Enhanced RhoA signaling stabilizes E-cadherin in migrating epithelial monolayers

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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 have raised several issues that I am confident you can address in a revised version of the paper. I would direct you to their specific comments for details. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

The manuscript describes how tension generated at cell-cell junction during epithelial migration maybe regulated by activated RhoA. The authors first show that junctional tension is increased in migrating MCF7 cells, accompanied by an accumulation of F-actin, NMIIA and NMIIB at cell-cell junctions. Next, the authors report that a molecular probe that binds selectively to the active GTP-bound form of RhoA accumulates at migrating cell borders. A comparable junctional accumulation of the probe is visualized in the zebrafish embryo at an early developmental stage where cells undergo collective migration. The authors then search for the Rho-GEF that activates RhoA. They focused on p114RhoGEF and showed that it accumulates at cell-cell junctions in migrating cells and that KD of p114RhoGEF prevents accumulation of both RhoA-GTP and NMIIA. The RhoA-GTP probe is made of a domain of the adaptor protein Anillin that has been proposed to link RhoA-GTP to Myosin II. The authors show that endogenous Anillin co-accumulates with E-cadherin at migrating cell borders, and that its down-modulation prevents the accumulation of F-actin, NMIIA and NMIIB (Fig1), RhoAGTP and E-Cadherin (Fig4) at migrating cell borders. Rescue experiments with WT (Fig1, Fig4 Ecadherin) or using a chimeric construct made of α-catenin and the Anillin-RhoA-GTP binding domain (Fig4 RhoA-GTP probe), in order to recruit selectively RhoA-GTP to cell-cell junctions, reverse these inhibitory effects. Finally, FRAP experiments on E-Cadh-mCherry indicate that knock-down of Anillin reduces its stability at migrating cell borders. Overall, these data, mostly correlative, led the authors to propose that RhoA-GTP activation and recruitment to cell-cell junctions stabilizes E-Cadherin, whose accumulation counteracts the increased tension forces generated upon collective migration.

Comments for the author

In general, the study is well conceived and logically organized. Most of the data support the conclusions drawn by the authors. The findings are technically sound, however the study in its current form is not appropriate for publication in Journal of Cell Science. Important areas to be clarified are outlined below.

Major concerns:
1) Anillin, through its down-modulation or by using various constructs, is used to address RhoA signaling in regulating mechanical forces during migration. Yet, the authors did not study the impact of manipulating Anillin on junctional tension. The authors should perform the experiments shown in Fig1C and/or Fig1D upon Anillin depletion to assess directly whether Anillin and RhoA signaling is involved in regulating tension.

2) Likewise, the study model is collective migration but the experiment showing the effect of Anillin down-modulation on collective migration is missing, it should be reported. At the end-point of the migration experiment, when the two cell monolayers close the gap and stop migrating, do the authors observe a reversal to pre-migratory E-cadherin and RhoA-GTP levels?

3) In Fig1, the tensile forces appear much stronger at the migration front. Also, in Fig2 the authors show that accumulation of RhoA is faster and more prominent in the first cell rows at the front of migration. The authors need to indicate for all their images in which part of the monolayer they took their pictures and should comment on whether all their markers accumulate with the same spatial pattern within the migrating cell monolayer.

4) The authors need to comment on the GFP-AHPH probe that may not just mark the presence of GTP-RhoA. It could also recruit GTP-RhoA from the cytosolic pool revealed by their FRET probe (FigS2C and see also Minor comment 4).

5) The magnification of the images varies in all figures, which does not help the reader to fully appreciate the staining patterns. This is especially true when comparing the “RhoA-GTP probe”

(see major comment 7) and MyoIIA labeling in Fig3 D/F. Images of the same magnification as 3D need to be shown for 3F.

6) Fig3D, the quality of the images is very poor especially for LARG KD, where no increase in RhoA staining is visible. At time 0, the image appears out of focus. New images need to be shown, as they do not reflect the quantification. Does LARG accumulate at migrating cell borders?

