Profilin connects Actin Assembly with Microtubule dynamics

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Abstract

Profilin controls actin nucleation and assembly processes in eukaryotic cells. Actin nucleation and elongation promoting factors (NEPFs) such as Ena/VASP, formins and WASP family proteins recruit profilin:actin for filament formation; some of these are found to be microtubule-associated, making actin polymerization from microtubule-associated platforms possible. Microtubules are implicated in focal adhesion turn-over, cell polarity establishment and migration, illustrating the coupling between actin and microtubule systems. Here we demonstrate that profilin is functionally linked to microtubules with formins and point to formins as major mediators of this association. To reach this conclusion we combined different fluorescence microscopy techniques including super resolution microscopy with siRNA-modulation of profilin expression and drug treatments to interfere with actin dynamics. Our studies show that profilin dynamically associates with microtubules and that this fraction of profilin contributes to balance actin assembly during homeostatic cell growth and impacts on microtubule dynamics. Hence profilin functions as a regulator of microtubule (+)-end turn-over in addition to being an actin control element.

List of abbreviations

NEPFs	Actin nucleation and elongation factors
Ena/VASP	Enabled/vasodilator-stimulated phosphoprotein
WASP	Wiskott-Aldrich syndrome protein
Srv2/CAP	Cyclase-associated protein
Arp2/3	Actin-Related Proteins 2/3
+TIPs	Microtubule plus-end-tracking proteins
APC	Adenomatous polyposis coli protein
Dia 1	Diaphaneous 1
EB-protein	End binding protein
WHAMM	Wasp homolog associated with actin, membranes and microtubules
WASH	Wasp and Scar homolog
STED microscopy	Stimulated emission depletion microscopy
GFP	Green fluorescent protein
TIRF microscopy	Total internal reflection fluorescence microscopy
CytD	Cytochalasin D
Jasp	Jasplakinolide
FH1	Formin homology 1
FH2	Formin homology 2
SMIFH2	Small molecule inhibitor of formin homology 2
HDAC6	Histone deacetylase 6
α-TAT	α-tubulin acetyltransferase
PLA	Proximity ligation assay
Pfn	Profilin
GAPDH	Glyceraldehyde phosphate dehydrogenase
CTN-Pfn	Citrine-profilin

MAPs	Microtubule associated proteins
GST	Glutathione S-transferase

Introduction

Actin polymerization, the directional growth of actin filaments as a consequence of ordered addition of new actin subunits at the favored (+)-end of the filament, is a fundamental and tightly regulated process required for numerous cellular phenomena. The structural and biochemical asymmetry of the filaments form the basis for the directional force-generation. This is kinetically maintained by hydrolysis of ATP on the incoming actin subunit shortly after its association at the filament (+)-end (Melki *et al.*, 1996; Nyman *et al.*, 2002b) and is under control of an array of actin binding proteins where different (+)-end tracking proteins (commonly referred to as nucleation and elongation promoting factors; NEPFs) such as VASP and formins govern processivity and elongation speed (Bugyi and Carlier, 2010; Grantham *et al.*, 2012).

Although the importance of actin for cell migration and particularly for the advancement of the cell edge is well established e.g. (Pollard and Borisy, 2003; Le Clainche and Carlier, 2008), it has been known for long that directional cell movement typically also requires an intact microtubule system (Vasiliev *et al.*, 1970; Tint *et al.*, 1991; Kaverina *et al.*, 1998). It is now clear that the two force-generating systems operate closely together to coordinate cell architectonics and behavior (Small *et al.*, 2002; Rodriguez *et al.*, 2003; Chesarone *et al.*, 2010; Rottner *et al.*, 2010). However, many details of the actin-microtubule interplay remain to be resolved, not least with respect to the possible coordination of microtubule-dependent cargo transport and actin polymerization, e.g. (Martin *et al.*, 2005; Shen *et al.*, 2012). Here we address this issue with a focus on profilin and its role as a central control-component of actin dynamics and assembly.

The profilin:actin complex represents the single most important species of polymerization-competent non-filamentous actin in most cells (Kaiser *et al.*, 1999; Lindberg *et al.*, 2008; Pernier *et al.*, 2016) where it operates by providing ATP-bound actin to NEPFs in support of the propulsion of various intracellular structures as well as formation and elongation of surface protrusions such as lamellipodia and filopodia (Suetsugu *et al.*, 1998; Hajkova *et al.*, 2000; Grenklo *et al.*, 2003; Li *et al.*, 2008). Actin dissociating from filament (-)-ends is sequestered by cofilin and, via not fully understood processes involving the cyclase associated protein (Srv2/CAP) and coronin e.g. (Chaudhry *et al.*; Bertling *et al.*, 2007; Mikati *et al.*,

2015), the actin monomers re-associate with profilin, are recharged with ATP and ready for a new round of NEPF-controlled polymerization. Recent observations demonstrate that profilin has a key-pivoting role to balance actin availability for different NEPF-driven actin assembly processes particularly by favoring formin and VASP driven polymerization over Arp2/3-dependent filament formation (Henty-Ridilla and Goode, 2015; Rotty *et al.*, 2015; Suarez *et al.*, 2015). The role of profilin as a central coordinator of actin filament barbed-end growth is also emphasized by observations made in vitro (Pernier *et al.*, 2016).

As with actin, the microtubule system is subject to extensive dynamics also in interphase cells where microtubule (+)-ends extend through the cytoplasm towards the cell edge. Not only are the microtubule (+)-ends engaged in association/dissociation of α/β-tubulin heterodimers but also harbor a dynamic complex of associated proteins, the so called +TIPs (Akhmanova and Steinmetz, 2008; Gupta *et al.*, 2014) involved in controlling microtubule (+)-end dynamics and function. Of these the adenomatous polyposis coli protein (APC) and the end binding (EB)-proteins have been shown to interact with Diaphaneous (Dia)-related formins, i.e. a major actin NEPF-family (Wen *et al.*, 2004; Okada *et al.*, 2010). Hence the +TIP-complex forms a molecular link to actin organization. Other components that are likely to participate in organizing actin dynamics in association with microtubules are WHAMM and WASH which are microtubule-binding members of the WASP superfamily of proteins and operate along with Arp2/3 to nucleate actin polymerization (Campellone *et al.*, 2008; Gad *et al.*, 2012; Shen *et al.*, 2012; Blom *et al.*, 2015). Moreover, formins have also been observed to associate along microtubule polymers and to influence their stability (Bartolini *et al.*, 2008; Bartolini *et al.*, 2012; Thurston *et al.*, 2012).

We previously noted a partial colocalization of profilin with microtubules in human fibroblasts (Grenklo *et al.*, 2004) and later reported that the proper distribution of profilin mRNA depends on microtubules (Johnsson and Karlsson, 2010). Profilin (unless otherwise stated profilin refers to the ubiquitous profilin isoform I) has been located in a broad range of cultured cells by the use of different profilin antibodies and fluorescence microscopy e.g. (Mayboroda *et al.*, 1997; Grenklo *et al.*, 2004; Li *et al.*, 2008). Typically the protein takes a general distribution, sometimes in a fenestrated pattern of fine dots, and accumulates towards the perinuclear region as well as in a thin ‘band’ along the leading cell edge. In the current study we show that profilin influences the control of microtubule dynamics further adding to

the conjecture of a close actin-microtubule interrelationship and underscoring profilin as a unique regulator of force-generation and cellular behavior in eukaryotes.

Results

Profilin codistributes with microtubules

Studies of the specific subcellular localization of profilin is complicated by an apparent over-all fluorescence, reflecting its abundance and juxta-membrane accumulation separately or in complex with actin (profilin:actin). This prevents the visualization of specific but less common cytoplasmic niches other than the plasma membrane where the protein may be located. To circumvent the problem with the disguising diffuse fluorescence, we introduced a brief detergent treatment under microtubule stabilizing conditions before the cells were fixed (Figure 1A). With the over-all diffuse fluorescence dramatically reduced, a partial labeling of the microtubule system was clearly revealed. Independent employment of the proximity ligation assay (Soderberg *et al.*, 2006) without detergent pretreatment and using different subsets of profilin:actin antibodies in combination with antibodies to tubulin and kinesin further supported a localization of profilin at or in close proximity with microtubules (Figures 1B, C and Supplementary Figure 1). This was also observed by super-resolution stimulated emission depletion (STED) microscopy where the profilin staining was found to decorate a majority of the microtubules in a fine-dotted pattern (Figure 1D).

