JCB

Microtubules promote intercellular contractile force transmission during tissue folding

Clint Ko, Vardges Tserunyan, and Adam Martin

Corresponding Author(s): Adam Martin, Massachusetts Institute of Technology

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March 7, 2019

Re: JCB manuscript #201902011

Dr. Adam C Martin
Massachusetts Institute of Technology
31 Ames St Building 68, Room 459
Cambridge, MA 02142

Dear Adam,

Thank you for submitting your manuscript entitled "Microtubules stabilize intercellular contractile force transmission during tissue folding". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that all three reviewers have substantial enthusiasm for the topic and the initial observation. However, all also think some of the major conclusions require more substantiation. Reviewer 1 points out the lack of a direct mechanistic connection between effects in microtubules, myosin, actin and junctions, and some lack of clarity about the directionality of the effects. They offer some solid suggestions for firming this up. Reviewer 2 and 3 echo these concerns, with a particular emphasis on effects on adherens junctions. Reviewer 3 also offers alternate explanations and wants more clarity on the effect on cell shape.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication.

Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript 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.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, 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. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Mark Peifer, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

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

This manuscript addresses the understudied issue of how microtubules influence contractile events. The authors use the well-characterized model of mesoderm invagination in the early fly embryo. Imaging both MTs and the minus-end-binding protein, patronin, they identify a pool of MTs that concentrate in the contractile medial-apical zone as mesodermal cells invaginate. Strikingly, this patronin-decorated network undergoes periodic condensation that appears to be influenced by RhoA-dependent actomyosin contractility. In turn, MT integrity and patronin are necessary for invagination. The authors propose that this represents a myosin-induced MTOC that serves to preserve the force-balancing of contractile networks across the epithelium, by maintaining the mechanical connection between the contractile medial-apical network and AJ. Specifically, condensation of myosin networks and the initial apical constriction of cells appears to begin unaffected, but later in the developmental process the medial-apical Myosin networks seem to "fracture", something that the Martin group reported earlier when actin dynamics were disrupted. This they describe as a separation of myosin II from junctions.

The manuscript in its current form contains many interesting observations, supported by clear data of high quality. I strongly suspect that the authors are onto something interesting, but at the moment the depth of its analysis is somewhat limited for the audience of JCB.

Specific comments

1) Actomyosin attachment to AJ. It looks to me that the medial apical (acto)myosin network is fracturing in the MT/patronin-disrupted cells. At some general level, you could argue that this reflects separation between the contractile network and AJ. However, this formulation implies that there might be some defect in the molecular apparatus(es) that couple the actomyosin network to cadherins. But this is not yet explored in the current manuscript. The data shown could alternatively be explained by a defect in the cortical actin itself, such that it is more predisposed to stress-induced rupture, and consequent dislocation of myosin condensates.
- One experiment that the authors could try would be to image the cortical F-actin itself with one of the F-actin sensors that are now readily available. It would be especially interesting to study early events at the cell-cell junctions (perhaps rupture does occur here as a first event?).
- A second question is how MTs might be having these effects. I appreciate that this is a complicated problem. Some possibilities that could be considered include RhoA signalling itself (which, as the authors know, can be influenced by MTs in diverse ways, and can now be visualized by a variety of sensors, including the kinase-dead ROCK that the Martin lab have used previously); and also MT-binding proteins that can influence actin dynamics, such as mDia and APC.

2) I am uncomfortable with the proposal that myosin sets up a MTOC. Agreed, the medial-apical condensates of patronin are disrupted, but at the current level of spatial and temporal resolution of the experiments this could reflect a role for myosin in concentrating minus-ends by advection, rather than creating a nucleation site. To make this conclusion, they really more direct tests of MT nucleation. These could include live-cell imaging for MT growth, especially after the MT network is first depolymerized with e.g. colchicine.

Small questions:

1) How long were drugs injected for? Was this acute manipulation of the cytoskeleton or something longer, where secondary effects were more likely to have occurred?

2) For clarity, when did the fracturing occur? Presumably after the 5 min period when they studied initial apical constriction.

3) Fig 5. Would it be helpful to have some vertical views of the embryos, to judge the degree of tissue folding (or its failure)? These are in the supplement and could be moved up. This is for patronin RNAi and I would recommend emphasizing this a little more, given that it provides the authors with a molecular handle for their analysis.

4) The authors conclude that there is no disorganization of the apical cortex, but this is based only on ROCK staining and E-cadherin, so doesn't pursue characterization in great detail.

