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ADF/Cofilin: a functional node in cell biology

Barbara W. Bernstein and James R. Bamburg

Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO 80523-1870, USA

Recent findings have significantly expanded our understanding of the regulation of actin-depolymerizing factor (ADF)/cofilin proteins and the profound multifaceted impact that these well-established regulators of actin dynamics have on cell biology. In this review we discuss new aspects of previously documented regulation, such as phosphorylation, but also cover novel recently established modes of regulation and functions of ADF (also known as destrin)/cofilin. We now understand that their activity responds to a vast array of inputs far greater than previously appreciated and that these proteins not only feed back to the crucially important dynamics of actin, but also to apoptosis cascades, phospholipid metabolism, and gene expression. We argue that this ability to respond to physiological changes by modulating those same changes makes the ADF/cofilin protein family a homeostatic regulator or 'functional node' in cell biology.

Introduction

Members of the ADF/cofilin family of actin-binding proteins, which are essential in eukaryotes, have long been known to play a key role in actin-filament dynamics in cells and to have highly complex and interesting modes of regulation. However, several recent publications have once again thrust members of this family into the limelight, this time more than ever as central figures in cell biology. The new revelations are in some cases significant expansions or clarifications of previously identified modes of regulating ADF/cofilin activity, but in other cases they are completely novel mechanisms of regulation and function, some not even directly related to actin assembly (Figure 1a). This review explains the implications of these discoveries for a broader understanding of how cofilin impacts cell behavior. Cofilin is emerging as an agent of cellular homeostasis (Figure 1b). This is not too surprising considering the well-established importance of actin dynamics in numerous vital cell processes and cofilin as a key regulator of those dynamics. We first provide background on the distribution and functions of ADF/cofilin family proteins, then discuss the new modes of regulation, and finally explain how these findings reinforce our suggestion that ADF/cofilin proteins are a functional node in cell biology.

Distribution of ADF/cofilin proteins

The essential ADF/cofilin proteins are expressed in all eukaryotes with three forms in mammals: ADF, cofilin-1 (the major form in non-muscle tissue) and cofilin-2 (the

major form in muscle). ADF and cofilin-1 have similar actin-dynamizing activities that differ quantitatively – ADF being somewhat more efficient in monomer sequestering and cofilin being more efficient in nucleation and severing (Box 1). Silencing and rescue experiments of cells in culture show that either protein can rescue most normal behaviors [1]. However, knockout of the cofilin-1 gene is embryonic lethal in mice [2], whereas the only gross deficit in ADF knockout mice is postnatal blindness at about 4 weeks [3]. Although some additional differences in ADF and cofilin have come to light and will be discussed below, we will mostly focus on cofilin-1 and refer to this molecule as 'cofilin'. We do this because cofilin-1 is the most ubiquitous and has been studied much more widely due to its essential role in development. The ADF/cofilin proteins differ enough that their cellular compartmental localizations and regulation are thought to represent functional specializations, but they are qualitatively similar in many respects.
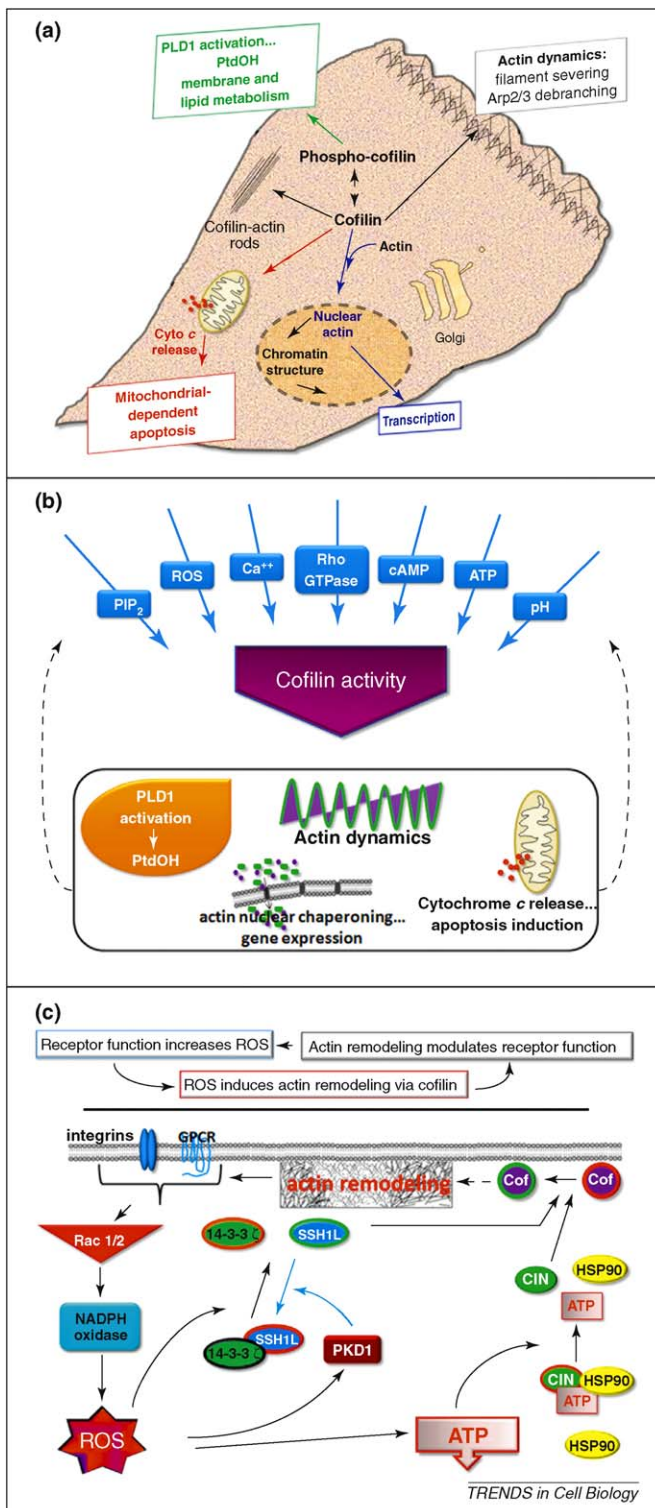
Functions of cofilin
Modulation of actin dynamics
Cofilin is best known as a regulator of actin filament non-equilibrium assembly and disassembly (Box 2). Whether cofilin promotes actin assembly or disassembly depends upon the concentration of cofilin relative to actin and the relative concentrations of other actin-binding proteins [4]. *In vitro* studies have demonstrated that if the ratio of cofilin/actin subunits in a filament is low ($<1/100$ of the K_D), persistent filament severing results [5]. At a high cofilin/actin ratio, cofilin severs rapidly and transiently and then stabilizes filamentous actin (F-actin) in a twisted form [6]. At even higher cofilin/actin ratios *in vitro,* cofilin can nucleate filaments through severing and through forming an as yet to be elucidated structure with actin monomers [5]. It remains to be determined if and exactly how this concentration dependence contributes to the impressive spatial coordination of actin dynamics that underlies highly motile regions such as lamellipodia and neuronal growth cones [7]. The presence of the many other actin-binding proteins might dampen or eliminate such behaviors. One would need to know the instantaneous relative protein concentrations in microregions to predict all behaviors, but these are currently impossible to ascertain.

