OPINION

Global treadmilling coordinates actin turnover and controls the size of actin networks

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Abstract | Various cellular processes (including cell motility) are driven by the regulated, polarized assembly of actin filaments into distinct force-producing arrays of defined size and architecture. Branched, linear, contractile and cytosolic arrays coexist *in vivo*, and cells intricately control the number, length and assembly rate of filaments in these arrays. Recent *in vitro* and *in vivo* studies have revealed novel molecular mechanisms that regulate the number of filament barbed and pointed ends and their respective assembly and disassembly rates, thus defining classes of dynamically different filaments, which coexist in the same cell. We propose that a global treadmilling process, in which a steady-state amount of polymerizable actin monomers is established by the dynamics of each network, is responsible for defining the size and turnover of coexisting actin networks. Furthermore, signal-induced changes in the partitioning of actin to distinct arrays (mediated by RHO GTPases) result in the establishment of various steady-state concentrations of polymerizable monomers, thereby globally influencing the growth rate of actin filaments.

The actin cytoskeleton, referred to as the 'actin factory' (REF. 1), is organized into intracellular 'assembly workshops' (also known as actin arrays), which produce the cell cortex, cell adhesions, membrane protrusions, contractile stress fibres and cytosolic arrays (FIG. 1a). The architecture, size, site and timing of actin network assembly are tightly controlled in many of the essential cellular processes such as morphogenesis[2–11], the immune response[12] and intracellular pathogen motility[4,13]. These networks — which consist mostly of linear filament bundles or branched filament arrays — are formed by polarized assembly of actin filaments, which is triggered by RHO GTPase-activated machineries[14]. Defects in network regulation are linked to a number of pathologies such as cancer cell metastasis as well as immune, hearing, cognitive and age-related disorders[15–20]. In any given cell, the various networks coexist and exchange subunits with a common pool of actin monomers. Monomer addition to and removal from the filaments takes place at filament ends. This monomer–polymer exchange is individually regulated in each network to generate network-specific polarized assembly.

Linear networks are assembled either by processive formins[21] or by Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) proteins[22]. Such networks are found in filopodia, stress fibres and integrin-based focal adhesions (FIG. 1). Branched filament arrays are initiated by the actin-related protein 2/3 (ARP2/3) complex[23–26], which is itself activated by Wiskott–Aldrich syndrome protein (WASP) family proteins. Branched networks are important for lamellipodia extension, pathogen propulsion, endocytosis, exocytosis, endoplasmic reticulum (ER)–Golgi trafficking and other processes (FIG. 1a). Bundled antiparallel filaments stabilized by myosin II form contractile structures. These are involved in dorsal and ventral stress fibres and filament bundles associated with focal adhesions[27] (FIG. 1a). In addition to their geometry, the size and shape of these different structures are also tightly regulated. For instance, filopodia generally do not extend beyond a few tens of microns[28–30], whereas the lamellipodia of crawling keratocytes maintain a constant shape during cell migration[31,32]. Thus, the number, lengths and rates of elongation of actin filaments in these cellular extensions are intricately controlled, and actin filament dynamics define both their size and geometry at the subcellular and cellular scales. Given that these diverse actin networks operate simultaneously in cells, it is to be expected that functional crosstalk between the assembly workshops exists. Over the past decade, important advances have been achieved in our understanding of the mechanisms by which cells regulate and balance the turnover of various coexisting actin networks.

The fundamental concept of treadmilling lies at the heart of actin network turnover. Treadmilling was first discovered and validated at the scale of a population of pure actin filaments assembled at a steady state in the presence of ATP[33]. ATP–actin monomers associate with the barbed end (also known as the plus end) of an actin filament and slowly hydrolyse to ADP–actin as the monomers 'age' in the filament. As a result, the core of the filament (away from the barbed end) consists of ADP–actin subunits. Actin filaments are maintained out of equilibrium because the subunit that dissociates from the filament end may not be ATP-bound, but ADP–inorganic phosphate (P_i)- or ADP-bound, and thus is energetically different from the associating ATP-bound monomer. Instead, filaments coexist with actin monomers at a 'steady state', referred to as treadmilling[33]. During treadmilling, filament disassembly by net loss of ADP–actin subunits at the pointed ends (also known as minus ends) is balanced by net barbed-end growth from ATP–actin monomers. As a result, the polymerizable actin monomer concentration remains constant. Note that net growth at barbed ends would be impossible if actin polymerized in a thermodynamically reversible fashion, as it does when bound

to ADP. If all monomers were ADP–globular actin (G-actin), they would only exhibit slow exchange reactions with the terminal subunits at either end of filaments, and no net growth of filaments would be observed. Treadmilling has been demonstrated to account for actin-based cell motility at the scale of an individual network[34,35]. Here, we argue that the concept of treadmilling described so far for individual filaments and networks can be extended to the whole cell, such that treadmilling occurs globally over all of the coexisting filaments. We refer to this as 'global treadmilling' and argue that this concept explains the distribution, the evolution as well as the maintenance of all coexisting actin networks in a cell.

Each cellular actin network grows by directed assembly of actin monomers (G-actin) at the barbed ends of individual filaments (in the network) growing against membranes in force-producing processes (FIG. 1a,b). Their assembly rate depends on the amount of available polymerizable monomeric actin. Monomer concentrations higher than the critical concentration for barbed-end assembly are required for barbed-end growth. Pointed ends are never observed to grow *in vivo*, indicating that free ATP–G-actin is at a concentration lower than the critical concentration for pointed-end assembly and/or that polymerizable monomers are

