Profilin Interaction with Actin Filament Barbed End Controls Dynamic Instability, Capping, Branching, and Motility

Graphical Abstract

Highlights

- The binding of profilin to barbed ends accounts for its effects on cell migration
- Profilin enhances length fluctuations of actin filaments by destabilizing barbed ends
- Profilin competes with capping protein at filament barbed ends
- Profilin competes with polymerases and filament branching machineries at barbed ends

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In Brief
Pernier et al. demonstrate how the binding of profilin to actin filament barbed ends affects actin homeostasis during cell migration. Profilin competes with and coordinates the function of barbed end regulators including Capping Protein, polymerases such as formin or VASP, and WASP-Arp2/3 filament branching machineries.

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Profilin Interaction with Actin Filament Barbed End Controls Dynamic Instability, Capping, Branching, and Motility

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SUMMARY

Cell motility and actin homeostasis depend on the control of polarized growth of actin filaments. Profilin, an abundant regulator of actin dynamics, supports filament assembly at barbed ends by binding G-actin. Here, we demonstrate how, by binding and destabilizing filament barbed ends at physiological concentrations, profilin also controls motility, cell migration, and actin homeostasis. Profilin enhances filament length fluctuations. Profilin competes with Capping Protein at barbed ends, which generates a lower amount of profilin-actin than expected if barbed ends were tightly capped. Profilin competes with barbed end polymerases, such as formins and VopF, and inhibits filament branching by WASP-Arp2/3 complex by competition for filament barbed ends, accounting for its as-yet-unknown effects on motility and metastatic cell migration observed in this concentration range. In conclusion, profilin is a major coordinator of polarized growth of actin filaments, controlled by competition between barbed end cappers, trackers, destabilizers, and filament branching machineries.

INTRODUCTION

Motile and morphogenetic processes are driven by polarized assembly of actin filaments, which generates protrusive or compressive forces against cellular membranes. Filament growth rate is controlled by the concentration of polymerizable monomeric actin that associates to barbed ends and by the activity of regulatory proteins at barbed ends (Carlier et al., 2015). Profilin, an essential actin-binding protein present in cells in the range 10–80 μM (dos Remedios et al., 2003; Witke et al., 2001), is a central player in actin-based motility, because profilin-actin complex feeds filament assembly selectively at barbed ends (Pollard and Cooper, 1984) and supports formin-mediated rapid processive barbed end assembly (Kovar et al., 2003; Romero et al., 2004). Thus, like free G-actin, profilin-actin is in dynamic equilibrium with F-actin at barbed ends. This is in contrast with β-thymosin, which forms non-polymerizing complexes with actin that are in rapid equilibrium with G-actin but not with F-actin.

While the cellular function of profilin is thought to be linked to its binding G-actin, elusive effects of profilin in motile and metastatic processes cannot easily be explained within this simple view. Injection of profilin inhibits lamellipodium motility and formation of the lamellipodial branched filaments (Cao et al., 1992; Rotty et al., 2015; Suarez et al., 2015). Consistently, profilin is downregulated in invasive metastatic breast cancer cells (Joy et al., 2014; Lorente et al., 2014) and its overexpression reduces their migration (Roy and Jacobson, 2004). These counterintuitive facts prompted us to take a new look at profilin.

Profilin associates with the barbed face of actin, which is exposed on both G-actin and F-actin at the filament barbed end. Profilin binds G-actin with high affinity (K_G = 0.1 μM), and barbed end F-actin with relatively lower affinity (K_F = 20 μM), promoting enhanced filament disassembly (Bubb et al., 2003; Courtemanche and Pollard, 2013; Jegou et al., 2011; Kinosian et al., 2002). The consequences of profilin’s interaction with barbed ends on filament assembly dynamics and profilin’s resulting competition with other barbed end regulators are explored here.

We find that profilin enhances fluctuations in the length of filaments. The extensive disassembly events are balanced by an increased amount of profilin-actin feeding barbed ends at steady state. We next reveal that profilin controls actin homeostasis by competing with Capping Protein (CP) at barbed ends, with formin and with WH2-domain-containing barbed end trackers such as VopF. Finally, profilin binding to barbed ends inhibits filament branching by WASP proteins and Arp2/3 complex and resulting actin-based motility. Proteins that track barbed ends such as VopF, WASP, formins, similarly inhibit filament barbed end branching by Arp2/3 complex. The reported “anti-capping” and “anti-branching” activities of profilin, which affect motility, are explained by competitive interplay of regulators at barbed ends.

RESULTS

Profilin Enhances Length Fluctuations of Actin Filaments in ATP

Actin filaments, like microtubules, use nucleotide hydrolysis associated with assembly to generate metastable dynamic polymers. Rapid disassembly of the ADP/GDP subunits in the core of
the polymer is prevented by a stable ATP/GTP cap at the growing plus/barbed end (Carlier et al., 1984). Dynamic instability is milder in actin than in microtubules (Hill, 1986; Ranjith et al., 2009; Stukalin and Kolomeisky, 2006; Vavylonis et al., 2005). Yet, fluctuations in the length of individual filaments, exceeding the low “length diffusivity” of reversible polymerization, have been detected (Fujiwara et al., 2002; Kuhn and Pollard, 2005).

Profilin was predicted to enhance length fluctuations by promoting faster filament disassembly (Jegou et al., 2011). To measure length fluctuations in the region of the monomer concentration $C_{SS}$ at which the net rate of filament barbed end growth is null, we first examined how the rate of barbed end elongation, $J$, at varied G-actin concentrations ($C$) is affected by profilin (Figure 1A). Only barbed ends contribute in $J(C)$ since pointed ends do not interact with profilin and disassemble extremely slowly. The dual activity of profilin is revealed by the data. In a range of profilin concentrations sufficient to convert G-actin into profilin-actin, barbed end growth proceeds equally well from profilin-actin or G-actin. At a range of higher concentrations (10–100 $\mu$M), profilin-enhanced dissociation from barbed ends promotes an increase in $C_{SS}$ ($J(C_{SS}) = 0$) from 0.1 $\mu$M up to 1 $\mu$M at 50 $\mu$M profilin, and 1.3 $\mu$M profilin-actin at 100 $\mu$M profilin. This means that an enhanced flux of profilin-actin association to barbed ends balances enhanced disassembly at steady state. The increase in $C_{SS}$ is the signature of the destabilization of filament barbed ends by profilin. In measurements of F-actin at steady state described later (Figures 4C, 4D, and 5E), the same values of $C_{SS}$ are found for profilin-actin co-existing at steady state with F-actin.

Single-filament kinetics using microfluidics-assisted microscopy of filaments immobilized at their pointed ends by spectrin-actin seeds confirmed the destabilization of barbed ends by profilin seen in bulk solution measurements (Figure 1B, symbols). Computed rates of filament growth using parameters for profilin binding to ATP-bound barbed ends (Table S1) confirm the experimental data (Figure 1B).
We then compared fluctuations in length in the absence and presence of free profilin in the vicinity of CSS (from Figures 1A and 1B) at a range of growth rates of $-2$ to $+2$ subunits/s (Figures 1C and 1D). The diffusion coefficient $D$ was $10 \pm 2$ subunits$^2$/s for non-capped filaments at steady state in the absence of profilin, one order of magnitude higher than for capped filaments ($0.31 \pm 0.2$ subunits$^2$/s, the detection limit). Over a wide range of elongation rates, fluctuations are enhanced by profilin, most extensively below CSS, as predicted (Vavylonis et al., 2005), where the maximal value of $D$ was increased up to 5-fold at $30 \mu$M profilin (Figure 2B). These features are reproduced in simulated kinetics of growth of individual filaments (Figures 1E and 1F), using the same parameters as in Figure 1B (Table S1; see Supplemental Experimental Procedures, Equation 1).

In conclusion, profilin promotes a mild form of “dynamic instability” in actin, by amplifying the effects of catastrophes above the critical concentration, and of rescues below the critical concentration. The filament monomer-polymer exchanges are largely dominated by profilin-actin exchanges at barbed ends exclusively.

**Effect of Profilin on Barbed End Assembly from ATP-, ADP-, and AMPPNP-Actin**

Profilin affects barbed end assembly differently depending on the nature of the actin-bound nucleotide (Figure 2A). While profilin slowed down barbed end assembly at 3 \mu M ATP-G-actin, consistent with the results in Figure 1A, it inhibited assembly at 3 \mu M ADP-G-actin, eventually causing barbed end disassembly at the same rate as in the absence of actin. Thus, filaments are unable to elongate from profilin-ADP-actin. This was confirmed in single-filament assays (Figure 2B). Finally, profilin inhibited filament growth from AMPPNP-actin. However, the barbed

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**Figure 2. Nucleotide Dependence of the Effect of Profilin**

(A) Effect of profilin on the rate of filament elongation in ATP, AMPPNP, and ADP. Conditions as in Figure 1A, with 3 \mu M G-actin. Dark green curve: in ADP, no G-actin.

(B) Single filament experiments at 3 \mu M ADP-actin. Data points from Jegou et al. (2011). The solid line is computed using parameters from Table S1, assuming that the barbed end on-rate constant for profilin-ADP-actin is the same as for ADP-actin ($2.6 \mu$M$^{-1}$s$^{-1}$). Error bars are standard deviations.

(C) Effect of profilin on barbed end assembly in ADP (C) and AMPPNP (D). Blue curves, no profilin; red curves, 100 \mu M profilin.
ends remained blocked by profilin-AMPPNP-actin and no depolymerization of AMPPNP-F-actin was observed at high profilin.

