Single-filament kinetic studies provide novel insights into regulation of actin-based motility

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ABSTRACT **Polarized assembly of actin filaments forms the basis of actin-based motility and is regulated both spatially and temporally. Cells use a variety of mechanisms by which intrinsically slower processes are accelerated, and faster ones decelerated, to match rates observed in vivo. Here we discuss how kinetic studies of individual reactions and cycles that drive actin remodeling have provided a mechanistic and quantitative understanding of such processes. We specifically consider key barbed-end regulators such as capping protein and formins as illustrative examples. We compare and contrast different kinetic approaches, such as the traditional pyrene-polymerization bulk assays, as well as more recently developed single-filament and single-molecule imaging approaches. Recent development of novel biophysical methods for sensing and applying forces will in future allow us to address the very important relationship between mechanical stimulus and kinetics of actin-based motility.**

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INTRODUCTION

Motile processes develop on time scales of 1 s to several tens of seconds, which reflects the range of relevant kinetic parameters governing intracellular actin assembly dynamics. The chemotactic ability of a cell to respond rapidly to environmental changes depends entirely on the rapid remodeling of its actin cytoskeleton (Carlier *et al.*, 2015). By means of kinetic regulation, intrinsically slower processes are accelerated, and faster ones decelerated, to match rates observed in vivo. Identifying these kinetic mechanisms is therefore a principal step in reconstituting motile processes from individual cell components and mathematical modeling of cellular behavior for gaining a quantitative understanding of biological processes (Shekhar *et al.*, 2014).

A simple example of kinetic regulation of actin dynamics is the 100-fold difference in rate of turnover/treadmilling of actin filaments in vitro compared with the in vivo rate in motile processes. Similar to the acceleration of the rate-limiting step in the ATPase cycle of myosin by actin (Lymn and Taylor, 1971), actin-depolymerizing factor (ADF)/cofilin enhances pointed-end depolymerization, which is the rate-limiting step of the filament turnover cycle. ADF cooperatively binds to and destabilizes actin–actin bonds in ADP–F-actin, resulting in enhanced disassembly of ADP–F-actin from pointed ends (Figure 1). ADF/cofilin thus establishes a larger stationary pool of polymerizable ATP–actin monomers (C_{SS}), leading to higher monomer flux associating to barbed ends ($k_{+B}.C_{SS}$, where k_{+B} is the association rate constant of actin monomers at the barbed end and C_{SS} is the steady-state actin monomer concentration) and hence faster protrusion rates. The destabilization of actin–actin bonds also facilitates filament severing (Maciver *et al.*, 1991). Note, however, that severing by itself (e.g., mediated by sonic vibration or by a potent severer like Cordon-bleu) does not affect the value of C_{SS}. If a kinetic screen for a protein-enhancing treadmilling had been designed before ADF/cofilin's discovery, ADF/cofilin would certainly have been found (Le Clainche and Carlier, 2008). It has recently been shown that ADF further enhances filament disassembly by synergizing with other factors like Aip1 (Nadkarni and Brieher, 2014; Gressin *et al.*, 2015), Coronin (Mikati *et al.*, 2015), and Twinfilin and Srv2/CAP (Johnston *et al.*, 2015).

Other examples of differences between in vivo and in vitro rates include dissociation of capping protein (CP) from barbed ends, which is intrinsically very slow but three orders of magnitude faster in lamellipodia (Miyoshi *et al.*, 2006), consistent with a lowered affinity. This was demonstrated by the discrepancy between apparent K_d (~100 nM) of *Dictyostelium* CP in whole-cell extracts and 100-fold

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Abbreviations used: ADF, actin depolymerizing factor; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CapZIP, CapZ-interacting protein; CARMIL, capping protein Arp2/3 myosin i linker; CP, capping protein; C_{SS}, steady-state actin monomer concentration; F-actin, filamentous actin; FH2, formin-homology 2 domain; G-actin, globular (monomeric) actin; k_{+B}, association rate constant of actin monomers at the barbed end; K_d, equilibrium dissociation constant; WH2, WASP-homology 2 domain.

