JJBS

Volume 4, Number 1, January 2011
ISSN 1995-6673
Pages 1 - 12

Jordan Journal of Biological Sciences

Redundant and Non-Redundant Functions of Actin Depolymerizing Factor (ADF) and Cofilin in Metastasis- Review

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Received: 3 November 2010; accepted in revised form 19 December 2010

Abstract

Tumor cell motility is the hallmark of invasion and an essential step in metastasis. Cellular changes that occur during the progression of cancer affect proteins that drive actin dynamics; these changes modulate cell cycle progression and lead to more invasive cancers. Actin depolymerizing factor (ADF)/cofilins (actin dynamizing proteins) and their regulatory proteins are involved in the initiation of early steps in cell motility. ADF/cofilins play important roles in various stages of cancer progression including cell polarization and polarized migration, escape from apoptosis, and secretion of metalloproteases, all of which are important in metastasis. Vertebrates express ADF, cofilin-1 and cofilin-2, and even though ADF and cofilin have many qualitatively similar biochemical properties, they differ quantitatively in actin interaction and in some types of regulation and, thus, are not functionally identical. This review compares the activities of these two proteins with respect to how they may function during tumor cell invasion. Understanding the molecular pathways of tumor invasion will provide new diagnostic approaches and targets for the treatment of metastatic cancer.

الملخص

تعتبر حركة الخلايا السرطانية السمة المميزة و الخطوة الأساسية في تكوين الأورام الخبيثة. تؤثر التغيرات الخلوية التي تحدث خلال تطور السرطان في البروتينات التي تتحكم بألياف الاكتين، و هذه التغيرات تعدل من دورة حياة الخلية و تؤدي الى تحول الورم من أولي إلى ثانوي أو خبيث. تلعب البروتينات (ADF/cofilin) و البروتينات المتحكمة بهم دورا مهما في استهلال حركة الخلايا و كذلك تلعب دورا أساسيا في تطور مرض السرطان الخبيث من خلال التحكم بشكل الخلايا و هجرتها و تجنب موت الخلايا المبرمج و إفراز الإنزيمات المحللة للنسيج ما بين الخلايا. تتكون عائلة (ADF/cofilin) في الفقاريات من ثلاث بروتينات هي: ADF و cofilin-1 و cofilin-2 ، يتشابه ADF و cofilin-1في كثير من الصفات البيوكيميائية النوعية و لكن يختلفوا في الصفات البيوكيميائية الكمية وأيضا من حيث تنظيمهما مما يجعلهما غير متطابقين من حيث الوظيفة. تهدف هذه النشرة إلى مقارنة دور كل من ADF و cofilin-1 في عملية تكوين الأورام الخبيثة مما يساعد في فهم تكون الأورام الخبيثة و بالتالي على تطوير علاجات جديدة لمرض السرطان.

Keywords: Actin Dynamics, Cell Motility, Polarization, Cancer, Invadopodia, LIM Kinase, Slingshot.

1. Introduction

Cell polarization and movement are fundamental features of embryonic development in multicellular organisms. They are required for establishing tissue patterns and in other processes such as the response of macrophages to pathogens, tissue repair (Nubler-Jung *et al.*, 1987) and *in vitro* wound healing (Grande-Garcia *et al.,* 2007). They are also important for disease processes such as the metastasis of tumor cells. Cells change their shape and migrate in response to guidance cues (Nobes and Hall, 1999). It is generally accepted that cell movement is mostly dependent on the dynamic reorganization of the actin cytoskeleton, which entails polymerization of actin at the leading edge, and actin bundling and myosin-based contractility at the rear (Machesky and Cooper, 1999; Ishizaki *et al.,* 2001).

Actin polymerization and depolymerization must be regulated spatially and temporally to produce motility. Polymerization requires the formation of free barbed ends (the fast-growing ends), the production of which is tightly regulated *in vivo* (DesMarais *et al.,* 2005). New free barbed ends can arise from uncapping or severing of existing actin filaments (F-actin) and *de novo* nucleation (Zwolak *et al.,* 2010). These processes are dependent upon actin-binding proteins (ABP), of which the members of the ADF/cofilin (AC) family of proteins are essential (reviewed in Maciver and Hussey, 2002; Ono, 2003; Bernstein and Bamburg, 2010).

Vertebrates express three isoforms of the AC family: actin depolymerizing factor (ADF) (Bamburg *et al*., 1980), cofilin-1 (Nishida *et al.*, 1984) and cofilin-2 (Ono *et al.*, 1994). Most researchers working with AC proteins focus

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on the role of cofilin-1 during cell motility, mainly because cofilin is the major form present (Wang *et al.*, 2008; Oser and Condeelis, 2009; Quintela-Fandino *et al.*, 2010). However, even though ADF and cofilin have many qualitatively similar biochemical properties, they differ quantitatively in their interactions with actin as well as in some aspects of their regulation and, thus, are not functionally identical. This current review aims at comparing the activities of these two proteins during tumor cell invasion and will try to answer one important question: do ADF and cofilin have redundant and/or nonredundant activities during metastasis?

