

Characterization Of Coronin 2A And Its Role In Regulating Cofilin Activity

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Abstract

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(under the direction of Dr. James E. Bear)

Coronins are conserved F-actin binding proteins that are important for motility and actin dynamics. Mammalian Coronin proteins can be broken down into three subtypes: Three Type I Coronins (consisting of Coronin 1A, 1B and 1C), two Type II Coronins (Coronin 2A and 2B), and one Type III Coronins (POD/Coronin 7). Each of these types has distinct localization patterns in the cell. Type I Coronins primarily localize to lamellapodial F-actin structures found at the leading edge of cells. Unlike type I Coronins, the type II Coronin 2A is excluded from the leading edge and localizes to stress fibers and focal adhesions. Studies on POD suggest that it primarily localizes to the Golgi apparatus. Depletion of Coronin 2A in MTLn3 cells decreases cell motility and focal adhesion turnover. Surprisingly, none of the pathways known to regulate focal adhesion turnover are affected by Coronin 2A depletion. Depletion of Coronin 2A does however increase phospho-Cofilin suggesting that misregulation of Cofilin may affect adhesion dynamics. Slingshot-1L, a Cofilin-activating phosphatase, localizes to focal adhesions and interacts with Coronin 2A. Depletion of Coronin 2A reduces Cofilin activity at focal adhesions as measured by barbed end density and actin turnover. Consistent with this idea, expression of an active mutant of Cofilin

bypasses the defects in cell motility and focal adhesion disassembly seen upon Coronin 2A depletion. These results implicate both Coronin 2A and Cofilin as new factors that can regulate focal adhesion turnover. Enforced expression of Coronin 2A induces the formation of structures that are similar to Cofilin-Actin rods. These are ordered aggregates that form in certain cells in response to stress, such as ATP-depletion. Like Cofilin-Actin rods, Coronin 2A rods also contain Cofilin and Actin, but unlike Cofilin-Actin rods, Coronin 2A rods stain positively with phalloidin. These data suggest that Coronin 2A may form submit Cofilin-Actin rod intermediates that require further investigation.

Dedication

This work is dedicated to my grandma Marie Schwacke who turned 100 years old this year. Her unbelievable longevity showed me that what I have been able to accomplish over the last six years is just a drop in the bucket of what I will hopefully be able to in my lifetime. Also to my family, friends, and Gwendolyn, the love of my life. Without them, my work would be for naught.

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My friends and family have always been there for me. Supporting me in the bad times and making the great times even better. I love you all.

Lastly, to my girl, Gwendolyn: I am the man I am today because of you. You brighten my day and make all the bad in the world mean nothing. I love you forever.

Table of Contents

TABLE OF CONTENTS.....	vi
LIST OF FIGURES.....	viii
LIST OF ABBREVIATIONS.....	x
CHAPTER 1: Introduction.....	1
Microtubule targeting of focal adhesions.....	4
Calpain cleavage of Talin.....	4
Myosin II based contraction.....	5
FAK-Dynamin II interactions.....	6
Stress fiber-focal adhesion interactions.....	6
Cofilin regulation.....	7
Chronophin.....	11
Slingshot.....	12
Coronins.....	12
CHAPTER 2: Coronin 2A regulates focal adhesion turnover through the Cofilin Pathway.....	17
Summary.....	17
Introduction.....	18
Results.....	21
Discussion.....	46

Materials and Methods.....	50
CHAPTER 3: Ectopic expression of Coronin 2A induces Cofilin-Actin rod formation.....	56
Summary.....	56
Introduction.....	57
Results.....	60
Discussion.....	70
Materials and Methods.....	73
CHAPTER 4: Conclusions and future prospects.....	77
REFERENCES.....	87

List of Figures

Figure 1: Diagram of two-dimensional cell migration.....	2
Figure 2: Diagram of Mammalian Coronin structure.....	13
Figure 3: Coronin 2A localizes to F-actin stress fibers and focal adhesions, but is excluded from the leading edge	22
Figure 4: Depletion of Coronin 2A impairs cell motility, but does not affect lamellipodial dynamics.....	24
Figure 5: Depletion of Coronin 2A increases focal adhesion size, decreases focal adhesion number, and decreases focal adhesion disassembly.....	28
Figure 6: Depletion of Coronin 2A increases P-Cofilin levels, but has no affect on other focal adhesion turnover protein components.....	30
Figure 7: EB1 dynamics in cells depleted of Coronin 2A.....	32
Figure 8: Coronin 2A interacts with Slingshot-1L and co-localizes at focal adhesions.....	34
Figure 9: Active mutants of Slingshot increase focal adhesion disassembly and partially bypass decreases in focal adhesion disassembly caused by depletion of Coronin 2A.....	37
Figure 10: Cofilin localizes to the proximal end of focal adhesions in fixed and live cells....	40
Figure 11: Coronin 2A depletion leads to reduced barbed end density and actin turnover at focal adhesions.....	42
Figure 12: Expression of Cofilin S3A rescues cell motility and focal adhesion disassembly defects caused by depletion of Coronin 2A.....	45

Figure 13: Ectopic expression of Coronin 2A induced rod-like aggregates that contain Cofilin and Actin.....	61
Figure 14: Ectopic expression of Coronin 2A-TagRFP does not affect Cofilin dephosphorylation upon ATP-depletion.....	63
Figure 15: Ectopic expression of Coronin 2A does not affect ATP-depletion induced Cofilin dephosphorylation or cell proliferation.....	65
Figure 16: Coronin 2A is immobile and Cofilin is mobile in Coronin 2A-induced rods.....	68

List of Abbreviations

FAK	Focal Adhesion Kinase
ER	Endoplasmic Reticulum
RNAi	Ribonucleic acid interference
ADF	Actin Depolymerizing Factor
P-Cofilin	Serine 3 phosphorylated Cofilin
F-actin	filamentous actin
AIP1	Actin-interacting protein 1
LPS	Lipopolysaccharide
Srv2	suppressor of <i>rasVall4</i>
CAP	Cyclase associated protein
Memo	Mediator of ErbB2-driven cell motility
HRG	Herugulin
PLD1	Phospholipase D1
PA	Phosphatidic acid
TPI	Triose-Phosphate Isomerase
PKD1	Protein Kinase D1
PXN	Paxillin
LIMK	LIM Kinase
TESK	TES Kinase

Coro2A-GFP	Coronin 2A-Green Fluorescent Protein
shCoro2A	short hairpin RNA against Coronin 2A
shNS	short hairpin RNS against non-specific sequence
qRT-PCR	quantitative real-time polymerase chain reaction
SSH1L-GFP	Slingshot-1L-Green Fluorescent Protein
BE	Barbed End
FA	Focal Adhesion
FRAP	Fluorescence recovery after photobleaching
ROS	Reactive Oxygen Species

Chapter 1: Introduction

Cell migration in multicellular organisms is required for many processes, such as embryonic development, the formation of the immune synapse, and epithelial tissue repair. The F-actin cytoskeleton is a major structural component in cells that is modulated during cell migration. Inappropriate changes in the actin cytoskeleton can lead to disease states such as cancer, muscular dystrophy or Alzheimer's disease. Within any cell, there are many proteins that perform specific functions to regulate the integrity of F-actin structures. While many of the proteins that interact or affect F-actin have been identified, how these proteins coordinate their activities are still being determined.

The process of two dimensional cell migration can be broken down into four steps (Outlined in Figure 1) (reviewed in [1, 2]). The precise regulation of each step is imperative for coordinated migration. First, a cell extends a membrane protrusion in the direction of migration. The formation of this membrane protrusion, or leading edge, establishes cellular asymmetry, giving the cell a front and back or tail. The protruding leading edge, also known as the lamellipodia, is full of densely packed branched F-actin networks that provide the force required to push the cell membrane forward. In the second step, integrin-based adhesions form at the periphery of the cell and attach the cell to the substratum (usually to an extracellular matrix). Many proteins become incorporated or removed from these adhesions to affect their stability, size, turnover and dynamics. These changes can occur through lipid or protein signaling, protein modifications, or combinations of these events. Reports suggest

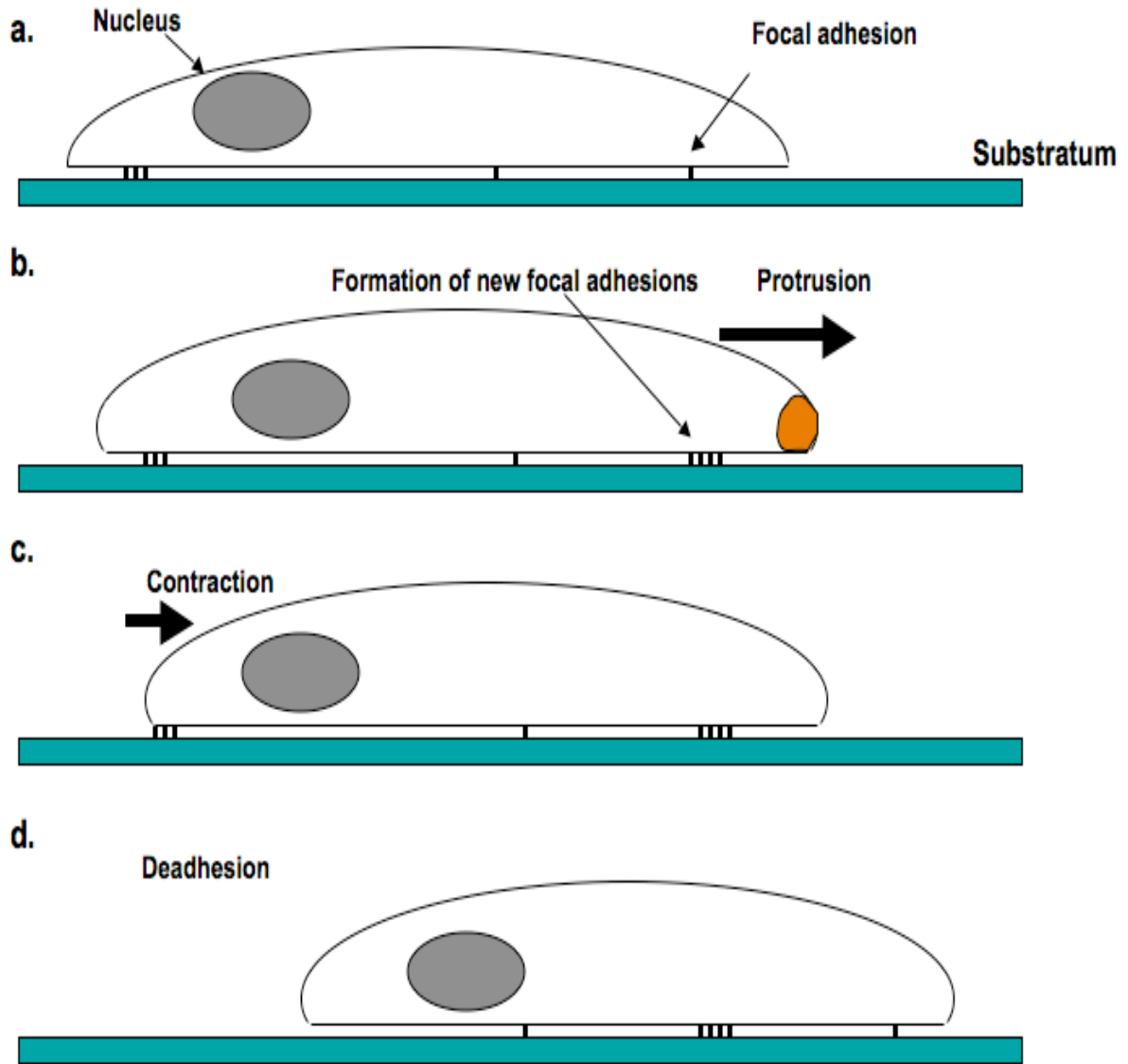


Figure 1. Diagram of two dimensional cell migration

(a) Resting cell.

(b) Stimuli induces leading edge protrusion and the formation of new focal adhesions.

(c) Myosin-based contraction pulls the cell forward.

(d) De-adhesion at the rear of the cell and the cell moves forward.

that at least 150 proteins localize to focal adhesions [3]. These adhesions either mature into larger interior adhesive plaques, called focal adhesions, or are disassembled depending on the environment of the adhesion and how the cell is responding to internal and external signaling events. The third step in cell migration occurs through myosin-based contractile forces along F-actin filaments through the focal adhesions. This is most commonly observed in the internal lamella region of the cell where the focal adhesions are connected to bundled-actin filaments called stress fibers. Lastly, the cell detaches at the rear of the cell allowing the cell to proceed in a forward direction. At this point the cell disengages the focal adhesion-stress fiber-substratum connections and the cell rear moves in the direction of migration. In actuality, all of these processes act in concert with one another and are continuously occurring during cell migration. Signaling molecules, like the Rho family of GTPases, affect all of these events by inducing the polymerization/depolymerization of F-actin networks that produce the forces required for membrane protrusion, changes in focal adhesion signaling proteins and focal adhesion dynamics, and myosin-based contraction [1].

Up to now, most studies on cell migration have primarily focused on the roles of lamellipodial proteins required for whole cell motility (reviewed in [4]), but there is growing interest in what proteins are involved in regulating focal adhesion dynamics and what proteins affect cell motility by changing cell adhesion. Studies on proteins that are known to regulate focal adhesion dynamics, like Focal adhesion kinase (FAK) [5] [6], ACF7 [7, 8], and Arg [9] [10], have shown that there are dramatic changes in cell speed and how cells move when focal adhesions are misregulated. In disease states, other focal adhesion localized proteins more than likely affect focal adhesion dynamics as well due to protein misregulation or changes in the level of protein expression, but they have not been thoroughly tested.

Mechanisms involved in focal adhesion formation, maturation, and disassembly are incomplete, but some of the requirements for focal adhesion disassembly have arisen (reviewed in [11]). These include microtubule targeting events[6, 12, 13], Calpain-cleavage of Talin and probably other focal adhesions proteins like Vinculin [14-18], Myosin II based contraction [19, 20], and FAK-Dynamin II interactions[6]. This list is incomplete and how these mechanisms coordinate with one another has not been addressed at all. Another mechanism that has not been thoroughly investigated is how do the interactions between F-actin and focal adhesion proteins change during focal adhesion turnover?

Microtubule targeting of focal adhesions

Microtubule networks, another core cytoskeletal component, target focal adhesions for disassembly. The plus-end of microtubules “touch” focal adhesions, usually multiple times, leading to disassembly [12, 13, 21]. This phenomenon has been observed under various cell contexts, but what is actually on the tips of microtubules that induce disassembly remains a mystery. Since microtubules interact with Endoplasmic Reticulum (ER) tubules [22], it may be possible that ER tubules are targeted to focal adhesions by microtubules. ER tubules could then release calcium at the focal adhesion, which induces signaling events required for focal adhesion disassembly. While this is just a hypothesis, calcium has been shown to regulate the functions of focal adhesion proteins, including Calpain, a calcium dependent protease known to cleave focal adhesion proteins.

Calpain-cleavage of Talin

For many years, it was known that western blots for Talin displayed a cleavage product caused by protein cleavage by Calpain [14-16]. The role of this event remained illusive until experiments showed that depleting cells of Calpain by RNAi displayed significantly decreases cell motility and focal adhesion disassembly [17, 23]. It is unclear as to why this is required for focal adhesions to disassemble. Protein degradation of these cleaved focal adhesion proteins is a likely event, but direct evidence of this is lacking. Other studies suggest that cleavage of PTP1B, a tyrosine phosphatase, by Calpain is required for invadopodia formation and that the cleaved product(s) of PTP1B have function that is/are in needed for proper invadopodia disassembly [24]. Invadopodia are invasive, adhesive structures that display some similar characteristics to that of focal adhesions. While the role of Calpain at focal adhesions is clearly important, further investigation is needed to elucidate the role of Calpain and the cleaved protein products in focal adhesions and how this affects turnover and focal adhesion dynamics.

Myosin II based contraction

The balance between actin polymerization and myosin-based contraction is required for cell movement to occur, but myosin contraction is also important for focal adhesion disassembly. Inhibition of Myosin with ML-7 decreases focal adhesion disassembly ~12 fold [20]. While focal adhesion assembly is misregulated by diminishing myosin II activity by either depletion of myosin II with RNAi or with inhibitors like blebbistatin [25, 26], the contraction along stress fibers connected is also needed for tail retraction. How this activity promotes focal adhesion disassembly is still unclear.

FAK-Dynamin II interactions

FAK is an extremely complex protein that has been shown to have many effects on cell migration, cancer progression and tissue homeostasis. FAK functions as a hub for protein-protein interactions and modifications such as tyrosine phosphorylation. It is likely that FAK structural and/or signaling properties are required for focal adhesion turnover, but the mechanism is yet to be identified. Mouse embryonic fibroblasts from FAK knockout animals display impaired motility and cell spreading [5]. These cells display significantly less focal adhesion disassembly compared to wild type cells [20]. Conditional gene targeting of FAK in mouse models of aggressive breast cancers display delayed cancer initiation and fewer metastases [27]. These are indications that regulation of focal adhesion dynamics through FAK activity may be important for cancer metastasis to occur. Other in vitro analysis shows that FAK regulation is important for focal adhesion turnover. Nocodazole washout experiments, a model used to synchronize focal adhesion disassembly, show that FAK is dephosphorylated and interacts with Dynamin 2, a protein that is known to regulate endocytosis, during focal adhesion disassembly [6]. Disassembly of FAK positive focal adhesions can be diminished in cells expressing dominant negative Dynamin 2. Dynamin 2 activity may be a useful way to turnover focal adhesions by either recycling proteins to new sites of adhesion or removing proteins by protein degradation. While these are possibilities, Dynamin 2 may have other unknown function that affect focal adhesion turnover.

Stress fiber-focal adhesion interactions

Many proteins that localize to focal adhesions, including Talin, Vinculin, Zyxin, and α -actinin, have one or more F-actin binding sites. These F-actin binding components of

focal adhesions appear to be important for connecting F-actin stress fibers to focal adhesions, but the role of these interactions has not been completely addressed. Forces generated by myosin-based contractility can cause conformational changes in at least some of these focal adhesion proteins, leading to interactions with other proteins and changes in signal transduction [28, 29]. Up to now, little is known about how focal adhesion-F-actin interactions are regulated. One mechanism is that Calpain cleavage of focal adhesion proteins may disrupt the entire complex and promote protein degradation of other focal adhesion proteins. While this has not been thoroughly proven, protein cleavage could either promote the degradation of focal adhesion proteins or a change in the conformation upon cleavage may affect F-actin binding.

Another study looked at focal adhesion proteins and F-actin interactions by speckle microscopy. In these studies, certain focal adhesion proteins resided predominantly with F-actin or with the integrins that were directly linked to the extracellular matrix. Focal adhesion proteins have been shown to sometimes stably associate with integrin based structures, while at other times move with F-actin retrograde flow. This has been referred to as a “clutch” like mechanism that describe how focal adhesion proteins affect F-actin interactions. [30]. It was not completely clear if this mechanism dissociates the stress fiber from the focal adhesion or if the interactions were just modified in some unknown way. Regardless, it is interesting to think about how focal adhesion proteins bind with F-actin and what regulates these interactions.

Cofilin Regulation

Actin depolymerizing factor (ADF)/Cofilin family of proteins (hereafter referred to as Cofilin) are widely studied, extremely complex, abundant proteins that are essential for mammalian development and cell motility through their ability to bind to G- and F-actin (reviewed in [31]). Studies focused on the F-actin regulatory activity of Cofilin have shown that active Cofilin binds preferentially to ADP-Actin filaments causing a change in the twist of the filament. This destabilizes the filament, inducing severing and/or depolymerization. Cofilin activity is regulated in many ways, but mostly commonly by phosphorylation at Serine 3. The phosphorylation of Cofilin by LIM or TES kinases inhibits Cofilin from binding either G- or F-actin [32, 33]. This is reverted to an active, dephosphorylated state by either the Slingshot family [34] or Chronophin [35] phosphatases. Other forms of regulation include interacting with PI(4,5)P2 [36] or changes in intracellular pH [37, 38]. All of these forms of regulation affect Cofilin's ability to bind to F-actin by either interacting with the F-actin binding site or affecting the structure of the protein [39]. There is also increasing data that protein-protein interactions affect Cofilin severing/depolymerization activity and other non-F-actin severing associated activities as well.

Beyond being phosphorylated, Serine 3 phosphorylated Cofilin (P-Cofilin) is further inhibited by interacting with 14-3-3 proteins [40]. This interaction with P-Cofilin is believed to isolate Cofilin in the cytoplasm of the cell. Since Slingshot, a Cofilin activating phosphatase, also interacts with 14-3-3 [41] and 14-3-3 proteins associated with Slingshot can dimerize [42], it is possible that a complex of these proteins form in which Slingshot phosphatase activity can dephosphorylate Cofilin, promoting its severing activity. Slingshot also binds directly to F-actin suggesting that Slingshot may not only activate Cofilin by dephosphorylation, but can properly localize Cofilin to F-actin structures to promote severing

[34]. Intriguingly, *in vitro* studies suggest the opposite to occur. In these assays, actin filaments pre-incubated with Slingshot decreases Cofilin-F-actin binding, a requirement for Cofilin severing activity [43]. While this may be true, another explanation is that an overabundance of Slingshot may saturate the F-actin binding sites for Cofilin, which prevents optimal Cofilin-F-actin stoichiometry from occurring. Slingshot can also bundle F-actin [43], which may reduce Cofilin's affinity for filaments. Another explanation is other F-actin and/or Slingshot interacting proteins may be needed to precisely localize Cofilin severing activity in a spatial-temporal manner to more tightly regulate where and when Cofilin is activated. For example, Coronin 1B has been shown to localize Slingshot the back of the lamellipodia [44]. Without this precise localization, a larger fraction Cofilin remains phosphorylated and actin turnover at the back of the lamellipodia is reduced. Other proteins may perform similar roles in regulating Slingshot and/or Cofilin activity in a spatial-temporal manner.

Other proteins that directly interact with Cofilin increase Cofilin severing activity. Actin-interacting protein 1 (AIP1), also known as WDR1, a protein that contains two seven-bladed β -propellers, can directly interact with Cofilin [45]. It was initially believed that AIP1 capped barbed ends and that this allowed for more severing to occur and prevent polymerization at the barbed end. While AIP1 does potentiate Cofilin's ability to diminish F-actin, it is not by capping barbed ends. Unlike, Gelsolin, AIP1, in the context of *in vitro* depolymerization/repolymerization assays, was unable to prevent polymerization at newly formed barbed ends [46]. How this works mechanistically is still undetermined and requires further investigation. New data suggest that under certain circumstances, like lipopolysaccharide (LPS)-induced stress in macrophage cell lineages, Caspase-11 is able to

synergize with AIP1 and amplify Cofilin severing activity [47]. Caspase-11 interacts with AIP1, enhancing its activity so that suboptimal quantities of AIP1 are capable of aiding in Cofilin severing activity. Caspase-11 has no effect on regulating Cofilin activity in the absence of AIP1, but Caspase-11 does interact with F-actin. It is unclear if the interaction of the Caspase-11-AIP1 complex with F-actin or with Cofilin or both is needed to increase F-actin fragmentation.

Srv2/CAP (suppressor of *rasVal14*/cyclase associated protein) (referred to as CAP) also increases Cofilin severing activity, but in a much different fashion [48]. Cofilin bound to ADP-G-actin exhibits decreased severing activity. CAP promotes the exchange of ADP for ATP, which also releases the actin monomer from Cofilin. In vitro, Cofilin severing increases in the presence of CAP. CAP may also increase Cofilin severing activity by unknown mechanisms that have yet to be identified.

