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PtdIns(4,5)P-restricted plasma membrane localization of FAN is involved in TNF-induced actin reorganization

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The WD-repeat protein factor associated with nSMase activity (FAN) is a member of the family of TNF receptor adaptor proteins that are coupled to specific signaling cascades. However, the precise functional involvement of FAN in specific cellular TNF responses remain unclear. Here, we report the involvement of FAN in TNF-induced actin reorganization and filopodia formation mediated by activation of Cdc42. The pleckstrin-homology (PH) domain of FAN specifically binds to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P), which targets FAN to the plasma membrane. Site-specific mutagenesis revealed that the ability of FAN to mediate filopodia formation was blunted either by the destruction of the PtdIns(4,5)P binding motif, or by the disruption of intramolecular interactions between the PH domain and the adjacent beige and Chediak-Higashi (BEACH) domain. Furthermore, FAN was shown to interact with the actin cytoskeleton in TNF-stimulated cells via direct filamentous actin (F-actin) binding. The results of this study suggest that PH-mediated plasma membrane targeting of FAN is critically involved in TNF-induced Cdc42 activation and cytoskeleton reorganization.

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Introduction

TNF is a pleiotropic and broadly active cytokine exerting its effects on many cell types inside and outside of the immune system. TNF orchestrates a wide range of biological functions including host defense against intracellular pathogens, septic shock, development of autoimmune diseases, wound healing and tumor defense (Locksley *et al*, 2001; Wajant *et al*, 2003). Two distinct cell surface receptors have been identified for TNF, TNF-receptor I (TNF-RI) and TNF-receptor II (TNF-RII) (Pfeffer *et al*, 1993; Vandenabeele *et al*, 1995). We and others have demonstrated a critical role for TNF-RI in the majority of biological effects promoted by TNF (Wiegmann *et al*, 1992; Neumann *et al*, 1996). By binding to TNF-RI, TNF mediates diverse cellular events in various cell types from proliferation to apoptosis as a result of engaging different cellular compartments and signaling cascades. These include JUN N-terminal kinase (JNK), NF-κB, caspase cascades, acidic sphingomyelinase (aSMase) and mitogen-activated protein kinases (MAPK). The majority of the known effects of TNF are initiated by binding of various adaptor molecules to the cytoplasmic death domain (DD) of TNF-RI (Chen and Goeddel, 2002; Wajant *et al*, 2003).

However, next to DD binding adaptor proteins, TNF-RI binds additional factors that may be important for TNF signaling (Boldin *et al*, 1995; Adam-Klages *et al*, 1998). For example, a more membrane-proximal neutral SMase (nSMase) activation domain (NSD) of TNF-RI has previously been recognized as a distinct functional domain that is both required and sufficient for the activation of nSMase (Adam *et al*, 1996). The only protein identified so far that binds to the NSD is the adaptor protein, factor associated with nSMase activity (FAN) (Adam-Klages *et al*, 1996). Bioinformatic analysis classified FAN as a WD-repeat containing protein, a growing family of regulatory proteins that are involved in signal transduction. Besides a C-terminal cluster of WD repeats mediating binding to the NSD of TNF-RI (Adam-Klages *et al*, 1996), FAN also contains a BEACH (beige and Chediak-Higashi) domain with unknown function. Structural analysis of neurobeachin (Nbea) that contains a BEACH domain homologous to the BEACH domain of FAN revealed that this domain is associated with a novel, weakly conserved pleckstrin-homology (PH) domain (Jogl *et al*, 2002). Biochemical studies and structural analysis suggested that the PH and BEACH domains interact to function as a single unit, forming a prominent groove at the interface that may be used for the recruitment and interaction with downstream binding partners (Jogl *et al*, 2002).

Originally shown to mediate TNF-induced activation of nSMase, FAN has been suggested to play an important role in the regulation of major inflammatory cellular responses to TNF (Adam-Klages *et al*, 1996). However, mice deficient for FAN do not exhibit any striking abnormalities, except a delay in cutaneous barrier recovery, suggesting a physiological role of FAN in epidermal barrier repair (Kreder *et al*, 1999). In addition, FAN has been implicated in TNF- and CD40-mediated induction of apoptosis (Segui *et al*, 1999, 2001), lysosomal permeabilization (Werneburg *et al*, 2004), IL-6

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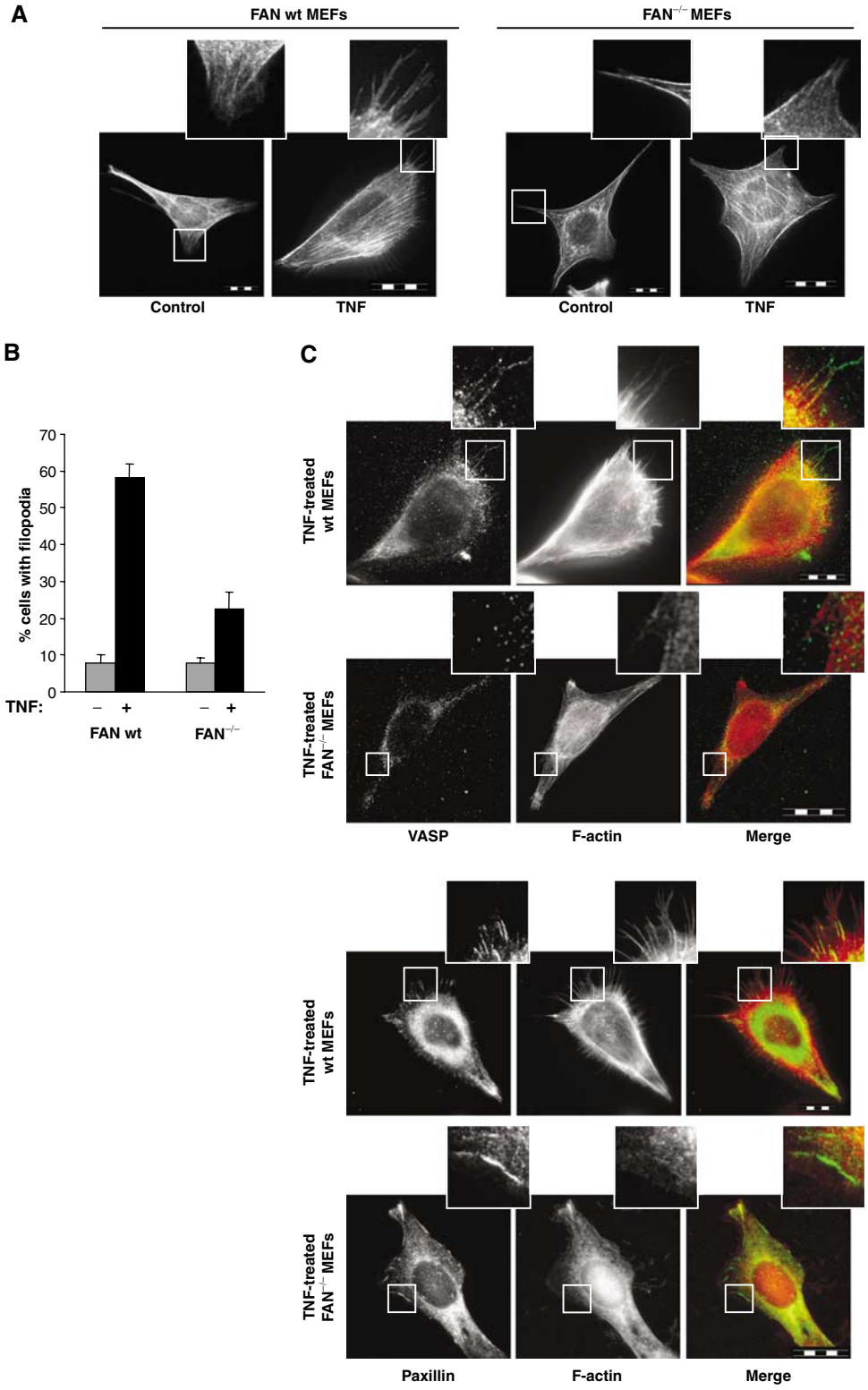

Figure 1 Impaired filopodia formation in FAN $^{-/-}$ fibroblasts after TNF stimulation. (**A**) TNF-induced filopodia formation in FAN wild-type (wt) MEFs. FAN wt and FAN$^{-/-}$ MEFs were stimulated with TNF for 10 min or left untreated and stained for F-actin using AlexaFluor568-conjugated phalloidin. Insets are enlargements of the boxed area. Scale bar, 20 μm. (**B**) Cells in panel A were quantified for filopodia formation. Cells were scored positive when presenting at least five filopodia. For each experiment, >100 cells were evaluated, and values are represented as mean±s.d. of at least three independent experiments. (**C**) TNF-induced filopodia contain filopodial markers. FAN wt and FAN$^{-/-}$ MEFs were stimulated with TNF for 10 min and stained for F-actin using AlexaFluor568-conjugated phalloidin (red) and VASP or paxillin using specific antibodies following incubation with AlexaFluor488-conjugated secondary antibodies (green). Scale bar, 20 μm.

