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# Cease-fire at the leading edge: new perspectives on actin filament branching, debranching and cross-linking

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## Abstract

Membrane protrusion at the leading edge of migrating cells is driven by the polymerization of actin. Recent studies using advanced imaging techniques raised a lively controversy about the morphology of these filaments; however, common ground between the two sides now appears to have been found. Here we discuss how the controversy has led to a deeper consideration of the architecture of actin networks underlying cell migration, and has helped define new challenges that lie ahead.

### Keywords

actin; Arp2/3 complex; cell motility; lamellipodium; electron microscopy

## Introduction

Cell motility is critically important for development, inflammation, and metastasis. To migrate, cells extend sheet-like projections known as lamellipodia, which are filled with polymerized actin filaments. Most of these filaments are oriented with their barbed (or fast-growing) ends facing the leading edge and their less dynamic pointed ends toward the cell interior [Pollard and Borisy 2003]. Polymerization is thought to occur exclusively at the barbed ends, and as a result, the leading edge is pushed forward to accommodate the growing filaments (Fig 1). In fact, in fast moving cells, the actin network stays relatively stationary compared to the substratum as the cell moves [Theriot and Mitchison 1991], showing that actin polymerization in the cell front exerts pushing forces which finally lead to protrusion.

A wealth of genetic evidence shows that new actin filaments in the lamellipodium are nucleated by the Arp2/3 complex together with activating proteins in the WASp/Scar/WAVE family [Machesky and Insall 1998; Rogers et al. 2003]. In vitro, WASp and Arp2/3 complex associate with the side of a pre-existing “mother” filament, and subsequently nucleate a new “daughter” filament which elongates at a 70° angle relative to the mother (Fig 2A) [Mullins et al. 1998]. After nucleation, Arp2/3 complex remains associated with the pointed end of the daughter filament and the side of the mother, creating a stable branch junction. A similar mechanism involving Arp2/3 complex-mediated nucleation - activated by ActA instead of WASp - underlies formation of the branched actin filament networks that

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propel the pathogen *Listeria monocytogenes* in infected cells [Theriot et al. 1992]. Motility of this nature can further be reconstituted in vitro using a system of only five purified proteins [Loisel et al. 1999; Wiesner et al. 2003], demonstrating that the basic machinery of polymerization is sufficient to produce force. A similar system is believed to drive internalization of endocytic vesicles in both yeast and mammals [Kaksonen et al. 2005; Liu et al. 2009; Michelot et al. 2010], although the extent to which actin-based force generation is required for endocytosis in mammalian cells is still being worked out [Boulant et al. 2011].

## The Lamellipodium: Branched or Unbranched?

Nearly simultaneous with the discovery that Arp2/3 complex produces branched filaments in vitro came in vivo electron micrographs from Tatyana Svitkina and colleagues [Svitkina et al. 1997], revealing a dense network of branched actin filaments in lamellipodia (Fig 2B). Further, Arp2/3 complex was localized to the branch junctions in these networks by immuno-EM [Svitkina and Borisy 1999], and the branches adhered very closely to the 70° angle observed in vitro. This remarkable similarity between the in vitro effects of Arp2/3 complex and the in vivo ultrastructure at leading edges gave traction to the “dendritic nucleation” model [Mullins et al. 1998; Pollard et al. 2000], in which new filaments are generated at the leading edge as branches. The model poses that WASp and a preexisting filament together activate Arp2/3 complex, resulting in the formation of a branch [Machesky et al. 1999]. This results in a dense gel of actin that resists deformation and leads to collective protrusion of a wide cell edge rather than individual filopodial (or finger-like) projections. In time, the network is disassembled at the rear of the lamellipodium, recycling components for new rounds of assembly.

