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Cofilin is required for polarization of tension in stress fiber networks during migration

Stacey Lee and Sanjay Kumar
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. They reviewed in the context of your prior submission to another journal with 2/3 the same and 1 new reviewer. They generally are very positive and have a few specific and focused requests. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This is a significantly improved version of a manuscript, which reports the roles of ADF/cofilins in the assembly of functional actin stress fibers. The authors demonstrate that depletion of cofilin-1

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results in formation of aster-like nodes along transverse arcs, and in diminished fusion of arcs with each other as they flow towards the cell center. This study also provides evidence that in the absence of cofilin-1, transverse arcs are under lower prestress, and that cofilin-1 -depleted cells fail to polarize, even after a chemokine stimulus.

The authors have satisfactorily addressed majority of my previous concerns.
However, there are still some relatively small issues (mainly with the knockdown-rescue experiments) that should be addressed before the study is suitable for publication.

Comments for the author

Major comment:

The knockdown-rescue experiments presented in Fig. S2 are slightly confusing. From the figure legend, I assume that the authors have compared here the phenotypes of control and knockdown cells overexpressing EGFP-cofilin. This is not the 'standard' way of performing rescue experiments. Instead, the authors should compare the phenotypes of control cells (not expressing the rescue construct), knockdown cells (not expressing the rescue construct), and knockdown cells (expressing the EGFP-cofilin rescue construct). Assuming that the rescue works, the phenotype of rescue cells should be similar to the control cells. By comparing the data presented in Fig. 1 and Fig. S2, this appears to be the case for 'nucleus morphology' and 'cell spread area'. However, based on Fig. 1 the 'focal adhesion circularity' is approximately 0.6 in control cells, 0.65 in knockdown cells, and (based on Fig. S2D) approximately 0.8 in the knockout rescue cells. This actually indicates that the EGFP-cofilin construct does not rescue the focal adhesion phenotype of cofilin-1 knockdown cells. Thus, the authors should repeat the knockdown-rescue experiments in a way that they would compare the phenotypes of EGFP-cofilin-1 expressing knockdown cells to the ones of control and knockdown cells not expressing this rescue construct. Moreover it would be informative to carry out the knockdown-rescue experiments for traction force assay. It is also possible that the EGFP-cofilin-1 fusion protein is not fully functional, and in this case the authors may consider using a rescue construct, which contains a smaller N-terminal epitope tag.

Minor comments:

1. The authors state in the 'Results' section (lines 197-199) that, "Interestingly, we did not see cofilin-dependent differences in the localization of ppMLC despite the measured differences in SF prestress (Fig. S3A-B, D)". However, on lines. 272-274, the authors state "To test this hypothesis, we first examined the localization of ppMLC, the active (contractile) form of NMMII, and found that that the nodal regions were also devoid of ppMLC (Fig. 5A, blue arrowheads)". These statements are somewhat contradictory with each other, and the text should be accordingly revised.

2. In Fig. S5A, it would be informative to include enlarged ROIs, demonstrating the lack of nodal networks in EGFP-Cofilin-3A transfected cofilin KD and NT cells.

Reviewer 2

Advance summary and potential significance to field

This manuscript presents new mechanistic details pertaining to the role of cofilin in cell polarization and contractile force generation. The authors were careful in their conclusions and did not overstate their data sets. It is rare to see, but the title is exactly what their data shows.

Comments for the author

I have no further suggestions. The authors provided sufficient data rescuing several of the major phenotypes of cofilin KD cells with WT expression.

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Reviewer 3

Advance summary and potential significance to field

In this manuscript, Lee and Kumar characterize the role of cofilin in stress fiber assembly and mechanics, and in the emergence of cell polarity. The authors first show that cofilin KD alters cell morphology, and specifically stress fiber organization. Then, they show the intriguing result that cofilin KD increases overall cell tractions, but decreases tension in individual stress fibers, and argue that this is due to a higher number of less contractile fibers. Dynamically, they show that cofilin KD cells form a more inter-connected network containing asters, which does not coalesce into thicker stress fibers. Finally, they show that cofilin KD cells fail to form a polarized stress fiber structure. The manuscript provides a thorough study of the interaction between cofilin, stress fiber dynamics and mechanics, and cell polarity. It presents an interesting mechanism by which the balance between actin polymerization and contractility may drive the emergence of polarity. It also helps to reconcile previous findings in the literature by proposing a reasonable model of the process. The manuscript has already undergone one round of revision at another journal, and the authors carried out important experiments (and specifically rescue experiments) to address reviewer concerns. In my view, most concerns were raised by the reviewers in this first round of reviews, and the authors have responded adequately by providing new data, and by adjusting some of their claims and statements in the text. As such, I only have a few comments:

Comments for the author

1. The term “polarization of tension” employed by the authors may be a bit confusing. Forces within cells are always balanced and are therefore not polarized per se. Tension can indeed be polarized by aligning in a specific direction, but that is not exactly what is being described here. I understand that the authors are fully aware of this, and with this term they rather refer to changes in the distribution of fibers with different amounts of tension. However, perhaps they could come up with another way to describe their results that could be more precise.