   If the concentration of the cofilin–actin complex exceeds some crucial threshold, yet another mode of regulation sets in. This occurs when cofilin is overexpressed or when almost all cells types are energetically or oxidatively stressed, the exception being cells expressing low levels

Corresponding author: Bernstein, B.W. (Barbara.Bernstein@ColoState.edu)

Figure 1. Diverse functions of cofilin including actin dynamics and beyond. (a) Diagram showing several of the functions of cofilin (cof) within the cell that are described in more detail throughout the text. (b) Many of the factors that regulate cofilin activity are themselves modulated by cofilin and phospho-cofilin. Thus cofilin has a homeostatic role. (c) An example of homeostatic feedback regulation is the oxidative-stress cycle. Reactive oxygen species (ROS), made as a result of signaling pathways or from other sources, activate cofilin phosphatases through different mechanisms discussed in the text. The resulting active cofilin can restructure the cortical actin and receptor interactions, modulating signaling. In all figures in this article, symbols surrounded by a red line are inactive, and those surrounded by a green line are active.

of cofilin (Figure 1a). Excessive levels of active cofilin drive the formation of cofilin–actin bundles or 'rods' [8] that sequester a large fraction of the total cofilin, thus rendering cofilin incapable of promoting disassembly or severing. This additional autoregulatory mode will be explained in more detail below.

Modulation of actin filament branching
Cofilin is now understood to have a role that underlies the rapid assembly and disassembly of long unbranched filaments in the lamellipodia of motile cells. This role derives from the recently discovered ability of cofilin to compete directly with the Arp2/3 complex and to reduce dramatically the affinity of Arp2/3 complex for filaments [6]. The Arp2/3 complex, consisting of two actin-related proteins and five others, nucleates the growth of new filaments at a distinctive 70° angle relative to pre-existing filaments, thus generating a dendritic network typical of the lamellipodium [9]. Newly reported results from *in vitro* studies show that cofilin enhances the removal of these branches [6] (Figure 1a). The Arp2/3 complex is involved in the forward movement of cells that is crucial for processes as fundamental as embryonic development. The cyclical appearance and disappearance of Arp2/3 branched filaments *in vivo* might result in part from the spatial and temporal coordination of cofilin activation and cofilin interaction with Arp2/3 (i.e. the generation of active cofilin at a time and place in the cell that renders it effective in modulating Arp2/3-induced actin branch growth). Regulation of cofilin within the leading edge of migrating cells will be discussed more fully below.

Chaperoning of actin to nucleus
Several studies have now pointed to functions of cofilin that are surprisingly unrelated to actin-assembly regulation. The first is that of chaperoning actin to the nucleus (Figure 1a). Actin itself has no nuclear-localization sequence but its binding partner cofilin does. Because actin is recognized to have important functions in chromatin remodeling,

Box 2. Background information on cofilin modulation of actin dynamics

Subunit release is both a function of nucleotide binding to actin and the structural state of the filament following assembly [80–82]. For mature filaments *in vitro* at steady-state, actin^{ATP} adds to the growing barbed end and actin^{ADP} is released from the pointed end in a process called 'treadmilling' [4] during which ATP is hydrolyzed and the γ phosphate is released. However, immediately after assembly, recent *in vitro* work shows that the filaments are found in a less-stable twisted form from which subunits dissociate rapidly; in this case they dissociate from the barbed end [81]. The filaments undergo spontaneous transitions between the twisted and untwisted forms before assuming the more stable untwisted form ~2 hours after assembling [83]. The binding of cofilin to these mature filaments has been suggested to destabilize them by inducing or stabilizing a twist [84]. Whether cofilin binds to the filaments depends on the release of inorganic phosphate (Pi) after actin^{ATP} hydrolysis because Pi binds antagonistically to cofilin [85]. In addition, release of Pi is increased ~10-fold by cofilin binding [86]. Cofilin's ability to depolymerize faster at higher pH is thought to result from the pH dependence of Pi release [45] because the binding of Pi is stronger at low pH (6.5) than high pH (8.0) (Ref. [85]).

