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FHOD1 interaction with nesprin-2G mediates TAN line formation and nuclear movement

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Abstract

Active positioning of the nucleus is integral to division, migration, and differentiation of mammalian cells[1]. Fibroblasts polarizing for migration orient their centrosomes by actin-dependent nuclear movement[2]. This nuclear movement depends on nesprin-2 giant (N2G), a large, actin-binding outer nuclear membrane component of transmembrane actin-associated (TAN) lines that couple nuclei to moving actin cables[3]. Here, we identify the diaphanous formin FHOD1 as an interaction partner of N2G. Silencing FHOD1 expression or expression of fragments containing binding sites of N2G or FHOD1 disrupted nuclear movement and centrosome orientation in polarizing fibroblasts. Unexpectedly, silencing of FHOD1 expression did not affect the formation or rearward flow of dorsal actin cables required for nuclear positioning. Rather, N2G-FHOD1 interaction provided a second connection to actin cables essential for TAN line formation and thus nuclear movement. These results reveal a unique function for a formin in coupling an organelle to actin filaments for translocation and suggest that TAN lines require multi-point attachments to actin cables to resist the large forces necessary to move the nucleus.

Diaphanous related formins (DRFs) constitute a family of Rho GTPase regulated proteins that regulate actin and microtubule cytoskeletons, thereby affecting multiple and diverse cellular processes[4, 5]. Most DRFs stimulate nucleation and/or elongation of linear actin filaments required for structures such as filopodia, lamellipodia and contractile rings. Despite similar domain organization and sequence homology to other formins, the DRF FHOD1 does not nucleate or elongate actin filaments, but rather bundles them[6]. FHOD1's bundling activity requires an actin binding region in the N-terminal regulatory domain and

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AUTHOR CONTRIBUTIONS G.G.G. and O.T.F. conceived the study, designed experiments and wrote the manuscript. S.K., R.Z., S.A. and G.W.L., I.S. designed and conducted experiments and discussed and interpreted the data together with O.T.F. and G.G.G.

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dimerization mediated by the FH2 domain[6]. Consistent with the biochemistry, expression of constitutively active FHOD1 (FHOD1 ΔC) lacking the C-terminal autoinhibitory domain in cells induces formation of thick actin cables that are decorated by the formin, another property that distinguishes FHOD1 from other DRFs[7, 8]. While recent reports imply that FHOD1 is hijacked during infection by various pathogens[9, 10] and contributes to adhesion maturation[11], cellular functions of FHOD1 remain largely unexplored.

RESULTS

Since our previous results indicated that (i) the structure and protein interactions of the FHOD1 N-terminus are distinct from other DRFs[12] and (ii) this domain is essential for actin cable formation by FHOD1 ΔC[7, 13], we sought to identify binding partners of the N-terminal domain. A yeast two-hybrid screen using residues 1-339 of human FHOD1 as bait identified residues 1340-1678 of human N2G as an interaction partner (Fig. 1a). Consistent with this interaction, GST-N2G 1340-1678 but not GST alone pulled down HA-tagged FHOD1 1-339 from HEK293T cell lysates (Fig. 1b). GST-N2G 1340-1678 also bound specifically to HA-FHOD1 WT and HA-FHOD1 ΔC (residues 1-1109). Importantly, HA-FHOD1 WT also immunoprecipitated with full length endogenous N2G (Fig. 1c).

To further map the FHOD1 binding site in N2G, fragments spanning the length of mouse N2G were tested by yeast two-hybrid for interaction with FHOD1 1-339. This mapping revealed that fragment H (residues 1130-1724) encompassing the region identified in the original yeast two-hybrid screen was the only region of N2G that interacted with FHOD1 1-339 (Fig. 1d). Fragments containing the C-terminus of FHOD1 did not interact with the H fragment or adjacent I or J fragments, the latter of which contains the N2G actin-binding calponin homology (CH) domains (Supplementary Fig. 1a-c). An N2G H fragment efficiently coimmunoprecipitated with HA-FHOD1 WT when coexpressed in 293T cells (Supplementary Fig. 1d).

