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Peer Review File

Leiomodin 2 is a processive pointed-end elongator of actin filaments

Corresponding Author: Professor Shashank Shekhar

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

Parts of this Peer Review File have been redacted as indicated to maintain confidentiality.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfactorily addressed my concerns.

Reviewer #3

(Remarks to the Author)
Biswas, Nature Comm: Leiomodin2 is a processive pointed-end elongator of actin filaments
Signed: Tom Pollard

This revised manuscript presents an interesting and important discovery about the regulation of actin polymerization, namely that the protein Lmod2 regulates the elongation of the slow growing, pointed end of actin filaments. The TIRF movies are convincing, and I agree with the authors' interpretation of the movies. Contrary to the concerns of another reviewer, this process has not been reported previously and helps explain the formation of muscle sarcomeres. The responses to review strengthen the paper.

I am enthusiastic about publication of the work but remain disappointed about the authors' lack of understanding of their data, which results in inaccurate, speculative interpretations that will confuse readers. I hope my suggestions in this review will help the authors resolve the issues in a second revision. I also note missed opportunities and suggested how to improve the presentation.

Data presentation and interpretation

Fig 1. As noted in the first review, the measurements by fluorescence microscopy are elegant and convincing. As I suggested, moving parts of former Fig 3A here as panel F is an improvement. The new data on elongation of free pointed ends by ATP-actin monomers is an important addition. The linear dependence on actin monomer concentration allows measurement of the two rate constants k+ and k-, which are similar to earlier work. This validates the accuracy of the authors' methods. However, the authors do not consider k-.

The parallel experiment with pointed ends associated with Lmod is the heart of the paper. As I explained in the first review, this curved plot reveals that elongation involves two linked reactions. The authors fit the data with an incomplete model (see below Lines 659-668). For readers without kinetics expertise, I suggest a more concise but more complete presentation of the data:

"Filaments anchored at their barbed ends by CP elongated their pointed ends at rates proportional to the actin monomer concentration. The slope of the plot gives the association rate constant of 1.8 ± 0.2 μM-1s-1 and the Y-intercept gives the dissociation rate constant of about 0.7/s, both similar to previous measurements (ref)."

"In contrast, with Lmod bound to the pointed end, the elongation rate depends on the actin monomer concentration only at

low actin monomer concentrations and plateaus at ~2/s at high actin concentrations. This is typical of a reversible second order reaction linked to a reversible first order reaction that is rate limiting at high actin concentrations. Note that the Y-intercept is zero, which shows that Lmod bound to the pointed end inhibits subunit dissociation, k1-." (This is not explained in the text.)

At this point the text should explain separately the following three points:

(1) Elongation mechanism at low actin: this is the simple bimolecular binding of actin monomers with pointed ends associated with Lmod with approximately the same k+ as bare pointed ends. This is not a trivial result; what is the mechanistic interpretation? Lines following 159 speculate that "Lmod may overcome this elongation barrier by either destabilizing the P-1's D-loop binding to P subunit or reorienting subdomain 2 of the terminal subunit." This speculation is incorrect. The rate constant is the same as a free pointed end. Similarly, "enhanced elongation at low G-actin concentrations (<2 μM) through structural remodeling of the pointed end" is incorrect. The higher rate is due to inhibition of subunit dissociation. On the other hand, material in the sentence "Lmod may overcome this elongation barrier by either destabilizing the P-1's D-loop binding to P subunit or reorienting subdomain 2 of the terminal subunit" would be helpful in the section on the rate limiting first order reaction

(2) Why is k- zero? This important observation is ignored. It likely means that Lmod inhibits subunit dissociation, a feature that may help explain other aspects of its mechanism in the next paragraph. This explains the higher rates of elongation than bare pointed ends: "Lmod-bound pointed ends elongated faster than free pointed ends," because subunits dissociated slower than from free pointed ends.

(3) What is the rate limiting first order reaction? The authors offer a possible mechanism, Lmod stepping along the filament. However, to help readers understand the data, the authors must think about and explain what the data tells us and does not reveal about events at the pointed end on the plateau. The mechanism is not known at this point, but the data has some clues about why this first order reaction (actually sum of two first order reactions) limits elongation.

Here are some processes for the authors to consider: The first order reaction is likely to be reversible with the sum of k2+ and k2- equal to 2/s, as explained during the first round of review. This reaction does not slow the second order bimolecular association reaction at actin monomer concentrations <1 μM when new subunits add at <1/s, but it limits elongation to ~2/s at higher monomer concentrations. Lmod on the pointed end is unlikely to affect actin monomers, so the mysterious reaction must affect the other partner in the bimolecular reaction, the pointed end. If the reaction blocks the pointed end site for monomer addition after a new subunit occupies the pointed end, it must be reversible and transient, because monomers continue to add at up to 2/s. This reaction is likely to be complicated, as noted above: "Lmod might limit the elongation rate by stabilizing the interaction of the D-loop of subunit P-1 with subunit P." You might speculate briefly how "stepping" might limit the rate of subunit addition at high actin monomer concentrations. Is it the same or different from previous work such as Vavylonis et al. Mol Cell 2006?

Modeling the data in Fig 1F. From pages 659-668. The model is incomplete.

"We therefore developed a two-step kinetic model for Lmod2-mediated elongation." (Actually, the first round of review explained how to analyze this data. This kinetic model is not newly developed, since it has been used for decades to analyze two step enzyme reactions.)

"The first step involves rapid, diffusion-limited association of G-actin to the Lmod2-bound pointed end, with a concentration-dependent rate $k_1 = k_1' [C]$." (Do not use the rate constant k_1 for a rate. The statement is incorrect, Drenckhahn, JBC 1986 showed that elongation at the pointed end is not diffusion limited.)

"We speculate that this is followed by a second, slower step (rate k_2) that is independent of G-actin concentration." (This formulation is wrong; it does not include the reverse rate constants for either reaction. The following approximation leaving out these reactions gives a good fit to the data simply because k1- is close to zero, but the formulation is incomplete.)

"We propose this second step may involve Lmod2 stepping to track the elongating filament end, similar to mechanisms described for formin-mediated barbed end elongation (Ref?). Based on this model, the average time it takes one elongation cycle (What is the evidence that the two reactions are coupled to form a cycle rather than being independent?) to be completed is given by (1/k1 + 1/k2) and the average elongation rate is given by r = (k1*k2)/(k1+k2). By fitting this equation to the experimental data (Fig. 1F), we show that a two-step model suffices in explaining our experimental observations. Moreover, it allows us to extract the two rates (these are rate constants) k1', k2 as 1.8 ± 0.2 μM-1s-1, 2.0 ± 0.1 s-1 respectively." (Fitting a model with all four rate constants would be more appropriate and would give the same result.)

Fig 2 on the effect of tropomyosin is mostly ok, except that the text does not attempt to explain the plateau at high tropomyosin concentration. What might be going on? Fig 2 does not fit into a logical sequence of data presentation. Fig 3 on profilin should follow Fig. 1 followed by Fig 6 A-F as new Fig 3. Thereafter would be a good place for Fig. 2 as Fig 4.

Fig. 3 covers the second important mechanistic finding. The multiple effects of profilin on elongation at the pointed end are surprising in some ways. Panel B has more complete data than old Fig 3A for a range of concentrations of 1:1 mixtures of actin and profilin. Without Lmod pointed ends do not elongate or even shorten as expected from prior work. With Lmod, the behavior is much like Fig 1F but with a slightly lower plateau of about 1.5/s. Again k1- must be zero; k1+ seems to be less than for actin alone. The text should have a better description of the data for actin-profilin: "elongation is proportional to [AP]

at low actin concentrations with k1+ = * and k1- = 0. The elongation rate plateaus at high AP concentrations but with a lower sum of rate constants of about 1.5 /s." (Is this what you call "attenuated elongation rates observed with increasing profilin (Fig. 3C)"? Please explain more clearly,) Panel C is a missed opportunity to test a range of profilin concentrations; only the 5 μM concentration adds to the data in panel B. Old Fig 3A had additional data with a range of actin concentrations with an excess of profilin. Panel F is a surprising new observation that must be telling us something about the mechanism, but this is not explored. How does profilin have these effects? Furthermore, does depolymerization by free profilin effect require the PRD of Lmod?

Line 255: "requires its WH2 domains for pointed-end polymerization" is ambiguous. Explain the experiment.

Line 265: knowing the actin monomer concentration is required to understand "(C) Lmod2-mediated elongation rate of filament pointed ends" since the reaction has two phases. Which phase?

Line 273: the "single exponential decay functon(lines) to determine Lmod2 dissociation rate." It is the dissociation rate constant not the rate.

Lines 275 and 645: "Dissociation rate" is really the "Dissociation rate constant for the exponential decay." Same problem on lines 655-6.

Line 277, Fig 4: Should be "repeated filament nucleation and elongation from…" Panel 4G is a nice addition to the paper!