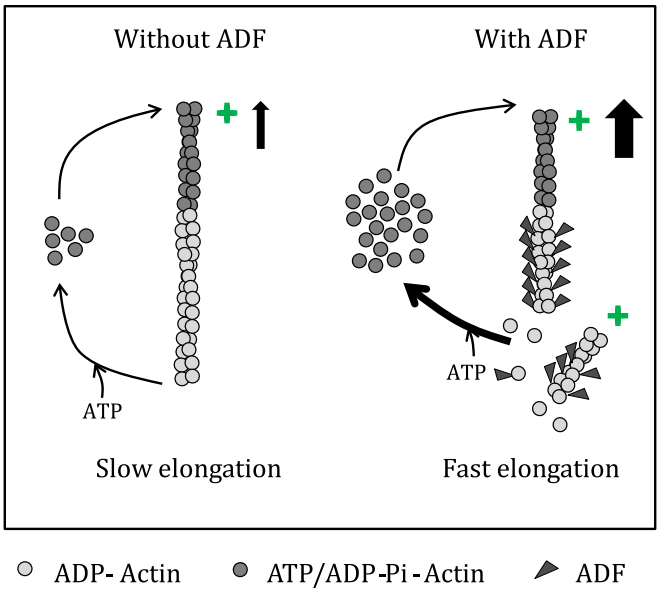

FIGURE 1: ADF enhances filament turnover rate. ADF enhances the rate-limiting of step of filament depolymerization during the treadmilling cycle. It does so by cooperatively binding the ADP–F-actin subunits and enhancing their disassembly at pointed ends by destabilizing actin–actin bonds in the filament. Similarly, ADF enhances spontaneous filament fragmentation. As a result of enhanced depolymerization, ADF enhances the stationary pool of monomeric ATP–actin, leading to a faster elongation rate.

lower K_d for purified *Dictyostelium* CP (Schafer *et al.*, 1996). Similarly, formins exhibit long dwell times at the barbed ends in vitro, resulting in much longer filaments than observed in formin-mediated cellular processes. We will show examples in which kinetic control of the duration of formin and CP residence at the barbed end is elicited either by an allosteric mechanism or competition between various actin-binding motifs present in a variety of proteins.

The regulation of rates at the single-filament level has profound implications for defining the functional diversity of filament networks. Coordinated turnover of various actin arrays in the same cell suggests the existence of timers, phasing kinetic steps, and retroactive loops. It is therefore very important to study the kinetics of individual reactions before exploring how they are integrated into more complex cycles. Here we present appropriate kinetic approaches for analysis of the mechanisms that govern reactivity of actin filament barbed ends, comparing their respective strengths and drawbacks and providing a few illustrative cases. We also discuss the role of kinetics in quantitative understanding of motile processes.

EXAMPLES OF PROCESSES THAT NEED ACCELERATION OR DECELERATION

Some intrinsic reactions occurring at the barbed end of an actin filament are so slow that their kinetic up-regulation must occur to explain faster dynamics seen in vivo. For example capping protein binds barbed ends with very high affinity, dissociating from barbed ends with half-life of ~25 min in vitro (Schafer *et al.*, 1996). Long dwell times can be both an advantage and a disadvantage. In the bulk cytoplasm and in quiescent nonmotile cells, stable capping prevents unproductive energy consumption due to actin monomer–polymer exchange. In motile regions of the cell, however, a more dynamic interaction of CP with barbed ends is required to allow efficient growth of dendritic filament arrays.

A search for cellular factors to enhance dissociation of CP from barbed ends first led to polyphosphoinositides like phosphatidylinositol 4,5-bisphosphate (Schafer *et al.*, 1996). Recently a class of proteins referred to as "uncappers" have been shown to rapidly release CP from capped filaments. By allosterically binding CP bound to the barbed end, uncappers reduce CP's affinity for the barbed end, thus enhancing its dissociation (Figure 2a). Proteins containing the uncapper CapZIP motifs include CARMIL, CIN85, Duboraya, and FAM21. These proteins act in a site-directed manner (Fujiwara *et al.*, 2014) in close association with machineries assembling branched filaments with Arp2/3 complex.