2. Cancer invasion and metastasis

Cancer is a group of diseases characterized by unregulated cell growth and invasion and a spread of cells from primary sites to other body sites. It involves dynamic changes in the genome; certain mutations produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function (Ruddon, 2007).

There are six hallmarks of cancer: growth signal autonomy, evasion of growth inhibitory signals, evasion of apoptosis, unlimited replicative potential, angiogenesis and invasion and metastasis (Ruddon, 2007). Metastasis remains the cause of 90% of deaths from solid tumors (reviewed in Gupta and Massagué, 2006).

Metastasis is believed to emerge from a genetically diversified cancer-cell population under the selective pressures of the surrounding environment. If proved to be true, this may explain why millions of cells might be released by a tumor into the circulation every day, but few of these cells will colonize a distant organ (reviewed in Gupta and Massagué, 2006). In order to achieve a successful metastasis, cancer cells have to invade basement membrane, intravasate into the blood stream, disseminate through the circulation, and extravasate into distant sites (Ruddon, 2007).

Invasive tumor cells acquire a migratory phenotype and exhibit a distinct gene expression profile, called the invasion signature, in which genes associated with proliferation and apoptosis are downregulated, while a set of motility-related genes are coordinately upregulated (Philippar *et al.,* 2008). In invasive mammary carcinoma cells, for example, the invasion signature includes a network of actin-regulatory proteins, including the Arp2/3 complex and cofilin that drive formation of membrane protrusions important for invasion, motility, and chemotaxis (Wang *et al.*, 2007).

3. Actin organization during metastasis

Tumor cell motility is the hallmark of invasion and an essential step in metastasis (reviewed in Gupta and Massagué, 2006). Cellular changes that occur during the progression of cancer affect proteins that drive actin dynamics; these changes modulate cell cycle progression and lead to more invasive cancers. Spatially controlled assembly of actin filaments, in response to stimuli, generates cell protrusions called lamellipodia, filopodia, and invadopodia. Motile cells use these extensions to explore the extracellular space and find their way toward their targets (reviewed in Carlier *et al.,* 2003).

3.1. Actin Cytoskeleton

3.1.1. Actin structure and dynamics

Globular (G-) monomeric actin, a 43 KDa protein, has a bound adenine nucleotide that lies in a deep cleft in the center of G-actin. It is occupied by ATP, ADP-Pi, or ADP depending on nucleotide exchange factors and the assembly state of the subunit (reviewed in dos Remedois *et al.,* 2003). Under physiological salt concentrations G-actin assembles to form F-actin (Pfaendtner *et al.,* 2010).

The conventional view of the actin filament is a two-start, right-handed long-pitch helix. In this model, there are 12-14 monomers per half turn and a half pitch of 360-390 Å. Actin filaments have a distinct structural polarity that was first noticed when the filaments were decorated with the heavy meromyosin or S1 fragments of myosin, both of which appear as arrowheads along the filament (Huxley, 1963; Nachmias and Huxley, 1970; Svitkina and Borisy, 1998), giving rise to the "barbed" and "pointed" end nomenclature used to identify opposite filament ends. Assembly studies on decorated actin filaments showed the barbed end to be the faster growing end (Fujiwara *et al.,* 2002).

Actin polymerization *in vitro* shows an initial delay (lag phase) that is due to the instability of actin dimers and the slow formation of stable actin trimers (nucleation). This phase is followed by an elongation phase during which actin monomers are assembled into filaments that grow from both ends, but faster at the barbed end. A lag phase in assembly does not occur *in vivo* because spontaneous nucleation is suppressed by actin monomer sequestering proteins. G-actin is incorporated into a growing filament in its ATP-bound form. ATP is then hydrolyzed to ADP-P_i. A conformational change accompanies the release of the phosphate, which is a much slower step *in vitro*. At a monomer concentration between the critical concentration of the barbed end (0.1 μM) and the pointed end (0.7 μM), the barbed end grows while the pointed end shrinks, resulting in a steady-state process called treadmilling (Fujiwara *et al.,* 2002). Although there is no change in polymer mass, befitting the term "steady state," individual filaments may grow faster or slower than average, resulting in a dynamic filament population.

3.2. Actin organization at the leading edge of migratory cells

3.2.1. Structure of actin cytoskeleton in lamellipodia and filopodia

Cells reach out with various membrane protrusions as they crawl and interact with their environment. Lamellipodia (Figure 1) contain an actin network within 1-3 μm of the leading edge of a migratory cell characterized by adjacent zones of actin filament polymerization and depolymerization. Lamellipodia protrude when the actin filaments, at the leading edge, are short and highly branched or cross-linked; lamellipodia contain transmembrane proteins that can adhere to substrata allowing cells to gain traction and move (Schafer, 2004). Behind the lamellipodium is a lamella (Figure 1) which

extends 10-15 µm from near the leading edge towards the cell interior (Delorme *et al.,* 2007).

