COMMENTARY

Competition and collaboration between different actin assembly pathways allows for homeostatic control of the actin cytoskeleton

Jeremy D Rotty[1,2] and James E Bear[1,2,*]

[1]UNC Lineberger Comprehensive Cancer Center; University of North Carolina at Chapel Hill; Chapel Hill, NC USA
[2]Department of Cell Biology and Physiology; University of North Carolina at Chapel Hill; Chapel Hill, NC USA

ABSTRACT. Tremendous insight into actin-associated proteins has come from careful biochemical and cell biological characterization of their activities and regulation. However, many studies of their cellular behavior have only considered each in isolation. Recent efforts reveal that assembly factors compete for polymerization-competent actin monomers, suggesting that actin is homeostatically regulated. It seems that a major regulatory component is competition between Arp2/3-activating nucleation promoting factors and profilin for actin monomers. The result is differential delivery of actin to different pathways, allowing for simultaneous assembly of competing F-actin structures and collaborative building of higher order cellular structures. Although there are likely to be additional factors that regulate actin homeostasis, especially in a cell type-dependent fashion, we advance the notion that competition between actin assembly factors results in a tunable system that can be adjusted according to extracellular and intracellular cues.

KEYWORDS. actin, Arp2/3 complex, Ena/VASP, formin, motility, profilin

Dynamic actin-based structures play important roles in nearly every aspect of eukaryotic life such as motility, vesicular trafficking and cell division. Though monomeric actin

('G-actin') above the critical concentration can spontaneously assemble actin filaments ('F-actin') *in vitro*, it has been appreciated for many years that specific actin assembly factors regulate the generation of F-actin in cells.[1] Among these factors is the Arp2/3 complex, which nucleates branched 'daughter' filaments from the sides of pre-existing 'mother' filaments, giving rise to the branched dendritic networks found at the lamellipodia, phagocytic cup, and endocytic patches.[2] Nucleation promoting factors (NPFs) are required for Arp2/3 complex activation and also deliver the initial actin monomers that trigger daughter filament nucleation.[1] Although the Arp2/3 complex is the only assembly factor known to generate this type of branched actin, many classes of actin assembly factors generate and/or elongate unbranched actin, including the Ena/VASP,[3] formin[4] and spire[5] protein families. These various nucleation/assembly factors contribute to specific F-actin structures. Fission yeast provides a good example, where the Arp2/3 complex generates endocytic actin patches while formins assemble actin cables and the contractile ring.[6] F-actin in mammalian cells is not so starkly segregated, with certain formin[7-9] and Ena/VASP[10,11] proteins contributing to stress fibers and filopodia while the Arp2/3 complex generates the bulk of the lamellipodial dendritic actin[12] (**Fig. 1A**). In yeast as well as mammals, actin assembly factors are active in the same cytoplasm, and their activities must be precisely balanced to allow normal cellular responses to occur. The biochemical and structural mechanisms of all of these factors, and their contribution to cellular function have been well studied separately, but how they work together in a common cytoplasm is poorly understood.

The emerging view of the actin cytoskeleton is that it is homeostatically regulated and that all assembly factors in the cell must compete for a finite pool of monomeric actin. Filamentous actin and monomeric actin exist simultaneously in cells, implying an F-actin:G-actin ratio that must be remarkably constant, but could be expected to shift according to extracellular or intracellular cues. As described above, actin is polymerized into actin filaments of varying geometries by assembly factors, crosslinking proteins and monomer-binding proteins (e.g. profilin). All of these processes utilize a common cellular pool of G-actin to make actin filaments. It has long been appreciated that cellular G-actin levels are maintained through an autoregulatory feedback mechanism.[13] Furthermore, it has been shown that the G-actin pool can homeostatically regulate cellular signaling based on its size relative to F-actin,[14] indicating the importance of a tightly regulated F-actin: G-actin ratio. Insights generated in fission yeast further support the idea that actin assembly is homeostatic. When the