2. Part of the authors’ interpretation of the data is the fact that cofilin removes short and branched actin filaments during arc maturation.
The authors themselves acknowledge that they have not been able to directly observe this process, so this remains as an untested hypothesis. Therefore I would suggest to tone down the abstract in the sentence that states “cofilin contributes to symmetry breakage by removing low-tension actomyosin filaments”. It is fine to mention this as a reasonable hypothesis in the model (figure 7), but it should not be mentioned in the abstract as a hard fact.

3. Figure 4C-D: The specific localization of alpha-actinin and tropomyosin at nodes is very hard to assess from the figures, as only overlay images are shown. Some sort of quantification would be required.

4. The same thing can be said for the claimed absence of co-localization between nodes and ppMLC.

First revision

Author response to reviewers' comments

We would like to thank the reviewers for their constructive comments, which we have addressed through additional analysis and changes to the manuscript text. We believe that these revisions have strengthened the manuscript and are optimistic that the reviewers will find our submission suitable for publication in the *Journal of Cell Science*. The major changes to the manuscript are summarized below and point-by-point responses to each of the reviewer comments are inline on the following pages.

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1. **Additional analysis characterizing the crosslinker-rich nodes in cofilin-depleted cells.** In order to better characterize the nodal morphologies in the cofilin knockdown (KD) cells, we have included additional panels in Fig. 4C and D and a new supplementary figure (now Fig. S4). The panels in Fig. 4C, D depict regions of crosslinker-rich nodes in representative cofilin KD cells and of the typical alternating, sarcomeric crosslinker morphology in control non-targeting (NT) cells. We have included both single- channel and composite images showing α-actinin, tropomyosin, ppMLC, or F-actin localization and have also included line scans drawn along the nodal transverse arc networks to better illustrate the changes in staining intensity. These scans provide additional support for the idea that local changes in the microstructure of transverse arcs brought about by cofilin depletion can result in deficiencies in the long- range contractile behavior of the cell.

2. **Changes and rearrangements to the main figures and supplementary figures.** To incorporate the additional analysis above, we have made the following changes or additions to the manuscript figures:
   a. **Figure 5**: We have included a new panel (B) showing the ppMLC and α-actinin fluorescence intensity along line scans in nodal regions in cofilin KD and NT cells. These line scans better depict the crosslinker-rich, myosin-devoid nodes, characterized by large peaks in α-actinin fluorescence and dips in ppMLC fluorescence.
   b. **Figure S4**. We have included four new panels depicting nodal ROIs in cofilin KD cells, and α- actinin (Fig. S4A, B) or tropomyosin (Fig. S4D, E) fluorescence intensities along line scans traced over nodes.
   c. **Figure S5**: The panels that were formerly in Fig. S4B-D (FilaminA and Arp3 localization) were moved to a new supplementary figure (Fig. S5). No changes were made to the content.
   d. **Figure S6**: (Formerly Fig. S5.) Fig. S6A now includes ROI insets showing normal transverse arc morphology in cofilin KD cells rescued with ectopic expression of EGFP-cofilin.

3. **Change to the term "polarization of tension."** We now refer to the idea of a polarized distribution of forces as "polarization of tension distribution" to prevent any confusion in terminology. We have made changes to the manuscript accordingly.

Reviewer 1 Advance Summary and Potential Significance to Field:

This is a significantly improved version of a manuscript, which reports the roles of ADF/cofilins in the assembly of functional actin stress fibers. The authors demonstrate that depletion of cofilin-1 results in formation of aster- like nodes along transverse arcs, and in diminished fusion of arcs with each other as they flow towards the cell center. This study also provides evidence that in the absence of cofilin-1, transverse arcs are under lower prestress, and that cofilin-1 -depleted cells fail to polarize, even after a chemokine stimulus.

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actually indicates that the EGFP-cofilin construct does not rescue the focal adhesion phenotype of cofilin-1 knockdown cells. Thus, the authors should repeat the knockdown-rescue experiments in a way that they would compare the phenotypes of EGFP-cofilin-1 expressing knockdown cells to the ones of control and knockdown cells not expressing this rescue construct. Moreover, it would be informative to carry out the knockdown-rescue experiments for traction force assay. It is also possible that the EGFP-cofilin-1 fusion protein is not fully functional, and in this case the authors may consider using a rescue construct, which contains a smaller N-terminal epitope tag.

