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Filopodia formation induced by active mDia2/Drf3

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Summary:

Filopodia are rod-shaped cell surface protrusions composed of a parallel bundle of actin filaments. Since filopodia frequently emanate from lamellipodia, it has been proposed that they form exclusively by the convergence and elongation of actin filaments generated in lamellipodia networks. However, filopodia form without Arp2/3-complex, which is essential for lamellipodia formation, indicating that actin filaments in filopodia may be generated by other nucleators. Here we analysed the effects of ectopic expression of GFP-tagged full length or a constitutively active variant of the human formin mDia2/Drf3. In contrast to the full-length molecule, which did not affect cell behaviour and was entirely cytosolic, active Drf3 lacking the C-terminal regulatory region (Drf3 Δ DAD) induced the formation of filopodia and accumulated at their tips. Low expression of Drf3 Δ DAD induced rod-shaped or tapered filopodia, whereas overexpression resulted in multiple, club-shaped filopodia. The clubs were filled with densely bundled actin filaments, whose number but not packing density decreased further away from the tip. Interestingly, clubs frequently increased in width after protrusion beyond the cell periphery, which correlated with increased amounts of Drf3 Δ DAD at their tips. These data suggest Drf3-induced filopodia form and extend by *de novo* nucleation of actin filaments instead of convergent elongation. Finally, Drf3 Δ DAD also induced the formation of unusual, lamellipodia-like structures, which contained both lamellipodial markers and the prominent filopodial protein fascin. Microarray analyses revealed highly variable Drf3 expression levels in different commonly used cell lines, reflecting the need for more detailed analyses of the functions of distinct formins in actin cytoskeleton turnover and different cell types.

Materials and Methods:

Expression constructs.

Sequences coding for N- and C-terminal fragments of human Drf3 were amplified from the cDNA clones BX649186 and BC048963 respectively, which were obtained from the Resource Centre of the German Human Genome Project (RZPD, Berlin). PCR fragments were combined using the internal BstXI restriction site and fused into the BglII and EcoRI sites of pEGFP-C1 (Clontech, Palo Alto, USA). Primers were as follows: DRF3 fwd GAGAGGATCCAAGATGGAACGGCACCAGCC, Drf3 rev GAGGAATTCTTAATACGGTTTATTAC, Drf3 internal fwd GAGAGATCTATGGAGGAGAGGAGC, Drf3 internal rev GAGAAGATCCACGGCTTTGGCCAATAAGGAA.

The C-terminally deleted active Drf3 (Drf3 Δ DAD) was generated by replacement of a PCR-amplified ClaI/SalI fragment (primers: Drf3mut fwd GAGGAAGATATTGAAGAGAAGAAATCGATTAAGA and Drf3mut rev CGCGTCGACTTTATTAATCACCTCAGTCTTCATTTCTAATAAACG) lacking residues 1056-1110.

Cells and Transfections. B16-F1 mouse melanoma cells (ATTC CRL-6323) and VA-13 human lung fibroblasts (ATCC CCL-75.1) were grown in DMEM high glucose (Gibco) supplemented with 10% FCS (EU, PAA, Austria), 2mM glutamine and penicillin/streptomycin, and transfected over night using Superfect (Quiagen) and FuGene (Roche) according to manufacturers' instructions, respectively. B16-F1 cells were subsequently seeded onto acid-washed glass coverslips, coated with 25 μ g/ml laminin, allowed to spread for app. 2-3 hours, and subjected to video microscopy or fixed as detailed below. Control and stable Nap1 knockdown VA-13 cell lines were maintained as described (Steffen

et al., 2004), plated on acid-washed glass coverslips coated with fibronectin (25µg/ml), and subjected to video microscopy 12-16 hours later.

Light Microscopy.

For fluorescence microscopy, cells expressing EGFP-tagged Drf3 were fixed with 4% formaldehyde (PFA) in phosphate-buffered saline (PBS) for 20 mins, extracted with 0.1% Triton X-100 for 1 min, and stained with Alexa594-coupled phalloidin (Invitrogen, Karlsruhe, Germany) or monoclonal antibodies directed against p16/ArpC5 (Olazabal *et al.*, 2002), cortactin (clone 289H10) or Abi-1, kindly provided by Giorgio Scita (Innocenti *et al.*, 2004). For fascin staining using monoclonal antibody 55K2 (Santa Cruz Biotechnology), cells were fixed with methanol. Alexa594-coupled secondary antibodies were from Invitrogen (Karlsruhe, Germany). The generation of monoclonal anti-cortactin antibodies will be described elsewhere (Lai *et al.*, unpublished).

Live cells were observed in an open heated chamber (Warner Instruments, UK) at 37°C on an inverted microscope (Axiovert 135TV, Zeiss, Jena, Germany) equipped for fluorescence and phase contrast microscopy, and with shutters (Optilas, Puchheim, Germany) in the transmitted and epifluorescence light paths controlled by an homemade interface. Data were acquired with a back-illuminated, cooled charge-coupled device camera (TE-CCD 800PB; Princeton Scientific Instruments, Princeton, USA) driven by IPLab software (Scanalytics, Fairfax, USA). Data were stored as 16-bit digital images and processed using IPLab, ImageJ and Adobe Photoshop CS software (Adobe Systems, Mountain View, USA).