Last year, J.V. Small and colleagues employed state-of-the-art sample preparation and 3D electron tomography techniques to determine actin architecture at the leading edges of four different cell types, and challenged a key component of the dendritic nucleation hypothesis: that any branching occurs in vivo at all [Urban et al. 2010]. Small suggested that the branches seen in earlier studies were artifacts of the critical-point drying method of sample preparation. For a while, it seemed as if differences in methodology might be the only explanation for the disparate results [Higgs 2011]. However, in September of this year, Svitkina’s group reported a reanalysis of the primary data from the Small study [Yang and Svitkina 2011], and reported the presence of numerous branches in the raw images (Fig 2C). Further, the branch angle adhered closely to the 70° angle observed previously in vitro and in vivo using the critical-point drying technique. In addition, the branch junctions were of bulbous shape, similar to cyro-EM structures of Arp2/3 complex junctions formed in vitro [Rouiller et al. 2008]. In his response, Small conceded that - based on his reanalysis of the data - all of these major points were correct [Small et al. 2011] but pointed out that branches are still more infrequent than reported previously. They also pointed out that the array is somewhat less dense than had been previously observed.

Now that there seems to be consensus on this issue, we can ask, what have we learned and what questions remain to be answered regarding actin ultrastructure at the leading edge?

## Moving forward

How has our view of the lamellipodial cytoskeleton changed by the recent findings mentioned above? Do all new filaments in the lamellipodium arise as branches? The tomogram shown by Small and colleagues annotates 208 barbed ends at the leading edge and 225 branch points in an area immediately adjacent to this. Small et al. also highlighted subsets of interconnected filaments. The ratio of branches to filaments within this subset is 90% or more, further supporting the dendritic nucleation model of filament formation in

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which essentially every new filament originates as a branch off a pre-existing filament. Thus, at this time, there does not seem to be any in vivo data supporting a mechanism for new filament nucleation other than that proposed by the original dendritic nucleation model.

Nevertheless, the magnificent quality of the EM-tomograms presented by Small and colleagues has shifted our focus to additional aspects of the dendritic nucleation model: (1) The abundance of filament overlaps relative to branch junctions places a renewed emphasis on the role of **cross-linking proteins** as key mechanical elements of the lamellipodium, perhaps more important than the role Arp2/3 complex has been suggested to play in this capacity. (2) Long segments of filaments lacking branches suggests that filament elongation may be quite rapid compared to branching, highlighting the importance of filament **elongation factors** such as Ena/VASP proteins and formins at the leading edge. (3) Using the branches identified by Small et al., we have determined that branch density decreases with distance from the leading edge (Fig 3), suggesting that **debranching factors** may have a prominent role in pruning the dendritic network as it flows back in the lamellipodium. Each of these three major points is discussed in greater detail below and is depicted in an inclusive model (Fig 4).

## Cross-linking proteins

The new data have shifted our view on the biophysics of motility. In silico modeling of cell motility depends on a dense gel of actin driving a load forward [Dayel et al. 2009; Ditlev et al. 2009]. Networks of long uncoupled filaments do not exhibit the mechanical integrity of the *in vivo* cytoskeleton [Palmer et al. 1999], nor do they sustain motility [Loisel et al. 1999]. Likely, the cross-over points observed by Small's group are stabilized by cross-linking proteins that act as girders to rigidify the actin meshwork. While a variety of actin cross-linking proteins with seemingly overlapping functions (e.g. α-actinin, fascin, fimbrin, filamin, and myosin) could act synergistically to strengthen and maintain network integrity [Tseng et al. 2002], filamin is most suited to generating the criss-crossed filaments observed. Filamin has long been known to induce gelation of actin filaments, even at low molar ratio [Janmey et al. 1990], and is necessary for motility in vivo [Cox et al. 1995; Cunningham et al. 1992]. Arp2/3 complex and filamin could therefore serve complementary roles in filament nucleation and mechanical strengthening, respectively, in the lamellipodium [Flanagan et al. 2001; Nakamura et al. 2002]. Another factor to consider is Coronin, which has in vitro bundling activity [Cai et al. 2007; Goode et al. 1999] and in vivo localizes by immuno-EM to filament 'junctions', presumed to be branches [Cai et al. 2008]. However, since critical-point drying was used in this study, it is possible that some of these junctions instead represent filament cross over points. Answering these questions will require biophysical modeling studies as well as the marriage of electron tomography with protein localization studies and/or super-resolution light microscopy to identify the specific factors present at filament crossovers.