5) It seems a little odd to have the actin-disruption data in Fig 7, when it is first mentioned much earlier.

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

This paper identifies a microtubule array organized by an actomyosin network and a role for microtubules in the transmission of apical actomyosin forces for invagination of the Drosophila ventral furrow. The study mainly focuses on the MT minus end binding protein Patronin. Patronin is

shown to uniquely localize to the apicomedial domain of apically constricting cells, in contrast to junctional localization in neighboring ectodermal cells, and Patronin organizes a stabilized, non-centrosomal MT network in the apicomedial domain. Actin and Rho pathway signaling are important for Patronin recruitment to the apicomedial domain, and apicomedial Patronin puncta coalesce and disperse with apicomedial myosin pulses. Depletion of Patronin, or inhibition of MTs, has minimal effects on the myosin pulsing or apicolateral junctions, but lead to transient, abnormal spatial separations of actomyosin pulses and a failure to invaginate the tissue. Overall, the paper is interesting for (i) identifying a MT network organized by actomyosin contractions and (ii) for showing a role for these MT networks in controlling the transmission of myosin-based forces between cells. The following points should be addressed.

1. For Patronin RNAi, was the penetrance of the apical acetylated tubulin microtubule reduction similar to the fairly low penetrance of the myosin network separations and tissue folding disruption (11/44 embryos)?

2. For the late cellularization embryo depleted of Patronin in Fig S3B, apicolateral E-cadherin accumulation seems to be reduced. Thus, the authors' over-state that there are "no defects" in apical adherens junction assembly. As shown by others and by the data in the current paper, these defects are transient however, and thus may not affect the authors' overall conclusions.

3. Although MTs might extend from the apical organizing centers to AJs as the authors discuss, it seems MTs could also extend downwards into the cell and perhaps integrate with lateral microtubule networks, or nucleus-associated networks. Such integration could stiffen the tissue and thereby increase force transmission from the apical actomyosin pulses. Since the authors have not defined how the MTs enhance force transmission, various possible mechanisms should be acknowledged. In particular, the authors should be more open to possibilities in the Results section, where they discuss how MTs could affect actomyosin assembly or AJ positioning or that "A final possibility is that microtubules regulate the connection between actomyosin networks and the adherens junctions", and in a following section, "A remaining possibility was that microtubules mediate the connection between actomyosin and adherens junctions". Since there is no data provided for such a connection, the authors should make it clear that there may be a number of ways the MTs could be contributing.

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

Tissue biogenesis often involves folding of an epithelial sheet. It is well-accepted that this tissue-level morphogenesis is achieved by contractility at the level of the cell. The cortical actomyosin cytoskeleton undergoes contractility, and the apical aspect of the cell becomes smaller, while the basolateral parts of such cells are not (as) contractile; differential contractility in the apical domain transforms columnar cells into wedge or cone shapes. The translation of cell behavior to the tissue level requires that contractile forces are conveyed throughout the tissue via intercellular junctions. The mechanism of connection between the apical actomyosin cytoskeleton and adherens junctions is unknown. Here, Ko and colleagues extend published work on the roles of microtubules in this coupling. They find that wholesale microtubule (MT) perturbations and depletion of the MT-minus-end-binding protein Patronin cause defects in apical cytoskeletal organization and in tissue folding.

Some of the authors' conclusions are not well-founded, and the overall effect of perturbing actin-junction linkage and tissue folding, without perturbing apical constriction is very puzzling. I offer

Major and Minor considerations to hopefully bring some clarity.

Major points:

1) Why doesn't the tissue fold if the apical aspect of the cells decreases (Figure 5A-C)? The cells must stay columnar instead of becoming wedge-shaped. That cell shape defect seems to be at least as much of a problem as actin-junction connection.

2) Why does Patronin depletion have a more severe effect than the MT drugs on the rate of apical contraction, slowing it down apparently (according to timepoints shown in Figure S3)?

3) It's fine to say the MT perturbations don't (grossly) perturb adherens junction assembly, as in the Results section heading, but the authors' data do not support the assertion that "There were no defects in apical E-cadherin polarity in either case [of MT drug treatment or Patronin depletion] (Fig. 5 F; Fig. S3, B and C)." I can see in 5F that cadherin label goes much further down the basolateral surface, and is brighter. (Assuming identical image acquisition and display), in late cellularization, there is also much more cadherin in the basolateral region following patronin depletion, and even more at the apical region. (S3C is not effective at showing anything, with the way the images are cropped and with the orientation of the basolateral surface with the axis of lowest optical resolution.) This may relate to overall cell shape (see #1).