These results identify an association of FHOD1 with N2G mediated by the N-terminus of FHOD1 and residues 1340-1678 in N2G. This region of N2G spans three predicted spectrin repeats (SRs 10-12) and part of a fourth (SR13). Interestingly, SRs 11-13 were previously identified as the second most evolutionary conserved set of spectrin repeats in N2G[14, 15]. Alignment of these repeats reveals a higher evolutionary conservation (28-54%) than the ~20% conservation that is generally observed between unrelated SRs[14, 15] (Fig. 1e, Supplementary Fig. 1e). Consistent with a specialized function of the FHOD1 interacting region in N2G, the region is not conserved in nesprin-1G[14, 15]. To identify specific N2G SRs involved in interaction with the N-terminus of FHOD1, we used various GST-tagged fragments of N2G SRs 10-13 to pull down HA-FHOD1 1-339 expressed in HEK293T cells. This analysis showed that fragments of N2G containing SRs 11-12 associated with FHOD1 1-339, while individual SRs did not associate (Fig. 1e). This identifies SRs 11-12 of N2G as the interaction site for FHOD1.

N2G is a ~800 kDa outer nuclear membrane protein essential for nuclear movement and thus centrosome orientation in migrating fibroblasts[3]. In contrast to many nuclear movements that are dependent on microtubules, N2G mediates actin-dependent nuclear movement in

starved fibroblasts stimulated by lysophosphatidic acid (LPA) or serum. We tested whether FHOD1 participated in N2G functions in nuclear movement and centrosome orientation by reducing its expression in NIH3T3 fibroblasts with four different siRNAs (Supplementary Fig. 2). Expectedly, ~ 60% of cells treated with control siRNA (to GAPDH) displayed LPA-stimulated centrosome orientation towards the wound edge (Fig. 2a,b) [3, 16]. In contrast, reduction of FHOD1 expression by each of the four siRNAs reduced centrosome orientation to about 35%, the level observed in unstimulated cells[17]. LPA-stimulated centrosome orientation results from active actin-dependent rearward movement of the nucleus while microtubules maintain the centrosome at the cell centroid[2, 16]. Analysis of nuclear and centrosome positions revealed that FHOD1 depletion blocked rearward nuclear positioning without affecting the position of the centrosome (Fig. 2c). Centrosome orientation and nuclear movement in FHOD1 depleted cells were rescued by re-expression of full length FHOD1 WT or constitutively active FHOD1 ΔC, but not by FHOD1 340-1164 lacking the N2G interacting region (Fig. 2d-f). Consistent with the critical role of nuclear positioning for fibroblast wound closure, migration of NIH3T3 cells into wounds was significantly reduced upon FHOD1 depletion (Fig. 2g,h). These results indicate that FHOD1 is required for actin-dependent nuclear movement and suggest that its interaction with N2G is important for this function.

To test directly whether FHOD1-N2G interaction was required for centrosome orientation and nuclear movement, we expressed the interacting regions of FHOD1 or N2G in starved NIH3T3 fibroblasts before stimulating them with LPA. Importantly, expression of the N2G H fragment containing SRs 11-12 that interact with FHOD1 potently disrupted LPA-stimulated centrosome orientation and rearward nuclear positioning (Fig. 2i-k). Similarly, FHOD1 1-339, which interacts with N2G and localizes to the nucleus and the cytoplasm[12], also acted as a dominant negative of these processes (Fig. 2i-k). We conclude that the interaction of FHOD1 with N2G is essential for centrosome orientation and rearward nuclear movement.

In NIH3T3 fibroblasts, actin-dependent nuclear movement is mediated by the assembly of N2G containing TAN lines that couple the nucleus to dorsal actin cables[3]. As reported[3], LPA-stimulated NIH3T3 fibroblasts treated with scrambled siRNA rapidly developed dorsal actin cables over the nucleus (Fig. 3a,c). Despite prevention of centrosome orientation and nuclear movement (Fig. 2b,c), FHOD1 depletion did not affect LPA-induced dorsal actin cables as measured by their numbers over the nucleus or the total intensity of nuclear or cytoplasmic phalloidin fluorescence (Fig. 3b-e). Normal formation of dorsal actin cables was also observed in FHOD1-depleted cells stimulated with serum, even though serum failed to stimulate centrosome orientation in these cells (Supplementary Fig. 3). Importantly, LPA-stimulated retrograde actin cable flow, which drives nuclear movement[2, 3], was unaffected by depletion of FHOD1 (Fig. 3f,g). FHOD1 is thus not essential for dorsal actin cable formation or retrograde flow during nuclear movement.

