RESEARCH ARTICLE

FHOD1 regulates stress fiber organization by controlling the dynamics of transverse arcs and dorsal fibers

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ABSTRACT

The formin FHOD1 (formin homology 2 domain containing protein 1) can act as a capping and bundling protein in vitro. In cells, active FHOD1 stimulates the formation of ventral stress fibers. However, the cellular mechanisms by which this phenotype is produced and the physiological relevance of FHOD1 function are not currently understood. Here, we first show that FHOD1 controls the formation of two distinct stress fiber precursors differentially. On the one hand, it inhibits dorsal fiber growth, which requires the polymerization of parallel bundles of long actin filaments. On the other hand, it stimulates transverse arcs that are formed by the fusion of short antiparallel actin filaments. This combined action is crucial for the maturation of stress fibers and their spatio-temporal organization, and a lack of FHOD1 function perturbs dynamic cell behavior during cell migration. Furthermore, we show that the GTPase-binding and formin homology 3 domains (GBD and FH3) are responsible for stress fiber association and colocalization with myosin. Surprisingly, a version of FHOD1 that lacks this domains nevertheless retains its full capacity to stimulate arc and ventral stress fiber formation. Based on our findings, we propose a mechanism in which FHOD1 promotes the formation of short actin filaments and transiently associates with transverse arcs, thus providing tight temporal and spatial control of the formation and turnover of transverse arcs into mature ventral stress fibers during dynamic cell behavior.

KEY WORDS: Actin cytoskeleton, Contractility, FHOD1, Intracellular organization, Myosin, Stress fibers

INTRODUCTION

Diaphanos-related formins (DRFs) form an evolutionarily conserved family of actin regulators, which are involved in numerous cellular processes, such as morphogenesis, cell division and cell polarity. In general, formins are well known for their ability to act as processive actin nucleators or ‘leaky cappers’ for actin filaments – they catalyze the nucleation of new actin filaments and stay attached to the barbed end of the growing filament (Kovar and Pollard, 2004; Higashida et al., 2004). However, the efficiency of actin nucleation and of subsequent processive monomer addition varies greatly among individual family members (Paul and Pollard, 2009). In addition to their nucleation activity, several formins have been described to have other modulatory roles, such as actin filament bundling or actin severing and de-polymerization (Harris et al., 2006; Harris et al., 2010), suggesting that they might also play additional roles in cells (Harris et al., 2004; Chhabra and Higgs, 2006; Chesarone et al., 2010).

For multiple family members, the highly conserved formin homology 2 (FH2) domain was shown to be sufficient to catalyze actin nucleation in vitro. The FH1 domain is thought to promote processive filament elongation by recruiting profilin-bound monomeric actin (Sagot et al., 2002; Paul and Pollard, 2008; Romero et al., 2004). Two additional modules shared among DRFs – the C-terminal diaphanous autoregulatory domain (DAD) and the N-terminal diaphanous inhibitory domain (DID) region – interact with one other, keeping the protein in the inactive conformation (Alberts, 2001; Faix and Grosse, 2006; Schönichen and Geyer, 2010). In mDia1 (DIAPH1), this auto-inhibitory intramolecular interaction was shown to be released by the binding of the Rho GTPase RhoA to the C-terminal GTPase-binding domain (GBD), thus stimulating the actin-nucleating activity of this formin and promoting actin polymerization into stress fibers from focal adhesions (Watanabe et al., 1997; Hotulainen and Lappalainen, 2006). ROCK, another prominent effector of RhoA, phosphorylates myosin II and increases the motor activity of myosin to promote the bundling and contractility of existing actin filaments (Ishizaki et al., 1997). Thus, RhoA controls stress fiber formation through the concerted activation of at least two downstream effectors, Rho kinase (ROCK) and mDia1 (Watanabe et al., 1999).

The formin FHOD1 (formin homology 2 domain containing protein 1) is also thought to play a role in stress fiber formation (Koka et al., 2003). FHOD1 shares a similar domain organization with that of other DRFs and is also activated by a mechanism involving the release of auto-inhibition (Schönichen et al., 2006). The truncation of the C-terminal amino acids 1012–1164, which include the DAD region and a short sequence of unknown function (called ‘X’), leads to constitutive activation of this formin and results in the formation of prominent stress fibers throughout the cell (Koka et al., 2003; Gasteier et al., 2003). The RhoA effector ROCK phosphorylates FHOD1 at three residues within this C-terminal region (S1131, S1137, T1141), disrupting the inhibitory interaction between the N-terminal FH3 domain (DID in mDia1) and the DAD region (Takeya et al., 2008). Similar to the C-terminal truncation mutant, the overexpression of the corresponding triple phosphomimetic mutant induces the formation of thick linear stress fibers, suggesting that ROCK-mediated phosphorylation is involved in the activation of FHOD1.
expression results in the collapse of the cellular stress fiber meshwork and causes defects in cell migration and cell spreading. Together, our work supports a model in which FHOD1 plays a central role in the spatial and temporal coordination of cellular stress fiber dynamics.