4) It seems like the major problem is consistency of apical contraction - in controls, cell size has low variance; with the MT perturbations, area variance is high in images (why is this not captured in Figure 5B?). Perhaps either the cells continue past a certain point and contract down more than controls, or neighbors don't react to contraction of some cells which keep getting smaller while the neighbors are pulled large. Is this a problem of mechanosensation?

Minor points:

1) Might mesoderm cell shape be constrained by something (abnormal) going on in the rest of the embryo?

2) The authors' assertion in the Introduction that throughout the literature "many of these studies involved depolymerizing or eliminating the entire microtubule cytoskeleton, making it difficult to determine if microtubules play other roles." is strange given their use of entire-microtubule-cytoskeleton perturbations too.

3) Figure 1D is confusing. It should not be a line graph, since the range across the x axis is not continuous (i.e. time or space) but rather bins of normalized intensity. And then the top brackets make spatial designations.

4) To support the authors' conclusion that "Patronin depletion dramatically reduced visible bundles of apical acetylated-Tubulin in mesoderm cells," Figure 2D needs magnified en face views to accompany the tissue-level display - so readers can inspect and appreciate parallel/horizontal bundles.

5) To reference the concept that RhoA regulates contractility via regulating F-actin, a review article or a foundational research paper would be more appropriate than the 2005, '07, '15 and '18 papers. Watanabe... Narumiya et al EMBOJ 1997 and Evangelista... Boone et al Science 1997 come to mind.

6) Was intensity in Figure 4B and D normalized to apical area?

7) In Figure 3E and 5D, the y axis label "r" should be labeled more helpfully; i.e. what's being compared. "r" is the units.

8) The authors should check for verb tense / typos i.e. "foci... organize(s)"

Response to reviewer comments:

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

This manuscript addresses the understudied issue of how microtubules influence contractile events. The authors use the well-characterized model of mesoderm invagination in the early fly embryo. Imaging both MTs and the minus-end-binding protein, patronin, they identify a pool of MTs that concentrate in the contractile medial-apical zone as mesodermal cells invaginate. Strikingly, this patronin-decorated network undergoes periodic condensation that appears to be influenced by RhoA-dependent actomyosin contractility. In turn, MT integrity and patronin are necessary for invagination. The authors propose that this represents a myosin-induced MTOC that serves to preserve the force-balancing of contractile networks across the epithelium, by maintaining the mechanical connection between the contractile medial-apical network and AJ. Specifically, condensation of myosin networks and the initial apical constriction of cells appears to begin unaffected, but later in the developmental process the medial-apical Myosin networks seem to "fracture", something that the Martin group reported earlier when actin dynamics were disrupted. This they describe as a separation of myosin II from junctions.

The manuscript in its current form contains many interesting observations, supported by clear data of high quality. I strongly suspect that the authors are onto something interesting, but at the moment the depth of its analysis is somewhat limited for the audience of JCB.

> We would like to thank the reviewer for their positive remarks and carefully reading our manuscript. Their suggestions and comments were constructive and valuable. We have addressed as many of their points as possible and this has strengthened our manuscript.

Specific comments

1) Actomyosin attachment to AJ. It looks to me that the medial apical (acto)myosin network is fracturing in the MT/patronin-disrupted cells. At some general level, you could argue that this reflects separation between the contractile network and AJ. However, this formulation implies that there might be some defect in the molecular apparatus(es) that couple the actomyosin network to cadherins. But this is not yet explored in the current manuscript. The data shown could alternatively be explained by a defect in the cortical actin itself, such that it is more predisposed to stress-induced rupture, and consequent dislocation of myosin condensates.
- One experiment that the authors could try would be to image the cortical F-actin itself with one of the F-actin sensors that are now readily available. It would be especially interesting to study early events at the cell-cell junctions (perhaps rupture does occur here as a first event?).

> We agree with the reviewer and did as they suggested. We imaged F-actin in both live and fixed embryos and show that the fracture occurs between the medioapical domain of myosin and the junctions (Fig. 6 D). Moreover, we measured a significant difference in the lifetime of holes or tears in the apical F-actin meshwork after microtubule disruption, whereas control embryos repair holes more quickly due to actin turnover (Jodoin et al., 2015) (Fig. 6, D-E). This supports our model of microtubules promoting the connection between actomyosin and AJs. This result also suggests that actin turnover, or some other aspect of F-actin behavior/organization, is regulated by microtubules.

