Catastrophic actin filament bursting by cofilin, Aip1, and coronin

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Running title: A catastrophic mode of actin disassembly
Abstract

Cofilin is an actin filament severing protein necessary for fast actin turnover dynamics. Coronin and Aip1 promote cofilin mediated actin filament disassembly, but the mechanism is somewhat controversial. An early model proposed that the combination of cofilin, coronin, and Aip1 disassembled filaments in bursts. A subsequent study only reported severing. Here, we used electron microscopy to show that actin filaments convert directly into globular material. A monomer trap assay also shows that the combination of all three factors produces actin monomers faster than any two factors alone. We show that coronin accelerates the release of inorganic phosphate from actin filaments and promotes highly cooperative cofilin binding to actin to create long stretches of polymer with a hypertwisted morphology. Aip1 attacks these hypertwisted regions along their sides, disintegrating them into monomers or short oligomers. The results are consistent with a catastrophic mode of disassembly, not enhanced severing alone.

Introduction

The rapid disassembly of actin filaments is necessary for the dramatic shape changes that accompany fundamental cellular processes such as cell division, endocytosis, and cell motility. Cofilin is an actin filament severing protein that is necessary for fast actin disassembly in cells(1). It binds to F-actin cooperatively (2-8) and alters filament twist (5,9) which disrupts contacts between subunits (10-14) and changes filament mechanics (10,15-19). This alternative twist is stable, but junctions between occupied and unoccupied polymer are weak causing filaments to break at or near the junction to produce two intact filaments with conservation of polymer mass (7,20-22). As a consequence, actin filaments are most susceptible to cofilin-mediated severing at intermediate occupancies of cofilin that maximize the number of heterotypic junctions (8,22-25).

While cofilin is necessary for actin disassembly, additional factors can contribute to the reaction. Coronin, Aip1 (also known as Wdr1), and Cyclase Associated Protein all enhance cofilin mediated actin disassembly, and each of them plays an important role in controlling actin turnover rates across a range of actin networks in a wide variety of cells (26-33). Each factor promotes cofilin-mediated severing (31,34,35). A key question is whether they can also alter the mechanism of filament disassembly (36). For example, cyclase associated protein accelerates actin subunit dissociation from the minus end of actin filaments (37,38) while Aip1 accelerates subunit loss from both ends of the filament (25).

One of the more striking departures from filament severing was seen with the combination of cofilin, coronin, and Aip1. The main motivation for studying them together with cofilin came from biochemical complementation which showed that both factors were required to disassemble Listeria actin comet tails (26). Subsequently, high speed imaging of single actin filaments with a temporal resolution of less than 20 ms showed an abrupt loss of actin polymer mass per event (39). The creation of two daughter filaments from a single mother filament could not be detected under these conditions, even with those high frame rates. The interpretation was that stretches of actin polymer were disintegrating into monomers and short oligomers in a single step referred to as a burst. An alternative interpretation is that the triple mix of cofilin, coronin, and Aip1 simply severs actin filaments, but one of the daughter filaments cannot be detected. In this study, we used electron microscopy as an orthogonal approach to further investigate filament disassembly in the presence of cofilin, coronin, and Aip1.

Results.

Cofilin-mediated severing reactions produce two intact actin filaments with no immediate loss in total actin polymer mass. In contrast, the triple mix of cofilin, coronin, and Aip1 is hypothesized to produce actin monomers and very small oligomers with an immediate loss of actin polymer mass (39). These two alternative models of filament disassembly can be distinguished by imaging actin filaments in the
electron microscope before and shortly after adding actin disassembly factors to pre-formed actin filaments. We have optimized a protocol with the goal to trap actin filaments undergoing disassembly to visualize and understand how actin polymer lost as a function of cofilin, coronin, and Aip1. Our basic protocol was to polymerize a solution of actin for a brief period time, add the disassembly factors, add glutaraldehyde to the reaction mixture before blotting the sample onto glow discharged EM grids, wash the grid to remove unbound proteins, stain with uranyl acetate, and image the sample in the electron microscope.

Figure 1 shows an overview of actin filaments before and after treating them with various combinations of cofilin, coronin, and Aip1. Figure 1A shows a set of low and high magnification views of a 10µM solution of actin that was induced to polymerize for one minute prior to fixation. Note that these are young filaments, and the reaction is still in the rapid assembly phase. We chose 10µM of actin to induce rapid polymerization without a lag phase. Figure 1B shows representative fields when a 10µM solution of actin is polymerized for one minute and then subsequently treated with 5µM cofilin, 2 µM coronin, and 0.2µM Aip1 for an additional 60 seconds prior to fixing and visualizing the entire reaction by electron microscopy. Concentrations of cofilin, coronin, and Aip1 were picked to mimic the estimated ratios of these factors to actin in various cell types and tissue extracts (25,26,40). While many long filaments form during the first minute of assembly (Figure 1A), no polymer remains after 60 seconds in cofilin, coronin, and Aip1 (Figure 1B). Higher magnification views (Figure 1A-B, lower panels) of the same reactions before and after treating actin filaments with the disassembly cocktail for one minute show globular material with a few, very-short oligomers in a field of view (compare Figures 1C and 1D). Otherwise, the products of this disassembly reaction did not appear much different than images of the three disassembly factors alone in the absence of any actin (Figure 1C). The rapid loss of all actin filaments required all three factors because filaments were readily found after treating one-minute old actin filaments with any two of three actin disassembly factors (Figure 1D-F).

