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Multicomponent Uncapping of Actin Filaments by Twinfilin and Formin

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

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Abstract

Multicomponent Uncapping of Actin Filaments by Twinfilin and Formin

By Vishal Reddy

The actin cytoskeleton plays a major role in determining cell shape, polarity, and flexibility as well as driving various cellular processes. Capping Protein (CP) arrests growing actin filament barbed ends by halting both polymerization and depolymerization. However, until CP is removed, the barbed end cannot be accessed by other actin-binding proteins, thus the intermediate step of uncapping must occur to dissociate CP. Though this process occurs rapidly in cells, laboratory experiments have revealed a relatively longer dwell time for CP on barbed ends. The disparity between observed rates *in vivo* and *in vitro* is thought to be due to the action of a class of actin-binding proteins known as uncappers. The effects of twinfilin (mTwinfilin-1) and formin (mDia1) on uncapping rates was tested using microfluidics-assisted Total Internal Reflection Microscopy (mf-TIRF). The effects of filament nucleotide state, a commonly understood influencer of actin filament biochemistry, were also accounted for. We found that twinfilin increased uncapping rates of both ADP and ADP-P_i state filaments by 8-fold and 3-fold respectively, saturating at a concentration of 5 μM twinfilin. We observed formin's effects on ADP and ADP-P_i filaments uncapping as a linear increase in uncapping rate with a maximum observed increase of 37-fold and 5-fold respectively at 200 nM formin. ADP and ADP-P_i filaments also appeared to possess different CP dissociation rates in absence of other proteins as well, with ADP filaments generally dissociating CP 3-fold faster than ADP-P_i. When present together, twinfilin and formin displayed a synergistic effect on uncapping rates for ADP and ADP-P_i filaments, and we observed a rate increase by about 270-fold and 8-fold respectively, for 5 μM twinfilin and 200 nM formin. Taken together, these results suggest that actin filament barbed ends in

the ADP state are more prone to uncapping than barbed ends in the ADP-P_i state due to a combination of weaker CP binding and increased susceptibility to the action of uncappers such as twinfilin and formin.

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I would like to extend my greatest appreciation to my advisor, Dr. Shashank Shekhar, for guiding me through the last two years by helping me develop the professional and academic skills required for research. I would also like to thank my mentor Ankita for taking the time and effort to train me and fuel my curiosity for my research. Lastly, I would like to thank the members of my committee for their time and engagement with my education and interests.

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Introduction

The dynamic regulation of the actin cytoskeleton is vital for various cellular processes including cell migration[1], endocytosis[1], lamellipodial formation[2], cytokinesis[3], and maintaining cell shape[14]. To sustain these processes, actin filaments experience rapid assembly and disassembly, processes that are governed by a multitude of actin-binding proteins within the cell. Three main divisions of actin-binding proteins that regulate actin dynamics within Eukaryotes are polymerases, depolymerases, and cappers.

Within cells, actin can exist as either monomeric G-actin or filamentous F-actin. When in its G-actin state, actin is bound to either ATP or ADP, the former of which facilitates polymerization to F-actin[15]. F-actin consists of long double-helical strands of actin monomers which are added to either the barbed (+) end or pointed (-) end of actin filaments. Upon polymerization, newly added monomers hydrolyze their bound ATP to ADP-P_i ($t_{1/2}$ ~ 2 sec)[16]. This is followed by P_i release which transforms the monomer from ADP-P_i to ADP ($t_{1/2}$ ~ 350 sec)[17]. The change in overall nucleotide state over time is commonly referred to as the aging of actin filaments. The ATP hydrolysis and release of P_i from F-actin subunits have been shown to drastically alter the interactions between actin and the various actin-binding proteins.

Rapid polymerization of actin filaments provides the force against cell membranes to drive processes of cell motion and division[18]. Filament nucleation and polymerization are directly proportional to the number of polymerizable monomers within the cell[30]. A protein known as profilin serves to maintain this pool of monomers by sequestering G-actin[31]. A class of proteins known as polymerases function to increase rates of actin polymerization within cells. One such protein, formin, attaches to the barbed ends of F-actin and accelerates polymerization by capturing nearby profilin-bound monomers with its long poly-proline regions[32]. Formins tend to be found within the

inner surface of the cell membrane and thus directly contribute to the intracellular force generation of actin filaments[33].

As actin filaments grow in length, the efficiency of their force generation decreases. This is due in part to the depletion of the active pool of monomers as well as the increased susceptibility of longer filaments to buckling[34], the bending of filaments. To alleviate this issue, cells must rapidly depolymerize the older, longer filaments and divert monomers to newer, shorter force-generating filaments. The depolymerization process is catalyzed by depolymerases such as twinfilin. Twinfilin, a member of the actin depolymerization factor homology family is a depolymerase of actin filaments in mammals[7]. At low concentrations of actin, twinfilin has been shown to outcompete free monomers for barbed end binding[4, 13]. Thus, twinfilin is a depolymerase that can function even in the presence of growth-promoting conditions.

Between the stages of polymerization and depolymerization exists a transient period in which actin filaments are arrested in a stable state. Capping Protein (CP) arrests growing filament barbed ends by halting both polymerization and depolymerization. This stabilization by CP allows for the funneling of free actin monomers away from older, longer filaments to shorter force-generating filaments[2]. By stabilizing the faster-growing barbed end, CP prevents monomers from accessing the barbed end. While capped, filaments are still able to depolymerize from the slower-growing pointed end. These actions in tandem work to funnel monomers from capped filaments to growing filaments. However, to continue polymerization or initiate depolymerization from the barbed end, CP must first dissociate, a relatively slow process as determined *in vitro* ($t_{1/2}$ ~ 30min)[6]. Despite CP's high binding affinity and slow dissociation kinetics *in vitro*, CP experiences rapid turnover in cells[6]. This finding is hypothesized to be the result of the action of various Capping Protein Interaction (CPI) motif proteins such as the CARMILs[20] and V1/myotrophin[25] and Actin Depolymerizing Factor Homology (ADF-H) proteins such as Twinfilin 1[4] and ADF/cofilins[21].

Previous work regarding capping protein dynamics has shown that mouse twinfilin 1, a protein composed of two ADF-H domains and a CPI-motif-containing tail, can accelerate filament uncapping sixfold *in vitro*[4]. Furthermore, adding V1, a protein known to sequester CP, led to nearly a 48-fold increase in uncapping rate compared to buffer alone[4]. However, even this increase in dissociation rate ($t_{1/2}$ ~ 40s)[12] is dwarfed by the rapid rates of CP turnover *in vivo* ($t_{1/2}$ ~ 1.7 s)[12], an increase by three orders of magnitude from *in vitro* controls. By a separate process, the formin mDia1 has been shown to form a complex on the barbed end by simultaneously binding with CP[9, 10]. This barbed-end-formin-capping-protein (BFC) complex exists for a brief period before resolving to either a capped or formin-bound barbed end[9]. Furthermore, recent work from our lab showed that twinfilin can join formin and CP at the barbed end to form a tripartite complex[11]. Considering these discoveries, we investigated how formin and twinfilin might together influence uncapping rates of filaments in both ADP and ADP-P_i states and observed the effects of the concentration of twinfilin and formin on these rates. We found that twinfilin and formin both accelerate CP dynamics individually and synergize to dramatically increase rates together. The maximum observed rate increase of this synergy far surpassed previous in vitro studies, falling within a single order of magnitude of in vivo rates. Twinfilin on its own appears to be a rather inefficient uncapper compared to formin. Additionally, uncapping rates depend on the concentrations of twinfilin and formin as well as the nucleotide state of the filaments' barbed ends. Overall, ADP-P_i barbed ends appear to dissociate CP far slower than ADP barbed ends and are more resistant to the effects of uncappers such as twinfilin and formin.

These observations reveal a previously unknown synergy in the multicomponent process of filament uncapping which can achieve rapid CP turnover similar to *in vivo* rates. Furthermore, the isolation of nucleotide state in the experiments overturn the understanding that CP has stronger affinity for ADP

filaments illustrating the gaps in our understanding of actin aging in relation to other cellular processes.