- A second question is how MTs might be having these effects. I appreciate that this is a complicated problem. Some possibilities that could be considered include RhoA signalling itself (which, as the authors know, can be influenced by MTs in diverse ways, and can now be visualized by a variety of sensors, including the kinase-dead ROCK that the Martin lab have used previously); and also MT-binding proteins that can influence actin

dynamics, such as mDia and APC.

> We showed that the localization of Rho Kinase and the downstream output of the Rho pathway, myosin activation, were all normal after disrupting microtubules (Fig. 5, A-E; Fig. S3 A; Fig. S4 B). In addition, we provide evidence that the primary defect of microtubule disruption is not due to an inability to establish polarized, functional adherens junctions (Fig. 5, F-H; Fig. 6 B; Fig. S3, B-C). We have now examined F-actin dynamics, using Utr as a live reporter of F-actin and demonstrated that microtubule disruption leads to more unstable (longer-lived) fractures and holes in the apical F-actin meshwork near cell junctions (Fig. 6, D-E), similar to a defect in actin turnover. Finally, we examined whether known F-actin-binding proteins that localize to junctions were mis-localized after microtubule disruption. After taxol injection, we observed that junctional Dia localization was unaffected but that Cno lost its localization (Fig. 6, G-H). We cannot rule out the possibility that the activity of Dia at junctions is affected, and that might explain the phenotype we observe. Because the repairs of actomyosin network separations we observe is not consistent with *cno* mutants (Sawyer et al., 2009), we don't think that loss of Cno at junctions is the causative agent for the furrowing phenotype; rather, it may be a consequence of repeated actomyosin network separations.

2) I am uncomfortable with the proposal that myosin sets up a MTOC. Agreed, the medial-apical condensates of patronin are disrupted, but at the current level of spatial and temporal resolution of the experiments this could reflect a role for myosin in concentrating minus-ends by advection, rather than creating a nucleation site. To make this conclusion, they really more direct tests of MT nucleation. These could include live-cell imaging for MT growth, especially after the MT network is first depolymerized with e.g. colchicine.

> We entirely agree that myosin contractility could concentrate microtubule minus ends and Patronin structures by advection and have added this interpretation to the Results. However, it is quite apparent that microtubules are organized and that this occurs in a center in the medioapical domain, which is why we refer to it as a microtubule organizing center. To make this point stronger and to determine whether the behavior of this center is similar to other MTOCs, we imaged microtubules with higher spatiotemporal resolution to visualize CLIP170 comets (Fig. 2, D and E), which track with plus-end growth. We observe microtubule growth emanating from medioapical patches towards cell junctions. This observation combined with the clear polarity and enrichment of microtubule binding proteins (Fig. 1 B; Fig. 2 C), suggests that this is a microtubule-organizing center.

Small questions:

1) How long were drugs injected for? Was this acute manipulation of the cytoskeleton or something longer, where secondary effects were more likely to have occurred?

> Imaging typically occurred within minutes of injection. We varied the timing of injection as well and did not observe significant differences in phenotype. The timing of injection is now explained more explicitly in the revised manuscript (lines 256-257).

2) For clarity, when did the fracturing occur? Presumably after the 5 min period when they studied initial apical constriction.

> Yes, we consistently observed the fractures 5 minutes after image acquisition (which occurred within minutes of drug injection). Initial phases of tissue folding appeared normal but as apical myosin accumulated at greater levels, a coherent supracellular myosin network was unable to form because of network separations. We made this more explicit in the revised manuscript (lines 301-304; 321-325).

3) Fig 5. Would it be helpful to have some vertical views of the embryos, to judge the degree of tissue folding (or its failure)? These are in the supplement and could be moved up. This is for patronin RNAi and I would recommend emphasizing this a little more, given that it provides the authors with a molecular handle for their analysis.

> Cross-section views were present with the *en face* views in Fig. 5 A but were hard to see in the original submission. We've made those easier to see and the different views are now clearly labeled. We chose not to focus on *patronin* RNAi because the penetrance of the phenotype was much lower and interpretations are further complicated by the fact that only Patronin-depleted embryos exhibited an initial heterogeneity in apical cell area (described in Takeda et al., 2017).

4) The authors conclude that there is no disorganization of the apical cortex, but this is based only on ROCK staining and E-cadherin, so doesn't pursue characterization in great detail.

> We reworked the organization of the last Results section and looked additionally at the apical F-actin meshwork and the localization of Dia and Cno.

