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Jean-Michel Bellanger, Catherine Astier, Claude Sardet, Yasutaka Ohta, Thomas P. Stossel, et al..
The Rac1-and RhoG-specific GEF domain of Trio targets filamin to remodel cytoskeletal actin. *Nature Cell Biology*, 2000, 2 (12), pp.888-892. 10.1038/35046533 . hal-02196838

HAL Id: hal-02196838

<https://hal.science/hal-02196838v1>

Submitted on 10 Jan 2025

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The Rac1- and RhoG-specific GEF domain of Trio targets filamin to remodel cytoskeletal actin

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Rho GTPases control actin reorganization and many other cellular functions. Guanine nucleotide-exchange factors (GEFs) activate Rho GTPases by promoting their exchange of GDP for GTP. Trio is a unique Rho GEF, because it has separate GEF domains, GEFD1 and GEFD2, that control the GTPases RhoG/Rac1 and RhoA, respectively. Dbl-homology (DH) domains that are common to GEFs catalyse nucleotide exchange, and pleckstrin-homology (PH) domains localize Rho GEFs near their downstream targets. Here we show that Trio GEFD1 interacts through its PH domain with the actin-filament-crosslinking protein filamin, and localizes with endogenous filamin in HeLa cells. Trio GEFD1 induces actin-based ruffling in filamin-expressing, but not filamin-deficient, cells and in cells transfected with a filamin construct that lacks the Trio-binding domain. In addition, Trio GEFD1 exchange activity is not affected by filamin binding. Our results indicate that filamin, as a molecular target of Trio, may be a scaffold for the spatial organization of Rho-GTPase-mediated signalling pathways.

Small GTPases of the Rho family have diverse effects on cellular structure and function¹. Depending on the cell type, specific Rho GTPases induce particular surface protrusions generated by actin-remodelling reactions that change cell shapes and influence cell adhesion and locomotion^{2,3}. Rho GTPases also affect the cell cycle and gene transcription, and participate as intermediates in malignant transformation caused by mutant Ras GTPases^{4,5}. Small GTPases are molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state. GEFs activate GTPases by accelerating the exchange of GDP for GTP. Rho GEFs invariably share the DH domain, which is responsible

for catalytic guanine nucleotide-exchange activity, and the PH domain, which is probably important for determining where in the cell GEFs operate^{6,7}. Trio is the founding member of an emerging family of Rho GEFs that exchange guanine nucleotides on more than one Rho GTPase⁸. One GEF domain of Trio (Trio GEFD1) activates the small GTPases RhoG and Rac, whereas the other (Trio GEFD2) acts on RhoA, indicating that this Rho GEF may link several GTPase pathways *in vivo*^{9,10}. Recent genetic studies using *Drosophila* and *Caenorhabditis elegans*^{11–15} have established Trio as a key component of the intracellular signalling pathway that regulates axon guidance and cell migration in the nervous system. In