In some cells, when filaments are severed, conditions favor assembly and the generation of barbed ends for polymerization [87]. A high concentration of pointed-end capping proteins favors assembly after severing whereas barbed-end capping proteins favor disassembly. The roles of other actin-binding proteins in modulating cofilin's effects on actin dynamics *in vivo* are discussed later.

in the formation of heterogeneous nuclear ribonucleoprotein complexes, and in gene expression [10,11], we consider that the ability to enable actin nuclear functions is one of the crucial cellular roles of cofilin. Moreover, plant ADF could have a specific effect on gene regulation *per se* in addition to its ability to chaperone actin [12].

Release of cytochrome c
The second function of cofilin that is not related to the regulation of actin assembly comes from a proteomic study in which cofilin was found to translocate to mitochondria after staurosporine induction of apoptosis in a neuroblastoma cell [13] (Figure 1a). In neutrophils, cofilin oxidation and its mitochondrial translocation was found also to induce apoptosis [14]. Cofilin translocation appears necessary for the opening of the mitochondrial permeability transition pore and subsequent release of cytochrome *c*, an early step in apoptosis. Actin is not carried with cofilin to mitochondria. Neither translocation to mitochondria nor the release of cytochrome *c* requires actin binding, although apoptosis is blocked by mutating the actin-binding domain [13]. Apoptotic death in tissues is preferable to necrosis in that it reduces damage to surrounding cells. Curiously, CAP1, an actin–cofilin binding protein (see below), also moves to mitochondria after apoptosis induction and, whereas overexpression of wild-type CAP1 alone does not induce apoptosis, it potentiates cofilin-induced apoptosis [15]. These effects require both the actin-binding domain of CAP1 and its mitochondrial targeting domain. CAP1-knockdown in HeLa cells makes them resistant to mitochondrial-dependent apoptosis. The mitochondrial targeting and functioning of cofilin and CAP1 are not unique among actin-binding proteins. Gelsolin, an actin severing and capping protein, with very different regula-

tion from cofilin, also translocates to mitochondria but, unlike cofilin, gelsolin is thought to inhibit cytochrome *c* release by closing the voltage-dependent anion channel [16]. These properties of gelsolin, CAP1, and cofilin tie together the cytoskeleton and apoptosis, as does actin turnover itself. A reduction in actin dynamics is thought to cause a decrease in mitochondrial membrane potential and increased levels of reactive oxygen species (ROS) [17].

Activation of phospholipase D1
Another unexpected and important new cofilin function is also the first assigned to phospho-cofilin, previously described only as an inactive form. It is novel in that it involves neither the direct regulation of actin assembly nor the binding of an actin regulatory protein. This function of phospho-cofilin is the direct activation of phospholipase D1 (PLD1; Figure 2a) (Ref. [18]), an enzyme essential for chemotaxis [19] – the movement of cells along chemical gradients. Chemotaxis figures centrally in processes such as neutrophil tracking of pathogens and cell migration toward growth factors (cancer cells, neuronal development). The key role of cofilin in these processes has previously focused on the two-stage activation of cofilin through release from phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P_2)-inhibitory binding and dephosphorylation [4]. The activation of PLD1 increases the levels of phosphatidic acid (PtdOH). This activation was demonstrated to be downstream of a G-protein-coupled receptor, the muscarinic cholinergic receptor [18]. Rac activation at the leading edge, a process essential for neutrophil chemotaxis, requires the precise localization of an atypical guanine exchange factor, DOCK2, that depends upon both PtdIns(3,4,5)P_3 and PtdOH [20]. Interestingly, this now suggests that cofilin has the dual job of ensuring that Rac is properly positioned on the membrane by providing a supply of PtdOH and then controlling actin dynamics. What could be more efficient than having a single component in the cell with such a diversity of functions? This arrangement ensures that the two activities needed are simultaneously present because they both reside in one molecule.