Prompted by these results we decided to analyze the microtubule-profilin association further with an approach where cells, prior to lysis, were incubated with the microtubule stabilizing and destabilizing drugs Taxol and Nocodazole, respectively. The resulting extracts were then centrifuged to partition microtubules with their associated components from the rest of the material. Western blotting of the samples showed co-sedimentation of profilin with the microtubules after Taxol-treatment (Figure 2A). In contrast, the corresponding samples of non-drug treated cells, or cells exposed to Nocodazole, displayed dramatically less profilin in the pelleted fraction, essentially confirming the immuno-histochemical results of a profilin-microtubule interaction. Densitometry of the Western blot result demonstrated a ca 4 times increased amount of profilin in the pelleted material after Taxol-treatment compared to untreated cells (Figure 2B). Based on the above results we conclude that a fraction of total cellular profilin is associated with the microtubule system. We then decided to over-express a profilin-citrine fusion construct in order to increase the yield in co-immunoprecipitation experiments where antibodies to GFP/citrine were employed. Under such conditions, tubulin

was detected as a binding-partner to the fusion-protein, and this result was corroborated by Total Internal Reflection Fluorescence (TIRF)-microscopy of cells where the profilin-citrine fusion was found to codistribute with the microtubules (Figure 2C-E).

Tubulin has been captured from a brain tissue extract on a profilin column (Witke *et al.*, 1998), but no compelling evidence has been presented for a direct interaction between the two proteins. This issue was addressed here by in vitro experiments where profilin was either combined with preformed microtubules or with tubulin prior to the onset of polymerization, (Supplementary Figure 2). Since no interaction or influence of profilin was observed in these assays, we conclude that the observations above reflect an indirect recruitment of profilin to the microtubule system.

Modulating actin dynamics shifts profilin to and fro the microtubule compartment

We then turned to investigate whether the association of profilin with actin was related to its codistribution with the microtubules. Exposing cells to Cytochalasin D (CytD), which blocks filament barbed end elongation or Jasplakinolide (Jasp), which increases actin polymerization by stabilizing the filaments led to an increase of profilin along microtubules (Figure 3A and B). The shifted localization of profilin to the microtubule-based compartment was readily observed by standard fluorescence microscopy using demembranation prior to fixation as described above (Figure 1). That the two drugs did not cause a variable result despite their different interference with actin polymerization is likely to reflect that we locate both profilin and profilin:actin by the microscopy analysis. Interfering with actin polymerization by CytD will increase the concentration of monomeric actin and therefore augment formation of profilin:actin, while the filament stabilizing effect of Jasp may have the opposite effect on the pool of non-filamentous actin and thereby increase the concentration of "free" profilin. This interpretation explains why we observed an increased amount of profilin associated with microtubules after both drug treatments. In order to test whether a massive upregulation of lamellipodia formation with major actin polymerization and engagement of profilin would alter profilin's codistribution with microtubules we exposed the cells to AlF_4. This is known to dramatically increase actin polymerization due to extensive activation of Rho GTPases and thereby cause formation of broad lamellipodia at advancing cell edges (Hahne *et al.*, 2001). Accordingly, the AlF_4-treated cells typically displayed an upregulated motility under these conditions, and, this was coupled to a dramatic decrease of profilin codistribution with microtubules (Figure 4). Hence we conclude that the actin polymerization status balances the

location of profilin to the microtubule system, extending the emerging view of profilin as a pivot for actin monomers between different assembly organizations in the cell (Henty-Ridilla and Goode, 2015).

Possible profilin-microtubule linker molecules

There are several actin assembly components that may be involved in connecting profilin to the microtubule system. The WASP-related protein WHAMM is one of those. It is known to bind microtubules and operates as an actin nucleation promoting factor due to a W-domain that is preceded by a proline-rich sequence (Campellone *et al.*, 2008; Rottner *et al.*, 2010), and Supplementary Figure 3A and B). Formins represent another family of proteins in this category of microtubule-binding components that carries a proline-rich sequence (Rottner *et al.*, 2010; Thurston *et al.*, 2012). By virtue of the well documented polyproline-binding activity of profilin, we hypothesized that either or both of these actin NEPFs were involved in linking profilin and/or profilin:actin to the microtubules. While the interaction between profilin and formins is well established it was necessary to test whether profilin expresses a similar interaction property for WHAMM. To that end a GST-fusion of a truncated WHAMM construct (WHAMM/C; residues 559-809) that contains a polyproline-sequence was expressed in E.coli and used for pull-down experiments from cell lysates followed by Western blot analysis with antibodies to profilin. The result showed that under these conditions profilin indeed is an interaction partner to WHAMM (Supplementary Figure 3C).

Like other WASP subfamily of proteins, WHAMM requires the Arp2/3-complex to function as an actin nucleator. Since this group of actin NEPFs constitutes a central mechanism for balancing profilin-controlled actin assembly (Rotty *et al.*, 2015; Suarez *et al.*, 2015), we analyzed this activity with respect to the profilin-microtubule interaction using the Arp2/3 inhibitor CK-666 and siRNA down-regulation of WHAMM expression, respectively. However, none of these approaches resulted in an altered distribution of profilin to microtubules as observed after fluorescence microscopy of pre-demembranated B16 cells (Supplementary Figure 3D), suggesting that WHAMM/Arp2/3 is not a major component in the recruitment of profilin/profilin:actin to the microtubules.

We then turned to formins. This family of actin NEPFs has been shown to distribute along microtubules (Thurston *et al.*, 2012) and are independent of Arp2/3 for their NEPF-activity. Incubation of the cells with the formin inhibitor SMIFH2, which binds to formin FH2-

domains and interferes with their binding to both microtubules and filamentous actin (Rizvi *et al.*, 2009; Goldspink *et al.*, 2013), led to a significant decrease of the profilin codistribution with microtubules, strongly indicating formins as central component for recruiting profilin/profilin-actin to microtubules (Figure 5). Of note in this context is that we kept the SMIFH2-exposure low (25 µM, 30 min) in order to avoid confounding effects as reported by (Isogai *et al.*, 2015).

The role of formins as profilin-microtubule linker molecules was then further examined by siRNA down-regulation of Dia 1 and 2. Of these, the expression of Dia 2 is the least abundant in B16 cells (Block *et al.*, 2008). A reduced expression of either of the two isoforms separately did not result in a significant influence on the extent of profilin along the microtubules. However, by combining the two siRNAs causing simultaneous downregulation of both formins, a reduction in microtubule-associated profilin was scored (Figure 5C-E) in agreement with the result after the SMIFH2-exposure reported above. The reduced colocalization seen after combining the siRNAs was less extensive than after treatment with SMFH2, suggesting that other variants of formins may contribute to the microtubule-recruitment of profilin.

Based on the above results we conclude that formins play a central role in distributing profilin to microtubules and that this localization of profilin shifts with the activity status of the cell. The ability to associate with microtubules is shared by most formins as is their profilin/profilin:actin-binding capacity, suggesting that the association of profilin to microtubules via formins is a general phenomenon, see further below and the discussion.

Profilin impacts on microtubule growth dynamics

The view that microtubules control cell polarity and migration was put forward already during the 1970's (Vasiliev *et al.*, 1970) and in support of this conjecture several studies have since then reported on an intimate relationship between the microtubule and actin microfilament systems (see introduction and references in (Coles and Bradke, 2015). Together with our observations above, this led us to assess whether profilin affected the organization of the microtubule array. We used siRNA to deplete profilin and analyzed two criteria for microtubule dynamics: α-tubulin acetylation and growth characteristics of microtubule ends. The former reflects microtubule stability; the longer individual polymers exist the more their α-tubulin subunits become acetylated, which allowed us to use this post-translational

modification as a “timer” for microtubule turn-over under control and profilin depleted conditions.

Comparison of extracts from control cells with those transfected with profilin siRNA revealed a significant increase of acetylated α-tubulin in the latter (Figure 6 and Supplementary Figure 4). Analysis of the expression of HDAC6 and α-TAT, the principal enzymes responsible for tubulin deacetylation and acetylation, respectively, did not reveal any variation compared to cells transfected with control siRNA (data not shown). We therefore conclude that cells expressing less profilin contain a larger fraction of long-lived microtubules.

To investigate if modulating profilin expression had an effect on microtubule (+)-end growth, we transiently transfected cells with GFP-labeled End Binding protein 3 (EB3) in combination with control siRNA or profilin siRNA (Figure 7 and Supplemental Movies 1-2). Tracing >160 microtubule ends, respectively, in the two categories of transfected cells demonstrated that down-regulation of profilin expression increased microtubule growth rate 1.6 times. Reasoning that more rapidly growing microtubules might be reflected by a shorter distance between polymer ends and the leading edge of extending lamellipodia led us to measure this distance for >600 microtubules in approximately 40 cells (Figure 8). The result showed a major decrease of the distance between microtubule ends and lamellipodial tips; for control cells 50% of the microtubules ended within 0.5 µm from the edge while in cells expressing reduced amounts of profilin this fraction had increased to 80%, further underscoring the view that profilin plays a role in the control of microtubule growth.

