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Functions of cofilin in cell locomotion and invasion

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Abstract

Recently, a consensus has emerged that cofilin severing activity can generate free actin filament ends that are accessible for F-actin polymerization and depolymerization without changing the rate of G-actin association and dissociation at either filament end. The structural basis of actin filament severing by cofilin is now better understood. These results have been integrated with recently discovered mechanisms for cofilin activation in migrating cells, which led to new models for cofilin function that provide insights into how cofilin regulation determines the temporal and spatial control of cell behaviour.

Most eukaryotic cells migrate by binding to the extra-cellular matrix using a motility cycle in which actin polymerization supplies the pushing force for protrusion of the leading edge to establish the direction of migration. During classic amoeboid cell migration, the motility cycle includes actin polymerization-driven formation of a protrusion in the new direction of migration, attachment to the substratum, generation of traction force and retraction of the tail. These events are dependent on actomyosin-mediated contractile force production and the actin cytoskeleton, which is dynamic and undergoes repeated cycles of actin polymerization and depolymerization in a spatially and temporally coordinated pattern (FIG. 1). Furthermore, the cytoskeleton has an essential role in establishing the internal cell architecture that controls cell migration. Other forms of movement such as blebbing[1] and rolling[2] have been described, but the importance of actin polymerization in these rare forms of motility is not well understood, and the involvement of cofilin in these types of cell motility has not been extensively studied. Therefore, our Review discusses the role of cofilin in cell locomotion and invasion in which actin polymerization is the pushing force for protrusion.

The actin-binding proteins involved in each of the steps of the motility cycle include cofilin, integrin–talin complexes, the actin-related protein 2/3 (ARP2/3) complex, formins, profilin, capping protein and myosin, among others (reviewed in REFS 3–7). Cofilin functions in the first step to initiate asymmetric actin polymerization and in later steps to recycle actin filaments. The involvement of cofilin in controlling the temporal and spatial extent of actin dynamics is seen in processes as diverse as tumour metastasis in mice[8], cytokinesis in

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SUPPLEMENTARY INFORMATION See online article: S1 (box)

yeast[9], morphogenesis in flies[10], neuronal plasticity in rats[11], as well as during inflammation[12]. The ADF/cofilin protein family consists of ADF (actin depolymerizing factor), cofilin 1 and cofilin 2. Cofilin 1 (hereafter referred to as cofilin) is the most abundant and ubiquitous member of the family in vertebrate non-muscle tissues and the only one required for viability[13]. The development of new technologies and their application to measure cofilin activity *in vivo* (BOX 1; Supplementary information S1 (box)) has provided new insights into its regulation and challenged previous models of cofilin function in cell migration.

Recent reviews have highlighted the biochemistry and cell biology of the ADF/cofilin protein family in cell migration, chemotaxis and cancer[8,13,14]. Here, we focus on recent results describing mechanisms of cofilin activation at different subcellular locations that led to new models for cofilin function in migrating cells.

Functions of cofilin

Microscopy studies revealing the interaction of F-actin with cofilin have shown that cofilin severs actin filaments but does not enhance depolymerization from the pointed end of the filament[9,15–17]. Conclusions from early biochemical experiments that cofilin increases the dissociation rate of G-actin from the pointed end of the filament[18,19] were incorrect, because they were not taking into account the number of filament ends created by cofilin severing[9,16]. Submicromolar concentrations of cofilin produce free barbed ends by severing existing filaments, and these new ends can be used to nucleate actin polymerization[20]. In addition, at micromolar concentrations, cofilin nucleates actin polymerization directly[15]. By creating new actin filaments through severing, cofilin supports ARP2/3 complex-mediated actin branching, as branches nucleated by the ARP2/3 complex are tenfold more stable on such recently polymerized filaments than on older filaments[20,21]. Cofilin also dissociates ARP2/3 complex-produced branches from older actin filaments[22]. Moreover, cofilin is involved in supporting contractility at the cell rear through local F-actin depolymerization[23] and in regulating actomyosin assembly by inhibiting binding of myosin II to F-actin[24].

Thus, cofilin has a central role in controlling actin dynamics, by catalysing actin polymerization and actin depolymerization through its severing activity, as well as by inducing dendritic nucleation and debranching.

Structural understanding of cofilin function

Several recent studies have advanced our understanding of the structural basis of cofilin-mediated severing — a process by which non-covalent bonds between actin molecules in F-actin are broken (FIG. 2). Cofilin increases the torsional dynamics of F-actin, and this can be propagated to regions of the filament that are not bound to cofilin[25,26]. A key finding is that cofilin severs F-actin at the junction between undecorated and cofilin-decorated regions of F-actin[27]. This observation, combined with a high-resolution structure of the cofilin-decorated actin filament[25,28], revealed that cofilin binding to F-actin disrupts the longitudinal interface in the filament (double-stranded actin filaments are defined by two interfaces: longitudinal and diagonal) while forming a ‘bridge’ to maintain the connection between interfaces. However, the cofilin-induced change in one of the four subdomains of G-actin, subdomain 2, disorders it and increases the flexibility of the F-actin filament. These changes in subdomain 2 cooperatively propagate into the undecorated filament regions[29]. The absence of cofilin in these regions makes the filament unstable due to the absence of the cofilin-mediated longitudinal stabilization, resulting in filament severing[25]. However, the discovery in protozoan (for example, *Toxoplasma gondii*) of cofilin family members that sever F-actin to about the same degree as their mammalian homologues with no detectable stable F-actin binding suggests additional mechanisms for severing, possibly involving

increased sequestration of G-actin monomers[30]. Further studies will be required to investigate whether there is a common severing mechanism in protozoa and higher organisms.

Polymerization or depolymerization of F-actin?

