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Review

Actin polymerization machinery: the finish line of signaling networks, the starting point of cellular movement

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Abstract. Dynamic assembly of actin filaments generates the forces supporting cell motility. Several recent biochemical and genetic studies have revealed a plethora of different actin binding proteins whose coordinated activity regulates the turnover of actin filaments, thus controlling a variety of actin-based processes, including cell migration. Additionally, emerging evidence is highlighting a scenario whereby the same basic set of actin regulatory proteins is also the convergent node of different signaling pathways emanating from extracellular stimuli, like those from receptor tyrosine kinases. Here, we will focus on the molecular mechanisms of how the machinery of actin polymerization functions and is regulated, in a signaling-dependent mode, to generate site-directed actin assembly leading to cell motility.

Key words. Actin dynamics; signaling complex; lamellipodia; RhoGTPases; actin-binding proteins.

Introduction

The dynamic assembly of actin filaments in response to extracellular signals is at the base of a wide range of fundamental cellular processes through which living cells change shape, extend protrusions like lamellipodia and filopodia, or wrap around a particle, as in a phagocytic cup [1–3]. The bulk turnover of actin subunits is 100–200 times faster in cells than with pure actin, pointing to a complex regulation in vivo. A large repertoire of actin-binding proteins consistently regulates the dynamic assembly and spatial organization of actin filaments, thus orchestrating the motile behavior of cells. Among these are proteins that (i) promote the nucleation of actin, such as the Arp2/3 complex or formins, that (ii) affect the depolymerization of filaments, such as the actin-depolymerizing factor (ADF/cofilin) family and that (iii) associate to monomeric actin, such as profilin and beta-thymosin, and that (iv) cap the ends of filaments. Coordination and integration of the activities of this basic set of proteins is essential to control site-directed actin polymerization in vivo [1–3]. Additionally, complexity is emerging with the discovery that these proteins are, in turn, targets of various signaling pathways emanating from diverse extracellular stimuli, such as those from the receptor tyrosine kinase (RTK) family. Within these pathways, Rho GTPase family members play a key role, acting as molecular switches on which signaling inputs converge and are transduced into a coordinated array of output events regulating site-directed actin dynamics required for cell motility [4–6].

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Here, we will examine the most recent evidence highlighting the molecular mechanisms controlling the basic machinery of actin polymerization. Additionally, with the aim of providing examples of how the machinery of actin polymerization is linked and controlled by signaling cascades emanating from Rho GTPases, we will further focus on those pathways and macromolecular complexes permitting a proper temporal and spatial regulation of Rac-mediated actin dynamics leading to membrane protrusion, the first step in the establishment of cell locomotion.

Actin treadmilling

Actin, a 43-kDa globular protein, is the most abundant protein in eukaryotes. In physiological medium, actin can undergo polymerization into helical filaments. Actin filaments are polarized, characterized by a fast-growing (plus or barbed) end and a slow-growing or minus end. Actin assembly is coupled with continuous ATP hydrolysis. Actin is an ATPase. Under physiological conditions, MgATP-bound G-actin is incorporated into growing filaments at the barbed end. ATP-actin is then converted in ADP-actin by slow hydrolysis as actin monomers are shifted along the filament toward the pointed ends. Thus, actin assembly at steady state can be described by an ATPase cycle featuring the energetic imbalance between the fast and slow ends of filaments linked to the irreversible hydrolysis of ATP. This cycle comprises three elementary steps: (i) net depolymerization from the pointed end, releasing ADP-G-actin; (ii) exchange of ATP for bound ADP, restoring ATP-G-actin, which (iii) undergoes net assembly at the barbed end. The kinetic constants of all these steps determine both the concentrations of each species at steady state and the turnover rate of actin filaments [7, 8]. It is noteworthy that the concentration of ATP-G-actin, which is kept stationary during this cycle, is intermediate between the critical concentrations (Cc = the minimal G-actin concentration required for assembly at the ends of filaments, resulting from the ratio of rate constants of dissociation and association at actin filament ends) at the barbed and pointed ends. Irreversible hydrolysis of the bound ATP associated with polymerization is at the origin of treadmilling and destabilizes the filaments [3]. Treadmilling is crucial in actin motility. The polymerized array of actin filaments displays a very rapid turnover. Filaments are oriented so that the growing barbed ends face the plasma membrane, while the pointed ends, where depolymerization occurs, are at the rear [9–11] (fig. 1). Notably, a similar architectural and dynamic organization of actin polymers is also used by a number of intracellular pathogens, such as *Listeria monocytogenes*, which hijack components of the actin-treadmilling machinery to generate comet tails for their propulsion. Indeed, characterization of the mechanisms driving *Listeria* or *Shigella* propulsion has been instrumental in uncovering key molecules regulating actin dynamics [12–14]. This arrangement suggests also that an insertional polymerization mechanism is at the base of force production needed for locomotion. Finally, the observation that the rate of barbed end growth equals the speed at which lamellipodia extend and *L. monocytogenes* moves provided the first direct support for actin polymerization as the driving force in motility [15, 16].

The rate of actin treadmilling is controlled by a diversified array of actin-binding proteins

In vitro studies using purified actin showed that depolymerization from the pointed end is slow and represents the limiting step in the treadmilling rate. As a result, the steady-state concentration of ATP-G-actin is very close to the critical concentration of barbed ends, so that barbed end growth balances pointed end depolymerization [17, 18]. Notably, the growth of barbed ends in in vivo processes, such as during formation of lamellipodia, which are extending plasma membrane protrusions in the direction of migration, is two orders of magnitude faster. This implies that the concentration of monomeric actin is maintained at higher values by factors controlling the dynamics of assembly at the two ends of the actin filament [3]. These factors are proteins that generally bind either to monomeric G-actin or to the filament, thereby regulating the rate of turnover and imposing spatial restrictions required for site-directed actin dynamics. These actin 'regulatory proteins' affect the kinetic/thermodynamic parameters of actin assembly at one or the other end of the filament in various fashions (figs 1, 2).

Instrumental in understanding the biochemical contributions of various actin regulatory proteins has been the in vitro reconstitution of actin-based motility of either bacteria or functionalized beads [19, 20]. These approaches led to the identification of the essential proteins for actin-based motility: actin, the actin nucleator complex Arp2/3, an Arp2/3 activator, an actin depolymerizing factor (ADF/cofilin) and a capping protein [21]. The movement is more effective when profilin is also included [21]. Interestingly, the same proteins are required for lamellipodia formation in *Drosophila S2* cells [22]. The fact that five essential proteins are sufficient to reconstitute actin-based propulsion of a particle strongly supports the notion that the regulated treadmilling of actin filaments, coupled to site-directed catalytic generation of new filaments, is at the origin of actin-based movements [23] (see also table 1).