We used a FRET-based assay to measure changes in actin polymer mass in the presence of cofilin (25). Unlike pyrene, the FRET assay is not perturbed by cofilin binding to F-actin but only reports on depolymerization. We used the FRET assay to compare disassembly of actin as a function of three disassembly factors. A 2µM solution of actin was polymerized to steady state and mixed with various combinations of the three disassembly factors. In the presence of 1.25 µM cofilin, we observed a 30-40% decrease in polymer mass, consistent with the fact that cofilin severs actin filaments and binds ADP-actin monomers that are lost from filament ends with an affinity of 150nM (6,41) (Figure 2A, green line). The addition of 0.1 µM Aip1 increased the initial disassembly rate approximately five times, and the final extent of depolymerization was the same as with cofilin alone (Figure 2A, yellow line) consistent with previous results (25). When 0.75 µM coronin was added to cofilin, it led to an apparent increase in polymer mass and a decrease in the depolymerization rate which is consistent with coronin’s ability to stabilize actin filaments (42) and suppress cofilin mediated filament severing (34,43) (Figure 2A, blue line). When all three factors were combined with actin, we obtained near complete loss of polymer mass (Figure 2A, red line). Along with a rapid rate of depolymerization, the extent of disassembly in the presence of all three factors was greater than that obtained by any other combination of factors. This result indicates that the triple mix of cofilin, coronin, and Aip1 rapidly depolymerizes actin more rapidly and to a greater extent than cofilin alone or cofilin in combination with either Aip1 or coronin alone.

The FRET assay measures loss of actin polymer mass. We also wished to directly measure the rate of actin monomer production. We therefore developed a new monomer trap assay that reports on actin monomers. We found that Vitamin D Binding Protein (DBP), which binds exclusively to actin monomers (44,45),
quenches the fluorescence of actin monomers labeled with Oregon Green (compare the purple line to the yellow line at time 0 in figure 2B). We took advantage of this property to monitor the rate of production of actin monomers in the presence of different combinations of disassembly factors. Adding DBP alone to a solution of F- actin labeled with Oregon Green leads to a slow decrease in fluorescence intensity as actin subunits are lost from the two ends of the filaments (Figure 2B purple line). Cofilin accelerated the rate of monomer production in the presence of DBP consistent with cofilin severing and the production of more ends that can shrink (Figure 2B, green line). Adding capping protein, which binds to actin filament plus ends with nanomolar affinity (47), to cofilin slowed the rate of monomer production relative to cofilin alone as expected (Figure 2B blue line). In contrast, adding coronin and Aip1 along with cofilin resulted in a very rapid production of actin monomers as indicated by the precipitous drop in Oregon Green fluorescence to its final value in less than 100 seconds (figure 2B, red line).

Although we were now convinced that the product of the disassembly reaction was largely actin monomer, to further distinguish the reaction from filament severing, we performed seeding reactions with the end products of depolymerization (Fig.2C). Severed short filaments would promote polymerization and diminish the lag phase of actin assembly. However, actin monomer or fragments with occluded ends would not serve as seeds. Unseeded pyrene actin showed a canonical lag phase prior to polymerization (Fig.2C, purple line) whereas seeds of cofilin, cofilin-coronin or cofilin-Aip1-severed F-actin filaments yielded a more effective seeding mixture consistent with the results of the FRET and electron microscopy (green, blue and yellow lines respectively). However, products of the 3X mix reaction displayed the same lag phase as monomeric actin (red line) indicating that they were incompetent to seed new actin assembly.

To gain greater insight into the mechanism by which the 3X mix of factors was able to rapidly disassemble actin with the generation of primarily actin monomer and products that were unable to seed new actin assembly, we varied the concentration of each factor in the presence of fixed concentrations of the remaining two factors. As expected, the rates of depolymerization increased with increasing concentrations of both cofilin and Aip1 (Fig.2D&F). However, coronin appeared to act best at a ratio of 5-12.5:1 cofilin:coronin and disassembly was less efficient at lower ratios (Fig.2E). This is consistent with coronin’s ability to bind and stabilize F-actin and also to bind antagonistically with cofilin (42,43,46).

We estimated an apparent critical concentration of actin assembly using fluorescence imaging to detect the formation of actin filaments. We call this an apparent critical concentration because fluorescence imaging will not detect short oligomers. For these experiments, we used higher concentrations of cofilin (20 µM) and Aip1 (5 µM) to be closer to saturation. It would be better to use an even higher concentration of Aip1 for these measurements, but we are not able to obtain a sufficient amount of Aip1 to do so. The reaction mixtures were incubated at room temperature for 4 hours to come to steady state and then imaged. Fluorescence micrographs presented in figure 3 show the onset of overt actin filament formation in the presence of the actin disassembly factors. Estimates of apparent critical concentrations are listed in Table 1. We estimate the apparent critical concentration of pure actin to be 0.4 µM, which is consistent with previous measurements using other methods (47). The combination of all three disassembly factors as well as the cofilin + Aip1 both raised the apparent critical concentration to 2.4 ≤Cc ≤ 4.8 µM. In addition, we also detected a modest increase in the apparent critical concentration by cofilin alone or in combination with coronin (~ 0.9 µM). The cofilin effect on the apparent critical concentration was greater than that detected by Andrianantoandro, et al (23) but less than that reported by Chen, et al (48).