A central question is what determines whether polymerization or depolymerization results from cofilin-mediated severing of the actin filament. Several studies have shown, both *in vitro* and *in vivo*, that the relative concentration of G-actin is a key determinant. Because dissociation of both ADP•G-actin and ATP•G-actin at the pointed end is slow, and the association rate for ATP•G-actin at the barbed end is 15–40 times higher, net polymerization occurs at sites of actin filament severing at physio logical ATP•G-actin concentrations[31]. Cyclase-associated protein (CAP) can accelerate the exchange of cofilin-associated ADP•G-actin with ATP•G-actin to help ensure the local supply of polymerization-competent G-actin and to support cofilin-induced actin polymerization[32]. However, ATP•G-actin concentrations in migrating cells are thought to be mostly maintained by profilin, which catalyses the exchange of nucleotides on G-actin and sustains free ATP•G-actin at a concentration of about 1 μM *in vivo*[33]. Recently, CAP has been shown to accelerate cofilin-dependent actin filament severing at a neutral pH[34], providing a mechanism for increased actin filament dynamics without changes in intracellular pH. Under conditions of low G-actin concentrations (as, hypothetically, could occur in cells that undergo prolonged and continuous cell migration), severing by cofilin would cause net actin depolymerization. However, G-actin concentrations that are low enough to achieve net depolymerization are not found in the most commonly studied migrating cells, such as fibroblasts and epithelial cells, which suggests that net polymerization is the initial result from cofilin severing *in vivo*[35]. In addition, cofilin increases the concentration of G-actin by promoting actin depolymerization and thus contributes to the maintenance of physiological G-actin levels[36,37].

Although the relative concentrations of active cofilin and ATP•G-actin help to determine the relative balance of actin polymerization and depolymerization that is likely to occur *in vivo* upon local cofilin activation, this balance can also be regulated by additional actin-modulating proteins such as AIP1 (actin interacting protein 1), MENA, RHOC, CAP, coronin and gelsolin (reviewed in REFS 13,14,38). AIP1 can tip the balance from polymerization towards net depolymerization[39,40]. In *Caenorhabditis elegans*, AIP-1 has been shown to potentiate cofilin severing[41], but AIP 1 also favours net cofilin-induced actin filament disassembly by capping the barbed ends of severed filaments to prevent their elongation and reannealing in both *Xenopus laevis* and *C. elegans*[42,43]. However, ENA/VASP (enabled/vasodilator-stimulated phosphoprotein) proteins such as MENA, which have anticapping activity, have been reported to maintain free barbed ends that have been generated by cofilin at the leading edge of cell protrusions[44]. RHOC has been shown to regulate cofilin phosphorylation through ROCK (RHO-associated protein kinase) and LIMK (LIM-domain kinase) to determine the location and amount of cofilin activity and actin polymerization at invadopodium and lamellipodium compartments[45,46]. Gelsolin has actin filament severing and barbed end capping activity and can cause net depolymerization. Coronin can regulate cofilin activity in two ways: preventing its binding to new actin filaments and enhancing its binding to old ADP•G-actin filaments.

In addition to the functions of cofilin in actin remodelling, a role for cofilin in the regulation of phopholipase D1 (PLD1; which catalyses the hydrolysis of phosphatidilcholine to choline and phosphatidic acid) has been proposed[47].

Beyond these recent insights into the functions of cofilin, progress has been made in our understanding of cofilin cellular localization and how it is locally activated in migrating cells.

The localization of cofilin

Cofilin is a small protein of 19 kDa that freely diffuses within cells and can be found in multiple cellular compartments, including the cytoplasm and nucleoplasm. Live-cell imaging of GFP–cofilin expressed in unstimulated carcinoma cells[48,49] and HeLa cells[50] suggests that cofilin is uniformly distributed. However, immunofluorescence microscopy of fixed carcinoma cells revealed that the two major forms of cofilin — cofilin and cofilin that is phosphorylated on Ser3 — are differentially distributed in the cell[51]. Ser3 is the major phosphorylation site involved in the regulation of cofilin, as phosphorylation at that residue inhibits all cofilin–actin interactions, including binding to G-actin, and it inhibits severing of F-actin. Phosphorylation of other cofilin residues also regulates its activity. For example, protein kinase Cα (PKCα)-dependent phosphorylation at Ser23 and Ser24 terminates histamine degranulation[52], and phosphorylation at Tyr68 by constitutively active SRC can promote the ubiquitin-mediated degradation of cofilin[53].

Antibodies specific to total cofilin and cofilin phosphorylated at Ser3, which allows the analysis of dephosphorylated cofilin[51], revealed that these cofilin forms show differences in their cellular distribution. Non-phosphorylated cofilin is found in locomotory and invasive protrusions such as lamellipodia in motile epithelial cells and invadopodia in motile carcinoma cells, whereas phosphorylated cofilin is more uniformly distributed throughout the cytoplasm, except at the leading edge[46,51,54]. In general, cofilin is active at the tip of the leading edge protrusion of migrating cells and inactivated by Ser3 phosphorylation 1 μm behind the leading edge[50]. These results are in line with the findings that inhibition of cofilin activity causes defects in protrusion, cell polarity and chemotaxis, the latter of which is dependent on directional protrusion formation[46,54–56].

Cofilin that is not phosphorylated at Ser3 is found in regions of the cell where it is involved in rapid, reversible binding interactions (for example, on the order of seconds for F-actin[57] and milliseconds for G-actin[58]) that are associated with the cofilin activity cycle. These involve interactions with F-actin, phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) at the plasma membrane, cortactin in invadopodia and diffusible G-actin heterodimers in the cytoplasm[59]. The binding of cofilin to PtdIns(4,5)P_2, cortactin and G-actin inhibits cofilin severing activity, and the binding of cofilin to F-actin is limited to tropomyosin-free filaments near the plasma membrane[60]. Overall, these binding interactions not only serve to regulate cofilin activity but also contribute to the positioning of cofilin once it is activated.

Cofilin is also found in the nucleus[61,62], where it can form stable heteropolymers with F-actin. However, this property is not thought to be involved in rapid actin dynamics or the regulation of cofilin activity during cell motility[13]. To fully understand how the location and timing of cofilin activation are influenced by these interactions, we must understand how cofilin is activated.

The mechanisms of cofilin activation

There are three important mechanisms that regulate the activation of cofilin: its dephosphorylation at Ser3; its release from PtdIns(4,5)P_2; and its release from cortactin (FIGS 3–5). These mechanisms have been studied in detail, and their relative effects on cofilin activity and the resulting behaviour of motile cells have been revealed. However, these activation mechanisms are not mutually exclusive, and how they are coordinated in motile cells is the subject of intense investigation.

