The Diaphanous-related Formin FHOD1 Associates with ROCK1 and Promotes Src-dependent Plasma Membrane Blebbing

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Diaphanous-related formins (DRFs) mediate GTPase-triggered actin rearrangements to regulate central cellular processes, such as cell motility and cytokinesis. The DRF FHOD1 interacts with the Rho-GTPase Rac1 and mediates formation of actin stress fibers in its deregulated form; the physiologically relevant activities and molecular mechanisms of endogenous FHOD1, however, are still unknown. Here we report that FHOD1 physically associates via the N-terminal part of its FH2 domain with the central domain of ROCK1. Although FHOD1 does not affect the kinase activity of ROCK1, the DRF is an efficient substrate for phosphorylation by ROCK1. Co-expression of FHOD1 and ROCK1 results in the generation of nonapoptotic plasma membrane (PM) blebs, to which the DRF is efficiently recruited. Blebbing induced by FHOD1 and ROCK1 depends on F-actin integrity, the Rho-ROCK cascade, and Src activity and is reminiscent of the recently described PM blebs triggered by expression of Src homology 4 (SH4) domain PM targeting signals. Consistently, endogenous FHOD1 is required in SH4 domain expressing cells for efficient PM blebbing and rounded cell morphology in two-dimensional cultures or three-dimensional matrices, respectively. Efficient association of FHOD1 with ROCK1, as well as recruitment of the DRF to blebs, depends on Src activity, suggesting that the functional interaction between both proteins is regulated by Src. These results define a role for endogenous FHOD1 in SH4 domain-induced blebbing and suggest that its activity is regulated by ROCK1 in a Src-dependent manner.

In response to intra- and extracellular cues, remodeling of the submembranous cytoskeleton constantly adjusts the plasma membrane (PM)² of eukaryotic cells. These cytoskeletal reorganizations are primarily controlled by small Rho-GTPases and their downstream signaling cascades, resulting in distinct types of invaginations or protrusions, depending on the specific set of GTases and effectors involved. In addition to well-described PM protrusions, such as lamellipodia and filopodia (1), under certain conditions, cells display on their surface highly dynamic rounded structures referred to as PM blebs (2). PM blebbing results from local destabilization of the cortical actin meshwork that causes expansion of the PM due to the osmotic pressure of the cell interior. Following this expansion phase, blebs typically briefly remain static before local actin polymerization and actin-myosin contraction events are thought to guide retraction of the bleb (3–7). PM blebbing has long been observed as an early event in apoptotic and necrotic processes (8–10). More recently, nonapoptotic PM blebs were identified to play roles in distinct cellular processes, such as cytokine release, cytokinesis, embryonic stem cell motility, or cancer cell invasion (11–14). Although PM blebbing seems to follow the common overall scheme of expansion and retraction, mechanistic differences exist between distinct types of nonapoptotic PM blebs, in particular in regard to the stimulus that initiates blebbing. Typically, blebbing is induced in a three-dimensional environment by yet to be identified stimuli and is thought to facilitate directed cell movement of, for example, tumor or germ cells (11, 14–16). More amenable for molecular analysis, several model systems have been described in which cell blebbing is efficiently observed under two-dimensional cell culture conditions. Deficiency in the actin-binding protein filamin A (5, 6) or the tumor suppressor p53 (17) as well as expression of the Dia-interacting protein DIP (18) or an effector loop mutant of active Rac1 (19) can cause efficient PM blebbing. We recently reported that expression of SH4 membrane-targeting domains, corresponding to an 18-aa short peptide with N-terminal acylation that mediates PM targeting of, for example, Src

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2 The abbreviations used are: PM, plasma membrane; DRF, Diaphanous-related formin; FH1, -2, and -3, formin homology 1, 2, and 3, respectively; SH4, Src homology 4; aa, amino acid(s); CHO, Chinese hamster ovary; TRITC, tetramethylrhodamine isothiocyanate; HA, hemagglutinin; WT, wild type; IP, immunoprecipitation; RNAI, RNA interference; GFP, green fluorescent protein; MLC, myosin light chain; DAD, Diaphanous autoregulatory domain.
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kinase family members, also induces PM blebbing in two-di-
menSional as well as three-dimensional cultures. In addition,
SH4 domain-induced blebbing correlates with accelerated cell
invasion and rounded cell morphology in three-dimensional
matrices (20). Virtually all types of blebs analyzed so far share
the dependence on the small GTPase Rho and its effector kinase
ROCK (2). SH4 domain blebbing additionally involves activity
of the protooncogene Src. How precisely actin destabilization
and subsequent polymerization is achieved, however, has not
been addressed for individual types of blebbing.

Of the four classes of actin nucleators described so far
(Arp2/3 complex, Spire proteins, cordon-bleu, and formins),
the last have been implicated in PM blebbing. Formins are mod-
ular proteins containing characteristic formin homology (FH)
domains 1 and 2 that mediate binding to profilin as well as actin
nucleation and bundling, respectively, and a less well defined
FH3 domain. Actin nucleation mediated by formins results in
unbranched filaments and occurs at the barbed end, at which the
formin persists for protection from capping proteins (21). A
subgroup of formin proteins, the Diaphanous-related formins
(DRFs), contain an additional GTPase binding site and function
in Rho-GTPase-regulated pathways (see Refs. 21 and 22 for a
review). DRFs are autoinhibited by an intramolecular inter-
action between their C-terminal Diaphanous autoinhibitory
domain (DAD) and the N terminus (23). Activation of DRFs
includes release of their autoinhibition, achieved by binding to
the GTPase and/or other cellular interaction partners (24–26).
The mammalian DRF family comprises the Dia, Daam, FRL,
and FHOD formins that are distinct in their GTPase specificity,
tissue distribution, and biological function. Regarding PM bleb-
ing, Dia1 (an interaction partner and regulator of Rho) and
mDia2 (an interaction partner of Cdc42, RhoA, and Rif) were
shown to be critical for bleb formation of MDA-MB-435 cancer
cells in three-dimensional matrices and DIP-expressing fibro-
blasts in two-dimensional ones, respectively (15, 18). The role
of, for example, Rac-interacting DRFs, such as FHOD1, in PM
blebbing has not yet been addressed.