The activation of PLD1 by phospho-cofilin has implications more far-reaching than chemotaxis. The generation of PtdOH through phosphorylation of diacylglycerol (DAG) was recently shown to release Rac-GDP from the guanine nucleotide exchange inhibitor Rho-GDI [21]. Such release, if also true for PLD1-generated PtdOH, could be part of a positive-feedback cycle from Rac-GTP to p21 activated kinase 1 (PAK1) to LIM kinase (LIMK), the predominant enzyme that phosphorylates cofilin (Figure 2a). Active LIMK generates more phospho-cofilin that then activates PLD1 and produces more PtdOH. The likelihood of a more direct cycle is quite high because PtdOH is known to stimulate PAK1 whose mediation of increased phospho-cofilin drives PtdOH generation [22]. Thus all the nuances of the phospho-regulation of cofilin now interweave with cellular phospholipid metabolism and put cofilin in the center of a growing functional cycle that even extends to ROS synthesis. Phospho-cofilin can negatively feed forward via PtdOH, Rac-GTP, and NADPH oxidase to induce ROS (Figures 1c, 2a), and ROS is now known to decrease phospho-cofilin levels as described below.
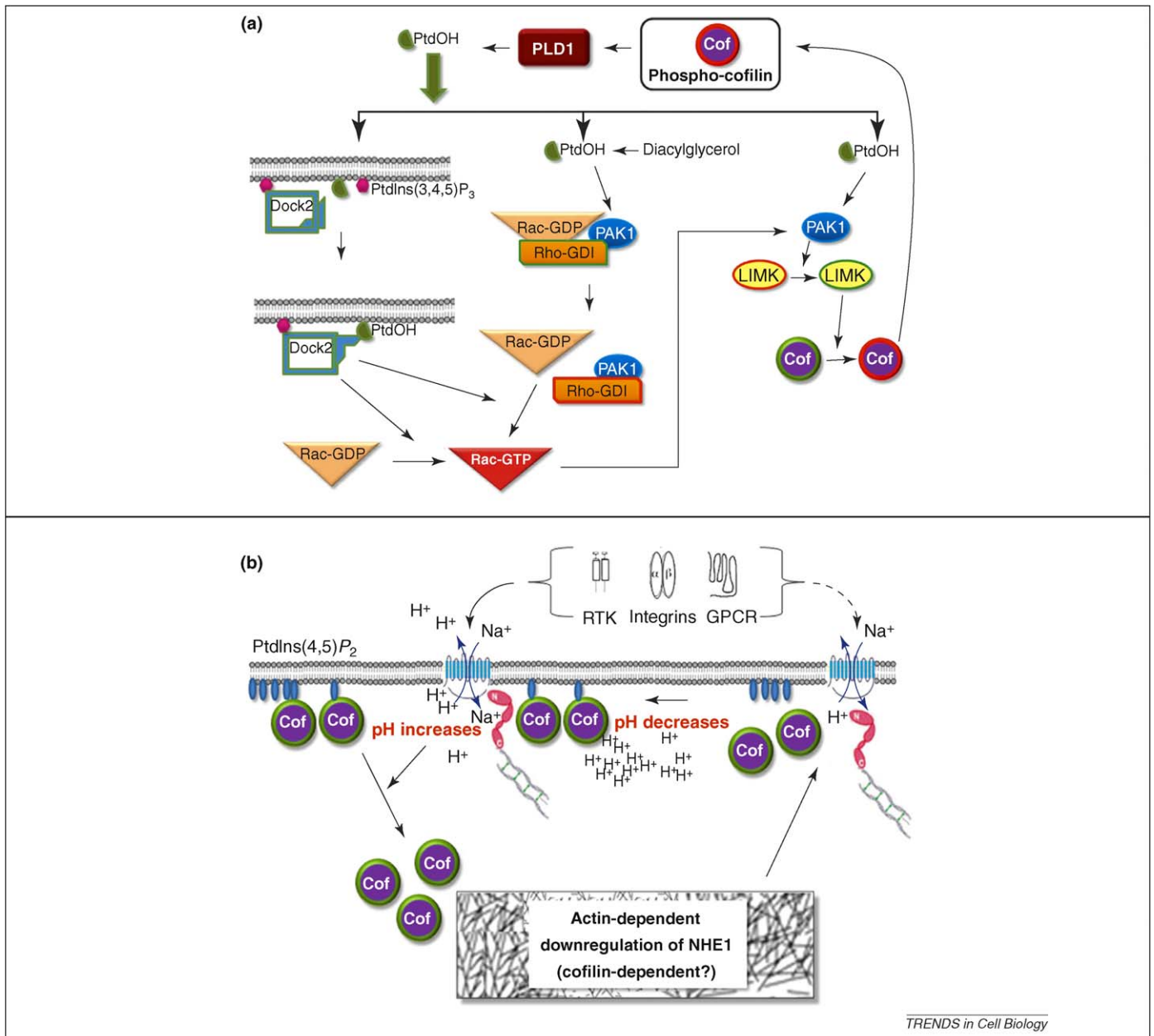
Figure 2. Recently discovered roles for cofilin in lipid metabolism and signaling and in pH-regulated actin remodeling. **(a)** Phospho-cofilin is an activator of phospholipase D1 (PLD1) and is thus a regulator of phosphatidic acid (PtdOH) production. PtdOH plays multiple roles in the activation of Rac1, a Rho-family GTPase, as well as directly activating PAK1, a kinase that is also downstream of active Rac1. PAK1 is an activator of the cofilin kinase LIMK1. Thus a phospho-cofilin/PtdOH positive feedback cycle exists. **(b)** Many receptors along with intracellular acidity activate the Na$^+$/H$^+$ exchanger (NHE1), linked via molecules in the ezrin/moesin/radixin family (shown in pink) to the actin cytoskeleton. The influx of Na$^+$ and efflux of H$^+$ locally elevates pH that enhances the release of cofilin from its inhibitory membrane binding to PtdIns(3,4)P$_2$. Released cofilin could then contribute to actin remodeling that can then decrease the flux of the NHE1 and reduce the pH. Thus cofilin is a probable regulator of pH homeostasis.

Regulation of cofilin

Phosphorylation of Ser3

Phosphorylation of cofilin on Ser3 inhibits its binding to G-actin (monomeric actin) and F-actin (Box 3). Research on this regulation has focused on the signaling pathways involved [23,24]. This work has exploded and now fills complex schematics of review articles with multiple, cross-talking pathways of two kinase families – LIM kinases and Tes kinases [4]. LIMK1 is a target of Rho GTPases via PAK1, PAK2, PAK4, and MRCKα (myotonic dystrophy kinase-related Cdc42-binding protein kinase, Ref. [25]). LIM kinase 2 (LIMK2) is a target of Rho GTPases via ROCK1 and ROCK2 and MRCKα. TES kinases can be regulated through cell-adhesion [26] and

other upstream factors [27] but have been much less studied. Cofilin is activated by two phosphatase enzymes: chronophin, highly specific for cofilin, and slingshot 1L (SSH1L), which also recognizes LIMK1 and coronin 1B as substrates [28]. Each of these phosphatases is regulated by different upstream signaling pathways [4].