Cofilin Ser3 dephosphorylation

Dephosphorylation of cofilin Ser3 was the first activation mechanism to be well characterized[63,64] (FIG. 3). Unphosphorylated cofilin, but not cofilin phosphorylated at Ser3, can bind actin and promote its polymerization and depolymerization *in vitro*[65]. In addition, S3D-cofilin (a phosphorylation mimic in which Ser3 was replaced with Asp) fails to bind to and sever F-actin, whereas S3A-cofilin (a mutant in which Ser3 was replaced with Ala) does[64,65]. Slingshot (SSH) was shown to be a major phosphatase responsible for dephosphorylating cofilin at Ser3 (REF. 66), and chronophin (CIN)[67] was recently identified as a cofilin-specific phosphatase. Although the Ser phosphatases PP1 and PP2A[68] can also dephosphorylate cofilin at Ser3, these phosphatases have broader substrate specificity than SSH or CIN.

Several recent studies have provided insights into the regulation of SSH1 activation, localization and scaffolding[69]. For example, coronin 1B was found to interact with SSH1 and target it to lamellipodia, thereby increasing cofilin activity at lamellipodia. A direct interaction may also take place between coronin 1B and cofilin, but further studies are needed to confirm this[70]. In neuregulin-stimulated breast carcinoma cells, the 14-3-3 family of regulatory proteins has been shown to inhibit F-actin-mediated activation of SSH1 through Ser978 dephosphorylation, which prevented the accumulation of SSH1 at the lamellipodium, thereby increasing the pool of inactive, phosphorylated cofilin[71]. Recently, protein kinase D (PKD) proteins[72,73] were shown to phosphorylate SSH1 at Ser978, which leads to 14-3-3 recruitment and PAK4 (p21-activated kinase 4) activation[73] and thus LIMK1 activation[74]. This suggests that PKD proteins can inhibit cofilin dephosphorylation by inactivating SSH1 and activating LIMK 1. Calcineurin has also been shown to trigger cofilin dephosphorylation through the activation of SSH1 (REF. 75), whereas Ca^{2+}/calmodulindependent protein kinase II (CaMKII) negatively regulates SSH1 (REF. 76), which suggests that CaMKII and calcineurin act as a switch controlling Ca^{2+}-dependent cofilin activation. Moreover, β-arrestin scaffolding has been shown to promote cofilin dephosphorylation following protease-activated receptor 2 (PAR2) stimulation, which activates CIN and antagonizes LIMK1 (REF. 77). These pathways are summarized in FIG. 3.

The rapid dephosphorylation of cofilin at Ser3 following stimulation with the G protein-coupled receptor ligands formylmethionine-Leu-phenylalanine (fMLF) and interleukin-8 (IL-8)[78] is required to initiate chemotaxis towards these ligands in leukocytes[79,80]. It was demonstrated that the generation of free actin filament barbed ends in these cells requires cofilin activation[79]. This was regulated predominately through a RAC2-dependent pathway in mouse neutrophils, as cofilin was not dephosphorylated in response to fMLF in RAC2-knockout cells. Overexpression of CIN rescued cofilin dephosphorylation under these circumstances[80]. Similarly, dephosphorylation of cofilin downstream of fMLF can be induced by the activation of the phosphatase SSH2 through repression of glycogen synthase kinase 3 (GSK3)[81]. PI3K can promote the insulin-induced activation of SSH1 and cofilin dephosphorylation in breast carcinoma cells, whereas PTEN antagonizes this effect, indicating a role for PtdIns(3,4,5) P_3 in this pathway[82].

LIMK1 and LIMK2 (REFS 45,83) as well as TES kinase 1 (TESK1) and TESK2 (REFS 84,85) phosphorylate co filin at Ser3 *in vivo*. In crawling cells, LIMKs are the most well-studied kinases and have been proposed to be the dominant kinase in the regulation of actin dynamics[69]. TESKs are thought to be involved in integrin signalling during focal adhesion formation[69]. LIMK1 and LIMK2 are activated by phosphorylation at Thr508 and Thr505, respectively, by various kinases, including ROCK[86] PAK1, PAK2, PAK4 (REFS 87,88), MRCKα[89] and MAPK-activated protein kinase 2 (REF. 90). The balance of activity between kinases and phosphatases that target cofilin is crucial in determining the amount

and location of co filin activity in migrating cells[91]. However, it is not always accurate to describe this balance, as we have in FIG. 3, by simply illustrating that LIMKs inhibits cofilin, whereas phosphatases activate it. Mathematical simulations based on known rate constants of reactions in the cofilin activity cycle indicate that LIMK-dependent cofilin phosphorylation in the context of other regulatory interactions, including cofilin dephosphorylation and actin monomer and PtdIns(4,5)P $_2$ binding, can actually amplify cofilin activity locally. Modelling predicts that increasing LIMK activity in a spatially enclosed system, such as the leading edge, can amplify the cofilin severing response and cofilin-dependent barbed end production in the presence of these other regulatory events, even though LIMKs are generally assumed to deactivate cofilin[59].

Although dephosphorylation at Ser3 is required for cofilin activity, it is not always sufficient for cofilin activation in a closed system in which other key regulatory events of the cofilin activity cycle can take place[51]. Thus, the amount of cofilin that is not phosphorylated on Ser3 does not directly reflect the level of cofilin activity. Other mechanisms that regulate cofilin activation, including its release from its inhibitory interaction with PtdIns(4,5)P_2 and cortactin, must be considered.