FHOD1 is currently one of the least well understood DRFs
on the molecular and cellular level. Although interacting with
Rac1, a constitutively active FHOD1 variant induces formation
of F-actin stress fibers in a Rho-ROCK-dependent manner and
coordinates F-actin and microtubule networks (27–32). Expression of active Rac1 recruits FHOD1 to the PM but seems
insufficient for its activation (29). FHOD1 shares typical fea-
tures with other DRF family members, such as overall domain
organization, multimerization, and autoinhibition (32–34), but
its GTPase interaction and FH3 domains are not well charac-
terized. By which mechanism autoinhibition of FHOD1 is
released and which biological activities endogenous full-length
FHOD1 exerts is not entirely clear; however, recent results
imply that phosphorylation of the FHOD1 DAD by ROCK facil-
itates activation of the DRF and stress fiber formation in epi-
thelial cells (35, 36).

Here we describe that FHOD1 directly interacts with ROCK1
and find that both proteins synergize to promote PM blebbing.
ROCK1/FHOD1-induced blebs displayed characteristics simi-
lar to the recently described blebbing induced by SH4 domains,
including the requirement for Src activity. Interestingly, both
endogenous FHOD1 and ROCK1 were required for efficient
SH4 domain-induced blebbing. Since both physical and func-
tional interactions between FHOD1 and ROCK1 required Src
activity, these results imply that Src acts as key regulator of the
functional interplay between FHOD1 and ROCK1 in SH4
domain-induced PM blebbing.

EXPERIMENTAL PROCEDURES

Cell Lines, Fixation, Immunostainings, and Microscopy—
CHO, HeLa, COS-7, and SYF cell lines were cultivated in
α-minimum Eagle’s medium or Dulbecco’s modified Eagle’s
medium (Invitrogen), respectively, supplemented with 10%
fetal calf serum (5% for COS-7 cells) and antibiotics. Stable
CHO cell lines inductively expressing CHO-N18-HASPB-GFP
were described previously (20), and transgene expression was
achieved by adding 1 μg/ml doxycycline for 24 h as described.
SYF−/− cells and SYF + c-Src cells (37) were kindly provided by
Klemens Rottner (HZI Braunschweig) with permission from
Phillipe Soriano (Fred Hutchinson Cancer Research Center,
Seattle, WA). Fixation, immunostainings, and microscopy were
performed as described previously (20).

Antibodies and Reagents—Antibodies were as follows: mouse
anti-α-tubulin clone B-5-1-2 (Sigma), mouse anti-c-Myc
(9E10), rat anti-HA (3F10), and mouse anti-c-Src (B-12) (all
from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit
anti-c-Src (Tyr(P)418) (BIOSOURCE), rabbit anti-FHOD1 (30),
rabbit anti-HA (Y-11) (Santa Cruz Biotechnology), rabbit anti-
MLC2 (Cell Signaling), rabbit anti-MLC (Ser(P)19) (Cell Signal-
ing), goat anti-mouse IgG Alexa-Fluor-350 (Invitrogen), goat
anti-rabbit IgG Alexa-Fluor-350 (Invitrogen), goat anti-mouse
IgG Alexa-Fluor-488 (Invitrogen), goat anti-rabbit IgG Alexa-
Fluor-488 (Invitrogen), goat anti-mouse IgG Alexa-Fluor-568
(Invitrogen), goat anti-rabbit IgG Alexa-Fluor-568 (Invitro-
gen), goat anti-mouse IgG Alexa-Fluor-660 (Invitrogen), and
goose anti-rabbit IgG Alexa-Fluor-660 (Invitrogen). Reagents
used were as follows: Alexa-Fluor-350 phalloidin (Invitrogen);
Alexa-Fluor-660 phalloidin (Invitrogen); blebbistatin (Calbio-
chem); cycloheximide (Calbiochem); cytochalasin D (Calbio-
chem); doxycycline (Sigma); Hoechst 33258 (Sigma); Iatrucu-
lin B (Calbiochem); LinMount (Linaris E6004); nocodazole
(Calbiochem); phalloidin-fluorescein isothiocyanate (Sigma);
phalloidin-TRITC (Sigma); PP1, PP2, and PP3 (Calbiochem);
recombinant human tumor necrosis factor α (PromeGa); and
Y-27632 (Calbiochem).