Nuclear movement in NIH3T3 fibroblasts depends on the assembly of N2G along dorsal actin cables to form TAN lines that couple the nucleus to moving actin cables. To test for a potential role of FHOD1 in TAN line formation, we investigated whether FHOD1 localized to these structures. Because antibodies for localizing FHOD1 under conditions that preserve

TAN lines were unavailable, we localized expressed RFP-FHOD1 constructs. Simultaneous visualization of N2G TAN lines by expression of GFP-mini-N2G (GFP-mN2G) or anti-N2G antibody and RFP-FHOD1 WT or ΔC revealed that FHOD1 was associated with dorsal actin cables and colocalized with TAN lines (Fig. 4a; Supplementary Fig. 4a). Importantly, in cells depleted of FHOD1, TAN line formation was strongly suppressed as assessed by either expressing GFP-mN2G or staining endogenous N2G, even though dorsal actin cables were evident over the nucleus (Fig. 4b,c and Supplementary Fig. 4b,c). These results indicate that FHOD1 is a component of TAN lines required for their formation.

FHOD1 has two actin interacting domains: one in its FH2 domain that appears to bind actin barbed ends and one in its N-terminus (residues 340-569), termed N-terminal actin binding site (ABS), that is required for FHOD1 to decorate actin cables[6, 8]. Since FHOD1 1-339 containing the N2G binding site but lacking the N-terminal ABS inhibited nuclear positioning and centrosome orientation (Fig. 2g-i), we tested the requirement of the N-terminal ABS for FHOD1 function in nuclear positioning by expressing FHOD1 1-569, which contains the N2G interacting site and the N-terminal ABS (Fig. 5a), in FHOD1-depleted cells. FHOD1 1-569 completely rescued centrosome orientation and partially rescued rearward nuclear positioning (Fig. 5b-d). These results were surprising because they suggested that the formin's FH2 domain was not absolutely required for rearward nuclear movement. To test this further, we prepared a chimeric construct (NCH, Fig. 5a) composed of the N2G interacting site in FHOD1 (1-339) and the well-characterized, actin-binding CH domains of α-actinin. Strikingly, NCH rescued centrosome orientation completely and rearward nuclear positioning partially when expressed in FHOD1-depleted cells (Fig. 5b-d). No rescue of these parameters was observed in FHOD1 silenced cells expressing the CH domains of α-actinin alone (Fig. 5a-d). Critically, both FHOD1 1-569 and the NCH chimera colocalized with dorsal actin cables above the nucleus (Fig. 5e). Coupled with our earlier results that the N2G binding fragment of FHOD1 (1-339) alone acted as a dominant negative (Fig. 2i-k), these results establish that the N-terminal N2G interacting site and ABS is the minimal domain of FHOD1 required for centrosome orientation and rearward nuclear positioning.

It was surprising that the FH2 domain, which defines formins, seemed dispensable for centrosome orientation and nuclear positioning. Yet, both FHOD1 1-569 and the chimera NCH, did not fully rescue nuclear positioning. To test if the FH2 domain might contribute to this function, we expressed in FHOD1-depleted cells a full length FHOD1 construct containing a point mutant (FHOD1 I705A, Fig. 5a) in a conserved residue in the FH2 domain that governs actin activity by DRFs[18]. In constitutively active FHOD1 ΔC, the I705A mutation prevented the normal stimulation of actin cable assembly of the WT protein (Supplementary Fig. 5; also see [9, 10]). FHOD1 I705A rescued centrosome orientation but only partially restored rearward nuclear positioning (Fig. 5b-d), suggesting that for full rearward nuclear positioning, both the N-terminal ABS and the FH2 domain are required.

DISCUSSION

The previous model for TAN lines[3, 19] hypothesized that the nucleus and the overlying dorsal actin cables are solely connected by the CH domains of N2G. Our current data

suggest that the soluble, cytoplasmic protein FHOD1 plays an essential role in linking the outer nuclear membrane protein N2G to actin cables (Fig. 5f). This model posits that FHOD1 enhances the interaction between N2G and the actin cable by providing N2G with a second physical link to actin. One end of FHOD1 (residues 1-339) connects to N2G by binding to SR11-12 that are unique to N2G; the other end of FHOD1's N terminus (residues 340-569) interacts with the actin cable through its ABS (residues ~400-530[6, 8]). We propose that the N- terminal ABS is critical for forming TAN lines as constructs lacking this site did not rescue FHOD1 depletion and a FHOD1 construct containing only the N2G binding site was dominant negative for nuclear movement. Additionally, a FHOD1 construct that contained both the N2G interaction site and the N-terminal ABS fully rescued centrosome orientation and largely rescued nuclear movement, as did a chimeric construct containing FHOD1's N2G-interacting domain and the CH domains from α-actinin.