In the absence of profilin, J(C) plots obtained in ADP and AMPPNP were linear as expected, with critical concentration values of 1.5 μM and 0.14 μM, respectively (Figures 2C and 2D). In the presence of 100 μM profilin, ADP-F-actin depolymerized at a high rate (55 subunits/s) independent of ADP-G-actin, consistent with Figure 2A. In contrast, AMPPNP-F-actin depolymerized at a 10-fold enhanced rate in the absence of actin, in agreement with Courtemanche and Pollard (2013), but the addition of AMPPNP-G-actin gradually led to total blockage of barbed ends, consistent with Figure 2A. In conclusion, profilin binds G-actin and barbed ends in various bound nucleotide states, but only profilin-MgATP-actin supports barbed end assembly.

### Capping Protein and Profilin Compete at Filament Barbed Ends: Implication in the Control of the F-Actin/G-Actin Ratio and Free Profilin Concentration

In live cells, most profilin is thought to be bound to G-actin (Kaiser et al., 1999). This view is based on the implicit assumption that barbed ends are fully capped in the bulk cytoplasm, which prevents the participation of profilin-actin in barbed end growth. In these conditions, profilin is now in equilibrium with G-actin only at the critical concentration for pointed end assembly, leading to 88% of total profilin being present in profilin-actin complex (Equation 2, Supplemental Experimental Procedures). Typically, in a cell containing 50 μM profilin, 43 μM profilin-actin would be present (Sirotkin et al., 2010), which would support transient barbed end growth of newly formed filaments at a rate of 1 μm/s, and formin-bound barbed ends at 5–10 μm/s. These rates are much higher than those observed so far, which suggests that the amount of profilin-actin is lower than predicted by strong capping.

How does the interaction of profilin with barbed ends interfere with the function of CP, the most ubiquitous and abundant barbed end capper? CP is required in motile processes such as lamellipodia (Edwards et al., 2014). CP binds terminal actin subunits with a Kd of 0.1 nM (Wear et al., 2003). Tuning the extent of barbed end capping in the cytoplasm is essential. Over 90% of filaments must be capped to maintain a high concentration of actin monomers available for transient localized barbed end assembly in motility (Carlier and Pantaloni, 1997; Hug et al., 1995; Walsh et al., 1984). However, capping of 100% barbed ends inhibits all actin-based movements. Clearly, the potential competition between profilin and CP and its consequences in motility have to be addressed.

Binding of CP (1 nM) to the growing barbed ends of single filaments was slowed down by profilin (Figure 3A), consistent with profilin binding to terminal ATP-F-actin (Kd = 29.3 ± 1.7 μM), in competition with CP (Table S1). Similarly, profilin slowed down binding of CP in ADP-F-actin depolymerization assays (Figure S1A). In contrast, depolymerization of CP-capped filaments was unaffected by up to 100 μM profilin (Figure S1B). Hence, profilin inhibits CP association to barbed ends but does not uncap CP from barbed ends, in agreement with Bubb et al. (2003). These effects are observed in a physiologically relevant range of concentrations of profilin (dos Remedios et al., 2003) and of free CP, since the major fraction of cellular CP (total concentration 1–2 μM) is sequestered by myotropin/V1 (see Discussion).

How does the competition between profilin and CP affect the distribution of the filament population between the capped (blocked) and non-capped (dynamic) state? To address this issue, we measured the steady state amount of F-actin at different concentrations of CP and profilin in the range 0–10 μM (Figures 3B and S1C). In the absence of profilin, CP caused partial depolymerization of 0.5 μM F-actin, corresponding to the increase in the critical concentration from 0.1 to 0.6 μM, its value for pointed end assembly (Figure 3B inset, blue symbols). The major change in critical concentration (Walsh et al., 1984) occurs between 90% capping (1 nM CP) and 99% capping (10 nM CP). Addition of increasing amounts of profilin to filaments containing between 10 and 100 nM CP did not promote a linear decrease in F-actin leading to complete disassembly, as observed when barbed ends are strongly capped by gelsolin (orange triangles in Figure 3B; Equation 2, Supplemental Experimental Procedures). Strikingly, only partial F-actin disassembly was recorded (Figures 3B and S1C). A stationary level of profilin-actin, which increased with CP, was established at 10 μM profilin in dynamic equilibrium with the remaining F-actin (Figure 3B inset, red symbols). In conclusion, by antagonizing capping of barbed ends by CP, profilin maintains active monomer-polymer exchange at a fraction of barbed ends. The thermodynamic data thus agree with the kinetic data.

These results were corroborated by sedimentation assays. SDS-PAGE analysis and pyrenyl-fluorescence measurements of profilin-actin in the supernatants of F-actin (20 μM) capped by either CP or gelsolin, (Figure 3C) confirm that profilin (50 μM) promotes complete depolymerization of gelsolin-capped filaments but only partial depolymerization in the presence of CP, leaving 80% profilin free.

To confirm that the difference in behavior of profilin with CP-capped and gelsolin-capped filaments results specifically from its ability to interact with barbed ends, we used thymosin β4 as a passive G-actin sequesterer that does not interact with actin filaments. In contrast with profilin, thymosin β4 caused identical depolymerization of F-actin when filaments were capped by either CP or gelsolin (Figure 3D).

In conclusion, the ability of profilin to compete with CP lowers the fraction of profilin in the actin-bound state and imposes a higher amount of free profilin than expected in conventional views. Free profilin can thus compete effectively with other barbed end binding proteins. A diagram summarizing the distributions of F-actin, free profilin, and profilin-actin in various states of barbed ends and at physiologically relevant concentrations of all proteins is shown as an illustration (Figure 3E).

### Profilin Competes with Barbed End Tracking Proteins and Formin

How does profilin also compete with polymerases that track barbed ends? Potential candidates include formins (Goode and Eck, 2007) and multimeric WH2 domain proteins such as VogF and VASP (Breitsprecher et al., 2008; Hansen and Mullins, 2010; Pernier et al., 2013). The WH2 domain of formins and the WH2 domains display steric clashes with profilin binding to the barbed face of terminal F-actin (Carlier et al., 2015). On the other hand, association of profilin-actin to the FH1 domain of formin is...
essential for rapid processive assembly by formins (Kovar et al., 2006; Romero et al., 2004). Thus, the effects of profilin on formin function are potentially complex. Excess of free profilin inhibits processive elongation of filaments by formin (Kovar et al., 2006). Profilin also inhibits FH2 (Higgs, 2005; Scott et al., 2011). Thus, inhibition of formin by profilin may not be due only to displacement of profilin-actin from the FH1 domain.

How profilin affects the kinetics of FH1-FH2 of mDia1 association to barbed ends was addressed in microfluidics-assisted total internal reflection fluorescence (TIRF) microscopy assays (Figure 4A). The free barbed end of spectrin-actin initiated filaments was exposed briefly to FH1-FH2 in the presence of varying concentrations of profilin, before being exposed to profilin-actin only. Free and formin-bound barbed ends were discriminated by their rate of barbed end growth in profilin-actin (Figure S2A). Profilin inhibited binding of formin to barbed ends in a saturation fashion, consistent with a mutually exclusive binding scheme and a binding constant of profilin of 34 μM for barbed ends.

The effect of profilin on the kinetics of processive assembly was analyzed. Whether formin was (Figure S2B) or was not anchored (Figure 4B), a bell-shaped dependence of the rate of processive assembly on profilin was observed. Effective processive depolymerization was observed at high profilin. In the absence of actin, profilin enhanced depolymerization of FH1-FH2 bound filaments, in agreement with data obtained with anchored formin (Jegou et al., 2013). Controls run in comparison with free barbed ends are shown. Together, the data demonstrate that profilin destabilizes the barbed ends without displacing FH1-FH2. Remarkably, while formin “protects” barbed ends from destabilization by profilin in the presence of actin, it amplifies profilin-induced destabilization in the absence of actin.

Figure 3. Profilin Competes with CP at Barbed Ends
(A) Time course of the fraction of individual filaments that get capped for the first time, in the presence of 1 nM CP and 0, 20, or 60 μM profilin, and G-actin at concentrations ensuring a growth rate of 10 subunits/s (see Supplemental Experimental Procedures). Inset: Pseudo first-order rate constant for CP binding versus profilin, representing binding of profilin to ATP-bound barbed ends (Kd = 29.25 ± 1.75 μM).
(B) F-actin assembled at steady state (2.3 μM F-actin, 2% pyrenyl labeled) in the absence and the presence of CP, and profilin as indicated. Inset: concentration of actin monomers at steady state versus CP, in the absence (blue sigmoidal curve) or the presence of 5 μM profilin (red curve). Data derived from main frame. Orange triangles, gelsolin in place of CP.
(C) Profilin-actin complex in the supernatants of F-actin assembled at 20 μM in the presence of either 100 nM CP (blue symbols) or gelsolin at a 1:300 ratio to actin (red symbols). Closed and open symbols represent values derived from pyrene fluorescence and SDS-PAGE (top panel), respectively.
(D) Unassembled actin in supernatants of F-actin assembled in the presence of thymosin β4 or profilin, and CP or gelsolin. Note that in contrast to profilin, thymosin β4 sequesters actin identically when filaments are capped by CP or by gelsolin. Top panel: SDS-PAGE of the samples.
(E) Distribution of F-actin, profilin-actin, and free profilin in a medium containing 50 μM total actin and 50 μM total profilin, with various states of barbed ends.