We thank the reviewer for this suggestion. The reviewer is correct to point out that the focal adhesion circularity measurements reported in Fig. 1 are different from that in Fig. S2. We would first like to note that different imaging modalities used to obtain the data in Fig. 1 (cofilin KD and NT cells) and Fig. S2 (cofilin KD + EGFP-cofilin and NT + EGFP-cofilin) may account for the apparent discrepancies in focal adhesion circularity. Specifically, we used a widefield microscope for Fig. 1 and a confocal microscope for Fig. S2. Thus, comparisons of qualitative or large-scale measurements, such as the presence of nuclear abnormalities, cell polarity, and cell spread area may be roughly made between the data in Fig. 1 and Fig S2. As expected, we find that the relative proportions of polarized cells and abnormal nuclei are in the cofilin- KD-rescue experiment are similar to that of the NT control in Fig 1. The same is true for the cell spread area. However, focal adhesion measurements between the two sets of data are not as directly comparable given the smaller size of focal adhesions, which makes their imaging more susceptible to differences in focal planes, background fluorescence, and other imaging settings. These may explain the apparent discrepancies in the average measured focal adhesion circularities across experiments. Thus, it is more informative to make comparisons of focal adhesion circularities within a set of images acquired from the same microscope. In doing so, we see that (1) cofilin KD cells have higher focal adhesion circularities than NT cells, and (2) the distributions of average focal adhesion circularities are similar across NT and cofilin KD cells that are transfected with ectopic cofilin, as expected.

Next, our rationale for comparing the phenotypes of cofilin KD cells transfected with EGFP-cofilin and control NT cells transfected with EGFP-cofilin (vs. the more standard control of naïve or NT cells not transfected with a construct) was to rule out any overexpression effects of cofilin-EGFP. In initial tests, we observed approximately 5% of transfected NT cells displaying spike-like, EGFP-cofilin-positive aggregates in the cytosol. These aggregates did not colocalize with or otherwise correlate with stress fibers (SFs). We hypothesized that these aggregates were due to cofilin overexpression as this effect was only observed in the NT cells with the highest EGFP fluorescence intensities and was never observed in EGFP-cofilin transfected KD cells or NT cells with low to moderate levels of EGFP-fluorescence. Indeed, others have shown that cofilin oligomerization is a means to sequester excess cofilin to promote actin polymerization (Goyal et al., 2013; Pfannstiel et al., 2001). It is likely that the cofilin aggregates that we observed were due to the massive overexpression of cofilin and subsequent oligomerization to rebalance actin polymerization levels in the cell. Therefore, in order to rule out any overexpression effects, we compared the KD-rescues to NT cells expressing low to moderate levels of EGFP-cofilin. As shown in Fig. S2, there were no differences across several phenotypes between the KD-rescue cells and the NT-EGFP-cofilin cells. Moreover, many of these phenotypes in the cofilin KD-rescues and NT + EGFP-cofilin were also comparable to those observed in NT cells not transfected with EGFP-cofilin (measurements for NT controls are shown in Fig. 1). We are thus confident that transfection with EGFP-cofilin in the KD cells produces a sufficient rescue in KD cells and minimal overexpression artifacts in NT cells moderately expressing the construct.

Moreover, despite the oligomerization of EGFP-cofilin in cells overexpressing the EGFP-cofilin construct, there is no consistent evidence that the N-terminal fluorophore tag affects the functionality of cofilin. Lai et al. conducted a particularly extensive validation of this EGFP-cofilin construct as well as the constitutively active S3A and dominant negative S3D cofilin mutant constructs. They found that cells transfected with the constitutively active cofilin S3A and dominant negative phosphomimetic cofilin S3D had similar recovery rates in the lamellipodium in FRAP experiments. They also used biochemical assays and TIRF microscopy in live cells to show that EGFP-cofilin could sever actin at a similar rate and manner as untagged cofilin. Furthermore, in a co-immunoprecipitation assay, EGFP-cofilin was found to bind to actin in cell extracts (Lai et al., 2008). We have included the citation to Lai et al.2008 on Line 324 of the manuscript. Thus, we

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are confident that the ectopic EGFP-cofilin has similar functionality to endogenous cofilin.

Finally, while redoing the morphological characterization experiment and traction force measurements with cofilin KD, NT, and cofilin KD+EGFP-cofilin rescue cells would certainly provide additional proof that varying cofilin expression levels are responsible for the phenomena that we observe, to do so would require many more months of additional experiments, which would be effectively impossible given the ongoing COVID-19 shutdowns. At best, these studies would confirm our findings without changing the overall conclusions of the study. Overall, the controls and validations of our cofilin KD experiments, including using two different cofilin KD cell lines each transduced with different shRNAs and rescue experiments, show that our results are not likely to be due to off-target effects from cofilin depletion. We are thus confident that our main findings may be attributed to the expression levels of cofilin.