Another recently discovered uncapping mechanism is via the formation of a transient ternary complex, in which CP and a barbed end–tracking protein simultaneously bind a barbed end. In the process, each of them lowers the other's affinity, thus enhancing CP's dissociation from the barbed end (Figure 2b). This form of uncompetitive inhibition, opposed to the mutually exclusive binding scheme, is kinetically effective in displacing CP by other barbed end trackers. Examples include uncapping of CP by WH2 domain–containing VopF and Enabled/vasodilator-stimulated phosphoprotein or of FH2 domain–containing formins (Pernier *et al.*, 2013; Bombardier *et al.*, 2015; Shekhar *et al.*, 2015). In a reciprocal manner, CP can also displace formin from the barbed end. This recent discovery disproved the previously held view that CP and formin bind to barbed ends in a mutually exclusive manner (Zigmond *et al.*, 2003; Moseley *et al.*, 2004; Kovar *et al.*, 2005; Bartolini *et al.*, 2012). Formin-anchored filament elongation is essential in organelles like filopodia. However, formin detachment from filaments has to be accelerated to prevent uncontrolled elongation of filaments due to the long dwell times of formins on barbed ends. In yeast, Bud14 rapidly displaces formin Bnr1 from growing barbed ends (Chesarone *et al.*, 2009), and Smy1 dampens elongation by interacting with the FH2 domain of formin Bnr1 (Chesarone-Cataldo *et al.*, 2011). Similarly, CP association to a formin-bound barbed end has recently been shown to accelerate formin dissociation from the barbed end (Bombardier *et al.*, 2015; Shekhar *et al.*, 2015). This reaction may underlie the reported filopodial regulation by CP (Sinnar *et al.*, 2014).

Another unexpected and interesting case is the synergy between Formin2 and Spire in actin assembly. These two proteins should antagonize and compete at barbed ends. Spire uses its WH2 domains to cap barbed ends. Formin2 by itself is a poor nucleator of actin filament and associates to barbed ends unusually slowly. However, Formin2 nucleates efficiently in the presence of Spire. When Spire is bound to barbed ends, its exposed N-terminal KIND domain associates with the C-terminal tail of Formin2, which allows fast recruitment of Formin2 at barbed ends and immediate onset of processive assembly coupled to displacement of Spire from barbed ends (Montaville *et al.*, 2014). The group of Bruce Goode has reported other examples of such a synergy between a nucleation-promoting factor and an elongator, including pairs of mDia1 and APC (Breitsprecher *et al.*, 2012), Bud6 and formin Bni1 (Graziano *et al.*, 2011).

Whereas some reactions need to be speeded up to match in vivo rates, others need to be slowed down. Proteins like CP or formins often associate extremely rapidly to filament barbed ends in a diffusion-limited manner. Their rate of association can be reduced by an inhibitor that binds in rapid equilibrium to barbed ends. Our recent experiments identified this function in the ubiquitous protein profilin. Profilin binds both G-actin (with high affinity) and F-actin (with lower affinity). Profilin binds barbed ends of