Severing and depolymerization

ADF/cofilin are ubiquitous, conserved actin-binding proteins [24]. Their role is to enhance the treadmilling rate of

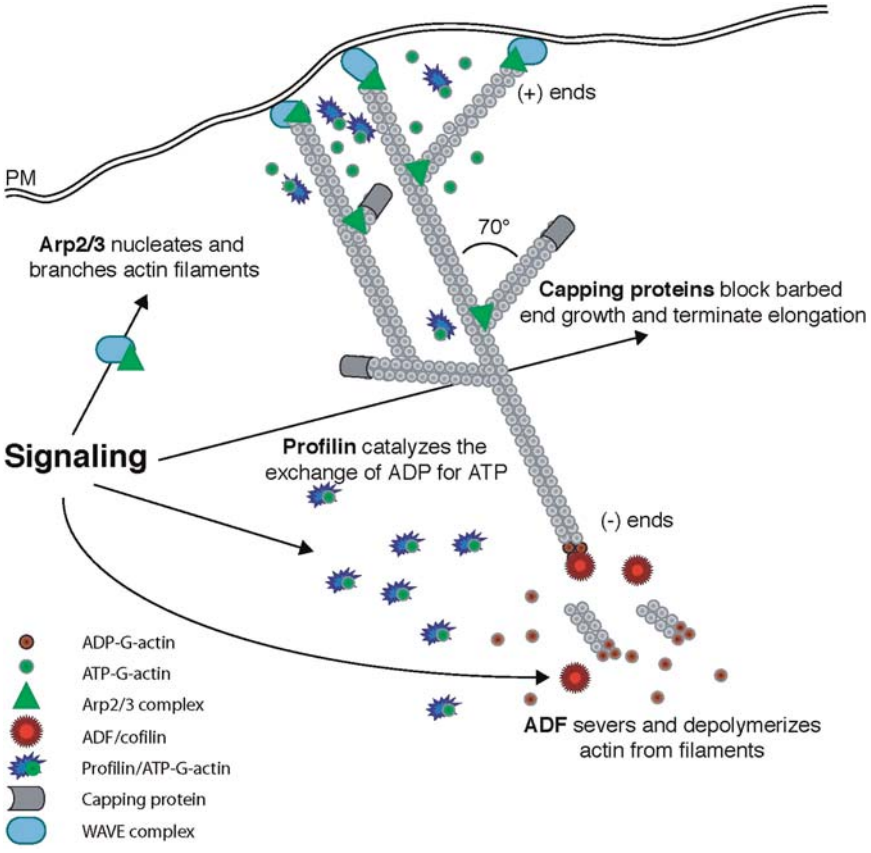

Figure 1. The actin polymerization machinery triggers dendritic nucleation for protrusion at the leading edge. The activated Arp2/3 complex nucleates and branches actin filaments at the leading edge, pushing the membrane forward. Capping proteins control the half-life of filaments, and by blocking a large fraction of barbed ends, promote site-directed elongation of uncapped filaments. ADF/cofilin promotes dissociation of ADP-actin from filament pointed ends and severs preexisting filaments, generating new barbed ends. Profilin catalyzes the exchange of ADP for ATP on monomeric actin molecules, which become available for new polymerization at barbed ends.

actin filaments through their ability to bind preferentially to ADP-bound forms of G- and F-actin and thus changing the rate of actin assembly. ADF specifically increases the rate of depolymerization of ADP-F-actin from the pointed ends, triggering a greater flux of ADP-G-actin, which increases the steady-state concentration of ATP-G-actin. This, finally, results in an enhancement of the rate of barbed end elongation balancing the high rate of pointed-end disassembly. Additionally, ADF/cofilin may generate new barbed ends by severing preexisting filaments [25]. Thus, by these mechanisms, ADF increases the overall rate of barbed-end growth at steady state, promoting actin-based motility [17, 26]. The activity of ADF is regulated by reversible phosphorylation of a serine residue in the N-terminal region (dephosphorylation = activation) [27]. A Rac-regulated LIM kinase inactivates ADF [28] (see also below). Conversely, the human hSSHs, like their homologue in *Drosophila*, Slingshot, have been recently shown to act as the activating phosphatases. In vitro experiments showed that SSH and hSSHs are able to dephosphorylate purified P-cofilin, and the loss of ssh function in *Drosophila* was found to increase the level of phospho-cofilin (P-cofilin). Additionally, SSH and hSSH expression has been shown to suppress actin reorganization induced by the expression of LIM kinase 1 and to reduce the level of P-cofilin in cultured cells [29].

Interactors of monomeric G-actin

Profilin and its *Drosophila* functional homologue Ciboulot are members of the actobindin family of proteins [30, 31], which are involved in actin polymerization. Profilin specifically binds ATP-G-actin in a complex that associates exclusively with barbed ends [32, 33], shifting in this way the distribution of G-actin at steady state. The pool of ADF-ADP-G-actin derived from pointed ends is converted into an ATP-bound profilin-actin complex, which associates at barbed ends, enhancing the treadmilling (figs 1, 2). In vitro, treadmilling is accelerated 125-fold by the synergistic effects of ADF and profilin [3, 34].