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secretion (Malagarie-Cazenave *et al*, 2004), interaction with the WD protein RACK1 (Tcherkasowa *et al*, 2002) and regulation of cardiac cell death (O'Brien *et al*, 2003). However, mechanistic insights into FAN function in TNF-RI signaling are still missing.

Previously, the involvement of the membrane proximal region of TNF-RI in TNF-induced actin polymerization has been suggested (Peppelenbosch *et al*, 1999). As this membrane proximal region included NSD, the FAN binding site, we hypothesized that FAN is involved in TNF-mediated reorganization of the actin cytoskeleton. TNF is a potent modulator of the actin cytoskeleton in various cell types. It induces the formation of actin containing structures like stress fibers, lamellipodia and filopodia (Puls *et al*, 1999) and affects chemotaxis and motility (Leibovich *et al*, 1987; Postlethwaite and Seyer, 1990; Cumberbatch *et al*, 1997; Puls *et al*, 1999; Banno *et al*, 2004; Kutsuna *et al*, 2004; Lokuta and Huttenlocher, 2005). The modulation of the actin cytoskeleton by the TNF-RI signaling complex appears to be mediated by small GTPases like Cdc42. However, upstream molecules present in the TNF-RI signaling complex that link TNF-RI to these processes have not yet been identified.

We show here that murine embryonic fibroblasts (MEF) deficient for FAN display defective TNF-induced actin reorganization and filopodia formation. Mechanistically, this function of FAN requires the PH domain that localizes FAN to the plasma membrane via specific binding to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P) and is essentially required for FAN function in TNF-induced actin reorganization.

Results

Filopodia formation is impaired in FAN-deficient fibroblasts

TNF has been shown to induce reorganization of the actin cytoskeleton and the formation of filopodia (Puls *et al*, 1999). To determine if FAN is involved in TNF-induced actin cytoskeleton reorganization, serum-starved subconfluent MEFs from wild-type (wt) and FAN-deficient ($FAN^{-/-}$) mice (Kreder *et al*, 1999) were stimulated with TNF for 10 min and stained for filamentous actin (F-actin) using labeled phalloidin. Both wild-type and FAN-deficient cells contain little polymerized actin without TNF stimulation (Figure 1A). In wild-type MEFs, TNF treatment significantly induced the formation of protrusive structures resembling filopodia (Figure 1A and B) (Kozma *et al*, 1995; Nobes and Hall, 1995). In contrast, strongly decreased filopodia formation was observed in TNF-treated $FAN^{-/-}$ MEFs upon TNF treatment (Figure 1A and B), suggesting an involvement of FAN in the formation of these protrusive structures.

Staining of cells for the filopodial protein VASP and for paxillin, which localizes to focal complexes associated with protrusive structures like filopodia (Figure 1C), identified the observed TNF-induced structures as filopodia (Hall, 1998; Puls *et al*, 1999; Kaverina *et al*, 2002; Mejillano *et al*, 2004), which is additionally confirmed by confocal microscopy analysis shown in Supplementary Figure S1A. It is important to note that FAN deficiency does not lead to a general inability to respond to TNF. Both wild-type and $FAN^{-/-}$ MEFs show equal activation of NF-κB (Figure 2A) and JNK (Figure 2B), in response to TNF stimulation, which is in line with previous observations suggesting unaffected MAP kinase signaling in $FAN^{-/-}$ cells (Lüschen *et al*, 2000). Transient expression of FAN in $FAN^{-/-}$ MEFs could completely restore the capability of TNF to induce filopodia formation (Figure 2C and D). When $FAN^{-/-}$ MEFs were transfected with a GFP-FAN expression plasmid TNF-induced filopodia formation was only observed in GFP-FAN transfected $FAN^{-/-}$ MEFs (Figure 2D). The impact of FAN on TNF-induced actin reorganization was further examined in Swiss 3T3 fibroblasts by using double-stranded short interference RNA (siRNA) to reduce the expression of FAN. Compared with FAN expression in non-transfected cells or cells transfected with control scrambled (scr)-siRNA, FAN expression was reduced by approximately 98% in FAN-siRNA2 and 75% in FAN-siRNA1 transfected cells, as measured by real-time RT–PCR (Figure 2E). The potency of FAN-siRNAs was additionally examined in Western blot analysis (Supplementary Figure S2A). As shown in Figure 2F, downregulation of FAN in Swiss 3T3 fibroblasts resulted in significantly decreased filopodia formation after TNF treatment. Together, these findings identify FAN as an important mediator of TNF-induced actin reorganization and filopodia formation.

FAN signals upstream of Cdc42 in TNF-induced filopodia formation

Actin reorganization is controlled by members of the Rho family small GTPases (Nobes and Hall, 1999; Bishop and Hall, 2000). Cdc42 has been implicated as a crucial effector molecule downstream of TNF initiating actin reorganization and particularly the formation of filopodia (Puls *et al*, 1999; Gadea *et al*, 2004). Indeed, transient expression of a dominant-negative form of Cdc42, Cdc42N17, abrogated filopodia formation in wild-type MEFs after TNF stimulation, underscoring the central role of Cdc42 in this process (Figure 3A and B). Unlike in wild-type MEFs, TNF failed to induce Cdc42 activation in $FAN^{-/-}$ MEFs (Figure 3C and D), indicating that FAN is required for TNF-induced Cdc42 activation. In contrast, activation of Cdc42 and filopodia formation by different stimuli such as PDGF or bradykinin was not affected in $FAN^{-/-}$ MEFs (Figure 3E and Supplementary Figure S2C, respectively) suggesting a specific role of FAN in TNF-induced actin reorganization. Introduction of Cdc42L61, a constitutively active form of Cdc42 (Puls *et al*, 1999), in combination with RacN17, a dominant-negative form of Rac1 inhibiting lamellipodia formation (Supplementary Figure S2B), induced filopodia formation in both wild-type and FAN-deficient cells (Figure 3F and G), confirming an intact actin cytoskeleton machinery in $FAN^{-/-}$ cells downstream of Cdc42. It is important to note that the loss of FAN did not affect the activation of other Rho GTPases such as Rac1 and RhoA by TNF (Figure 3H), indicating a specific role for FAN in TNF-induced activation of Cdc42.

Besides its role in filopodia formation, Cdc42 has been shown to be essential for maintaining cell polarity for forward movement by reorientation of the Golgi apparatus in the direction of movement (Nobes and Hall, 1999; Etienne-Manneville and Hall, 2001). A possible impact of FAN deficiency on cell polarity was examined by analyzing reorientation of the Golgi apparatus in a wound-scratch test after TNF treatment. Reorientation analysis was assessed by

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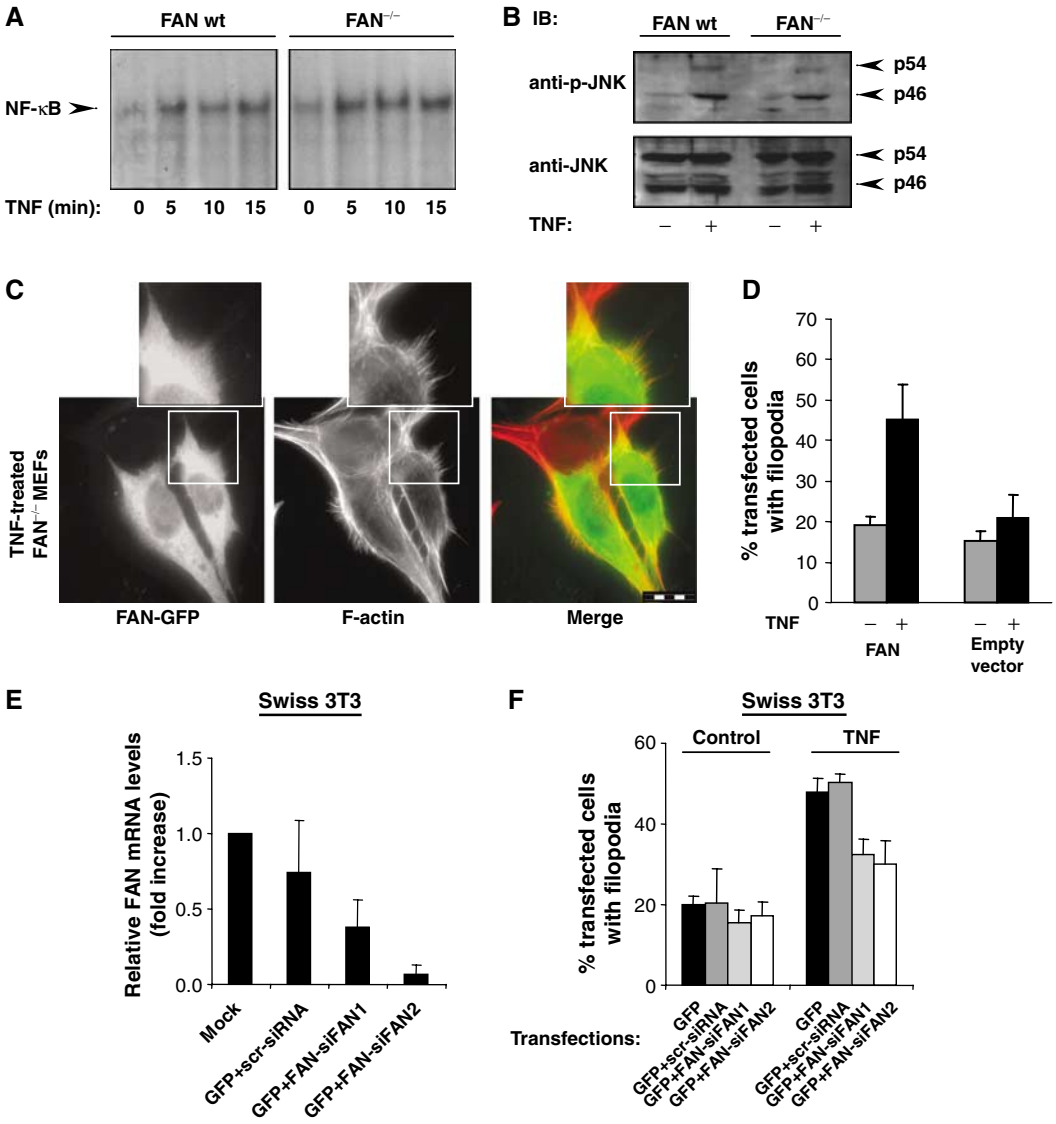

