An mDia1-INF2 formin activation cascade facilitated by IQGAP1 regulates stable microtubules in migrating cells

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INTRODUCTION

Formins are multidomain proteins that regulate the dynamics and organization of both actin filaments and microtubules (MTs) in a variety of cellular functions requiring coordinated action of the cytoskeleton (Bartolini and Gundersen, 2010; Chesarone et al., 2010; Breitsprecher and Goode, 2013). Formins nucleate and elongate unbranched actin filaments through the activities of two domains: formin homology 1 (FH1), which binds to actin-bound profilin, and formin homology 2 (FH2), which is involved in actin polymerization. The FH2 domain of formins dimerizes, and this is essential for formin association with the barbed end of growing actin filaments (Moseley et al., 2004; Zigmond, 2004; Otomo et al., 2005). Of the 15 vertebrate formins, the largest subgroup is the diaphanous-related formins (DRFs), characterized by an N-terminal GTPase-binding domain (GBD), an adjacent diaphanous inhibitory domain (DID), and a C-terminal diaphanous autoregulatory domain (DAD). In many cases, the binding of a Rho-GTPase to the GBD regulates DRF activation by releasing an intramolecular inhibitory interaction between the DID and the DAD domains that keeps the molecule inactive (Alberts, 2001; Amano et al., 2003; Otomo et al., 2005, 2010). However, both activation and deactivation steps are often incomplete or kinetically slow when tested with purified components, suggesting that full activation may require convergence of multiple inputs (Li and Higgs, 2003; Maiti et al., 2012).

Multiple formins regulate microtubule (MT) arrays, but whether they function individually or in a common pathway is unknown. Lysophosphatidic acid (LPA) stimulates the formation of stabilized detyrosinated MTs (Glu MTs) in NIH3T3 fibroblasts through RhoA and the formin mDia1. Here we show that another formin, INF2, is necessary for mDia1-mediated induction of Glu MTs and regulation of MT dynamics and that mDia1 can be bypassed by activating INF2. INF2 localized to MTs after LPA treatment in an mDia1-dependent manner, suggesting that mDia1 regulates INF2. Mutants of either formin that disrupt their interaction failed to rescue MT stability in cells depleted of the respective formin, and the mDia1-interacting protein IQGAP1 regulated INF2's localization to MTs and the induction of Glu MTs by either formin. The N-terminus of IQGAP1 associated with the C-terminus of INF2 directly, suggesting that mDia1 regulates INF2. INF2 localized to MTs after LPA treatment in an mDia1-dependent manner, suggesting the possibility of a tripartite complex stimulated by LPA. Supporting this, the interaction of mDia1 and INF2 was induced by LPA and dependent on IQGAP1. Our data highlight a unique mechanism of formin action in which mDia1 and INF2 function in series to stabilize MTs and point to IQGAP1 as a scaffold that facilitates the activation of one formin by another.
Formins also act as positive regulators of MT organization and stability (Bartolini and Gundersen, 2010; Chesarone et al., 2010). In many cases, activation of formins generates a subset of selectively stabilized MTs that accumulates posttranslational modifications of tubulin, such as deetyrosinated (or Glu) and acetylated tubulin (Palazzo et al., 2001a, 2004; Gundersen et al., 2004; Goulilhari et al., 2005; Andres-Delgado et al., 2012; Thurston et al., 2012). These modified MTs act as specialized tracks for kinesin-dependent transport of cargos such as vimentin intermediate filaments and endocytic vesicles (Kreitzer et al., 1999; Lin et al., 2002; Reed et al., 2006; Konishi and Setou, 2009; Wickstrom et al., 2010). In some cases, the formin activity toward MTs is independent of the actin polymerization activity (Bartolini et al., 2008; Cheng et al., 2011; Andres-Delgado et al., 2012; Daou et al., 2014; Roth-Johnson et al., 2014).

The FH2 domain of DRFs binds MTs and is typically implicated in the MT activity, although other domains also interact with MTs (Bartolini and Gundersen, 2010; Gaillard et al., 2011). The formin FH2 activity toward MTs seems to be regulated by an autoinhibitory mechanism similar to that toward actin. Active Rho GTP stimulates mDia1’s activity to stabilize MTs (Palazzo et al., 2001a, 2004; Goulilhari et al., 2005, 2008), and MT stability can be induced by mDia1 and other formin constructs lacking the DID domain or by expressing their DAD domains (Palazzo et al., 2001a, 2004; Andres-Delgado et al., 2012; Thurston et al., 2012; Daou et al., 2014).

The relatively large number of vertebrate formins raises the question of whether individual formin family members regulate discrete aspects of the actin and MT cytoskeletons. Indeed, it was proposed that individual formin family members might regulate the distinct arrays of actin filaments found in cells (Chhabra and Higgs, 2007), and there is now abundant evidence for this (Chesarone et al., 2010). However, recent studies also reveal that multiple formins cooperate in the regulation of individual actin arrays. For example, FMNL1 and mDia1 have been implicated in the signaling pathway regulating actin assembly during polar body extrusion (Zhang et al., 2015), and both mDia1 and mDia2 RNAs inhibit nuclear actin assembly in NIH3T3 fibroblasts (Baarlink et al., 2013). INF2 was shown to regulate lamellipodial actin dynamics by opposing Rho/mDia-mediated actin polymerization (Sun et al., 2013).