In conclusion, down-regulation of profilin expression results in more long-lived microtubules that extend their (+)-end tips with increased speed towards the cell edge compared to cells under non-perturbed profilin conditions. Together, these experiments therefore suggest that under non-disturbed homeostatic growth, profilin increases microtubule dynamics by increasing the rate and/or frequency by which microtubules depolymerize from their (+)-ends. We conclude that profilin is not only a regulator of actin polymerization and a pivot, balancing different actin assembly forms but also has a role in the control of microtubule dynamics, affecting microtubule extension into the peripheral region of advancing cell edges.

Discussion

Cell motility is a highly combinatorial phenomenon involving numerous signaling and force-generating processes where the dynamically organized microtubule and microfilament systems function as ultimate and coordinated generators of directional force (Pollard and Borisy, 2003; Rodriguez *et al.*, 2003; Field and Lenart, 2011). Here, we provide evidence that profilin, a central control component of actin assembly, well-known as a regulator of actin polymerization (Carlsson *et al.*, 1977); reviewed by (Witke, 2004; Jockusch *et al.*, 2007; Karlsson and Lindberg, 2007) and recently proposed to balance different NEPF-driven filament assembly-formations (Rotty *et al.*, 2015; Suarez *et al.*, 2015) and coordinate filament barbed end growth (Pernier *et al.*, 2016; Shekhar *et al.*, 2016), also is linked to the control of microtubule dynamics via an indirect interaction mediated by formins.

That a thoroughly characterized protein such as profilin, which has been studied since the 1970's now is found to distribute to the discrete and easily recognized microtubule array in cultured cells may appear unexpected. However, a retrospective scrutiny of earlier localization studies of profilin by fluorescence microscopy from other laboratories (Buss *et al.*, 1992; Mayboroda *et al.*, 1997) as well as from our published work (Hájková, 1999; Skare *et al.*, 2003) reveals that such a microtubule localization was hinted long before we reported that profilin indeed partially codistribute with microtubules in human fibroblasts (Grenklo *et al.*, 2004). More recently this was confirmed by (Bender *et al.*, 2014) who detected a codistribution of profilin with microtubules in platelets and fibroblasts and, in agreement with our observations here, observed an increased microtubule acetylation in profilin-deficient platelets. The reason why the connection of profilin to microtubules has not been generally acknowledged before, is probably caused by the random profilin distribution observed by standard fluorescence microscopy of cultured cells (see introduction) together with the strong bias for profilin as a major actin regulator, as demonstrated for a range of cell types (Carlsson *et al.*, 1977; Lassing and Lindberg, 1988; Ballweber *et al.*, 1998; Kaiser *et al.*, 1999; Hajkova *et al.*, 2000; Lu and Pollard, 2001; Grenklo *et al.*, 2003) including platelets (Markey *et al.*, 1981). To our knowledge, the data presented here are the first providing evidence that profilin plays a role in controlling microtubule growth dynamics and hence contributes to the co-ordination of the actin and microtubule systems, see model (Figure 9).

We could not connect the microtubule-associated and WASP-related protein WHAMM with the profilin-microtubule interaction. As other WASP-family proteins, WHAMM is an Arp2/3-dependent NEPF; it has been suggested to cause intracellular membrane modulation and trafficking between ER and Golgi (Campellone *et al.*, 2008), and recently was linked to autophagosome biogenesis via nucleation and formation of an ER-tethered actin comet tail-like assembly process (Kast *et al.*, 2015). Here we showed that profilin binds a WHAMM fragment, which contains a poly-proline sequence motif typically known to recruit profilin/profilin:actin (Bjorkegren *et al.*, 1993) and essential for efficient profilin:actin-dependent filament growth e.g. (Lu and Pollard, 2001; Yarar *et al.*, 2002; Grenklo *et al.*, 2003). Together this makes WHAMM an ideal candidate as a connecting molecule between profilin and microtubules, and currently we cannot unequivocally exclude that it has such a function, for instance in the Golgi region (Dong *et al.*, 2000) or other similarly confined areas of the cell.

In contrast our data identify microtubule-associated formins as major profilin/profilin:actin recruiting components. The ability to associate with microtubules is shared by most formins and occurs via a formin homology 2 (FH2)-domain (Bartolini *et al.*, 2008; Bartolini *et al.*, 2012; Thurston *et al.*, 2012). Similarly, their capacity to bind profilin/profilin:actin is common among them and depends on their FH1-domain which contains a poly-proline sequence. Hence, the formins are central actin NEPFs along with the WASP/WAVE and Ena/VASP families of proteins. Among the formins, Diaphaneous (Dia)-1 and -2 take a special role in the context of microtubules since in addition to their association along the polymers they have been identified as binding partners to the adenomatous polyposis coli (APC) protein (Bartolini and Gundersen, 2010; Okada *et al.*, 2010), a component of the +TIP-complex controlling the (+)-end of microtubules. Furthermore, APC has been shown to cooperate with Dia to induce rapid actin polymerization from profilin:actin. Hence, formin-microtubule interactions can both occur directly via interactions along the polymer and indirectly via +TIPs.

The distribution of profilin along the microtubules is in agreement with the view that actin polymerization contributes to membrane tubulation along microtubules (Campellone *et al.*, 2008); in fact the profilin we detect along microtubules may represent profilin:actin as much as profilin alone. Not least the differential effect of the drug treatments and motility upregulation by AlF4 suggests that profilin:actin contribute to this pool of profilin.

Observations that formins impact on microtubule stability in NIH 3T3 fibroblasts (Bartolini *et al.*, 2012; Thurston *et al.*, 2012) along with an increased Dia 1 association to microtubules after treatment with latrunculin (Bartolini *et al.*, 2012), which interferes with actin polymerization and causes derangement of filamentous actin are in agreement with the results presented here. We show that reducing actin dynamics by CytD and Jasp causes profilin/profilin:actin to accumulate along the microtubules. Furthermore, in support of a cooperation with microtubule-associated formins we observe an influence of profilin on microtubule stability. Together these observations point to a scenario where microtubule-associated and stabilizing formins recruit profilin and profilin:actin to the polymer. In this situation the polymer-stabilizing effect imposed by the formin is balanced by its engagement as an actin NEPF at actin filament barbed ends generated in the vicinity. This, in turn, is dependent on the availability of profilin:actin. Hence, when there is less profilin expressed the more formin molecules remain microtubule-associated as is reflected in more stable polymers as we observed here.

It is also possible that some of the profilin molecules derive from translation of microtubule-associated profilin mRNA (Johnsson and Karlsson, 2010) similarly to what has been reported for the intermediary protein peripherin (Chang *et al.*, 2006). The extent by which microtubules serve as platforms for actin-driven membrane modulations is likely to vary with conditions and cell type. For instance in platelets as reported by (Bender *et al.*, 2014) the association of profilin/profilin:actin with microtubules seems to serve as a localized reservoir for actin in a polymerization-ready form from which actin is recruited for the massive filament formation typically seen upon platelet activation (Markey *et al.*, 1981; Karlsson *et al.*, 1984).

Clearly, our observation that profilin plays a role in controlling the dynamics of microtubule ends is difficult to reconcile with its presence along the entire polymer length. As discussed above, we have seen that reducing profilin expression by siRNA increased tubulin acetylation, which is indicative of more stable microtubules, and by imaging of cells transfected with EB3-GFP an increased microtubule growth was observed after lowered profilin concentration. Congruent with the latter we also measured a closer average distance between the cell edge and microtubule tips that extended towards lamellipodia, in direction of edge-advancement. Collectively this means that under non-perturbed conditions, profilin reduces microtubule growth by contributing to the frequency and/or pausing by which microtubule (+)-ends

undergoes catastrophe, see model Figure 9. Such scenarios would result from lowering the rate of addition of new GTP-charged α/β-tubulin subunits. Possibly profilin combines with +TIP-interacting formins (Okada *et al*., 2010) to exert such a function in parallel to its "gatekeeping" role to direct formation of different actin assembly forms (Henty-Ridilla and Goode, 2015). It cannot be excluded that the increased microtubule dynamics observed in profilin depleted cells was due to a general derangement of the microfilament system, resulting in less confined space for microtubule growth. However, based on our microscopy analysis we consider this unlikely, since lamellipodia formation and phalloidin-staining remained largely unperturbed over the time-frame when the measurements were performed. Moreover, for the live cell imaging care was taken to select cells of wild-type morphology with clearly recognizable lamellipodia and where individual microtubules could be identified and measured for their elongation.