Actin dynamics are amplified in breast cancer cells in response to EGF receptor activation (reviewed in [49]). Specific chemotactic cues activate Cofilin to induce F-actin reorganization and directed movement toward the stimuli. Recently, the protein Memo (mediator of ErbB2-driven cell motility) was shown to interact with Cofilin [50]. Memo, an ErbB2 and PLC δ interacting protein, directly interacts with Cofilin and slightly enhances Cofilin severing activity in a Herugulin (HRG) dependent manner. These two proteins co-localize at the leading edge and probably remodel leading edge actin structures near ErbB2 receptors that have been activated by HRG.

Although most studies up to date have depicted P-Cofilin as being soluble and in an inactive conformation, some studies indicate that there are proteins that interact with and are activated by binding to P-Cofilin [51]. Previous work showed that activation of

Phospholipase D1 (PLD1) downstream of muscarinic receptor activation leads to Rho-kinase activation and stress fiber formation. Studies by Han et al. showed that PLD1 directly interacts with Cofilin in a phospho-specific manner. The PLD1-P-Cofilin interaction induces an accumulation of F-actin stress fibers by PLD1-mediated hydrolysis of phosphatidylcholine in cell membranes to phosphatidic acid (PA).

Another study found that Cofilin and Triose-Phosphate Isomerase (TPI) interactions provide glycolytic fuel to Na,K-ATPases, increasing channel activation in response to Rho activation by lysophosphatidic acid[52]. In these studies, Cofilin was shown to interact with the Na,K-ATPase channel via co-immunoprecipitation. While these interactions were not shown to be direct, some of the protein complex contains P-Cofilin. This is only suggestive that the complex activity is caused by P-Cofilin and not dephosphorylated Cofilin, but the activation of the Na,K-ATPase does not occur in cells expressing S3A-Cofilin, an unphosphorylatable mutant of Cofilin. In vitro analyses of either P-Cofilin and/or S3D-Cofilin, a phospho-mimetic, are needed to determine the requirement of P-Cofilin in for Na,K-ATPase activation.

Increasing data has shown that Cofilin is regulated by protein-protein interactions. While the list of Cofilin interacting proteins is short and the mechanisms of how the known interacting proteins function are mostly unclear, these new data suggest that Cofilin functions in the cell are numerous and regulate many cellular activities.

Chronophin

Chronophin, a novel haloacid dehalogenase family phosphatase, has been shown to dephosphorylate Cofilin under precise conditions [53] [54]. Chronophin is highly expressed

in the brain and under normal conditions is sequestered by Hsp90 and therefore unable to interact with Cofilin. Under the cellular stress of ATP-depletion, Chronophin is released from Hsp90, becomes activated, and promotes the rapid dephosphorylation of Cofilin. This in turn leads to a stress response by the cell to form Cofilin-Actin rod aggregates, which are found associated with fibril tangles in Alzheimer's disease brain specimens [55].

Slingshot

Slingshot phosphatases provide Cofilin dephosphorylation under physiological conditions. Slingshot can be inactivated by C-terminal phosphorylation by Protein Kinase D1 (PKD1) [56] on one of two 14-3-3 consensus binding Serine residues. The interaction with 14-3-3 prevents Slingshot from binding to F-actin, inhibiting phosphatase activity [41]. Signaling events, such as Rac activation, lead to Calcineurin activation. Calcineurin, a calcium dependent phosphatase, can dephosphorylate the C-terminal phosphorylation sites and activate Slingshot [57]. This promotes Cofilin severing and induces F-Actin polymerization at the leading edge. While the events at the leading edge of have been well documented, Slingshot regulation of Cofilin at other F-actin locations remains unstudied.

Coronins

Coronins are highly conserved, F-actin binding proteins. They are named for the crown-like localization pattern seen in Dictyostelium [58]. Mammalian Coronins have six members that can be broken down into three types. Type I Coronins (Coronin 1A, 1B, and 1C), type II Coronins (Coronin 2A and 2B), and type III Coronin (POD/Coro7) have only been recently examined and current studies are producing new information about Coronin

Coronin structure

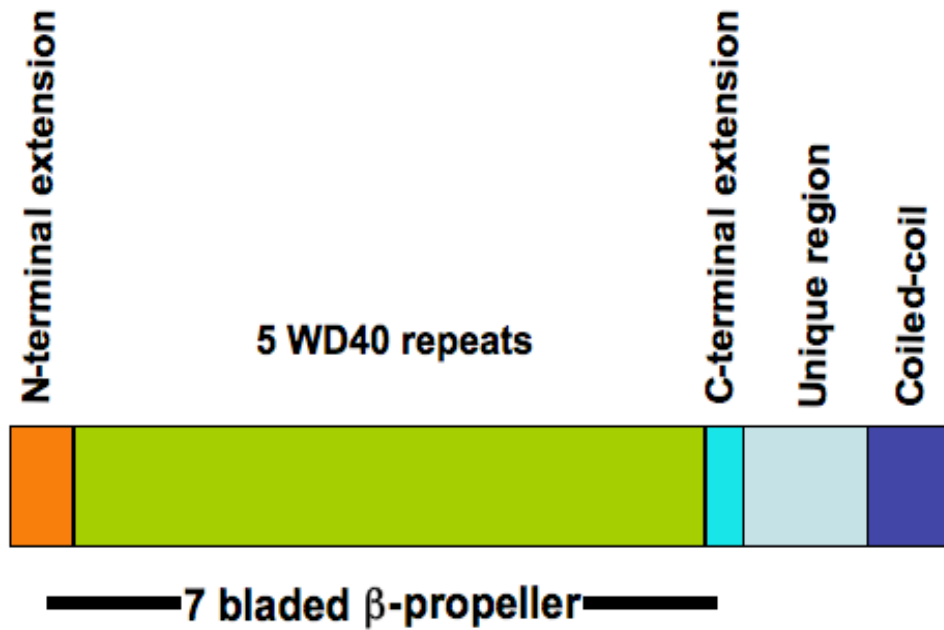


Figure 2. Diagram of Mammalian Coronin structure

function (reviewed in [59]). Type I and type II Coronins have the same basic domains (Figure 2). A region of five WD40-repeats, with β -sheet secondary structure are flanked by C- and N-terminal extensions. X-ray crystallography diffraction patterns indicate that parts of the C- and N-terminal extensions fold into two non-consensus β -sheets and together with the WD40 repeats, the domain folds into a β -propeller structure [60]. In other proteins, such as the beta subunit of heterotrimeric G proteins [61], these β -propeller domains have been shown to be important for protein-protein interactions. Studies with the type I Coronin, Coronin 1B, have verified the importance of this domain by identifying a single residue found on the surface of the β -propeller required for F-actin binding [62]. Besides the β -propeller structure, Coronins also have a unique region and a coiled-coil domain. So far, there is no identified function of the unique region in any of the mammalian Coronins. The coiled-coil domain, like in other proteins, is important for oligomerization [44]. The coiled-coil domain of Coronin 1A forms a trimer [63]. While no other Coronin coiled-coil has been verified to form a trimer, structural modeling suggests that the other type I Coronins form trimers as well. The type III Coronin, POD/Coro7 (hereafter referred to as POD), has a slight variation in structure from the type I or II Coronins. Pod lacks a coiled-coil domain, but has structural repeats of the remaining domains.

The expression patterns of each of the Coronins are distinct as well. Coronin 1A is hematopoietic specific and Coronin 1C is transcriptionally regulated downstream of ERK signaling [64, 65]. Coronin 1C also is upregulated in increasing malignancy grades of glioma cancers [66]. Coronin 1B and POD are both ubiquitously expressed. Coronin 2A is predominantly found in epithelial tissues, especially the testis, and Coronin 2B is mostly in neuronal cells [44].

Even though the structural components of Coronins are highly conserved throughout evolution, the localization patterns of the types of Coronins have diverged by gene expansion in metazoans. Predominantly, type I Coronins localize to the leading edge of the cell [44], POD is found at the Golgi apparatus [67], while the type II Coronins localize to F-actin stress fibers and focal adhesions [68]. Most studies have focused on the type I Coronins, which have retained most of the same activities and localization patterns as seen in *Dictyostelium*. These proteins localize to the lamellipodia of the cell where they can interact with the Arp2/3 complex, a protein complex required for F-actin branching. At these branch points, Coronins (especially Coronin 1B) promotes the debranching of F-actin filaments by activating Cofilin near these branch points and replacing the Arp2/3 complex with itself, destabilizing the F-actin filaments [44, 62, 69, 70]. This action is important for the recycling of the dendritic meshwork at the leading edge of cells needed for cellular protrusions. POD is important for Golgi organization [71], but studies have not identified key functions. Very little is known about type II Coronins. Coronin 2A, also known as IR10, can interact with N-Cor complex, a transcriptional repressor complex [72], but a role for Coronin 2A in regulating F-actin has not been addressed. Coronin 2B, also known as ClipinC, localizes to F-actin stress fibers and at focal adhesions in SH-SY5Y neuroblastoma cells [68]. Coronin 2B also co-immunoprecipitates with Vinculin, but any role of this interaction has not been determined.

Here, we show that Coronin 2A, similar to Coronin 2B, localizes to F-actin stress fibers and focal adhesions. We perform an initial characterization of Coronin 2A and determine its function in regulating focal adhesion dynamics through the Slingshot-Cofilin pathway. Furthermore, enforced expression of Coronin 2A induces the formation of Cofilin-

Actin rod aggregates that may be important for affecting neuronal activity in response to stress.

Chapter 2

CORONIN 2A REGULATES FOCAL ADHESION TURNOVER THROUGH THE COFILIN PATHWAY

Summary

Coronins are conserved F-actin binding proteins that are important for motility and actin dynamics. Unlike type I Coronins, Coronin 2A localizes to stress fibers and focal adhesions, and is excluded from the leading edge. Depletion of Coronin 2A in MTLn3 cells decreases cell motility and focal adhesion turnover. Surprisingly, none of the pathways known to regulate focal adhesion turnover are affected by Coronin 2A depletion. Depletion of Coronin 2A does however increase phospho-Cofilin suggesting that misregulation of Cofilin may affect adhesion dynamics. Slingshot-1L, a Cofilin-activating phosphatase, localizes to focal adhesions and interacts with Coronin 2A. Depletion of Coronin 2A reduces Cofilin activity at focal adhesions as measured by barbed end density and actin turnover. In both fixed and live cell contexts, Cofilin localizes to the proximal end of focal adhesions. While expression of wild-type Cofilin in Coronin 2A-depleted cells has no major affect on focal adhesion dynamics, expression of an active mutant of Cofilin bypasses the defects in cell motility and focal adhesion disassembly. These results implicate both Coronin 2A and Cofilin as new factors that can regulate focal adhesion turnover.

Introduction

Precise control of cell-matrix adhesion is necessary for cell migration to occur. Fibroblasts lacking Paxillin (PXN) or Focal Adhesion Kinase (FAK), two core focal adhesion components, display aberrant adhesion and decreased cell motility in vitro. Embryos deficient in these genes also lack proper mesoderm formation leading to embryonic lethality [5, 73]. In many cancer cell types, focal adhesion proteins display either modulated protein expression or inappropriate regulation. For example, FAK is overexpressed in many cancer cell types and is responsible for hyper-phosphorylation of other focal adhesion proteins [74]. Conversely, targeted disruption of the FAK gene in breast cancer models show that FAK is required carcinoma formation and metastasis [27]. Other focal adhesion proteins have altered expression profiles in cancer models as well. Recent data suggest that the EGF-induced switch from tensin-3 expression to the expression of the anti-adhesive molecule cten leads to increased metastasis of mammary tumor cells [75]. These observations indicate that proper focal adhesion dynamics are critical for morphogenesis and play a significant role in cancer progression.

Coronins are highly conserved F-actin binding proteins that are important for cell motility and actin dynamics [69, 76, 77]. Mammalian genomes contain at least six Coronin genes that can be separated into three types: Type I (Coronin 1A, 1B and 1C), Type II (Coronin 2A and 2B), and Type III (Coro7/POD) [59]. Each of the Coronins displays different tissue expression patterns with at least one type I Coronin and POD expressed in all tissues and cell types. Type II Coronins show a more restricted expression pattern with strong enrichment in tissues containing epithelial and neuronal cell types [44].

The subcellular localization of each of the types of Coronins is also quite distinct. Type I Coronins localize primarily to the lamellipodia and some vesicular structures [44, 78], type II Coronins localize to stress fibers and focal adhesions [68], and POD localizes to the Golgi apparatus [67]. Type I Coronins have a clear role in regulating cell motility, while the function of type II Coronins remains unknown. Type I Coronins such as Coronin 1B coordinately regulate Arp2/3 and Cofilin activities in lamellipodia [69]. Coronin 1B targets Arp2/3-containing branches and replaces Arp2/3 at branches leading to network remodeling [70]. In addition, Coronin 1B is required for proper targeting of Slingshot-1L, an activating phosphatase for ADF/Cofilin proteins to the rear of lamellipodia [69]. It is unclear if type II Coronins execute similar functions at other cellular locations.

ADF/Cofilin proteins (hereafter referred to as Cofilin) promote actin dynamics by several mechanisms including severing actin filaments [31]. Depletion of Cofilin by RNAi increases stress fiber thickness, decreases F-actin retrograde flow, and impairs whole cell motility [79, 80]. Several pathways have been identified that regulate Cofilin activity. Cofilin is inactivated by interacting with PI(4,5)P₂ on membranes [36], conformational changes induced by intracellular pH shifts [38], or via phosphorylation of Serine 3 by Lim kinase (LIMK) or Tes kinase (TESK) [32, 33]. All of these events prevent Cofilin from binding to F-actin. Activation of Cofilin can be achieved by the dephosphorylation of Serine 3 by the Slingshot and Chronophin phosphatases [34, 35]. Interestingly, both LIMK and Slingshot-1L share a common localization to focal adhesions [81, 82]. Despite the localization of these Cofilin regulatory proteins to focal adhesions, Cofilin activity has never been directly implicated in focal adhesion dynamics.

Several mechanisms are known to regulate the disassembly of focal adhesions. These include microtubule targeting of focal adhesions [13], Talin cleavage by Calpain [23], changes in Myosin II-generated tension [20], and changes in FAK phosphorylation and interactions with Dynamin [6]. However, the inter-relationship among these mechanisms is poorly understood. An unexplored mechanism could involve Cofilin-mediated actin filament severing activity at focal adhesions. Here, we have investigated the role of Coronin 2A in regulating Cofilin activity at focal adhesions and the subsequent effects on whole cell motility.

Results

Coronin 2A localizes to stress fibers and focal adhesions, but not lamellipodia

To investigate the function of Coronin 2A (also known as IR10, ClipinB, WDR2, Coronin 4 and CRN5), we expressed Coronin 2A tagged with GFP (Coro2A-GFP) in MTLn3 cells, a mammary adenocarcinoma cell line. Coro2A-GFP co-localizes along F-actin stress fibers and with Vinculin at focal adhesions (Fig. 3A). Interestingly, Coronin 2A does not co-localize with Coronin 1B (Fig. 3A) or Coronin 1C (data not shown) at the leading edge of cells and is excluded from the lamellipodial region of the cell. These observations, along with the absence of type I Coronins from focal adhesions (data not shown), indicate that type I and type II Coronins have distinct properties that determine protein localization.

Endogenous Coronin 2A has the same localization pattern as Coro2A-GFP (Fig. 3B).

Interestingly, Coronin 2A only localizes to some of the internal focal adhesions that have stress fibers attached, but does not localize to focal complexes found near the leading edge in the lamellipodia. Other focal adhesion markers, such as Talin and FAK, show similar partial co-localization with Coronin 2A at focal adhesions (data not shown).

Coronin 2A-depleted cells display decreased cell speed, but show no change in

lamellipodial dynamics

To address the functional role of Coronin 2A, we developed an shRNA against rat Coronin 2A (shCoro2A) delivered by lentivirus to deplete this gene product. To control for the effects of lentiviral infection, we also used a non-targeting shRNA (shNS). To verify that any effects were not due to off-target silencing, we developed a rescue construct that encodes both shCoro2A

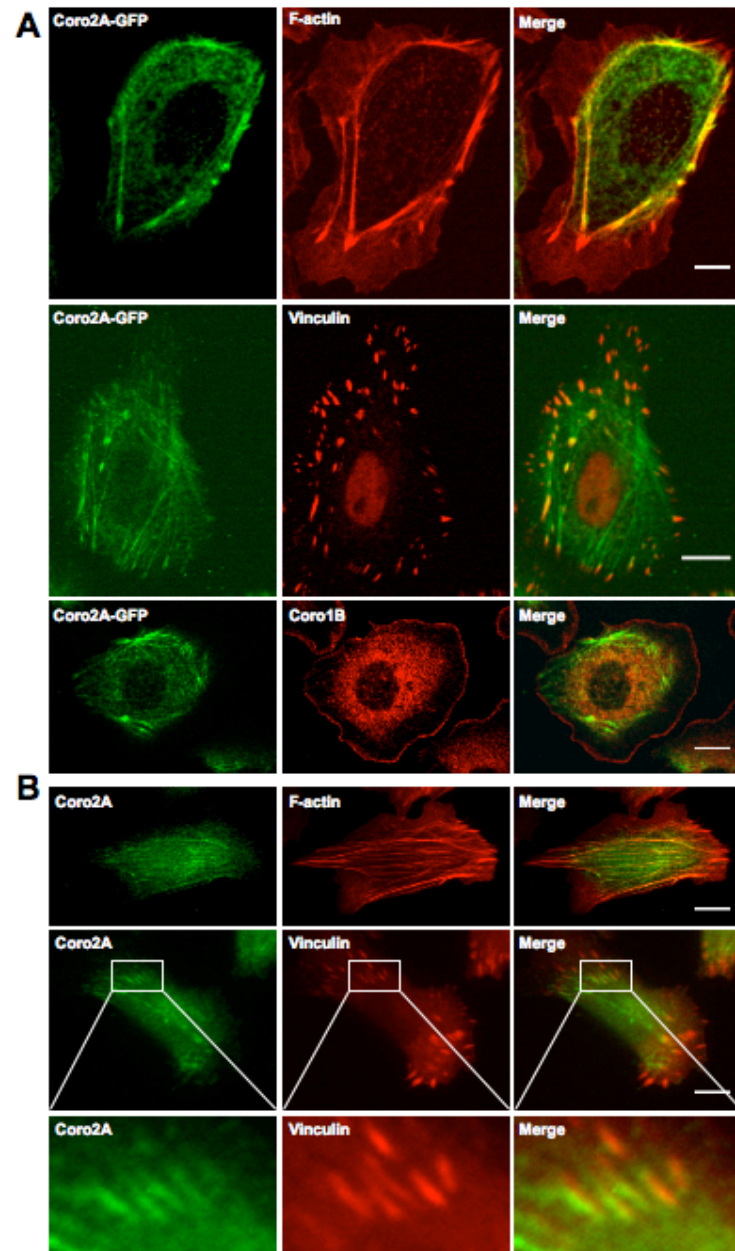


Figure 3. Coronin 2A localizes to F-actin stress fibers and focal adhesions, but is excluded from the leading edge.

(A) MTLn3 cells expressing Coronin 2A-EGFP were stained with Alexa568-Phalloidin (top panel) or with antibodies against either Vinculin (middle panel) or Coronin 1B (bottom panel). Bar = 5 μ m

(B) Immunofluorescence of endogenous Coronin 2A (green) with either Alexa568-Phalloidin (top panel) or a Vinculin antibody (middle panel). (Bottom panel) Inset of Coronin 2A and Vinculin from middle panel show Coronin 2A localizes to focal adhesions.

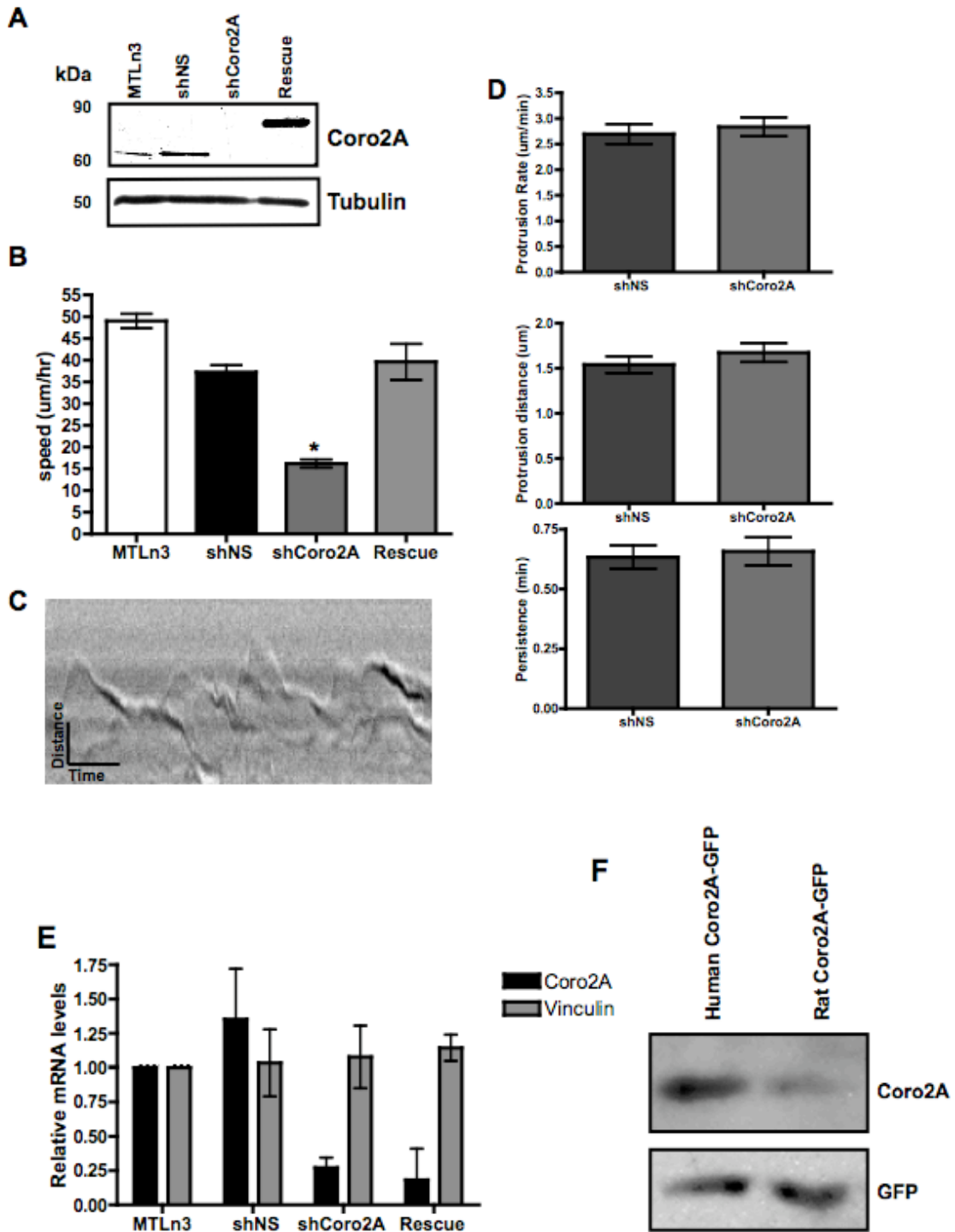


Figure 4. Depletion of Coronin 2A impairs cell motility, but does not affect lamellipodial dynamics.

(A) MTLn3 cells were infected with lentivirus that expressed shRNAs against a non-specific sequence (shNS), Coronin2A (shCoro2A) or shCoro2A that also coexpressed human Coro2A-GFP (Rescue). Lysate were blotted for Coronin 2A and Tubulin as a loading control.