Electron Microscopy.

For negative stain electron microscopy cells were grown on formvar films and processed essentially as described by (Auinger & Small, 2007). Briefly, cells were fixed for 2 mins in a mixture of Triton X-100 and glutaraldehyde in a cytoskeleton buffer (CB: 10mM MES,

150mM NaCl, 5mM EGTA, 5mM glucose, 5mM MgCl₂; pH 6,1), with added 1µg/ml phalloidin and post-fixed in 2% glutaraldehyde in the same buffer, including 1µg/ml phalloidin. They were then stained in a mixture of sodium silicotungstate (2%) and aurothioglucose (1%) and examined in an FEI morgagni electron microscope operating at 80kV.

DNA Microarray Hybridization and Analysis.

Cell lines used in this experiment were HeLaS3 (human cervix carcinoma CCL-2.2), A431 (human epidermoid carcinoma CRL-1555), VA-13 (human fibroblast SV40-transformed CCL-75.1), Caco-2 (colon carcinoma HTB-37), B16-F1 (mouse melanoma CRL-6323), NIH3T3 (mouse embryo fibroblast CRL-1658) and Swiss 3T3 (mouse embryo fibroblast CCL-92), and grown essentially as recommended by ATCC. Total RNA of 2×10^6 cells of each cell line was isolated and processed as described (Czuchra *et al.*, 2005), except that the Gene Chips were MOE 430 2.0 for murine and HG U133 2.0 for human samples, respectively, and analysed using Gene Chip Operating Software GCOS 1.4 (Affymetrix). Data for haematopoietic samples (PBM = human peripheral blood monocytes; THP-1, human acute monocytic leukaemia, TIB202) were extracted from the NCBI GEO data set GSE3280 (Gebhard *et al.*, 2006).

Introduction:

Non-muscle cells contain a large pool of globular monomeric actin, which upon appropriate stimuli can reversibly polymerize into filaments to alter cell shape and morphology. These processes involve Rho-family GTPases, which drive cell locomotion by regulating adhesion and protrusion at the front and de-adhesion/retraction at the rear (Hall, 1998, Small *et al.*, 1996). The two most prominent and best characterized protrusive organelles are lamellipodia and filopodia (Pollard & Borisy, 2003, Small *et al.*, 2002). According to our current knowledge, actin filaments in cells are nucleated by two major machineries, Arp2/3-complex and formins (Pollard, 2007). After activation by so-called nucleation promoting factors such as N-WASP or Scar/WAVE-complex proteins (Stradal & Scita, 2006), Arp2/3-complex is thought to amplify the generation of barbed, fast-growing actin filament ends through the formation of branches, the fate and dwell-time of which in live cells is still under debate (Goley & Welch, 2006, Resch *et al.*, 2002).

Formins nucleate actin filaments by a mechanism different from Arp2/3 complex (Faix & Grosse, 2006, Goode & Eck, 2007, Pollard, 2007). These dimeric multi-domain proteins recruit profilin-actin complexes by virtue of their proline-rich formin homology (FH) 1 - domains and *de novo* assemble linear actin filaments by their adjacent FH2 domains (Pruyne *et al.*, 2002). As processive motors, formins remain tightly bound to the fast growing barbed end of the actin filament adding monomers for elongation (Kovar & Pollard, 2004, Romero *et al.*, 2004). Diaphanous-related formins (Drfs) constitute a conserved subfamily that act as effectors of Rho-family GTPases (Watanabe *et al.*, 1997). The core FH1 and FH2 domains are flanked by a large N-terminal regulatory region containing the Diaphanous-inhibitory domain (DID) and an adjacent GTPase binding domain (GBD). In addition, they harbour a small C-terminal Diaphanous-autoregulatory domain (DAD). Binding of an activated Rho-GTPase to GBD releases an inhibitory intramolecular interaction between DID and DAD, leading to an

activated state (Alberts, 2001, Watanabe *et al.*, 1999). In addition, this release of autoinhibition appears to enable proper cellular localization (Seth *et al.*, 2006). Collectively, these observations suggest that specific cellular F-actin nucleators are employed to fulfil different cellular functions.

In contrast to lamellipodia, which are composed of a dense criss-cross meshwork of actin filaments generated by the Arp2/3 complex (Pollard & Borisy, 2003, Small *et al.*, 2002, Steffen *et al.*, 2006), filopodia are rod-shaped cell surface protrusions built of parallel actin filaments that are cross-linked into compact bundles that elongate by actin incorporation at their tips (Applewhite *et al.*, 2007, Faix & Rottner, 2006, Mallavarapu & Mitchison, 1999). Filopodia typically extend a few microns beyond the cell periphery and are found in a wide range of evolutionary distant organisms such as mammalian cells and *Dictyostelium* amoebae (Mitchison & Cramer, 1996, Schirenbeck *et al.*, 2005, Small, 1988). However, despite the large number of proteins implicated in filopodia formation our knowledge about the precise molecular mechanisms underlying their formation is still preliminary (Faix & Rottner, 2006, Gupton & Gertler, 2007). They can be formed downstream of Rho GTPases such as Cdc42 and Rif, but their relative relevance and the downstream signaling cascades are less well defined (Hall, 1998, Pellegrin & Mellor, 2005). For instance, although Cdc42 can efficiently induce filopodium formation, it is not essential for this process (Czuchra *et al.*, 2005). Similarly, N-WASP, which was originally believed to bridge Cdc42 signalling to Arp2/3-complex-mediated filopodia formation, is dispensable for this process (Lommel *et al.*, 2001, Snapper *et al.*, 2001). Finally, since filopodia frequently emerge from lamellipodial structures (Small *et al.*, 1999), it has been hypothesized that lamellipodia serve as precursors for filopodia (Biyasheva *et al.*, 2004, Mejillano *et al.*, 2004). In the model coined ‘the convergent elongation model of filopodia formation’, filopodial actin filaments arise from the dendritic network of Arp2/3-nucleated filaments by selective elongation, coalescence, and bundling by