In the past year this work has taken a few new interesting twists and turns [29–31], providing a more detailed understanding of the upstream regulators of the kinases and phosphatases that turn off and on cofilin binding to actin (Figure 1c and Figure 2a): (i) cysteine residues in the scaffolding protein 14-3-3ζ are targets of **oxidative stress**, thus releasing SSH1L from its inhibiting complex [29]; (ii) alternatively, **oxidative stress** also can inhibit SSH1L by

Box 3. Structure of human cofilin

The residues shown in Figure I in yellow are ones that are in the actin-contact regions (right side of the molecule in the side view), those in cyan are in the PtdIns(3,4)P_2-binding region [79], and those in dark blue are in the nuclear localization sequence. Also shown is the Ser3 regulatory phosphorylation site, the two Lys residues (K126 and K127) to which Ser3 binds when it is phosphorylated, His133 that confers pH-sensitivity to the PtdIns(3,4)P_2-binding, and Tyr68 that serves as a Src substrate for phosphorylation prior to ubiquitination and degradation of cofilin.
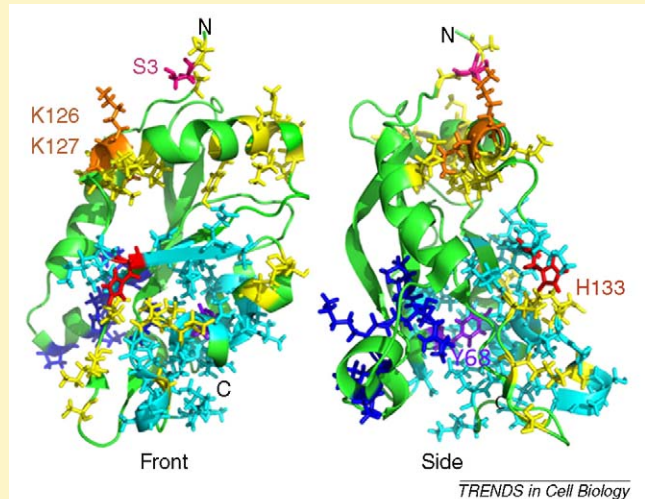

Figure I. Front and side views of human cofilin.

activating protein kinase D1 (PKD1) phosphorylation of SSH1L [30,32], that creates the phosphorylated sites in SSH1L to which 14-3-3ζ binds [33]; (iii) **energetic stress** (i.e. ATP depletion) frees chronophin from an inhibitory complex with HSP90 (Ref. [31]); (iv) fluctuations in intracellular **calcium** have the capacity to activate or inactivate cofilin through SSH1L and LIMK [4]. In neurons the calcium-sensitive upstream factors are the following: calcineurin, an activator of SSH1L; CaMKII, a modulator of a synaptic GTPase activating protein, synGAP; and RasGRF1, a RAS guanine exchange factor [34]. (v) **cAMP**/protein kinase A can activate LIMK1 directly in mouse embryonic fibroblasts [35]; however elevated cAMP might also lead to cofilin dephosphorylation indirectly [36,37]; (vi) type I and II **coronin**s have different localizations, but depletion of either results in increased phospho-cofilin; phospho-coronin 1B, like cofilin, is activated by SSH1L [38]. Coronin 1B is found in the leading edge; whereas coronin 2A is predominantly a stress fiber and focal adhesion protein [39].

Tyrosine phosphorylation and ubiquitination

Tyrosine 68 (Y68) is a residue common to mammalian, chicken, and *Xenopus* cofilin but is not present in mammalian or chicken ADF (Box 1). Phosphorylation of Y68 was shown this year to be another mode of cofilin regulation [40]. It does not affect actin-dynamizing activity but, when 293T cells are transfected with v-Src, Y68 phosphorylation does increase ubiquitination and proteosome degradation enough to reduce cofilin levels and cell spreading. Surprisingly F-actin levels also decline, showing the difficulty of predicting the state of actin assembly based

only on the levels of cofilin. The relationship between cofilin level and cell migration is biphasic [41]. A moderate increase in cofilin accelerates cell migration, clearly a factor in metastasis, but greater increases reverse this effect.

pH and phosphatidylinositol (4,5) bisphosphate

A greater understanding of other modes of cofilin regulation has also been achieved. It has long been recognized that **pH** *in vitro* [42,43] and *in vivo* [44,45] modulates mammalian but not yeast cofilin activity. The *in vivo* mechanism was recently clarified. Frantz *et al.* demonstrated that the ability of cofilin to act as a cellular pH sensor, with higher activity at higher pH, involves the well-established inhibition of cofilin activity by binding to PtdIns(4,5)P_2 (Figure 2b) [46]. They used a fibroblast lacking H^+ efflux and thus incapable of generating the transient barbed-end increase needed for membrane protrusion in response to growth factors, integrin engagement, and wounding. They demonstrated that the cofilin H133A mutant, that is pH-unresponsive with limited PtdIns(4,5)P_2 binding, but still capable of actin severing *in vitro*, rescues the growth factor response of fibroblasts lacking H^+ efflux. By contrast, the S3A cofilin mutant, that cannot be inactivated by phosphorylation but can be inactivated by protonation of H133 and so has strong PtdIns(4,5)P_2 binding at the lower pH of resting cells, cannot rescue the growth factor response in fibroblasts lacking H^+ efflux. Thus they confirmed suggestions of cofilin as a coincidence detector and brought together two modes of cofilin regulation previously considered to be separate – pH and PtdIns(4,5)P_2 binding. If a 'sticky' mutant of cofilin (D122K) with increased PtdIns(4,5)P_2 affinity is overexpressed in migrating fibroblasts, chemotaxis is disrupted, confirming the importance of cofilin release [47].