We next turned to examining the mechanism of actin disassembly. The 3X mix of cofilin, coronin, and Aip1 rapidly converts actin
polymer to monomer. We and others have already imaged the disassembly process using fluorescence microscopy, which also suggested a rapid dissolution of F-actin into monomers (39,43). Electron microscopy could provide additional insight into the mechanism if we could capture images of actin filaments in the process of disassembling. Working together, two people can reliably fix the sample within five seconds of adding the depolymerizers to a solution of F-actin. In figure 4A, solutions of actin were polymerized for 1 minute. Cofilin, coronin, and Aip1 were then added to these filaments and the reactions fixed after 5, 10, or 30 seconds. The low magnification images show the rapid loss of actin polymer mass over time (figure 4A, upper panels). The higher magnification images show filament architecture at these early time points (figure 4A, lower panels). Within 5 seconds of adding the triple mix, long sections of actin polymer were losing coherent structure. By ten seconds, filaments appeared shorter and were disintegrating all along their length. By 30 seconds, most of the polymer mass was gone and the few remaining filaments were short with a highly compromised, disorganized structure. A gallery of images showing dissolution of actin polymer structure in the presence of cofilin, coronin, and Aip1 is provided in supplementary figure 1.

The speed of filament disintegration as well as the profound disruption of filament architecture makes it difficult to understand what is happening during the run-up to an Aip1 mediated disassembly event. Previous results with Listeria showed that the comet tails were stable in the presence of coronin and cofilin alone but disassembled upon addition of Aip1 (26). This, and the observation that coronin suppresses cofilin-mediated filament severing (43) led us to think that we could split the reaction into two steps. In the first step, we copolymerized 10 μM actin in the presence of 5 μM cofilin and 2 μM coronin and subsequently added Aip1 in a second step to disassemble these filaments. The upper panels in Figure 4B show three different magnifications of actin filaments that were assembled in the presence of cofilin and coronin for one minute. The lower panels in Figure 4B show images at three magnifications of the products of the reaction after adding Aip1 to filaments that were copolymerized in the presence of coronin and cofilin. The filaments appeared stable in the first step (Figure 4B, upper panels) and underwent dramatic disassembly within 30 seconds after treating with Aip1 (Figure 4B, lower panels). Thus, at the level of filament architecture that can be assessed by electron microscopy with negative stain, the two-step reaction disassembled actin filaments through the same pathway as adding all three factors at the same time.

We examined the structure of stable actin filaments assembled in the presence of cofilin and coronin alone for 90 seconds (Figures 5A and 5B). Actin filaments can be described as a helix comprised of two protofilaments (47). In projection in the electron microscope the two protofilaments appear thin when they are on top of one another and thick when the two protofilaments are side-by-side. The distance from one thick region to the next is termed the crossover distance, which is 37 nm in pure actin and is determined by the configuration of actin subunits within the filament. The diameter of pure actin is approximately 7 nm. When actin filaments were polymerized in the presence of coronin and cofilin, we frequently found thin filaments of entirely normal twist adjacent to filaments that appeared thicker, with an altered twist and a shortened crossover distance. Figure 5B shows a representative tomogram with two filaments of distinct twists, with dots indicating the positions of crossover distances on the filaments. It is possible to distinguish individual protomers within the filament in an enlarged version of the tomogram shown in Supplementary Figure 2.

Measuring the crossover distances over many filaments produced a bimodal distribution with one peak corresponding to ~37 nm which is expected for pure actin and a second peak at ~27 nm which is expected for cofilin-actin (Figure 5C). Measuring filament caliber also produced a bimodal distribution with one peak at ~7 nm expected for pure actin and a second peak at ~11 nm which is expected for cofilin-actin...
Figure 5D). Figure 5E shows the relationship between the crossover distance and filament caliber. Filaments that are thin (~7 nm) have the normal ~37 nm crossover distance while filaments that are thick (~11-12 nm) have a shortened crossover distance. These results are consistent with those of McGough who showed that cofilin binding to the sides of actin filaments at equilibrium alters the configuration of the filament, which can be seen with shortened crossover distance (5). Based upon figure 5E, we designated those segments of actin polymer that were thin and had a long crossover distance to be of “normal twist”. Those segments of polymer that were thick and had a shortened crossover distance were designated as “hypertwisted” due to their appearance relative to normal filaments (compare the filaments in figures 5A and 5B where the normal twist is marked with blue dots and hypertwisted segments are marked with red dots).

We used these definitions of normal twist and hypertwist to analyze the population of filaments that were formed by polymerization in the presence of cofilin and coronin. Figure 5F shows that the length distribution of filaments that are 100% hypertwisted and 100% normal twisted is the same suggesting that the two different populations of filaments have similar stabilities and that we are analyzing twist in filaments of similar length. Next, we measured crossover distances along the entire length of 300 different filaments. Figure 5G is a histogram showing the number of filaments with given percentage of hypertwist. The histogram shows a bimodal distribution where filaments are most likely 100% normal twist or 100% hypertwisted. The data in Figure 5G was replotted in Figure 5H to show the length of each filament and the relative amount of normal twist versus hypertwist. Each bar along the abscissa in figure 5H represents one filament, and the value on the ordinate is the length of that filament. Blue versus red bars indicate the portion of that filament that is normally twisted versus hypertwisted. This analysis shows that about one third of the filaments are in the normal conformation, about one third are entirely hypertwisted, and the remaining third of filaments of the filaments consist of co-

alternating segments of normal and hyper-twist. For those filaments that contained both twists, the transition between normal and hypertwist is sharp (figure 5I & J) as shown previously by electron microscopy with high precision measurements (11,49). If cofilin was binding randomly on filaments, we would expect a single population of filaments with mixed twists. That filaments are completely saturated or entirely bare indicates that cofilin’s binding on actin is highly cooperative. Cooperative cofilin binding to aged F-actin in the ADP state has been described many times (2-8,23). However, calculations of expected cluster sizes based upon cofilin cooperative binding predicts only small cluster sizes (10,23). Here, we are seeing whole filaments either saturated or devoid of cofilin. In addition, the filaments in this experiment are at most only 90 seconds old, and yet half of the total polymer is in the hypertwisted state.