Microtubule +-ends have been implicated in focal adhesion turnover (Krylyshkina *et al.*, 2003) and our data show that profilin contributes to the capacity of microtubule ends to probe advancing lamellae by increasing their turn-over in migrating cells. Definitely, there is a lot more to understand concerning profilin function in this context.

Materials and Methods

Cell culturing

Mouse melanoma B16-F1 and HEK293T cells were cultured in DMEM (Thermo Scientific) supplemented with 10% fetal calf serum (FCS) at 37°C in the presence of 5% CO_2.

Antibodies

Antibodies used were: against α-tubulin from Abcam, ab7291 and ab18251, made in mouse and rabbits respectively (used at dilutions of 1:2000, for Western blot and 1:200 for microscopy), ab1173 against Dia 1 (1:1000) and against GAPDH, ab8245 (1:40 000); against kinesin, K1005 (1:50); against Ac-tubulin, T6793 (1:500), against the profilin I N-terminal, P7749 (1:100) and β-actin, A5441 (1:4000) from Sigma and against profilin I (own laboratory, raised in rabbits, 1:2000); against β-arrestin from Santa Cruz Biotechnology, sc9182 (1:20) and sc-393499 (1:100) against Dia 2; against GFP from Roche, 11814460001 (1:2000); HRP-conjugated against mouse-Ig from Dako, P0447 (1:2000) and against rabbit-Ig from Pierce, 1858415 (1:1000); FITC-conjugated against mouse and rabbit Ig from Jackson ImmunoResearch laboratories, 705-095-003 (1:400) and 711-095-152 (1:1000), respectively; and TRITC-conjugated against rabbit-Ig from Thermo Fisher Scientific, 115-025-062 (1:400).

siRNA transfection and Western blot analysis

B16 cells were transfected with a siRNA cocktail of four different profilin siRNA-duplexes (50 nM each, Darmacon: LQ-062705-01; for sequences see Supplementary Figure 4) or a non-targeting control siRNA (Darmacon: D-001910-03) using Lipofectamine 2000 (Invitrogen). Dia 1 and 2 siRNAs were from Santa Cruz Biotechnology, sc-35191 and sc-155883, respectively. For transfections 25 nM of each was used. The cells were lysed after 24 h, in Passive Lysis Buffer (Promega, 31655601) unless otherwise stated, and the protein concentration determined by Bradford protein assay. After SDS-PAGE electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Hybond-C Extra; Amersham Bioscience) and identified by primary antibodies and HRP-conjugated secondary antibodies. The membranes were developed using West Dura HRP substrate (Pierce), images were captured on a ChemiDoc (Bio-Rad) using Quantity One software for densitometry.

Microtubule partitioning assay

B16 cells cultured overnight were trypsinized (Thermo Scientific) and suspended in DMEM (Gibco) supplemented with 10% FCS. After adjustment to a final concentration of 2x 10^6 cells per ml, they were treated with either 35 µM Taxol (Paclitaxel, Sigma; T7402) for 4 minutes at 37°C or 66 µM Nocodazole (Fluka: 74151) for 15 minutes at 37°C and then centrifuged at 1200 x g in an Eppendorf centrifuge for 30 seconds. The pelleted cells were resuspended in 200 µl PEM buffer (80 mM Pipes, pH 6.9, 5 mM EGTA, 1 mM $MgCl_2$) supplemented with 0.5% Triton X-100, 5µl/ml leupeptin (Sigma: L0649). The extracts were centrifuged at 1800 x g for 30 seconds, the resulting pellets stocked at - 20°C and the supernatants precipitated with acetone over night at - 20°C. Subsequently, the precipitated supernatant material was collected by centrifugation, resuspended in 200 µl of SDS-PAGE sample buffer and boiled immediately in parallel with the original pellet material after similar sample preparation and volume adjustment to allow for direct comparison of the two fractions.

Co-immunoprecipitation

B16 cells transfected with either pEGFP C1 or CTN-Pfn, encoding EGFP or citrine-profilin, respectively, were lysed in 200 µl 20 mM Tris-HCl pH 6.9, containing 0.5% NP-40, 2 M glycerol, 10% DMSO, 1 mM $MgCl_2$, 2 mM EGTA, 200 µM sodium orthovanadate, 5 µg/µl leupeptin and 500 µM PMSF (Wersinger and Sidhu, 2005). The cell extracts were centrifuged at 10 000 x g for 5 minutes, 50 µl of the supernatant were boiled in SDS-PAGE sample buffer and saved as control (input), while the remaining extract volume was pre-cleared with 40 µl Sepharose 4B (Pharmacia Fine Chemicals: 51-1870-04; 50% slurry) for 30 min and then incubated with 40 µl Nanobody^{GFP}-beads (camelid antibodies against GFP, e.g. (Kirchhofer *et al.*, 2010) coupled to NHS-activated agarose (Pierce: 26200) as described by (Holmberg *et al.*, 2014). After incubation, the beads were washed four times with buffer before boiling in 60 µl SDS-PAGE sample buffer and loaded onto an SDS-page.

GST fusion protein purification and pull-down assay

Glutathione S-transferase (GST)-WHAMM/CC (amino acid residues 559 to 809), GST-WASP/VCA (amino acid residues 442 to 502) and GST alone were expressed in *Escherichia coli* and purified using Glutathione-Sepharose beads (GE Healthcare). For the GST pull-down with GST-WHAMM and GST-WASP, HEK293T cells were lysed in 10 mM Tris HCl pH 7.5, 0.5% Triton X-100, 10% Glycerol, 100 mM NaCl and 1% Aprotinin (lysis buffer). The

cell lysates were centrifuged at 13 000 rpm in a bench-top microcentrifuge for 15 min at 4 °C. The supernatant was collected and incubated with the pre-treated beads for 60 min end-over-end at 4 ºC. The beads were washed three times with lysis buffer and then analyzed by SDS-PAGE and electro-transferred to nitrocellulose filters (GE Healthcare).

Citrine-Profilin fusion construct

Citrine was PCR amplified from plasmid pRSETb-Citrine (Generous gift from Roger Tsien, 55) using primers containing a restriction site for Pst I and encoding linker residues (Forward: TAT CTG CAG TCT GGG TCT AGT GGT TCT ATG GTG AGC AAG GGC GAG; Reverse: ATC CTG CAG TGA CCC GCC CTT GTA CAG CTC GTC CAT G), gel purified and digested with Pst I. The human profilin cDNA (Pfn1, GeneID: 5216) was introduced into pEGFPc2 (Clontech, GenBank Accession number: U57606), followed by excision of the EGFP-gene, religation and subsequent Pst I cleavage. The gel purified and Pst I digested citrine-cDNA was then ligated into the opened profilin gene in the pc2-vector to generate the internal fusion-gene encoding the citrine-profilin fusion protein (CTN-Pfn) where the citrine C- and N-termini are fused via linker residues to Q79 and D80, respectively, in profilin. The object with this construct was to avoid to link the fluorophore-protein to the amino- or carboxy-termini of profilin since these positions may interfere with profilin's interaction with poly-L-proline (Wittenmayer *et al.*, 2000). As aimed for, CTN-Pfn retains full binding capacity for poly-L-proline, whilst its interaction with actin is reduced. The in vitro characterization of CTN-Pfn will be published elsewhere (Nejedla et al, manuscript in preparation).

Microtubule protein preparation, co-sedimentation and assembly assays

For details see (Draberova *et al.*, 2010). Briefly, porcine brain tubulin and thermostable MAPs containing MAP2 and tau were isolated from microtubule protein (MTP-2). For co-sedimentation, Taxol-stabilized microtubules (6 μM tubulin) were mixed with profilin, MAPs or BSA (final concentrations 0.10-0.13 mg/ml), incubated for 30 min at 37°C and the mixture (120 μl) was centrifuged for 20 min at 25 °C on a 4 M glycerol cushion (800 μl) in 80 mM PIPES pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, containing 10 μM Taxol in a MLS50 rotor (Beckman Coulter, Fullerton, CA, USA) at 43,000 rpm. The protein content of the starting mixtures, supernatants and pellets was resolved 12.5% SDS-PAGE. Pellets were resuspended in sample buffer which equaled 0.5x the supernatant volume. The tubulin assembly was monitored by turbidimetry at 350 nm and 37°C of samples containing 20 μM tubulin in 80

mM PIPES pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP and 3.0 M glycerol plus 20 µl of profilin (25 µM final concentration) in 10 mM NaCl, 5mM Tris, pH 7.8, 10% glycerol. Final sample volume was 100 µl. Control samples contained 20 µl of thermostable MAPs (1.91 mg/ml) or the profilin buffer alone.