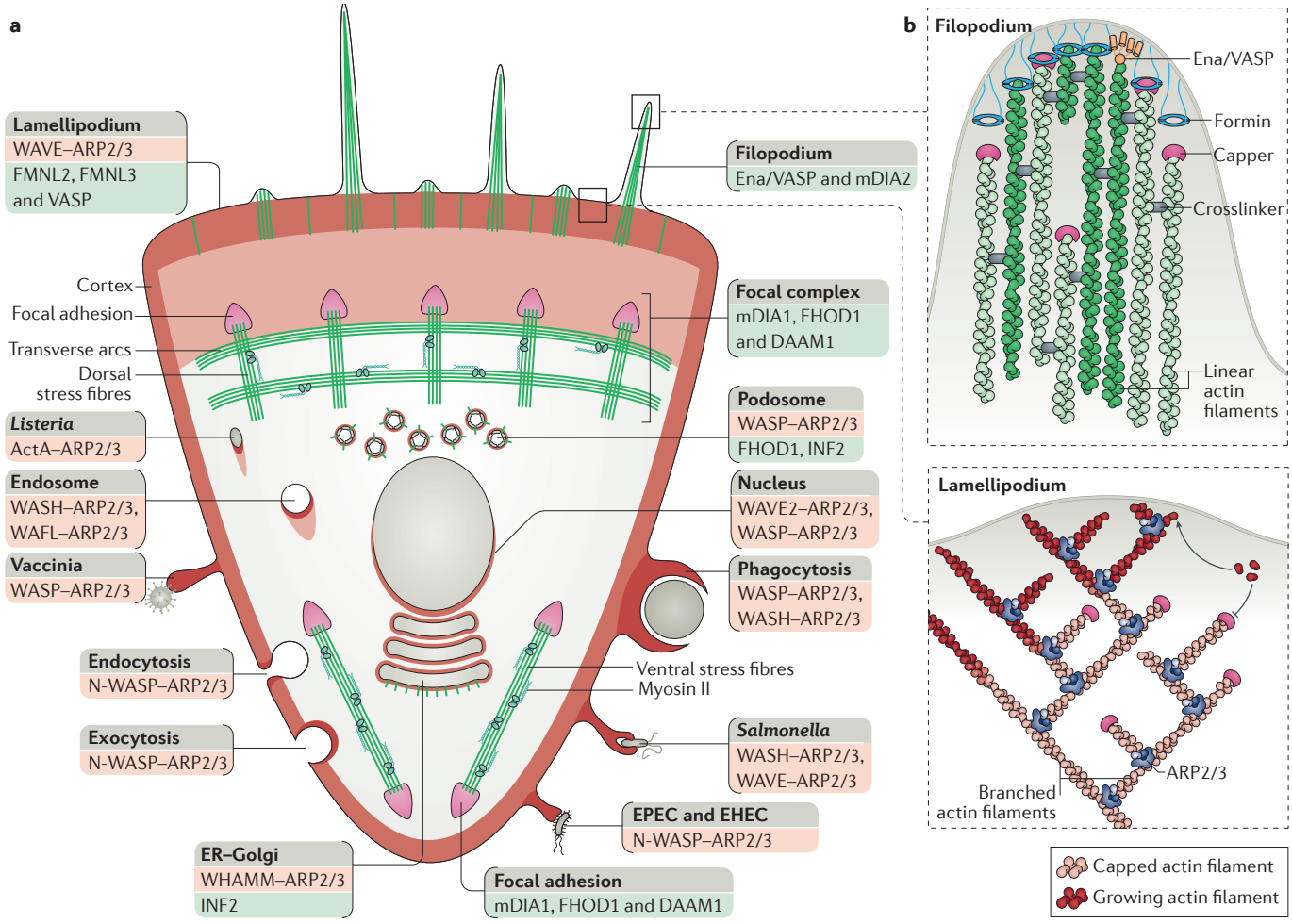


Figure 1 | **Actin assembly workshops in cells. a** | Branched actin networks (red) are assembled by the actin-related protein 2/3 (ARP2/3) complex, the activity of which is regulated by various RHO GTPase-activated proteins of the Wiskott–Aldrich syndrome protein (WASP) family (highlighted by red shading): WASP family verprolin-homologous protein 1 (WAVE1)–WAVE3 at the tip of lamellipodia (see REF. 158 for a review); neural WASP (N-WASP) at the neck of clathrin-coated endosomes and around exocytic vesicles[159], in pedestals associated with enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC)[160] and in vaccinia virus-induced actin tails[161]; WASP and SCAR homologue (WASH) and WAVE at sites of *Salmonella* entry[162]; WASH and WASP and FKBP-like (WAFL) in early endosome formation[163]; WASP homologue associated with actin, membranes and microtubules (WHAMM) in endoplasmic reticulum (ER)–Golgi transport[24]; WASP in podosomes[164]; and WASP and WAVE2 in phagocytic vesicle formation[165] and in actin structures around the nucleus[166]. Linear actin bundles (green) are assembled by RHO/CDC42-activated formins (highlighted by green shading): mammalian diaphanous homologue 2 (mDIA2) at the tips of filopodia[142]; formin-like protein 2 (FMNL2) and FMNL3 at the tips of lamellipodia[167]; inverted formin 2 (INF2) at ER and Golgi membranes[168]; mDIA1, formin homology 2 domain-containing 1 (FHOD1) and Dishevelled-associated activator of morphogenesis 1 (DAAM1) at focal complexes and adhesions[169]; and FHOD1 and INF2 in podosomes[169]. Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) proteins are actin polymerases that track filament barbed ends at the tips of filopodia and lamellipodia and at focal adhesions[29,85]. Antiparallel filaments in contractile structures such as stress fibres are associated with non-muscle myosin II. **b** | View of dendritic and linear actin networks to show how the capping of filaments takes place, which leads to their transition from active growth to an arrest of filament growth[170]. Filament growth can be arrested either by binding of a capping protein (capper) to the barbed end or by the simultaneous binding of formin and capping protein. The darker colour indicates filament barbed ends that are actively growing, whereas the lighter colour indicates filaments that are capped and no longer grow. ActA, actin assembly-inducing protein.

unable to support pointed-end growth. Treadmilling maintains the monomer concentration above the critical concentration for barbed-end assembly. The energy needed for polarized filament growth is provided by the hydrolysis of ATP bound to actin monomers. A number of accessory actin-binding proteins are present in cells and regulate filament treadmilling. As demonstrated both *in vivo*[36–38] and in *in vitro* reconstituted motility assays[39], actin-binding proteins increase the total monomer concentration at steady state, thereby leading to the fast treadmilling that is normally observed *in vivo*[40]. The activity of actin-binding proteins is locally regulated by signalling and this can greatly influence the growth of defined arrays. When diverse actin assembly machineries operate simultaneously in cells, each array contributes to the global actin dynamics with a specific impact dependent on its size, architecture and intrinsic kinetic parameters. This interplay results in the steady-state concentration of actin monomers.

In this article, we emphasize how the acknowledged fundamental concepts of actin self-assembly and its regulation are sufficient to account for recent observations that suggest that various actin networks exhibit homeostatic behaviour. We propose that a combination of global treadmilling and signal-induced shifts in actin steady states controls the balance between different coexisting actin arrays. In this model, the steady-state concentration of polymerizable monomers is the most important factor that defines the growth rate and size of these arrays, which are required for specific mechanical properties of cells.

The polymerizable pool of actin

The amount of polymerizable monomers defines the growth rate of actin filaments. Polymerizable actin monomers could not exist in large amounts *in vivo* owing to the presence of physiological salt concentrations, which promote massive polymerization (the G-actin to filamentous actin (F-actin) transition). Upon polymerization of a pure actin solution, only 0.1 μM G-actin would remain in solution, which is referred to as the steady-state ATP–G-actin concentration; the remaining actin would be F-actin. These pure actin filaments at steady state would undergo very slow barbed-end growth because the steady-state monomer concentration is only a few nanomolar above the critical concentration for the assembly of ATP–actin monomers at filament barbed ends (0.08 μM).

However, the fast barbed-end growth rates observed *in vivo* indicate that the steady-state concentration of polymerizable actin monomers is actually much higher than expected (in the range of a few micromoles). How can this be explained? Key regulatory proteins control the nature and concentration of polymerizable actin monomers by binding actin monomers (the most notable example is profilin), filament ends (the capping proteins (uncappers)) and filament sides (the filament-stabilizing and severing proteins) (BOX 1; see also TABLE 1). Actin monomers can exist in three forms: as free G-actin, profilin–actin and β-thymosin–actin. Only G-actin and profilin–actin can bind to filament ends and therefore represent the population of polymerizable actin monomers. β-Thymosin–actin represents the sequestered, non-polymerizable G-actin. The steady-state concentration of polymerizable monomers reflects the energetics of monomer–polymer exchanges, which is similar, if not identical, to the critical concentration (that is, the monomer–polymer equilibrium dissociation constant) for reversible polymer assembly in physics. In other words, the lower the monomer concentration, the higher the stability (lifetime) of the filaments and the slower the rate of their barbed-end growth.

The role of filament disassembly

The steady-state monomer concentration can be increased by enhancing filament disassembly. In treadmilling of filaments assembled from pure actin, the rate of barbed-end assembly is limited by monomer regeneration as a result of slow disassembly ($0.1\ s^{-1}$) at the pointed ends (FIG. 2a). The disassembly rate can be increased *in vivo* mainly through the activity of the actin depolymerizing factor (ADF; also known as destrin)/cofilin family of proteins. In line with this, in several cell types, proteins of the ADF/cofilin family have been shown to enhance motility and filament treadmilling (see REF. 41 for a review). They often act in combination with the ADF cofactor actin-interacting protein 1 (AIP1)[41], twinfilin and Srv2/adenylyl cyclase-associated protein (CAP) proteins[42–48].

The name of the ADF/cofilin protein family originated from the ability of its members to both bind to filaments and promote the partial disassembly of pure actin filaments assembled *in vitro*[49–52]. It was also recognized in early studies that they weakly sever filaments[50,53]. The exact mechanism for the biological function of ADF/cofilin is still debated. Both severing and partial depolymerization were proposed to result from the binding of ADF/cofilin to filaments, which weakens lateral interactions in the filament (see REF. 41 for a review). Biochemical studies reveal that ADF/cofilin binds ADP–actin with high specificity, with a preference for ADP–G-actin versus ADP–F-actin. This result implies that ADF-decorated filaments are less stable than standard filaments, which is confirmed by experimental evidence for an ADF-enhanced critical concentration for the assembly of ADP–actin[47]. ADF/cofilin proteins preferentially bind cooperatively to ADP–F-actin subunits rather than to newly assembled ATP–F-actin or ADP–P_i–F-actin stretches close to barbed ends[47,54–57]. ADF/cofilin-induced structural change[55] and destabilization of filaments leads to their severing[55,58,59], preferentially at filament bends and at the boundaries between bare and ADF/cofilin-coated sections of filaments[60]. Severing has been demonstrated to increase with an increase in ADF concentration[53,61].