In a series of molecular dynamic simulations using NMR structure [48], the structural basis for the independence of pH and phosphorylation regulation was deduced [46]. Increased pH has little effect on cofilin's overall structure but, as shown, deprotonates His133 that reduces PtdIns(4,5)P_2 binding to that of the H133A mutant. The inhibition of phospho-cofilin binding to actin was also explained: the phosphorylation of Ser3 enables salt bridges to form between itself and two residues, Lys126 and Lys127 that are involved in actin binding of dephospho-cofilin [48] (Box 3).

Oxidation

Several years ago it was found that the major intracellular reducing agent, glutathione, when oxidized, can oxidize and dimerize cofilin *in vitro* through inter-molecular disulfide bonding (Figure 3a). Moreover, the dimerized cofilin no longer has actin depolymerizing or severing activity [49]. Klemke *et al.* have extended oxidation as a regulatory mechanism to cofilin *in vivo; however*, in their studies, intra- rather than inter-molecular disulfide bonds form [50]. In T cells the generation of a C39–C80 intra-molecular bond, delivered by the oxidative burst of granulocytes, does not eliminate actin binding, but increased F-actin results, suggesting a new mode of cofilin–actin inhibition – oxidation
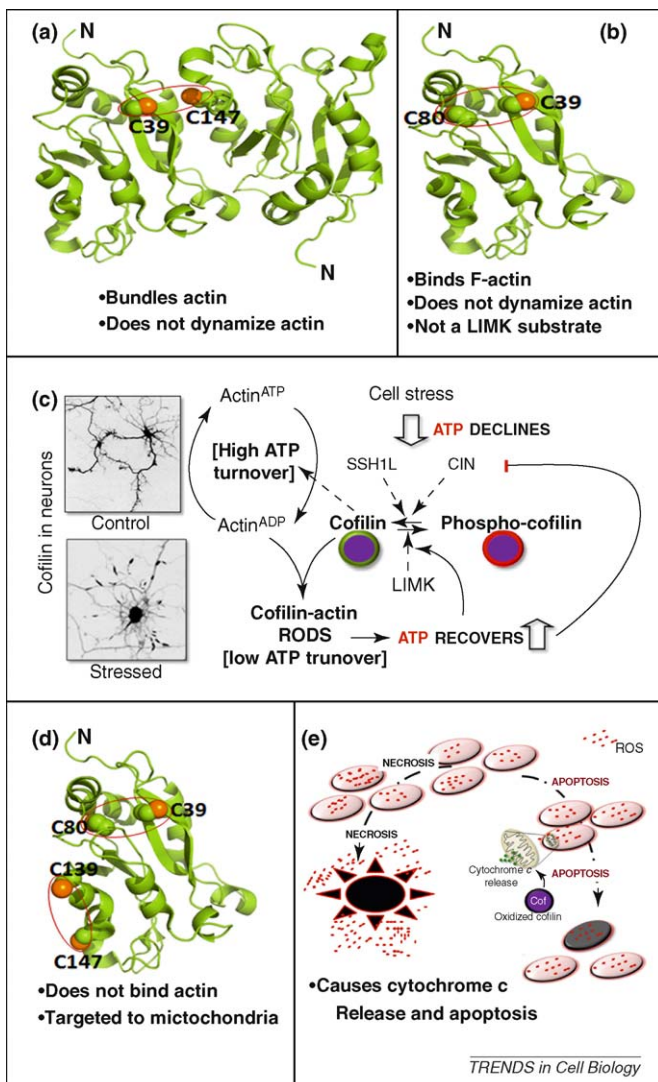
Figure 3. Cofilin as a mediator of oxidative stress. **(a)** Hypothetical model of a cofilin dimer linked by a disulfide bridge between C39 of one cofilin and C147 of another. This dimer promotes actin bundling *in vitro*, and is formed by oxidized glutathione [49], but was not detected *in vivo* following cell exposure to taurine chloramine, the major oxidant in neutrophils [14]. Instead, taurine chloramine induces one or two intramolecular disulfide bonds from the four cofilin cysteine residues. **(b)** The intramolecular disulfide bond between C39 and C80. The two residues are not close enough to form a disulfide bond without some alteration in the protein structure, that causes a loss of actin-dynamizing activity without eliminating F-actin binding. **(c)** Cofilin–actin rods form when abnormally high levels of active cofilin and ADP–actin are reached, as in the response to oxidative stress and ATP decline. The inverted images show cofilin-immunostained neurons under control and stressed conditions. Rods can sequester a large percentage of the active cofilin, thus slowing the decline in ATP brought about by cofilin-stimulated actin turnover and its associated ATP hydrolysis – and such turnover consumes considerable ATP. The ability to maintain some critical level of ATP is necessary for the cell to recover as stress subsides. Thus cofilin contributes to energy homeostasis. **(d)** The fully oxidized cofilin, with disulfide bonds between C39–C80 and C139–C147, does not bind to F-actin but is targeted to the mitochondrial outer membrane. Preliminary modeling of the structure showed a disruption in the alpha-5 helix that includes C147. Two F-actin-binding residues (Box 3) reside nearby, and the alteration in structure probably accounts for the loss of F-actin binding. **(e)** Fully oxidized cofilin **(d)** is targeted to the mitochondrial outer membrane where it causes leakage of cytochrome c and activation of an apoptotic cascade. Apoptosis causes much less tissue damage than necrosis, a process responsible for inflammation, increased ROS, and extensive cell death.

(Figure 3b). Further studies on the activity of oxidized cofilin were performed with taurine chloramine, the primary oxidant generated by activated neutrophils [14]. Both C39–C80 and C139–C147 intra-molecular disulfide bonds form in taurine chloramine-treated lymphoma cells in which

cofilin is a main target of oxidation (Figure 3d) [14]. Neither disulfide bond alone inhibits cofilin–actin binding *in vitro*, but actin binding is eliminated when both internal disulfide bonds form. Furthermore, mutation of any one of these cysteines or Ser3 phosphorylation blocks cofilin induction of cytochrome c release from mitochondria and apoptosis induced by taurine chloramine (Figure 3e). Because anti-oxidants inhibit staurosporine-induced apoptosis in neurons [51], cofilin oxidation could also be the mechanism for cytochrome c release induced by staurosporine in neuroblastoma cells [13].