With respect to MTs, which generally form only a single array in cells, there is perhaps an even stronger case for formin collaboration. Most of the formin family members regulate MTs, and there are cases in which multiple formins are required to generate a subset of stable MTs (Bartolini and Gundersen, 2010). For example, in breast carcinoma cells, mDia1, mDia2, and mDia3 function nonredundantly for cortical MT capture (Daou et al., 2014). In T-cells, FMNL1, mDia1, and the closely related formin INF2 are all necessary for the generation of stable deetyrosinated microtubules (Glu MTs), which are essential for centrosome reorientation to the immunological synapse (Andres-Delgado et al., 2012). Nonetheless, whether the formins in these cases exert their effects on MTs sequentially or are required independently is unknown.

Two formins that regulate MT stability, mDia1 and INF2, are known to interact through their DAD and DID domains, respectively (Sun et al., 2011, 2013). The interaction of INF2 with mDia1 has been suggested to negatively regulate mDia1’s action on actin polymerization, but whether it is necessary for regulating MTs is unknown (Sun et al., 2011, 2013). In this study, we addressed whether mDia1 and INF2 function individually or together on MTs using the well-established NIH3T3 fibroblast system, in which Rho induces stable MTs through the formin mDia1 (Cook et al., 1998; Palazzo et al., 2001a). We find that mDia1 functions upstream of INF2, stimulating its localization to MTs. The interaction between mDia1’s DAD and INF2’s DID is required for stable MT formation. Both formins interact with the adaptor protein IQGAP1, and IQGAP1 is required for stable MT formation, INF2 localization to MTs, and promotion of mDia1 and INF2 interaction in vivo. These results suggest a novel mechanism of formin activation in which mDia1 activates INF2’s MT-stabilizing activity through the scaffolding function of IQGAP1.

RESULTS
INF2 is required for lysophosphatidic acid stimulation of Glu MTs

We tested the role of INF2, implicated in the stabilization of MTs in vitro (Gaillard et al., 2011) and T-cells (Andres-Delgado et al., 2012), in the formation of Glu MTs in the well-characterized NIH3T3 fibroblast system. Reducing INF2 expression with two different small interfering RNA (siRNA) oligonucleotides significantly inhibited the percentage of cells with Glu MTs without noticeably affecting dynamic tyrosinated (Tyr) MTs (Figure 1, A–C). The reduction in Glu MTs was comparable to that observed in mDia1 knockdown, and knocking down both formins did not further reduce Glu MTs (Supplemental Figure S1, A and B). Unlike in T-cells, knocking down mDia1, INF2, or both did not affect centrosome reorientation in cells at the edge of wounded monolayers (Figure 1, C and D, and Supplemental Figure S1B). Of note, knocking down either formin also resulted in a significant increase in MT dynamics by increasing MT growth and shrinkage rates and decreasing pausing (Figure 1E, Supplemental Table S1, and Supplemental Movies S1–S3).

Active forms of INF2 induce stable Glu MTs

Next we tested the ability of INF2 to form de novo stable Glu MTs in serum-starved cells that have few Glu MTs (Cook et al., 1998; Palazzo et al., 2001a). Overexpression of full-length INF2 only weakly induced Glu MTs, suggesting that INF2’s MT stabilizing activity is negatively regulated by intramolecular inhibition. Indeed, fragments of INF2 containing the FH2 domain but lacking the N-terminal regulatory domain (FH2DAD, FH1FH2, and FH2) all induced stable Glu MTs above background levels (Figure 2, A–C), indicating that the FH2 alone is capable of generating Glu MTs. The FH2DAD was the only fragment that induced Glu MTs extending to the cell periphery, suggesting that peripheral MT stabilization may require the DAD domain, perhaps to induce endogenous INF2. Consistent with this idea, expression of INF2-DAD stimulated Glu MT formation (Figure 2, D and E). The Glu MTs induced by INF2-DAD, FH1FH2, and FH2 alone were all resistant to nocodazole-induced depolymerization, confirming their stability (Figure 2, F–I). INF2 may also be regulated negatively by membrane association, as expression of FH2-DAD constructs containing a C-terminal consensus motif for prenylation did not strongly induce Glu MTs (Supplemental Figure S2).

Mutation of three leucine residues (Leu767, Leu777, Leu856) to alanine in the DAD of INF2 abrogates its in vitro actin depolymerization but not polymerization activity (Chhabra and Higgs, 2006). Conversely, mutation of Lys792 in the FH2 domain of INF2 to alanine diminishes actin polymerization activity without affecting actin severing and depolymerization (Ramabhadran et al., 2012). We introduced the same mutations alone or in combination in green fluorescent protein (GFP)–INF2 and found that the triple Leu767,777,856, Lys792, or the combined mutants rescued stable Glu MT formation in INF2-depleted cells as well as wild-type INF2 (Supplemental Figure S3A). To confirm this, we expressed an INF2–FH2 construct containing the Lle453/Lys792 mutant shown to severely affect actin polymerization in vitro. Both the Lys792 and the Lle453/Lys792 double mutant retained...
INF2 localizes to Glu MTs in a lysophosphatic acid–dependent manner