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at steady state (2 \mu M actin) as a function of profilin in the absence and the presence of 44 nM FH1-FH2. Inset: effect of FH1-FH2 on F-actin at 80 nM profilin. (B) Profilin first assists, then inhibits formin-based rapid elongation at high profilin concentration. Filaments initiated from immobilized spectrin-actin seeds with free (red) or formin-bound (black) barbed ends are exposed to a flow containing profilin only (open symbols) or profilin with 0.5 \mu M G-actin (closed symbols). Barbed end elongation rates are monitored; N = 40–50 filaments. Error bars are SEM. (C) Profilin and formin antagonize in controlling the steady-state of actin assembly. Amount of F-actin at steady state in the absence and the presence of VopF (red, 50 nM; orange, 70 nM) and profilin. Inset: Effect of VopF on F-actin at 52 \mu M profilin.

The above kinetic data are confirmed by the thermodynamic data. Formin, VopF, and VASP all maintain a low critical concentration (high stability) of barbed ends (Pernier et al., 2013; Romero et al., 2004). Here we show that formin (Figure 4C) as well as VopF (Figure 4D) similarly antagonize the destabilizing effect of profilin at barbed ends by increasing the amount of F-actin (Experimental Procedures). The general view that reactivity of barbed ends is controlled by competitive binding is not new. VopF uncaps CP from barbed ends using its WH2 domains, the dissociation of CP being enhanced by VopF via a transient low-affinity ternary complex with barbed ends (Pernier et al., 2013). Formin uncaps CP using the same molecular mechanism (Shekhar et al., 2015).

**Profilin Binding to Barbed Ends Inhibits Filament Branching by N-WASP with Arp2/3 Complex and Actin-Based Motility**

The intriguing effects of profilin on cell motility reported earlier cannot be explained merely by its competition with barbed end trackers. In particular, the selective inhibition of lamellipodium (Cao et al., 1992), the disappearance of the WAVE-Arp2/3 branched filament array at 40 \mu M profilin (Rotty et al., 2015), and the inhibition of reconstituted propulsion of *Listeria* in the range of 10–50 \mu M profilin (Loisel et al., 1999) correlate with the inhibition of filament branching by profilin (Machesky et al., 1999; Rodal et al., 2003; Suarez et al., 2015). This effect required profilin’s ability to bind actin, its binding to poly-L-proline being dispensable (Rotty et al., 2015; Suarez et al., 2015). However, only binding of profilin to G-actin was considered in previous works.

We explored how profilin affects in vitro propulsion of N-WASP coated beads. Upon increasing profilin, the length of the actin tails decreased (Figure 5A) and branching density declined (Figures 5A and 5B). At 50 \mu M profilin, 60% of the beads moved only 2-fold slower than at 10 \mu M profilin (Figure S3A). Alexa 488-labeled Arp2/3 bound to N-WASP-coated beads identically at 3 or 50 \mu M profilin, testifying that only Arp2/3 incorporation in the tail is inhibited. Increasing the concentration of CP from 10 to 30 nM increased bead velocity by 22% at 20 \mu M profilin without restoring the original tail morphology. In summary, profilin inhibits filament branching by N-WASP-Arp2/3, corroborating recent reports (Rotty et al., 2015; Suarez et al., 2015).

Profilin also inhibited filament branching in spectrin-actin seeded polymerization assays with soluble VCA-Arp2/3, corroborating early (Machesky et al., 1999) and recent (Suarez et al., 2015) observations (Figure 5C). While 60 \mu M profilin slows down free barbed end growth by 2.2-fold, in the presence of Arp2/3 inhibition was much stronger than expected if only barbed end growth was inhibited (computed dashed curve in Figure 5C). The possibility that profilin competes with the WH2 domain of VCA for binding G-actin has been proposed (Suarez et al., 2015). Within this hypothesis, increasing VCA should balance out this effect. No reversal of the effect of 30 \mu M profilin was seen even by increasing the amount of VCA up to 10-fold (Figure S3B). Suarez et al. (2015) proposed that the direct competition between profilin and VCA for binding G-actin...
accounted for the inhibition of branching competing directly with VCA for binding G-actin, but they actually found a 5-fold decrease in affinity of VCA for G-actin under conditions (1 mM actin, 20 mM profilin) where a 200-fold decrease was predicted by a mutually exclusive binding scheme. In conclusion, both our and Suarez et al.’s data exclude that profilin inhibits branching only by displacing G-actin from VCA. Profilin also inhibited filament branching in single-filament assays (Suarez et al., 2015) (Figure 5D).

Inhibition of Arp2/3-mediated dendritic meshworks, irrespective of the nature of the branching protein (WAVE, N-WASP, VCA, ActA), takes place in a concentration range (5–100 μM) at which profilin binds to filament barbed ends, suggesting that profilin inhibits filament branching at barbed ends (Pantaloni et al., 2000). However, profilin might also bind and inhibit Arp2/3 complex (Mullins et al., 1998), a possibility weakened by the absence of profilin in large complexes in subcellular fractionation experiments (Kaiser et al., 1999). To distinguish between the two possibilities, we figured that if profilin simply inhibits Arp2/3, the destabilization of barbed ends by profilin at steady state should be unaffected by VCA-Arp2/3. In contrast, we find that the presence of VCA and Arp2/3 restores a higher level of F-actin (lower monomer concentration) in the presence of high amounts of profilin (Figure 5E). Thus, VCA and Arp2/3, like formin or VopF (Figures 4C and 4D), antagonize barbed end destabilization induced by profilin, supporting the view that filament branching by VCA-Arp2/3 takes place at barbed ends, at variance with the proposed side-branching model (Amann and Pollard, 2001a; Blanchin et al., 2000).

Live TIRF Microscopy Analysis of Assembly of Branched Filaments Reveals that Barbed End Branching Prevails over Side Branching

The side-branching model was tempered by the conspicuous observation that branching was favored in the region that appeared close to the barbed end where ADP-Pi subunits were thought to facilitate side branching (Amann and Pollard, 2001b). However, no experimental evidence has established a role of bound nucleotide in branching. This hypothesis, which had been discarded previously (Blanchin et al., 2000), was further disproved, as no massive increase in branching activity

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**Figure 5. Profilin Inhibits Filament Branching by Arp2/3 Complex and Resulting Actin-Based Motility**

(A) Double fluorescence (Alexa 594-actin and Alexa 488-Arp2/3) images of N-WASP-coated beads propelling in the reconstituted motility assay in the presence of profilin.

(B) Branching density ratio derived from integrated fluorescence intensity of Arp2/3 and actin along the comet tail.

(C) Effect of 60 μM profilin on barbed end growth initiated by 0.3 nM spectrin-actin seeds in the presence of 3 μM MgATP-G-actin and 0.16 μM VCA, in the absence and the presence of 46 nM Arp2/3 complex. Controls (no profilin) in dimmer colors. Dashed lines are calculated using a model (see Supplemental Experimental Procedures) in which filament branching is unaffected by profilin and filaments grow at the standard growth rate (dim red) and at a rate 55% lowered by profilin (bright red).

(D) Images of filaments branched with VCA and Arp2/3 complex at different concentrations of profilin after 1,000 s. Red dots, branch junctions.

(E) Destabilization of filaments by profilin is relieved by VCA-Arp2/3. Assembled F-actin in the presence of profilin and 50 nM VCA, with or without 30 nM Arp2/3. Inset: Increase in F-actin upon addition of Arp2/3 complex (30 nM) to F-actin pre-assembled with VCA and without (blue) or with (red) 60 μM profilin.
was measured on F-ADP-Pi filaments (Le Clainche et al., 2003) or in the presence of BeF3 (here, Figure S4). However, several reports showed clear evidence of side branching off F-ADP filaments (Smith et al., 2013; Risca et al., 2012), with a very low frequency of $3 \times 10^{-5}$ M$^{-1}$ s$^{-1}$ (Smith et al., 2013). How two mechanisms of branching could co-exist is not understood from available data.

We reasoned that different morphologies of individual dendritic structures initiated from a single nucleus would be obtained within the “side-branching only” versus the “end-branching only” mechanisms. While end branching generates a dichotomous fractal morphology, side branching increases the branching density on older, longer exposed regions of the filaments, generating a more bushy morphology (Figure 6A).

Because filaments are helical, helical filament branching develops 3D arborescent structures. Observation of filament branching in a constrained 2D geometry introduces biases and limits the rotational freedom of filaments (see Supplemental Experimental Procedures). To optimize the comparison of kinetic information derived from bulk solution and single-filament TIRF measurements of filament branching, we analyzed TIRF recordings of the spontaneous assembly of G-actin into non-anchored filaments branching and growing at a constant rate (see Supplemental Experimental Procedures). The following observations were made (Figure 6B). The first branching event $B_0$ occurs very early following nucleation, at a distance $d_0$ from the mother filament pointed end of less than 0.8 μm, leading to a large number of symmetric V-shaped structures (Figure 6B; Movies S1 and S2). Notably, if branching occurred mainly from the side of filaments, the branching frequency derived from the value of $d_0$ would generate such densely branched filaments that individual branches would be unresolvable in TIRF. In contrast, the next branching event $B_1$ on the mother filament takes place at a 3-fold larger distance $d_1$ from $B_0$ (Figure 6C). Moreover, the distances $d_1$, $d_2$, and $d_3$ between consecutive branching points $B_0$, $B_1$, $B_2$, and $B_3$ along the same mother filaments (see Supplemental Experimental Procedures) had the same value of 2.5 ± 0.3 μm (925 ± 100 subunits) (N = 250, 150, and 50 for $d_1$, $d_2$, and $d_3$ respectively (Figure 6C).