Dissociation of the cofilin–PtdIns(4,5)P_2 inhibitory complex

Cofilin is known to be inactivated by its interaction with PtdIns(4,5)P_2 at the plasma membrane[92,93]. This follows a general mechanism whereby membrane lipids have been shown to bind various actin regulatory proteins such as profilin[33] and gelsolin[94] that also interact with PtdIns(4,5)P_2 to inhibit their function[95]. In migrating cells, the hydrolysis of PtdIns(4,5)P_2 by phospholipase C (PLC) to form inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) can release cofilin from its inhibitory interaction with the lipid, resulting in the local activation of F-actin filament severing, protrusion and cell polarity[49,96] (FIG. 4). In addition, the rapamycin-induced targeting of phosphoinositide 5-phosphatase to the plasma membrane, which results in dephosphorylation of PtdIns(4,5)P_2, is sufficient to release cofilin from the plasma membrane and initiate the formation of cellular protrusions[49]. This indicates that PtdIns(4,5) P_2 binding to cofilin directly inhibits cofilin activity at the plasma membrane. Moreover, new protrusions are initiated by the local photoactivation of caged S3C-cofilin (a mutant in which Ser3 was replaced with Cys), and these protrusions determine the direction of cell migration[97]. This suggests that the local release of cofilin from PtdIns(4,5)P_2 binding does not only serve as an activation switch for protrusion initiation, but that cofilin locally activated in response to external stimuli causing hydrolysis of PtdIns(4,5)P_2 can drive chemotaxis and determine the direction of cell migration[49,55,56,96].

An additional regulatory overlay in the activation of cofilin by release from PtdIns(4,5)P_2 is the involvement of local pH changes. Cofilin has long been recognized as a pH-sensitive actin-binding protein. Cofilin activity *in vitro* increases in the range of physiological pH from pH 6.8 to 7.2 (REFS 98–100) and, in fibroblasts, a rise in intracellular pH is necessary for cofilin-regulated actin dynamics[101]. His133 of cofilin is unprotonated at higher pH, which induces structural changes that decrease its binding affinity for other proteins, including cortactin[102,103] (FIG. 2). The intracellular pH is regulated by the Na^+–H^+ exchanger 1 (NHE1), an ubiquitously expressed transmembrane protein that exchanges extracellular Na^+ for intracellular H^+ (REF. 104). The local increase in pH at the cytoplasmic side of the plasma membrane, which results from the recruitment and activation of NHE1, decreases cofilin-dependent clustering of PtdIns(4,5)P_2 (REF. 105). This could lead to changes in PtdIns(4,5)P_2 density and PLC-dependent lipid hydrolysis *in vivo* (FIG. 4). In this context, the pH-regulated clustering interaction between cofilin and PtdIns(4,5)P_2 would act as a pH biosensor, connecting changes in intracellular pH to local protrusion and

motility in crawling cells in response to growth factor stimulation[102]. This pH-dependent cofilin activation pathway may contribute to the spread of metastatic tumours[106], which require the activation of cofilin for tumour cell motility and chemotaxis[8,107,108]. However, the release of cofilin from PtdIns(4,5)P_2 by lipid hydrolysis is not absolutely dependent on increasing pH, as the ectopic delivery of an active phosphoinositide 5-phosphatase to the plasma membrane is sufficient for the activation of cofilin and membrane protrusion[49]. A new signalling pathway for the activation of cofilin that involves release from PtdIns(4,5)P_2 is summarized in FIG. 4.

Dissociation of the cofilin–cortactin inhibitory complex

The binding of cofilin to cortactin also negatively regulates cofilin activity, and this mechanism seems to be specific to invasive protrusions, such as invadopodia[109–112] (FIG. 5). Initially, the release of cofilin from cortactin in invadopodia has been correlated with cortactin phosphorylation[113]. More recently, it was shown that cortactin phosphorylation is catalysed by Arg kinase in tumour cells[114], but this does not directly regulate co filin–cortactin binding, as both the phosphorylated and dephosphorylated forms of cortactin bind equally well to cofilin[103]. Further analysis demonstrated that cortactin phosphorylation results in the recruitment of NHE1 to the invadopodium core, causing a local increase in the intracellular pH. This increase in pH releases cofilin from its inhibitory binding to cortactin, resulting in the local activation of cofilin severing and actin polymerization in invadopodia. This mechanism has been shown to regulate the oscillatory protrusion of invadopodia and subsequently tumour cell invasion in two dimensions and three dimensions, as the submembrane compartment undergoes oscillatory changes in the intracellular pH[103]. Interestingly, cofilin activation at the leading edge of locomotory protrusions (for example, lamellipodia) and in invasive protrusions (invadopodia) requires local pH changes to enable the release of cofilin from its inhibitory binding partners, PtdIns(4,5)P_2 and cortactin, respectively. Whether this common pattern extends to cofilin in other cellular compartments, and how these different compartments are coordinated in their activation of cofilin during cell migration, remains to be investigated.

The array treadmilling protrusion model

A well-known model for actin polymerization during protrusion in migrating cells is the array treadmilling protrusion model (FIG. 6a). This model was proposed on the basis of *in vitro* biochemical data and suggests that the ARP2/3 complex is the central organizer of actin dynamics during protrusion[115,116].

One interpretation of this model is that cofilin functions exclusively as an actin depolymerization factor that depolymerizes actin filaments at the back of the protrusion to yield G-actin. In this way, cofilin functions only to generate G-actin to sustain steady-state ARP2/3 complex-mediated dendritic nucleation of actin polymerization at the leading edge of the cell[69]. Cofilin-dependent actin depolymerization coupled to ARP2/3 complex activity may be required for the extension of the dendritic actin network to power protrusion, the first step of the motility cycle of migrating cells (FIG. 1).

The array treadmilling protrusion model should be revised to incorporate recent observations about the dynamics of actin polymerization in protrusions and cofilin functions in particular.

The treadmilling protrusion model should include cofilin severing and cofilin nucleation that together produce free actin filament barbed ends at the leading edge for actin polymerization and protrusion initiation. For example, cofilin itself functions in early polymerization that precedes ARP2/3 complex-mediated dendritic nucleation[117] (FIG. 6b).

Furthermore, the different shapes of protrusions, ranging from flat lamellipodia to needle-shaped invadopodia, are not explained by the array treadmilling protrusion model. The local release of cofilin from PtdIns(4,5)P_2 at membranes and from cortactin within the invadopodium core, which leads to its activation, can define the boundaries of actin polymerization into complex shapes. For example, during invadopodium formation, a RHOC–ROCK dependent pathway increases LIMK1 activity surrounding the invadopodium core.

