FAN mediates TNF-induced actin reorganisation

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Contents

Abstract

The WD repeat protein FAN is a member of the family of TNF receptor adapter proteins that are coupled to specific signalling cascades. In contrast to the other known adapter proteins that bind to the death domain of TNF-RI, FAN has been shown to interact with a membrane-proximal NSD domain. Though initially shown to promote nSMase activation by TNF, the precise functional involvement of FAN in specific cellular responses upon TNF stimulation remained unclear. In this work the role of FAN during TNF signalling was analysed. The data presented here show that FAN is involved in TNF-induced filopodia formation which is dependent on the activation of Cdc42. The N-terminal fold of FAN was identified in this study as a PH domain which specifically binds to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P) and 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 BEACH domain, suggesting a functional contribution of the BEACH domain. In order to mediate TNF-induced actin reorganisation, FAN was shown to interact with the actin cytoskeleton in TNF-stimulated cells by binding directly to F-actin, thus providing a scaffold linking TNF signalling to actin reorganisation. FAN deficiency affected TNF-induced fibroblast motility and *in vivo* migration of Langerhans cells demonstrating the impact of FAN-mediated actin reorganisation for TNF-stimulated motility. The results of this study identify FAN as a central mediator of TNF-induced actin reorganisation and show that PH-mediated plasma membrane targeting of FAN is critically involved in linking TNF signalling to actin cytoskeleton reorganisation.

Zusammenfassung

Das WD Protein FAN gehört zur Familie der TNF-Rezeptor Adapterproteine, die an spezifische Signaltransduktionskaskaden gekoppelt sind. Im Gegensatz zu den anderen bekannten Adapterproteinen, die an die Death Domain des TNF-Rezeptors I binden, interagiert FAN mit der membranproximalen NSD Domäne. Obwohl anfangs schon gezeigt wurde, dass FAN an der durch TNF stimulierten Aktivierung der nSMase beteiligt ist, ist die genaue Funktion von FAN bei den spezifischen zellulären Effekten von TNF nicht bekannt. In dieser Arbeit wurde die Rolle von FAN im TNF Signalweg untersucht. Die hier vorgestellten Daten zeigen, dass FAN an der TNF-induzierten Bildung von Filopodien über die Aktivierung von Cdc42 beteiligt ist. Der N-terminale Bereich von FAN wurde in dieser Arbeit als eine PH Domäne identifiziert, die spezifisch an Phosphatidyl-Inositol-4,5-Phosphat (PtdIns(4,5)P) bindet und FAN an die Plasmamembran rekrutiert. Die gezielte Mutagenese der FAN Primärstruktur zeigte, dass die Funktion von FAN bei der TNF-induzierten Bildung von Filopodien von der PtdIns(4,5)P-Bindung oder der intramolekularen Interaktion zwischen der PH und der BEACH Domäne abhängig ist, was eine funktionelle Beteiligung der BEACH Domäne vermuten lässt. Es wurde außerdem gezeigt, dass FAN über direkte Bindung an F-Aktin mit dem Aktinzytoskelett interagiert, um TNF-induzierte Aktinreorganisation zu modulieren. So kann eine Plattform gebildet werden, die TNF-Signale mit der Reorganisation des Aktinzytoskeletts verbindet. Das Fehlen von FAN beeinträchtigte die TNF-induzierte Motilität von Fibroblasten und auch die Migration von Langerhanszellen *in vivo*, was die Bedeutung der durch FAN vermittelten Reorganisation des Aktinzytoskeletts für die TNF-induzierte Zellmotilität unterstreicht. Die Ergebnisse dieser Arbeit identifizieren FAN as zentralen Faktor bei der TNF-induzierten Aktinreorganisation und zeigen, dass die PH-vermittelte Rekrutierung von FAN an die Plasmamembran für die Verknüpfung der TNF-Signalkaskade mit der Reorganisation des Aktinzytoskeletts notwendig ist.

Abbreviations

Only abbreviations are listed that have not been described in the text

aa	amino acids
bp	basepares
BSA	bovine serum albumin
C-terminal	carboxyterminal
DMSO	dimethylsulfoxide
E.coli	*Escherichia coli*
DTT	Dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FBS	fetal bovine serum
GFP	green fluorescent protein
FITC	fluorescein-5-isothiocyanat
GDP	guanosine bisphosphate
GST	glutathione *S*-transferase
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
HRP	horse radish peroxidase
IF	immunofluorescence
IB	immunoblot
IL-1	interleukin-1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilo base
kDa	kilo dalton
LPS	lipopolysaccheride
min	minute
N-terminal	aminoterminal
ORF	open reading frame
MEFs	mouse embryonic fibroblasts
MHC	major histocompability complex
NA	numerical aperture
NaOVa	Sodium orthovanadate
ON	over night
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethanesulphonyl fluoride
rpm	rotations per minute
RT	room temperature
SDS	sodiumdodecylsulfat
SDS-PAGE	SDS polyacrylamid gel electrophoresis
sek	second
TEMED	N,N,N',N'-tetramethyl diamine
Tris/HCl	tris[hydroxymethyl]aminoethane
TRITC	tetramethylrhodamine isothiocyanat

Introduction

TNF signalling – a double-edged sword

TNF (tumour necrosis factor) is a pleiotropic and broadly active cytokine exerting its effects on many cell types inside and outside of the immune system. Originally identified as a factor inducing necrosis in tumours, TNF has now been recognised as a major proinflammatory mediator involved a wide range of biological functions including host defence against intracellular pathogens, wound healing and tumour defence (Locksley et al., 2001; Wajant et al., 2003). However, several pathologic effects like septic shock, development of autoimmune diseases and rheumatoid arthritis are also associated with TNF, making research on TNF signalling important for therapeutic approaches (Choo-Kang et al., 2005). TNF is mainly produced by macrophages, but also by a variety of other tissues including lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue. TNF is primarily produced as a transmembrane protein that is released via proteolytic cleavage by the metalloprotease TACE (TNF alpha converting enzyme). TNF exerts an extreme spectrum of biological responses, and most cells show at least

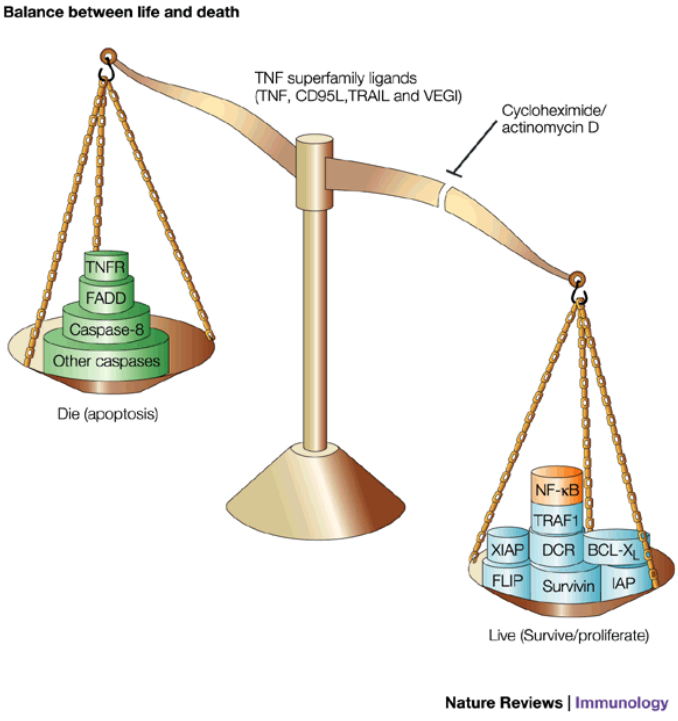

Fig. 1. Balance between life and death mediated by TNF-superfamily members. The composition and activation status of TNF receptor adapter proteins and additional downstream factors determines the outcome of TNF superfamily signalling. From (Aggarwal, 2003)

some TNF responsiveness. A general aspect of signal transduction of all TNF superfamily members is the delicate balance between proliferative signalling and the induction of apoptosis, which is modulated by the unique composition of adapter proteins (Fig. 1).

Two distinct cell surface receptors have been identified for TNF, TNF-RI (TNF-receptor I) and TNF-RII (TNF-receptor II). TNF-RII expression is limited to cells of the immune system and tightly regulated. In contrast, TNF-RI can be found on many different cell types, albeit with great variations in receptor numbers on the cell surfaces (Pfeffer et al., 1993; Vandenabeele et al., 1995). TNF-RI plays a crucial role in the majority of biological effects promoted by TNF (Neumann et al., 1996; Wiegmann et al., 1992), and signalling mechanisms of TNF-RI have been studied most extensively.

Signalling is triggered by binding of TNF to the receptors at the cell surface. A common feature of the receptors of the TNF superfamily is their lack of enzymatic activity. Signal transduction cascades are initiated by recruiting adapter proteins to the intracellular domains which translate the signalling events to specific cellular responses. Despite the initial thought that binding of TNF induces receptor trimerisation as a key activation event during TNF signalling, more recent work has shown TNF-receptors and other members of the TNF superfamily to rather form preassembled complexes. These complexes undergo further conformational change upon ligand binding leading to initiation of the downstream signalling events (Chan et al., 2000).

Signal transduction pathways controlled by TNF-RI – a question of adapters

By binding to TNF-RI, TNF induces diverse cellular effects ranging from inflammatory responses and proliferative signals to apoptosis by modulation of various cellular signal transduction cascades. How these divergent responses mediated by one single type of receptor are discriminated is not fully understood yet. The effect of TNF stimulation on intracellular signalling pathways depends on the composition of distinct adapter proteins forming the TNF-RI signalling complex (Fig. 2). Recruitment of TRADD (TNF receptor-associated death domain protein) to the death domain of TNF-RI provides a scaffold for binding of TRAF2 (TNF receptor-associated factor 2) and RIP (receptor-interacting kinase) leading to activation of the NF-κB (nuclear factor

kappa B) pathway. In an uninduced state, NF-κB dimers are complexed with I-κB (inhibitor of NF-κB) proteins that mask their nuclear localisation sequence, thereby retaining NF-κB proteins in the cytoplasm.

For activation and nuclear translocation of NF-κB, TNF stimulates proteosomal degradation of I-κB by recruiting and activating the IKK (I-κB kinase) complex (Perkins, 2000). Activated IKK complex is able to phosphorylate the regulatory domain of I-κB leading to its degradation and subsequent liberation of NF-κB proteins. Activation of NF-κB can lead to activation of a huge number of inflammatory-related genes

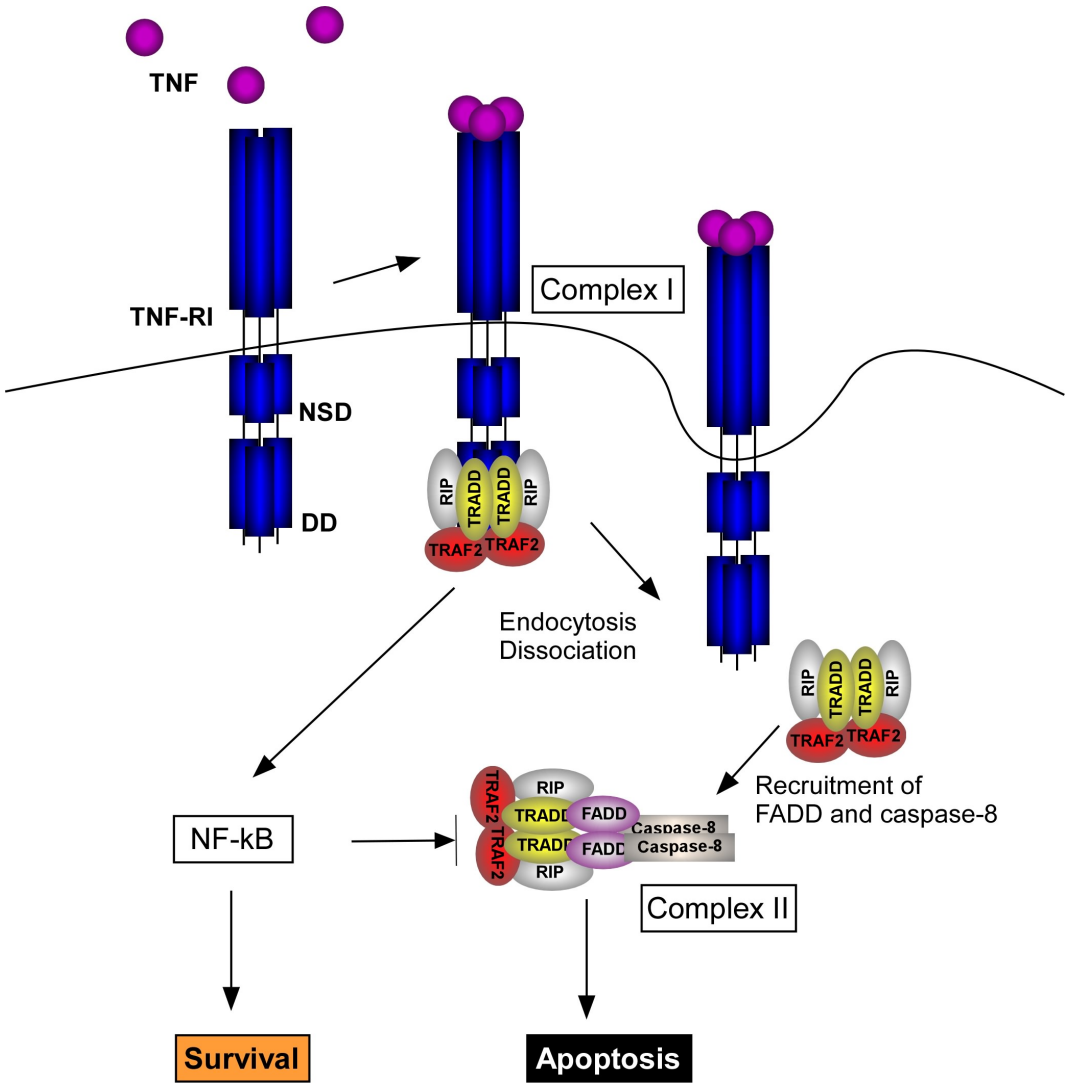

Fig. 2. TNF-RI signals via two complexes. After binding of TNF to TNF-RI, TRADD, RIP and TRAF2 are recruited. The membrane-bound complex I signals for NF-kB activation leading to inflammatory responses and cell survival, whereas complex II, which is formed after dissociation from the receptor and recruitment of FADD and caspase-8, induces apoptosis. Modified from (Micheau and Tschopp, 2003).

and has been implicated in most of the TNF-mediated responses.

Recruitment of TRAF2 to the death domain also induces activation of MAP (mitogen-activated protein) kinases and the distantly related JNK (c-Jun N-terminal kinase) kinases. By interacting with MKK7 (MAP kinase kinase 7), TRAF2 can stimulate phosphorylation of JNK leading to the translocation of phospho-JNK into the nucleus and subsequent activation of the transcription factors c-Jun and ATF2. Although JNK activation has been implicated in TNF-induced apoptosis (Varfolomeev and Ashkenazi, 2004) as well as in upregulation of collagenases and E-selectin, the importance of this pathway for TNF-mediated cellular responses remains unclear (Wajant et al., 2003). Additional recruitment of RIP to TRAF2 leads to activation of the p38 MAPK signalling pathway as an important event in TNF-mediated inflammatory responses resulting in upregulation of IL-1 and IL-6 production.

As member of the death domain containing receptors, TNF-RI is able to trigger the apoptotic machinery in a variety of cell lines. In contrast to other related death receptors like CD95, the apoptosis-inducing capability of TNF-RI is masked by strong NF-κB activation, both pathways being inhibitory to each other. When NF-κB signalling is blocked, TNF-RI can signal apoptosis by recruiting TRADD and FADD (Fas-associated death domain) and caspase-8 to the death domain which leads to downstream initiation of the apoptotic cascade. Recent studies added a further level of complexity to the molecular signal transduction mechanisms of TNF-RI by showing that the association of the adapter protein complex with the receptor also determines the signal outcome (Muppidi et al., 2004). Association of the TNF-RI signalling complex at the cell membrane favours NF-κB activation, but subsequent dissociation of the adapter complex from the receptor probably after endocytosis induces recruitment of the apoptosis-inducing factors leading to apoptosis (Fig. 2).

TNF also modulates activity of sphingomyelinases (SMases), enzymes that hydrolyse the membrane lipid sphingomyelin (SM). Cleavage of SM produces ceramide, a neutral lipid implicated in diverse signalling pathways. Ceramide has been shown to be involved in stress responses, apoptosis and inflammation, but the underlying mechanisms remain elusive (Andrieu-Abadie and Levade, 2002; Kolesnick and Krönke, 1998). TNF-induced ceramide generation can contribute to activation of caspases and also lysosomal proteases like cathepsins (Heinrich et al., 1999). Both sphingomyelin and ceramide are important for membrane dynamics and lipid raft generation. Many

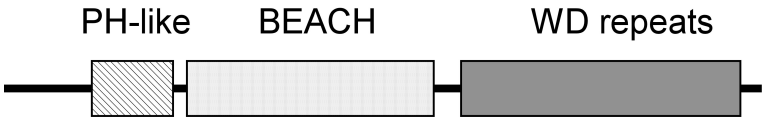

Fig. 3. Domain structure of FAN. Besides the seven C-terminal WD repeats that have been shown to mediate interaction with TNF-RI, FAN contains a BEACH domain with unknown function. Structural analysis identified a N-terminal PH-like fold which is associated with the BEACH domain. Numbers indicate amino acid positions.

signalling proteins have been shown to either reside or be translocated into lipid rafts during signal transduction, and modulation of lipid rafts plays a critical role regulating CD95 capping and TNF-RI signalling (Cremesti et al., 2002).