Minor comments:

1. The authors state in the 'Results' section (lines 197-199) that "Interestingly, we did not see cofilin-dependent differences in the localization of ppMLC despite the measured differences in SF prestress (Fig. S3A-B, D)". However, on lines. 272-274, the authors state "To test this hypothesis, we first examined the localization of ppMLC, the active (contractile) form of NMMII, and found that that the nodal regions were also devoid of ppMLC (Fig. 5A, blue arrowheads)". These statements are somewhat contradictory with each other, and the text should be accordingly revised.

We thank the reviewer for pointing out these seemingly contradictory statements. The key to resolving the apparent contradiction lies in the fact that each statement refers to a different subtype of SF (ventral SFs vs transverse arcs). In lines 198-200 and Fig. S3, we measured the normalized ppMLC intensity along the SF that was spanning the gap of the U-pattern, which can be characterized as a ventral SF given that it may form adhesions at the to arms of the U-pattern only. Consistent with our past work, the localization of ppMLC along ventral SFs was somewhat uniform when imaged with a confocal microscope (Kassianidou et al., 2017; Lee et al., 2018) and more punctate, but still decorating the entire SF, when imaged at higher resolutions (Fig. 5A, S3). We only observed nodal regions in regions along transverse arc SFs, a different subtype of SF. In NT cells imaged using superresolution microscopy, transverse arcs were observed to have uniformly punctate ppMLC localizations, similar to ventral SFs (Fig. 5A, NT cells). In the cofilin-depleted cells, while ppMLC localization along transverse arcs is punctate in along some length of the SF, it is also interspersed with areas markedly devoid of ppMLC, which correspond to the nodal regions.

We have edited the manuscript as follows:

> Interestingly, we did not see cofilin-dependent differences in the localization of ppMLC along the ventral SF spanning the gap of the U-pattern, despite the measured differences in SF prestress (Fig. S3A-B, D) (Line 198-200).
>
> To test this hypothesis, we first examined the localization of ppMLC, the active (contractile) form of NMMII, and found that that the nodal regions in transverse arcs were also devoid of ppMLC (Fig. 5A, B, blue arrowheads) (Lines 281-283).

2. In Fig. S5A, it would be informative to include enlarged ROIs, demonstrating the lack of nodal networks in EGFP-Cofilin-3A transfected cofilin KD and NT cells.

> We have included enlarged ROIs in Fig. S5A (now Fig. S6A) demonstrating the lack of nodal networks in EGFP-Cofilin-S3A transfected cells in cofilin KT and NT cells. Fig. S6A is reproduced below.

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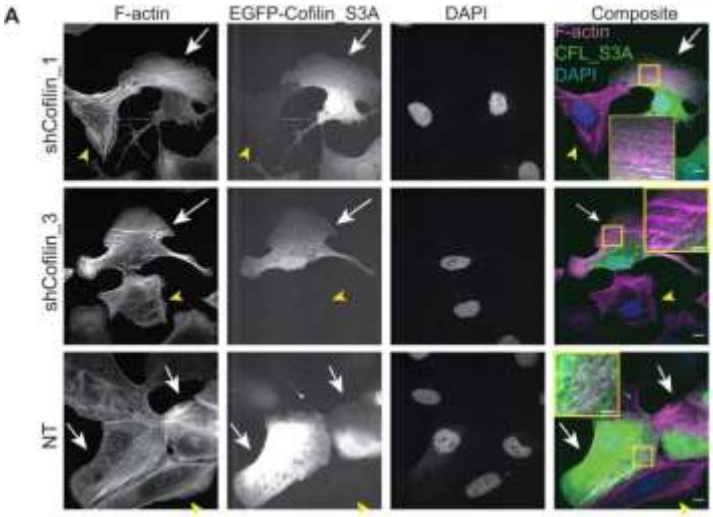

Figure S6. Cofilin KD cells regain a polarized SF phenotype when transfected with constitutively active cofilin. (A) shCofilin_1, shCofilin_3, and NT cells transfected with cofilin_S3A (constitutively active, non-phosphorlyatable), indicated by white arrows. Yellow arrows indicate non-polarized cells not transfected with cofilin_S3A.

Reviewer 2 Advance Summary and Potential Significance to Field:

This manuscript presents new mechanistic details pertaining to the role of cofilin in cell polarization and contractile force generation. The authors were careful in their conclusions and did not overstate their data sets. It is rare to see, but the title is exactly what their data shows.

Reviewer 2 Comments for the Author:

I have no further suggestions. The authors provided sufficient data rescuing several of the major phenotypes of cofilin KD cells with WT expression.

We are pleased the reviewer finds our revisions satisfactory and thank them for their time.