Figure 2 FAN deficiency specifically affects TNF-induced filopodia formation. (**A**) FAN deficiency does not affect TNF-induced NF-κB activation. A total of 5×10^6 FAN wt or FAN$^{-/-}$ MEFs were stimulated with TNF for the indicated times, and analyzed for NF-κB binding activity. (**B**) FAN deficiency does not affect TNF-induced JNK activation. A total of 10^5 FAN wt or FAN$^{-/-}$ MEFs were stimulated with TNF for 15 min, and total cell lysates were used for immunoblotting (IB) and probed for phosphorylated and total JNK using specific antibodies. (**C**) Overexpression of FAN restores TNF-induced filopodia formation in FAN$^{-/-}$ MEFs. FAN$^{-/-}$ MEFs were transfected with pEGFP-FAN (green), stimulated with TNF for 10 min and stained for F-actin using AlexaFluor546-conjugated phalloidin (red). Scale bar, 20 μm. (**D**) Quantitation of FAN$^{-/-}$ MEFs transfected with pEGFP-FAN or pEGFP empty vector bearing filopodia. For each experiment, >100 transfected cells were evaluated, and values are represented as mean ± s.d. of at least three independent experiments. (**E**) siRNA-mediated downregulation of FAN. Swiss 3T3 fibroblasts were mock transfected or transfected with siRNAs against FAN (FAN-siRNA1, FAN-siRNA2) or scrambled siRNA (scr-siRNA) as control, and analyzed for FAN mRNA levels using LightCylcler PCR. Data are represented as mean ± s.d. of triplicates of two independent experiments. (**F**) siRNA-mediated FAN knockdown abrogates TNF-induced filopodia formation. Swiss 3T3 fibroblasts were transfected with FAN-siRNA1, FAN-siRNA2 or scr-siRNA, together with pEGFP empty vector. GFP fluorescence was used to visualize transfected cells, and >100 transfected cells were evaluated for filopodia formation as described in panel D.

specific staining of the Golgi apparatus using anti-Rab6 antibody, and the percentage of cells with the Golgi facing toward the wound was evaluated at indicated time points in the presence of TNF. Wild-type MEFs showed enhanced reorientation of the Golgi apparatus upon TNF treatment (see also Supplementary Figure S4A), whereas this effect was markedly reduced in FAN$^{-/-}$ MEFs (Figure 4A and B), which is consistent with a defective TNF-induced activation of Cdc42 in these cells.

The PH domain targets FAN to the plasma membrane via binding to PtdIns(4,5)P

The identification of FAN as a mediator of TNF-induced filopodia formation allowed us to assess selectively the functional roles of the single domains of FAN using several deletion mutants (Figure 5A). Besides the WD repeats that are essential for TNF-RI interaction, crystal structure determination of neurobeachin and sequence comparison with FAN identified the N-terminal part of FAN as a novel, weakly

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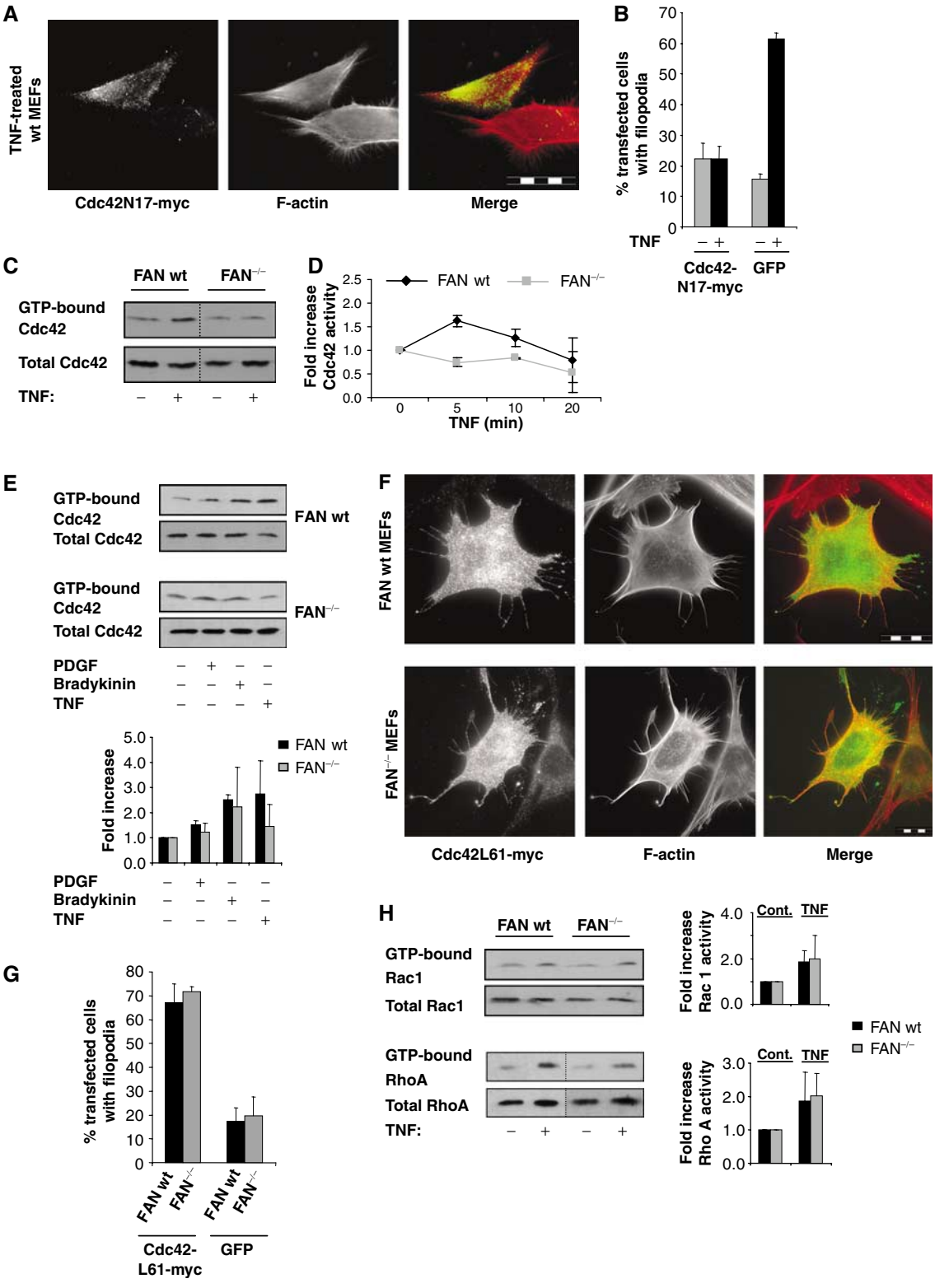
conserved PH domain, which is associated with the neighboring BEACH domain (Jogl *et al*, 2002). However, the functional significance of these two domains has not yet been elucidated. One of the important features of actin modulating proteins is membrane localization in order to integrate membrane receptor signals to actin cytoskeletal reorganization (Sechi and Wehland, 2000). Often, membrane targeting of actin modulating proteins is achieved by binding to phosphoinositides via PH or PH-like domains (Hogan *et al*, 2004; Olsten *et al*, 2004; Canton *et al*, 2005). As shown in Figure 5B, a FAN-GFP fusion protein colocalizes with TNF-RI at the plasma membrane. In order to investigate the possible role of the PH domain in targeting FAN to the plasma membrane, deletion mutants lacking either the PH domain or different parts of FAN were expressed in COS-7 cells. Confocal microscopy revealed that removal of amino-acid



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residues 1–275, which include the N-terminal PH domain, abrogates membrane association of FAN and colocalization with plasma membrane-associated TNF-RI (Supplementary Figure S3A), whereas deletion of the C-terminal WD repeats or the BEACH domain has no effects on the subcellular distribution of FAN (Figure 5C and D; Supplementary Figure S3B and C). These findings implicate the novel PH domain to be responsible for plasma membrane association of FAN.