proteins of the filopodium tip complex (Korobova & Svitkina, 2008, Svitkina *et al.*, 2003). However, recent loss of function studies of Arp2/3-complex or its activators using RNAi in B16-F1 mouse melanoma and human T cells indicated that filopodium formation can still occur normally in the absence of lamellipodia (Gomez *et al.*, 2007, Steffen *et al.*, 2006). Consistently, ablation of WAVE-complex components by gene disruption in *Dictyostelium* also showed no effects on filopodia formation (Steffen *et al.*, 2006). Collectively, these data imply that filopodial actin filaments may exclusively be formed through nucleators other than the Arp2/3-complex. In line with this finding, in *Dictyostelium* cells, the Diaphanous-related formin dDia2 was shown to be critical for filopodium formation (Schirenbeck *et al.*, 2005). Most notably, GFP-tagged dDia2 was shown to surf on distal tips of filopodia as they elongated (Schirenbeck *et al.*, 2005). From the large number of formin isoforms in mammalian cells, so far only two Drfs, namely mDia1/Drf1 and mDia2/Drf3, have been implicated in the assembly of filopodial actin filaments (Faix & Grosse, 2006). An active, N-terminally truncated form of mDia1 was reported to accumulate at the tips of filopodia-like structures in *Xenopus* fibroblasts (Higashida *et al.*, 2004), although this has not been confirmed by other studies. More strikingly, the Cdc42- and Rif-effector mDia2/Drf3 was linked to filopodia assembly and localized to their tips after co-overexpression with the active forms of these GTPases (Pellegrin & Mellor, 2005, Peng *et al.*, 2003, Wallar *et al.*, 2006). Finally, RNAi-mediated knockdown of mDia2/Drf3 was recently reported to impair not only filopodia, but also lamellipodia protrusion in B16-F1 cells. It was accordingly proposed that mDia2/Drf3 is recruited to the lamellipodium by direct interaction with the WAVE-complex component Abi-1, and drives the formation as well as the convergence of lamellipodia into filopodia (Yang *et al.*, 2007).

Here, we have characterized a novel, constitutively active version of human Drf3 as compared to the full-length formin by light and electron microscopy methods. Evidence is provided that the exaggerated filopodia assembly induced by active Drf3 is mediated through *de novo* actin

nucleation, not convergent elongation, and occurs in the virtual absence of functional WAVE-complex. Finally, expression patterns of this and related Drfs in a number of frequently used cell types were determined.

Results and Discussion:

We have previously shown that the product of a Drf3 cDNA (IMAGE clone BC048963) fused to EGFP localized to filopodia tips (Faix & Rottner, 2006). Although this cDNA was erroneously annotated as full-length, it lacked a 5'-sequence encoding 263 N-terminal amino acid residues, including N-terminal regulatory regions implicated in autoinhibition and potential surfaces of interaction with other cellular factors. Thus, we have now generated an EGFP-construct harbouring the full-length sequence (residues 1-1110). Similar to previous observations (Yang *et al.*, 2007), full-length Drf3, which could be readily detected in cell extracts at its expected molecular weight using anti-EGFP antibodies (not shown), was cytosolic and did not interfere with the motility of B16-F1 cells (Figure 1A, Supplementary Movie 1). This is consistent with data using myc-tagged murine Drf3 proteins (Pellegrin & Mellor, 2005, Wallar *et al.*, 2006), but not the EGFP-tagged filopodium formin dDia2 in *Dictyostelium* (Schirenbeck *et al.*, 2005).

It was reasonable to assume that the subcellular distribution of our ectopically expressed full length Drf3 was due to an intramolecular interaction between its regulatory DID and DAD domains, as shown for instance for mDia1 (Seth *et al.*, 2006). To study the localization and dynamics of an active Drf3 protein, we removed a small C-terminal region encompassing the DAD-domain (Drf3 Δ DAD), as indicated in Figure 1B. Transient expression of this active Drf3 variant caused a dramatic induction of filopodia formation, with dozens of individual filopodia formed at a given time (Figure 1C, Supplementary Movie 2). In addition, the truncated formin strongly accumulated at the tip of each filopodium, suggesting actin polymerisation to be powered at the tips of these structures by the ectopically expressed protein (Figure 1C, Supplementary Movie 2). Filopodia formation in most cells occurred at the expense of lamellipodia, indicative of a mutual antagonism between lamellipodia and filopodia formation in this motile cell type (Supplementary Movie 2 and not shown).

