (Figure 3D). To exclude the involvement of Rock, an alternative downstream effector of Rho in regulating SD protein trafficking, we inhibited Rock using Y27632. This treatment did not restore the membrane trafficking of podocin or nephrin, supporting the assertion that the function of
INF2 in maintaining the membrane trafficking of SD proteins is mediated by counterbalancing the activity of the otherwise Rho-activated mDia.

**INF2 Directs Lipid Raft–Mediated Lamellipodial Trafficking of SD Proteins**

The trafficking of the nephrin-podocin protein complex to the SD membrane is a lipid raft–mediated process.4 Podocin, a prohibitin homology domain containing protein with affinity for lipid rafts, helps recruit nephrin to the membrane and serves as a scaffolding protein for SD complex organization.32–34 To understand the role of INF2 in maintaining nephrin-podocin complex trafficking, we investigated the interaction of INF2 with podocin and nephrin, as well as the lipid raft scaffolding protein caveolin-1.35 As shown in Figure 4, A and B, in HEK 293T cells that overexpressed specific SD proteins, endogenous INF2 was co-immunoprecipitated with overexpressed hemagglutinin (HA)-podocin, but was immunoprecipitated by nephrin only in the presence of HA-podocin. This finding indicates that podocin mediates an association of INF2 with nephrin. The interactions of endogenous INF2 with endogenously lipid raft components podocin and caveolin-1 were verified by co-immunoprecipitation in differentiated podocytes (Figure 4C). We observed that INF2 co-localizes with both podocin and caveolin-1 along the lamellipodial edge adjacent to the cortical actin meshwork (Figure 4D). With INF2 knockdown, caveolin-1 and podocin, as well as lipid raft components labeled by cholera B toxin, are no longer detected at the plasma membrane edge but rather relocatealize to intracellular vesicular structures (Figure 4E). Although INF2 knockdown did not prevent association of the SD proteins nephrin and podocin with detergent resistant membranes, we did observe a qualitative increase in the fraction of nephrin in non-detergent resistant membranes (Figure 4F). Taken together, these results suggest that INF2 plays a necessary role not only in the trafficking of lipid rafts to the surface but also the recruitment of nephrin to the rafts.

**INF2 Preserves Lamellipodial Trafficking in Response to Rho/mDia Activation**

As shown in Figure 5, when we activated Rho by expressing constitutive active mutant of Rho (CA-RhoA), we found prominent stress fibers (panel A) with loss of cortical actin meshwork and lamellipodia (panel B, quantification) compared with control cells expressing wt-RhoA, which were characterized by a well-developed cortical actin. In cells expressing active mDia2ΔGBD (constitutively active mDia2 that lacks the Rho GBD and is therefore incapable of auto-inhibiting its actin polymerization activity), we found a diminished actin meshwork at the retracted lamellipodia and aggregation of active mDia2 near the cell membrane (panel A, arrows). By contrast, cells expressing full-length mDia2 (autonomous mDia2 as a control) showed a well-developed cortical actin network. Compared with the control cells with a fine distribution of podocin along the lamellipodia, the podocytes with Rho (CA-RhoA) or mDia (mDia2ΔGBD) activation showed a failure in membrane trafficking of nephrin (Figure 6A) and podocin (Figure 6C). When INF2 was coexpressed with CA-RhoA or mDia2ΔGBD, we observed restoration of lamellipodial morphology and membrane trafficking of nephrin (Figure 6A) and podocin (Figure 6C, arrows). We quantified the membrane trafficking of nephrin by measuring the fraction of surface biotinylated protein. We observed a statistically significant decrease in the membrane trafficking of nephrin in cells with active RhoA or mDia2ΔGBD (Figure 6B; P<0.05 CA-RhoA versus wt-RhoA; P<0.05 mDia2ΔGBD versus full-length mDia2), both of which could be restored partially by coexpression of wild-type (wt) INF2 (P<0.05 CA-RhoA+wt-INF2 versus CA-RhoA; P<0.05 mDia2ΔGBD +wt-INF2 versus mDia2ΔGBD). Furthermore, we found that the coexpression of INF2 variant 3, a truncated transcription variant of only INF2-DID without direct actin-binding activity, was sufficient to rescue the disrupted membrane trafficking of nephrin and podocin. This result highlights the importance of INF2’s mDia-antagonizing effects, as opposed to the intrinsic actin polymerization/dem polymerization activity of INF2, in maintaining the lamellipodial biology of podocytes.