7) Fig3D is entitled “GTP-RhoA”, in the legend it says “GFP-RhoA (detecting GTP-RhoA)” and in the text only GTP-RhoA is mentioned. Did the authors really use GFP-RhoA as indicted in the figure legend or is it Anillin-GFP as in Fig2A (GFP-AHPH)? GFP-RhoA can not be used to label the GTP-bound form of RhoA, as GFP-RhoA is mainly GDP-bound. If it is not a labeling mistake, then the authors need to show staining with GFP-AHPH.

8) Finally, there seems to be a discrepancy between the numbers shown in 4F and 4G for the KD samples. The KD0 and KD12 curves indicate that the plateau of recovery are much higher compared to the Ctrl0, yet the bar graph indicates a similar immobile fraction. The authors need to explain how they extracted the immobile fraction values from the Recovery curves?

Minor comments:
1) Figure legend 1A,B indicates the use of Caco-2 cells, I believe the authors meant MCF-7 cells. Figure legend 1(E-J), “Myosin IIB (GI)” should be “Myosin IIB (GJ). Figure legend 3(J-K), it is written “Anillin increases AJ in migrating…”. Should not it be “Anillin increases at AJ in migrating…”?

2) Fig1G, the authors show the accumulation of NMIIB together with that of F-actin and NMIIA at migrating cell borders and conclude that cortical actomyosin may contribute increased contractility during migration. Yet, in their previous work (Priya et al, 2015) with MCF7 cells, they showed that although NMIIB accumulates at ZA, its down-regulation did not affect RhoA or RhoA-GTP accumulation at cell junctions. The authors could consider analyzing the impact of the down-regulation of NMIIA or NMIIB on RhoA-GTP accumulation, or tune down their conclusion by referring to their previous observation.

3) Fig3C, the WB of total p114 indicates that there is an increase in p114 levels at 6hrs as the loading control signal is much lower when compared to the control. The authors need to show a representative WB and the quantification of their replicates that match their conclusion.

4) FigS2C, the use of FRET reporter activity nicely confirmed the up-regulation of RhoA-GTP in migrating cells. However, in contrast with the Anillin-based probe, the FRET probe shows a strong accumulation of RhoA activation away from the cell junctions. The authors should comment on the different patterns displayed by their two probes, and the possibility, or not, that activated RhoA away from cell junctions could play a role in the regulation of cortical mechanical forces in migrating cells.

5) In light of the model proposed by the authors whereby RhoA activation stimulates E-cadherin stabilization and accumulation at migrating cell borders, could they comment on the sequential accumulation of the different molecular actors (E-cadh, p114, RhoA, Anillin, MyoIIA) upon migration, and the existence of positive feedback loop?

Reviewer 2

Advance summary and potential significance to field

The authors demonstrate the importance of GTP-RhoA recruitment for actomyosin network and E-cadherin association with cell contacts during the migration of MCF7 monolayer, as well as during zebrafish epiboly. These results are important for the understanding of RhoA dynamics during epithelial remodeling, and will be of great interest for the readers of Journal of Cell Science.

Comments for the author

The experiments seem to be performed carefully. However, some of the data analysis procedures or presentation need to be further clarified. Additionally, some figures definitely need to be

remodeled, and the conclusion / discussion to be further developed.

Major comments :
1) Figure S1 : It would be good to show lamellipodia in the context of the monolayer as well; it is difficult to be convinced only on the base of a close view on one cell. By the way, why such emphasis is made about cryptic lamellipodia (Figure S1, Movie 1) if they are not used, studied, at all in the core of the study ? Does the level of GTP-RhoA at cell contacts during MCF7 migration impact the formation or the dynamics of cryptic lamellipodia ?

2) Page 3, last paragraph: why discussing a data of the Figure 4D here ? Why not showing monolayers stained for E-cadherin at 0 and 12hr to start Figure 1 ?