TNF-RI induces activation of acidic and neutral SMases (aSMase and nSMase) via its two distinct cytoplasmic domains. ASMase activation is mediated by the death domain of TNF-RI, and is implicated as cell-type specific modulator of TNF-induced cell death (Adam-Klages et al., 1998). One aSMase, SMPD1, is localised to lysosomes and plasma membrane, and its genetic deficiency is responsible for development of the Niemann-Pick disease (Ferlinz et al., 1991; Schuchman et al., 1991). Activation of nSMase by TNF is transduced by the nSMase activation domain (NSD) of TNF-RI (Adam et al., 1996) and has been implicated in TNF-mediated inflammatory responses and induction of apoptosis (Andrieu-Abadie and Levade, 2002; Cremesti et al., 2002). Recently, one nSMase, nSMase3 or SMPD4, has been cloned that is responsive to TNF (Krut et al., 2006). However, the role of nSMase3 during TNF signalling still remains largely undefined.

The adapter protein FAN

The majority of the known effects of TNF are initiated by binding of different adapter proteins to the cytoplasmic death domain of TNF-RI (Chen and Goeddel, 2002; Wajant et al., 2003). However, TNF-RI binds additional factors that may be important for TNF signalling (Adam-Klages et al., 1998; Boldin et al., 1995). The more membrane-proximal NSD of TNF-RI has previously been recognised as a distinct functional domain for the activation of nSMase (Adam et al., 1996).

The only protein identified so far that binds to the NSD is the adapter protein FAN (factor associated with nSMase activity) (Adam-Klages et al., 1996). Overexpression of full-length FAN enhanced nSMase activity in TNF-treated cells, while truncated mu-

tants of FAN produced dominant-negative effects. 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 which are necessary for 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 (Fig. 3). Structural analysis of neurobeachin (Nbea), a protein 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).

PH domains have been first described as internally repeated motifs of the haematopoietic protein pleckstrin (Tyers et al., 1988). Despite low sequence homology PH domains share a common protein structure which is composed of a seven-stranded β-sandwich terminated with a C-terminal α-Helix. (Lemmon and Ferguson, 2000). Many proteins that have been identified to contain PH domains are involved in regulatory and signal transduction pathways. A prominent feature of many PH-domains is their ability to interact with phospholipids at cellular membranes which is mediated by conserved basic residues inside the β-sheets of the PH domain. This enables for signal-dependent targeting of proteins to different membrane compartments inside the cell (Cozier et al., 2004). In addition, binding of the PH domain to membrane lipids can induce a conformational change influencing the activation of the host protein (Cozier et al., 2004; Welch et al., 2002). The role of the putative PH-domain of FAN has not been investigated yet.

Functional implications of FAN action

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 (Fig. 4) (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, studies with FAN-deficient mouse embryonic fibroblasts (MEFs) have implicated FAN in TNF and CD40 mediated apoptosis (Segui et al., 1999; Segui et al., 2001) but failed to shed light on the molecular mechanisms of FAN action. Similarly, use of truncated FAN or FAN-deficient MEFs has suggested the involvement of FAN in several different processes like IL-6 secretion (Malagarie-Cazenave et al., 2004), regulation of cardiac cell death (O'Brien et al., 2003), lysosomal permeabilisation (Werneburg et al., 2004) and lysosomal size (Möhlig et al., 2007). However, mechanistic insights into FAN function in TNF-RI signalling are still missing.

Previously, the involvement of the membrane proximal region of TNF-RI in TNF-induced actin polymerisation has been suggested (Peppelenbosch et al., 1999). As this

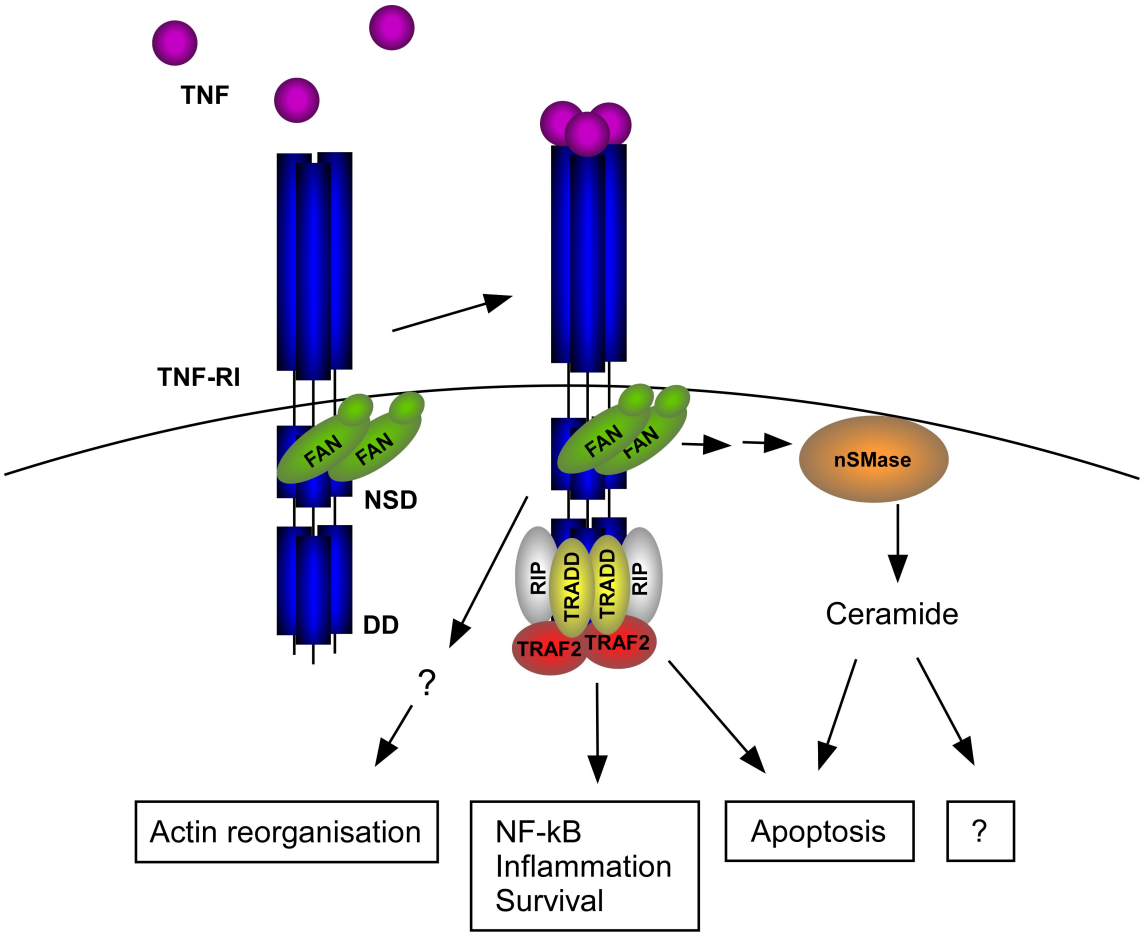

Fig. 4. Role of FAN in TNF-RI signalling. FAN binds to the plasma membrane with its N-terminal PH domain and is associated with the NSD of TNF-RI. TNF stimulation leads to activation of nSMase via FAN action, leading to the generation of ceramide which has been implicated in modulating TNF-induced apoptosis. Whereas apoptotic and inflammatory signalling are dependent on adapter proteins like TRADD, TRAF2 and RIP binding to the DD, the pathways how TNF regulates actin reorganisation remain elusive.

membrane proximal region included the NSD, the FAN binding site, we hypothesised that FAN is involved in TNF-mediated reorganisation of the actin cytoskeleton.

Actin cytoskeleton reorganisation – dynamically shaping the cell

The cytoskeleton which comprises a network of three types of protein filaments (actin, microtubules and intermediate filaments) is constantly remodelled as the cell communicates with its environment. Networks of actin filaments localised prominently at the cell periphery are especially sensitive to signals at the membrane-cytoplasm interface, and reorganisation of the actin cytoskeleton is crucial for many cellular processes including cytokinesis, endocytosis, vesicle trafficking, cell morphology, polarisation and motility. Remodelling of the actin cytoskeleton is driven by continuous polymerisation and depolymerisation of actin filaments and is controlled by many different actin-binding proteins (ABPs) that initiate or terminate polymerisation, sequester monomeric actin and link filaments to each other or to the membrane (Winder and Ayscough, 2005). Intracellular and extracellular signals which are transduced by receptors and downstream signalling cascades influence reorganisation of the actin cytoskeleton leading to complete cellular responses like cell migration (Pollard and Borisy, 2003).

Migration of cells is a multistep process, which starts with protrusion of the leading edge and formation of stable attachments near the leading edge. These focal complexes which mature into focal adhesions then serve as traction points to generate the propulsive force needed for subsequent retraction of the cell rear and forward movement of the cell (Fig. 5) (Webb et al., 2002). Protrusion of the leading edge is driving by actin polymerisation resulting in the formation of lamellipodia and filopodia. Lamellipodia are broad flat structures composed of a branched network of actin filaments and are necessary to generate the traction force at the leading edge (Small et al., 2002). In contrast, filopodia contain long, unbranched, bundled actin filaments forming thin finger-like protrusions that dynamically extend and retract from the cell body. Filopodia function in sensing environmental cues to guide directed migration of cells or axons (Wood and Martin, 2002).

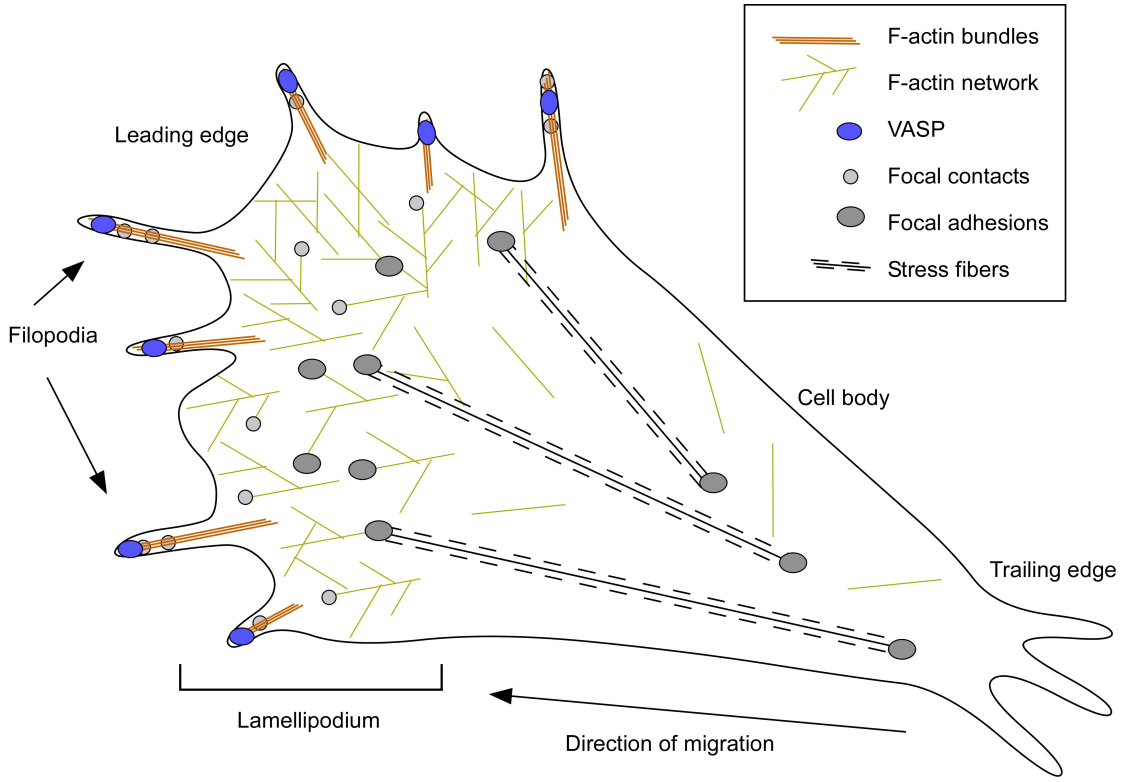

Fig. 5. Model of a migrating fibroblast. During cellular migration a network of branched actin filaments are formed at the leading edge leading to broad protrusion of the membrane forming a lamellipodium. Filopodia are formed containing long actin bundles with VASP and additional proteins located at their tips. Focal contacts and focal adhesions provide the adhesion at filopodia and lamellipodia necessary for retraction of the cell body. Stress fibers contain actin filaments and myosin II providing the contractile tension for cell body retraction. Modified from (Nicholson-Dykstra et al., 2005)

Central regulators of actin reorganisation are the small GTPases of the Rho family. By cycling between an active GTP-bound and an inactive GDP-bound state, GTPases act as molecular switches to control complex cellular processes. Action of a small number of Rho GTPase family proteins is regulated by an increasing number of activators (guanine nucleotide exchange factors, GEFs) and inactivators (GTPase-activating protein, GAPs) which convey spatial and temporal specificity (Etienne-Manneville and Hall, 2002; Hall, 1998). Of the more than 20 members of the Rho family of small GTPases Cdc42, Rac and Rho have been initially identified to regulate actin reorganisation and are best characterised (Nobes and Hall, 1995). Studies in Swiss 3T3 fibroblasts, which show very low background actin polymerisation in a serum-starved state and are thus suitable for actin studies showed that each GTPase mediates actin polymerisation into a specific structure. Injection of Cdc42 induces formation of filopodia, Rac lamelli-

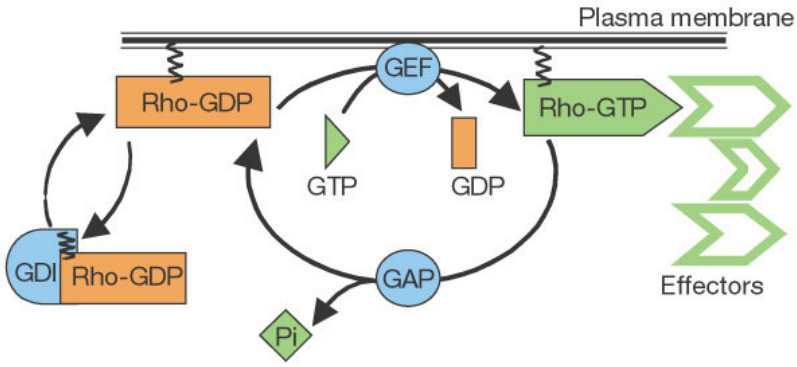

Fig. 6. The Rho GTPase cycle. The GTPases cycle between an inactive GDP-bound and an active GTP-bound conformation. In the active state, they interact with downstream effectors. Rho GTPase activity is tightly regulated by a large number of proteins which fall into three classes. GEFs (guanine nucleotide exchange factors) catalyse exchange of GDP for GTP thus promoting activitation of the GTPases. GAPs (GTPase-activating protein) enhance GTP hydrolysis leading to inactivation of the GTPases, and GDIs (ganine nucleotide exchange inhibitors) inhibit GTPase activation by blocking membrane association. From (Etienne-Manneville and Hall, 2002).

podia and Rho stress fibers, long contractile bundles of actin filaments and myosin. However, since then more GTPases and several isoforms (Rac 1, 2, 3 and Rho A, B, C) have been identified with overlapping roles in the formation of the different actin structures (Aspenström et al., 2004). For example, formation of filopodia can be regulated independently of Cdc42 by the recently identified Rho GTPase Rif (Pellegrin and Mellor, 2005).

The formation of filopodia is crucially dependent on the presence of Ena/VASP proteins. The Ena/VASP family includes three mammalian members, Mena (Mammalian enabled), VASP (Vasodilator-stimulated phosphoprotein) and EVL (Ena-VASP-like), which bind polymerised actin (F-actin) and are localised to places of growing actin filaments like the distal tips of lamellipodia and filopodia. Initially thought to promote actin-driven motility, the role of the Ena/VASP family proteins remains controversial. On a molecular level, however, the picture emerges that Ena/VASP proteins, by associating with actin filaments, promote filament elongation by anti-capping and bundling activity, thus favouring formation of long, unbranched filaments like those needed for filopodia formation (Krause et al., 2002; Schirenbeck et al., 2006).

TNF-induced actin reorganisation – shaping the immune system

In contrast to the well-studied role of TNF as key player in innate immunity, little is known about the actin-modulatory capacities of TNF. However, complete immune responses include processes like wound healing and chemotaxis which require reorganisation of the actin cytoskeleton to influence cell shape, cell polarisation and motility. TNF has been shown to modulate motility and chemotaxis by reorganisation of the actin cytoskeleton in different cell types ranging from keratinocytes, epithelial cells, endothelial cells and fibroblasts to immune cells like neutrophils and dendritic cells (Banno et al., 2004; Cumberbatch et al., 1997; Kutsuna et al., 2004; Lokuta and Huttenlocher, 2005; Postlethwaite and Seyer, 1990; Wojciak-Stothard et al., 1998). It remains largely unclear, however, how TNF influences actin reorganisation on a molecular level. Also, the participation of actin reorganisation itself for signal transduction events during TNF receptor signalling as has been shown for apoptosis induction by CD95 remains unclear (Fais et al., 2005).

Cytokines like TNF and IL-1 have been shown to induce the formation of filopodia in fibroblasts (Puls et al., 1999). Filopodia formation by TNF seems to be mediated by Cdc42, but the signalling events leading to Cdc42 activation are unknown. Activation of Cdc42 appears to be independent of classical TNF-activated signalling pathways like JNK and NF-κB. Also, the adapter proteins TRADD, RIP and TRAF2, which bind to the death domain of TNF-RI, have been excluded to participate in TNF-mediated actin reorganisation (Puls et al., 1999). Rather, the involvement of the membrane proximal region of TNF-RI including the FAN binding site in TNF-induced actin polymerisation has been suggested (Peppelenbosch et al., 1999), thus making FAN an interesting candidate to signal TNF-mediated actin reorganisation.

