Twinfilin bypasses assembly conditions and actin filament aging to drive barbed end depolymerization

Shashank Shekhar, Greg Hoeprich, Jeff Gelles, and Bruce Goode

Corresponding Author(s): Bruce Goode, Brandeis University

Review Timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission Date</td>
<td>2020-06-03</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>2020-08-07</td>
</tr>
<tr>
<td>Revision Received</td>
<td>2020-10-06</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>2020-10-21</td>
</tr>
<tr>
<td>Revision Received</td>
<td>2020-10-27</td>
</tr>
</tbody>
</table>

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August 7, 2020

Re: JCB manuscript #202006022

Prof. Bruce L Goode
Brandeis University
Biology
Rosenstiel Center
415 South Street
Waltham, MA 02454

Dear Bruce,

Thank you for submitting your manuscript entitled "Twinfilin accelerates or slows depolymerization of actin filament barbed ends depending upon their nucleotide state." Please accept our apologies for the delay in the processing of your manuscript.

The manuscript was assessed by expert reviewers, whose comments are appended to this letter. Overall, the reviewers were enthusiastic about the study and we invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the major concern shared by reviewers is regarding the results presented in Figure 2. Reviewer #1 asks to repeat these experiments using buffer containing BeF3 in order to mimic ADP-Pi. Both reviewers also point out that the measured depolymerization rate at barbed end is much slower than in previous reports and ask for explanation of these results. Additionally, reviewer #1 notes a difference in depolymerization rates between Figure 3E and Figure 1C. We feel these points are important and must be addressed by both experiments and textual revisions.

We agree with reviewer #2 that the data presented in Figure 3 makes an important contribution to the paper, thus, this should be retained.

Please be sure to also include a point-by-point rebuttal for all the items raised by the reviewers.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Reports may have up to 5 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.
IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers’ comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Bill Bement, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, the authors investigate the effect of twinfilin on actin filaments in function of the nucleotide bound to actin subunits at the barbed end (ADP-Pi or ADP). The rational for doing these experiments is based on discrepancy on the role of twinfilin in actin disassembly between two labs (Goode and Romet-Lemonne labs) using the same method. One lab (Goode) proposed that twinfilins accelerate the rate of barbed end depolymerization where the Romet-Lemonne lab proposed that twinfilins do not increase the rate of barbed end depolymerization.
First, I would like to acknowledge the efforts made by these authors to reconcile their observations with the data from the other lab. However, I have some major concerns/comments that the authors should address before publication.

1) It is not clear why the authors were thinking that the discrepancy came from the nucleotide state of the subunits at the barbed ends since the two labs used previously the same experimental system. Therefore, at the beginning of the result section, the authors should better explain why they did investigate the effect of twinfilin in function of the nucleotide bound to the barbed end of the actin filament.

2) The first experiment, Figure 1 is consistent with twinfilin slowing down the rate of actin filament depolymerization at the barbed end from 7.9 to 5.6 subunits/second.

3) The second experiment is done in phosphate buffer (50 mM Pi). A necessary control is that the phosphate buffer is acting only on the actin filament nucleotide and not on the interaction of twinfilin with actin filaments independently of the nucleotide. In general, BeF3 is better suited for such experiments. The authors should add BeF3 in their reaction mixture to generate ADP-BeF3 filament (mimicking ADP-Pi) and test if they confirm the effect of twinfilin made with the Pi buffer.

4) In Figure 2d, in absence of twinfilin, actin filaments seem to depolymerize very slowly (0.3 subunits by second). The reported rate of depolymerization at the barbed end for ATP subunits is 1.4 subunits by second (about 5 time faster, Pollard, 1986). The authors should explain/discuss this difference (this is a major issue here). In presence of twinfilin, the rate of depolymerization increases up to 2.2 subunits by second. This is now close to the reported rate of depolymerization at the barbed end (see above). The authors should also challenge these parameters in a regular buffer supplemented with BeF3.

The conclusion made by the authors from these experiments is: "The in vitro experiments predict that these concentrations of twinfilin would strongly accelerate depolymerization. ». However, in their experiments in presence of twinfilin, actin filaments do not depolymerize faster than previously measured rate of depolymerization at the barbed end. The actin filaments seem to depolymerize faster because in absence of twinfilin for some reason their actin filaments are depolymerizing very slowly. This is a major drawback of this study.