Constructs—The cDNA library from human peripheral
blood mononuclear cells used in the yeast two-hybrid screen
and cloned in the pGAD3S2X vector has been already described
(38). DNA constructs for expression in yeast of the wild type
(WT) and deleted forms (ΔC, 1–863, 1–807, and 1–611) of
FHOD1 fused to LexA as well as mammalian expression con-
structs for HA-tagged FHOD1 and FHOD1-ΔC variants have
been described previously (33). Plasmids for expression of Myc-
tagged ROCK1 and ROCK1-Δ3 were gifts from Shuh Narumiya
(Kyoto University Faculty of Medicine). The expression con-
structs for fusion proteins of wild-type or activated GTPases
as well as for N18-HASPB-GFP and N18-Yes-WT-GFP were
described earlier (20, 29). The C3-GFP plasmid as well as
expression plasmids for Myc-tagged GTPases were kindly

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briefly, 250 μl of whole cell lysate derived from a 10-cm dish, which was grown with HeLa cells at 90% confluence were pre-treated with 25 μl of Protein G-Sepharose beads (50% suspension) for 30 min. IP was performed by the addition of 7 μl of mouse anti-c-Myc antibody which was coupled to 30 μl of Protein G-Sepharose beads (50% suspension). For the kinase reaction, beads were suspended in 30 μl of kinase assay buffer (39), containing 4 μCi of [γ-32P]ATP, 10 mM ATP, 3 or 5.6 μg of the substrates MLC (Sigma) or FHOD1, respectively, and either 5.5 or 6-well format. On the following day, cells were harvested for microscopic analysis and Western blot analysis of knockdown efficiency.

**In Vitro Kinase Assay—Immunoprecipitation (IP) of Myc-tagged ROCK1** was performed as described previously (8). Briefly, 250 μl of whole cell lysate derived from a 10-cm dish, which was grown with HeLa cells at 90% confluence were pre-treated with 25 μl of Protein G-Sepharose beads (50% suspension) for 30 min. IP was performed by the addition of 7 μl of mouse anti-c-Myc antibody which was coupled to 30 μl of Protein G-Sepharose beads (50% suspension). For the kinase reaction, beads were suspended in 30 μl of kinase assay buffer (39), containing 4 μCi of [γ-32P]ATP, 10 mM ATP, 3 or 5.6 μg of the substrates MLC (Sigma) or FHOD1, respectively, and either 5.5 μl of solvent control or ROCK inhibitor (Y-27632; final concentration 10 μM) was added for specificity control. Following 30 min of shaking at 30 °C, the kinase reaction was stopped, subjected to separation by SDS-PAGE, and transferred to Western blotting to a polyvinylidene fluoride membrane, which was finally incubated for 24 h with a photosensitive film.

**RNAs—For targeted knockdown of gene expression, double-stranded RNA oligonucleotides were transfected into target cells by lipofection.** For transfection, 3.0 ml of whole cell lysate derived from a 10-cm dish, which was grown with HeLa cells at 90% confluence were pre-treated with 25 μl of Protein G-Sepharose beads (50% suspension) for 30 min. IP was performed by the addition of 7 μl of mouse anti-c-Myc antibody which was coupled to 30 μl of Protein G-Sepharose beads (50% suspension). For the kinase reaction, beads were suspended in 30 μl of kinase assay buffer (39), containing 4 μCi of [γ-32P]ATP, 10 mM ATP, 3 or 5.6 μg of the substrates MLC (Sigma) or FHOD1, respectively, and either 5.5 μl of solvent control or ROCK inhibitor (Y-27632; final concentration 10 μM) was added for specificity control. Following 30 min of shaking at 30 °C, the kinase reaction was stopped, subjected to separation by SDS-PAGE, and transferred to Western blotting to a polyvinylidene fluoride membrane, which was finally incubated for 24 h with a photosensitive film.

**Drug Treatment—HeLa, SYF cells, or SYF + c-Src cells expressing ROCK1 either alone or in combination with FHOD1, N18-Yes-GFP, or N18-HASPB-GFP were synchronized in PM bleb formation.** Therefore, membrane blebbing was efficiently abrogated by treatment with Y-27632 (SYF cells, 10 μM; HeLa cells, 90 μM) for 2 h. After extensive washing of the cells, de novo bleb formation was allowed for 3 h in medium containing a solvent control or Y-27632 (SYF cells, 10 μM; HeLa cells, 90 μM), 100 μM blebbistatin, 2 μM cytochalasin D, 25 μM latrunculin B, 384 μM nocodazole, or 1–10 μM PP1. Subsequently, the cells were fixed and stained for F-actin or α-tubulin (20, 40). Drug treatment was controlled for efficient disruption of the targeted structures and reversibility within 3 h after wash out of the drug.

**Induction of Apoptosis—For induction of apoptosis, HeLa cells grown on coverslips were treated for 3 h with a combination of 50 μg/ml recombinant human tumor necrosis factor α and 5 μg/ml cycloheximide.** Apoptotic HeLa cells were determined by an Annexin V-mediated surface-specific stain of externalized phosphatidylserine. Therefore, living cells were carefully washed with AB buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2) and incubated with staining solution consisting of AB buffer containing fluorescently labeled Annexin V-Alexa-Fluor-568 at a dilution of 1:50 (v/v) for 15 min at room temperature. Subsequently, cells were fixed and stained for F-actin.

**Co-immunoprecipitation—To analyze FHOD1-ROCK1 association by co-immunoprecipitation, 2 × 105 COS-7 cells were co-transfected with 10 μg of the indicated plasmid DNA using the electroporation method.** PP1, PP2, or PP3 was added to transfected cells for 4 h with PP1 and PP3 in normal conditions or overnight with PP2 at 37 °C in serum-starved conditions (Dulbecco’s modified Eagle’s medium supplemented with 0.1% bovine serum albumin and 10 mM HEPES, pH 7.5). 48 h after transfection, cells were lysed in assay buffer (25 mM Tris-HCl (pH 8), 2 mM EDTA, and 150 mM NaCl) containing 1% Triton for 30 min at 4 °C. The cleared lysate was precipitated with an anti-Myc antibody in the presence of protein G-Sepharose and incubated overnight at 4 °C. The immunoprecipitates were then analyzed by Western blotting.