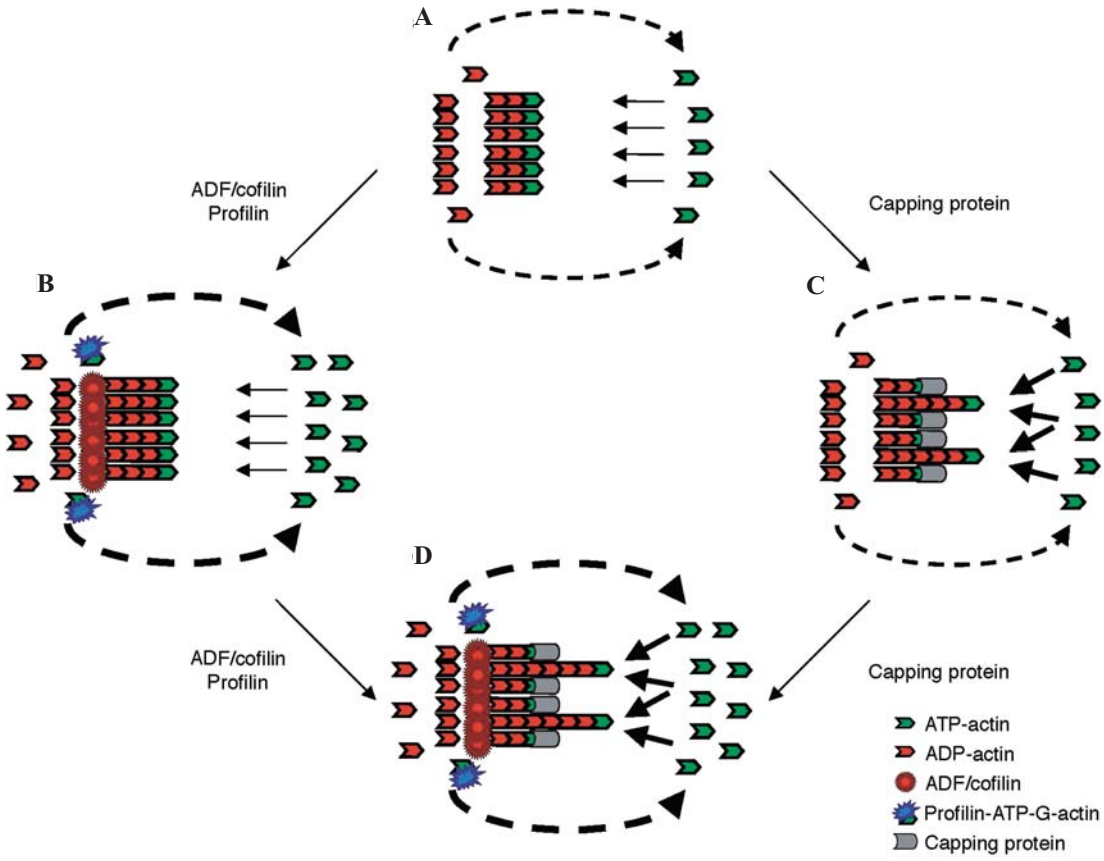

Figure 2. Regulation of treadmilling in motility. ADF/cofilin and capping proteins act in a coordinated manner to enhance barbed-end growth and accelerate membrane protrusions. (*A*) Treadmilling in the absence of ADF/cofilin, profilin and capping protein. The pool of ATP-G-actin is limited, and the rate of polymerization at the barbed ends is slow. (*B*) ADF/cofilin alone, by enhancing depolymerization from pointed ends, increases the pool of G-actin available for polymerization at the barbed ends, thereby increasing the steady state of barbed-end growth. Profilin synergizes with ADF/cofilin by promoting the exchange of nucleotides on G-actin. (*C*) Capping proteins, by blocking a large number of barbed ends, funnel G-actin, deriving from the depolymerization from the pointed ends, to the few uncapped filaments. (*D*) Cumulative effects of ADF/cofilin-profilin and capping proteins.

Barbed-end cappers

Capping proteins are required for efficient motility of many cells [35, 36]. Cells contain a variety of barbed-end capping proteins. Gelsolin and its relatives (severin, adseverin/scinderin, villin and fragmin) bind very tightly to barbed ends (Kd = 10^{-11}–10^{-12} M) and sever filaments. Other weaker, non-severing capping proteins (Kd = 10^{-8}–10^{-9} M) are proteins such as β2 (CP), the homolog of CapZ in non muscle cells, and CapG [3, 37–39].

Capping proteins contribute to the regulation of actin treadmilling in multiple ways. First, by capping a large fraction of barbed ends, they establish a high steady-state concentration of monomeric actin, deriving from depolymerization at pointed ends. This may, in turn, feed growth, in a spatially restricted and signaling-dependent fashion, of a few uncapped filaments that individually grow faster. Second, by arresting the growth of filaments, they regulate their lifetime and average length in lamellipodia [3, 38] (figs 1, 2). Thus, in vivo, their regulated dissociation is proposed to provide a source of free filament ends to funnel actin polymerization. This is consistent with a model where signal-regulated and spatially-restricted capping/uncapping of existing filaments provides sites of actin filament elongation, thus highlighting a role for actin filament barbed-end capping proteins as critical targets of signaling cascades leading to actin-based motility [40, 41] (see also below). Uncapping, for instance, was found to occur in cells at sites of active actin assembly, including protruding lamellae and rocketing vesicles [41, 42]. Moreover, recently, the balance between capping and anti-capping activities has been proposed to be critical in determining how cells ‘choose’ between two distinct modes of organizing the architecture of actin-based protrusions leading to the formation of either lamellipodia or filopodia. Mejillano and colleagues showed that depletion by RNA interference (RNAi) of capping protein (CP), a ubiquitous, highly conserved barbed-end capper [37, 43], suppressed lamellipodia, and induced the explosive formation of filopodia in several cell lines.

Table 1. Regulators of Rac-dependent actin remodeling. The proteins involved in these processes were categorized, from top to bottom, as actin binding proteins, Rac-GEFs (Rac-guanine nucleotide exchange factors), downstream effectors of Rac involved in lamellipodia formation and nucleation promoting factors downstream of Rac.

Protein	Biochemical properties	Predicted cellular function(s)	Selected refs
ADF/cofilin	increases the rate of pointed end depolymerization, severs filaments, enhances treadmilling	regulates lamellipodium protrusion	[25, 26, 34]
Profilin	promotes nucleotide exchange on actin	promotes polymerization	[32–34]
Gelsolin	severs filaments and caps the barbed end, regulated by Ca^{2+} and low pH	enhances lamellipodium protrusion and membrane ruffling	[39, 155, 156]
Capping protein	caps the barbed end	promotes actin-based motility	[22, 37, 40]
Eps8	caps barbed ends upon interaction with Abi	enhances actin-based motility	[53]
Arp2/3	amplifies actin filament barbed ends upon interaction with the VCA domain of WAS familiy proteins	essential for actin filament assembly in lamellipodia	[1, 61, 62, 72, 73, 75]
Eps8	part of a Rac-GEF complex (Sos-1, Abi, PI3-K)	activates Rac	[56–58, 159]
Sos-1	Ras-GEF, Rac-GEF	activates Ras, activates Rac	[56–58, 159, 160]
Swap-70	Rac-GEF	activates Rac	[119]
Tiam-1	Rac-GEF	activates Rac	[120]
Dock180/ELMO	Rac-GEF complex	activates Rac	[122–124]
Abi	part of a Rac-GEF complex (Sos-1, Eps8, PI3-K)	activates Rac	[56–58, 159]
IRSp53	bundles actin filaments, interacts with WAVEs	implicated in linking RhoGTPases to downstream effectors	[131–134, 161]
Abi	binds to Nap1 and WAVE	essential component of a WAVE-containing complex	[59]
Sra-1/PIR121	binds specifically to RacGTP	essential component of a WAVE-containing complex	[60, 138, 140]
Nap1	binds to Sra-1/Pir121 and Abi1	essential component of a WAVE-containing complex	[59, 140]
WAVEs	nucleation promoting factors, activate the Arp2/3 complex	essential for Arp2/3 complex activation at the lamellipodium tip	[59, 60, 69, 131, 161]

