JCB

Local Monomer Levels and Established Filaments Potentiate Non-Muscle Myosin 2 Assembly

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June 22, 2023

Re: JCB manuscript #202305023

Dr. Jordan Robert Beach
Loyola University Chicago
2160 S 1st Ave
Maywood, Illinois 60153-3328

Dear Dr. Beach,

Thank you for submitting your manuscript entitled "Local Monomer Levels and Established Filaments Potentiate Non-Muscle Myosin 2 Assembly" to the Journal of Cell Biology. Your manuscript was assessed by three expert Reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

The Reviewers are overall enthusiastic about your work and feel that it provides an important advance in our understanding of myosin filament assembly. Most of the comments are relatively straightforward requests for changes to text and figures to clarify the presentation of results, improve quantification of the data, or add more analysis to the Discussion. Please also take into consideration Reviewer #3's point regarding manuscript organization and data presentation. Reviewer #3 also suggests NM2A depletion and overexpression assays to manipulate filament density and formation rate. We agree this would make for an interesting addition to the work that would likely strengthen the overall message regarding the key role of monomer availability in controlling NM2 filament assembly, but we don't feel that it is essential. However, if you have these data in hand or are able to complete the experiments in a reasonable time frame, we do encourage you to add them to the manuscript.

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 an Article is < 40,000, 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.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://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. Articles may have up to 5 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.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. If your paper will include cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and

allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find 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,

Greg Alushin, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

The actomyosin cytoskeleton powers cell migration and cell shape change. Despite decades of experiments across biological scales, key questions still remain about how individual myosin motor proteins assemble first into filaments and then into larger order structures. In earlier work the authors and others used enhanced resolution imaging to visualize individual myosin filaments live. Here they substantially extend this work, revealing important new insights into how myosin filament assembly and super-assembly is regulated and defining in exquisite quantitative detail how the process unfolds. This work provides important new insights into a key cell biological process and will be of broad interest to biochemists, cell and developmental biologists. However, there are places in the current version where it is difficult to follow the logic the authors used to draw certain conclusions. If these issues were addressed, I think this would be a great addition to the JCB.

Figure 1. Here the authors provide evidence that NM2 filament appearance does not appear to be driven by either Calcium spikes or RhoA signaling. This was interesting and surprising. However, in Fig. 1B, I found it difficult to distinguish the Ca+ signal-could they make this clearer?

Fig 1D,E The authors are generally rigorously quantitative. I found the correlation of filament appearance with tail retraction an interesting idea but did the authors do any quantification to support this observation?

I found Fig 3 interesting and impressive. I think it deserves a bit more elaboration. The authors state: "This includes both seemingly disorganized actin and higher density bundled actin (Fig. 3G)" It would be helpful if they annotated their images with some examples of each.

The authors efforts to modulate levels of "free myosin" were very interesting, but I found the logic a bit hard to follow. In Figure 2 they find that reducing actin dynamics reduces filament appearance rate-this made sense to me. However, they then built on this by inhibiting ROCK. They state "treatment of cells with ROCK inhibitor (Y27632) resulted not only in disassembly of actomyosin structures, but robust assembly of nascent NM2 filaments in the lamella". First, I was a bit surprised that ROCK inhibition doesn't affect filament appearance-could they walk through this logic-shouldn't it reduce the pool of active myosin? They then combine inhibition of actin dynamics with inhibition of ROCK, and this restores filament appearance. Is 4d ROCKi vs ROCKi+JL or its it JL vs ROCKi+JL. Did they quantify control vs ROCKi ?

The experiments in Fig 6 and 7 are very cool!

The authors state: "Similar to the fixed results, most of the pre-partition or two puncta data contained multiple NM2 filaments. This number increased upon detectable partitioning and post-partitioning states (Fig. 7G)". It might be better to say "appeared to increase" as they overlap. They also should define nascent vs mature

I'd love to hear the authors speculate about how stacks continue to grow after partitioning as work dating back to the Borisy lab's

pioneering work suggests large scale stacks can assembly-perhaps add this to the figure 8 model.