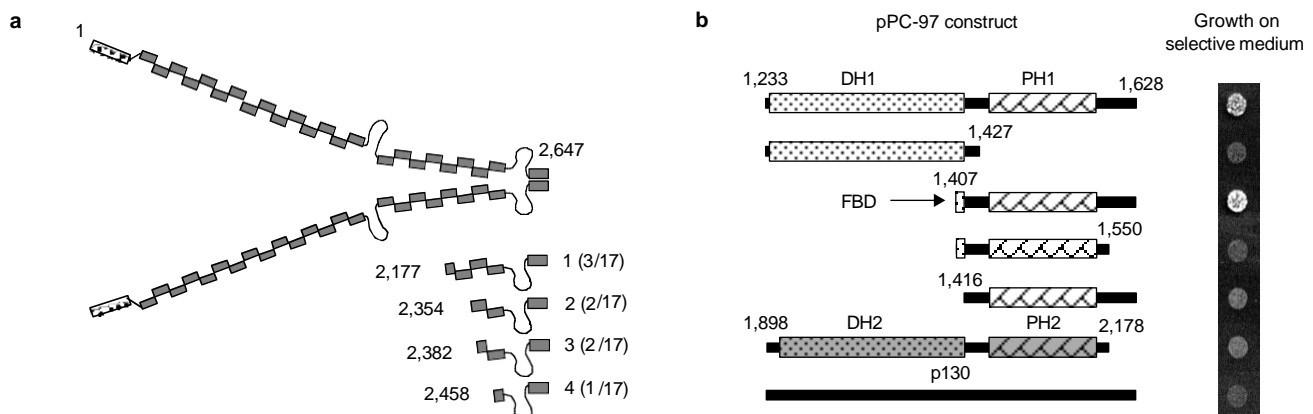


Figure 1 **Trio GEFD1 interacts specifically with filamin A.** **a**, The rod-like structure (grey rectangles) of filamin A (punctuated rectangle represents the actin-binding domain) and the four filamin segments corresponding to cDNAs isolated from the two-hybrid screen are represented with their relative frequencies in brackets. **b**, The

pPC97 constructs used to determine the specificity of the Trio–filamin interaction and to map the filamin-binding domain (FBD) within Trio are represented with the results of interaction assays carried out with the fil2 segment on His⁻ medium supplemented with 20 mM 3-amino triazole.

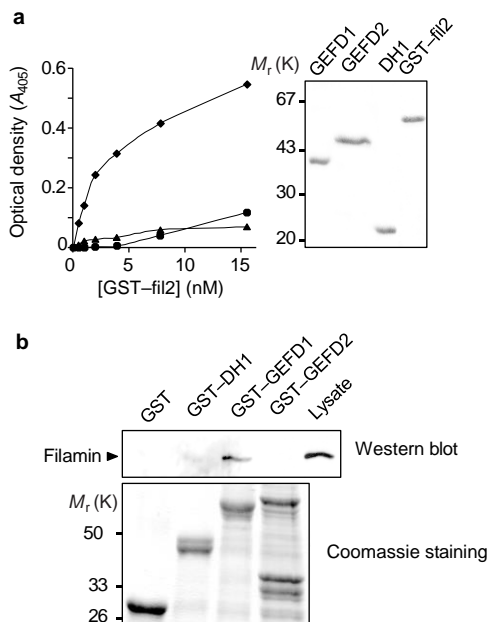


Figure 2 Trio GEFD1 binds to filamin *in vitro*. **a**, Left panel, the ability of recombinant GST-fil2 to bind to Trio GEFD1 (diamonds), Trio DH1 (triangles) or Trio GEFD2 (circles) was measured by ELISA. Right panel, Coomassie staining of the purified proteins used in these experiments. **b**, Upper panel, HeLa cell lysates were incubated with the indicated recombinant GST-fusion proteins. Retained filamin was then detected by western blotting. Lower panel, each GST construct was first analysed by SDS-PAGE.

addition, the Rac- and RhoG- specific Trio GEFD1 has an important role in this process¹². These findings highlight the importance of defining the molecular mechanisms that underlie the regulation of the activity of Trio, and especially of its first GEF domain.

Because actin reorganization requires spatially controlled assembly, disassembly and architectural rearrangement of actin filaments, Rho GTPases that affect it must initiate reactions that ultimately involve actin-binding proteins that function within particular cell domains. Accordingly, although little evidence supports direct effects of GTPases on actin remodelling, accumulating research findings are revealing downstream effects of GTPases on actin-regulating proteins such as myosin kinases, cofilin-regulating kinases, Wiskott-Aldrich-syndrome protein (WASP)-family proteins, phosphoinositide kinases, and proteins of the filamin family¹⁶⁻²². Filamins are of particular interest, as they have several effects on the actin cytoskeleton. They crosslink actin filaments into orthogonal networks or parallel bundles, tie cortical actin-filament networks to diverse plasma-membrane receptors, and act as a scaffold for intracellular signalling cascades^{20,23-25}. Here we show that Trio also binds to filamin and that this interaction has specific consequences for remodelling of the actin cytoskeleton in response to Trio *in vivo*.

