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FHOD1 coordinates actin filament and microtubule alignment to mediate cell elongation

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Abstract

Diaphanous-related formins (DRFs) are actin nucleators that mediate rearrangements of the actin cytoskeleton downstream of specific Rho GTPases. The DRF Formin Homology 2 Domain containing 1 (FHOD1) interacts with the Rac1 GTPase and induces the formation of and associates with bundled actin stress fibers. Here we report that active FHOD1 also coordinates microtubules with these actin stress fibers. Expression of a constitutive active FHOD1 variant in HeLa cells not only resulted in pronounced formation of FHOD1-actin fibers but also caused marked cell elongation and parallel alignment of microtubules without affecting cytokinesis of these cells. The analysis of deletions in the FH1 and FH2 functional regions revealed that the integrity of both domains was strictly required for FHOD1's effects on the cytoskeleton. Dominant-negative approaches demonstrated that filament coordination and cell elongation depended on the activity of the Rho-ROCK cascade, but did not involve Rac or Cdc42 activity. Experimental depolymerization of actin filaments or microtubules revealed that the formation of FHOD1-actin fibers was a prerequisite for the polarization of microtubules. However, only simultaneous disruption of both filament systems reversed the cell elongation induced by activated FHOD1. Thus, sustained cell elongation was a consequence of FHOD1-mediated actin–microtubule coordination. These results suggest filament coordination as a conserved function of mammalian DRFs.

Keywords: Diaphanous-related formin; FHOD1; Microtubule polarization; Actin stress fibers; Cell elongation

Introduction

Formins are multidomain proteins that modulate cytoskeletal architecture. Conserved in eukaryotes from yeast to mammals, they control cell polarity during processes such as morphogenesis, motility, cytokinesis, and differentiation by organizing the actin cytoskeleton and microtubules (MTs) [1–3]. Formins are characterized by their highly conserved formin homology domains FH1 and FH2, which are required for interactions with cellular ligands to mediate effector functions. While the FH1 domain consists of a characteristic accumulation of proline residues, FH2 domains are complex structures with a specialized fold that positions lasso and loop structures for crucial protein interactions [4,5]. Some formins additionally contain a less conserved FH3 domain that might determine their subcellular localization [6]. Among the formin protein family, a subgroup termed Diaphanous-related formins (DRFs) has recently attracted considerable attention. This stems from the ability of DRFs to physically interact with small Rho GTPases and to function as GTPase effectors. GTPase binding occurs at a GTPase binding domain (GBD) at the N-terminus of the DRF and releases the autoinhibitory interaction of the GBD with the Diaphanous autoinhibitory domain (DAD) at the far C-terminus of the protein [7].

Functional consequences of DRF activation include cytoskeletal rearrangements and transcriptional activation of specific promoters such as the serum response element

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(SRE) [8,9]. These effects are directly mediated by the conserved ability of DRFs to nucleate actin polymerization. DRFs nucleate actin from the barbed end resulting in the formation of short, unbranched actin filaments, thus employing a mechanism that is distinct from that of other nucleators such as the Arp2/3 complex [10]. The FH2 domain represents the minimal domain sufficient for nucleation [11–15], and profilin binding to the FH1 domain enhances nucleation for some but not all DRFs analyzed. Correspondingly, for DRFs such as mDia1, fragments containing both domains are more efficient in nucleating actin than the isolated FH2 domain [11,16,17].

Distinct DRF-small GTPase pairs have been identified in mammalian cells that confer specific phenotypes upon activation of the DRF by the GTPase. This is best understood for the interaction of RhoA-C with the DRF effector mDia1, where activation of mDia1 results in the formation of actin stress fibers and concomitant transcriptional activation of the SRE [8,9,18]. These effects are dependent on the kinase activity of another Rho effector, the ROCK kinase that facilitates bundling of nucleated filaments into stress fibers. Similarly, the yeast DRFs Bni1 and Bnr1 interact with Rho GTPases to cause formation of thick actin cables [19]. In contrast, RhoD associates with another DRF effector, hDia2C, to regulate endosome motility [20]. For Cdc42, the specific interaction with mDia3 was demonstrated to facilitate formation of microspikes [21]. In contrast, the role of the two Rac-interacting DRFs identified thus far, FRL and FHOD1, as Rac effectors is less clear. While FRL is exclusively expressed in macropahages, where it modulates actin organization [22], the FHOD1 could represent a more general DRF effector of Rac because of its ubiquitous expression pattern [23].

Additionally, DRFs also modulate the organization of the MT network. The fission yeast Cdc12p drives formation of the medial actomyosin ring [24], while For3p organizes actin and MTs for polarized growth and symmetric cell division [25,26]. Similarly, the *S. cerevisiae* DRFs Bni1p and Bnr1p are essential for mitotic spindle organization [12,16,19,25,27,28]. These effects are believed to represent indirect consequences of DRF-induced actin cable formation. In mammalian cells, mDia1 and mDia2 are localized partially to the cleavage furrow of mitotic cells to facilitate cytokinesis [9,29]. This localization is likely a consequence of the ability of these DRFs to associate with stable, detyrosinated MTs that are further stabilized by DRF binding [30]. Depending on the cell system used, different consequences of DRF activation on MT organization were observed. In NIH3T3 cells, activated mDia1 induced formation of an array of stabilized MTs that were partially decorated by the DRF without apparent involvement of the actin filament system or ROCK [30]. However, in HeLa cells, activation of mDia1 causes alignment of MTs with actin stress fibers resulting in marked cell elongation. Interestingly, this cell elongation was primarily driven by MTs, whereas actin filaments appeared dispensable for sustained cell elongation [31]. Thus, actin filaments and the MT network functionally cooperate in different cell types and Rho GTPases with their effectors bridge actin and MT organization. The mechanism as well as physiological relevance of the various DRF-mediated alterations of MT organization is still unknown.