Experimental Techniques

1. Microfluidics-assisted TIRF microscopy

The main technique that we used to address the questions described above is called microfluidics-assisted TIRF (mf-TIRF) microscopy[35, 36]. This approach has two parts: microfluidics and TIRF. Total Internal Reflection Fluorescence microscopy (TIRF) uses an evanescent wave to selectively illuminate and excite fluorophores in a very shallow region immediately above the coverslip, i.e., around the depth of 100 nm near the surface. It not only reduces background fluorescence but also allows visualization of fluorescently labeled individual actin filaments and single protein molecules interacting with these filaments.

The working principle underlying TIRF microscopy is illustrated in figure 1. A laser beam traveling at a high incidence angle through a glass slide reaches the interface of the two media with different refractive indices, it either gets refracted into the second medium or reflected at the interface depending on the difference in the refractive indices of the two media and the incidence angle. At an angle greater than the critical angle, the incident light reflects entirely into the first medium.

Although light no longer travels into the second medium when the angle of incidence is greater than the critical angle, the reflected light generates a highly restricted electromagnetic field (called the evanescent field) adjacent to the interface, in the lower refractive index medium. The evanescent field decays exponentially in intensity with the distance from the interface. The field extends at most up to 200 nm thickness. Fluorophores located in the vicinity of the glass-liquid surface can be excited by the evanescent field. However, because of the exponential decay of the evanescent field intensity, the excitation of fluorophores is restricted to a region that is typically less than 200 nm in thickness. The objects farther than 200 nm from the interface do not get illuminated which leads to

the reduction of the background fluorescence. This enables a higher signal-to-noise ratio as compared to conventional wide-field fluorescence microscopy.

Although this technique allows observation of fluorescently labeled actin filaments, the data analysis is complicated by the free diffusion of actin filaments in the plane of the image. Therefore, we used a modified version of the traditional TIRF technique by combining it with microfluidics. We made a flow cell using a polydimethylsiloxane (PDMS) chamber which has three inlets and one outlet and placed it on the top of the coverslip. In addition to its moldability, the PDMS chamber has the benefits of being inert and hydrophobic, not allowing interactions with our proteins nor swelling when exposed to aqueous solutions. Actin filaments in the chamber can then be exposed to solutions with different biochemical conditions by connecting the protein reservoirs to the chamber's inlets. The computer-controlled regulator allows fast and reliable control of pressures and flow rates in various ranges. Therefore, we can vary the flows in the flow cell in real-time. The flow helps align the filaments parallel to the coverslip. Since the filaments are aligned parallel to the coverslip, a single attachment point at the barbed end, or the pointed end is sufficient to keep the filament in the evanescent field. The flow also aligns the filament in a nearly straight line which makes the measurement of filament length and determining the location of filament ends straightforward and accurate.

Another advantage that microfluidics offers is the ability to rapidly change the biochemical conditions to which filaments are being exposed at a given time. This is achieved by rapidly changing the specific flow being flowed into a chamber through different inlets. The continuous flow of fresh solutions helps maintain constant protein concentration throughout the flow cell and allows for the acquisition of more accurate data.

The field of view contains hundreds of filaments aligned parallel to the flow which have been exposed to the same conditions and have the same history. This makes data collection straightforward and high throughput. The drag force of flowing fluid in microfluidics can also be used as an experimental tool. The flowing fluid exerts a viscous drag along the contour of the filament which is maximum at the anchoring point and decreases along the filament length. This force applies a mechanical tension (piconewton range) on the filaments at their point of anchoring and can be used as a tool to analyze how force affects the activity of actin-binding proteins. The ability to apply force on hundreds of filaments in parallel provides a huge advantage as compared to optical tweezers where tensile forces can be applied on only one filament at a time.

Taken together this system has several advantages. It allows us to change biochemical conditions rapidly with no time delay as well as enables high throughput acquisition of 100's of filaments at the same time. Lastly, the flow helps align the filaments which makes the analysis of mf-TIRF data much simpler as compared to the conventional TIRF data.

1. Materials and Methods

Passivation of glass coverslips

Glass coverslips will first be cleaned by sonication in detergent for 20 minutes, followed by successive sonications in 1 M KOH and 1 M HCl for 20 minutes each and then in ethanol for another 20 minutes. The cleaned coverslips will then be dried under an N_2 stream and coated with a solution containing 2 mg/ml Biotin and 10 mg/ml PEG-silane dissolved in 80% ethanol adjusted to pH 2. The coated coverslips will then be left overnight in the oven at 70° C.

PDMS chamber set-up

A PDMS (Polydimethylsiloxane) chamber with three inlets and one outlet will be clamped onto a Biotin-PEG-silane coated coverslip, to form a microfluidic flow cell as shown in figure 2. The assembled flow cell will then be placed on the microscope stage. The chamber is connected to the microfluidics flow control system and the tubes from the microfluidics system will be connected to the inlets and the outlets of chamber making sure there is no bubble while flowing mf-TIRF buffer (10 mM imidazole pH 7.4, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.2 mM ATP, 25 mM DTT and 1 mM DABCO). The flow rate in the chamber will be monitored and controlled using the MAESLFO system. For experiments where pointed ends are free, biotinylated capping protein will be anchored on the streptavidin-coated coverslip surface and preformed actin filaments will be flowed in whose barbed ends are captured by surface-bound capping protein while their pointed ends will remain free. An example viewing frame is shown in figure 3. Actin filaments can be polymerized in KME buffer (100 mM KCl, 1 mM $MgCl_2$ 0.2 mM EGTA).

Image acquisition and analysis

Single-wavelength time-lapse TIRF imaging will be performed on a Nikon microscope in the Shekhar lab equipped with a 20-mW Argon laser, a 60x TIRF-objective, and an EMCCD camera. The focus will be maintained by the Perfect Focus System. The images will be acquired using the imaging software Elements. The acquired data will be analyzed in Fiji and the kymograph plugins will be used to draw kymographs of individual filaments. The kymograph allows rapid measurement of the point of uncapping for filaments as shown in figure 4.

Results

Twinfilin uncaps both ADP and ADP-P_i filament barbed ends

Previous studies have reported that twinfilin regulates CP dynamics by uncapping aged barbed ends[4]. To determine how the uncapping activity of twinfilin is influenced by the nucleotide state of actin filaments, we performed uncapping experiments using mf-TIRF. Preformed actin filaments (in ADP or ADP-P_i state) were introduced into the mf-TIRF chamber and captured at their barbed ends by coverslip-anchored CP. The dissociation of filaments from coverslip-bound CP caused the immediate disappearance of filaments from the field of view. We recorded the disappearance of actin filaments in the field of view over time. A cartoon of the experimental setup is shown in figure 5. Changes in the time-dependent survival fraction of CP-bound filaments were then used to determine the dissociation rate of CP from the barbed end.

To determine the effect of twinfilin on the uncapping of ADP-actin filaments, we exposed CP-anchored ADP filaments to TIRF buffer containing a range of twinfilin concentrations and measured the resulting barbed-end uncapping rates. We found that the barbed end uncapping rate increased with twinfilin concentration. Compared to the control, 5 μM mTwinfilin-1 increased the rate of CP's dissociation from ADP barbed ends by about 8-fold. Next, we investigated how twinfilin affects uncapping ADP-P_i filaments. Actin filaments were maintained in the ADP-P_i state by conducting the entire experiment in the presence of TIRF buffer supplemented with 50mM Pi. We found that similar to ADP filaments, the rate of uncapping of ADP-P_i filaments also increased with twinfilin concentration. Compared to the control, 5 μM mTwinfilin-1 increased the rate of CP's dissociation from ADP-P_i barbed ends by about 3-fold.

Comparing the uncapping rates of ADP and ADP-P_i barbed ends in the absence of twinfilin, we found that ADP filament barbed ends uncap faster (figures 6 & 7). This suggests that capping protein binds

more strongly to ADP-P_i barbed ends. Similarly in the presence of twinfilin, ADP filament barbed ends uncapped 4-fold faster compared to ADP-P_i filaments. Taken together, our data suggests that capping protein has a higher affinity for ADP-P_i barbed ends and twinfilin uncaps ADP filaments faster.