5) It seems a little odd to have the actin-disruption data in Fig 7, when it is first mentioned much earlier.

> The data in Fig. 7 B has been moved to S2 C and is mentioned earlier in the fourth section (lines 223-224).

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

This paper identifies a microtubule array organized by an actomyosin network and a role for microtubules in the transmission of apical actomyosin forces for invagination of the Drosophila ventral furrow. The study mainly focuses on the MT minus end binding protein Patronin. Patronin is shown to uniquely localize to the apicomedial domain of apically constricting cells, in contrast to junctional localization in neighboring ectodermal cells, and Patronin organizes a stabilized, non-centrosomal MT network in the apicomedial domain. Actin and Rho pathway signaling are important for Patronin recruitment to the apicomedial domain, and apicomedial Patronin puncta coalesce and disperse with apicomedial myosin pulses. Depletion of Patronin, or inhibition of MTs, has minimal effects on the myosin pulsing or apicolateral junctions, but lead to transient, abnormal spatial separations of actomyosin pulses and a failure to invaginate the tissue. Overall, the paper is interesting for (i) identifying a MT network organized by actomyosin contractions and (ii) for showing a role for these MT networks in controlling the transmission of myosin-based forces between cells. The following points should be addressed.

> We thank the reviewer for their helpful comments and have addressed the points below.

1. For Patronin RNAi, was the penetrance of the apical acetylated tubulin microtubule reduction similar to the fairly low penetrance of the myosin network separations and tissue folding disruption (11/44 embryos)?

> We indicated how many embryos displayed the reduction of acetylated-Tubulin staining (line 154; 8/8 embryos). We think that the lower penetrance of the myosin network separation phenotype is due to variability on how disorganized the microtubule cytoskeleton was. We observed many cases where the tissue still folds even though microtubules appeared disorganized (presence of apical centrosomes; Fig. S1 C), suggesting that there may be a threshold of microtubule network disorganization above which the tissue is no longer able to fold.

2. For the late cellularization embryo depleted of Patronin in Fig S3B, apicolateral E-cadherin accumulation seems to be reduced. Thus, the authors' over-state that there are "no defects" in apical adherens junction assembly. As shown by others and by the data in the current paper, these defects are transient however, and thus may not affect the authors' overall conclusions.

> We agree that the original language in the manuscript overstated the effect of microtubule perturbation on adherens junction assembly or localization; we have changed the writing to reflect this. With data that was present in the original submission as well as new data we've added to the revision, we have shown both qualitatively and quantitatively that E-cadherin is present, polarized, and functional (Fig. 5, F-H; Fig. 6, B-D; Fig. S4 A). We have shown that β-catenin is present apically in Patronin-depleted embryos (Fig. S3 B). The phenotype of depleting adherens junction components (Martin et al., 2010) is different than the actomyosin separation phenotype we observe. While we cannot rule out that some aspect of adherens junctions are defective (especially after repeated instances of actomyosin separations from junctions), we believe the evidence listed in the revised manuscript support our argument that the primary defect does not occur at junctions.

3. Although MTs might extend from the apical organizing centers to AJs as the authors discuss, it seems MTs could also extend downwards into the cell and perhaps integrate with lateral microtubule networks, or nucleus-associated networks. Such integration could stiffen the tissue and thereby increase force transmission from the apical actomyosin pulses. Since the authors have not defined how the MTs enhance force transmission, various possible mechanisms should be acknowledged. In particular, the authors should be more open to possibilities in the Results section, where they discuss how MTs could affect actomyosin assembly or AJ positioning or that "A final possibility is that microtubules regulate the connection between actomyosin networks and the adherens junctions", and in a following section, "A remaining possibility was that microtubules mediate the connection between actomyosin and adherens junctions". Since there is no data provided for such a connection, the authors should make it clear that there may be a number of ways the MTs could be contributing.

> In the revised manuscript we have improved the spatial/temporal resolution of our CLIP170 imaging to show that microtubules clearly grow from the medioapical MTOC out to the junctions. In addition, we have imaged F-actin to show that fractures in the supracellular network occur between the medioapical myosin and the junctions and are longer-lived (not repaired as quickly as WT). Finally, we have revised the writing in the manuscript to acknowledge other possible mechanisms.