Whether MTs are also modulated by mammalian DRFs other than mDia1 is an open question. The DRF FHOD1 is one candidate with the potential for cytoskeletal remodeling that specifically binds to and is activated by the Rac1 GTPase in vitro and in vivo [32,33]. Rac is known to regulate the organization of both actin filaments and MTs [34,35]. FHOD1 may serve as effector for some of the effects of Rac on actin, since activated FHOD1 causes the formation of thick actin stress fibers that are decorated by the DRF. These alterations are paralleled by morphological changes of the cell [32,36,37]. Since these effects strictly depend on the activity of the Rho effector ROCK, and FHOD1 does not induce Rho activity [32], the DRF may link signaling pathways controlled by Rac and Rho downstream of the GTPases to coordinate cytoskeletal remodeling. However, the involvement of MTs in these processes has not yet been addressed. In this study, we therefore set out to analyze the effects of FHOD1 activation on MT organization. While active FHOD1 induced cell elongation in both NIH3T3 and HeLa cells, only HeLa cells displayed marked alignment of MTs with FHOD1-induced actin stress fibers. MT alignment was dispensable for stress fiber formation but MT polarization was strictly dependent on the presence of FHOD1-actin fibers. This coordination of actin filaments and MTs by FHOD1 required the FH1 and FH2 domains of the DRF as well as the activity of the Rho-ROCK cascade. Interestingly, simultaneous disruption of both actin filaments and MTs was necessary to overcome FHOD1-induced cell elongation. Thus, FHOD1 coordinates actin filaments and MTs in HeLa cells.

Materials and methods

Expression constructs

Expression plasmids for GFP-tagged GTPases and for the various HA-tagged FHOD1 proteins used were described earlier [32]. Deletions of the FH1 and FH2 domains in the full-length and ΔC constructs, respectively, were introduced by ligating the *Afl*III/*Bam*HI digested PCR amplicons into the *Nco*I/*Bam*HI restriction sites of the EF-HA plink vector [38] following standard cloning protocols. All PCR products were generated using gene-specific oligonucleotides (MWG Biotech AG) and high-fidelity polymerase (Roche). The correctness of the deletions was verified by sequencing in all clones. For GFP.FHOD1 fusions, PCR amplicons encoding full-length FHOD1 (aa 1–1164) or C-terminal truncated FHOD1 (aa 1–1010)

were introduced into the *Eco*RI restriction site of the pEGFP C2 expression vector (Clontech). The expression plasmid for YFP-paxillin was a kind gift of Inna Grosheva.

Cells and transfections

NIH3T3 and HeLa cells were maintained in standard DMEM low and high medium, respectively. Media were complemented with 10% fetal calf serum, 2 mM glutamin, 50 μg/ml penicillin, and 50 U/ml streptomycin at standard conditions. All media components were purchased from Life Technologies. Prior to transfection, cells were plated onto glass coverslips and grown to 60–80% confluence over night. HeLa and NIH3T3 cells were transfected with a total of 1 μg plasmid DNA using Metafectene (Biontex) according to the manufacturer's instructions and were further processed 24–30 h post transfection. For disruption of actin filaments or MTs, HeLa cells were treated with 4 μM cytochalasin D (Sigma), 400 ng/ml nocodazole (Calbiochem), or both for 8 min, fixed and subjected to immunohistochemical analysis. To inhibit ROCK, cells were treated with 30 μM Y-27632(Calbiochem) for 60 min.

Immunofluorescence microscopy

For immunofluorescence, cells were fixed with 3% paraformaldehyde (PFA) for 15 min at room temperature, except for microtubule staining, which was performed after fixation with cold methanol. After permeabilization with PBS/0.1% Triton X-100 for 2 min, cells were blocked with PBS/1% BSA for 30 min. For indirect immunofluorescence, primary antibodies were diluted 1:200–1:500 in PBS/1% BSA and incubated for 45 min at room temperature. Mouse (monoclonal HA-probe F-7) or rabbit (polyclonal HA-probe Y-11) anti-HA antibodies were obtained from Santa Cruz Biotechnology. The anti-tubulin mouse monoclonal antibody (B-5-1-2) was purchased from Sigma. After 4 washing steps with PBS, fluorescent secondary antibodies (Alexa 488, Alexa 568, or Alexa 350 obtained from Molecular probes) were diluted 1:1000 in PBS/1% BSA and incubated for 45 min. Following extensive washing with PBS, cells were mounted with Histogel Histoprime (Linaris Biologische Produkte). For F-actin staining, fixed and lysed cells were treated with FITC or TRITC conjugated phalloidin (Sigma) for 5 min and extensively washed with PBS. Nuclei were counterstained with Hoechst (Sigma). Indirect fluorescence images were monitored with an Olympus IX-70 microscope and processed using Adobe Photoshop software. Quantitative analysis of cell elongation was carried out by measuring the lengths of the long and short axes using the Soft Imaging System analysis program. Statistical verification of the observed differences was ascertained with the Student's *t* test. Confocal images were collected using a confocal laser scanning microscope (Leica TCS.NT system, Leica) attached to a DM IRB inverted microscope with a PLAPO 63 × 1.32 oil immersion objective.

Western blotting

For Western Blot analysis, HeLa or NIH3T3 cells were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.6; 5 mM EDTA; 150 mM NaCl; 0,1% Triton X-100) and sonicated. Cleared lysates (10,000 × *g*, 15 min) corresponding to 5×10^5 cells per lane were separated on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane. The FHOD1 rabbit polyclonal antiserum was raised after immunization with two peptides (MAGGEDRGDGEVSC and KPSSEEGKKSRRSLEC) which are present in human and mouse FHOD1 sequences but no other FHOD1 variants found in the database. As a loading control, 14-3-3 γ was detected by a polyclonal (C-16) antibody (Santa Cruz). Following incubation with primary and secondary antibodies, proteins were detected using the supersignal detection kit (Pierce) according to the manufacturer's instructions.

Results

Activated FHOD1 induces actin stress fiber formation in NIH3T3 and HeLa cells and leads to cell elongation

Activation of the mammalian DRF mDia1 provokes distinct cytoskeletal changes in NIH3T3 and HeLa cells [30,31]. To analyze the effects of FHOD1, we therefore compared the phenotypes of FHOD1 activation in both cell lines. NIH3T3 and HeLa cells were transfected with constructs encoding for HA-tagged versions of wild type FHOD1 protein (FHOD1 wt) or a constitutive active variant lacking the C-terminal DAD (FHOD1ΔC). First, cell morphology, F-actin organization, and FHOD1 localization were monitored. In both cell types, FHOD1 wt was distributed throughout the cytoplasm of transfected cells and had no marked effect on cell shape or actin filaments (Fig. 1A, panels 1, 3 and 5, 7). In contrast, overexpression of FHOD1ΔC caused the formation of thick actin stress fibers oriented along the long axis of the cells that were decorated by the DRF (panels 2, 4 and 6, 8). Additionally, overexpression of FHOD1ΔC but not FHOD1 wt caused elongation of both cell types as compared to non-transfected cells. To validate this influence on cell elongation, the length of the long and short axes of NIH3T3 and HeLa cells expressing FHOD1ΔC or FHOD1 wt, respectively, were compared to that of control cells transfected with the empty vector control. Differences in cell length and length/diameter ratios as evaluated by Student's *t* test analysis are presented in Fig. 1B. NIH3T3 cells expressing FHOD1ΔC had a mean length of the long axis of 81.6 μm with a standard deviation of 28.6 μm ($n = 45$). This was greater than that of cells expressing FHOD1 wt (62.3 μM ± 22.1 μm, $n = 50$, $P < 0.0003$) and significantly greater than the mean length of cells transfected with the empty expression vector alone (48.1 μm ±17.9 μm, $n = 50$,