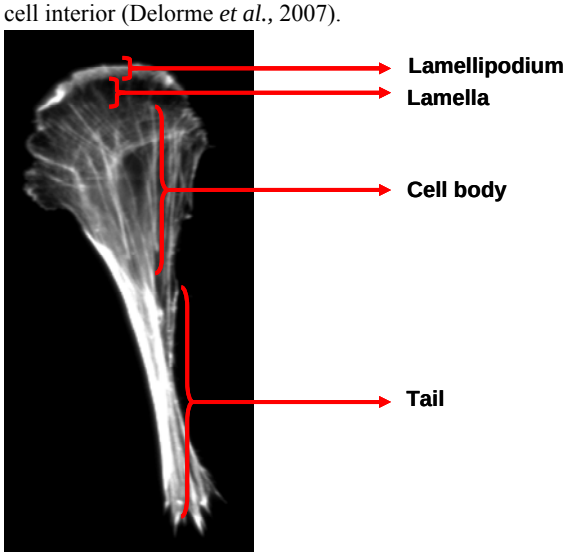

Figure 1. Cell regions of a migratory cell. Polarized chick embryo cardiac fibroblast was fixed and stained for F-actin with fluorescently-labeled phalloidin. The cell has a lamellipodium which is the actin-rich region at the front, followed by a lamella (faint region), a cell body and the tail at the rear. Scale bar, 10 µM.

Filopodia are long thin membranous protrusions (0.5 to several µm in length) containing bundles of actin filaments of near uniform polarity (Mallavarapu and Mitchison, 1999). F-actin in filopodia is cross-linked with fascin (Machesky and Li, 2010). The barbed ends of the actin filaments in filopodia are distal to the cell body, i.e., toward the tip of the filopodium (Cramer *et al.,* 1997, Svitkina *et al.,* 1997). Filopodia seem to be used by many cell types as a sensing organelle to explore the extracellular environment and the surface of other cells, identify appropriate targets for adhesion, and then transform guidance cue signals into intracellular signals for traction force generation (Xue *et al.,* 2010).

3.2.2. Regulation of actin cytoskeleton in lamellipodia and filopodia

Elongation of the barbed ends (pointing towards the membrane) of actin filaments drives membrane protrusion. The assembly and disassembly of the actin filament/network at the lamellipodia has been formulated into a "dendritic-nucleation model" (Mullins *et al.,* 1998). In this model, actin assembles predominantly at the leading edge because the concentration of uncapped barbed ends is both high and rate-limiting for polymerization. There are three proposed mechanisms for the formation of the dendritic filament array: 1) Arp2/3 complex, activated by WASp or another activator, binds laterally along pre-existing filaments on ATP- or ADP-Pi-actin subunits, promoting growth of a new filament at a ~70° angle to the original filament; 2) barbed end branching through the interaction between the Arp2/3 complex and the barbed end of a filament; 3) filamin-mediated cross-linking of the actin meshwork which is a non-Arp2/3 process (reviewed in Small *et al.,* 2008; Bugyi and Carlier, 2010). These may all work to some extent within the same lamellipodium or be used to dramatically different extents by different cell types, or by the same cell under different conditions.

Replenishment of the pool of G-actin for polymerization occurs by depolymerization of the network in regions more distant to the cell edge. Two likely mechanisms contribute to the rapid depolymerization of F-actin. The first is the removal of the Arp2/3 complex from pointed ends, facilitating depolymerization (Gupton *et al.,* 2005). The other mechanism is actin filament severing by ADF/cofilin (Delorme *et al.,* 2007) and/or gelsolin (Larson *et al.,* 2005).

In contrast to the lamellipodium, a lamella contains non-homogeneous regions of actin polymerization and depolymerization. In cells without a lamellipodium, the lamella reaches all the way to the leading edge (Ponti *et al.,* 2005). Tropomyosin (TM) and myosin II are signature molecules of the lamella and are absent from lamellipodia (Ponti *et al.,* 2004). Long TM-decorated actin filaments comprise the contractile actomyosin network, which promotes contraction in central cell regions, and which, in turn, restricts lamellipodium formation to specific persistent sites at the cell edge. The junction between the lamellipodium and lamella is characterized by substrate adhesions that translate actomyosin contraction in the lamella into cell pulling forces on the extracellular matrix (ECM) (Ponti *et al.,* 2004; Gupton *et al.,* 2005).

According to the convergent-elongation model of filopodia formation (Svitkina *et al.,* 2003), filopodia arise from the lamellipodial network by filament elongation from specific precursor sites, which are generated by lateral translocation and conversion of a dendritic array of actin filaments. This model suggests a dependence of filopodia formation on pre-existing lamellipodia. However, other studies have found that cells silenced for expression of Arp2/3 and WASp exhibited normal filopodial protrusion; these filopodia might be a function of the formin proteins, known nucleators of actin filaments (Peng *et al.,* 2003; Steffen *et al.,* 2006).

3.3. Actin organization in invadopodia

3.3.1. Structure of actin cytoskeleton in invadopodia

Cell migration through the ECM involves proteolytic degradation of ECM components. This proteolysis is mediated by specialized actin-rich membrane structures adherant to ECM called invadopodia and podosomes. Cell types that form podosomes include monocytic, endothelial, and smooth muscle cells, whereas invadopodia are formed by transformed and invasive cancer cells (Furmaniak-Kazmierczak *et al.,* 2007; Linder, 2007). Recent studies show that invading tumor cells at sites of ECM degradation form cell membrane protrusions 8 µm wide and 2 µm deep into the ECM containing multiple slender invadopodia, each about 0.8 µm diameter and 2 µm in length (Artym *et al.,* 2006). The formation of invadopodia has been correlated to the degree of tumor cell invasiveness (Artym *et al.,* 2009).