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

In this study, Quintanilla et al. investigated how nonmuscle myosin II assembles into bipolar filaments and higher order structures in the lamella of migrating cells. The authors conclude that the myosin II assembly is typically triggered by local retraction of lamellipodia and can be stimulated by higher local availability of myosin monomers. To link these phenomena, the authors propose that cell edge retraction can increase local concentration of myosin II monomers, and thus stimulate the filament assembly. On the other hand, they find that calcium fluxes and Rho activation, which are generally considered to be the main triggers of myosin assembly, are not obviously involved in myosin assembly in their system. Likewise, the local organization of actin filaments at the sites of myosin filament assembly, which the authors revealed by correlative platinum replica EM and superresolution light microscopy, did not exhibit any features that correlated with and could favor myosin polymerization. Another important finding in this study is that myosin assembly seems cooperative, because several myosin filaments typically form a nascent cluster. Furthermore, the authors resolved the previously puzzling mechanism of local amplification of myosin filaments by showing that this amplification resulted from separation of preexisting clusters of myosin filaments, but not from splitting of an individual filament. Overall, the findings of this study significantly advance our understanding of the initial assembly of myosin II filaments, but also raise new previously unappreciated questions, which can serve as framework for future research. The study is performed at an exceptional level of technical quality using cutting edge technology and illustrated by gorgeous images and movies. The presented data solidly support the authors' conclusions.

There are several relatively minor concerns that need to be addressed.

1. p.2, l. 54. In the statement "We generated fibroblasts (13) with...", the relevance of ref. 13 is unclear. Were fibroblasts generated in ref. 13 or in this study? Although it becomes a bit clearer after reading Methods, it is still better to reword this sentence.
2. In Movie 1, one of the early frames seems to be out of sequence, because some filaments disappear and then appear again.
3. Fig. 1E. VASP is shown in grey not in purple, as the legend says.
4. Although the presented images reporting the relationships between the myosin filament formation and other events (calcium sparks, Rho activation, edge retraction) are informative, quantification of these data would be helpful. What fraction of each of these events is followed by filament formation? How often does Rho activation precede or coincide with filament formation? It seems that the kymograph in figure 1C shows both of events.
5. In figure 3G, the panel 4 is flipped relative to the original orientation in figure 3B.
6. p. 6, l. 127. It would help to add a brief explanation of how EGFP-actin was used as a standard given that actin polymerizes. A more detailed description in Methods is also recommended.
7. It would be nice to discuss a few additional points in Discussion, such as:
- What causes the retraction of the leading edge to induce myosin assembly? A common thinking is that you need myosin to retract the edge.
- How to reconcile the statement on p. 9, ll. 186-187 ("we speculate that leading edge retractions serve to locally concentrate myosin monomers by reducing the local actin pore size") with the EM data that do not obviously show smaller pore size at the sites of myosin filament assembly?
- It would be interesting to expand the discussion on how a mechanosensitive feedback that alters local actin can lead to cooperative myosin assembly (p. 11, l. 208). In the present form, it contradicts one of the main conclusions that local actin status is not important. An appealing idea is that myosin II has higher affinity for actin filaments under tension (e.g. PMID: 22022566, PMID: 22339860). Such tension can be generated by the first myosin filament, and then the tense actin filaments attract more myosin monomers. Such conformational changes would not be detectable by PREM, which would explain the apparent conflict. Along the same lines, it would be nice to give some additional explanation, rather than simply citing papers, of how mechanosensation can lead to canonical signaling and RLC phosphorylation.
8. p. 11, l. 231 indicates some missing methods than need to be added.
9. p. 12, l. 247: typo in the concentration of latrunculin.

Reviewer #3 (Comments to the Authors (Required)):

Non-muscle myosin-2 (NM2) motors are force generators that power a vast range of biological processes at the subcellular, cellular, and tissue scales. NM2 exists as an inactive "monomer" (a hexamer of two heavy chains and four light chains), which can also assemble into mechanically active bipolar "filaments", not unlike the thick filaments first observed in the sarcomere. In this paper, Quintanilla et al. set out to deepen our understanding of how NM2 monomers assemble into bipolar filaments, and how these filaments grow to build higher order structures. The authors use state-of-the-art super-resolution and live imaging approaches, as well as a combination of drug perturbations, genetic and optogenetic manipulation, and molecular counting to follow the details of filament formation and expansion. The resulting datasets are assembled into visually striking and informative figures that (in most cases) clearly communicate the raw data and quantitative analysis. While there are several papers in the

literature that have used similar approaches to interrogate related questions about NM2 (including the PI's postdoctoral work), the paper under review takes the imaging and analysis a step further, and in doing so, illuminates new mechanistic insight. From the perspective of this Reviewer, the most interesting results are those indicating that NM2 monomer concentration controls filament assembly (Fig. 1D,E; Fig. 4; Fig. 5). While this finding is somewhat expected as most/all assembly processes will be second-order with regard to subunit concentration, it is impressive to see confirmation of this point in living cells. It also suggests that in future studies, biologists need to focus on uncovering activities that control the availability of NM2 monomers for building filaments. The authors also report interesting new data on the progression of filament growth and splitting/partitioning that enables maturation of NM2 contractile networks. Below I list a few points that I hope the authors will consider toward improving the manuscript.