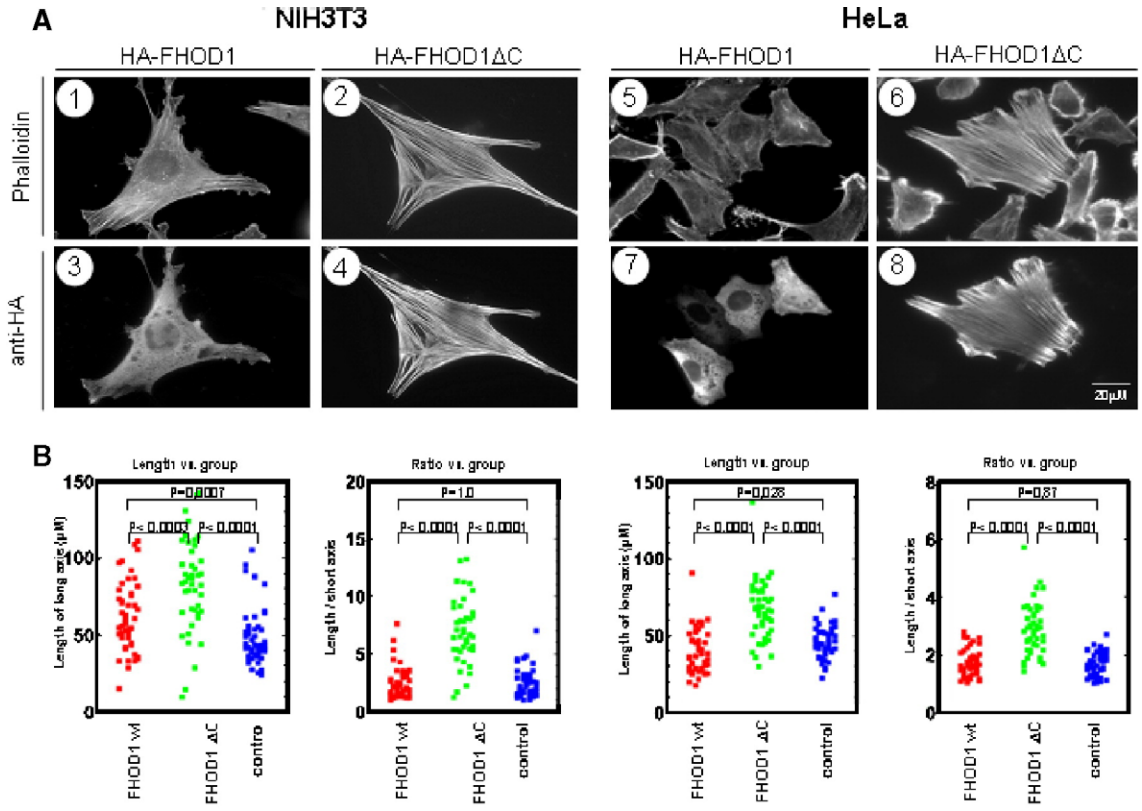

Fig. 1. Activation of FHOD1 induces morphological changes and formation of actin stress fibers in NIH3T3 and HeLa cells. (A) Immunofluorescence of HeLa and NIH3T3 cells transfected with active FHOD1 (HA-FHOD1ΔC) and corresponding F-actin staining. HeLa and NIH3T3 cells overexpressing FHOD1ΔC are elongated and form thick stress fibers that FHOD1ΔC associates with. (B) Quantitative analysis of cell elongation induced by FHOD1ΔC in HeLa and NIH3T3 cells. Cells were transfected with the indicated FHOD1 expression constructs and the respective proteins were visualized by immunofluorescence. The lengths of the long and short axis of about 50 cells were measured (left panels) and plotted with the ratios of long versus the short axis (right panels). *P* denotes the respective *P* values (Student's *t* test). Cells expressing active FHOD1 display a significant increased length–diameter ratio if compared to lengths of FHOD1 wt expressing and control cells.

$P < 0.0001$). The ratios of the long to the short axis were calculated for FHOD1ΔC expressing cells as 6.98 ± 2.85 in comparison to 2.45 ± 1.38 for FHOD1 wt ($P < 0.0001$) and 2.45 ± 1.2 ($P < 0.0001$) for mock-transfected cells. For HeLa cells, the average length of the long axes of FHOD1ΔC expressing cells was 66.7 µm ± 18.9 µm ($n = 45$), which was significantly greater than that of FHOD1 wt expressing cells 39.8 µm ± 14.4 µm ($n = 45$, $P < 0.0001$) or of mock-transfected cells 45.6 µm ± 10.6 µm ($n = 45$, $P < 0.0001$). Here, the ratios were defined as 2.6 ± 0.4 for FHOD1ΔC and for FHOD1 wt and mock-transfected cells as 1.7 ± 0.5 ($P < 0.0001$) and 1.7 ± 0.3 ($P < 0.0001$), respectively. Thus, FHOD1ΔC but not FHOD1 wt caused a significant increase in cell length and length/diameter ratio in both cell lines. However, net cell elongation was much more pronounced in HeLa as compared to NIH3T3 cells.

FHOD1 overexpression has no effect on cytokinesis

Formins have been implicated in cell division [39] and some cells analyzed here contained more than one nucleus. Since blocking cytokinesis has profound effects on cell morphology, we therefore analyzed whether expression of FHOD1 wt or FHOD1ΔC had a direct effect on cell division. Hoechst staining of the nuclei of transfected NIH3T3 and HeLa cells revealed individual cells containing two nuclei. However, such cells were also observed in untransfected control cells. Quantification of multinucleated cells (Fig. 2B) demonstrated that approximately 10% of all non-transfected cells contained more than one nucleus in these experiments. Importantly, an identical percentage of multinucleated cells was detected in the FHOD1 wt or FHOD1ΔC expressing populations. Effects of FHOD1ΔC on cell division were therefore excluded as cause for the observed cell elongation.