5) The experiments in Figure 3 is very nice but somehow disconnected from the main message of the paper. This experiment is very preliminary and will require a lot of work to be able to conclude on what is going on. Therefore, these experiments should be included in a different manuscript studying the assembly of actin filaments in presence of disassembly factors.

For example, these experiments should be repeated with higher monomer concentration (1.5 μM for example) because 0.5 μM is near the critical concentration and twinfilin may change the Cc for free barbed end, allowing formin filament to grow and free barbed end to disassemble.

The authors should include a movie of control experiments with a flow of twinfilin buffer only after flow of mDia1.

In addition, actin filament growing with formin-attached seems darker? why?

Formin-filaments are growing at 30 subunits by seconds. The association rate constant at the barbed end in presence of mDia1 is around 45 μM-1 s-1 (Kovar et al., Cell 2006). At 0.5 μM actin monomer, the BE polymerization should be around 20 subunits/seconds. Here the actin filaments are growing at 32 subunits by seconds, it seems a little fast.

The subunits, in the actin filament that depolymerizes, have been polymerized for at least 600 s.
Accordingly (based on Figure 1), these subunits should be loaded with ADP so twinfilin should slow down their depolymerization to 3.6 subunits/seconds. In Figure 3e, the authors report a rate of depolymerization at the barbed ends in presence of 5 µM twinfilin of around 7 subunits by second, higher than in Figure 1C.

In Figure 3e, very low concentration of twinfilin (25nM) blocks actin assembly at the barbed end since the rate of BE polymerization is near 0, why?

In the movie, some actin filament with free barbed ends (growing slowly) do not depolymerize in presence of twinfilin? why?

To really understand these experiments a labeled version of twinfilin seems necessary.

6) The cartoon Figure 4 is interesting but the effect of twinfilin on ADP-Pi or ADP actin filament will work only if twinfilin stays bound the depolymerizing barbed end. I do not think the authors have demonstrated this property. In addition the cartoon includes capping proteins, but the experiments in this manuscript do not include capping proteins.

Reviewer #2 (Comments to the Authors (Required)):

This paper by Shekar et al., is short and to the point. Careful measurements including a comparison of conventional and microfluidic assisted TIRF show the effects of Twinfilin on the barbed end of actin filaments. They clarify that differences in observation may have been due to conditions used because the effect of mTwf1 on barbed ends is nucleotide dependent: mTwf1 slows depolymerization of ADP-actin barbed ends and accelerates depolymerization of ADP-Pi actin. They also nicely demonstrate that sequestration of monomers by mTwf1 is negligible at best. Overall, it is a clear demonstration of the only know barbed end depolymerizing factor. The paper is clearly written, though there are a number of editing mistakes (dropped words, for example). My concerns are minor and listed in order of appearance:

In the abstract the statement "presumed to depolymerize only after" should be tempered with primarily instead of only, for example.

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First paragraph of Results and Discussion: A description here of the incubations after polymerization would be very helpful to the reader, instead of making them search through the methods.

Figure 1. I'm curious why 3 um profilin were used for conventional TIRF experiments an 5 um for the mfTIRF, as stated in the figure legend. It's also not clear if this is correct because no value is given for conventional in the methods and 4uM is given for mfTIRF.

The depolymerization rates in Figure 2 are painfully slow compared to ATP actin. 1 - is this similar to rates reported for depoly in the presence of Pi? Please address this. 2 - the difference between figure 1 and 2 rates and the slowness in figure 2 is easy to miss because the scales are different in the 1ef and 2bc. This feels misleading.

The dose dependence experiment in Figure 2 is very nice. It is well used to suggest mechanism and consider cellular conditions. It could also be used to explain the choice of 5 uM in several of the
Measuring filament growth/shrinkage in the presence of mDia1 and mTwf1 demonstrates nicely that the mTwf1 does not appreciably sequester monomers. It would help the reader to state clearly that the rate of mDia mediated growth is the same before and after addition of mTwf1. Also a clear statement that the effect is not sensitive to the concentration of mTwf1 would strengthen/clarify the result. The data are convincing and important and the movie is beautiful.

Figure legend 3 refers to a thick line as a fit. This should be in 3e not 3d.

The scale of change is discussed/presented two ways and should be made uniform. In figure 2e it is plotted as fold change. In figure 3f it is plotted as percent change. Either is fine

It’s not clear if the sentence "The unique ability of twinfilin to drive barbed end depolymerization under assembly-promoting conditions may arise from an ability to suppress ATP-actin subunit exchange at barbed ends while permitting ADP-Pi and ADP actin subunit exchange." Is speculation or based on published data. Please either add references or clarify that it is not known.