To understand the role of coronin in cooperative loading of cofilin to produce hyper twisted filaments, we performed a set of experiments by varying the time and the sequence of coronin & cofilin addition during actin polymerization (Figure 6). Figure 6A shows actin co-polymerized with coronin and cofilin (same condition as Figure 3, step 1 & Figure 5). Figure 6B shows the products of a modified-step 1 reaction when actin is first co-polymerized with coronin for 60 sec before the addition of cofilin for another 90 seconds. Both reactions were capable of producing long stretches of hyper twist regions. Therefore, coronin can act on the filament before cofilin to promote cofilin binding consistent with previous results using Listeria actin comet tails and single filaments (26,50). To determine if coronin can facilitate cooperative cofilin loading in aged actin filaments, we prepolymerized actin for 30 min before adding coronin & cofilin for 90 sec (Figure 6C). We imaged over 300 filaments under the electron microscope and counted the number of normal twist, hyper twist, and mixed twist filaments. We found that hyper twist filaments are rare (Figure 6C, lower left graph) and a high percentage of filaments are thicker but with normal twist (Figure 6C, lower right graph). Thus, coronin does not facilitate
cooperative cofilin loading onto aged filaments. We also observed severing of naked normal twist filaments (Figure 6C, upper left panels). However, none of the thicker normal twist filaments are associated with severing (Figure 6C, upper right panels). We have previously demonstrated that coronin-decorated filaments are thicker but retain the normal helical twist (42). When coronin was left out of the reaction (Figure 6D), we observed frequent severing of aged filaments that are normal twist (Figure 6D, upper & lower left panels). Only a very small fraction of hypertwisted regions can be found in the absence of coronin (Figure 6D, lower right graph). Thus, cooperative cofilin loading is facilitated by coronin only in polymerizing or newly polymerized filaments that are either ATP or ADP•Pi bound but not in aged filaments that are mostly ADP bound.

We investigated coronin’s effects on phosphate release from actin as a possible explanation for coronin’s enhancement of cofilin binding. For this we used a phosphate release assay that uses the substrate 2-amino-6-mercaptopo-7-methylpurine ribonucleoside (51). The substrate undergoes a shift in absorbance during phosphorolysis that enables spectroscopic monitoring of the rate of phosphate release. By this assay we found that cofilin alone accelerated the rate of phosphate release from F-actin, consistent with previous results using actophorin which is the cofilin homolog in Acanthamoeba (52) (Figure 7A). Increasing amounts of coronin alone also accelerated the rate of phosphate release from actin (Figure 7B). Copolymerization of cofilin and coronin with actin achieved even faster rates of phosphate release than either factor on its own (Figure 7C). Some rates of phosphate release under different conditions are summarized in Figure 7D. These results show that coronin can accelerate Pi release from F-actin, which would help drive cofilin binding to produce the highly cooperative changes in F-actin structure on young filaments.

The distribution of normal and hypertwisted sections of actin that formed as filaments were polymerizing in the presence of cofilin and coronin provided an opportunity to test if Aip1 was disassembling actin at heterotypic junctions or along the length of hypertwisted segments. We hypothesized that it was the hypertwisted stretches of actin filaments that underwent catastrophic disassembly in the presence of Aip1. If true, then actin filaments still visible by EM during the disassembly reaction should either have normal, 37 nm crossover distances or they should be in the process of bursting, but there should be very few filaments with the shortened 27 nm crossover distance. To test this, 10 μM actin was copolymerized in the presence of 2 μM coronin and 12 μM cofilin which produced filaments with normal twist and hypertwist as expected (Figure 8A, marked with an N for Normal and H for Hyper twists). To such a sample, 0.2 μM Aip1 was added for 5 seconds followed by rapid fixation of the filaments and visualization by EM (Figure 8B). Under these bursting conditions, in every case where we could assign a crossover distance, it was 37 nm (these filaments are marked with an N for Normal in the figure). All other sections of polymer were disintegrating as seen previously in Figure 3 with no describable order to these “polymer” segments (marked with a B for Bursting in the figure). Disintegration of polymer is also evident in the gallery of images in supplemental figure 1. We were unable to detect any filaments with shortened crossovers as they underwent pronounced disassembly. These results are consistent with the hypothesis that Aip1 can target hypertwisted segments of F-actin along their length and not just heterotypic boundaries alone (25).

Under these bursting conditions, the disassembly products are either monomeric or short oligomers. However, it is difficult to determine whether severing also occurs during the bursting reaction due to the high percentage of bursting products. To assess the possibility that severing might occur concurrently with bursting, we reduced the fraction of actin filaments containing hyper twist regions by changing the ratio of actin to cofilin (Figure 9A). We decreased the concentration of cofilin from 12 μM to 4 μM and kept the concentration of actin and coronin the same as for figures 4-6. Under this new sub-stoichiometric ratio of
cofilin to actin, we still observed highly cooperative long stretches of cofilin-loaded hyper twist regions. But now, we have fewer hypertwist regions and a lot of naked, normal twist filaments (Figure 9A). Thus, if severing occurs during a bursting reaction, it should be more readily observed. Figure 9B shows the bursting reaction upon the addition of Aip1 for 10 and 60 sec. We found that bursting (marked as B in Figure 9B, left panel) happens to a small fraction of filaments within 10 sec but naked and normal twist long actin filaments remains (Figure 9B, left panel). However, if we wait for the reaction to go for 60 sec, we observed many shorter normal twist filaments (Figure 9B, middle panel), which appear to be the products of severing (Figure 9B, right panel). Thus, severing can occur in the same bursting reaction mixture on filaments that are not loaded cooperatively with cofilin.