Activation of p190 RHO guanine nucleotide exchange factor (p190 RHO GEF; which activates RHOC) outside the core and of p190 RHO GTPase-activating protein (GAP; which deactivates RHOC) within the core restricts RHOC activity to a ring surrounding the invadopodium core. As RHOC activates LIMK1, this concentrates active cofilin (that is, not phosphorylated on Ser3) within the core and inactive Ser3 phosphorylated cofilin outside the core. The same mechanism has been shown to regulate spatial confinement of cofilin at the leading edge of locomotory protrusions. The restriction of cofilin activity to the core of the invadopodium[45] or at the tip of the leading edge[46] confines actin polymerization and F-actin turnover to this region. As a result, optimized focal invadopodial and lamellipodial protrusions and retraction oscillations are achieved during tumour cell invasion[38,45,103].

Evidence shows that a fraction of active cofilin that functions in filament severing is found adjacent to the cytoplasmic surface of the plasma membrane, whereas Ser3 phosphorylated cofilin is more distant from the plasma membrane[46,49,51,56,96,102]. This adds an additional dimension to the location of cofilin activity in the array treadmilling protrusion model, placing cofilin activity at the front of the extending protrusion (FIG. 6b).

The current array treadmilling protrusion model does not take into account the synergistic interaction between cofilin and the ARP2/3 complex, in which cofilin supplies newly polymerizing actin filaments on which the ARP2/3 complex-nucleated branches are more stable thereby placing these branches at the cell membrane[20,35,118].

In addition, the inhibitory effect of tropomyosin on cofilin activity at the base of locomotory protrusions, where tropomyosin is bound to actin filaments, prevents the severing and depolymerization of actin filaments at a region where the severing activity of cofilin has been proposed to occur in the array treadmilling protrusion model[60] (FIG. 6b).

Notably, cofilin, but not the ARP2/3 complex, is required for the early actin polymerization response to growth factor stimulation and the resulting initiation of protrusions in migrating cells[49,96,102,117]. These results further highlight the importance of cofilin as an actin polymerization factor at the leading edge of locomotory protrusions.

Finally, cofilin, but not the ARP2/3 complex, is involved in directional protrusion during chemotaxis in cancer cells[55,97,117], which is consistent with studies in fibroblasts showing that the ARP2/3 complex is not required for chemotaxis[119]. These results are consistent with the finding that ARP2/3 complex-dependent array treadmilling is not part of the early actin polymerization phase involved in chemosensing at the leading edge[55,117].

New models for cofilin function in migrating cells

New models for the initiation of cofilin activity in locomotory and invasive protrusions, are shown in FIGS 4–6. The cofilin activity cycle (FIG. 6c) is a common feature of both invasive and locomotory protrusions and is common to cell types as diverse as leukocytes and tumour cells[120].

Two major additions have been made to the array treadmilling protrusion model. First, the model now suggests that a significant fraction of active cofilin associates with plasma membrane-adjacent actin filaments at the leading edge of locomotory protrusions[49,55,59] (FIGS 4,6b) and not only at the back of the lamellipodium as previously suggested (FIG. 6a). This is consistent with a role of cofilin in initiating protrusion activity. In addition, cofilin is localized at the cortactin-containing core of invasive protrusions (FIGS 5,6b).

Second, an activation step that spatially determines cofilin activation is now central to new models for cofilin function during protrusion. In locomotory protrusions, this mechanism includes the local action of NHE1 and PLC leading to the release of active cofilin from the plasma membrane (FIG. 4). In invasive protrusions, cortactin and NHE1 regulate the localized activation of cofilin at the invadopodium core. Cortactin phosphorylation by SRC and Arg kinase recruits NHE1 (a step thought to involve ERM (ezrin–radixin–moesin) proteins), and the resulting rise in pH releases cofilin from its inhibitory interaction with cortactin and local cofilin activation (FIG. 5).

On the basis of these new models, it is now possible to clarify inconsistencies that have complicated our understanding of cofilin function *in vivo*. A common cofilin activity cycle that describes cofilin activity in crawling cells (FIG. 6c) explains how results obtained from various cell types that begin the cycle at different starting points can suggest that there are distinct mechanisms of cofilin regulation depending on cell type. This confusion largely stems from the relatively low resolution of methods that are used to analyse cofilin activity *in vivo*. Due to the rapid movement of cofilin between the cytosol, plasma membrane and cortactin- and F-actin-rich compartments it seems that cofilin activity is uncoupled from these regulatory events in each compartment. Cofilin localization to these subcellular compartments cannot be distinguished using standard light microscopy, as the distance between these compartments is within the diffraction limited spot of conventional light microscopes. Determining the localization and movement of cofilin in these different compartments and where cofilin is active requires high-resolution imaging methods as described in BOX 1 (see also Supplementary information S1 (box).

For example, because increased cofilin phosphorylation at Ser3 accompanies the initiation of its activity cycle, it has been proposed that cofilin phosphorylation is required for actin polymerization and that dephosphorylated cofilin is only involved in actin depolymerization. However, the new models derived from high-resolution imaging reveal that increased levels of cofilin phosphorylation are a result of cofilin activation by dephosphorylation-independent mechanisms[51] (FIGS 4–6), which can lead to actin polymerization as described above. It has been proposed that cofilin phosphorylation is involved in recycling cofilin back to the initial starting point in its activity cycle (FIG. 6c) and in spatially restricting cofilin activity to determine the shape of protrusions[45,59].

Manipulating the expression or activity of a component of the cofilin activity cycle without measuring the corresponding change in output (that is, the cofilin-dependent actin filament free barbed end transient) can also lead to inconsistent data. For example, increasing LIMK activity in a crawling cell may inactivate cofilin globally. If an increase in cell protrusion activity is observed after increasing LIMK, it is sometimes mistakenly concluded that cofilin inhibits protrusion. However, cofilin activity at the leading edge can increase following a rise in LIMK activity as cofilin is restricted to this location, leading to increased protrusion. This relationship can only be detected by directly measuring cofilin-generated barbed end transients[59,91] (BOX 1, Supplementary information S1 (box)).