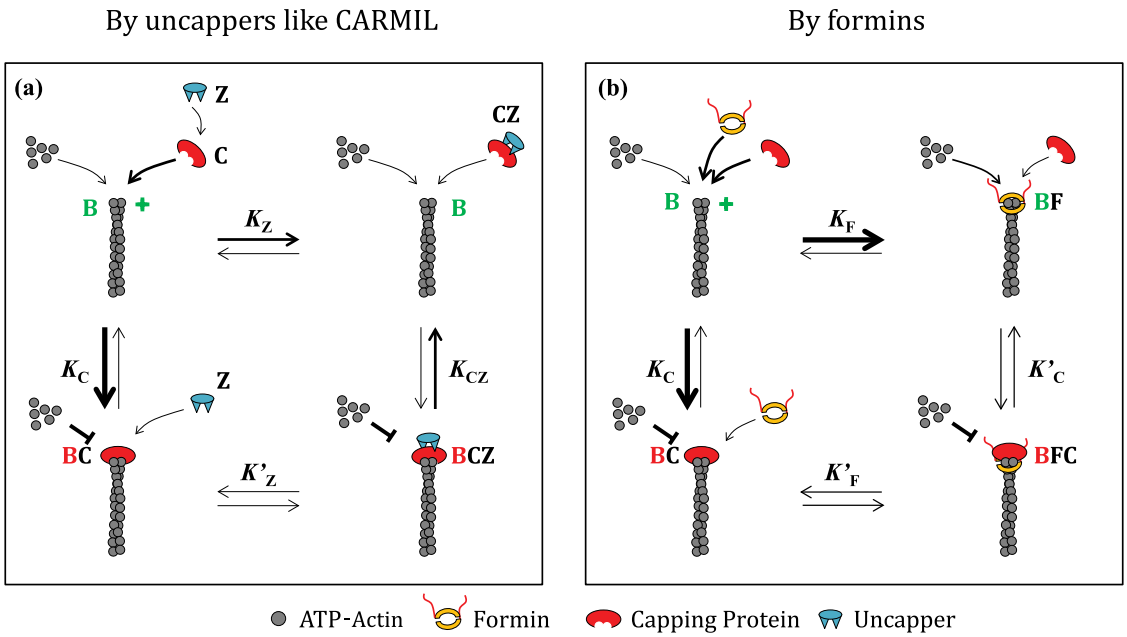

FIGURE 2: Two mechanisms for rapid uncapping of CP-capped barbed ends. (a) Scheme 1: uncapping by uncappers. Filament barbed ends (B) bind CP (C) with high affinity (K_C = 0.1 nM). CP makes a complex, CZ, with CapZIP (Z), which also caps barbed ends, albeit with affinity lower than CP (K_{CZ} = 38 nM). CP dissociates very slowly from barbed ends, k_{-C} = 0.0003 s^{-1}, whereas CZ dissociates much more rapidly, k^B_{-CZ} = 0.095 s^{-1} (Fujiwara *et al.*, 2010). Although K_{CZ} was not experimentally determined, since the other equilibrium constants were measured, K_{CZ} was calculated from detailed balance ($K_C K'_Z = K_Z K_{CZ}$). At low concentration of CP, addition of Z to capped filaments (BC) leads to formation of a transient BCZ complex, followed by dissociation of CZ, leaving free uncapped barbed ends. At higher CP concentrations, adding Z leads to formation of CZ in amounts sufficient to bind barbed ends, and barbed ends stay capped by CZ (BCZ) in a more dynamic equilibrium than by C alone. Red indicates paused state, and green indicates elongating state. Arrow thickness signifies the magnitude of the reaction rate. (b) Scheme 2: uncapping by a barbed-end tracker like formin. Barbed ends (B) bind CP (C) and formin (F) with high affinity and slow dissociation rates. Both proteins can be bound simultaneously in the ternary complex BFC, with enhanced dissociation rates of both F (k'_{-F}) and C (k'_{-C}) within this complex. On addition of either C to BF or F to BC, the distribution of conformational states after transient formation of BFC depends on the relative values of k'_{-F} and k'_{-C} (Shekhar *et al.*, 2015). Note that in the standard mutual exclusion scheme (direct competition) only B, BC, and BF states exist.

ADP-F-actin filaments with K_d = 1–25 μM (Kinosian *et al.*, 2002; Jegou *et al.*, 2011) and enhances depolymerization (Bubb *et al.*, 2003). Similar K_d values have been found for profilin binding to ATP and ADP-Pi barbed ends (Jegou *et al.*, 2011), and a higher value of ~225 μM was found for AMPPNP barbed ends (Courtemanche and Pollard, 2013). Thus profilin competes with CP and barbed end–tracking proteins (Pernier *et al.*, 2016), thus slowing down their association to barbed ends.

BULK-SOLUTION KINETICS AND SINGLE- FILAMENT KINETICS: COMPLEMENTARY APPROACHES TO ADDRESS THE SAME QUESTIONS

Traditionally, bulk kinetic assays have been used for quantifying rate constants of interaction between two or more proteins. Commonly used bulk approaches include changes in light scattering, fluorescence intensity, and anisotropy. The behavior of all molecules is averaged out in the monitored output. The advantage of these methods is that rate constants are easily and rapidly derived, assuming that all molecules are identical. A commonly used assay for F-actin assembly exploits the 20-fold increase in fluorescence intensity of pyrenyl-labeled actin (Kouyama and Mihashi, 1981) or 7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole–labeled actin (Detmers *et al.*, 1981) associated with the transition from the G-actin to the F-actin state. The increase in fluorescence intensity is proportional to total polymer mass and therefore provides quantitative evaluation of time-dependent rate of assembly of G-actin into F-actin. Rate parameters can then be extracted using appropriate mathematical modeling of the assembly kinetics.