To determine if coronin can facilitate cooperative cofilin loading at a low cofilin concentration that usually favors severing (2,15,23,53), we decreased the concentration of cofilin and varied the concentration of actin to a ratio similar to that used for Figure 9. Since we are using a lower concentration of actin at 2.5 µM, we increased the reaction time to 2 hours to allow the step 1 copolymerization reaction to reach a steady state. Under this new sub-stoichiometric condition for cofilin to actin, we still can find long stretches of hypertwist (Figure 10A). Thus, coronin facilitates cooperative loading of cofilin across an order of magnitude of cofilin concentrations (1-12 uM) as long as it is present during actin polymerization. These stable hypertwisted filaments burst upon the addition of Aip1 even for 5 sec (Figure 10B). The left panel of Figure 10B shows a bursting filament next to an intact normal twist filament. The instantaneous nature and the total structural loss for the entire actin filament (Figure 10B, right panel) resembles bursting of hypertwist filaments prepared using 12 uM cofilin in Figure 8B. Aip1 also induced severing of naked normal twist filaments (Figure 10C). Thus, Aip1 disintegrates long stretches of cofilin-saturated domains in a single burst while severing occurs at regions of low cofilin occupancy and normal twist.

Discussion.

Our results show that the combination of cofilin, coronin, and Aip1 induce a highly cooperative mode of actin disassembly that is distinct from filament severing. Using electron microscopy, we showed that the triple mix produced globular material consistent with a rapid conversion of actin polymer to monomer, which is further supported by our monomer trap assay. Experimentally separating the disassembly reaction into discrete steps showed that coronin and cofilin induced highly cooperative cofilin loading to generate long stretches of actin in the hypertwisted configuration that is indicative of cofilin binding (5). Hypertwisted actin rapidly disintegrated to monomer upon addition of Aip1. We showed that coronin accelerates Pi release from actin filaments which provides an explanation for how coronin promotes cofilin binding, and our electron microscopy further supports the idea that Aip1 attacks the sides of filaments occupied by cofilin (25,54).

We imagine two alternative mechanisms for how Aip1 converts cofilin-saturated polymer to monomer. In the first, a severing event is followed by rapid depolymerization from the end. The alternative possibility is that hypertwisted segments are disintegrating into monomers and short oligomers along their lengths. In this case, filament disassembly results from disruption of both intra- and interprotofilament contacts. We currently favor this second model because our electron micrographs show long stretches of polymer disintegrating all at once suggesting loss of interprotofilament adhesions. High resolution molecular EM shows that cofilin alters protomer contacts both along a protofilament (11) and also between the protofilaments (14). Aip1 does not displace cofilin from the filament (25), so it might further distort contacts between subunits, perhaps by shoving cofilin deeper into the filament, as suggested before (55). We continue to refer to this mode of actin disassembly as “bursting” to reflect the fast conversion of polymer to monomer and to distinguish it from severing and sequential loss of subunits from filament ends.
Another group did not see bursting or rapid conversion from polymer to monomer in the presence of cofilin, coronin, and Aip1 (43). Using TIRF imaging, they reported only severing reactions. The authors offered two possible explanations for the discrepancy between their study and the first description of filament bursting (39). One suggestion was that differences in filament attachment to substrates accounted for the differences in disassembly behavior. However, here we show long stretches of polymer disintegrating all at once using filaments in free solution with no connections to a substrate and no filament bundling. The second suggestion was that differences in labeling the actin with fluorescent probes could account for differences in disassembly mechanism. However, here we can distinguish severing from bursting reactions using unlabeled actin filaments and electron microscopy. If not filament attachment or different fluorochromes, then what? In the Jansen et al study, the authors presented only one time-lapse sequence in which the rate of acquisition was fast enough to be able to distinguish severing events from bursting events or rapid depolymerization from filament ends. Bursting reactions are fast, hence the word “bursting”, which refers to the abruptness of the event. Slow sampling rates will miss them. In addition to the issue of temporal undersampling, the authors did not analyze the data in a manner that could have detected bursting events. Examination of figure 1a in the study by Jansen et al shows rapid loss of polymer mass in the presence of cofilin, coronin, and Aip1, but the data was not analyzed in such a fashion to reveal mechanism. Finally, Jansen et al looked primarily at disassembly reactions in the presence of low concentrations (nanomolar) of the three actin disassembly factors. Bursting occurs under more physiological conditions where the disassembly factors are present at hundreds to thousands of nanomolar concentrations. Overall, however, we are in agreement that coronin promotes highly cooperative cofilin binding and that the combination of the three factors accelerates disassembly.

Our results provide new insight into the role of coronin in cofilin mediated actin disassembly. Coronin promotes cofilin binding to Listeria comet tails (26) and filaments in vitro (50), but it occupies the cofilin binding site on F-actin (42). Coronin has a higher affinity for ATP-actin than ADP actin (34,46) while cofilin has a higher affinity for ADP actin. We showed that coronin stimulates phosphate release from F-actin which would allow cofilin to displace coronin from the filament. By rapidly converting newly polymerized actin into the ADP bound state, coronin helps paint actin filaments for cofilin loading. Phosphate release alone, however, does not account for the full effect of coronin on cofilin binding because cofilin loads with higher cooperativity on to actin when copolymerized with coronin than on to aged filaments. In addition, bursting events occurred with much higher frequency using filaments assembled in the presence of cofilin and coronin than with aged filaments and cofilin alone. Coronin might further alter actin structure to further enhance cofilin binding. Alternatively, time dependent aging effects might stabilize filaments (56) and steer disassembly away from cooperative bursting and towards severing, although some think age dependent stabilization is an artifact (57). Either way, the electron microscopy presented here is consistent with previous results showing that coronin alters the pathway of disassembly towards bursting (39), but it does not affect the final outcome because coronin had no effect on the critical concentration.