The model figure should be modified to highlight the contributions from this paper, not the capping protein portions. They could be left but dimmed out, perhaps?

Information about the data acquisition rate(s) is needed.
Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, the authors investigate the effect of twinfilin on actin filaments in function of the nucleotide bound to actin subunits at the barbed end (ADP-Pi or ADP). The rationale for doing these experiments is based on discrepancy on the role of twinfilin in actin disassembly between two labs (Goode and Romet-Lemonne labs) using the same method. One lab (Goode) proposed that twinfilins accelerate the rate of barbed end depolymerization where the Romet-Lemonne lab proposed that twinfilins do not increase the rate of barbed end depolymerization.

First, I would like to acknowledge the efforts made by these authors to reconcile their observations with the data from the other lab. However, I have some major concerns/comments that the authors should address before publication.

We thank the reviewer for their positive and kind words about our study. The reviewer states the two labs in the earlier studies used the same method (Hakala et al, 2019; Johnston et al, 2015). We would like to clarify that the two groups had used different types of TIRF microscopy in their studies. The Romet-Lemonne lab had used microfluidics-assisted TIRF where only one end of the actin filament is anchored, while the Goode lab had used conventional (open flow) TIRF methodology, in which filaments are attached to the glass coverslip all along their lengths by incorporation of biotin-actin. In the recent preprint study from the Romet-Lemonne and Lappalainen labs (Hakala et al, 2019), it was suggested that the manner of filament attachment could have led to the discrepancy, i.e., that this difference in techniques might account for the difference in results. This prompted us to directly compare Twinfilin effects at the barbed ends of filaments by the two methods, side-by-side.

1) It is not clear why the authors were thinking that the discrepancy came from the nucleotide state of the subunits at the barbed ends since the two labs used previously the same experimental system. Therefore, at the beginning of the result section, the authors should better explain why they did investigate the effect of twinfilin in function of the nucleotide bound to the barbed end of the actin filament.

We have added a brief explanation of this rationale to the beginning of the Results. As mentioned above, we initially questioned whether the discrepancy might be due to the different TIRF approach used (conventional vs. microfluidics-assisted TIRF). However, we also carefully reviewed our previous publications on Twinfilin, and noticed that we did not age the actin filaments as long as the Hakala study, leading us to consider whether we had fully converted the actin subunits to ADP-actin. This prompted us to directly compare Twinfilin effects on filaments in the two different nucleotide states (ADP and ADP+Pi).

2) The first experiment, Figure 1 is consistent with twinfilin slowing down the rate of actin filament depolymerization at the barbed end from 7.9 to 5.6 subunits/second.

Correct.

3) The second experiment is done in phosphate buffer (50 mM Pi). A necessary control is that the phosphate buffer is acting only on the actin filament nucleotide and not on the interaction of twinfilin with actin filaments independently of the nucleotide. In general, BeF3 is better suited for such experiments. The authors should add BeF3 in their reaction mixture to generate ADP-BeF3 filament (mimicking ADP-Pi) and test if they confirm the effect of twinfilin made with the Pi buffer.

We thank the reviewer for suggesting this experiment. In the revised manuscript, we show that Twinfilin accelerates barbed end depolymerization in the presence of BeF3, similar to its effect in the presence of 50 mM Pi (Figure 2e).

4) In Figure 2d, in absence of twinfilin, actin filaments seem to depolymerize very slowly (0.3 subunits by second). The reported rate of depolymerization at the barbed end for ATP subunits is 1.4 subunits by second (about 5 time faster, Pollard, 1986). The authors should explain/discuss this difference (this is a major issue here). In presence of twinfilin, the rate of depolymerization increases up to 2.2 subunits by second. This is now close to the reported
rate of depolymerization at the barbed end (see above). The authors should also challenge these parameters in a regular buffer supplemented with BeF3.