Notably, concomitant removal of CP and ENA/VASP, barbed-end interactors promoting rapid-debranching [44], functionally antagonizing capping activity [45], resulted in ruffling rather than filopodia formation [40]. This led to a model for selection between lamellipodia and filopodia in which CP may negatively regulate, in an Ena/VASP-dependent fashion, the latter type of membrane protrusions. Additionally, the activities of the gelsolin/villin family members, as well as the activity of CapG and CapZ, are tightly regulated by signaling-dependent molecules, such as phosphatidylinositol-4,5-bisphosphate (PIP2) [46, 47], which controls a variety of key regulators of the machinery of actin polymerization [48–50]. Finally, gelsolin and gelsolin-like proteins are strictly controlled by Ca^{2+} levels and pH [46, 51].

Recently, a new class of actin barbed-end capping protein was described and characterized revealing potential novel modality through which signals may regulate actin capping [52, 53]: the eps8-family [54, 55]. Eps8, the prototype of this family, is a typical modular protein that participates in signal transduction from the small GTPase Ras to Rac, leading to actin remodeling [56]. Eps8 interacts with both the guanine nucleotide exchange factor Sos-1 and the adaptor Abi-1 in a multi-protein complex including also p85, the regulatory subunit of phosphatidylinositol 3-kinase [57]. This complex is crucial for activation of the Rho GTPase, Rac, as witnessed by the observation that genetic removal of Eps8 impairs growth factor-induced, Ras-dependent GTP loading of Rac and the ensuing actin remodeling. The Eps8 'output' function resides in the C-terminal 'effector' region, which binds to Sos-1, unmasking its Rac-GEF activity, and associates with F-actin in vitro [58]. In vivo, Eps8 co-localizes with a variety of F-actin-rich structures, including lamellipodia. Most notably, the isolated effector domain of Eps8 and of its family members was shown to be able to cap barbed ends with an affinity in the nM^{-1} range [53], comparable to canonical cappers [3]. Despite this, Eps8-fam-

ily proteins do not share any similarity with other capping proteins. This indicates that the Eps8 family members define a new class of barbed-end cappers, which may exert their biochemical activity through peculiar and unique mechanisms. The association to Abi1, a physiological interactor of Eps8, leads to its activation, revealing novel modalities of regulation of capping through protein: protein interactions [53]. This indicates that the full length protein is, at least in vitro, autoinhibited. Thus, Eps8 and Abi1 may contribute to the activation of Rac by entering a Sos-1-based activating complex, and acting as a capping assembly, they may participate in Rac downstream pathways, dictating the site and the spatial organization of the dendritic actin meshwork. It is of note that the Eps8/Abi-1 complex appears to be functional also in actin polymerization-based events, which are not regulated by Rho GTPases, indicating that this complex may participate in a wider variety of actin-based processes, independent of its ability to activate Rac [53]. Interestingly, Abi1 has been assigned a role in actin assembly downstream of Rac affecting the site-restricted activation of WAVE2 in a complex together with Nap1 and PIR121/Sra-1 [59, 60]. Thus, Eps8 and Abi-1 seem to sit at the heart of a multilayered system of regulation of the actin cytoskeleton, raising the interesting possibility that the two molecules are engaged in multi-protein complexes which are remodeled during signal propagation through the controlled replacement of subunits, while keeping core components, an issue that needs to be further investigated.

De novo actin nucleation: branching versus linear

To balance the effect of capping, site-directed nucleation mechanisms must operate to keep a constant number of uncapped, growing barbed ends. Two cellular factors have been shown to generate new filaments in a site-directed, signaling-controlled fashion. The Arp2/3 complex [1, 3, 61] branches actin filaments in the lamellae of migrating cells [62]. Formins, instead, directly bind filament barbed ends, and, independent of the Arp2/3 complex, catalyze processive growth of un-branched actin filaments [63– 65].

The Arp2/3 complex branches filaments

The Arp2/3 complex consists of seven conserved polypeptides comprising the two actin-related proteins Arp2 and Arp3, and the subunits p40, p34, p21, p20 and p16 [66]. The Arp2/3 complex is located in motile regions of the cell and in the actin tails of propelling vesicles and pathogens [23, 61]. Electron microscopy analysis of the branched filament array in lamellipodia and actin comet tails has shown that the Arp2/3 sits at the branch junctions of filaments [62]. Accordingly, biochemical and microscopic experiments established that the Arp2/3 complex branches new actin filaments (daughter filaments) at 70° angles from pre-existing filaments (mother filaments) [61, 67, 68]. Notably, Arp2/3 alone displays no activity on actin polymerization in vitro and only association to an 'nucleation promoting factor' (NPF) leads to initiation of filament branching [1, 23].

Proteins of the WASP (Wiskott-Aldrich syndrome protein) and WAVE (WASP family verprolin-homologous) families are the major activators of the Arp2/3 complex. They also serve as coincident detectors of signaling from the Rho GTPases, Cdc42 and Rac, or from other signaling molecules (PIP2) or adaptors (Grb2, Nck) to the Arp2/3 complex [23, 69, 70] (see also below). All WASP and WAVE proteins display a similar modular organization. They contain a conserved C-terminal 'effector' domain (VCA) and a more diverse N-terminal region responsible for targeting and regulation of the C-terminal domain. The VCA domain initiates polymerization of new actin filaments by bringing together actin monomers and the Arp2/3 complex [1, 23, 69, 70]. Within the VCA domain, a verprolin homology domain (V) binds G-actin monomers [71], an intermediate conserved cofilin homology domain (C) has been implicated in driving the conformational changes of the Arp2/3 complex, necessary for its activation [72–74], and the C-terminal acidic region (A) binds directly the Arp2/3 complex [75]. The ternary complex formed by G-actin-VCA-Arp2/3 represents the minimal activating unit capable of nucleating new actin filaments. Additionally, it interacts with mother filaments to initiate the formation of daughter filaments [1, 23]. How exactly the activated Arp2/3 complex interacts with a filament to initiate branching has been the subject of intense debate and investigation in recent years. Two possible models have been proposed: the first one favors the possibility that the Arp2/3 complex branches by binding to the side of pre-existing filaments [66, 67, 76]; the second, instead, proposes that branching can occur only at the barbed ends of growing filaments, [3, 61, 77]. Whatever the case, a consensus has been reached that the Arp2/3 complex generates a branched array of actin filaments in vitro. The dendritic character of lamellipodial network was revealed by the use of metal-replica electron microscopy techniques. Actin appeared to be organized into networks with the highest density at the leading edge, where actin filaments are extremely branched and characterized by an abundance of free barbed ends in a characteristic brush-like appearance. [40, 62, 78]. Nevertheless, by employing different electron microscopy techniques, several additional studies have indicated that lamellipodia are composed of a network of relatively long and unbranched actin filaments, at least in more proximal lamellipodial regions [79, 80]. Such a structure may arise from rapid daughter filament de-branching catalysed by ATP hydrolysis on Arp2 [81].