Conclusions

Recent studies have confirmed that cofilin is a central component of actin dynamics in migrating cells because it catalyses either actin polymerization or depolymerization through its severing activity and due to its ability to catalyse dendritic nucleation stability and debranching. We now understand the structural basis of cofilin-mediated severing, which explains how cofilin can lead to both polymerization and depolymerization in a single severing event. Cofilin is active at the leading edge of locomotory protrusions of migrating cells, and inhibition of its activity causes defects in protrusion, cell polarity and chemotaxis. This conclusion is supported by the discovery that there are three main mechanisms by which cofilin is activated: the dephosphorylation of cofilin at Ser3; the release of cofilin from PtdIns(4,5)P_2; and the release of cofilin from cortactin. In particular, the PtdIns(4,5)P_2-dependent regulatory mechanism, which is governed by stimulated PLC activation, is important for the spatial and temporal control of cofilin activation required for the initiation of the locomotory protrusion, cell polarity and chemotaxis. The integration of these regulatory mechanisms within the cofilin activity cycle has allowed us to add refinements to the array treadmilling model of protrusion dynamics and presents an opportunity, as illustrated in the new models of cofilin function, to understand the temporal and spatial shaping of cell protrusions during cell migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

Motility cycle	A series of steps that enables cell movement. The first step is the formation of a directional protrusion driven by actin polymerization, followed by adhesion, contractile tension and tail retraction.
Amoeboid cell migration	A common type of motility whereby cells extend actin polymerization-dependent protrusions in the direction of migration.
Chemotaxis	Polarized cell migration in response to extracellular, soluble cues. Chemotaxis is characterized by the directional extension of a locomotory protrusion towards the source of a chemoattractant.
Pointed end	The end of the actin filament that is characterized by a slow growing rate.
Barbed ends	The fast growing ends of actin filaments that are characterized by a fast 'on rate' and high affinity for ATP•G-actin.
Invadopodium	Invasive protrusion that is extended by actin polymerization and is involved in extracellular matrix degradation.
Lamellipodium	A common locomotory protrusion that is extended by actin polymerization.

Histamine degranulation	Release of the pro-inflammatory molecule histamine from intracellular granules.
Crawling cells	Cells that use the motility cycle for locomotion.
Focal adhesion	Macromolecular complexes involved in cell–extracellular matrix interactions.

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Box 1

High-resolution methods to study cofilin in migrating cells*

Fluorescence resonance energy transfer (FRET)

The dynamics of the dissociation of active cofilin from the plasma membrane can be monitored by FRET between GFP–cofilin and mCherry-CAAX[49] in living eukaryotic cells. The use of expressed GFP-tagged cofilin in eukaryotic cells has been validated: GFP-cofilin interacts with actin in cell extracts (using co-immunoprecipitation); it severs actin filaments *in vitro* (as shown by co-sedimentation assays); and has been observed to fragment individual actin filaments in an *in vitro* assay (using total internal reflection (TIRF) microscopy)[121]. However, in budding yeast[122] and fission yeast[9], the GFP–tagged cofilin fusion protein is not fully functional and had to be overexpressed to rescue cofilin deletion phenotypes.

These results suggest that it is important to validate the use of GFP–cofilin fusion proteins in each cell type by an independent method such as antibody-based FRET.

The binding of endogenous cofilin to F-actin can also be measured by antibody-based FRET (for example, between immunolabelled endogenous cofilin and F-actin[46,49,113] in fixed cells). These two FRET signals, GFP and/or mCherry and antibody-based FRET, can be studied independently during cell protrusion even though they are within the diffraction limited spot of light microscopes. Both of these independent methods yield similar results in eukaryotic cells[49,113].

Fluorescence loss in photobleaching (FLIP)

The mobility of plasma membrane- and F-actin-bound cofilin can be measured by bleaching cytosolic GFP–cofilin. Using appropriate conditions, mobility differences between the plasma membrane- and F-actin-bound fractions are quantified[49,96]. Fluorescence recovery after photobleaching (FRAP) experiments can be used to analyse the lateral mobility of plasma membrane-bound cofilin.

Bimolecular fluorescence complementation (BiFC)

Binding of cofilin to F-actin and G-actin can be monitored using Venus fluorescent protein (a derivative of YFP) that is split between actin and cofilin. It does not distinguish between F-actin- and G-actin-bound cofilin. The actin–cofilin binding induced by this approach is irreversible[123].

Proximity ligation assay (PLA)

This method can be used to study F-actin and G-actin binding to endogenous cofilin at single-molecule resolution[46]. PLA uses antibodies conjugated to oligonucleotides that form complementary segments to a circularization probe, which are *in situ* ligated and circularly amplified. The amplified DNA can be detected by dye-labelled probe complementation. PLA has an extremely high signal-to-noise ratio for the detection of protein–protein binding *in situ*[124].

Caged cofilin

A constitutively active S3A cofilin phosphorylation mimetic mutant (in which Ser3 was replaced with Asp) is chemically caged using a photolabile moiety. Upon photocleavage, activated cofilin is released. This technique can be used to initiate focal activation of cofilin in live cells and has been applied to validate the model presented in FIG. 4. The

*Images for this box are available in Supplementary information S1 (box).

system is not reversible, and therefore the potential effects on cells from the accumulation of active cofilin must be considered[97].

Cofilin-dependent barbed ends *in situ*

By labelling actin barbed ends with fluorescently tagged G-actin, cofilin severing and/or barbed end generation activity can be measured *in situ*[113,117].