(1) My main concern with this paper is how it is written. Several negative results are presented throughout (in Fig. 1 and 3), which leaves the reader feeling that the authors are panning around without testing a specific hypothesis. The experiments on calcium signaling (Fig. 1B), RhoA signaling (Fig. 1C), and the PREM images (Fig. 3) are inconclusive. The PREM dataset in particular adds no information to the story. In the case of the RhoA signaling experiment (Line 61-62), this might not even be negative data, but rather a failure to detect low levels of RhoA at the beginning of filament assembly events. By rewriting/editing the narrative to focus on a clear hypothesis and the positive findings (monomer concentration controls filament assembly and the partitioning data), the authors could create a more coherent and linear paper that would serve as a valuable addition to the literature.

(2) It seems odd that Fig. 2C is plotted with a logarithmic y-axis given that most/all of the data are clustered between 0.5 and 1. Explain or consider replotting on standard linear axis.

(3) Figures 2/4/5 - Have the authors tried using KD/KO/overexpression in an attempt to manipulate NM2A filament spatial density or formation rate? One could simply overexpress NM2A, sort for high, medium, and low expressers and then score filaments at steady state (fixed) or the appearance rate in timelapse data. I realize that the 2017 NCB paper from Beach used overexpression and KD of NM2A to interrogate filament growth and partitioning, but not de novo appearance.

(4) Figure 5 - There is no quantification included for this experiment and as presented this comes across as an n=1 result. The authors could include quantification of the recruitable and endogenous NM2 signal levels in the ROI over time, and the lag time between these increases (images in 5C). One might predict that photoactivating in more distal regions of the cell would give rise to a greater lag in the accumulation of endogenous NM2. Please indicate how many times this experiment was performed.

(5) Figure 6 - This figure is confusing. As presented the data in panel G seem to be comparing "apples and oranges". Fig. 6H top row shows new filaments forming throughout the lamella, whereas the bottom row shows a single cluster growing over a similar but not identical timeframe. The spatial scales of the top vs. bottom row are entirely different which adds to confusion. But what are the events that are plotted in H? An increase in signal equivalent to 60 GFPs? Either de novo or in a cluster? To clarify this figure, I think it would be interesting if the authors showed intensity vs. time plots for multiple individual events observed in the raw timelapse images. The 60 GFP step increments should be apparent in these records.

(6) Figure 7 - "Sub-resolution" is not a biological designation, as it refers to the performance of the microscopy. It is also ambiguous given the different scales of diffraction-limited and super-resolution imaging that have been applied to this problem (here and in the literature). The authors should consider a non-technical label here.

Re: JCB manuscript #202305023

We thank the reviewers for their helpful and insightful comments and critiques. We feel the manuscript is significantly improved and hope that we have sufficiently addressed their concerns. There are minor text edits throughout the manuscript that were not tracked, but major additions to the text and author responses below are colored in blue.

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

The actomyosin cytoskeleton powers cell migration and cell shape change. Despite decades of experiments across biological scales, key questions still remain about how individual myosin motor proteins assemble first into filaments and then into larger order structures. In earlier work the authors and others used enhanced resolution imaging to visualize individual myosin filaments live. Here they substantially extend this work, revealing important new insights into how myosin filament assembly and super-assembly is regulated and defining in exquisite quantitative detail how the process unfolds. This work provides important new insights into a key cell biological process and will be of broad interest to biochemists, cell and developmental biologists. However, there are places in the current version where it is difficult to follow the logic the authors used to draw certain conclusions. If these issues were addressed, I think this would be a great addition to the JCB.

Thank you for the critical reading of the manuscript and we're glad you appreciated the "exquisite quantitative detail" and think that it "provides important new insights into a key cell biological process". We're very proud of this work!

1) Figure 1. Here the authors provide evidence that NM2 filament appearance does not appear to be driven by either Calcium spikes or RhoA signaling. This was interesting and surprising. However, in Fig. 1B, I found it difficult to distinguish the Ca+ signal-could they make this clearer?

Response:

We apologize for the difficulty.

First, we have updated the figure with enhanced contrast to more clearly mark calcium spikes.

Second, we have added images demonstrating that the calcium spike fills the entire lamella and is not spatially localized in a manner that would facilitate localized myosin 2 assembly. This does not rule out a contribution to regional myosin 2 assembly but argues against a localized contribution.

Finally, we have quantified appearance rate of nascent myosin 2 clusters in the lamella before and after treatment with a specific MLCK inhibitor (peptide18), where we fail to observe significant changes in appearance rate. Collectively, these data argue against calcium-MLCK being a major contributor to myosin 2 appearance in fibroblast lamella.