Many PH domains are known for their ability to bind to distinct phosphoinositides, which is often utilized in combination with additional protein–protein interaction as a mechanism for signal-dependent membrane targeting (Lemmon and

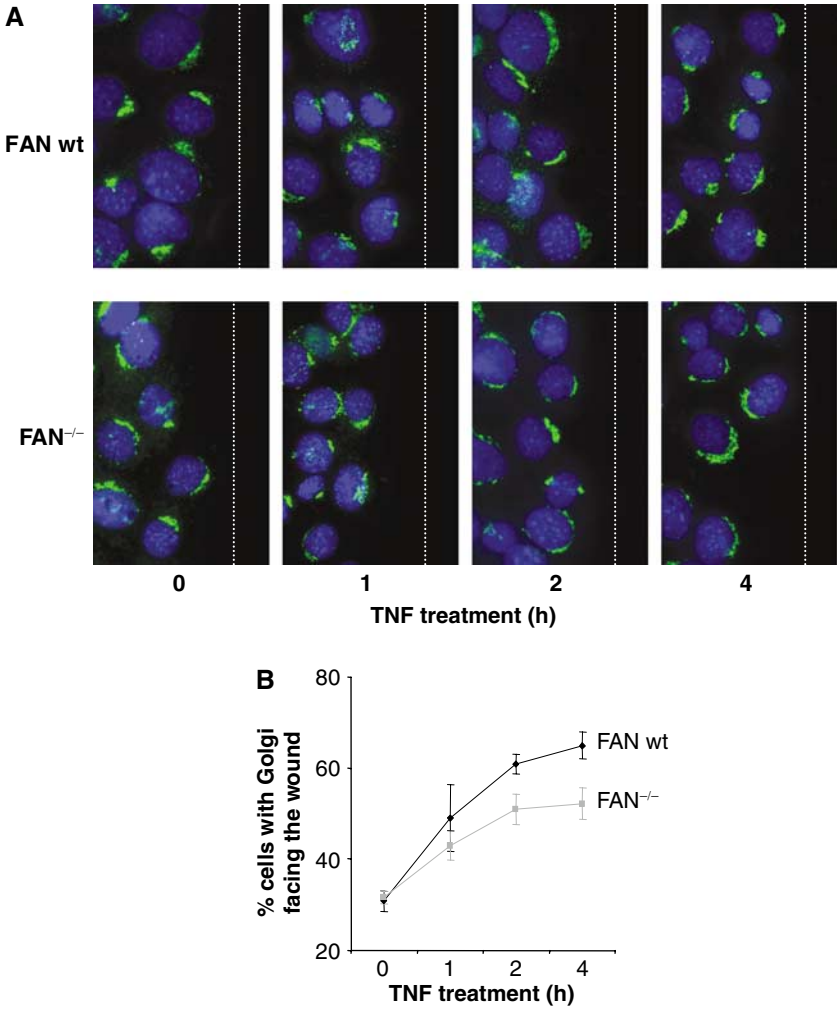

Figure 4 Impaired Golgi apparatus reorientation in $FAN^{-/-}$ MEFs. (**A**) Golgi reorientation in a scratch-wound test. After scratching of confluent layers of FAN wt and $FAN^{-/-}$ MEFs (0 h), cells were immediately treated with TNF and stained after the indicated times for the Golgi apparatus using anti-Rab6 antibody and Hoechst 33258 to visualize the nuclei. The dotted line indicates the direction of the wound. (**B**) Quantification of Golgi reorientation. Cells with the Golgi orientated toward the wound were scored positive at the indicated times. For each experiment, >100 cells were evaluated. Results are represented as mean $\pm$ s.d. of at least three independent experiments.

Figure 3 FAN mediates TNF-induced filopodia formation through Cdc42 activation. (**A**) TNF-induced filopodia formation is blocked by overexpression of dominant-negative Cdc42 (Cdc42N17). FAN wt MEFs were transfected with an myc-tagged dominant-negative Cdc42 (Cdc42N17-myc), treated with TNF for 10 min and stained for F-actin using AlexaFluor568-conjugated phalloidin (red) and anti-myc (green) to visualize filopodia and myc-tagged Cdc42N17, respectively. (**B**) Quantitation of FAN wt MEFs bearing filopodia transfected with Cdc42N17 or GFP and stimulated with TNF. For each experiment, >100 transfected cells were evaluated, and values are represented as mean $\pm$ s.d. of at least three independent experiments. (**C**) TNF-induced activation of Cdc42. A total of 4×10^6 FAN wt or $FAN^{-/-}$ MEFs were stimulated with TNF for 5 min or left untreated. Activated GTP-bound Cdc42 was precipitated from total lysates and detected on Western blot using a Cdc42-specific antibody. An aliquot of the total lysate used for precipitation was analyzed for total Cdc42 content in cell lysates. (**D**) Cdc42 activation after TNF stimulation was quantified at indicated times by Western blotting using AlphaEasy FC software (Alpha Innotech, San Leandro, USA). Data are shown as mean $\pm$ s.d. of three independent experiments. (**E**) Activation of Cdc42 by PDGF and bradykinin. FAN wt and $FAN^{-/-}$ MEFs were stimulated with PDGF or bradykinin for 10 min or left untreated. Activated GTP-bound Cdc42 was detected as in panel C and quantified as in panel D. (**F**) Constitutively active Cdc42 overcomes the defective TNF-induced filopodia formation in $FAN^{-/-}$ MEFs. FAN wt and $FAN^{-/-}$ MEFs were transfected with an myc-tagged constitutively active Cdc42 (Cdc42L61-myc) and stained for F-actin (red) and myc-Cdc42L61 (green). Cells were cotransfected with dominant-negative RacN17 to avoid Rac activation by Cdc42 (Nobes and Hall, 1995) (see also Supplementary Figure S2B). (**G**) Quantitation of FAN wt and $FAN^{-/-}$ cells bearing filopodia transfected with Cdc42L61 or GFP as in panel B. (**H**) TNF-induced activation of Rac1 and RhoA. FAN wt and $FAN^{-/-}$ MEFs were stimulated with TNF for 10 min or left untreated, and activated GTP-bound GTPases were precipitated from total lysates and detected on Western blot using specific antibodies. Results were quantified as in panel D.