INF2 fragments containing the FH2 domain bind to MTs directly and stabilize them against depolymerization in vitro (Supplemental Figure S4; Gaillard et al., 2011), indicating that MT stabilization in cells might be related to direct MT binding. To test this, we analyzed the localization of endogenous INF2 in starved cells before and after stimulation with lysophosphatic acid (LPA) to induce stable Glu MTs. Epifluorescence microscopy showed that INF2 accumulated in a pericentriolar region coincident with Glu MTs only in cells stimulated with LPA (Figure 3A). Of importance, this localization was strongly reduced in cells treated with high doses of nocodazole to induce complete depolymerization of dynamic and stable MTs, suggesting that INF2 associated with MTs. To critically test this possibility, we used total internal reflection fluorescence (TIRF) microscopy, which illuminates a narrow (∼100–200 nm) section of the ventral surface of the cell and was previously used to localize Glu MTs at the ventral surface (Wen et al., 2004). By TIRF microscopy, we found that INF2 appeared as linear streaks after LPA stimulation that colocalized preferentially with Glu MTs (Figure 3, B and C, and Supplemental Figure S5). LPA-dependent association of INF2 with MTs was confirmed by enrichment of the INF2 signal on Triton X-100–resistant Glu MTs by Western blot analysis and immunofluorescence of the insoluble cytoskeletal fraction (Figure 3, D and E). Together these results show that INF2 is required for LPA stimulation of stable Glu MTs in NIH3T3 fibroblasts, its activity toward MTs depends on its FH2 domain (but not its actin polymerization activity), and it localizes to Glu MTs in an LPA-dependent manner.

INF2 functions downstream of mDia1

The involvement of two formins in the stabilization of Glu MTs downstream of LPA raised two possibilities: 1) the two formins function in series, with both of them regulating the same subset of MTs and one of them functioning upstream of the other, or 2) the two formins function in parallel, with both of them inducing a subset of stable Glu MTs. To critically test this possibility, we used total internal reflection fluorescence (TIRF) microscopy, which illuminates a narrow (∼100–200 nm) section of the ventral surface of the cell and was previously used to localize Glu MTs at the ventral surface (Wen et al., 2004). By TIRF microscopy, we found that INF2 appeared as linear streaks after LPA stimulation that colocalized preferentially with Glu MTs (Figure 3, B and C, and Supplemental Figure S5). LPA-dependent association of INF2 with MTs was confirmed by enrichment of the INF2 signal on Triton X-100–resistant Glu MTs by Western blot analysis and immunofluorescence of the insoluble cytoskeletal fraction (Figure 3, D and E). Together these results show that INF2 is required for LPA stimulation of stable Glu MTs in NIH3T3 fibroblasts, its activity toward MTs depends on its FH2 domain (but not its actin polymerization activity), and it localizes to Glu MTs in an LPA-dependent manner.
DAD domains of both formins induced Glu MTs in starved cells but not in cells depleted of their parent molecules (Figure 4A). Of interest, whereas INF2-DAD induced Glu MTs in mDia1-depleted cells, mDia1-DAD failed to induce Glu MTs in INF2-depleted cells. These results suggest that INF2 activity is downstream of mDia1 and that activation of INF2 is sufficient to induce Glu MTs in the absence of mDia1.

We also examined whether the DAD domains could rescue the increased MT dynamics observed in cells depleted of each formin (Figure 1E). Neither DAD domain rescued the increased MT growth or shrinkage rates or decreased pausing in cells depleted of their parent molecules (Figure 4B). Nonetheless, INF2-DAD significantly suppressed these parameters in mDia1-depleted cells, and mDia1-DAD failed to suppress them (Figure 4B, Supplemental Table S2, and Supplemental Movies S4–S12). These results lend further support to the idea that INF2 functions downstream of mDia1 in regulating MTs.

To test the relationship between mDia1 and INF2 further, we examined the localization of each formin in the absence of the other. We detected no obvious alteration in the localization of endogenous mDia1 in INF2-depleted cells (Supplemental Figure S6). However, the accumulation of INF2 along the length of MTs as detected by TIRF was lost in cells deprived of mDia1 (Figure 4C). Thus the localization of INF2 on MTs requires mDia1.

The DID of INF2 has been shown to interact directly with the DAD of mDia1 (Sun et al., 2011), potentially providing an explanation for how activated mDia1 may function upstream to activate INF2. To test this idea, we took advantage of variants in INF2’s DID that cause focal segmental glomerulosclerosis (FSGS) and disrupt this interaction (Brown et al., 2010; Sun et al., 2011). We attempted to rescue Glu MT formation in INF2-depleted cells by expressing wild-type INF2 or these disease variants. Unlike their wild-type counterpart, neither E184K nor R218Q INF2 restored levels of Glu MTs in INF2-depleted cells (Figure 4, D and E). Consistent with this result, mDia1ΔDAD, which cannot bind to INF2 (Sun et al., 2011), failed to rescue MT stability in cells depleted of mDia1 (Figure 4F). Together these results strongly support a role for mDia1 in the regulation of INF2 activity toward MTs and suggest that the interaction between mDia1-DAD and INF2-DID is required for this regulation.