Results

Trio GEFD1 binds to filamin A. A yeast two-hybrid screen using the Trio GEFD1 domain as bait isolated 17 putative Trio interactors. Sequence analysis of eight complementary DNAs revealed that they all encoded truncated polypeptides corresponding to the carboxy-terminal part of the human protein named ABP-280 when first cloned and now designated filamin 1 or filamin A (Fig. 1a).

Filamin A is the principal human non-muscle isoform of this protein family. It is a flexible homodimer of relative molecular mass 280,000 (M_r , 280K) containing an amino-terminal actin-binding

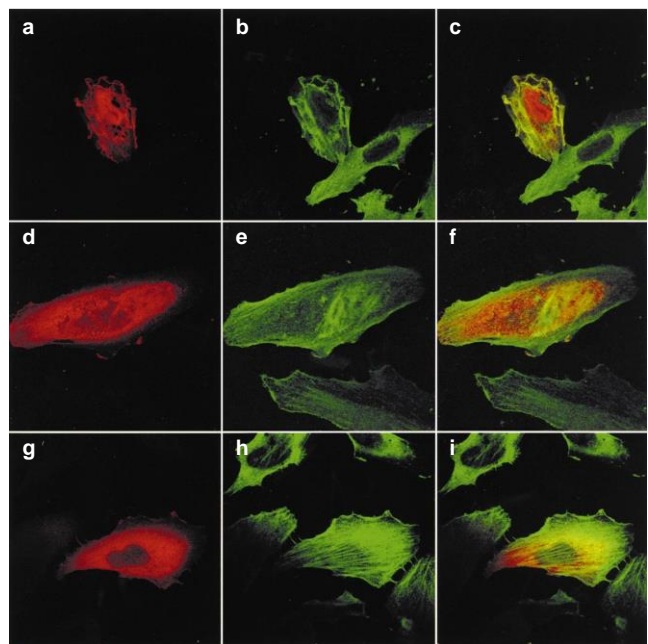


Figure 3 Localization of Trio and filamin in Trio GEFD1-induced membrane ruffles. HeLa cells transfected with haemagglutinin-tagged Trio GEFD1 (HA-Trio GEFD1; **a-c**), HA-Trio DH1 (**d-f**) or HA-Trio GEFD2 (**g-i**) were fixed and permeabilized 15 h after transfection. Filamin staining is shown in **b, e** and **h**. **a, d** and **g** show staining with a polyclonal anti-HA antibody (Zymed) for the corresponding HA-Trio constructs. Filamin was detected with FITC-coupled anti-mouse immunoglobulins; Trio constructs were visualized using TRITC-coupled anti-rabbit immunoglobulins. Cells were then submitted to confocal microscopy; selected slices are shown in separate staining and as merged images (**c, f, i**).

domain that precedes 24 immunoglobulin-like β -sheet motifs, the last of which (repeat 24) represents all or part of the self-association domain of the molecule²⁶. The longest filamin cDNA obtained in our two-hybrid screen corresponds to repeats 21-24, encompassing amino acids 2,177-2,647 (which we have designated fil1). The smallest cDNA includes part of repeat 23 and the whole of repeat 24 (amino acids 2,458-2,647), which we have defined as fil4. Fil4 is sufficient on its own for tight binding to Trio GEFD1 in the two-hybrid system.

We investigated the specificity of the interaction of Trio GEFD1 with filamin A and sought to identify the minimum binding domain within Trio by testing the binding of the filamin segment referred to as fil2 (amino acids 2,354-2,647; Fig. 1a) to other baits in the two-hybrid assay (Fig. 1b). Neither the Trio GEFD2 domain nor the pRB-related pocket protein p130 (ref. 27) interacted with fil2. Fil2 did not bind to the Trio DH1 domain, indicating that the catalytic domain of Trio GEFD1 is not the region that is responsible for filamin binding. This analysis defines a new PH1-containing functional domain on Trio, the filamin-binding domain (FBD, amino acids 1,407-1,628; Fig. 1b).