Proteins modulating cofilin–actin interactions
The number and nuances of the proteins modulating cofilin–actin interactions continues to grow. These proteins include tropomyosins, cortactin, CAP1/Srv2p, coronins, and Aip1 (actin-interacting protein; reviewed in Ref. [52]). The plethora of **tropomyosins** encoded by four genes and alternative splicing extends to over 40 isoforms. The better-known longer forms are competitors of cofilin for F-actin and stabilize filaments, whereas at least one of the shorter forms is a cofilin collaborator in enhancing actin dynamics ([53]; reviewed in Ref. [54]). In contrast, **cortactin**, an actin-binding protein of the cell cortex, competes with cofilin for binding to actin subunits. However, cortactin binds preferentially to ATP and ADP·Pi subunits of actin; whereas cofilin binds preferentially to ADP–actin [4]. In addition, in the highly specialized invadopodium compartment of metastatic cells, cofilin binds cortactin in a complex with Arp2/3, the Arp2/3 activator N-WASp, and the N-WASp activator Nck1. Cofilin is released for the essential function of severing after phosphorylation of a **cortactin** tyrosine [55].

A third type of interaction with cofilin is seen with **CAP1/Srv2p**, found in all eukaryotes, and Aip1. As with Aip1, CAP1/Srv2p alone has no effect on actin stability but does potentiate cofilin-stimulated filament turnover [56]. CAP1/Srv2p was first identified as part of the yeast adenylyl cyclase complex [57]. It displaces cofilin from actin^{ADP} [58]. This displacement enhances nucleotide exchange because cofilin binding to G-actin retards exchange. Such *in vitro* studies of actin with two proteins can give us useful information, but we are still far from a clear understanding of how the combination of these proteins in a particular cellular compartment might interact to effect actin regulation.

Another three-protein study, this a recent one on yeast **coronin** (Crn1), illustrates well how the complexity of actin regulation, involving nucleotide states, combined with a third protein (Crn1), can amplify the polarizing effects of cofilin on actin turnover [59]. The report also helps to resolve a number of apparently conflicting reports on coronin's effects on actin. Crn1 has three domains: a N-terminal β-propeller domain that binds F-actin, a middle domain, and a C-terminal coiled-coil (CC) domain that modulates the Arp2/3 complex. The β-propeller and CC domains allow Crn1 to have opposing effects on cofilin/actin interaction. The effect that prevails depends upon the nucleotide bound to actin. The CC domain competitively binds to ATP/ADP·P$_i$-actin, thus reducing the already low binding of cofilin to this actin. In ADP-rich actin regions,

the β-propeller domain synergizes cofilin severing. When the dominant inhibitory effects of the CC domain are negated by phosphorylation, Crn1 can switch from its filament protective role to its cofilin- synergizing role via the β-propeller domain. Additional insight into the probable behavior of some of these proteins in intact cells can be gained *in vitro* when still more proteins are included.

Reconstituted systems are a step toward the complex environment of the cell. Such a system, that contained cofilin, Aip1, and coronin1a, revealed a surprising new mechanism for cofilin depolymerization of actin [60]. Aip1 only potentiates the severing activity of cofilin; it does not sever on its own (reviewed in Ref. [52]). In this system of Aip1, coronin1a, and cofilin, designed to study disassembly mechanisms on a single-filament level, the fluorescent-labeled actin disassembles in a pattern of successive bursts not previously described and not observed with cofilin alone. Because cofilin has been shown to disrupt subunits between adjacent helical strands of actin in filaments [61], and Aip1 enhances cofilin severing and depolymerizing activity by binding along cofilin-decorated actin [62], one plausible mechanism suggested for the bursting activity is weakening of intra-filament strand interactions and the severing and removal of single-stranded actin subunits in a depolymerizing burst [60]. 'Cooperative strand separation' is supported by the fact that cofilin severing by itself does not alter the binding of the barbed-end capping protein CapZ, but cofilin severing in the presence of coronin and Aip1 abrogates CapZ binding. This suggested unwinding of actin filament strands has been seen in electron micrographs [63].

Concluding remarks
Cofilin: a functional node in cell biology
The integration of our current understanding of cofilin regulation and cellular function leads inevitably to the conclusion that cofilin is a functional node in cell biology. The activity of cofilin is modulated by almost any perturbation or fluctuation of normal cell physiology, and in turn it has the potential to correct or dampen those fluctuations (Figure 1b,c; Figure 2). Actin binding by cofilin is inhibited by phosphorylation mediated by a wide range of factors, depending upon protein and ion concentrations in microdomains of compartments that differ from cell to cell: calcium, ROS, cAMP, PtdOH, ATP levels, and RhoGTPases downstream of many transmembrane receptors (e.g. integrin receptors, receptor tyrosine kinases, G-protein coupled receptors). Cofilin binding to and/or dynamizing of actin is also inhibited directly by oxidation (Figure 3). Inhibition by PtdIns$(4,5)P_2$ is decreased by elevated pH (Figure 2b). In turn PtdIns$(4,5)P_2$ hydrolysis and subsequent DAG increase and phosphorylation probably drive a feed-forward cycle in which phosphorylated DAG (PtdOH) increases the activity of PAK and LIMK, thus generating more phospho-cofilin (Figure 2a). Through PLD1, increased phospho-cofilin results in perpetuation of the phospholipid cycle, along with its many ramifications. Cofilin feeds back and acts as a functional node by correcting physiological perturbations through its ability to regulate actin dynamics that modulate the following: (i) pH through Na^+/H^+ exchanger (NHE1) insertion and activity (Figure 2b) [64,65], (ii) ionic fluxes