Reviewer 3 Advance Summary and Potential Significance to Field:

In this manuscript, Lee and Kumar characterize the role of cofilin in stress fiber assembly and mechanics, and in the emergence of cell polarity. The authors first show that cofilin KD alters cell morphology, and specifically stress fiber organization. Then, they show the intriguing result that cofilin KD increases overall cell tractions, but decreases tension in individual stress fibers, and argue that this is due to a higher number of less contractile fibers. Dynamically, they show that cofilin KD cells form a more inter-connected network containing asters, which does not coalesce into thicker stress fibers. Finally, they show that cofilin KD cells fail to form a polarized stress fiber structure. The manuscript provides a thorough study of the interaction between cofilin, stress fiber dynamics and mechanics, and cell polarity. It presents an interesting mechanism by which the balance between actin polymerization and contractility may drive the emergence of polarity. It also helps to reconcile previous findings in the literature by proposing a reasonable model of the process. The manuscript has already undergone one round of revision at another journal, and the authors carried out important experiments (and specifically rescue experiments) to address reviewer concerns. In my view, most concerns were raised by the reviewers in this first round of reviews, and the authors have responded adequately by providing new data, and by adjusting some of their claims and statements in the text. As such, I only have a few comments:

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Reviewer 3 Comments for the Author:

1. The term "polarization of tension" employed by the authors may be a bit confusing. Forces within cells are always balanced and are therefore not polarized per se. Tension can indeed be polarized by aligning in a specific direction, but that is not exactly what is being described here. I understand that the authors are fully aware of this, and with this term they rather refer to changes in the distribution of fibers with different amounts of tension. However, perhaps they could come up with another way to describe their results that could be more precise.

We thank the reviewer for this comment and have changed the language in the manuscript to mitigate potential confusion. Whereas before we referred to the idea of the distribution of forces that would result in a polarized, migrating cell as "polarization of tension", we now refer to this concept in the manuscript as "polarization of tension distribution."

2. Part of the authors' interpretation of the data is the fact that cofilin removes short and branched actin filaments during arc maturation. The authors themselves acknowledge that they have not been able to directly observe this process, so this remains as an untested hypothesis. Therefore, I would suggest to tone down the abstract in the sentence that states "cofilin contributes to symmetry breakage by removing low-tension actomyosin filaments". It is fine to mention this as a reasonable hypothesis in the model (figure 7), but it should not be mentioned in the abstract as a hard fact.

We have replaced the indicated sentence with the following:

Here, we provide evidence for a model in which the actin-severing protein cofilin participates in symmetry breakage by removing low-tension actomyosin filaments during transverse arc assembly. (Lines 31-33).

3. Figure 4C-D: The specific localization of alpha-actinin and tropomyosin at nodes is very hard to assess from the figures, as only overlay images are shown. Some sort of quantification would be required.

For clarity, we have included additional panels in Fig. 4C and D, reproduced below, showing the single- channel images (F-actin and α-actinin (Fig. 4C) or tropomyosin (Fig. 4D)) of the region of interest. From these single channel images, the reader may better observe the α-actinin clusters that forms nodes in the transverse arcs of the cofilin-depleted cells as well as the tropomyosin-devoid holes in the nodes, which are markedly different compared to the smaller, punctate sarcomeric α-actinin and uniform tropomyosin localizations in NT cells.

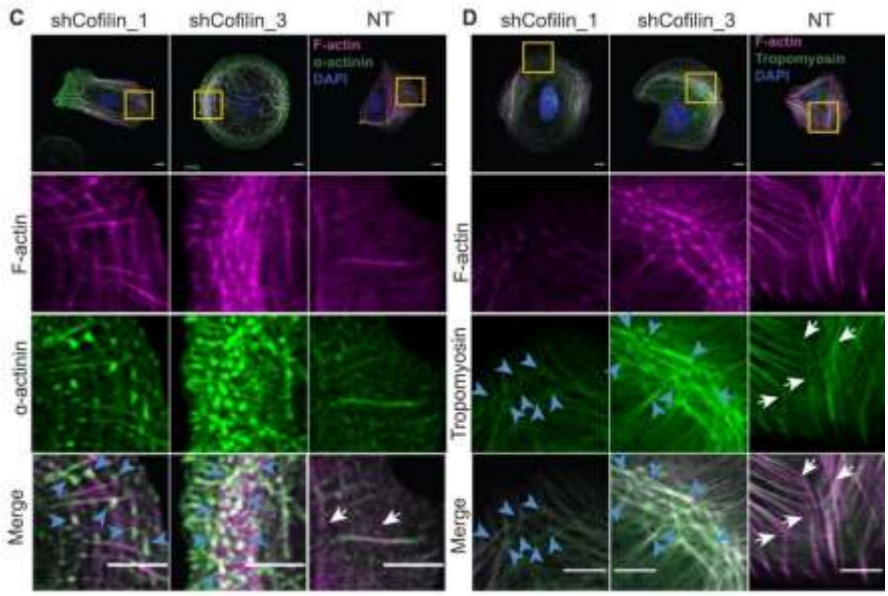

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Figure 4. Transverse arcs in cofilin KD cells have altered morphodynamics. Confocal images of cells stained for F-actin (magenta), DAPI (blue), and (C) α-actinin (green) or (D) tropomyosin (green). Blue arrowheads point to nodal intersections. White arrows point to transverse arc-dorsal SF intersections in NT cells. Scale bars: 10 μm. See also Fig. S4.