**Mutations Disassociating INF2 from mDia Impair INF2’s Rescuing Effects on Lamellipodial Trafficking**

We used the Swiss-model protein structure homology-modeling server (http://swissmodel.expasy.org/)36 to model the INF2-DID homodimer/mDia-DAD interaction based on the crystal structure of mDia1-DID/DAD complex (MMDB: 38033, PDB: 2BAP; Figure 7). Previously, we found that mutations changing amino acid residues E184 and R218 cause human FSGS.21 These residues localize at the interface of INF2-DID homodimer and might stabilize the spatial configuration of the dimer and facilitate binding of INF2 to mDia-DAD. Both E184K and R218Q disrupt the INF2/mDia association as shown by both co-immunoprecipitation and yeast two-hybrid studies.18 In addition, M1041 and L1044 in mDia are key residues that localize to the DID/DAD binding interface and mediate the direct interaction of DID and DAD. Mutations engineered to alter these DAD residues also inhibit INF2-DID/mDia-DAD binding.18 To determine whether maintenance of cortical actin dynamics and trafficking of SD proteins depend on the mDia-DAD/INF2-DID interaction, we introduced mutations into INF2 (E184K, R218Q, human disease-causing mutations) or mDia (M1041A, L1044A). We sought to determine whether these mutations influenced the ability of INF2 to rescue the altered SD protein trafficking caused by active mDia.

In cells expressing full-length mDia2, SD proteins nephrin and podocin (Figure 6, A and C) were targeted to the lamellipodial membrane. In cells expressing active mDia2 mutants mDia2ΔGBD or M1041A and L1044A, nephrin was retained in cytosolic fraction of cells (Figure 6A, by Western blotting analysis of surface biotinylation of nephrin) and podocin was
confining intracellular vesicular structures (Figure 6C). The disrupted trafficking of the proteins by mDia2ΔGBD was restored by the coexpression of WT-INF2. However, the E184K and R218Q INF2 mutants did not restore the membrane trafficking of nephrin (Figure 6B, fraction of nephrin on surface of cells: \(P > 0.05\) mDia2ΔGBD+E184K-INF2 versus mDia2ΔGBD; \(P > 0.05\) mDia2ΔGBD+R218Q-INF2 versus mDia2ΔGBD) and podocin (Figure 6C). In addition, wt-INF2 did not rescue the altered trafficking induced by mutant mDia2 M1041A and L1044A (\(P > 0.05\) mDia2 M1041A &L1044A+wt-INF2 versus mDia2 M1041A &L1044A).

Thus, the specific interaction between INF2-DID and mDiaDAD is essential for mediating the anti-Rho/mDia property of INF2 in preserving cortical actin dynamics and the trafficking of SD proteins in podocytes.

Abnormal Distribution of SD Proteins in Patients with FSGS Carrying R218Q Mutation

To determine whether disease-causing mutations of INF2 lead to failure in the trafficking of SD proteins in patients with FSGS who have these mutations, we immunostained a kidney biopsy section from a patient with FSGS who had an R218Q mutation.
for podocin and nephrin (proband of pedigree FG-JN2). We previously demonstrated that this mutation prevents the interaction of INF2 with mDia. As shown in Figure 8, in both a nonproteinuric person (normal control) and in a kidney biopsy specimen from a patient with FSGS that was secondary to hypertension and obesity, both podocin (in green) and nephrin (in red) assume a linear or continuous distribution along the capillary of the glomerulus with a high degree of colocalization. However, in the glomerulus from the patient with FSGS who carried the INF2 R218Q mutation (R218Q-FSGS), both the proteins show a granular or discontinuous pattern of distribution.