Our model has implications for how N2G connects to dorsal actin cables during nuclear movement. We previously showed that the actin binding ability of N2G's CH domains was essential for TAN line formation [3]. Our current data stress that the actin binding capability of FHOD1 is also necessary for N2G to form TAN lines and move the nucleus. This implies that a multivalent connection between the nesprin and the actin cable are required to resist the force generated by moving such a large organelle as the nucleus. Such a role for FHOD1 may be analogous to that played by the multiple actin binding proteins that mediate connections between membrane integrin receptors in focal adhesions and actin filaments in stress fibers [20, 21]. Additionally, the association of FHOD1 with N2G puts its N-terminal ABS in proximity to the CH domains of N2G and this should increase the avidity of N2G interaction with actin filaments and enhance capture of actin cables as they move over the nucleus. Finally, connecting N2G to actin cables via FHOD1 may allow regulation as interactions of the FHOD1 N-terminus with its C-terminal autoinhibitory domain or activating GTPases could affect FHOD1-N2G interactions.

The multivalent feature of the model seems at odds with the rescue of TAN line formation and nuclear movement in N2G depleted cells by mN2G, which lacks the FHOD1 interacting site but contains CH domains[3]. However, since mN2G was overexpressed in these rescue studies, the high levels of CH domains available for interacting with actin cables likely compensate for the multivalent attachment through a single N2G.

A detailed structure of N2G and FHOD1 in association with actin filaments awaits higher resolution studies. Nonetheless, the extended structure of SR proteins and the conserved 5 nm length of SR repeats makes a prediction about the geometry of N2G relative to the actin filament when it is bound via its CH domains and FHOD1's N-terminal ABS. The CH domains and the FHOD1 interacting region (SRs 11-12) in N2G would be expected to be separated by as much as 50 nm. Given that the N2G-interacting site and the ABS in the N-terminus of FHOD1 likely span less than 10 nm[12], this predicts that N2G bound to actin via its CH domains and FHOD1 will lie nearly parallel to the long axis of the actin filament (as depicted in Fig. 5f) rather than perpendicular as predicted in earlier models of N2G interaction with actin filaments[1, 22].

The involvement of FHOD1 in moving nuclei is a unique function for a formin. Most formins stimulate actin filament elongation by processively binding the barbed end of the actin filament through their FH2 domain. Although FHOD1's FH2 domain is highly conserved compared to other DRFs and behaves as if it binds to actin barbed ends, it does not seem to stimulate actin polymerization in vitro or in cells[6]. Instead, the main biochemical activity of FHOD1 is in bundling actin filaments and binding along their length and these activities require the unique N-terminal ABS[6, 8]. FHOD1's FH2 domain contributes to bundling by establishing the dimeric nature of FHOD1. Our results suggest that FHOD1's FH2 domain may not contribute directly to its activity in TAN line formation. Yet, the lack of complete rescue of nuclear movement with FHOD1 constructs bearing FH2 domain mutations or deletions suggests that it may contribute, perhaps by promoting dimerization to additionally stabilize TAN line structure by cross-linking adjacent nesprins.