Recombinant GEFD1 binds to human filamin *in vitro*. An enzyme-linked immunosorbent assay (ELISA)-based solid-phase binding assay reported saturable high-affinity ($K_{d,app} \approx 5$ nM) binding of Trio GEFD1 to both the C terminus of recombinant human filamin A, which was previously designated as fil2 (Fig. 2a) and to purified chicken-gizzard filamin²⁸ (data not shown). In contrast, Trio DH1 and Trio GEFD2, which lack the Trio FBD, did not bind to fil2. In a separate approach, recombinant glutathione-S-transferase (GST)-tagged GEFD1, but not GST-Trio DH1 or GST-Trio GEFD2, bound to filamin from HeLa-cell lysates (Fig. 2b).

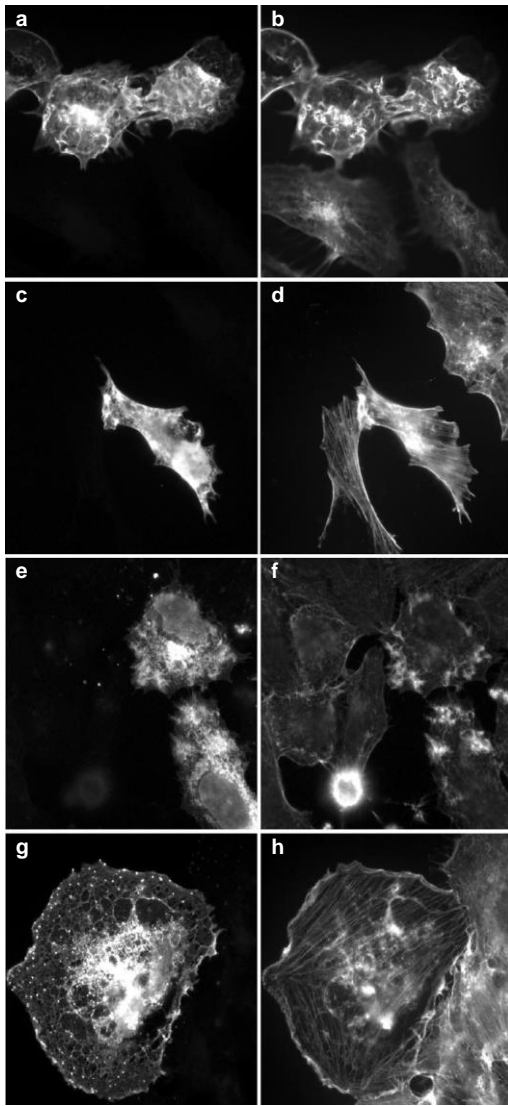


Figure 4 Filamin A expression is required for Trio GEFD1-induced actin-cytoskeleton modification. Filamin A-replete (A7) cells were transfected with HA-Trio GEFD1 cDNA (**a, b**), and filamin A-deficient (M2) cells were transfected with HA-Trio GEFD1 cDNA (**c, d**), GFP-RhoG(V12) (**e, f**) or GFP-Rac(V12) (**g, h**) 4 days after plating. After fixation and permeabilization, expression of GFP-GTPases was directly visualized (**e, g**), whereas overexpressed Trio GEFD1 was detected using the anti-HA 12CA5 monoclonal antibody followed by FITC-coupled anti-mouse immunoglobulins (**a, c**). Filamentous actin was stained with rhodamine-phalloidin (**b, d, f, h**).

These data confirm the two-hybrid results and establish a strong and specific interaction between the first GEF domain of Trio and the C terminus of filamin A.