A 25-fold increase in depolymerization rate at pointed ends — while the average filament length was seen to decrease less than twofold — was also reported and proposed to result from the destabilization of actin–actin interactions in the filament[47,61]. The enhanced rate of depolymerization of ADF-bound subunits provides a direct explanation for the observed partial depolymerization and for the measured increase in the steady-state concentration of ATP–actin monomers[47,48,62–65], which are responsible for faster treadmilling in solutions of pure actin.

Severing by itself is not thermodynamically equivalent to depolymerization. By producing as many barbed ends as pointed ends, severing increases the fluxes of assembly and disassembly of pure F-actin identically (FIG. 2b). This effect would not change the steady-state concentration of polymerizable actin monomers[47,66,67] (see REF. 68 for a review) (FIG. 2b). As proof of this, the potent severing protein cordon-bleu (COBL) does not affect monomer concentration[61]. Notably, however, ADF/cofilin has been shown to increase the steady-state pool of polymerizable monomers[40]. It has been suggested that destabilization of actin–actin bonds in the filament leads to enhanced disassembly and an increased monomer pool (FIG. 2c). This view has been

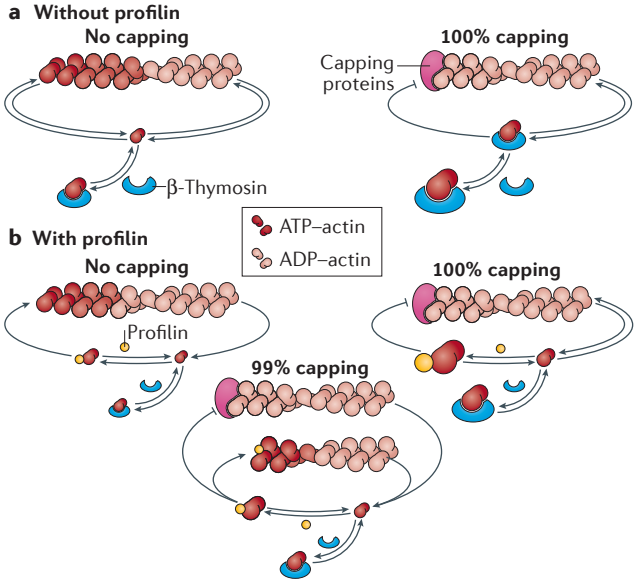
Box 1 | **Control of the pool of actin monomers**

Cappers increase the steady-state concentration of monomers
Capping proteins (cappers) bind tightly to filament barbed ends and block all monomer (globular actin (G-actin)) association–dissociation reactions. In cells, capped filaments depolymerize from their pointed ends, enhancing the steady-state monomer concentration that transiently[34] feeds the newly created barbed ends (this is known as the 'funnelling hypothesis' (REF. 40)). The increase in growth rate of non-capped filaments occurs if 90–100% of bulk filaments are capped.

Sequestration does not affect the monomer steady-state value
β-Thymosins are the major ATP–G-actin sequestering proteins in vertebrates[150]. They are abundant (100–300 μM) in erythropoietic[151] and embryonic cells[152,153] and are present in lower amounts (2–20 μM) in most other cell types[154,155]. The addition of a β-thymosin to a filamentous actin (F-actin) solution causes filament depolymerization into non-polymerizable β-thymosin–actin, which reaches equilibrium with G-actin at its steady-state value. This can be described by the equation $[TA]_{SS} = [T]_{total}.[A]_{SS}/([A]_{SS} + K_T)$, in which $[TA]_{SS}$ is the steady-state concentration of β-thymosin–actin; $[T]_{total}$ is the total β-thymosin concentration; $[A]_{SS}$ is the steady-state G-actin concentration and K_T is the equilibrium dissociation constant for the binding of β-thymosin to G-actin. Consequently, more β-thymosin–actin is present when barbed ends are capped owing to the higher steady-state value of G-actin (see the figure). However, β-thymosin addition does not change the steady-state ATP–G-actin concentration[156] (although alternative views have been expressed[143]).

Profilin–actin is the major polymerizable monomeric actin species
Profilin is a major regulator of actin assembly that binds ATP–G-actin with high affinity. Profilin–actin exclusively feeds barbed-end growth[157], thus pointed ends only disassemble in the presence of profilin. As a result, profilin enhances treadmilling. Profilin–actin is energetically equivalent to G-actin in monomer–polymer exchanges, it greatly lowers the steady-state concentration of free G-actin and it represents the main form of polymerizable G-actin (see the figure). At high concentrations (>10 μM), profilin also competes with capping protein at barbed ends. Therefore, 90% of profilin would be bound to G-actin if 100% of barbed ends were capped (see the equation above), whereas a high concentration of profilin downregulates the level of capping and of profilin–actin (see the figure and the main text).

Filament stabilizers decrease monomer concentrations
Proteins such as myosin, tropomyosin and calponins, which bind more specifically to F-actin than to G-actin, stabilize filaments by shifting the monomer–polymer equilibrium towards a lower steady-state concentration of monomers that is associated with slower filament dynamics.



	F-actin	G-actin	β-Thymosin–actin
No capping	98 μM	0.1 μM	1.9 μM
100% capping	91.9 μM	0.6 μM	7.5 μM

Total actin concentration	Total thymosin β4 concentration
100 μM	20 μM

	F-actin	G-actin	β-Thymosin–actin	Profilin–actin
10 μM profilin				
No capping	99.8 μM	0.001 μM	0.020 μM	0.1 μM
100% capping	83.33 μM	0.6 μM	7.5 μM	8.57 μM
99% capping	94.6 μM	0.07 μM	1.31 μM	4 μM
50 μM profilin				
No capping	98.95 μM	0.002 μM	0.040 μM	0.1 μM
100% capping	49 μM	0.6 μM	7.5 μM	42.9 μM
99% capping	95.29 μM	0.01 μM	0.2 μM	4.5 μM

questioned, with severing alone proposed to account for the biological function of ADF[69]. However, recent single-filament experiments show that the rate of depolymerization is indeed enhanced in the presence of both ADF and its cofactor AIP1 (REF. 70). Further studies of the effect of ADF/cofilin on the treadmilling rate of single filaments should clarify the mechanisms that are responsible for the biological function of ADF.

In vivo, ADF has been shown to enhance treadmilling in dendritic arrays[47,48,71] by promoting an increase in the amount of monomers[48]. In this context, newly created barbed ends in a branched network grow transiently before being capped by heterodimeric capping protein. The majority of pointed ends are embedded in branch junctions and therefore cannot depolymerize until debranching or severing occurs (FIG. 2d). Debranching is accelerated by ADF/cofilin[72], by proteins of the coronin family[73] (see REF. 74 for a review), which bind to various ARP2/3 isoforms selectively[75], and by glia maturation factor (GMF)[76], a protein of the ADF homology family that enhances the dissociation of ADP–ARP2 from the daughter filament[77,78] (FIG. 2d). Together, these activities determine the number of filament barbed and pointed ends and their contributions to the steady-state concentration of polymerizable monomers.

Incidentally, an arrest of motility was reported at high ADF concentrations[47]. This result is consistent with a block in treadmilling caused by saturating concentrations of ADF owing to a shift from steady state to a true equilibrium between ADF–ADP–F-actin and ADF–ADP–G-actin[47], which may explain the freezing of actin in 'rods' (reviewed in REF. 79). In conclusion, the biochemical properties of ADF/cofilin account for its cellular function.