FIGURE 1. (*See next page*). Functional consequences of monomer competition by actin assembly pathways. (**A**) Monomer competition allows for simultaneous assembly of diverse actin filament populations in the same cytoplasm. NPF-bound actin promotes dendritic actin nucleation and generation of lamellipodia while profilin-actin promotes stress fiber and filopodia elongation. (**B**) Homeostatic regulation of actin monomers allows distinct assembly pathways to collaboratively build cellular structures. In this example, NPF-actin promotes Arp2/3 complex branch nucleation while Ena/VASP is recruited to the daughter filament barbed end to elongate the growing filament using profilin-actin. (**C**) Collaboration and competition between actin assembly pathways may fine tune cellular processes by regulating the balance between nucleation of new filaments and elongation of existing filaments. Wild Type cells strike a balance between nucleation and elongation, while being capable of enhancing overall motility upon stimulation. Ena/VASP disruption leads to a denser dendritic actin network, shorter actin branches and enhanced cell motility speed, suggesting that nucleation is favored over branch elongation. Conversely, disruption of the formin FMNL2 makes branch elongation less efficient leading to decreased cell motility speed. Finally, disruption of Arp2/3 complex activity leads to loss of the branched actin network and a shift toward profilin-actin dependence and generation of long, unbranched protrusions containing Ena/VASP and focal adhesion proteins.

FIGURE 1. (*See previous page*).
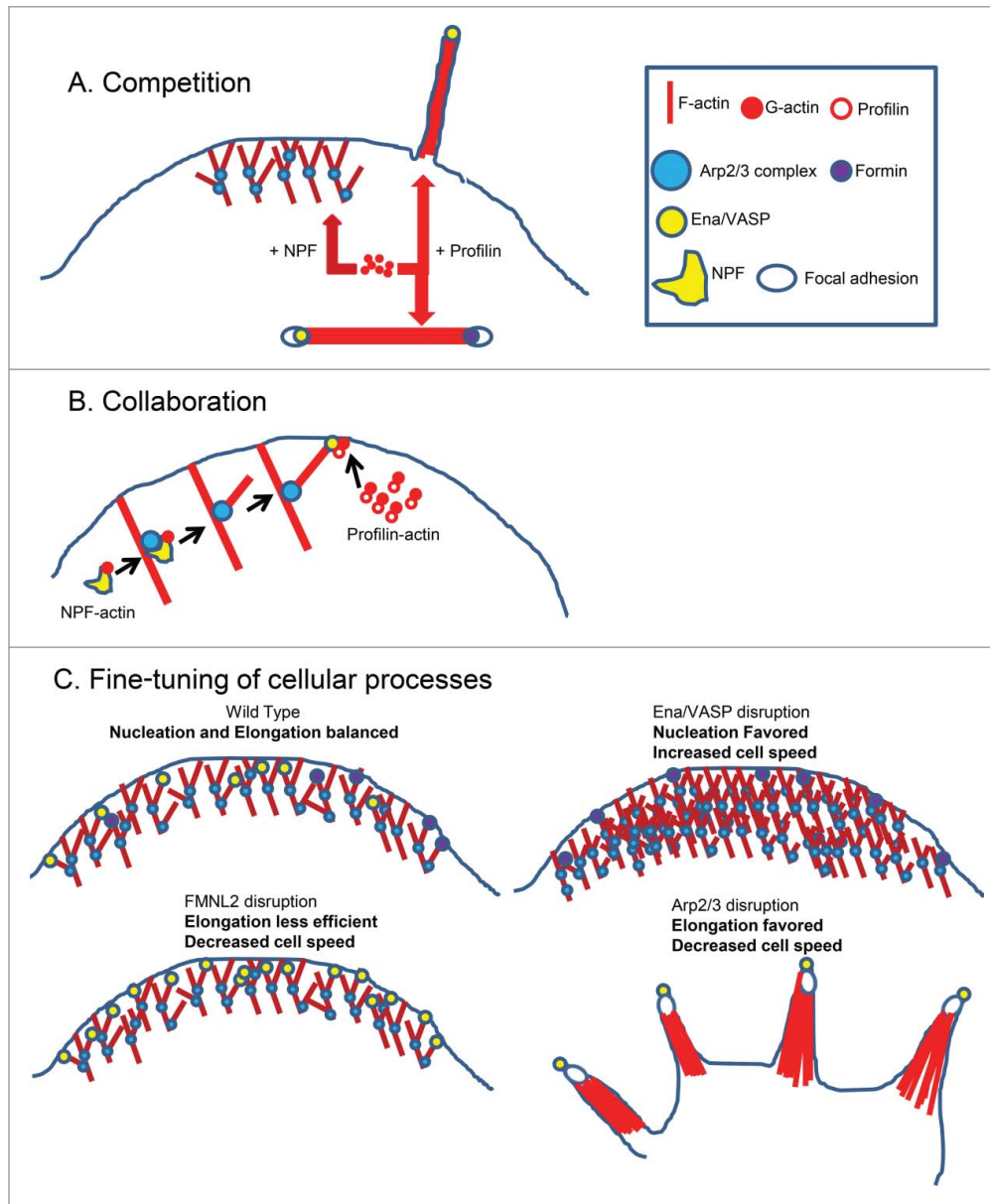
Arp2/3 complex is disrupted in this system, endocytic patches are compromised, as expected.[15] Surprisingly, actin cable formation is also enhanced, via a compensatory increase in formin activity.[15] These data illustrate that actin exists in a finite assembly-competent pool, and that the activities of competing assembly pathways are precisely balanced under normal conditions.