Aim of the study

The function and molecular mode of action of the TNF-RI-interacting adapter protein FAN still remain elusive. As TNF is involved in a variety of central immune responses and resulting diseases, further elucidation of the signalling mechanisms underlying TNF action are of great interest. Reorganisation of the actin cytoskeleton is central to many processes involved in cellular immune responses, but little is known about modulation by cytokines like TNF. Despite previous studies suggesting involvement of

FAN in a variety of cellular processes, details of FAN function in TNF-RI signalling are missing. This work aimed to unravel the molecular mechanisms and function of FAN during TNF signalling. Using FAN-deficient MEFs, TNF-dependent actin reorganisation and subcellular localisation of FAN and different FAN variants were investigated. Interaction studies with overexpressed or recombinantly expressed FAN were applied in order to decipher the molecular mechanisms of FAN action. In order to gain insight into the role of the different domains of FAN, deletion mutants were examined for their subcellular localisation and actin reorganisation capacity. The results of this study identify FAN as mediator of TNF-induced actin reorganisation important for cell polarisation and motility. By interacting with membrane phospholipids, the TNF-RI and the actin cytoskeleton machinery FAN links TNF-RI signalling to reorganisation of the actin cytoskeleton.

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, Berlin, Germany) and 10% fetal bovine serum. HEK 293FT cells were cultured as described above in DMEM / 10% fetal bovine serum supplemented with 2 mM L-glutamine, non-essential amino acids and 10 mM sodium pyruvate (Biochrom). Cells were transfected using ExGENE (Fermentas, St. Leon-Rot, Germany) (MEFs) or the calcium phosphate method (COS-7 and HEK 293FT cells) (Wigler et al., 1978).

Antibodies and Reagents

All chemicals were purchased from Sigma (München, Germany) or Roth (Karlsruhe, Germany) unless indicated otherwise.

Table 1. Antibodies used in western blotting

antibody	isotype	supplier	dilution
GST	rabbit polyclonal	Amersham Biosciences (Freiburg, Germany)	1:3000
JNK / p-JNK	rabbit polyclonal	Cell Signaling Technology (Danvers, USA)	1:1000
TNF-RI	rabbit polyclonal	Santa Cruz Biotechnologies (Heidelberg, Germany)	1:200
Cdc42	rabbit polyclonal	Santa Cruz Biotechnologies	1:200
Rac1	mouse monoclonal	Upstate (Dundee, UK)	1:1000
RhoA	rabbit polyclonal	Santa Cruz Biotechnologies	1:200
p-Paxillin	rabbit polyclonal	Santa Cruz Biotechnologies	1:200
His	mouse monoclonal	Quiagen (Hilden, Germany)	1:1000
Bax	rabbit polyclonal	BD Biosciences (Heidelberg, Germany)	1:2000
VASP	rabbit polyclonal	Axxora (Lörrach, Germany)	1:2000
GFP	mouse monoclonal	BD Biosciences	1:1000

Table 2. Antibodies used in immunofluorescence

antibody	isotype	supplier	dilution
Myc	mouse monoclonal	Invitrogen (Karlsruhe, Germany)	1:200
VASP	rabbit polyclonal	Axxora (Lörrach, Germany)	1:500
Paxillin	mouse monoclonal	BD Biosciences	1:500
I-A^d (MHC-II) (FITC conjugated)	mouse monoclonal	BD Biosciences	1:200
TNF-RI (PE conjugated)	mouse monoclonal	R&D Systems (Wiesbaden, Germany)	1:50

DNA constructs

For construction of FAN-GFP-fusion proteins, open reading frames (ORF) encoding human FAN and different variants of FAN were amplified by PCR and cloned into pEGFP-C3 vector (Invitrogen, Karlsruhe, Germany). For production of recombinant GST-tagged FAN full length protein, the ORF of FAN was cloned into the prokaryotic

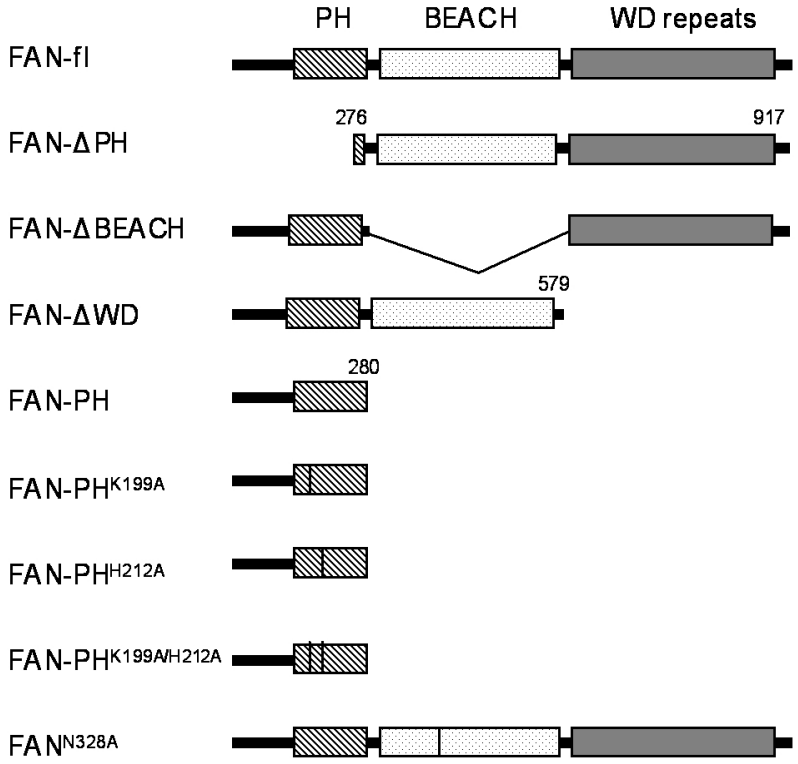

Fig. 7. FAN deletion constructs and mutations used in this study. Numbers indicate aminacid positions.

expression vector pGEX-4T3 (Amersham Corp., Freiburg, Germany). Similarly, the part of the FAN ORF coding for the PH-domain (aa 1-275) of FAN or different mutants were cloned into pET-20b vector (Novagen/Merck Biosciences, Darmstadt, Germany). FAN-GST fusion proteins for eukaryotic expression and GST-pull down experiments were constructed by cloning the ORFs of different FAN-mutants into pRK-GST vector. All constructs were verified by sequence analysis. An overview of the ORFs used in this study is given in Fig. 7.

Table 3. DNA constructs used in this work
pEGFP-C3 (EGFP n-term., eukaryot. expression)

ORF name	amino acids	MW (kDa)	size (bp)	5' primer	3' primer
FAN-fl	1-917	90	2751	FAN-XhoIfl-5'	FAN-HindIII-3'
FAN-ΔPH	275-917	64	1926	FAN-XhoI-dII-5'	FAN-HindIII-3'
FAN-ΔBeach	Δ280-579	62	1854	FAN-dBEACH-5' FAN-XhoIfl-5'	FAN-HindIII-3' FAN-dBEACH-3'
FAN-ΔWD	181-579	40	1197	FAN-XhoIfl-5'	FAN-HindIII-dWD2
FAN-PH	179-279	10	294	FAN-XhoI-dI-5'	FAN-PH-3'
FAN-mut10 (K199A)	179-279	10	294	FAN-XhoI-dII-5' FAN-mut10-5'	FAN-mut10-3' FAN-PH-3'
FAN-mut11 (H212A)	179-279	10	294	FAN-XhoI-dII-5' FAN-mut11-5'	FAN-mut11-3' FAN-PH-3'
FAN-mut12 (K199A/ H212A)	179-279	10	294	FAN-XhoI-dII-5' FAN-mut11-5' (Template FAN-mut11)	FAN-mut11-3' FAN-PH-3'
FAN-mut13 (K199A/H212A)	1-917	90	2751	FAN-XhoIfl-5' FAN-mut13-5'	FAN-mut13-3' FAN-HindIII-3'
FAN-mut4 (N328A)	1-917	90	2751	From (Jogl et al., 2002)	

pRK-GST (GST n-term, eukaryot. expression)

ORF name	amino acids	MW (kDa)	size (bp)	5' primer	3' primer
FAN-fl	1-917	90	2751	FAN-XbaI-fl-5'	FAN-HindII-3'
FAN- ΔPH	275-917	64	1926	FAN-XbaI-dII-5'	FAN-HindIII-ph-3'
FAN- ΔBeach	Δ280-579	62	1854	FAN-XbaI-fl-5' (Template FAN-ΔBeach-GFP)	FAN-HindII-3'
FAN- ΔWD	1-579	58	1737	FAN-XbaI-fl-5'	FAN-HindIII-dWD2

pET-20b (His-tag c-term., prokaryot. expression)

ORF name	amino acids	MW (kDa)	size (bp)	5' primer	3' primer
FAN-PH	179-279	10	294	FAN-HindIII-dII-5'	FAN-PH-XhoI-3'
FAN-mut10 (K199A)	179-279	10	294	FAN-HindIII-dII-5' (template FAN-mut10-GFP)	FAN-PH-XhoI-3'
FAN-mut11 (H212A)	179-279	10	294	FAN-HindIII-dII-5' (template FAN-mut11-GFP)	FAN-PH-XhoI-3'
FAN-mut12 (K199A/ H212A)	179-279	10	294	FAN-HindIII-dII-5' (template FAN-mut12-GFP)	FAN-PH-XhoI-3'

pGEX-4T3 (GST-tag n-term., prokaryot. expression)

ORF name	amino acids	MW (kDa)	size (bp)	5' primer	3' primer
FAN-fl	1-917	90	2751	FANfl-XhoI-5'	FAN-Not1-3'

Table 4. List of primers used to generate DNA-constructs used in this work

Primer name	Sequence (5'-3')
FAN-dBEACH-3'	CAAACTTTTTAGCTATGTGGCAATGTA
FAN-dBEACH-5'	ACATACCTAAAAAGTTTGTCCCAGACC
FAN-HindIII-3'	CCCCCCCCCAAGCTTTTAATACTGCAATTTCCA-GAATAT
FAN-HindIII-dWD2	CCCCCCAAGCTTTTAAAACTTTGGGGTGATCCTTCG
FAN-HindIII-ph-3'	CCCCCCCCCAAGCTTTTAATCTCTATCTTGAGGTT-CATAG
FAN-mut10-3'	CACCATTTCTGCTGCGCATTCCAT
FAN-mut10-5'	ATGGAATGCGCAGCAGAAATGGTG
FAN-mut11-3'	CGTGATGCACACTGCTCCAGGATT
FAN-mut11-5'	AATCCTGGAGCAGTGTGCATCACG
FAN-mut13-3'	GGTAGATGTCGGAACACAGATC
FAN-mut13-5'	GATCTGTGTTCCGACATCTACC
FAN-Not1-3'	CCCCCCCCCGCGGCCGCTTAATACTGCAATTTCCA-GAAT
FAN-PH-3'	CCCCCCCCCAAGCTTGATCTCTATCTTGAGGTTCATAG
FAN-PH-XhoI-3'	CCCCCCCCCCTCGAGATCTCTATCTTGAGGTTCATAG
FAN-XbaI-dII-5'	CCCCCCCCCTCTAGAATGCAAGATAGAGATGATCTC-TAT
FAN-XbaI-fl-5'	CCCCCCCCCTCTAGAATGGCGTTTATCCGGAAGAAG
FAN-XhoI-dI-5'	CCCCCCCCCCTCGAGATGAGAACAT-CATTTGACAAAAACAGG
FAN-XhoI-dII-5'	CCCCCCCCCCTCGAGATGCAAGATAGAGATGATCTC-TAT
FAN-XhoI-fl-5'	CCCCCCCCCCTCGAGATGGCGTTTATCCGGAAGAAG

Western blotting

Samples were heated in SDS sample buffer for 5 minutes at 100°C and centrifuged 2 min at 20,000 x g. Polyacrylamid gels (10 – 12 %) were started at 120 mV and continued at 180 mV in SDS running buffer. After blotting gels on nitrocellulose membranes (Protran, Schleicher & Schuell) for 90 min in blot transfer buffer, membranes were blocked for 30 min in blocking buffer and incubated with primary antibody for one hour. After washing and incubation with secondary antibody for one hour, signals were detected on film (Amersham Inc.) using enhanced chemiluminescence (ECL reagent, Pierce). If appropriate, signals were quantified using AlphaEasy FC software (Alpha Innotech, San Leandro, USA).

Table 4. Buffers used for western blot

SDS sample buffer (5x)	25% glycerol 0.6 M Tris-HCl 144 mM SDS 0.1% brome phenol blue
Blot transfer buffer	25 mM Tris-HCl 190 mM glycine 20% methanol
S-PBS	120 mM NaCl 10 mM NaH_2PO_4 30 mM K_2HPO_4 pH 7,6
Blocking buffer	10 mM Tris-HCl 150 mM NaCl 5% milk powder 2% BSA 0,1% Tween-20 pH 7,4-7,6
SDS running buffer (1000 ml)	190 mM glycine 20 mM Tris-Base 0,1% SDS

Immunofluorescence

Cells grown on coverslips were stimulated with TNF (100 ng/ml) for 10 minutes 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 hr without permeabilisation and washed twice with PBS. For intracellular staining, cells were permeabilised with 0.1% saponin during blocking, and incubated with the appropriate primary and secondary antibodies in PBS/0.1% saponin for 1 hr each. For F-actin staining, cells were incubated with AlexaFluor568- or AlexaFluor488-conjugated phalloidin in PBS/0.1% saponin for 30 min. Cells were mounted on glass slides and examined using an Olympus IX81 fluorescence microscope (Objective: 60x PLAPO oil, NA 1.4) 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).

Paxillin and JNK phosphorylation

After stimulation with TNF, MEFs were washed twice in PBS, scraped off the plate and lysed in TNE buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, 1% NP40, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, pH 7.4) for 30 min on ice. After clearing by centrifugation, equal amounts of lysate were analyzed by SDS-PAGE and western blotting using phospho-specific antibodies against phosphorylated paxillin or phosphorylated JNK.

GTPase activation assay

To assess activated GTPases in cell lysates, an assay was used which exploits the specific binding of the CRIB domain from human PAK1 to GTP-bound Cdc42 and Rac1 (Malliri et al., 2002; Sander et al., 1998). Cells were treated with TNF (100 ng/ml), PDGF (20 ng/ml), bradykinin (400 ng/ml) or left untreated, and were lysed by scraping cells from the plate in lysis buffer (25 mM HEPES, 1% NP40, 10 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 5 mM NaF, 1 mM NaOVa, 1 mM PMSF, 1 µg/ml aprotinin, pH 7.5). Lysates were cleared shortly by centrifugation at 20,000 x g for 5 min. All steps were done on ice due to the instability of the GTP bound to the GTPases. Equal amounts of lysate were incubated for 30 min with 20 µg GST-CRIB fusion protein, which was recombinantly expressed in *E.coli* (O/N at 30°C) and coupled to glutathione-sepharose beads (Amersham Corp., Freiburg, Germany). After precipitation by centrifugation, beads were washed four times with wash buffer (25 mM HEPES, 1% NP40, 30 mM $MgCl_2$, 40 mM NaCl, 1 mM DTT, pH 7.5), eluted in SDS-sample buffer and subjected to SDS-PAGE and western blot. GTP-bound and total Cdc42 or Rac1 levels were then detected using specific antibodies. For RhoA activation, the same principle was applied using the RhoA-binding domain of Rhotekin (Ren et al., 1999) and RhoA-specific antibody for immunoblot analysis.

Protein expression and purification

For expression of the FAN-PH domain fused to a His tag, the part of FAN containing the PH domain (residues 1-275) was cloned into the pET20b vector (Novagen/Merck Biosciences, Darmstadt, Germany) and expressed in *E.coli BL21* at 37°C for 3h with 0.1 mM IPTG. After resuspension of bacterial pellet in lysis buffer (20 mM Tris,

500 mM NaCl, 10 mM imidazole, 1% Triton X100, pH 7.5) and subsequent lysis using a combination of lysozyme treatment (1 µg/ml for 30 min) and sonication (3 x 30 sec), lysates were cleared by centrifugation at 20,000 x g for 30 min. Soluble protein was bound to nickel-agarose affinity beads (Novagen/Merck Biosciences, Darmstadt, Germany) and after thorough washing 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 GST-FAN expression, the ORF of FAN was cloned into the pGEX-4T3 vector (Amersham Biosciences (Freiburg, Germany), and protein expression was induced in *E.coli BL21* with 0.1 mM IPTG at 30°C for 5 h. Cells were resuspended in lysis buffer (50 mM Tris, 270 mM sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM β-ME, 1% Triton X100), lysed as described above, and cleared lysates were incubated with glutathione-sepharose beads. After washing in lysis buffer containing 500 mM NaCl, bound protein was eluted in a buffer containing 50 mM Tris (pH 7.5), 270 mM sucrose, 10 mM β-ME, 0.1 mM EGTA and 20 mM glutathione.

Protein-lipid overlay assay

The protein-lipid overlay assay was performed as described previously (Dowler et al., 2000). A nitrocellulose membrane spotted with 1 µl of lipid solution containing 0.5 nmol of phospholipids was blocked for 1 hr 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.

GST pull down assay

$3x10^6$ HEK 293FT cells were transiently transfected with the DNA constructs coding for the indicated GST fusion proteins. After 24 hrs 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-X100, 1 mM DTT and 1mM PMSF at pH 7.5 for 30 min on ice, and centrifuged for 30 min at 20,000 x g. Supernatants were incubated with 25 µl glutathione-sepharose 4B beads (Amersham Corp., Freiburg, Germany) for 1 hr at 4°C.

Beads were washed extensively, resuspended in SDS sample buffer and analysed by SDS-PAGE and immunoblotting.