Trio GEFD1 and filamin localize in Trio-induced actin structures. As previously described in Swiss 3T3 cells⁹, Trio GEFD1 induces the formation of a distinctive population of serpiginous actin-rich ruffles on the dorsal surface of HeLa cells, as shown by rhodamine-phalloidin staining (data not shown), and accumulates in these structures (Fig. 3a). Filamin and Trio GEFD1 concentrated morphologically in these actin-rich structures, as visualized by confocal microscopy (Fig. 3c). Expression of Trio DH1 in HeLa cells did not affect the morphology of the actin cytoskeleton, indicating that the PH1 domain of Trio may be absolutely required for Trio GEFD1 function *in vivo* (Fig. 3d). Confocal analysis showed that

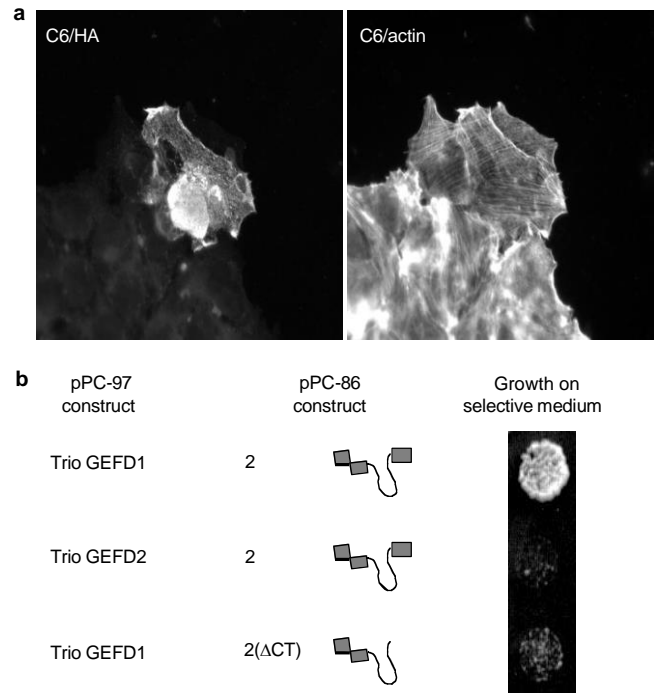


Figure 5 The capacity of Trio GEFD1 to remodel the actin cytoskeleton is directly linked to its binding to filamin. **a**, Filamin-deficient cells replete with C-terminally truncated filamin (C6) were transfected with HA-Trio GEFD1 cDNA 4 days after plating. They were then treated as described in Fig. 4. Results are representative of three independent experiments. **b**, The capacity of Trio GEFD1 to interact with the fil2 segment (amino acids 2,354–2,647) or with the corresponding C-terminally truncated construct fil2(ΔCT) (lacking amino acids 2,354–2,535) was measured by two-hybrid assay. The interaction between Trio GEFD2 and fil2 is shown as a negative control.

Trio DH1 exhibited diffuse cytoplasmic staining and did not co-localize with filamin (Fig. 3d–f). Similarly, the PH-domain-containing Trio GEFD2, which promotes formation of actin stress fibres⁹ and does not bind to filamin, did not co-localize with filamin in HeLa cells (Fig. 3i).

Trio GEFD1-induced ruffle formation requires the presence of filamin A. We examined Trio GEFD1-mediated induction of actin-cytoskeleton modification in a cell line (M2) derived from a human malignant melanoma that does not express minimal detectable filamin messenger RNA or protein. M2 cells are extremely defective in translational locomotion and exhibit extensive and prolonged membrane blebbing, a phenotype that is reversed by stable transfection of filamin A cDNA (A7 subline)²⁹. In the majority (75%) of transfected A7 cells, expression of Trio GEFD1 induced the characteristic dorsal ruffles previously observed in 3T3 fibroblasts (Fig. 4a, b). Expression of Trio GEFD1 in filamin-deficient M2 cells, however, although it induced a slight increase in peripheral actin staining in one-third of the transfected cells, failed to elicit the distinctive dorsal ruffles observed in A7 cells (Fig. 4c, d). This filamin-dependent effect seems to be specific for Trio GEFD1, as expression of the constitutively activated forms of the Trio GEFD1 targets Rac and RhoG, which are GEF-independent, promoted remodelling of the actin cytoskeleton independently of the presence of filamin (Fig. 4e–h). In this case, 72% of RhoG(V12)- and 73% of Rac(V12)-transfected M2 cells exhibited GTPase-specific modification of the actin cytoskeleton.