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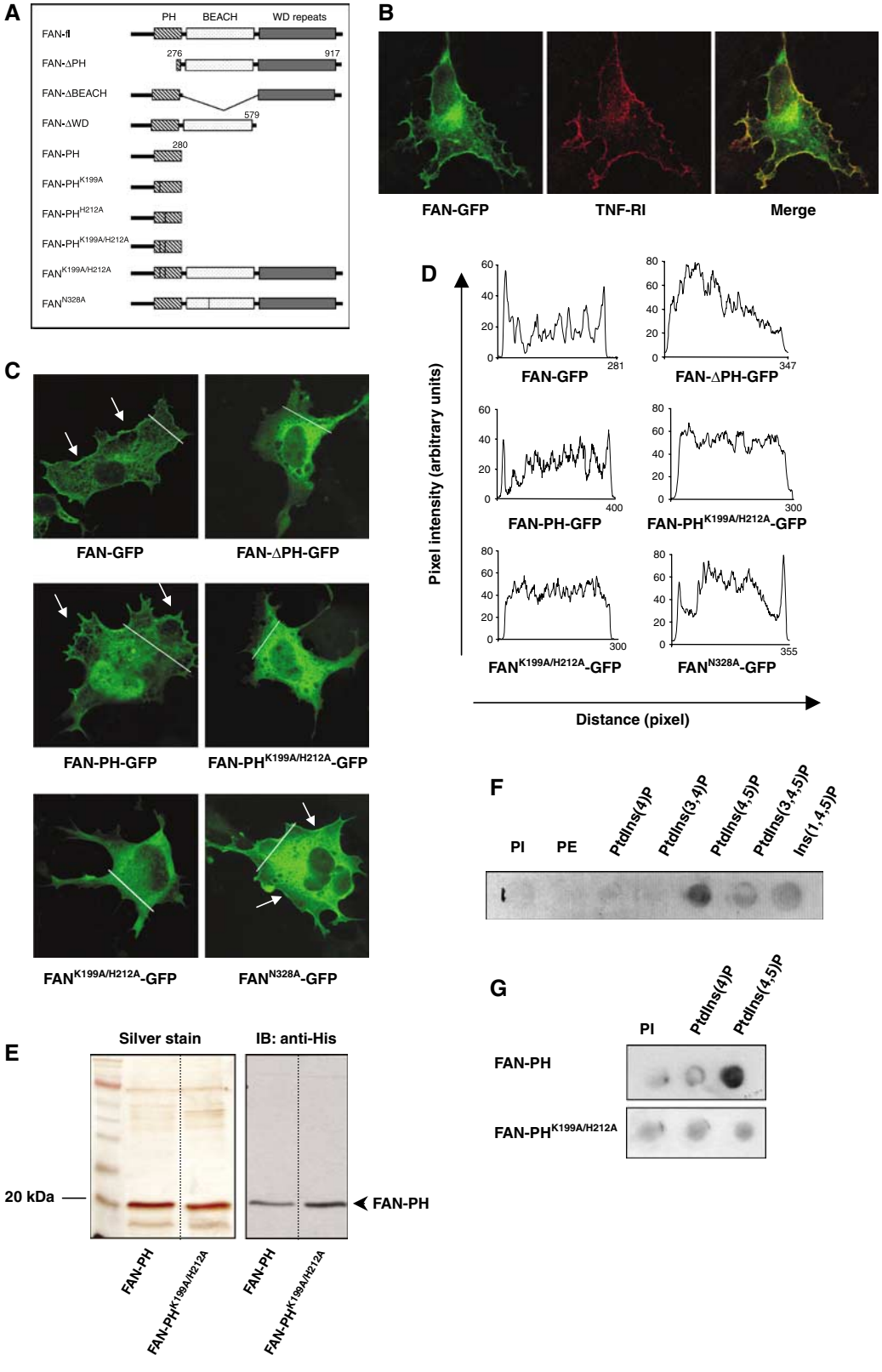
Ferguson, 2000; Cozier *et al*, 2004). To test if the N-terminal part of FAN has phospholipid binding ability, recombinant FAN-PH was expressed in *Escherichia coli* as His-tag fusion protein and affinity purified (Figure 5E). In a lipid overlay assay, membranes spotted with different lipids were incubated with recombinant FAN-PH protein and bound protein was detected by Western blot using anti-His antibody. As shown in Figure 5F, the recombinant FAN-PH-domain protein bound specifically to PtdIns(4,5)P. No binding could be observed either to related phosphoinositides or to different phospholipids like phosphatidylcholin or phosphatidylethanolamine.



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It has been proposed that positively charged conserved residues in the basic binding pocket of several PH domains mediate the interaction of PH domains with phosphoinositides (Lemmon and Ferguson, 2000; Cozier *et al*, 2003; Edlich *et al*, 2005). Therefore, mutations in the residues K199 and H212 of FAN were introduced that are located in the basic binding pocket of the FAN PH domain, which was determined by sequence alignment with PH domains of well-characterized proteins like phospholipase C (Lemmon *et al*, 1995). Recombinant double mutant FAN (Figure 5E) failed to bind to PtdIns(4,5)P (Figure 5G), indicating that the mutated basic residues are essential for phospholipid binding of the PH domain of FAN.

PtdIns(4,5)P is mainly located at the plasma membrane and serves as anchor for membrane targeting of proteins (Lemmon *et al*, 1996). Confocal microscopy of FAN-GFP expressing cells revealed that the membrane localization observed with FAN-PH and FAN-full length (fl) expressing cells is reduced in cells expressing the PH double mutant of FAN either as PH domain alone (FAN-PH$^{K199A/H212A}$) or as full-length protein (FAN$^{K199A/H212A}$) (Figure 5C and D). A control mutation located outside the PH domain (N328) had no effect on membrane localization. Thus, the weakly conserved PH domain of FAN displays PtdIns(4,5)P binding properties characteristic of classical PH domains, which are necessary and sufficient for plasma membrane association of FAN.

Plasma membrane association of FAN is indispensable for TNF-induced filopodia formation

To address the functional significance of plasma membrane targeting by the PH domain, PH deletion mutants of FAN were investigated for mediating TNF-induced filopodia formation. FAN$^{-/-}$ MEFs were transfected with FAN-GFP or with the PH deletion mutant, FAN-ΔPH-GFP, stimulated with TNF and stained for F-actin to visualize filopodia. As already shown in Figure 2C, expression of full-length FAN in FAN-deficient cells led to the induction of filopodia after TNF stimulation. In contrast, cells expressing FAN-ΔPH did not form filopodia after TNF stimulation (Figure 6A and B). Furthermore, reconstitution of FAN-deficient cells with the double mutant FAN$^{K199A/H212A}$ did not rescue filopodia formation. As FAN$^{K199A/H212A}$ is a loss-of-function-mutant with regard to both PtdIns(4,5)P binding and plasma membrane targeting (Figure 5C and G), this observation suggest a causative link of PH-directed plasma membrane targeting of FAN and TNF-induced filopodia formation.

Finally, a possible contribution of the BEACH domain to TNF-induced filopodia formation was examined. By NMR analysis we have previously suggested that the BEACH domain and the PH domain interact to form a functional unit (Jogl *et al*, 2002). Indeed, as shown in Figure 6B, FAN devoid of the BEACH domain (FAN-ΔBEACH) failed to restore filopodia formation after TNF treatment of FAN-deficient cells. Similarly, an internal BEACH mutation (FAN^{N328A}) that has been shown to disrupt the BEACH/PH interaction (Jogl *et al*, 2002) did not rescue filopodia formation (Figure 6B). Thus, the PH domain modulates TNF-induced filopodia formation not only by plasma membrane association but also through cross-talk with the BEACH domain.

FAN connects the plasma membrane to the actin cytoskeleton

A fundamental property of many plasma membrane-associated proteins, including ERM (ezrin-redaxin-moesin) and WASP (Wiskott–Aldrich syndrome protein)-WAVE (WASP family verprolin homologous protein) protein families, is their association with the actin cytoskeleton (Bretscher *et al*, 2002; Takenawa and Suetsugu, 2007). Thus, we hypothesized that FAN as an adaptor protein associated with both TNF-RI and the plasma membrane might interact with the actin cytoskeleton complex in order to provide a regulated link from plasma membrane-associated TNF-RI to cortical filamentous actin.

To determine the possible interaction partners of the actin cytoskeleton machinery, HEK 293 cells were transiently transfected with FAN-GST or GST alone, and examined by GST pull-down assays. As shown in Figure 7A, actin was co-precipitated upon TNF treatment with ectopically expressed GST-FAN but not GST. Additionally, weak co-precipitation of VASP was observed, a member of the ENA/VASP family of actin-associated proteins, which is located at filopodial tips and has recently been shown to promote F-actin bundling required for filopodia formation (Reinhard *et al*, 2001; Schirenbeck *et al*, 2006). As this effect was dependent on TNF treatment, these results suggest that the interaction between FAN and the actin cytoskeleton is tightly regulated by TNF. Consistently, immunoprecipitation of actin and VASP resulted in co-precipitation of ectopically expressed GST-FAN after TNF treatment, corroborating the interaction observed in the GST pull-down assay. GST-FAN, VASP or actin did not co-precipitate with Bax (proapoptotic Bcl2 protein), excluding immunoprecipitation conditions which favor nonspecific binding (Figure 7B).

In order to unravel the nature of FAN interaction with actin cytoskeleton machinery, FAN/actin direct interaction was analyzed in an *in vitro* F-actin binding assay using recombinant FAN-GST and F-actin. As shown in Figure 7C, FAN-GST

Figure 5 The PH domain directs FAN to the plasma membrane via specific binding to PtdIns(4,5)P. (**A**) A schematic representation of the FAN variants. (**B**) Colocalization of plasma membrane TNF-RI and FAN. COS cells were transfected with pEGFP-FAN (green) and TNF-RI lacking functional DD to avoid toxic effects (Tcherkasowa *et al*, 2002). Cells were stained for TNF-RI (red) without permeabilization for selective visualization of plasma-membrane TNF-RI, and analyzed by confocal microscopy. (**C**) PH-mediated plasma membrane localization of FAN. Confocal images of COS cells transiently expressing pEGFP-FAN or different deletion mutants of FAN. Arrows indicate membrane staining of pEGFP-FAN. (**D**) Quantification of membrane staining in panel C. Intensity profiles along the indicated white lines of the pictures in panel C were generated using ImageJ (Abramoff *et al*, 2004). (**E**) Expression and purification of recombinant His-tagged FAN-PH. FAN-PH and FAN-PH$^{K199A/H212A}$ were recombinantly expressed and affinity purified. Equal amounts were analyzed by SDS–PAGE following silver staining or immunoblot (IB) analysis with anti-His antibody. (**F**) Lipid overlay assay using purified FAN-PH protein. The recombinantly expressed PH domain of FAN was incubated with a nitrocellulose membrane spotted with different phospholipids. Bound protein was detected using anti-His antibody. (**G**) Lipid overlay assay as in panel E using purified FAN-PH and FAN-PH$^{K199A/H212A}$ protein. Equal amounts of protein were incubated with phospholipid-spotted membranes and detected using anti-His antibody. PI, phosphatidylinositol; PE, phosphatidylethanolamine; PtdIns(4)P, phosphatidylinositol-4-phosphate; PtdIns(3,4)P, phosphatidylinositol-3,4-phosphate; PtdIns(4,5)P, phosphatidylinositol-4,5-phosphate; PtdIns(3,4,5)P, phosphatidylinositol-3,4,5-phosphate; Ins(1,4,5)P, inositol-1,4,5-phosphate.