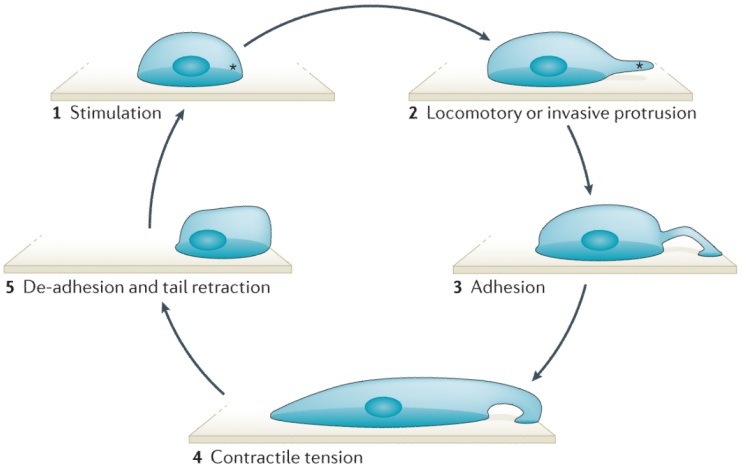

Figure 1. Steps of the cell motility cycle

An external or internal signal stimulates the local and asymmetric polymerization of actin (depicted by an asterisk) (1). Polymerizing actin pushes against the cell membrane to form a locomotory or invasive protrusion (2). The attachment of the protrusion to the extracellular matrix initiates adhesion formation at the leading edge and signals to the contractile machinery of the cell to initiate contraction (3). Contractile tension distorts the cell into an elongated shape (4). Release of the rear adhesion allows tail retraction and locomotion of the cell (5).

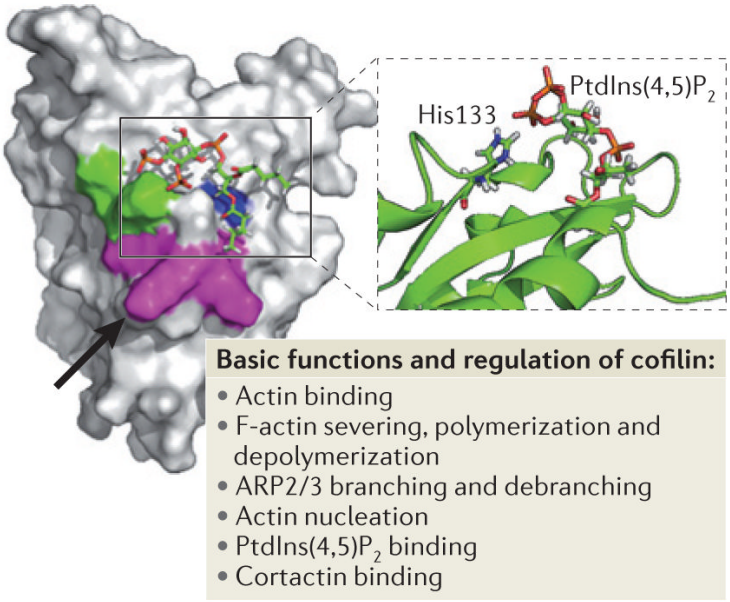

Figure 2. Domain structure of cofilin and its main functions

Key amino acids involved in the regulation of cofilin function are Asp122 and His133. The black arrow points to Asp122 located underneath Leu126 (in magenta). Asp122 regulates binding of cofilin to phosphatidylinositol-4,5-bisphophate (PtdIns(4,5)P_2). A cofilin mutant in which Asp122 has been replaced with Ala has increased binding affinity for PtdIns (4,5)P_2 and this results in the inhibition of actin polymerization, cell protrusion and migration. His133 decreases cofilin affinity for PtdIns(4,5)P_2 when deprotonated. Substitution of His133 with Ala in cofilin causes loss of H^+ binding and increases steady-state actin polymerization and cell protrusion. Lys132 (which is involved in actin binding) and His133 are shown in green, Lys125, Lys126, and Lys127 (which are alternative residues for PtdIns(4,5)P_2 binding) are shown in magenta and Phe15 and Leu99 are shown in blue. The figure is modified, with permission, from REF. 102 © (2008) Rockefeller University Press.

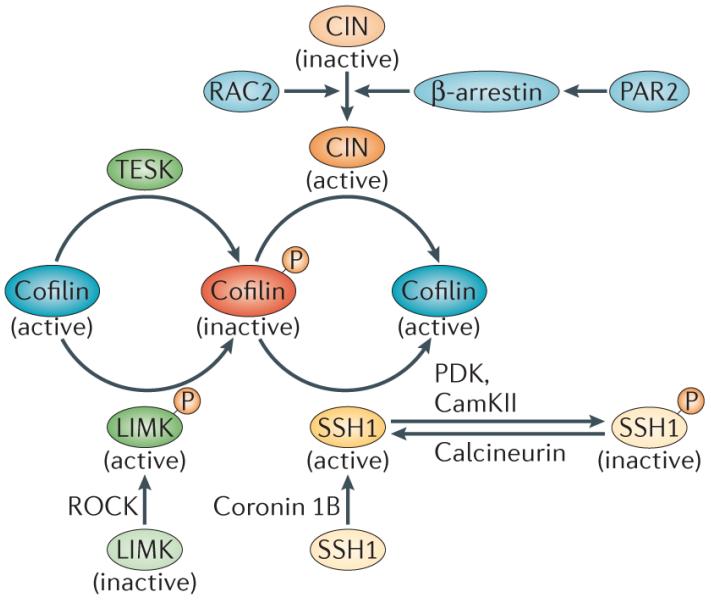

Figure 3. Regulatory mechanisms of cofilin phosphorylation and dephosphorylation

Cofilin phosphorylation at Ser3 is driven by LIM-domain kinase (LIMK) and TES kinase (TESK). LIMK is activated by ROH-associated protein kinase (ROCK). Dephosphorylation of cofilin at Ser3 is mediated by the phosphatases chronophin (CIN) and slinghshot 1 (SSH1).The localization and activation of SSH1 is mediated by coronin 1B. Phosphorylation of SSH1 by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and phosphoinositide-dependent protein kinase (PDK) results in its inactivation. Calcineurin activates SSH1 by dephosphorylating it. CIN is activated by its interaction with β-arrestin, which is activated by protease-activated receptor 2 (PAR2). In neutrophils, RAC2 has been shown to induce cofilin activation in a CIN-dependent manner.