3) Page 4, first paragraph: does this increase in tension concern several cell rows ? Which ones ? In fact, it seems that increase in tension, as it is presented in 1A, occurs preferentially within the first rows of cells, close to monolayer margin, which is not consistent with the images of AHPH signal showed in 2A.

4) Figure 1C: it would be nice to have images to visualize the recoil, in addition to the graph.

5) In the figure legend of Figure 1, why Caco2 cells are mentioned ? I didn't see any Caco2 cells in the manuscript nor figures.

6) Figure 1D: it is difficult to distinguish between magenta and blue signals. Moreover, it would be good to show images of separate channels in addition to merged channels to better assess alpha18 increase. Does this increase in alpha 18 occur in the global monolayer, or in specific cell rows ?

7) Figure 1C-D: how many events or cells among three experiments have been used for these quantifications ?

8) Authors mention that the 3 NMII paralogs are expressed in MCF7 but only present the data for MyoIIA and MyoIIB; why not MyoIIC ?

9) Figure 1 F-G: does the increase of MyoIIA and IIB also occur at cell contacts in the monolayer margin ?

10) Figure 1E-J: authors show here data about anillin-KD but they are not mentioned at all in the result section for Figure 1; why ??

11) Figure 2A: the authors should use a color-coding of the AHPH signal to avoid confusion.

12) Page 5, second paragraph, and Figure 2A: do differences exist for AHPH junctional recruitment between cell rows from the monolayer margin? It seems to be the case from the images in 2A. In fact, quantifications according to cell rows are shown in Figure 2B but not described in the result section. Looking more carefully at the graph 2B, there are inconsistencies between graph2B and images presented in 2A: in 2A, it seems that cell rows 1 to 3-4 do not exhibit much junctional AHPH whereas cell rows 4-5 to 15 do. Is it the case? However, in the graph 2B, quantifications show a strongest junctional AHPH in cell rows 1 to 5. Could the authors comment on this?
By the way, could the authors add in this figure a scheme clearly explaining the cell row delimitations to avoid reader confusion.

13) Figure 2A, time point 0h: why is there AHPH signal at "lamellae of leader cells"? As far as I understood, cells "were grown to confluence in silicon moulds that were then removed to allow the cells to migrate"; what exactly is the time 0h then?

14) Figure S2B: an anillin-KD was used here but not described in the result section; why?

15) Figure S2C: the authors should explain with in details the FRET experiments and data for the non-specialist readers.

16) Figure 2D-E: the authors should detail more these data and explain what are the ZI and ZII zones for naive readers.
Moreover, why does AHPH appear as “puncta at the medial-apical surface of the cells “in zebrafish, and not in MCF7 monolayers anytime? Could the authors comment on this?
In addition, why is there an increase of junctional AHPH at the zebrafish monolayer margin ZI 8hpf and not in MCF7 monolayer margin 3h, 12h? In fact, it seems that there is an opposite tendency between MCF7 cells and zebrafish: in zebrafish, strong increase of junctional AHPH in ZI compared to ZII (significantly different in 2D), but in MCF7 cells (2A) no real change in the very first rows compared to the other (at least for the images in 2A).

17) Page 5, end of the third paragraph: is there a correlation between AHPH junctional recruitment and enhancement of contractile tension at AJs? Could this be quantified somehow?

18) Figure 3A: Could the authors show p114Rho-GEF staining on large monolayers as well, like in 2A ? Where does this increase take place, according to the cell row delimitations mentioned in the context of Figure 2 ?

19) Figure 3D: the image of GTP-RhoA under p114 KD is astonishing; it looks like GTP-RhoA is totally excluded from cell contacts, or accumulated close to contacts; in addition, cytoplasmic GTP-RhoA signal seems to increase then. Is it the case ? Could the authors comment on these?
Authors mention Ect2 in the text; what happens for Ect2 in MCF7 monolayers ?

20) Page 6, last paragraph: “depleting anillin by RNAi reduced junctional GTP-RhoA in premigratory monolayers and this was restored by expression of an RNAi-resistant transgene”. Why there is no data showed for these results ? Why mentioning them in this part of result section then ?