## Elongation Factors

As Small and colleagues show, many of the actin filaments in lamellipodia are over a micron in length. This is in contrast to the original version of the dendritic nucleation model which proposed that, after nucleation by Arp2/3 complex, filaments grew briefly at their barbed ends before being capped by capping proteins [Pollard et al. 2000]. The identification of long filaments by EM, and of filament elongation factors (formins and Ena/VASP proteins) at the leading edge calls for a reconsideration of this view. Formins processively cap the barbed ends of growing actin filaments [Evangelista et al. 2002; Kovar and Pollard 2004; Moseley et al. 2004; Zigmond et al. 2003], and in the presence of profilin, formins can accelerate barbed end growth many fold over growth at free barbed ends [Kovar 2006; Romero et al. 2004]. This makes formins strong candidates for generating some of the long

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filaments observed by EM. However, the role of formins in lamellipodial protrusion remains poorly understood. The only members of the formin family that have been identified at the lamellipodial leading edge at endogenous expression levels are mDia1 and mDia2 [Watanabe et al. 1997; Yang et al. 2007], and both have been shown to promote cell motility [Shi et al. 2009; Yang et al. 2007]. Although some other formins such as FMNL2, DAAM1 and FHOD1 are also implicated in cell migration [Ju et al. 2010; Kitzing et al. 2010; Koka et al. 2003], their endogenous localization patterns and potential functional roles in lamellipodia remain unknown. In addition, potential roles at the leading edge for the other ten mammalian formins remain open. By comparison, proteins in the Ena/VASP family are well established as integral components of the actin-machinery at the leading edge. Early studies showed that members of this family are strongly enriched at the lamellipodial plasma membrane [Rottner et al. 1999], and that localization correlates directly with rate of lamellipodium protrusion [Bear et al. 2000; Bear et al. 2002; Rottner et al. 1999]. Moreover, targeting of Ena/VASP proteins to the leading edge led to the formation of longer filaments and reduced filament branching densities [Bear et al. 2002]. Recently, biochemical analyses showed that Ena/VASP proteins greatly accelerate filament elongation, comparable to formins, and that they protect barbed end growth from capping proteins when clustered on surfaces [Breitsprecher et al. 2008; Breitsprecher et al. 2011; Hansen and Mullins 2010]. Thus, enhanced elongation of filaments may be responsible for the correlation between Ena/VASP localization at the leading edge and rate of lamellipodial protrusion. In the future, it will be a great challenge to physicists, biochemists and cell biologists to unravel the complex interplay between nucleation and elongation factors, and to determine how regulated changes in their local activation and attenuation might produce differing actin architectures and forces.

## Debranching Factors

The data from Small and colleagues reveal that there are surprisingly few short branches, and that branch density in the actin networks decreases with distance inward from the leading edge (Fig 3). This suggests that filaments must be debranched as the network matures. Branched filaments generated by Arp2/3 complex in vitro are highly stable over many minutes, suggesting that rapid debranching in vivo would require additional factors [Gandhi et al. 2010]. At least three different classes of proteins have been implicated in removing Arp2/3 complex branch junctions in vitro: the actin severing/disassembly protein ADF/cofilin [Blanchoin et al. 2000; Chan et al. 2009], a structural cousin of ADF/cofilin that binds Arp2/3 complex instead of actin, GMF (glia maturation factor) [Gandhi et al. 2010; Nakano et al. 2010], and the filament-bundling and Arp2/3 complex-interacting protein Coronin [Cai et al. 2008]. In addition, there is evidence that nucleotide hydrolysis on the Arp2 and Arp3 subunits of Arp2/3 complex is a prerequisite for debranching [Martin et al. 2006]. What remains to be seen is whether debranching is simply a byproduct of global disassembly that occurs in the network or occurs as a separate remodeling phase prior to filament disassembly. A related question is how the lamellipodium becomes remodeled into the unbranched lamellum [Burnette et al. 2011; Hotulainen and Lappalainen 2006]. Yeast GMF has a very potent debranching effect on Arp2/3 complex generated networks [Gandhi et al. 2010; Nakano et al. 2010], but it is not yet known whether mammalian GMFs have the same activity. It will also be important to determine whether mammalian GMF co-localizes with ADF/cofilin and cooperates in the disassembly phase of lamellipodial dynamics, or localizes closer to the leading edge and has a role in earlier remodeling events. Another possibility is that different GMF proteins contribute to each of these processes, and indeed there are two GMF paralogues expressed in mammals, GMF-β and GMF-γ, which appear to be differentially regulated [Ikeda et al. 2006; Nakano et al. 2010]. Like GMF, ADF/cofilin exhibits a strong debranching activity in vitro [Blanchoin et al. 2000; Chan et al. 2009]. As ADF/Cofilin severs and depolymerizes filaments in vivo and in vitro [Carlier et al. 1997;