Our studies in mammalian cells reinforced the notion that actin is homeostatically regulated. Genetic ablation of Arp2/3 complex function in fibroblasts, via conditional deletion of the *Arpc2* gene (denoted *Arpc2*−/−), abolished lamellipodia and dramatically changed F-actin organization.[16] *Arpc2*−/− cells protruded via bundled filopodial-like structures that contained core focal adhesion proteins (vinculin),

actin assembly factors (Mena, VASP) and bundled actin filaments. Although the organization of actin filaments was dramatically changed, total F-actin levels remained unchanged in *Arpc2−/−* cells.[16] Both formins and Ena/VASP proteins elongate actin filaments more efficiently using profilin-actin,[17-20] so we reasoned that disrupting profilin-1 expression via shRNA-mediated knockdown would disrupt both assembly pathways. Profilin-1 depleted *Arpc2−/−* cells have a significantly reduced spread cell area and a dramatically reduced F-actin level compared to profilin-containing *Aprc2−/−* cells.[16] To our surprise, *Arpc2−/−* cells treated with the pan-formin inhibitor SMIFH2[21] maintained overall F-actin levels comparable to both untreated *Arpc2−/−* and WT cells.[16] However, when we disrupted Ena/VASP function via intracellular sequestration (FP4-mito[22]) in *Arpc2−/−* cells, there was a striking reduction in both F-actin levels and spread cell area.[16] Based on these data, we identified Ena/VASP proteins as the fundamental compensatory mechanism in *Arpc2−/−* cells, as well as establishing that F-actin in these cells required profilin-bound actin. It is perhaps not so surprising that the cell has a means of compensating for loss of a major cytoskeletal regulator, though we expect to find cell types and/or contexts where compensation does not occur or occurs by different mechanisms.

Upon examining our data further, a second idea emerged based on our observation that profilin-1 depletion in otherwise normal WT cells elicited changes consistent with enhanced Arp2/3 complex function. These included a larger lamellipodia, higher levels of Arp2/3 complex at the cell edge, and a wider band of both actin and Arp2/3 complex at the lamellipodia.[16] Important observations made via *in vitro* TIRF assays,[23] biochemical studies[24,25] and microinjection[26] experiments through the years indicated that the Arp2/3 complex nucleates daughter filaments less efficiently in the presence of profilin-actin. With these studies in mind, we wondered whether increasing profilin levels would introduce changes consistent with Arp2/3 complex inhibition. Our cells were not amenable to stable profilin overexpression so

instead we acutely increased profilin-1 concentration via microinjection. Exogenous profilin disrupted lamellipodia and decreased Arp2/3 complex localization to the cell periphery long after the initial injection.[16] We also observed that the F-actin generated in response to profilin microinjection tended to be in the form of stress fibers rather than protrusive structures.[16] Together these data imply a redistribution of monomeric actin from the lamellipodia to internal stress fibers. Interestingly, *Arpc2−/−* cells were largely resistant to profilin microinjection, as expected, since it is already strongly reliant upon profilin-actin to generate F-actin.[16]