2) Fig 1D,E The authors are generally rigorously quantitative. I found the correlation of filament appearance with tail retraction an interesting idea but did the authors do any quantification to support this observation?

Response:

This is a valid point. Unfortunately, we spent more time trying to confidently quantify this argument than we did for the rest of the rebuttal data combined. Unfortunately, we struggled to do so. The biggest issue is that nearly all of our fibroblast cells have a staggered tail release with multiple adhesions and stress fibers releasing in a sequential manner. Therefore, defining the time/window of tail retraction becomes quite difficult. We tried collecting additional data at higher temporal resolution, tracking nuclear translocation, various substrate patterns to restrict multiple tails or multiple lamella, photopatterned dots and low fibronectin coating to limit tail retraction time and hope for quicker tail retractions, and photoablation of tails to induce tail retraction. We hope the quantitation throughout the rest of the manuscript demonstrates our desire and ability to quantify imaging data when we are confident we can do it accurately.

But we could not confidently put numbers to this reproducible qualitative observation. We can assure you that we are more frustrated than you by this failure.

We have kept the existing text in place that states this is a “qualitative correlation”. If the reviewer has additional suggestions to quantify this we are definitely open to hearing them.

3) I found Fig 3 interesting and impressive. I think it deserves a bit more elaboration. The authors state: "This includes both seemingly disorganized actin and higher density bundled actin (Fig. 3G)" It would be helpful if they annotated their images with some examples of each.

Response:

We agree. We have updated the CLEM data with new examples and annotations (now in Figure 1) to help guide the reader in the different structures we see.

4) The authors efforts to modulate levels of "free myosin" were very interesting, but I found the logic a bit hard to follow. In Figure 2 they find that reducing actin dynamics reduces filament appearance rate-this made sense to me. However, they then built on this by inhibiting ROCK. They state "treatment of cells with ROCK inhibitor (Y27632) resulted not only in disassembly of actomyosin structures, but robust assembly of nascent NM2 filaments in the lamella". First, I was a bit surprised that ROCK inhibition doesn't affect filament appearance-could they walk through this logic-shouldn't it reduce the pool of active myosin? They then combine inhibition of actin dynamics with inhibition of ROCK, and this restores filament appearance. Is 4d ROCKi vs ROCKi+JL or its it JL vs ROCKi+JL. Did they quantify control vs ROCKi ?

Response: we have broken this up in sections:

4A) “First, I was a bit surprised that ROCK inhibition doesn't affect filament appearance-could they walk through this logic-shouldn't it reduce the pool of active myosin?”

Response:

Yes, this was a striking observation we made previously that conflicted with our expectations and was touched upon in the initial discussion. In brief, ROCKi should absolutely reduce the pool of active 6S myosin. We believe it can simultaneously push the pool of inactive “10S” myosin over a filament formation threshold, as previous data has demonstrated that unphosphorylated myosin can still form filaments (Kendrick-Jones and Citi 1987), albeit at a higher concentration.

We have re-written the Discussion to more clearly explain this model.

4B) Is 4d ROCKi vs ROCKi+JL or its it JL vs ROCKi+JL?

Response:

4D is pre treatment versus post treatment of cells treated with ROCKi + JL. If you stall actin dynamics with JL, you stall myosin filament appearance. This would be blocking D in Model Figure 8. You can overcome this deficit, however, by sending more monomer into the system using ROCKi (enhancing C in Model Figure 8).

4C) Did they quantify control vs ROCKi ?

Response:

We did not quantify this important control experiment in the original manuscript but have now added this data to Fig 1, demonstrating that ROCK inhibition results in an enhancement of lamellar filament appearance.

5) The experiments in Fig 6 and 7 are very cool!

Response:

Thank you. We agree.

6) The authors state: "Similar to the fixed results, most of the pre-partition or two puncta data contained multiple NM2 filaments. This number increased upon detectable partitioning and post-partitioning states (Fig. 7G)". It might be better to say "appeared to increase" as they overlap. They also should define nascent vs mature

Response:

We agree and have added “appeared to increase” to that sentence.

Regarding nascent and mature - We agree. we have now defined nascent in the first sentence of the Results as the “earliest frame a bipolar structure is identifiable”. We previously defined “mature” NM2A filaments as having ~30 monomers and 60 MHCs based on published and cited literature (Results; section “Established NM2 filaments potentiate filament assembly”; 2nd paragraph).

7) I'd love to hear the authors speculate about how stacks continue to grow after partitioning as work dating back to the Borisy lab's pioneering work suggests large scale stacks can assembly-perhaps add this to the figure 8 model.