FHOD1 function in nuclear movement resembles that recently described for the formin INF2 in binding ER membranes and contributing to their scission by deforming them in an actin-dependent fashion[23]. There are 15 formin family members in mammals and it will be interesting to test whether other members of this family also function as proteins that mediate force transmission between the actin cytoskeleton and membranes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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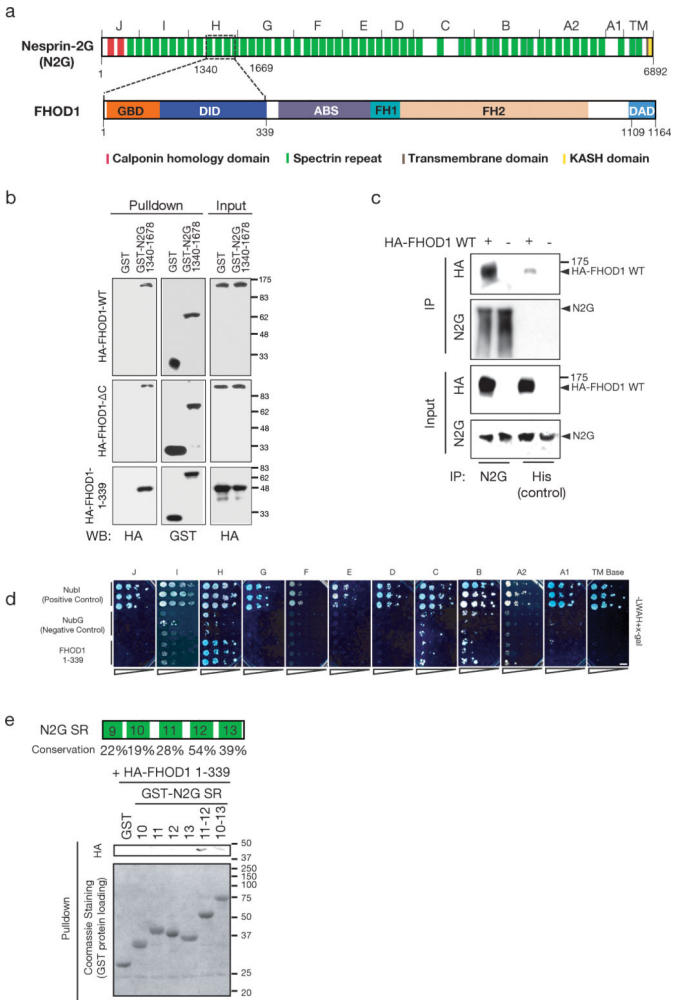

Figure 1.

FHOD1 interacts with N2G. (**a**) Schematic representation of the interaction site between human FHOD1 and N2G identified by yeast two hybrid is shown mapped onto mouse N2G and is indicated by the dotted box. The letters above N2G refer to fragments used for the directed yeast two hybrid in d. Domains in FHOD1 are: GBD, GTPase binding domain; DID, Diaphanous inhibitory domain; ABS, actin binding site; FH1, formin homology 1 domain; FH2, formin homology 2 domain; DAD, Diaphanous autoregulatory domain. (**b**) Pull down of HA-FHOD1 constructs with GST-N2G 1340-1678. HEK293T cell lysates containing the indicated HA-FHOD1 constructs were pulled down with GST-N2G 1340-1678 or GST and analysed by western blotting (WB) with HA or GST antibody. (**c**) Coimmunoprecipitation of HA-FHOD1 WT with antibody to endogenous N2G (or unrelated His antibody as a control) from lysates of transfected 293T cells. Immunoprecipitates were analysed by western blotting with antibodies to HA and N2G. (**d**) Directed membrane yeast two hybrid with the N2G fragments as baits and FHOD1 1-339 as prey and positive and negative controls. Triplicates at increasing dilution are shown. Only fragment H interacted above background level with FHOD1 1-339. Bar, 5 mm. (**e**) Pull down of HA-FHOD1 1-339 with indicated SRs from the interacting region of N2G. The evolutionary conservation of the residues in each of the SRs is indicated (see Methods and Supplementary Fig. 1).

Lysates from 293T cells expressing HA-FHOD1 1-339 were pulled down with the indicated GST- tagged N2G SR constructs or GST alone and analysed by Western blotting with an antibody to HA. Coomassie staining is shown for GST loads.