In a companion paper, Suarez et al. found that antagonism between profilin and the Arp2/3 complex regulated actin monomer usage and, therefore, the balance between formin- and Arp2/3 complex-generated actin structures. They showed that fission yeast overexpressing profilin could not form Arp2/3 complex-dependent endocytic patches but still competently generated formin-dependent actin structures.[27] Conversely, they showed that increasing actin (and thereby decreasing the relative amount of profilin-actin) enhanced patch formation and blocked formin-generated structures.[27] These experiments, together with our studies in fibroblasts, supported the overall insight that profilin and the Arp2/3 complex are mutually antagonistic.

With these results in hand, the authors of this study used elegant TIRF microscopy and biochemical assays to gain greater mechanistic insight into this relationship. They confirmed that profilin suppresses Arp2/3 complex branch nucleation and that this behavior was dependent only upon profilin's ability to bind free monomeric actin.[27] However, purified formin could only robustly generate F-actin in the presence of the Arp2/3 complex if profilin was present.[27] Furthermore, both profilin's poly-proline binding and actin-binding activities were required for formin activity in these assays.[27] Finally, and importantly, the authors also demonstrated that nucleation promoting factors (NPFs) and profilin compete with one another for free actin.[27] It is noteworthy that data generated from *in vitro* TIRF microscopy, fission yeast, and mammalian cell culture have

all converged to argue for an antagonistic relationship between profilin and the Arp2/3 complex.

Our work and that of Suarez et al. indicate that the competition between profilin and NPFs for actin monomers is an ancient and fundamental mechanism that solves the problem of dividing the pool of actin monomers among distinct F-actin assembly pathways (**Fig. 1A**). However, yeast lack Ena/VASP homologues and respond to loss of Arp2/3 complex function by inducing formin-based actin assembly,[15] while F-actin in our spread *Arpc2−/−* cells is largely indifferent to the formin inhibitor SMIFH2.[16] These findings argue that each model system, and potentially each cell type, possesses a unique strategy for distributing actin monomers. Though there are generalizable lessons to be learned about the interplay among actin regulatory proteins, the field will need to bear in mind that the insights generated from different cells and contexts may yield slightly different results.

So what advantage does monomer competition confer to eukaryotic cells? The most obvious interpretation of these findings suggests that different pools of polymerization competent monomers can be added differentially to distinct F-actin populations (**Fig. 1A**). Keeping these pathways segregated based on the type of actin required for optimal activity (e.g., NPF- or profilin-bound) confers specificity to each process as well as reserving a subpopulation of the total actin monomer pool for assembly by a given pathway. The cellular outcome of this is that distinct pools of F-actin can be built simultaneously by competing actin assembly pathways in the same cytoplasm.

The second potential consequence of monomer competition is that it allows branched and unbranched actin assembly pathways to operate collaboratively in the same cellular compartment (**Fig. 1B**). It is tempting to speculate that competition between profilin and NPFs for actin monomers may tune the ratio of branch elongation to branch nucleation. The high local concentration of profilin feeding into these elongating branches may effectively block further NPF activity after the initial nucleation event, thereby spatially constraining the actively elongating actin filaments from daughter filament nucleation. The lamellipodial actin network is mainly composed of branched actin polymerized by the Arp2/3 complex.[12] However, both Ena/VASP[28,29] and the formin FMNL2[30] have also been shown to localize to the lamellipodial edge. FMNL2 has been observed via *in vitro* TIRFM to elongate Arp2/3 complex-generated daughter filaments in a profilin-dependent fashion.[30] Ena/VASP proteins antagonize capping protein, a relationship that affects both the length and density of actin filaments in the dendritic network.[28] Recent work investigating the regulation of actin dynamics in dendritic spines revealed that the Arp2/3 complex and VASP/FMNL2 are spatially segregated,[31] supporting a model wherein the former nucleates new actin filaments and the latter factors elongate the resulting daughter filaments. Presumably both activities are required to form dendritic spines of the correct size and shape.