**Yeast Two-hybrid Analysis—All of the procedures used to analyze interactions in the two-hybrid system were performed in the L40 yeast reporter strain as previously described in detail (41).** The screening of the peripheral blood mononuclear cell cDNA library was performed using the WT LexA-FHOD1 fusion as bait. Plasmid DNA was rescued from positive clones and tested for specificity by retransformation into L40 with WT FHOD1 or the Ras extraneous target as described previously (41).

**Invasion Assay—For invasion assays, CHO-N18-HASPB-GFP cells were treated with control or FHOD1-specific RNAi, and SH4 domain expression was induced by the addition of doxycycline as described above.** Transwell inserts (Greiner Bio-One) were coated with 50 μl of growth factor-reduced Matrigel (BD Biosciences). 8,500 cells were seeded and allowed to adhere before the lower chamber was filled with 1 ml of minimum Eagle’s medium containing 0.5% fetal bovine serum and the upper chamber was filled with 200 μl of medium. Invasion assays were stopped after 24 h, and cells were fixed with 8% paraformaldehyde and visualized using rhodamine-phalloidin and 4’,6-diamidino-2-phenylindole. Invasion assays were analyzed by taking z intervals of 5 μm with a ×20 objective using confocal microscopy (Leica TCS SP2). For quantification, the amount of invaded cells in each optical section with a distance of more than 10 μm from the membrane from nine randomly chosen fields was counted. For imaging cell morphology of invaded cells, single confocal sections were taken with a ×20 objective.
**FHOD1 in Membrane Blebbing**

**A**

![Diagram of FHOD1 and ROCK1 proteins]

**B**

| + His | - His | β-Gal | pLex    | pGad    |
|-------|-------|-------|---------|---------|
|       |       |       | FHOD1 wt | Ras     |
|       |       |       | FHOD1 wt | ROCK1 368-1357 |
|       |       |       | FHOD1 wt | ROCK1 368-1357 |

**C**

| + His | - His | β-Gal | pLex    | pGad    |
|-------|-------|-------|---------|---------|
|       |       |       | FHOD1 ΔC | ROCK1 368-1357 |
|       |       |       | FHOD1 1-863 | ROCK1 368-1357 |
|       |       |       | FHOD1 1-807 | ROCK1 368-1357 |
|       |       |       | FHOD1 1-611 | ROCK1 368-1357 |

**D**

| Myc-ROCK1 | HA-FHOD1 | wt | Δ3 | wt | ΔC | wt | ΔC | wt | Δ3 |
|-----------|----------|----|----|----|----|----|----|----|----|
| ROCK1-wt  |          |    |    |    |    |    |    |    |    |
| ROCK1-Δ3  |          |    |    |    |    |    |    |    |    |
| FHOD1-wt  |          |    |    |    |    |    |    |    |    |
| FHOD1-ΔC  |          |    |    |    |    |    |    |    |    |

**IP α-Myc**

**Input**
RESULTS

ROCK1 and FHOD1 Physically Interact—In a search for FHOD1-interacting proteins, we performed a yeast two-hybrid screen using full-length FHOD1 as a bait and a human peripheral blood mononuclear cell cDNA library. One of the positive clones that interacted with FHOD1 was identified as a fragment encompassing aa 368–1357 of ROCK1, as indicated by growth on medium lacking histidine as well as activation of the β-galactosidase reporter gene (Fig. 1, A and B). The specificity of this interaction was validated by the lack of interaction of FHOD1 or ROCK1 with the Ras and Raf negative controls, respectively (Fig. 1B). The analysis of various FHOD1 fragments indicated that the constitutively active FHOD1ΔC (which lacks aa 1011–1164 and thus its autoregulation domain) can also interact with ROCK1 and mapped the ROCK1 interaction to the N-terminal region of the FHOD1 FH2 domain (aa 612–807) (Fig. 1C). Co-immunoprecipitation experiments from COS-7 cells expressing epitope-tagged ROCK1 and FHOD1 confirmed the specific association of the proteins (Fig. 1D). WT FHOD1 as well as FHOD1ΔC also associated efficiently with the constitutively active ROCK1Δ3 (aa 1–727) variant, indicating that aa 368–727 of ROCK1 are required for the association with ROCK1. Association with ROCK1 was also detected for FHOD1 variants lacking the entire FH1 domain (ΔFH1, lacking aa 570–610) or the core region of the FH2 domain in the C-terminal part (ΔFH2, lacking aa 807–866) (data not shown). Together, FHOD1 physically associates with ROCK1 in yeast and mammalian cells, and this interaction is governed by the N-terminal half of the FHOD1 FH2 domain and the central core region of ROCK1.