Notwithstanding its immense contributions to kinetics, this technique has certain limitations. First, some proteins, such as ADF or profilin, bind labeled actin with reduced affinity and may affect its fluorescence (Malm, 1984; Carlier *et al.*, 1997). Second, because this method only measures the amount of polymerized actin, it is difficult to identify events like nucleation, annealing, and severing. The method applies to reactions in which F-actin is a soluble polymer. However, reaction rates may differ when the polymer is side bound or end anchored to a membrane. Third and most important, bulk-solution "self-averaging" approaches fall short of identifying molecular mechanisms that occur at the scale of individual filaments (like length fluctuations) or in analysis of vectorial/processive reactions. Their application is also limited when nonhomogeneous populations (or rare subpopulations) exist. In these cases, what was an advantage turns out to blur the real thing. Single-filament studies, which emerged in the cytoskeleton field 15 years ago, overcame these limitations using total internal reflection fluorescence microscopy for observing real-time branching of actin filaments (Amann and Pollard, 2001; Mahaffy and Pollard, 2006) and assembly dynamics at barbed and pointed ends (Kuhn and Pollard, 2005).

Over the years, single-filament kinetics has evolved into a choice tool to analyze how individual regulators regulate barbed ends. Either the presence of a regulator at the barbed end can be identified by its effect on the elongation rate or the protein can be directly labeled fluorescently and observed by single-molecule fluorescence approaches. Each approach has its strengths and limitations.

In the first approach, changes in filament elongation rate provide a kinetic probe to detect the binding of a ligand. These changes are used to characterize the underlying kinetic mechanisms. A detailed kinetic analysis at several ligand concentrations is required to establish whether the monitored change in growth rate is strictly coupled to association-dissociation of the ligand at filament ends or occurs in a subsequent isomerization step. Thus a wealth of information is derived regarding the molecular mechanism of interaction of the ligand with barbed ends. However, not all barbed end–binding proteins cause a drastic change in elongation rate, making their detection difficult. Examples include barbed end–tracking proteins such as VopF, which does not exhibit a detectable change in elongation rate but is detected by its uncapping activity (Pernier *et al.*, 2013). The classical use of competitive inhibitors thus can reveal the interaction of a "mute" ligand with barbed ends. In addition, two proteins bound simultaneously to the barbed end might show the same phenotype as when only one of them is bound; for example, simultaneous binding of CP and formin to barbed ends arrests filament growth in the same manner as CP alone does (Shekhar *et al.*, 2015).

In the second approach, single-molecule fluorescence imaging is used to visualize interaction of fluorescently labeled proteins with the filaments (Smith *et al.*, 2013a). Direct observation of uncapping of CP-capped filaments by a labeled CARMIL fragment (Fujiwara *et al.*, 2010) and measurement of filament branching kinetics by Arp2/3 (Smith *et al.*, 2013a,b) have exploited this approach. However, it should be kept in mind that due to lack of single actin subunit–level resolution (resolution limited to 160 nm or 50 subunits), just visualizing a fluorescent spot at the end of a filament by itself does not necessarily mean that the protein is actually interacting at the barbed face of the last terminal subunits at the barbed end. The protein could actually be bound in a 50 subunits–range away from the end, on the side of the filament. In addition, fluorescence labeling by itself might affect the protein's binding activity to actin. Finally, in single-molecule imaging, only low amounts of labeled protein can be used to avoid nonspecific adsorption of molecules to the coverslip, as well as to ensure that there is only one fluorescent molecule per unit diffraction-limited detection volume (approximately nanomolar concentration; Loveland *et al.*, 2012). These drawbacks limit its application. In standard open-flowcell setups, analysis of rapid reactions is prevented by the large dead time (tens of seconds) between perturbing the sample conditions and recording observations.

Microfluidics-assisted fluorescence microscopy has helped overcome majority of these limitations and facilitated a high-throughput study of actin kinetics, in particular elucidating the mechanism of inorganic phosphate release in ATP hydrolysis on F-actin (Jegou *et al.*, 2011). First, the biochemical conditions to which the filaments are being exposed can be changed in <1 s dead time, at least 10-fold faster than in a traditional open-flowcell; second, growth rates can be monitored in a large range of ligand concentrations. The rapid simultaneous observation of a large filament population ($n > 100$) leads to straightforward and accurate evaluation of rate constants. Compared to single-molecule fluorescence imaging, this approach also allows working at higher concentrations, except with proteins that strongly adsorb to the surface and may artifactually bind filaments.