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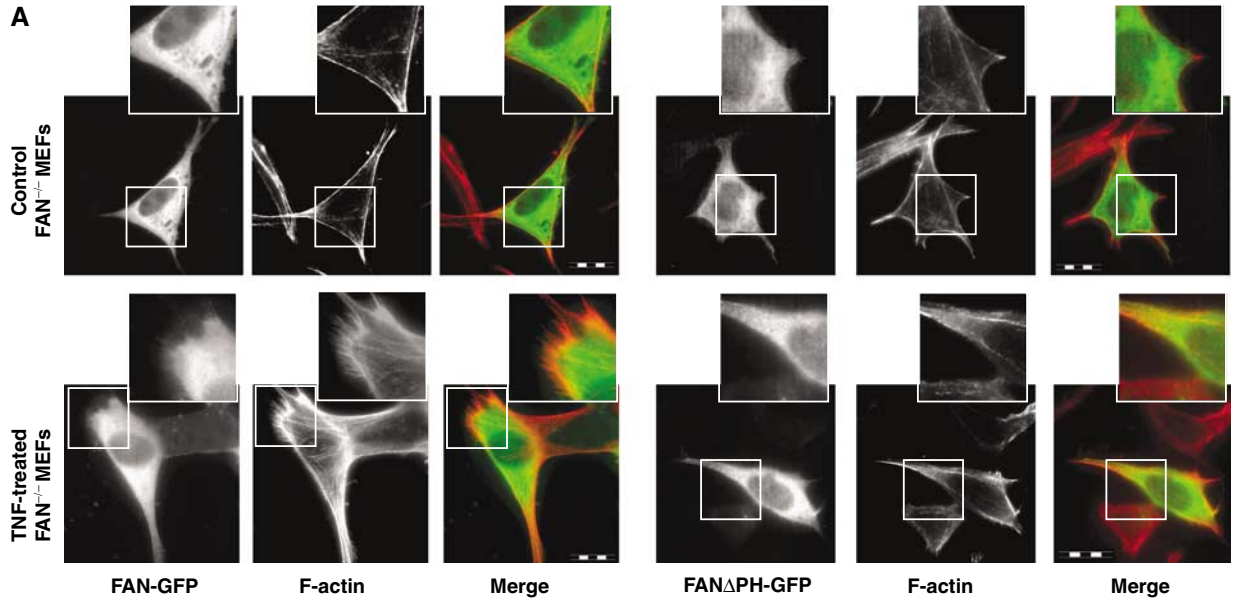

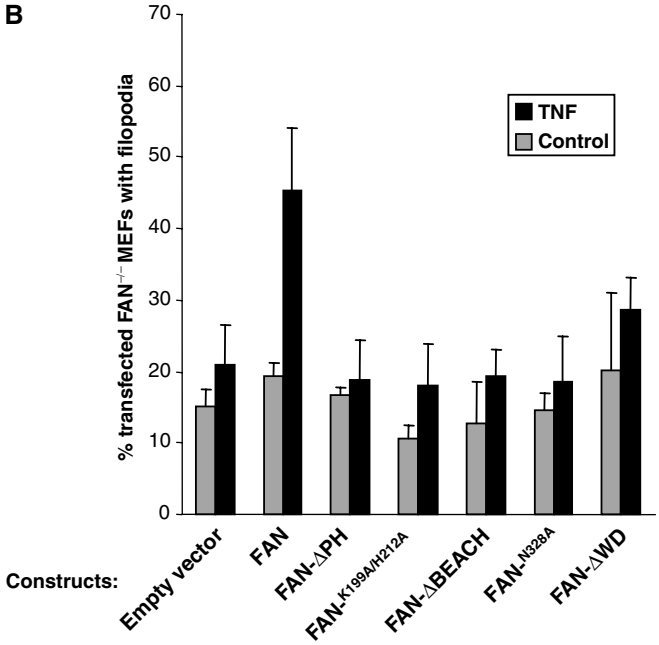

Figure 6 Plasma membrane association of FAN is indispensable for TNF-induced filopodia formation. (**A**) FAN$^{-/-}$ MEFs transfected with pEGFP-FAN or pEGFP-FAN-ΔPH (green) were stimulated with TNF for 10 min or left untreated. Cells were stained for F-actin using AlexaFluor546-conjugated phalloidin (red) to visualize filopodia. Insets are enlargements of the boxed area. Scale bar, 20 μm. (**B**) FAN$^{-/-}$ MEFs transfected with the indicated pEGFP-FAN fusion constructs were stimulated with TNF for 10 min or left untreated. Cells were stained for F-actin using AlexaFluor568-conjugated phalloidin and quantified for filopodia formation. For each experiment, >100 transfected cells were counted, and results are represented as mean ± s.d. of at least three independent experiments.

but not GST alone was co-sedimented with F-actin, demonstrating a direct F-actin binding capability of FAN. The observed interactions provide a functional link between FAN and the actin cytoskeleton machinery, which may result in co-precipitation of proteins involved in filopodia formation like VASP, as shown in Figure 7A and B.

Further investigations on the impact of the PH domain of FAN on the actin binding capacity in HEK 293 cells revealed that deletion of the PH domain, which abrogates plasma membrane association of FAN (Figure 5C) also abolished the interaction of FAN with actin, whereas binding to TNF-RI remained unaffected (Figure 7D). Finally, no actin binding was detected with FAN-PH alone, suggesting that PH modulates FAN actin binding by promoting correct subcellular localization at the plasma membrane.

Discussion

In contrast to other TNF receptor adaptor proteins such as TRADD, FADD, RIP or TRAF2, FAN does not bind to, or interact with death domains, and its precise function in specific cellular responses to TNF remained largely unclear.

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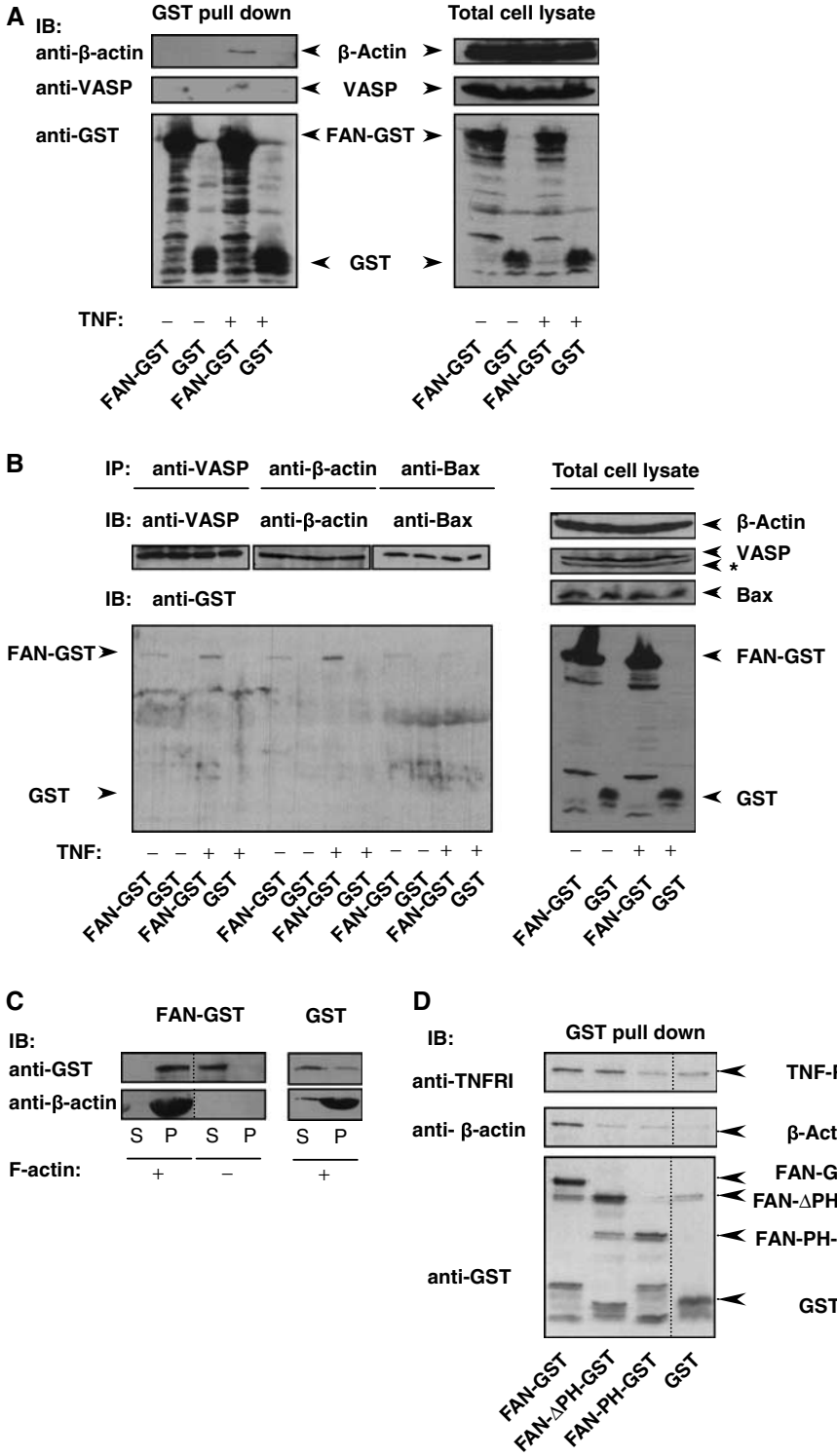