21) Figure 4C: anillin-KD cells do not upregulate GTP-RhoA at cell contacts in migrating cells, but a basal level of junctional GTP-RhoA remains; would anillin be required only during mechanical stimulation ?

22) Page 7, first paragraph: I don’t understand why data 1E-J are presented in Figure 1 since they are not used at the beginning of the result section but only page 7. Same remark for S2B. This is really disagreeable for the readers; remodeling of these figures is absolutely required.

23) The authors should explain in more details in the text why AHDM does not allow rescue of junctional GTP-RhoA.

24) Figure 4D: under anillin-KD, there is a decrease of E-cadherin at 0 and 12hr but no gross impact on the MCF7 monolayer; why? In fact, it looks more like a global decrease of E-cadherin level when anillin expression is inhibited: there is not accumulation of E-cadherin at cell contacts neither in the cytoplasm; did the authors check the E-cadherin level in anillin-KD cells ?

25) Conclusion / Discussion should be more developed.

Minor points:
1) Figure S1: why the “A” annotation ? There is no other item in this Figure.
2° Page 12: is plasmocin from Invitrogen or Invivogen ? I didn’t find it on the Invitrogen (ThermoFisher) website.
2) Figure legend 2: (GFP-AHPH_ should be I guess (GFP-AHPH).
3) Page 5 third paragraph: “… where it subsequently increased essed”: what is “essed” for?

First revision

Author response to reviewers' comments

Reviewer 1

Comments for the Author:

In general, the study is well conceived and logically organized. Most of the data support the conclusions drawn by the authors. The findings are technically sound, however the study in its current form is not appropriate for publication in Journal of Cell Science. Important areas to be clarified are outlined below.

General Response

We thank the reviewer for these supportive and helpful comments. We've endeavoured to address each of their points, with new data and analyses where appropriate. This includes an additional three new main figures.

Major concerns:

1) Anillin, through its down-modulation or by using various constructs, is used to address RhoA signaling in regulating mechanical forces during migration. Yet, the authors did not study the impact of manipulating Anillin on junctional tension. The authors should perform the experiments shown in Fig1C and/or Fig1D upon Anillin depletion to assess directly whether Anillin and RhoA signaling is involved in regulating tension.

Response: This is a very good point, for which we thank the reviewer. To address this, we examined anillin KD cells, KD cells reconstituted with AH-α-catenin, and controls, and measured tissue mechanics with:

i) Bayesian inversion stress microscopy, to evaluate overall stresses in the epithelial layer; and

ii) AJ recoil (marked with E-cadherin-GFP) after laser ablation, as a measure of tension at the AJ.

Both assays show that the rise in epithelial tension associated with migration is reduced by anillin KD and restored by AH-α-catenin. This confirms our hypothesis that RhoA signaling at AJ contributes to the increased epithelial tension associated with collective migration.

This new data is included in a new Figure 6.

2) Likewise, the study model is collective migration but the experiment showing the effect of Anillin downmodulation on collective migration is missing, it should be reported.

a) Response: To address this, we evaluated the movement of migrating cells within the monolayer using time-lapse imaging to track their nuclei labelled with Hoechst 33342. We examined two features of migration: i) the speed of cells (studying cells in the first 12-15 rows from the edge of the artificial wound, which are the subpopulation that move during our experiments); and ii) Measuring the extent to which the tracks of individual cells cross: we use this as a measure of the orderliness of cell migration, reasoning that the more orderly the migration the less frequently would tracks cross.

Strikingly, we found that anillin KD cells moved more quickly and in a less orderly fashion than did control cells, and these changes were corrected when junctional RhoA was selectively restored by expression of AH-α-catenin. This suggests that junctional RhoA signaling does, indeed, influence epithelial collective migration. This notion was supported by an analysis of p114 RhoGEF KD cells (which are unable to upregulate junctional RhoA signaling on migration). We hypothesize that junctional RhoA signaling may restrain the motility of constituent cells to promote coordinated, orderly cell migration.