Line 283: Another missed opportunity. The "ABS3 (WH2) mutant elongated filaments at ~50% of wild-type rates (0.64 ± 0.06 vs. 1.42 ± 0.50 subunits·s–1 at 2 μM G-actin; Fig. 4C)." Why is the rate slower? Smaller k1+ or larger k1- or something about the first order reaction? Some insight could be informative.

Lines 291-293: Renucleation "probability" or "rate" are more appropriate than "renucleation efficiency." Efficiency is a thermodynamic term.

Fig 5. Four similar panels in one row would save space.

Fig 6 A-F: the subject matter is more appropriate paired with Figs 1 and 3.

Line 360: "(E) Rate of elongation (mean ± SD) of filament pointed ends from coverslip-anchored Lmod2 and fluorescently labelled 549-Lmod2 bound to distal pointed ends." Is the concentration of actin monomers again 2 μM?

Line 385 and Fig 6E: This is a nice addition, but something is wrong. Both rates should be about 1/s. The rate with Lmod agrees with Fig 1F but without Lmod the rate is very far less than Fig 1F. I am skeptical about "likely due to monomer depletion in the elongation mixture." Is this true? If it were, it should slow elongation with and without Lmod proportionally. Knowing the concentrations of the proteins, the nucleation and elongation rates, volume of the chamber and the time after mixing, are monomers really depleted?

Line 347, Fig 6G and line 406: Replace "Lmod2 elongates the filament" with "Actin-profilin elongates pointed end associated with Lmod2".

Line 367: Sentence is repeated.

Line 369: Revise as "Following nucleation by CapZ, the barbed end of the filament remains capped and anchored in the Z disc by alpha-actinin. The filament pointed end grows and is capped by Tmod1."

Line 418: revise as "explains our experiments in mice where expressing an…."

Line 421: "the traditional model of actin filament treadmilling…" I guess the authors could not resist including the treadmilling model, which the reviewers suggested discarding. This sentence is unnecessary.

Style: The presentation would be better with separate sections for results and discussion. As suggested above, much of the interesting data requires better descriptions to help readers lacking expertise in kinetics to understand its meaning.

The text is prone to constructions that are inaccurate or confusing. Simpler constructions would be better. Here are examples from the abstract with suggestions for revision. More can be done on the rest of the text.

Line 23: Revise "enabling elongation even under profilin-rich, cytosol-like conditions that otherwise promote depolymerization of free pointed ends" as "enabling elongation even in the presence of profilin concentrations found in cytoplasm that otherwise would promote depolymerization of free pointed ends."

Line 25: Revise "Strikingly, Lmod2's activity persists in the presence of tropomyosin, confirming its physiological role" as "Strikingly, Lmod2's activity persists in the presence of tropomyosin, a component of thin filaments in muscle."

Line 28: Revise "A human dilated cardiomyopathy-associated mutation ablates Lmod2's polymerase activity, directly linking this mechanism to cardiac dysfunction" as "A mutation of Lmod2 associated with human dilated cardiomyopathy eliminates

Lmod2's polymerase activity, showing that it is important for cardiac function." (Avoid using nouns as adjectives.)

Line 29: Revise "Our work establishes pointed-end polymerization as a novel cellular mechanism of actin assembly and provides a mechanistic basis for Lmod2-related muscle diseases" as "Our work establishes that polymerization of actin filaments pointed ends contributes to the formation of muscle sarcomeres and explains how mutations of Lmod2 contribute to muscle diseases." (Claims of novelty are probably not acceptable; avoid nouns as adjectives.

Here are examples from the main text.

Line 36: "depolymerization is thought to occur primarily at filament pointed ends by dissociation of ADP-actin monomers." This is far from certain. Uncapped barbed ends depolymerize much faster, so given proteins that inhibit capping protein as well as ref 8 and 9, the depolymerization of short actin filament fragments in cytoplasm is uncertain.

G-actin and F-actin are jargon. Better to use actin monomer and actin filament, which anyone can understand.

Line 40: clarify "to barbed but not pointed ends" as "to barbed ends but not pointed ends of pure actin filaments."

Reviewer #4

(Remarks to the Author)
In the manuscript entitled "Leiomodin-2 is a processive pointed-end elongator of actin filaments", Bissau and colleagues characterize the role of Lmod2 as a processive actin polymerase. The manuscript is clearly written, and the experimental work appears technically sound, with conclusions that are generally supported by the data. However, while the study touches on biologically relevant questions, particularly in the context of cardiac muscle physiology, it suffers from a significant lack of novelty that ultimately limits its overall impact. Moreover, there are critical experimental concerns regarding the cardiac function analyses that must be addressed.

Major Concerns:

Lack of Novelty:
The key mechanistic insight—that Lmod2 enhances actin polymerization at the pointed end of thin filaments—has been previously described. Notably, Pappas et al. (PNAS, doi:10.1073/pnas.1508273112) demonstrated that expression of GFP-Lmod2 significantly increases actin incorporation at the pointed ends in cardiomyocytes, suggesting an active role for Lmod2 in promoting pointed-end polymerization. The current study does not sufficiently distinguish its findings from this prior work or provide novel mechanistic insights beyond what has already been reported.

Insufficient Controls and Characterization in the Cardiac Context:
The manuscript attempts to connect Lmod2 function to cardiomyopathy; however, the experimental data supporting this link are incomplete and lacking in rigor:
The sarcomeric localization of both GFP-Lmod2 and GFP-Lmod2-ABS3 should be demonstrated, ideally by co-staining with established sarcomeric markers.
The assessment of cardiac function is incomplete. Parameters such as septal wall thickness and cardiac output are missing and should be included for a comprehensive evaluation.
The use of echocardiography without isoflurane anesthesia raises concerns. The authors should clarify and justify this choice, particularly in terms of potential stress-induced variability.
Histological analyses of the hearts are absent. The authors should include both H&E and Masson's Trichrome staining to evaluate cardiac tissue architecture and fibrosis.
Finally, there is no direct evidence presented that sarcomeric contractile force is altered. This is a critical aspect of the proposed mechanism and should be addressed using appropriate biophysical or functional assays (e.g., tension measurements in isolated muscle fibers or cardiomyocytes).
Summary:
While the manuscript addresses a biologically important protein, the lack of novelty in the mechanistic findings and the absence of essential controls and functional data in the cardiac analysis significantly limit the impact of the study in its current form. Additional experiments are necessary to strengthen the authors' conclusions and to elevate the significance of the findings.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
Biswas revised 2604
Signed: Tom Pollard

I have been happy to work with the authors on the interpretation of these important experiments since December 2024. This second revision is close to being ready for acceptance and now has valuable support from structural studies by the Dominguez lab.

The authors were contentious and thorough in responding to the second round of review. This third version focuses appropriately on the biophysical analysis of the elongation of the pointed end of actin filaments associated with Lmod2. It can be revised without further experiments to address one major problem and numerous small problems explained below.

The authors know about the structures in the Brotzman/Dominguez companion paper. A short paragraph on what they learned about the mechanism from those structures might be interesting.

Major problem:

Line 114: The Michaelis-Menten formulation is inappropriate for analyzing the elongation of actin filaments with Lmod2 on the pointed end. Not only are the M-M assumptions inappropriate, but the derived parameters Km and Vmax do not correspond to any of the measured kinetic parameters. On the other hand (as explained in two previous reviews and used by the authors in the response to review), equations for a second order reaction linked to a reversible first order reaction are appropriate, and simple inspection of the data gives k+1 = 0.8 subunits μM-1s-1, k-1 = 0, and k+2 + k-2 = 2.2 subunits s-1. Future experiments will be necessary to evaluate the mechanism of the first order reactions and to measure k+2 and k-2. Explain the analysis by this method in the text paragraph beginning on line 139.

Minor problems requiring revision of the text:

Line 15 following (abstract): the abstract should include the most important mechanistic discovery the two step elongation mechanism for Lmod on pointed ends.

Line 17 should be revised. I suggest “While barbed-end polymerization is well-established, profilin has long been assumed to inhibited pointed-end elongation in cells.”

Line 20 should be revised. I suggest “…processive actin polymerase for pointed end elongation.”

Line 66 should be revised. I suggest “…cause one form of nemaline myopathy.”

Line 69-70: I suggest “… polymerize actin filaments at their PEs by actin monomers alone or bound to profilin, a condition…”

Line 71: “directly visualized individual Lmod2 molecules”. The Dominguez paper shows that two Lmod2 molecules participate.

Line 77: Omitted the crucial information that Lmod is biotinylated.

Line 113: The units are incorrect: k+1 = 0.8 subunits μM-1s-1 and k-1 = 0.5 subunits s-1. You used excessive significant figures here and elsewhere.

Line 171: “… 549-Lmod2-bound PEs elongated faster than free PEs (Fig. 2C)” disagrees with Fig 1G where the rates are about the same with 2 μM actin monomers.

Line 176: I know Dominguez calls the barbed end groove a cleft, but it is really a groove compared with the deep nucleotide binding cleft.

Lines 176-77: Ambiguous, because you do not specify whether the groove is on the incoming monomer or the ends of the filament.

Line 181: “…mostly unaffected2.” What do you mean by “mostly.” Ref 2 is the wrong reference. Better would be ref 3.