Most branching events occurring in the plane of observation generated mother and daughter filaments of equal length (Movies S1 and S2). Branching at an angle from the plane generated bright dots often leading to late emergence of already long filaments (see Supplemental Experimental Procedures). Rare side-branching events were identified (arrows in Figure 6B bottom panel and Movie S1, right panel), so that the distance $d_1$...
appeared essentially conserved, within the SD, over 200 s following the appearance of B1. Note that monitoring of spontaneous assembly from G-actin facilitates the evidence for barbed end branching in the early steps of assembly when little F-actin has assembled. The probability of side branching increases as F-actin accumulates.

These data support the view that branching occurs mainly at barbed ends at a constant frequency as the filament is growing at constant rate. The contribution of side-branching events is too small to bias the evidence for the main process in the period of time investigated. The value of the frequency of barbed end branching, $k_{bb} = 0.9 ± 0.1 \text{mM}^{-1} \text{s}^{-1}$, was derived from the measured filament growth rate (9 subunits/s) and the average distance between branching points (925 subunits), and assuming all the Arp2/3 (12.5 nM) to be in an active complex with VCA. The small value of $d_0$ compared with $d_1$, $d_2$, and $d_3$ suggests that barbed end branching of very short filaments prevents their loss by total disassembly.

The frequency of side branching was evaluated using a two-color fluorescence assay (Movie S3). Pre-assembled Alexa 488-actin filaments (green actin) were flushed in the chamber together with Alexa 594-G-actin (red actin), VCA, and Arp2/3. The frequency of side branching, $k_{sb}$, derived from the linear time dependence of the side branching of red filaments off the side of green filaments (Figure 6D and Supplemental Experimental Procedures, Equation 3) was $10.5 \times 10^{-3} \text{mM}^{-1} \text{s}^{-1}$, in satisfactory agreement with Smith et al. (2013) and with Figure 2D in Risca et al. (2012). Newly initiated red filaments branched in arborescent structures displaying the same morphology as in Figure 6B. Note that if only side branching was imposed to accommodate the data in Figure 6B, producing one branching event every 925 subunits on average, the frequency of side branching would have to be $1.08 \times 10^{-2} \text{mM}^{-1} \text{s}^{-1}$, that is, 10-fold higher than the actual value measured for $k_{sb}$.

The Arp2/3 Branching Machinery Competes with Proteins Tracking Barbed Ends

If filaments branch at barbed ends, proteins tracking or capping barbed ends should also compete with VCA-Arp2/3. This was actually observed. In a bulk solution assay, VopF inhibited filament branching, like profilin (Figure 7A). In TIRF assays, filaments elongating in the presence of barbed end-bound VopF
or FH1-FH2 of mDia1 failed to branch at barbed ends (Movie S4).
The time course of F-actin assembly and the general pattern of branched filaments were dramatically different from the densely branched patterns observed in the absence of barbed end binding reagents. Only side branching occurred with the expected $t^2$ dependence due to the fact that the amount of F-actin exposed to side branching increases as filaments grow (Figures 7B and 7C and Equation 4, Supplemental Experimental Procedures).

Finally, in the presence of $80 \mu$M profilin, barbed end branching vanished and side branching supported the rare remaining branching events (Figures 7B and 7C). The same value of $k_{sb}$ was derived from analysis of the samples containing either formin or VopF or profilin as in Figure 6D.

The barbed end polymerase VASP harbors “anti-capping” properties (Bear et al., 2002; Breitsprecher et al., 2008; Hansen and Mullins, 2010) using its WH2 domains, as VopF does. VASP was also suggested to be an “anti-branching” factor (Bear et al., 2002; Skoble et al., 2001). To get mechanistic insight into this behavior of VASP, we reconstituted actin-based propulsion of beads coated with the Listeria protein ActA, a functional homolog of N-WASP (Boujemaa-Paterski et al., 2001; Skoble et al., 2000). VASP binds FPPPP repeats in ActA (Niebuhr et al., 1997) and enhances Listeria motility by an unknown mechanism (Laurent et al., 1999; Loisel et al., 1999; Smith et al., 1996).

ActA-coated beads propelled by sustained assembly of dense Arp2/3-branched actin tails. Addition of VASP in the medium promoted a dramatic change in the morphology of tails into long unbranched actin bundles strikingly similar to formin-induced tails (Benanti et al., 2015; Romero et al., 2004) and propulsion was 3-fold faster, indicating that VASP inhibits filament branching by ActA-Arp2/3 by tracking filament barbed ends (Figure 7D and Movie S5).

**DISCUSSION**

This work reveals a new face of profilin: Its interaction with actin filament barbed ends has profound effects on assembly dynamics, actin homeostasis, and resulting motility. Profilin promotes large fluctuations of filament length that evoke mild dynamic instability. Profilin stands as a major competitor of barbed end regulators such as CP, formins, WH2 domain proteins that track barbed ends, or the N-WASP-Arp2/3 filament branching machinery. Filament branching takes place mainly via association of WASP-Arp2/3 with terminal barbed end subunits, explaining the persistent polarity of the lamellipodial network. Profilin appears as a major coordinator of actin filament polarized growth in cell migration and developmental processes. Finally, our results clarify the still elusive aspects of “anti-capping” and “anti-branching” regulation of actin filament dynamics (Rotty et al., 2015).

**Profilin Enhances Filament Barbed End Dynamics and Resulting Length Fluctuations**

At the steady state of actin assembly, length fluctuations resulting from the different dynamics of ADP-Pi and ADP-actin are enhanced by profilin by a factor of ~5–10. This mild dynamic instability affects the length distribution of filaments, by promoting total disassembly of short filaments. In our simulations, at an average growth rate of 1 subunit/s, more than 60% of nucleated filaments disappear in a few minutes at 30 $\mu$M profilin due to length fluctuations, versus less than 30% in the absence of profilin.

**Filament Assembly from Profilin-Actin Requires MgATP**

While actin assembles well in filaments regardless of the nature of the bound nucleotide (ATP, ADP, or AMPPNP) and associated divalent metal ion (Mg$^{2+}$ or Ca$^{2+}$), barbed end growth is observed only from profilin-MgATP-actin. Filament elongation from profilin-actin requires a drop in affinity of profilin following association of each profilin-actin to the barbed end. Profilin has a low affinity for terminal AMPPNP-F-actin, yet barbed end growth from profilin-AMPPNP-actin fails to proceed. Consistently, the isoenergetic square describing association of actin and profilin at barbed ends (Pantaloni and Carlier, 1993; Yarmola and Bubb, 2006) is satisfied in AMPPNP but not in MgATP. Perhaps cleavage of the $\gamma$-phosphoester bond of ATP on the terminal or penultimate subunit facilitates the structural change leading to dissociation of profilin from the barbed end.

**Physiological Relevance of the Competition between Profilin and Barbed End Binding Proteins**

CP is the major capping protein in cells. We find that in a concentration range of 10–100 $\mu$M profilin and 1–100 nM CP, competition between profilin and CP at barbed ends results in a lower amount of profilin-actin co-existing with F-actin and CP than in conventional views based on strong capping. In turn, the fraction of profilin in the free state is higher than expected. Do these results have physiological significance given the abundance and much higher affinity of CP than profilin for barbed ends? We believe they do, first because active CP is present at a few nanomolar, since 98% of the total amount of CP (1 $\mu$M) is maintained inactive in a high-affinity complex ($K_0 = 7$ nM) with myotrophin/ V1, present at 3 $\mu$M (Edwards et al., 2014; Fujiiwara et al., 2014; Takeda et al., 2010). Second, in the range of 90–100% capped filaments, a drop of only a few percent promotes a massive change in the steady state of actin assembly (Walsh et al., 1984; Perrier et al., 2013). Estimates can be found for the amount of unassembled actin monomers in cells, but the concentrations of (polymerizable) profilin-actin and free profilin are not well known (Moseley and Goode, 2006; Sirotnik et al., 2010).

Nevertheless, the measured rates of transient filament growth in motile processes match a concentration of a few micromolar profilin-actin, consistent with our proposed scenario in which profilin competes with CP at barbed ends. Competition may be expected as well between profilin and CPs such as Eps8, IQGAP1, or CapG, which bind barbed ends with affinities in the nanomolar range.

Kinetic and steady state F-actin measurements show that, at high concentration, profilin inhibits binding of formin or VopF to barbed ends. We confirm and build on observations of inhibition of filament branching by profilin made by Machesky et al. (1999), Rotty et al. (2015), and Suarez et al. (2015) to show that it is by binding to barbed ends that profilin inhibits filament branching by VCA, N-WASP, and ActA with Arp2/3 complex. The modest profilin-induced decrease in affinity of VCA for actin (Suarez et al., 2015) suggests that an active ternary complex forms between profilin, actin, and VCA, as reported for profilin, actin,
and β-thymosin/WH2 domains (Xue et al., 2014; Yarmola and Bubb, 2004). Inhibition of branching by binding of profilin to barbed ends is consistent with early observations (Cao et al., 1992) that, upon injection in cells, profilin in contrast with standard sequestering agents promotes selective disassembly of lamellipodial arrays. Our data support the view that injection of profilin abrogates barbed end branching at the leading edge, leading to loss of sustained formation of new filaments, loss of contacts between the membrane and the cytoskeleton, and subsequent pointed end disassembly of the array. Increasing profilin should also lower the extent of barbed end capping, which synergizes in slowing down migration. The facts that excess profilin slows down the motility of cancer cells (Roy and Jacobson, 2004) and abrogates a lamellipodial network in control cells while increasing F-actin in Arpc2−/− cells (Rotty et al., 2015) are consistent with our data.