(B) Time-lapse microscopy of MTLn3, shNS, shCoro2A, and Rescue cell lines was used to determine single cell speed, depicted in graph. Error bars represent 95% confidence intervals. Asterisk indicates $p < 0.0001$ by t-test.

(C,D) Representative kymograph of a protruding MTLn3 cell. Time (y-axis) and protrusion distance (x-axis) were used to calculate (D) protrusion rate, protrusion distance, and persistence. Error bars represent 95% confidence intervals.

(E) Real-Time PCR data showing relative mRNA levels of Coronin 2A and Vinculin in MTLn3 cells expressing shNS, shCoro2A, or Rescue. Note: Rescue is shCoro2A that co-expresses human Coronin2A-EGFP. The real-time probe for Coronin2A does not recognize the human gene.

(F) Lysates from 293FT cells expressing human Coronin2A-GFP or rat Coronin2A-GFP were immunoblotted for GFP and with the Coronin 2A antibody. The Coronin 2A antibody recognizes the human protein better than the rat protein.

indicates that shCoro2A-expressing cells have nearly undetectable amounts of Coronin 2A protein (Fig. 4A). This result was confirmed by qRT-PCR analysis (Fig. 4E). Although cells expressing the rescue construct appear to have higher than endogenous levels of Coronin 2A protein, separate pilot experiments indicate that this antibody recognizes human Coronin 2A more strongly than rat Coronin 2A (Fig. 4F) indicating that the level of rescue protein expression is in the physiological range.

Since Coronin 2A localizes to F-actin stress fibers and focal adhesions, we examined the motility of cells that were depleted of Coronin 2A. Single cell tracking indicated that Coronin 2A-depleted cells have substantially reduced cell speed relative to control cells. Re-expression of human Coronin 2A-GFP restored cell speed to the level of control cells indicating that the effects caused by the expression of shCoro2A are due to the specific depletion of Coronin 2A (Fig. 4B). The cells depleted of Coronin 2A display protrusion and retraction at the leading edge, but do not display much overall translocation. We used kymography to determine if there were any differences in lamellipodial dynamics caused by the depletion of Coronin 2A (example in Fig. 4C). Protrusion rate, protrusion distance, and persistence (Fig. 4D) are all unaffected by Coronin 2A depletion, suggesting that the decrease in cell motility is not due to alterations in lamellipodial dynamics.

Cells depleted of Coronin 2A have defects in focal adhesion turnover

Since there are defects in cell motility in the Coronin 2A-depleted cells and Coronin 2A localizes to focal adhesions, the size and number of these structures were quantified. Quantification of focal adhesions in cells expressing either shNS or shCoro2A indicate that depletion of Coronin2A increases focal adhesion size and decreases the total number of focal

adhesions per cell (Fig. 5A, B, example images in C). To measure cell spreading, we used the ACEA-RT CES system that measures changes of impedance caused by cells interacting with a microelectrode within the surface of the dish. Coronin 2A-depleted cells showed a slight, but not statistically significant ($p=0.1726$ for MTLn3 vs. shCoro2A, $p=0.0661$ for shNS vs. shCoro2A) increase in cell spreading compared to control cells (Figure 5D). This indicates that adhesion formation and cell spreading appear to be normal in Coronin 2A-depleted cells.

Larger focal adhesions in the Coronin 2A-depleted cells indicate that focal adhesion dynamics may be affected. To investigate possible changes in focal adhesion turnover, Coronin 2A-depleted cells that co-express GFP-Paxillin were used to monitor focal adhesion assembly and disassembly [20]. Compared to control cells, focal adhesion assembly is not affected in the Coronin 2A-depleted cells (Figure 5E), but the rate of focal adhesion disassembly decreases by half (Fig. 5F). Together, these data indicate that Coronin 2A plays a significant role in controlling focal adhesion dynamics.

Adhesion defects caused by the depletion of Coronin 2A are mediated through the Cofilin pathway

To determine the cause of focal adhesion turnover defects in the Coronin 2A-depleted cells, we examined various cellular pathways and processes that regulate focal adhesion turnover. There are no significant differences in Talin cleavage by Calpain, FAK phosphorylation at Y397 or phospho-MLC (P-MLC) levels (Fig. 6A). The frequency of microtubule targeting to focal adhesions is decreased in Coronin 2A-depleted cells, but this appears to be caused by slightly decreased microtubule growth rates (Fig. 7 A, B) and not due to microtubule stability

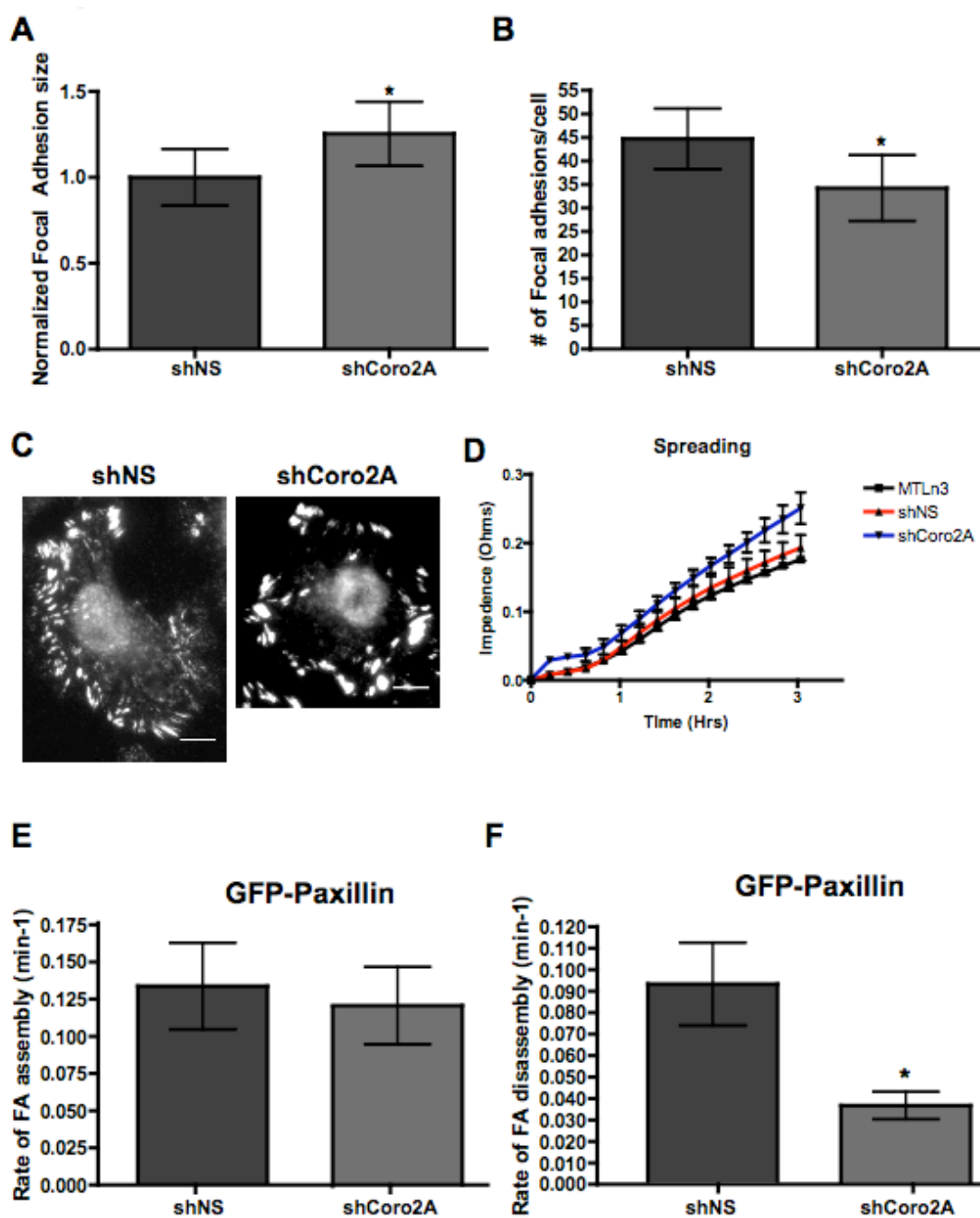


Figure 5. Depletion of Coronin 2A increases focal adhesion size, decreases focal

adhesion number, and decreases focal adhesion disassembly. (A) Focal adhesions size and (B) number were measured in MTLn3 cells expressing shNS or shCoro2A. Vinculin positive focal adhesions were used for the quantifications. Focal adhesion size was normalized against neighboring uninfected cells on the same coverslip. Asterisks indicate $p=0.0424$ for focal adhesion size and $p=0.0286$ for focal adhesion number by t-test.

(C) Representative Vinculin immunofluorescence for MTLn3 cells expressing shNS or shCoro2A used for quantifications in A and B. Bar = $5\mu\text{m}$

(D) Cell spreading as measured by change of impedance with the ACEA RT-CES system.

Equal number of MTLn3, shNS, and shCoro2A cells were plated in triplicate. $p=0.1726$ for MTLn3 vs. shCoro2A and $p=0.0661$ for shNS vs. shCoro2A by t-tests.

(E,F) Average rates of focal adhesion (E) assembly or (F) disassembly visualized by GFP-Paxillin in cells expressing either shNS or shCoro2A. Cells were imaged once a minute for 30 min. Changes of fluorescent intensity were used to determine focal adhesion assembly and disassembly rates. Asterisk indicates $p<0.001$ by t-test.

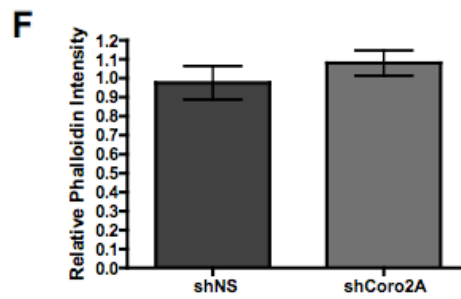
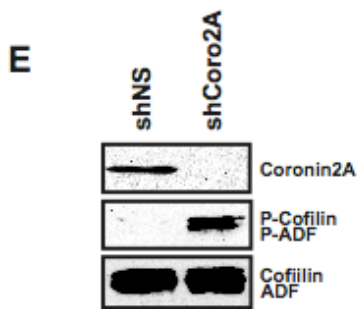
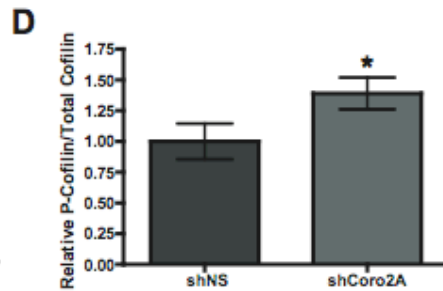
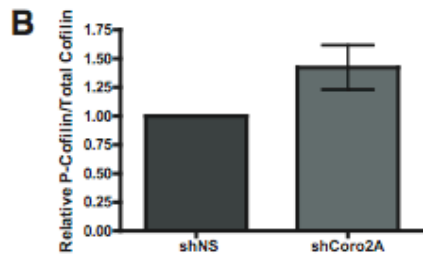
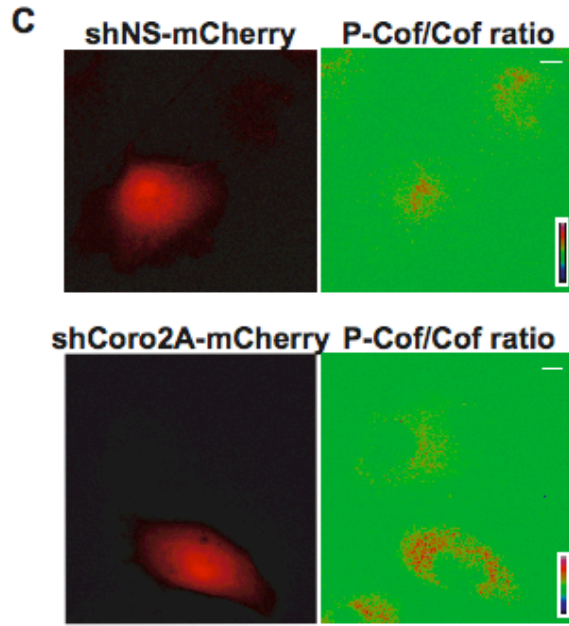
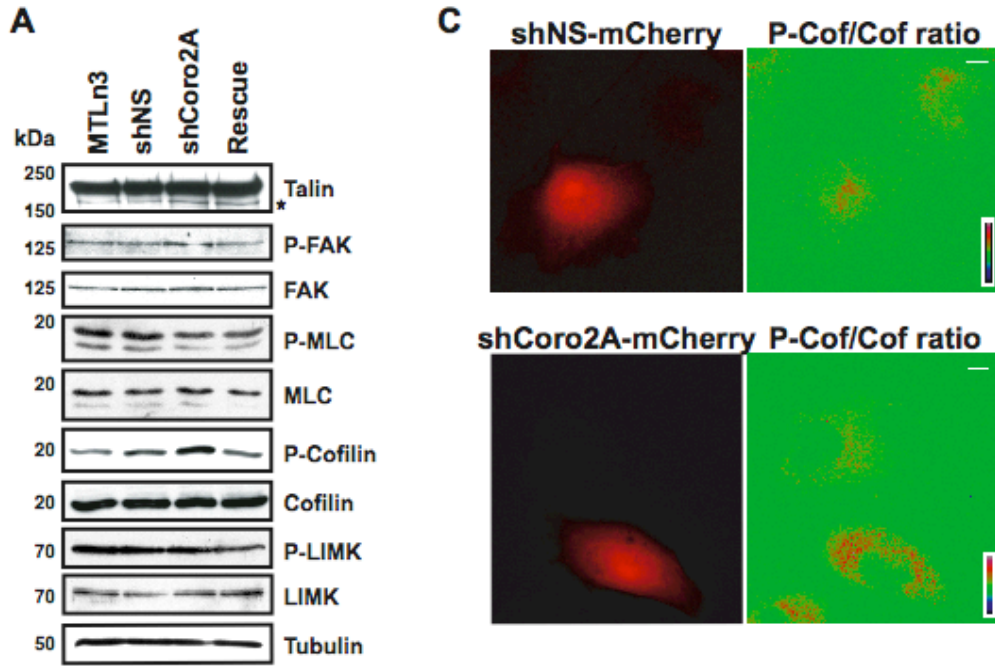


Figure 6. Depletion of Coronin 2A increases P-Cofilin levels, but has no effect on other

focal adhesion turnover protein components. (A) Representative immunoblots of MTLn3, shNS, shCoro2A, and Rescue cell lysates for Talin, P-FAK, FAK, P-MLC, MLC, P-Cofilin, Cofilin, P-LIMK, LIMK, and Tubulin (loading control). Asterisk indicates Calpain-induced Talin cleavage product.

(B) Quantification of P-cof vs Total Cofilin blots. Error bars represent 95% confidence intervals.

(C) Representative ratiometric images of P-cof/Cofilin for shNS and shCoro2A cells. Images were normalized to neighboring uninfected cells. Bar = 5 μ m

(D) Quantification of ratiometric images in C. Asterisk indicates $p=0.0001$ by t-test. Error bars represent 95% confidence intervals.

(E) MTLn3 cell lysates expressing either shNS or shCoro2A were immunoblotted with an antibody that recognizes both ADF and Cofilin or P-ADF and P-Cofilin. Immunoblot for Coronin 2A shows effective reduction in shCoro2A expressing cells.

(F) Cells expressing either shNS or shCoro2A were fixed and stained with Alexa488-phalloidin. Phalloidin intensities were normalized against uninfected neighboring cells.

$P=0.0572$

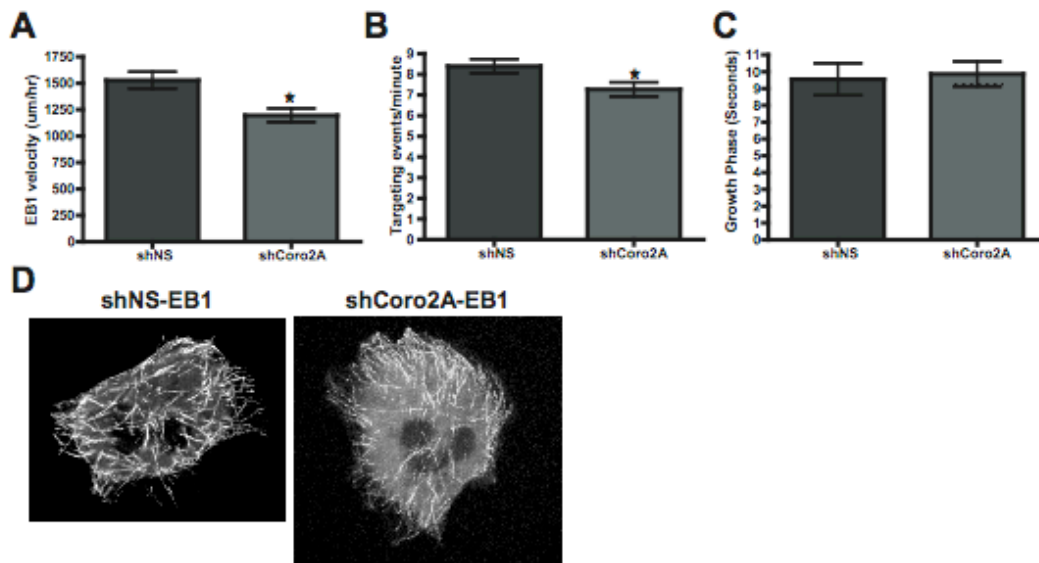


Figure 7. EB1 dynamics in cells depleted of Coronin 2A.

(A) Microtubule growth rate was measured by tracking EB1-GFP velocity. $p < 0.001$.

(B) Cells expressing shNS-TRFP-Actin or shCoro2A-TRFP-Actin with EB1-GFP were monitored for occurrences of EB1-GFP in the proximity of the terminal ends of actin stress fibers. This was counted as a targeting event. $p < 0.001$.

(C) Growth phase is the amount of time an EB1-GFP spot is visible on a growing microtubule.

(D) Example of maximum intensity projections show EB1-GFP paths. Expression of shCoro2A has no effect on linearity of paths.

or inability to properly target focal adhesions (Fig. 7 C, D). These observations suggest Coronin 2A may control focal adhesion turnover through a different pathway.

Since Coronin 1B regulates Cofilin activity at the leading edge of the cell [69], we investigated whether Coronin 2A has a similar role in regulating Cofilin phosphorylation at focal adhesions. Depletion of Coronin 2A leads to roughly a 1.5-fold increase in phosphorylated Cofilin (P-Cofilin) by both western blots (Fig. 6A,B) and ratiometric immunofluorescence (Fig. 6C,D). ADF phosphorylation is also increased in the Coronin 2A-depleted cells (Fig. 6E). To determine if the increased phosphorylation of Cofilin was due to increased LIMK activity in Coronin 2A-depleted cells, we blotted for phosphorylated (active) LIMK and observed no change upon Coronin 2A depletion (Fig. 6A). Consistent with a failure to activate LIMK, Coronin 2A depletion does not cause a global change in F-actin content as measured by changes in phalloidin intensity (Fig. 6F).

Cofilin's activating phosphatase Slingshot-1L (SSH-1L) has been shown to localize to the leading edge of cells, and similar to other reports [82], we observed SSH1L-GFP localizing to focal adhesions (Fig. 8A). Coronin 2A and Slingshot-1L also co-localize at some but not all focal adhesions (Fig. 8B). By co-immunoprecipitation, Coronin 2A interacts with Slingshot-1L (Fig. 8C). This interaction is independent of Slingshot's phosphatase activity since co-immunoprecipitation occurs with a phosphatase inactive mutant of Slingshot (CS) equally as well as wild-type Slingshot (Fig. 8C). Furthermore, endogenous Coronin 2A can co-immunoprecipitate with Slingshot1L-GFP indicating that this interaction is not mediated by GFP dimerization or by an interaction with GFP (Fig. 8D).

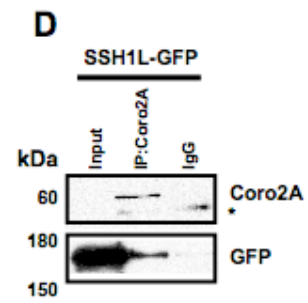
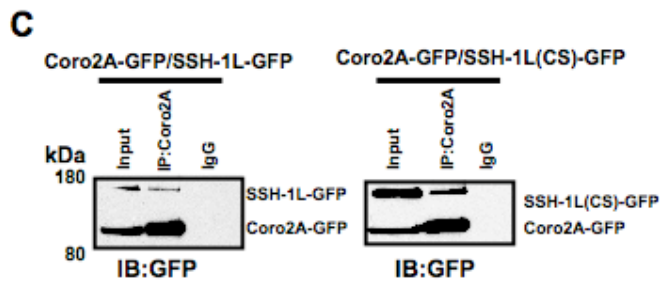
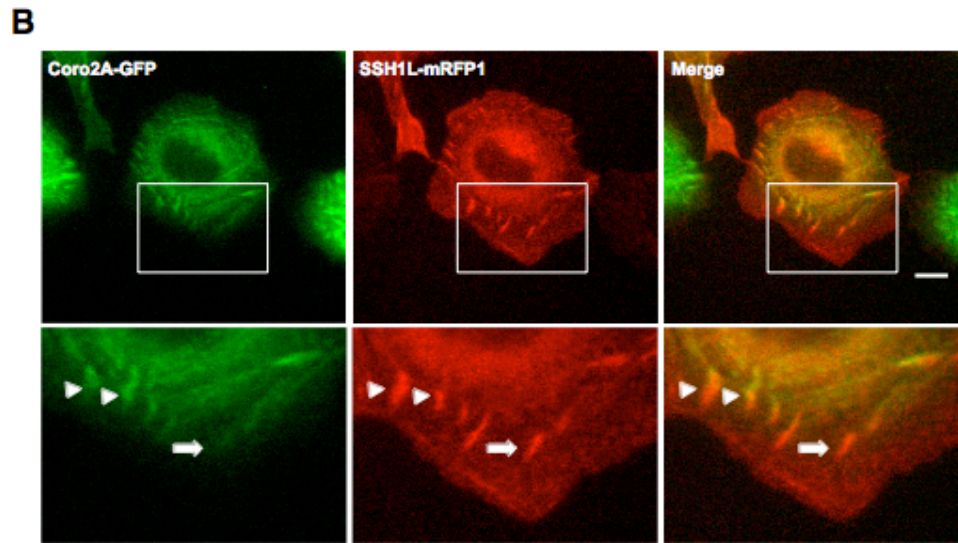
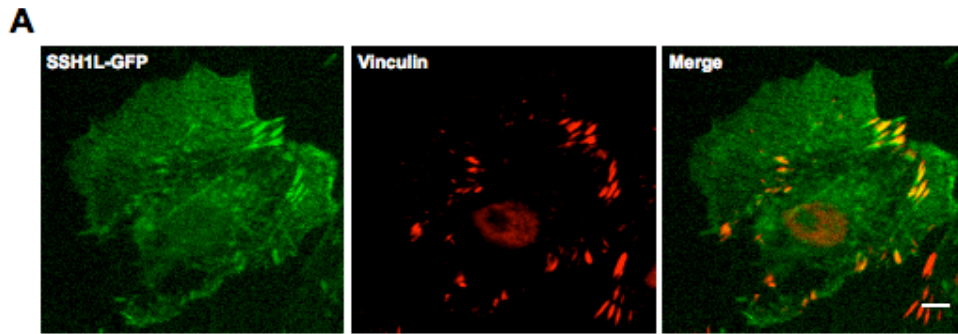


Figure 8. Coronin 2A interacts with Slingshot-1L and co-localizes at focal adhesions.