Competition at barbed ends

Apart from the regulators of filament depolymerization dynamics and their direct modulators, other regulators that target barbed ends also affect the

Table 1 | **Regulators of actin dynamics**

Protein	Interaction with actin	Main functions	Binding constant*	Competition with other factors	Rate constant of actin monomer association with barbed ends‡
β-Thymosins	ATP–G-actin	Sequestering	1 μM (REF. 150)	Profilin	NA
Profilin	ATP–G-actin	Forms profilin–actin complex that can bind to barbed ends	0.1 μM (REF. 94)	β-Thymosin	10 $\mu M^{-1}.s^{-1}$ (REF. 97)
	ADP–G-actin	Forms profilin–actin complex but does not bind to filaments	1 μM (REF. 156)	β-Thymosin	
	Barbed ends (ADP or ADP–P_i)	Destabilizes barbed ends	20 μM (REFS 94,96)	Barbed-end regulators	
ADF/cofilin	ADP–G-actin	Binds and assembles in filaments (critical concentration of ADF–ADP–G-actin assembly into ADF–ADP–F-actin filaments = 4.5 μM)	0.1 μM (REF. 47)	ND	NA
	ATP–G-actin	Binds poorly	2 μM (REF. 47)	ND	
	ADP–F-actin	Disassembly factor	NA	Tropomyosin, myosin	
Capping protein	Barbed ends	Blocks barbed ends	0.1 nM (REF. 92)	Formin, profilin, regulators with WH2 domains	NA
Formins (for example, mDIA1)	Barbed ends	Processive assembly	0.001 nM (REF. 89)	Profilin, capping protein, regulators with WH2 domains	50–100 $\mu M^{-1}.s^{-1}$ (REFS 65,129)
VASP or VopF (WH2 domain)	ATP–G-actin	Nucleates filaments	0.1 μM (VopF)[81]	Profilin	10 $\mu M^{-1}.s^{-1}$ (VopF)[81] to 30 $\mu M^{-1}.s^{-1}$ (VASP)[22]
	Barbed ends	Barbed-end tracking	10 nM (VASP)[22] to 20 nM (VopF)[81]	Profilin and barbed-end capping proteins	
Spire (WH2 domain)	ATP–G-actin	Nucleates filaments if at low molar ratio to actin and sequesters G-actin at a range of actin concentrations	0.1 μM (REF. 171)	Profilin	NA
	Barbed ends	Capping	3–5 nM (REF. 88)	Barbed-end capping proteins	
Tropomyosin	F-actin	Filament stabilizer	1 mM (REF. 172)	ADF	NA
Calponin	G-actin	ND	0.15 μM (REF. 173)	ND	NA
	F-actin	Filament stabilizer	0.05–2 μM (REF. 173)	ADF	
VCA of WASP (WH2 domain)	ATP–G-actin	VCA–actin assembles to barbed ends as for profilin–actin (profilin-like mechanism)	0.5–1 μM (REFS 174,175)	Profilin	ND
	Barbed ends	Capture and tracking	ND	Barbed-end regulators	

ADF, actin depolymerizing factor; F-actin, filamentous actin; G-actin, globular actin; mDIA1, mammalian diaphanous homologue 1; NA, not applicable; ND, not determined; P_i, inorganic phosphate; VASP, vasodilator-stimulated phosphoprotein; VCA, verprolin-homology, cofilin-homology and acidic domain; WASP, Wiskott–Aldrich syndrome protein; WH2 domain, Wiskott–Aldrich homology 2 domain. *The binding constant for the interaction of the indicated protein with G-actin or filament barbed ends. ‡The rate constant of association of a complex of the indicated protein and actin monomers with filament barbed ends.

steady-state amount of polymerizable monomers. It is now becoming clear that these barbed end-binding regulators simultaneously engage in a complex, competitive crosstalk at barbed ends. These regulators are discussed in detail below.

Competition and antagonism between capping proteins and polymerases. Various proteins such as formins[65], VASP[80] and VopF[81] act as polymerases at filament barbed ends. Formins track terminal subunits at barbed ends via a dimeric formin homology 1 (FH1)–FH2 domain as the filament grows for tens of minutes[21]. They enhance filament elongation rates by up to tenfold for mammalian diaphanous homologue 1 (mDIA1; also known as DIAPH1) alone[65], and even further when in synergy with cytoplasmic linker protein 170 (CLIP-170; also known as CLIP1)[82]. Surface cell antigen 2 (Sca2), a bacterial protein from pathogenic *Rickettsia* species, mimics formins functionally (if not structurally)[83] and uses profilin–actin to facilitate bacterial motility[13,84]. Proteins of the Ena/VASP family are slower polymerases that bind to barbed ends using a multimeric Wiskott–Aldrich homology 2 (WH2) domain structure[22,29,80,85]. The pathogen proteins VopF from *Vibrio cholerae*[81] and BimA from *Burkholderia mallei*[86] similarly track barbed ends via their WH2 domains (Supplementary information S1 (box)). By maintaining filament barbed ends in an active state of growth, these polymerases antagonize and prevent the binding of capping proteins. In other words, they impose a lower steady-state concentration of monomers than if all barbed ends are capped.

Many barbed end-binding proteins compete with each other, either directly or via a transient ternary complex comprising the two regulators bound to barbed ends simultaneously[65,81,87–89] (see REF. 90 for a review). The mutually reduced affinity of each ligand for the barbed end in the ternary complex enables faster dissociation of one protein by the other. Formins thus uncap barbed ends that are bound by capping protein by a mechanism that is different from that used by uncapping proteins ('uncappers') — such as capping

protein, ARP2/3 and myosin I linker protein (CARMIL; also known as LRRC16A) or FAM21 — which interact directly with capping protein (through their capping protein interaction (CPI) motifs) to form a complex that then acts as a low-affinity dynamic capper[91,92] (see REF. 93 for a review).

Profilin competes with capping protein and other barbed-end regulators. The ubiquitous actin monomer-binding protein profilin also binds to filament barbed ends, albeit with a lower affinity than for G-actin (binding constants of 20 μM and 0.1 μM, respectively). Profilin binding to barbed ends enhances the dissociation of actin from barbed ends; that is, it destabilizes the filament[94–96]. As a result, in solutions of pure actin, as the free profilin concentration increases from 1 μM to 100 μM, the profilin–actin concentration increases from 0.1 μM to 1.3 μM (REF. 97). This novel function of