Catastrophic actin filament bursting could explain the exponential decay kinetics of Listeria actin comet tails (58). A combination of bursting and severing might also help explain the two distinct actin turnover rates detected in the actin cortex (59). A catastrophic mode of filament disassembly would produce many actin monomers but few filament ends. Eliminating filaments without creating ends would explain why actin does not incorporate into the disassembling Listeria actin comet tail (58). In contrast, severing does not produce monomers, but it does produce filament ends which can the grow in the presence of actin monomer. In this case, cofilin is an actin assembly factor that stimulates polymerization by generating open
barbed ends that rapidly grow (60,61). We propose that it is only through the action of auxiliary factors like Aip1, coronin, and CAP that cofilin works to replenish the actin monomer pool and to prevent the cell from filling up with short, capped filaments that have limited morphogenetic power.

Experimental Procedures.

**FRET assay:** Aliquots of labeled actin were diluted to 20 μM in G buffer (pH 7.4) and spun the next day at 227,900 X g for 20 minutes. 35-40% Tetramethylrhodamine labelled actin and 12-15% Oregon green 488 labelled actin were premixed. For depolymerization reactions in the presence of the bursting factors actin was prepolymerized at 10 μM by the addition of 1x F-buffer (10 mM HEPES 7.8, 50 mM KCl, 0.5 mM EGTA, 1 mM MgCl2, 1 mM ATP). The final concentration of actin in the reaction was 1 μM. Spectroscopic monitoring of fluorescence of OG488 (λEx = 490 nm, λEm = 530 nm) over time was used to report on disassembly on a Spectramax M2 fluorimeter (Molecular Devices). Final concentrations of disassembly proteins were 1.25 μM cofilin, 0.75 μM coronin and 0.1 μM Aip1, unless indicated otherwise in graphs. Data was normalized using values of 1 as control actin polymer (in 1xF buffer) and 0 as control actin monomer (in G-buffer).

**Monomer generation assay:** Actin for this assay was prepared identically as the FRET assay except for the exclusion of TMR-actin from the reaction. TMR-actin was replaced by unlabelled G-actin. 1 μM Vitamin D-binding protein (Athens Research and Technology) was added to monomeric or polymeric actin to sequester actin monomer and the fluorescence was monitored (λEx = 490 nm, λEm = 530 nm) over time on a Spectramax M2 fluorimeter (Molecular Devices). Final concentrations of disassembly proteins were 1.25 μM cofilin, 0.75 μM coronin and 0.1 μM Aip1, unless indicated otherwise in graphs. Data was normalized using values of 1 as control actin polymer (in 1xF buffer) and 0 as control actin monomer (in G-buffer).

**Critical concentrations.** Fluorescence microscopy was used to detect the minimum concentration of actin necessary for filament formation. Actin, 20% labeled with Cy5, were mixed with the different actin disassembly factors at the concentrations indicated in the results section in 1X F-Buffer containing 0.4% methylcellulose, 100 nM catalase, 200 nM glucose oxidase, and 1 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. The reaction mixtures were incubated at room temperature for 4 hours in order to reach steady state. At the end of the incubation period, glucose was added to a final concentration of 40 mM from a 400 mM stock. The glucose completes the photo buffer, but it adding after the incubation period prevents acidification of the solution from production of gluconic acid. A 2 μl aliquot was removed from the reaction and placed on a glass slide. A 22X22 mm glass coverslip was placed on top of the sample drop followed by pressing down on the coverslip, hard, to make a very thin layer of sample. The reaction on the slide was then imaged using standard widefield fluorescence on a Zeiss Axioimager with a 63X 1.4 numerical aperture objective, a Hamamatsu Orca camera, and 2X2 binning. Five images were collected per sample, and each reaction was repeated three times. FIJI was used to prepare images for figures. We estimated the critical concentration to be the midpoint between the lowest actin concentration where filaments could be detected and the highest actin concentration where filaments could not be detected.

**Seeding assay:** Actin filaments treated with various combinations of depolymerizers as described in the FRET assay. The reaction was allowed to proceed to completion and 1/10 of the reaction (125 nM) was used to seed new pyrene G-actin assembly (25% labelled, 1 μM, preincubated in G-buffer). Fluorescence was monitored (λEx = 365 nm, λEm = 410 nm) over time.

**Phosphate Release:** Phosphate release from F-actin was measured using purine nucleoside phosphorylase described by Webb (51). Briefly, 20 uM G•actin was polymerized in the presence of varying concetrations of cofilin and coronin described in the text in reaction buffer (20mM Tris pH7.5, 50 mM KCl, 2mM MgCl2, 1mM EGTA, 1mM ATP, 0.2 mM methylthioguanosine, and 1 unit of purine nucleoside phosphorylase). Phosphate release
was monitored by measuring the increase in optical density at 360 nm using a Spectramax M2 plate reader. The OD 360 nm signal was corrected for scattered light from actin polymerization by subtracting the signal obtained at 60 nm in the absence of purine nucleoside phosphorylase.

Negative Stain Electron Microscopy: To measure actin filament crossover distances, 10 μM G•actin was copolymerized with 12 μM cofilin +/- 2 μM coronin for 90 seconds. Samples were fixed for 30 seconds with 0.1% glutaraldehyde, transferred to glow-discharged, carbon-coated EM grids for 10 seconds and then washed two times in polymerization buffer before staining with 2% uranyl acetate. To monitor filament disassembly in presence of Aip1, actin was copolymerized with cofilin and coronin using concentrations indicated in the results. Aip1 was then injected into the sample to a final concentration of 0.2 μM before fixing with glutaraldehyde at various times thereafter as described above. Electron micrographs were acquired using Tecnai G² Spirit BioTWIN electron microscope at 120kV.