Formin uncaps both ADP and ADP-P_i filament barbed ends

Prior studies have suggested that the mechanism by which formin uncaps filament barbed ends involves its ability to form complexes with capped filament barbed ends[9]. These barbed-end-formin-capping-protein (BFC) complexes can further resolve into capped barbed ends and formin-bound barbed ends. A cartoon of this mechanism is shown in figure 8. However, the efficacy of formin as an uncapper on both ADP and ADP-P_i filaments remains unclear. We once again used mf-TIRF microscopy to determine how formin affects uncapping rates. We first tested formin's effects on ADP filament uncapping. The ADP filaments were formed in KME buffer and left on the bench for 30 minutes to polymerize and age (allow P_i release). These filaments were then captured from their barbed ends using capping protein anchored on the surface and exposed to a solution containing formin (mDia1). We systematically changed the concentration of formin and measured the barbed end uncapping rates (figures 9 & 10). We found that the rates of barbed end uncapping increased linearly with a maximum rate of 0.01016 s^{-1} at 200 nM formin, a 37-fold increase from buffer alone. We then sought to answer how formin affects ADP-P_i filament uncapping. The ADP-P_i filaments were formed in TIRF buffer supplemented with 50 mM P_i and incubated for 30 minutes to polymerize. These filaments were then captured using capping protein anchored on the surface and exposed to a solution containing formin. Filaments were maintained in the ADP-P_i state by supplementing TIRF buffer with 50 mM P_i. The uncapping rates were measured with increasing concentrations of formin. Interestingly, unlike the ADP filaments, the ADP-P_i uncapping rate appears to saturate around 100 nM formin with a maximum mea[s]ured rate of 0.00065 s^{-1} at 200 nM formin, a nearly 5-fold increase from buffer alone. Overall, we were able to show that formin can accelerate uncapping rates of ADP

filaments and ADP-P_i filaments, however, the trends with increasing concentrations of formin differ between the two nucleotide states. Furthermore, the slower rate of uncapping for ADP-P_i filaments remains consistent with the findings for twinfilin. This supports the possibility that capping protein has a higher affinity for ADP-P_i barbed ends over ADP barbed ends. The data also seem to suggest that the nucleotide state of barbed ends not only affects capping protein affinity but also formin affinity as well, with ADP filaments having a greater affinity for formin than ADP-P_i filaments.

Twinfilin and Formin synergize to accelerate uncapping for both ADP and ADP-P_i filament barbed ends

Recent studies involving the so-called BFC complexes have shown that twinfilin, with its ability to accelerate capping protein dissociation from filament barbed ends, can bias the BFC complex towards the formin-bound state[11]. We sought to elaborate on this finding by investigating the uncapping rates of ADP and ADP-P_i filaments in the presence of twinfilin and formin. We once again used mf-TIRF to measure these rates. The ADP filaments were formed in KME buffer and left on the bench for 30 minutes to polymerize and age (allow P_i release). These filaments were then captured from their barbed ends using capping protein anchored on the surface and exposed to a solution containing formin and twinfilin. We maintained the amount of twinfilin at the saturating concentration of 5 μM, systematically changed the formin concentration, and measured the rates (figures 11, 12). We found that the uncapping rates increased drastically from twinfilin and formin in isolation with a maximum measured rate of 0.07204 s^{-1} at 200 nM formin and 5 μM twinfilin, nearly a 270-fold increase from buffer alone. We then tested the effect of the twinfilin/formin combination on ADP-P_i uncapping rates. The ADP-P_i filaments were formed in TIRF buffer supplemented with 50 mM P_i and incubated for 30 minutes to polymerize. These filaments were then captured using capping protein anchored on the surface and exposed to a solution containing formin and twinfilin. Filaments were maintained in the ADP-P_i state by supplementing TIRF buffer with 50mM P_i. The uncapping rates

were measured with a constant 5 μM twinfilin and increasing concentrations of formin (figures 11, 12). We found that the uncapping rates for ADP-P_i filaments in the presence of both twinfilin and formin increased from twinfilin and formin in isolation, though not to the same extent as for ADP filaments (figure 13). We measured a maximum rate of .00105s^{-1} at 5 μM twinfilin and 200 nM formin, nearly an 8-fold increase from buffer alone.

Discussion

The regulation of CP dynamics involves the action of various families of actin-binding proteins. Though once thought to depend primarily on CPI-motif proteins such as CARMILs, recent discoveries suggest that competition for the barbed end is a more potent driver of CP dissociation than direct interaction with CP[4]. Though on its own twinfilin appears to be a rather inefficient uncapper, saturating around 5 μM for an 8-fold increase in uncapping rate, in tandem with cofactors such as formins and myotrophins, its uncapping ability improves significantly. Compared to the fastest previously observed 180-fold increase for CARMIL (mCAH3)[20], twinfilin (mTwinfilin-1) aided by formin (mDia1) displayed a dramatic 270-fold increase for ADP filaments with no sign of saturation.

Despite its classification as a depolymerase, twinfilin displays unique stabilization abilities similar to cappers[23]. Twinfilin has been shown to increase barbed end depolymerization of ADP-Pi filaments while slowing barbed depolymerization of ADP filaments[13]. The observed disparity between twinfilin uncapping rates between ADP and ADP-P_i filaments would suggest that twinfilin uncapping is more common for filaments that have been capped long enough to have ADP actin subunits at their barbed ends. These older filaments, no longer contributing force to the membrane, would need to be quickly uncapped and depolymerized to continue funneling monomers to free barbed ends. Twinfilin could aid in uncapping, supported by V1/myotrophin, to allow other depolymerization agents, such as cofilins and SRV2/CAP, access to the barbed end.

Though formin serves as an effective polymerase, due to both its location on the membrane and its high polymerization rate, it remains a poor nucleator of actin filaments[28]. Thus, it has been hypothesized that formin requires pre-nucleated filaments to achieve maximal efficiency. One such method by which formin could acquire pre-nucleated barbed ends would be via forming BFC complexes with capped filaments. Since filament capping in cells is a stochastic process[22],

filaments of any length and location in the cell are equally likely to be capped. Such events could prove detrimental to force generation when smaller filaments near the membrane are capped prematurely. Thus, the mechanism of formin uncapping could provide such filaments with a route to be rescued from their capped state. The role of twinfilin in this tripartite uncapping system would increase the likelihood that BFC complexes resolve to a formin-bound state[11] and as a result are able to continue rapid polymerization at leading edges.

The data also suggests that in addition to binding with higher affinity to CP, ADP-P_i actin filaments are also less susceptible to the action of uncappers *in vitro*. This finding conflicts with previous work showing a much lower affinity for CP on ADP-P_i barbed ends *in vivo*[24], indicating the role of other players in the regulation of filament capping and uncapping. Though the aging of actin filament subunits is generally stochastic with a slow rate[16, 17], it has been shown that the rate of P_i release is faster at filament ends by 300-fold[19]. If capped filaments retain this rapid release rate, the biochemical properties of capped barbed ends could be more variable than previously thought.

The assessment of the synergy of twinfilin and formin reveal the fastest recorded in vitro uncapping rates, nearly doubling the previous highest rates observed by CARMILs. The individual contributions by twinfilin and formin allow each protein to bridge the uncapping process directly to depolymerization, to recycle of actin monomers, or polymerization, to continue force generation at the cell membrane, respectively. Lastly, the slow process of actin aging appears as a rate limiting step to uncapping. The acceleration of actin aging, whether by other actin binding proteins or structural factors at the barbed end, could serve as the final factor in rapid CP dynamics in cells.

Future Directions

Other actin-binding proteins have been shown to play a role in governing the mechanics of the barbed end. In addition to CARMILs, cofilin and SRV2/CAP have been shown to accelerate filament uncapping, while V1/myotrophin has been shown to sequester capping[26]. Cofilin's mechanism appears to differentiate from both formin and twinfilin in that the protein decorates the sides of filaments prior to uncapping[21].

On the other hand, CAP's uncapping effects are less well-documented. The C-terminal end appears to be able to uncap but at a much lower rate than twinfilin. While its N-terminal end appears to aid in twinfilin's depolymerase activity[28], it does not appear to have any significant effect on uncapping rates. Interestingly, N-CAP does not significantly increase twinfilin's uncapping ability as well.