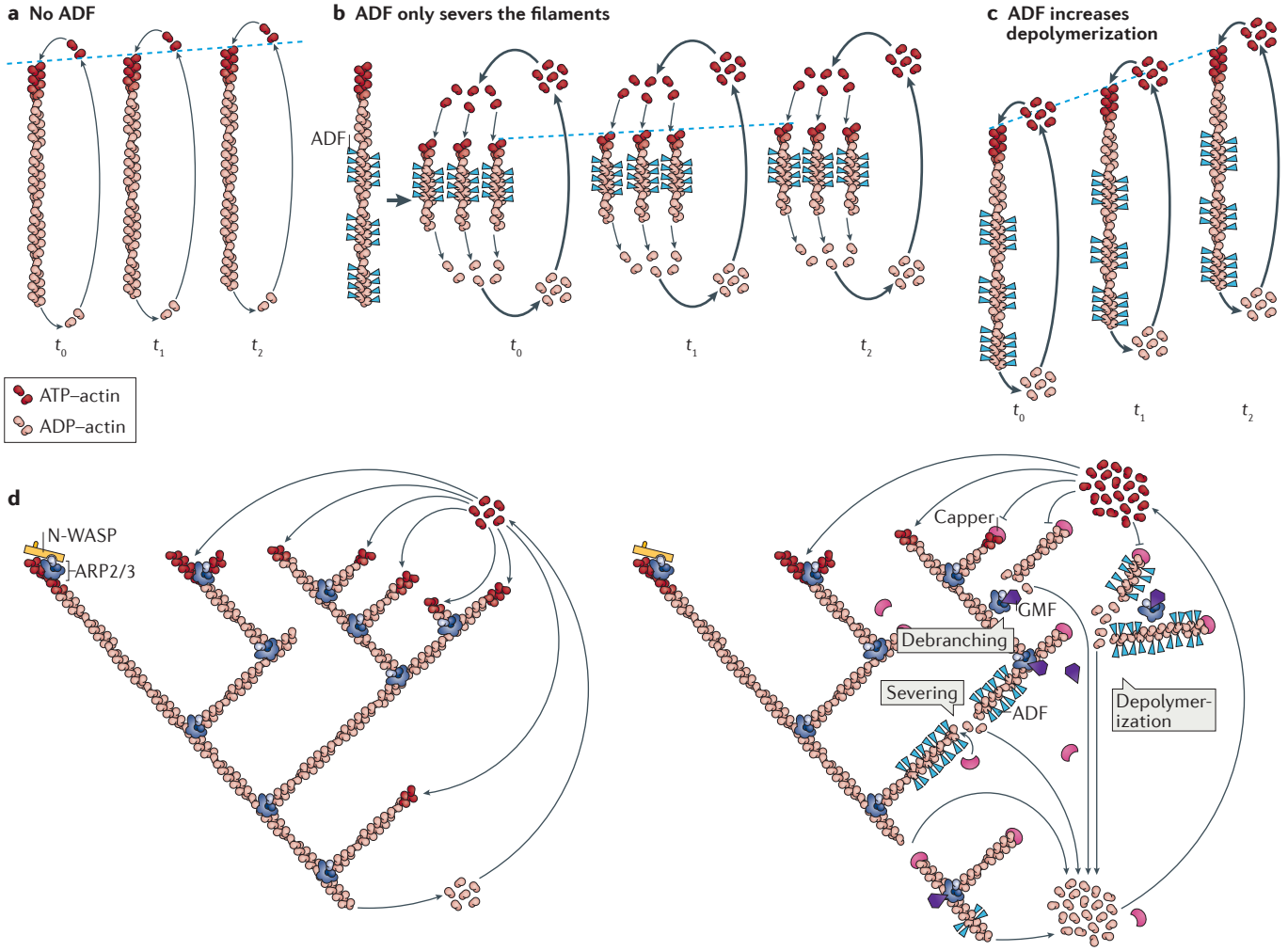


Figure 2 | **The role of ADF/cofilin in the regulation of treadmilling of actin filaments. a–c** | Potential effects of actin depolymerizing factor (ADF)/cofilin on the concentration of actin monomers and filament treadmilling. The treadmilling cycle in a population of filaments is represented by a time-lapse schematic of a representative (average) filament at steady state at three sequential time points: t_0, t_1 and t_2. The slope of the dashed blue line represents the treadmilling rate. In the absence of ADF (part **a**), treadmilling is slow owing to the rate-limiting slow pointed-end depolymerization. Severing of filaments by ADF following its binding to the sides of filaments (part **b**) promotes the same *n*-fold increase in the number of filament barbed and pointed ends. The *n*-fold higher flux of depolymerization is thus balanced by an equally increased polymerization flux. Hence, neither the rate of depolymerization per pointed end nor the rate of elongation per barbed end are affected by severing, thus the steady-state concentration of free globular actin (G-actin) remains unchanged and treadmilling is slow. ADF, by destabilizing actin–actin bonds in the filament, can also enhance the rate constant of pointed-end disassembly (part **c**). This leads to an increased flux of disassembling monomers and increases the steady-state concentration of free G-actin and filament treadmilling. **d** | Treadmilling in dendritic arrays. In a dendritic array comprising actin, neural Wiskott–Aldrich syndrome protein (N-WASP) and actin-related protein 2/3 (ARP2/3) only (left), most pointed ends are embedded in branch junctions, do not disassemble and therefore do not feed the actin monomer pool. A large number of barbed ends (9 barbed ends in the illustrated case) grow from monomers that are produced by a single slowly disassembling pointed end, causing extremely slow elongation rates of the individual free barbed ends. In the cellular context (right), a large fraction of barbed ends are capped and thus the remaining few free barbed ends can grow faster. In addition, capping proteins (cappers) cooperate with ADF and debranching factors from the coronin and glia maturation factor (GMF) family[170] to promote treadmilling: filament severing by ADF (severing) and filament debranching by coronin or GMF (debranching) followed by capping of the newly formed barbed ends increases the number of pointed ends that can disassemble; disassembly of pointed ends (depolymerization) can be further promoted by ADF. This disassembly increases the steady-state concentration of actin monomers and promotes the growth of uncapped barbed ends. The treadmilling rate of the dendritic array is therefore governed by the frequencies of branching, barbed-end capping and debranching, as well as by the severing and depolymerizing activities of ADF[150].

profilin has an important bearing on the control of the reactivity of filament barbed ends and of the steady-state concentration of polymerizable monomers.

It is generally understood that the majority of cellular profilin is bound to G-actin, based on measurements of free and actin-bound profilin eluted from size-exclusion chromatography of *Acanthamoeba castellanii* extracts[98]. This is indeed the case *in vitro* when pure F-actin filaments are fully capped (100% capping) (BOX 1). However, a high concentration of profilin was shown to compete with capping protein at barbed ends[97]. In light of this result, even a slight decrease in the fraction of capped barbed ends has important thermodynamic consequences (see BOX 1). Profilin will no longer cause disassembly of F-actin into profilin–actin as efficiently as it does when 100% of the barbed ends are capped (see BOX 1), and a profilin–actin concentration of only a few micromolar will coexist with capped and profilin-bound barbed ends. In other words, in the presence of capping protein, profilin limits the maximal concentration of profilin–actin that can coexist with filaments. In addition, at high profilin concentrations, a large fraction of total profilin remains unbound. The equilibrium of profilin–actin with unbound profilin and G-actin in turn imposes a concentration of free G-actin in the nanomolar range. Profilin–actin thus remains the major form of polymerizable monomeric actin.

In cells, the total concentration of capping protein is a few micromoles, but its effective concentration is reduced to 10–50 nM owing to sequestration by myotrophin (MTPN; also known as V1) (see REFS 93,99 for reviews), ensuring that 99.0–99.8% of filaments are capped instead of 99.99%, which facilitates downregulation of the extent of capping. Therefore, in physiological ranges of 10–100 μM profilin and 10–50 nM capping protein, the profilin–actin concentration is only a few micromolar. The observed rate of formin-based filament assembly *in vivo*[100] is consistent with this low concentration of profilin–actin. Correlated with this observation, a large fraction of profilin is unbound (free). Unfortunately, the fractions of free and actin-bound profilin are not yet known in most mammalian cells, and we expect future studies to uncover these.

The dependence of assembled F-actin on the ratio between profilin and either formin, VASP or VopF provided evidence for profilin competing with these barbed-end regulators[97]. At high concentrations, profilin destabilizes filaments; that is, it imposes a high steady-state concentration of profilin–actin ($[PA]_{SS}$). By contrast, assembly-promoting factors (such as formin, VASP and VopF) impose a low profilin–actin concentration; that is, a higher total amount of F-actin (FIG. 3a). The extent of displacement of barbed-end regulators by profilin depends on the affinity of the regulator for barbed ends (FIG. 3b; TABLE 1).

Competitive regulation of filament branching at barbed ends. The WASP family–ARP2/3 filament-branching machinery promotes and maintains dendritic actin arrays, such as those initiated and maintained in lamellipodia by the WASP family verprolin-homologous protein (WAVE)–ARP2/3 complex. Although the inhibition of lamellipodial actin arrays by high concentrations of profilin has been known for over two decades[101], the mechanism remains unclear. Recently, it has been demonstrated that filament branching is inhibited by profilin *in vitro*[102–104]. The inhibition was shown to require the ability of profilin to bind actin, and two different mechanisms for this inhibition have been proposed. First, profilin might compete with the WH2 domain of WASP[103] for binding to free actin monomers, a reaction that is essential for branching[105]. The second mechanism invokes the direct competition between profilin and the WH2 domain of WASP proteins for the barbed end of the mother filament, indicating that filament branching takes place at filament barbed ends. Filament branching has been reported to occur mainly from the side of a pre-existing filament[106,107], whereas a recent study showed that both side and end branching might be supported by the binding of the WASP WH2 domain to either terminal or core subunits of the filament[97]. Interaction of profilin with filament barbed ends would then competitively inhibit binding of the WASP–ARP2/3 complex to the mother filament barbed end, which is the first step in branching. In this model, at high profilin concentrations, only binding to the sides of filaments would occur. The fact that branching is also inhibited by the barbed-end trackers VopF and formin[97] and by the functional antagonism between capping proteins and WASP–ARP2/3 (REF. 108) lends support to this mechanism (see Supplementary information S2 (box) for a detailed discussion of the mechanisms of filament branching).