(A) Slingshot 1L-GFP localizes to focal adhesions. MTLn3 cells transfected with SSH1L-GFP were fixed and stained with Vinculin antibodies. Bar = 5 μ m

(B) MTLn3 cells expressing Coronin 2A-GFP and SSH1L-mRFP1 co-localize at focal adhesions. Inset of Coronin 2A-GFP and SSH1L-mRFP1 positive focal adhesions (arrowheads). Some SSH1L-mRFP positive focal adhesions contain little to no Coronin 2A-GFP (arrows).

(C) Lysates from 293FT cells with enforced expression of Coro2A-GFP and Slingshot-1L-GFP (SSH1L-GFP) or SSH1L (CS)-GFP were immunoprecipitated with a Coronin 2A antibody and immunoblotted for GFP.

(D) Coimmunoprecipitation of endogenous Coronin 2A with SSH1L-GFP.

Immunoprecipitation performed the same as in (C). Immunoblots were probed with Coronin 2A and GFP antibodies. Asterisk indicates antibody immunoglobulin reacting with the secondary antibody.

One obvious mechanism by which Coronin 2A might affect the Cofilin pathway at focal adhesions is to serve as a targeting subunit for Slingshot-1L at this location. However, SSH1L-GFP targets focal adhesion structures equally well in control and Coronin 2A-depleted cells (Fig. 9A). Since the depletion of Coronin 2A does not affect Slingshot-1L localization to focal adhesions, we examined the dynamics of SSH1L-GFP at focal adhesions in cells expressing either shNS or shCoro2A. The rate of focal adhesion disassembly as marked by SSH1L-GFP was similar to the rate observed with GFP-Paxillin and displayed the same ~2 fold decrease in Coronin 2A-depleted cells compared to control cells (Fig. 9C).

Another possibility is that Coronin 2A regulates Slingshot-1L activity at focal adhesions. Although Slingshot-1L regulation is incompletely understood, phosphorylation of two Serine residues in its C-terminus (S937 and S978) and subsequent 14-3-3 binding have been shown to inhibit its activity [41, 42, 56]. We examined if SSH1L-S937A, S978A (abbreviated as SSH1L-2SA), a non-phosphorylatable mutant with higher phosphatase activity, had an effect on focal adhesion disassembly. Similar to wild-type Slingshot-1L, SSH1L-2SA-GFP localizes to focal adhesions in both control and Coronin 2A-depleted cells (Fig. 9B). Expression of the active SSH1L-2SA mutant increases focal adhesion disassembly rates by ~2-fold. Interestingly, depletion of Coronin 2A in the SSH1L-2SA expressing cells produces an intermediate result. The rate of disassembly is lower than that with the expression of the mutant alone, but higher than Coronin 2A depletion in the presence of wild-type Slingshot-1L (Fig. 9C). This result suggests that increased Slingshot activity can compensate for Coronin 2A depletion, but that Coronin 2A may also have an effect on focal adhesion dynamics downstream of Slingshot-1L.

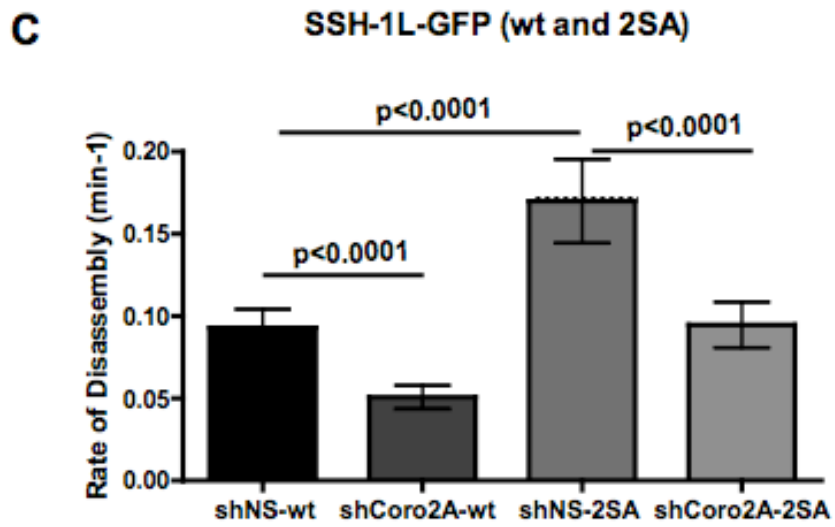
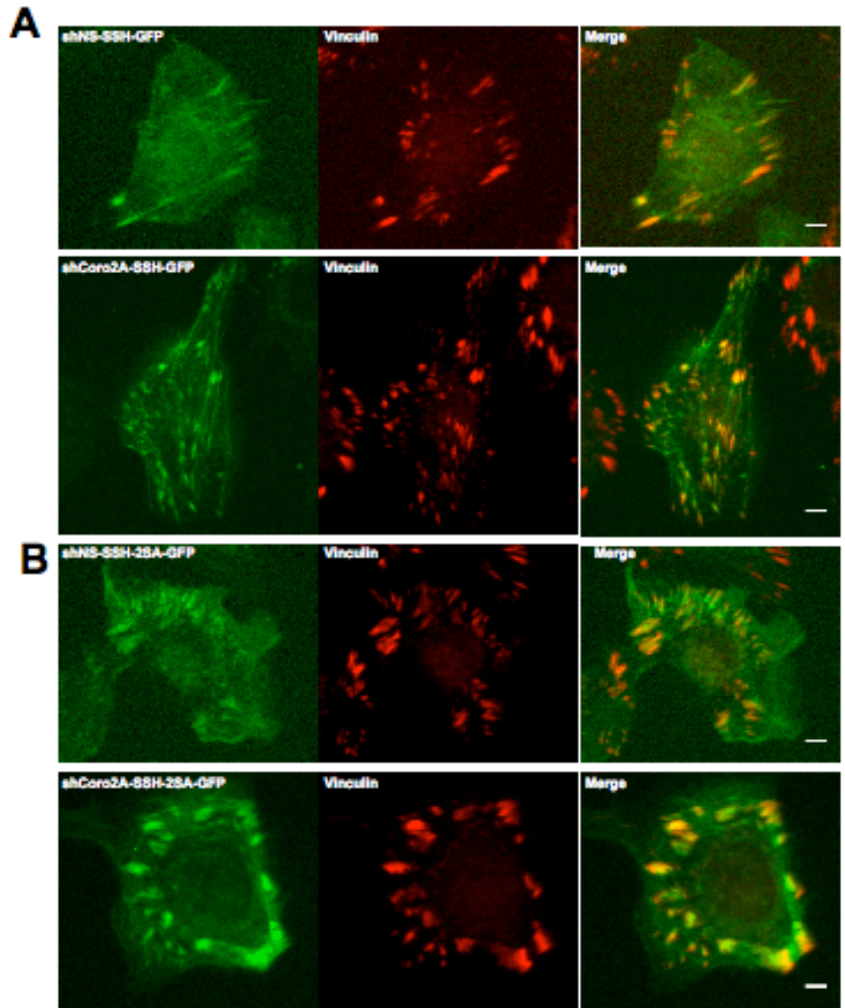


Figure 9. Active mutants of Slingshot increase focal adhesion disassembly and partially bypass decreases in focal adhesion disassembly caused by depletion of Coronin 2A.

(A) SSH1L-GFP localizes to focal adhesions in shNS and shCoro2A expressing cells. Cells were co-stained with Vinculin antibodies. Bar = 5 μ m

(B) SSH1L-2SA-GFP localizes to focal adhesions in shNS and shCoro2A expressing cells. Cells were costained with Vinculin antibodies.

(C) Focal adhesion disassembly rates, as done in Fig. 3, visualized by SSH1L-GFP or SSH1L-2SA-GFP (active) in cells expressing either shNS or shCoro2A. Error bars represent 95% confidence intervals.

Cofilin localizes to the proximal end of some focal adhesions

While proteins that regulate Cofilin activity, like Slingshot, LIM kinase and TESK, localize to focal adhesions, it remains unclear if Cofilin also localizes to focal adhesions. Cells that are permeabilized with Saponin prior to fixation display punctate spots of Cofilin at the proximal end of some FAK-positive focal adhesions (Fig. 10A). MTLn3 cells expressing GFP-Paxillin and Cofilin-TagRFP were employed to further characterize Cofilin at focal adhesions. By TIRF microscopy, spots of Cofilin-TagRFP localize to the proximal end of the focal adhesion (Fig 10B). In some cases, the appearance of Cofilin at the base of a focal adhesion is followed by focal adhesion disassembly (Fig 10B, middle and bottom panels).

Active Cofilin can bypass focal adhesion and motility defects caused by the depletion of Coronin 2A

Since Cofilin localizes to some focal adhesions, assays were performed to determine if Cofilin severing activity occurs at focal adhesions. We used two approaches to evaluate the activity status of Cofilin in situ upon Coronin 2A depletion. The actin filament severing activity of Cofilin generates free barbed ends that can be detected by the incorporation of labeled G-actin in permeabilized cells [83]. Thus, the presence of free barbed ends can be used as a surrogate marker for Cofilin activity, although barbed ends at this structure may arise from other mechanisms such as anti-capping protein activity (eg. VASP). To measure the density of actin filament barbed ends at focal adhesions, we labeled free barbed ends and visualized them relative to the focal adhesion marker Vinculin. The relative density of free barbed ends at Vinculin-positive focal adhesions (reported as the ratio of barbed ends (BE) to focal adhesion (FA) staining) is significantly diminished in Coronin 2A-depleted cells (Fig.

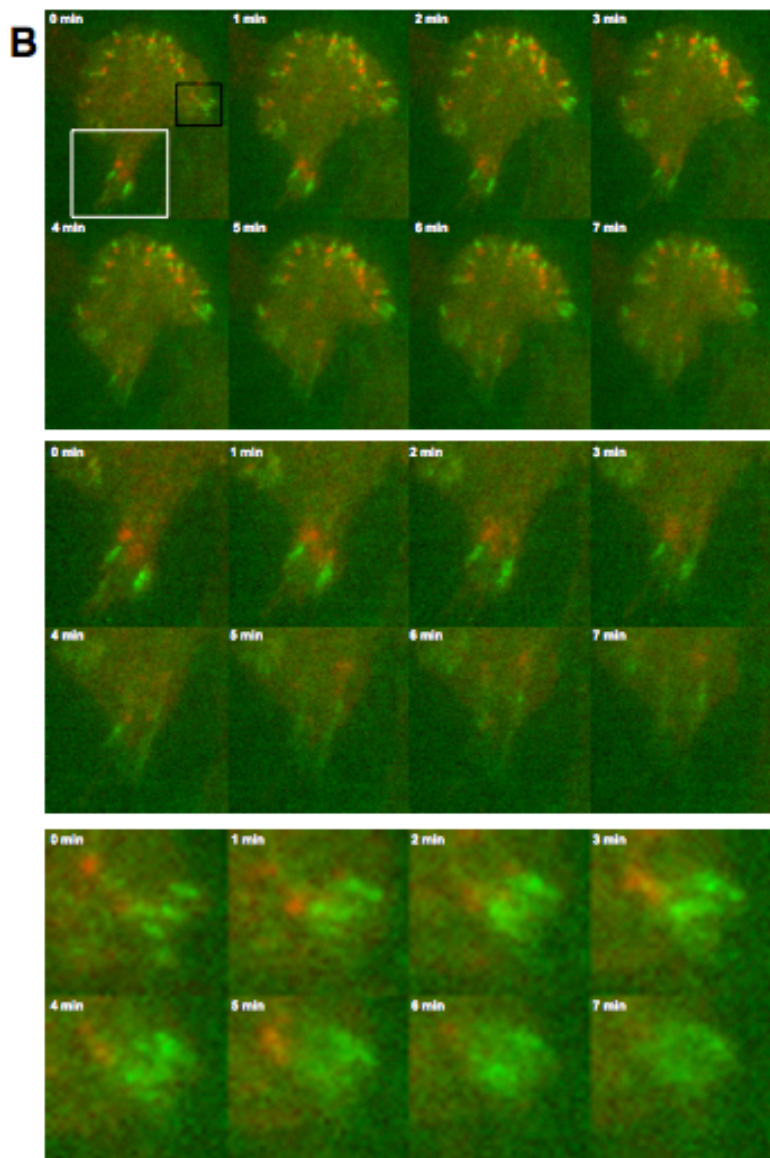
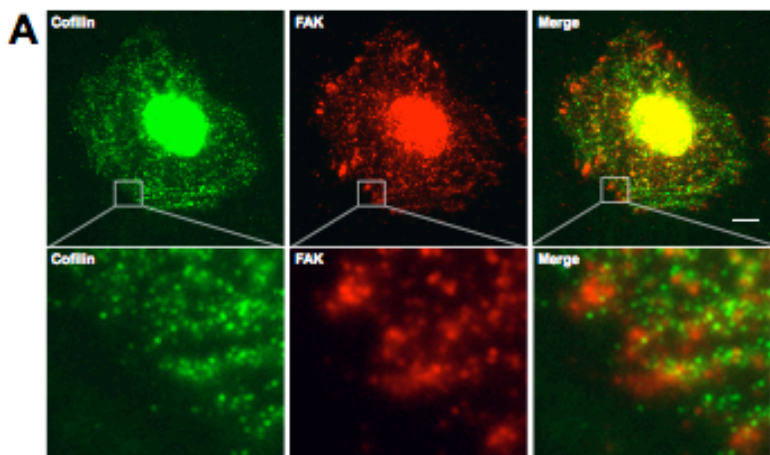


Figure 10. Cofilin localizes to the proximal end of focal adhesions in fixed and live cells.

(A) Immunofluorescent images of saponin permeabilized MTLn3 cells stained for Cofilin and FAK. Bar = 5 μ m

(B) Montage of live-cell images of cell expressing shNS-GFP-Paxillin and Cofilin-TagRFP.

Images were taken every minute. Cofilin-TagRFP localizes to the base of GFP-Paxillin positive focal adhesions leading to focal adhesion disassembly. (Middle panel), enlarged view of white box inset. (Bottom panel), enlarged view of black box inset.

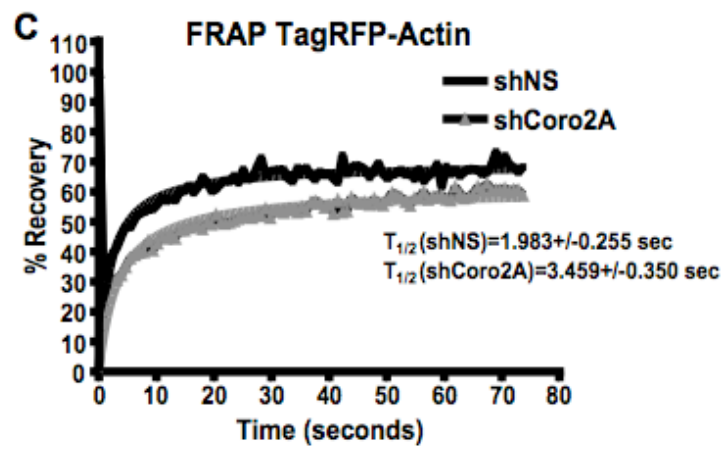
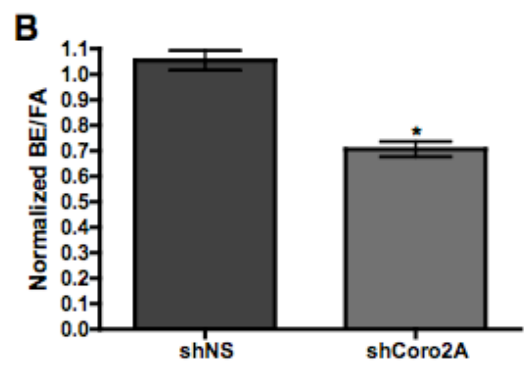
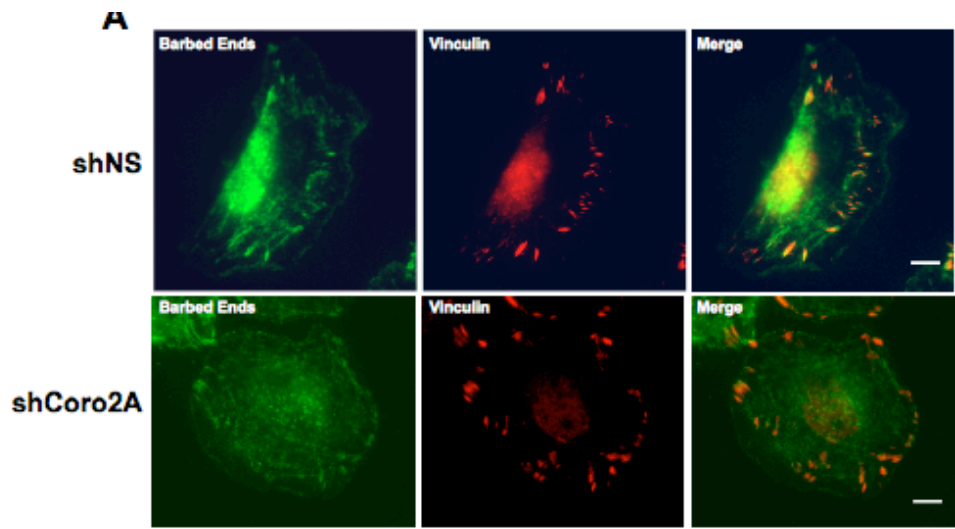


Figure 11. Coronin 2A depletion leads to reduced barbed end density and actin turnover at focal adhesions.

(A) Representative images of OregonGreen-Actin incorporated into free barbed ends at Vinculin stained focal adhesions in MTLn3 cells expressing shNS-TagRFP-Actin or shCoro2A-TagRFP-Actin. Bar = 5 μ m

(B) Graph depicting the barbed end/focal adhesion average fluorescence intensities. These results were normalized to intensity values of uninfected MTLn3 cells on the same coverslip. Error bars indicate 95% confidence intervals. Asterisk indicates $p < 0.001$.

(C) Graph of fluorescence recovery after photobleaching (FRAP) of TagRFP-Actin at focal adhesions in MTLn3 cells expressing either shNS or shCoro2A. $T_{1/2}$ values indicate time required for 50% recovery of fluorescence. $n=9$ cells was used to calculate recovery rate.

11A, B). In addition, fluorescence recovery after photobleaching (FRAP) of TagRFP-Actin was performed to monitor any changes in actin dynamics near focal adhesions. Coronin 2A depleted cells display significantly decreased recovery rates of labeled actin at focal adhesions compared to control cells (Fig 11C). Along with the increased phosphorylation of Cofilin in Coronin 2A-depleted cells, reduced density of barbed ends at focal adhesions and the interaction of Coronin 2A and Slingshot-1L, these data strongly suggest that Coronin 2A modulates focal adhesion turnover through the Cofilin pathway.

To directly determine if decreased Cofilin activity is responsible for the decreased cell motility in the Coronin 2A-depleted cells, we tested whether an active mutant of Cofilin (Cofilin S3A) could bypass these defects. Expression of Cofilin S3A in Coronin 2A-depleted cells restores whole cell motility to that of control cells (Fig. 12A). To test whether the focal adhesion turnover defect present in the Coronin 2A-depleted cells was due to insufficient Cofilin activity, we expressed either wild-type or S3A Cofilin in the Coronin 2A-depleted cells. Expression of the wild-type Cofilin was unable to rescue the defect in focal adhesion disassembly observed with Coronin 2A-depletion (Fig. 12B). However, expression of Cofilin S3A in Coronin 2A-depleted cells restored focal adhesion disassembly rates to the rate of control cells (Fig. 12B). Expression of Cofilin S3A alone has no effect on whole cell motility or focal adhesion turnover, suggesting that the bypass effect of this mutant is specific to Coronin 2A-depletion. Together, these data indicate that Coronin 2A affects focal adhesion disassembly through the activation of Cofilin.

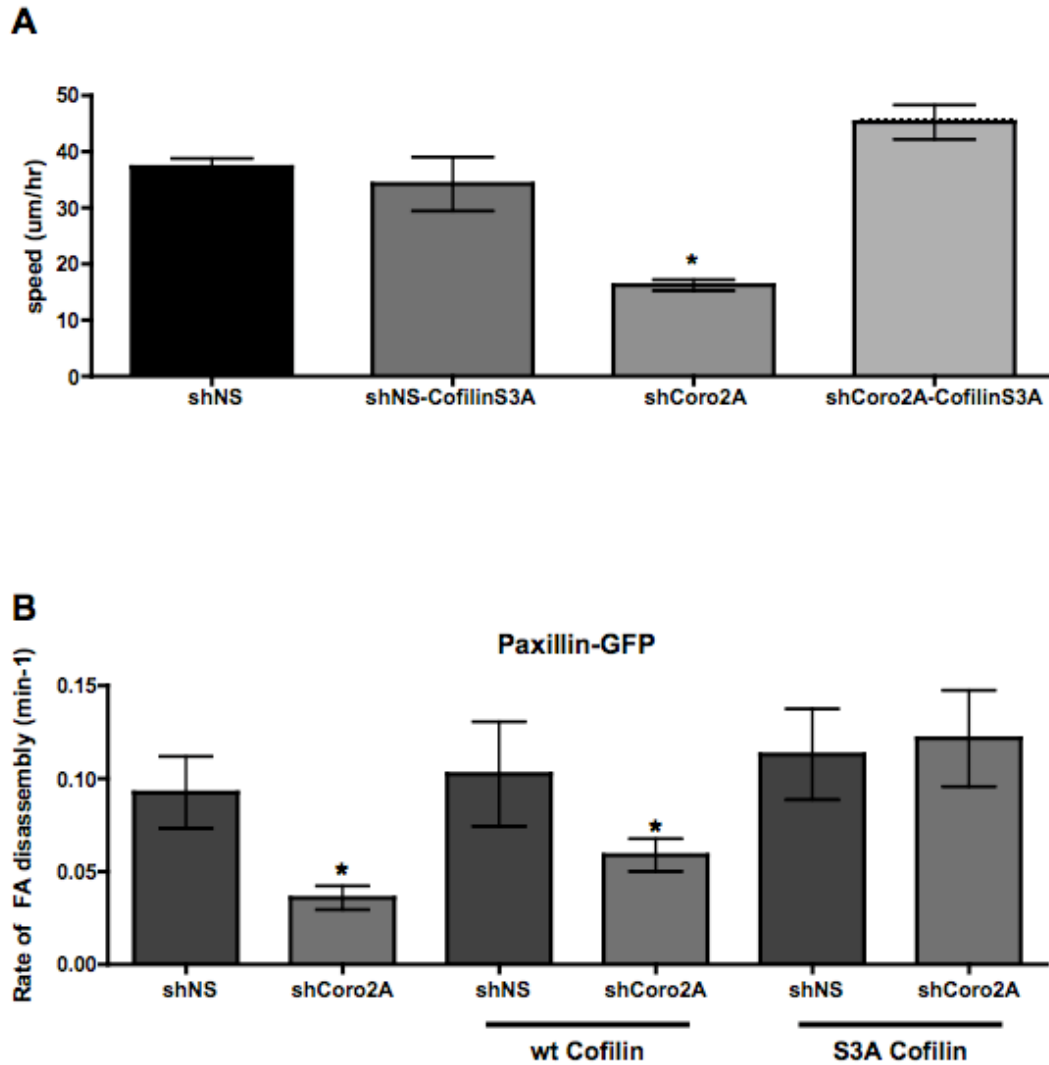


Figure 12. Expression of Cofilin S3A rescues cell motility and focal adhesion disassembly defects caused by depletion of Coronin 2A.