Data availability.
All data are contained within the article and accompanying supporting information.

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Conflict of Interest Statement.
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions.
VT and WMB performed the electron microscopy. VT performed the phosphate release assay. AVN performed the spectroscopy and developed the monomer trap assay. WMB estimated apparent critical concentrations. All authors analyzed results and wrote the manuscript.

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**Figure Legends.**

Figure 1. Overview of actin filament disassembly in the presence of cofilin, coronin, and Aip1 using electron microscopy. In all panels, actin is polymerized for 1 minute before any additional steps. (A) A 10 uM solution of G-actin was induced to polymerize by adding salt and ATP. The sample was fixed and prepared for visualization in the electron microscope. The upper panels show low magnification views while the lower panels are at higher magnification. (B) Electron micrographs of the products produced by adding 5 uM cofilin, 2 uM coronin, and 0.2 uM Aip1 to filaments for 60 seconds. The upper panels show low magnification views while the lower panels are at higher magnification. (C) Image of 5 uM cofilin, 2 uM coronin, and 0.2 uM Aip1 alone (no actin). (D) Morphology of actin filaments that were incubated for an additional 60s in the presence of 5 uM cofilin and 0.2 uM Aip1. (E) Morphology of actin filaments that were incubated for an additional 60s in the presence of 2 uM coronin and 0.2 uM Aip1.
Morphology of actin filaments that were incubated for an additional 60s in the presence of 5 uM cofilin and 2 uM coronin.

Figure 2. The bursting reaction proceeds with rapid kinetics to generate monomer and/or products incompetent to seed actin assembly. (A) Disassembly kinetics of 1 µM actin was monitored by loss of FRET signal in the presence of various combinations of factors. (B) Rate of monomer production was monitored by quenching of Oregon green -488 actin by Vitamin D- binding protein over time (C) Pyrene-based seeding reaction using the products of reaction (A) showed that products of the bursting reaction are not competent to seed new actin assembly. (D) Dose response curves of cofilin in the presence of 0.75 µM cofilin and 0.1 µM Aip1 shows increasing rates of disassembly with increasing amounts of cofilin (E) Lower ratios of coronin: cofilin produces more efficient disassembly whereas higher ratios may stabilize actin filaments. (F) Initial rates (120 s) of disassembly with varying concentrations of Aip1 and fixed amounts of cofilin and coronin show increased rates of disassembly with increasing amounts of Aip1. Representative data from n=3 experiments is shown.

Figure 3. Estimation of apparent critical concentrations of actin assembly in the presence of actin disassembly factors. Fluorescent micrographs showing the onset of filament formation in the presence of actin disassembly factors as the actin concentration increases from left to right. The concentrations were 20 µM Cofilin, 2 µM Coronin, and 5 µM Aip1. Actin concentrations are µM and listed above each column. Scale bar in the lower right panel applies to all the images. The experiments were repeated three times with identical results. Approximate critical concentrations estimated from these results are listed in table 1.

Figure 4. Long stretches of actin polymer disintegrate in the presence of coronin, cofilin, and Aip1. (A) Actin filaments, one minute old, were mixed with cofilin, coronin, and Aip1 and then fixed at the times indicated. The panels are images of the reaction products at various times after adding the triple mix of disassembly factors. The upper panels show low magnification views while the lower panels are at higher magnification. Scale bars are 100 nm. Segments of actin polymer are losing coherent structure as early as five seconds. (B) Actin filament structure is intact in the presence of cofilin and coronin alone. Each consecutive upper panel shows higher magnification images of actin filaments that were polymerized in the presence of cofilin and coronin for one minute. Adding Aip1 to minute-old filaments copolymerized with cofilin and coronin results in the rapid disintegration of polymer into monomers and short oligomers. Each consecutive lower panel shows higher magnification images of the products of the reaction. Scale bars are 200 nm.

Figure 5. Cooperative changes in filament structure in the presence of cofilin and coronin. (A) Electron micrograph of actin filaments assembled in the presence of cofilin and coronin for 90 seconds. Red and blue dots along the filaments mark the crossover points. (B) One section of an electron tomogram showing the crossover points on two different filaments. (C) Histogram of crossover distances seen in actin filaments polymerized in the presence of cofilin and coronin for 90 seconds shows two different populations. (D) Two populations of filament widths form in the presence of cofilin and coronin. (E) Relationship between crossover distance and caliber shows that two populations of actin filaments form in the presence of cofilin and coronin. (F) Length distribution of filaments that consist either entirely of normal or hyper twist. (G) Histogram showing the percentage of hyper twist in 300 different filaments assembled in the presence of cofilin and coronin. (H) Length and portion of hyper twist of the same 300 filaments. Each bar is one filament. (I) An example of a filament containing both normal twist and hyper twist. Crossover distances were measured along the filament in the direction of the arrow and used to generate the graph for filament 1 in (J) which shows the sharp transitions between normal and hyper twisted configurations in two representative filaments. Scale bar is 25 nm in A and 50 nm in B and I.