Figure 1. FHOD1 physically interacts with ROCK1. A, schematic representation of the domain organization of FHOD1 and ROCK1. Residues are highlighted by their position number; black lines indicate parts of the proteins required for FHOD1–ROCK1 interactions. B, yeast two-hybrid analysis. L40 yeast strains expressing the indicated pairs of FHOD1 and ROCK constructs fused to LexA (left column) or Gal4AD (right column) were analyzed for histidine auxotrophy and β-galactosidase activity. Double transformants were patched on selective medium with histidine (+His) and were replica-plated on medium without histidine (−His) and on Whatman filters for subsequent β-galactosidase assays. Growth in the absence of histidine and expression of β-galactosidase activity indicated interaction between hybrid proteins. The specificity of the binding was verified by the absence of respective reporter gene activation in cells expressing LexA-FHOD1 in combination with the Gal4AD-Raf hybrid. C, yeast two-hybrid analysis as described in B but with different FHOD1 constructs for mapping of its interaction with ROCK1. Parts of the FH2 domain of FHOD1 are important for its interaction with ROCK1. D, co-immunoprecipitation of FHOD1 and ROCK1. Lysates (input) from COS-7 cells expressing the indicated HA-tagged FHOD1 and Myc-tagged ROCK1 constructs were subjected to IP with an anti-Myc or anti-HA antibodies. FHOD1ΔC (aa 1–1010) and ROCK1Δ3 (aa 1–727) are constitutively active variants.

FHOD1 and ROCK1 Synergize for Efficient PM Blebbing—To address potential functional consequences of the interaction of FHOD1 with ROCK1, both proteins were co-expressed in HeLa cells (Fig. 2). 24 h post-transfection with expression constructs for HA-tagged FHOD1 and Myc-tagged ROCK1, cells were fixed, stained for F-actin and the overexpressed proteins, and then analyzed by microscopy. Expectedly (8, 9), cells expressing ROCK1 alone (Fig. 2, B) displayed the typical cyto-plasmic localization of the DRF and normal F-actin organization or in vector-transfected control cells (13.3 ± 1.6% and 9.2 ± 3.0% of all cells, respectively). Co-expression of FHOD1 with ROCK1, however, markedly changed cell morphology, resulting in the generation of a large number of relatively small PM blebs. On average, most of the cells showed more than 12 blebs/cell (approximately 77% compared with 39% of solely ROCK1-expressing cells; Fig. 2D), and the size of individual PM blebs was relatively reduced (~75% smaller than 3.5 μm in diameter, as compared with 49% in ROCK1-expressing cells; Fig. 2C). Importantly, virtually all cells (92.8 ± 3.7%) that were positive for ROCK1 and FHOD1 displayed PM blebbing, demonstrating a significant increase in PM blebbing efficacy when compared with the expression of ROCK1 alone (Fig. 2B). Although the subcellular localization of ROCK1 in blebbing cells remained unchanged upon co-expression with FHOD1, the DRF was efficiently recruited to the bleb membrane and lumen in the presence of ROCK1. Blebs generated in the presence of both ROCK1 and FHOD1 were more enriched in F-actin than ROCK1 blebs (Fig. 2A, i). PM blebbing induced by ROCK1 or by ROCK1 and FHOD1 did not result in rapid cell death, exposure of phosphatidylserine, or nuclear condensation and was thus unrelated to apoptosis (Fig. S1). We conclude that FHOD1 and ROCK1 synergize to efficiently generate nonapoptotic PM blebs.

FHOD1/ROCK1 PM Blebbing Depends on Rho Signaling, F-actin Integrity, and Src Activity—To obtain a more detailed picture of the signaling pathway that controls FHOD1/ROCK1 stimulated PM blebbing, we first analyzed the involvement of individual Rho-GTPases (Fig. 3). FHOD1 was co-expressed together with ROCK1 and a GFP fusion protein of either dominant negative Rac1 (Rac1-N17), Cdc42 (Cdc42-N17), the Clos- tridium botulinum C3 transferase (for inactivation of Rho), or a GFP control. Expression of dominant negative Rac1 or dominant negative Cdc42 had no inhibitory effect on PM blebbing but rather caused an increase in blebbing. In contrast, inhibition of Rho efficiently prevented PM blebbing induced by ROCK1 alone or by ROCK1 and FHOD1 (Fig. 3, A and B). Consistently, inhibition of ROCK activity with Y-27632 potently abolished PM blebbing induced by ROCK1 alone or in combination with FHOD1 (Fig. 3C). Both types of blebs also displayed comparable sensitivity to F-actin disruption, with latrunculin B being more efficient than cytochalasin D. In contrast, disruption of microtubules by nocodazole had no effect. However, the use of further pharmacological inhibitors revealed statistically significant differences between blebs induced by ROCK1 alone or in conjunction with FHOD1; blebbistatin, an inhibitor of the myosin II motor protein, reduced blebbing in both cases. However, ROCK1 blebs were markedly more sensitive to the treatment than ROCK1/FHOD1 blebs. Finally, the Src inhibitor PP1 had virtually no effect on ROCK1 blebs but reduced PM blebbing of cells co-expressing ROCK1
and FHOD1 to levels achieved by ROCK1 only. This suggests that PM blebs generated in the presence of FHOD1 and ROCK1 represent protrusions governed by a machinery including Src that is dispensable for the generation of blebs by ROCK1 alone.