Line 189: The plateau is clearly less than 1.5 subunits/s.

Line 190: What assumptions were used to calculate the saturation of actin with profilin?

Lines 193-196 should be in the discussion.

Line 194: Typo: “(ue.g. Lmod2’194 s stepping)”

Line 199: Do any of the polyproline sequences in the PRD meet the criteria for binding profilin (Petrella 1996 Biochemistry)?

Line 205: Testing the effect of profilin is important. However, this analysis is compromised because the fraction of actin with bound profilin is not constant across this concentration range of equimolar actin and profilin. The M-M analysis is even less appropriate under these conditions than in Fig 1G with just actin.

Line 224: A fiduciary is someone who manages money or property for someone else. I think you mean “fiducial mark.”

Line 236: What is biphasic about the curve in Fig 3G? It looks like saturation binding curve with a Kd around 1 μM.

Line 236: “elongation persisted at the physiological Tpm1.1:actin ratio characteristic of muscle thin filaments (i.e., 1:7).” The ratio that matters is TM:polymerized actin. You only know the total actin monomer concentration. On the other hand, if you knew the affinity of TM for actin filaments under your conditions, you could use the TM concentration to estimate the saturation of the very low concentration of filaments.

Line 243: Specify whether the binding sites are for actin monomers or filaments.

Line 247: do you know that the point mutations in the region corresponding to Tmod1’s ABS1 change the affinity of this region for actin? If not, you cannot draw conclusions from this experiment.

Line 265: A t test assumes equal variances. Not true here.

Line 284: Similar to what?

Line 308: Start new paragraph with “Our results….”

Line 312: “….the elongation rate plateaus,….”

Line 313: “…suggesting rate limiting, first order, conformational changes.” You do not know if these conformational changes are in actin or Lmod2 or both. Might note “Linked conformational changes following macromolecular binding are very common.”

Line 315-317: “alternates between monomer delivery and a subsequent rearrangement or stepping event.” This is only one possibility and “delivery” is ambiguous. Is “delivery” actin monomer binding to Lmod2 or association of this monomer with the pointed end? You do not know if delivery to the pointed end or another uncharacterized conformational change is the rate limiting step.

Line 320: Rather than “quantitative analysis” I suggest “The data in Fig. 1G shows that…”

Line 324: “...inhibit subunit dissociation.”

Line 378: “Lmod-mediated elongation would however generate filaments containing ATP–actin at both ends…” I am skeptical. The maximum elongation rates of ~2/s is only 7 times faster than the ATP hydrolysis rate (0.3/s), so few ATP-subunits would accumulate.

Line 408: “…depolymerization mediated by actin turnover factors such as cyclase-associated protein and cofilin.” Are they active against actin filaments saturated with tropomyosin? Not cofilin.

Line 520: Is recombinant Tpm1 properly acetylated on its N-terminus?

Line 553: How was Lmod biotinylated? In the text it would be better to say “biotinylated Lmod was anchored to streptavidin adsorbed to the coverslip” rather than “Lmod was adsorbed.”

We thank the reviewers for their valuable comments that have greatly helped us improve the manuscript. Our point-by-point responses (in blue) to the Reviewer's comments/concerns (italics) are listed below. Major changes in the revised manuscript have been highlighted in yellow.

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed my concerns.

Thank you for your kind words about our manuscript.

Reviewer #3 (Remarks to the Author):

Biswas, Nature Comm: Leiomodin2 is a processive pointed-end elongator of actin filaments
Signed: Tom Pollard

1. This revised manuscript presents an interesting and important discovery about the regulation of actin polymerization, namely that the protein Lmod2 regulates the elongation of the slow growing, pointed end of actin filaments. The TIRF movies are convincing, and I agree with the authors' interpretation of the movies. Contrary to the concerns of another reviewer, this process has not been reported previously and helps explain the formation of muscle sarcomeres. The responses to review strengthen the paper.
   I am enthusiastic about publication of the work but remain disappointed about the authors' lack of understanding of their data, which results in inaccurate, speculative interpretations that will confuse readers. I hope my suggestions in this review will help the authors resolve the issues in a second revision. I also note missed opportunities and suggested how to improve the presentation.

   We sincerely appreciate the reviewer's enthusiastic support of our work and their recognition of its significance. We also thank the reviewer for their careful and insightful analysis of our data. In response to the reviewer's concerns regarding the mechanistic interpretation, we have extensively revised the manuscript to clarify our analysis of the elongation kinetics, reduce speculative statements, and more clearly distinguish conclusions supported by the data from areas that remain unresolved. Please see our detailed responses below.

   Please note that we have minimized the discussion of possible structural rearrangements caused by Lmod, as these aspects are addressed in greater detail in a complementary manuscript from the Dominguez laboratory that is also under consideration at Nature Communications.

   Data presentation and interpretation
2. Fig 1. As noted in the first review, the measurements by fluorescence microscopy are elegant and convincing. As I suggested, moving parts of former Fig 3A here as panel F is an improvement. The new data on elongation of free pointed ends by ATP-actin monomers is an important addition. The linear dependence on actin monomer concentration allows measurement of the two rate constants k+ and k-, which are similar to earlier work. This validates the accuracy of the authors' methods. However, the authors do not consider k-.

We now provide the values of k_{-1} and k_{+1} for free PE elongation in the manuscript text and in Figure 1 legend. Following text has been added to the manuscript :

> *"Free pointed ends elongated at rates proportional to the actin monomer concentration. The slope of the plot gives the association rate constant of 0.79 ± 0.07 subunits.μM.s^{-1} and the Y-intercept gives the dissociation rate constant of about 0.51 ± 0.06 s^{-1}, both similar to previous measurements (Kuhn and Pollard, 2005; Pollard, 1986)"*

Following text has been added to Figure 1 caption:

> *"linear fit yielding k_{+1} = 0.79 ± 0.07 subunits.μM.s^{-1} and k_{-1} = 0.51 ± 0.06 subunits.s^{-1}".*

3. The parallel experiment with pointed ends associated with Lmod is the heart of the paper. As I explained in the first review, this curved plot reveals that elongation involves two linked reactions. The authors fit the data with an incomplete model (see below Lines 659-668).

   Following the reviewer's suggestion, we considered a two-step model containing two linked reactions for Lmod-mediated elongation of pointed ends. Moreover, we assume that both the reactions are reversible. In the first step, actin monomers are delivered to the Lmod-bound pointed end with forward and backward rates of k_{+1} and k_{-1} respectively. This reaction is followed by a reversible first-order reaction characterized by rate constants k_{+2} and k_{-2} respectively (likely involving Lmod stepping).

$$E + A \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EA^{*}$$

   Assuming steady-state conditions for the intermediate complex EA, we derived the expression below for the elongation rate as a function of actin monomer concentration that depends on all four rate constants $(k_{+1}, k_{-1}, k_{+2}, k_{-2})$. This expression predicts that at low actin concentrations ([M]), the elongation rate varies linearly with monomer concentration, whereas at high actin concentrations it approaches a plateau determined primarily by the forward rate of the second step. Both limits are consistent with our experimental observations.

$$V = \frac{k_{+1} * k_{+2} * [M] - k_{-1} * k_{-2}}{k_1 * [M] + k_{-1} + k_2 + k_{-2}} \qquad (1)$$

   Note that the elongation rate depends upon actin monomer concentration as well as four different parameters. Based on our experimental observation that Lmod-bound pointed ends do not exhibit any measurable elongation in the absence of G-actin, we infer that k-1 ~0, which simplifies the above expression to:

$$V = \frac{k_{+1} * k_{+2} * [M]}{k_1 * [M] + k_2 + k_{-2}} \qquad (2)$$

   The different limits of this expression are given below:

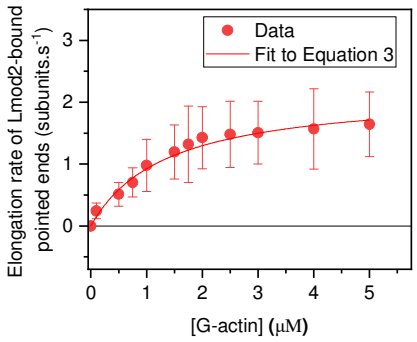
   1. At low values of M, the elongation rate becomes:

$$V = \left(\frac{k_{+1} * k_{+2}}{k_{+2} + k_{-2}}\right) * M \qquad (3)$$

In other words, the elongation rate varies linearly with M at low actin concentration, consistent with our experimental observations (and as pointed out by the reviewer).

2. At very high actin monomer concentration, V approaches k_{+2}, which is also consistent with the observed plateau in elongation rate in our experiments.