Immunoprecipitation

$3x10^6$ HEK 293FT cells were transiently transfected with the DNA constructs coding for the indicated fusion proteins. After 24 hrs cells were stimulated with TNF (100 ng/ml) for 10 min or left untreated, lysed in TNE lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, 1% NP40, protease inhibitor cocktail (Roche, Darmstadt, Germany), pH 7.4) for 30 min on ice, and centrifuged for 30 min at 20,000 x g. 600 µg of supernatants were incubated with the indicated primary antibodies (2 µg (VASP, actin), 1 µg (Bax)) for 2 hrs at 4°C. After addition of 25 µl of GammaBind Sepharose Beads (Amersham Corp., Freiburg, Germany) and incubation for 1 hr at 4°C, beads were extensively washed, resuspended in SDS sample buffer and analysed by SDS-PAGE and immunoblotting.

Cell polarity determination

Cell polarity was examined by analysing reorientation of the Golgi apparatus in a wound-scratch test (Kupfer et al., 1982; Nobes, 2000). MEFs were grown on 6-well plates to confluent monolayers. After inducing a scratch using a white pipette tip, MEFs were immediately incubated with TNF (100 ng/ml) and fixed after incubation for the indicated times using 3% paraformaldehyde for 20 min. The Golgi apparatus was stained using anti-Rab6 antibody, and nuclei were counterstained using Hoechst 33258. The percentage of cells with their Golgi orientated towards the wound was evaluated under a fluorescent microscope. Numbers represent means of triplicates of three independent experiments each with > 100 cells counted.

F-actin cosedimentation assay

The F-actin cosedimentation assay was performed using the Actin Binding Protein Biochem Kit (Cytoskeleton, Inc., Denver, USA) according to the manufactures instructions. Non-muscle actin was polymerised for 1 hr at room temperature in actin polymerisation buffer. Polymerised actin (23 µM) 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 x g for 90 min. FAN-GST and GST protein was detected in supernatants and pellets using anti-GST antibody after immunoblotting.

Migration of Langerhans cells out of mouse ear epidermal sheets

Emmigration of Langerhans cells (LCs) from the mouse ear epidermis after injection of TNF was measured indirectly by staining LCs in the ear epidermis and measuring their density (Cumberbatch et al., 1994). Male and female BALB/c mice (n=10 (wt), n=9 ($FAN^{-/-}$ MEFs)) were anesthetised, and approximately 25 µl (50 ng) TNF in PBS/0.1% BSA or PBS/0.1% BSA as negative control was injected intradermally into the ear. After 2 hrs ears were harvested and split into dorsal and ventral halves. The dorsal halves were incubated with 0.2 mM EDTA in PBS at 37°C for 1.5 hr to separate the epidermis from dermis. After preparation of the epidermis using forceps, epidermal sheets were fixed in cold acetone at -20°C for 20 min. LCs in epidermal sheets were stained for 30 min using FITC-conjugated anti-MHC-II antibody (anti-I-A^d) diluted 1:200 in PBS/0.1% BSA. Stained epidermis was mounted on glass slides in PBS and analysed using an Olympus IX81 confocal microscope (UPLSAPO 60 x water objective, NA 1.2). Due to the relative thickness of the epidermal sheets, z-stacks of 12 images were generated and images are displayed as total intensity projections of all 12 images of the z-stack. For quantification, LCs were counted in epidermal sheets in triplicates for each sample (n=10 (wt), n=9 ($FAN^{-/-}$)). LC density was calculated as number of LCs/mm2 and expressed as changes in percent of control.

Motility analysis using time-lapse video microscopy

MEFs were seeded on 12-well plates O/N and imaged at 37°C using a Leica inverted microscope DMIRE2 with CO_2 supply and capture software FW4000. Phase contrast pictures were taken every 15 min over 10 hrs, and movies were generated from the pictures using the microscope software. Migration paths of individual cells were tracked in the movies using DIAS Analysis software (Soll Technologies Inc., Iowa, USA). From the migration paths total path length, net path length and overall speed were calculated using DIAS software. For each condition, at least 12 cells were tracked in four independent experiments.

Electrophoretic Mobility Shift Assay (EMSA) (NF-κB activity)

Electrophoretic mobility shift assays were performed by Katja Wiegmann as described previously (Kashkar et al., 2006) using the NF-κB-specific oligonucleotides (Applied Biosystems, Darmstadt, Germany) end-labeled with γ-^{32}P-ATP (Amersham Corp., Freiburg, Germany).

Results

Filopodia formation is impaired in FAN-deficient fibroblasts

TNF has been shown to induce reorganisation of the actin cytoskeleton and the formation of filopodia (Puls et al., 1999). To determine if FAN is involved in TNF-induced actin cytoskeleton reorganisation, serum-starved subconfluent MEFs from wild type (wt) and FAN-deficient ($FAN^{-/-}$) mice (Kreder et al., 1999) were stimulated with TNF for 10 minutes and stained for polymerised (filamentous) actin (F-actin) using

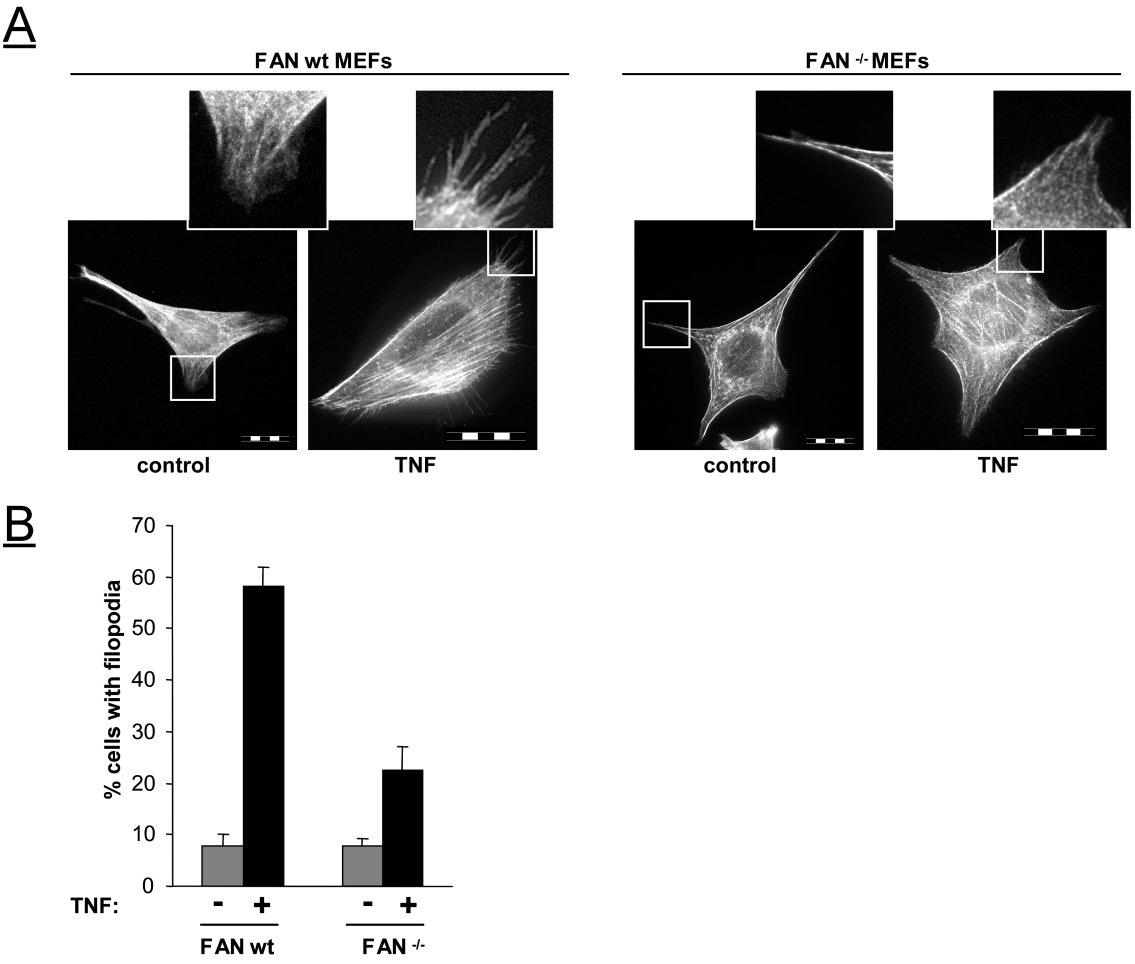

Fig. 8. Impaired filopodia formation in $FAN^{-/-}$ MEFs after TNF stimulation. A, TNF-induced filopodia formation in FAN wildtype (wt) MEFs. FAN wildtype (wt) and $FAN^{-/-}$ MEFs were stimulated with TNF for 10 minutes or left untreated and stained for F-actin using AlexaFluor568-conjugated phalloidin. Insets are enlargements of the boxed area. Bar = 20 µm. B, Cells of (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 ± SD of at least three independent experiments.

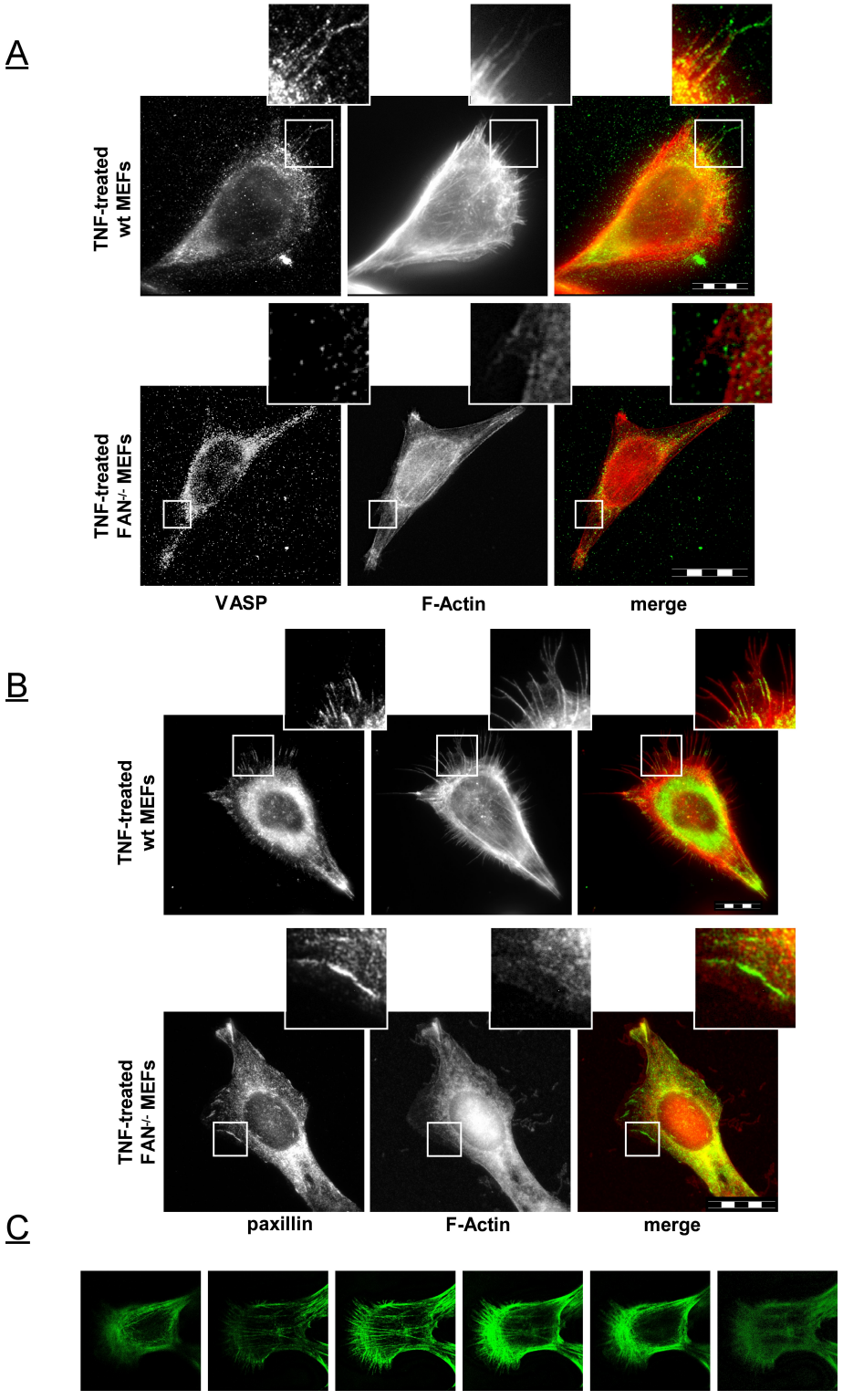

Fig. 9. Filopodial markers identify TNF-induced protrusive structures as filopodia. FAN wt and FAN$^{-/-}$ MEFs were stimulated with TNF for 10 minutes and stained for F-actin (red) and VASP (A) or paxillin (B) using specific antibodies (green). Bar = 20 µm. C, Z-stack of a TNF-treated FAN wt fibroblast. MEFs were treated with TNF for 10 minutes and stained with for F-actin (green) to visualise filopodia. Confocal pictures along the z-axis were taken to exclude that the protrusions are adherent to the substrate and thus rather represent retraction fibers than filopodia.

labelled phalloidin. Both wild type and FAN-deficient cells contain little polymerised actin without TNF stimulation (Fig. 8A). In wild type MEFs, TNF treatment induced the formation of protrusive structures resembling filopodia (Fig. 8A-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 (Fig. 8A-B), suggesting an involvement of FAN in the formation of these protrusive structures.

To confirm the filopodia phenotype of the thin protrusive structures seen after TNF treatment, FAN wt MEFs were stained for the filopodial protein VASP and for paxillin, which localises to focal complexes associated with protrusive structures like filopodia (Fig. 9A-B). Localisation of VASP and paxillin in the observed TNF-induced structures

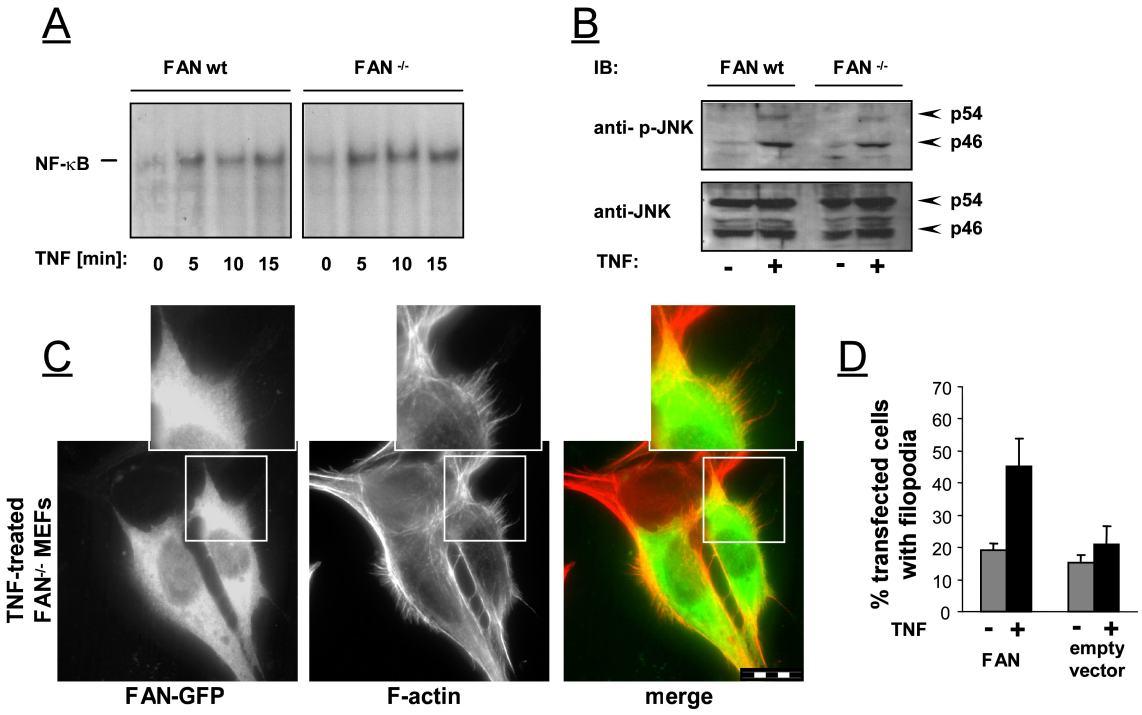

Fig. 10 FAN deficiency specifically affects TNF-induced actin reorganisation.
A, FAN deficiency does not affect TNF-induced NF-κB activation. 5x10^6 FAN wt or FAN$^{-/-}$ MEFs were stimulated with TNF for the indicated times and analysed for NF-κB binding activity. B, FAN deficiency does not affect TNF-induced JNK activation. 10^5 FAN wt or FAN$^{-/-}$ MEFs were stimulated with TNF for 15 minutes, 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 minutes and stained for F-actin (red). 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 ± SD of three independent experiments.

identified them as filopodia (Hall, 1998; Kaverina et al., 2002; Mejillano et al., 2004; Puls et al., 1999). By using confocal microscopy, a z-stack of a TNF-stimulated cell was generated to exclude that the structures represent retraction fibers, actin-containing structures which resemble filopodia but remain adhesive to the substratum after cell body retraction (Fig. 9C) (Mitchison, 1992).

It is important to note that FAN deficiency does not lead to a general inability to respond to TNF. Both wildtype and $FAN^{-/-}$ MEFs show equal activation of classical TNF-inducible targets like NF-κB (Fig. 10A) and JNK (Fig. 10B) in response to TNF stimulation. This is in line with previous observations showing also unaffected MAP kinase signalling in $FAN^{-/-}$ cells (Lüschen et al., 2000).