Thus, the Arp2/3 complex mediates an amplification of barbed ends close to the membrane, allowing efficient protrusion and creating a structure which can rapidly reorganize into a more complex array of actin filaments by debranching, capping and bundling activities.

Formins nucleate unbranched actin filaments

Formins have been characterized as nucleators of unbranched actin filaments and implicated in several motile events, such as actin cable formation in yeast [65, 82, 83], assembly of actin filaments in the cytokinetic ring [84], focal adhesions and adherens junctions [85], in cell migration and ruffling [86, 87], and filopodia formation [88].

Formins are a family of proteins comprising several members, sharing a common domain organization that includes the functional formin homology domains FH1 and FH2, which are conserved in all eukaryotes [65, 89]. The FH2 domain is sufficient to nucleate actin in vitro, while the FH1 domain is required for actin nucleation in vivo and for binding to profilin [90–92]. This latter interaction has been recently shown to play a prominent role in the mechanism of nucleation induced by mammalian formins. In the absence of profilin, formins bind actin filament barbed ends and, by weakly blocking them, act in a manner that resembles canonical actin capping proteins. Conversely, when bound to profilin, the FH1-FH2 module of mDia1 accelerated the hydrolysis of ATP on F-actin filaments, increasing by 15-fold the rate of polymerization at barbed ends [93].

Formins induce processive polymerization, using free energy derived from the acceleration of the hydrolysis of ATP coupled with actin polymerization. This, taken together with the fact that formins are involved in and controlled by a variety of signaling pathways [65], points towards a role in the generation of actin-driven membrane protrusions.

Signalling pathways leading to actin-based motility

A prototypical example: growth factor-induced lamellipodia formation

Integration and coordination of the activities of all the proteins interacting with and affecting the different steps of actin polymerization is needed to promote cell motility. The same set of actin regulatory proteins is also the target of a number of signaling pathways, which transduce, in a temporally and spatially controlled fashion, extracellular cues enabling a cell to respond to its environment. Among these responses, the acquisition of a motile phenotype has been the subject of intense investigation. The ability of cells to move is essential for a number of fundamental physiological processes, including tissue morphogenesis, embryonic development and immunological response. Additionally, regulated cell motility is often linked to pathology, first and foremost cancer.

Cell migration is a multistep process requiring tightly regulated and coordinated dynamic changes of the actin cytoskeleton. These include de novo and site-directed polymerization of actin filaments at the periphery of cells, precise control of filament lengths and dynamics, and the turnover of cell-cell and cell-substrate adhesions. These changes of the cytoskeleton are usually initiated in response to ligand binding, which can be growth factors or proteins of the extracellular matrix, to cell surface receptors. Among these changes, the extension of membrane protrusions, which often assume the shape of actin-rich, polarized lamellae driving the movement of an entire cell, is considered the initial event in the acquisition of a migratory phenotype. The nature of RTK-mediated signaling leading to membrane protrusion has indeed been explored in great detail in recent years and has become a paradigmatic model for unraveling the molecular complexity of the signaling network governing actin-based motility.

Among the signaling proteins controlling this event, Rho-GTPases have emerged as central players. Although Rho GTPases have been implicated in a number of different fundamental cellular processes, such as cell-cycle progression, gene transcription and vesicular trafficking, they have been shown to be essential for the regulation of actin remodeling [94–96]. Historically, Rac, Cdc42 and RhoA are the three GTPases, whose activities and functional roles in driving distinct actin dynamics-based processes have been most extensively investigated. Notably, for instance, while the overall rate of actin assembly in a forming lamellipodium induced by extracellular cues is mostly dependent on Rac, the initiation of the assembly processes and its site-restricted localization are mainly regulated by Cdc42, while RhoA is required for generating contractile forces for productive cell translocation. Thus, proper coordination of the activities of all these key transducers of signaling is required for efficient cell migration. Due to space limitations and the availability of a number of excellent, recent papers covering the functional diversity of various Rho GTPases, the upcoming section of this review will specifically focus on the mechanisms through which a Rac-dependent signaling network functions in vivo, highlighting the importance of a relatively small set of key proteins in generating a highly integrated and coordinated molecular circuitry (see also table 1).

RhoGTPases: masterminds of actin-based cell motility

The family of Rho-GTPases consists of 22 members [97]. Like all GTPases, they cycle between an active (GTP-

bound) and an inactive (GDP-bound) conformation (fig. 3a). GTP loading and its subsequent hydrolysis are regulated by guanine-nucleotide exchange factors (GEFs), and GTPase activating proteins (GAPs), respectively. Another class of proteins, guanine nucleotide dissociation inhibitors (GDIs), modulate the distribution of Rho GTPases between membrane(s), which is the site where these proteins are biologically active, and the cytosol [6, 98]. In principle, activation of Rho GTPases could proceed through modulation of any of these regulatory proteins; in vivo, however, regulation of GEF activity appears to be of paramount importance. Notably, the numbers of GEFs far exceed that of Rho GTPases and are thought to act promiscuously towards several of the members of this family of proteins. Alternatively, different GEFs may exert specific roles depending on the signaling pathways and the specific cellular context.