Podosomes usually consist of a core column of actin filaments that extends upwards from the ventral cell surface into the cytoplasm forming a characteristic ring-like structure, whereas invadopodia are long finger-like membrane extensions containing a meshwork of microfilaments that penetrate into the ECM from the

ventral side of the plasma membrane (Ayala *et al.,* 2006; Weaver, 2006). Invadopodia are enriched in actin and actin-associated proteins such as cortactin, Arp2/3, WASp, cofilin, and capping proteins; other proteins enriched in invadopodia include dynamin, cell adhesion molecules, such as integrins, tyrosine kinases, small GTPases, and soluble and membrane-bound proteases (Artym *et al.,* 2006). Invadopodia enhance the invasive potential of cells by concentrating metalloproteinases (ECMMPs) and serine proteases that degrade the ECM (Furmaniak-Kazmierczak *et al.,* 2007).

The broad spectrum of proteins that localize to invadopodia and the function of invadopodia in ECM degradation defines the invadopodium as a unique cellular structure characterized by coordinated interaction and interplay of molecules for cell adhesion, actin nucleation and polymerization, directed protease trafficking, endocytosis, and exocytosis. The extracellular matrix degradation coupled with internalization suggests that invadopodia actively remodel their surrounding microenvironment (Weaver, 2006; Artym *et al.,* 2010).

3.3.2. Regulation of actin cytoskeleton in invadopodia

The current model for invadopodia formation has several steps. The initial stages of assembly involve the formation of a primary invadopodial membrane process that extends from the ventral cell membrane toward the ECM. The tip of the invadopodia flattens as it interacts with the ECM, and it undergoes constant rapid ruffling. Invadopodia precursors are assembled by actin polymerization machinery containing Cdc42, WASp, the Arp2/3 complex, and cortactin in response to extracellular stimuli (Yamaguchi *et al.,* 2005; Weaver, 2006, Artym *et al.,* 2010). Cofilin severing increases the number of free barbed ends to initiate actin polymerization, which enhances further invadopodial growth (reviewed in Oser and Condeelis, 2009). Finally these structures gather matrix metalloproteinases to mature into functional invadopodia (Yamaguchi *et al.,* 2010). *In vitro*, invadopodia are induced to form by plating cells on 3D matrix cushions in the presence of growth factors, and are typically recognized by colocalization of invadopodia markers with degradation of the surrounding fluorescently-labeled ECM (Figure 2) (Weaver, 2006, Artym *et al.,* 2009).

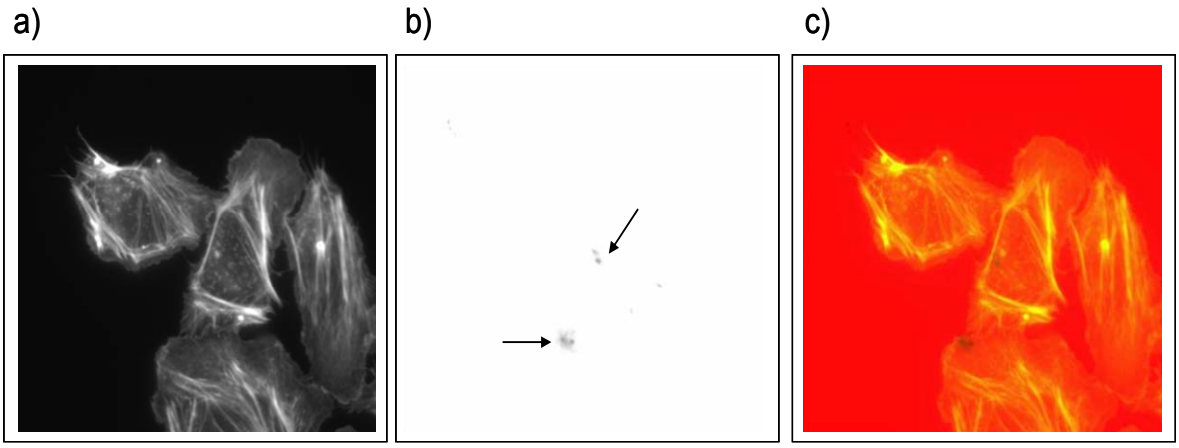

Figure 2. Invadopodia formation in mammary adenocarcinoma MTLn3 cell. (a) MTLn3 breast cancer cells assemble actin filaments to invade into a fluorescently-labeled gelatin film. (b) Matrix digestion is evident as the dark sites where the gelatin has been digested by invadopodia. (c) Merged image of a and b, actin filaments (green) and fluorescently-labeled gelatin (red). Scale bar, 10 μM.