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Hotulainen et al. 2005; Lappalainen and Drubin 1997; Maciver et al. 1998], it remains to be seen whether it works specifically on Arp2/3 complex-actin contacts or changes actin structure in a manner refractory to maintaining the branch. Finally, Coronin exhibits a weaker effect on debranching, increasing the frequency of debranching events only 2–3 fold [Cai et al. 2008], but nonetheless may contribute to this process in vivo. In particular, it will be critical to examine how these three factors, GMF, ADF/cofilin, and Coronin function together in debranching.

### Conclusions and Perspectives

The basic mechanism of Arp2/3 complex-dependent nucleation and branching has been conserved in organisms as diverse as yeast and humans. This raises an important question, which is how the lamellipodium diverged from simpler Arp2/3 complex- and actin-containing structures such as yeast endocytic patches. It is tempting to speculate that the more complex actin structures such as a lamellipodium evolved through modification of branch frequency and filament length, as well as through the expression of new and diverse filament cross-linkers. This underscores the importance of studying the architectures of cytoskeletal networks in a wide range of model systems. Only through such comparisons will the basic design principles of network formation be revealed, and the relevant molecular differences between networks that account for the major differences in their organization and physical properties. These questions pose exciting challenges for the coming years. Further, this recent debate serves as an important reminder that seemingly irreconcilable views can in fact reveal deeper truth, when both sides relentlessly pursue to that end.

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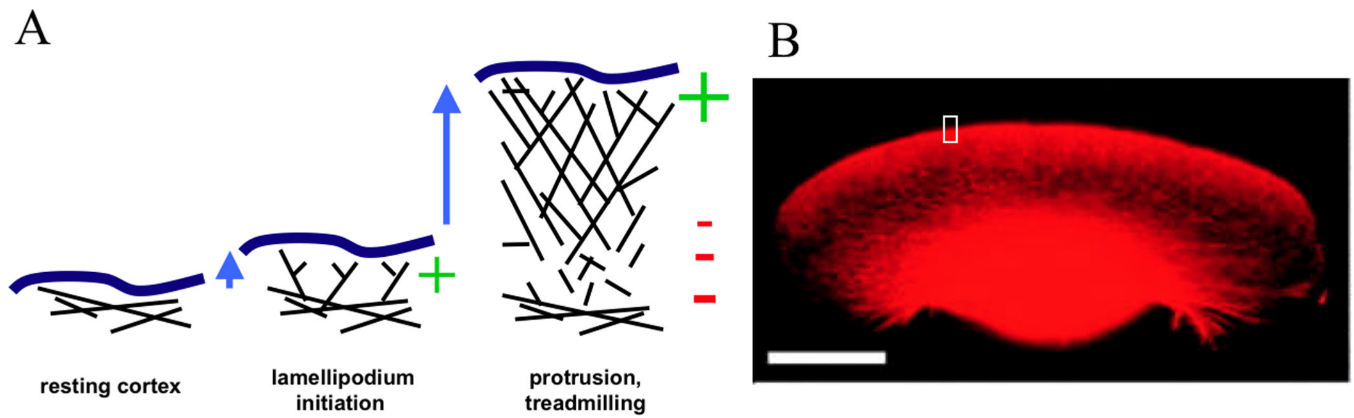