From RTKs to Rac

Seminal studies by Ridley, Hall and co-workers showed that signals induced by growth factors [i.e. epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin] resulting in membrane ruffling

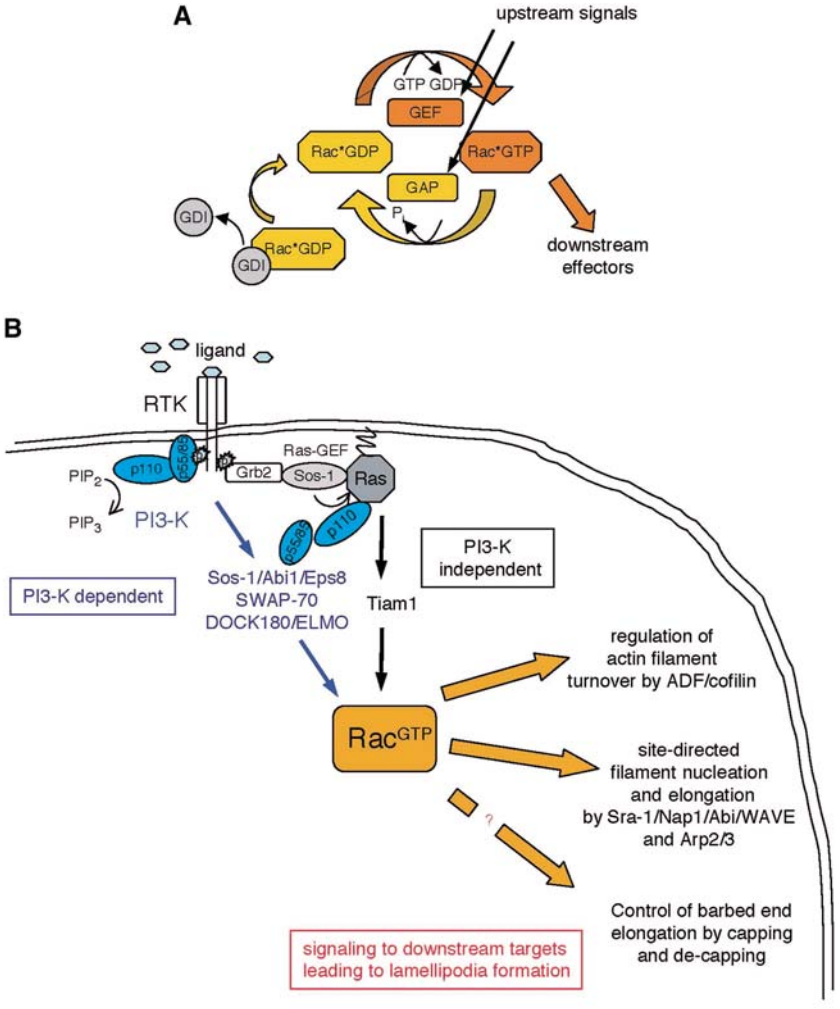

Figure 3. (*A*) Rac activity is controlled by GEFs, GAPs and GDIs. Rac, like all GTPases, cycles between an inactive GDP-bound form and an active GTP-bound form. The cycle is tightly regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Activated (GTP-loaded) Rac relays signals to its downstream effectors. (*B*) Schematic illustration of the molecular signaling pathways leading to Rac activation and subsequent lamellipodia formation. Rac becomes activated (GTP loaded) by a variety of GEFs, which, in turn, are regulated by diverse upstream signaling events, like those emanating from RTKs. Except for Tiam1, the dependency on PI3-K was demonstrated. Once Rac is activated, it elicits actin polymerization, leading to lamellipodia formation by controlling several downstream pathways. The spatially controlled actin polymerization, at the plasma membrane is achieved by relaying the activation signal via the Sra-1/Nap1/Abi/WAVE complex to Arp2/3 complex-catalyzed nucleation. Furthermore, Rac modulates ADF/cofilin-mediated actin depolymerization from the pointed end. Finally, the generation of free barbed ends, e.g. resulting from uncapping, is a prerequisite for Arp2/3-mediated nucleation. This process most likely can also becontrolled by Rac, although the mechanism of regulation remains poorly defined (for details, see text).

strictly depend on Rac1 [99]. The initial step of growth factor binding to RTKs leads to receptor dimerization and subsequent activation of its cytoplasmic kinase domain. The ensuing auto-phosphorylation of tyrosine residues on the receptor C-terminal tail enables binding of a variety of SH2-domain-containing adaptor proteins, thus leading to the generation of signaling platforms composed of multiprotein complexes [100–102]. This makes it possible to greatly diversify and amplify the initial signaling input, leading to regulation of biological outputs as different as cell-cycle progression, differentiation and actin remodeling. In this latter case, the recruitment of the SH2-domain-containing heterodimeric enzyme phosphatidylinositol 3-kinase (PI3-K) is crucial for linking growth factor stimulation to Rac activation.

Class IA PI3-Ks are composed of a p110 catalytic subunit, which uses phosphatidylinositol 4, and 5 phosphate (PIP2) to generate phosphatidylinositol 3,4,5 phosphate (PIP3), and a regulatory (p85/p55) subunit [103]. Specific tyrosine-phosphorylated residues of the receptor mediate high affinity binding of the SH2-domain of the regulatory (p85/p55) subunit, whereupon the catalytic (p110) moiety is recruited and activated [104, 105]. Additional complexity to this mode of activation came with the demonstration that the catalytic p110 subunit can directly associate to another critical small GTPase, Ras. Ras is recruited and activated by a complex consisting of Grb2, an adaptor molecule containing SH3 and SH2 domains, the latter of which bind to the activated and phosphorylated tails of RTKs [106–108], and Sos-1, a dual guanine nucleotide exchange factor for Ras and Rac (fig. 3b, see also below). Thus, concomitant binding of PI3-K to GTP-loaded Ras, via its catalytic subunit, and to RTK, via p85, is thought to cooperate for its optimal activation. The enzymatic product of this lipid kinase, PIP3, has in turn been implicated in actin-based cell migration in at least two ways: recently, PI3-K has been proposed to generate a gradient of PIP3 at the leading edge of protruding membranes [109], critical for recruitment of downstream targets, such as WAVE2 [110]. This, however, was not sufficient to elicit actin polymerization in the presence of dominant negative Rac [110]. Furthermore, it has to be pointed out that the precise mechanisms of how the spatial orchestration of this highly polar membrane lipid is achieved, and the extent of its contribution in generating membrane protrusions remains to be defined. One interesting possibility to account for this comes from the observation of the involvement in directional migration of the tumor suppressor PTEN, a phosphoinositide 3′-specific phosphatase that dephosphorylates PIP3 and P(3,4)P2 to P(4,5)P2 and P(4)P, respectively [111–113]. In chemotacting *Dictyostelium* cells, for instance, PTEN and PI3-K exhibit reciprocal patterns of spatial localization [114]. PI3-K is enriched at the leading edge of extending protrusions where PTEN is excluded but persists at the sides and the back of the cell [114]. Thus, it is reasonable to assume that the spatially restricted activation of PI3-K and PTEN generates and maintains a PIP3 gradient at the leading edge of motile cells, critically contributing to the regulation of site-directed actin dynamics.