FHOD1 induces longitudinal alignment of actin stress fibers and microtubules in HeLa but not in NIH3T3 cells

Next, we analyzed the effects of FHOD1 activation on the organization of the MT network. HeLa and NIH3T3 cells were transfected with expression constructs for FHOD1ΔC, stained for F-actin and tubulin and analyzed by confocal microscopy (Fig. 3). FHOD1ΔC expressing cells were easily identified by the formation of pronounced actin stress fibers and cell elongation. In NIH3T3 cells

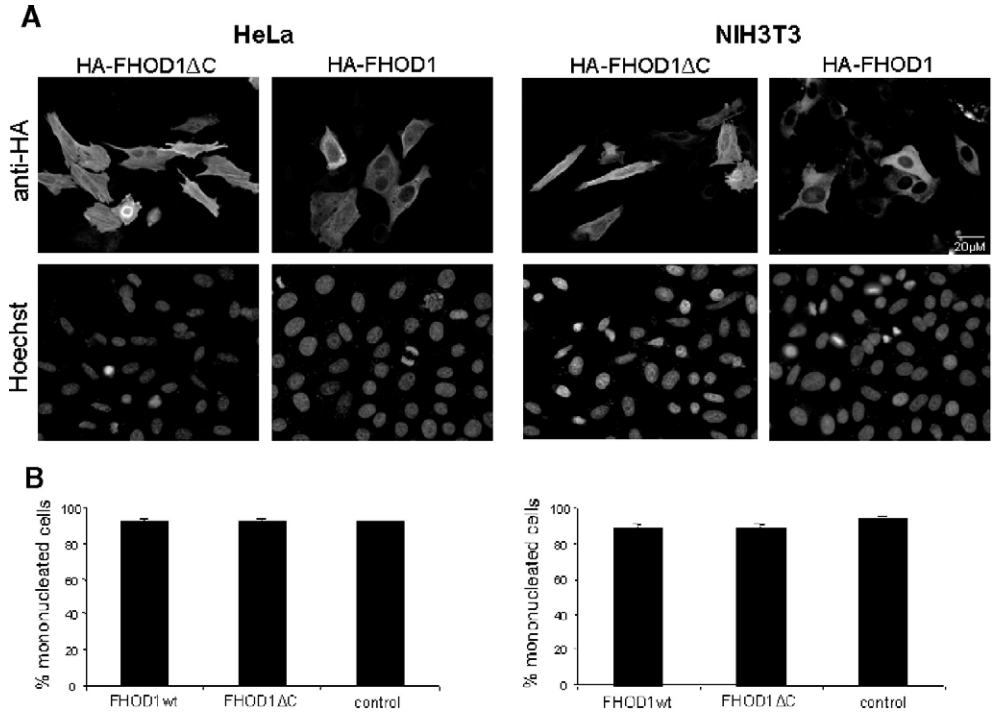

Fig. 2. FHOD1 does not affect cytokinesis. (A) HA immunofluorescence of HeLa and NIH3T3 cells transfected with the indicated expression constructs for HA-tagged FHOD1 (upper panels) and Hoechst staining to reveal the nuclei (lower panels). (B) Quantitative analysis of the effects of FHOD1 on cell division. Depicted are percentages of cells containing one nucleus for cells expressing FHOD1 wt, FHOD1ΔC, or non-transfected control cells. Values represent averages with the indicated standard deviations from three independent transfections in which more than 100 cells were evaluated each.

(Fig. 3A, upper panels), expression of FHOD1ΔC did not lead to overall changes in MT organization and no significant alignment of MTs with actin stress fibers was observed. Only at the very cell periphery, some alignment of MTs to individual, prominent F-actin bundles could be detected. In contrast, the MT network in HeLa cells was drastically changed by overexpression of active FHOD1. Following the distribution of FHOD1-actin fibers, MTs were oriented along the long axis of the cell and significantly overlapped with actin stress fibers (right panels). These results demonstrate the ability of FHOD1 to coordinate MTs with actin filaments in dependence of the cellular environment. Given that we were overexpressing FHOD1 variants, the levels of endogenous FHOD1 proteins in both cells may be a critical parameter for the outcome of these experiments. However, no significant differences in the overall amounts of endogenous FHOD1 protein were detected in anti-FHOD1 Western blot analysis of lysates from non-transfected HeLa and NIH3T3 cells. Of note, several protein species were detected in both cells lines. The slowest migrating populations correspond to the expected sizes of full-length human (118 kDa) and mouse (130 kDa) FHOD1 proteins. In both HeLa and NIH3T3 cells, two major protein species, possibly reflecting yet uncharacterized posttranslational modifications, were detected. Additional faster migrating protein species may represent splice variants and/or degradation products of full-length FHOD1.

MT alignment and cell elongation in HeLa cells by FHOD1 depends on the integrity of the FH1 and FH2 domains

Since the effects of FHOD1 activation on MTs were most pronounced in HeLa cells, this cell line was used for all subsequent analyses. To get first mechanistic insights, we asked whether the conserved FH1 and FH2 domains of FHOD1 shown to be essential for rearrangements of the actin cytoskeleton [32] were also required for alignment of MTs. To this end, FHOD1 wt and ΔC constructs bearing single deletions of these domains were expressed in HeLa cells and the MT arrangement and distribution of the respective FHOD1 proteins were revealed by immunostaining and confocal images were taken of middle and bottom sections of the cells (Fig. 4A). As seen before, expression of FHOD1 wt or its derivatives carrying FH1 or FH2 deletions, respectively, had no effect on MT organization when compared to non-transfected cells. Consequently, cells expressing these FHOD1 variants were not elongated as compared to control cells (Fig. 4B). In contrast, expression of FHOD1ΔC induced the alignment of MTs with FHOD1-actin fibers and cell elongation (Figs. 4A and 4B). Of note, both effects were fully abrogated upon deletion of either the FH1 or the FH2 domain. Interestingly, analysis of the subcellular distribution of FHOD1 and tubulin at various z sections of the same cell revealed marked differences. In sections near the bottom of these cells (upper panels), all FHOD1 proteins analyzed except FHOD1ΔC were distrib-