Our results support the following mechanistic view. In live cells, the concentration of polymerizable actin monomers results from the regulated cycles of assembly and disassembly of actin filaments (Carlier et al., 2015; Danuser et al., 2013; Xue and Robinson, 2013). The pools of capped, free, and tracker-bound barbed ends, free G-actin, profilin-actin, and free profilin are in a complex dynamic equilibrium. The pool of polymerizable monomeric actin is replenished, i.e., non-finite, and profilin orchestrates actin homeostasis. This view differs from the one in which several filament assembly machineries compete for a finite pool of actin monomers (Suarez et al., 2015).

Filament Branching Occurs Mainly via Association of VCA-Arp2/3 to Filament Barbed Ends

Evidence for barbed end branching by VCA-Arp2/3 is provided by the inhibition of branching by profilin and proteins that track filament barbed ends (formin, VopF, VASP), and by thermodynamic data showing that the destabilization of filament barbed ends by profilin is antagonized by VCA-Arp2/3. Consistently, capping of barbed ends is energetically more costly in the presence of VCA-Arp2/3 (Pantaloni et al., 2000) and gelsolin-capped filaments fail to stimulate branching (Figure 4 in Boujemaa-Patet- ski et al., 2001). Other reported inhibitors of branching may also act at barbed ends. Our analysis of live imaging of filament branching clarifies conflicting views regarding barbed end branching and side-branching mechanisms in providing estimates of the frequency of each process.

Analyses of filament branching were derived from fluorescence microscopy of fixed filaments, branched in the presence of phalloidin (Blanchin et al., 2000) or from electron microscopy images of short branched filaments (Pantaloni et al., 2000). Often, filaments were tethered to the coverslip while being exposed to VCA and Arp2/3 (Aman and Pollard, 2001b; Risca et al., 2012; Smith et al., 2013). Most studies concentrated on analysis of side-branching events, favored when barbed ends were capped (Smith et al., 2013). Yet, in Figure 1D of Risca et al. (2012), 20% of surface-tethered red filaments display identifiable barbed end branching, consistent with a very low frequency \( k_{\text{br}} \) of 0.06–0.11 \( \mu M^{-1} \text{s}^{-1} \) (branching occurred at a distance of one-quarter of the length of green filaments assembled over 70–120 s).

In our experiments, in contrast, filaments are not tethered to the coverslip while being exposed to VCA and Arp2/3. The rotational freedom of the nucleating and growing barbed ends is therefore closer to a situation in which filaments branch in 3D in solution, e.g., in pyrene-actin fluorescence assays of branched filament assembly. Hence, inhibition of branching by VopF or profilin is recorded both in bulk solution kinetics and in live fluorescence microscopy (compare Figures 7A and 7C, 5C and 7C, respectively), consistent with the view that barbed end branching is the predominant pathway. We find that immobilization of filaments by a streptavidin-biotin link appreciably impairs barbed end reactivity, slowing down both the growth rate and the branching frequency (Figure S5 and Movie S6).

Presumably, the same structural organization of the Arp2/3 subunits at the branched junction is built via either end branching or side branching. How can the same protein-protein contacts be eventually established via each pathway? One possibility is that the observed structural change of several actin subunits of the mother filament at the branch junction (Rouiller et al., 2008) is facilitated in barbed end branching. Another possibility is that the WH2 domain of VCA uses its ability to capture barbed ends (Co et al., 2007) in the branching reaction. The plasticity of the filament (Galkin et al., 2010) might allow side branching via insertion of the WH2 domain into the core of the filament, as WH2 domain proteins such as Spire or Cobl do (Carlier et al., 2013). The preferential side branching on the convex face of curved filaments (Risca et al., 2012) is suggestive of such a possibility. Further biochemical and structural experimentation and modeling are required to test this hypothesis.

In vivo, filament branching is catalyzed by small-size WASP proteins that localize at membranes in protrusive, compressive, or adhesive processes or at the surface of a pathogen where filament barbed ends abut. Barbed end branching ensures the persistence of polarized dendritic arrays and allows the growth of mother and daughter branches to equally contribute to production of force. Assuming a barbed end branching frequency of 1 \( \mu M^{-1} \text{s}^{-1} \), and cellular concentrations of 1–5 \( \mu M \) profilin-actin and 0.1–0.2 \( \mu M \) Arp2/3, the branching distance would be between 0.1 and 0.5 \( \mu M \), in satisfactory agreement with measurements in lamellipodia (Iwasa and Mullins, 2007). Our conclusions have profound implications regarding the molecular mechanism by which dendritic structures are formed in numerous processes dependent on WASP family proteins and Arp2/3 complex and on the associated physical mechanism of force production.

**EXPERIMENTAL PROCEDURES**

**Proteins**

Actin from rabbit muscle was isolated in G form, pyrenyl labeled on cysteine 374, and Alexa 488-, Alexa 594-, or biotin-labeled on lysines (Thermo Scientific). Arp2/3 was purified from ovine brain. Recombinant mouse profilin 1, N-WASP VCA, CP, gelsolin, VopF (Pernier et al., 2013), recombinant VASP (Laurent et al., 1999), ActA (Cicchetti et al., 1999), mDia1 FH1-FH2 (Romero et al., 2004), and biotinylated SNAP-tagged FH1-FH2 (Shekhar et al., 2015) were used.

**Kinetic Measurements of Filament Barbed End Growth**

Initial rates of filament barbed end growth or disassembly were monitored using the change in pyrenyl-actin fluorescence in a Safas Xenius spectrolfluorimeter (Safas). For details see Supplemental Experimental Procedures.
Measurements of Assembled and Unassembled F-Actin at Steady State

F-Actin (labeled with 2% pyrenyl) was incubated overnight at 4°C in the dark in the presence of regulatory proteins. Fluorescence intensity was converted into F-actin amounts using standards. The concentration of unassembled actin reflected the thermodynamic stability of F-actin in the presence of effectors acting antagonistically at barbed ends. For details, see Supplemental Experimental Procedures.

TIRF Microscopy of Single Filaments

Microfluidics-assisted TIRF microscopy was used to monitor the kinetics of filament growth or depolymerization using an Olympus IX71 microscope with a 60x oil objective and a Cascade EMCCD (Photometrics) camera (Jegou et al., 2011, 2013). Filament assembly was initiated from spectrin-actin functionalized glass coverslips or from anchored forms (Shekhar et al., 2015). A standard open chamber TIRF method was used to monitor the kinetics of individual filament branching and growth in the presence of actin, VCA, and Arp2/3 complex. For details, see Supplemental Experimental Procedures.

Numerical Simulations of Filament Elongation

Simulations were performed with a program written in C (free BloodshedDevC++) following a Gillespie algorithm. The length of individual filaments was computed over time for a population of at least 50 filaments for a given set of kinetic parameters. The same analysis of length fluctuations was performed on simulated and experimental data. See Supplemental Experimental Procedures for details.

Bead Motility Assay

Experiments were conducted as described previously (Wiesner et al., 2003), except for CP replacing gelsolin. For details, see Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, one table, and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.12.024.

AUTHOR CONTRIBUTIONS

M.F.C. designed the project, performed bulk solution experiments, and wrote the paper; J.P. performed and analyzed bulk solution and single-filament experiments; S.S. performed and analyzed single-filament and motility assays; A.J. performed and analyzed length fluctuations measurements; B.G. provided technical help and performed bulk solution measurements.

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Supplemental Information

Profilin Interaction with Actin Filament
Barbed End Controls Dynamic Instability, Capping, Branching, and Motility

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Supplemental Information

Profilin interaction with actin filament barbed end controls dynamic instability, capping, branching and motility

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Supplemental Figures

Figure S1. Profilin competes with CP for barbed end binding but does not uncap CP, related to Figure 3.

A. Time course of dilution-induced depolymerization of 2% labeled pyrenyl-labeled F-actin in F buffer containing indicated amounts of CP alone (left panel) or CP + 100 µM profilin (right panel).

B. Time course of dilution-induced depolymerization of 2% labeled pyrenyl-labeled, CP-capped, F-actin in F buffer containing the indicated amounts of profilin. Traces are shifted vertically for readability.

C. Measurements of F-actin (2.15 µM total actin, 2% pyrenyl-labeled) at steady state in absence (blue) and presence of CP at 4 nM (red) and 50 nM (green). The pyrenyl-actin fluorescence intensities are converted into mass amount of F-actin.
Figure S2. Competition between profilin and formin, related to Figure 4.

A. Kymographs of filaments corresponding to Figure 4A. Filaments were grown off immobilized spectrin-actin seeds in the presence of profilin-actin and exposed to formin in the presence of 50 µM profilin for 10s, followed by profilin-actin only. Left panel shows a filament that has not bound formin during the short exposure period and resumes slow growth following removal of formin. Right panel shows a filament that has bound formin as appears from the fast growth following removal of formin. Note that during exposure to formin + profilin, the two filaments depolymerize to different extents, which is also an indication of whether formin is (fast depolymerization, right panel) or is not bound (slower depolymerization, left panel)

B. Biphasic effect of profilin on formin. Profilin increases, then decreases the rate of processive elongation without causing dissociation of the filament from formin. The growth rate of filaments initiated from anchored formins was measured at 0.5 µM actin and profilin as indicated. Data are comparable to those obtained with filaments initiated from immobilized spectrin-actin seeds (Figure 4B). Error bars are S.E.M.
Figure S3. Inhibition of filament branching by profilin, related to Figure 5.