Depending on expression levels of the active formin, filopodia showed distinct morphologies. In strongly expressing cells, filopodia with club-shaped distal ends were observed (Figure 1D), whereas in cells exhibiting moderate expression tapered filopodia, similar in morphology to constitutively formed filopodia, were seen (Figure 1E). Similar clubs were recently described upon over-expression of an N-terminally deleted mDia2 variant (Yang *et al.*, 2007). Club-shaped distal ends suggest the protrusion of these filopodia results from imbalanced biochemical activities between the active formin at the tip and other tip or shaft factors required for filament bundling and length regulation. The question is whether the formin at the tip generates filaments by continuous nucleation, or whether its activity is restricted to mere elongation of pre-existing filaments, as proposed by the convergent elongation model. Yang *et al.* (2007) concluded club-shaped filopodia to be formed by a combination of convergent elongation in the tip with increased depolymerization in the shaft. However, our observations argue against this hypothesis as follows: First, filopodia induced at the cell periphery were frequently observed to thicken after they had separated from the surrounding cellular network (Figure 2). Filopodia thickening frequently coincided with an increase of fluorescence intensity of the GFP-tagged formin in the tip (Figure 2 and not shown), and comparison of mDia2 and phalloidin fluorescence revealed a robust correlation between active mDia2 and filament mass in the filopodium (Figure 3).

Electron microscopy of whole-mount specimens showed that the clubs contained bundles of closely packed actin filaments that decreased in number with distance from the tip (Figures 4B, C), giving rise to the tapered appearance. These structures were distinct from filopodia formed in non-transfected control B16-F1 cells (Figure 4A) that showed an uniform diameter of 0.1-0.2 μ m. In addition, the shafts of Drf3-induced filopodia consisted of a moderate number of long parallel filaments apparently protected from depolymerization (Figure 4B). Counts of individual filaments in Drf3-induced tips as indicated in Figure 5A revealed a linear correlation between tip width and filament number (Figure 5B), with a constant packing

density (Figure 5C). These observations and those on phalloidin labelling showed that tip thickening as observed by video microscopy (Figure 2) was not due to splicing of a constant number of filaments at their tips, but instead an increase in filament number, through additional nucleation events. Finally, the activity of the formin was apparently so strong that clubs branching off parent clubs could occasionally be observed (Figure 4D); this evidently occurs in the absence of nucleation by Arp2/3-complex, which does not localize to filopodia (Korobova & Svitkina, 2008, Svitkina & Borisy, 1999).

Collectively, these data can only be explained by continuous nucleation activity at the filopodium tip, presumably mediated by the formin, and concomitant depolymerization further behind. This mechanism is inconsistent with the “convergent elongation” of a limited number of pre-existing lamellipodial into filopodial filaments, and is in line with other findings showing that filopodia can form in the absence of lamellipodia (Gomez *et al.*, 2007, Steffen *et al.*, 2006). Yang *et al.* (2007) recently described an inhibition of lamellipodia formation upon depletion of mDia2/Drf3 and an interaction between mDia2/Drf3 and the WAVE-complex subunit Abi-1. They concluded accordingly that this formin not only is essential for filopodia formation *per se*, but drives them indirectly through elongating lamellipodial filaments and/or providing the mother filaments for Arp2/3 mediated generation of lamellipodia networks, in the absence of which filopodia formation would not occur. To test whether the active Drf3 variant (Drf3 Δ DAD) used here required lamellipodial filaments as precursors of filopodial filaments, we used VA-13 fibroblast cells stably suppressed for the WAVE-complex component Nap1, previously established to lack lamellipodia (Steffen *et al.*, 2004). The same cells were already shown to form filopodia upon injection of constitutively active Cdc42 (Steffen *et al.*, 2006), but formin functions such as that of Drf3 have not been explored in these cells. Mock siRNA-treated controls expressing Drf3 Δ DAD formed filopodia tipped by the EGFP-tagged formin as expected, although the response was not as pronounced as observed in B16-F1 (Supplementary Movie 3). More importantly, Nap1 knockdown cells,

which displayed strongly reduced levels of all WAVE-complex subunits including Abi (Steffen *et al.*, 2006) formed numerous Drf3 Δ DAD-driven filopodia (Supplementary Movie 4), indicating not only that this response can occur in the absence of functional WAVE-complex, but also that lamellipodial filaments are dispensable for the formation of Drf3-induced filopodia.