As suggested, we now measure the depolymerization rate at the barbed end in the presence of BeF3, and observe a similar rate of depolymerization (0.30 ± 0.13 subunits s⁻¹). Pollard's original estimates of barbed end depolymerization rate (1986) were made by other, more indirect methods. Later, the Pollard group itself employed TIRF microscopy to directly observe filaments shortening over time, and reported a much slower barbed end depolymerization rate, 0.89 subunits s⁻¹ (Kuhn and Pollard, 2005). More recently, Jegou et al., (2011) repeated these experiments using mF-TIRF, which improves the accuracy of measuring filament length changes over time, and observed an even slower rate, 0.16 s⁻¹. Some of the differences in these reported rates can be due to the method of attachment of filaments; in mF-TIRF the filaments are only attached at their pointed ends, whereas in conventional TIRF they are attached all along their lengths. Additional variables that can affect the rate include: (i) the percentage of labeled (vs. unlabeled) actin used, (ii) precisely how the actin is labeled (which dye, and whether the dye is coupled to a single Cys residue, or random lysines), (iii) temperature, and (iv) photodamage during TIRF, which can cause a subset of actin subunits to undergo photo-dimerization (Niedermayer et al, 2012). For these reasons, there is often some variability observed in the rates of polymerization and depolymerization between different studies and different groups. Importantly, our experiments are internally controlled, that is, we are comparing the rates of depolymerization where the only variable is the presence or absence of Twinfilin. Further, we observed similar rates of depolymerization by Twinfilin using conventional (open flow) TIRF and in mF-TIRF.

The conclusion made by the authors from these experiments is: "The in vitro experiments predict that these concentrations of twinfilin would strongly accelerate depolymerization. ». However, in their experiments in presence of twinfilin, actin filaments do not depolymerize faster than previously measured rate of depolymerization at the barbed end. The actin filaments seem to depolymerize faster because in absence of twinfilin for some reason their actin filaments are depolymerizing very slowly. This is a major drawback of this study.

Please see above.

5) The experiments in Figure 3 is very nice but somehow disconnected from the main message of the paper. This experiment is very preliminary and will require a lot of work to be able to conclude on what is going on. Therefore, these experiments should be included in a different manuscript studying the assembly of actin filaments in presence of disassembly factors.

We retained Figure 3 in the manuscript, as instructed by the Editor and encouraged by reviewer #2. These results provide a clear demonstration that Twinfilin induces depolymerization at free barbed ends under assembly-promoting conditions, while formin-capped filaments in the same reactions continue to grow. Further, they end speculation that monomer sequestration by Twinfilin underlies its depolymerization effects.

For example, these experiments should be repeated with higher monomer concentration (1.5 µM for example) because 0.5 µM is near the critical concentration and twinfilin may change the Cc for free barbed end, allowing formin filament to grow and free barbed end to disassemble.

As suggested, we have now performed the experiments at two higher G-actin concentrations (3 and 10 µM). At 3 µM G-actin, we show that mTwf1 continues to drive barbed end depolymerization, albeit at a slower rate compared to at 0.5 µM G-actin. In the presence of very high (10 µM) G-actin, mTwf1 neutralizes growth at barbed ends, and filaments undergo only very slow net depolymerization. These effects support our proposed working model for twinfilin's effects at barbed ends, which involves twinfilin molecules binding to the filament end and inducing short, processive depolymerization runs, then dissociating.
The authors should include a movie of control experiments with a flow of twinfilin buffer only after flow of mDia1. The data appear in Figure 2e (0 µM Twinfilin, i.e., buffer control). As requested by the reviewer, we have now added Supplement video S2.

In addition, actin filament growing with formin-attached seems darker? why?

This is correct. Filaments elongated by formins are dimmer (i.e., darker), because profilin binds unlabeled actin monomers with a higher affinity than labelled actin monomers (Kovar et al., 2006). This leads to fewer labeled actin subunits being incorporated into the filament when a formin is bound to the barbed end, making them dimmer.

Formin-filaments are growing at 30 subunits by seconds. The association rate constant at the barbed end in presence of mDia1 is around 45 µM-1 s-1 (Kovar et al., Cell 2006). At 0.5 µM actin monomer, the BE polymerization should be around 20 subunits/seconds. Here the actin filaments are growing at 32 subunits by seconds, it seems a little fast.

Our rate was 31.7 subunits s⁻¹ (for 0.5 µM G-actin), i.e., 63.4 subunits s⁻¹ µM⁻¹. This is a bit faster than the 46.9 subunits s⁻¹ µM⁻¹ rate reported in Kovar et al., 2006. However, it is fairly close to rates we have observed and reported for mDia1, typically ~55 subunits s⁻¹ µM⁻¹ (Breitsprecher et al., 2012). As mentioned above, there are many contributing factors to the variation observed in the absolute polymerization (and depolymerization) rates measured in TIRF between different labs. There are some differences between our assays and the Kovar study, which may account for the small discrepancy in rates for mDia1. These include: (1) their mDia1 construct was GST tagged, whereas ours was 6His tagged; and (2) we used 2 µM profilin with 0.5 µM G-actin, whereas they used 3 µM profilin with 1 µM G-actin (a different ratio of profilin to actin, possibly leading to different concentrations of profilin-actin, actin, and free profilin in the reactions). In addition, variations in temperature can have a significant effect on polymerization rate.