(A) Graph of average cell speeds of MTLn3, shNS, shCoro2A, shNS-CofilinS3A and shCoro2A-CofilinS3A expressing cells, as done in Fig. 2. Error bars indicate 95% confidence intervals. Asterisk indicates $p < 0.001$.

(B) Graph of average rates of focal adhesion disassembly visualized by GFP-Paxillin in cells expressing either shNS or shCoro2A along with wild-type (wt) or S3A Cofilin-mRFP1, as done in Fig. 3. Error bars indicate 95% confidence intervals. Asterisk indicates $p < 0.001$.

Discussion

Considerable evidence points to the important role that Coronins play in cell motility, but the function of type II Coronins, such as Coronin 2A, has never been addressed. Unlike the more well characterized type I Coronins, Coronin 2A localizes to stress fibers and focal adhesions, and is excluded from the leading edge. Our data indicate that Coronin 2A plays a key role in regulating whole cell motility and focal adhesion turnover. Depletion of this gene leads to insufficient Cofilin activity at focal adhesions and reduced adhesion disassembly in the interior region of the cell. This effect may be mediated in part through a Coronin 2A-Slingshot-1L interaction that regulates Slingshot (and therefore Cofilin) activity. Punctate spots of Cofilin are also found at the proximal end of internal focal adhesions. Consistent with a role of Cofilin localization and activation in these processes, defects in motility and focal adhesion turnover induced by depletion of Coronin 2A can be bypassed by an active mutant of Cofilin.

Our data highlight the striking diversity of localization and function amongst the mammalian Coronin family of proteins. Previous data from our lab indicate that the primary function of type I Coronins (eg. Coronin 1B) is to interact with the Arp2/3 complex and Slingshot-1L at the rear of the lamellipodia and remodel the dendritic meshwork [69, 70]. Like Coronin 1B, Coronin 2A interacts with Slingshot-1L. This indicates that Cofilin regulatory activity is conserved between type I and II Coronins. Despite this similarity, type I Coronins and Coronin 2A have distinct spatial distributions. Coronin 2A localizes to stress fibers and focal adhesions in the central region of the cell and is excluded from the leading edge, while type I Coronins are concentrated in this compartment. It remains unclear as to

what causes these differences in localization patterns and elucidating this will require further investigation.

A significant conclusion from these studies is that the activation of Cofilin's severing activity is an important factor in focal adhesion disassembly. Previous studies on TESK1 have shown a requirement of Cofilin phosphorylation in order for proper spreading to occur. Other studies in monocytes showed that S3A Cofilin inhibited tail retraction, but neither of these studies addressed the role of Cofilin in regulating focal adhesion dynamics [84]. While many studies have linked Cofilin activity to actin turnover at specific structures such as the dendritic meshwork, stress fibers and myofibril arrays [85], our data are the first to directly implicate Cofilin activity in the turnover of focal adhesions. One simple model for the direct involvement of Cofilin in this process is that actin filaments in stress fibers connected to focal adhesions must be severed to initiate, propagate or complete the turnover of the adhesion. Data from others has already indicated that the linkage between stress fibers and focal adhesion components, such as Paxillin, is capable of slipping in a clutch-like fashion [30]. Thus, Cofilin severing might reflect an alternate pathway by which the linkage between stress fibers and focal adhesions can be regulated. Since some studies have indicated that Cofilin severs actin filaments in bundled structures poorly [86], there may be a requirement for bundle remodeling near the point of Cofilin action in order for this mechanism to function efficiently.

One intriguing question that arises from these studies is how does Coronin 2A modulate Cofilin activity at focal adhesions? Based on the increased levels of phosphorylated Cofilin observed upon Coronin 2A depletion and the selective ability of S3A (but not wild-type) Cofilin to bypass the motility and adhesion defects, it seems likely that Coronin 2A

affects Cofilin activity at least in part via its phosphorylation status. The interaction between Coronin 2A and Slingshot-1L supports this notion, but it is important to point out that Coronin 2A does not simply target Slingshot-1L to focal adhesions. Furthermore, Coronin 2A is unlikely to exclusively regulate Slingshot-1L activity through controlling the phosphorylation of the C-terminal Serine residues as the enhanced focal adhesion disassembly triggered by the activated Slingshot-1L 2SA mutant would have been completely unaffected by Coronin 2A depletion in this case. Thus, Coronin 2A may control Slingshot-1L activity by an unknown mechanism. Alternately, Coronin 2A may affect Cofilin by a Slingshot-1L independent mechanism such as through an effect on actin filament structure in the focal adhesion or the direct enhancement of Cofilin activity as was described recently for Coronin 1A in an in vitro system [87]. These and other possibilities will need to be addressed in future experiments.

Previous studies uncovered multiple mechanisms for focal adhesion turnover and it is worth considering how Cofilin-based severing might be integrated with some of these other mechanisms. One significant mechanism of turnover is the Calpain-dependent cleavage Talin [17, 18]. This event permanently breaks a key linkage between integrin receptors and other components in the focal adhesion plaque. Cofilin-based actin filament severing might serve a similar purpose at the proximal end of the focal adhesion and may synergistically accelerate adhesion turnover. Another important mechanism of adhesion assembly and disassembly is Myosin-based contractility. The isometric tension generated by this mechanism is important for the maturation of nascent contacts near the leading edge and is also important for detachment of adhesions at the trailing edge [20, 88, 89]. Cofilin-based severing could directly impact Myosin-based contractility by modifying actin filament structure near the

adhesion. It is unclear how these mechanisms and others such as microtubule contact and FAK/Dynamin interactions function together to drive focal adhesion turnover.

In addition to the complication of multiple interrelated mechanisms of focal adhesion turnover, it is clear that different mechanisms may dominate in particular cellular compartments and in different cell types. Cells must balance the formation and dissolution of adhesions in an asymmetric manner to promote efficient cell migration. Thus, adhesion turnover at the front of the cells may utilize a distinct set of turnover mechanisms than those at the trailing edge of the cells. Since Coronin 2A is excluded from the periphery of the cell, it seems likely that it would participate only in turnover of centrally located adhesions. Finally, it is important to note that expression of Coronin 2A is not detectable in fibroblasts and thus its role in focal adhesion turnover may be specific for certain cell types such as epithelial cells. However, various Slingshot and LIMK isoforms are ubiquitously expressed, so regulated Cofilin activation may be an intrinsic part of focal adhesion turnover in all cells. Considering the profound effect focal adhesion turnover kinetics has on overall cell migration, it will be critical to elucidate this process in detail in order to understand migration-dependent processes such as the immune response and cancer metastasis.

Materials and Methods

Molecular cloning- pLL5.0 base vector and pLL5.0-shNS-GFP/mCherry were described in [69]. shRNA sequences were designed to target rat, but not human, Coronin 2A (shCoro2A). Oligonucleotides were annealed and ligated in HpaI and XhoI sites in pLL5.0. Coronin 2A shRNA sequence: GGAACGTCTTGGACATCAT. A rescue construct was made by PCR amplifying human Coronin 2A and cloning it into EcoRI and BamHI sites in pLL5.0-shCoro2A. The following cDNAs were PCR amplified and inserted in pLL5.0-shNS-GFP and pLL5.0-shCoro2A-GFP with the sites indicated: Paxillin (GFP-PXN; double blunt ligation into RI/SbfI site), Slingshot-1L (wt or CS)(SSH1L-GFP; MfeI/BglII into EcoRI/BamHI in pLL5.0), Cofilin S3A (EcoRI/BamHI) and TagRFP-Actin (EcoRI/SbfI). cDNAs for SSH1L and Cofilin S3A were PCR amplified, digested with Sall/SacII or EcoRI/BamHI, respectively, and ligated into pML2-mRFP1. SSH1L-2SA-GFP was made by site directed mutating serine 937 and serine 978 to alanines. PCR primers sequences are available upon request.

Antibodies and reagents- GST-Coronin 2A short tail (amino acids 493-526) was produced in *E. coli*, purified using Glutathione-Agarose beads, and used to inject rabbits for the production of polyclonal antibodies for Coronin 2A (Covance). Purified antibodies were isolated by applying serum to MBP-Coronin 2A short tail that was immobilized on an Ultralink biosupport (Pierce). Antibodies were eluted from the column with both high and low pH solutions and tested for recognition of Coronin 2A protein by immunoblotting, immunofluorescence, and immunoprecipitation. The following antibodies were purchased and used at the dilutions as indicated: α -Tubulin clone DM1A (Sigma; WB 1:1000), GFP

clone JL-8 (Clontech; WB 1:5000) or (Roche; IP 1 μ l), Talin (Sigma; WB 1:5000), FAK (Millipore; WB 1:1000, IF 1:200), P-FAK (Biosource; WB 1:1000), Cofilin (Cytoskeleton Inc.; WB 1:500) or (Mab22 (a gift from Jim Bamberg) IF 1:100), P-Cofilin (Biosource, WB 1:1000, or 4321 (a gift from Jim Bamberg), IF 1:70)), LIMK (Cell Signaling; WB 1:1000), P-LIMK (ECM Biosciences; WB 1:1000), Myosin Light Chain (MLC) 2 (Cell Signaling; WB 1:1000), and P-MLC (Cell Signaling; WB 1:1000). Coronin 1B rabbit polyclonal antibody is described in [44]. Cy2, Cy5, RhodamineRedX, and HRP conjugated secondary antibodies are from Jackson Immunoresearch Laboratories. Immobilon-P PVDF is from Millipore. Recombinant Rat EGF was purchased from Sigma. Rat tail Collagen was purchased from BD Biosciences. 100X Pen Strep Glutamine (PSG), α -MEM, DMEM, Alexa Fluor 488,568 and 647 labelled Phalloidins, OregonGreen- and Alexa Fluor 647-Actin were purchased from Invitrogen. Fetal Bovine Serum (FBS) was purchased from Hyclone.

Cell Culture and shRNA lentiviral production- MTLn3 cells (a gift from John Condeelis, described in [90]), a rat mammary adenocarcinoma cell line, were grown in α -MEM containing 5% FBS and 1X PSG. 293FT cells were grown in DMEM containing 10% FBS and 1X PSG. Retroviral and lentiviral production was performed as previously described in [69]. MTLn3 cells were infected with retrovirus or lentivirus for 4 hours and then the media was changed. Effects of lentiviral infections were examined by Western blot after 3-4 days.

ACEA RT-CES experiments- After background measurements were taken (100 μ l of media), 5000 cells in 100 μ l was added to each well (three experiments, done in triplicate).

Impedance measurements were taken every 2 minutes for 3 hours and quantified in Prism (Graph Pad, Inc.)

Single Cell Tracking- MTLn3 cells were either uninfected or infected with lentiviruses encoding shNS, shCoro2A, rescue, shNS-Cofilin S3A-GFP or shCoro2A-Cofilin S3A-GFP. These cells were plated on 50 µg/mL rat tail Collagen coated Bioptechs Delta T dishes for 16-18 hrs. Time-lapse microscopy was performed on an Olympus IX-81 inverted microscope (10X objective) with a Hamamatsu CCD camera (model c4742-80-12AG). Cell speed was measured with Tracking software (Andor Bioimaging) or Slidebook software (Intelligent Imaging Innovations) using manual tracking mode. Graphs displayed were made in Prism (Graph-Pad,Inc.).

Immunoprecipitations- 293FT cells were transfected with plasmids as indicated in the figures. An 80-90% confluent 6cm dish of cells was lysed in 1mL of 1% Triton X-100 in PBS. Lysates were spun at 14K at 4°C for 5 minutes. Approximately 1µg of GFP or Coronin 2A antibodies were used to immunoprecipitate in combination with 20 µl of Protein A or G Sepharose beads (Pierce or GE Lifesciences, respectively). Immunoprecipitated proteins were run by SDS-PAGE, transferred to PVDF (Millipore), and immunoblotted for Coronin 2A or GFP.

Focal adhesion assembly and disassembly experiments- MTLn3 cells infected with shNS-GFP-Paxillin, shCoro2A-GFP-Paxillin, shNS-SSH1L-GFP, shCoro2A-SSH1L-GFP, shNS-SSH1L-2SA-GFP, or shCoro2A-SSH1L-2SA-GFP were plated as in single cell tracking

experiments. Cells were imaged on a Nipkow-type spinning disk confocal scan head (Yokogawa CSU-10) with a 60X 1.45 NA objective. Images were taken 1 frame every minute for 40 minutes. In 4 cells over 2 experiments, at least 12 focal adhesions per cell were analyzed with ImageJ software. These adhesions fit the criteria that 1) were not localized to the edge of a protruding lamellipodia, and 2) localized to either the tail or internal region of the cell. The intensity of GFP in each frame was used to determine rates of focal adhesion assembly and disassembly as in [20]. All images were corrected for photobleaching.

Focal adhesion and Cofilin live-cell imaging- MTLn3 cells infected with shNS-GFP-Paxillin and Cofilin-TagRFP were plated as in focal adhesion assembly and disassembly conditions. Total internal reflectance microscopy (Olympus) was used to illuminate fluorescent proteins in close proximity to the coverslip. Images of Cofilin and Paxillin were taken every minute for 30 minutes.

Immunofluorescence- MTLn3 cells were plated on acid-washed coverslips coated with 50 μ g/mL rat tail collagen. Cells were fixed with 4% PFA, or pre-permeablized in Permeablization buffer (20 mM Hepes, pH 7.5, 138 mM KCl, 4mM MgCl₂, 3 mM EGTA, 0.2 mg/ml of saponin, 1 mM ATP, 1% BSA) for 30 seconds followed by 4% PFA fixation for 10 minutes. After 3 washes with PBS, cells were permeablized with 0.1% TritonX-100 in PBS for 5 minutes. Cells are blocked for 15 minutes in PBS containing 5% normal goat serum (Jackson Labs.) and 5% fatty-acid free BSA. Primary antibodies were applied to cells in PBS containing 1% BSA for 1hr. If needed, diluted primary antibody solutions also

contained AlexaFluor 488, 568, or 647 Phalloidin (Invitrogen). Cells are washed 3 times in PBS. Cy2, Cy5, RhodamineRedX conjugated secondary antibodies were diluted to 1:400 in 1% BSA in PBS and applied to the coverslips for 1hr. After 3 washes in PBS, the coverslips are mounted onto slides with Fluoromount G (Electron Microscopy Sciences). Barbed-end experiments were done as in [83]. Briefly, cells are permeablized in Permeablization buffer containing 2 μ M OregonGreen or Alexa Fluor 647 Actin (Invitrogen) for 20 seconds. Cells are then fixed and processed as above.

Fluorescence recovery after photobleaching- MTLn3 cells infected with shNS-TagRFP-actin or shCoro2A-TagRFP-actin were photobleached at focal adhesions with a 405nm laser for 30ms. TagRFP fluorescence intensities were monitored every 0.784 seconds on an Olympus FV1000 microscope with a 60X 1.2NA Olympus objective. 9 cells were analyzed for each condition. All images were corrected for overall photobleaching. Images were analyzed in ImageJ.

P-Cofilin/Cofilin Ratiometric imaging- MTLn3 cells were fixed with 4% PFA and processed as in immunofluorescence experiments. Fields containing both an infected cell expressing either shNS or shCoro2A-mCherry and an uninfected cell were imaged in the linear range of the camera. Fluorescent images of P-Cofilin and Cofilin were collected and processed using the ImageJ plugin Ratio plus. Relative intensities were obtained by normalizing the values to the uninfected neighboring cells.

EB1-GFP experiments- MTLn3 cells expressing either shNS-EB1-GFP or shCoro2A-EB1-

GFP with or without TagRFP-Actin were imaged either every second for EB1-GFP dynamics and microtubule growth rate studies, or every 3 seconds for focal adhesion targeting. Focal adhesion targeting was determined by EB1-GFP tracking down an actin stress fiber and terminating at the base of the stress fiber (considered to be a targeting event). EB1-GFP velocity (rate of microtubule growth) was determined by tracking the centroid of the EB1-GFP spot over time. The distance divided by time was used to determine EB1 velocity. Growth phase was determined to be the amount of time an EB1-GFP spot is observed over the course of a movie. Since EB1-GFP localization requires a growing microtubule, this was used to estimate microtubule stability.

Chapter 3

Ectopic expression of Coronin 2A induces Cofilin-Actin like rod formation

Summary

In response to environmental stress, cells have adapted protective mechanisms to promote cell survival. Events, such as ischemic injury or ATP-depletion, have been shown to induce the formation of Cofilin-Actin rod aggregates. These rods form to reduce ATP consumption by approximately fifty percent, therefore promoting cell survival. Up to now, Cofilin and Actin are the only known proteins found in stable rods. Here, we show that ectopic expression of Coronin 2A induces the formation of aggregates similar to Cofilin-Actin rods. Ectopic Coronin 2A induced rods contain Cofilin and Actin, but unlike ATP-depletion induced Cofilin-Actin rods, they stain positively with phalloidin. Previous fluorescence recovery after photobleaching (FRAP) experiments showed that ATP-depletion induced Cofilin-Actin rods contain very stable, immobile Cofilin. FRAP analysis indicates that ectopic Coronin 2A induced rods contain mobile Cofilin and immobile Coronin 2A. Ectopic expression of Coronin 2A also reduces the amount of time needed for Cofilin-GFP rods to initiate upon ATP-depletion without increasing the rate of Cofilin dephosphorylation. These data are indications that ectopic Coronin 2A expression may induce the formation of unstable Cofilin-Actin rod intermediates or precursors that form in response to environmental stress.

Introduction

Organisms undergo a constant barrage of environmental insults and genetic mutations that impair a cell's ability to function properly. Some of these stresses require the cell to stop normal function, repair itself if possible, and then resume normal cellular activities. If it is unable to do this, most of the time the cell will become apoptotic and die. In most tissues, this is not a problem because neighboring cells can divide and replace the apoptotic cells. Neurons and other terminally differentiated cells, however, do not have the luxury of replacing dying cells due to the fact that they are unable to replicate. To compensate, these cells have developed mechanisms used to increase the likelihood of cell survival (reviewed in [91]).

A model of neuronal stress, soluble β -amyloid protein precursor, which mimics Alzheimer's disease [92], induces the formation of Cofilin-Actin rod aggregates that are found to associate with fibril tangles in neurons of these mice. Other in vitro studies have shown that environmental stresses, such as ATP-depletion (sodium azide and 2-deoxyglucose treatment) or exposure to reactive oxygen species (ROS) [54, 55, 93], can also induce the formation of Cofilin-Actin rods. Physiologically, this impairs many cellular functions due to decreased Actin dynamics. As an example, analysis of neurons containing Cofilin-Actin rods display impaired synaptic activity and reduced mitochondrial function [93]. Even though cellular activities are impaired in cells that contain rods, relief of acute stress back to physiological conditions can promote rod disassembly and return cellular function back to normal. However, long-term exposure to environmental stress conditions locks Cofilin-Actin rods into stable aggregates that are thought to terminally prevent normal cellular function [55].

Recently, some of the mechanisms of rod formation have been uncovered. Cofilin-Actin rods contains only dephosphorylated Cofilin [55]. For this to occur, Cofilin phosphatases must be activated in response to stress conditions, which leads to Cofilin dephosphorylation. Experimental studies have implicated Chronophin as the major contributor of dephosphorylation during ATP-depleting conditions [54]. Under normal physiological conditions, Chronophin is held in an inactive conformation by interacting with Hsp90. This is an ATP-dependent interaction. Upon ATP-depletion, Chronophin is released and can then dephosphorylate Cofilin leading to Cofilin-Actin rod formation [54].

Conversely, Slingshot is activated in cells exposed to ROS, by either hydrogen peroxide treatment or increased intracellular ROS production [94]. Normally, Slingshot is inactivated by interacting with 14-3-3 proteins. Once cells are exposed to ROS, 14-3-3 proteins become oxidized and Slingshot is released from the complex. Slingshot is then able to bind to F-actin promoting Cofilin dephosphorylation [94]. While Cofilin dephosphorylation is required, other stress response cofactors may be important to organize the Actin and/or Cofilin in the correct arrangement needed for rod formation.

Coronin 2A is an F-actin binding protein that localizes to F-actin stress fibers and focal adhesions. Recent studies have shown that depletion of Coronin 2A inhibits focal adhesion disassembly through an active Cofilin dependent mechanism (see Chapter 2). Data show that Coronin 2A interacts with Slingshot and regulates its activity at focal adhesions. Cells depleted of Coronin 2A contain higher P-Cofilin protein levels, display impaired motility, and have less free barbed ends and F-actin turnover at focal adhesions. These data implicate Coronin 2A as a regulator of Cofilin activity at focal adhesions, but how Coronin 2A-Slingshot-Cofilin signaling occurs is not known. We also noticed that ectopic expression

of Coronin 2A induces rod like aggregates that contain Cofilin and Actin. Here, we investigate the role of Coronin 2A in Cofilin-Actin rod formation under ATP-depleting conditions.

Results

Ectopic expression of Coronin 2A induces the formation of rod aggregates that contain Cofilin and Actin

To determine the localization pattern of Coronin 2A, we expressed Coronin 2A-GFP or Coronin 2A-TagRFP in Rat2 fibroblasts and HeLa cells, respectively. While most cells expressed relatively low levels of Coronin 2A, cells with high levels formed rod like aggregates (Fig. 13A, B). These rods are appeared to be similar to Cofilin-Actin rods found in Alzheimer's disease neurons or neurons that have been depleted of ATP in vitro. Immunofluorescence of these rods display similarities and differences to Cofilin-Actin rods. Like Cofilin-Actin rods, Coronin 2A-induced rods contain both Cofilin and Actin, but do not contain several other Actin-binding proteins such as Vinculin, Vasp, or Coronin 1B (Fig. 13A, B). Unlike Cofilin-Actin rods, Coronin-2A induced rods stain positively with phalloidin (Fig. 13A). Since Cofilin and phalloidin compete for the same binding site on F-actin, the Cofilin and Actin in Coronin 2A rods must have a different, unstable conformation compared to Cofilin-Actin rods.