Response:

This is a great idea. We have updated the model (Figure 8) and Discussion/legend to include stack formation. We believe stacks and clusters are driven by the same molecular mechanism, with the actin movements dictating cluster formation vs stack formation. After multiple myosin 2 filaments are present in a small stack, moving actin filaments pull apart the myosin 2 filaments. If the actin moves in a parallel fashion, the myosin 2 filaments remain in register and generate stacks. If the actin moves in a non-parallel disordered manner, the myosin 2 filaments follow suit, generating clusters. Actin crosslinkers in the rear lamellar likely support the parallel movements of the actin filaments, thereby enhancing stack formation.

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

In this study, Quintanilla et al. investigated how nonmuscle myosin II assembles into bipolar filaments and higher order structures in the lamella of migrating cells. The authors conclude that the myosin II assembly is typically triggered by local retraction of lamellipodia and can be stimulated by higher local availability of myosin monomers. To link these phenomena, the authors propose that cell edge retraction can increase local concentration of myosin II monomers, and thus stimulate the filament assembly. On the other hand, they find that calcium fluxes and Rho activation, which are generally considered to be the main triggers of myosin assembly, are not obviously involved in myosin assembly in their system. Likewise, the local organization of actin filaments at the sites of myosin filament assembly, which the authors revealed by correlative platinum replica EM and superresolution light microscopy, did not exhibit any features that correlated with and could favor myosin polymerization. Another important finding in this study is that myosin assembly seems cooperative, because several myosin filaments typically form a nascent cluster. Furthermore, the authors resolved the previously puzzling mechanism of local amplification of myosin filaments by showing that this amplification resulted from separation of preexisting clusters of myosin filaments, but not from splitting of an individual filament. Overall, the findings of this study significantly advance our understanding of the initial assembly of myosin II filaments, but also raise new previously unappreciated questions, which can serve as framework for future research. The study is performed at an exceptional level of technical quality using cutting edge technology and illustrated by gorgeous images and movies. The presented data solidly support the authors' conclusions.

We appreciate the reviewers careful eye for detail in catching some inconsistencies and helping to improve the manuscript.

There are several relatively minor concerns that need to be addressed.

1. p.2, l. 54. In the statement "We generated fibroblasts (13) with...", the relevance of ref. 13 is unclear. Were fibroblasts generated in ref. 13 or in this study? Although it becomes a bit clearer after reading Methods, it is still better to reword this sentence.

Response:

We agree this was confusing. We have updated the text. The parental fibroblasts were generated previously and the CRISPR-KI lines were generated for this project.

2. In Movie 1, one of the early frames seems to be out of sequence, because some filaments disappear and then appear again.

Response:

Very nice catch. This has been corrected. We accidentally flipped early timepoints in a manual rebuild.

3. Fig. 1E. VASP is shown in gray not in purple, as the legend says.

Response:

Thank you for catching this. We have updated this figure with new examples from data with the Rho biosensor and this VASP data has been moved to supplemental with correct text.

4. Although the presented images reporting the relationships between the myosin filament formation and other events (calcium sparks, Rho activation, edge retraction) are informative, quantification of these data would be helpful. What fraction of each of these events is followed by filament formation? How often does Rho activation precede or coincide with filament formation? It seems that the kymograph in figure 1C shows both of events.

Response:

We agree more quantification here is helpful.
Regarding calcium: Please see Reviewer 1 comment 1
Regarding RhoA activation: We have now quantified the number of nascent myosin 2 filament appearances that are clearly preceded by active RhoA (~15%), display active RhoA signal after myosin 2 appearance(~30%) , or never display any active RhoA (~55%). We have also added kymograph examples of each. These data are included in Figure 1.
Regarding leading edge retractions: We have quantified the number of nascent myosin 2 filament appearances that are discernibly preceded by a leading edge retraction (~80%). These data are now included in Figure 2.

5. In figure 3G, the panel 4 is flipped relative to the original orientation in figure 3B.
Response:
Great catch - however, this no longer applies as CLEM data has been updated.

6. p. 6, l. 127. It would help to add a brief explanation of how EGFP-actin was used as a standard given that actin polymerizes. A more detailed description in Methods is also recommended.
Response:
We agree. We have updated text in the results to more clearly explain that we imaged low level EGFP-actin expression, similar to speckle microscopy, such that individual EGFP-actin molecules are spatially separated to allow for quantification.

The Methods “Cell culture and transfection” section previously included the following statement, which we think is sufficient.
“Cells transfected with EGFP-Actin for the molecular counting experiments were transfected 4-6 hours prior to imaging and plated immediately to achieve extremely low expression for single molecule identification.”