Balanced growth of networks

The various intracellular actin networks depicted in FIG. 1 coexist and turn over simultaneously in cells. Recent *in vivo* studies[102,109] have also shown that branched arrays and linear bundles cooperate with each other (see REF. 110 for a review). *In vivo* studies addressing the maintenance of actin homeostasis in yeast and mammalian cells[109,111] have revealed a balance between the activities of ARP2/3 and formins, the nucleators for branched and linear arrays, respectively.

A delicate balance exists between branched and linear networks. A powerful approach for studying the balance between branched and linear arrays is to externally perturb one network and observe the effect on the other. ARP2/3 depletion has been achieved by RNAi[112,113], genetically[5,7,113] and by subcellular sequestration[114–116]. These studies have consistently shown that formin-induced bundles are enhanced in the absence of ARP2/3, whereas the total amount of F-actin remains unchanged[102,113]. For example, chemical inhibition of ARP2/3 activity in nematode leukocytes causes the formation of formin-induced arcs of bundled filaments[117]. Abolishing the lamellipodial network by depletion of capping protein also promotes filopodia formation[118]. In the same way, ARP2/3 depletion results in morphological alterations in the lamellipodial network[119].

Similarly, formin inhibition by a small-molecule inhibitor (SMIFH2) did not affect the global level of cellular F-actin and increased actin assembly in ARP2/3-branched actin arrays, promoting lamellipodia formation and cell migration[102]. Depletion of the formin-like (FMNL) proteins reduces the rate of formation of lamellipodial protrusions, which contain both linear bundles and dendritic arrays, but does not fully abolish the dendritic meshwork[120] (Kage *et al.*, in the press). These results, presented schematically in FIG. 3, suggest that upon deletion of one assembly machinery, the F-actin redistributes into other structures and that these networks can be generated independently of each other. However, more extensive crosstalk between the various arrays that coexist in the cell surely exists. For instance, in fibroblastic formin-assembled structures, the filament treadmilling rate was reduced by depletion of ARP2/3 (REF. 102). Interplay between highly stable contractile fibres and other actin networks has also been observed. Inhibition of myosin II ATPase activity by blebbistatin revealed that the assembly of contractile actomyosin antagonizes the formation of

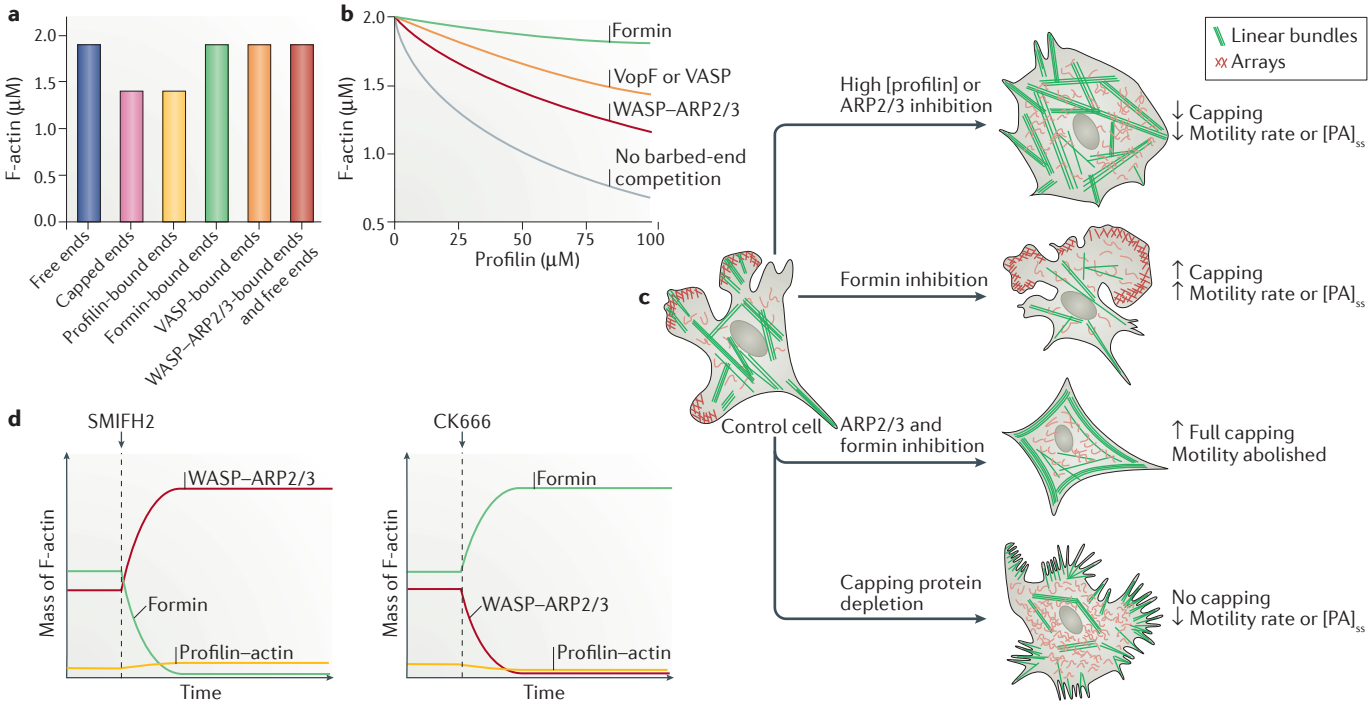


Figure 3 | **A global treadmilling model accounts for the functional balance between various actin networks in motile processes. a** | The energetics of assembly at filament barbed ends is controlled by competition between barbed-end regulators. The graph shows the measured *in vitro* concentration of filamentous actin (F-actin) (2 μM total actin) for various functional states of barbed ends: free, capped, profilin-bound, formin-bound, vasodilator-associated phosphoprotein (VASP)-bound and Wiskott–Aldrich syndrome protein–actin-related protein 2/3 (WASP–ARP2/3)-bound and free ends. Note that in the latter three states, barbed ends are bound to catalysts that affect the rate of assembly. **b** | Competition between profilin and barbed end-bound assembly factors. The destabilization of filament barbed ends by profilin is monitored by the decrease in F-actin (and concomitant increase in profilin–actin). How the F-actin concentration changes in the presence of increasing profilin concentrations is affected by the binding of F-actin assembly factors to barbed ends (in particular, it depends on the affinity with which the given assembly factor binds to barbed ends). In other words, profilin destabilizes filaments more easily when it outcompetes lower affinity barbed-end trackers. **c** | Schematic representation of actin arrays that are established following perturbation of actin regulators. These perturbations lead to changes in the steady-state concentration of polymerizable profilin–actin monomers ($[PA]_{SS}$) as well as the distribution of actin between different arrays and their organization, which as a result affect cell migration. Microinjection of a large amount of profilin inhibits ARP2/3 branched filaments and partially displaces capping protein from barbed ends; inhibition or depletion of ARP2/3 abolishes the formation of dendritic filaments. In both cases, formation of linear bundles is enhanced at the expense of dendritic arrays and migration is impaired. Inhibition of formins by the small-molecule inhibitor SMIFH2 increases lamellipodial ARP2/3 branched actin arrays, which promotes migration as well as causes the formation of actin bundles by other polymerases. Simultaneous inhibition of both ARP2/3 and formin causes an increase in capped cytoplasmic filaments as well as the formation of actin bundles by other polymerases. This treatment effectively abolishes migration. Depletion of capping protein abolishes lamellipodia and stabilizes filopodia and cytoplasmic bundles. Most actin is then cytoplasmic and consequently motility is severely impaired. **d** | Graphs schematically showing the relaxation process of actin upon either formin inhibition by treatment with SMIFH2 (left) or ARP2/3 inhibition by treatment with the inhibitor CK666 (right). Note that a large shift in F-actin mass from one array to another array occurs together with a minor change in the concentration of profilin–actin.

lamellipodial actin in epithelial cells[27] and stabilizes filopodial actin bundles in neuronal growth cones[121].