Trio GEFD1-induced ruffle formation requires its binding to filamin A. Expression of Trio GEFD1 also failed to induce actin-associated membrane changes in M2 cells stably transfected with a

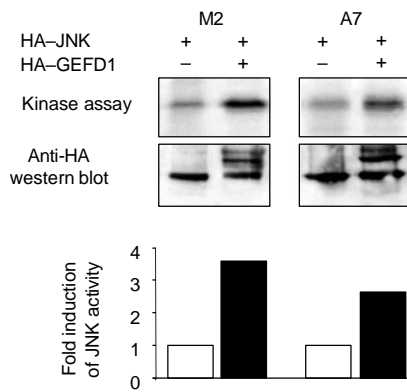


Figure 6 Filamin is dispensable for Trio GEFD1-elicited JNK stimulation. Filamin-deficient (M2) or filamin-replete (A7) cells were transfected with HA-JNK cDNA alone or with HA-Trio (GEFD1) cDNA. Two days after transfection, cells were lysed and JNK activity was monitored as described⁹. The histogram shows quantification of this activity using a Phosphorimager-coupled computer. Equivalent expression of transfected cDNAs was documented by western blotting using the 12CA5 monoclonal antibody. Data are representative of six independent experiments.

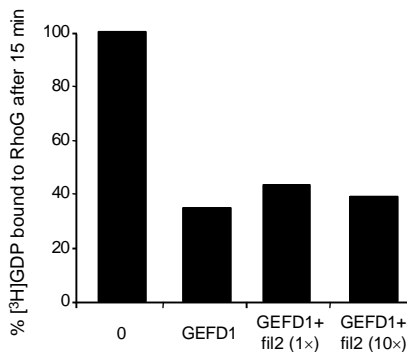


Figure 7 Filamin does not affect the GDP/GTP exchange activity of Trio GEFD1. The exchange activity of a submaximally effective concentration of Trio GEFD1 (28.5 nM) on RhoG (200 nM) was measured in presence or absence of an equal molar amount (1x) or a ten-fold excess (10x) of GST-fil2. Exchange activity is expressed as the percentage of [³H]GDP still bound to the GTPase after 15 min.

truncated construct of filamin A that lacks the most C-terminal 112 amino acids, which are responsible for the dimerization of filamin as well as for Trio binding²¹ (C6 cells, Fig. 5a). The requirement of these C-terminal 112 amino acids for Trio binding is also demonstrated by the two-hybrid data (Fig. 5b). These results show that the capacity of Trio GEFD1 to remodel the actin cytoskeleton is directly linked to its binding to filamin A.

Filamin A is dispensable for JNK activity stimulation by Trio GEFD1. In addition to its effect on the actin cytoskeleton, Trio GEFD1 activates a Rac-mediated pathway that leads to stimulation of c-Jun kinase (JNK)⁹. Previous studies have shown that filamin A binds to the JNK activator SEK1 and is necessary for JNK activation by tumour-necrosis factor- α (TNF α)²⁴. We monitored the activation of ectopically expressed JNK by Trio GEFD1 in filamin-deficient M2 and filamin-replete A7 cells (Fig. 6). Expression of Trio GEFD1 promoted comparable JNK activation in both filamin-expressing and -deficient cells.

Filamin does not affect the exchange activity of Trio GEFD1. An equal molar amount or a ten-fold excess of either GST-fil2 or purified chicken-gizzard filamin (data not shown) did not affect the

exchange activity of a submaximally effective concentration of Trio GEFD1 on RhoG (Fig. 7) or Rac (data not shown).

Discussion

We have identified a new functional domain of 220 amino acids on Trio that is responsible for its binding to filamin. This FBD consists of the PH1 domain of Trio flanked by short N- and C-terminal sequences. These sequences resemble those of the N-terminal PH domain of the Rac-specific GEF, Tiam, which function in concert with adjacent sequences to localize Tiam at the plasma membrane³⁰. Whether these surrounding sequences physically participate in filamin binding or are necessary for the structural integrity of the Trio PH1 domain is not clear at present.