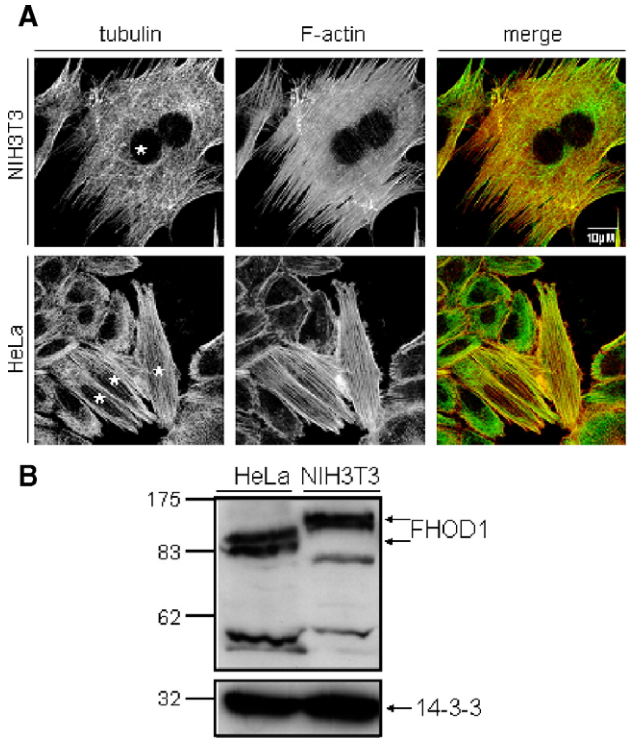

Fig. 3. FHOD1 induces the alignment of actin stress fibers and MTs in HeLa cells. (A) Confocal microscopy analysis of effects of FHOD1ΔC expression on actin filaments and MT network in NIH3T3 and HeLa cells. NIH3T3 (upper panels) and HeLa cells (lower panels) were transfected with an expression plasmid for HA-FHOD1ΔC and stained for tubulin (left) and F-actin (middle). Asterisks indicate cells expressing FHOD1ΔC. The merged images (right) show colocalization of MTs and actin filaments in HeLa cells, while in NIH3T3 cells, F-actin and MTs are not aligned. (B) Western blot analysis for the expression levels of endogenous FHOD1 in HeLa and NIH3T3 cells. Lysates of 5×10^5 HeLa or NIH3T3 cells were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with an antibody against FHOD1 (upper panels) or 14-3-3 protein as a loading control.

uted diffusely in the cytoplasm and did not significantly colocalize with MTs. In contrast, expression of FHOD1ΔC led to MT-FHOD1-actin fiber alignment. The opposite scenario was observed in sections across the middle of the same cells revealing the perinuclear area. FHOD1 proteins that did not induce stress fibers accumulated at this localization and overlapped significantly with perinuclear MTs. In contrast, FHOD1ΔC was virtually absent form this area. These results suggest that activation of FHOD1 triggers a relocalization of the protein from a perinuclear compartment that is enriched in MTs. Given the limited resolution of these confocal micrographs and the dense protein packing at this subcellular localization, our data do not allow to conclude on a specific interaction/colocalization of FHOD1 with perinuclear MTs. Together, these data demonstrate that the coordination of MTs with FHOD1-actin fibers as well as cell elongation requires the FH1 and the FH2 domain of the DRF. These results further indicated that the formation of FHOD1-actin fibers is the driving force for the alignment of MTs.

FHOD1-induced stress fiber formation and MT alignment depend on the activity of the Rho pathway in HeLa cells

We have shown previously in NIH3T3 cells that the formation of FHOD1-actin stress fibers depends on the Rho-ROCK cascade and requires both the FH1 and FH2 domains [32]. We here tested whether FHOD1-induced alignment of MTs along actin stress fibers and cell elongation are controlled by the same pathway in HeLa cells. First, the involvement of the small GTPases Rac1, Cdc42, and Rho in the formation of FHOD1-actin fibers was investigated by individually blocking their activity (Fig. 5A). Overexpression of dominant-negative Rac1N17.GFP or Cdc42N17.GFP did not affect stress fiber formation induced by FHOD1ΔC (panels 1–6). In contrast, when Rho was inhibited by overexpression of C3 transferase, FHOD1ΔC failed to induce actin stress fibers (panel 7) and did not associate with F-actin (panels 8 and 9). Comparable inhibition of FHOD1ΔC-induced changes was also obtained when cells were treated with Y-27632 (60 min at 30 μM), a specific inhibitor of ROCK (panels 10–12). Pronounced membrane ruffling upon Y-27632 treatment reflects the indirect activation of the Rac GTPase [40]. Thus, similar to NIH3T3 cells, FHOD1 induces stress fibers via the Rho-Rock cascade in HeLa cells. We next analyzed the effects of GTPase inhibition on MT alignment (Fig. 5B). Again, inhibition of either Rac1 or Cdc42 had no effect and MTs aligned along FHOD1ΔC induced actin stress fibers (panels 1–6). Importantly, MT organization was indistinguishable from non-transfected cells when the formation of FHOD1-actin stress fibers was prevented by overexpression of C3 transferase (panels 7–9) or treatment with Y-27632 (panels 10–12). Similar results were obtained for cell elongation (Fig. 5C): Blocking Rho and ROCK activity fully abrogated FHOD1ΔC-induced cell elongation, while inhibition of Rac and Cdc42 had no effect. Since FHOD1ΔC does not affect the activity of Rho [32], the coordination of MTs with actin filaments by FHOD1ΔC is controlled via the Rho-ROCK pathway downstream of the GTPase and these rearrangements induce cell elongation.

Actin filaments and MTs are required for FHOD1-induced elongation of HeLa cells

The data presented above suggested that the formation of FHOD1-actin fibers drives the coordination of MTs as well as cell elongation. To address which filament system is responsible for the observed cell elongation, we next disrupted actin filaments or MTs by treatment with cytochalasin D (cytD) or nocodazole (nocod), respectively (Fig. 6A). Disruption of MTs upon treatment with nocodazole (400 ng/ml for 8 min) had no significant effect on the formation of FHOD1-actin fibers. MT depolymerization was complete in some but not all areas of the cell shown in Fig. 6A, yet, FHOD1-actin fibers were readily detectable throughout irrespective of the amounts of MTs still present.