In conclusion, diverse experimental perturbations of any one of the cellular actin networks promote the redistribution of actin into other networks. The mechanisms driving this redistribution and the favouring of one network over others have only recently begun to be understood. A key molecular player has been the ubiquitous actin-binding protein profilin.

Profilin controls the balance between linear and branched networks. A number of *in vivo* studies have suggested that profilin orchestrates the balance between branched and unbranched filaments. Increasing cellular profilin by microinjection promoted the disappearance of the lamellipodial network and increased the formation of filopodial as well as contractile F-actin bundles[101,102]. Conversely, profilin depletion promoted an increase in the lamellipodial array (and a higher branching density)[102]. This result might also explain the inverse correlation between the expression level of profilin 1 and the metastatic potential of human breast cancer cells[122–125], which relies on dendritic network activity. In plant cells, similar versatile effects in response to varying the levels of vegetative profilin isoforms (in this case on plant size) have been reported[126–128].

It was thought that the versatile effects of profilin result from the fact that profilin–actin is the sole form of monomeric actin that supports rapid processive filament assembly in linear arrays by formins[65,129], whereas assembly of dendritic arrays does not require profilin and would be better supported by free G-actin. According to this hypothesis, two pools of actin monomers, G-actin and profilin–actin, would be used to assemble dendritic and linear filaments, respectively[103,109]. However, this explanation fails to account for the concentration-dependent effects of profilin on motility; at low concentrations (0–5 μM), profilin enhances the rate of actin-based propulsion of *Listeria monocytogenes* in cell extracts[130]

as well as in reconstituted motility assays[39], but at higher concentrations (>10 μM) it slows down motility[39,97], which is in line with the lamellipodial inhibition by excess profilin in cells[101,102].

The above data can be explained by the fact that profilin binds to G-actin with high affinity and to filament barbed ends with lower affinity[97]. The low affinity of profilin for filament ends might also explain why regulators such as the formins, which bind barbed ends with high affinity, are less easily displaced by profilin than those that bind with lower affinity, such as WH2-domain proteins (FIG. 3b; TABLE 1). Moreover, the inhibition of filament branching *in vivo* at high profilin concentrations is remarkably similar to *in vitro* results obtained when free profilin is present in controlled excess over profilin–actin[97]. This similarity suggests that the added excess of cellular profilin remains as free profilin, whereas, in the conventional view that filaments are strongly capped *in vivo*, it should fully convert into profilin–actin by depolymerizing filaments. Finally, silencing profilin expression in epithelial cells promotes a 29% decrease in F-actin[131]. This result would be expected only if large amounts of free profilin are present in those cells and compete with capping protein. In this case, deletion of profilin would restore a higher level of barbed-end capping. At the same time, more actin would be sequestered by β-thymosin and, consequently, much less F-actin would be assembled (see BOX 1).

Mechanisms of actin homeostasis

We have so far focused on the molecular mechanisms that control the assembly and the size of individual actin networks. In this section, we address the key question of how cells control the distribution of actin between these coexisting actin networks and coordinate their turnover. In particular, it is important to understand how changes in the concentration of polymerizable monomeric actin (profilin–actin) are coordinated with changes in the distribution of filaments among various functional states in live cells. An answer to this question is currently unavailable. However, we propose that the concept of actin treadmilling, which has thus far been used to describe the dynamics of single filaments and individual motile processes (for example, array treadmilling)[34,35], can be further extended to the whole cell. By incorporating the aforementioned insights into barbed-end regulation, we demonstrate that 'global treadmilling' can establish the balance between actin networks and that it helps explain how actin homeostasis occurs at the scale of a whole cell.

Global treadmilling predicts actin network coordination. Actin filament dynamics are controlled by the competitive binding of capping proteins, destabilizers and assembly catalysers. Filament assembly is supported by profilin–actin produced in a sustained fashion by monomer disassembly from filament pointed ends. In such a global treadmilling cycle, the average rate of filament disassembly V_{depol} and the average filament barbed-end assembly rate V_{pol} balance each other. The solution of the equation $V_{pol} = V_{depol}$ defines the steady-state concentration of profilin–actin, $[PA]_{SS}$. In this global treadmilling cycle, V_{pol} and V_{depol} can be expressed as follows:

$$V_{pol} = \Sigma k_{+i}[B_i].([PA]_{SS} - C_C^{B})$$
$$V_{depol} = \Sigma k_{-i}[P_i]$$

in which C_C^{B} is the critical concentration for barbed-end assembly of polymerizable actin monomers (that is, profilin–actin). $[B_i]$ is the concentration of filament barbed ends in state i (where i can refer to free, capped, dynamically capped, formin-bound, VASP-bound, WASP–ARP2/3-bound or profilin-bound); $[P_i]$ represents the concentration of filament pointed ends (the subscript i refers to the biochemical nature of pointed ends: free, ADF-bound or tropomyosin-bound) that disassemble at rate k_{-i}. $[P_i]$ depends on debranching factors. The values of k_{+i} are the intrinsic rate constants for association of profilin–actin to a barbed end in state i. For example, this value is low ($10\ \mu M^{-1}.s^{-1}$) for free barbed ends, is typically high ($100\ \mu M^{-1}.s^{-1}$) for formin-bound barbed ends and is zero for capped barbed ends.

Signalling pathways locally control the number and state of barbed ends by activating specific barbed-end regulators (FIG. 1). The energetics of each array contribute to the established value of $[PA]_{SS}$. We assume that the total amount of actin is constant, which is consistent with *in vivo* data[102]. In cells, barbed ends exist predominantly in a capped form[34] (FIG. 1b). Nevertheless, the fraction of filaments in the capped state is a crucial parameter in global treadmilling and can be regulated by sequestration of capping proteins, by uncapping[90–93], by the extent of competition between the cappers, by the signal-controlled stimulators of barbed-end assembly (polymerases) and by filament stabilizers as well as destabilizers.

The global treadmilling framework also makes predictions about the cellular response to perturbations of the actin cytoskeleton. Abrogation of the assembly of a given type of network translates into its disassembly, which is accomplished by the redistribution of actin into other networks and, as a consequence, the establishment of a new steady-state concentration of profilin–actin. The redistribution follows a general relaxation process (that is, the return of a system to equilibrium following a perturbation) (REF. 132 and references therein), similar to the shift in equilibrium of a chemical reaction driven by a change in pressure or temperature (in cellular terms, this change is instigated by signalling). The change in concentration of profilin–actin to the new steady-state value during the relaxation process, although quite small, is linked to a massive exchange of F-actin between different networks (FIG. 3c,d).

Assembly and maintenance of dendritic arrays is typically coupled to a higher level of capping — implying a slightly higher value of $[PA]_{SS}$ — than for the assembly of formin-induced linear bundles[133] (BOX 1). Consequently, in the global treadmilling model, abrogation of the dendritic actin meshwork will lead to a decrease in $[PA]_{SS}$ in the system, resulting in a slower filament assembly rate. This has in fact been observed *in vivo*[102]. Conversely, abrogation of highly stable filaments (for example, actomyosin bundles, which contribute to lowering the $[PA]_{SS}$ (see BOX 1)) promotes relaxation towards a higher level of $[PA]_{SS}$, which can then feed less stable networks, and an increased treadmilling rate is expected to result from the higher level of $[PA]_{SS}$, promoting motility. Thus, the value of $[PA]_{SS}$ established in each state lies at the heart of the treadmilling cycle through which the respective activities of coexisting branched filaments, linear bundles, capped filaments and contractile actomyosin bundles are coordinated.

In a hypothetical situation in which ARP2/3, VASP and formins are all inhibited simultaneously, the model predicts that cellular actin would then assemble into cytosolic, disorganized capped filaments. In conclusion, we propose that the concept of treadmilling of actin is central and sufficient to account for the coordinated balance between cellular actin arrays. In this model, the various actin networks behave as communicating vessels. Their relative filament number and F-actin mass are controlled by signalling, which is translated into specific values of $[PA]_{SS}$.