**DISCUSSION**

Point mutations in the INF2-DID can cause isolated FSGS or FSGS together with Charcot-Marie-Tooth disease. INF2 antagonizes Rho-activated actin assembly by binding and inactivating mDia formin proteins. Ultrastructural analysis of an INF2-mutant kidney biopsy specimen showed abnormal aggregation of actin filaments, a common consequence of Rho signaling activation in cultured cells. These findings led us to hypothesize that disease-causing mutations of INF2 could lead to unregulated activation of Rho-actin dynamics in podocytes, possibly through unbalanced actin polymerizing activity of mDia, a target for INF2.

In exploring INF2’s role in regulating actin-dependent features of podocytes in culture, we noticed changes in ruffle morphology. In podocytes depleted of INF2 using siRNA, we observed loss of lamellipodia and prominent parallel actin bundles. The actin reorganization induced by INF2 depletion could be reversed by DN-Rho, Rho inhibitor, or DN-mDia, suggesting that INF2 antagonizes Rho/mDia signaling. We found no evidence of a direct effect of INF2 knockdown on Rho/Rac/CDC42 activity. We did note an increase in the aggregation of mDia at the retracted lamellipodia. Thus, we speculate that INF2 knockdown may lead to actin reorganization caused by uncontrolled mDia activity. Lamellipodia are areas of active actin polymerization and depolymerization turnover and vesicle trafficking, mDia, the major Rho effector mediating actin elongation and stress fiber formation, localizes to lamellipodia. In these studies, we found that selective Rho/mDia activation in podocytes using a constitutively active Rho or mDia leads to more pronounced stress fibers and loss of cortical actin at lamellipodial edges. These changes were reversed by expression of INF2.

In parallel with changes in lamellipodial actin dynamics, we observed altered trafficking of SD complex proteins nephrin and podocin. In cultured podocytes, we observe a fine distribution of SD proteins along the lamellipodial membrane. However, in INF2-depleted podocytes demonstrating a retracted lamellipodia, SD proteins lose their membrane distribution and are trapped in cytoplasmic vesicles. Trafficking of SD protein complex is a lipid raft–dependent process. The SD is a lipid raft–enriched membrane in which the critical SD protein nephrin is recruited. mutations disrupting the
raft localization of podocin or the nephrin/podocin interaction disassociate nephrin from the raft-mediated trafficking pathway, disrupt its membrane localization, and cause congenital proteinuria.\textsuperscript{33,38} We found that INF2 interacts with podocin and caveolin-1, proteins both involved in lipid raft–mediated trafficking; this finding suggests a direct role for INF2 in lipid raft–mediated SD protein trafficking. In addition to podocin and caveolin-1, recent studies have also identified INF2’s association with raft proteins MAL and MAL2, which mediate membrane trafficking of functional molecules in Schwann cells,\textsuperscript{24} T lymphocytes,\textsuperscript{39} Madin-Darby canine kidney cells, and HepG2 hepatocytes.\textsuperscript{39} Here we found that the association of nephrin with INF2 depended on the presence of podocin, indicating the involvement of INF2 in podocin-mediated trafficking of nephrin. We also found that the depletion of INF2 decreased the recruitment of nephrin to lipid rafts and the mobility of the rafts during the surface targeting of SD proteins. Therefore, by connecting actin dynamics and lipid raft–based transporters, INF2 appears to serve as an essential protein in driving the trafficking of SD proteins in podocytes.