4. ADF/cofilin proteins during metastasis

The first step of migration is actin polymerization, which drives the formation of cell protrusions, which adhere to the extracellular matrix, define the direction of migration, and initiate cell crawling (Nobes and Hall, 1999). ADF/cofilin proteins and their regulatory proteins modulate actin assembly in most mammalian cells and are involved in the initiation of the early steps in the motility cycle (reviewed in Van Troys *et al.*, 2008). ADF/cofilin proteins have molecular masses between 13-19 kDa (reviewed in Bamburg, 1999). ADF was named because of its ability to depolymerize F-actin and form a complex with G-actin in a 1:1 ratio, while cofilin was named because it co-sediments with F-actin. However, these are pH-dependent activities, and both proteins can bind and co-sediment with F-actin as well as sever filaments and increase dynamics of turnover, but to different extents.

ADF/cofilin proteins bind preferentially to ADP-F-actin (Kudryashov *et al*., 2010). They enhance the turnover of actin subunits in actin filaments by binding to a slightly twisted form of F-actin, thus stabilizing the twisted state, enhancing severing and providing more filaments ends to nucleate growth or enhance disassembly (Andrianantoandro and Pollard, 2006). After disassembly, Srv2/CAP and profilin induce a rapid exchange of ATP for ADP on the G-actin, releasing ADF/cofilin proteins for recycling to F-actin to repeat their dynamizing effects (Bertling *et al.,* 2007). Profilin, a major ATP-actin binding protein, promotes the addition of actin onto filament barbed ends (Didry *et al.*, 1998).

Of the three ADF/cofilin isoforms, ADF is the most efficient in turning over actin filaments (Vartiainen *et al.,* 2002). Cofilin-1 is more efficient than ADF in nucleation and severing of F-actin (reviewed in Bernstein and Bamburg, 2010). However, the severing activity of cofilin-

1 shows a biphasic concentration dependence, first increasing, and then decreasing with cofilin concentration, i.e., at high molar ratio with actin, cofilin-1 promotes filament assembly (Pavlov *et al.,* 2007). Cofilin-2 has weaker F-actin depolymerization activity than ADF and cofilin-1 and promotes filament assembly, rather than disassembly (Chen *et al.,* 2004; Nakashima *et al.,* 2005).

In cultured cells, the housekeeping functions of cofilin that are blocked by cofilin knockdown can be rescued by expressing ADF; the opposite is also true, cofilin can rescue ADF down regulation (Hotulainen *et al.,* 2005). In contrast, in more complex conditions such as during specific developmental or physiological processes, different AC isoforms display distinct effects which demonstrate that these isoforms have qualitatively similar but quantitatively different effects on actin dynamics (Lehman *et al.,* 2000; Ono *et al.,* 2003; Gurniak *et al.,* 2005).

4.1. Structure of ADF/cofilin

ADF and cofilin from a single organism share about 70% sequence identity (reviewed in Bamburg, 1999). Vertebrate AC proteins have a nuclear localization sequence (NLS) which allows them to chaperone actin into the nucleus (Matsuzaki *et al.,* 1988; Iida *et al.,* 1992). ADF/cofilin proteins have a characteristic single domain called the ADF-homology domain (ADF-H) (reviewed in Van Troys *et al.,* 2008). The ADF-H consists of a four stranded mixed β-sheet surrounded by four α-helices (Lappalainen *et al.*, 1997; reviewed in Maloney *et al.,* 2008).

Cofilin-1 has four cysteine (Cys) residues (C39, C80, C139 and C147) that are targets of oxidation and can form specific intramolecular disulfide bonds (C39-C80 and C139-C147). The formation of both intramolecular disuflide bonds is required to eliminate actin binding (Klamt *et al.,* 2009). Oxidized cofilin-1 gets translocated to mitochnodria where it causes the release of cytochrome c, an early step in apoptosis (reviewed in Bernstein and Bamburg, 2010). ADF has seven Cys residues but only three (C39, C80 and C147) are conserved with those in cofilin-1 (reviewed in Bernstein and Bamburg, 2010). Thus, ADF might not be targeted to mitochondria during oxidative stress, even though this has not been directly tested.

Recently, cofilin-1 was found to be a substrate for v-Src (activated Src tyrosine kinase) phosphorylation on tyrosine (Y) 68 (Y68) (Yoo *et al.,* 2010). This post-translational modification of cofilin makes it a target for the ubiquitin-proteosome pathway. Cofilin degradation affects its cellular functions on actin dynamics such as enhanced cell spreading and the G-actin/F-actin ratio (Yoo *et al.,* 2010). ADF has a phenylalanine (F) residue at position 68 (F68) and thus is not a substrate for Src-mediated phosphorylation and accompanying ubiquitin degradation (reviewed in Bernstein and Bamburg, 2010).