Moreover, PIP3 has been shown to regulate the activation of Rac. The first indication that PI3-K can act upstream of Rac came from the observations that interference with PI3-K activity, using either pharmacological inhibitors, such as wortmannin, or dominant negative PI3-K constructs, led to the complete abrogation of PDGF-induced, Rac-dependent membrane ruffling. Ectopic expression of a constitutively active form of Rac1 could overcome this inhibition [104, 115–117], thus supporting an epistatic relationship between PI3-K and Rac. The biochemical mechanisms underlining this cross-talk turned out to be a much more difficult nut to crack. With the availability of in vivo assays capable of measuring the levels of GTP-loaded Rac, it became apparent that activation of PI3-K was functionally linked to Rac activation, providing the first biochemical evidence of a PI3-K-Rac pathway. The missing molecular links in this cascade were GEFs. The structural features of GEFs, which are frequently characterized by a modular organization with a variety of protein:protein interaction motifs, strengthen the possibility that these molecules may be capable of intercepting a variety of incoming signals from RTK, leading to Rac activation. A number of Rac-specific guanine nucleotide exchange factors were shown to be regulated by binding to PIP3 [118] (fig. 3b). For instance, genetic removal of Swap-70, a GEF for Rac, was shown to reduce ruffling in response to pharmacological doses of PDGF [119]. Similarly, PIP3-independent and -dependent GEFs, such as Tiam1 and Vav2/3, have been shown to play a role in Ras and RTK-mediated Rac activation, respectively [120, 121]. A peculiar mode of action for Rac-specific guanine nucleotide exchange factors was demonstrated in the case of Dock180, an unconventional Rac-GEF devoid of the Dbl homology domain present in all other known mammalian Rac-GEFs. Dock180 can mediate GTP loading of Rac in vitro [122, 123]. In vivo, however, the formation of a bipartite DOCK180/ELMO complex is required for its function [124]. The assembly into multimolecular complexes as a modality of regulation of GEFs involved in RTK-to-Rac signaling is also exemplified in the case of Sos-1. Sos-1, a dual GEF for Ras and Rac, was proposed to act as a Rac-GEF when engaged in a complex with Eps8 and Abi1 (also called E3b1), two adaptor molecules and phosphatidylinositol-3,4,5-triphosphate [57]. Accordingly, interference with any of the components of the complex reduces or abrogates membrane ruffling induced by RTK activation.

The final step: how Rac mediates lamellipodia formation

Several downstream targets of Rac have been extensively investigated for their ability to contribute to actin dynamics. Emerging evidence supports a model whereby Rac, through binding to a variety of distinct effectors, not only coordinates the activities directly affecting the rate of actin treadmilling and the architectural organization of the actin meshwork within the lamellipodium, but also imposes a strict spatial control on actin polymerization, generating the physical force pushing the membrane forward. Three major modes through which Rac regulates actin dynamics can be categorized, all contributing to enhanced protrusion of the membrane: de novo nucleation of actin filaments from monomers and filament elongation, resulting in the formation of a branched array of filaments by proteins such as the Arp2/3 complex; acceleration of actin turnover by proteins such as cofilin; regulated exposure of existing barbed ends after dissociation of high affinity filament capping proteins, such as gelsolin (fig. 1, see also above). Whatever the case, all the emerging evidence points to a scenario whereby Rac-mediated control of site-directed actin dynamics is achieved through the transmission of signal via macromolecular complexes that, to different extents, are required to physically link Rac to the basic machinery of actin polymerization, permitting a precise spatio-temporal coordination of the turnover of actin filaments.

Site-directed filament elongation: a matter of Arp2/3 and WAVE

In vitro, the rate-limiting step of actin polymerization is the assembly of free actin monomers into a trimer [1]. This, in vivo, is enhanced by several factors that stimulate nucleation [70]. A family of regulatory proteins, including WASP, N-WASP and WAVEs (1, 2 and 3), participate in these processes by relaying signaling from Cdc42 and Rac to the actin nucleation machinery – the Arp2/3 complex [2, 70]. These proteins possess a common modular organization that includes the highly conserved C-terminus capable of binding to and activating the Arp2/3 complex [69]. A variety of biochemical and structural biological evidence defined the modality through which signaling is conveyed to regulate WASP and N-WASP, particularly in the case of Cdc42 [70, 125]. Conversely, only recently the mechanisms that control WAVE proteins have begun to be clarified.

WAVE-mediated actin nucleation at the lamellipodium tip was assumed early on to function downstream of Rac [126], although the molecular linkage remained elusive, since WAVE proteins are incapable of direct binding to GTP-bound Rac. Furthermore, in contrast to WASPs, whose autoinhibitory conformation is released synergistically by binding of GTP-Cdc42 and PIP_2 in in vitro actin polymerization assays [127–129], full-length WAVE is fully active in this assay [130]. This suggested the possibility that regulation of WAVE proteins by GTP-loaded Rac could be exerted by controlling its localization, rather than its activity, thus allowing spatially restricted, Arp2/3-mediated actin nucleation at the leading edge of cells. Consistent with this, WAVE proteins were shown to assemble into multiprotein complexes at the lamellipodium tip, which exert a tight regulation either on their activity and/or on their stability and localization. The first link between WAVE2 and Rac was found to be mediated by the insulin receptor substrate IRSp53. However, while a complex including Rac-IRSp53-WAVE2 could be reconstituted in vitro, it did not significantly affect the nucleation promoting function of WAVE2 [131]. Additionally, no genetic evidence has been so far provided of a requirement of IRSp53 in Rac-mediated, WAVE-dependent ruffling. Finally, IRSp53 has also been shown to be a downstream target of the small GTPase Cdc42, leading to filopodia formation, pointing to a much more complex scenario [132–134]. Thus, the functional and physiological role exerted by IRSp53 on WAVE activity is far from being established, and more investigation in this direction will be needed.