RESULTS

FHOD1 depletion leads to the collapse of the stress fiber meshwork in U2OS cells

To study the cellular mechanism by which FHOD1 induces stress fibers in cells, we used RNAi to deplete the endogenous formin. The efficient knock-down of the protein was confirmed by western blotting (supplementary material Fig. S1A). First, transverse arcs and dorsal stress fibers were quantified in RNAi-treated cells. We distinguished between these distinct stress fiber types based on their pattern of association with focal adhesions (Hotulainen and Lappalainen, 2006): actin arcs are not linked to focal adhesions but instead are linked to dorsal stress fibers, whereas the distal ends of dorsal stress fibers are associated with focal adhesions, and their proximal ends are associated with actin arcs. After FHOD1 depletion, both well-organized actin arcs and dorsal stress fibers were observed less frequently (Fig. 1A, yellow arrow, 1B). These findings were confirmed with four individual siRNA oligonucleotides to exclude off-target effects (supplementary material Fig. S1B). As reported previously (Small et al., 1998; Hotulainen and Lappalainen, 2006), transverse arcs were located on the dorsal cell surface (Fig. 1C). We next investigated whether FHOD1 plays a role in the spatial organization of arcs within individual cells. We restricted our morphometric measurements to those cells that were still able to form arcs, and we found that FHOD1 depletion decreased the arc-covered cell area (Fig. 1D; see supplementary material Fig. S1C for definition of area quantification). Furthermore, the average actin fluorescence intensity was reduced in this area, suggesting that the density of arcs is reduced as well (Fig. 1E). Interestingly, we observed a substantial increase in the number of cells that contained prominent stellate accumulations of actin fibers (Fig. 1F–H). The exclusive presence of the focal adhesion marker paxillin at the distal ends of the individual fibers and the strong myosin II staining in the center suggested that these unusual structures might be generated by the collapse of the meshwork of transverse arcs and dorsal stress fibers (Fig. 1G,H). Live-cell imaging revealed that these structures are only formed transiently (Fig. 1I), leaving behind the less dynamic peripheral actin bundles described above (Fig. 1A, yellow arrow). In addition to the clear long-term effects of FHOD1 depletion on actin network organization, cytochalasin D washout experiments revealed impaired stress fiber recovery in cells lacking FHOD1, suggesting that this formin is not only crucial for long-term network organization but also for the dynamic de novo formation of stress fibers (supplementary material Fig. S2). These striking defects in actin stress fiber organization were accompanied by a significant decrease in migration efficiency (supplementary material Fig. S3A–D).

The observed disruption in actin network organization was also paralleled by alterations in the size of focal adhesions. We quantified this effect by measuring the average number and size of focal adhesions in confocal micrographs of cells expressing paxillin fused to the fluorescent protein mKate (Fig. 2A). We found that the average size of focal adhesions in FHOD1-depleted cells was...
Fig. 1. Stress fiber organization is perturbed by the depletion of FHOD1. (A) Representative confocal images of the actin cytoskeleton in fixed siRNA-treated U2OS cells. The types of stress fiber were identified by F-actin (Rhodamine–phalloidin) and paxillin staining (focal adhesion marker, data not shown). Green and red arrows indicate dorsal stress fibers and transverse arcs, respectively. The yellow arrow points to thick peripheral stress fiber bundles. Scale bars: 20 μm. (B) The percentage of cells with dorsal stress fibers (dSF), transverse arcs (TA), both stress fiber types (dSF + TA) or thick peripheral actin bundles only (peripheral bundles; yellow arrow in A). Data show the mean ± s.d. from three experiments (n=360 cells for each condition). (C) The 3D organization of transverse arcs in U2OS cells. Depicted is the maximum projection of a confocal Z-stack (F-actin), in which the Z coordinates are indicated by the color bar. Scale bar: 10 μm. (D) Arc-covered lamellum regions were measured based on confocal Z-stacks (F-actin) and were normalized to the whole-cell adhesion area (ImageJ, free-hand line tool) (see supplementary material Fig. S1C for an illustration of the analysis process). Only cells that contained arcs were included in the analysis. Data represent the mean ± s.e.m. n=90 cells for each condition from three experiments. (E) The mean actin intensity in arc-covered lamellum regions from D. a.u. arbitrary units. Red lines represent the mean values. (F) Quantification of cells with stellate stress fiber aggregates. Data show the mean ± s.d. from three experiments. n=360 cells for each condition. (G) A representative image of a stellate stress fiber aggregate in a FHOD1-depleted cell. Maximum projection of a confocal Z-stack showing F-actin (Rhodamine–phalloidin, red) and focal adhesions (anti-paxillin antibody, green). Scale bar: 10 μm. (H) Actin aggregates contain myosin IIA. A representative maximum projection of a confocal Z-stack of a FHOD1-depleted cell expressing EGFP–NMHC IIA (green). F-actin was visualized by Rhodamine–phalloidin staining (red). Myosin IIA is excluded from most of the straight actin fibers (white arrowheads), whereas strong localization is detected within the aggregate. Scale bar: 10 μm. (I) Representative time-lapse confocal images depicting the disassembly of an actin aggregate in an EGFP–actin-expressing FHOD1-depleted cell. Scale bar: 10 μm. n=20 movies. White boxes, areas of the images that are enlarged in a separate image. ***P<0.001, **P<0.01, *P<0.05.
significantly decreased (Fig. 2B) compared with those of control (ntsiRNA) cells, whereas the number of focal adhesions per cell was significantly increased (Fig. 2C). By contrast, the average cell area was not altered after formin depletion [ntsiRNA, 1740 μm² ± 123.8; FHOD1 siRNA, 1679 μm² ± 97.05 (mean ± s.e.m.)]. This apparent change in the organization of focal adhesions was accompanied by only moderately perturbed cell spreading (Fig. 2D,E; supplementary material Fig. S3E). We found that the early phase of U2OS cell spreading on collagen type I was significantly impaired, as reflected by a smaller average adhesion area compared with control cells (Fig. 2D,E, 30 min). Interestingly, this difference was lost at a later time-point (Fig. 2D,E, 60 min), suggesting compensation by other, possibly Rac1-mediated, mechanisms (Price et al., 1998; del Pozo et al., 2000).