4.2. Tissue/Cellular Distribution of ADF/cofilin

The relative expression levels of the three ADF/cofilin proteins (ADF, cofilin-1 and cofilin-2) vary in a spatial and temporal-specific manner (Gurniak *et al.,* 2005). During the development of mice, cofilin-1 is the predominant one and it remains ubiquitously expressed in most adult tissues (Gurniak *et al.,* 2005). ADF is expressed at low levels during mouse embryonic development with the highest levels found in embryonic heart and the adaxial region of somites of E10.5 embryos (Gurniak *et al.,* 2005). ADF is upregulated after birth in epithelial and endothelial tissues (Gurniak *et al.,* 2005; Vartiainen *et al.,* 2002). In the chick system, which has only ADF and cofilin-2, ADF becomes post-natally upregulated in the nervous system and in epithelial and endothelial tissues such as intestine, kidney and testis (Bamburg and Bray, 1987).

In late embryogenesis and after birth, cofilin-2 replaces cofilin-1 in striated muscle, becoming the major isoform expressed in differentiated skeletal muscle and cardiac muscle (reviewed in Van Troys *et al.,* 2008). The human cofilin-2 gene yields two mRNAs with identical coding sequence. The first (cof-2b) is strongly expressed in skeletal muscle and heart and the second (cof-2a) is expressed at lower levels in other tissues (Thirion *et al.* 2001; reviewed in Maloney *et al.,* 2008).

ADF and cofilin often show diffuse immunostaining in resting cells; they get translocated upon stimulation to the leading edge of motile cells such as *Dictyostelium*, neutrophils, HL-60 cells and chick cardiac fibroblasts (Bamburg and Bray, 1987; Aizawa et al., 1995; Suzuki et al., 1995; Djafarzadeh and Niggli 1997; Dawe et al., 2003). Inactive cofilin is found to be highest in the perinuclear region in motile colon adenocarcinoma NRK39 and LS180 cell lines, whereas active cofilin is localized with actin filaments at the periphery (Nowak *et al.,* 2010). In resting breast adenocarcinoma MTLn3 cells, cofilin is distributed diffusely in the cytoplasm. However, upon stimulation with epidermal growth factor (EGF), cofilin is recruited to the leading edge (Chan *et al.,* 2000). A similar situation is observed in morphologically polarized neurons, which have a higher ratio of total cofilin to the inactive phospho-cofilin in the growth cones of their more rapidly extending axons, compared with growth cones of slower growing minor processes (Garvalov *et al.,* 2007). In spontaneously polarizing chick cardiac fibroblasts, cofilin is required for the formation of oriented actin-filament bundles in the cell body, a process needed to coordinate the spatial location of the cell rear and front during fibroblast polarization (Mseka *et al.,* 2007).

4.3. Regulation of ADF/cofilin

ADF and cofilin from metazoans are complexly regulated. One principal mechanism is their inhibition by phosphorylation on a serine residue near the N-terminus (Ser3 of encoded sequence). The phosphorylated form does not bind to either G- or F-actin (Ressad *et al.,* 1998; Blanchoin *et al.,* 2000; Bamburg and Wiggan, 2002). There are two major kinase families that phosphorylate ADF/cofilin proteins in animal cells, the LIM kinases (LIMK) and testicular kinases (TESK). LIMKs are downstream effectors of the Rho-family GTPases, they can be activated by phosphorylation by the Rac- and Cdc42-activated kinase PAK, or by the Rho kinase ROCK (reviewed in Van Troys *et al.,* 2008). The dephosphorylation of ADF and cofilin is regulated by several phosphatases, particularly the slingshot phosphatase (SSH) family and chronophin (CIN), also

known as pyridoxal-5-phosphate phosphatase (Figure 3) (Huang *et al.,* 2006).

G- and F-actin binding of ADF/cofilin is inhibited by phosphoinositides, because the phosphoinositide-binding site, a large positively charged surface, overlaps with the G- and F-actin binding sites. ADF/cofilin proteins do not display phosphoinositide specificity; i.e., they bind phosphatidylinositol 4,5-bis-phosphate ($PI4,5P_2$), phosphatidylinositol-3,4-bis-phosphate ($PI3,4P_2$) and phosphatidyinositol 3,4,5-tris-phosphate ($PI3,4,5P_3$) and have been proposed to act as a sensor for PIP_2-density on the plasma membrane (Zhao *et al.,* 2010). The $PI4,5P_2$-hydrolysing enzyme phospholipase C (PLC) releases active cofilin from the membrane in various stimulated cells (Matsui *et al.,* 2001; Zhou *et al.,* 2007).