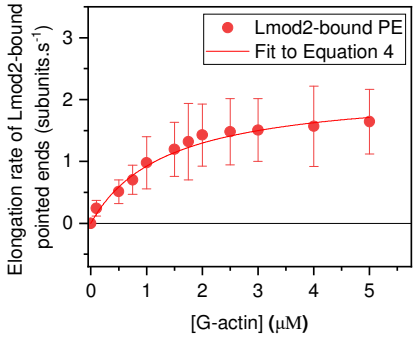
While the goodness of the fit is high ($R^2 \approx 0.98$, see graph below), the inferred parameters have large uncertainties (k_{+1}= 553.7 ± 61606.6 $s^{-1}.\mu M^{-1}$, k_{+2} = 2.18 ± 0.54 s^{-1}, k_{-2}= 749.3 ± 84018.9 s^{-1}) suggesting that we cannot reliably constrain three independent parameters when fitting this expression to our experimental data.



To address this, we considered a reduced form of the expression. The elongation rate can be written in a Michaelis–Menten-like form:

$$V = \frac{V_{max} * [M]}{K_M + [M]} \qquad (4)$$

where $V_{max} = k_{+2}$ and $K_M = (k_{+2} + k_{-2})/k_{+1}$. This reduces the elongation rate to a function of two parameters, namely, V_{max} and K_M, that we can uniquely determine by fitting our data to this expression (see graph below):



As shown, this expression fits the data well ($R^2 \approx 0.98$) and yields $V_{max} = 2.176 \pm 0.20\ s^{-1}$ and $K_M = 1.36 \pm 0.26\ \mu M$. However, because K_M depends on multiple unknown rate constants, we cannot uniquely determine k_{+1} and k_{-2} from these data alone.

In summary, we conclude that while the full reversible two-step model is consistent with the data, we are currently unable to extract all the rate constants from our experimental measurements. Therefore, to avoid overinterpretation and maintain clarity for the reader, we have limited the presentation in the manuscript to the reduced Michaelis–Menten form while noting that the behavior is consistent with a linked two-step mechanism. We thank the reviewer for prompting us to consider this kinetic framework more explicitly.

[Redacted]

For readers without kinetics expertise, I suggest a more concise but more complete presentation of the data:
"Filaments anchored at their barbed ends by CP elongated their pointed ends at rates

proportional to the actin monomer concentration. The slope of the plot gives the association rate constant of 1.8 ± 0.2 μM-1s-1 and the Y-intercept gives the dissociation rate constant of about 0.7/s, both similar to previous measurements (ref)."

"In contrast, with Lmod bound to the pointed end, the elongation rate depends on the actin monomer concentration only at low actin monomer concentrations and plateaus at ~2/s at high actin concentrations. This is typical of a reversible second order reaction linked to a reversible first order reaction that is rate limiting at high actin concentrations. Note that the Y-intercept is zero, which shows that Lmod bound to the pointed end inhibits subunit dissociation, k1-." (This is not explained in the text.)

As advised by the reviewer, we have now added the following text (with minor modifications from the wording suggested by the reviewer) to more clearly convey this interpretation:

> "*We next asked whether elongation at Lmod-bound PEs depends on G-actin concentration, as it does for free PEs. To measure elongation rates of Lmod-bound PEs, we used the strategy shown in Fig. 1E. Elongation rates of free PEs were determined by exposing actin filaments anchored to coverslip-bound biotin-CP to labeled G-actin (no filaments were captured on the coverslip in the absence of coverslip-anchored biotin-CP; movie S6). Free pointed ends elongated at rates proportional to the actin monomer concentration. The slope of the plot gives the association rate constant of 0.79 ± 0.07 subunits.μM.s-1 and the Y-intercept gives the dissociation rate constant of about 0.51 ± 0.06 s-1, both similar to previous measurements (Kuhn and Pollard, 2005; Pollard, 1986). In contrast, the elongation rate of Lmod-bound PEs depends on the actin monomer concentration only at low actin monomer concentrations and plateaus at ~2.2 ± 0.2 subunits·s⁻¹ at high actin concentrations. This is typical of a reversible second order reaction linked to a reversible first order reaction that is rate limiting at high actin*

concentrations. We speculate that this rate-limiting step may reflect a process associated with Lmod2 tracking the growing PE, such as a conformational transition or stepping reaction, analogous to gating steps previously described for formins (Vavylonis et al., 2006).

Interestingly, we observed that at G-actin concentrations below ~2 μM, Lmod-bound PEs elongated faster than free PEs (Fig. 1G). Importantly, Lmod-bound PEs elongated even at G-actin concentrations below the critical concentration for the free PE (0.6 μM), i.e., under conditions where the free PE depolymerizes. Linear fits restricted to this low-concentration regime yielded a slope, with $k+1 = 0.84 \pm 0.08$ subunits·μM^{-1}·s^{-1}, comparable to the G-actin association rate constant measured here for free PE (Fig. 1G) or reported previously (Kuhn and Pollard, 2005; Pollard, 1986). Importantly, the y-intercept of the fit was close to zero ($k_{-1} \approx 0$), indicating that Lmod binding suppresses subunit dissociation from the pointed end. This is consistent with our experimental observation that Lmod-bound PEs do not undergo any depolymerization in the absence of free G-actin. Thus, enhanced net elongation of Lmod-bound PEs at low actin concentrations arises primarily from suppression of subunit dissociation rather than a substantial increase in the association rate."

4. At this point the text should explain separately the following three points:
(1) Elongation mechanism at low actin: this is the simple bimolecular binding of actin monomers with pointed ends associated with Lmod with approximately the same k+ as bare pointed ends. This is not a trivial result; what is the mechanistic interpretation? Lines following 159 speculate that "Lmod may overcome this elongation barrier by either destabilizing the P-1's D-loop binding to P subunit or reorienting subdomain 2 of the terminal subunit." This speculation is incorrect. The rate constant is the same as a free pointed end. Similarly, "enhanced elongation at low G-actin concentrations (<2 μM) through structural remodeling of the pointed end" is incorrect. The higher rate is due to inhibition of subunit dissociation. On the other hand, material in the sentence "Lmod may overcome this elongation barrier by either destabilizing the P-1's D-loop binding to P subunit or reorienting subdomain 2 of the terminal subunit" would be helpful in the section on the rate limiting first order reaction.

   We agree that the enhanced net elongation of Lmod-bound pointed ends at low actin concentrations arises primarily from inhibition of subunit dissociation rather than an increase in the association rate constant. As mentioned above, we have added the following text to the manuscript to make this point explicit:

   "Importantly, the y-intercept of the fit was close to zero ($k_{-1} \approx 0$), indicating that Lmod suppresses subunit dissociation from the pointed end. This is consistent with our experimental observation that Lmod-bound PEs do not undergo any depolymerization in the absence of free G-actin. Thus, enhanced net elongation of Lmod-bound PEs at low actin concentrations arises primarily from suppression of subunit dissociation rather than a substantial increase in the association rate."

In line with the reviewer's suggestion, we have also removed the speculative discussion regarding D-loop interactions and structural rearrangements from this section. Please note that we have minimized the discussion of structural mechanisms, as these aspects are addressed in greater detail in a complementary manuscript from the Dominguez laboratory that is also under consideration at Nature Communications. We have instead limited the discussion in this part of the manuscript to the experimentally supported conclusion that Lmod suppresses subunit dissociation at the pointed end.

5. (2) Why is k- zero? This important observation is ignored. It likely means that Lmod inhibits subunit dissociation, a feature that may help explain other aspects of its mechanism in the next paragraph. This explains the higher rates of elongation than bare pointed ends: "Lmod-bound pointed ends elongated faster than free pointed ends," because subunits dissociated slower than from free pointed ends.

   We agree with the reviewer that the near-zero value of k_{-1} is an important observation. We have now directly measured subunit dissociation from Lmod-bound pointed ends and find that k_{-1} is effectively zero under our experimental conditions.

   While the molecular basis for this suppression of pointed-end depolymerization is not directly resolved by our data, we note that a previous cryo-EM study showed that the LRR domain of tropomodulin simultaneously interacts with the terminal three actin subunits at the pointed end (Carman et al., 2023). Based on this, we suggest that Lmod2 may achieve pointed-end stabilization through a similar mode of interaction involving its LRR domain. Such interactions could prevent subunit dissociation and thereby enhance net pointed-end elongation, as observed here (Fig. 1G). We have now included the following text in the manuscript:

   > *"A recent cryo-EM study showed that the LRR domain of tropomodulin simultaneously interacts with the terminal three actin subunits at the PE (Carman et al, 2023). Based on this, we propose that Lmod2 might achieve PE stabilization through a similar interaction of its LRR domain with terminal actin subunits. Such binding would prevent subunit dissociation and thereby enhance net PE elongation as observed here (Fig. 1G)."*

6. (3) What is the rate limiting first order reaction? The authors offer a possible mechanism, Lmod stepping along the filament. However, to help readers understand the data, the authors must think about and explain what the data tells us and does not reveal about events at the pointed end on the plateau. The mechanism is not known at this point, but the data has some clues about why this first order reaction (actually sum of two first order reactions) limits elongation.