A. Average velocity of N-WASP coated beads (closed circles, left axis) and fraction of motile beads (open squares, right axis) as a function of profilin (conditions as in Figure 5A). Error bars are standard deviations.

B. Inhibition of filament branching by profilin is not relieved by increasing amounts of VCA. Spectrin-actin seeded barbed end growth was monitored in the presence of 2.78 µM actin (2% pyrenyl-labeled) and 0.2 µM VCA and the following additions: none (dim blue); 32 µM profilin (dim red); 25 nM Arp2/3 (dark blue without profilin, bright red with 32 µM profilin; pale and dark green with 32 µM profilin and 0.1 or 1 µM VCA respectively). Changes in VCA did not affect growth rate in the presence of 32 µM profilin.
Figure S4. Filament branching is not facilitated off F-ADP-Pi, related to Figure 6.

Spontaneous assembly of actin in branched filaments is monitored using the pyrenyl-actin fluorescence assay, with or without 100 µM BeF₃⁻. The time course of branched filament assembly is not modified by BeF₃⁻, a structural analog of Pi. Note the 17% lower fluorescence of pyrenyl-ADP-BeF₃⁻-F-actin as compared to pyrenyl-ADP-F-actin (Combeau and Carlier, 1989). Normalization of the BeF₃ curve restores a curve identical to the control in absence of BeF₃⁻, demonstrating that an identical number of branching events have occurred in both assays.
Figure S5. The barbed ends of filaments growing in a coverslip-tethered state via a streptavidin-biotin link display reduced reactivity and branching frequency, related to Figure 6.

Time lapse images of assembly of branched filaments with 0.9 µM actin (10% Alexa 488-labeled) without (A) and with (B) 7.5 % biotinylated actin, 50 nM VCA and 12.5 nM Arp2/3 complex. Coverslips in A and B were identically treated with biotin-PEG-silane and streptavidin (see Supplemental Experimental Procedures). Filaments grow at 9 su/s (A) and 6 su/s (B).
Supplemental Movies

Movie S1. Filament branching by VCA-Arp2/3 complex, related to Figure 6B.
TIRFM visualization of the assembly of single filaments branching with 0.9 µM actin (10 % Alexa 488-labeled), 50 nM VCA and 12.5 nM Arp2/3 (corresponding to Figure 6B). Scale bar 2 µm. Time in s.

Movie S2. Filament branching by VCA-Arp2/3 complex, related to Figure 6C.
TIRFM visualization of the assembly of single filaments branching with 0.9 µM actin (10 % Alexa 488 labeled), 50 nM VCA and 12.5 nM Arp2/3 (analysis in Figure 6C) Scale bar 10 µm. Time in s.

Movie S3. Filament side branching by VCA-Arp2/3 complex, related to Figure 6D.
TIRFM visualization of the assembly of single filaments branching with 0.9 µM actin (10 % Alexa 488 labeled). At t = 0.9 µM actin (10 % Alexa 594 labeled) was flown into the reaction chamber together with 50 nM VCA and 12.5 nM Arp2/3. The boxed area is shown in Figure 6D. Scale bar 10 µm. Time in s.

Movie S4. Effect of barbed-end interacting proteins on filament branching, related to Figure 7.
Filament branching by VCA-Arp2/3 complex in the presence of 3 µM profilin.
TIRFM visualization of the assembly of single filaments branching with 0.9 µM actin (10 % Alexa 488 labeled), 200 nM VCA and 50 nM Arp2/3 in presence of 3 µM profilin. The boxed area is shown in Figure 7B. Scale bar 10 µm. Time in s.

Inhibition of filament barbed end branching by VopF.
TIRFM visualization of the assembly of single filaments branching with 0.9 µM actin (10 % Alexa 488 labeled), 3 µM profilin, 200 nM VCA, 50 nM Arp2/3, 200 nM VopF. The boxed area is shown in Figure 7B. Scale bar 10 µm. Time in s.

Inhibition of filament barbed end branching by formin.
TIRFM visualization of the assembly of single filaments branching with 0.9 µM actin (10 % Alexa 488 labeled), 3 µM profilin, 200 nM VCA, 50 nM Arp2/3 and 15 nM FH1-FH2 mDia1. The boxed area is shown in Figure 7B. Scale bar 10 µm. Time in s.

Inhibition of filament barbed end branching by 80 µM profilin.
TIRFM visualization of the assembly of single filaments branching with 0.9 µM actin (10 % Alexa 488 labeled), 200 nM VCA, 50 nM Arp2/3 and 80 µM profilin. The boxed area is shown in Figure 7B. Scale bar 10 µm. Time in s.

Movie S5. VASP competes with filament branching at barbed ends, related to Figure 7.
Phase contrast movies of ActA-coated beads in a reconstituted motility assay containing 7 µM F-actin, 2 µM profilin, 100 nM Arp2/3, 3.5 µM ADF and 200 nM gelsolin, in absence or presence of 100 nM VASP. The boxed area is shown in Figure 7D. Scale bar 10 µm. Time in min.

Movie S6. Effect of filament anchoring on barbed end branching, related to Figure 6 and S5.
Filament branching by VCA-Arp2/3 complex using biotin-PEG passivation.
TIRFM visualization of the assembly of single filaments branching with 0.9 µM actin (10 % Alexa 488 labeled), 50 nM VCA and 12.5 nM Arp2/3 (corresponding to Figure S5A) Scale bar 2 µm. Time in s.
Inhibition of filament barbed end branching by filaments anchoring.
TIRFM visualization of the assembly of single filaments branching with 0.9 µM actin (10 % Alexa 488 and 7.5% labeled), 50 nM VCA and 12.5 nM Arp2/3 (corresponding to Figure S5B) Scale bar 2 µm. Time in s.
**Supplemental Table**

**Table S1. Parameters used in numerical simulations, related to Figure 1.**

| Rate Constant | Value | Unit       | Reference                      |
|---------------|-------|------------|--------------------------------|
| $k_{\text{on}}$ ATP-actin | 10    | $\mu$M$^{-1}$ s$^{-1}$ | (Pantaloni et al., 2000) |
| $k_{\text{off}}$ ATP-actin | 1     | s$^{-1}$    | (Pantaloni et al., 2000) |
| $k_{\text{off}}$ ADP-Pi-actin | 0.2   | s$^{-1}$    | (Jegou et al., 2011) |
| $k_{\text{off}}$ ADP-actin | 5.8   | s$^{-1}$    | (Jegou et al., 2011) |
| $K_D^\text{prof-ATP-BE}$ | 29.25 | $\mu$M     | this paper                     |
| $K_D^\text{prof-ADP-Pi-BE}$ | 5.9   | $\mu$M     | (Jegou et al., 2011)          |
| $K_D^\text{prof-ADP-BE}$    | 28.1  | $\mu$M     | (Jegou et al., 2011)          |
| $k_{\text{off}}$ prof-ATP-actin | 5     | s$^{-1}$    | this paper                     |
| $k_{\text{off}}$ prof-ADP-Pi-actin | 4.7   | s$^{-1}$    | (Jegou et al., 2011)          |
| $k_{\text{off}}$ prof-ADP-actin | 51.6  | s$^{-1}$    | (Jegou et al., 2011)          |
| $k_{\text{cleave}}$           | 0.3   | s$^{-1}$    | (Blanchoin and Pollard, 2002) |
| $k_{\text{BE cleave}}$        | $\leq$ 0.3 | s$^{-1}$    | this paper                     |
| $k_{\text{BE prof cleave}}$   | $\leq$ 0.3 | s$^{-1}$    | this paper                     |
| $k_{\text{Pi release}}$       | 0.007 | s$^{-1}$    | (Jegou et al., 2011)          |
| $k_{\text{BE Pi release}}$    | 1.8   | s$^{-1}$    | (Jegou et al., 2011)          |
| $k_{\text{BE prof Pi release}}$ | 6.8   | s$^{-1}$    | (Jegou et al., 2011)          |
Supplemental Experimental Procedures

**Kinetic measurements of barbed end growth rate and J(C) plots**

A filament solution (2.5 µM F-actin) used as seeds was diluted into F buffer containing various concentrations of G-actin and profilin. The same 2% pyrenyl-labeled actin solution was used to prepare the G-actin and the F-actin solution used as seeds (Carlier et al., 1984). This low fraction of pyrenyl-actin reduced the bias in the interpretation of the fluorescence changes in terms of amounts of F-actin, which results from the low affinity of profilin for G-actin in which cysteine 374 is modified ((Malm, 1984)). As noted by Bubb et al. (Bubb et al., 2003), the error is negligible in the depolymerization assays.

Stock solutions of 10 µM MgATP-G-actin were prepared by addition of 20 µM MgCl₂ and 0.2 mM EGTA to CaATP-G-actin, and kept on ice for a few hours.