Proximity Ligation Assay (PLA) and Fluorescence Microscopy

B16 cells cultured on coverslips pre-coated with 25 µg/ml laminin (Sigma, L2020) for 2 hours at 37°C, were fixed for 20 minutes at 37°C with 4% formaldehyde (Sigma) in PBS containing 5 mM EGTA and then demembranated with 0.1% Triton X-100 in PBS-EGTA for 10 minutes at RT. The proximity ligation assay (PLA) was carried out using the Duolink PLA in situ kit (Olink Biosciences) following the manufacturer's protocol. The cells were incubated with primary avian antibodies generated against cross-linked profilin:actin (Nyman *et al.*, 2002a) and affinity purified on immobilized actin and profilin, respectively, (previously referred to as AI, AII, PI and PII where the roman number indicates two different animals immunized as described in (Grenklo *et al.*, 2004; Li *et al*., 2008)). Along with these profilin/profilin:actin antibodies used at a concentration of 50 µg/ml, anti-α-tubulin (generated in mice), anti-α-tubulin (rabbit), anti-kinesin and anti-β-arrestin were added for 1 hour at RT followed by incubation with species-specific secondary antibodies conjugated with oligonucleotides (PLA-probes MINUS and PLUS). To allow for visualization of microtubules simultaneously with the PLA-signal the buffer solution containing fluorescent oligonucleotides was supplemented with a FITC-labeled secondary antibody recognizing rabbit anti-tubulin. The coverslips were mounted in ProLong Gold (Molecular Probes) and microscopy was performed using a Leica DMLB microscope equipped with 63x/1.32 objective lens and a DC350F CCD camera (Leica Microsystems). To compare signal density between different samples, four representative areas were selected per cell in each experiment; two in lamellipodia and two in the center of the lamella, each being a circle of 6.6 μm^2. The total number of distinct dots in those was determined, and the mean values obtained by the different PLA labelings were compared.

To visualize profilin distribution along microtubules by fluorescence microscopy, B16 cells were cultured on coverslips, washed with PBS, and permeabilized for 40 s in PEM buffer containing 0.5% Triton X-100, before fixation for 20 min in PEM containing 1% dimethyl sulfoxide and 3.7% formaldehyde (Raynaud-Messina *et al.*, 2004; Bouissou *et al.*, 2009).

Drug treatment as indicated was performed prior to permeabilization with 0.1% DMSO (vehicle), 0.5 µM Cytochalasin D, 0.05 µM Jasplakinolide, 25 µM SMIFH2, 50 µM CK-666 for 30 min, or with a mixture of 50 µM $AlCl_3$ and 30 mM NaF for 30 and 60 min to generate AlF_4. The samples were then incubated with SiR-actin (Tebu-Bio, SC001), primary and secondary antibodies as indicated, mounted and observed using an Axiovert 200M fluorescence microscope (Zeiss) equipped with a climate chamber, an EC-Plan-Neofluar 63x/1.4 objective lens and DG-4 light source (Sutter Instrument, CA). Images were captured with a Cascade 1K camera (Roper). Profilin-microtubule codistribution in leading lamellae was quantified using the ImageJ plugin image correlation analysis (Mander's colocalization coefficient). Slidebook software was used to measure the distances between microtubule ends and outer edges of the lamellipodia by drawing a straight line from each microtubule end to the edge which was marked by Rhodamine-phalloidin (Sigma: P1951, 1:200).

Live cell imaging of EB3-GFP transfected cells

B16 cells were cultured on laminin-coated glass-bottom dishes (MatTek: P35GC-1.5-10-C). After 24 hours, the cells were transfected with 20 ng/µl EB3-GFP, 5nM siGloRNA (Dharmacon: D-001630-02) and siRNA cocktail (as above) and cultured for an additional 24 hours. Then, cells expressing siGloRNA were identified for EB3-GFP recording at 37^{o}C in the presence of 5% CO_2, using the same microscope and settings as for fluorescence microscopy. Images were acquired at 5-seconds intervals during 5 min periods.

TIRF microscopy

Cells transiently expressing the citrine-profilin fusion were culture on glass as for live imaging (above), fixed with formaldehyde and after detergent-demembranation and labeling with tubulin antibodies they were observed in TIRF-mode with a AxioObserver D1 Laser TIRF 3 system (Carl Zeiss), equipped with an alpha Plan-Apochromate 63x/1.46 oil objective lens, OPSL (488 nm/20 mW) and DPSS (561 nm/20 mW) lasers, and the 52HE, 488 nm and 86HE, 561 nm shift free filter sets. Images were captured with an Axiocam MRm rev.3 camera (Carl Zeiss) and the system was controlled by ZEN blue 2011 software.

Super-resolution multicolor imaging (STED)

STED was performed with a Leica TCS SP8 3X STED microscope, equipped with a 100x NA 1.4 STED WHITE objective. Optimal excitation wavelengths were selected from a broadband white-light fiber laser source (tunable range 470-670 nm). Stimulated emission depletion was

performed with a high power fiber laser at 775 nm that achieves a lateral resolution of sub-50 nanometer.

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Figure Legends

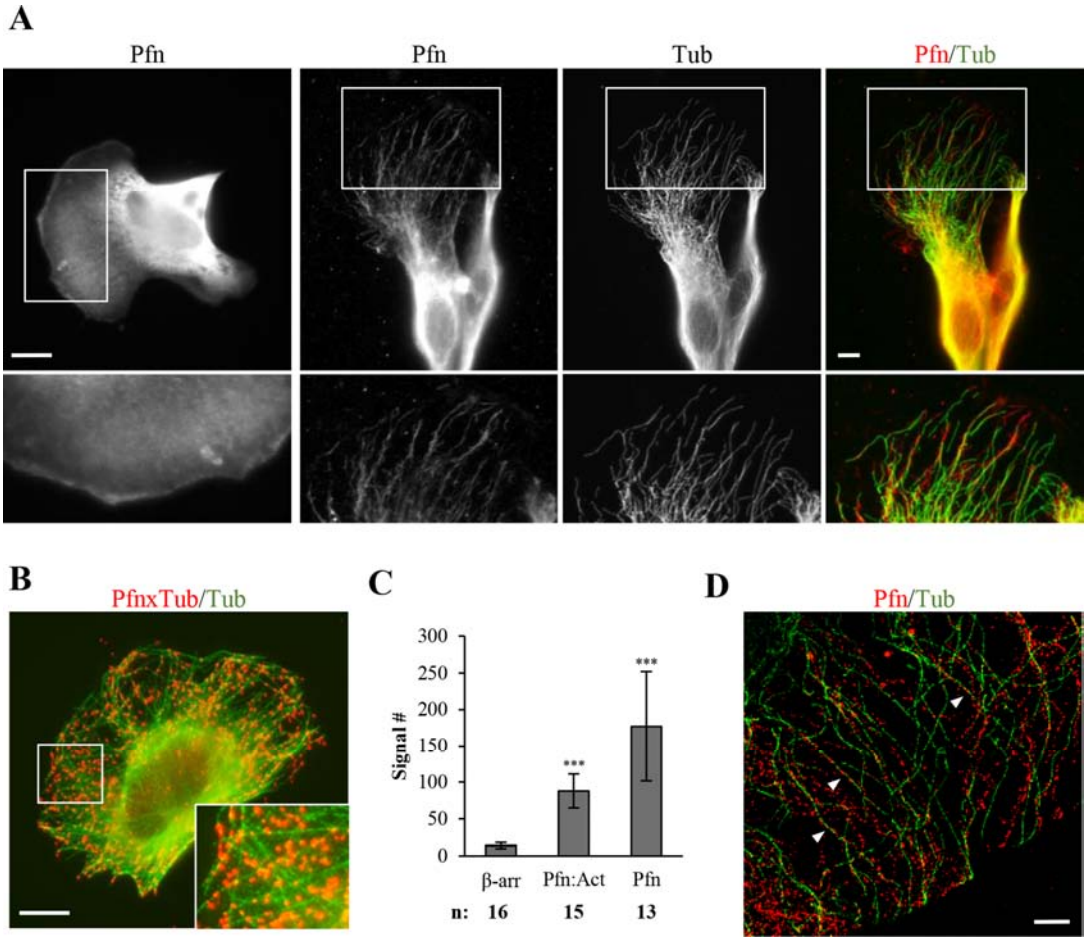

Figure 1. Profilin codistributes with microtubules.