We are wondering if the reviewer was expecting this detail in the “Molecular counting” section of the Methods and perhaps future readers will feel the same. Therefore, we have also added “(see "Cell culture and Transfections" for additional details)” to the end of the first sentence in “Molecular counting” to guide readers back to the other details.

It would be nice to discuss a few additional points in Discussion, such as:
7) What causes the retraction of the leading edge to induce myosin assembly? A common thinking is that you need myosin to retract the edge.
Response:
This is a great suggestion. However, we believe there is conflicting literature on the contribution of myosin 2 here, with some data supporting a role for myosin 2 in edge retraction (directly or indirectly) and other data seeing no significant contribution (Ryan and Vavylonis, 2012 BioJ). We believe most data supports a complex contribution from adhesion, actin polymerization, and membrane tension (Nickaeen and Mogilner Plos computational 2017, Ji Danuser NCB 2018, Guathier and Sheetz 2012), although clearly these components are difficult to be uncoupled from myosin 2. Provided this complexity and ambiguity we have chosen not to delve deeply into this process here, but have briefly added these concepts and citations to the Discussion.

8) How to reconcile the statement on p. 9, ll. 186-187 ("we speculate that leading edge retractions serve to locally concentrate myosin monomers by reducing the local actin pore size") with the EM data that do not obviously show smaller pore size at the sites of myosin filament assembly?
Response:

Thank you for this comment. It is clear we need to do a better job of what we think is going on with the actin. Our data suggests that static actin architecture alone is insufficient to dictate or predict where NM2 filaments will assemble. In contrast, dynamic actin networks that decrease pore size can trap NM2 monomers to transiently increase local monomer levels and enable filament formation. We have updated the text throughout the manuscript to try to convey this model more clearly. Importantly, we clearly state in the Discussion that features of static actin (twist, tension state, etc.) are beyond the resolution of our PREM imaging and could certainly still contribute.

9. It would be interesting to expand the discussion on how a mechanosensitive feedback that alters local actin can lead to cooperative myosin assembly (p. 11, l. 208). In the present form, it contradicts one of the main conclusions that local actin status is not important. An appealing idea is that myosin II has higher affinity for actin filaments under tension (e.g. PMID: 22022566, PMID: 22339860). Such tension can be generated by the first myosin filament, and then the tense actin filaments attract more myosin monomers. Such conformational changes would not be detectable by PREM, which would explain the apparent conflict. Along the same lines, it would be nice to give some additional explanation, rather than simply citing papers, of how mechanosensation can lead to canonical signaling and RLC phosphorylation.

Response:

We completely agree and appreciate the reviewer's comments and suggestions. We have added text and citations in the Results and Discussion more explicitly acknowledging the limits of our PREM imaging and that actin tension, twist, etc might be enhancing myosin assembly.

10. p. 11, l. 231 indicates some missing methods than need to be added.

Response:

Apologies. These methods have been updated.

11. p. 12, l. 247: typo in the concentration of latrunculin.

Response:

Thank you, this is corrected.

Reviewer #3 (Comments to the Authors (Required)):

Non-muscle myosin-2 (NM2) motors are force generators that power a vast range of biological processes at the subcellular, cellular, and tissue scales. NM2 exists as an inactive "monomer" (a hexamer of two heavy chains and four light chains), which can also assemble into mechanically active bipolar "filaments", not unlike the thick filaments first observed in the sarcomere. In this paper, Quintanilla et al. set out to deepen our understanding of how NM2 monomers assemble into bipolar filaments, and how these filaments grow to build higher order structures. The authors use state-of-the-art super-resolution and live imaging approaches, as well as a combination of drug perturbations, genetic and optogenetic manipulation, and molecular counting to follow the details of filament formation and expansion. The resulting datasets are assembled into visually striking and informative figures that (in most cases) clearly communicate the raw data and quantitative analysis. While there are several papers in the literature that have used similar approaches to interrogate related questions about NM2 (including the PI's postdoctoral work), the paper under review takes the imaging and analysis a step further, and in doing so, illuminates new mechanistic insight. From the perspective of this Reviewer, the most interesting results are those indicating that NM2 monomer concentration controls filament assembly (Fig. 1D,E; Fig. 4; Fig. 5). While this finding is somewhat expected as most/all assembly processes will be second-order with regard to subunit concentration, it is impressive to see confirmation of this point in living cells. It also suggests that in future studies, biologists need to focus on uncovering activities that control the availability of NM2 monomers for building filaments. The authors also report interesting new data on the progression of filament growth and splitting/partitioning that enables maturation of NM2 contractile networks. Below I list a few points that I hope the authors will consider toward improving the manuscript.

We are glad that the reviewer finds our work “impressive” and appreciate their critical feedback in improving the manuscript.