MgADP-G-actin was prepared by adding 0.2 mM ADP, 20 µM MgCl₂, 0.2 mM EGTA, 1 mM glucose, 1 µM Ap₅A and 15 U/ml hexokinase to a solution of 10 µM CaATP-G-actin 1:1 complex freed from ATP by two consecutive Dowex-1 treatments (Mockrin and Korn, 1980). MgAMPPNP-G-actin was prepared similarly except without Ap₅A and replacing ADP by 1 mM AMPPNP. Hexokinase and glucose were maintained in all AMPPNP-containing G and F buffers to eliminate traces of ATP (Motojima and Yoshida, 2003), (MONTAVILLE et al., 2014). Trace amounts of ATP in AMPPNP cause transient ATP-like behavior in bulk solution studies of actin assembly (Romero et al., 2004), and are likely to introduce long term ATP effects in TIRF assays, in which the very low number of filaments fails to exhaust ATP rapidly. F-AMPPNP-actin seeds were prepared by adding 0.1 M KCl and 1 mM MgCl₂ to a 10 µM MgAMPPNP-G-actin solution.

**Single filament microscope observations using microfluidics**

Filament barbed end growth was initiated from immobilized spectrin-actin seeds, by flowing in 15% Alexa-488 labeled 1µM MgATP-G-actin in order to reach filaments of ~5µm in length. Filaments were then exposed to a solution of various actin, CP, formin and profilin concentrations for 5 minutes before the image acquisition is started in TIRF. Alternatively, filaments were initiated from immobilized biotinylated SNAP-tagged formin (Shekhar et al., 2015).

For length fluctuations measurements, images were acquired every 30 seconds in epifluorescence exposing anchored filaments for 27 seconds to a solution of a defined actin and profilin concentrations, using 15% labeled actin, and for 3 seconds to a solution of identical actin and profilin concentrations but using unlabeled actin. Images are acquired during exposure to unlabeled actin, at maximum flow rate to ensure proper alignment of the filament relative to the glass surface and minimize background noise. Fluorescence exposure time is set to 500ms. Images are processed using ImageJ and the KymoToolbox plugin (available upon request to fabrice.cordelieres@u-bordeaux.fr) to obtain length versus time kymographs. Filament length variations were measured by fitting the pixel intensity profile close to the barbed-end by Gaussian survival function (8, 9). Only filaments traces that last more than 5 consecutives images are kept for analysis. Using numpy/python, the mean square displacement is then computed $\text{MSD}(\Delta t) = \langle (x(t+\Delta t)-x(t))^2 \rangle$ and fitted by $(a^{*}\Delta t)^2 + 2*b^{*}\Delta t + c$, where a is the mean elongation rate, b the diffusion coefficient and c a constant that emerge from the experimental noise (mainly from signal to noise ratio (originating from actin labeling fraction of actin and background noise) and unfocusing). We ensure that no drift of
the setup that would artificially add-up to the mean elongation rate is present, by tracking the position of the pointed end.

To analyze the inhibition of CP association to barbed ends by profilin, the elongation of filaments was monitored in epifluorescence using the method described above, exposing filaments to a solution of labeled actin, profilin, and Capping Protein, and to a solution of identical protein content but with unlabeled actin. Filament contrast was improved with KymoToolbox, and a kymograph was generated for each filament using MultipleKymograph in ImageJ. For each filament, the time of the first pausing event was observed directly on the kymograph. The actin concentrations varied, in order to have an elongation rate of approximately 10 subunit/second (in the non-capped state), regardless of the profilin concentration. In the absence of profilin, the rate of capping at 1nM CP was found to be independent of the observed elongation rate (comprised between 0.6 and 1.4 subunit/second, using different actin concentrations).

**Numerical simulations**

Simulations were performed using a Gillespie algorithm, which can be summarized as follows. Based on the simulated filament’s composition, and the nucleotide-state of its barbed end, all possible events (addition/loss of subunit, cleavage of ATP, etc.) are listed and their probabilities are computed according to their reaction rate constants. The time of occurrence of the next event is determined following the generation of a random number by the computer program, and the nature of the event that occurs is subsequently determined, following the generation of another random number. The composition of the filament is modified accordingly, and the process is then repeated.

For each simulated filament, the total concentrations of profilin and ATP-actin are fixed, and the concentrations of profilin-actin, free actin and free profilin are computed analytically (using $K_D$=0.1 µM for profilin-MgATP-G-actin). The effective reaction rate constants that depend on these concentrations are also computed, assuming that profilin is in rapid equilibrium with the barbed end (Jégou et al. 2011). For example, the effective ADP-actin off rate constant is

$$v_{off}^{ADP-actin} = k_{off}^{ADP-actin} + (k_{off}^{prof-ADP-actin} - k_{off}^{ADP-actin}) \frac{[profilin]}{([profilin]+K_D^{prof-ADP-BE})}$$

(equation 1) where $k_{off}^{ADP-actin}$ is the barbed end off-rate constant for ADP-actin, $k_{off}^{prof-ADP-actin}$ is the barbed end off-rate constant for profilin-ADP-actin, and $K_D^{prof-ADP-BE}$ is the dissociation constant for profilin at an ADP barbed end.

The possible events that can occur are the following. The terminal subunit can dissociate from the barbed end. ATP-G-actin can bind to the barbed end. Profilin-ATP-G-actin can bind to the barbed end. If the terminal subunit is ATP-actin, it can cleave its ATP. If the terminal subunit is ADP-Pi-actin, it can release its Pi. All ATP-actin subunits within the filament can cleave their ATP. All ADP-Pi-actin subunits within the filament can release their Pi.

The program keeps track of the nucleotide state of each subunit in the filament, throughout the simulation.

Only barbed end dynamics were considered. The initial filament state in our simulations was an ADP-filament with 3 terminal ATP-subunits at the barbed end. In all the conditions that we have used in this article, steady-state was reached within a few seconds. The average elongation rates (Fig. 1B) were computed on a population of 50 filaments, for profilin concentrations multiple of 0.5 µM. For length fluctuations (Fig. 2D) the MSD analysis was applied to a population of 200 filaments, with the same time step as for experimental data.
Measurements of unassembled actin at steady state as a probe of the regulated state of barbed end dynamics: barbed end capping, tracking and destabilization.

In a physiological ionic strength solution containing ATP, actin is assembled at a steady state in which F-actin co-exists with G-actin. The steady state concentration of G-actin (often called critical concentration) is determined by monomer-polymer exchanges at the barbed end pointed ends, which in pure actin are dominated by barbed end dynamics. The concentration of G-actin at steady state is regulated in various ways.

- **Capping of barbed ends and resulting enhanced sequestration of G-actin**
  
  Strong barbed end capping proteins like gelsolin bind very tightly to filament barbed ends (K_D lower than 10^{-10} M). The total blockage of barbed end dynamics leaves only pointed ends free for monomer-polymer exchanges. The concentration of G-actin co-existing with F-actin thus equals the critical concentration for actin assembly at the pointed end = 0.6 to 0.7 µM.

Sequestration of G-actin (non-polymerizable monomeric actin) is determined by the equilibrium between free G-actin and the added sequestering protein. At a total concentration of sequestering agent [S_0], the amount of sequestered actin [SA] is expressed by:

\[ [SA] = [S_0].[A_{SS}]/([A_{SS}] + K_S) \]  

where [A_{SS}] represents the concentration of free G-actin at steady state, which itself is determined by monomer-polymer exchanges, and K_S is the equilibrium dissociation constant for the SA complex (K_S = [S].[A]/[SA]). The value of K_S being generally in the submicromolar to micromolar range, the amount of sequestered actin varies with the value of [A_{SS}]. Typically, the amount of sequestered actin increases upon capping the barbed ends.

Profilin makes a complex with G-actin that participates in barbed end assembly but not in pointed end assembly. As a result, when barbed ends are not capped, profilin promotes a decrease in the contribution of free G-actin in monomer-polymer exchanges at barbed ends (Pantaloni and Carlier, 1993), while when 100% barbed ends are strongly capped, it sequesters G-actin as described by equation 2.

- **Destabilization of barbed ends by profilin**
  
  Profilin at high concentration (10 to 100 µM) binds and destabilizes barbed ends by increasing the dissociation rate of terminal actin subunits. As a result, profilin establishes a high steady state level of polymerizable profilin-actin monomers, and lowers the steady state level of F-actin (this paper).

- **Barbed end tracking by formins and multimerized WH2 domains in Ena/VASP or VopF/VopL**
  
  Barbed end trackers like formin and VopF change the kinetics of barbed end assembly-disassembly but by themselves do not affect the steady-state G-actin concentration which remains the same as for free barbed ends (Pernier et al., 2013; Romero et al., 2004).

- **Changes in the steady state level of actin assembly probe the competitive binding of regulators to barbed ends**
  
  When two proteins that bind barbed ends in a competitive fashion and regulate monomer-polymer exchanges with different specific effects are present together with F-actin, the level of monomeric actin reached at steady state depends on the fraction of barbed ends bound to each protein, that is on the amount of each protein and on their respective affinities.
for the barbed end. Consistently, measuring how the steady state level of unassembled actin imposed by known barbed end regulator R1 varies upon addition of another R2 provides insight into competitive binding of R1 and R2 to barbed ends. This test is used in this work to analyze how profilin competes at barbed ends with Capping Protein, formins, VopF and VCA-Arp2/3 branching machinery.

Modeling of polymerization in branched filaments and effect of profilin (Figure 5C)
The amount of F-actin is computed numerically, for time increments of 0.2 seconds. The initial barbed end concentration is that of spectrin-actin seeds. Branching is modeled by considering a constant rate of formation of new barbed ends, per concentration of F-actin, and per concentration of G-actin. To illustrate what would be the effect of profilin on elongation alone, the rate of formation of new barbed ends was left unchanged, and the elongation rate was decreased by 55%. Photobleaching was taken into account by considering a constant decrease of the pyrene fluorescence efficiency per unit of time and per concentration of F-actin.