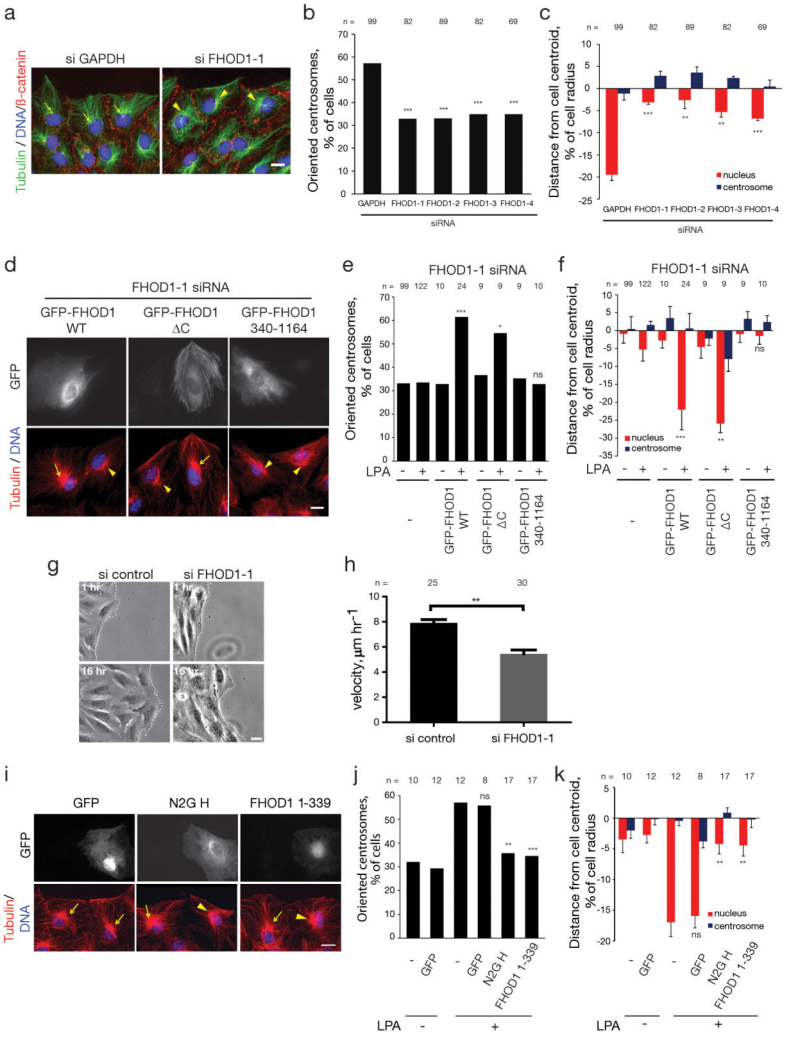

Figure 2.

FHOD1 is required for nuclear movement. (**a**) Immunofluorescence images of LPA-stimulated, wounded monolayers of NIH3T3 fibroblasts depleted of GAPDH or FHOD1. The wound is towards the top in this and all subsequent figures. Arrows and arrowheads indicate oriented and non-oriented centrosomes, respectively. (**b**) Quantification of centrosome orientation in the experiment shown in a. Centrosome orientation between the leading edge and nucleus was scored as described[2, 16]; random orientation is 33% by this measure. (**c**) Quantification of centrosome and nucleus position along the front-back axis in LPA- stimulated NIH3T3 fibroblasts depleted of GAPDH or FHOD1. The cell centroid is defined as “0”; positive values, toward the leading edge; negative, away. Data in a-c are from 3 experiments; n = number of cells analysed per experiment in this and subsequent panels. (**d**) Immunofluorescence images of LPA-stimulated, wounded monolayers of FHOD1-1 siRNA treated NIH3T3 fibroblasts re-expressing the indicated FHOD1 constructs. Arrows indicate oriented centrosomes; arrowheads, non-oriented centrosomes. (**e**) Quantification of centrosome orientation in the experiment shown in d. (**f**) Analysis of centrosome and nucleus position in the experiment shown in d. Data in d-f are from 3

experiments. **(g)** Images from a phase contrast movie of NIH3T3 fibroblast migrating into wounds after treatment with FHOD1-1 siRNA or scrambled siRNA control. The dashed line shows the wound edge. **(h)** Velocity of wound closure in NIH3T3 fibroblasts treated with FHOD1-1 siRNA or scrambled siRNA control. Data are from 3 individual experiments. (**i**) Immunofluorescence images of LPA-stimulated, wounded monolayers of NIH3T3 fibroblasts expressing interacting regions of N2G or FHOD1. Arrows indicate oriented centrosomes; arrowheads, non-oriented centrosomes. (**j**) Quantification of centrosome orientation in the experiment shown in i. (**k**) Analysis of centrosome and nucleus position in the experiment shown in i. Data in i-k are from 4 experiments. Bars, a, d, g, i: 10 μm. Error bars for c,f,h,k: SEM. The n number of cells analysed is shown in (b, c, e, f, h, j, k) ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; ns, not significantly difference by Fisher's exact test (b,e,j) and two-tailed t-test (c,f,h,k).