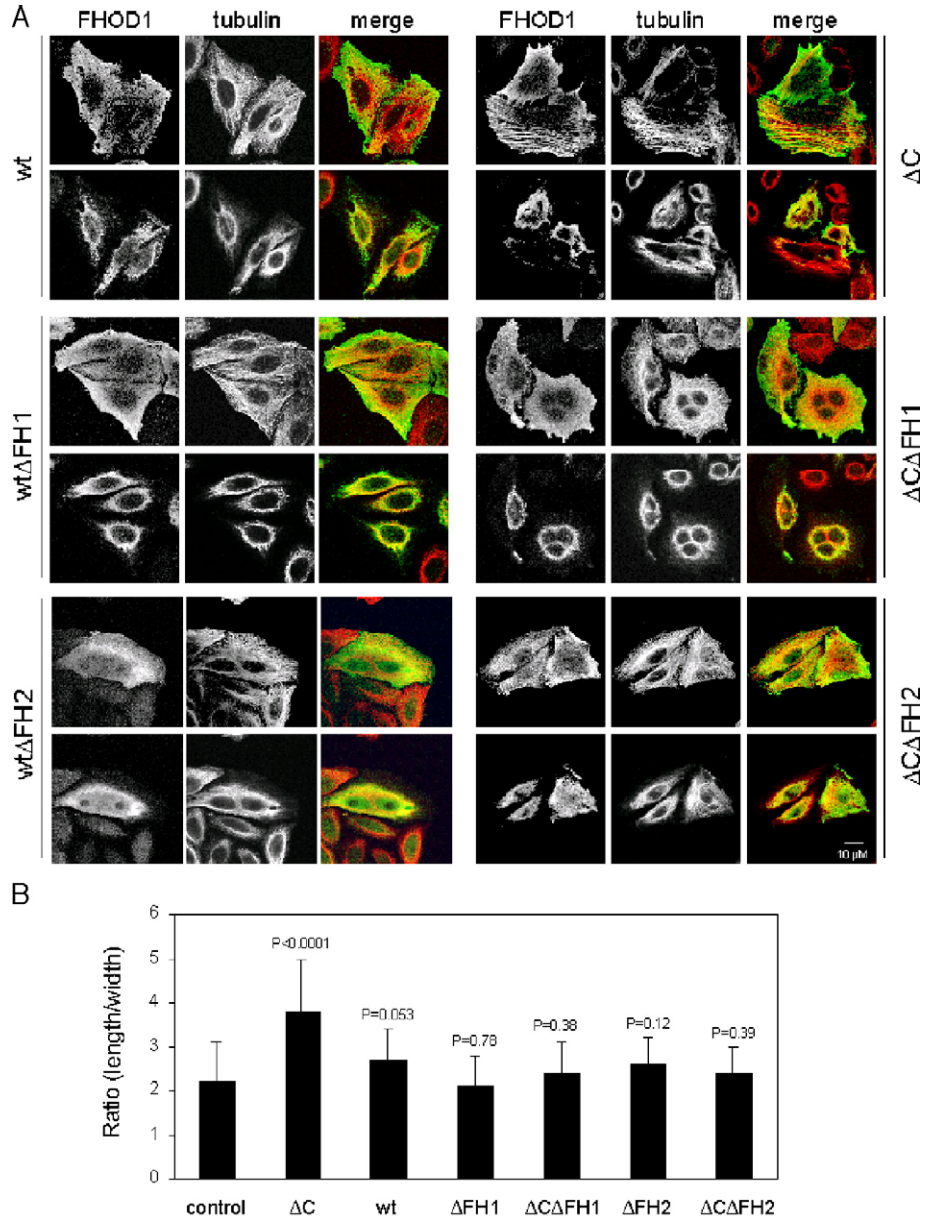

Fig. 4. FHOD1ΔC-induced MT alignment depends on the integrity of the FH1 and FH2 domains. (A) Confocal analysis of HeLa cells transfected with the indicated FHOD1 expression plasmids. 20 h post transfection and following fixation, FHOD1 and MTs were stained with rabbit anti-HA and mouse anti-tubulin antibodies, respectively. Confocal images taken at the bottom (upper panels) and the nuclear region of the cells (lower panels) are shown. (B) Statistical evaluation of cell elongation induced by the different FHOD1 expression plasmids. The length of the long and short axes of over 40 cells of each group was measured on immunofluorescent images. The statistical significance of the differences observed is denoted by the *P* values from Student's *t* test analysis.

Even complete MT disruption did not affect FHOD1's effects on F-actin. In contrast, depolymerization of F-actin by cytD treatment (4 μM for 8 min) had significant effects on MT organization. As described before [32], disruption of actin filaments also desintegrated FHOD1-actin fibers and FHOD1 was found in punctate accumulations in the cytoplasm that partially colocalize with actin aggregates resulting from incomplete depolymerization of F-actin filaments. While MTs were not depolymerized under this condition, no marked orientation along the long axis of the cell could be observed, indicating that the actin filament disruption abrogated the MT polarization induced by FHOD1ΔC. Interestingly neither cytD nor nocod alone was sufficient to revert the elongation of FHOD1ΔC expressing cells over the investigated time frame (Fig. 6B). This was only achieved upon simultaneous treatment of the cells with both drugs. Under these conditions, FHOD1ΔC was found predominantly in the cytoplasm, in which actin fibers and MTs were efficiently depolymerized and the cells were not elongated as compared to control cells. Importantly, the distribution of YFP-paxillin in cells treated as in Fig. 6A revealed that the retraction observed in the presence of cytD and nocod did not result from the disruption of focal adhesion complexes (Fig. 6C). These