V1/Myotrophin's sequestration effects also seem to provide a level of regulation against premature capping of growing filaments. On its own, it has been shown to sequester capping protein without directly displacing it from barbed ends[25]. In tandem with twinfilin, it has also been shown to significantly increase uncapping rates[4]. V1's involvement could potentially bring CP dissociation observed by twinfilin and formin to even faster turnover rates. Interestingly, while CP remains highly conserved in eukaryotes, V1 is notably absent from the plant and fungal kingdoms[25].

Lastly, twinfilin 2b, an isoform found predominantly in skeletal muscle and the heart[29], could be tested for uncapping activity to observe tissue-specific differences in isoform activities.

Figures

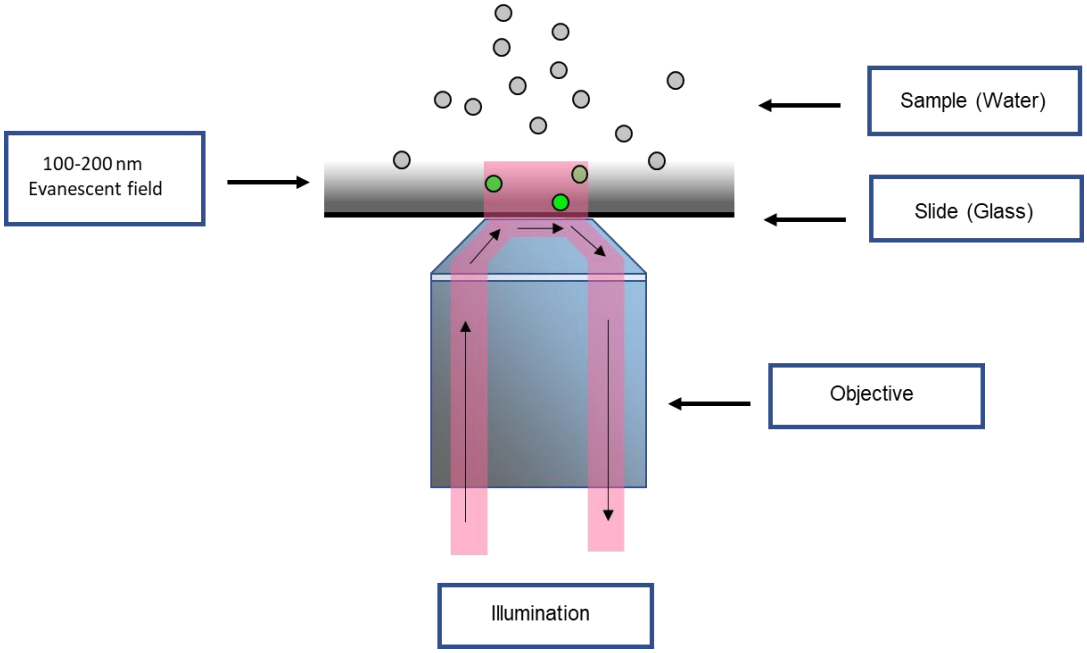

Figure 1: Schematics of Total Internal Reflection Fluorescence microscopy.

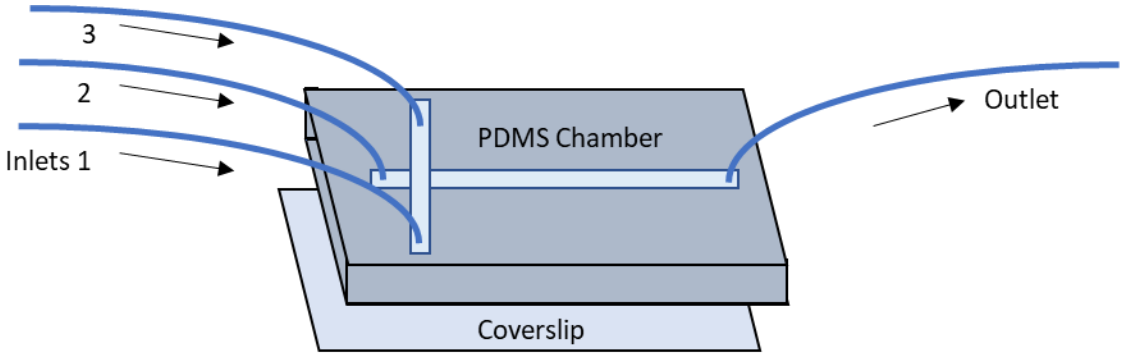

Figure 2: Schematic of PDMS flowcell setup. A PDMS microfluidics chamber and a coverslip are assembled to form a flow cell, with 3 inlets and one outlet.

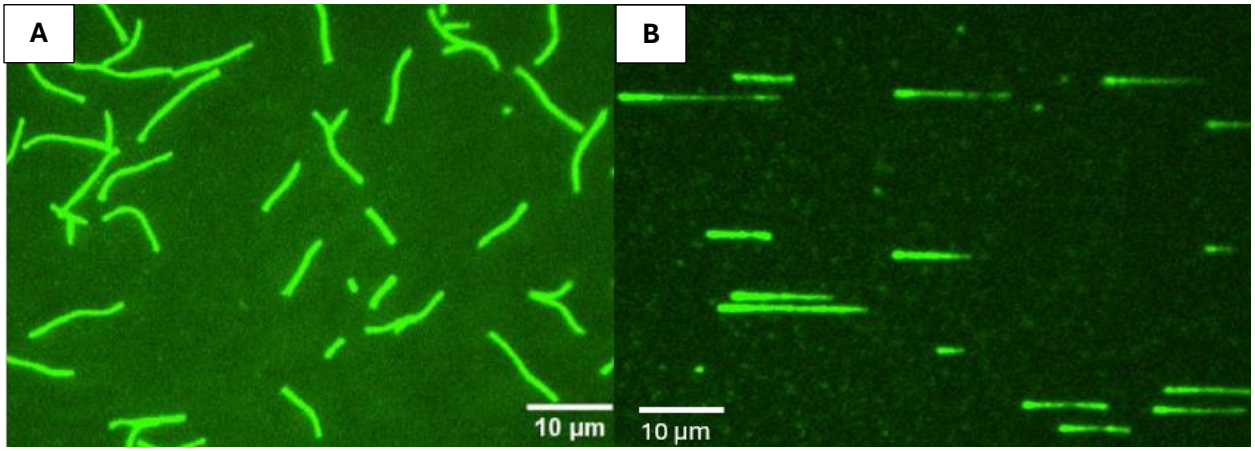

Figure 3: Representative fields of view. A, Conventional TIRF. **B,** mf-TIRF (right)

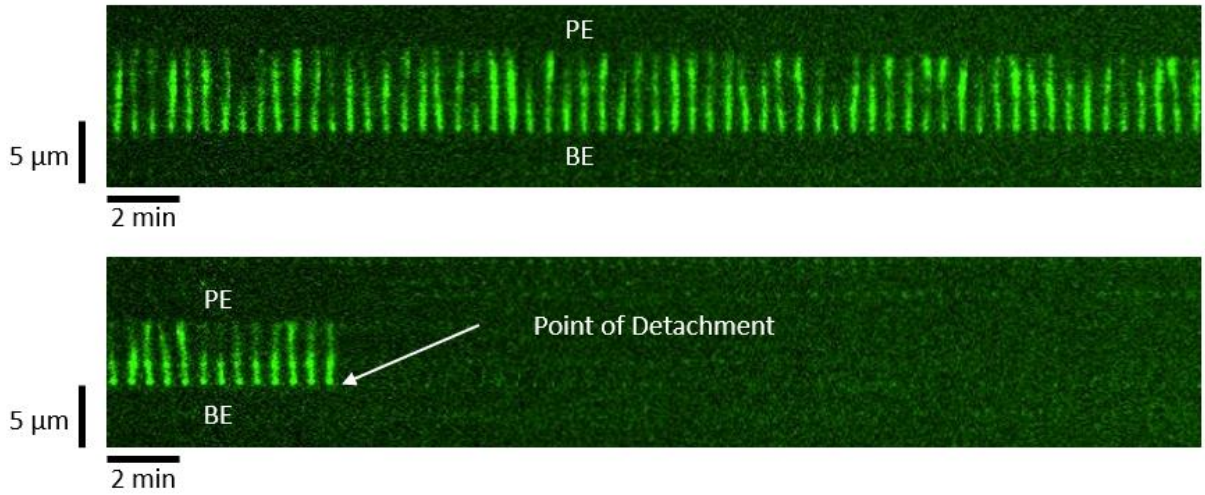

Figure 4: Example kymograph montage. Filaments are anchored by their barbed ends (BE) via capping protein while the pointed ends (PE) are free. Analysis conducted by measuring the time points of detachment of filaments with a minimum length of 5 µm. Filaments that remain until the end of the video are considered to have not been uncapped while those that detach are considered to have uncapped.