FUTURE OF SINGLE-FILAMENT KINETIC ASSAYS

Combining the microfluidics approach with multicolor single-molecule imaging should prove invaluable in the future. Transient exposure of filaments to high concentrations of labeled molecules will be possible, and the ability to wash out the unbound labeled molecules will eliminate the background of free labeled molecules. Several fluorescently labeled proteins may be monitored simultaneously (Smith *et al.*, 2013b), taking in vitro systems ever closer to in vivo–like situation in which multiple proteins work together. To achieve this, improved passivation and labeling methodologies will have to be developed. More sophisticated designs of microfluidic devices will also be required. Data collected in complex schemes will foster novel modeling approaches that need quantitative assessments of rate constants. Along this line, a successful prediction of the spatiotemporal dynamics of filopodia was made using reaction rate constants of barbed-end regulators (Mogilner and Rubinstein, 2005).

The single-filament assays and bulk-solution assays allow the study of actin assembly dynamics at two extreme size scales. A novel approach might be found at the crossover of the two scales. Observing rare labeled filaments in solutions containing unlabeled filaments has been used to understand actin rheology (Kas *et al.*, 1994; Murrell and Gardel, 2012). Inspired by this assay, the kinetic behavior of individual labeled filaments placed in a flow containing unlabeled actin and a cocktail of defined regulatory proteins is now at hand and would reveal how individual filaments behave when placed in in vivo mimicking conditions.

Single-filament assays have also enabled the study of mechanical properties at the scale of individual filaments (Jegou *et al.*, 2013). Biophysical methods designed both to measure and apply forces in the pico- to nano-Newton range have renewed the interest in the mechanochemical basis of cell motility, allowing studies of force-dependent binding strengths. A number of actin-binding proteins interact with the sides of the filaments, either stabilizing (e.g., tropomyosin) or destabilizing (e.g., ADF/cofilin) the polymer. Most single-filament kinetic measurements have been done in the absence of load, on unstretched/uncompressed filaments. However, filaments in cells often grow under tension. Tensile forces might affect actin assembly, as well as the association/dissociation reactions of regulators with filaments. Specifically, the elongation rate of a formin-bound barbed end increases under a pulling force (Jegou *et al.*, 2013). How the complexes dissociate upon application of force will provide insights into the molecular mechanism of complex formation: either a simple bimolecular reaction or a two-step process in which isomerization of a first low-affinity complex in rapid equilibrium strengthens the interaction. Corresponding slip bonds and catch bonds have been defined (Marshall *et al.*, 2003), as well as catch–slip bonds (Sundd *et al.*, 2011), which govern actin disassembly (Lee *et al.*, 2013). Applying a pulling force on the filament increases the dissociation of formin from the barbed end (unpublished results). In contrast, applying a pulling force on cadherin–catenin complexes on filaments stabilizes the binding (Buckley *et al.*, 2014). Tension generated in the actin cytoskeleton can have secondary indirect effects on the kinetics of interaction between filaments and other actin-binding proteins. For example, applying tension to an actin filament has been shown to cause reduction in severing by ADF (Hayakawa *et al.*, 2011). As another example, tension generated by actomyosin stretches talin in focal adhesions, enhancing its binding to vinculin (Ciobanasu *et al.*, 2014). In exploring how the binding kinetics of other important side-binding proteins such as tropomyosins or the formin processive walk are affected by mechanical strain on the filament, either tension or torque should reveal as-yet-unknown aspects of their binding mode to actin. Gelsolin and

Spire, the actin filament cappers, bind the side of a filament, followed by filament severing and capping of the newly formed barbed end. It will be interesting to know whether applying tension on the filament affects association of gelsolin to the filament. Alternatively, filaments can be bent mechanically. Local filament curvature was found to affect Arp2/3 based filament branching (Risca *et al.*, 2012).

In conclusion, a wealth of new information is expected to come from the application of novel and more extensive kinetic approaches to actin dynamics. Obvious consequences in the structural biology of actin and quantitative modeling of normal and pathological cell processes are anticipated.

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