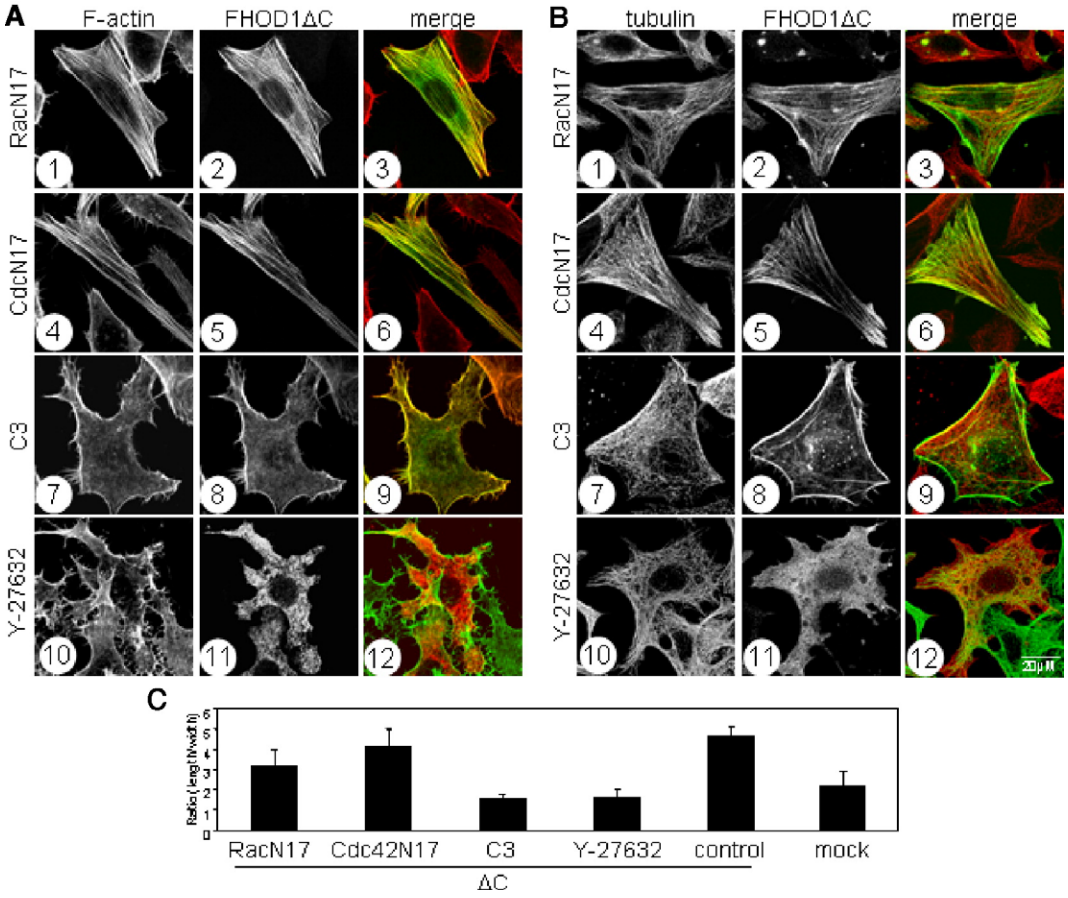

Fig. 5. FHOD1-induced stress fiber formation, MT alignment, and cell elongation depend on the activity of the Rho pathway in HeLa cells. FHOD1ΔC proteins were co-expressed in HeLa cells with transdominant-negative Rac (RacN17), Cdc42 (Cdc42N17), C3 transferase (C3) or treated with ROCK inhibitor (Y-27632). Following fixation, cells were stained for FHOD1 and F-actin (A) or tubulin (B). In (C), the length to width ratios are shown for the cells analyzed in (A) and (B) as well as for cells expressing FHOD1ΔC or untransfected control cells (mock). Depicted are the mean values of at least 40 cells with the indicated standard error of the mean.

results revealed that F-actin is critical for the polarization of MTs induced by activated FHOD1 and demonstrate that both actin filaments and MTs are necessary for sustained elongation of FHOD1ΔC expressing cells.

Discussion

In this study, we present evidence for the coordination of MTs with actin stress fibers upon overexpression of a constitutive active version of the DRF FHOD1. FHOD1ΔC caused the longitudinal alignment of MTs in parallel to FHOD1-actin stress fibers. This polarization of MTs coincided with marked cell elongation. These effects required both the FH1 and FH2 domains of the DRF and were dependent on the Rho-ROCK cascade but not of Cdc42 or Rac1. Finally, only simultaneous disruption of actin filaments and MTs reversed cell elongation induced by activated FHOD1. Thus, formation of FHOD1-actin stress fibers determines the coordination of MTs and both filament systems are required for the sustained cell elongation observed upon activation of FHOD1.

FHOD1 has been characterized as a Rac-interacting protein that, upon activation, causes the formation of thick actin stress fibers that are coated by the DRF. Additionally, active FHOD1 variants induce transcription from the SRE [32,33,37]. Although this remains to be demonstrated experimentally, based on the findings for other DRFs, these effects are assumed to reflect the ability of FHOD1 to cause nucleation of actin filaments. Whether FHOD1 also causes alteration of cellular filament systems other than actin has not yet been addressed. The present study demonstrates that FHOD1 has the ability to coordinate MT and actin filaments, resulting in the orientation of MTs along the long axis of cells in parallel to FHOD1-actin fibers as well as net cell elongation. Of note, these effects were markedly more pronounced in HeLa than in NIH3T3 cells, where only moderate effects of FHOD1 on the organization of MTs were observed. These differences are reminiscent of results reported for mDia1, which also causes MT alignment alongside stress fibers in HeLa cells, while these effects are hard to be appreciated in NIH3T3 cells [30,31]. How the cellular environment may affect the ability of FHOD1 and mDia1 to coordinate MTs with actin filaments remains