As mentioned above, most Drf3 Δ DAD overexpressors formed filopodia at the expense of lamellipodia, causing a virtually exclusive accumulation of the formin variant in the tips of finger-like plasma membrane extensions. However, in a small number of cells still capable of lamellipodia formation, active Drf3 also appeared enriched in the tip region of these protrusive sheet-like structures (see Supplementary Movie 5 and also Yang *et al.*, 2007). Notably, the dynamics of the active formin in the front region of these protrusions differed from canonical lamellipodial tip factors such as VASP (Rottner *et al.*, 1999) or WAVE-complex components (Steffen *et al.*, 2004, Stradal *et al.*, 2001). More specifically, the width of Drf3 enrichment was more variable than observed with the former components, and active Drf3 appeared to display more rapid lateral movements (see Supplementary Movie 3), which may be linked to tight association of the formin with laterally polymerising filaments (unpublished data). To test whether the structures accumulating Drf3 Δ DAD at their tips indeed constituted lamellipodia, Drf3 overexpressors were counterstained for the actin cytoskeleton with phalloidin or antibodies specific for the Arp2/3-complex subunit ArpC5 (p16). These data showed Drf3 enriched at the tip of actin filament networks reminiscent of lamellipodia (Figure 6), that were labelled by Arp2/3-complex antibodies (Figure 6), and by reagents specific for Arp2/3-complex activators, WAVE-complex (Abi) and cortactin (Figure 7). These data confirmed that formins and the Arp2/3-complex-machinery can in principle co-exist in the same subcellular location. Interestingly however, the same structures were also prominently labelled with the filopodial actin bundling protein fascin (Figure 7), that is normally enriched in microspikes and required for their formation in B16-F1 cells (Vignjevic

et al., 2006). These Drf3 Δ DAD-induced “lamellipodia” may therefore represent hybrids of lamellipodia and filopodia. Future studies will have to establish whether mDia2 and potentially other formins are components of genuine lamellipodia and whether they are indeed required for the nucleation of filaments in both lamellipodia and filopodia.

One important prerequisite for future analyses of formin function in mammalian cells and tissues is information about their relative expression in different cell types. Thus, we performed expression profiling of Drfs and other relevant actin regulators by microarray analyses of B16-F1 cells and two commonly used murine fibroblast cell lines (Swiss and NIH 3T3) as well as different human cell lines (Figure 8). Interestingly, Drf1 (Dia1) was expressed in all studied lines, whereas Drf2 and Drf3 were much less abundant in most cell lines. Specifically, and quite surprisingly, Drf3 was almost absent in our murine lines, including B16-F1 used in a recent RNAi study (Yang *et al.*, 2007), and in NIH 3T3 fibroblasts, as previously reported (Peng *et al.*, 2003, Tominaga *et al.*, 2000). In contrast, expression was observed in the human fibroblast cell lines VA-13 and epithelial-like lines HeLaS3 and Caco-2 as well as the leukaemia line THP-1. Thus, these human cell lines might be more appropriate for interference with endogenous Drf3 functions in future studies. Less surprisingly, the Arp2/3-complex, as monitored by the Arp3 subunit, was expressed at comparable levels in all lines, corroborating its key function in numerous actin-based processes (Goley & Welch, 2006). The expression levels of the Arp2/3-complex activators of the WASP and WAVE families, the latter known to be essential for lamellipodium protrusion (Stradal & Scita, 2006), were also consistent with previously published data. The expression pattern of haematopoietic WASP and ubiquitous N-WASP was mutually exclusive (Stradal *et al.*, 2004), and ubiquitous WAVE2 was more abundant in our lines than the more neuronal isoform WAVE1, and most prominently expressed in the motile B16-F1 cells. Finally, mRNA encoding for WAVE3, known to be largely restricted to the nervous system (Stradal *et al.*,

2004), was exclusively detected in VA-13 cells, the significance of which remains unknown. However, these results await confirmation by Western Blotting using isoform-specific antibodies.

In conclusion, we have shown that the active Drf3 variant Drf3 Δ DAD used here can prominently associate with both filopodia tips and lamellipodia-like structures. The strong filopodia induction observed upon Drf3 Δ DAD over-expression correlated with generation of club-like structures containing numerous actin filaments formed by *de-novo* nucleation. Future functional studies should address the relative relevance of Drf3 and potential additional formins in filopodia and perhaps lamellipodia formation in different cell types.

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Legends to Figures:

Figure 1. Expression of active Drf3 Δ DAD induces the formation of filopodia. (A) EGFP-tagged full-length Drf3 is entirely cytoplasmic. (B) Schematic illustration of the used Drf3 Δ DAD construct lacking amino acid residues 1056-1110. GBD: GTPase-binding domain; DID: Diaphanous-inhibitory domain; DD: Dimerisation domain; CC: Coiled coil; FH: Formin-homology domain; DAD: Diaphanous-autoinhibitory domain. (C) Drf3 Δ DAD expressing cell displaying numerous filopodia. (D) and (E) Filopodial protrusions formed in high and low Drf3 Δ DAD expressors, respectively. Scale bar in C is 10 μ m and valid for A and C. Scale bars in D and E are 5 μ m.

Figure 2. Spontaneous thickening of Drf3 Δ DAD-induced filopodia. Panels of time-lapse sequences of parts of Drf3 Δ DAD-overexpressers showing filopodia after having separated from the cell periphery. Note thickening of filopodia tips and concomitant increase in intensity (A) or widening of the fluorescence signal (arrows in B-D). Bar in D is valid for all panels and corresponds to 2 μ m.