**FHOD1 promotes transverse arc formation and turnover into ventral stress fibers**

In order to study the cellular mechanism by which FHOD1 affects the cellular stress fiber organization, we performed cytochalasin D washout experiments using cells expressing FHOD1 mutants. Compared with control EGFP-transfected cells, overexpression of wild-type FHOD1 did not significantly alter stress fiber recovery dynamics (Fig. 3A,B). To study the effect of activated FHOD1, we used a point mutation (FHOD1 V228E) that induces an open protein conformation and strongly stimulates stress fiber formation in cells (Schulte et al., 2008). In contrast to C-terminally truncated constitutively active FHOD1 (FHOD1 1–1011), this mutant retains an intact C-terminus. After cytochalasin D washout, FHOD1 V228E induced an enhanced generation of dorsal transverse arcs as compared with the EGFP control and wild-type FHOD1 (Fig. 3A,B). This observation is further supported by an analysis of confocal F-actin image stacks, which showed that FHOD1 V228E induced enhanced F-actin staining on the dorsal cell surface (Fig. 3C).

In addition, live-cell imaging during cytochalasin D washout revealed that the enhanced arc formation was paralleled by an accelerated transformation of arcs into straight stress fibers (Fig. 3C–E; supplementary material Movie 1). This can explain
Fig. 3. Active FHOD1 promotes transverse arc formation and enhanced maturation into linear stress fibers. The acute effects of FHOD1 on stress fiber formation. Cells expressing EGFP, EGFP–FHOD1 wild-type (FHOD1 WT) and active EGFP–FHOD1 V228E (FHOD1 V228E) were treated with cytochalasin D (2 μM for 90 min) to disrupt actin filaments. Stress fiber recovery was stimulated by drug washout. Cells were either fixed and stained with Rhodamine–phalloidin (F-actin) (A–C) or subjected to confocal time-lapse imaging of the actin cytoskeleton (D–F). (A) Upper panel, 3D organization of stress fibers. Depicted are representative maximum projections of confocal Z-stacks (F-actin) 30 min after washout, in which the Z-coordinates are indicated by the color bar. Lower panel, enlarged areas from the boxed regions of the upper panels. Scale bar: 10 μm. (B) Arc-covered lamellum regions (shown as a percentage of the total cell adhesion area) in fixed cells 30 min after drug washout (see supplementary material Fig. S1C for an illustration of the analysis process). Data show the mean±s.e.m. of 100 cells for each condition from three experiments. (C) FHOD1 V228E enhances stress fiber formation on the dorsal side of the cell. Z-sections (middle) were generated from confocal Z-stacks (F-actin) along the lines indicated by the red arrows in the maximum projections (ImageJ, MultipleKymograph tool) (left). Scale bar: 10 μm. Mean actin intensity was measured on the dorsal (red dotted line) or ventral (green dotted line) side, as indicated in the schematic (middle), and was plotted as the ratio of dorsal:ventral (right). n=67 and 85 cells for the two conditions, respectively (three independent experiments; red lines indicate the mean). (D) Representative time-lapse confocal images of stress fiber recovery after cytochalasin D washout in cells expressing mCherry–actin and EGFP–FHOD1 constructs (not shown). Acquisitions started immediately after washout. Scale bars: 10 μm. (E) Time-point of the first linear stress fiber appearance in time-lapse images. n=40, 33 and 44 cells for the three conditions, respectively (three experiments; red lines indicate the mean). (F) The data from D were plotted as a histogram. n.s., not significant. ***P<0.001, **P<0.01.
the known long-term effect of enhanced stress fiber formation after the overexpression of constitutively active FHOD1 mutants (Koka et al., 2003; Gasteier et al., 2003; Takeya and Sumimoto, 2003; Schulte et al., 2008). Our data now show that FHOD1 stimulates the generation of transverse arcs and that constitutively active FHOD1 causes these to mature more rapidly into linear stress fiber bundles (Fig. 3D–F).

We next examined the effect of FHOD1 activation or inactivation on dorsal stress fibers. In cells lacking this formin, we found that the average growth rate of dorsal stress fibers and the average maximal fiber length were significantly increased (Fig. 4A,B). Furthermore, in FHOD1-depleted cells, the majority of mature dorsal stress fibers bent or buckled instead of growing straight and perpendicular to the cell edge as in control cells (Fig. 4C,D; supplementary material Movie 2). This suggests that the formation of dorsal stress fibers is stimulated in the absence of FHOD1 and that their regulation is perturbed, leading to aberrant turnover dynamics. Conversely, dorsal stress fibers were observed less frequently, and their average length was decreased after cytochalasin D washout, if activated FHOD1 was expressed (Fig. 4E–G). In contrast to other formins, such as mDia1, which was proposed to stimulate actin polymerization by processive...
actin filament elongation (Higashida et al., 2004), this inhibitory effect of FHOD1 might be due to an alternative mechanism involving filament end capping.

**FHOD1 localizes to contractile anti-parallel actin arrays at sites of myosin association**

To gain more insight into the mechanism of FHOD1-mediated actin arc formation we next studied its intracellular localization by using confocal scanning and total internal reflection fluorescence (TIRF) microscopy at low expression levels. Wild-type FHOD1 is largely cytosolic; however, because of the increased signal-to-background ratio in TIRF microscopy, we found that wild-type FHOD1 was distributed in an irregular punctate pattern, with some preference for ventral stress fibers. These structures are known to form anti-parallel actin-filament arrays [i.e. arrays of mixed actin filament polarity (Naumanen et al., 2008)] (Fig. 5A, yellow arrow). Actin arcs, which also contain anti-parallel actin arrays, undergo rapid retrograde flow along the dorsal side of the cell and are therefore hardly detected using TIRF microscopy. The constitutively active mutant FHOD1 1–1011 was strongly associated with both ventral stress fibers and arcs and therefore was easily detected in the complete cell volume by using confocal scanning microscopy (Fig. 5B, yellow and green arrows, respectively).