Active Forms of ROCK1 and Src Localize in FHOD1/ROCK1-induced PM Blebs—We next asked whether ROCK1 and Src act locally during bleb formation and if they are active in PM blebs induced by expression of ROCK1 alone or in combination with FHOD1. To localize the activity of ROCK1, the staining pattern of endogenous myosin light chain (MLC), a major substrate of ROCK, was investigated. Overall, MLC was detected at the PM and along stress fibers in control cells (vector) and was found enriched in bleb membrane and lumen in ROCK1- and ROCK1/FHOD1-expressing cells (Fig. 4A). This distribution was indistinguishable from that of the phosphorylated, active pMLC (Fig. 4B). Together and consistent with the inhibitory effects of Y-27632, these results suggest that ROCK1 acts locally at both types of blebs to provide contractility. The analysis of Src distribution revealed a pattern reminiscent of microtubules and a significant localization of the kinase to ROCK1 as well as ROCK1/FHOD1 PM blebs (Fig. 4C). The use of a phosphospecific antibody to detect active Src revealed a marked accumulation of phospho-Src at the PM of control cells and a moderate presence at ROCK1 PM blebs. In line with the observed sensitivity to the Src inhibitor PP1 (Fig. 3C), active Src was significantly more enriched in PM blebs induced by co-expression of ROCK1 and FHOD1 (Fig. 4D). These results suggested that efficient PM blebbing induced by ROCK1 and FHOD1 involves the localized activity of ROCK1 as well as of Src. Based on this requirement for Rho-ROCK, F-actin, and Src, FHOD1/ROCK1 blebs appear strikingly related to recently described blebs induced by overexpression of SH4 membrane-targeting domains (20).

The Association between ROCK1 and FHOD1 Is Regulated by Src Activity—Since FHOD1 and ROCK1 functionally synergized for efficient PM blebbing in a Src-dependent manner, we next addressed whether Src is involved in the regulation of the physical association between FHOD1 and ROCK1 and performed co-precipitation experiments in the presence of increasing amounts of the Src inhibitor PP1 (Fig. 5A, left). Of note, treatment with PP1, which interfered with FHOD1/ROCK1-mediated PM blebbing (Fig. 3C) substantially reduced the association of FHOD1 with WT or Δ3 ROCK1 proteins without affecting FHOD1 or ROCK1 expression levels. Similar interference with the FHOD1-ROCK1 interaction was also obtained with the structurally unrelated Src inhibitor PP2 (Fig.
of the association between FHOD1 and ROCK1 was observed upon treatment with the inactive structural homologue PP3 (Fig. 5B). Of note, also endogenous FHOD1 protein was found to associate with ROCK1 in a Src-dependent manner (Fig. 5C). These results were confirmed by localization analyses in intact cells; although FHOD1 was efficiently recruited to the PM and to blebs in the presence of ROCK1, interference with blebbing by inhibition of Src activity abrogated PM localization in the presence of the kinase (Fig. 5D). The localization of ROCK1 as well as PM blebbing induced by expression of ROCK1 alone, however, were unchanged upon inhibition of Src activity (Fig. 5, D and E). We conclude that Src activity is essential for the efficient association between FHOD1 and ROCK1 in mammalian cells and governs the recruitment of FHOD1 to the PM in the presence of ROCK1.

FHOD1 Does Not Affect Overall ROCK1 Activity but Is a ROCK1 Substrate—To address if overall ROCK1 activity was affected by co-expression of FHOD1, we scored its ability to phosphorylate a MLC substrate in vitro following immunoprecipitation from transfected cells (Fig. 6A). Although negative controls (lanes 1 and 6) expectedly did not yield phosphorylation of MLC, active ROCK1/H90043 generated high levels of pMLC (lane 5). WT ROCK1 (lane 2) resulted in lower but significant pMLC levels that were reduced to background in the presence of the specific ROCK1 inhibitor Y-27632 (lane 4). Co-expression of FHOD1 with ROCK1 (lane 3) had only minor effects and did not result in elevated ROCK activity, ruling out that FHOD1 functionally synergizes with ROCK1 via general up-regulation of its kinase activity. To test whether FHOD1 could instead be subject to regulation via phosphorylation by ROCK1, we repeated the kinase reaction using recombinant purified FHOD1 as substrate for immunoprecipitated ROCK1 (Fig. 6B). Indeed, potent phosphorylation of full-length FHOD1 as well as of a truncated FHOD1 fragment were observed when FHOD1 protein was added to immunoprecipitates containing active ROCKΔ3 (lane 4). This reaction was fully inhibited by the addition of Y-27632 (lane 5) and much more pronounced than nonspecific background phosphorylation observed in the absence of ROCK1 (lane 2). Immunoisolated WT ROCK1 only displayed weak activity in this assay, resulting in a slight

FIGURE 3. Enhancement of PM blebbing by FHOD1 requires F-actin, Rho signaling, and Src activity. A, confocal micrographs of HeLa cells co-transfected with expression plasmids of either GFP or dominant negative forms of GTPases (Rac1-N17, Cdc42-N17) fused to GFP or GFP-tagged C3 transferase for inhibition of Rho activity. After 24 h, cells were fixed and stained for transiently expressed proteins and F-actin. *, cells expressing all three transgenes; scale bars, 10 μm. B, percentages of blebbing HeLa cells treated and processed as described in A (arithmetic means of at least three independent experiments ± S.D. with over 100 cells counted per condition). C, percentages of blebbing HeLa cells transfected with plasmids encoding ROCK1 and FHOD1 as indicated. 24 h post-transfection, cells were pretreated with 90 μM Y-27632 for 2 h to remove already formed PM blebs, washed, and incubated in the presence of the indicated drugs for an additional 3 h (90 μM Y-27632, 2 μM cytochalasin D, 25 μM latrunculin B, 100 μM blebbistatin, 384 μM nocodazole, 50 μM PP1). Subsequently, cells were fixed, stained for the transiently expressed proteins and F-actin, and analyzed for PM blebbing (arithmetic means of at least three independent experiments ± S.D. with over 100 cells counted per condition). p values indicate statistical significance as determined by Student’s t test.
increase in phosphorylation of the truncated FHOD1 fragment but not the full-length protein. Thus, recombinant FHOD1 can be efficiently phosphorylated by active ROCK1 in vitro.