Figure 3. B16-F1 cell over-expressing EGFP-tagged Drf3 Δ DAD and counterstained for phalloidin. Line-scans show fluorescence intensities as measured for F-actin and Drf3 Δ DAD in the tip regions of three distinct filopodia of variable prominence. Colour codes used in the curves at the bottom (curves A, B, C) highlight the different measurements as indicated in the images at the top. A robust correlation between Drf3 Δ DAD and F-actin amounts is observed in these filopodia. Bar equals 3 μ m.

Figure 4. Filopodia ultrastructure in control and Drf3 Δ DAD overexpressing cells. Transmission electron micrographs of negatively stained whole mount cytoskeletons of B16-F1 control cell (A) or Drf3 Δ DAD overexpressors (B-D). (A) Typical control filopodium formed in non-transfected B16-F1 cell, not displaying filopodial thickening in the tips frequently observed with Drf3 Δ DAD-induced filopodia (B-D). Note prominent actin filament accumulation in filopodia clubs, and a low number of long, linear filaments along the shafts (B, C). (D) shows representative example of filopodial club, branching off another. Scale bars are 500nm.

Figure 5. Analysis of filament numbers in Drf3 Δ DAD-induced filopodia. B16-F1 cells over-expressing EGFP-tagged Drf3 Δ DAD were subjected to manual counting of filaments crossing white lines drawn across tip or shaft regions as indicated (A). Scale bar equals 500nm. (B) Filament numbers as assessed from tips of filopodial clubs plotted versus tip widths (N=22). (C) Comparison of filament numbers in tips and shafts of Drf3 Δ DAD-induced filopodia as indicated, which corresponded on average to 10.68 ± 1.19 and 10.42 ± 1.13 filaments per 100nm, respectively.

Figure 6. Drf3 Δ DAD can target to the tip of a lamellipodium-like structure. EGFP-tagged Drf3 Δ DAD accumulation at the tips of lamellipodial actin filament networks, as stained by phalloidin (top), and harbouring Arp2/3-complex (bottom) as indicated. Bars are 10 μ m.

Figure 7. Drf3 Δ DAD-induced lamellipodia-like structures contain Arp2/3-complex activators and fascin. B16-F1 cells expressing EGFP-tagged Drf3 Δ DAD were counterstained for the WAVE-complex component Abi, cortactin and fascin as indicated. Note prominent co-

accumulation of all Drf3 Δ DAD with the respective component. Asterisk in fascin image highlights typical label on microspikes (Vignjevic *et al.*, 2006) embedded into the lamellipodium of a neighbouring, non-transfected cell. Scale bar equals 10 μ m.

Figure 8. Microarray analyses of cell lines as indicated. (A) Expression levels of Drf1-3 normalized to β -actin. Note most prominent Drf3 expression in HeLaS3, VA-13 and THP-1. (B) Arp3, (C) N-WASP/WASP and (D) WAVE isoform expression as normalized to β -actin. Black solid and dashed lines in A, C and D indicate average background levels $\pm$ standard deviation from all Gene Chips.

References:

- Alberts, A. S. (2001) Identification of a carboxyl-terminal diaphanous-related formin homology protein autoregulatory domain. *J Biol Chem*, **276**, 2824-2830.
- Applewhite, D. A., Barzik, M., Kojima, S., Svitkina, T. M., Gertler, F. B. & Borisov, G. G. (2007) Ena/VASP proteins have an anti-capping independent function in filopodia formation. *Mol Biol Cell*, **18**, 2579-2591.
- Auinger, S. & Small, J. V. (2007) Correlated Light and Electron Microscopy of the Cytoskeleton. In: *Methods in Cell Biology, Basic Biological Electron Microscopy* (ed. T. D. Allen). Academic Press.
- Biyasheva, A., Svitkina, T., Kunda, P., Baum, B. & Borisov, G. (2004) Cascade pathway of filopodia formation downstream of SCAR. *J Cell Sci*, **117**, 837-848.
- Czuchra, A., Wu, X., Meyer, H., van Hengel, J., Schroeder, T., Geffers, R., Rottner, K. & Brakebusch, C. (2005) Cdc42 is not essential for filopodium formation, directed migration, cell polarization, and mitosis in fibroblastoid cells. *Mol Biol Cell*, **16**, 4473-4484.
- Faix, J. & Grosse, R. (2006) Staying in shape with formins. *Dev Cell*, **10**, 693-706.
- Faix, J. & Rottner, K. (2006) The making of filopodia. *Curr Opin Cell Biol*, **18**, 18-25.
- Gebhard, C., Schwarzfischer, L., Pham, T. H., Schilling, E., Klug, M., Andreesen, R. & Rehli, M. (2006) Genome-wide profiling of CpG methylation identifies novel targets of aberrant hypermethylation in myeloid leukemia. *Cancer Res*, **66**, 6118-6128.
- Goley, E. D. & Welch, M. D. (2006) The ARP2/3 complex: an actin nucleator comes of age. *Nat Rev Mol Cell Biol*, **7**, 713-726.
- Gomez, T. S., Kumar, K., Medeiros, R. B., Shimizu, Y., Leibson, P. J. & Billadeau, D. D. (2007) Formins regulate the actin-related protein 2/3 complex-independent polarization of the centrosome to the immunological synapse. *Immunity*, **26**, 177-190.
- Goode, B. L. & Eck, M. J. (2007) Mechanism and function of formins in the control of actin assembly. *Annu Rev Biochem*, **76**, 593-627.
- Gupton, S. L. & Gertler, F. B. (2007) Filopodia: the fingers that do the walking. *Sci STKE*, **2007**, re5.
- Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science*, **279**, 509-514.
- Higashida, C., Miyoshi, T., Fujita, A., Ocegueda-Yanez, F., Monypenny, J., Andou, Y., Narumiya, S. & Watanabe, N. (2004) Actin polymerization-driven molecular movement of mDial1 in living cells. *Science*, **303**, 2007-2010.
- Innocenti, M., Zucconi, A., Disanza, A., Frittoli, E., Areces, L. B., Steffen, A., Stradal, T. E., Di Fiore, P. P., Carlier, M. F. & Scita, G. (2004) Abi1 is essential for the formation and activation of a WAVE2 signalling complex. *Nat Cell Biol*, **6**, 319-327.
- Korobova, F. & Svitkina, T. (2008) Arp2/3 Complex Is Important for Filopodia Formation, Growth Cone Motility and Neuritegenesis in Neuronal Cells. *Mol Biol Cell*.
- Kovar, D. R. & Pollard, T. D. (2004) Insertional assembly of actin filament barbed ends in association with formins produces piconewton forces. *Proc Natl Acad Sci U S A*, **101**, 14725-14730.
- Lommel, S., Benesch, S., Rottner, K., Franz, T., Wehland, J. & Kuhn, R. (2001) Actin pedestal formation by enteropathogenic Escherichia coli and intracellular motility of Shigella flexneri are abolished in N-WASP-defective cells. *EMBO Rep*, **2**, 850-857.
- Mallavarapu, A. & Mitchison, T. (1999) Regulated actin cytoskeleton assembly at filopodium tips controls their extension and retraction. *J Cell Biol*, **146**, 1097-1106.