   Here are some processes for the authors to consider: The first order reaction is likely to be reversible with the sum of k2+ and k2- equal to 2/s, as explained during the first round of review. This reaction does not slow the second order bimolecular association reaction at actin monomer concentrations <1 μM when new subunits add at <1/s, but it limits elongation to ~2/s at higher monomer concentrations. Lmod on the pointed end is unlikely to affect actin monomers, so the mysterious reaction must affect the other partner in the bimolecular reaction, the pointed end. If the reaction blocks the pointed end site for monomer addition after a new subunit occupies the pointed end, it must be reversible and transient, because

monomers continue to add at up to 2/s. This reaction is likely to be complicated, as noted above: “Lmod might limit the elongation rate by stabilizing the interaction of the D-loop of subunit P-1 with subunit P.” You might speculate briefly how “stepping” might limit the rate of subunit addition at high actin monomer concentrations. Is it the same or different from previous work such as Vavylonis et al. Mol Cell 2006?

We thank the reviewer for this important point. We agree that the plateau in elongation rate reflects a first-order process that limits elongation at high actin monomer concentrations. As suggested, this step is likely to be reversible. While the precise molecular basis of this transition is not resolved by the present data, we have revised the manuscript to discuss the possibility that it reflects a conformational or positional change of Lmod at the pointed end (e.g., a “stepping” or gating process), while avoiding more specific structural speculation. We have removed the earlier discussion of D-loop interactions, as these are not directly supported by the kinetic data and are better addressed by the complementary structural study from the Dominguez laboratory. We now focus the discussion on the kinetic constraints revealed by the data.

7. Modeling the data in Fig 1F. From pages 659-668. The model is incomplete.

   “We therefore developed a two-step kinetic model for Lmod2-mediated elongation.” (Actually, the first round of review explained how to analyze this data. This kinetic model is not newly developed, since it has been used for decades to analyze two step enzyme reactions.)

   We agree, and have removed the claim that it’s a new model.

8. “The first step involves rapid, diffusion-limited association of G-actin to the Lmod2-bound pointed end, with a concentration-dependent rate $k_1 = k_1' [C]$.” (Do not use the rate constant k_1 for a rate. The statement is incorrect, Drenckhahn, JBC 1986 showed that elongation at the pointed end is not diffusion limited.)

   “We speculate that this is followed by a second, slower step (rate k_2) that is independent of G-actin concentration.” (This formulation is wrong; it does not include the reverse rate constants for either reaction. The following approximation leaving out these reactions gives a good fit to the data simply because k1- is close to zero, but the formulation is incomplete.)

   “We propose this second step may involve Lmod2 stepping to track the elongating filament end, similar to mechanisms described for formin-mediated barbed end elongation (Ref?). Based on this model, the average time it takes one elongation cycle (What is the evidence that the two reactions are coupled to form a cycle rather than being independent?) to be completed is given by (1/k1 + 1/k2) and the average elongation rate is given by r = (k1*k2)/(k1+k2). By fitting this equation to the experimental data (Fig. 1F), we show that a two-step model suffices in explaining our experimental observations. Moreover, it allows us to extract the two rates (these are rate constants) k1', k2 as 1.8 ± 0.2 μM-1s-1, 2.0 ± 0.1 s-1 respectively.” (Fitting a model with all four rate constants would be more appropriate and would give the same result.)

All of this text has now been removed. Please see our response to point 3 above for the detailed discussion about using a model with four rate constants.

9. Fig 2 on the effect of tropomyosin is mostly ok, except that the text does not attempt to explain the plateau at high tropomyosin concentration. What might be going on?

   This dataset has now been moved to Figure 3G. At present, we do not have a definitive explanation for the plateau observed at high tropomyosin (Tpm) concentrations. A likely explanation is saturation of actin filament sides by Tpm, such that further increases in Tpm concentration do not produce additional effects on elongation. Another possibility is the interaction between Lmod and Tpm. However, the available data do not allow us to distinguish between these possibilities. We anticipate that future experiments using Lmod mutants defective in Tpm binding will help clarify this mechanism. We have now added the following text to the manuscript to clarify this:

   > *"While Tpm1.1 reduced elongation rates in a concentration-dependent, biphasic manner (Fig. 3G), elongation persisted at the physiological Tpm1.1:actin ratio characteristic of muscle thin filaments (i.e., 1:7). The elongation rate plateau at higher Tpm concentrations likely reflects saturation of Tpm's binding to filament sides and/or Lmod. While the underlying mechanism for this Tpm-dependent reduction in elongation is currently unclear, future experiments using Lmod2 variants lacking the Tpm-binding site may help resolve this question."*

10. Fig 2 does not fit into a logical sequence of data presentation. Fig 3 on profilin should follow Fig. 1 followed by Fig 6 A-F as new Fig 3. Thereafter would be a good place for Fig. 2 as Fig 4.

    We thank the reviewer for this helpful suggestion regarding figure organization. We have rearranged the figures to improve the logical flow of the manuscript. In particular, the profilin data now follows the Lmod elongation experiments, which we believe provides a clearer progression from baseline elongation to the effects of regulatory factors. We now present the labeled Lmod experiments (previously Fig. 6) as Figure 2, as this establishes the core mechanistic findings of the study before introducing additional modulators like profilin and Tpm.

11. Fig. 3 covers the second important mechanistic finding. The multiple effects of profilin on elongation at the pointed end are surprising in some ways. Panel B has more complete data than old Fig 3A for a range of concentrations of 1:1 mixtures of actin and profilin. Without Lmod pointed ends do not elongate or even shorten as expected from prior work. With Lmod, the behavior is much like Fig 1F but with a slightly lower plateau of about 1.5/s. Again k1- must be zero; k1+ seems to be less than for actin alone. The text should have a better description of the data for actin-profilin: "elongation is proportional to [AP] at low actin concentrations with k1+ = * and k1- = 0. The elongation rate plateaus at high AP concentrations but with a lower sum of rate constants of about 1.5 /s." (Is this what you call "attenuated elongation rates observed with increasing profilin (Fig. 3C)"? Please explain more clearly,) Panel C is a missed

opportunity to test a range of profilin concentrations; only the 5 □M concentration adds to the data in panel B. Old Fig 3A had additional data with a range of actin concentrations with an excess of profilin. Panel F is a surprising new observation that must be telling us something about the mechanism, but this is not explored. How does profilin have these effects? Furthermore, does depolymerization by free profilin effect require the PRD of Lmod?

We thank the reviewer for this detailed and insightful analysis of the profilin data. In response, we have substantially revised this section. We now explicitly describe that, in the presence of profilin, elongation of Lmod-bound pointed ends is proportional to profilin–actin concentration at low actin concentrations and saturates at higher concentrations, with a reduced plateau compared to actin alone. Consistent with the reviewer's interpretation, our data indicate that subunit dissociation is strongly suppressed under these conditions ($k_{-1} \approx 0$). Unfortunately, the limited number of data points in this regime does not allow reliable determination of k_{+1}.

Further, the reduced plateau at higher actin–profilin concentrations indicates that the rate-limiting first-order step (i.e., the second step in the linked reaction scheme) is affected by profilin. This suggests that profilin does not simply reduce monomer availability, but instead modulates the actin-independent transition at the pointed end that limits elongation at high monomer concentrations. However, the structural or biochemical basis of this effect remains unclear. We have therefore refrained from further speculation in the manuscript.

Based on the reviewer's suggestion, we have now re-included the dataset with excess profilin over a range of actin concentrations as Supplementary Figure S4.

The reviewer has also raised important questions regarding the role of the PRD. Experiments with the PRD-deficient mutant may help resolve how profilin influences the second step of the reaction. These experiments are ongoing and will be part of the follow-up study.

Following text has now been added to the results section to clarify profilin's role:

> *"Lmod2-nucleated filaments continued to elongate from the PE in the presence of equimolar profilin and G-actin, though their elongation rates were reduced compared to filaments grown with G-actin alone (Fig. 3A–C, movie S9). At low actin concentrations (< 2 µM) elongation rates increased approximately linearly with actin concentration, whereas at actin concentrations above 2 µM, elongation rates saturated at ~1.5 subunits·s⁻¹, lower than the plateau observed in the absence of profilin (Fig. 1G). When profilin was added in excess to G-actin, ensuring that >97% of the actin monomers were bound to profilin, a similar qualitative dependence on total actin concentration was observed, but the elongation rate at saturation decreased further (Fig. 3C, S4). This reduced plateau suggests that profilin influences the actin-independent, rate-limiting step in elongation possibly by perturbing Lmod2-dependent transitions (for e.g. Lmod2's stepping) at the pointed end, thereby slowing this step. However, the molecular basis of this effect remains unclear."*

12. Line 255: "requires its WH2 domains for pointed-end polymerization" is ambiguous. Explain the experiment.

This line has been removed in the revised manuscript.

13. Line 265: knowing the actin monomer concentration is required to understand "(C) Lmod2-mediated elongation rate of filament pointed ends" since the reaction has two phases. Which phase?

    Monomer concentrations are now clearly defined in the figure legends.

14. Line 273: the "single exponential decay function(lines) to determine Lmod2 dissociation rate." It is the dissociation rate constant not the rate.

    Thank you for pointing this out. We have now corrected this throughout the manuscript.

15. Lines 275 and 645: "Dissociation rate" is really the "Dissociation rate constant for the exponential decay." Same problem on lines 655-6.
    Thank you for pointing this out. We have now corrected this throughout the manuscript.