**Fig. 5. FHOD1 localizes to contractile stress fibers with anti-parallel actin arrays.** (A) FHOD1 localizes to ventral stress fibers (yellow arrow). TIRF images of EGFP–FHOD1 (green) and RFP–actin (red). Ventral stress fibers are attached to the cell cortex at both ends and therefore are entirely visible in the TIRF field. Scale bar: 20 μm. (B) Constitutively active FHOD1 1–1011 localizes to the distal ends of dorsal stress fibers. Maximum projections of confocal Z-stacks are shown. Overlay: F-actin (red, phalloidin staining), EGFP–FHOD1 (1–1011) (green), paxillin staining (blue). The yellow arrow shows a ventral stress fiber associated with the substrate at both ends. The orange arrow shows the main portion of a dorsal stress fiber. The red arrow shows the distal end of a dorsal stress fiber. The green arrow shows a transverse arc. Scale bar: 10 μm. (C) Myosin II localization at the distal ends of dorsal stress fibers at the interface with focal adhesions (red and white arrowheads). Confocal single-plane images of F-actin (Rhodamine–phalloidin, red), anti-NMHC IIA (green) and anti-paxillin (blue) staining. Scale bar: 10 μm. (D) Active FHOD1 V228E colocalizes with myosin on contractile stress fibers. Confocal images of EGFP–FHOD1 V228E (green) and myosin (mCherry–NMHC IIA, red) colocalization on transverse arcs. Yellow arrows, the punctate pattern of FHOD1 and myosin colocalization. Representative images of n=60 cells from four experiments. Scale bar: 10 μm.
Interestingly, active FHOD1 did not bind to the majority of dorsal stress fiber structures, which are thought to be composed of parallel actin arrays (i.e. unidirectional actin filament arrays) (Fig. 5B, orange arrows). Thus, the preferential binding of FHOD1 to ventral stress fibers and actin arcs compared with dorsal fibers suggests that FHOD1 selectively binds to anti-parallel actin arrays. However, FHOD1 did bind to the distal ends of dorsal fibers that overlapped with focal adhesions (Fig. 5B, red arrows), as did myosin (Fig. 5C, red and white arrowheads; supplementary material Fig. S4A), suggesting coordinated functionalities of the two proteins in these areas.

Arcs are formed in the lamellipodium by the bundling and fusion of short \( \alpha \)-actinin-bound actin filaments that undergo rapid retrograde flow (Hotulainen and Lappalainen, 2006; Burnette et al., 2011; Tojkander et al., 2012). Myosin II is incorporated into these actin filament bundles, generating an alternating pattern with \( \alpha \)-actinin. Because FHOD1 associates with stress fibers, it might play a role in coordinating those processes. Previously, it was unclear whether FHOD1 cooperates with \( \alpha \)-actinin-mediated filament assembly or myosin-mediated contractility. Here, we found that activated FHOD1 (EGFP–FHOD1 V228E) substantially colocalized with myosin and was excluded from \( \alpha \)-actinin-enriched regions (Fig. 5D; supplementary material Fig. S4B). Thus, our results suggest that FHOD1 might cooperate with myosin to regulate the formation of contractile stress fibers.

**Recruitment of FHOD1 to stress fibers and myosin colocalization requires an N-terminal targeting region**

The stress fiber localization of FHOD1 is mediated by sequences within its N-terminal region (1–573) that consists of multiple parts, including the GBD, FH3 domain, linker and helical domain (Takeya and Sumimoto, 2003; Schönichen et al., 2013). The FH3 domain adjacent to the GBD bears the capacity to interact with the C-terminal DAD to mediate auto-inhibition (Takeya and Sumimoto, 2003; Schönichen et al., 2006; Schulte et al., 2008). The helical domain was suggested to be responsible for FHOD1 side-binding to actin filaments in vitro and might therefore mediate filament bundling (Schönichen et al., 2013). However, the precise N-terminal region that is responsible for the stress fiber localization of the protein had not been determined. To address this, we generated various FHOD1-truncation mutants and analyzed their subcellular localization (Fig. 6A,B; supplementary material Fig. S5B).

As expected, full-length activated FHOD1 V228E robustly associated with stress fibers, similar to the previously described constitutively active mutant FHOD1 1–1011 (Schulte et al., 2008; Koka et al., 2003; Gasteier et al., 2003; Takeya and Sumimoto, 2003) (Fig. 6A,B). In agreement with previous studies, the entire N-terminus (1–573) was found along stress fibers (Fig. 6B). Here, we show that GBD alone (1–115) was predominantly cytosolic, whereas GBD-FH3 (1–339) displayed a distinct association with stress fibers (Fig. 6B) and colocalization with myosin II (Fig. 6C). By contrast, a construct lacking these domains (340–1164) was again mostly cytosolic, as judged by confocal microscopy (data not shown), and did not localize along stress fibers. However, using TIRF microscopy, we found a weak but reproducible accumulation of FHOD1 340–1164 along the entire length of focal adhesions (Fig. 6B; supplementary material Fig. SSC). Interestingly, when stress fiber formation was stimulated acutely by cytochalasin D washout, this N-terminal truncation mutant strongly accumulated in the lamellipodium (Fig. 6D). Lamellipodia were rarely observed after long-term expression of FHOD1 340–1164; however, if they were present, we also observed enrichment of this mutant in this cell region in steady-state conditions (data not shown). Together, these observations show that the GBD-FH3 domains can mediate the stress fiber association of FHOD1 and its colocalization with myosin II independent of the two domains that were previously proposed to mediate F-actin interaction – the FH2 domain (617–1011) and the helical domain (396–573) (Pruyne et al., 2002; Schönichen and Geyer, 2010; Schönichen et al., 2013). In agreement with this, a construct containing only the linker region and the helical domain (340–573) localized mostly diffusely in the cells (supplementary material Fig. S5B).

As shown recently, truncation of the entire N-terminus (574–1164) effectively ablated the stress fiber association of FHOD1 (Fig. 6B) (Schönichen et al., 2013). Here, the majority of this truncated FHOD1 was cytosolic, but it was also found in dot-like accumulations throughout the cell body, with a preference for focal adhesions, suggesting that this construct has a tendency to form aggregates but nevertheless retains some subcellular-targeting functionality (Fig. 6B).