IQGAP1 is required for formin-induced Glu MTs

Despite the fact that mDia1’s DAD interacts directly with INF2’s DID in vitro (Sun et al., 2011), its expression did not induce Glu MTs in serum-starved cells in the absence of mDia1 (Figure 4B). This may reflect the low affinity of interaction between mDia1-DAD and INF2-DID (Sun et al., 2011). We hypothesized that in vivo full-length mDia1 was necessary to bring its DAD into the proximity of INF2, perhaps by binding a protein that bridges the two formins. IQGAP1 is such a candidate bridging molecule: it interacts specifically with activated mDia1,
whether IQGAP1 was required to generate stable Glu MTs. Compared to wild-type (WT) mouse embryonic fibroblasts (MEFs), MEFs from IQGAP1-knockout (KO) mice exhibited almost no detectable Glu MTs by immunofluorescence or Glu tubulin by Western blot (Figure 5, A and B). This phenotype represented defective MT stabilization, as shown by lack of acetylated MTs, another tubulin posttranslational modification associated with MT stability, and the finding that Glu MT levels could be rescued with the MT stabilizer Taxol (Supplementary Figure S7). Lack of Glu MTs was not due to reduced levels of mDia1 or INF2, which in fact were up-regulated compared with WT MEFs (Figure 5B). Of importance, reexpression of IQGAP1 in IQGAP1 KO cells rescued Glu MT levels in these cells (Supplementary Figure S8, A and B). Acute depletion of IQGAP1 by siRNA oligonucleotides also reduced Glu tubulin levels biochemically and Glu MTs by immunofluorescence (Figure 5, C and E). Conversely, overexpression of IQGAP1 in serum-starved cells induced formation of Glu MTs (Figure 5, F and G). Thus IQGAP1 is required for LPA-stimulated Glu MT formation and is sufficient to induce Glu MTs when overexpressed.

As in cells depleted of mDia1, INF2 failed to localize to MTs in cells depleted of IQGAP1 (Figure 5H). However, whereas we detected INF2 along the length of stable Glu MTs, endogenous IQGAP1 only localized to cortical dynamic MTs in NIH3T3 cells (Supplementary Figure S9, A–C). Reexpression of IQGAP1 in IQGAP1 KO MEFs induced INF2 localization to Glu MTs (Supplementary Figure S8C). This result suggests that IQGAP1 may be necessary to activate INF2’s MT-stabilizing activity. To test this, we determined whether IQGAP1 was required for the induction of Glu MTs by mDia1’s DAD domain. mDia1-DAD domain failed to induce Glu MTs when expressed in cells depleted of IQGAP1 (Figure 5I). In addition, IQGAP1 overexpression failed to induce Glu MTs in mDia1- or INF2-depleted cells (Figure 5J). These observations suggest that IQGAP1 is necessary for mDia1’s activation of INF2 and that IQGAP1 cannot stimulate MT stability without the formins.

We confirmed that endogenous INF2 and IQGAP1 interacted by communoprecipitation (Figure 6A). Active mDia1 interacts with the C-terminus of IQGAP1 (Brandt et al., 2007), so it was of interest to determine whether INF2 interacted with the same or a different site on IQGAP1. We found that full-length INF2 and INF2-FH2C, but not INF2-N, interacted with IQGAP1 by pull-down assays using recombinant or overexpressed proteins (Figure 6B-D). INF2-FH2C interacted with an N-terminal fragment of IQGAP1 but not a C-fragment (Figure 6E). Of importance, using recombinant...
whether this complex might form in cells, we used in situ proximity ligation (PLA) assays that generate punctate signals when antibodies to the two interacting species are within 30–40 nm (Soderberg et al., 2006). First, we confirmed that the PLA signal (measured as number of puncta per cell) depended on the presence of mDia1 and INF2 primary antibodies and mDia1 expression (Figure 7, A–D). Next we observed that INF2/mDia1 PLA puncta increased upon LPA addition (Figure 7, E and F) and that INF2/mDia1 puncta were significantly reduced in immortalized MEFs from IQGAP1 KO mice (Figure 7, G and H). These results indicate that LPA induces the association of INF2 and mDia1 in cells and that this depends on IQGAP1.

DISCUSSION

One of the crucial questions for how formins regulate MT stability is whether their action is direct or is mediated by downstream events. A number of formins, including mDia1, mDia2, and INF2, have been shown to bind to MTs and enhance their stability in vitro (Bartolini et al., 2008; Gaillard et al., 2011; Thurston et al., 2012) and in vivo (Palazzo et al., 2001a; Wen et al., 2004; Bartolini et al., 2008; Andres-Delgado et al., 2012; Thurston et al., 2012; Daou et al., 2014). Previously mDia1 was shown to interact with the MT plus end–tracking proteins EB1 and APC, suggesting that the formation of a complex might be involved in generating stable MTs (Gundersen et al., 2004; Wen et al., 2004). Here we found that a second formin, INF2, implicated in MT stability in T-cells, was also required for MT stability and regulation of MT dynamics in NIH3T3 fibroblasts and, critically, functioned downstream of mDia1. Either formin was necessary to reduce MT growth and shrinkage rates while increasing pausing, suggesting that at steady state, Glu MT stability is critically dependent on reduction of MT dynamics as if stabilized by a “capping” mechanism indicated by previous observations (Cook et al., 1998; Infante et al., 2000; Palazzo et al., 2001a). Consistent with a more proximal effect on MTs, INF2 localized to stable MTs, and this localization required mDia1 and IQGAP1. These data suggest a revised model for MT stabilization by formins in which one formin (mDia1) activates a second formin (INF2), allowing it to bind to and stabilize MTs through its FH2 domain.