16. Line 277, Fig 4: Should be "repeated filament nucleation and elongation from…" Panel 4G is a nice addition to the paper!

    Thank you. We have now revised the text accordingly:

    > *"To exclude the possibility that this increased detachment rate arose from impaired coverslip attachment of ΔWH2 rather than faster PE dissociation, we performed re-nucleation experiments. Following filament dissociation, repeated filament nucleation and elongation were observed from the same spots. Comparable re-nucleation efficiencies were observed at the initial Lmod2 locations for WT (44.5%) and ΔWH2 (45.4%) (Fig. 4F, fig. S4, movie S12), indicating that the faster detachment of ΔWH2-nucleated filaments reflects reduced PE processivity rather than compromised coverslip attachment."*

17. Line 283: Another missed opportunity. The "ABS3 (WH2) mutant elongated filaments at ~50% of wild-type rates (0.64 ± 0.06 vs. 1.42 ± 0.50 subunits·s−1 at 2 μM G-actin; Fig. 4C)." Why is the rate slower? Smaller k1+ or larger k1- or something about the first order reaction? Some insight could be informative.

    We thank the reviewer for this thoughtful question. We agree that the reduced elongation rate observed for the ABS3 (WH2) mutant is an important observation. However, the current data at a single G-actin concentration do not allow us to distinguish whether this effect arises from changes in the bimolecular association step (k_{+1}), subunit dissociation (k_{-1}), or the rate-limiting first-order transition.

    As part of a follow-up work, we are currently conducting experiments to measure elongation rate as a function of actin concentration, which we expect will help address these questions. To avoid overinterpretation based on the current data, we have limited the discussion in the manuscript to describing the observed reduction in elongation rate without assigning a specific mechanism. Experiments do dissect this mechanism further are ongoing.

18. Lines 291-293: Renucleation “probability” or “rate” are more appropriate than “renucleation efficiency.” Efficiency is a thermodynamic term.

    “Efficiency” has been changed to rate.

19. Fig 5. Four similar panels in one row would save space.

    We thank the reviewer for this suggestion. In revising the manuscript, we have removed this figure and the associated in vivo experiments to maintain a sharper focus on the biochemical reconstitution and mechanistic analysis presented in this study.

20. Fig 6 A-F: the subject matter is more appropriate paired with Figs 1 and 3.

    We thank the reviewer for the comment. We have now moved figure 6 (experiments with labeled Lmod) to figure 2.

21. Line 360: “(E) Rate of elongation (mean ± SD) of filament pointed ends from coverslip-anchored Lmod2 and fluorescently labelled 549-Lmod2 bound to distal pointed ends.” Is the concentration of actin monomers again 2 µM?

    Yes, the G-actin concentration is 2 µM, we have now clarified the concentrations in the figure legend.

22. Line 385 and Fig 6E: This is a nice addition, but something is wrong. Both rates should be about 1/s. The rate with Lmod agrees with Fig 1F but without Lmod the rate is very far less than Fig 1F. I am skeptical about “likely due to monomer depletion in the elongation mixture.” Is this true? If it were, it should slow elongation with and without Lmod proportionally. Knowing the concentrations of the proteins, the nucleation and elongation rates, volume of the chamber and the time after mixing, are monomers really depleted?

    This has now been fixed. We have now changed the experiment schematic to make sure that we first capture Lmod-nucleated filaments (to which Lmod is still bound at pointed ends), followed by a flow of G-actin. During the second G-actin flow, no Lmod is present. With this configuration, we find that Lmod-bound ends grow faster than free pointed ends, as expected. These data are presented in Fig. 2C.

23. Line 347, Fig 6G and line 406: Replace “Lmod2 elongates the filament” with “Actin-profilin elongates pointed end associated with Lmod2”.

    We have moved that portion of the figure 6 to figure 2 and renamed the figure as “Direct visualization of Lmod2 during PE elongation”.

24. Line 367: Sentence is repeated.

    The repeated line is removed from the current version.

25. Line 369: Revise as “Following nucleation by CapZ, the barbed end of the filament remains capped and anchored in the Z disc by alpha-actinin. The filament pointed end grows and is capped by Tmod1.”

    The revised text reads as “Following nucleation, filament remains capped at its BE by CapZ and gets anchored in the Z disc via alpha-actinin. The filament PE gets capped by Tmod1.”

26. Line 418: revise as “explains our experiments in mice where expressing an….”

    We have removed all the in vivo experiments and data from the current version of the manuscript.

27. Line 421: “the traditional model of actin filament treadmilling…” I guess the authors could not resist including the treadmilling model, which the reviewers suggested discarding. This sentence is unnecessary.

    We have now removed all mentions of the word treadmilling from the manuscript.

28. Style: The presentation would be better with separate sections for results and discussion. As suggested above, much of the interesting data requires better descriptions to help readers lacking expertise in kinetics to understand its meaning.

    We have now reorganized the manuscript with separate results and discussion section.

29. The text is prone to constructions that are inaccurate or confusing. Simpler constructions would be better. Here are examples from the abstract with suggestions for revision. More can be done on the rest of the text.

    Line 23: Revise “enabling elongation even under profilin-rich, cytosol-like conditions that otherwise promote depolymerization of free pointed ends” as “enabling elongation even in the presence of profilin concentrations found in cytoplasm that otherwise would promote depolymerization of free pointed ends.”

    This has been changed.

    Line 25: Revise “Strikingly, Lmod2’s activity persists in the presence of tropomyosin, confirming its physiological role” as “Strikingly, Lmod2’s activity persists in the presence of tropomyosin, a component of thin filaments in muscle.”

    We respectfully disagree with this framing and would like to keep this sentence as is.

    Line 28: Revise “A human dilated cardiomyopathy-associated mutation ablates Lmod2’s polymerase activity, directly linking this mechanism to cardiac dysfunction” as “A mutation of Lmod2 associated with human dilated cardiomyopathy eliminates Lmod2’s polymerase activity, showing that it is important for cardiac function.” (Avoid using nouns as adjectives.)

We apologize, since the cardiomyopathy-associated mutation neither ablates nor eliminates Lmod's elongation, our prior phrasing was incorrect. We have now replaced this with "*greatly reduce Lmod2's PE elongation activity*". We have now edited it as follows:

> *"Remarkably, human dilated cardiomyopathy-associated mutations in Lmod2 greatly reduce Lmod2's PE elongation activity, providing a potential mechanism for disease progression, underscoring the essential role of its actin polymerase activity in formation and maintenance of muscle sarcomeres."*

Line 29: Revise "Our work establishes pointed-end polymerization as a novel cellular mechanism of actin assembly and provides a mechanistic basis for Lmod2-related muscle diseases" as "Our work establishes that polymerization of actin filaments pointed ends contributes to the formation of muscle sarcomeres and explains how mutations of Lmod2 contribute to muscle diseases." (Claims of novelty are probably not acceptable; avoid nouns as adjectives.

The sentence that the reviewer is referring to has been deleted. It has now been revised to: *"Remarkably, human dilated cardiomyopathy-associated mutations in Lmod2 greatly reduces Lmod2's PE elongation activity, providing a potential mechanism for disease progression, underscoring the essential role of its actin polymerase activity in formation and maintenance of muscle sarcomeres."*

Here are examples from the main text.

Line 36: "depolymerization is thought to occur primarily at filament pointed ends by dissociation of ADP-actin monomers." This is far from certain. Uncapped barbed ends depolymerize much faster, so given proteins that inhibit capping protein as well as ref 8 and 9, the depolymerization of short actin filament fragments in cytoplasm is uncertain.

We thank the reviewer for this important point. It is correct that uncapped barbed ends can depolymerize faster than pointed ends under certain conditions, particularly in the absence of actin monomers.

Our statement was intended to reflect typical conditions in cellular actin networks, where barbed ends are either capped or uncapped and exposed to relatively high concentrations of actin monomers, which favor barbed-end assembly. Under these conditions, net depolymerization would primarily be expected to occur at pointed ends.

30. G-actin and F-actin are jargon. Better to use actin monomer and actin filament, which anyone can understand.

    All mentions of F-actin have been replaced with "actin filaments." We have retained the term G-actin but now define it explicitly at first mention as "monomeric actin (G-actin)" to ensure clarity for non-experts.

31. Line 40: clarify "to barbed but not pointed ends" as "to barbed ends but not pointed ends of pure actin filaments."

Done.

Reviewer #4 (Remarks to the Author):

In the manuscript entitled “Leiomodin-2 is a processive pointed-end elongator of actin filaments”, Bissau and colleagues characterize the role of Lmod2 as a processive actin polymerase. The manuscript is clearly written, and the experimental work appears technically sound, with conclusions that are generally supported by the data. However, while the study touches on biologically relevant questions, particularly in the context of cardiac muscle physiology, it suffers from a significant lack of novelty that ultimately limits its overall impact. Moreover, there are critical experimental concerns regarding the cardiac function analyses that must be addressed.