Finally, monomer competition may allow for the fine-tuning of cellular processes (**Fig. 1C**). Lamellipodial protrusion rates can vary dramatically between cell types, or within the same cell when faced with different contextual cues. For example, keratocytes protrude much more rapidly (2.4 microns/min)[32] compared to fibroblasts (ranging from 0.08 to 0.89 microns/min),[32,33] despite the structure and function of the lamellipodia being nearly identical across much of the metazoan lineage. Disruption of Ena/VASP activity yields a denser dendritic network with shorter filaments, and cell speed is enhanced.[28] Conversely, disruption of FMNL2 negatively impacts lamellipodial protrusion and cell speed.[30] As branch nucleation requires NPF-actin and filament elongation is more efficiently accomplished by profilin-actin, subtle alterations in the balance of NPF-bound actin to profilin-bound actin may fine-tune protrusion rates by adjusting the balance of filament nucleation and elongation at the leading edge. The observations yielded by Suarez et al.[27] and Rotty et al.[16] suggest that this balance is tunable, and that the set point may be different in distinct cell types based on the operant factors in a particular cell. Since we know that a balance is struck between profilin-

and NPF-dependent actin assembly pathways in these systems, a fascinating future direction will be defining the mechanical, cytoskeletal and signaling pathways that collaborate to tune the balance between them.

Our data agrees well with the emerging view of actin as a homeostatic system. However, our work has focused exclusively upon actin assembly, and we recognize that other factors will also affect actin homeostasis. When discussing dynamic actin structures, it is important to realize that assembly and disassembly are fundamentally linked. F-actin disassembly and/or severing via ADF/cofilin yields polymerization competent monomers[12,34] as well as yielding free polymerization-competent barbed ends.[35,36] The role of the cofilin phosphatase Slingshot[37,38] and the coronin[39] family of proteins may be major factors in sensing F-actin and in regulating the size of the G-actin pool. Furthermore, the activities of cofilin and the Arp2/3 complex are synergistic[34,40] and both are required for proper lamellipodia function,[41-43] so it seems that polymerization-competent actin generated by disassembly near membranes may be primed for the nucleation of new daughter filaments. In fact, this 'recycling' has recently been directly observed.[44] In addition to disassembly, F-actin bundling (via fascin, filamin, α-actinin, etc.), myosin contractility and actin monomer sequestration (via thymosin-β4) can all be expected to affect G-actin levels and F-actin production. Indeed, thymosin-β4-bound actin has been shown to contribute differentially to the lamellipodial actin network through formins, revealing an additional axis of competition for G-actin.[44] Furthermore, certain isoforms of the F-actin binding protein tropomyosin increase F-actin levels in adipose cells, indicating that the F-actin to G-actin ratio can potentially be modulated by F-actin binding proteins as well as actin assembly factors.[45] However, this effect may depend upon the ability of different tropomyosin isoforms to bind preferentially to F-actin generated by distinct formin isoforms.[46] This combined activity seems to impart specific characteristics to the resulting tropomyosin-bound actin filament, implying that not all actin filaments are functionally equivalent (for more extensive review, see Gunning et al.[47]). Each of these actin-binding proteins is itself regulated by upstream signaling pathways, as well as spatial cues that recruit them to particular locations within the cell. Many of these factors will vary (in expression as well as activity) based on the type of cell being assayed. Understanding the actin cytoskeleton at a systematic level represents both a challenge and opportunity for the field moving forward. There can be little doubt that a global understanding of actin regulation will yield significant cell biological insights, as well as pointing us toward the interactions, crosstalk and feedback mechanisms that might be leveraged for the treatment of pathophysiological conditions.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

FUNDING

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