In addition to functional and localization data, this sequential model for formin action on MTs is supported by interaction data on mDia1, INF2, and IQGAP1. A previous study showed that the DAD domain of mDia1 interacted with the DID domain of INF2 (Sun et al., 2011), and we found that this interaction was essential for proteins, we found that the interaction between INF2-FH2C and IQGAP1-N was direct (Figure 6F). Together these data show that each of the three components, mDia1, INF2, and IQGAP1, associate via nonoverlapping sites, potentially forming a trimeric complex (Figure 6G).

We attempted to isolate a complex by sucrose gradient fractionation and coimmunoprecipitation but did not detect one, suggesting that it may form transiently or may not resist cell lysis. To assess
IQGAP1 may act to bring mDia1 and INF2 together to facilitate interaction between their DAD and DID. Indeed, our data showing that IQGAP1 is required for induction of stable Glu MTs by LPA or the DAD domains of either formin strongly support such a model. In addition, our studies show that INF2 and IQGAP1 interact through sites that would allow simultaneous binding of mDia1. Despite this, we were unable to isolate a stable complex containing the three proteins. Nonetheless, we used PLA to show that mDia1 and INF2 interacted in vivo and that this interaction was stimulated by LPA and required IQGAP1. Together these results suggest that the interaction between mDia1, INF2, and IQGAP1 is transient and may release INF2 once it has been activated.

To our knowledge, these data are the first to suggest that formins function in series to regulate the formation of a cytoskeletal array. Previous studies implicated multiple formins in regulating both the actin and MT cytoskeletons but did not show that two formins functioned sequentially to induce a single cytoskeletal change. This result raises the question of whether INF2 is a downstream effector of mDia1 only or whether other formins can converge in the activation of INF2 to regulate MT stability, thus allowing activation of MT stability downstream of multiple stimuli. It is also possible that the mDia1-INF2 pair can be activated to regulate actin structures in addition to stable MT arrays. For example, INF2's DID interaction with mDia1's DAD was shown to inhibit the polymerization of actin by mDia1 (Sun et al., 2011, 2013). Formins are known to interact with other regulators of the cytoskeleton, including MT plus end–tracking proteins EB1 and APC (Gundersen et al., 2004), MT-modifying enzyme HDAC6 (Destaing et al., 2005), and actin regulators APC (Moseley et al., 2007; Webb et al., 2009; Okada et al., 2010) and spire (Rosales-Nieves et al., 2006; Dahlgaard et al., 2007; Quinlan, 2013). Perhaps the selected recruitment of these cytoskeletal regulators downstream of distinct signaling pathways contributes to the formation of distinct cytoskeletal arrays.

Unlike loss of MT stability, no effect on centrosome reorientation was detected in either mDia1- or INF2-silenced cells, suggesting that, unlike T-cells, the activity of these formins on Glu MTs has no functional consequence on the polarization of the centrosome/nuclear axis in NIH3T3 cells at the edge of a wounded monolayer. T-cells seem to depend on Glu MTs for centrosome orientation, but repeated studies show that this not the case in fibroblasts (Palazzo et al., 2001a; Bartolini and Gundersen, 2006). We cannot
fully explain this discrepancy, but it may reflect differences in the mechanisms of centrosome orientation in the two systems: in NIH3T3 fibroblasts, the nucleus is moved rearward to orient the centrosome (Gomes et al., 2005; Luxton et al., 2010; Luxton and Gundersen, 2011), whereas in T-cells, the centrosome is moved (Ritter et al., 2013).

Our work is not the first to implicate IQGAP1 as a scaffolding protein for regulators of MTs. For example, CLIP-170 and APC interact with IQGAP1 to mediate the transient capture of MTs at cortical regions (Watanabe et al., 2004). Similarly, CLASP2, which also interacts with IQGAP1, is involved in MT stability in migrating cells (Drabek et al., 2006) and might regulate the association of IQGAP1 with MTs and EB1 (Watanabe et al., 2009). Induction of Glu MTs in starved cells was shown to occur upon GSK3 inhibition caused by mDia-mediated novel protein kinase C activation, suggesting positive regulation of CLASP2/IQGAP1 complex formation by formin function (Eng et al., 2006). Comprehensive in vitro and in vivo analysis will be required to understand the sequence of the association of all these proteins and whether they assemble into a MT “stabilosome.”