Ectopic expression of Coronin 2A potentiates ATP-depletion induced Cofilin-Actin rod formation

Recently, a Cofilin-Actin rod formation model system has been developed in HeLa cells [54]. In this model, a HeLa cell line stably expressing Cofilin-GFP is exposed to ROS or ATP-depleting conditions. This induces the formation of Cofilin-GFP rods over the course of thirty minutes [54, 94]. This can be visualized by monitoring GFP incorporation into the rod structures. We have made a similar cell line by stably expressing Cofilin-GFP in HeLa

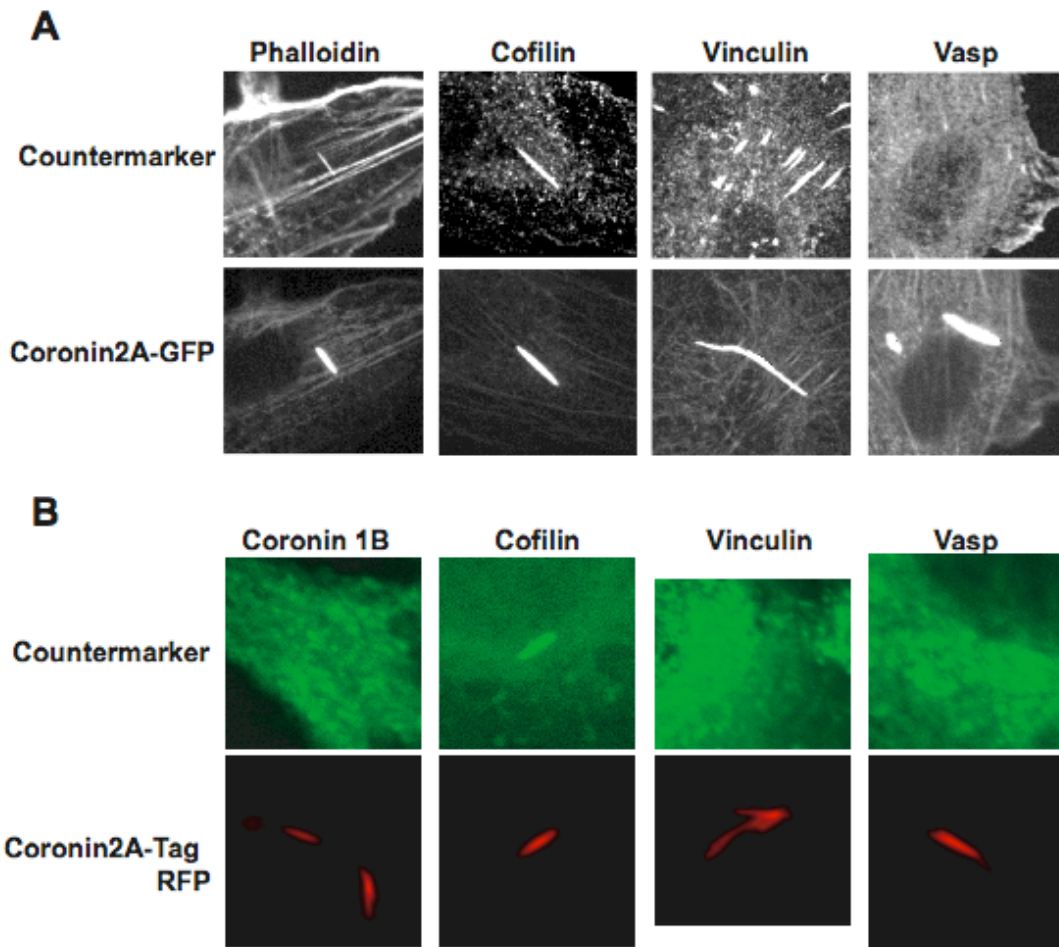


Figure 13. Ectopic expression of Coronin 2A induced rod-like aggregates that contain Cofilin and Actin.

(A) Rat2 cells expressing high levels of Coronin 2A-GFP form rod-like aggregates.

Representative images of cells containing Coronin 2A-GFP rods co-stained for F-actin with Alexa568-Phalloidin, Cofilin, Vinculin, or Vasp.

(B) HeLa cells expressing high levels of Coronin 2A-TagRFP. Representative images of cells containing Coronin 2A-TagRFP rods co-stained for Coronin 1B, Cofilin, Vinculin, or Vasp.

cells via lentiviral transduction. These cells were then FACS sorted for cells expressing similar levels of Cofilin-GFP. Upon ATP-depletion, these cells form Cofilin-Actin rods at similar rates to those seen in ([54])(Fig. 14B). Cofilin-GFP expressing HeLa cells co-expressing Coronin 2A-TagRFP display a rapid increase in the number of cells containing Cofilin-Actin rods compared to Cofilin-GFP alone expressing cells (Fig. 14A, B). Interestingly, Coronin 2A-TagRFP rods were not found in the great majority of these cells (between 90-95%) (Fig. 14E). Also, ATP-depletion did not induce an increase in the number of cells with Coronin 2A-TagRFP rods (Fig. 14E). On average, these HeLa cells are expressing less than 1 fold over endogenous levels of Coronin 2A indicating that these aggregates are not caused by massive overexpression of Coronin 2A (Fig. 15A). We next employed kymography to determine if Coronin 2A expression affects rod growth rates or lengths. Analyses indicate that there is no significant change in Cofilin-GFP rod growth rate or rod length (Fig. 14C, D).

Ectopic Coronin 2A expression does not affect Cofilin dephosphorylation upon ATP-depletion

One necessary requirement for Cofilin-Actin rod formation is the dephosphorylation of Cofilin. Since Coronin 2A interacts with Slingshot and depletion of Coronin 2A in MTLn3 cells increases P-Cofilin levels (Fig. 6 A, B, C, D, E), one could presume that ectopic Coronin 2A expression may increase Cofilin dephosphorylation through the Slingshot pathway. This is not the case. Coronin 2A expression has no effect on Cofilin dephosphorylation under ATP-depleting conditions (Fig. 15A). Another explanation for ectopic Coronin 2A expression inducing rod formation is that Coronin 2A itself causes a

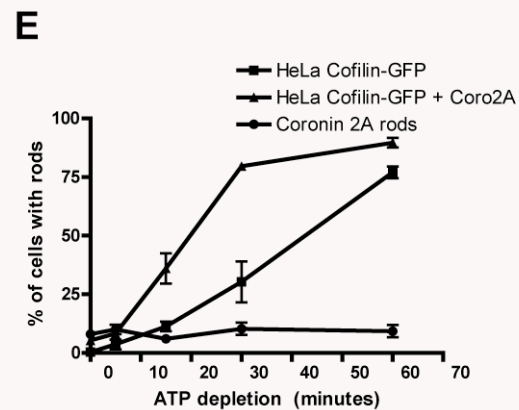
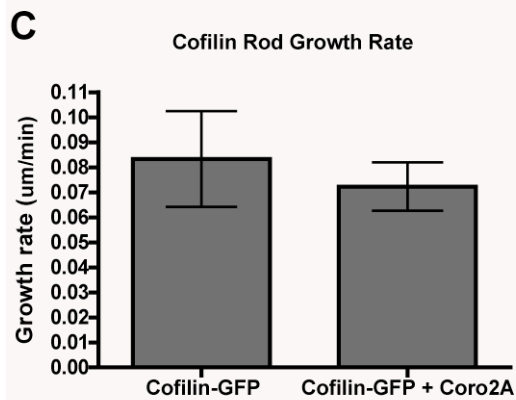
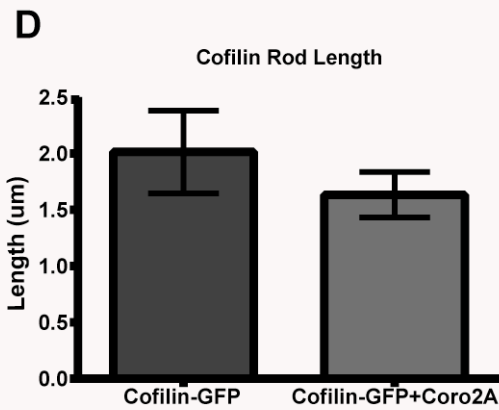
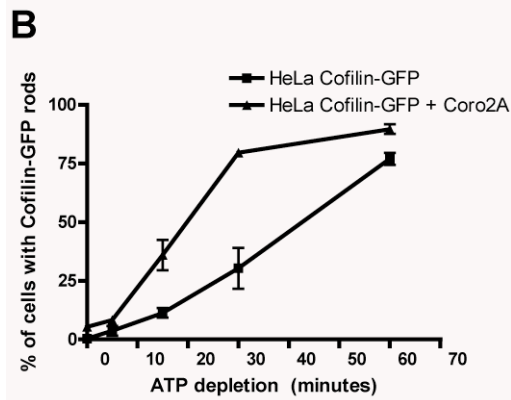
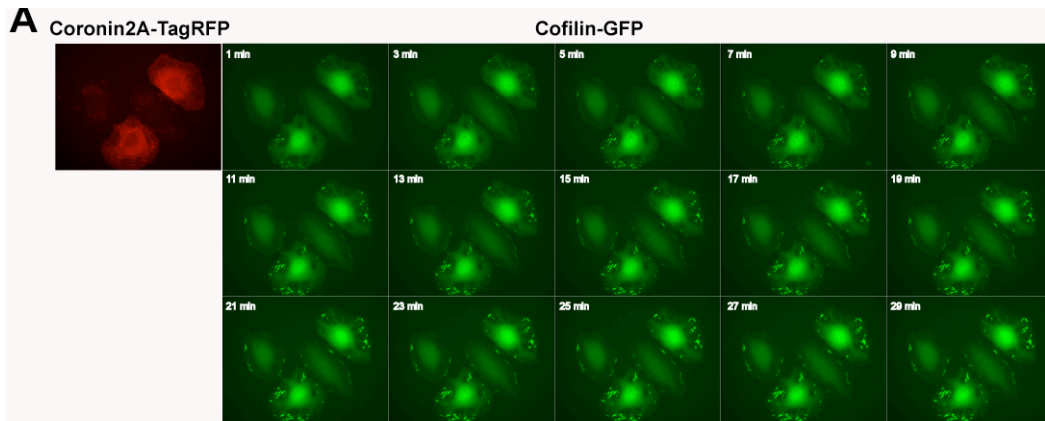


Figure 14. Ectopic expression of Coronin 2A-TagRFP does not affect Cofilin dephosphorylation upon ATP-depletion.

(A) HeLa cells expressing Cofilin-GFP with and without Coronin 2A-TagRFP were ATP-depleted. Representative Immunoblots of Coronin 2A, P-Cofilin and Cofilin.

(B) Quantification of immunoblots (n=3) monitoring P-Cofilin/Cofilin ratios. Time course of ATP-depletion shows no change in Cofilin dephosphorylation caused by the expression of Coronin 2A.

(C) Ectopic expression of Coronin 2A-TagRFP does not affect cell cycle. Growth curves show no change in cell number over the course of 3 days (n=3).

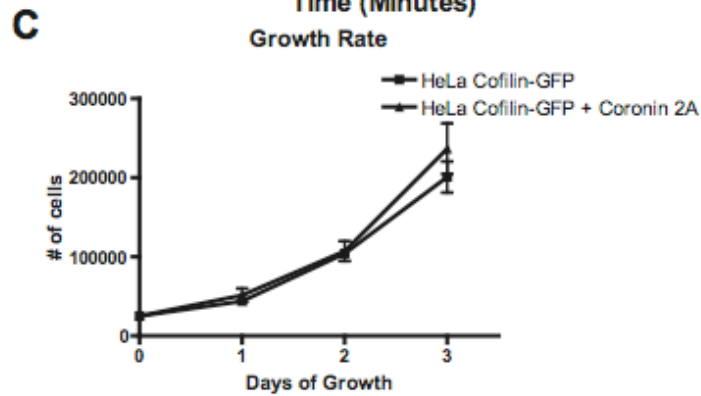
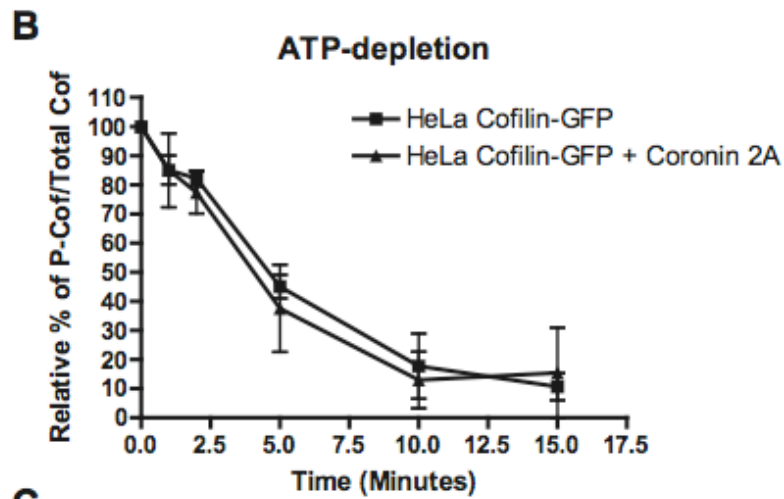
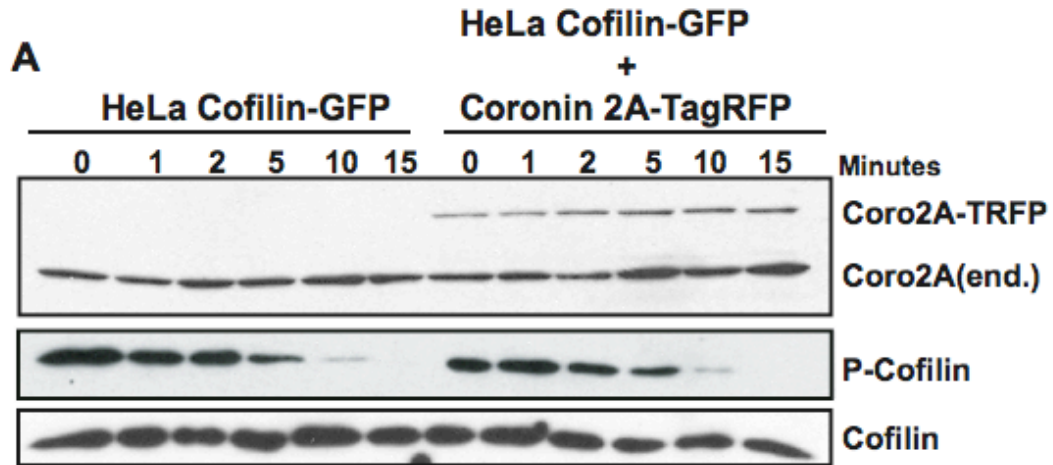


Figure 15. Ectopic expression of Coronin 2A does not affect ATP-depletion induced Cofilin dephosphorylation or cell proliferation.

(A) ATP-depletion time course of HeLa cells expressing Cofilin-GFP with or without Coronin-2A-TagRFP. Representative immunoblots for Coronin 2A, P-Cofilin, and Cofilin.

(B) Quantification of P-Cofilin/Cofilin ratios from immunoblots in (A). n=3. Error bars are standard errors.

(C) Growth curves for HeLa cells expressing Cofilin-GFP with or without Coronin 2A-TagRFP. n=3.

stress response. As an estimation for cellular stress, cell proliferation was monitored over the course of three days. In these experiments, cell growth rate is the same in cells expressing Cofilin-GFP with or without co-expressing Coronin 2A-TagRFP (Fig. 15C), indicating that ectopic expression of Coronin 2A does not induce a stress response.

Coronin 2A is immobile and Cofilin is mobile in Coronin 2A-induced rods

To further characterize similarities and differences between Coronin 2A rods and Cofilin-Actin rods, we performed fluorescence recovery after photobleaching (FRAP) experiments. As seen in previous studies [93], FRAP analysis of Cofilin-Actin rods caused by ATP-depletion show that Cofilin-GFP present in these structures is very immobile (Fig. 16A, top panel). Coronin 2A rods containing either endogenous Cofilin or Cofilin-GFP show a similar characteristic in that they display very little fluorescence recovery after photobleaching (Fig. 16A, middle and bottom panels). This is an indication that Coronin 2A is in a stable conformation in rod structures. Interestingly, Cofilin-GFP mobility is dramatically increased in Coronin 2A rods compared to ATP-depletion induced rods (Fig. 16A, top and bottom panels) and almost completely recovers back to prebleaching fluorescence intensity. These data show striking differences between Coronin 2A rods and Cofilin-Actin rods.

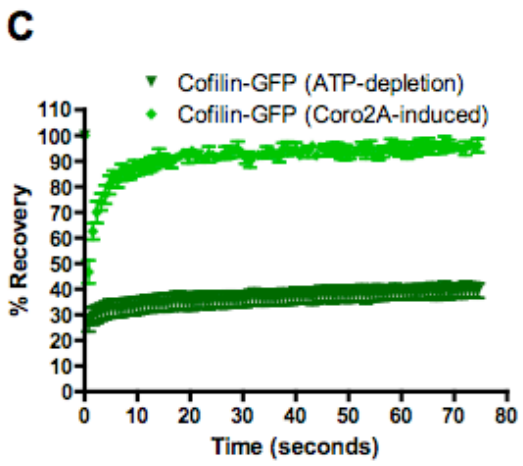
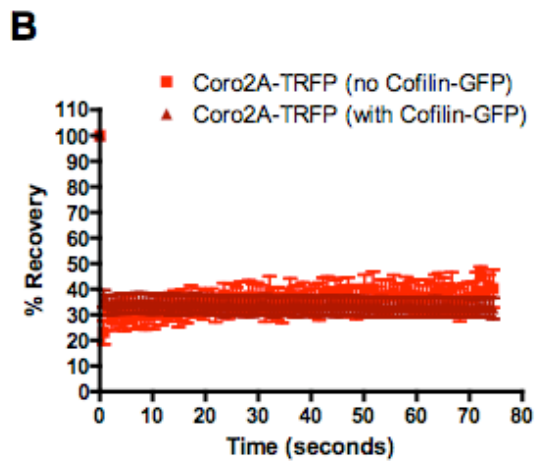
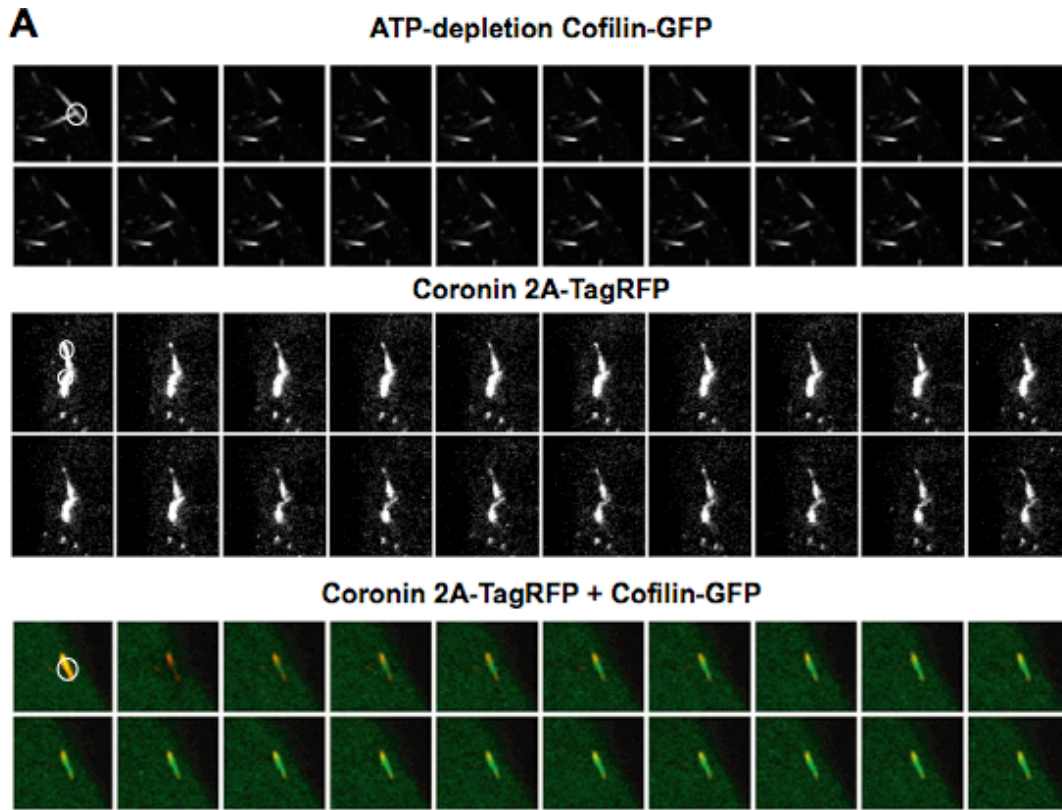


Figure 16. Coronin 2A is immobile and Cofilin is mobile in Coronin 2A-induced rods

(A) Top panel, HeLa cells expressing Cofilin-GFP were treated with 10 μ M sodium azide and 6 μ M 2-deoxyglucose for 30 minutes to induce Cofilin-GFP rods. Middle panel, HeLa cells that contain Coronin 2A-TagRFP rods due to high ectopic expression. Bottom panel, HeLa cells that contain Coronin 2A-TRFP (shown in red) and Cofilin-GFP (shown in green) induced by Coronin 2A expression. Rods were bleached (white circle) with a 405 nm laser for 30ms. Each frame is 0.784 seconds.

(C) Quantification of Coronin 2A-TagRFP FRAP results. n=8, error bars represent s.e.m.

(D) Quantification of Cofilin-GFP FRAP results

Discussion

Here we show that ectopic expression of Coronin 2A can induce the formation of rod aggregates that are similar to Cofilin-Actin rods. These aggregates contain Cofilin, Actin and Coronin 2A, but not other actin binding proteins such as Vinculin, Vasp, or Coronin 1B. Ectopic expression of Coronin 2A-TagRFP potentiates Cofilin-Actin rod formation in ATP-depleting conditions even in cells that do not contain Coronin 2A rods. This is not due to elevated Slingshot activity because expression of Coronin 2A has no effect on Cofilin dephosphorylation upon ATP-depletion. Coronin 2A rods differ from Cofilin-Actin rods because they contain mobile Cofilin, while Cofilin-Actin rods induced by ATP-depletion contain very stable Cofilin.

Cofilin-Actin rods are found associated with fibril tangles in Alzheimer's disease brain sections [55]. Little is known about the actual physiological conditions that promote the formation and stability of rods. From the data presented here, we show that upon ATP-depletion, ectopic expression of Coronin 2A has no effect on Cofilin dephosphorylation, but does potentiate rod formation. This may indicate a novel function of Coronin 2A in regulating Cofilin. While it has not been shown, it is possible that Coronin 2A may interact directly with Cofilin or indirectly through Slingshot to promote Cofilin-F-actin binding. How localized concentrations of Cofilin saturate F-actin in rods is also unknown and may be facilitated by Coronin 2A. Further studies will be needed to test these ideas.

Coronin 2A rods and Cofilin-Actin rods show similarities and differences. Both contain Actin and Cofilin, but in different conformations. This is shown in two ways. First, in Coronin 2A rods, the Cofilin-Actin interaction is unstable as documented by the ability to stain actin filaments with phalloidin. Since phalloidin and Cofilin compete for the same F-

actin binding site, stable association between Cofilin and F-actin prevents phalloidin staining in the rods [95]. This is what is seen in Cofilin-Actin rods induced by ATP-depletion [55], but is not seen in Coronin 2A rods. Second, FRAP of Cofilin-GFP rapidly recovers in Coronin 2A-TagRFP rods. Conversely, Cofilin is very stable and does not recover in Cofilin-Actin rods induced by ATP-depletion. Unknown changes within the rods under ATP-depletion conditions affect Cofilin-Actin interactions. Rapid exchange of F-actin within Coronin 2A-TagRFP rods may be occurring as well. Determining why and how Coronin 2A affects Cofilin-F-actin interactions will require further investigation.

As seen in the FRAP experiments, the Coronin 2A in rods is very stable indicating that it is in a locked conformation. While this is very interesting, the physiological role of Coronin 2A in Cofilin-Actin rod formation remains elusive. Since Coronin 2A interacts with Slingshot, it is surprising that depletion of Coronin 2A affects Cofilin rod formation under ATP-depleting conditions, because these conditions favor Chronophin as the activating phosphatase of Cofilin [54]. Ectopic expression of Coronin 2A potentiating Cofilin-Actin rod formation under these conditions may indicate that Coronin 2A is a cofactor aiding in rod formation. Loss of function experiments are needed to determine the role of Coronin 2A in these processes. In contrast, Coronin 2A regulation of rod formation is more likely to occur in response to ROS, which induces Slingshot activation [94]. ROS experiments also need to be performed to determine if Slingshot-Coronin 2A interactions affect Cofilin dephosphorylation and/or Cofilin-Actin rod formation.

Previous data from our laboratory indication Coronin 2B is the predominant type II Coronin in the brain [44]. While Cofilin-Actin rods have been shown to form in epithelial cells types, the role of type II Coronins in Alzheimer's disease is physiologically more

relevant. As proof of principal, ectopic expression of Coronin 2B induces the formation of Cofilin-Actin like rods as well (data not shown). This result opens the door for determining if Coronin 2B plays a role in regulating impaired neuronal function through the formation of Cofilin-Actin rods in Alzheimer's disease brains.