We next studied the functional role of the FHOD1 N-terminal stress fiber localization domain and the helical domain. Surprisingly, in long-term expression as well as cytochalasin D washout experiments, FHOD1 340–1164, which lacks the stress-fiber-targeting GBD-FH3 domains (Fig. 6B), nevertheless stimulated the formation of thick linear stress fibers to a similar extent as the constitutively active FHOD1 V228E (Fig. 7A–C). FHOD1 340–1164 still encompasses the helical domain (396–573) that was suggested to bind to actin filaments in vitro through side-binding, and therefore it might mediate filament bundling (Schönichen et al., 2013). Thus, the helical domain might play a functional role in stress fiber formation. Indeed, the truncated mutant that lacks the helical domain (FHOD1 574–1164) failed to stimulate stress fiber formation (Fig. 7A,B).

Thus, FHOD1 can induce stress fibers independently of the N-terminal GBD-FH3 domains (1–339), which are responsible for stress fiber targeting. Our results therefore suggest that the helical domain (396–573) plays a key role in the enhanced stress fiber formation shown by the FHOD1 340–1164 mutant, potentially by mediating the accumulation of the protein in the lamellipodium (Fig. 6D), which might lead to the formation of short actin filament bundles through its proposed role in actin bundling (Schönichen et al., 2013).

We next characterized the spatial organization of the thick actin stress fiber bundles that are induced by activated FHOD1 V228E in more detail by using confocal F-actin image stacks. We found that the majority of those bundles were localized on the ventral side of cells, where they associated with focal adhesions on both ends. Thus, those bundles were mainly composed of ventral stress fibers (Fig. 7D, left). Interestingly, we also observed a minor fraction of thick actin bundles that formed an arch-like structure, being dorsally localized only in their central region (Fig. 7D, right, white arrow) and ventrally localized in the flanking fiber ends (Fig. 7D, right, white arrowhead), which were associated with focal adhesions. Thus, while sharing partial dorsal localization with actin arcs, these arch-like structures also showed properties of ventral stress fibers.

**The C-terminal FHOD1 domain is required for the efficient formation of ventral stress fibers**

The C-terminal DAD region of FHOD1 mediates its auto-inhibitory conformation by interacting with the N-terminal FH3 domain (Takeya and Sumimoto, 2003; Schönichen et al., 2006).
The C-terminally truncated FHOD1 construct (FHOD1 1–1011) was therefore used in previous studies as a model for constitutively active FHOD1 with an open conformation. To test whether the FHOD1 C-terminus might play additional functional roles, we compared the stress fiber formation of FHOD1 1–1011 with that of the FHOD1 point mutant (V228E), which contains the entire C-terminus. Similar to FHOD1 1–1011, FHOD1 V228E is in the active conformation because this mutation blocks the intramolecular auto-inhibitory interaction (Schulte et al., 2008). We analyzed the effect of these FHOD1 constructs on actin organization by quantifying the number of cells that contain an increased number of mature ventral stress fibers (Fig. 8A). As expected, wild-type FHOD1 only weakly enhanced the formation of ventral stress fibers, whereas the FHOD1 1–1011 mutant significantly stimulated their formation almost threefold as compared with EGFP (Fig. 8A). Interestingly, the FHOD1 V228E mutant had an even more pronounced effect on the formation of ventral stress fibers. This was surprising, as confocal imaging and cross-correlation analysis of FHOD1 fluorescence with the corresponding actin signals revealed that the FHOD1 V228E mutant was only weakly associated with stress fibers as compared with FHOD1 1–1011 (Fig. 8B,C).

Although FHOD1 1–1011 lacks the three C-terminal residues that can be phosphorylated by the upstream regulator ROCK,
these sites are still present in FHOD1 V228E. To study the effects of ROCK inhibition on the localization of these mutants, we combined acute pharmacological inhibition of the kinase with live-cell TIRF microscopy. We found that wild-type FHOD1 rapidly dissociated from actin fibers after the addition of the ROCK inhibitor Y-27632 (50 μM for 5 min). This suggests that the phosphorylation of FHOD1 by ROCK antagonizes constitutive de-phosphorylation, thereby allowing dynamic control of the association of FHOD1 with stress fibers (Fig. 8D,E). As expected, FHOD1 1–1011 remained mostly at stress fibers after ROCK inhibition. However, FHOD1 V228E dissociated substantially from stress fibers after Y-27632 treatment, similar to wild-type FHOD1 (Fig. 8D, arrows, 8E). This finding suggests that, even in the FHOD1 V228E mutant, phosphorylation of the C-terminus still controls the actin-binding function that is mediated by the N-terminal GBD-FH3 domains. Taken together, our observations suggest that the C-terminus plays not only an important role in FHOD1 auto-inhibition, but also a more direct functional role in the stimulation of stress fiber formation by FHOD1.

DISCUSSION

Here, we show that FHOD1 controls the coordinated maturation of stress fibers by its distinct effects on two dynamic precursor stress fiber types: it stimulates the formation of transverse arcs and inhibits the growth of dorsal fibers. These combined activities of FHOD1 lead to the efficient formation of less-dynamic ventral stress fibers. Our detailed analysis of this process offers novel mechanistic insight into the role of FHOD1 in the dynamic processes that build contractile actin structures in cells.