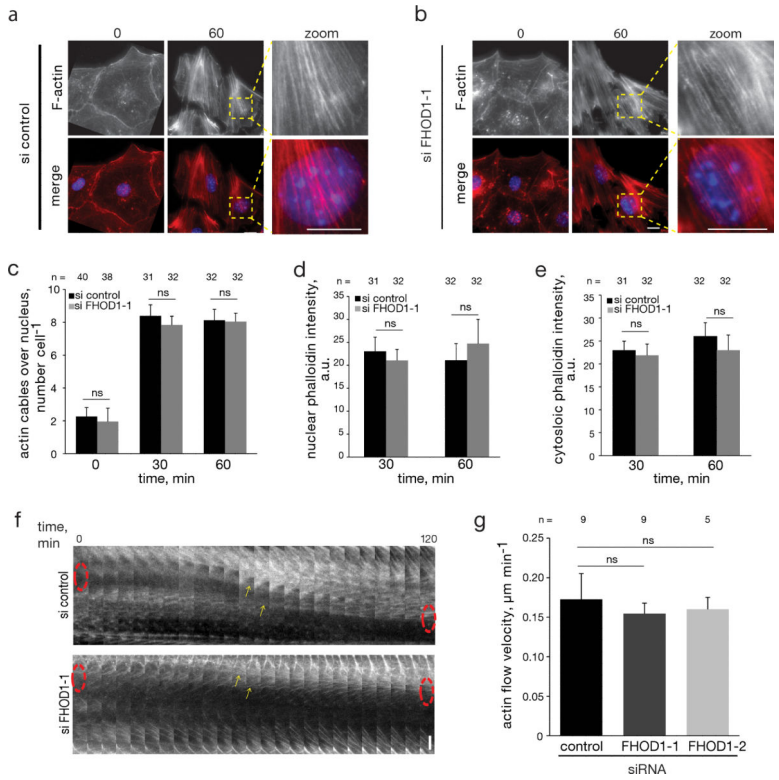

Figure 3.

FHOD1 is dispensable for formation of dorsal actin cables and retrograde actin flow. (**a, b**) Fluorescence images of F-actin (phalloidin) and DNA (DAPI) in LPA-stimulated NIH3T3 fibroblasts treated with (a) control or (b) FHOD1 siRNAs. Time in min after LPA stimulation is shown at top. Zoomed images of the outlined regions in the 60 min time point show dorsal actin cables over the nucleus. (**c-e**) Quantification of (c) the number of dorsal actin cables above nuclei, (d) nuclear phalloidin intensity, and (e) cytosolic phalloidin intensity in NIH3T3 fibroblasts treated with control or FHOD1 siRNAs and stimulated with LPA for the indicated time. Data in a-e are from 5 experiments; n = number of cells analysed per experiment in these and subsequent panels. (**f**) Kymographs from movies of Lifeact-GFP stably expressed in NIH3T3 fibroblasts treated with control or FHOD1-specific siRNA. Time (min) is shown above the kymograph; each panel is 5 min. Arrows, retrogradely moving dorsal actin cables; dashed circles, position of nucleus. (**g**) Velocity of actin cable retrograde flow in NIH3T3 fibroblasts treated with control or FHOD1 siRNAs determined from kymographs as in (f). Data are from 3 experiments. Bars, a,b: 10 µm; f, 5 µm. Error bars (c-e, g), SEM. The n number of cells analysed is shown in (c-e, g). ns, not significantly different by two-tailed t-test.

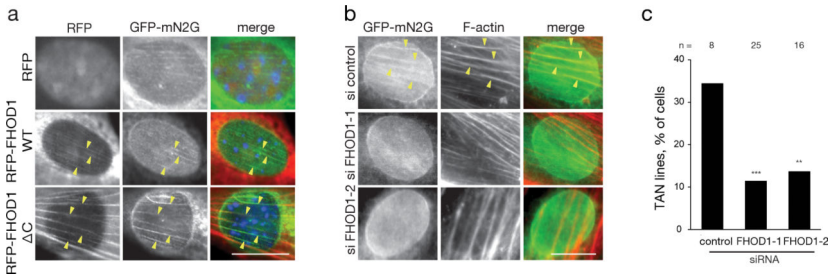

Figure 4.