Major Concerns:

32. Lack of Novelty:

The key mechanistic insight—that Lmod2 enhances actin polymerization at the pointed end of thin filaments—has been previously described. Notably, Pappas et al. (PNAS, doi:10.1073/pnas.1508273112) demonstrated that expression of GFP-Lmod2 significantly increases actin incorporation at the pointed ends in cardiomyocytes, suggesting an active role for Lmod2 in promoting pointed-end polymerization. The current study does not sufficiently distinguish its findings from this prior work or provide novel mechanistic insights beyond what has already been reported.

We respectfully disagree with the reviewer’s assessment of novelty. As noted by the reviewer, previous studies, including work from our group and others, have shown that upon expression of Lmod, thin filaments become longer in intact cardiac muscle, both in culture and *in vivo*. However, while these studies demonstrated this phenomenon, they have not been able to define the underlying mechanism.

The only way to determine mechanism is through biochemical reconstitution of pointed-end elongation of actin filaments using purified proteins, where elongation by labeled Lmod can be directly observed in real time. This is precisely the approach taken in the present study. Importantly, in none of the previously published work could the mechanism of processivity be discovered. In contrast, our experiments directly demonstrate that Lmod2 remains associated with the pointed end while promoting elongation, thereby establishing a processive mechanism for pointed-end growth. In summary, while prior work provided important in vivo and cellular observations, the present study delivers the first direct mechanistic insight into how Lmod2 regulates actin filament elongation.

33. Insufficient Controls and Characterization in the Cardiac Context

The manuscript attempts to connect Lmod2 function to cardiomyopathy; however, the experimental data supporting this link are incomplete and lacking in rigor:

The sarcomeric localization of both GFP-Lmod2 and GFP-Lmod2-ABS3 should be demonstrated, ideally by co-staining with established sarcomeric markers. The assessment of cardiac function is incomplete. Parameters such as septal wall thickness and cardiac output are missing and should be included for a comprehensive evaluation.

The use of echocardiography without isoflurane anesthesia raises concerns. The authors should clarify and justify this choice, particularly in terms of potential stress-induced variability.

Histological analyses of the hearts are absent. The authors should include both H&E and Masson's Trichrome staining to evaluate cardiac tissue architecture and fibrosis. Finally, there is no direct evidence presented that sarcomeric contractile force is altered. This is a critical aspect of the proposed mechanism and should be addressed using appropriate biophysical or functional assays (e.g., tension measurements in isolated muscle fibers or cardiomyocytes).

While we agree with the reviewer that these are important directions for future investigation, the primary focus of this study, however is on the biochemical analysis of Lmod2-induced elongation. Therefore, these in vivo data were not included in the manuscript. We agree that it is always useful to demonstrate that processes occur in vivo; however, this is not the central focus, nor the novelty, of the present work. Rather, the strength of this study lies in defining the molecular mechanism using controlled biochemical approaches. In the revised manuscript, we have therefore removed all in vivo experiments (former Figure 5 and associated analyses) and have refocused the paper exclusively on the mechanistic biochemical findings. We note that inclusion of all the experiments requested by the reviewer, such as detailed cardiac functional measurements, histological analyses, and additional localization studies would amount to a substantial body of work that could constitute a separate study in itself.

34. Summary:

While the manuscript addresses a biologically important protein, the lack of novelty in the mechanistic findings and the absence of essential controls and functional data in the cardiac analysis significantly limit the impact of the study in its current form. Additional experiments are necessary to strengthen the authors' conclusions and to elevate the significance of the findings.

We respectfully disagree. While the in vivo data requested by the reviewer are important, they are beyond the scope of the current manuscript which is the biochemical mechanism of pointed-end elongation by Lmod.

Reviewer #3 (Remarks to the Author):

Biswas revised 2604

Signed: Tom Pollard

I have been happy to work with the authors on the interpretation of these important experiments since December 2024. This second revision is close to being ready for acceptance and now has valuable support from structural studies by the Dominguez lab.

The authors were contentious and thorough in responding to the second round of review. This third version focuses appropriately on the biophysical analysis of the elongation of the pointed end of actin filaments associated with Lmod2. It can be revised without further experiments to address one major problem and numerous small problems explained below.

The authors know about the structures in the Brotzman/Dominguez companion paper. A short paragraph on what they learned about the mechanism from those structures might be interesting.

We sincerely thank the reviewer for their enthusiastic support of our work and for recognizing its significance in understanding actin filament regulation. We are very grateful for their thoughtful suggestions and advice.

Major problem:

1. Line 114: The Michaelis-Menten formulation is inappropriate for analyzing the elongation of actin filaments with Lmod2 on the pointed end. Not only are the M-M assumptions inappropriate, but the derived parameters Km and Vmax do not correspond to any of the measured kinetic parameters. On the other hand (as explained in two previous reviews and used by the authors in the response to review), equations for a second order reaction linked to a reversible first order reaction are appropriate, and simple inspection of the data gives k+1 = 0.8 subunits μM-1s-1, k-1 = 0, and k+2 + k-2 = 2.2 subunits s-1. Future experiments will be necessary to evaluate the mechanism of the first order reactions and to measure k+2 and k-2. Explain the analysis by this method in the text paragraph beginning on line 139.

   We have now removed all references to Michaelis-Menten kinetics in the main text and figure legends, and have replaced the text with the following text in the main text.

   “Free pointed ends elongated at rates proportional to the actin monomer concentration. The slope of the plot gives the association rate constant k_{+1} = 0.8 ± 0.1 subunits.μM^{-1}.s^{-1} and the Y-intercept gives the dissociation rate constant k_{-1} = 0.5 ± 0.1 subunits.s^{-1}, both similar to previous measurements (Kuhn and Pollard, 2005; Pollard, 1986). In contrast, the elongation rate of Lmod-bound PEs increased with actin monomer concentration only at low actin concentrations and approached a plateau of ~1.6 ± 0.5 subunits·s^{-1} at higher actin concentrations. This behavior is consistent with a reversible second-order association reaction linked to a reversible first-order transition that becomes rate-limiting at high actin concentrations. While the saturation elongation rate depends on both k_{+2} and k_{-2}, the forward and reverse rates of the first-order transition, only the sum, not the individual values of k_{+2} and k_{-2}, can be determined from our

data alone. The rate-limiting transition may reflect a process associated with Lmod2 tracking the growing PE, such as a conformational transition or stepping reaction, analogous to gating steps previously described for formins (Vavylonis et al., 2006)."

Minor problems requiring revision of the text:

1. Line 15 following (abstract): the abstract should include the most important mechanistic discovery the two step elongation mechanism for Lmod on pointed ends.

   We have now added the following text to the manuscript: "Kinetic analysis indicates that Lmod2-mediated elongation proceeds through a linked two-step mechanism, in which monomer addition is followed by a first-order transition at the Lmod2-bound pointed end that limits elongation at high actin concentrations."

2. Line 17 should be revised. I suggest "While barbed-end polymerization is well-established, profilin has long been assumed to inhibited pointed-end elongation in cells."

   Thank you for your suggestion. We have considered this carefully but prefer to keep the sentence unchanged.

3. Line 20 should be revised. I suggest "…processive actin polymerase for pointed end elongation."

   Done.

4. Line 66 should be revised. I suggest "…cause one form of nemaline myopathy."

   Done. We have now replaced the text with "cause a form of nemaline myopathy".

5. Line 69-70: I suggest "… polymerize actin filaments at their PEs by actin monomers alone or bound to profilin, a condition…"

   Done.

6. Line 71: "directly visualized individual Lmod2 molecules". The Dominguez paper shows that two Lmod2 molecules participate.

   We have now removed the word "individual".

7. Line 77: Omitted the crucial information that Lmod is biotinylated.

The sentence now contains the word “biotinylated”.

8. Line 113: The units are incorrect: k+1 = 0.8 subunits μM-1s-1 and k-1 = 0.5 subunits s-1. You used excessive significant figures here and elsewhere.

   Thank you. We have corrected the units and reduced excessive significant figures throughout the manuscript.

9. Line 171: “… 549-Lmod2-bound PEs elongated faster than free PEs (Fig. 2C)” disagrees with Fig 1G where the rates are about the same with 2 μM actin monomers.

   The mean elongation rate of Lmod2-bound PEs is only slightly faster than free PEs in Fig. 1G. Therefore, we have now edited the text as “549-Lmod2-bound PEs elongated slightly faster than free PEs.

10. Line 176: I know Dominguez calls the barbed end groove a cleft, but it is really a groove compared with the deep nucleotide binding cleft.

    We understand the reviewer’s point. However, for consistency, we have retained this wording.

11. Lines 176-77: Ambiguous, because you do not specify whether the groove is on the incoming monomer or the ends of the filament.

    We have now edited it as “Profilin binds to the hydrophobic cleft (H-cleft) at the BE of actin monomers (G-actin).”

12. Line 181: “…mostly unaffected2.” What do you mean by “mostly.” Ref 2 is the wrong reference. Better would be ref 3.

    We have removed the word “mostly”. We have also moved the reference #2 next to the text where we describe that majority of monomers are thought to be bound to profilin in cells. For BE elongation by profilin-actin, we have now included a review of ours instead.