Interestingly, FHOD1 was preferentially associated with actin structures that have anti-parallel orientation, such as ventral stress fibers and transverse arcs. By contrast, FHOD1 was largely absent from dorsal fibers, which mainly consist of
parallel actin-bundles (Cramer et al., 1997; Svitkina et al., 1997; Pellegrin and Mellor, 2007). This preference of FHOD1 for anti-parallel actin fibers is shared by myosin II (Verkhovsky and Borisy, 1993), which has to bind such anti-parallel fibers to generate contractile forces (Clark et al., 2007). The only region close to dorsal stress fibers where there was association of FHOD1 and myosin II overlapped substantially with focal adhesions, suggesting that a subpopulation of anti-parallel-oriented actin filaments, albeit with currently unknown function, might be present in that region. It is currently unclear how FHOD1 might be able to associate selectively with anti-parallel actin bundles. One possibility would be by the binding of FHOD1 to myosin II, either directly or indirectly through adapter proteins. This idea is supported by our observation that FHOD1 is colocalized with myosin-II-rich stripes and not with α-actinin within stress fibers. The N-terminal GBD-FH3 region is required for the colocalization of FHOD1 with myosin II and might thus mediate such coordinated recruitment.

Both myosin II and FHOD1 are activated by phosphorylation that is mediated by the serine/threonine kinase ROCK (Amano et al., 1996; Kimura et al., 1996; Hannemann et al., 2008; Takeya et al., 2008). The upstream activator of ROCK, the small GTPase RhoA, is known to form spatial activity gradients that correlate with cell motility, cell shape and polarity (Pertz et al., 2006; Nalbant et al., 2009). Thus, in these cellular processes, the RhoA–ROCK pathway is a likely candidate to coordinate both the spatio-temporal organization of stress fiber formation through FHOD1 and the contractility of these stress fibers through myosin II.

Although the regulation of FHOD1 by ROCK-mediated phosphorylation was proposed previously (Hannemann et al., 2008; Takeya et al., 2008), the functional role of this modification was not fully explored. On the one hand, the effect of ROCK on the subcellular localization of FHOD1 and the timescale at which ROCK mediates FHOD1 regulation were unknown. On the other hand, experiments that use C-terminal truncation mutants or phosphomimetic mutations are difficult to interpret because they might affect other functions of the C-terminus that are not directly related to auto-inhibition. Indeed, the C-terminal DAD region of the formin mDia1 was recently suggested to play an active role in filament nucleation (Gould et al., 2011). Here, we show that, in comparison to the C-terminal truncation (FHOD1 1–1011), the point mutation V228E is an even stronger activator of stress fiber formation, although it shows significantly less colocalization with...
stress fibers. This suggests that the C-terminus of FHOD1 indeed plays additional roles in FHOD1 function, potentially by augmenting actin monomer recruitment, as reported for the formins mDia1 and FMNL2 (Gould et al., 2011; Block et al., 2012). Alternatively, the sensitivity of the FHOD1 association with stress fibers to ROCK inhibition suggests that dynamic binding and unbinding of FHOD1 to stress fibers, mediated by cycles of phosphorylation and dephosphorylation, might be necessary to activate FHOD1 function fully.

One of the most intriguing observations in this study was that FHOD1 has at least two distinct cellular functions: (1) stimulating stress fibers that contain anti-parallel filaments (arcs and ventral stress fibers) and (2) inhibiting the growth of stress fibers that contain long parallel filaments (dorsal stress fibers). Our studies give important novel insight into the molecular basis of how FHOD1 could perform those functions: (1) We were able to narrow down the region of FHOD1 that mediates the association of the protein with stress fibers and its colocalization with myosin II to amino acids 1–339. (2) We found that a truncation mutant lacking the GBD-FH3 domains accumulated in the lamellipodium and along the entire length of focal adhesions. (3) We found that persistent association of FHOD1 with stress fibers is not necessary for the stimulation of stress fiber formation. (4) Further truncation of a helical domain (396–573) that was recently suggested to mediate actin filament bundling in vitro (Schönichen et al., 2013) prevented the FHOD1-mediated stimulation of stress fibers. Together, these observations suggest that the helical, FH1-, FH2- and C-terminal domains are crucial for both the stimulation of arcs by FHOD1 and for their turn-over into ventral stress fibers. Previous studies proposed that the short bundled actin filaments that form transverse arcs originate from the lamellipodium (Hotulainen and Lappalainen, 2006; Burnette et al., 2011; Tojkander et al., 2012). Therefore, the lamellipodial localization of the highly active truncation mutant 340–1164 during acute arc stimulation suggests that FHOD1 might mediate the bundling of preformed short actin filaments at the leading edge of the cell through its helical domain, and that these filaments are then efficiently assembled into transverse arcs at the interface with the lamellum.

Classically, formins are thought to act as processive actin-capping proteins that can stimulate actin polymerization (Zigmund et al., 2003; Shemesh et al., 2005b; Oromo et al., 2005). However, recent biochemical studies show that purified active FHOD1 inhibits actin polymerization in addition to filament bundling (Schönichen et al., 2013). In cells, dorsal stress fibers require rapid filament elongation at the associated focal adhesion, which is mediated by the efficient processive capping protein mDia1 (Hotulainen and Lappalainen, 2006; Oakes et al., 2012). At these focal adhesions, FHOD1 might act as a capping protein, which would inhibit the growth of associated dorsal stress fibers by competing with mDia1. Our observation that a FHOD1 mutant lacking the stress-fiber-targeting GBD-FH3 domains associates more prominently with focal adhesions in steady-state conditions further supports this idea. In the lamellipodium, linear filaments are generated by mDia2 or FMNL2 (Yang et al., 2007; Tojkander et al., 2012; Block et al., 2012). Here, FHOD1 might enrich the pool of short actin filaments either by permanent or slow processive capping. Concomitantly, FHOD1 might promote transverse arc assembly by bundling those preformed short filaments. Thus, it is intriguing that FHOD1 by the same molecular activity might control opposing processes at dorsal stress fibers and arcs. Finally, whereas the central and C-terminal domains of FHOD1 are responsible for robust filament formation, the N-terminal stress-fiber-targeting region (1–339) might facilitate the precise spatial targeting of FHOD1 to ‘fine tune’ its cellular function. These processes in the generation of mature contractile stress fibers are summarized in a working model that integrates the two proposed molecular functions of FHOD1—filament capping and bundling—into a dynamic process that is tightly controlled in space and time (supplementary material Fig. S6).