The subunits, in the actin filament that depolymerizes, have been polymerized for at least 600 s. Accordingly (based on Figure 1), these subunits should be loaded with ADP so twinfilin should slow down their depolymerization to 3.6 subunits/seconds. In Figure 3e, the authors report a rate of depolymerization at the barbed ends in presence of 5 µM twinfilin of around 7 subunits by second, higher than in Figure 1C.

As mentioned above, the higher rates we observe in these experiments are likely due to the presence of 2 µM profilin. Free profilin increases the rate of depolymerization at barbed ends, both in absence and presence of G-actin (Figure 4b in Pernier et al, 2016). Importantly, in presence of 2 µM profilin, mTwf1 strongly increases the rate of depolymerization, in a concentration-dependent manner, so its contribution to depolymerization is not in question. However, profilin may indeed be making a contribution, which we now openly discuss in the manuscript.

In Figure 3e, very low concentration of twinfilin (25nM) blocks actin assembly at the barbed end since the rate of BE polymerization is near 0, why?

Again, profilin and mTwf1 may work together to promote barbed end depolymerization, which is likely why a low concentration of mTwf1 (25 nM) has a strong effect in this assay, i.e., when profilin is present. Importantly, because formin protects barbed ends from mTwf1, the elongation rates of formin-bound actin filaments does not show any noticeable effect from mTwf1 (across a range of concentrations, 25 nM to 5 µM).

In the movie, some actin filament with free barbed ends (growing slowly) do not depolymerize in presence of twinfilin? why?

In addition, variation
This has been reported in Niedermayer et al., 2012, and may be due to occasional photo-dimerization of actin subunits in the filament.

To really understand these experiments a labeled version of twinfilin seems necessary.

Labeled mammalian Twinfilin (mTwf1) experiments would admittedly improve our understanding of the mechanism. For this reason, we SNAP-tagged and dye labeled mTwf1. Unfortunately, the fusion protein was extremely prone to sticking to coverslip surface, which precluded experiments in which we monitored its localization during barbed end depolymerization events.

6) The cartoon Figure 4 is interesting but the effect of twinfilin on ADP-Pi or ADP actin filament will work only if twinfilin stays bound the depolymerizing barbed end. I do not think the authors have demonstrated this property. In addition the cartoon includes capping proteins, but the experiments in this manuscript do not include capping proteins.

We agree. Our model is that Twinfilin stays processively associated with the depolymerizing barbed end, and (as the reviewer points out) this is likely the only way to explain the activities of Twf1 we observe. This property was demonstrated for yeast Twf1 in Johnston et al., 2015. Although we have not yet demonstrated this for mouse Twf1, because of technical obstacles encountered with a SNAP-mTwf1 construct sticking (mentioned above), we feel that it is reasonable in putting a model together to lean on the observations made with yeast Twf1. We have clarified this assumption in the figure legend.

In this concluding model, we felt it was important to frame our results into the larger picture on twinfilin function at barbed ends, and present readers with a more integrated view. Otherwise, readers may be confused about how the twinfilin’s nucleotide-sensitive depolymerization effects at barbed ends are to be considered in the context of its previously reported regulatory effects on capping protein. To clarify which parts of the model are new versus based on previous work, we have taken the suggestion of reviewer #2 and ‘dimmed’ the capping protein (CP) steps in the model. We have also added more references to the previous work in the caption.

Reviewer #2 (Comments to the Authors (Required)):

This paper by Shekar et al., is short and to the point. Careful measurements including a comparison of conventional and microfluidic assisted TIRF show the effects of Twinfilin on the barbed end of actin filaments. They clarify that differences in observation may have been due to conditions used because the effect of mTwf1 on barbed ends is nucleotide dependent: mTwf1slows depolymerization of ADP-actin barbed ends and accelerates depolymerization of ADP-Pi actin. They also nicely demonstrate that sequestration of monomers by mTwf1 is negligible at best. Overall, it is a clear demonstration of the only know barbed end depolymerizing factor. The paper is clearly written, though there are a number of editing mistakes (dropped words, for example). My concerns are minor and listed in order of appearance:

In the abstract the statement "presumed to depolymerize only after" should be tempered with primarily instead of only, for example.