13. Line 189: The plateau is clearly less than 1.5 subunits/s.

    Thank you for pointing this out. We have now corrected it to 1.3 subunits/s.

14. Line 190: What assumptions were used to calculate the saturation of actin with profilin?

A K_d of profilin-G-actin binding of 0.1 μM was used for these calculations. We have now added the following information to the main text: “assuming a K_d of 0.1 μM”

15. Lines 193-196 should be in the discussion.

    Done.

16. Line 194: Typo: “(ue.g. Lmod2’194 s stepping)”

    Corrected.

17. Line 199: Do any of the polyproline sequences in the PRD meet the criteria for binding profilin (Petrella 1996 Biochemistry)?

    Thank you for directing us to this paper. This is part of ongoing work in which we are exploring the role of the PRD and profilin in Lmod2-mediated elongation. We are also investigating whether profilin directly binds Lmod2’s PRD.

18. Line 205: Testing the effect of profilin is important. However, this analysis is compromised because the fraction of actin with bound profilin is not constant across this concentration range of equimolar actin and profilin. The M-M analysis is even less appropriate under these conditions than in Fig 1G with just actin.

    We agree with the reviewer that the fraction of actin bound to profilin is not constant across the equimolar actin/profilin concentration range. To address this, we already provided data under conditions in which actin is saturated with profilin (~97% bound) in Fig. 3C and Supplementary Fig. 4. We have also removed the Michaelis–Menten analysis.

19. Line 224: A fiduciary is someone who manages money or property for someone else. I think you mean “fiducial mark.”

    Done.

20. Line 236: What is biphasic about the curve in Fig 3G? It looks like saturation binding curve with a Kd around 1 μM.

    We have removed the word “biphasic”.

21. Line 236: “elongation persisted at the physiological Tpm1.1:actin ratio characteristic of muscle thin filaments (i.e., 1:7).” The ratio that matters is TM:polymerized actin. You only know the total actin monomer concentration. On the other hand, if you knew the affinity of TM for actin filaments under your conditions, you could use the TM concentration to estimate the saturation of the very low concentration of filaments.

    We agree with the reviewer. We have edited the text as “We found that Tpm1.1 reduced elongation rates in a concentration-dependent manner (Fig. 3G).”

22. Line 243: Specify whether the binding sites are for actin monomers or filaments.

    We considered this suggestion but would like to keep this sentence unchanged.

23. Line 247: do you know that the point mutations in the region corresponding to Tmod1’s ABS1 change the affinity of this region for actin? If not, you cannot draw conclusions from this experiment.

    Whether the region in Lmod2 corresponding to Tmod1’s ABS1 binds actin has been debated. A recent study reported that mutations in this region reduce actin binding by an isolated Lmod2 peptide (Larrinaga et al., 2026; PMID: 41641537), whereas an earlier study using isothermal titration calorimetry did not detect binding between the corresponding Lmod2 region and actin (Boczkowska et al., 2015; PMID: 26370058). We used the same quadruple mutant described by Larrinaga et al. and found that these mutations do not measurably affect Lmod2-mediated PE elongation or filament dissociation in our single-filament assay. Consistent with this, ABS1 of Lmod2 was not observed in the cryo-EM structures of Lmod2-bound pointed ends in the companion study from the Dominguez laboratory (Brotzman et al.). Nevertheless, we agree that the precise role of this region requires further investigation.

    We have now added the following text in the Results and in the Discussion sections to clarify this point.

    Results section

    “To test the importance of Lmod2’s N-terminal region corresponding to Tmod1’s ABS1, we used an Lmod2 quadruple mutant which was recently suggested to reduce ABS1’s affinity for actin(Larrinaga et al., 2026). These mutations in ABS1 (Fig. 1A) had no measurable effect on Lmod2-mediated PE elongation rates or filament dissociation in our single-filament assay (Fig. 4C–E), suggesting that Lmod2’s ABS1 domain is dispensable for PE elongation in vitro.”

    Discussion section

    “Lmod shares substantial domain similarity with Tmod, including an N-terminal region corresponding to Tmod’s ABS1, the LRR domain, and tropomyosin-binding sites(Fowler and Dominguez, 2017). Despite these similarities, Lmod and Tmod have distinct effects at pointed ends: Lmod promotes pointed-end elongation, whereas Tmod caps pointed ends. While there is

general agreement on the role of the LRR domain, the existence of a bona fide ABS1 in Lmod2 is debated. A human Lmod2 construct corresponding to Tmod1's ABS1 (human Lmod2 residues 51–102; Tmod1 residues 50–101) did not bind monomeric actin by isothermal titration calorimetry(Boczkowska et al., 2015). A recent study, however, suggested that ABS1 in Lmod2 is N-terminally shifted, corresponding to mouse Lmod2 residues 43–88(Larrinaga et al., 2026). This study reported that the quadruple mutant used here reduces actin binding by an isolated ABS1 peptide in vitro and leads to longer thin filaments when introduced into full-length Lmod2 in cells(Larrinaga et al., 2026). We find that these mutations do not measurably affect Lmod2-mediated PE elongation in vitro (Fig. 4C–E), suggesting that this region may contribute to Lmod2 function through an as yet unidentified mechanism. Consistently, while ABS1 in Tmod1 plays a critical role in sterically blocking subunit exchange at the pointed end(Carman et al., 2023), the corresponding region was not observed in Lmod2-bound PE structures (Brotzman et al.). Taken together with previous work, our results suggest that although both Lmod and Tmod similarly use their LRR domains to bind filament pointed ends, they have diverged substantially in the regions N- and C-terminal to the LRR to support distinct functions, elongation and capping, respectively."

24. Line 265: A t test assumes equal variances. Not true here.

    Thank you for pointing this out. We have now used Welch's t-test to compare mean elongation rates between wild-type Lmod2 and the mutants, because the variances between groups were unequal. The differences between WT and the ΔWH2/DCM* mutants remain statistically significant using Welch's t-test. We have added these details to the figure legend.

25. Line 284: Similar to what?

    We have now combined this paragraph with the preceding paragraph to make it obvious what "Similar" was referring to.

26. Line 308: Start new paragraph with "Our results…."

    Done.

27. Line 312: "….the elongation rate plateaus,…."
    Done.

28. Line 313: "…suggesting rate limiting, first order, conformational changes." You do not know if these conformational changes are in actin or Lmod2 or both. Might note "Linked conformational changes following macromolecular binding are very common."

    We could not identify the exact phrase quoted by the reviewer in the current manuscript.

29. Line 315-317: “alternates between monomer delivery and a subsequent rearrangement or stepping event.” This is only one possibility and “delivery” is ambiguous. Is “delivery” actin monomer binding to Lmod2 or association of this monomer with the pointed end? You do not know if delivery to the pointed end or another uncharacterized conformational change is the rate limiting step.

    We have now edited the text as follows “Although the exact structural basis of this step remains unresolved, our data are consistent with a model in which the Lmod2-bound PE alternates between monomer addition and a subsequent rate-limiting transition, which may involve a conformational change, Lmod2 stepping or another unresolved transition at the pointed end. In this model, elongation is limited not by monomer availability but by Lmod2-dependent transitions at the filament end.”

30. Line 320: Rather than “quantitative analysis” I suggest “The data in Fig. 1G shows that…”

    The following sentence has been changed.

31. Line 324: “...inhibit subunit dissociation.”

    Thank you for pointing this out. We have fixed this.

32. Line 378: “Lmod-mediated elongation would however generate filaments containing ATP–actin at both ends…” I am skeptical. The maximum elongation rates of ~2/s is only 7 times faster than the ATP hydrolysis rate (0.3/s), so few ATP-subunits would accumulate.

    Thank you for bringing this up. We agree that the ATP/ADP-Pi cap generated by Lmod-mediated elongation is expected to be small.

33. Line 408: “…depolymerization mediated by actin turnover factors such as cyclase-associated protein and cofilin.” Are they active against actin filaments saturated with tropomyosin? Not cofilin.

    We appreciate the reviewer’s concern and are aware that Tpm protects actin filaments from cofilin. However, previous *in vitro* experiments have shown that it does not completely eliminate the ability of cofilin to bind and sever/depolymerize the actin filaments (Jansen and Goode, MBoC, 2019; Dhar et al, PLoS Genet, 2025).

34. Line 520: Is recombinant Tpm1 properly acetylated on its N-terminus?

Yes, recombinant full-length Tpm1.1 has an N-terminal extension Ala-Ser to mimic the native N-terminal acetyl group.

35. Line 553: How was Lmod biotinylated? In the text it would be better to say “biotinylated Lmod was anchored to streptavidin adsorbed to the coverslip” rather than “Lmod was adsorbed.”

    The Lmod2 biotinylation method is already described in the Methods section under “Purification, biotinylation and dye labeling of Lmod2.”

    In the text, we now say “we immobilized biotinylated SNAP-tagged mouse full-length Lmod2 on streptavidin-coated, PEG-silane–passivated coverslip within an mf-TIRF chamber”.