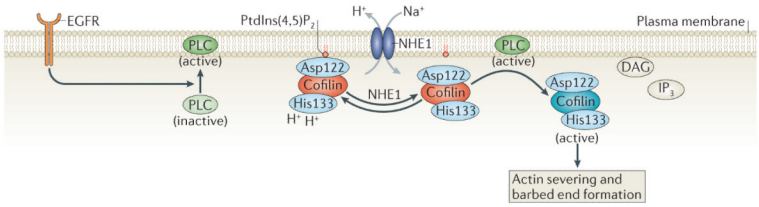

Figure 4. A new model for the activation of cofilin at the leading edge of locomotory protrusions

Inactive cofilin is bound to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) at the plasma membrane through its Asp122 residue. Na^+–H^+ exchanger 1 (NHE1) can increase the intracellular pH by exchanging intracellular H^+ for extracellular Na^+. This causes the deprotonation of cofilin at His133, which alters its binding affinity for PtdIns(4,5)P_2. An increased pH facilitates phospholipase C (PLC)-mediated hydrolysis of PtdIns(4,5)P_2 to diacylglycerol (DAG) and inositol-1,4,5,-trisphosphate (IP_3) and the release of cofilin from PtdIns(4,5)P_2. Epidermal growth factor receptor (EGFR) stimulates PLC activity, which in turn increases cofilin activation. See BOX 1 for methods describing how to study cofilin mobility as well as cofilin binding to the plasma membrane and F-actin.

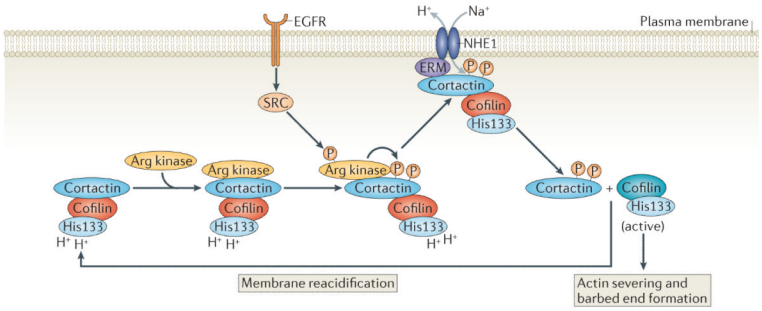

Figure 5. A new model for the activation of cofilin in invasive protrusions

Tyr phosphorylation of cortactin by Arg kinase, which is activated downstream of epidermal growth factor receptor (EGFR), regulates the interaction between the Na^+–H^+ exchanger 1 (NHE1) and cortactin. The recruitment of phosphorylated cortactin to NHE1 may be mediated by ERM (ezrin–radixin–moesin) proteins. NHE1 increases the intracellular pH, which induces the release of cortactin-bound cofilin.

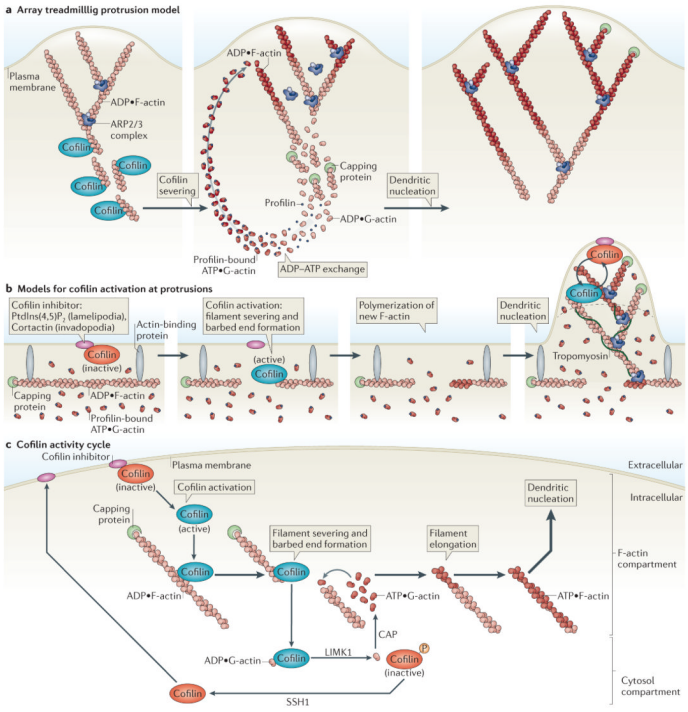

Figure 6. Models for cofilin function

a | Array treadmilling protrusion model. Cofilin severs and depolymerizes actin filaments at the base of the lamellipodium, thereby supplying G-actin monomers for steady-state actin polymerization in conditions of G-actin depletion. Dendritic nucleation is mediated by the actin-related protein 2/3 (ARP2/3) complex. **b** | Models for cofilin activation at protrusions. Release of cofilin from its inhibitor (phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) at lamellipodia or cortactin at invadopodia) at the plasma membrane increases severing of actin filaments, generating free barbed ends that define the sites of dendritic nucleation by the ARP2/3 complex. G-actin monomers are supplied from an abundant pre-existing G-actin pool. The ARP2/3 complex mediates dendritic nucleation. Tropomyosin limits cofilin action, as it inhibits binding of cofilin to F-actin. This confines cofilin severing to the tip of protrusions (dotted black line), where cofilin continues its cycles of activation and deactivation due to the local cofilin activity cycle. **c** | Cofilin activity cycle. Following its dephosphorylation or its release from inhibitory binding partners, activated cofilin binds to and severs actin filaments, generating barbed ends that are used for actin polymerization. LIM kinase 1 (LIMK1) and cyclase-associated protein (CAP) accelerate the dissociation of ADP•G-actin–cofilin complexes, and this increases the concentration of ATP•G-actin monomers available for filament elongation at the newly formed barbed ends. The capping of new barbed ends by actin interacting protein 1 (AIP1) (not shown) promotes cofilin-dependent filament disassembly. LIMK1 phosphorylates cofilin and thus inactivates it, leading to the release of bound G-actin. Upon the activation of slingshot 1 (SSH1) , cofilin is dephosphorylated and recycled to the inhibitory compartment. Figures are not drawn to scale. See BOX 1 for methods describing how to study cofilin activation.