If FAN is specifically involved in TNF-induced filopodia formation, expression of FAN in $FAN^{-/-}$ MEFs should restore the capability of TNF to induce filopodia. When $FAN^{-/-}$ MEFs were transfected with a GFP-FAN expression plasmid which lead to a transient expression of GFP-FAN, TNF-induced filopodia formation was only observed in GFP-FAN transfected $FAN^{-/-}$ MEFs, which could be identified by GFP fluorescence (Fig. 10C). Quantification of cells bearing filopodia showed that transient expression of FAN in $FAN^{-/-}$ MEFs could restore the capability of TNF to induce filopodia formation almost completely. These findings identify FAN as an important mediator of TNF-induced actin reorganisation and filopodia formation.

FAN signals upstream of Cdc42 in TNF-induced filopodia formation

Actin reorganisation is controlled by members of the Rho family small GTPases (Bishop and Hall, 2000; Nobes and Hall, 1999). Cdc42 has been implicated as a crucial effector molecule downstream of TNF initiating actin reorganisation and particularly the formation of filopodia (Gadea et al., 2004; Puls et al., 1999). Indeed, transient expression of a dominant-negative form of Cdc42, Cdc42N17, abrogated filopodia formation in wildtype MEFs after TNF stimulation underscoring the central role of Cdc42 in this process (Figs. 11A-B). These data indicate that FAN acts upstream of Cdc42. Thus, wildtype and $FAN^{-/-}$ MEFs were examined for activation of Cdc42 after treatment with TNF using a pull down assay which specifically detects GTP-bound Cdc42. Unlike in wildtype MEFs, TNF treatment failed to induce Cdc42 activation in $FAN^{-/-}$ MEFs (Figs. 11C-D), indicating that FAN is required for TNF-induced activation of Cdc42.

To exclude a defect in filopodia formation downstream of Cdc42 in FAN$^{-/-}$ MEFs, a constitutively active form of Cdc42 (Cdc42L61) was introduced (Puls et al., 1999). As Cdc42 also activates Rac1, cotransfection of dominant negative Rac1 (RacN17) is necessary to avoid extensive formation of lamellipodia (Fig. 12C) (Nobes and Hall, 1995). Expression of constitutively active Cdc42 lead to the formation of filopodia in both wildtype and FAN$^{-/-}$ cells (Figs. 12A-B) confirming an intact actin cytoskeleton machinery in FAN$^{-/-}$ cells downstream of Cdc42.

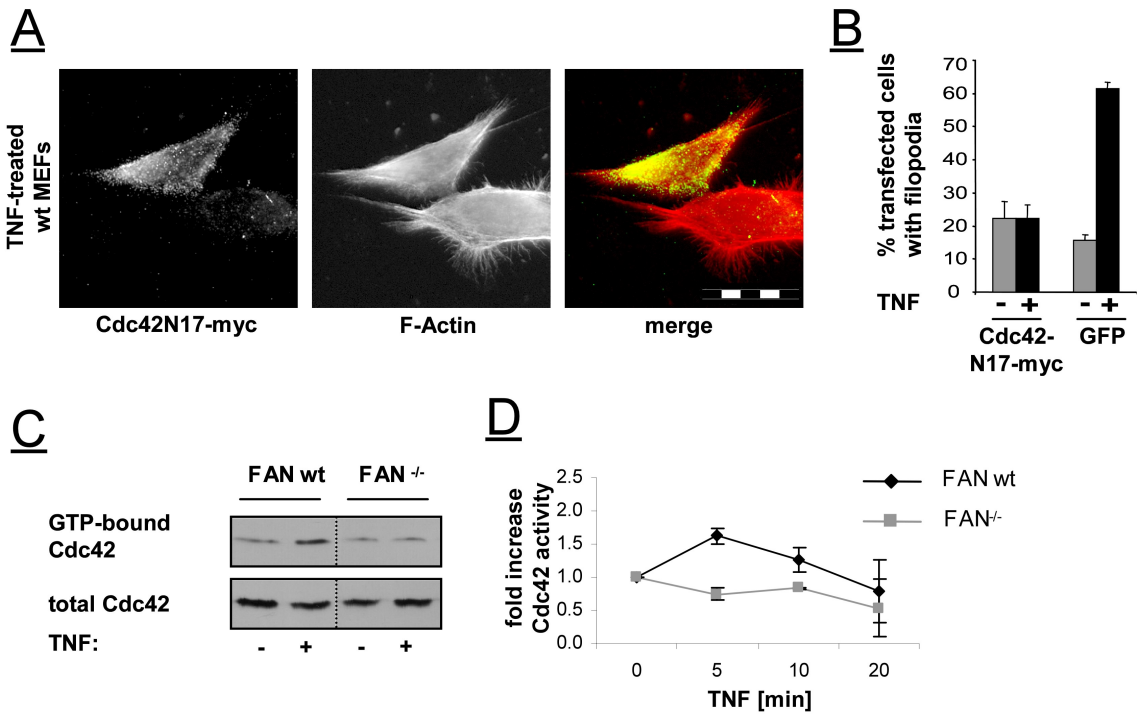

Fig. 11. FAN signals upstream of Cdc42 in TNF-induced filopodia formation.
A, Cdc42N17 abrogates TNF-induced filopodia. FAN wt MEFs were transfected with a myc-tagged version of dominant-negative Cdc42 (Cdc42N17), treated with TNF for 10 minutes or left untreated and stained for F-actin (red) to visualize filopodia and anti-myc to visualize myc-tagged Cdc42N17 (green).
B, Quantitation of FAN wt MEFs bearing filopodia transfected with Cdc42N17 or GFP and stimulated with TNF. For each experiment, >100 transfected cells were counted, and results are represented as mean ± SD of at least three independent experiments.
C, TNF-induced activation of Cdc42. 4x10^6 FAN wt or FAN$^{-/-}$ MEFs were stimulated with TNF for 5 minutes 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 analysed for total Cdc42 content in cell lysates.
D, Cdc42 activation after TNF stimulation was quantified at indicated times by western blotting. Data are shown as mean ± SD of three independent experiments.

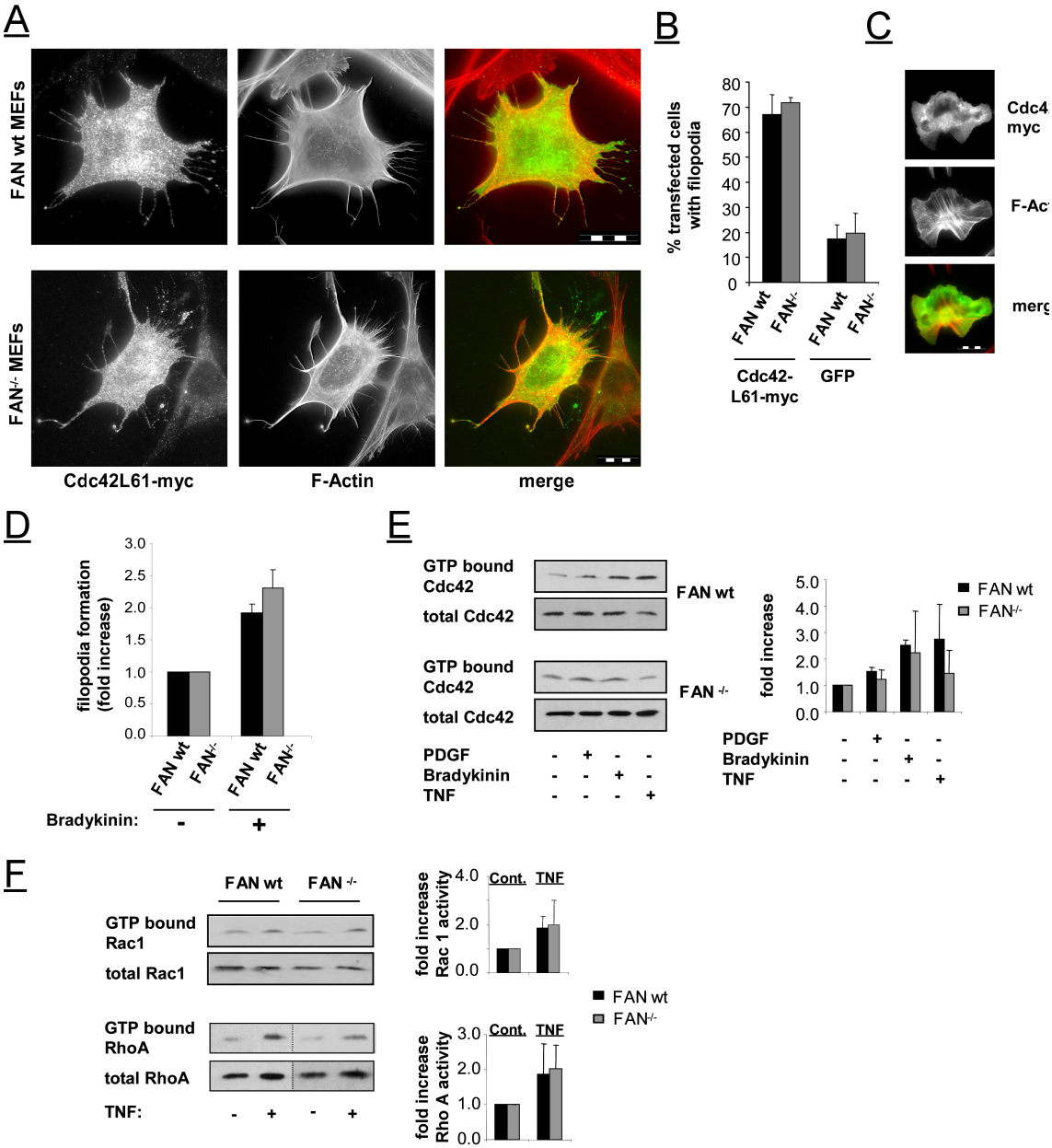

Fig. 12. FAN specifically mediates TNF-induced activation of Cdc42. A, Constitutively active Cdc42 overcomes the defective TNF-induced filopodia formation in FAN$^{-/-}$ MEFs. FAN wt and FAN$^{-/-}$ MEFs were transfected with a myc-tagged constitutively active Cdc42 (Cdc42L61-myc) and stained for F-actin (red) and myc-Cdc42L61 (green). Cells were cotransfected with dominant-negative RacN17 to avoid Rac activation by Cdc42 (see also C). B, Quantitation of FAN wt and FAN$^{-/-}$ cells bearing filopodia transfected with Cdc42L61 or GFP as in (A). C, Transfection of Cdc42L61 without RacN17 results in formation of extensive lamellipodia due to Rac activation by Cdc42L61. D, Quantification of FAN wt and FAN$^{-/-}$ cells bearing filopodia after bradykinin treatment (400 ng/ml) for 10 minutes. E, Activation of Cdc42 by PDGF and bradykinin. FAN wt and FAN$^{-/-}$ MEFs were stimulated with PDGF (20 ng/ml) or bradykinin for 10 minutes or left untreated. Activated GTP-bound Cdc42 was detected as in Fig. 4C and quantified as in Fig. 4D. E, TNF-induced activation of Rac1 and RhoA. FAN wt and FAN$^{-/-}$ MEFs were stimulated with TNF for 10 minutes 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 (E).

To test if FAN deficiency does also affect filopodia formation induced by different stimuli other than TNF, FAN wt MEFs and FAN$^{-/-}$ MEFs were stimulated with bradykinin, a well established inducer of Cdc42-dependent filopodia in fibroblasts (Kozma et al., 1995). Bradykinin could equally induce filopodia in FAN$^{-/-}$ MEFs and FAN wt MEFs, showing that FAN deficiency has no effect on bradykinin-induced filopodia formation (Fig. 12A). Similarly, activation of Cdc42 after treatment with bradykinin or PDGF was not impaired in FAN$^{-/-}$ MEFs, whereas TNF could not activate Cdc42 (Fig. 12B). 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. Both wildtype and FAN$^{-/-}$ MEFs showed activation of Rac1 and RhoA after treatment with TNF (Fig. 12F). These data reveal a specific role for FAN only in TNF-induced activation of Cdc42.

Defective Cdc42 activation in FAN$^{-/-}$ MEFs also affects cell polarisation

Besides its role in filopodia formation, Cdc42 has been shown to play a central role in regulating cell polarity (Etienne-Manneville, 2004). Polarisation of cells for forward movement can be assessed by analysing reorientation of the Golgi apparatus in the direction of movement (Etienne-Manneville and Hall, 2001; Nobes and Hall, 1999). As activation of Cdc42 is impaired in FAN$^{-/-}$ cells, a possible impact of FAN deficiency on cell polarity was examined by analyzing Golgi reorientation in a wound-scratch test.

After staining of the Golgi apparatus and the nucleus, the percentage of cells with the Golgi facing towards the wound was evaluated at indicated time points. As TNF treatment enhances reorientation of the Golgi (Fig. 13D) and FAN function depends on TNF, cell polarisation of FAN wt and FAN$^{-/-}$ MEFs was compared in the presence of TNF. Indeed, reorientation of the Golgi apparatus after TNF treatment was markedly reduced in FAN$^{-/-}$ MEFs compared to FAN wt MEFs (Fig. 13B-C).

These data show that the TNF-dependent enhancement of cell polarisation is abolished in FAN$^{-/-}$ MEFs, which is consistent with a defective TNF-induced activation of Cdc42 in these cells.

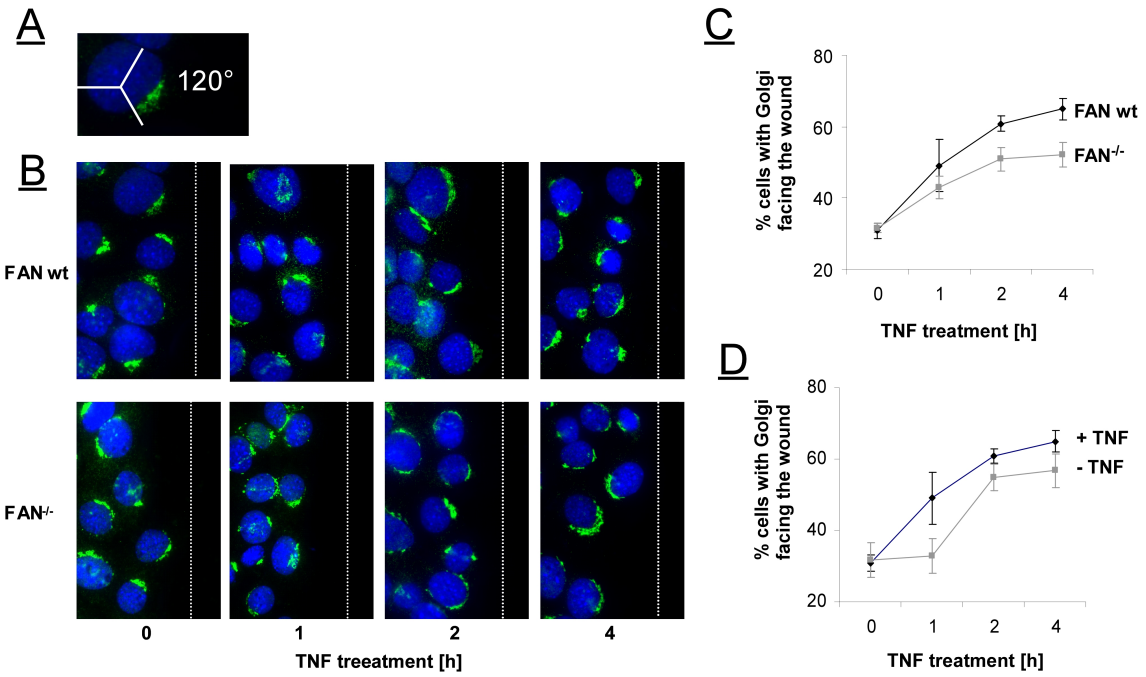

Fig. 13. Impaired Golgi apparatus reorientation in FAN$^{-/-}$ MEFs. A, To quantify Golgi reorientation, cells were divided into 120° sections and scored positive when the Golgi was inside the section facing towards the wound. B, Golgi reorientation in a scratch-wound test. After scratching of confluent layers of FAN wt and FAN$^{-/-}$ MEFs (0 hr), 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. C, Quantification of Golgi reorientation in FAN wt and FAN$^{-/-}$ MEFs. Cells with the Golgi orientated towards the wound were scored positive at the indicated times. For each experiment, >100 cells were evaluated. Results are represented as mean ± SD of at least three independent experiments. D, Golgi reorientation is enhanced in the presence of TNF. FAN wt MEFs treated with TNF or left untreated were quantified for Golgi reorientation as in (C).