In addition to the single-channel panels in Fig. 4C-D, we have also included additional analyses, which are included in Fig. S4, reproduced below. Specifically, we generated line scans along transverse arcs in cofilin KD and NT cells in both the α-actinin and F-actin/phalloidin channels to visualize changes in fluorescence intensity in the respective channels. Line scans in cofilin KD cells in the α-actinin channel show large variations in fluorescence intensity, with fluorescence intensity at the nodes as high as 3 times the maximum fluorescence intensity along transverse arcs in control NT cells. In contrast, line scans in NT cells in the α- actinin channel show a much smaller variation in fluorescence intensity, with fluctuations corresponding to sarcomeric structures along the transverse arc (Fig. S4A, B).

We have included the following description in the main text:

> The fluorescence intensity of α-actinin at these nodes was as high as three times the fluorescence intensity of α-actinin along transverse arcs in comparable regions in NT cells (Fig. S4B). (Lines 241-243).

Additionally, we generated line scans along transverse arcs in the tropomyosin and F-actin channels and found large variations in tropomyosin fluorescence intensity in nodal regions in cofilin KD cells, with dips in intensity corresponding to the tropomyosin-devoid nodes. On the other hand, in NT cells, tropomyosin intensity exhibited less variation (Fig. S4D, E).

We have included the following description in the main text:

> However, we found that nodes in transverse arcs, which are less mature SFs compared to ventral SFs (measured in Fig. S3A, C), were specifically devoid of tropomyosin (Fig. 4D, S4D, blue arrowheads). Furthermore, line scans in these nodal regions exhibited large variations in tropomyosin fluorescence intensity (Fig. S4E). In contrast, transverse arcs in NT cells had mostly continuous tropomyosin decoration, with minimal variation in fluorescence intensity (Fig. 4D, S4D, E, white arrows) (Lines 260- 265).

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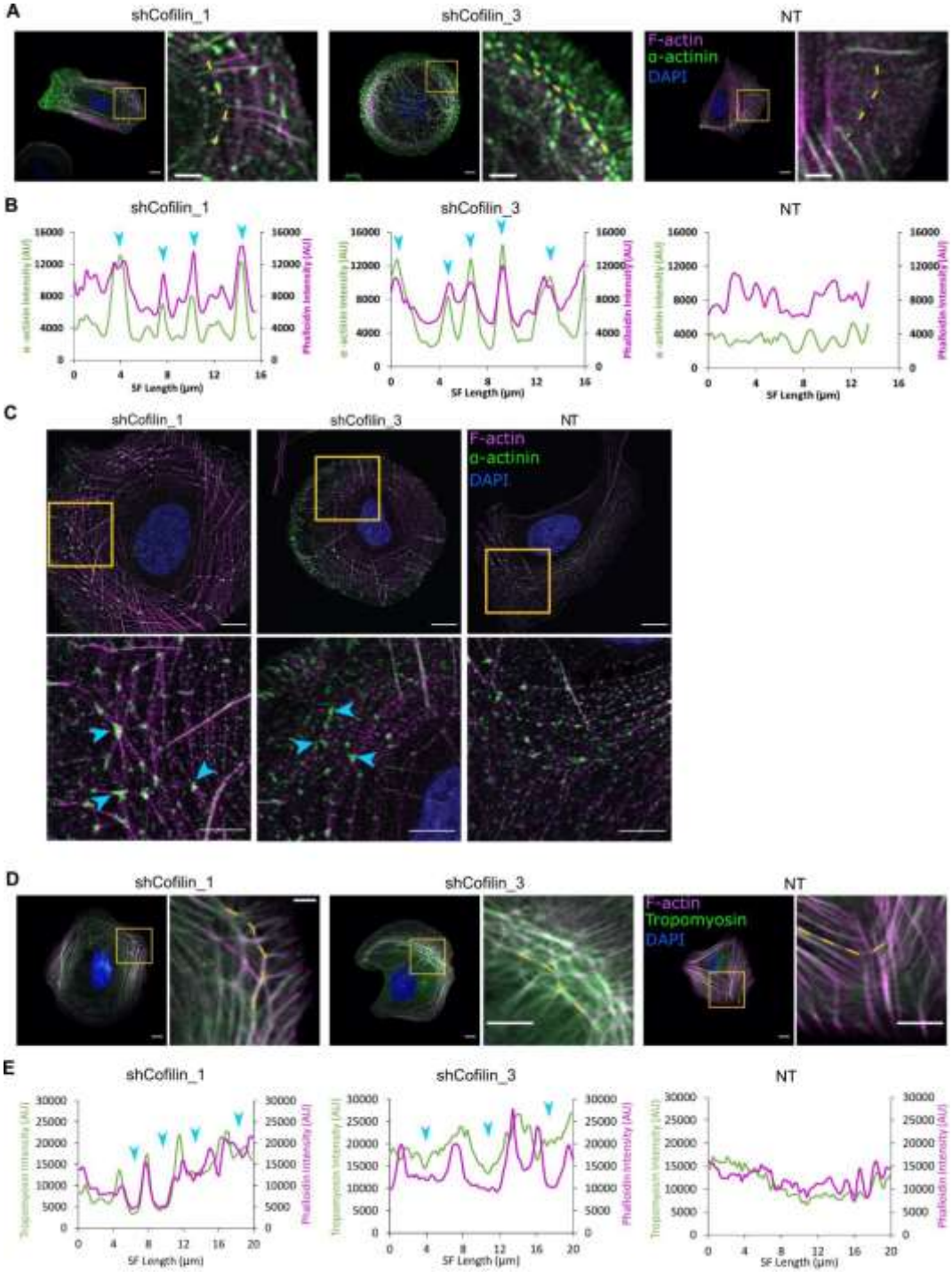