Mutations in INF2’s DID domain that disrupt the interaction between INF2 and mDia1 have been implicated in the etiology of FSGS and Charcot–Marie–Tooth (CMT) diseases (Brown et al., 2010; Boyer et al., 2011; Sun et al., 2011, 2013). Our results show that these mutations also prevent the formation of stable Glu MTs, raising the possibility that lack of MT stability may contribute to these diseases. CMT hereditary neuropathy refers to a group of disorders caused by a variety of mutations affecting axonal function, the insulating myelin coating, or both, whereas FSGS is primarily a glomerular disorder that causes renal dysfunction and in which the affected genes encode regulators of the actin cytoskeleton in podocytes. Although loss of INF2-mediated inhibition of Rho/mDia-driven actin polymerization in the foot process of podocytes is one mechanism proposed for mutant INF2 in the pathogenesis of FSGS (Sun et al., 2011, 2013), the precise role of INF2 mutations in the etiology of CMT and FSGS is largely unexplained. MTs are known to maintain the shape and integrity of podocyte major processes, and disruption of MT stability is predicted to indirectly affect the delivery of regulators of actin dynamics in the foot processes. In addition, loss of MT stability by mutant INF2 might equally be crucial for maintenance of axonal integrity at the onset of CMT. Examination of MT stability in tissue or cells isolated from patients may reveal whether loss of MT stability by mutated INF2 correlates with the onset of neuropathy and/or kidney dysfunction.

MATERIALS AND METHODS
Plasmids and reagents
DNA constructs expressing the following DNA fragments tagged with c-myc or glutathione S-transferase (GST) at their amino terminus were described previously (Andres-Delgado et al., 2010, 2012; Madrid et al., 2010): myc-FH2DAD (amino acids [aa] 532–1010); myc-FH1FH2 (aa 266–955); myc–WT FH2 (aa 532–955) or with the Lys792Ala or triple Leu976, Leu986Ala, or combined mutations (Lys792Ala/Leu976, Leu977, Leu986Ala, or combined mutations (Lys792Ala/Leu976, Leu977, Leu986Ala) were made by cloning INF2 sequences from the corresponding constructs in pSPORT6 (Madrid et al., 2010) in the pEGFP-C1 expression vector. DNA constructs expressing FH2DADC fragments with mutations in the CAAX box of INF2-1 (FH2DADC-AIVQ and FH2DADC-CIVL) or with the carboxyl-terminal end of INF2-1 substituted by that of INF2-2 (splicing variant 2 without CAAX box; FH2DAD-N) were achieved by PCR using a perfectly matched 5′ oligonucleotide primer and a 3′ oligonucleotide primer containing the appropriate modification and cloning the amplification fragment in the plasmid pCR3.1 (ThermoFisher Scientific, Waltham, MA).
was made by cloning the myc-tagged FH-2DADC1 fragment of INF2-1 in pGEX-4T-1. His-tagged-mDia1-FH2DAD (aa 748-1203) was a kind gift of Robert Grosse (University of Heidelberg, Heidelberg, Germany; Brandt et al., 2007). GFP-mDia1 lacking the DAD domain was generated using standard PCR procedures using a template kindly provided by S. Narumiya (Kyoto University, Kyoto, Japan). GFP-INF2-E184K and -R218Q mutants were constructed using a QuikChange Site-Directed Mutagenesis kit (Stratagene, San Diego, CA) according to the manufacturer’s protocols. Cherry-mDia1FH1COOH was generated as described (Okada et al., 2010). GFP-IQGAP1, GFP-IQGAP1-C (1950–1657), and -N (1–550), as well as GST-IQGAP1-N and -C plasmids, were kindly provided by Geri Kreitzer (Cornell University, Ithaca, NY) and George Bloom (University of Virginia, Charlottesville, VA). All constructs were verified by sequencing. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. LPA was obtained from Avanti (Alabaster, AL).

**Antibodies**

Antibodies used include polyclonal rabbit 301 generated against INF2 peptide SVKEGAQRKWAALKEKLGPC (amino acids 2–19), mouse monoclonal supernatant (1E4) against the same sequence, and rabbit polyclonal anti-INF2 (Proteintech, Rosemont, IL). The INF2 peptide was synthesized in an automated peptide synthesizer (Abimed, Langerfeld, Germany) coupled to keyhole limpet hemocyanin (ThermoFisher Scientific) and injected in rabbits and mice. Spleen cells from immunized mice were fused to myeloma cells and a hybridoma clone (E4) selected that produced antibody that recognized INF2 in extracts from Cos-7 cells transiently expressing myc-tagged INF2. The specificity of rabbit antiserum and the E4 monoclonal antibody was assayed by immunoblot (IB) and immunofluorescence (IF) analyses. Other antibodies used include mDia1 (clone 51; 1:100 for IF, 1:500 for WB; BD Biosciences, San Jose, CA), rat anti–tyrosinated tubulin (YL-1/2; 1:10 for IF, 1:1000 for IB), rabbit anti-Glu tubulin (1:500 for IF, 1:10,000 for IB; Gundersen et al., 1984), mouse (1:8000; ThermoFisher Scientific) or rabbit (FL-335; 1:1000; Santa Cruz Biotechnology, Dallas, TX) anti–glyceraldehyde 3-phosphate dehydrogenase, mouse anti–β-actin (C4; 1:100), mouse anti-pericentrin (1:100; BD Biosciences), mouse anti-Myc (9E10; 1:100; Santa Cruz Biotechnology), mouse anti–acetylated tubulin (6-11B-1; 1:100), mouse anti–α-tubulin (DM1A; 1:1000), rabbit
anti-GFP (Millipore, Billerica, MA), mouse (10 μg/ml for IF; 1 μg/ml for IB) and rabbit (1:250 for IF; 1:1000 for IB) anti-IQGAP1 antibodies (kind gifts of George Bloom), and rabbit anti-IQGAP1 (H-109; 1:100 for IF; 1:500 for IB; Santa Cruz Biotechnology). For endogenous immunolocalization, a mouse anti-IQGAP1 from BD Biosciences was used (1:100).