FHOD1 is essential for TAN line formation. (**a**) Fluorescence images of the indicated RFP-FHOD1 constructs or RFP as a control and GFP-mN2G (a TAN line marker) on the dorsal surface of wound edge NIH3T3 fibroblasts. Arrowheads, FHOD1 colocalizing with mN2G in TAN lines. (**b**) Fluorescence images of GFP-mN2G and F-actin (phalloidin) on the dorsal surface of wound edge NIH3T3 fibroblasts treated with control or FHOD1 siRNA. Arrowheads, TAN lines with colocalized GFP-mN2G and F-actin. (**c**) Quantification of the frequency of wound-edge NIH3T3 fibroblasts with TAN lines following treatment with the indicated siRNAs. Data are from 3 experiments; n = number of cells analysed per experiment in (c). Bars, a,b: 10 μm. (c) ***, P<0.001; **, P<0.01 by Fisher's exact test.

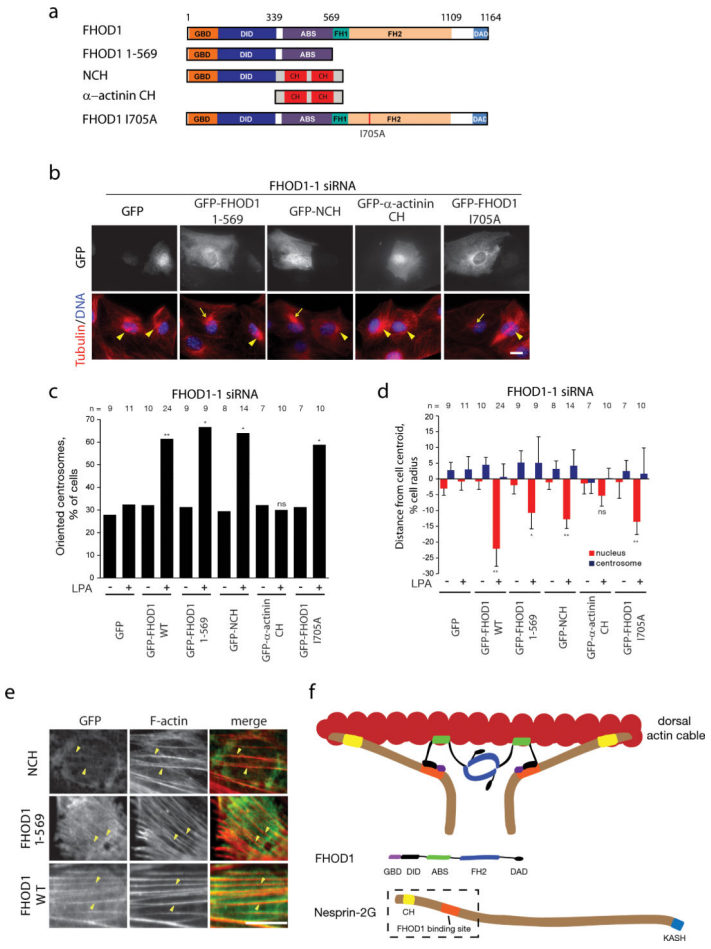

Figure 5.

The N-terminal actin binding site of FHOD1 provides N2G with an additional contact to actin filaments required for TAN line formation. (**a**) Schematic of constructs used. (**b**) Immunofluorescence images of LPA-stimulated, wounded monolayers of FHOD1-1 siRNA treated NIH3T3 fibroblasts expressing the indicated constructs and stained for GFP, Tyr tubulin, and DNA (DAPI). Arrows indicate oriented centrosomes; arrowheads, non-oriented centrosomes. (**c**) Quantification of centrosome orientation in the experiment shown in (b). (**d**) Analysis of centrosome and nucleus position in the experiment shown in (b). Data in b-d are from 3 experiments; n = number of cells analysed per experiment. (**e**) Immunofluorescence images of the indicated GFP constructs and F-actin (phalloidin) over nuclei of wound-edge NIH3T3 fibroblasts depleted of FHOD1. Arrowheads, examples of expressed GFP protein colocalizing with dorsal actin cables over the nucleus. (**f**) Model of multivalent connection of N2G to actin filaments established by FHOD1-N2G interaction. N2G's paired CH domains provide one connection to the actin filament; FHOD1 associated with N2G provides a second actin filament binding site through its N-terminal ABS. FHOD1 is enlarged relative to N2G to allow depiction of its domains. Bars, b,e: 10 μm. Error bars d: SEM. **, $P < 0.01$; *, $P < 0.05$; The n number of cells analysed is shown in (c, d) ns, not significantly different by Fisher's exact test (c) and two-tailed t-test (d).