**Fig. 1.**

(A) Model of actin ultrastructure in the lamellipodium. From the network of cortical actin filaments (*black*), dendritic nucleation and polymerization pushes the cell membrane (*blue*) forward. Filaments are debranched and disassembled at the rear of the protruding lamellipodium. Regions of assembly (*green plus signs*) and disassembly (*red minus signs*) are shown. (B) Fluorescence light micrograph of a migrating *Xenopus laevis* fibroblast that was fixed and stained with Rhodamine-phalloidin. Reproduced with permission from Svitkina and Borisy [Svitkina and Borisy 1999]. A small region of the lamellipodium represented by the cartoon in *A* is indicated (*white box*).

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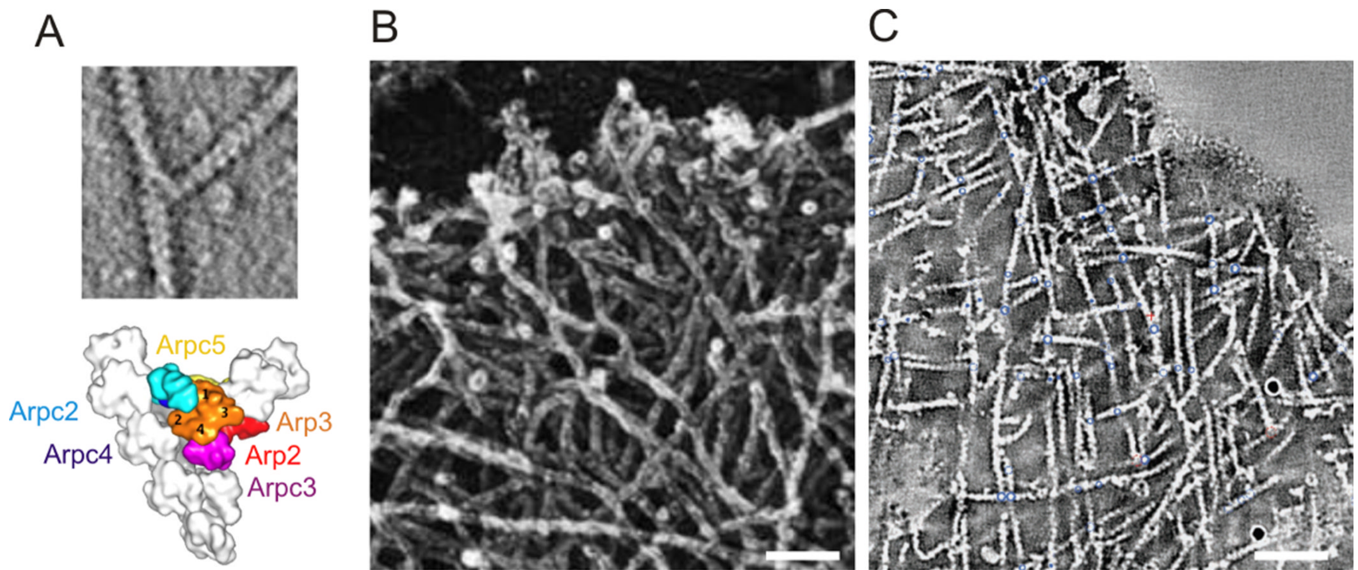


**Fig. 2.**

Electron micrographs of actin filaments assembled by Arp2/3 complex in vitro and branched actin networks at the leading edge of cells. (A) Arp2/3 complex-containing actin filament branches assembled in vitro using purified proteins. Reproduced from [Rouiller et al. 2008] with permission. (*Top*) Tomogram of a branch junction formed by purified *A. castellanii* Arp2/3 complex. (*Bottom*) Branch 3D reconstruction based on EM-tomography data. (B) Lamellipodium architecture visualized by EM tomography in Urban et al [Urban et al. 2010]. Lamellipodium architecture visualized by EM after critical-point drying in Yang et al [Yang et al. 2007]. Scale = 100 nM, for both images. Reproduced with permission.