An alternative mechanism to control the activity of WAVEs via Rac was recently proposed [135]. WAVE1 was found to be kept inactive through its association with three other proteins: Nap1, an NCK-associated protein [136], PIR121/Sra-1, identified as a Rac effector [137, 138] and HSPC300 [135]. This complex was unable to stimulate actin polymerization in in vitro assays. Addition of active Rac relieved this inhibition by inducing the disassembly of the inhibitory Nap1-PIR121 sub-complex from the WAVE1-HSPC300 unit, which was then capable of actin nucleation [135]. More recently, in vivo and in vitro experiments challenged this attractive inhibitory model arguing, instead, for a positive mode of regulation exerted by the assembly of WAVE into a Abi1-Nap1-PIR121 complex. According to these latter studies, Abi1 was shown to interact directly with the WHD domain of WAVE2, to increase WAVE2-mediated actin polymerization activity, and to mediate the assembly of a WAVE-Abi1-Nap1-PIR121 complex [59]. Unexpectedly, in vitro reconstituted WAVE1- and WAVE2-Abi1-Nap1-PIR121 complexes were as active as the WAVE1- or 2-Abi1 sub complexes in stimulating Arp2/3, and addition of activated Rac either in vitro or in vivo did not disrupt the complex [59, 60]. Furthermore, the integrity of the macromolecular complex was shown to be also required for proper targeting to the leading edge of membrane protrusions, as witnessed by the observation that all the components of the WAVE-based complex are dynamically re-localized to the leading edge of membrane protrusions, where de novo actin polymerization occurs [60]. This latter evidence is in agreement with previous and independent studies on the localization of Abi1 and

WAVE during membrane protrusion [69, 139]. The sum of these data suggest a model whereby the WAVE-Abi1-Nap1-PIR121/Sra-1 signaling complex needs to be recruited to lamellipodia upon Rac activation, thus leading to site-directed nucleation of actin filaments. Further support to this came from a number of RNAi studies performed in different model systems. Systematic, individual ablation of WAVE, Nap1, PIR121/Sra-1 and Abi1 gene products in *Drosophila* S2 Schneider cells [22, 140] and in mammalian cells [59, 60] led to a complete loss of lamellipodia formation. Moreover, mutations of the homologues of Sra-1/PIR121 or Nap1/Kette in plants resulted in a phenotype similar to the one caused by mutations in the Arp2/3 complex [141–144]. Thus, all together, these data support the notion that PIR121/ Sra-1, Nap1/Kette and Abi play positive, rather than inhibitory roles in WAVE/Scar regulation. An additional role exerted by this multimolecular complex was demonstrated by the observation that an intact Sra-1/Nap1/ Abi/Wave complex is a prerequisite for the stability of WAVE [22, 59, 60, 140, 145]. Interestingly, reintroduction of GFP-tagged WAVE2, which was not degraded, could not restore lamellipodia formation in Nap1 knockdown cells in response to Rac, highlighting the requirement of an integral complex for proper signal transduction from Rac to WAVE [60].

Regulation of actin filament turnover

In addition to the control on site-directed, de novo actin nucleation, Rac has been shown to mediate a signaling cascade leading to the regulation of the actin depolymerizing factor ADF/cofilin. This pathway has been extensively characterized and shown to occur through a 'canonical' kinase cascade. One of the first Rac effectors identified is PAK (p21 activated kinase), a serine/threonine kinase, whose activity is induced following association to GTP-loaded Rac. PAK in turn can phosphorylate a number of different substrates, including LIM kinase [146], leading to its upregulation. LIM kinase is then capable of directly regulating the activity of ADF/cofilin by controlling its phosphorylation status [147]. ADF/cofilin displays constitutive actin severing and depolymerizing activity once it is in a non-phosphorylated form, promoting actin filament turnover. Consistent with this view, ADF/cofilin has been shown to be required for cell motility [148, 149]. Further support for this came from studies in single cells. RNAi-mediated reduction of cofilin in *Drosophila* S2 cells led to accumulation of filamentous actin at the cell cortex [22], strengthening its role in promoting actin dynamics during plasma membrane protrusion. Rac may, therefore, modulate filament turnover by directly controlling ADF/cofilin activity. An additional level of ADF/cofilin regulation might be exerted through its association with the lipid second messenger PIP2. In vitro and in vivo experiments indicated that binding of PIP2 to ADF/cofilin prevents its interaction to actin [150]. Notably, Rac has been proposed to positively regulate both the PI4,P5 kinase [151], a lipid kinase, whose catalytic activity is responsible for the production of PIP2, and a lipid phosphatase, like synaptojanin II [152]. This suggests that coordinated regulation of these proteins by Rac may control the local levels of PIP2, possibly generating PIP2 gradients along the breath of the lamellipodium. This may, in turn, be instrumental in modulating the activities, in a spatially-restricted fashion, of proteins such as ADF/cofilin and/or capping proteins.

Control of barbed end elongation by capping and de-capping

The availability of free barbed ends for the Arp2/3-based actin nucleation machinery is fundamental to promote motility. Hence, dissociation of barbed-end cappers accompanies filament elongation [153]. Additionally, by blocking a large fraction of barbed ends, cappers establish a high steady-state concentration of monomeric actin, thus actively feeding the growth of free barbed ends. Finally, by arresting the growth of filaments, they regulate their life-time and average length in various motile actin structures such as lamellipodia.

Signaling leading to actin-based motility is thought to regulate the interaction of cappers with barbed ends, thus providing sites where actin polymerization generates propulsive force [41, 50, 154]. Consistently, gelsolin, a filament capping protein, has been implicated in the regulation of the actin cytoskeleton downstream of Rac. Gelsolin null fibroblasts displayed reduced ruffling in response to epithelial growth factor (EGF) stimulation [155]. Furthermore, the uncapping event of gelsolin from actin was found to occur at sites of active actin assembly [41, 156]. Conversely, in *Drosophila* S2 cells, RNAi-mediated reduction of gelsolin had no effect on lamellipodia formation [22]. This may be due to the fact that a variety of different capping proteins may contribute to this phenotype. These proteins may play specific roles depending on the cellular context and the signaling pathways in which they are implicated. In support of this view, genetic removal of either murine gelsolin or CapG only results in mild physiological alterations that are restricted to the tissues where the expression of these capping proteins is high [157, 158]. Finally, the discovery of the existence of a new family of actin capping proteins, like the Eps8 family [52, 53], involved in different steps of the cascade regulating actin remodeling, indicates that multiple mechanisms may have evolved to precisely regulate this activity in a tissue-specific and signaling-dependent manner. Clearly, more investigations will be needed to unravel the molecular basis of the connection between key signaling molecules, such as Rac, and capping proteins: an exciting challenge for the years to come.

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