Endogenous FHOD1 Is Specifically Involved in SH4 Domain PM Blebbing—The involvement of Src activity observed for PM blebbing induced by ROCK1/FHOD1 or SH4 domains has not yet been reported for other types of PM blebs. Since these results relied on the use of pharmacological Src inhibitors, we sought to further validate the requirement of Src for SH4 domain-induced blebbing in cells lacking Src expression. To this end, SYF cells that lack expression of Src, Yes, and Fyn (SYF−/−) or SYF cells with reconstituted Src expression (SYF + c-Src) were used. Expectedly, expression of the Yes SH4 domain fused to GFP (N18-Yes-GFP) caused pronounced PM blebbing in more than 60% of the transfected cells in SYF + c-Src cells, whereas only background blebbing activity was observed in SYF−/− cells lacking Src (Fig. 7, A and B). SH4 domain-induced blebbing in SYF + c-Src cells was suppressed by PP1 in a dose-dependent manner. Similar results were
obtained in SYF cells co-expressing ROCK1/FHOD1 (data not shown).

These results now allowed us to test whether endogenous FHOD1 is involved in Src-dependent PM blebbing induced by SH4 domains. To this end, FHOD1 expression was efficiently reduced by specific RNAi-mediated knockdown in SYF/c-Src cells (Fig. 7E, top). Importantly, PM blebbing was significantly reduced albeit not abrogated from 64.5 ± 1.4% in the control to 39.3 ± 7.1% upon reduction of FHOD1 expression (Fig. 7, C and D). Similar results were obtained in N18-HASPB-GFP-expressing CHO cells; although the SH4 domain induced efficient PM blebbing in cells treated with control RNAi (79.6 ± 4.3%), reduction of FHOD1 expression significantly decreased the number of blebbing cells (42.9 ± 16.8%) (Fig. 7, C–E, middle). Such ~2-fold reduction in blebbing efficiency was also observed under knockdown conditions where FHOD1 was undetectable by Western blotting (data not shown), suggesting that FHOD1 is critically involved in but is not essential for SH4 domain-induced PM blebbing.

To analyze the specificity of FHOD1 for SH4 domain-induced blebs, we next tested the involvement of endogenous FHOD1 in various types of PM blebbing by RNAi in HeLa cells (Fig. 7E, bottom). In line with the nonapoptotic nature of ROCK1/FHOD1 blebs, FHOD1 knockdown had no effect on the morphology (data not shown) and frequency of apoptotic blebs induced by tumor necrosis factor α/cycloheximide treatment (Fig. 7F). Similarly, FHOD1 expression levels were not limiting for PM blebbing induced by overexpression of ROCK1 alone (Fig. 7G), confirming that PM blebs induced by ROCK1 or ROCK1/FHOD1 require distinct machineries. Since FHOD1 physically interacts with Rac1 and a specific Rac1 effector loop
mutant (L61A37) has been reported to induce PM blebbing (19, 27, 29), we also tested if FHOD1 knockdown affects PM blebbing induced by Rac1-L61A37. Although this Rac1 variant efficiently induced large numbers of relatively small nonapoptotic PM blebs, FHOD1 RNAi caused only a slight and statistically nonsignificant reduction of blebbing (Fig. 7H). Together, these results define FHOD1 as a DRF specifically involved in PM blebbing induced by SH4 domains.

We next sought to address whether the support of SH4 domain-induced PM blebbing by FHOD1 had functional consequences. PM blebbing can facilitate invasion of tumor cells that use a rounded, amoeboid mode of motility in three-dimensional environments. This mode of invasion is in contrast to the mesenchymal type of cell movement used by other tumor cells, where more elongated cells achieve motility by proteolytic degradation of the surrounding matrix (42). Since SH4 domain-expressing cells adopt a rounded cell morphology in a three-dimensional environment and invade into three-dimensional Matrigel (20), we analyzed cell invasion and morphology of these cells treated with control or FHOD1-specific RNAi (Fig. 8). Surprisingly, we noted in three independent experiments that efficient reduction of FHOD1 expression significantly enhanced invasion of SH4 domain-expressing cells into Matrigel (Fig. 8, A and B). Importantly, enhanced cell invasion upon FHOD1 knockdown coincided with a relative decrease in cells that displayed rounded cell morphologies (Fig. 8C). Instead, the majority of the cells adopted an elongated cell shape with pronounced differences in the length of the long and short axis of the cell. Such elongated cells were 2–5 times more frequent upon FHOD1 knockdown as compared with RNAi control-treated cells. We conclude that FHOD1 is involved in the regulation of cell morphology and invasion efficiency of SH4 domain-expressing cells in three-dimensional matrices.

**DISCUSSION**

We report here the physical interaction of the DRF FHOD1 with the Rho effector kinase ROCK1. Initially identified as direct interaction partners by a yeast two-hybrid screen, both proteins were found to associate in intact cells, and FHOD1 was recruited to the PM in the presence of the kinase. At the functional level, co-expression of both proteins resulted in efficient PM blebbing in a Rho-ROCK-, F-actin-, and Src-dependent manner.