Materials and Methods

Molecular cloning- pLL5.0 base vector was described in [69]. pLL5.0-TagRFP was made by PCR amplifying TagRFP from pTagRFP-Actin (Evrogen) and replacing GFP with TagRFP in the BamHI and SbfI restriction enzyme sites. cDNAs were PCR amplified and inserted in either pLL5.0-GFP or pLL5.0-TagRFP with the sites indicated: Coronin 2A (EcoRI/BamHI), and Cofilin (EcoRI/BamHI).

Antibodies and reagents- Coronin 2A antibody production and purification were described previously in Chapter 2. The following antibodies were purchased and used at the dilutions as indicated: Coronin 2A (NC540; WB 1:250), Cofilin (Cytoskeleton Inc.; WB 1:500, IF 1:100), P-Cofilin (Biosource; WB 1:1000), Vinculin (hVin1; Sigma; IF 1:200), Coronin 1B rabbit polyclonal antibody is described in [44]; IF 1:100), Vasp (2010 was described in [96]; IF 1:400). Cy2, Cy5, RhodamineRedX, and HRP conjugated secondary antibodies are from Jackson Immunoresearch Laboratories. Immobilon-P PVDF is from Millipore. Fibronectin was purchased from BD Biosciences. 100X Pen Strep Glutamine (PSG), DMEM, and AlexaFluor568-Phalloidin were purchased from Invitrogen. Fetal Bovine Serum (FBS) was purchased from Hyclone.

Cell culture and shRNA production- 293FT and HeLa cells were grown in DMEM containing 10% FBS and 1X PSG. Lentiviral production was performed as previously described in [69]. Briefly, 293FT cells are grown to 75% in a 6cm dish. Lentiviral expression plasmids are transfected into cells with Fugene6. After cells are fluorescent, media is changed. After one day, viral media is applied to 30-50% confluent HeLa cells in a

6-well dish. After 4 hours, equal volumes of fresh media are added to the viral media. After 16-24 hours, media is replaced and cells are grown and expanded. After 2 days, fluorescent cells are FACS sorted for cells expressing similar levels of GFP.

ATP-depletion induced Cofilin dephosphorylation assay- HeLa cells expressing Cofilin-GFP with and without expressing Coronin 2A-TagRFP were plated in a 6 well dish overnight. Cells were washed one time with PBS and one time with DMEM with no glucose containing 10% FBS and 1X PenStrepGlut. DMEM media without glucose media was added to cells for two hours to starve cells of ATP. DMEM media without glucose containing 10 μ M Sodium Azide and 6 μ M 2-Deoxyglucose were applied to cells for times indicated. Cells were lysed in PBS/1% Triton-X 100 containing protease inhibitors and 50 mM sodium fluoride. Equal amounts of proteins were run by SDS-PAGE, transferred to PVDF and immunoblotted for Cofilin and P-Cofilin. Densitometry readings were taken and averaged. Error bars indicate S.E.M. for all runs. n=3.

ATP-depletion induced rod formation assay- HeLa cells were plated in Biotechs Delta T dishes coated in 20 μ g/mL Fibronectin for 16-18 hrs. Cells were treated as done in Cofilin dephosphorylation experiments. Time-lapse microscopy was performed on an Olympus IX-81 inverted microscope (40X, 0.60NA objective) with a Hamamatsu CCD camera (model c4742-80-12AG) and a Prior Lumen200Pro epifluorescence system. Images were captured with Slidebook software (Intelligent Imaging Innovations). Kymographic analysis was performed with the ImageJ plugin Multiple Kymograph. Graphs displayed were made in Prism (Graph-Pad,Inc.).

Immunofluorescence- Cells were plated on Fibronectin coated coverslip overnight. Cells were fixed with 4%PFA for 10 minutes or room temperature methanol for 2 minutes. Cells were permeablized with 0.1% Triton-X 100 in PBS for 5 minutes. Cells were then blocked in 5% fatty acid-free BSA/1% normal goat serum in PBS for 15 minutes. Primary antibodies were applied for 1-2 hours. Cells were washes 3 times with PBS and secondary antibodies were applied for 1 hour. Cells were then mounted to coverslips.

Coverslips were imaged on a Nipkow-type spinning disk confocal scan head (Yokogawa CSU-10) with a 60X 1.45 NA objective. Representative images are shown in figures.

Fluorescence recovery after photobleaching of Cofilin-GFP and Coronin 2A-TagRFP- Cells expressing Cofilin-GFP, Coronin 2A-TagRFP or both were plated on 20 $\mu\text{g}/\text{mL}$ Fibronectin coated coverslips overnight. Cofilin-GFP expressing cells were depleted of ATP with 10 μM Sodium Azide and 6 μM 2-Deoxyglucose for 30 minutes prior to imaging. Coronin 2A-TagRFP cells were not treated. Cells were photobleached at rods with a 405nm laser for 30ms. GFP and/or TagRFP fluorescence intensities were monitored every 0.784 seconds on an Olympus FV1000 microscope with a 60X 1.2NA Olympus objective. An n=8 was used for each condition. All images were corrected for overall photobleaching. Images were analyzed in ImageJ.

Growth rate experiments- 25,000 cells were counted and plated into a 35mm culture dish. After 24, 48, and 72 hours, cells were trypsinized and counted. All cell counting was done in triplicate and the average value was used in quantification of cell growth rates. n=3. Graphs

were made with Prism software (Graph-Pad,Inc.).

Chapter 4

Conclusions and Future Directions

Over the course of these studies, we have performed a basic characterization of Coronin 2A and determined its function in regulating focal adhesion turnover. In contrast to Coronin 1B, Coronin 2A localizes to F-actin stress fibers and focal adhesions and is excluded from the leading edge, but like Coronin 1B, Coronin 2A can interact with Slingshot-1L, an activating phosphatase for Cofilin. The affects caused by Coronin 2A depletion are at least in part due to improper regulation of Slingshot needed to dephosphorylate and activate Cofilin. This is indicated by cells that are depleted of Coronin 2A contain higher levels of P-Cofilin, reduced free barbed ends at focal adhesions, and decreased F-actin flow at the ends of stress fibers. Besides a decrease in phosphatase activity, Slingshot and Coronin 2A appear to work combinatorially in the activation of Cofilin at focal adhesions by an unknown mechanism. While the role of Coronin 2A in regulating focal adhesion turnover is clear, many questions about Coronin 2A function(s) and how focal adhesion turnover works mechanistically remain unanswered.

Ectopic expression of Coronin 2A also has an effect on Cofilin regulation. High levels of ectopic expression induces the formation of rod like aggregates that are very similar to Cofilin-Actin rods. Cofilin-Actin rods form in response to stress conditions including ischemic injury, ATP-depletion, or the presence of reactive oxygen species. Low level

ectopic expression of Coronin 2A potentiates Cofilin-Actin rod formation in response to ATP-depletion, even in cells that do not contain Coronin 2A rods. While these data suggest that Coronin 2A may be important for Cofilin-Actin rod formation, there are clear differences in the Cofilin and Actin structures in Coronin 2A-induced rods. These preliminary studies on Coronin 2A and stress response have given glimpses of information that may be important in effecting stress induced Cofilin-Actin rod formation, but many questions remain.

These new data on Coronin 2A/Cofilin localizations and functions may be explained by the following model (Figure 17). In the case of focal adhesion disassembly, Coronin 2A may perform two independent roles to regulate Cofilin activity. First, an interaction between Coronin 2A and Slingshot is required to temporally and spatially control Cofilin dephosphorylation. This is observed by increased Cofilin phosphorylation upon Coronin 2A depletion. Second, Coronin 2A could affect Cofilin binding/severing of F-actin by directly interacting with the filaments. Since stress fibers are composed of bundled filaments, Coronin 2A may function to unbundle or at least increase the accessibility for Cofilin to bind and sever actin filaments. This idea that Coronin 2A-F-actin interactions affect Cofilin binding is supported by the Coronin 2A rod data. The presence of Coronin 2A at Cofilin rods inhibits Cofilin from stably associating with F-actin. Since Coronin 2A is in a fixed, immobile conformation, dissociation from the actin filaments may be required for Cofilin-F-actin binding to occur. While all of this is hypothetical, these data suggest that Coronin 2A affects Cofilin function in the cell through multiple mechanisms. Future studies are needed to elucidate all of these possible outcomes. In the following chapter, unanswered questions about Coronin 2A biological function will be presented. I will start by discussing similarities

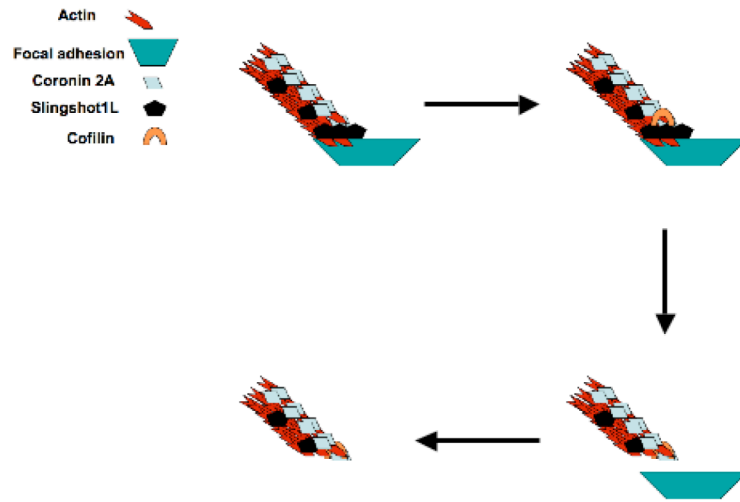


Figure 17. Model of Focal Adhesion disassembly.

and differences of Coronin 1B and Coronin 2A. This will be followed by how Coronin 2A-Slingshot-F-actin-Cofilin regulation can be addressed. Thirdly, the role of Coronin 2A and focal adhesion dynamics in the context of three-dimensional analysis and cancer physiology will be discussed. Finally, I will hypothesize about potential mechanisms involving type II Coronins in Cofilin-Actin rod formation.

The first thing that I noticed during my studies of Coronin 2A is the extreme differences in localization patterns of type I and type II Coronins, more specifically Coronin 1B and Coronin 2A. To recap, Coronin 1B is found at the leading edge of cells where it interacts with the Arp2/3 complex and is important for the disassembly of branched F-actin at the rear of the lamellipodia [44, 62, 69, 70]. In contrast, Coronin 2A is excluded from the leading edge and localizes along F-actin stress fibers and at focal adhesions. The almost complete segregation of these proteins is striking (Fig. 1A), but what factor(s) cause the differences in localization? Performing sequence alignments of the two proteins show that the β -propeller region has a relatively high amount of amino acid similarity (68 % similarity), while the unique and coiled-coil domains become more divergent (37% and 30% similarity, respectively). By creating chimeric Coronin proteins via swapping Coronin 1B and Coronin 2A domains and fusing them to GFP, we can determine any changes in localization of these proteins through fluorescence imaging. Theoretically, specific domains will change the localization of Coronin 2A to the leading edge of cells and/or Coronin 1B to stress fibers and/or focal adhesions. This may be caused by a few different reasons. For example, if there is an Arp2/3 complex binding site in Coronin 1B and not in Coronin 2A, the insertion of this region into Coronin 2A may localize it to the leading edge of cells. Some Coronin chimeras have been made [62], but they have only been documented in S2

cells and need further characterization in mammalian cell contexts. Previous Coronin 1B-2A chimeras were shown to affect F-actin cable formation, which was used to identify the F-actin binding site in Coronin 1B. While Coronin 2A still localizes to F-actin structures, it does not contain the same binding site as Coronin 1B [62].

Another possibility is that coiled-coil based oligomerization and/or other protein-protein interactions affect the size/shape of Coronin 2A making it too big to intercalate into the lamellipodia of the cell. Studies on cells that were microinjected with fluorescently labeled Ficolls of different molecular weights showed that bigger Ficoll particles were excluded from the lamellipodia of the cell and displayed a similar localization pattern to Coronin 2A [97, 98]. While this is only suggestive that Coronin 2A is in a large enough complex to be excluded from the lamellipodia, it still is possible. An indication that this may be true is that the great majority of Coronin 2A is found in the insoluble fraction of cells that have been lysed (data not shown). To test if Coronin 2A mega-oligomers exist, one could isolate these structures from cells and protein complex size could be estimated by gel filtration or size exclusion chromatography. From elutions that contain Coronin 2A-complexes, new binding partners could also be identified by mass spectrometry.

Another interesting finding from these studies is that Coronin 2A interacts with Slingshot. From colocalization data and Cofilin activity assays, these proteins more than likely interact and regulate Cofilin activation at focal adhesions. Coronin 2A is required for proper focal adhesion turnover as marked by GFP-Paxillin and Slingshot-GFP intensities. Interestingly, wild-type Slingshot over-expression does not affect focal adhesion disassembly in Coronin 2A-depleted cells. This is another indication that Coronin 2A is regulating Slingshot phosphatase activation. So, how does Coronin 2A regulate Slingshot activity? The

only known form of Slingshot inhibition is through Serine phosphorylation at two consensus 14-3-3 binding sites on the C-terminal end of the protein [41, 56, 99, 100]. Slingshot is sequestered to the cytoplasm by 14-3-3 proteins, diminishing Slingshot's ability to bind to F-actin, an interaction required for maximal Slingshot phosphatase activity [41]. Antibodies are theoretically available to determine if Slingshot is phosphorylated at the consensus 14-3-3 binding sites [41], however they have been difficult to obtain. Cell lysates from Coronin 2A-depleted cells could be immunoblotted with these antibodies to determine if this is one way Coronin 2A is regulating Slingshot phosphatase activity.

Since expression of Coronin 2A has an effect on focal adhesion disassembly in the presence of an unphosphorylatable, active mutant of Slingshot, it appears that mechanistically Coronin 2A has another function required for Cofilin activation other than through 14-3-3-Slingshot interactions. Another possibility is that since both Coronin 2A and Slingshot bind to F-actin (data not shown and [34, 100]), they may appropriately organize actin filaments for Cofilin binding/severing to occur. This may happen simultaneously with Cofilin dephosphorylation by Slingshot. To test these ideas, fluorescent actin filament severing assays can be used. In these assays, fluorescently labeled actin is polymerized into filaments in a flow chamber and various combinations of Coronin 2A, Slingshot and Cofilin can be added. The affects of protein activities can be monitored by changes in the F-actin fluorescence/structures using total internal reflectance microscopy [101, 102]. Since the activity state of Slingshot affects focal adhesion dynamics, mutant forms of Slingshot would also be useful to determine the role of coordinated Coronin 2A-Slingshot activities.

Other in vitro assays, such as F-actin cosedimentation or pyrene actin-based depolymerization assays, can be used to determine if the proteins involved bind/sever

synergistically, antagonize each other, or neither. In the F-actin cosedimentation assays, proteins that bind to actin filaments are pelleted by high-speed centrifugation, but if these proteins destabilize F-actin, like Cofilin, the actin becomes solublized and less actin is found in the pellet fraction. If Coronin 2A has an effect on Cofilin activation in the presence or absence of Slingshot, there will be increased amounts of actin in the soluble fraction. In pyrene actin depolymerization assays, polymerized pyrene actin is diluted below the critical concentration of polymerization and spontaneous depolymerization occurs. Cofilin activity increases the depolymerization by severing actin filaments [103, 104]. Including Coronin 2A and/or Slingshot into these reactions may affect the rate of actin depolymerization. These data may provide valuable information about how Coronin 2A-Slingshot-Cofilin regulation affects F-actin at focal adhesions.

A major caveat of cell culture experiments is that they are done on tissue culture plastic in a two-dimensional environment. Physiologically, epithelial cells are rarely in a two-dimensional context and even more rarely in the context of a surface as stiff as glass or plastic. This leads to the questions: What do focal adhesions look like in a three-dimensional context and is Coronin 2A function conserved in three-dimensions? Other groups have shown how cells adhere and migrate in three-dimensions is quite different than two-dimensional migration [105, 106], but very little is known about the adhesive structures in those contexts. Initial three-dimensional studies showed that focal adhesions do exist, but the composition of and signaling from the adhesions are quite distinct [107, 108]. As an example, in two-dimensions, FAK is highly phosphorylated at Y397, but this is mostly unphosphorylated in three-dimensions. Also, the integrins important for three-dimensional adhesion are different as well [107]. While it appears that stress fibers still exist in three-

dimensional assays [109], it is unclear if the same proteins reside on these actin structures. The first experiment is to determine the localization of Coronin 2A, Slingshot, and Cofilin in three-dimensions. If the change in the environments affects localization, it is important to know what these changes are. Immunofluorescence of other focal adhesion proteins, like Talin, would also be beneficial.

Next, does depletion of Coronin 2A decrease cell motility in three-dimensions through decreased focal adhesion disassembly? Since Coronin 2A has such an effect on adhesion dynamics in two-dimensions, it seems likely that the same would be true in three-dimensions. The problem still arises that the role of focal adhesions in a three-dimensional context is unknown. To estimate cancer cell motility, we can embed cells into a three-dimensional matrix, such as Matrigel or gelled Collagen, and monitor the movements of the cells over time. These experiments can also provide information about the importance of focal adhesions in a more physiological context. Assuming that depletion of Coronin 2A still inhibits cell motility by decreasing focal adhesion turnover, these cells should migrate less. A huge caveat is that we must assume that any motility defect is caused by the same defect that we see in a two-dimensional assay. Overcoming this issue will require repeating all of the two-dimensional assays in three-dimensions. While most if not all of the fixed-cell imaging is doable, live cell imaging may be difficult due to changes in the z-axis. Long acquisition time experiments, like focal adhesion turnover assays and whole cell migration, can be monitored by taking z-stacks, but experiments requiring rapid, short exposure times, like kymography, may be unobtainable.

The physiological role of Coronin 2A is the overall goal of this project. To truly determine the function of Coronin 2A in cancer cell migration, cells must be examined in

living animals. MTLn3 cells depleted of Coronin 2A can be injected into mammary fat pads of rats and migration of these cells can be monitored by intravital imaging [110]. Previous studies showed that MTLn3 cells can migrate along Collagen fibers to get to a blood vessel, a require step for metastasis [111]. If the role of Coronin 2A in adhesion in two-dimensions is similar in three-dimensions, cells will fail to translocate to the blood vessel preventing cancer cell invasion and metastasis. It is unclear if depletion of Coronin 2A would have any affect on the growth of a primary tumor in the mammary fat pad. This could be useful information in determining if Coronin 2A has any effect on mitogenic signaling.

On-going studies have indicated a role of Coronin 2A in promoting Cofilin-Actin rod formation in response to stress conditions. The HeLa cell based model of Cofilin-Actin rod formation has shown that ectopic expression of Coronin 2A enhances the speed at which rods are initiated in cells responding to ATP-depletion. Previous studies suggest that Chronophin is the primary source of Cofilin dephosphorylation under ATP-depleting conditions [54]. How does Coronin 2A, a protein that affects Slingshot activation, sensitize the ATP-depletion induced Cofilin-Actin rod formation pathway? Since ectopic Coronin 2A expression does not affect Cofilin dephosphorylation upon ATP-depletion, Coronin 2A may organize the Cofilin or F-actin or both into conformations that initiate rod formation. The first thing to determine is does Coronin 2A interact with Cofilin? By co-immunoprecipitation, this has not been shown to be true in my hands. Protein crosslinking may be needed prior to immunoprecipitation to see this interaction. It is also possible that this is an indirect effect through the Coronin 2A-Slingshot complex. Another possibility is that while Coronin 2A does not have a global affect on Cofilin dephosphorylation upon ATP-depletion, it may be possible that localized Cofilin dephosphorylation occurs. This may be

seen by P-Cofilin/Cofilin ratiometric immunofluorescence experiments. Over time, localized Cofilin dephosphorylation can be measured by subtracting the amount of P-Cofilin from total Cofilin in the cell.

In contrast to ATP-depletion conditions, Cofilin-actin rod formation in response to reactive oxygen species occurs in a different way. In this case, 14-3-3 proteins, that are normally inactivating Slingshot, are oxidized, which releases and activates Slingshot [94]. Slingshot can then bind to F-actin and rapidly dephosphorylate Cofilin, but what is the role of Coronin 2A in this process? To determine this, HeLa cells expressing Cofilin-GFP will be depleted of Coronin 2A and monitored for Cofilin-Actin rod formation upon exposure to ROS. If Coronin 2A is an important factor in Slingshot and/or Cofilin activation in this process, a lag or elimination of rod formation is expected.

Rods formed by ectopic Coronin 2A expression are characteristically different than ATP-depletion induced rods. For example, Cofilin and Actin structures do not stably associate in Coronin 2A-induced rods. Can the Coronin 2A-induced rods be used to gain more information about Cofilin-Actin rods? From analyses of Coronin 2A rods and Cofilin-Actin rods, the first major difference is that Coronin 2A rods stain positively with phalloidin. This is not seen in Cofilin-Actin rods [55]. Previous studies have focused on stable rods that have been exposed to ATP-depleting conditions for at least thirty minutes, if not longer. The question is do these rods stain positively with phalloidin while they are forming? If yes, this would be a strong indication the Coronin 2A rods may mimic Cofilin-Actin rod intermediates. Other proteins may also associate and be involved in rod assembly. Recent data suggest that the Lifeact peptide [112], an expressible marker of F-actin structures, is excluded from Cofilin-Actin rods [113]. It is unclear if Lifeact will mark Coronin 2A rods or

not. In the case of Cofilin-Actin rods, Cofilin completely saturates the phalloidin binding sites on F-actin. This is why phalloidin does not stain Cofilin-Actin rods. Since Coronin 2A rods stain positively with phalloidin, it is likely that Lifeact will also associate with these structures. The dynamics of the Cofilin in Coronin 2A rods suggests that it is not stably associating with the actin filaments. FRAP analysis of the actin filaments is needed to determine any differences in actin turnover within the rod. Rod formation assays can be used to determine the dynamic nature of Cofilin-Actin rod during formation. The Lifeact peptide can be used to determine if Cofilin is in stable association with F-actin or does the tight binding occur over time of ATP-depletion. These experiments can retrieve valuable information about how rods are formed.

Lastly, ectopic expression of Coronin 2B, a type II Coronin predominantly expressed in the brain, induces Cofilin-Actin rod like aggregates as well (data not shown). This is a much more interesting protein to study Cofilin-Actin rod biology due to the expression pattern of the protein. While I believe that similar results will be obtained with Coronin 2A, the physiologically relevant protein is Coronin 2B. These same experiments should be performed with Coronin 2B to verify these affects in response to stress. Depletion of Coronin 2B in neurons would also be a useful way to address these questions.

Here, I have described many unanswered questions about type II Coronin function. While the data presented in the earlier chapters has produced useful information about F-actin and focal adhesion regulation, more physiological environments can verify if the in vitro function occurs in living systems. These data have opened a new area of Coronin study that I hope will be beneficial for mankind and future experiments may identify more key physiological roles for type II Coronins.