- Mejillano, M. R., Kojima, S., Applewhite, D. A., Gertler, F. B., Svitkina, T. M. & Borisy, G. G. (2004) Lamellipodial versus filopodial mode of the actin nanomachinery: pivotal role of the filament barbed end. *Cell*, **118**, 363-373.
- Mitchison, T. J. & Cramer, L. P. (1996) Actin-based cell motility and cell locomotion. *Cell*, **84**, 371-379.
- Olazabal, I. M., Caron, E., May, R. C., Schilling, K., Knecht, D. A. & Machesky, L. M. (2002) Rho-kinase and myosin-II control phagocytic cup formation during CR, but not FcgammaR, phagocytosis. *Curr Biol*, **12**, 1413-1418.
- Pellegrin, S. & Mellor, H. (2005) The Rho family GTPase Rif induces filopodia through mDia2. *Curr Biol*, **15**, 129-133.
- Peng, J., Wallar, B. J., Flanders, A., Swiatek, P. J. & Alberts, A. S. (2003) Disruption of the Diaphanous-related formin Drf1 gene encoding mDia1 reveals a role for Drf3 as an effector for Cdc42. *Curr Biol*, **13**, 534-545.
- Pollard, T. D. (2007) Regulation of actin filament assembly by Arp2/3 complex and formins. *Annu Rev Biophys Biomol Struct*, **36**, 451-477.
- Pollard, T. D. & Borisy, G. G. (2003) Cellular motility driven by assembly and disassembly of actin filaments. *Cell*, **112**, 453-465.
- Pruyne, D., Evangelista, M., Yang, C., Bi, E., Zigmond, S., Bretscher, A. & Boone, C. (2002) Role of formins in actin assembly: nucleation and barbed-end association. *Science*, **297**, 612-615.
- Resch, G. P., Goldie, K. N., Krebs, A., Hoenger, A. & Small, J. V. (2002) Visualisation of the actin cytoskeleton by cryo-electron microscopy. *J Cell Sci*, **115**, 1877-1882.
- Romero, S., Le Clainche, C., Didry, D., Egile, C., Pantaloni, D. & Carlier, M. F. (2004) Formin is a processive motor that requires profilin to accelerate actin assembly and associated ATP hydrolysis. *Cell*, **119**, 419-429.
- Rottner, K., Behrendt, B., Small, J. V. & Wehland, J. (1999) VASP dynamics during lamellipodia protrusion. *Nat Cell Biol*, **1**, 321-322.
- Schirenbeck, A., Bretschneider, T., Arasada, R., Schleicher, M. & Faix, J. (2005) The Diaphanous-related formin dDia2 is required for the formation and maintenance of filopodia. *Nat Cell Biol*, **7**, 619-625.
- Seth, A., Otomo, C. & Rosen, M. K. (2006) Autoinhibition regulates cellular localization and actin assembly activity of the diaphanous-related formins FRLalpha and mDia1. *J Cell Biol*, **174**, 701-713.
- Small, J. V. (1988) The actin cytoskeleton. *Electron Microsc Rev*, **1**, 155-174.
- Small, J. V., Anderson, K. & Rottner, K. (1996) Actin and the coordination of protrusion, attachment and retraction in cell crawling. *Biosci Rep*, **16**, 351-368.
- Small, J. V., Kaverina, I., Krylyshkina, O. & Rottner, K. (1999) Cytoskeleton cross-talk during cell motility. *FEBS Lett*, **452**, 96-99.
- Small, J. V., Stradal, T., Vignat, E. & Rottner, K. (2002) The lamellipodium: where motility begins. *Trends Cell Biol*, **12**, 112-120.
- Snapper, S. B., Takeshima, F., Anton, I., Liu, C. H., Thomas, S. M., Nguyen, D., Dudley, D., Fraser, H., Purich, D., Lopez-Illasaca, M., Klein, C., Davidson, L., Bronson, R., Mulligan, R. C., Southwick, F., Geha, R., Goldberg, M. B., Rosen, F. S., Hartwig, J. H. & Alt, F. W. (2001) N-WASP deficiency reveals distinct pathways for cell surface projections and microbial actin-based motility. *Nat Cell Biol*, **3**, 897-904.
- Steffen, A., Faix, J., Resch, G. P., Linkner, J., Wehland, J., Small, J. V., Rottner, K. & Stradal, T. E. (2006) Filopodia formation in the absence of functional WAVE- and Arp2/3-complexes. *Mol Biol Cell*, **17**, 2581-2591.
- Steffen, A., Rottner, K., Ehinger, J., Innocenti, M., Scita, G., Wehland, J. & Stradal, T. E. (2004) Sra-1 and Nap1 link Rac to actin assembly driving lamellipodia formation. *Embo J*, **23**, 749-759.