**FIGURE 7.** *Endogenous FHOD1 is involved in Src-dependent PM blebbing.* A, confocal micrographs of SYF−/− cells depleted for endogenous c-Src, Yes, and Fyn or SYF + c-Src cells reconstituted with c-Src. Cells were transfected with plasmids encoding for GFP or N18-Yes-GFP and treated with solvent or the Src kinase inhibitor PP1 in the indicated amounts for 3 h. Cells were fixed and stained for F-actin. Scale bars, 10 μm. B, percentage of blebbing cells depicted in A. Values for GFP-expressing cells are shown as black bars, and values for N18-Yes-GFP cells are shown as white bars (arithmetic means of three independent experiments ± S.D. with over 100 cells counted per condition). C, confocal micrographs of SYF + c-Src cells transfected with an expression construct for N18-Yes-GFP or CHO-N18-HASPB-GFP cells after treatment with a nonspecific control (con) or FHOD1-specific (FHOD1) RNAi oligonucleotides. Cells were fixed and stained for F-actin. Scale bars, 10 μm. D, quantification of the experiments shown in C. p values indicate the statistical significance as evaluated by Student’s t test. E, Western blot analysis of knockdown efficiency. F, quantification of PM blebbing in RNAi-treated HeLa cells following induction of apoptosis by treatment with tumor necrosis factor α/cycloheximide (50 and 5 μg/ml) for 3 h. Depicted is the percentage of blebbing cells that stained positive for Annexin V. G, quantification of PM blebbing in HeLa cells expressing Myc-tagged ROCK1. H, quantification of PM blebbing in HeLa cells expressing Myc-tagged Rac1-L61A37. All values are the arithmetic mean of at least three independent experiments ± S.D. with over 100 cells counted per condition.
Endogenous FHOD1 regulates cell morphology and invasion efficacy in three-dimensional Matrigel. A, effect of FHOD1 knockdown on invasiveness of CHO-N18-HASPB-GFP cells into Matrigel. Shown are results from three independent experiments with values obtained for the RNAi control set to 1. B, Western blot analysis of knockdown efficiency. C, confocal micrographs of cells within Matrigel from the experiment analyzed in A. Shown are representative confocal sections of invaded cells with predominantly rounded morphology for the controls and elongated shape after FHOD1 knockdown. The merge image shows F-actin in red, N18-HASPB-GFP in green, and Hoechst 33268-stained nuclei in blue. Scale bar, 20 μm.
alone, which occurs via an FHOD1-independent mechanism, is not regulated by Src. Src has been reported to regulate cytoskeletal remodeling by various formins, including FHOD1 (44–47). Our results therefore underscore the general role of Src for DRF activity and suggest that Src may directly regulate protein interactions of DRFs.

It is important to note that PM blebbing was reduced but not abolished in the RNAi experiments despite strong reduction of FHOD1 protein levels and that, once reduced below an assumed critical concentration, further decrease in FHOD1 expression by more efficient knockdown did not result in further reduction of PM blebbing. We cannot exclude the possibility that such residual blebbing reflects the activity of a cellular FHOD1 pool refractory to RNAi by our experimental approach. More likely, even if FHOD1 is necessary for optimal PM blebbing, some other factors are sufficient to maintain appreciable blebbing in its absence. Whether this involves the closely related FHOD2, other DRF proteins, or additional actin regulators is currently being investigated. However, recent studies revealed that specific DRFs are involved in various types of PM blebbing; human Dia 1 is critical for blebbing and motility of MDA-MB-435 cancer cells in three-dimensional environments (15), and mammalian Dia 2 mediates blebbing upon overexpression of DIP (18). Thus, DRFs emerge as important regulators of PM blebbing, with individual family members apparently facilitating distinct types of membrane blebbing. This specificity may reflect the particular trigger for PM blebbing in individual cases, suggesting that GTPase specificity of a given DRF may determine in which kind of PM dynamization event it will be involved. An important open question is what exactly the DRFs contribute to facilitate the blebbing process. Although this remains formally to be demonstrated for FHOD1, the enrichment of F-actin and the DRF at the bleb base and lumen, respectively, indicates that its involvement in blebbing may reflect nucleation of new actin filaments, which may synergize with ROCK-mediated contractility for bleb retraction. Since actin nucleation is a conserved feature of DRFs, this may apply to many different types of blebbing. The involvement of individual DRFs in select types of blebbing indicates that, depending on the individual DRF involved, blebbing could also be facilitated by less well conserved DRF activities, such as bundling of actin filaments (48) or overall activation of Rho signaling (15).

Another important issue is the physiological relevance of FHOD1-mediated PM blebbing. Since SH4 domain expression promotes PM blebbing and cell invasion in Matrigel, we hypothesized that FHOD1 knockdown would diminish invasion. Instead, an increase in invasion efficacy was observed that correlated with the adoption of a more elongated rather than rounded cell morphology. These changes in cell morphology were presumably facilitated by the weakening of PM–cortex interactions due to expression of the SH4 domain and by reduced contraction in FHOD1 knockdown cells. Similar changes in cell morphology of invading cells have already been reported, albeit in the opposite direction; tumor cells employing an elongated invasion mode readily adopt rounded cell morphology and blebbing motility when matrix degradation is inhibited (49). In analogy, our results may indicate that the reduction of FHOD1 expression not only caused morphology alteration but triggered the cells to switch to an elongated mesenchymal mode of motility. These results emphasize DRFs as versatile regulators of cell morphology and invasiveness and warrant a detailed analysis of the specific role of DRFs for the invasive potential of individual types of cancer cells.

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