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 (Fig. 7 in Materials and Methods). 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 conserved PH domain which is associated with the neighbouring 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 localisation in order to integrate membrane receptor signals to actin cytoskeletal reorganisation (Sechi and Wehland, 2000). Often, membrane targeting of actin modulating proteins is achieved by binding to phosphoinositides via PH or PH-like domains (Canton et al., 2005; Hogan et al., 2004; Olsten et al., 2004). When FAN is expressed as GFP-fusion protein, colocalisation with TNF-RI at the plasma membrane can be observed (Fig 14A, upper panel). 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 differ-

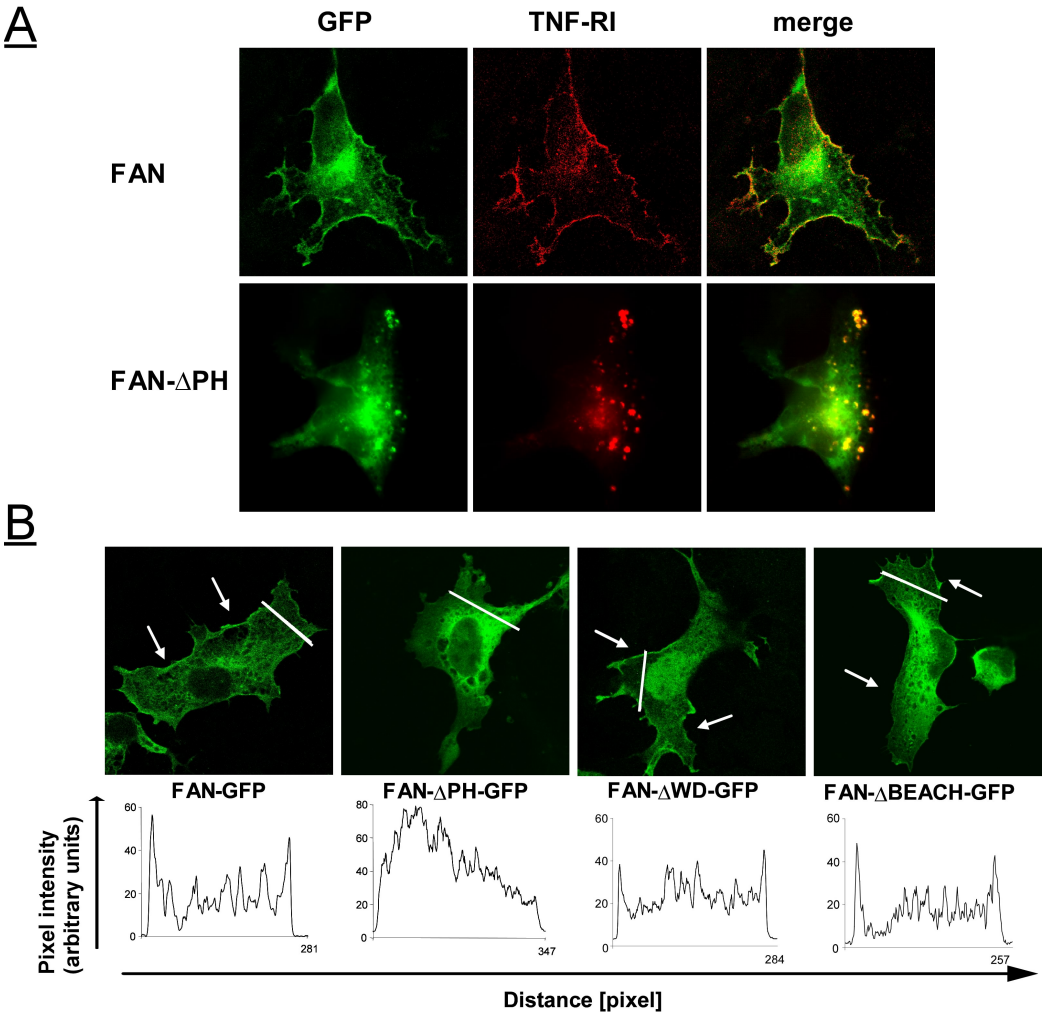

Fig. 14. The PH domain directs FAN to the plasma membrane. A, Colocalization of plasma membrane TNF-RI and FAN. COS cells were transfected with pEGFP-FAN or pEGFP-FAN-ΔPH (green) and TNF-RI lacking a functional death domain to avoid toxic effects (Tcherkasowa et al., 2002). Cells were stained for membrane or intracellular TNF-RI (red), and analysed by confocal microscopy. B, Membrane localisation of FAN. Confocal images of COS cells expressing pEGFP-FAN or different deletion mutants of FAN. Arrows indicate membrane staining of pEGFP-FAN. For quantification of membrane staining, intensity profiles along the indicated white lines were generated using ImageJ (Abramoff et al., 2004)

ent parts of FAN were expressed in COS-7 cells as GFP fusion proteins.

Confocal microscopy revealed that removal of amino acid residues 1-275 which in-

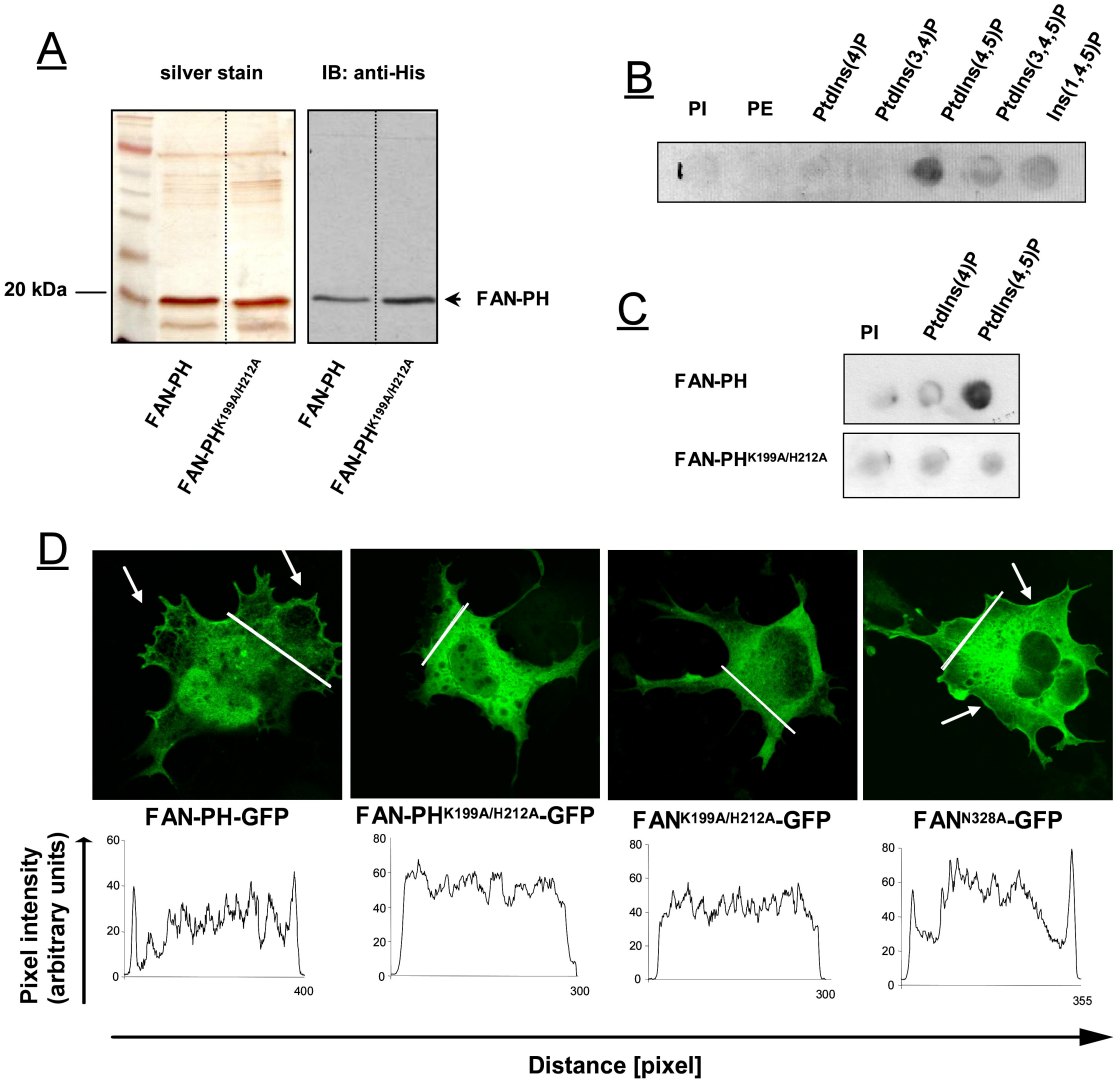

Fig. 15. The PH domain of FAN specifically binds PtdIns(4,5)P. A, Expression and purification of recombinant His-tagged FAN-PH proteins. FAN-PH and FAN-PH$^{K199A/H212A}$ were recombinantly expressed and affinity-purified. Equal amounts were analyzed by SDS-PAGE following silver staining or immunoblot (IB) analysis with anti-His antibody. B, Lipid overlay assay using purified FAN-PH protein. The recombinantly expressed PH domain of FAN was incubated with a nitrocellulose membrane spotted with different phopholipids. Bound protein was detected using anti-His antibody. C, Lipid overlay assay as in (B) 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. D, Membrane localisation of FAN-PH depends on PtdIns(4,5)P binding. Confocal images of COS cells expressing pEGFP-FAN-PH or different point mutantions of FAN. Arrows indicate membrane staining of pEGFP-FAN. Intensity profiles along the white line were generated as in Fig. 14B. 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

clude the N-terminal PH domain abrogates membrane association of FAN and colocalisation with TNF-RI at the plasma membrane (Fig. 9A, lower panel). Rather, FAN-ΔPH colocalises with intracellular TNF-RI, which is localised into vesicles {Bradley, 1995 #59, thus showing that deletion of the PH domain does not affect the overall ability of FAN to interact with TNF-RI. Deletion of the C-terminal WD-repeats or the BEACH domain had no effects on the subcellular distribution and plasma membrane association of FAN (Fig. 14B). These findings show that the N-terminal part containing the novel PH domain is necessary for plasma membrane association of FAN.

Many PH domains are known for their ability to bind to distinct phosphoinositides which is often utilised in combination with additional protein-protein interaction as a mechanism for signal-dependent membrane targeting. To test if the N-terminal part of FAN has phospholipid binding ability, recombinant FAN-PH was expressed in *E.coli* as His-tag fusion protein and affinity-purified (Fig. 10A). 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 Fig. 10B, 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.

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 (Cozier et al., 2003; Edlich et al., 2005; Lemmon and Ferguson, 2000). 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-characterised proteins like phospholipase C (Lemmon et al., 1995). After expression and purification of the FAN-PH domain harbouring the two mutations (FAN-PH$^{K199A/H212A}$) (Fig. 15A), the recombinant FAN PH protein and the mutated protein were tested for their lipid binding ability in a lipid overlay assay. In contrast to wild type FAN-PH protein, FAN-PH$^{K199A/H212A}$ protein failed to bind to PtdIns(4,5)P (Fig. 15C), 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 localisation 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$^{K199A/H212A}$) (Fig. 15D). A control mutation located outside the PH domain (N328) had no effect on membrane localisation. 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 Fig. 10C, expression of full-length FAN in FAN-deficient cells lead to the induction of filopodia after TNF stimulation. In contrast, FAN$^{-/-}$ MEFs expressing FAN-ΔPH did not form filopodia after TNF stimulation (Figs. 16A-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 (Figs. 15C-D), 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 Fig. 16B, 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 (Fig. 16B). Thus, the PH domain modulates TNF-induced filopodia formation not only by plasma membrane association but also through cross-talk with the BEACH domain.

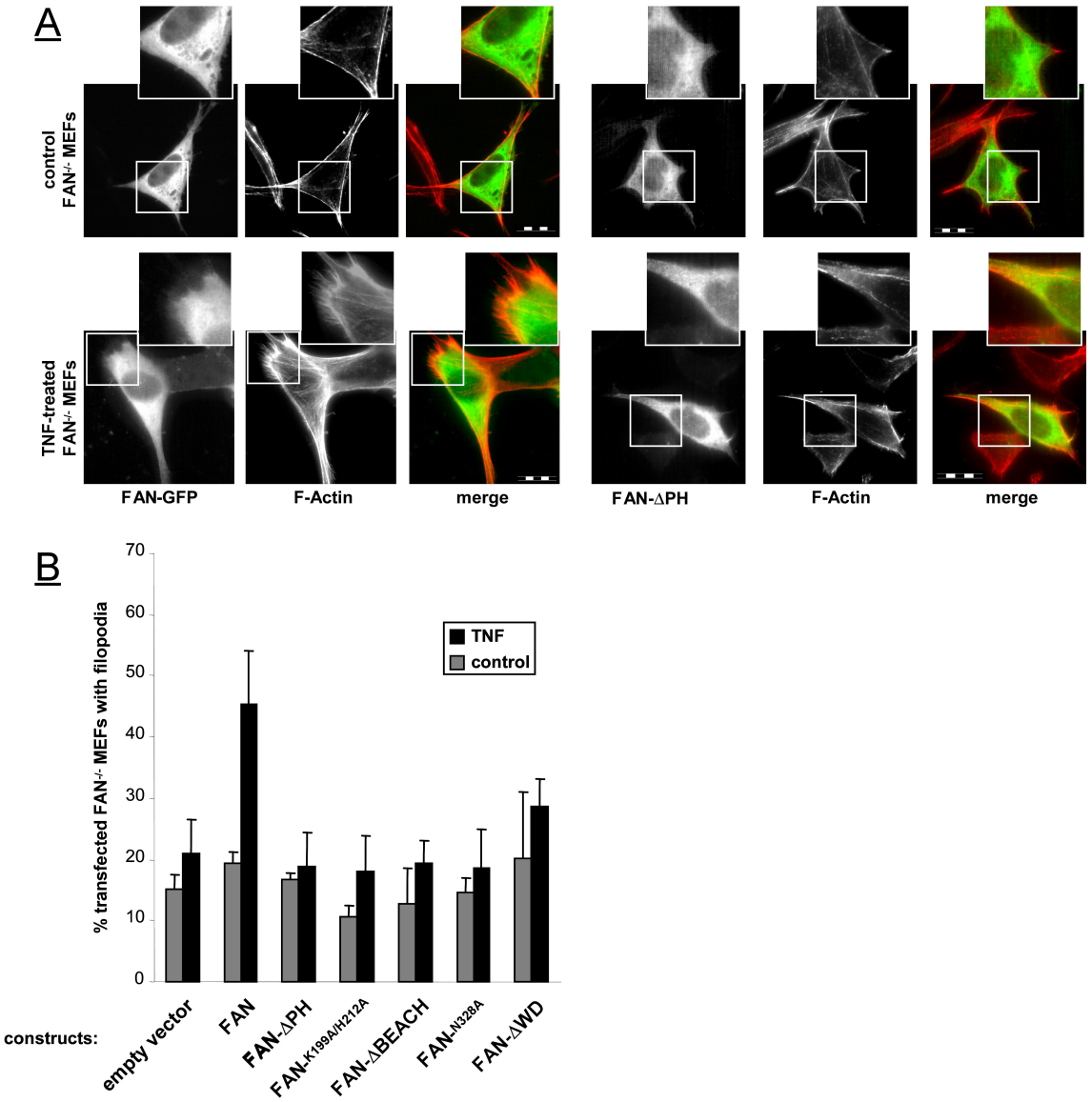

Fig. 16. Plasma membrane association of FAN is indispensable for TNF-induced filopodia formation. A, TNF-induced filopodia formation depends on the PH-domain. FAN$^{-/-}$ MEFs transfected with pEGFP-FAN or pEGFP-FAN-ΔPH (green) were stimulated with TNF for 10 minutes or left untreated. Cells were stained for F-actin (red) to visualize filopodia. Insets are enlargements of the boxed area. Bar = 20 µm. B, Quantification of filopodia formation after TNF treatment. FAN$^{-/-}$ MEFs transfected with the indicated pEGFP-FAN fusion constructs were stimulated with TNF for 10 minutes or left untreated. Cells were stained for F-actin and quantified for filopodia formation. For each experiment, >100 transfected cells were counted, and results are represented as mean ± SD of three independent experiments.

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 syndrom 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 hypothesised 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,

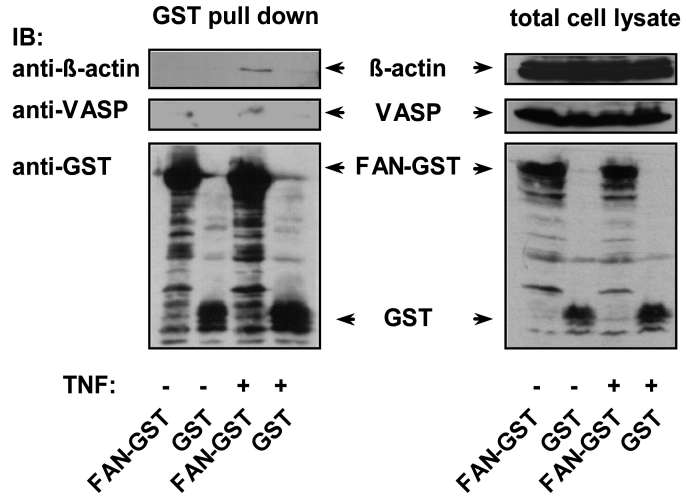

Fig. 17. Actin and VASP coprecipitate with FAN. GST pull down assay after TNF treatment. HEK 293 cells were transiently transfected with FAN-GST or GST alone and stimulated with TNF for 10 minutes or left untreated. Cells were lysed after 24 hrs and subjected to a GST pull down assay. Precipitates and total cell lysate were immunoblotted and probed with the indicated antibodies (IB) to detect coprecipitated proteins.

HEK 293 cells were transiently transfected with FAN-GST or GST alone, and examined by GST pull-down assays. As shown in Fig. 17, 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 regulated by

TNF. Consistently, immunoprecipitation of actin and VASP resulted in coprecipitation of ectopically expressed GST-FAN after TNF treatments, corroborating the interaction observed in the GST pull down assay.