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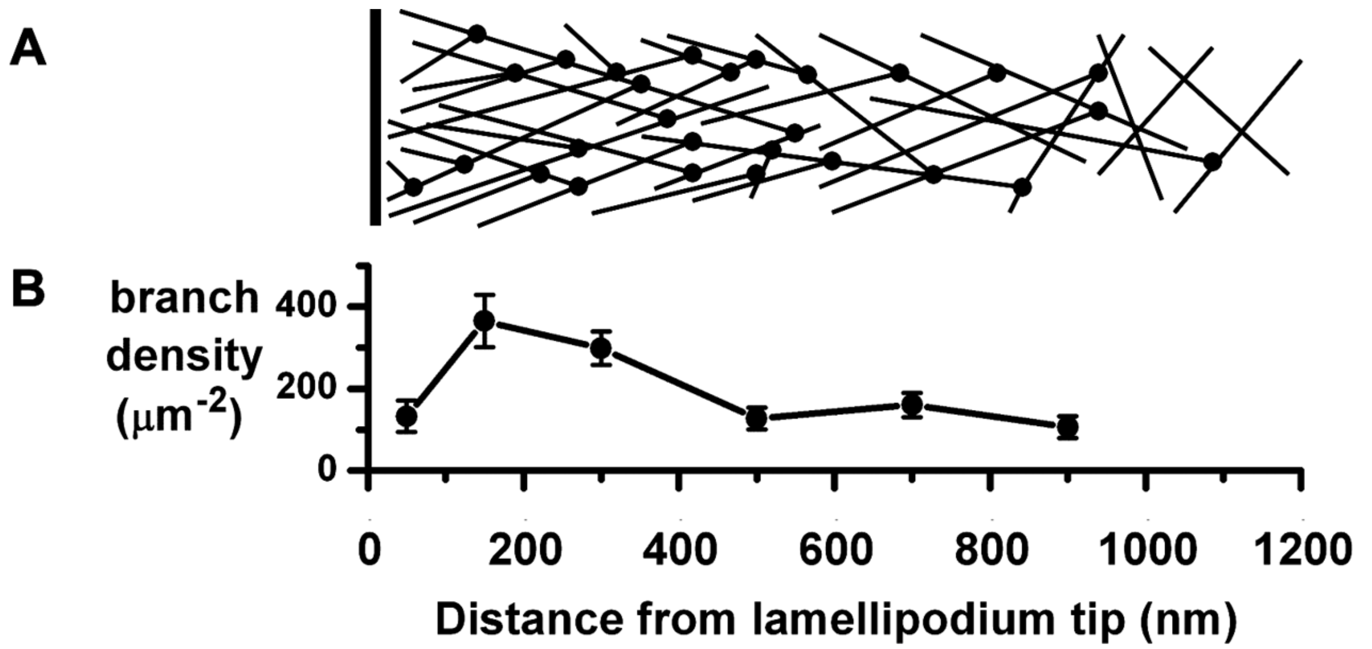


**Fig. 3.**

Distribution of actin filament branch junctions in the lamellipodium of an NIH 3T3 cell. (A) Cartoon representation of architecture in the lamellipodium. Black nodes represent filament branch junctions. (B) Variation in branch density with increasing distance from the cell edge, calculated from the images in Small et al [Small et al. 2011].