The altered distribution of SD proteins upon INF2 knockdown is consistent with recent findings in transgenic mice expressing constitutively active RhoA. These mice develop podocyte FP effacement, proteinuria, and FSGS.\textsuperscript{10,11} In
podocytes of these animals, SD protein distribution was granular and discontinuous, in contrast to the linear distribution normally seen. Wallar and colleagues have shown that controlled Rho/mDia activity is critical for the actin assembly and disassembly dynamics that drive membrane remodeling and directional endosome transport. Excess active mDia disrupts directional myosin-driven vesicle transport. Here we found that the impaired membrane trafficking of SD proteins caused by INF2 knockdown could be restored by dominant negative Rho or mDia, indicating that the failure in trafficking is mediated by uncontrolled Rho/mDia signaling after INF2 loss. This trafficking failure was not rescued by Y27632, an inhibitor for Rock, another downstream effector of Rho, suggesting the effect of INF2 deletion is specific to mDia. We introduced mutations that destroy the INF2-DID/mDia-DAD interaction into both the INF2 and mDia sequence to confirm the selective targeting of active Rho/mDia signaling by INF2 in preserving the lamellipodial trafficking. We found that the trafficking defect caused by active mDia could not be rescued by the disease-causing INF2 mutations E184K or R218Q, nor could wt INF2 rescue the effects of mDia–harboring mutations (M1041A and L1044A) that prevent the INF2–mDia interaction. Together, these results confirm a role for the interaction of the INF2 with mDia in maintaining normal podocyte function. In parallel, we found that SD protein trafficking disrupted by active mDia could also be rescued by INF2-DID, a domain that targets mDia-DAD without direct actin binding or processing functions. This result indicates that the role of INF2 in mediating lamellipodial trafficking is largely due to the mDia-antagonizing effect of INF2, instead of the direct activity of the protein on actin filaments.

In summary, on the basis of our studies, we believe that INF2 mutations lead to uncontrolled Rho signaling and unbalanced actin polymerization. We have demonstrated an essential role of INF2 as a modulator of mDia activity downstream of Rho. INF2 helps maintain lamellipodial actin dynamics and coordinate the trafficking of SD proteins, especially in response to Rho activation. Abnormal activation of Rho signaling has been identified in multiple forms of kidney injury, including diabetic nephropathy, acute ischemia, ischemia-reperfusion, and hypertensive kidney disease. In patients with INF2 mutations, uncontrolled mDia-mediated actin dynamics may predispose to podocyte injury. In the absence of normal INF2 activity, the changes in actin regulation in response to Rho activation may alter podocyte cytoskeletal function, impair the trafficking of SD proteins, and disrupt glomerular filtration function.

**CONCISE METHODS**

**Antibodies**

Rabbit anti-INF2 is a polyclonal antibody raised against amino acids 994–1273 of mouse INF2. Rabbit anti-mDia1/2 is a polyclonal antibody that recognizes both mDia1 and mDia2. Mouse-anti HA and peroxidase conjugate antibodies are from Cell Signaling Technology (Beverly, MA); guinea pig–derived antinephrin is a product of Progenbiotechnik (Germany); rabbit antipodocin is from Sigma-Aldrich Corp., mouse-anti Rho, mouse-anti CDC42, mouse-anti Rac1, and mouse-anti cortactin are from EMD Millipore (Billerica, MA); and mouse-anti Na+/K+ ATPase α1 and rabbit-anti caveolin-1 are from Santa Cruz Biotechnology, Inc. Mouse anti-HSP70 antibody (Heat Shock Protein 70) was obtained from Stressgen Bioreagents. Alexa Fluor labeled secondary antibodies and phalloidin are products of Molecular Probes.

**Plasmids**

pEGFP-mDia2 and pEGFP-mDia2ΔGBD were generated by H.N.H.’s laboratory (Department of Biochemistry, Geisel School of Medicine, Dartmouth University). pCMV6-AN-DDK-INF2 plasmid and pCMV6-AN-Myo-DDK-INF2 Variant3 (containing DID only) plasmid was obtained from OriGene Technologies (Rockville, MD). E184K, S186P, and R218Q mutations were introduced into INF2, and M1041A&L1044A mutations were introduced into mDia using a QuikChange site direct mutagenesis kit (Stratagene). Human podocin was cloned into pcDNA3.1 by PCR to generated HA-tagged podocin. Human nephrin was cloned into pcDNA3.1 by PCR (non-tagged). pEGFP-mDia1N3 (HindIII) was a generous gift from Dr. Shuh Narumiya, Department of Pharmacology, Kyoto University, Japan.