(A) B16 cells were stained with profilin (Pfn) or α-tubulin (Tub) antibodies. The general distribution of profilin as it is seen after fixation and detergent treatment is displayed in the picture to the far left. In striking contrast, as illustrated next, the profilin codistribution with microtubules becomes visible after pre-treatment with 0.1% Triton X-100 before fixation. Marked areas are shown at higher magnification underneath each panel. (B) The result of the proximity ligation assay (PLA) with a combination of profilin and tubulin antibodies. The microtubule system was visualized, using a tubulin antibody from a different species than for the PLA assay (see Materials and Methods). (C) The graph displays the signal density from three PLA-experiments where the tubulin antibodies were combined with antibodies as indicated: β-arrestin (β-arr; included as a control) and two different profilin/profilin:actin antibodies (Pfn/Pfn:Act, generated against cross-linked profilin:actin and affinity purified against profilin and actin, respectively). Signal # denotes total number of PLA-marks in four

regions of a total area of 26.4 µm^2 per cell. (D) The presence of profilin in a dotted pattern along microtubules (arrowheads) as seen by super-resolution STED microscopy. Cells were treated with DMSO as in Figure 3. Manders´ colocalization coefficient (Dunn *et al.*, 2011) was determined to 0.79 (27 cells; 3 independent experiments), i.e. 79% of the microtubules within the region were associated with profilin at least once along their lengths see also Figure 3 and Supplementary Figure 5. Student t-test, *** $p \leq 0.001$; n = number of cells (three independent experiments, approximately equal number of cells in each experiment); error bars indicate SEM. Scale bars: (A) 10 µm, (B) 25 µm, and (C) 2.5 µm.

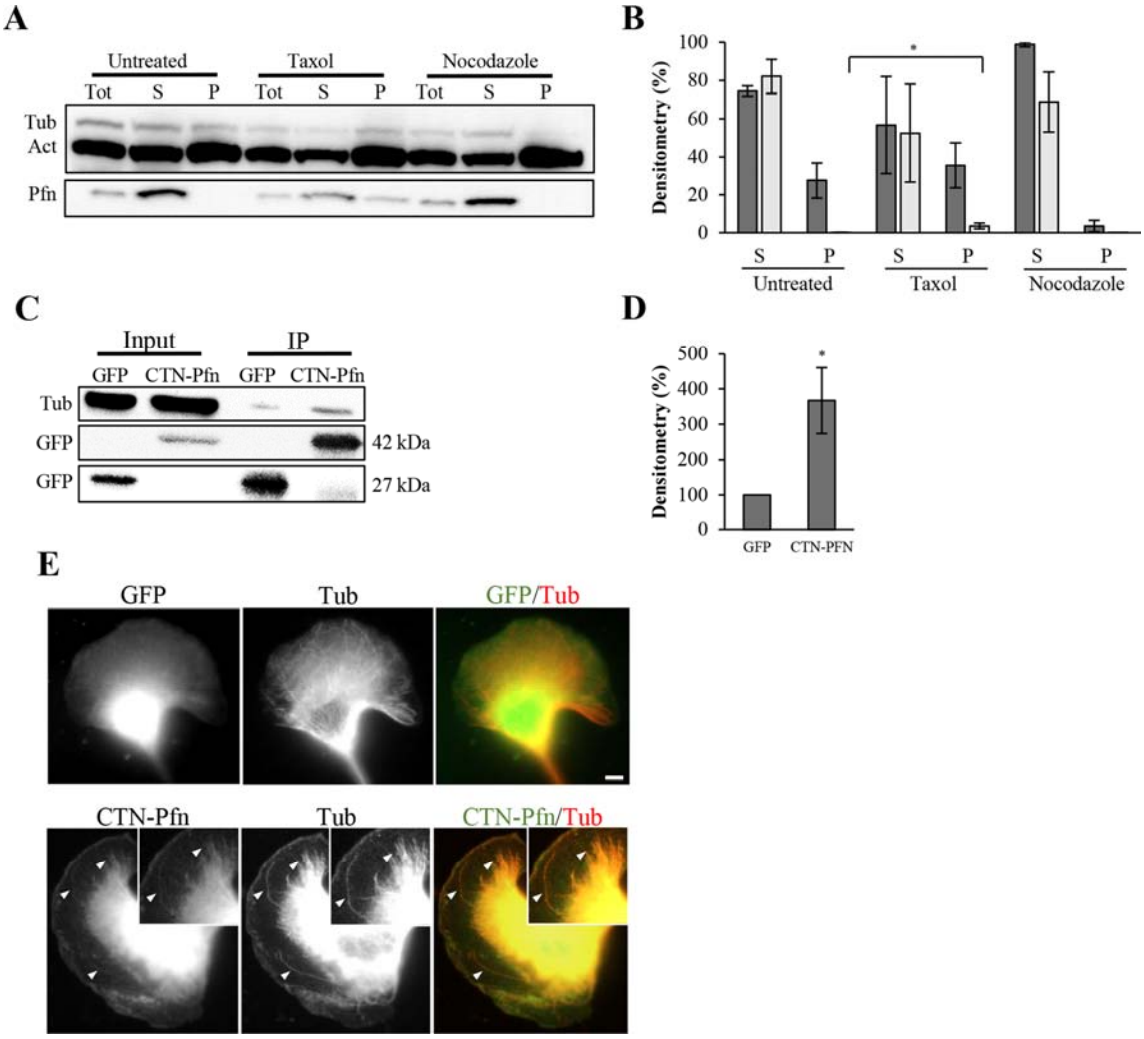

Figure 2. Profilin co-partitions with microtubules and co-immunoprecipitates with tubulin. (A) Cells were treated with Taxol or Nocodazole prior to lysis followed by centrifugation to analyze for microtubule co-partitioning of profilin by Western blot. Pellets are denoted (P), supernatants (S) and total extracts (Tot), and the protein bands are identified to the left: tubulin (Tub), actin (Act), and profilin (Pfn). (B) Densitometry of the tubulin (grey) and profilin (white) bands after analysis as in (A) and normalized against actin; three independent

experiments. Pelleted profilin can only be observed in extracts of Taxol-treated cells. (C) Co-immunoprecipitation analysis after expression of a citrine-profilin fusion (CTN-Pfn), cell lysis and incubation of the extracts (as indicated on top) with beads conjugated with GFP-antibodies followed by Western blot of the captured material with antibodies against tubulin and GFP (left). (D) Densitometry of the GFP/citrine-profilin bands after co-immunoprecipitation as in (C) upper row; GFP indicates the control cell extract; student t-test, * $p \leq 0.05$; three independent experiments, values were normalized against input and error bars indicate SEM. (E) TIRF microscopy was used to visualize codistribution of CTN-Pfn with microtubules after fixation and staining with tubulin antibodies. Arrowheads (lower panels) point to profilin localizing along microtubules, inset shows a region in higher magnification. Scale bars: 5 µm.