In summary, our study establishes a key role for the formin FHOD1 in the spatio-temporal control of the dynamics of the actin filament meshwork in adherent cells. FHOD1 promotes the efficient formation of transverse actin arcs in the leading edge and, by restricting the length of dorsal stress fibers, it promotes the coordinated turn-over of actin arcs into mature contractile stress fibers.

**MATERIALS AND METHODS**

**Cell culture and reagents**

Human U2OS osteosarcoma cells (ATCC HTB-96) were maintained in DMEM-GlutaMAX (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Pan-Biotec), 50 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies) at 37°C under 5% CO2. Prior to cell seeding, coverslips (1.5 glass, Thermo Scientific) and dishes (1.5 glass bottomed, MatTek Corporation) were coated with 10 μg/ml collagen type I (C8919, Sigma-Aldrich) for 1 h at 37°C and were washed with phosphate-buffered saline (PBS). Cells were treated with the mycotoxin cytochalasin D (2 μM for 90 min) (C8273, Sigma Aldrich) to allow complete depolymerization of F-actin. Treatment started 72 h after the transfection of cells with siRNAs or 16 h after the transfection of cells with EGFP or EGFP-FHOD1 constructs. The recovery of stress fibers was stimulated by five washing steps with growth medium. The selective ROCK inhibitor Y-27632 (Y0503) was purchased from Sigma-Aldrich.

** Constructs and siRNAs**

EGF-actin was provided by Melissa Rolls (Pennsylvania State University, PA) and mKate–paixillin was a gift from Eli Zamir (MPI Dortmund, Germany). EGFP–NMHCIA [Addgene Plasmid 11347 (Wei and Adelstein, 2000)] and mCherry–NMHCIA [Addgene Plasmid 35687 (Dulyaninova et al., 2007)] plasmids were obtained from Addgene. mCherry–α-actinin 1 and mCherry–actin constructs were generated by replacing the EGF from pEGFP–α-actinin 1 [Addgene plasmid 11908 (Edlund et al., 2001)] and pEGFP-actin with mCherry from pmCherry-C1 (Clontech) using the AgeⅠ/BsrGⅠ restriction sites. For FHOD1 localization studies, low expression EGFP–FHOD1 constructs (delCMV-EGFP–FHOD1) were used as described previously (Schönichen et al., 2013). Low cellular expression using a truncated less-efficient CMV promoter (delCMV) has been described in a previous study by Watanabe and Mitchison (Watanabe and Mitchison, 2002). The FHOD1 V228E mutant was generated by site-directed mutagenesis (QuikChange Mutagenesis Kit, Agilent Technologies). Truncated FHOD1 mutants were generated by PCR from wild-type delCMV-EGF/FHOD1 or FHOD1 V228E, respectively. Cells were transfected with plasmid DNA constructs using Lipofectamine 2000 (Life Technologies).

All siRNAs were purchased from Qiagen. The FlexiTube GeneSilence set (GS29109) recommended by Qiagen to specifically target human FHOD1 contained four different siRNAs (termed #2, #5, #6 and #7 by the supplier), which were used either in combination (referred to as siFHOD1) or as individual oligonucleotides: siFHOD1#2, 5'-CAGCGAGGAGGACCTCTACAA-3'; siFHOD1#5, 5'-AGGTTGACGCTATCTGGAA-3'; siFHOD1#6, 5'-CCCGCGGTGTCGCCATGCTA-3' and siFHOD1#7, 5'-CCGGTGACACCGAGCTCTCTA-3'. AllStars negative control siRNA (1027280) was used as control (ntriRNA). With the exception of wound healing assays, cells were transfected with 5 nM of the indicated siRNAs directly after plating.
(fast-forward transfection) using HiPerFect (Qiagen) and were replated 24 h after transfection. Experiments were performed 72 h after siRNA transfection. Knockdown efficiency was determined by western blotting.

**Immunofluorescence**

Cells were fixed with 4% formaldehyde in PBS (for 20 min at 37°C), washed with PBS and permeabilized using 0.2% Triton X-100 in PBS (for 10 min at room temperature). Samples were washed and incubated in blocking solution (2% bovine serum albumin in PBS for 1 h at room temperature) followed by incubation with anti-paxillin antibody (1:500 in blocking solution for 1 h; clone 349, BD Transduction Laboratories) or anti-NMICIIa antibody (1:200 in blocking solution for 1 h; ab24762, Abcam), respectively. The coverslips were washed and incubated with Alexa-Fluor-488- or Alexa-Fluor-633-conjugated goat-anti-mouse-IgG (1:500; Life Technologies) or Alexa-Fluor-488-conjugated goat-anti-rabbit-IgG (1:1000; Life Technologies) secondary antibody together with Rhodamine–phalloidin to visualize filamentous actin (1:1000; Life Technologies) in blocking solution (1 h at room temperature). Samples were washed and, if required, stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:2000, 10 min at room temperature; Sigma-Aldrich). The coverslips were mounted using ProLong Gold (Life Technologies).

**Microscopy**

Epifluorescence and phase-contrast imaging were performed with an Eclipse Ti inverted microscope (Nikon) equipped with a motorized stage, a built-in Perfect Focus System and a CoolSNAP HQ2 camera (Photometrics). Images were acquired using a ×20/0.45NA air objective or a ×60/1.40NA oil-immersion objective. Acquisition was controlled by NIS-Elements Imaging Software (Nikon). Random positions were chosen using the multi-position tool.