**RNA Interference**

Human podocytes were transfected with INF2-targeting Dicer-Substrate siRNA duplexes (TriFECTa kit from IDT) using Hyperfect transfection reagent (Qiagen) at a final concentration of 10 nM. DS Scrambled-Neg, a randomized RNA duplex sequence (not present in
human genome sequence) served as a negative control. The transfection efficiency was evaluated by Cy3 fluorescent-labeled transfection control duplex. The cells were processed for Western blotting, immunofluorescent stain, or immunoprecipitation 72 hours after the transfection.

Cell Culture
The conditionally immortalized human podocyte cell line developed by transfection with the temperature-sensitive SV40-T gene has been previously described.45 The podocytes were maintained in RPMI1640 with 10% FBS, Insulin-transferrin-selenium (Gibco), and 50 IU/ml penicillin-streptomycin at 33°C (permissive condition). Podocytes maintained at permissive condition were used for transfection experiments. To measure the endogenous interaction of INF2 with podocin and caveolin-1, podocytes were seeded on collagen I–coated plates and differentiated at 37°C for 2 weeks (nonpermissive condition). For the co-immunoprecipitation experiment, HEK 293T cells were maintained in DMEM with 10% FBS at 37°C. Experiments were performed with subconfluent cultures of podocytes to allow for the accurate analysis of lamellapodial structure. We cannot rule out the possibility that increased cell-cell contact might have additional effects on nephrin and podocin trafficking, either independent or dependent on INF2 function.

Lamellipodia Quantification
Immunofluorescent staining was performed to illustrate the expression and distribution of cortactin and actin architecture in podocytes with different treatments, as described elsewhere.25 Following the standard quantification method described previously,25 images were captured with a Cool-SNAPCF camera attached to a Nikon (Tokyo, Japan) Eclipse 80i microscope. Podocytes immunostained for cortactin and F-actin were used for the quantification of lamellipodia. Background fluorescence was subtracted from the image, and individual cells were then digitally outlined with Image J software (National Institutes of Health) to measure total cell fluorescence for cortactin or phalloidin staining. All areas outside the cell were cleared to best visualize the leading edges of lamellipodia. The fluorescence intensity within an entire cell was summed. Lamellipodia was defined as an actin-rich fringe with fluorescence intensity gradually declining with the distance from the edge in phalloidin-stained cells. Cell image intensity thresholds were set automatically by Image J with an isodata algorithm. The threshold pixels in lamellipodia were selected by drawing regions of interest, and the fluorescence intensities within the selected lamellipodia regions were summed. The fraction of a cell containing lamellipodia was expressed as a percentage of total cell area. The rim of cortactin staining at the leading edge was then digitally outlined and its length was expressed as a percentage of the total cell fluorescence. The summed length of lamellipodia was expressed as a percentage of total cell circumferences. Quantification was performed using a blind experimental procedure by an uninformed observer on unidentified samples.

Immunofluorescence Staining
Expression of nephrin and podocin were studied by indirect immunofluorescence in renal biopsy sections from an affected member of family FG-JN with autosomal dominant FSGS caused by an INF2 R218Q mutation,21 as well as a patient diagnosed with secondary FSGS (no INF2 mutation), and a portion of normal kidney tissue (contained within a nephrectomy specimen done for renal carcinoma) as a nonproteinuric control. Formalin-fixed, paraffin-embedded biopsy sections were de-waxed and rehydrated gradually through graded alcohols, followed by antigen retrieval through 3 minutes of autoclaving in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). The sections were then blocked in 10% FCS and

Figure 8. Abnormal distribution of SD proteins in patients with FSGS who carry R218Q mutation. Immunostaining of podocin (green) and nephrin (red) in renal biopsy sections from a nonproteinuric person (normal control), a secondary FSGS (control FSGS), and an individual with FSGS caused by an INF2 R218Q mutation (R218Q-FSGS).
sequentially incubated successively with rabbit antipodocin (dilution 1:100) and guinea pig anti-nephrin (dilution 1:100) antibodies, followed by Alexa Fluor 488-labeled goat anti-rabbit IgG (dilution 1:100) and Alexa Fluor 592-labeled goat anti-guinea pig IgG (dilution 1:100). After mounting, the sections were observed under a confocal microscope.