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

Tissue biogenesis often involves folding of an epithelial sheet. It is well-accepted that this tissue-level morphogenesis is achieved by contractility at the level of the cell. The cortical actomyosin cytoskeleton undergoes contractility, and the apical aspect of the cell becomes smaller, while the basolateral parts of such cells are not (as) contractile; differential contractility in the apical domain transforms columnar cells into wedge or cone shapes. The translation of cell behavior to the tissue level requires that contractile forces are conveyed throughout the tissue via intercellular junctions. The mechanism of connection between the apical actomyosin cytoskeleton and adherens junctions is unknown. Here, Ko and colleagues extend published work on the roles of microtubules in this coupling. They find that wholesale microtubule (MT) perturbations and depletion of the MT-minus-end-binding protein Patronin cause defects in apical cytoskeletal organization and in tissue folding.

Some of the authors' conclusions are not well-founded, and the overall effect of perturbing actin-junction linkage and tissue folding, without perturbing apical constriction is very puzzling. I offer Major and Minor

considerations to hopefully bring some clarity.

> We thank the reviewer for their constructive suggestions and comments. We think that addressing their points has greatly increased the clarity of the manuscript.

Major points:

1) Why doesn't the tissue fold if the apical aspect of the cells decreases (Figure 5A-C)? The cells must stay columnar instead of becoming wedge-shaped. That cell shape defect seems to be at least as much of a problem as actin-junction connection.

> Initially, everything appears normal (except for the cell size heterogeneity in Patronin RNAi) and cells constrict well. However, folding doesn't happen until cells reduce their apical area by more than 50 % (Martin et al., 2009). After apical myosin has accumulated and when folding would normally happen, myosin networks begin to separate from junctions. When these separations occur, cells lose their constricted state at the apical surface (evident in Fig. 6, A-B). The disruption of the apical actomyosin network is the most prominent phenotype and given its importance, we argue that this is what results in the tissue folding defect.

2) Why does Patronin depletion have a more severe effect than the MT drugs on the rate of apical contraction, slowing it down apparently (according to timepoints shown in Figure S3)?

> The montage was misleading due to incorrect time stamps, which have been fixed. One major difference between Patronin RNAi and other microtubule perturbations is that Patronin RNAi leads to an initial heterogeneity in apical cell area sizes before any appreciable myosin accumulation. The smaller cells constrict a lot and the bigger cells sometimes expand. One possible explanation for this effect has been proposed by Takeda et al. 2017, where a disorganized cortical microtubule network affects the ability of cells to mechanically resist stochastic fluctuations in cell constrictions.

3) It's fine to say the MT perturbations don't (grossly) perturb adherens junction assembly, as in the Results section heading, but the authors' data do not support the assertion that "There were no defects in apical E-cadherin polarity in either case [of MT drug treatment or Patronin depletion] (Fig. 5 F; Fig. S3, B and C)." I can see in 5F that cadherin label goes much further down the basolateral surface, and is brighter. (Assuming identical image acquisition and display), in late cellularization, there is also much more cadherin in the basolateral region following patronin depletion, and even more at the apical region. (S3C is not effective at showing anything, with the way the images are cropped and with the orientation of the basolateral surface with the axis of lowest optical resolution.) This may relate to overall cell shape (see #1).

> The cross-section images are misleading because cells are not oriented perfectly perpendicular to the apical surface; cells are curved. Therefore, it's difficult to assess whether changes in intensity along the apical-basal axis are due to a defect or simply the cell-cell interface moving in and out of plane. Because such a view is misleading and not very informative, we chose to remove them and instead quantified the ratio of junctional to medial E-cadherin intensity (Fig. 5 G), similar to a previous analysis we used (Jodoin et al., 2015). Figures S3 B and C were included so readers could qualitatively appreciate the presence of β-catenin and E-cadherin on the apical side of cells, despite the pitfalls of this sort of cross-sectional view as explained above.

4) It seems like the major problem is consistency of apical contraction - in controls, cell size has low variance; with the MT perturbations, area variance is high in images (why is this not captured in Figure 5B?). Perhaps either the cells continue past a certain point and contract down more than controls, or neighbors don't react

to contraction of some cells which keep getting smaller while the neighbors are pulled large. Is this a problem of mechanosensation?

> Area variation is only high in Patronin RNAi. In most cases where MT drugs are injected, initial apical cell area and rate of constriction are not as variable and close to control conditions.

Minor points:

1) Might mesoderm cell shape be constrained by something (abnormal) going on in the rest of the embryo?