Confocal laser-scanning microscopy was performed on a TCS SP5 AOBS system (Leica Microsystems) supported by LASAF software (Leica Microsystems) and equipped with an HCX PL APO ×63/1.4NA oil-immersion objective. The laser lines used for excitation were 488 nm (EGFP, Alexa Fluor 488), 561 nm (RFP, Rhodamine, mCherry) and 633 nm (Alexa Fluor 633). Confocal Z-stacks were acquired with 210-nm spacing, and maximum projections were generated with ImageJ software (http://rsbweb.nih.gov/ij/). Color-coded maximum projections of confocal Z-stacks were generated using the “Time Series Color Coder” macro (http://cmci.embl.de/downloads/timeseriescolorcoder) with a modified look-up table (LUT).

Time-lapse TIRF microscopy was performed on an Eclipse Ti-E (Nikon) inverted microscope with a motorized TIRF Illuminator Unit (Nikon), an Andor AOTF Laser Combiner (Andor Technology) and a Clara Interline CCD camera (Andor Technology). The laser lines used for the excitation of EGFP and mCherry were 488 nm and 561 nm, respectively. Images were acquired by using an Apo TIRF ×100/1.49NA oil-immersion objective (Nikon). Acquisition was controlled by Andor IQ Software (Andor Technology).

All microscopes were equipped with temperature-controlled incubation chambers. Time-lapse microscopy experiments were performed at 37°C in CO₂-independent medium [HBBS buffer, 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM MgCl₂ and 1 mM CaCl₂, supplemented with 10 μl/ml Oxyrase (Oxyrase)] with the indicated frame rates.

**Wound healing assay**

IBIDI culture inserts (IBIDI) were used to study wound-healing efficiency. Cells (8 × 10⁴) were seeded in each compartment of the culture inserts and were directly transfected with 10 nM siRNA using HiPerFect (Qiagen). The inserts were removed 72 h after siRNA transfection and the growth medium was replaced with growth medium supplemented with 10 mM HEPES. Cell migration was monitored by time-lapse phase-contrast microscopy (with a frame rate of 30 s). For each condition, ten different positions along the wound were recorded in each experiment. The migration efficiency was quantified by measuring the migrated distance (ImageJ). Lines were drawn along the wound edge at the start and end of the assay and the average movement was calculated from all positions.

**Cell adhesion and spreading**

For adhesion and spreading assays, cells were detached from culture dishes using Trypsin-EDTA and were plated onto collagen-type-I-coated glass coverslips in growth medium 72 h after siRNA transfection. After 15, 30 or 60 min, the coverslips were rinsed once with PBS, fixed with 4% formaldehyde and stained with DAPI (nuclei) and Rhodamine–phalloidin (F-actin). Epifluorescence images were acquired using a ×20/0.45NA oil objective. Adhered cells were quantified from 25 random regions for each condition and time-point. To quantify cell spreading, 20 random positions were acquired using a ×60/1.40NA oil-immersion objective for each condition, and the cell area was measured using ImageJ software. The focal adhesion size and number per cell were quantified from confocal images of cells expressing mKate–paxillin using ImageJ. Thresholds were set manually to isolate focal adhesions properly. The average area and number of focal adhesions per cell were quantified from binary images using the analyze particle tool in ImageJ. Particles smaller than 0.25 μm² and larger than 10 μm² were excluded to avoid nonspecific background signal and neighboring adhesions that were not properly separated.

**RNA isolation, reverse transcription and quantitative real-time PCR analysis**

Total RNA was extracted using the RNeasy Mini kit (Qiagen). Reverse transcription was performed with SuperScript® II Reverse Transcriptase (Life Technologies) according to the manufacturer’s protocols using 500 ng of total RNA. Quantitative real-time PCR was performed with a StepOnePlus™ Real-Time PCR system (Life Technologies) using the Fast SYBR® Green Master Mix (Life Technologies) and human FHOD1-specific primer pairs (forward, 5′-TACACGGTCACCCCTACTCAA-3′; reverse, 5′-AGTGCACTCGTCACATCGTA-3′). GAPDH, ACTB and RNR11S were used as housekeeping genes (QuantiTect Primer Assays, Qiagen). Relative quantification was performed using the efficiency-corrected relative quantification method (Pfaffl, 2001). Primer efficiencies were calculated for each primer pair by performing dilution series experiments.

**Western blot analysis**

Cells were washed once with ice-cold PBS and were lysed in ice-cold radioimmunoprecipitation assay buffer [50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (Roche)]. Insoluble cell debris were removed by centrifugation at 13,000 g for 10 min at 4°C. The protein concentration of the supernatants was determined by the Bradford method (BioRad). Equal amounts of total protein were mixed with 5×Laemmli sample buffer, boiled at 95°C for 10 min and separated by SDS-PAGE. After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Thermo Scientific) using a Biometra fastblot B34 blotting device (Biometra). Blots were blocked for 1 h at room temperature with 5% nonfat dry milk in TBS-T (20 mM Tris pH 7.6, 137 mM NaCl and 0.1% Tween-20) and incubated overnight at 4°C with the primary antibodies anti-FHOD1 (1:200, FM5321, ECM Bioscience) or anti-α-tubulin (1:20,000, clone B-5-1-2, Sigma-Aldrich). Membranes were washed three times with TBS-T and were incubated with HRP-conjugated anti-mouse secondary antibody (1:20,000; Santa Cruz Biotechnology) for 1 h at room temperature. After additional washing steps with TBS-T and TBS (20 mM Tris pH 7.6 and 137 mM NaCl), protein bands were visualized using ECL western blotting substrate (Pierce). Images were captured with a Fusion Fx7 system and were quantified with Bio-1D software (Pelog).

**Statistical analysis**

Statistical analysis

Independent two-tailed Student’s t-tests were used unless otherwise stated. In cases where there were unequal variances, Welch’s t-test was used. The resulting P-values are indicated in the figures and legends.
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