GST-FAN, VASP or actin did not coprecipitate with Bax (pro-apoptotic Bcl2 pro-

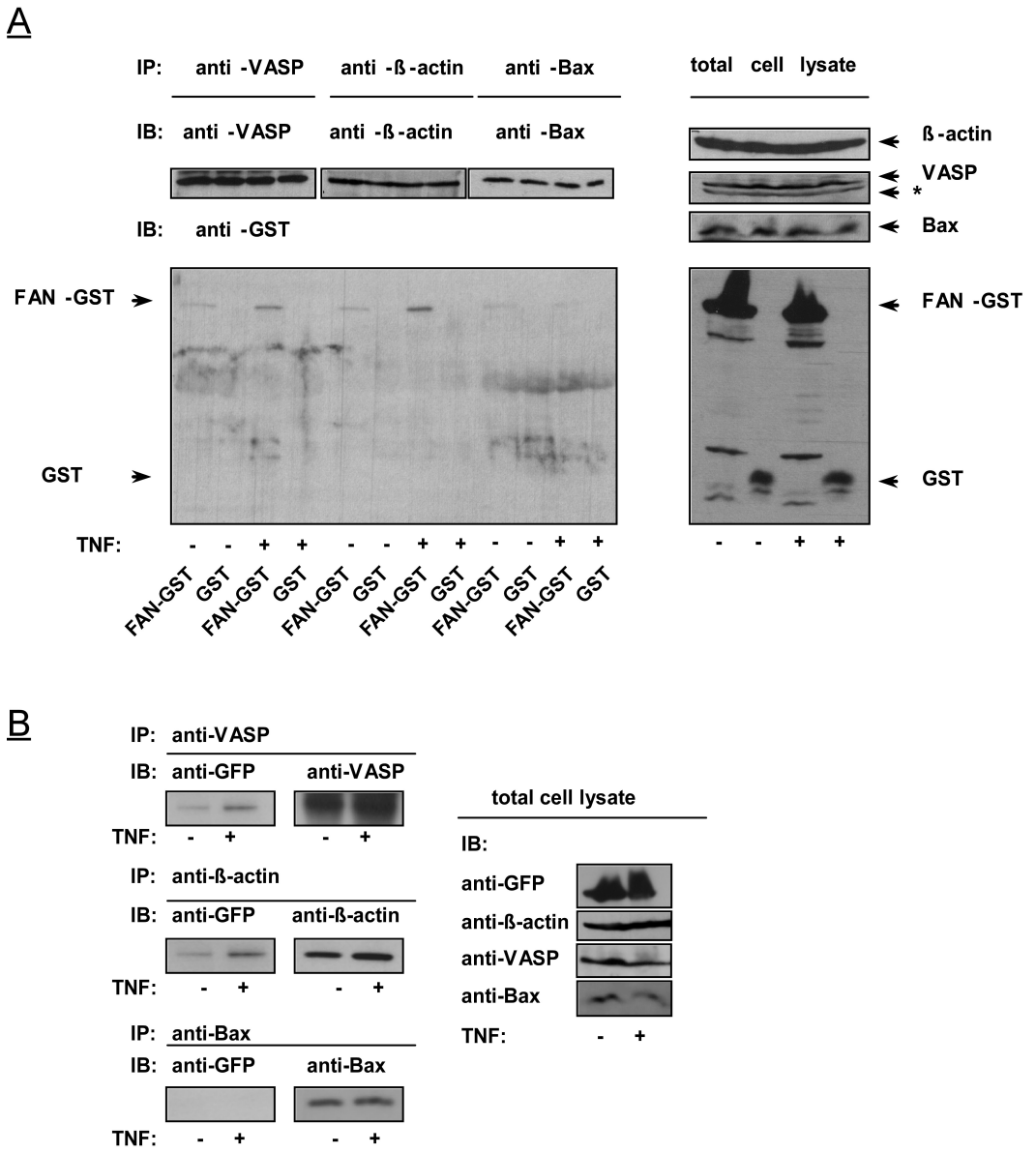

Fig. 18. FAN interacts with the actin cytoskeleton. A, Immunoprecipitation (IP) of VASP and actin after TNF treatment. Cells were transiently transfected with FAN-GST or GST alone and stimulated with TNF for 10 minutes or left untreated. Cells were lysed after 24 hrs, and IP was carried out using specific antibodies against actin, VASP or Bax. Precipitates and total cell lysate were immunoblotted and probed with the indicated antibodies. Asterisks (*) represent actin band recognised by actin-specific antibody before reprobing with VASP-specific antibody. B, IP after TNF treatment using FAN-GFP fusion proteins. Cells were transiently transfected with DNA constructs coding for FAN-GFP, stimulated with TNF for 10 minutes or left untreated. Lysates were immunoprecipitated as in (B).

tein) excluding immunoprecipitation conditions which favour non-specific binding (Fig. 18A). Furthermore, GFP-tagged FAN was immunoprecipitated in the same fashion as GST-tagged FAN, thus excluding any side effects of the protein tag in the precipitation assay (Fig. 18B).

In order to unravel the nature of FAN interaction with the actin cytoskeleton machinery, direct interaction of FAN with F-actin was analyzed in an *in vitro* F-actin binding assay using recombinant FAN-GST and F-actin. As shown in Fig. 19A, FAN-GST but not GST alone cosedimented with F-actin demonstrating direct F-actin binding of FAN. The observed interactions provide a functional link between FAN and the actin cytoskeleton machinery which may result in coprecipitation of proteins involved in filopodia formation like VASP as shown in Figs. 17-18.

As deletion of the PH domain of FAN abrogated membrane localisation and TNF-induced filopodia formation, the impact of the PH domain on the actin binding properties of FAN was investigated. Deletion of the PH domain also abolished the interaction of FAN with actin in a GST pull down assay whereas binding to TNF-RI remained un-

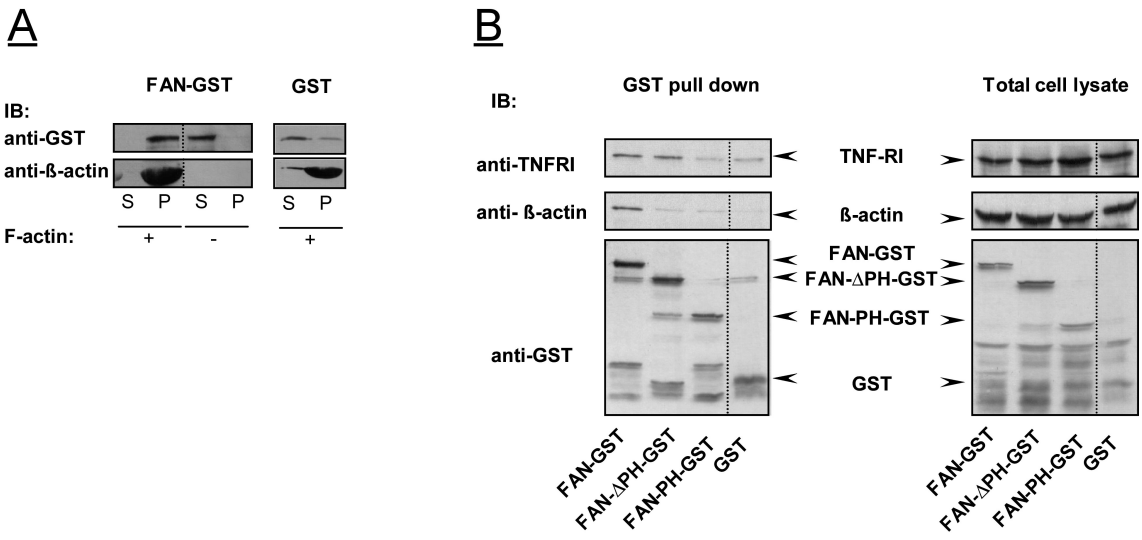

Fig. 19. The interaction of FAN with actin is dependent on the PH domain. A, F-actin cosedimentation assay. FAN-GST or control GST proteins were subjected to a F-actin sedimentation assay as described in Materials and Methods. Supernatants (S) and Pellets (P) were analysed by immunoblot (IB) using anti-GST and anti-β-actin antibodies. Samples without F-actin were included as binding control. B, GST pull down assay with cells ectopically expressing TNF-RI. HEK 293 cells were transiently cotransfected with full-length TNF-RI together with FAN-GST, FANΔPH-GST, FAN-PH-GST, or GST alone. After 24 hrs cells were lysed and subjected to a GST pull down assay.

affected (Fig. 19B). The PH domain alone does not interact with actin suggesting that PH modulates FAN actin binding by promoting correct subcellular localisation at the plasma membrane.

FAN deficiency affects TNF-dependent motility

Our data show that FAN connects the TNF-RI signalling complex to the actin cytoskeleton in order to transduce signals by TNF. Actin reorganisation and filopodia formation are essential processes during cellular motility (Ridley et al., 2003). As FAN-deficient cells show defects in activation of Cdc42 after TNF treatment leading to impaired filopodia formation and cell polarisation, motility of MEFs was analysed using live cell imaging. Migration of FAN wt and $FAN^{-/-}$ MEFs was monitored at 15 minutes intervals over 10 hours with an inverted microscope with CO_2 supply at 37°C. The migratory paths of individual cells were tracked to visualise motility of the cells. When the migratory paths of FAN wt and $FAN^{-/-}$ cells in the absence of TNF were compared, similar weak spontaneous migration was observed (Fig. 20A). TNF treatment increased random migration in wildtype fibroblasts, whereas $FAN^{-/-}$ cells did not display enhanced motility in response to TNF. Specifically, total path length, net path length and overall speed increased in FAN wt MEFs after TNF stimulation but not in the $FAN^{-/-}$ cells (Fig. 20B). These findings indicate that the defects in actin reorganisation and polarisation in $FAN^{-/-}$ cells affect cellular motility in response to TNF.

The regulation of cellular motility and focal complex turnover is linked to phosphorylation of paxillin (Petit et al., 2000; Tsubouchi et al., 2002), which is inducible by TNF (Brown and Turner, 2004; Fuortes et al., 1994; Hanna et al., 2001). As shown in Fig. 20C, western blot analysis using an antibody specific for tyrosine-phosphorylated paxillin revealed that TNF induces paxillin phosphorylation in wildtype cells, which was hardly discernible in $FAN^{-/-}$ cells. This observation confirms at the molecular level the FAN dependent phenotype of TNF-induced cellular motility.

TNF-dependent cell motility is especially important during an immune response. To analyse the effect of FAN deficiency on motility of immune cells in an *in vivo* situation, migration of Langerhans cells (LCs) was analysed in FAN$^{-/-}$ mice. LCs are a subset

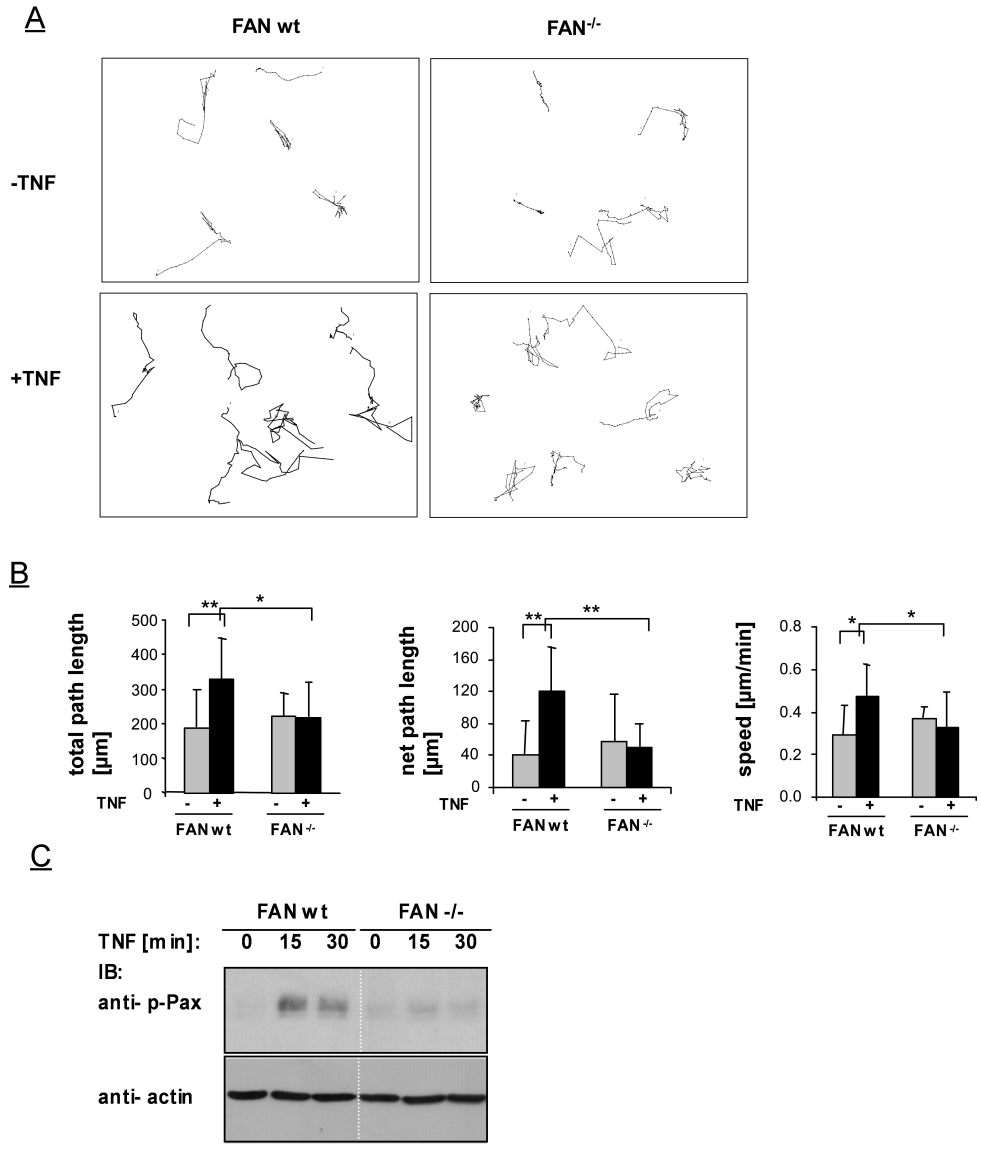

Fig. 20. Reduced random motility and paxillin phosphorylation in FAN$^{-/-}$ fibroblasts. A, Migratory behaviour of FAN wt and FAN$^{-/-}$ MEFs stimulated with TNF or left untreated. Pictures were taken every 15 min for 10 h at 37°C using a Leica inverted microscope with CO_2 supply. Representative migration tracks of individual FAN wt and FAN $^{-/-}$ MEFs are shown. B, Quantitation of migratory behaviour. Parameters from migration tracks were computed using DIAS software. Each bar represents the mean ± SD of at least four independent experiments. Total path length represents the length of the track, whereas net path length is the distance between starting and end point of a track. Significance was measured using a Student's t-test. * $p<0.05$, ** $p<0.01$. C, Impaired paxillin phosphorylation in FAN$^{-/-}$ MEFs. FAN wt and FAN$^{-/-}$ MEFs were stimulated with TNF for the indicated times, and phosphorylated paxillin was detected in total cell lysates by immunoblotting (IB) using phospho-specific anti-paxillin antibody. Actin reprobing ensured equal loading.

of dendritic cells residing in the epidermis. They are thought to function as first line defence in the skin sensing infection and emigrating into draining lymph nodes to elicit the appropriate immune response. This migration is dependent on TNF and IL-1 produced by keratinocytes, and injection of TNF into mouse ear epidermis has been used to study the migratory behaviour of LCs (Cumberbatch et al., 1997; Cumberbatch et al., 1994).

To analyse the impact of FAN during TNF-induced LC migration, wildtype and FAN$^{-/-}$ mice were injected with TNF or PBS into both ears, and ear epidermis was prepared after 2 hrs. Epidermal sheets were stained for MHC-II to visualise LCs (Fig. 21A). Both wildtype and FAN$^{-/-}$ ear epidermis contained comparable amounts of LCs without TNF treatment (Figs. 21A-B). Injection of TNF resulted in lower numbers of LCs in ear epidermis from wild type mice indicating migration of LCs out of the epidermis into draining lymph nodes. In contrast, TNF did not lead to emigration of LCs from ear epidermis from FAN$^{-/-}$ mice indicating a defect in TNF-induced LC motility. Thus, FAN deficiency also affects motility of immune cells like LCs in a physiological context.

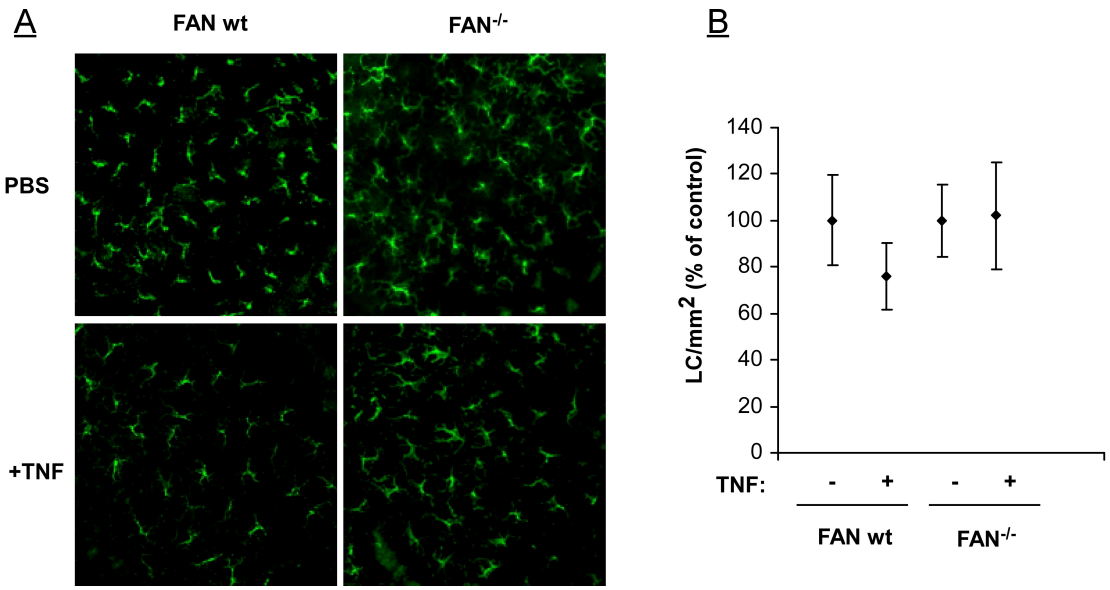

Fig. 21. Impaired TNF-dependent migration of Langerhans cells (LCs). A, Staining of LCs in mouse ear epidermis. Epidermal sheets of FAN wt and FAN$^{-/-}$ mice injected with TNF or PBS as control were stained with anti-MHC-II antibody to visualise LCs. Pictures represent total intensity projections of z-stacks taken with a confocal micrsocope. B, Quantification of LCs in epidermal sheets. Numbers are represented as mean number of LCs/mm^2 ± SD (n=10 (wt), n=9 (FAN$^{-/-}$)) and expressed as changes in percent of control.