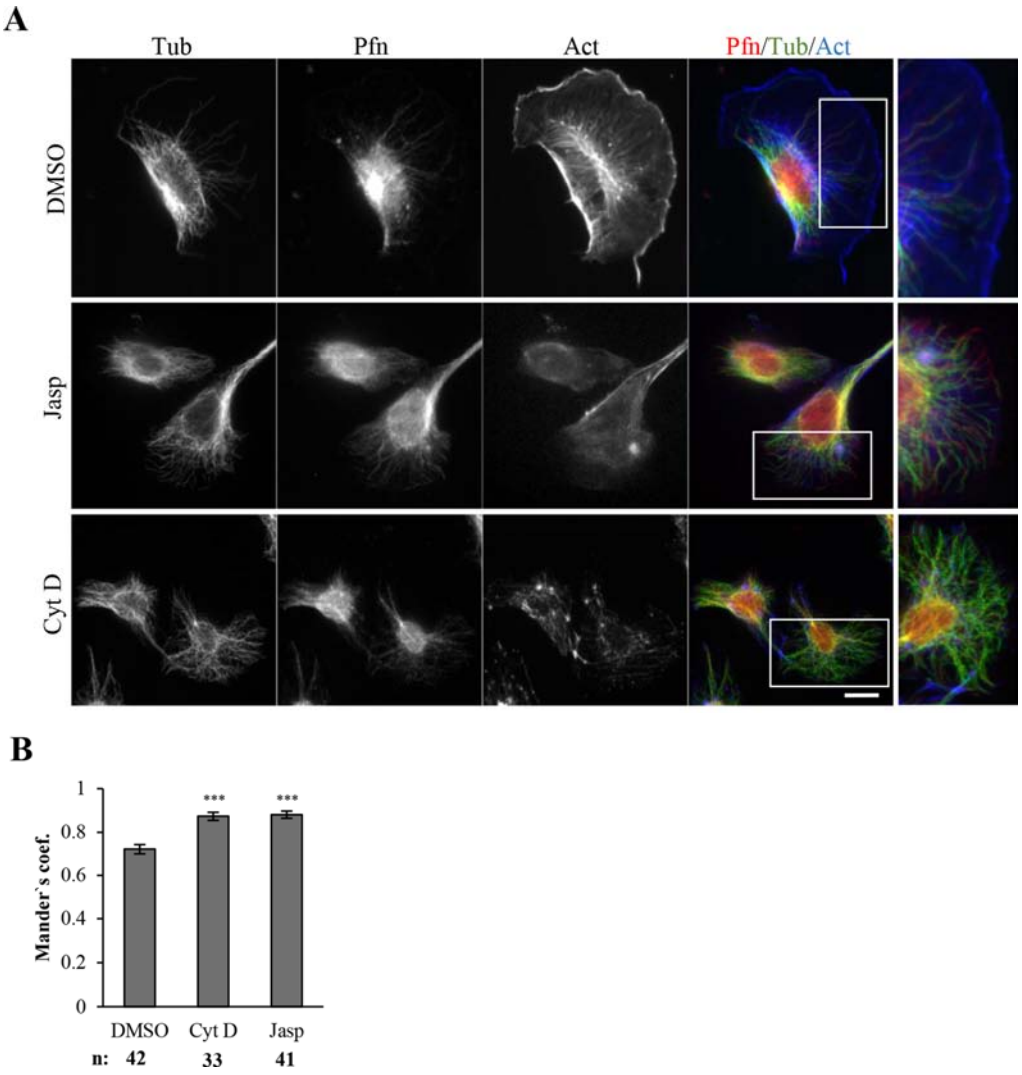

Figure 3. The profilin-microtubule association is influenced by the actin polymerization status.

(A) B16 cells were exposed to 0.1 % DMSO (vehicle), 0.5 µM Cytochalasin D (CytD) and 50 nM Jasplakinolide (Jasp) for 30 min followed by detergent pre-treatment and fixation as in (Figure 1A). The distribution of profilin (red) and tubulin (green) is displayed along with filamentous actin, using SiR-actin (blue); marked areas (merge only) are shown at higher magnification to the right. (B) The extent of profilin-microtubule colocalization after drug treatment as in (A) using Manders´ colocalization coefficient (Dunn *et al.*, 2011) as in Figure 1 and further explained in Supplementary Figure 5.; two independent experiments, statistics as denoted ain (Figure 1). Scale bar: (A) 10 µm

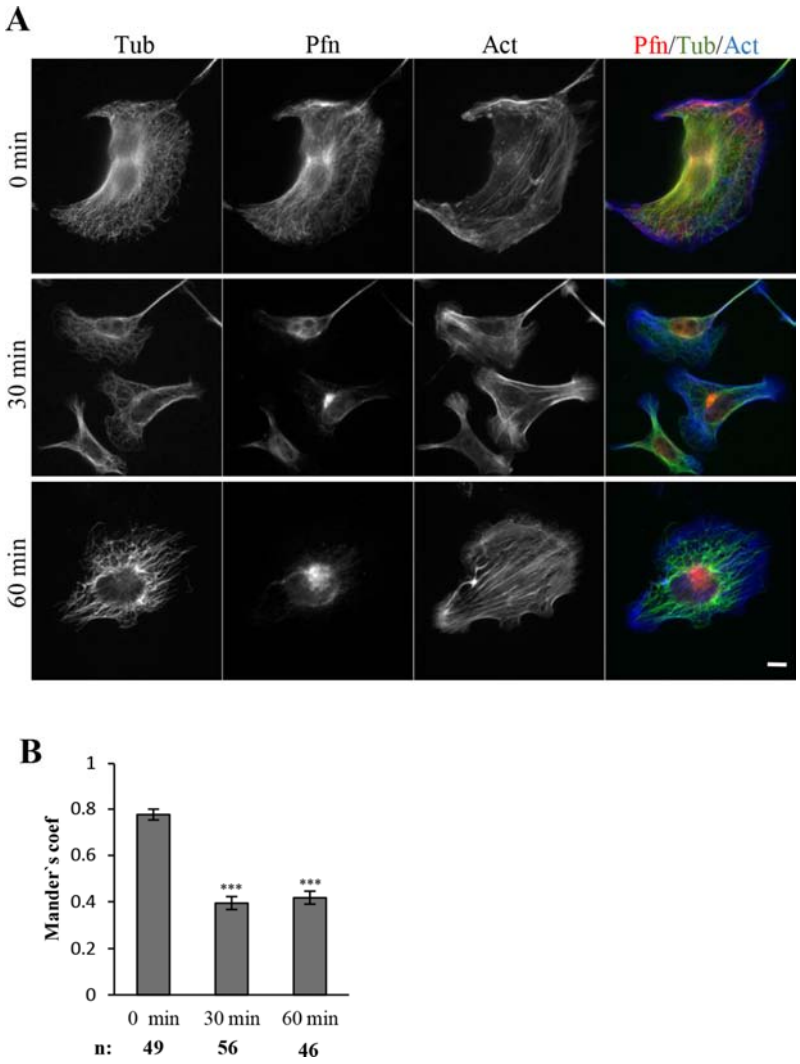

Figure 4. Increased lamellipodia formation decreases the profilin-microtubule co-distribution. (A) B16 cells were treated with AlF_4 (see Material and Methods) for 30 and 60 min, respectively, “demembranated” with Triton X-100, fixed and stained for profilin, tubulin and filamentous actin as in (Figure 3). (B) The bar diagram illustrates the profilin-microtubule

colocalization as in (Figure 3). Three independent experiments, statistics as in (Figure 1). Scale bar 10 µm.

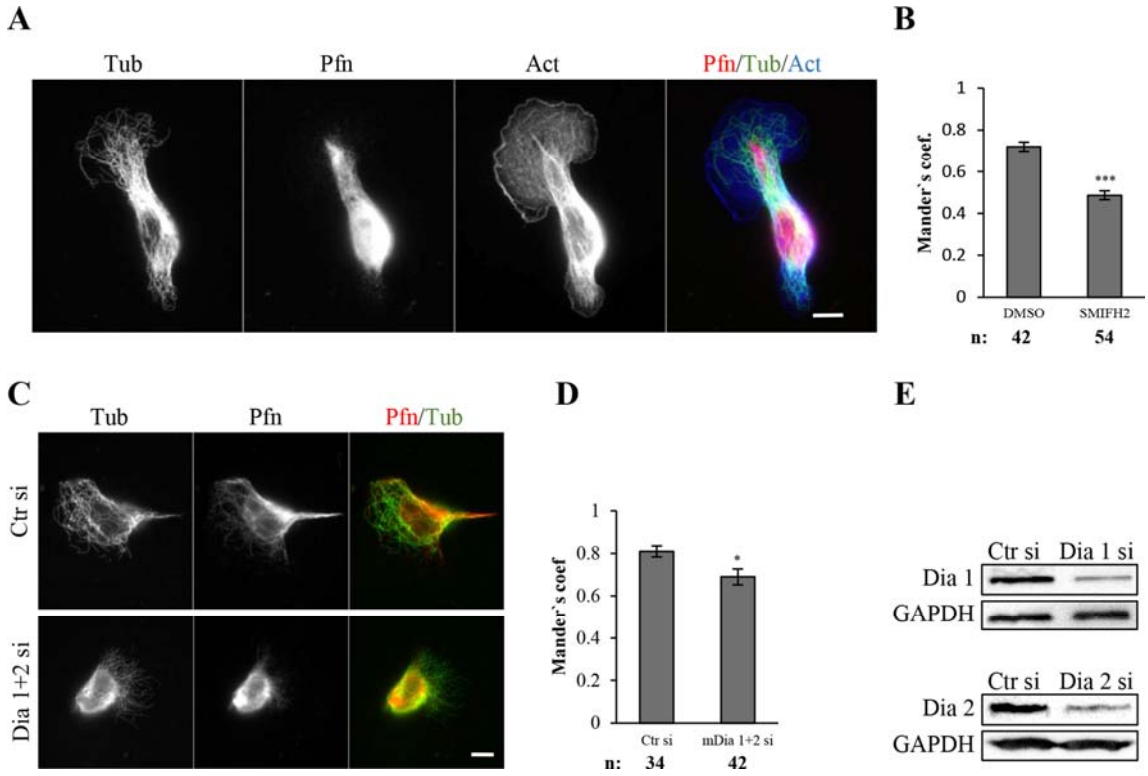

Figure 5. Formins are potential linkers between profilin and microtubules.

(A) B16 cells were treated with the formin inhibitor SMIFH2 (25 µM) for 30 min; otherwise same conditions and labelling as in (Figures 3 and 4). (B) The graph shows the colocalization analysis; two independent experiments, statistics as in (Figure 1). (C) Cells after transfection with the control siRNA (upper panels) and after transfection with pooled Dia 1 and 2 siRNAs (lower panels) were fixed and stained for tubulin and profilin as before. (D) Quantitation of the colocalization of profilin and microtubules as in Fig 3. Two separate experiments, n = number of cells, statistics as in (Figure 2). (E) Western blot showing reduced expression of mDia 1 and 2, respectively, after siRNA transfection; separate transfections of the two siRNAs. Scale bars: 10 µm.