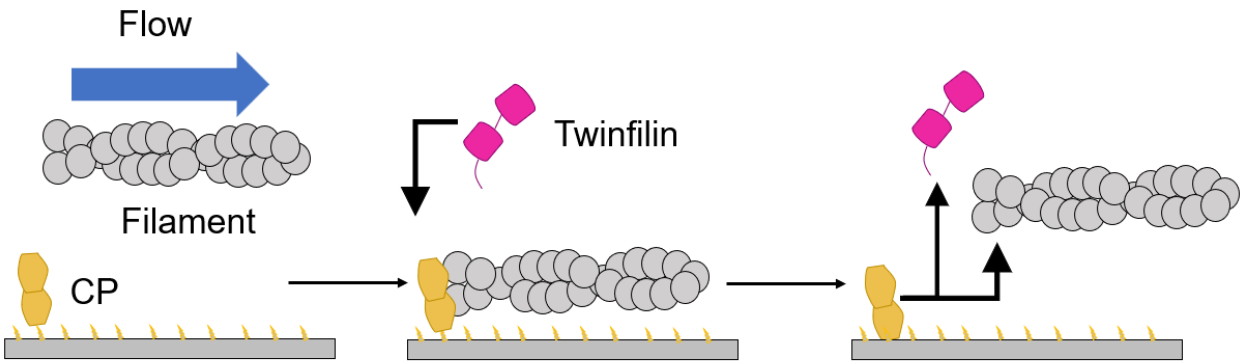

Figure 5: Cartoon of twinfilin uncapping experiment. First, preformed filaments are captured by anchored capping protein. Next, the anchored filaments are exposed to solutions containing various twinfilin concentrations. The uncapping rates are observed as the rate at which filaments disappear from the viewing frame.

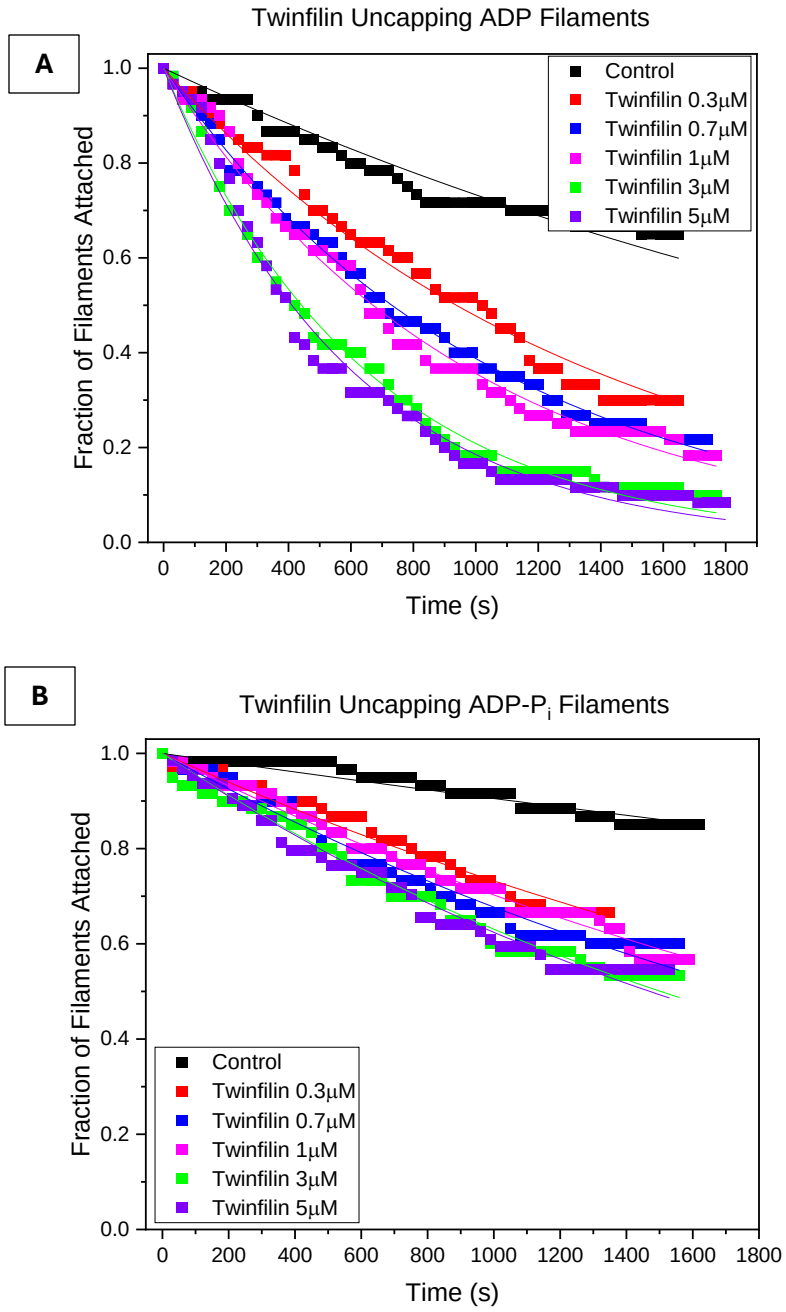

Figure 6: Twinfilin uncapping CDFs. A & B, Fraction of filaments attached to CP as a function of time in presence of varying concentrations of twinfilin. Experimental data (symbols) are fitted to a single-exponential function (lines) to determine CP dissociation rate. N = 60 filaments for all experiments.