> Constraining mesoderm cell shape change by altering what happens in the rest of the embryo results in a qualitatively different phenotype, which is not observed when microtubules are disrupted. Expansion of the contractile domain in DV, which slows mesoderm cell shape change (Heer et al., 2017), results in myosin rings on the apical surface (Chanet et al., 2017), which we do not observe after disrupting microtubules. In addition, expanding the contractile domain in DV does not consistently result in fractures in the supracellular myosin network (Chanet et al., 2017). Changing stiffness of neighboring cells does not lead to myosin network separations (Perez-Mockus et al., 2017). Furthermore, acute microtubule disruption during folding causes immediate fracture of the supracellular myosin network in the ventral furrow, suggesting that the effect is direct. We've now shown that Fog expression reorganizes the microtubule cytoskeleton when ectopically expressed in other cells (Fig. 4 F), suggesting that microtubules have a specific function in Fog expressing cells.

2) The authors' assertion in the Introduction that throughout the literature "many of these studies involved depolymerizing or eliminating the entire microtubule cytoskeleton, making it difficult to determine if microtubules play other roles." is strange given their use of entire-microtubule-cytoskeleton perturbations too.

> True, our perturbations would affect the entire microtubule network in cells. However, neither taxol nor Patronin RNAi eliminates microtubules through severing or depolymerization. Instead, microtubule organization is affected, while many MT polymers are still intact (Fig. S1 C; Fig. S3 D). Furthermore, we have shown that acute injection of microtubule drugs exhibited an immediate effect on the ventral furrow. However, we removed that sentence and reworked the introduction.

3) Figure 1D is confusing. It should not be a line graph, since the range across the x axis is not continuous (i.e. time or space) but rather bins of normalized intensity. And then the top brackets make spatial designations.

> In order to make Fig. 1D less confusing, we displayed the data as a histogram showing the % of cells we measured falling into bins of ratios of junctional to medial intensities.

4) To support the authors' conclusion that "Patronin depletion dramatically reduced visible bundles of apical acetylated-Tubulin in mesoderm cells," Figure 2D needs magnified en face views to accompany the tissue-level display - so readers can inspect and appreciate parallel/horizontal bundles.

> Magnified *en face* views of both apical and sub-apical regions have been added for clarity in Fig. 2 F.

5) To reference the concept that RhoA regulates contractility via regulating F-actin, a review article or a foundational research paper would be more appropriate than the 2005, '07, '15 and '18 papers. Watanabe... Narumiya et al EMBOJ 1997 and Evangelista... Boone et al Science 1997 come to mind.

> We added those references and the Otomo et al. 2005 work as well.

6) Was intensity in Figure 4B and D normalized to apical area?

> No, we did not normalize to apical area because we were interested in measuring absolute Patronin::GFP intensity at the apical cortex independently of apical cell area.

7) In Figure 3E and 5D, the y axis label "r" should be labeled more helpfully; i.e. what's being compared. "r" is the units.

> Done.

8) The authors should check for verb tense / typos i.e. "foci... organize(s)"

> Done.

May 16, 2019

RE: JCB Manuscript #201902011R

Dr. Adam C Martin
Massachusetts Institute of Technology
31 Ames St Building 68, Room 459
Cambridge, MA 02142

Dear Adam:

Thank you for submitting your revised manuscript entitled "Microtubules promote intercellular contractile force transmission during tissue folding". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). Please note the typo correction noted by Reviewer 2, and make some minor changes to the text to address the issues raised by Reviewer 3--no new experiments or data are necessary and there will be no need for re-review.

- Provide supplementary text as a separate, editable .doc or .docx file

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

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B. FINAL FILES:

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Mark Peifer, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

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

Overall, the authors have reasonably addressed the points I raised earlier and I think that the revised MS will interest the general audience of The JCB. As reviewer 2 noted, one valuable contribution of the manuscript is to identify a novel mode of MT organization that is clearly important for tissue folding in this system. It appears to work by promoting actin turnover and repair, allowing coupling of the medial-apical network with adherens junctions. It would, of course, be even more satisfying to have a mechanism for this effect, but this clearly will need to be the subject for another project.

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

The authors have effectively addressed my past concerns. This is an interesting and important study.

I noticed one typo in line 346: for "(Jodoin et al.)", the year is missing.

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

For their revision, the authors did a tremendous amount of work and the manuscript is substantially

strengthened. I have a few minor comments.

1) Is expression Patronin or Canoe regulated by Twist and/or Snail? If Twist and/or Snail have know consensus sequences, this could be quickly examined and mentioned, since the text now leads to that question.