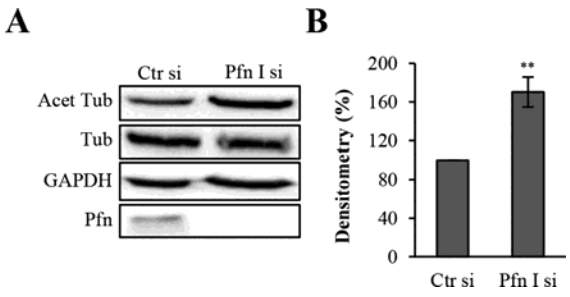

Figure 6. Down regulation of profilin expression increases acetylation of tubulin.
(A) Cells were transfected with control siRNA (Ctr si) or a cocktail of four different profilin I siRNA:s (Pfn I si), and resulting extracts were analyzed by Western blot for the presence of acetylated tubulin (Acet Tub), tubulin, glyceraldehyde phosphate dehydrogenase (GAPDH; loading control) and profilin. (B) Quantification of blotted bands by densitometry; mean value of four independent experiments. Statistics as in (Figure 1), ** $p \leq 0.01$.

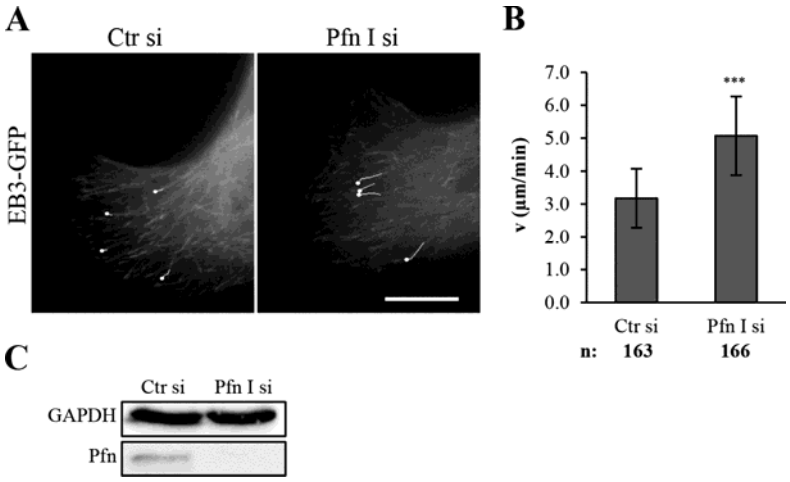

Figure 7. Profilin influences microtubule dynamics.
(A) Video clips (Supplementary Videos 1 and 2) at 5 s intervals of EB3-GFP-transfected B16 cells after subsequent transfection with control siRNA or Pfn I siRNA (left and right panel, respectively). Traces of the advancement of some microtubule plus ends over a period of 15 s are indicated. Note, longer distances after down regulation of profilin expression, which corresponds to increased microtubule growth-velocity as shown in the graph in (B). Only cells positive for siGloRNA, indicating siRNA transfection were selected for the measurements. The microtubule (+)-end growth rate was established by frame-by-frame analysis of the videos to ensure measurement of uninterrupted extension solely. Three independent experiments, 6 cells (two from each), n = number of microtubule plus ends (similar number per cell and experiment). Statistics as in (Figure 1). Scale bar: 10 µm. (C) Cell cultures used

for live cell imaging were lysed and analyzed by Western blot to verify reduced profilin expression.

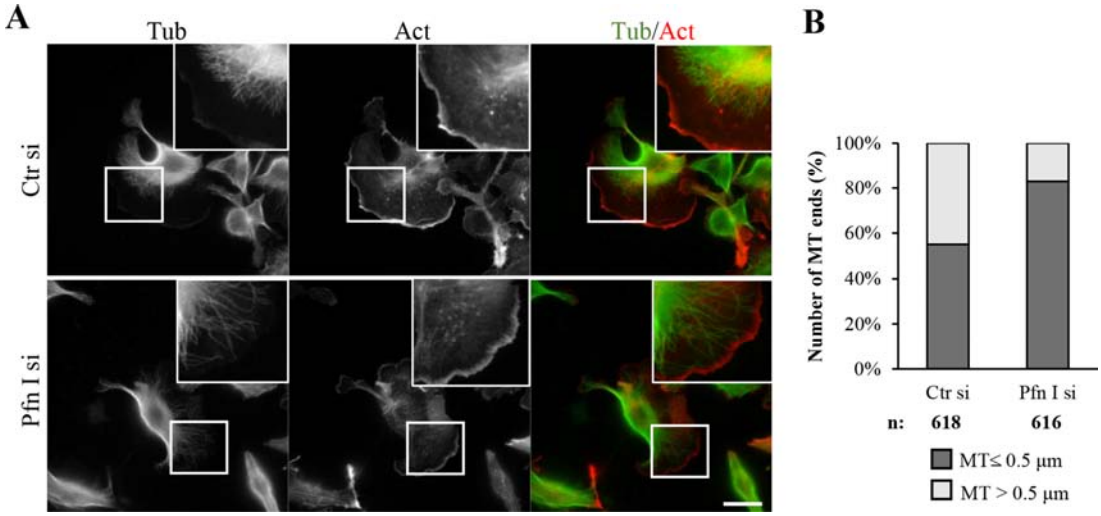

Figure 8. Microtubule ends reach closer to the cell edge after down regulation of profilin. (A) The B16 cells were transfected with control siRNA or profilin I siRNA and stained with tubulin antibodies and Rhodamine-phalloidin. The distance between the microtubule ends and the cell edge, defined as the outermost rhodamine-staining of the lamellipodia was measured by Slidebook software (see Material and methods). (B) The graph illustrates the number of microtubules that ends within 0.5 µm (gray) or at a longer distance from the edge (white), respectively; two independent experiments, approximately 40 cells, n = number of microtubules. Scale bar 10 µm.

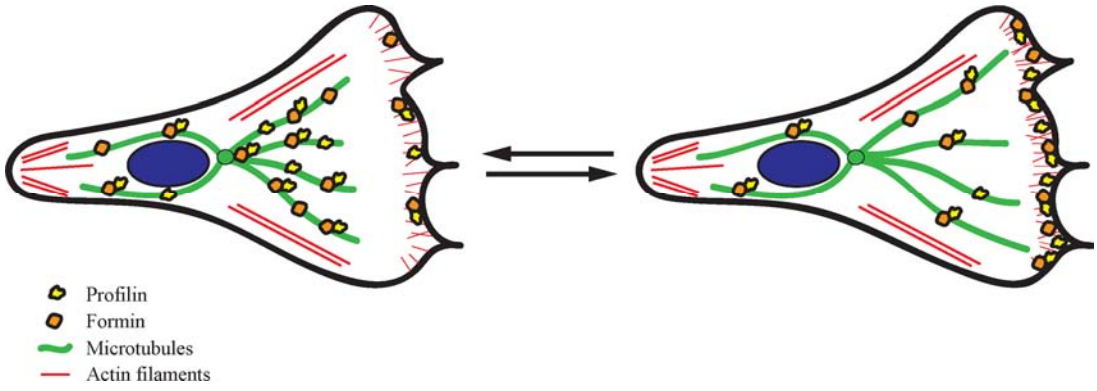

Figure 9. A cartoon, modelling profilin function in relation to microtubules based on the presented data. During homeostatic growth and motility, the presence of profilin (yellow) along microtubules (green) is balanced against the requirement for its documented role as a regulator of actin polymerization. Formins (orange) are major profilin-recruiting components

on the microtubules as indicated by experiments with SMIFH2 and siRNA, respectively. Additionally, formins and profilin are found at the cell periphery where they participate in actin regulation. Interfering with actin dynamics as with CytD and Jasp (see text) causes accumulation of profilin to microtubules, as also is reported for formins (Bartolini *et al.*, 2012). Depletion of microtubule-associated profilin after siRNA-transfection results in faster growth of microtubules and increased acetylation of tubulin. Similarly, stimulation of actin dynamics by AlF_4 reduced profilin association along the microtubules, increased tubulin acetylation and lamellipodia formation. We hypothesize a similar scenario in response to increased receptor activation. At the periphery, profilin cooperates with formins and other actin NEPFs for rapid elongation of the sub-membranous actin array and advancement of the cell front. The increased microtubule growth in the direction of the forward moving membrane allows for increased microtubule-dependent transport in support of the advancing edge. Our observations as summarized by this model therefore identify profilin as part of a molecular network that coordinates actin and microtubule functions in migrating cells.