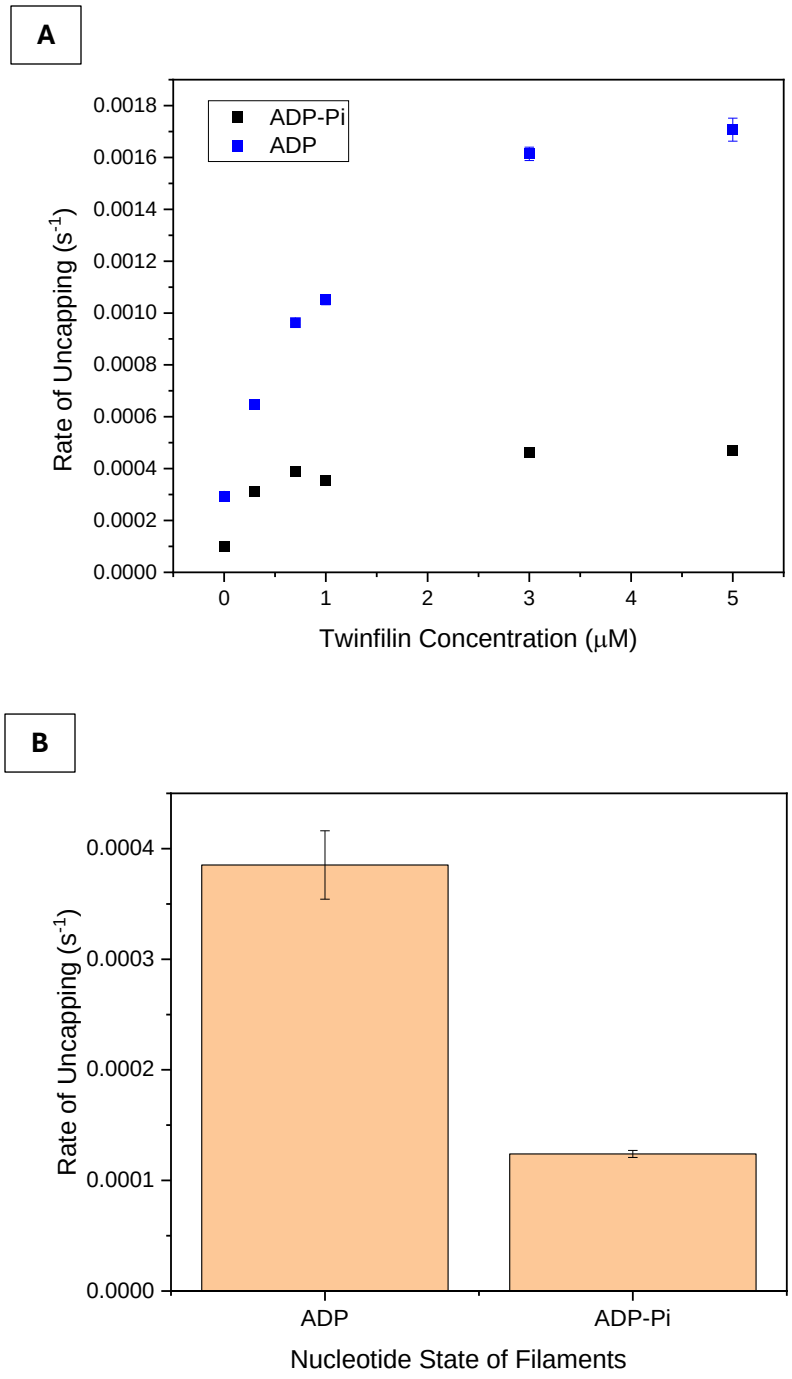

Figure 7: Quantification of twinfilin uncapping rates. Uncapping CDFs were fitted to the function $y = e^{-kx}$ to find k, the rate of uncapping. **A,** Uncapping rates for ADP and ADP-P_i filaments at various twinfilin concentrations is shown. **B,** Comparison of control rates for ADP and ADP-P_i filaments, TIRF 1X buffer and 1X P_i buffer is shown.

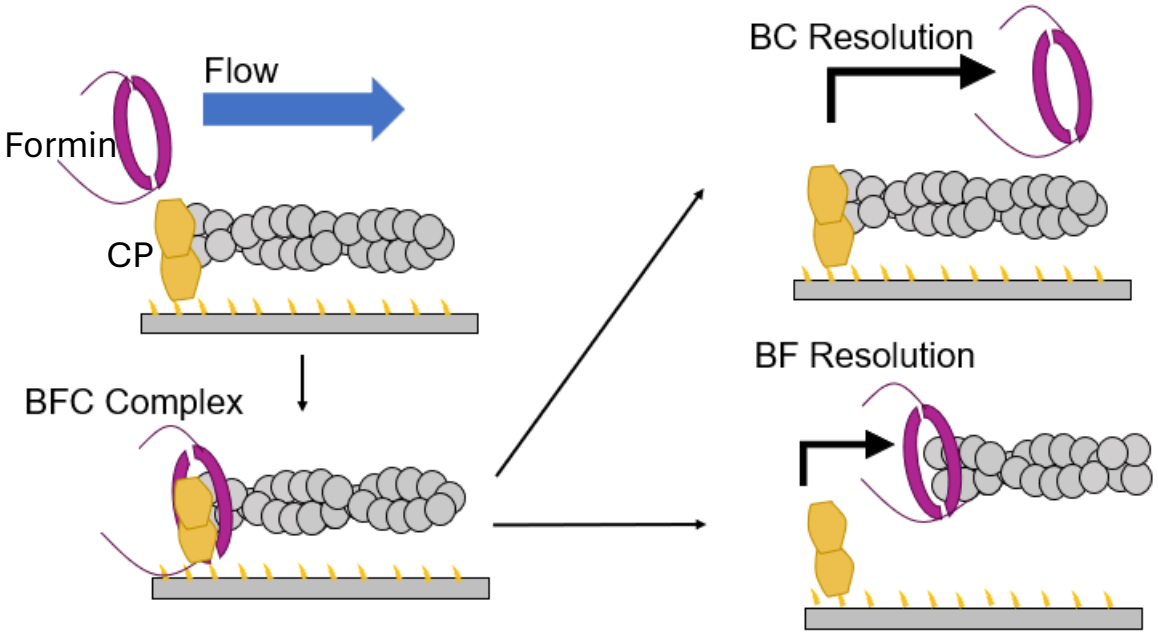

Figure 8: Cartoon of formin uncapping via BFC complex. First, pre-grown filaments anchored by CP are exposed to formin. Formin then creates BFC complexes on these anchored filaments. BFC complex resolves to either a barbed-end-formin (BF) state or barbed-end-capping-protein (BC) state, the former of which is observed as uncapping.

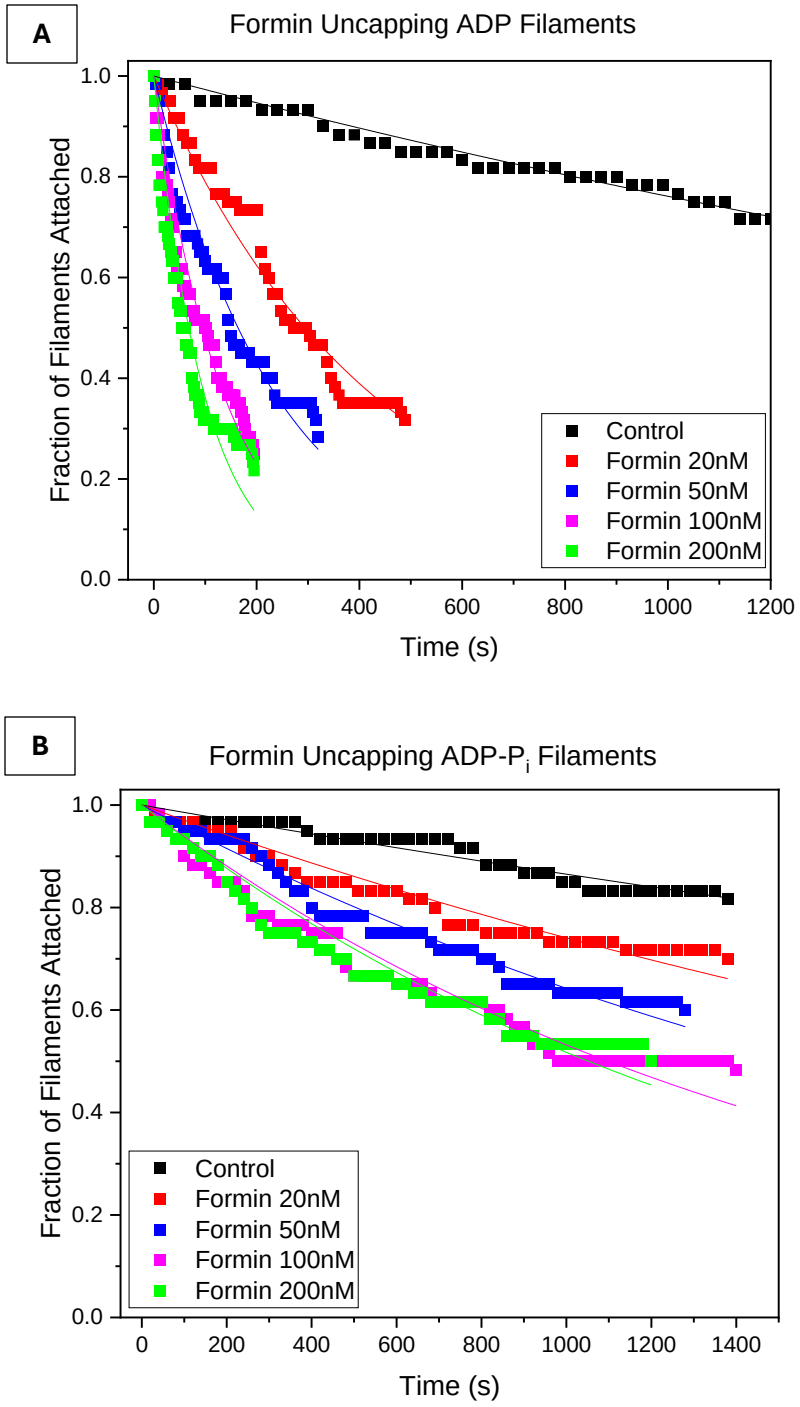

Figure 9: Formin uncapping CDFs. A & B, Fraction of filaments attached to CP as a function of time in presence of varying concentrations of formin. Experimental data (symbols) are fitted to a single-exponential function (lines) to determine CP dissociation rate. N = 60 filaments for all experiments.