References

1. Ridley AJ, MA Schwartz, K Burridge, RA Firtel, MH Ginsberg, G Borisy, JT Parsons, AR Horwitz: Cell migration: integrating signals from front to back. *Science* 2003;302:1704–1709.
2. Vicente-Manzanares M, DJ Webb, AR Horwitz: Cell migration at a glance. *J Cell Sci* 2005;118:4917–4919.
3. Zamir E, B Geiger: Components of cell-matrix adhesions. *J Cell Sci* 2001;114:3577–3579.
4. Pollard TD, GG Borisy: Cellular motility driven by assembly and disassembly of actin filaments. *Cell* 2003;112:453–465.
5. Ilic D, Y Furuta, S Kanazawa, N Takeda, K Sobue, N Nakatsuji, S Nomura, J Fujimoto, M Okada, T Yamamoto: Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 1995;377:539–544.
6. Ezratty EJ, MA Partridge, GG Gundersen: Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat Cell Biol* 2005;7:581–590.
7. Kodama A, I Karakesisoglou, E Wong, A Vaezi, E Fuchs: ACF7: an essential integrator of microtubule dynamics. *Cell* 2003;115:343–354.
8. Wu X, A Kodama, E Fuchs: ACF7 regulates cytoskeletal-focal adhesion dynamics and migration and has ATPase activity. *Cell* 2008;135:137–148.
9. Koleske AJ, AM Gifford, ML Scott, M Nee, RT Bronson, KA Miczek, D Baltimore: Essential roles for the Abl and Arg tyrosine kinases in neurulation. *Neuron* 1998;21:1259–1272.
10. Peacock JG, AL Miller, WD Bradley, OC Rodriguez, DJ Webb, AJ Koleske: The Abl-related gene tyrosine kinase acts through p190RhoGAP to inhibit actomyosin contractility and regulate focal adhesion dynamics upon adhesion to fibronectin. *Mol Biol Cell* 2007;18:3860–3872.
11. Broussard JA, DJ Webb, I Kaverina: Asymmetric focal adhesion disassembly in motile cells. *Curr Opin Cell Biol* 2008;20:85–90.
12. Kaverina I, K Rottner, JV Small: Targeting, capture, and stabilization of microtubules at early focal adhesions. *J Cell Biol* 1998;142:181–190.
13. Kaverina I, O Krylyshkina, JV Small: Microtubule targeting of substrate contacts promotes their relaxation and dissociation. *J Cell Biol* 1999;146:1033–1044.

14. Beckerle MC, K Burridge, GN DeMartino, DE Croall: Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell* 1987;51:569–577.
15. Beckerle MC, T O'Halloran, K Burridge: Demonstration of a relationship between talin and P235, a major substrate of the calcium-dependent protease in platelets. *J Cell Biochem* 1986;30:259–270.
16. O'Halloran T, MC Beckerle, K Burridge: Identification of talin as a major cytoplasmic protein implicated in platelet activation. *Nature* 1985;317:449–451.
17. Bhatt A, I Kaverina, C Otey, A Huttenlocher: Regulation of focal complex composition and disassembly by the calcium-dependent protease calpain. *J Cell Sci* 2002;115:3415–3425.
18. Huttenlocher A, SP Palecek, Q Lu, W Zhang, RL Mellgren, DA Lauffenburger, MH Ginsberg, AF Horwitz: Regulation of cell migration by the calcium-dependent protease calpain. *J Biol Chem* 1997;272:32719–32722.
19. Chrzanowska-Wodnicka M, K Burridge: Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol* 1996;133:1403–1415.
20. Webb DJ, K Donais, LA Whitmore, SM Thomas, CE Turner, JT Parsons, AF Horwitz: FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol* 2004;6:154–161.
21. Efimov A, N Schiefermeier, I Grigoriev, R Ohi, MC Brown, CE Turner, JV Small, I Kaverina: Paxillin-dependent stimulation of microtubule catastrophes at focal adhesion sites. *J Cell Sci* 2008;121:196–204.
22. Waterman-Storer CM, ED Salmon: Endoplasmic reticulum membrane tubules are distributed by microtubules in living cells using three distinct mechanisms. *Curr Biol* 1998;8:798–806.
23. Franco SJ, MA Rodgers, BJ Perrin, J Han, DA Bennin, DR Critchley, A Huttenlocher: Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat Cell Biol* 2004;6:977–983.
24. Cortesio CL, KT Chan, BJ Perrin, NO Burton, S Zhang, ZY Zhang, A Huttenlocher: Calpain 2 and PTP1B function in a novel pathway with Src to regulate invadopodia dynamics and breast cancer cell invasion. *J Cell Biol* 2008;180:957–971.
25. Choi CK, M Vicente-Manzanares, J Zareno, LA Whitmore, A Mogilner, AR Horwitz: Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat Cell Biol* 2008;10:1039–1050.

26. Tanaka M, T Abe, Y Hara: Roles of focal adhesions and fibronectin-mediated cohesion in proliferation of confluent fibroblasts. *J Cell Physiol* 2009;219:194–201.
27. Lahlou H, V Sanguin-Gendreau, D Zuo, RD Cardiff, GW McLean, MC Frame, WJ Muller: Mammary epithelial-specific disruption of the focal adhesion kinase blocks mammary tumor progression. *Proc Natl Acad Sci U S A* 2007;104:20302–20307.
28. Petch LA, SM Bockholt, A Bouton, JT Parsons, K Burridge: Adhesion-induced tyrosine phosphorylation of the p130 src substrate. *J Cell Sci* 1995;108:1371–1379.
29. Burnham MR, PJ Bruce-Staskal, MT Harte, CL Weidow, A Ma, SA Weed, AH Bouton: Regulation of c-SRC activity and function by the adapter protein CAS. *Mol Cell Biol* 2000;20:5865–5878.
30. Hu K, L Ji, KT Applegate, G Danuser, CM Waterman-Storer: Differential transmission of actin motion within focal adhesions. *Science* 2007;315:111–115.
31. Bamburg JR, BW Bernstein: ADF/cofilin. *Curr Biol* 2008;18:R273–5.
32. Yang N, O Higuchi, K Ohashi, K Nagata, A Wada, K Kangawa, E Nishida, K Mizuno: Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* 1998;393:809–812.
33. Toshima J, JY Toshima, T Amano, N Yang, S Narumiya, K Mizuno: Cofilin phosphorylation by protein kinase testicular protein kinase 1 and its role in integrin-mediated actin reorganization and focal adhesion formation. *Mol Biol Cell* 2001;12:1131–1145.
34. Niwa R, K Nagata-Ohashi, M Takeichi, K Mizuno, T Uemura: Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. *Cell* 2002;108:233–246.
35. Gohla A, J Birkenfeld, GM Bokoch: Chronophin, a novel HAD-type serine protein phosphatase, regulates cofilin-dependent actin dynamics. *Nat Cell Biol* 2005;7:21–29.
36. van Rheenen J, X Song, W van Roosmalen, M Cammer, X Chen, V Desmarais, SC Yip, JM Backer, RJ Eddy, JS Condeelis: EGF-induced PIP2 hydrolysis releases and activates cofilin locally in carcinoma cells. *J Cell Biol* 2007;179:1247–1259.
37. Bernstein BW, WB Painter, H Chen, LS Minamide, H Abe, JR Bamburg: Intracellular pH modulation of ADF/cofilin proteins. *Cell Motil Cytoskeleton* 2000;47:319–336.

38. Frantz C, G Barreiro, L Dominguez, X Chen, R Eddy, J Condeelis, MJ Kelly, MP Jacobson, DL Barber: Cofilin is a pH sensor for actin free barbed end formation: role of phosphoinositide binding. *J Cell Biol* 2008
39. Nagaoka R, K Kusano, H Abe, T Obinata: Effects of cofilin on actin filamentous structures in cultured muscle cells. Intracellular regulation of cofilin action. *J Cell Sci* 1995;108:581–593.
40. Gohla A, GM Bokoch: 14-3-3 regulates actin dynamics by stabilizing phosphorylated cofilin. *Curr Biol* 2002;12:1704–1710.
41. Nagata-Ohashi K, Y Ohta, K Goto, S Chiba, R Mori, M Nishita, K Ohashi, K Kousaka, A Iwamatsu, R Niwa, T Uemura, K Mizuno: A pathway of neuregulin-induced activation of cofilin-phosphatase Slingshot and cofilin in lamellipodia. *J Cell Biol* 2004;165:465–471.
42. Kligys K, J Yao, D Yu, JC Jones: 14-3-3zeta/tau heterodimers regulate Slingshot activity in migrating keratinocytes. *Biochem Biophys Res Commun* 2009
43. Kurita S, E Gunji, K Ohashi, K Mizuno: Actin filaments-stabilizing and -bundling activities of cofilin-phosphatase Slingshot-1. *Genes Cells* 2007;12:663–676.
44. Cai L, N Holoweckyj, MD Schaller, JE Bear: Phosphorylation of coronin 1B by protein kinase C regulates interaction with Arp2/3 and cell motility. *J Biol Chem* 2005;280:31913–31923.
45. Rodal AA, JW Tetreault, P Lappalainen, DG Drubin, DC Amberg: Aip1p interacts with cofilin to disassemble actin filaments. *J Cell Biol* 1999;145:1251–1264.
46. Ono S, K Mohri, K Ono: Microscopic evidence that actin-interacting protein 1 actively disassembles actin-depolymerizing factor/Cofilin-bound actin filaments. *J Biol Chem* 2004;279:14207–14212.
47. Li J, WM Briehner, ML Scimone, SJ Kang, H Zhu, H Yin, UH von Andrian, T Mitchison, J Yuan: Caspase-11 regulates cell migration by promoting Aip1-Cofilin-mediated actin depolymerization. *Nat Cell Biol* 2007;9:276–286.
48. Mattila PK, O Quintero-Monzon, J Kugler, JB Moseley, SC Almo, P Lappalainen, BL Goode: A high-affinity interaction with ADP-actin monomers underlies the mechanism and in vivo function of Srv2/cyclase-associated protein. *Mol Biol Cell* 2004;15:5158–5171.
49. Wang W, R Eddy, J Condeelis: The cofilin pathway in breast cancer invasion and metastasis. *Nat Rev Cancer* 2007;7:429–440.

50. Meira M, R Masson, I Stagljar, S Lienhard, F Maurer, A Boulay, NE Hynes: Memo is a cofilin-interacting protein that influences PLC γ 1 and cofilin activities, and is essential for maintaining directionality during ErbB2-induced tumor-cell migration. *J Cell Sci* 2009;122:787–797.
51. Han L, MB Stope, ML de Jesus, PA Oude Weernink, M Urban, T Wieland, D Roskopf, K Mizuno, KH Jakobs, M Schmidt: Direct stimulation of receptor-controlled phospholipase D1 by phospho-cofilin. *EMBO J* 2007;26:4189–4202.
52. Jung J, T Yoon, EC Choi, K Lee: Interaction of cofilin with triose-phosphate isomerase contributes glycolytic fuel for Na,K-ATPase via Rho-mediated signaling pathway. *J Biol Chem* 2002;277:48931–48937.
53. Huang TY, C Dermardirossian, GM Bokoch: Cofilin phosphatases and regulation of actin dynamics. *Curr Opin Cell Biol* 2006;18:26–31.
54. Huang TY, LS Minamide, JR Bamburg, GM Bokoch: Chronophin mediates an ATP-sensing mechanism for cofilin dephosphorylation and neuronal cofilin-actin rod formation. *Dev Cell* 2008;15:691–703.
55. Minamide LS, AM Striegl, JA Boyle, PJ Meberg, JR Bamburg: Neurodegenerative stimuli induce persistent ADF/cofilin-actin rods that disrupt distal neurite function. *Nat Cell Biol* 2000;2:628–636.
56. Eiseler T, H Doppler, IK Yan, K Kitatani, K Mizuno, P Storz: Protein kinase D1 regulates cofilin-mediated F-actin reorganization and cell motility through slingshot. *Nat Cell Biol* 2009
57. Wang Y, F Shibasaki, K Mizuno: Calcium signal-induced cofilin dephosphorylation is mediated by Slingshot via calcineurin. *J Biol Chem* 2005;280:12683–12689.
58. de Hostos EL, B Bradtke, F Lottspeich, R Guggenheim, G Gerisch: Coronin, an actin binding protein of Dictyostelium discoideum localized to cell surface projections, has sequence similarities to G protein beta subunits. *Embo J* 1991;10:4097–4104.
59. Uetrecht AC, JE Bear: Coronins: the return of the crown. *Trends Cell Biol* 2006;16:421–426.
60. Appleton BA, P Wu, C Wiesmann: The crystal structure of murine coronin-1: a regulator of actin cytoskeletal dynamics in lymphocytes. *Structure* 2006;14:87–96.
61. Siekhaus DE, DG Drubin: Spontaneous receptor-independent heterotrimeric G-protein signalling in an RGS mutant. *Nat Cell Biol* 2003;5:231–235.
62. Cai L, AM Makhov, JE Bear: F-actin binding is essential for coronin 1B function in vivo. *J Cell Sci* 2007;120:1779–1790.

63. Gatfield J, I Albrecht, B Zanolari, MO Steinmetz, J Pieters: Association of the Leukocyte Plasma Membrane with the Actin Cytoskeleton through Coiled Coil-mediated Trimeric Coronin 1 Molecules. *Mol Biol Cell* 2005;16:2786–2798.
64. Li Z, S Van Calcar, C Qu, WK Cavenee, MQ Zhang, B Ren: A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. *Proc Natl Acad Sci U S A* 2003;100:8164–8169.
65. Moreno-Bueno G, E Cubillo, D Sarrío, H Peinado, SM Rodríguez-Pinilla, S Villa, V Bolos, M Jorda, A Fabra, F Portillo, J Palacios, A Cano: Genetic profiling of epithelial cells expressing e-cadherin repressors reveals a distinct role for snail, slug, and e47 factors in epithelial-mesenchymal transition. *Cancer Res* 2006;66:9543–9556.
66. Thal D, CP Xavier, A Rosentreter, S Linder, B Friedrichs, A Waha, T Pietsch, M Stumpf, A Noegel, C Clemen: Expression of coronin-3 (coronin-1C) in diffuse gliomas is related to malignancy. *J Pathol* 2008;214:415–424.
67. Rybakin V, M Stumpf, A Schulze, IV Majoul, AA Noegel, A Hasse: Coronin 7, the mammalian POD-1 homologue, localizes to the Golgi apparatus. *FEBS Lett* 2004;573:161–167.
68. Nakamura T, K Takeuchi, S Muraoka, H Takezoe, N Takahashi, N Mori: A neurally enriched coronin-like protein, ClipinC, is a novel candidate for an actin cytoskeleton-cortical membrane-linking protein. *J Biol Chem* 1999;274:13322–13327.
69. Cai L, TW Marshall, AC Uetrecht, DA Schafer, JE Bear: Coronin 1B coordinates Arp2/3 complex and cofilin activities at the leading edge. *Cell* 2007;128:915–929.
70. Cai L, AM Makhov, DA Schafer, JE Bear: Coronin 1B antagonizes cortactin and remodels Arp2/3-containing actin branches in lamellipodia. *Cell* 2008;134:828–842.
71. Rybakin V, NV Gounko, K Spate, S Honing, IV Majoul, R Duden, AA Noegel: Crn7 interacts with AP-1 and is required for the maintenance of Golgi morphology and protein export from the Golgi. *J Biol Chem* 2006;281:31070–31078.
72. Yoon HG, DW Chan, ZQ Huang, J Li, JD Fondell, J Qin, J Wong: Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1. *Embo J* 2003;22:1336–1346.
73. Hagel M, EL George, A Kim, R Tamimi, SL Opitz, CE Turner, A Imamoto, SM Thomas: The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. *Mol Cell Biol* 2002;22:901–915.
74. Mitra SK, DD Schlaepfer: Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol* 2006;18:516–523.

75. Katz M, I Amit, A Citri, T Shay, S Carvalho, S Lavi, F Milanezi, L Lyass, N Amariglio, J Jacob-Hirsch, N Ben-Chetrit, G Tarcic, M Lindzen, R Avraham, YC Liao, P Trusk, A Lyass, G Rechavi, NL Spector, SH Lo, F Schmitt, SS Bacus, Y Yarden: A reciprocal tensin-3-cten switch mediates EGF-driven mammary cell migration. *Nat Cell Biol* 2007;9:961–969.
76. de Hostos EL, C Rehfuss, B Bradtke, DR Waddell, R Albrecht, J Murphy, G Gerisch: Dictyostelium mutants lacking the cytoskeletal protein coronin are defective in cytokinesis and cell motility. *J Cell Biol* 1993;120:163–173.
77. Foger N, L Rangell, DM Danilenko, AC Chan: Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. *Science* 2006;313:839–842.
78. Rosentreter A, A Hofmann, CP Xavier, M Stumpf, AA Noegel, CS Clemen: Coronin 3 involvement in F-actin-dependent processes at the cell cortex. *Exp Cell Res* 2007;313:878–895.
79. Hotulainen P, E Paunola, MK Vartiainen, P Lappalainen: Actin-depolymerizing factor and cofilin-1 play overlapping roles in promoting rapid F-actin depolymerization in mammalian nonmuscle cells. *Mol Biol Cell* 2005;16:649–664.
80. Sidani M, D Wessels, G Mouneimne, M Ghosh, S Goswami, C Sarmiento, W Wang, S Kuhl, M El-Sibai, JM Backer, R Eddy, D Soll, J Condeelis: Cofilin determines the migration behavior and turning frequency of metastatic cancer cells. *J Cell Biol* 2007;179:777–791.
81. Foletta VC, N Moussi, PD Sarmiere, JR Bamburg, O Bernard: LIM kinase 1, a key regulator of actin dynamics, is widely expressed in embryonic and adult tissues. *Exp Cell Res* 2004;294:392–405.
82. Soosairajah J, S Maiti, O Wiggan, P Sarmiere, N Moussi, B Sarcevic, R Sampath, JR Bamburg, O Bernard: Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin. *Embo J* 2005;24:473–486.
83. Chan AY, M Bailly, N Zebda, JE Segall, JS Condeelis: Role of cofilin in epidermal growth factor-stimulated actin polymerization and lamellipod protrusion. *J Cell Biol* 2000;148:531–542.
84. LaLonde DP, MC Brown, BP Bouverat, CE Turner: Actopaxin interacts with TESK1 to regulate cell spreading on fibronectin. *J Biol Chem* 2005;280:21680–21688.
85. Ono S: Mechanism of depolymerization and severing of actin filaments and its significance in cytoskeletal dynamics. *Int Rev Cytol* 2007;258:1–82.

86. Michelot A, J Berro, C Guerin, R Boujemaa-Paterski, CJ Staiger, JL Martiel, L Blanchoin: Actin-filament stochastic dynamics mediated by ADF/cofilin. *Curr Biol* 2007;17:825–833.
87. Kueh HY, GT Charras, TJ Mitchison, WM Briehner: Actin disassembly by cofilin, coronin, and Aip1 occurs in bursts and is inhibited by barbed-end cappers. *J Cell Biol* 2008;182:341–353.
88. Rottner K, A Hall, JV Small: Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr Biol* 1999;9:640–648.
89. Gupton SL, CM Waterman-Storer: Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. *Cell* 2006;125:1361–1374.
90. Chan AY, S Raft, M Bailly, JB Wyckoff, JE Segall, JS Condeelis: EGF stimulates an increase in actin nucleation and filament number at the leading edge of the lamellipod in mammary adenocarcinoma cells. *J Cell Sci* 1998;111:199–211.
91. Mehta SL, N Manhas, R Raghubir: Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Res Rev* 2007;54:34–66.
92. Maloney MT, LS Minamide, AW Kinley, JA Boyle, JR Bamburg: Beta-secretase-cleaved amyloid precursor protein accumulates at actin inclusions induced in neurons by stress or amyloid beta: a feedforward mechanism for Alzheimer's disease. *J Neurosci* 2005;25:11313–11321.
93. Bernstein BW, H Chen, JA Boyle, JR Bamburg: Formation of actin-ADF/cofilin rods transiently retards decline of mitochondrial potential and ATP in stressed neurons. *Am J Physiol Cell Physiol* 2006;291:C828–39.
94. Kim JS, TY Huang, GM Bokoch: Reactive Oxygen Species (ROS) Regulate a Slingshot-Cofilin Activation Pathway. *Mol Biol Cell* 2009
95. Yonezawa N, E Nishida, S Maekawa, H Sakai: Studies on the interaction between actin and cofilin purified by a new method. *Biochem J* 1988;251:121–127.
96. Bear JE, TM Svitkina, M Krause, DA Schafer, JJ Loureiro, GA Strasser, IV Maly, OY Chaga, JA Cooper, GG Borisy, FB Gertler: Antagonism between Ena/VASP Proteins and Actin Filament Capping Regulates Fibroblast Motility. *Cell* 2002;109:509–21.
97. Jones JD, K Luby-Phelps: Tracer diffusion through F-actin: effect of filament length and cross-linking. *Biophys J* 1996;71:2742–2750.
98. Luby-Phelps K, DL Taylor: Subcellular compartmentalization by local differentiation of cytoplasmic structure. *Cell Motil Cytoskeleton* 1988;10:28–37.

99. Nishita M, Y Wang, C Tomizawa, A Suzuki, R Niwa, T Uemura, K Mizuno: Phosphoinositide 3-kinase-mediated activation of cofilin phosphatase Slingshot and its role for insulin-induced membrane protrusion. *J Biol Chem* 2004;279:7193–7198.
100. Ohta Y, K Kousaka, K Nagata-Ohashi, K Ohashi, A Muramoto, Y Shima, R Niwa, T Uemura, K Mizuno: Differential activities, subcellular distribution and tissue expression patterns of three members of Slingshot family phosphatases that dephosphorylate cofilin. *Genes Cells* 2003;8:811–824.
101. Amann KJ, TD Pollard: Direct real-time observation of actin filament branching mediated by Arp2/3 complex using total internal reflection fluorescence microscopy. *Proc Natl Acad Sci U S A* 2001;98:15009–15013.
102. Kuhn JR, TD Pollard: Real-time measurements of actin filament polymerization by total internal reflection fluorescence microscopy. *Biophys J* 2005;88:1387–1402.
103. Blanchoin L, TD Pollard: Mechanism of interaction of Acanthamoeba actophorin (ADF/Cofilin) with actin filaments. *J Biol Chem* 1999;274:15538–15546.
104. De La Cruz EM: Cofilin binding to muscle and non-muscle actin filaments: isoform-dependent cooperative interactions. *J Mol Biol* 2005;346:557–564.
105. Torka R, F Thuma, V Herzog, G Kirfel: ROCK signaling mediates the adoption of different modes of migration and invasion in human mammary epithelial tumor cells. *Exp Cell Res* 2006;312:3857–3871.
106. Mayer C, K Maaser, N Daryab, KS Zanker, EB Brocker, P Friedl: Release of cell fragments by invading melanoma cells. *Eur J Cell Biol* 2004;83:709–715.
107. Cukierman E, R Pankov, DR Stevens, KM Yamada: Taking cell-matrix adhesions to the third dimension. *Science* 2001;294:1708–1712.
108. Cukierman E, R Pankov, KM Yamada: Cell interactions with three-dimensional matrices. *Curr Opin Cell Biol* 2002;14:633–639.
109. Byfield FJ, RK Reen, TP Shentu, I Levitan, KJ Gooch: Endothelial actin and cell stiffness is modulated by substrate stiffness in 2D and 3D. *J Biomech* 2009
110. Wang W, JB Wyckoff, S Goswami, Y Wang, M Sidani, JE Segall, JS Condeelis: Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors. *Cancer Res* 2007;67:3505–3511.
111. Wang W, G Mouneimne, M Sidani, J Wyckoff, X Chen, A Makris, S Goswami, AR Bresnick, JS Condeelis: The activity status of cofilin is directly related to invasion, intravasation, and metastasis of mammary tumors. *J Cell Biol* 2006;173:395–404.

112. Riedl J, AH Crevenna, K Kessenbrock, JH Yu, D Neukirchen, M Bista, F Bradke, D Jenne, TA Holak, Z Werb, M Sixt, R Wedlich-Soldner: Lifeact: a versatile marker to visualize F-actin. *Nat Methods* 2008;5:605–607.
113. Munsie LN, N Caron, CR Desmond, R Truant: Lifeact cannot visualize some forms of stress-induced twisted f-actin. *Nat Methods* 2009;6:317.