through the regulation of transmembrane protein activities [66], (iii) ATP levels through hydrolysis associated with actin turnover, and (iv) receptor delivery to the cell surface [67–71]. Unrelated to actin dynamics, cofilin also feeds back through phospho-cofilin stimulation of PtdOH synthesis [18] and the contribution of cofilin to gene expression through nuclear translocation of actin [10,11]. Thus we suggest that cofilin functions as a node, 'a centering point of component parts', because its own activity is controlled by perturbations that can all feed back to mitigate or exacerbate those fluctuations.

A prime example of the ability of cofilin to coordinate cell physiology is the formation of cofilin–actin rods (bundles of cofilin-saturated filaments), that occurs when cells are energetically stressed (Figure 3c). The rods appear when abnormally high levels of active cofilin and actin^{ADP} arise; this occurs with ATP decline [8]. Abnormal levels of active cofilin follow the release of chronophin from an inhibitory ATP–HSP90–chronophin complex when ATP drops [31]. Rod formation is exacerbated by further depression of ATP levels and rising ROS levels, that then activate the other cofilin phosphatase SSH1L [29] and drive the concentration of active free cofilin even higher. Stable rods in regions of restricted volume, such as neurites, sequester almost all the immunostainable cofilin present, thus reducing the considerable ATP consumed by actin turnover [72]. Hence rods are neuroprotective, but only transiently, because they cause neurite degeneration if they persist. Their transient preservation of ATP has the potential to contribute to the maintenance of normal oxidation potential, pH, calcium levels, and phospholipid metabolism (Figure 3).

Cofilin: linkage to disease
A variety of cellular stresses, resulting in ATP decline, cause rod development. These are the same kinds of stress that are associated with sporadic Alzheimer disease. Moreover, the $A\beta_{1-42}$ amyloid peptide, infamous in its soluble form for cognitive defects and in its filamentous form for aggregating in Alzheimer disease plaques, also induces rods. The same soluble forms of $A\beta_{1-42}$, when used at concentrations that impact cognitive function, generate rods in neuronal culture [73]. In addition to being something of an energy cushion, these rods in neurons also accumulate hyper-phosphorylated tau, perhaps serving as precursors of 'striated neuropil threads', a hallmark of Alzheimer disease [74].

If cofilin is a functional node in cell biology, as we are suggesting, it is not surprising that interference with its normal activity is highly likely to have severe repercussions. Repeatedly, the study of unrelated diseases uncovers aberrant regulation of cofilin as a culprit. Klemke and colleagues have suggested that the dysregulation of cofilin interaction with actin underlies the immune deficiencies prevalent in cancer [50]. They found that the production and release of peroxide from macrophages is intense enough to oxidize cofilin in T cells. A single intra-molecular disulfide bond renders it still capable of binding actin but incapable of dynamizing it (Figure 3b). This oxidized cofilin significantly increases F-actin levels, reduces motility, and thus is the probable basis for the suppressed immune response frequently associated with cancer. Kim *et al.*,

using experimentally higher levels of peroxide, found no oxidation of cofilin itself but instead oxidation of the scaffolding and SSH1L-inhibiting protein 14-3-3ζ (Ref. [29]). It is possible that the crucial difference between these studies is the fact that Kim *et al.* used HeLa cells stably transfected to express a cofilin protein. The overexpression of cofilin in HeLa cells enormously enhances the generation of cofilin–actin rods when the cells are challenged with peroxide. With a lower level of cofilin–G-actin complex, not favoring rod generation, cofilin might be more susceptible to oxidation that prevents actin depolymerization. Both groups found reduced levels of phospho-cofilin. In T cells, oxidized cofilin was reported to be a poor substrate for LIMK1, but the activation of SSH1L through oxidative release from 14-3-3ζ might also contribute to reduced phospho-cofilin levels because SSH1L activates cofilin directly.

Other examples of cofilin being involved in disease include HIV-1 infection and inherited cancer. It was recently reported that HIV-1 nef, directly or indirectly, potently inactivates cofilin through PAK2, presumably via LIMK phosphorylation. Cofilin inactivation in human T lymphocytes impairs their motility and allows pathogens to evade the immune system [75]. Alternatively, cofilin is normally inactive in resting CD4 T cells. In this case activation of cofilin through viral envelope–CXCR4 receptor signaling allows viral nuclear translocation by enhancing the turnover of the otherwise inhibitory cortical actin [76]. Another example is Carney syndrome, the most common cardiac tumor, that is associated with mutation of the adenylate cyclase regulatory protein encoded by the *Prkar1a* gene. The downstream effector responsible for abnormal motility in Carney syndrome was found to be cAMP–PKA activation of LIMK1 and cofilin hyper-phosphorylation [35].

Cofilin: future perspectives

Throughout this review we have emphasized how cofilin has, within limits, the capacity to sense and to mitigate a wide variety of physiological perturbations. This assessment is based on evidence that has been pieced together, often from several cell types. It lacks detailed data, particularly protein concentrations and interactions in dynamic processes, which are essential for a complete and clear determination of its value and validity. Technological developments, primarily in imaging, could soon allow the advancement needed for millisecond microdomain analysis of protein interactions and ion concentrations in single cells. Although the role of cofilin in an ever-expanding number of diseases supports its importance in homeostasis as a functional node, additional information is clearly essential.

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