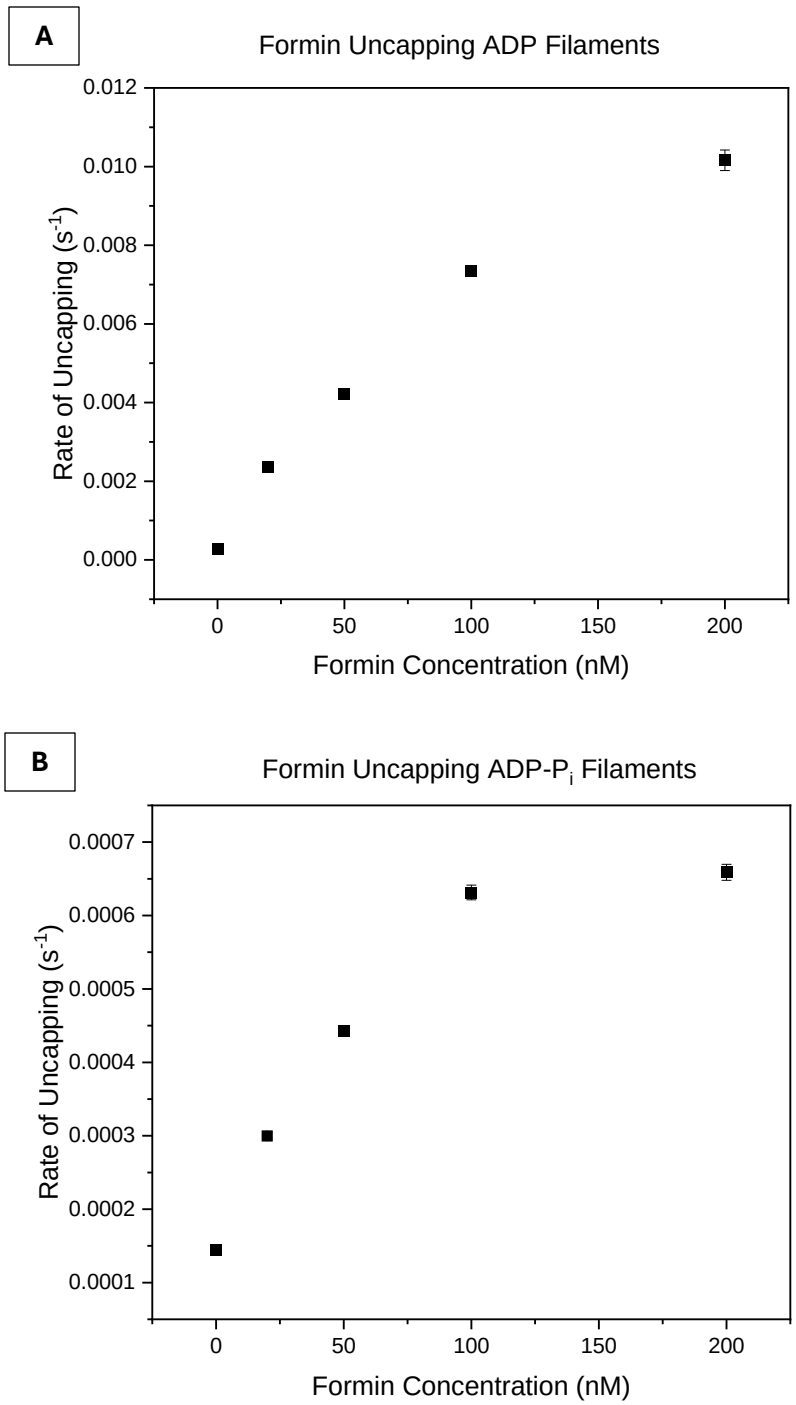

Figure 10: Quantification of formin uncapping rates. Uncapping CDFs were fitted to the function $y = e^{-Ax}$ to find A, the rate of uncapping. **A,** Uncapping rates for ADP filaments at various formin concentrations is shown. **B,** Uncapping rates for ADP-P_i filaments at various formin concentrations is shown.

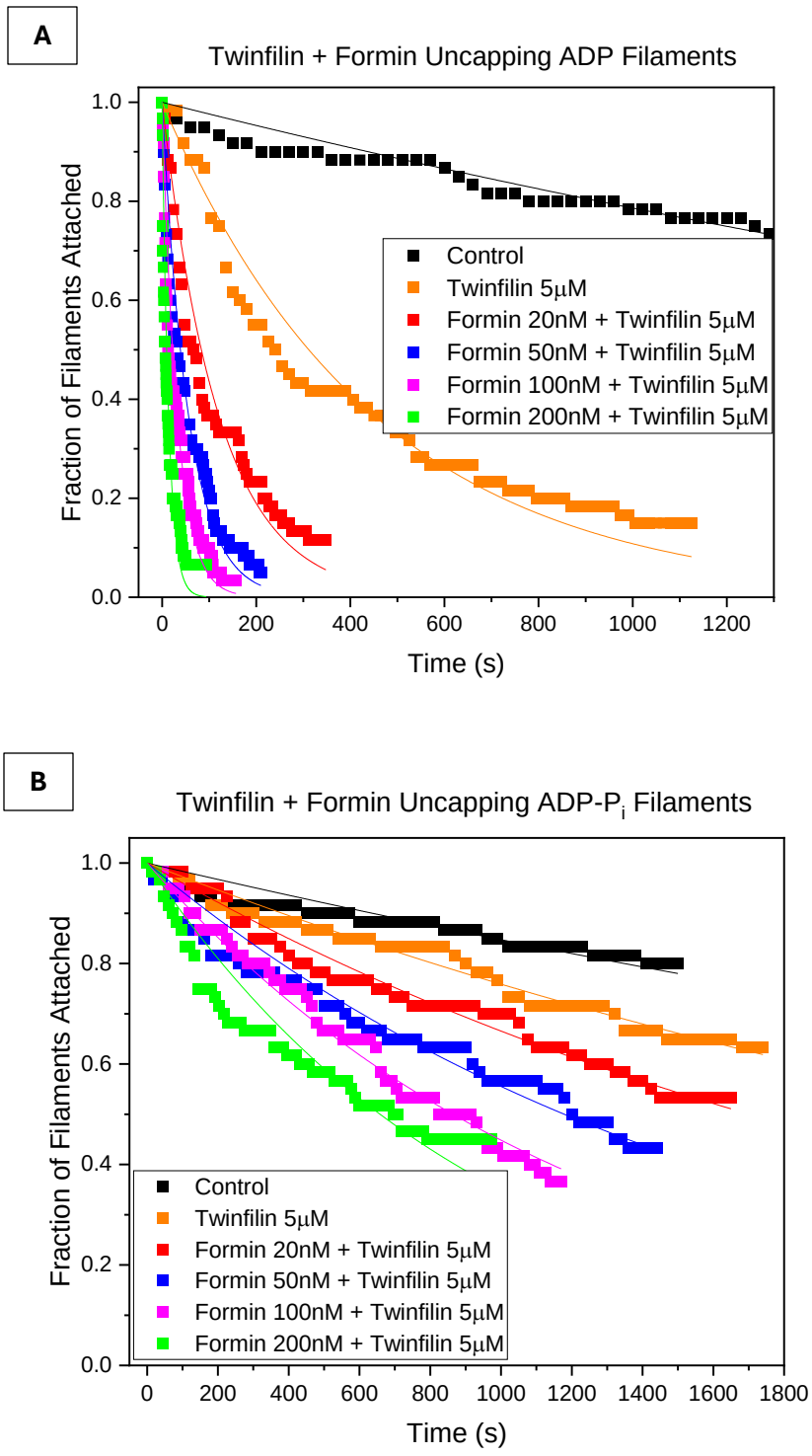

Figure 11: Formin + twinfilin uncapping CDFs. A & B, Fraction of filaments attached to CP as a function of time in presence of varying concentrations of formin. Twinfilin was kept constant at 5 μM. Experimental data (symbols) are fitted to a single-exponential function (lines) to determine CP dissociation rate. N = 60 filaments for all experiments.