Discussion

In contrast to other TNF receptor adapter 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. Here we show that FAN is crucial for the formation of filopodia and actin cytoskeleton reorganisation induced by TNF. A major finding of this study identifies the N-terminal domain of FAN as a PH-like domain which specifically binds PtdIns(4,5)P. This lipid interaction is essential for targeting FAN to the plasma membrane and for FAN-mediated TNF-induced actin reorganisation. FAN interacts with F-actin and VASP upon TNF treatment, thus linking TNF-RI signalling to the actin cytoskeleton machinery. 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 localisation. Thus, the molecular mode of PH domain action in FAN signalling is defined by two independent functional features, that is, targeting of FAN to the plasma membrane and correct inter-domain interaction between BEACH and PH regulating FAN function.

This study identified FAN as mediator of TNF-induced actin reorganisation and links the NSD of TNF-RI and FAN to a function in actin reorganisation. One of the central findings of this study demonstrates the crucial role of FAN in TNF-induced filopodia formation. The formation of filopodia seems to be a pivotal characteristic of actin reorganisation in fibroblasts induced by TNF (Gadea et al., 2004; Puls et al., 1999; Sugihara et al., 2002). Our data show that FAN-deficient MEFs exhibit reduced filopodia formation after stimulation with TNF compared to wild-type MEFs (Fig. 8). Some residual TNF-dependent induction of filopodia could still be observed in $FAN^{-/-}$ MEFs which may be attributed to a FAN-independent mechanism of TNF action. MAPK signalling has been implicated in TNF-induced actin reorganisation (Gadea et al., 2004; Kutsuna et al., 2004). It has been shown that TNF-induced MAPK signalling (Lüschen et al., 2000) and JNK activation (Fig. 10) remain unaffected by FAN deficiency which could account for the observed residual FAN-independent actin reorganisation. However, the considerably large reduction of filopodia formation in $FAN^{-/-}$ MEFs can be restored by reintroduction of FAN clearly identifying FAN as the main mediator of

TNF-induced filopodia formation and actin reorganisation. Although many studies demonstrate the various effects of TNF on actin reorganisation, specific downstream effectors which couple TNF signals to RhoGTPase activation and actin reorganisation have not been identified. A previous study already excluded the participation of the classical TNF-RI adapter proteins TRADD, RIP and TRAF2 which bind to the death domain of TNF-RI (Puls et al., 1999). A role for the membrane proximal domain of TNF-RI in actin reorganisation has been suggested in macrophages (Peppelenbosch et al., 1999). Using TNF receptor mutants, overall decrease in F-actin was attributed to both the death domain and the membrane proximal domain, whereas TNF-induced cortical actin reorganisation was dependent on the membrane proximal domain including the FAN binding site NSD. Here we provide evidence that identifies FAN as the first TNF-RI interacting adapter protein that has been directly linked to actin reorganisation induced by TNF.

It has already been suggested that TNF modulates actin reorganisation and filopodia formation by activation of Cdc42 (Gadea et al., 2004; Peppelenbosch et al., 1999; Puls et al., 1999). We show that FAN is crucial for TNF-induced activation of Cdc42, and specifically mediates activation of Cdc42 only by TNF but not by other stimuli like bradykinin and PDGF (Fig. 12). Whereas TNF-induced activation of Cdc42 was abrogated in $FAN^{-/-}$ cells, FAN deficiency did not seem to affect the activation of Rac1 and RhoA in TNF-treated MEFs. This implicates FAN as a TNF signalling factor selectively operating in the Cdc42 activation pathway. However, we cannot exclude cell type specific effects due to the complex and tissue-specific regulation and interplay of the RhoGTPases in different signalling cascades. Further experiments are needed to clarify the mode of Rho and Rac activation by TNF and FAN.

Cdc42 is an important mediator of filopodia formation and cell polarisation which are both important steps during cell migration (Etienne-Manneville, 2004). FAN-mediated activation of Cdc42 induced by TNF subsequently affects filopodia formation and cell polarisation (Fig. 8 and 13). Mice lacking FAN show a delay in cutaneous barrier repair which could indicate problems in wound closure, a process where TNF is crucially involved. We show in an *in vitro* wound/scratch assay that FAN mediates polarisation of cells which is an important prerequisite for wound closure (Fig. 13). This indicates that FAN could be important for TNF-mediated wound healing processes. Further experiments assessing polarisation and migration of keratinocytes and wound

closure in FAN-deficient mice are needed to reveal the role of FAN during wound healing.

Up to now only the C-terminal WD repeats of FAN have functionally characterised. The relevance of the recently identified BEACH domain and the proposed adjacent weakly conserved PH-like domain had not been resolved (Jogl et al., 2002). Here we show for the first time that the N-terminal fold next to the BEACH domain in FAN has features of a classical PH domain. By *in vitro* lipid interaction assays and subcellular localisation, the N-terminal fold of FAN was shown to specifically bind to PtdIns(4,5)P and target FAN to the plasma membrane (Fig. 15). Thus, despite low sequence homology to other PH domains, the lipid binding and membrane targeting properties identify the N-terminal fold of FAN as a PH domain. Analysis of FAN deletion mutants and PH mutants with disrupted phospholipid binding capacity demonstrate that the phosphoinositide interaction and membrane localisation are crucial for FAN-mediated TNF-induced filopodia formation. 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 localise 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 to direct actin polymerisation close to the plasma membrane (Insall and Weiner, 2001) and as an factor regulating the adhesion of the actin cytoskeleton to the plasma membrane (Raucher et al., 2000). FAN utilises its PtdIns(4,5)P-binding ability to associate with the plasma membrane (Fig. 14), where interaction with membrane TNF-RI and possibly additional proteins can occur (Tcherkasowa et al., 2002).

In this context, the PH domain may modulate the actin binding capacity of FAN in a dual way: first by correct subcellular localisation of FAN, and second by promoting a conformational change of FAN induced by binding to PtdIns(4,5)P, a mechanism which has been also described for N-WASP (neural Wiskott-Aldrich Syndrome Protein) and ERM (ezrin-radixin-moesin) proteins (Bretscher et al., 2002; Prehoda et al., 2000). This conformational change could have a liberating effect on the adjacent BEACH domain which is associated in close contact with the PH domain leading to activation of FAN (Jogl et al., 2002). Our results support this idea by showing that TNF-induced filopodia

formation requires the presence of the BEACH domain (Fig. 16). Most importantly, a mutation in FAN that has been shown to interfere with the interactions between the PH and BEACH domains can still localise to the plasma membrane (Fig. 15) but is not able to mediate TNF-induced filopodia formation (Fig. 16), demonstrating that the PH-BEACH interaction is important for TNF-induced actin reorganisation. Structural data of the FAN protein itself and further interaction studies with isolated FAN variants will shed more light on the interdomain interactions and its importance for FAN function.

In our data a direct interaction of FAN with Cdc42 could not be detected. Since FAN does not contain any known structural features that would suggest GTPase regulatory functions like GEF or GAP activity, actin reorganisation and Cdc42 activation by FAN seems to be modulated through a different mechanism. As shown in Figs. 17-19, FAN appeared as a functionally dormant, plasma membrane associated protein without actin cytoskeleton binding property. Upon TNF stimulation FAN interacted with the actin cytoskeleton machinery demonstrated by coprecipitation with actin and VASP. FAN binds directly to F-actin as demonstrated in an *in vitro* F-actin cosedimentation assay (Fig. 19). A fundamental property of many actin cytoskeleton modulators including proteins of the ERM and WASP/WAVE (WASP family verprolin homolgous) family is their association with the plasma membrane and the underlying cytoskeleton, con-

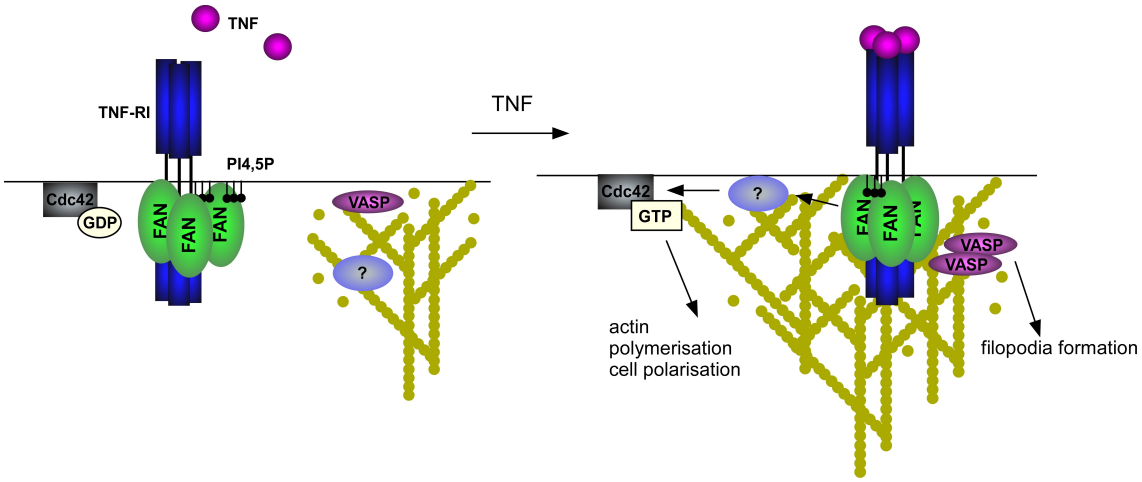

Fig. 22. Model of FAN-mediated TNF-induced actin reorganisation. In an uninduced state, FAN is localised to the plasma membrane by association with PtdIns(4,5)P but does not interact with the actin cytoskeleton. Upon TNF stimulation FAN interacts with the actin cytoskeleton leading to the formation of a signalling complex that links the TNF-RI to the cytoskeleton machinery. Cdc42 could be activated by FAN via local recruitment of one ore more unknown modulators leading to actin polymerisation and filopodia formation which is supported by the presence of VASP.

necting 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. Though ERM proteins were implicated very early as linkers between plasma membrane and microfilaments, no direct association with the actin cytoskeleton of the isolated protein could be detected at first. Subsequent research revealed that the F-actin-binding ability is masked in the dormant protein by association of the N- and C-terminal domains. PtdIns(4,5)P interaction and phosphorylation induces a conformational change leading to dissociation of the domains and F-actin binding (Bretscher et al., 2002). Similarly, FAN binds TNF-RI, associates with the plasma membrane via PtdIns(4,5)P binding (Fig. 15) and interacts with actin cytoskeleton machinery upon TNF treatment (Figs. 17-19). By linking the actin cytoskeleton machinery to TNF-RI, FAN could form a signalling platform which locally modulates TNF-induced actin reorganisation in a phosphoinositide-dependent fashion (Fig. 22). A similar mechanism has been recently shown for Toll-like receptor signalling where sorting of distinct adapter proteins is used to define the signalling pathways to be activated. The adapter protein TIRAP (TIR-domain-containing adaptor protein) has been shown to bind to PtdIns(4,5)P and selectively recruits the adapter protein MyD88 (myeloid differentiation primary-response protein 88) but not TRIF (TIR-domain-containing adaptor protein inducing IFN-β) to specialised regions of the plasma membrane where signalling complexes are assembled (Kagan and Medzhitov, 2006).

FAN was found to coprecipitate with the actin-binding protein VASP (Figs. 17-18). Recent evidence suggests VASP as an important factor to direct actin polymerisation to form filopodia instead of lamellipodia by its anti-capping and bundling activity (Mejillano et al., 2004). Thus, the presence of VASP could specifically favour filopodia induction at sites of TNF-induced actin reorganisation. Association with ENA/VASP proteins to mediate filopodia formation has also been shown for IRSp53 (insulin receptor substrate p53) which forms a complex with Mena leading to enhanced formation of filopodia (Krugmann et al., 2001). In contrast to IRSp53 that binds to Cdc42, we could not detect coprecipitation of Cdc42 with FAN. If FAN does not interact with Cdc42, activation of Cdc42 and subsequent actin reorganisation could also be induced by locally recruiting Cdc42-specific GEFs by FAN-mediated formation of the actin cytoskeleton-TNF-RI signalling scaffold. The identification of these yet unknown factors

will be an important step towards a more thorough understanding of the molecular action of FAN during TNF-induced activation of Cdc42.

Our results show that FAN binds directly to F-actin (Fig. 19A). However, FAN does not contain any known actin binding domains. As the considerably well-studied PH domains and WD-repeats are not known for their actin binding ability, the BEACH domain of FAN remains as candidate for mediating F-actin binding. BEACH domains have been found in proteins from all eukaryotes but little is known about their function (De Lozanne, 2003). The BEACH-containing protein LYST (lysosomal trafficking regulator, also known as Chediak-Higashi syndrome protein (CHS)) seems to be involved in lysosomal trafficking as cells defective in this protein display enlarged lysosomes and secretory effects, resulting in a rare lysosomal storage disorder (Chediak-Higashi syndrome) (Ward et al., 2002). A recent study showed that FAN-deficient fibroblasts also exhibited enlarged lysosomes (Möhlig et al., 2007). Vesicular trafficking is dependent on reorganisation of the actin cytoskeleton and also on Cdc42 activity (Ridley, 2006). Thus further experiments addressing the F-actin binding capacity of the BEACH domain of FAN and other BEACH proteins will be central to unravelling the function of BEACH domains in general.

Cellular motility is essentially involved in immune reactions, and actin reorganisation plays an important role in cellular motility. TNF-induced cell migration is important during an inflammatory response and affects also processes like chemotaxis of immune cells. As a central mediator of TNF-induced actin reorganisation, FAN was shown to affect TNF-induced motility of MEFs (Fig. 20). FAN deficiency results in ineffective interplay between membrane TNF-RI and the actin cytoskeleton which may lead to impaired actin reorganisation and migration. Furthermore, actin reorganisation and filopodia formation by Cdc42 are essential steps for migration of cells (Ridley et al., 2003). Impaired Cdc42 activation and subsequent filopodia formation in $FAN^{-/-}$ MEFs could also lead to reduced TNF-induced motility.

Dendritic cells are important mediators of the early phases of the inflammatory response as they take up antigen in the periphery and migrate to draining lymph nodes where they activate circulating T-cells (Mellman and Steinman, 2001). Migration of Langerhans cells (LCs), epidermal dendritic cells, from the epidermis of mouse ears into draining lymph node has been widely used as an *in vivo* model for dendritic cell migration during skin irritations (Cumberbatch et al., 1997). As shown in Fig. 21, FAN

is required for TNF-induced emigration of LCs from the ear epidermis, clearly placing the observed *in vitro* effects of FAN-mediated TNF-induced actin reorganisation in a physiological context. Although it is still the subject of discussion if LCs directly activate T-cells or have rather regulatory functions (Kissenpfennig and Malissen, 2006), these data show the impact of FAN on leukocyte motility under inflammatory conditions. It will be interesting to test the consequences of FAN-deficiency during migration of other immune cells, e.g. motility of isolated dendritic cells, neutrophils or macrophages in boyden chambers. In addition, analysis of FAN deficiency during murine infection models like experimental leishmaniasis (von Stebut, 2007) are important for further characterising the involvement of FAN-mediated migration in immune responses.

It has been proposed that PtdIns(4,5)P accumulates at plasma membrane lipid microdomains like lipid rafts, where it modulates the activity of proteins involved in regulating 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 localise to the plasma membrane (Wiegmann and Krönke, unpublished data). By modulating nSMase3 activity, FAN could influence membrane lipid composition and PtdIns(4,5)P distribution and thus modulate the dynamics of membrane microdomains and lipid rafts. Furthermore, lipid rafts have been implicated in modulation of cell motility (Golub and Caroni, 2005) and have been shown to be sites of enhanced actin polymerisation (Rozelle et al., 2000). Filopodia formation requires deformation and tubulation of the plasma membrane (Mattila et al., 2007). Thus, FAN-mediated modulation of nSMase activity resulting in subsequent alteration of membrane microdomains and fluidity could contribute to protrusion formation and motility induced by TNF.

It is not clear in which way FAN-mediated nSMase activation and actin reorganisation are connected. Either FAN-induced assembly of an actin-based signalling platform

at the sites of TNF stimulation could lead to local activation of nSMase and concomitant modulation of membrane dynamics important for protrusion formation. The effects of actin reorganisation on nSMase activation could be tested by disrupting the signalling platform using inhibitors of actin polymerisation and measuring activation of nSMase after TNF treatment. More evidence though point to an upstream or parallel function of nSMase activation during FAN-mediated actin reorganisation. As rafts have been shown to modulate cytoskeletal-membrane communication as well as TNF-RI signalling (Meiri, 2004; Muppidi et al., 2004), modulation of membrane lipids via FAN-dependent nSMase activity could participate in linking TNF signals to actin reorganisation. This is supported by a study from Hanna and coworkers who 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 (Hanna et al., 2001). Also, mutations of the PH or BEACH domains of FAN 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). Thus, it will be interesting to test a functional link between FAN and nSMase3 in TNF-induced actin reorganisation. For example, examining actin dependent responses like motility and chemotaxis in a nSMase deficient background will reveal the impact of nSMase3 on TNF signalling.

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

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