Protein purification, GST pull-down, and direct protein-binding assays
GST-tagged proteins (GST, GST-INF2-FH2DADC, GST-INF2-FH2DAD, GST-INF2-C, and GST-INF2-N) were expressed in Escherichia coli BL-21 and purified on agarose-coupled glutathione (GE Healthcare, Little Chalfont, United Kingdom) according to the manufacturer’s protocols. Histidine-mDia1-FH2DAD was purified as described (Bartolini et al., 2008). Purified proteins were dialyzed into HKCL buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and 140 mM KCl) using Slide-A-Lyzer dialysis cassettes (10,000 MWCO; ThermoFisher Scientific) overnight at 4°C. GST-pull-down assays were carried out by incubating glutathione-agarose–bound purified GST-tagged proteins (20 μg) with precleared whole-cell lysates prepared using NP-40 lysis buffer (20 mM Tris-base, pH 7.4, and 140 mM KCl) with precleared HKCL buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and 140 mM KCl) using Slide-A-Lyzer dialysis cassettes (10,000 MWCO; ThermoFisher Scientific) overnight at 4°C. GST-pull-down assays were carried out by incubating glutathione-agarose–bound purified GST-tagged proteins (20 μg) with precleared whole-cell lysates prepared using NP-40 lysis buffer (20 mM Tris-base, pH 7.4, and 140 mM KCl) with precleared HKCL buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and 140 mM KCl) using Slide-A-Lyzer dialysis cassettes (10,000 MWCO; ThermoFisher Scientific) overnight at 4°C. GST-pull-down assays were carried out by incubating glutathione-agarose–bound purified GST-tagged proteins (20 μg) with precleared whole-cell lysates prepared using NP-40 lysis buffer (20 mM Tris-buffered saline, pH 7.4). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA) and were preabsorbed to minimize cross-reaction with other species. Immunostained samples were observed using a Nikon TE2000 microscope with a 60×/1.45 objective and an Orca II ER charge-coupled device (CCD) camera (Hamamatsu, Hamamatsu, Japan) controlled by MetaMorph software. In experiments assessing Glu MT levels, injected cells or 200 cells randomly chosen at the edge of an artificial wound were counted to manufacturer’s protocols (Invitrogen). siRNA duplexes targeting mDia1 and INF2 were based on previously published sequences (Bartolini and Gunnersen, 2006; Madrid et al., 2010). IQGAP1 siRNA (5′-GGAUAUUUAACGUGGUAUdTdT-3′) was generated through the Dharmacon (Lafayette, CO) siRNA design algorithm (www.thermoscientificbio.com/design-center/), and a scrambled noncoding sequence provided by the manufacturer was used as a negative control. Knockdown efficiency and percentage of cells with Glu MTs or oriented centrosomes were analyzed 48 or 72 h after transfection as indicated in the figures. For cDNA expression for immunoprecipitation studies, NIH3T3 fibroblasts were transfected at 30% confluence in growth medium with Lipofectamine Plus (ThermoFisher Scientific) according to the manufacturer’s protocols and allowed to express for 24 h. For cDNA expression in cells previously silenced by siRNA transfection, cells were transfected 48 h after siRNA transfection and allowed to express an additional 24 h before fixation. For Western blot analysis, unless otherwise stated, cells were lysed in RIPA buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, 1 mM PMSF, and protease and phosphatase inhibitor cocktails) for 30 min. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Samples were boiled in sample buffer (50 mM Tris-HCl, pH 8, 120 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol [DTT], phenylmethylsulfonyl fluoride [PMSF], and protease and phosphatase inhibitor cocktails from ThermoFisher Scientific) from cells transfected with the indicated GFP constructs. Complexes were incubated on ice, and cell lysates were precleared by centrifugation at 4°C, and microinjected into nuclei of cells selected randomly from the edges of a wounded monolayer 2 h before fixation and immunostaining.

siRNA, cDNA transfection, and Western blot analysis
NIH3T3 fibroblasts were transfected with siRNA oligonucleotides (Shanghai GenePharma, Shanghai, China) using RNAiMax according to manufacturer’s protocols (Invitrogen). siRNA duplexes targeting mDia1 and INF2 were based on previously published sequences (Bartolini and Gunnersen, 2006; Madrid et al., 2010). IQGAP1 siRNA (5′-GGAUAUUUAACGUGGUAUdTdT-3′) was generated through the Dharmacon (Lafayette, CO) siRNA design algorithm (www.thermoscientificbio.com/design-center/), and a scrambled noncoding sequence provided by the manufacturer was used as a negative control. Knockdown efficiency and percentage of cells with Glu MTs or oriented centrosomes were analyzed 48 or 72 h after transfection as indicated in the figures. For cDNA expression for immunoprecipitation studies, NIH3T3 fibroblasts were transfected at 30% confluence in growth medium with Lipofectamine Plus (ThermoFisher Scientific) according to the manufacturer’s protocols and allowed to express for 24 h. For cDNA expression in cells previously silenced by siRNA transfection, cells were transfected 48 h after siRNA transfection and allowed to express an additional 24 h before fixation. For Western blot analysis, unless otherwise stated, cells were lysed in RIPA buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, 1 mM PMSF, and protease and phosphatase inhibitors), normalized for protein concentration by bicinchoninic acid assay, boiled in SDS sample buffer, separated by SDS–PAGE, and Western blotted. Incubation with primary antibodies was followed by incubation with the appropriate IR680- or IR800-conjugated secondary antibodies (1:10,000; Rockland Immunocyticals, Pottstown, PA), and final data acquisition and quantification were performed using an Odyssey imaging system (Li-COR Biosciences, Lincoln, NE).