PH domains are present in many proteins that are implicated in signal-transduction cascades and are essential for the proper subcellular localization of these proteins. Various PH domains bind *in vitro* to membrane phosphoinositides³¹, and Trio PH1 binds to phosphatidylinositol-4,5-bisphosphate³². Some PH domains, however, are also ligands for proteins, such as the heterotrimeric GTPase $\beta\gamma$ subunits or filamentous actin^{33,34}. We have shown that Trio PH1 establishes a functional molecular link between Trio and its particular effects on the actin cytoskeleton, through the protein filamin. Moreover, we have demonstrated that this association is required for Trio GEFD1-mediated remodelling of the actin cytoskeleton. In contrast, Trio GEFD1-elicited activation of JNK is independent of filamin, demonstrating a functional dichotomy between Trio GEFD1-induced downstream signalling pathways at the Rho GEF level. Similarly, the association of the Cdc42-specific GEF frabin with actin through its actin-binding domain is reportedly essential for formation of actin microspikes but not for JNK activation³⁵.

The extraordinary ability of Rho GTPases to coordinate functions of unrelated actin-binding proteins to remodel actin-based structures at the cell surface requires exquisite spatial coordination. A scaffold on which various components instructed by the GTPases could assemble is one mechanism by which this kind of organization could be accomplished, and filamin is an attractive candidate for providing such support. Filamins crosslink actin filaments into gel-like structures and also link the peripheral actin cytoskeleton to transmembrane receptors that are involved in physiological processes such as motility, adhesion or cell-shape changes^{23,25,36-38}. Mutations in the filamin A gene on the X chromosome are responsible for the human genetic disorder periventricular heterotopia, which results from migrational defects of a subset of cortical neurons during embryonic development³⁹. The fact that both Trio and filamin are involved in the control of neuronal development strongly reinforces the physiological significance of the Trio-filamin interaction presented here. Accordingly, filamin and Trio have been localized to nerve growth cones, where Rho GTPases have previously been proposed to control actin dynamics leading to growth-cone guidance^{12,40,41}.

In addition, filamin A binds to several GTPases, including Rac1, Cdc42, RhoA and RalA^{21,24}. Of these interactions, only the binding of RalA to filamin is GTP-dependent²¹, indicating that filamin has a specific effector function for RalA. We have shown that filamin is dispensable for cytoskeletal rearrangements induced by the unregulated activated forms of RhoG and Rac1. These data, together with our observation that filamin binding does not affect the *in vitro* exchange activity of Trio GEFD1, strongly support a scaffolding function for filamin in the pathway that links Trio to its distinctive effects on the actin cytoskeleton. In such a model, Rho GTPases would localize on filamin irrespective of their bound guanine nucleotide, and wait for activators such as Trio to turn them on and for other effectors to inactivate them. The juxtaposition of a Rac-activating GEF and Rac on a filamin template positions potential Rac activation to stimulate nucleation of actin polymerization⁴² at sites where newly assembled actin filaments can be crosslinked, ligated to membrane partners⁴³ or otherwise modified. □

Methods

DNA constructs.

For the two-hybrid experiments, cDNAs encoding different segments of Trio (Fig. 1b) were inserted into the pPC97 plasmid⁴⁴, as was a control pPC97-p130 construct²⁷. Recombinant GST-fusion proteins were engineered by subcloning appropriate cDNAs into the pGEX5X vector (Pharmacia). For expression in mammalian cells, cDNAs encoding GEFD1, DH1 or GEFD2 were inserted into the pSR α HA-Tag vector. GFP-tagged GTPase constructs were as described³. The pGEX-cjun and pSR α HA-JNK constructs were supplied by B. Dérjard (Univ. Nice, France).

Yeast two hybrid system.

Using the pPC97-GEFD1 construct as bait, we screened 3×10^5 clones of a human fibroblast cDNA library inserted into the pPC86 vector^{27,44}. The specificity of the rescued clones and mapping experiments were determined by spotting 10^5 cells co-transformed with pPC86-filaminCter clone 2 (fil2, amino acids 2,354–2,647) and pPC97-constructs on Leu⁻ Trp⁻ His⁻ plates supplemented with 20 mM 3-amino triazole.