(1) My main concern with this paper is how it is written. Several negative results are presented throughout (in Fig. 1 and 3), which leaves the reader feeling that the authors are panning around without testing a specific hypothesis. The experiments on calcium signaling (Fig. 1B), RhoA signaling (Fig. 1C), and the PREM images (Fig. 3) are inconclusive. The PREM dataset in particular adds no information to the story. In the case of the RhoA signaling experiment (Line 61-62), this might not even be negative data, but rather a failure to detect low levels of RhoA at the beginning of filament assembly events. By rewriting/editing the narrative to focus on a clear hypothesis and the positive findings (monomer concentration controls filament assembly and the partitioning data), the authors could create a more coherent and linear paper that would serve as a valuable addition to the literature.

Response:

We agree with the reviewer that there are always different ways to assemble a story from the data. We included the calcium and Rho signaling early in the story for multiple reasons. Calcium-MLCK and RhoA-ROCK are the canonical models for driving RLC phosphorylation and filament assembly. When we began presenting the other parts of this story at conferences and seminars, there was an inevitable question from the audience of “what about calcium and what about RhoA?”. We believe the readers of this manuscript will ask similar questions and we fear that removing the data will suggest we are dodging those questions.

Currently, we have performed additional experiments measuring lamellar filament appearance upon treatment with MLCK inhibitor (peptide 18; Fig 1) and ROCK inhibitor (Y27632; Fig 1; see also Reviewer 1 comment 1&4). We observe no difference in filament appearance upon inhibition of MLCK and an increase (not decrease) in filament appearance upon inhibition of ROCK. We have also quantified the appearance of RhoA relative to the appearance of a nascent myosin cluster, demonstrating that we often fail to observe RhoA prior to a nascent myosin appearance. Importantly, the reviewer is absolutely correct that like everyone, we are limited by the sensitivity and spatio-temporal resolution of the sensors we are using, and we have tried to make this caveat explicitly clear to the reader.

Collectively, these data show that the canonical calcium-MLCK pathway is not driving the majority of nascent filament appearances we observe in fibroblasts, and Rho signaling is more complex in its effect on nascent filament appearances. Both of these conclusions set the stage for and open the door for the additional mechanistic models we explore throughout the rest of the paper. Therefore, we prefer to leave these data in and the story as is, but are happy to iterate if the reviewers and editor advise otherwise.

(2) It seems odd that Fig. 2C is plotted with a logarithmic y-axis given that most/all of the data are clustered between 0.5 and 1. Explain or consider replotting on standard linear axis.

Response:

We apologize for this confusion. The explanation is that the data is normalized to pre-treatment, which was set to equal 1. In order for a x-fold increase to match an x-fold decrease, we used a log2 scale. However, we agree that this is not the most intuitive units to use and have updated to “fold change” relative to pre-treatment.

(3) Figures 2/4/5 - Have the authors tried using KD/KO/overexpression in an attempt to manipulate NM2A filament spatial density or formation rate? One could simply overexpress NM2A, sort for high, medium, and low expressers and then score filaments at steady state (fixed) or the appearance rate in timelapse data. I realize that the 2017 NCB paper from Beach used overexpression and KD of NM2A to interrogate filament growth and partitioning, but not de novo appearance.

Response:

This is a great idea and the reviewer is correct that based on our model we would expect differences in filament assembly kinetics and appearance rates. However, we also agree with the reviewer that this is an incremental extension of our previous data and we put this at the end of the list of experiments for this revision per the guidance of the editor, which we were not able to get to within the revision period.

(4) Figure 5 - There is no quantification included for this experiment and as presented this comes across as an n=1 result. The authors could include quantification of the recruitable and endogenous NM2 signal levels in the ROI over time, and the lag time between these increases (images in 5C). One might predict that photoactivating in more distal regions of the cell would give rise to a greater lag in the accumulation of endogenous NM2. Please indicate how many times this experiment was performed.

Response:

We appreciate the authors concerns here. Regarding n values - we initially did a robust series of photoactivation experiments in which we repeatedly observed the qualitative results presented in the original manuscript. However, we completely agree that more rigorous quantification is justified. We have now quantified 20 cells from 5 independent experiments and measured both recruitable and endogenous myosin intensity in the activation ROI, an ROI immediately behind the activation zone in the direction of retrograde flow, and a control region. We have updated Figure 4 with this quantitative support and the conclusion remains unchanged.

Regarding performing activation as function of distance from cell body, this is a very cool experiment. We were not able to try this in a reproducible manner for this project. But it’s a great idea for a future project.