2) I think my confusion was about the distinction between apical contractility and apical constriction. I see that the former is plenty active, but the latter fails (at least at a tissue-wide level). I think the authors do a good (better?) job making this distinction, but I feel it could be made even more explicitly.

3) S3B shows clearly that the mesoderm of a Patronin-depleted embryo underwent apical constriction, but I see other problems such as nuclei at different levels within cells, possibly cells that have lost apical contacts (or are crooked within the image plane), and a binucleate cell.

4) Since wholesale microtubule perturbations such as Taxol can be pleiotropic, the authors should test how junctional Canoe localization is affected by Patronin depletion. As the authors state "Canoe (Cno) [is] the Drosophila Afadin homologue which mediates linkages between F-actin and adherens junctions (Sawyer et al., 2009; Choi et al., 2016)." Does it perform this known function in response to the Patronin-based network or does its localization simply depend on microtubule dynamics?

5) Single fluorophore images such as Figure 2B, D, G, 3A, 4F, should be black on white to optimally convey information, instead of green on black.

6) Typo: space missing in 7 axis label for 5D "areareduction"

2nd Revision - Authors' Response to Reviewers: May 21, 2019

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

Overall, the authors have reasonably addressed the points I raised earlier and I think that the revised MS will interest the general audience of The JCB. As reviewer 2 noted, one valuable contribution of the manuscript is to identify a novel mode of MT organization that is clearly important for tissue folding in this system. It appears to work by promoting actin turnover and repair, allowing coupling of the medial-apical network with adherens junctions. It would, of course, be even more satisfying to have a mechanism for this effect, but this clearly will need to be the subject for another project.

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

The authors have effectively addressed my past concerns. This is an interesting and important study.

I noticed one typo in line 346: for "(Jodoin et al.)", the year is missing.

> Fixed.

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

For their revision, the authors did a tremendous amount of work and the manuscript is substantially strengthened. I have a few minor comments.

1) Is expression Patronin or Canoe regulated by Twist and/or Snail? If Twist and/or Snail have know consensus sequences, this could be quickly examined and mentioned, since the text now leads to that question.

> Neither is known to be a transcriptional target of Twist/Snail. We think that the tissue-type specific organization of Patronin that we observe is not due to direct transcriptional regulation. Instead, because embryonic transcription factors activate *fog* signaling in the mesoderm and endoderm during gastrulation, activation of actomyosin contractility in these tissues drive the formation of medioapical Patronin foci specifically in the mesoderm and endoderm. We added parts of this to the discussion (lines 402 – 408).

2) I think my confusion was about the distinction between apical contractility and apical constriction. I see that the former is plenty active, but the latter fails (at least at a tissue-wide level). I think the authors do a good (better?) job making this distinction, but I feel it could be made even more explicitly.

> Overall, addressing this distinction has clarified the story and model.

3) S3B shows clearly that the mesoderm of a Patronin-depleted embryo underwent apical constriction, but I see other problems such as nuclei at different levels within cells, possibly cells that have lost apical contacts (or are crooked within the image plane), and a binucleate cell.

> In all Patronin-depleted embryos, cells initiate apical constriction. Only about 20% of embryos imaged displayed a myosin network separation phenotype, which results in expanded cell areas at later stages of folding. The most penetrant phenotype is the heterogeneity in initial apical cell area, which is associated with abnormal nuclear positioning (Takeda et al., 2018). It's unclear to what extent abnormal nuclear positioning plays into the phenotypes we described in our manuscript; however, most embryos that displayed abnormal nuclear positioning still fold. It's possible that nuclear positioning is more sensitive to smaller changes in microtubule organization, but much larger perturbations on microtubule organization are required to perturb tissue folding and intercellular contractile force transmission.

4) Since wholesale microtubule perturbations such as Taxol can be pleiotropic, the authors should test how junctional Canoe localization is affected by Patronin depletion. As the authors state "Canoe (Cno) [is] the Drosophila Afadin homologue which mediates linkages between F-actin and adherens junctions (Sawyer et al., 2009; Choi et al., 2016)." Does it perform this known function in response to the Patronin-based network or does its localization simply depend on microtubule dynamics?

> We think this is an interesting point and would be great to study in future work. It remains to be seen how direct of an effect microtubules or microtubule organization/dynamics plays a role in Cno localization at junctions.

5) Single fluorophore images such as Figure 2B, D, G, 3A, 4F, should be black on white to optimally convey information, instead of green on black.

Done.

6) Typo: space missing in 7 axis label for 5D "areareduction"

The label has been fixed.