Cell culture and transfection.

HeLa cells were maintained as described⁸. Human filamin-deficient melanoma cell lines (M2), or sublines replete with full-length filamin (A7) or with filamin A truncated by 112 amino acids at its C terminus (C6) were maintained as described^{21,29}. Four days after plating, M2, A7 and C6 cells were transfected with lipofectamine reagent (Life Technologies) according to the manufacturer's instructions. HeLa cells plated on glass coverslips were transfected by the DEAE-dextran method.

In vitro binding assays.

Recombinant Trio constructs were excised from GST by factor Xa. ELISA experiments⁴⁵ were carried out by coating Trio domains at a concentration of 10 nM. After saturation, coated plates were incubated with serial dilutions of GST-fil2 for 2 h at 37 °C. An affinity-purified polyclonal anti-GST antibody was added at a concentration of 0.25 mg ml⁻¹, after which alkaline-phosphatase-labelled anti-rabbit immunoglobulins were added. Assays were monitored at 405 nm with *p*-nitrophenyl phosphate, and were conducted in triplicate.

For affinity precipitation of human filamin by recombinant GST-fusion proteins, lysates from 10⁸ HeLa cells were collected in NP40 lysis buffer (0.5 % NP40, 150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 mM NaVO₃, 10 mM Na₂P₂O₇, 20 mM NaF, 20 mM β -glycerophosphate and protease inhibitors). Lysates were incubated for 1 h with glutathione-sepharose 4B beads and the resulting supernatant was incubated for 3 h at 4 °C with 30 μ g of different GST-recombinant proteins immobilized on glutathione-sepharose. Beads were washed in excess buffer, and samples were resolved by SDS-PAGE (5%) and transferred to nitrocellulose membranes. The presence of filamin was then documented by immunoblotting using a monoclonal antibody (no. 1,680, Chemicon, Temecula, CA).

Immunofluorescence microscopy.

Twenty-four hours after transfection, cells were serum-starved, fixed and permeabilized as described¹⁰. Cells were stained with appropriate primary and secondary antibodies as indicated. Melanoma cells were observed under a DMR Leica microscope using a $\times 63$ planapochromat lens. Confocal analysis of HeLa cells transfected with Trio GEFD1, DH1 or GEFD2 was carried out using a Bio-Rad 1024 CLSM system under a Nikon Optiphot II upright microscope according to the manufacturer's instructions. All transfections were repeated at least 3 times and an average of 50 cells was examined each time.

JNK assays.

Cells were transfected with pSR α 3HA-JNK with or without pSR α HA-Trio GEFD1 and serum-starved 24 h after transfection; JNK assays were carried out as described⁹. Samples were analysed by SDS-PAGE and transferred onto a nitrocellulose filter. The relative rate of phosphorylation was quantified on a Phosphorimager screen using ImageQuant software.

In vitro GDP/GTP-exchange assays.

Guanine nucleotide-release assays were carried out as described⁸ using recombinant GST-Trio GEFD1 (28.5 nM) and GST-tagged Rho GTPases (200 nM). To measure the effect of filamin A on the exchange activity of Trio GEFD1, an equal molar amount (28.5 nM) or a ten-fold excess (285 nM) of GST-fil2 was pre-incubated with Trio GEFD1 for 20 min on ice before exchange assays were carried out on the appropriate GTPase. Data were obtained in duplicate.

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ACKNOWLEDGEMENTS

We thank S. Schmidt and S. Estrach for discussions, S. Diriong for technical assistance and P. Travo, Head of the CRBM Integrated Imaging Facility, for interest and support. Confocal analysis was carried out at the Centre Regional d'Imagerie Cellulaire, Montpellier, with the help of N. Lautredou. This work was funded by CNRS institutional grants, contracts from the Ligue Nationale contre le Cancer, the Association pour la Recherche contre le Cancer, and USPHS NIH grant HL19429. Correspondence and requests for materials should be addressed to A.D.