Figure 6. Generation of long stretches of hyper twist filaments requires coronin and depends on filament age. (A) Hyper twist (marked by H) and normal twist (marked by N) filaments were readily formed when
actin is polymerized in the presence of coronin and coflin. (B) Generation of long stretches of hyper twist (marked by H) and normal twist (marked by N) regions when coflin was added to 90 sec-old filaments polymerized in the presence of coronin. (C) Absence of long stretches of hyper twist regions when coflin was added to 30 min-old filaments polymerized in the presence of coronin. Severing (marked by S) can readily be detected at naked normal twist filaments (left panels). Thicker normal twist filaments corresponding to coronin decorated actin can also be found (right panels). Lower left graph shows that the presence of coronin cannot facilitate the generation hyper twist filaments in aged filaments. Lower right graph shows that a coronin decorates aged filaments and prevent them from severing. (D) Absence of long stretches of hyper twist regions when coflin was added to 30 min-old filaments (lower right graph). Severing (marked by S) of naked normal twist filaments (marked by N) is readily observed.

Figure 7. Coronin and coflin accelerate phosphate release from F-actin. (A) Rates of phosphate release from actin filaments in the presence of increasing concentrations of coflin alone and (B) coronin alone show accelerated phosphate release by the individual factors. (C) Together, coflin and coronin cooperate to enhance phosphate release more than each individual factor. (D) Comparison of rates of phosphate release in the presence of actin alone, 4 µM coflin, 4 µM coronin, or a combination of the two. Representative data from n= 4 experiments is shown.

Figure 8. Aip1 disintegrates hyper twisted polymer. Actin was prepolymerized in the presence of coronin and coflin in A and B for 90 seconds and then spiked with Aip1 for 5 seconds in B before fixing. 6A) Four representative images of actin filaments polymerized in the presence of coronin and coflin for 90 seconds. Segments of polymer with normal twist are marked “N” and hyper twisted polymer is marked “H”. (B) Actin, first assembled as in 6A, five seconds after adding Aip1. “Bs” mark regions where polymer segments are disintegrating. Unmarked filaments are not obviously bursting, but crossover distances cannot be assigned. Scales bars are 200 nm.

Figure 9. Generation of long stretches of hyper twist regions at sub-stochiometric actin to coflin ratio that are substrates for Aip1-induced bursting. (A) Actin was copolymerized with coronin and coflin at coflin to actin ratio of 0.4. Long and short stretches of hyper twist regions are readily detected. (B) Bursting of hyper twist regions at 10 sec after Aip1 addition and severing of naked normal twist regions by 60 sec.

Figure 10. Generation of stable hyper twist regions at low coflin concentration that are substrates for Aip1-induced bursting. (A) Coronin facilitates the formation of hyper twist filaments at 1 µM coflin. (B) Bursting of hyper twist filament (marked as B) but not a neighboring normal twist filament (marked as N) upon Aip1 addition for 5 sec. (C) Severing of undecorated normal twist filaments upon addition of Aip1 for 5 sec.
Figure 1
A

1-Step Depolymerization:

F actin + Triple Mix 5 sec
F actin + Triple Mix 10 sec
F actin + Triple Mix 30 sec

B

Step 1: Co-Polymerize Actin with Coronin & Cofilin (90 sec)

Step 2: Add Aip1 30 sec

Figure 4
Figure 5
Figure 6

A. Co-Polymerize 10 uM Actin with 2 uM Coronin & 12 uM Cofilin for 90 sec

B. Co-Polymerize 10 uM Actin + 2 uM Coronin for 60 sec then add 12 uM Cofilin for 90 sec

C. Pre-Polymerize 10 uM Actin for 30 min then add 2 uM Coronin & 12 uM Cofilin for 90 sec

Severing of Undecorated Normal Twist filaments

Coronin-Decorated Thick Normal Twist filaments

D. Co-Polymerize 10 uM Actin with 12 uM Cofilin in the absence of Coronin

Severing of Undecorated Normal Twist Filaments

Graphs showing the number of filaments:
- Normal Twist
- Mixed Twist
- Hyper Twist

Graphs comparing Naked, Mixed, and Coronin Saturated conditions.
Figure 7

| Actin  | Cofilin | Coronin | Rate of Pi Release | Fold Increase |
|--------|---------|---------|-------------------|---------------|
| 20 mM  | 0       | 0       | 0.002             | 1X            |
| 4 mM   | 0       | 4 mM    | 0.0025            | 1.25X         |
| 0      | 4 mM    | 0       | 0.006             | 3X            |
| 4 mM   | 0       | 4 mM    | 0.012             | 6X            |
**High Cofilin Young Filaments** -
Step 1: Co-polymerize Actin with Coronin & Cofilin for 90 sec
Cofilin : coronin = 6
Cofilin : Actin = 1.2
- Generation of Hyper Twist & Normal Twist Filaments

**High Cofilin Young Filaments** -
Step 2: Disassembly Reaction with AIP1 for 5 sec
Cofilin : Aip1 = 40
- Preferential Bursting of Hyper Twist Filaments
- NO Bursting of Normal Twist Filaments
A

10 μM Actin + 2 μM Coronin + 4 μM Cofilin Co-polymerize for 90 sec - Cofilin:Actin ratio at 0.4

B

10 μM Actin + 2 μM Coronin + 4 μM Cofilin Co-polymerize for 90 sec then add 0.3 μM Aip1

Figure 9
**Low Cofilin Step 1:**
Co-polymerize Actin with Coronin & Cofilin
Cofilin:coronin = 2; Cofilin:Actin = 0.4
- Hyper Twist & Normal Twist Filaments

**Low Cofilin Step 2:**
Disassembly Reaction with Aip1 for 5 sec
- Preferential Bursting of Hyper Twist Filaments
- NO Bursting of Normal Twist Filaments

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**Low Cofilin Step 2: Disassembly Reaction with Aip1 - Severing of Normal Twist Filaments**

Co-polymerize
2.5 uM Actin
+ 0.5 uM Coronin
+ 1.0 uM Cofilin
for 2 hr
then add
0.1 uM Aip1 for 5 sec
- Severing of undecorated Normal Twist Filaments

Figure 10