**Quantification of Cytosol to Membrane Transfer of RhoA and mDia**

Podocytes were transfected with INF2-targeting siRNA or control RNA duplex for 72 hours. After starvation in medium containing 0.5% FBS overnight, the cells were scraped and homogenized in cold homogenization buffer (10 mM Tris–HCl, pH 7.5; 1 mM EGTA; 1 mM MgCl2) supplemented with complete protease inhibitor cocktail (Roche LTD). Cells were disrupted by passing through a 22-gauge needle, and lysates were clarified by centrifugation at 4300 g for 10 minutes. Supernatants were subsequently centrifuged at 50,000 g and 4°C for 60 minutes to produce cytosolic and membrane fractions in a Beckman Optima TLX ultracentrifuge with TLA45 rotor (Beckman Coulter, Inc.). The membrane fraction was washed three times with the hypotonic buffer and dissolved in 1% SDS in buffer A (50 mM Tris–HCl, pH 7.5; 140 mM NaCl; 10% glycerol; 1% Triton X-100) supplemented with protease inhibitor cocktail. Protein concentrations were determined by using Bradford reagent. Equal amounts of protein were loaded on a 4%–20% SDS gel and separated by electrophoresis. Rho and mDia protein in the cytosolic and membrane fractions of cells were measured by Western blotting (mouse anti-Rho A, B&G, rabbit anti-mDia 1/2). HSP 70 and Na+/K+ ATPase were used as markers for cytosolic and membrane fractions, respectively. Protein band intensities were measured using ImageJ software. The fractionation of cytosolic to membrane-associated Rho and mDia was expressed as the percentage of total protein.

**Lipid Raft Fractionation**

Undifferentiated podocytes were transfected with INF2-targeting siRNA or control RNA duplex for 48 hours, followed by cotransfection of plasmids encoding nephrin and HA-podocin. Twenty-four hours later, lipid rafts were isolated from the cells by discontinuous sucrose density gradient ultracentrifugation, as described elsewhere. Cells were washed in ice-cold PBS and lysed in a modified lysis buffer: 1% Triton X-100 in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (25 mM MES, pH 6.5, 150 mM NaCl, 2 mM EDTA complemented with protease inhibitors) on ice. Lysates were homogenized and mixed with an equal volume of 85% (wt/vol) sucrose in MES buffer, and then overlaid with 5.5 ml of 36% sucrose and 2.5 ml of 5% sucrose. The gradients were subjected to ultracentrifugation at 250,000 g at 4°C for 18 hours with a 90-Ti rotor in a Beckman L8M ultracentrifuge (Beckman Coulter, Inc.). Fractions of 1 ml were collected from the top of the gradients. The detergent resistant microdomain (lipid rafts) and the nondetergent resistant microdomain (nonraft microdomain) were labeled by enrichment of caveolin-1 and Na+/K+ ATPase.

**Surface Biotinylation**

The surface trafficking of nephrin was measured by using the Pierce Cell Surface Protein Isolation Kit. After different treatments, podocytes grown on six-well plates were surface-biotinylated on ice using Sulfo-NHS-SS-biotin after different treatments, followed by lysis in modified radioimmunoprecipitation buffer. Lysates were incubated with streptavidin beads at 4°C and spun down to collect the supernatants as the cytosolic fraction. After washes, the streptavidin beads were eluted in equal volume of 1X SDS loading buffer as the surface fraction. The level of nephrin in cytoplasm and surface fractions of cells were analyzed by Western blotting. HSP 70 and Na+/K+ ATPase were used as controls for unbiotinylated and surface-biotinylated proteins, respectively.

**Others**

Rho activation assay and co-immunoprecipitation were performed as described elsewhere.

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**DISCLOSURES**

None.

**REFERENCES**

BASIC RESEARCH