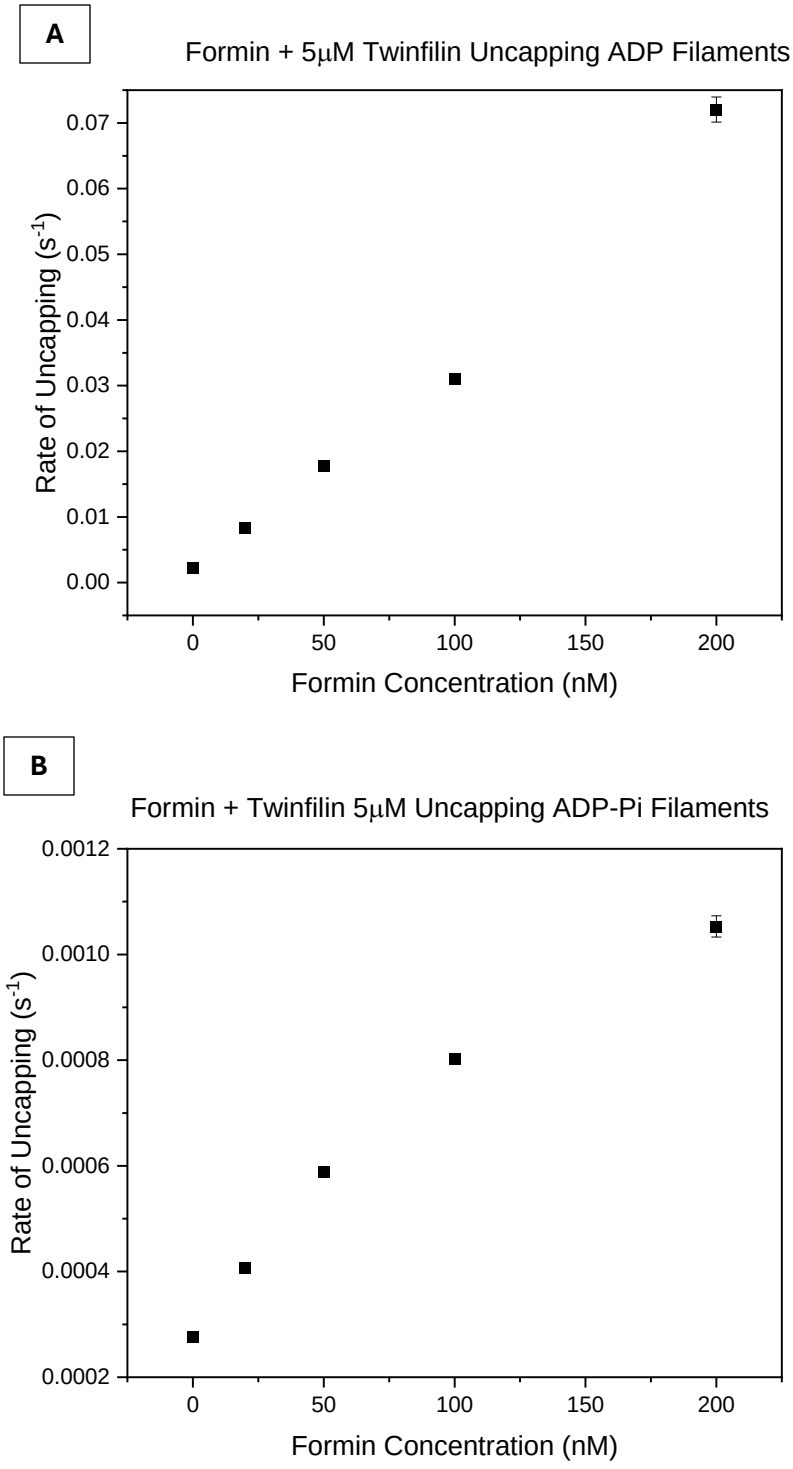

Figure 12: Quantification of formin + twinfilin uncapping rates: Uncapping CDFs were fitted to the function $y = e^{-Ax}$ to find A, the rate of uncapping. **A,** Uncapping rates for ADP filaments at various formin concentrations are shown. **B,** Uncapping rates for ADP-P_i filaments at various formin concentrations are shown.

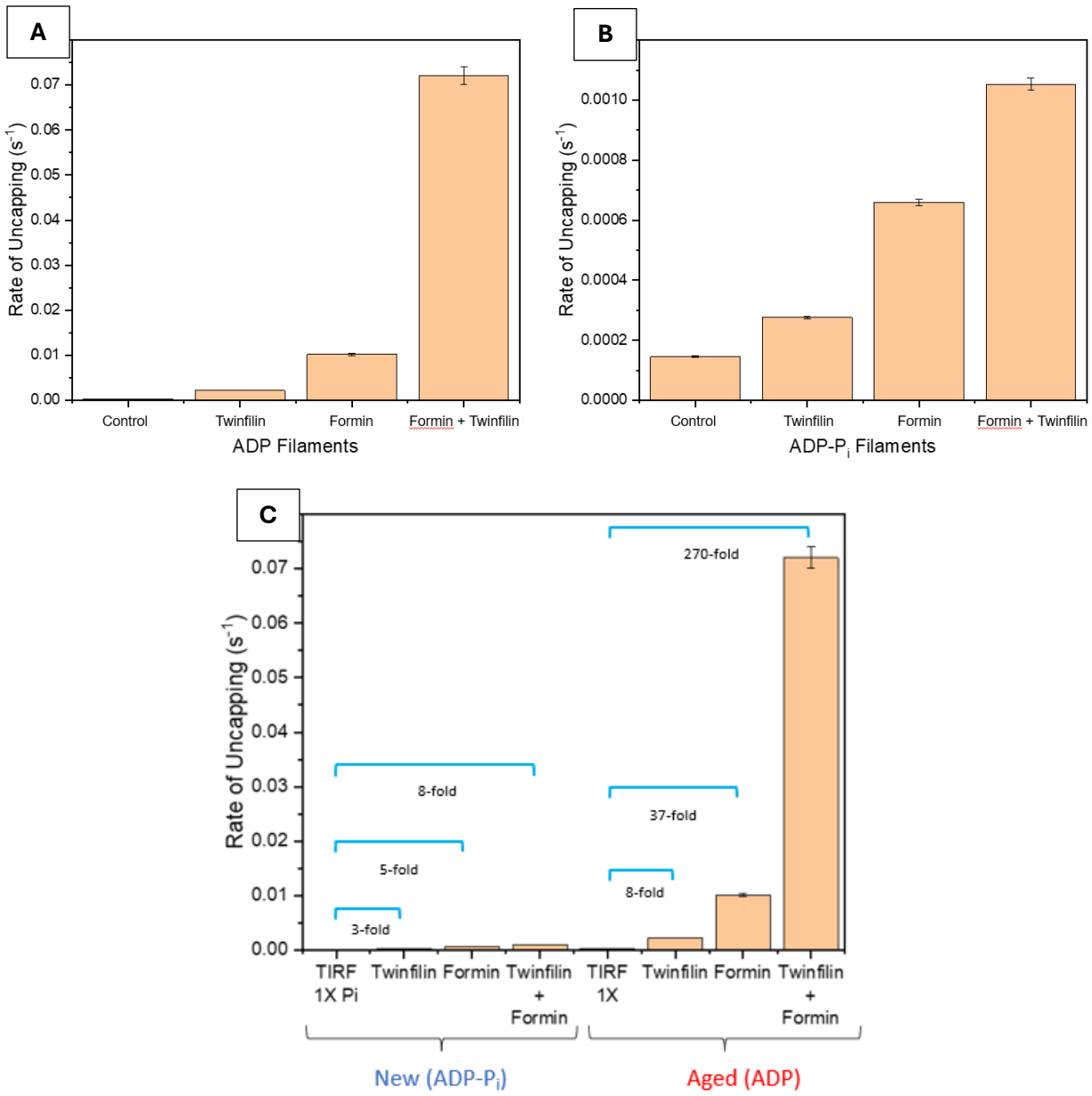

Figure 13: Comparison of uncapping rates: A & B, Side by side comparison of maximum observed uncapping rates for each experimental condition for ADP and ADP-P_i filaments separately. **C,** Side by side comparison of maximum rates for all conditions with fold changes from buffer controls. The maximum increase for twinfilin was at 5 μM twinfilin. The maximum increase for formin was 200 nM formin. The maximum increase for twinfilin + formin was at 5 μM twinfilin **+** 200 nM formin**.**

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