(5) Figure 6 - This figure is confusing. As presented the data in panel G seem to be comparing "apples and oranges". Fig. 6H top row shows new filaments forming throughout the lamella, whereas the bottom row shows a single cluster

growing over a similar but not identical timeframe. The spatial scales of the top vs. bottom row are entirely different which adds to confusion. But what are the events that are plotted in H? An increase in signal equivalent to 60 GFPs? Either de novo or in a cluster? To clarify this figure, I think it would be interesting if the authors showed intensity vs. time plots for multiple individual events observed in the raw timelapse images. The 60 GFP step increments should be apparent in these records.

Response:

We apologize for the confusion. We have updated the text and figure. This includes adding quantitative numbers for filament increases between frames to (G) to demonstrate how many more filaments are being added to clusters than are initiating nascent assembly events. We have also added a 3rd row of images to (G) which is the same lamellar area used to quantify the nascent appearance events but now displaying the number of filaments being added to ALL existing clusters. Finally, we have added quantitative data to (H) for the number of filaments being added to single clusters over time. When you sum the single cluster growth for all clusters, you obtain the filament growth for all clusters.

(6) Figure 7 - "Sub-resolution" is not a biological designation, as it refers to the performance of the microscopy. It is also ambiguous given the different scales of diffraction-limited and super-resolution imaging that have been applied to this problem (here and in the literature). The authors should consider a non-technical label here.

Response:

We agree that “sub-resolution” is a relative term. However, we struggle to come up with an alternative term that is not relative but conveys what we want to describe. Therefore, we have chosen to use the term “sub-resolution” but have more clearly defined it in the text. We have also removed it from the Abstract so that it is only used after we define it.

The main argument that the term is used for is to convey that fluorescent doublets cannot be assumed to be individual myosin 2 filaments, as previous works have suggested. Instead, our data suggests that the majority of fluorescent doublets are “small” filament stacks below the resolution of our imaging. To further convey this, we have added a supplemental figure (Fig. S3) demonstrating how many filaments of varying sizes (based on published EM data) can fit within the 2D pixels provided by our current imaging, noting this number would be even bigger when considering 3D voxels and 3D z-stacks.

January 9, 2024

RE: JCB Manuscript #202305023R

Dr. Jordan Robert Beach
Loyola University Chicago
Physiology
2160 S 1st Ave
Maywood, Illinois 60153-3328

Dear Dr. Beach,

Thank you for submitting your revised manuscript entitled "Local Monomer Levels and Established Filaments Potentiate Non-Muscle Myosin 2 Assembly." We would be happy to publish your paper in JCB pending minor changes in the text and 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 Articles is < 40,000, 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: Articles may have up to 10 main text figures. 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. Please add a scale bar for the images on the right panel of Fig. 1B.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

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. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments). If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

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."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial, please add a reference citation if possible.

6) 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
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. You currently exceed this limit but, in this case, we will be able to give you the extra space. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

9) Video legends: Should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments, concentration and duration, or transfection), the imaging method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.

10) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

11) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

12) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. Please note that ORCID IDs are required for all authors. At resubmission of your final files, please be sure to provide your ORCID ID and those of all co-authors.

14) Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.**

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Please contact the journal office with any questions at cellbio@rockefeller.edu.

Thank you for this very interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Greg Alushin, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

As I noted in my original review, the actomyosin cytoskeleton powers cell migration and cell shape change. Despite decades of experiments across biological scales, key questions still remain about how individual myosin motor proteins assemble first into filaments and then into larger order structures. In earlier work the authors and others used enhanced resolution imaging to visualize individual myosin filaments live. Here they substantially extend this work, revealing important new insights into how myosin filament assembly and super-assembly is regulated and defining in exquisite quantitative detail how the process unfolds. The authors have fully addressed all of my issues, and I think have also addressed those raised by the other reviewer. This work provides important new insights into a key cell biological process and will be of broad interest to biochemists, cell and developmental biologists.

Reviewer #3 (Comments to the Authors (Required)):

I really enjoyed reading this revised manuscript. The authors addressed my comments with additional explanation in the rebuttal, changes in figure layouts, additional quantification and data panels, and deeper clarification of the logic throughout. Collectively, these changes greatly improve the accessibility of this work; thanks to the authors for being responsive to the feedback. I expect that this paper will be well received by the readership of the JCB and I only have a couple of small edits, listed below.

Minor edits:
A typo on page 4 in the section entitled "Actin dynamics facilitate nascent..." EGFP-RhioBio should probably be RhoBio?

Pg. 10 - The authors state "Notably, placing EM-guided filaments within the voxels from our fluorescent imaging reveals that the number of filaments we quantify within our two-puncta structures is entirely feasible (Fig. S3)." Here in the narrative, please provide the two references used to generate the EM guided bounding boxes alluded to in this supplemental figure.