Thank you. We have tempered the statement by changing “only” to “primarily” in the abstract.

First paragraph of the introduction: The sentence "ADP-Pi and ADP F-actin have distinct conformations, dynamics, and binding partners." Should be referenced. Additionally, more current references would be appropriate for the sentence immediately following this one.
Thank you. We have now added more current references.

First paragraph of Results and Discussion: A description here of the incubations after polymerization would be very helpful to the reader, instead of making them search through the methods.

Thank you. We have edited this text to clarify the incubation conditions.

Figure 1. I’m curious why 3 um profilin were used for conventional TIRF experiments and 5 um for the mfTIRF, as stated in the figure legend. It’s also not clear if this is correct because no value is given for conventional in the methods and 4uM is given for mfTIRF.

The concentration of profilin we used in mf-TIRF experiments when preassembling the actin filaments was 5 µM; we have corrected the typographical error of 4 µM. In mf-TIRF experiments, it takes an extra 4-5 minutes for the solution (actin and profilin in TIRF buffer) to pass through the tubing and reach the flow cell. To prevent polymerization of G-actin during this dead time, we add a slightly higher concentration of profilin to more strongly suppress spontaneous actin nucleation. However, just to clarify, in the experiments in Figures 1 and 2, profilin is only present during the initial preassembly of the actin filaments, and is not present during the actual experiment, where we are monitoring the filament depolymerization rates in the presence and absence of mTwf1. We have clarified this point in the Methods.

The depolymerization rates in Figure 2 are painfully slow compared to ATP actin. 1 - is this similar to rates reported for depol in the presence of Pi? Please address this. 2 - the difference between figure 1 and 2 rates and the slowness in figure 2 is easy to miss because the scales are different in the 1ef and 2bc. This feels misleading.

For point “1”, please see our response to comment 4 from Reviewer 1. For point “2”, the scales are different because the data are acquired less frequently in Figure 2, where the depolymerization rates are slower; this was necessary to allow for a longer window of observation without photobleaching filaments. We have added a note in the caption to Figure 2 pointing out that a different scale was used compared to in Figure 1.

The dose dependence experiment in Figure 2 is very nice. It is well used to suggest mechanism and consider cellular conditions. It could also be used to explain the choice of 5 uM in several of the experiments.

Thank you.

Measuring filament growth/shrinkage in the presence of mDia1 and mTwf1 demonstrates nicely that the mTwf1 does not appreciably sequester monomers. It would help the reader to state clearly that the rate of mDia mediated growth is the same before and after addition of mTwf1. Also a clear statement that the effect is not sensitive to the concentration of mTwf1 would strengthen/clarify the result. The data are convincing and important and the movie is beautiful.

Thank you, we have added a statement in the text clarifying that the rate of formin-mediated growth is the same before and after adding different concentrations of mTwf1.

Figure legend 3 refers to a thick line as a fit. This should be in 3e not 3d.

Thank you. This has been fixed.

The scale of change is discussed/presented two ways and should be made uniform. In figure 2e it is plotted as fold change. In figure 3f it is plotted as percent change. Either is fine

Thank you. This has been fixed.
It's not clear if the sentence "The unique ability of twinfilin to drive barbed end depolymerization under assembly-promoting conditions may arise from an ability to suppress ATP-actin subunit exchange at barbed ends while permitting ADP-Pi and ADP actin subunit exchange." is speculation or based on published data. Please either add references or clarify that it is not known.

In this sentence, we have clarified that this was speculation about the molecular mechanism underlying the observed effects.

The model figure should be modified to highlight the contributions from this paper, not the capping protein portions. They could be left but dimmed out, perhaps?

Fixed. Thank you for this suggestion of dimming out the steps with capping proteins.

Information about the data acquisition rate(s) is needed.

Fixed. This information has been added to the Methods.
October 21, 2020

RE: JCB Manuscript #202006022R

Prof. Bruce L Goode
Brandeis University
Biology
Rosenstiel Center
415 South Street
Waltham, MA 02454

Dear Bruce,

Thank you for submitting your revised manuscript entitled "Twinfilin accelerates or slows depolymerization of actin filament barbed ends depending upon their nucleotide state". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Reports is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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5) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
   a. Make and model of microscope
   b. Type, magnification, and numerical aperture of the objective lenses
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