- Stradal, T., Courtney, K. D., Rottner, K., Hahne, P., Small, J. V. & Pendergast, A. M. (2001) The Abl interactor proteins localize to sites of actin polymerization at the tips of lamellipodia and filopodia. *Curr Biol*, **11**, 891-895.
- Stradal, T. E., Rottner, K., Disanza, A., Confalonieri, S., Innocenti, M. & Scita, G. (2004) Regulation of actin dynamics by WASP and WAVE family proteins. *Trends Cell Biol*, **14**, 303-311.
- Stradal, T. E. & Scita, G. (2006) Protein complexes regulating Arp2/3-mediated actin assembly. *Curr Opin Cell Biol*, **18**, 4-10.
- Svitkina, T. M. & Borisy, G. G. (1999) Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J Cell Biol*, **145**, 1009-1026.
- Svitkina, T. M., Bulanova, E. A., Chaga, O. Y., Vignjevic, D. M., Kojima, S., Vasiliev, J. M. & Borisy, G. G. (2003) Mechanism of filopodia initiation by reorganization of a dendritic network. *J Cell Biol*, **160**, 409-421.
- Tominaga, T., Sahai, E., Chardin, P., McCormick, F., Courtneidge, S. A. & Alberts, A. S. (2000) Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. *Mol Cell*, **5**, 13-25.
- Vignjevic, D., Kojima, S., Aratyn, Y., Danciu, O., Svitkina, T. & Borisy, G. G. (2006) Role of fascin in filopodial protrusion. *J Cell Biol*, **174**, 863-875.
- Waller, B. J., Stropich, B. N., Schoenherr, J. A., Holman, H. A., Kitchen, S. M. & Alberts, A. S. (2006) The basic region of the diaphanous-autoregulatory domain (DAD) is required for autoregulatory interactions with the diaphanous-related formin inhibitory domain. *J Biol Chem*, **281**, 4300-4307.
- Watanabe, N., Kato, T., Fujita, A., Ishizaki, T. & Narumiya, S. (1999) Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. *Nat Cell Biol*, **1**, 136-143.
- Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M. & Narumiya, S. (1997) p140mDia, a mammalian homolog of *Drosophila* diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *Embo J*, **16**, 3044-3056.
- Yang, C., Czech, L., Gerboth, S., Kojima, S., Scita, G. & Svitkina, T. (2007) Novel roles of formin mDia2 in lamellipodia and filopodia formation in motile cells. *PLoS Biol*, **5**, e317.

SUPPLEMENTARY INFORMATION

Supplementary Movies:

Movie 1. B16-F1 cell moving on laminin and expressing EGFP-tagged full-length human Drf3. Display rate is 330 fold. Bar is 10 μ m.

Movie 2. B16-F1 cell overexpressing EGFP-tagged, active Drf3. Display rate is 330 fold. Bar is 10 μ m.

Movie 3. Control siRNA-treated (Control RNAi) VA-13 fibroblast cell (Steffen *et al.*, 2006) transiently transfected with Drf3 Δ DAD. Display rate is 330 fold. Bar is 10 μ m.

Movie 4. Nap1 knockdown VA-13 fibroblast (Nap1 RNAi) transiently expressing Drf3 Δ DAD. Note prominent formation of filopodia tipped by EGFP-tagged Drf3 Δ DAD in spite of WAVE-complex loss of function and coincident lack of lamellipodia (Steffen *et al.*, 2004). Display rate is 330 fold. Bar is 10 μ m.

Movie 5. B16-F1 cell moving on laminin and expressing EGFP-tagged, active Drf3. Note the accumulation of Drf3 at the tips of both filopodia and lamellipodia-like structures. Display rate is 330 fold. Bar is 10 μ m.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8