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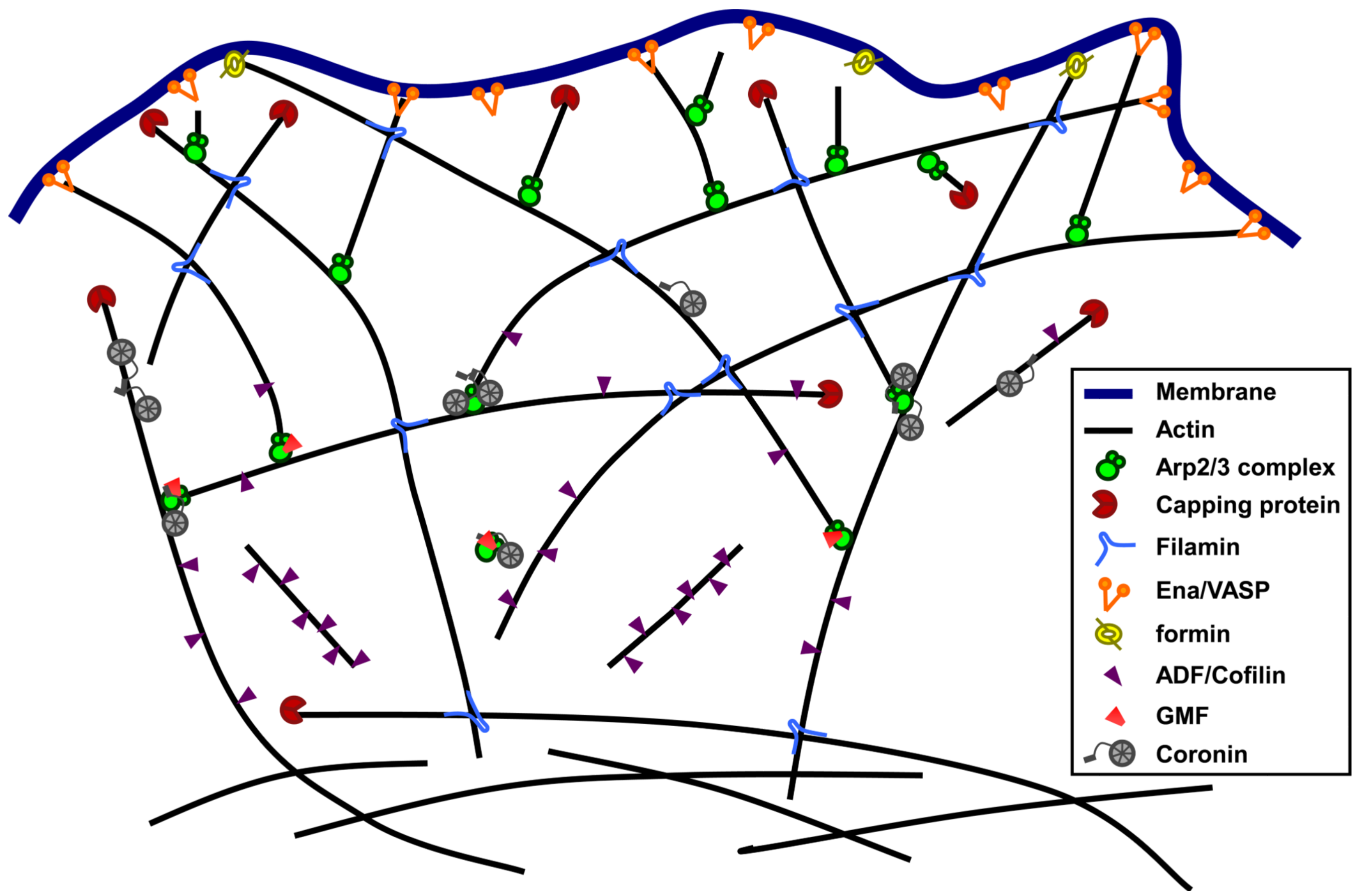


**Fig. 4.**

Model for regulation of actin filament architecture at the lamellipodium. Filaments are nucleated at the cell edge as dendritic branches by Arp2/3 complex and plasma membrane-associated WASp/Scar (*not shown*). Many of the free barbed ends generated are capped by capping protein binds, which terminates filament growth. Filament crossovers are stabilized by cross-linking proteins such as filamin. As the dendritic network ages and is left behind by the advancing leading edge, disassembly factors such as ADF/cofilin, GMF, and Coronin, debranch Arp2/3 complex-nucleated filaments. Severed filaments at the rear of the lamellipodium are either completely disassembled by ADF/cofilin, working together with Coronin and other factors (e.g. Aip1 and Srv2/CAP, not shown) or cross-linked by fimbrin, α-actinin, and myosin II (*not shown*) to generate the actin bundles and arcs found in the lamella.