Figure 7 FAN interacts with the cytoskeleton. (**A**) GST pull-down assay after TNF treatment. HEK 293 cells were transiently transfected with DNA constructs coding for FAN-GST or GST alone and stimulated with TNF for 10 min or left untreated. Cells were lysed after 24 h and subjected to a GST pull-down assay. Precipitates and total cell lysate were immunoblotted and probed with the indicated antibodies (IB) to detect co-precipitated proteins. (**B**) Immunoprecipitation (IP) of VASP and actin after TNF treatment. HEK 293 cells were transiently transfected with DNA constructs coding for FAN-GST or GST alone and stimulated with TNF for 10 min or left untreated. Cells were lysed after 24 h, and IP was carried out using specific antibodies against actin, VASP or Bax. Precipitates and total cell lysate were immunoblotted and probed with the indicated antibodies. Asterisks (*) represent actin band recognized by actin-specific antibody before reprobing with anti-VASP-specific antibody. (**C**) F-actin sedimentation assay. FAN-GST or control GST proteins were subjected to a F-actin sedimentation assay as described in Materials and methods. Supernatants (S) and pellets (P) were analyzed by immunoblot (IB) using anti-GST and anti-β-actin antibodies. Samples without F-actin were included as binding control. (**D**) GST pull-down assay with cells ectopically expressing TNF-RI. HEK 293 cells were transiently cotransfected with DNA constructs coding for full-length TNF-RI, together with FAN-GST, FANΔPH-GST, FAN-PH-GST, or GST alone. After 24 h, cells were lysed and subjected to a GST pull-down assay as in panel A.

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Here, we show that FAN is crucial for the formation of filopodia and actin cytoskeleton reorganization induced by TNF. A major finding of our study demonstrates specific binding of the FAN PH domain to PtdIns(4,5)P, which directs FAN to the plasma membrane. Additionally, FAN binds to the actin cytoskeleton machinery upon TNF treatment, which is modulated by PH action. FAN-PH mutants with a non-functional PtdIns(4,5)P binding site were unable to mediate TNF-induced filopodia formation. Furthermore, disruption of the intramolecular interaction between the PH and BEACH domains abrogated the actin modulatory function of FAN without affecting membrane localization. Thus, the molecular mode of PH domain action in FAN signaling is defined by two independent functional features, that is, targeting of FAN to the plasma membrane and correct interdomain interaction between BEACH and PH regulating FAN function.

Several proteins involved in modulating the actin cytoskeleton via regulation of Rho GTPases contain PH or PH-like domains with lipid binding properties (Bellanger *et al*, 2000; Ohta *et al*, 2006), which enable these proteins to localize correctly to the plasma membrane (Cozier *et al*, 2004; Lemmon, 2004). PtdIns(4,5)P has been shown to directly interact with a large number of regulators of the actin cytoskeleton and control their activity at the plasma membrane (Hilpela *et al*, 2004). Accumulating evidence also suggests PtdIns(4,5)P as a spatial marker for directing actin polymerization close to the plasma membrane (Insall and Weiner, 2001). Like other cytoskeletal modulators FAN contains a PH domain that can bind to PtdIns(4,5)P (Figure 5). FAN utilizes its PtdIns(4,5)P binding ability to associate with the plasma membrane, where interaction with plasma membrane TNF-RI and possibly additional proteins can occur (Tcherkasowa *et al*, 2002).

In this study FAN was identified as a TNF-RI binding adaptor protein, which promotes Cdc42 activation upon TNF stimulation (Figure 3). Since FAN does not contain any known Rho GTPase regulatory capacities like GAP (GTPase-activating protein) or GEF (guanine nucleotide-exchange factor) activity, further modulators of actin cytoskeleton might be the downstream target of FAN. As shown in Figure 7, FAN appeared as a functionally dormant, plasma membrane-associated protein without actin cytoskeleton binding property in untreated cells. Upon TNF stimulation FAN interacted with the actin cytoskeleton machinery demonstrated by co-precipitation with actin and VASP and by F-actin co-sedimentation (Figure 7A–C). A fundamental property of many actin cytoskeleton modulators including ERM and WASP/WAVE proteins is their association with the plasma membrane and the underlying cytoskeleton connecting the plasma membrane and plasma membrane proteins to the cortical cytoskeleton (Bretscher *et al*, 2002; Takenawa and Suetsugu, 2007). These proteins are mainly activated by extracellular signals, undergo conformational change and bind to the actin cytoskeleton. Similarly, FAN binds TNF-RI, associates with the plasma membrane (Figure 5) and interacts with actin cytoskeleton machinery upon TNF treatment (Figure 7). By interaction with the actin cytoskeleton machinery and TNF-RI, FAN could form a signaling platform, which locally modulates TNF-induced actin reorganization in a phosphoinositide-dependent manner, a mechanism similar to what has been recently shown for Toll-like receptor signaling (Kagan and Medzhitov, 2006). In this context, the PH domain may modulate the actin binding capacity of FAN in a dual way: first by correct subcellular localization of FAN and second by promoting a conformational change of FAN (Jogl *et al*, 2002) upon specific PtdIns(4,5)P binding (Figure 6B), as described for N-WASP (neural WASP) (Prehoda *et al*, 2000). In contrast to ERM proteins, which are mainly linked to the plasma membrane by additional membrane proteins, FAN can directly associate with the plasma membrane via its specific PtdIns(4,5)P binding capacity. The direct association with plasma membrane lipids enables FAN for immediate early reaction to plasma membrane lipid modification, probably resulting in modulation of its downstream binding partners.

It has been proposed that PtdIns(4,5)P accumulates at plasma membrane lipid-microdomains, where it modulates the activity of proteins that regulate the actin cytoskeleton (Chong *et al*, 1994; Honda *et al*, 1999; Laux *et al*, 2000). Sphingolipids are known to form liquid-ordered microdomains that segregate from the more fluid regions of membranes. In particular, sphingomyelin (SM) has been shown to form tight hydrophobic interactions with cholesterol and to play a key role in the formation of lipid rafts (Barenholz, 2004). FAN has been initially identified as the factor associated with nSMase activity, an enzyme belonging to the family of sphingomyelinases that hydrolyze SM to ceramide (Cer), and thereby alter membrane lipid composition (Cremesti *et al*, 2002). A recently identified novel nSMase3 was shown to be TNF responsive in a FAN-dependent manner (Krut *et al*, 2006), and to localize to the plasma membrane (K Wiegmann and M Krönke, unpublished data). By modulating nSMase3 activity, FAN could influence membrane lipid composition and PtdIns(4,5)P distribution, and thus modulate downstream signaling of TNF-RI.

FAN appears to be important for TNF-induced Cdc42 activation. It is worth noting that, next to neutral sphingomyelinases including nSMase3, Cdc42 is the first signaling component identified to be regulated by FAN (Figure 3). Strikingly, Hanna *et al* (2001) have previously reported that exogenous sphingomyelinase and synthetic C2-ceramide induce membrane association of Rho GTPases like Cdc42 and phosphorylation of paxillin and focal adhesion kinase. Thus, it will be interesting to test a possible functional link between FAN and nSMase3 in TNF-induced activation of Cdc42 and subsequent formation of filopodia. This idea is supported by the observation that mutations of PH or BEACH that disrupt the interaction between the PH and BEACH domains not only block TNF-induced filopodia formation but also destroy FAN-mediated activation of nSMase (Jogl *et al*, 2002).