Analysis of live TIRF recordings of assembly of branched filaments with VCA and Arp2/3 complex.
The kinetics of single filament assembly in branched structures was monitered by TIRF microscopy (Olympus IX71 inverted microscope, 60X TIRF objective, cascade II EMCCD camera). Coverslips and glass slides were sequentially cleaned by sonication with H₂O, ethanol, acetone, 1M KOH, H₂O for 20 min. Flow chambers were assembled and passivated with mPEG-silane - or biotin-PEG-silane in Figure S5 (Laysan Bio, Inc.). To anchor filaments for experiments with biotin-labeled actin (Figure S5), chambers were washed with G Buffer 0.1 % BSA and incubated 30 min with 1 mg/mL streptavidin (Thermo Scientific). In all experiments, following passivation chambers were sequentially washed with G Buffer supplemented with 0.3% methylcellulose (Sigma) and with actin (10 % Alexa488 or Alexa594, or 7.5 % Biotin labeled) Arp2/3, VCA, profilin, VopF, and formin at indicated concentrations.

The filament being a chiral helical polymer, daughter filament growth following consecutive branching events develops in 3D. Evenant wave microscopy provides a biased measurement of branching filaments in 2D. Only a minority of branches that grow within the 250 nm distance from the coverslip are fully seen. Filament observation has often been facilitated by anchoring using coverslips sparsely coated with myosin or using a biotin-streptavidin link to biotinylated actin. In this geometry, the rotational freedom and reactivity of barbed ends may be impaired, the growth of daughter filaments that point toward the glass surface is hampered and those that point away from the TIRF plane are not seen. To minimize these drawbacks, and optimize the rotational freedom of barbed ends, the coverslip surface was thoroughly passivated to avoid any adsorption of filaments. Addition of methylcellulose (0.32 %) avoided the diffusion of filaments away from the observation plane without inducing detectable bundling. Spontaneous assembly of actin in branched filaments was recorded at 1 µM G-actin (5 to10% Alexa488-labeled), 5-10 nM Arp2/3 and 50 nM VCA, at 1 frame every 5 or 10 s. The growth and branching of individual filaments into dendritic structures was monitored. It was verified that the rate of filament growth remained constant during the whole recording, testifying that the concentration of actively polymerizing monomeric actin did not decline during the experiment.
Due to brownian motion, some branches growing away from the plane of observation often flap down and become visible already with appreciable length at frame n, while only the branch junction was detectable as a bright dot or a kink on the mother filament at frame n-1, n-2… The bright dot at the origin of these branches was visible early in the closest visible vicinity of the barbed end (Movie S2). Conversely, sometimes visible daughter branches suddenly disappear due to rotation of the mother filament and re-appear later. These difficulties, linked to the phenomenology of the 3D-dendritic array, preclude an automatic quantitative analysis of the frequency of branching along the filament, and restrict the number of manually accessible parameters describing time dependent branching and growth.

Therefore, the mechanisms of filament side branching and barbed end branching were tentatively discriminated using the following parameters (see text for rationale). All movies were inspected frame by frame over periods of 10 min following assembly of the chamber to obtain reliable measurements of distances between consecutive branch junctions on the same mother filament as individual dendritic arrays develop from one filament nucleus. Due to the 2D geometry of observation of 3D branched filaments in TIRF, the ends of the growing filaments often fluctuate off the region of observation, making measurements of the lengths of mother and daughter branches unreliable, while this method was useful in EM observation of samples of branched filaments assembled in 3D in a test tube (Pantaloni et al., 2000). We opted for measurements of distances between consecutive branching points as a more reliable parameter. We used Image J to measure first the distance \( d_0 \) from the pointed end at which the first branching event \( B_0 \) occurred on each primary, so-called « ancestor » mother filament. We similarly measured the distances \( d_i \) between the consecutive branching points \( B_i-B_{i+1} \) (i= 0, 1, 2, 3) on each mother filament, when multiply branched structures have been formed. As dendritic arborescences develop, each daughter filament becomes a mother filament upon branching, and so on. The distance \( d_1 \) thus included i) the distance between the first (\( B_0 \)) and the second (\( B_1 \)) branch junctions on the « ancestor » mother filament ; ii) the distance between \( B_0 \) and the first branch junction (\( B'_1 \)) on the first generation daughter filament following its initiation from the ancestor mother filament, the distance between \( B'_1 \) and the first branch junction (\( B''_1 \)) on the second generation daughter filament, and so forth. Most measurements were performed on the first, second and third generation of branches, since the field of view later on became too crowded to extract reliable data. For the same reason, only values of \( d_i \) (i = 1 to 3, sometimes 4) corresponding to four (sometimes five) consecutive branching events on a given mother filament could be collected with reasonable confidence. Statistical significance of the data was met by cumulating the measurements extracted from five movies of identical samples performed consecutively with the same proteins the same day. Distributions of distances \( d_i \) collected in each movie (35 to 50 measurements of \( d_1 \) in each movie) were consistent with each other. The number of collected data declined from \( d_1 \) (N= 200 to 250) to \( d_4 \) (N=40 to 50). We finally measured, monitoring each individual filament frame by frame, how the value of \( d_1 \) varied between the time it was first recorded (upon \( B_1 \) event) and the latest time it could be clearly observed before the field of view was obscured by too many branched filaments growing from neighboring arborescent structures (generally between 7 and 10 min). At least 20 measurements of \( d_1 \) couples were recorded. These measurements had to be performed manually on a large number of movies (5 movies, see Movie S2 for example). Measured distances in pixels were converted in nm (one pixel= 260 nm without zoom, and 162 nm with zoom). The three series of data obtained for \( d_0, d_1 \) at early and late times and the \( d_i \) values were used to discriminate between side-branching and barbed
end branching (see text for details). To evaluate the frequency of side branching only, two methods were used.

In the first method, filaments pre-assembled from Alexa-488-G-actin (green filaments) were introduced in the flow cell together with VCA Arp2/3 and Alexa 594-actin (Red actin). For simplicity, the side branching reaction is described as a single step in which association of VCA-Arp2/3 complex with the side of a filament generates a branched junction.

The frequency of side branching \( k_{sb} \) was thus derived from the slope of the linear time dependence of the number of red filaments \( FY(t) \) initiated from the side of green filaments, using the following kinetic scheme:

\[
F + Y \rightarrow FY
\]

\[
d[FY]/dt = k_{sb}. [F].[Y]
\]

where \([F]\) represents the number of green F-actin subunits, derived from the total length of green filaments, \([Y]\) represents the concentration of branching VCA-Arp2/3 complex and \([FY(t)]\) the number of red filaments emerging from green filaments. \([F]\) and \([Y]\) are constant during the measurement period, hence \([FY(t)] = k_{sb}.[F].[Y] \cdot t\). The fact that the experimental data \([FY(t)]\) varied linearly with time validates the proposed description of side branching.

In the second method, filaments were assembled in the presence of either VopF or formin FH1-FH2 from mDia1, which both track barbed ends, and profilin-actin (1 \( \mu \)M 10% Alexa 594-labeled actin, 3 \( \mu \)M profilin), VCA and Arp2/3. In this case filaments branch only from the side of growing filaments. The rate of barbed end growth of filaments \( d[F]/dt = k_{+B}.(C-C_c) \) was measured and was constant, thus \((|F(t)| = \alpha \cdot t, \text{where } \alpha = k_{+B}.(C-C_c)\). Note that \(k+B\) differs with VopF and FH1-FH2. The number of side branching events was recorded and analyzed as above (equation 3), but taking into account the linear increase of \([F]\) with time, which leads to a quadratic time dependence of \([FY]\).

\[
[FY(t)] = (\frac{1}{2}). \alpha \cdot k_{sb}. [Y] \cdot t^2
\]

The value of \(k_{sb}\) was derived from the slope of \([FY(t)]\) versus \(t^2\). Again the linear experimental \(t^2\) dependence validates the proposed description of side branching.

**Bead motility assays**

N-WASP-coated 2\( \mu \)m diameter polystyrene beads (Wiesner et al., 2003) are introduced in a motility chamber that contains 7\( \mu \)M F-actin, 50nM Arp2/3, 10nM CP, 5\( \mu \)M ADF and varied amount of profilin. Bead movement is recorded for 40 minutes in phase contrast microscopy taking 1 frame every 20 seconds using Metamorph (Molecular Devices, Sunnyvale, CA). To reconstitute bead trajectories and extract bead mean velocities, bead positions in movies were automatically tracked using ImageJ plugin 2D Particle Tracker from the MOSAIC group (10). Only tracked trajectories that last for more than 10 consecutives images were kept for analysis. Beads that display an elongation rate lower than 0.1 \( \mu \)m/minute were considered as non-propelled beads. The same protocol was used with ActA-coated beads (Boujemaa-Paterski et al., 2001; Cicchetti et al., 1999).

For double fluorescence experiments, images were acquired in Epi-fluorescence of motility assays with Alexa594-Actin and Alexa488-Arp2/3. The images were processed in ImageJ using the rolling ball background subtraction algorithm to subtract the fluorescence background. The actin and Arp2/3 intensity profile was then measured along individual comets (Wiesner et al., 2003). Branching density was calculated as a ratio of Arp2/3 and
Actin along the comet length. For each condition, branching density was averaged over at least 10 comets.

**SDS-PAGE Analysis**
Quantitative analysis of amount of monomeric actin in supernatants in various biochemical conditions was quantified via ImageJ plugin for Gel Analysis and appropriate actin standards.
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