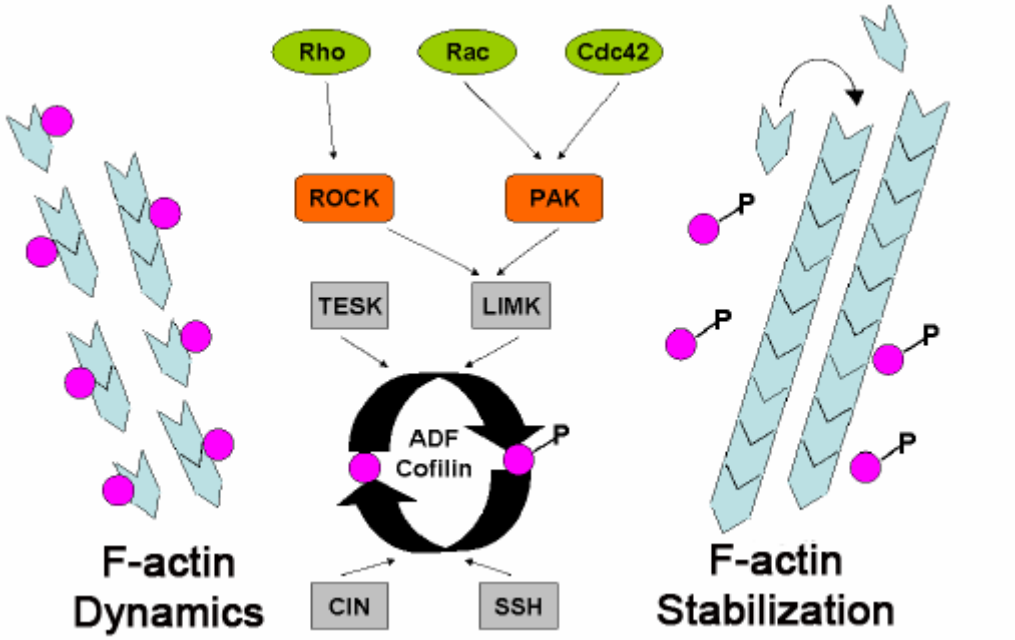

Figure 3. Regulation of ADF/cofilin. AC proteins enhance the turnover of actin filaments by inducing filament severing. LIMK and TESK phosphorylate AC at Ser3 inhibiting their actin-binding activity, while slingshot (SSH) and chronophin dephosphorylate AC proteins activating them.

AC proteins are pH-dependent in their interactions with F-actin; at pH< 7.0, both ADF and cofilin, when present in excess over actin, slightly increase the G-actin pool while binding and co-sedimenting with F-actin at a stoichiometry of 1:1 with actin subunits. Increasing intracellular pH results in more ADF colocalizing with G-actin, but the pH shift has little effect on cofilin, which remains mostly F-actin associated (Bernstein *et al.,* 2000).

The activities of ADF and cofilin are regulated by other mechanisms including interaction with actin-interacting protein 1 (Aip1) which binds the ADF/cofilin/actin complex. Aip1 enhances the ability of ADF/cofilin proteins to sever actin filaments, and may also accelerate depolymerization by capping their barbed ends (Okada *et al.,* 2002; Mohri *et al.,* 2006; Kile *et al.,* 2007). Other ADF/cofilin regulatory mechanisms include competition with tropomyosin for actin binding (DesMarais *et al.,* 2002; Bryce *et al.,* 2003; Kuhn and Bamburg, 2008), compartmentalization (Nebl *et al.,* 1996) and differential stabilization of ADF and cofilin mRNAs by the actin monomer pool (Minamide *et al.,* 1997).

Recently, it has been found that cofilin-1 is regulated by microRNAs (miRNAs) which are short (21-22 nucleotides) noncoding RNAs that bind the 3' untranslated region (UTR) of mRNAs and mainly repress translation. Two miRNAs (miR-103 or miR-107) repress cofilin-1 translation and when their levels are reduced in transgenic mouse model, cofilin proteins levels are increased accompanied with the formation of rod-like structures (Yao *et al.,* 2010). There is no sequence homology between miR-103 or miR-107 and ADF cDNA, suggesting a specific targeting of cofilin and not ADF by these miRNAs.

4.4. Role of ADF and cofilin during metastasis

Trying to decipher from the literature the specific role(s) of ADF and cofilin-1 during metastasis is challenging because most researchers, studying the role of these proteins during invasion, focus on cofilin-1. Although ADF and cofilin can substitute for one another for most housekeeping activities in cultured cells (Hotulainen *et al.,* 2005), this is not always the case during development. Cofilin-1 null mice are not viable despite the fact that ADF is upregulated (Gurniak *et al.,* 2005). In contrast, ADF null mice are viable but show abnormal corneal thickening, suggesting that cofilin-1 can rescue the lack of ADF except in corneal epithelial cells (Ikeda *et al.,* 2003). This finding demonstrates that AC isoforms are not completely redundant. However, in ureteric bud (UB) epithelium ADF and cofilin show considerable functional overlap; deletion of cofilin-1 in UB epithelium or an inactivating mutation in ADF had no effect on renal

morphogenesis, but simultaneous lack of both genes arrested branching morphogenesis at an early stage (Kuure *et al.,* 2010).

Silencing cofilin-1 in a highly invasive colorectal cancer cell line (Isreco1) did not interfere with its ability to undergo transwell migration across collagen in response to a chemotactic attractant, whereas silencing of ADF, which represented only 17% of the total ADF/cofilin, significantly inhibited transwell migration, strongly suggesting different cellular functions of each protein (Estornes *et al.,* 2007). ADF generates a larger actin monomer pool than cofilin (Yeoh *et al.,* 2002); thus activation of ADF in cell regions away from the leading edge could be necessary to provide a pool of subunits that maintains leading edge protrusion during migration.