Reorganization of the actin cytoskeleton in response to cytokines like TNF plays a central role in modulating the shape and behavior of cells. Cellular motility modulated by TNF has been shown to be especially important in epidermal repair and wound healing (Cumberbatch *et al*, 1997; Banno *et al*, 2004; Lokuta and Huttenlocher, 2005). The identification of FAN as a mediator of Cdc42 activation and cell polarization closes an important gap in our understanding of TNF-induced molecular pathways regulating cytoskeletal reorganization.

Materials and methods

Cell culture and transfection

Mouse embryonic fibroblasts (MEFs), Swiss 3T3 fibroblasts and COS-7 cells were cultured at 37°C in DMEM (Biochrom,

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Berlin, Germany) and 10% fetal bovine serum. HEK 293FT cells were cultured as described above supplemented with 2 mM L-glutamine, non-essential amino acids and 10 mM sodium pyruvate (Biochrom). For F-actin staining, cells were plated on coverslips and transfected using ExGENE (Fermentas, St Leon-Rot, Germany) (MEFs) or the calcium phosphate method (COS-7 and HEK 293FT cells).

Antibodies and reagents

Goat polyclonal anti-GST antibody was obtained from Amersham Corp. (Freiburg, Germany). Rabbit polyclonal antibody JNK and phospho-JNK was obtained from Cell Signaling Technology (Danvers, USA). Phycoerythrin-conjugated (PE) mouse anti-human TNF-RI monoclonal antibody was obtained from R&D Systems (Wiesbaden, Germany). Mouse anti-His antibody was obtained form Quiagen (Hilden, Germany). Rabbit polyclonal anti-Cdc42 and mouse monoclonal anti-RhoA antibodies were obtained from Santa Cruz Biotechnologies (Heidelberg, Germany). Mouse monoclonal anti-Rac1 antibody was obtained from Upstate (Dundee, UK). Rabbit polyclonal anti-VASP antibody was obtained from Axxora (Lörrach, Germany). Mouse monoclonal anti-β-actin and horseradish peroxidase (HRP) conjugates of anti-rabbit and anti-mouse IgG were obtained from Sigma (München, Germany). AlexaFluor-568 and AlexaFluor-488 conjugated phalloidin were purchased from Molecular Probes (Karlsruhe, Germany). Rabbit anti-Bax antibody was obtained from BD Biosciences (Heidelberg, Germany). All chemicals were purchased from Sigma (München, Germany), unless indicated otherwise.

DNA constructs

Open reading frames of genes encoding human FAN and different variants of FAN were cloned into pEGFP-C3 vector (Invitrogen, Karlsruhe, Germany) for immunofluorescence, into pRK-GST vector for GST pull-down experiments, or pET-20b vector (Novagen/Merck Biosciences, Darmstadt, Germany) or pGEX-4T3 vector (Amersham Corp., Freiburg, Germany) for prokaryotic protein expression. All constructs were verified by sequence analysis.

Immunofluorescence

Cells grown on coverslips were stimulated with TNF (100 ng/ml) for 10 min and washed twice with cold PBS. Cells were then fixed with 3% paraformaldehyde/PBS for 20 min and blocked with 3% bovine serum albumin in PBS for 30 min. For staining of surface TNF-RI, cells were incubated with PE-conjugated TNF-RI specific antibody for 1 h without permeabilization, and washed twice with PBS. For staining of the actin cytoskeleton, cells were permeabilized with 0.1% saponin during blocking, and incubated with AlexaFluor568- or AlexaFluor488-conjugated phalloidin in PBS/0.1% saponin. Cells were mounted on glass slides and examined using an Olympus IX81 fluorescence microscope or a Leica DMIRE2 confocal microscope. If appropriate, images were processed using CellP deconvolution software (Olympus SIS). For quantification of filopodia, >100 cells were evaluated under the microscope, and the percentage of cells that showed more than five filopodia was calculated (Gadea *et al*, 2004).

GTPase activation assay

The Cdc42/Rac1 activation assay was performed as described (Sander *et al*, 1998; Malliri *et al*, 2002). Briefly, cells were treated with TNF (100 ng/ml), PDGF (20 ng/ml), bradykinin (400 ng/ml) or left untreated, and were lysed before incubation with GST-CRIB fusion protein, which contains the GTPase binding domain from human PAK1B coupled to glutathione–Sepharose beads (Amersham Corp., Freiburg, Germany). After precipitation, beads were washed four times, eluted in SDS sample buffer and immunoblotted with antibodies against Cdc42 or Rac1. For RhoA activation, the same principle was applied using the Rho binding domain of Rhotekin (Ren *et al*, 1999) and RhoA-specific antibody for immunoblot analysis.

Protein expression and purification

The part of FAN containing the PH domain (residues 1–275) cloned into pET20b vector was expressed in *E. coli* at 37°C for 3 h. Soluble protein was bound to nickel–agarose affinity resin (Novagen/Merck Biosciences, Darmstadt, Germany) and eluted as described by the manufacturer in a buffer containing 20 mM Tris–HCl (pH 7.5), 500 mM NaCl, 1 mM PMSF and 100 mM imidazole. For production of FAN-GST, FAN cloned into pGEX-4T3 vector was expressed in *E. coli* at 25°C for 5 h and purified using glutathione–Sepharose 4B beads (Amersham Corp., Freiburg, Germany).

Protein–lipid overlay assay

The protein–lipid overlay assay was performed as described previously (Dowler *et al*, 2000). Briefly, a nitrocellulose membrane spotted with 1 µl of lipid solution containing 0.5 nmol of phospholipids was blocked for 1 h in 3% BSA in TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and incubated overnight with approximately 0.1 µg/ml of the indicated His-fusion proteins. After washing in TBST, membranes were incubated with monoclonal anti-His antibody and secondary HRP-conjugated antibody. Bound protein was detected by enhanced chemiluminescence.

F-actin sedimentation assay

The F-actin sedimentation assay was performed using the Actin Binding Protein Biochem Kit (Cytoskeleton, Inc., Denver, USA) according to the manufacturer's instructions. Briefly, polymerized actin was incubated with approximately 0.5 µg recombinant FAN-GST or control GST protein for 30 min at room temperature and centrifuged at 150 000 *g* for 90 min. FAN-GST and GST protein was detected in supernatants and pellets using anti-GST antibody after immunoblotting.

GST pull-down assay

A total of 3×10^6 HEK 293 cells were transiently transfected with the DNA constructs coding for the indicated proteins. After 24 h, cells were stimulated with TNF (100 ng/ml) for 10 min or left untreated, lysed in 50 mM Tris–HCl, 15 mM EGTA, 100 mM NaCl, 0.1% Triton X-100, 1 mM DTT and 1 mM PMSF at pH 7.5, and centrifuged for 30 min at 20 000 *g*. Supernatants were incubated with 25 µl glutathione–Sepharose beads for 1 h at 4°C. Beads were washed extensively, resuspended in SDS sample buffer and analyzed by SDS–PAGE and immunoblotting.

Real-time light-cycler RT–PCR

Swiss 3T3 fibroblasts were transiently transfected with two different predesigned siRNAs against murine FAN (NSMAF, Ambion Europe, Huntingdon, UK) (FAN-siRNA1 and 2) or control scrambled siRNA (scr-siRNA) using Hyperfect (Quiagen, Hilden, Germany). Total RNA was isolated 48 h after transfection and reverse transcribed into cDNA using Superscript IIITM First-Strand Synthesis Supermix for qRT-PCR (Invitrogen, Karlsruhe, Germany). A 2 µl (10%) volume of the appropriate cDNA samples was subjected to the Light Cycler real-time PCR using the LC FastStart DNA MasterPLUS SYBR Green 1 Kit (Roche) and sequence-specific oligonucleotide for FAN. For relative quantification, the FAN cDNA levels were determined relative to a calibrator cDNA and normalized for hypoxanthine-phosphoribosyl-transferase (HPRT) or for hydroxymethylbilane synthase (HMBS) gene expression (reference genes) employing the LightCycler Relative Quantification Software (Roche Diagnostics, Mannheim, Germany). Expression levels determined in untreated cells were set to 100%.

Electrophoretic mobility shift assay (NF-κB activity)

Electrophoretic mobility shift assays (EMSAs) were performed as described previously (Kashkar *et al*, 2007) using the NF-κB-specific oligonucleotides (Applied Biosystems, Darmstadt, Germany) end labeled with γ-^{32}P-ATP (Amersham Corp., Freiburg, Germany).

Cell polarity determination

Cell polarity was determined by analyzing reorientation of the Golgi apparatus in a wound-scratch test, essentially as described (Kupfer *et al*, 1982; Nobes, 2000). Briefly, after wounding, monolayers of MEFs were immediately incubated with TNF (100 ng/ml) and fixed at the indicated times. The Golgi apparatus was stained using anti-Rab6 antibody, and nuclei were counterstained using Hoechst 33258. The percentage of cells with their Golgi orientated toward the wound was evaluated under the microscope.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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