Figure S4. Transverse arc nodes are enriched in α-actinin and devoid of tropomyosin. (A) Confocal images of cofilin KD and NT cells stained for F-actin (magenta), α-actinin (green), and DAPI (blue). Images reproduced from Fig. 4C. (B) Line scans along transverse arcs (indicated by the dashed yellow line in panel A) in the α-actinin (green) and phalloidin (magenta) channels. Blue arrow heads indicate nodal regions of α- actinin clusters. (C) SIM images of cofilin KD and NT cells stained for F-actin (magenta), α-actinin (green), and DAPI (blue). Blue arrowheads point to α-actinin clusters at nodal points. White arrows point to small, periodic α-actinin clusters. (D) Confocal images of cofilin KD and NT cells stained for F-actin (magenta), tropomyosin (green), and DAPI (blue). Images reproduced from Fig. 4D. (E) Line scans along transverse arcs (indicated by the dashed yellow line in panel A) in the tropomyosin (green) and phalloidin (magenta) channels. Blue arrow heads indicate nodal regions devoid of tropomyosin. Scale bars for all panels: 10 μm, Inset: 5 μm.

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4. The same thing can be said for the claimed absence of co-localization between nodes and ppMLC.

We have added an additional panel in Fig. 5 to show line scans along transverse arcs in cofilin KD and NT cells in both the α-actinin and ppMLC channels to visualize changes in fluorescence intensity in the respective channels (Fig. 5B, reproduced below). The line scans indicate that decreases in ppMLC fluorescence intensity (gray line) correspond to the α-actinin-rich nodes (blue arrowheads). NT cells also have a similar pattern where decreases in ppMLC correspond to the α-actinin-rich regions, as would be expected in normal sarcomeric structures.