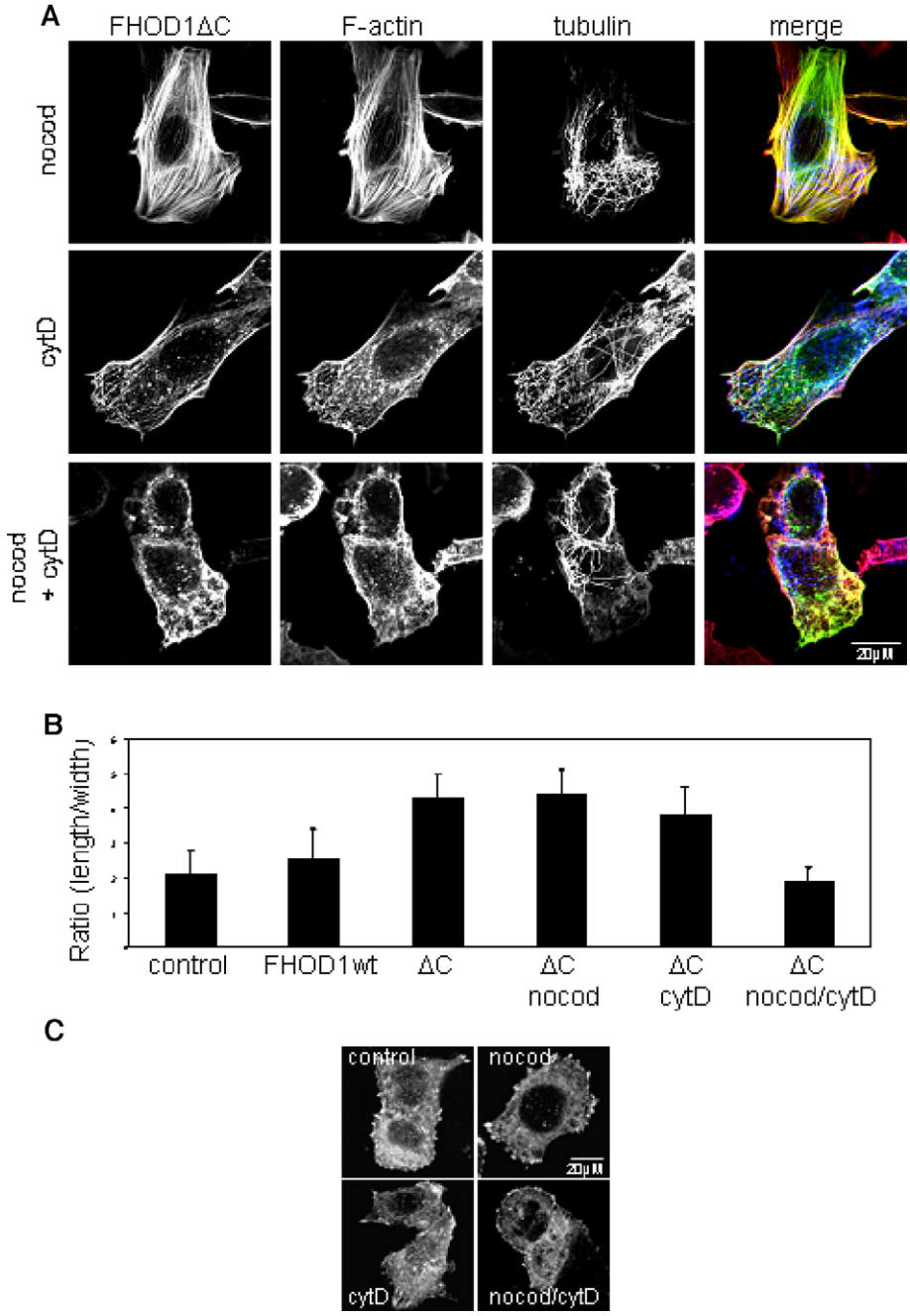

Fig. 6. Actin and MT networks are required for cell elongation. (A) Immunofluorescence analysis to monitor the involvement of actin and MT networks in cell elongation. HeLa cells were transfected with an expression construct for GFP-tagged FHOD1ΔC. 20 h post transfection cells were treated with cytochalasin D (4 μM), nocodazole (400 ng/ml), or both for 8 min, fixed with methanol and stained for F-actin and tubulin. The merged panels display the overlay picture of all three fluorescent channels. (B) Quantitative analysis of the influence on MTs and actin on cell length. The length of the long and the short axis of more than 40 cells processed as in (A) was measured and compared to non-treated FHOD1 expressing and control cells. *P* values from Student's *t* test analysis compared to the length/width ratio for FHOD1ΔC were 0.37 (ΔC nocod), 0.3 (ΔC cytD), <0.0001 (control and nocod/cytD), or <0.0001 (FHOD1 wt), respectively. (C) Immunofluorescence analysis of HeLa cells expressing YFP-paxillin. Treatment with cytD and/or nocod was carried out as described for panel A.

unclear. Different overall levels of endogenous FHOD1 could be excluded as reason for this cell type variability. The detection of truncated FHOD1 species, in particular, in NIH3T3 cells, however raised the possibility that the composition of FHOD1 populations and their regulation may vary between cell types. On the other hand, the differences between HeLa and NIH3T3 cells observed suggest that cell elongation can be mediated by distinct mechanisms: HeLa cells utilize both actin filaments and MTs while stretching of NIH3T3 cells in response to FHOD1 activation is significantly less dependent on MTs. In line with this scenario, the relative contribution of MTs to actin-mediated formation of cell protrusions as well as to cell migration vary significantly between different cell types

[41]. In conclusion, our results emphazise that MT coordination appears to be a common feature of mammalian DRFs and that HeLa cells serve as a suitable experimental system to study these effects.

Our results indicate that the alignment of MTs with FHOD1-actin stress fibers is driven by the formation of the DRF-coated actin cables. First, disruption of FHOD1-actin fibers by cytD abrogated MT polarization. Second, formation of FHOD1-actin fibers and MT alignment were governed by the same determinants. As previously described in NIH3T3 cells, induction of stress fibers by FHOD1 critically depended on the integrity of both, the FH1 and FH2 domains, also in HeLa cells and FHOD1 mutants lacking these domains failed to polarize MTs. Furthermore, our experiments in HeLa cells confirm the previous observation that FHOD1ΔC-induced actin rearrangements require the activity of the Rho-ROCK cascade but are independent of Rac or Cdc42 mediated upstream signaling. Of note, the block of FHOD1-actin fiber formation by C3 transferase or Y-27632 also fully abolished MT polarization. Thus, it seems plausible that the formation of these fibers is a prerequisite for MT coordination. How this is achieved by the DRF at the molecular level will be an interesting question for future studies. The requirement of the FH2 domain may be explained by its ability to nucleate actin polymerization, thereby providing the basis for the formation of FHOD1-actin fibers. One may speculate that the FH1 domain then serves for MT coordination. We did not analyze whether FHOD1 associates directly with MTs as described for mDia1 [30]. However, MTs were co-aligned alongside FHOD1-actin fibers but did not overlap with them and we failed to detect significant colocalization between activated FHOD1 and MTs. Thus, it appears more likely that MT coordination is mediated by the FH1 domain via an indirect mechanism rather than by direct physical contact.

Net cell elongation was a direct consequence of the actin–MT coordination by activated FHOD1. Our results demonstrate that pronounced elongation of HeLa cells coincides with marked MT alignment and polarization. A role for MTs in cell elongation is supported by the disruption experiments, where only depolymerization of both filament systems reversed FHOD1-induced cell stretching. These observations fit best with a model in which MT–actin coordination serves to induce pronounced cell elongation. In this scenario, coordinated polarization of both filament systems would provide oriented mechanical force to mediate the required morphological changes. Once the cell has attached to the substratum in its elongated shape, actin stress fibers as well as MT arrays alone are sufficient to sustain an elongated cell shape. The role of focal adhesions in this process deserves a more detailed analysis in future studies. Interestingly, FHOD1 resembles mDia1 in the overall effects of MT polarization and cell elongation, but the morphological changes induced by mDia1 were entirely dependent on MTs and depolymerization of actin had no effect on cell elongation. Furthermore, depolymerization of actin did not affect MT alignment in the case of mDia1 [31]. Thus, FHOD1 and mDia1 may mediate MT coordination via slightly different mechanisms. Such differences on the molecular level may be indicated by the fact that FHOD1-induced actin stress fibers appear thicker than those triggered by other DRFs and the unique property of FHOD1 to stably associate with these fibers.

Together, our results demonstrate that activated FHOD1 coordinates MTs via its ability to induce the formation of bundled actin stress fibers, resulting in cell elongation. These effects are mediated by the Rac-interacting DRF via targeting of the Rho-ROCK cascade. Thus, FHOD1 may be particularly well suited for the coordination of Rho and Rac signaling cascades. The functional interplay of actin filaments and MTs is central to many cellular processes such as cell movement and morphogenesis [42]. In terms of FHOD1 function, cell motility might be of special interest since cytoskeletal coordination during the process critically depends on the fine tuning between Rac and Rho signaling. Of note, FHOD1 has been suggested to enhance cell migration [36]. The role of actin–MT coordination for this and other activities of FHOD1 will be subject to future studies.

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