Epifluorescence and TIRF microscopy
NIH3T3 fibroblasts grown on coverslips were typically fixed in methanol at −20°C for 10 min to preserve MT integrity and rehydrated in TBS (10 mM Tris-buffered saline, pH 7.4). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA) and were preabsorbed to minimize cross-reaction with other species. Immunostained samples were observed using a Nikon TE2000 microscope with a 60×/1.45 objective and an Orca II ER charge-coupled device (CCD) camera (Hamamatsu, Hamamatsu, Japan) controlled by MetaMorph software. In experiments assessing Glu MT levels, injected cells or 200 cells randomly chosen at the edge of an artificial wound were scored as positive if >10 MTs were brightly stained by the Glu antibody. This cutoff was selected based on previous considerations (Cook et al., 1998; Palazzo et al., 2001a). For centrosome orientation, cells at the edge of an artificial wound were randomly selected and scored as previously described (Palazzo et al., 2001b). Both counts were preformed blinded. Data were quantified and expressed as means ± SEM from at least three independent experiments (>200 cells/experiment).

MT dynamics
NIH3T3 fibroblasts were infected with pMSCV-puro-tagRFP-C4 α-tubulin retrovirus to generate a red fluorescent protein (RFP)–tubulin stably expressing cell line. RFP-tubulin NIH3T3 cells were transfected with noncoding control siRNA (NC) or either siRNA to silence Dia1 (siDia1) or INF2 (siINF2) for 72 or 48 h and then transfected...
with enhanced GFP control, mDia1 DAD-EGFP (aa 1180–1255), or INF2 DAD-EGFP (aa 955–1010) for another 24 h. Live imaging of MT dynamics in transfected cells was performed at 37°C and 5% CO₂ for 5 min (5 s/frame) with a 100x PlanApo objective (numerical aperture 1.45) and an iXon X3 CCD camera (Andor, Belfast, United Kingdom) on a Nikon Eclipse Ti microscope controlled by Nikon’s NIS-Elements software (Nikon, Tokyo, Japan). Movies were analyzed by ImageJ using a manual tracking plug-in. Statistics among groups were performed by two-way analysis of variance (ANOVA test).

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Preparation of detergent-resistant cell fraction
Confluent starved cells before and after treatment with LPA for 2 h were scraped and washed twice in ice-cold phosphate-buffered saline before lysis in ice-cold fractionation buffer (10 mM 1,4-piperazineethanesulfonic acid, pH 6.8, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 0.5% Triton X-100, 1 mM DTT, and protease inhibitors from ThermoFisher Scientific) for 20 min on ice. The cell lysate was centrifuged at 4°C for 3 min at 15000 × g to isolate the soluble from the Triton X-100-insoluble fraction. Denaturing SDS loading buffer was added and matching aliquots of each protein boiled and loaded onto SDS–PAGE for Western blot analysis.

In situ location and function

Samples were fixed in methanol at −20°C and processed for Duolink in situ amplification as suggested by the manufacturer’s protocol (Onlink Bioscience, Uppsala, Sweden) using mouse anti-mDia1 (clone 51, 1:100; BD Biosciences) and rabbit anti-INF2 (301). Random images of stained samples were captured by epifluorescence microscopy using a 60× oil immersion objective, and PLA signals were quantified using custom software. Briefly, cell boundaries were drawn, and the number of PLA puncta and their sizes (in pixels) were determined by the software using thresholded images. Data were exported to Microsoft Excel or GraphPad Prism 4.0 for statistical analysis and plotting. All PLA experiments were performed at least three times, and for each experiment, 10 images were taken per condition using identical microscope and camera settings. In each experiment, a minimum of 50 cells were counted per condition and used for analysis.

Other methods
See Supplemental Materials and Methods for MT binding and stability assays and confocal microscopy.

ACKNOWLEDGMENTS
We thank Feng Ning Yuan for excellent technical assistance and members of the Gundersen lab for very helpful discussions and critical comments on the manuscript. We are particularly grateful to Wakam Chang for writing the software to count PLA puncta and David Sacks (National Institutes of Health, Bethesda, MD) for sharing IQGAP1-null MEFs. Our gratitude goes to George Bloom and David Sacks (National Institutes of Health, Bethesda, MD) for sharing IQGAP1 reagents. This work was supported by National Institutes of Health Grant GM105536 to G.G.G. and Geri Kreitzer for sharing IQGAP1 reagents. This work was supported to M.A.A. from the Ministerio de Economía y Competitividad, Spain.

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