Experimental evidence in support of the global treadmilling model. The global treadmilling model accounts well for the phenotypes observed when the actin cytoskeleton is perturbed in living cells (represented schematically in FIG. 3c). As described earlier, inhibition of the assembly of dendritic structures results in actin redistribution to all of the remaining networks with a turnover rate that depends on the change in the concentration of profilin–actin induced by the perturbation.

Inhibition of dendritic network assembly has been shown to enhance formin-initiated bundles in systems in which the activity of formins dominates over other actin-assembly activities[102,134]. However, in cases in which other actin networks (such as cytosolic capped filaments, adhesion-linked actin bundles, stress fibres or profilin-destabilized filaments) predominate, a simple increase in linear bundles following inhibition of ARP2/3 is not observed[135]. Similarly, consistent with the global treadmilling model, disruption of the highly stable actomyosin contractile structures (BOX 1) by blebbistatin results in the formation of less stable networks, including the formation of lamellipodia in epithelial cells, which normally do not form these structures[27], or filopodial bundles in the growth cones of neurons[121]. Importantly, here we assume that blebbistatin contributes to the observed phenotypes owing mainly to its ability to destabilize filaments by promoting the dissociation of myosin rather than by abolishing actomyosin contractility. If contractility is abolished while maintaining the myosin that is bound to and constitutively stabilizes F-actin bundles (such as following treatment with the myosin ATPase activity inhibitor butanedione monoxime), it is expected that the low concentration of profilin–actin will be maintained (BOX 1) and the formation of lamellipodia or filopodia will not be promoted. In support of this view, the formation of highly stable contractile stress fibres is associated with the disassembly of the less stable non-contractile fibres (which require a higher concentration of profilin–actin to be maintained)[136].

Finally, ectopic formation of branched filaments in the cytoplasm induced by overexpression of the constitutively active carboxy-terminal region of WASP proteins, the verprolin-homology, cofilin-homology and acidic domain (VCA) region, results in a large decrease of the lamellipodial network[6,133,137–140]. Furthermore, adding large amounts of formins or VCA–ARP2/3 to a reconstituted motility assay arrests actin-based propulsion of neural WASP (N-WASP)-functionalized beads[141]. These observations support the global treadmilling model as follows: by inducing the ectopic formation of actin arrays that lead to actively growing barbed ends, VCA–ARP2/3 imposes a low steady-state amount of profilin–actin, lower than that imposed by capping proteins. The concentration of polymerizable actin monomers may then be too low to sustain site-directed growth of new barbed ends that supports actin-based motile processes. A similar effect is expected for overexpression of constitutively active formin or VASP proteins. In line with this prediction, overexpression of the formin BNI1-related protein 1 (Bnr1) is lethal in yeast owing to the emergence of toxic, disorganized cable-like filaments[6], and overexpression of Ena/VASP inhibits the motility of fibroblasts[121]. On the contrary, in one report, overexpression of a construct comprising the FH1 and FH2 domains of FMNL3 resulted in filopodial extension[142]. This result could be explained by an inability of this truncated formin to sufficiently nucleate the excess of cytosolic filaments.

Alternative models of actin homeostasis. Previous models of actin network homeostasis rely on the assumption that a large exhaustible pool of polymerizable actin monomers (free G-actin and profilin–actin) exists in cells[103,109] (see REF. 143 for a review). This pool is expected to be consumed upon actin assembly into competing dendritic or linear arrays. The idea of an exhaustible pool of polymerizable actin monomers presents an attractive and intuitively simple interpretation of the apparently mutually exclusive assembly of linear and dendritic networks in cells. It differs from the view that actin is assembled at a regulated steady state in cells. The concept that filament treadmilling is important for regulating polarized filament assembly has not been considered in this alternative model. Instead, large changes in F-actin levels take place during assembly from the pool of monomers, presumably up to a thus far undefined, dynamic steady state. In addition, the maintenance of the G-actin pool by thymosin β4 and profilin (BOX 1) is at odds with this model. It has instead been assumed that the role of profilin is to specifically support the formin-mediated assembly of linear actin bundles and to inhibit filament branching by competing with the WH2 domain of WASP for binding to G-actin, whereas the binding of profilin to barbed ends has not been considered.

The idea of global treadmilling described in this Opinion article presents an independent and different conceptual approach to quantitatively account for thermodynamic and kinetic aspects of the distribution of actin in various networks and the coordinated filament turnover between these networks. In contrast to the above alternative model, here, the regulation of the level of barbed-end capping in various cellular actin arrays and the competition between regulators (including profilin) at filament barbed ends are the key factors that govern actin homeostasis and establish the concentration of profilin–actin that is characteristic of each cellular state. In this model, profilin–actin, not G-actin, is the main form of polymerizable actin monomers; the profilin–actin concentration relaxes between various steady states within a small (micromolar) concentration range, and this correlates with changes in the distribution and the state of filaments, which are governed by signalling cues. To identify which of these models better describes the mechanisms of actin homeostasis that operate *in vivo*, a greater understanding of actin species concentrations and their dynamics as well as further insights into the regulation of filament barbed end reactivity, in particular by WH2 domains[90] (Supplementary information S1,S2 (boxes)), are needed.

Conclusions and perspectives

To gain further insights into the homeostatic regulation of actin arrays, accurate *in vivo* measurements of the cellular concentration of profilin–actin and free profilin, the total filament number and the F-actin mass in various states are needed. These measurements would allow the use of mathematical modelling to reconstitute *in silico* the organization of the actin cytoskeleton into the various arrays generated upon the activation of defined signalling pathways or changes associated with different perturbations to the actin cytoskeleton[144]. However, so far the progress towards this goal has been limited.

The need to quantify the amount of G-actin *in vivo* is widely appreciated. Attempts to achieve this goal have been made in both migrating as well as non-migrating cells (REFS 145–147 and references therein). However, quantification of all forms of monomeric and assembled actin has so far proven to be technically difficult. To start with, fluorescent F-actin probes such as tagged LifeAct or utrophin may not bind identically to various F-actin structures, preventing accurate quantitative

measurement of F-actin mass. In addition, LifeAct has been shown to alter filament nucleation, elongation and ADF-induced severing[148]. In live-cell imaging, fluorescently labelled actin may not partition identically to the various F-actin arrays, free G-actin and actin in complex with regulatory proteins. Similarly, although fluorescence and electron microscopy have revealed the location of filament barbed ends, both have failed to provide a precise count of these owing to limited resolution. An additional limitation in our understanding of actin arrays *in vivo* stems from the fact that highly structured actin arrays such as lamellipodial and filopodial networks or stress fibres are more clearly visible than cytoplasmic, less organized fine arrays or shorter filaments, which end up being underestimated. Likewise, fluorescent phalloidin staining reveals all cellular F-actin, but short, rapidly depolymerizing filaments may be lost in the cell permeabilization step. Finally, the highly motile fraction of cellular actin may contain, in addition to G-actin, β-thymosin–actin, profilin–actin and very short, unstable and rapidly depolymerizing filaments. In addition to these limitations, an evaluation of the free G-actin, profilin–actin, free profilin and total β-thymosins in cells would need to be performed in a variety of cell types to fully understand actin homeostasis *in vivo*. Advances in live fluorescence imaging of actin dynamics in various networks, single-molecule tracking and Förster resonance energy transfer *in vivo*[149], and super-resolution microscopy make this achievement possible in the near future. Novel motility assays that combine controlled contributions of various barbed-end regulators will also prove instrumental.

Apart from quantifying the *in vivo* concentrations of the various actin forms, progress in biochemical analysis of the interplay of regulators at filament barbed ends is needed, and this calls for a deeper structural analysis of the complexes formed by these regulators with terminal actin subunits (see Supplementary information S1,S2 (boxes)). Finally, we believe that crosstalk between the signalling pathways that activate distinct actin networks may further control their coordinated dynamics and deserves further investigation in the future.

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Competing interests statement

The authors declare no competing interests.

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