Too much or too little cofilin activity inhibits membrane protrusions required for motility and chemotaxis. However, the literature in this area is sometimes confusing. For example, a moderate (two-to four-fold) overexpression of cofilin at the protein level increases the velocity of cell migration in *Dictyostelium* (Aizawa *et al.,* 1995) and human glioblastoma cells (Yap *et al.,* 2005), but higher levels of expression inhibit cell motility (Lee *et al.,* 2000). Expression of wild type or a non-phosphorylatable cofilin mutant in which serine3 has been mutated to alanine (S3A) increases melanoma cell invasion through a reconstituted basement membrane (Dang *et al.,* 2006). There are, however, several studies showing that cofilin is down regulated in other cancers and that its overexpression is antagonistic to invasion. Furthermore, expression of LIMK1 in human breast cancer cells, which should lower active AC, enhanced cell proliferation, invasiveness and *in vitro* angiogenesis (Bagheri-Yarmand *et al.,* 2006).

Further confusing information on the role of AC proteins comes from other cancer studies. In support to the more active cofilin in invasive cancers is the report that the amount of phosphorylated cofilin is decreased in cell lines derived from T-cell lymphoma (Jurkat) and carcinomas from the cervix (HeLa), colon (KM12) liver (HepG2) and kidney (COS1) (Nebl *et al.,* 1996). Increased levels of cofilin mRNA and protein are detected, using proteomic and cDNA microarray approaches, in clinical tumor samples of oral squamous-cell carcinoma (Turhani *et al.,* 2006), renal cell carcinoma (Unwin *et al.,* 2003), and ovarian cancer (Martoglio *et al.,* 2000); cofilin-1 was found to be significantly increased in the saliva of patients of head and neck squamous cell carcinoma (Dowling *et al.,* 2008). On the other hand, suppression of LIMK2 in human fibrosarcoma cells, which should increase active cofilin, limits their migration and efficiency to form dense colonies without affecting cell proliferation rate or viability (Suyama *et al.,* 2004; Vlecken and Bagowski, 2009).

These discrepancies are most readily explained if every cell has an optimum amount and activity of cofilin that is required to counter balance the effects of other proteins that antagonize cofilin's ability to sever and dynamize F-actin. Among these other proteins are tropomyosins, most of which bind and stabilize the untwisted form of actin and compete with ADF/cofilin for binding (DesMarais *et al.,* 2002), and cortactin, which binds to ATP and ADP-Pi forms of actin subunits in F-actin (Bryce *et al.,* 2005) and slows down the Pi release, which is necessary to generate the ADP-actin form recognized by ADF and cofilin (Oser *et al.,* 2009). Furthermore, cortactin directly binds cofilin, and releases it in a cortactin phosphorylation–dependent manner (Oser *et al.,* 2009). Thus different cell types could have cofilin activities that lie on opposite sides of the peak of a bell shaped curve that describes optimal cofilin activity for polarized migration. In some tumor cells, cofilin would need to be upregulated or activated to shift toward optimal activity whereas in other cell types this shift would require its inactivation or down regulation.

In addition or alternatively, in different cells the optimal activity of ADF and cofilin may need to be adjusted and will depend on the ratio of ADF/cofilin in each cell type or perhaps even within each cell. Also, it would be interesting to know how Aip1 changes along with ADF and cofilin in tumor cells. Mutational studies of Aip1 showed that the severing and capping activities are differently affected by point mutation, indicating that these activities might be uncoupled (Mohri *et al.,* 2006). Thus, further investigations are needed to understand which activity of Aip1 is required for ADF/cofilin-mediated metastasis. Further studies are also required to better understand the collaboration between tropomyosin isoforms, profilin, ADF/cofilin and Aip1 in organizing the actin cytoskeleton in tumor cells, as was done for studying the role of these proteins in sarcomeric actin organization (Yamashiro *et al.*, 2008).

Invasive cancer cells form invadopodia which aid in the cell's ability to escape from a surrounding basal lamina and pass across the endothelial cell barrier of capillaries (Artym *et al.,* 2009). The formation of invadopodia requires cofilin for initiation, stabilization and maturation (Yamaguchi *et al.,* 2005). Silencing cofilin expression interferes with long-lived invadopodia and decreases matrix degradation activity in metastatic carcinoma cells. When unpolarized highly metastatic breast tumor cells (MTLn3) become polarized by stimulation, unphosphorylated (active) ADF/cofilin is released from PI-4, 5-P_2 at the membrane. Growth factors, such as EGF, locally activate phospholipase C, cleaving membrane PI-4, 5-P_2 and freeing cofilin to sever filaments, giving rise to the increase in F-actin barbed ends that occurs about 1 min. after treatment; the formation of the barbed ends is necessary to initiate assembly of an invadopodium, the structure that is needed for tumor cells to escape into the vasculature (Mouneimne *et al.,* 2004; Sidani *et al.,* 2007, van Rheenen *et al.,* 2007).

In conclusion, determining the expression status of cofilin alone is insufficient to describe the characteristics of tumour cells, such as proliferation, migration and invasion. Rather, the balanced contribution of cofilin and ADF and other molecules in the ADF/cofilin regulatory pathways has to be taken into consideration. In addition, there is a great need of investigating not only single components of the ADF/cofilin pathways, but rather multiple key regulators and the final output of any single component.

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