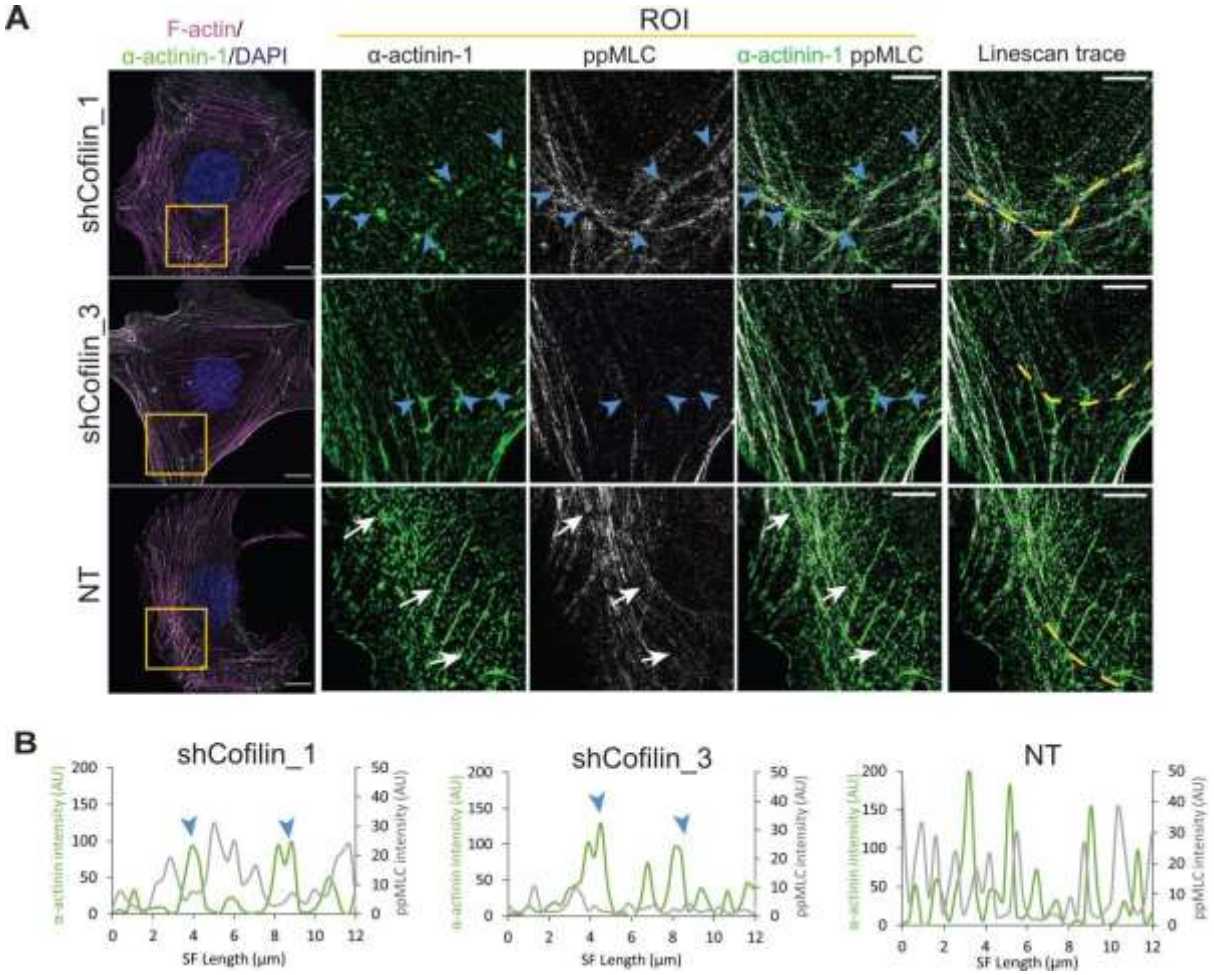

Figure 5. Transverse arcs in cofilin KD cells generate lower contractile forces. (A) SIM images of U2OS cells stained for α-actinin and ppMLC. Blue arrowheads point to α-actinin clusters and white arrows point to smaller, periodic α-actinin bands. Scale bar: 10 μm, ROI: 5 μm. Magenta: F-actin, green: α-actinin, white: ppMLC blue: DAPI. (B) Line scans along transverse arcs (indicated by the dashed yellow line in panel A) in the ppMLC (gray) and α-actinin (green) channels. Blue arrow heads indicate nodal regions devoid of ppMLC.

References

Goyal, P., Pandey, D., Brünnert, D., Hammer, E., Zygmunt, M. and Siess, W. (2013). Cofilin Oligomer Formation Occurs In Vivo and Is Regulated by Cofilin Phosphorylation. *PLoS One* **8**, e71769.

Kassianidou, E., Hughes, J. H. and Kumar, S. (2017). Activation of ROCK and MLCK tunes regional stress fiber formation and mechanics via preferential myosin light chain phosphorylation. *Mol.*

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Biol. Cell **28**, 3832–3843.

Lai, F. P., Szczodrak, M., Block, J., Faix, J., Breitsprecher, D., Mannherz, H. G., Stradal, T. E., Dunn, G. A., Small, J. V. and Rottner, K. **(2008). Arp2/3 complex interactions and actin network turnover in lamellipodia.** ***EMBO J.*** 27**, 982–992.**

Lee, S., Kassianidou, E. and Kumar, S. (2018). Actomyosin stress fiber subtypes have unique viscoelastic properties and roles in tension generation. *Mol. Biol. Cell* **29**, 1992–2004.

Pfannstiel, J., Cyrklaff, M., Habermann, A., Stoeva, S., Griffiths, G., Shoeman, R. and Faulstich, H. (2001). Human Cofilin Forms Oligomers Exhibiting Actin Bundling Activity. *J. Biol. Chem.* **276**, 49476– 49484.

Second decision letter

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AUTHORS: Stacey Lee and Sanjay Kumar
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I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The manuscript provides important new information on the role of cofilin in stress fiber assembly, and is in my opinion now acceptable for publication.

Comments for the author

The authors have satisfactorily addressed my previous concerns.

Reviewer 3

Advance summary and potential significance to field

The authors have adequately addressed my concerns and the manuscript is in my view now ready for publication.

Comments for the author

The authors have adequately addressed my concerns and the manuscript is in my view now ready for publication.

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