This data is included in a new Fig 7.

At the end-point of the migration experiment, when the two cell monolayers close the gap and stop migrating, do the authors observe a reversal to pre-migratory E- cadherin and RhoA-GTP levels?

b) Response: To minimize the duration of imaging (and its costs – both of photodamage to

the cells and our budget for the imaging) we imaged cells for ~4 hrs after the migrating fronts first contacted one another. During this period the cells that were at the very margins of the moving population re-established cell-cell contacts, but GTP-RhoA (GFP-AHPH) levels did not change. This could be because the monolayers did not re-establish confluence in this time. As can be seen from the data, which we include below for the reviewer, some gaps between cells were still evident. With respect, we preferred not to hold up resubmission of the MS for what we feel is an interesting, but somewhat peripheral, question. [NOTE: We have removed a figure which was provided for the referees in confidence.]

3) In Fig1, the tensile forces appear much stronger at the migration front. Also, in Fig2 the authors show that accumulation of RhoA is faster and more prominent in the first cell rows at the front of migration. The authors need to indicate for all their images in which part of the monolayer they took their pictures and should comment on whether all their markers accumulate with the same spatial pattern within the migrating cell monolayer.

Response:

a) The sites from which the magnified views in original Fig 2A were taken were also marked in the lower-magnification views. To clarify this, we have included a cartoon (in Fig S2C) that explains the zones which we analysed (as suggested by Reviewer 2) and also included in the figure's caption an explanation of where the high- magnification views fit in this schema.

Unless otherwise indicated, all the images and quantitative analysis was performed on cells within the first 7 rows of the migrating monolayer. We appreciate that this was often not clear, so it is now explicitly stated in each of the captions.

b) We include new data that compares the spatial patterns of epithelial tension and junctional RhoA signaling. Ideally, we would have liked to directly compare tension at AJ with RhoA signal levels, but this was technically unfeasible. (We would have had to use a FRET-based α-catenin tension sensor – rather than the destructive measure of junctional recoil – and the available fluorophores were not compatible with our GFP- AHPH-GFP cell line.)

Therefore, we simultaneously compared GFP-AHPH movies with patterns of monolayer stress, measured with Bayesian Inversion Stress Microscopy. It should be noted that these were performed on PDMS substrata (needed for the TFM from which BISM is derived) and GTP-RhoA levels were lower on these soft substrata than in the migration assays performed on glass. Nonetheless, cells migrated and upregulated junctional GTP-RhoA on PDMS as they did on glass. And in this experiment we saw that the regions of enhanced monolayer tension mapped broadly onto the regions where junctional AHPH increased. So, this suggests that there is a spatial relationship between these two parameters. Furthermore, quantitation of average stresses and average junctional AHPH indicated that RhoA activity at AJ increased earlier and faster than did the stresses.

This data is included in a new Fig 3.

4) The authors need to comment on the GFP-AHPH probe that may not just mark the presence of GTPRhoA. It could also recruit GTP-RhoA from the cytosolic pool revealed by their FRET probe (FigS2C and see also Minor comment 4).

Response: With respect, we think that it is unlikely that GFP-AHPH will recruit GTP- RhoA from the cytosolic pool to the membrane, because in our experience GFP-AHPH is cytosolic unless there is a pre-existing pool of GTP-RhoA at the membrane. Although the AHPH domain does have potential membrane-binding motifs (including a PH domain), in our earlier work we found that the construct was cytosolic when we inhibited RhoA (with C3-transferase) or when we introduced 2 point-mutations that ablated its ability to bind GTP-RhoA (Priya et al., Nat Cell Biol, 2015). Moreover, the PH domain alone was a poor membrane-localizer. (It should also be noted that the affinity of anillin for GTP-RhoA is one of the lowest of measured GTP-RhoA binders [e.g. Blumenstein and Ahmadian, JBC 2004].) Thus, the membrane localization of AHPH appears to reflect where GTP-RhoA is to be found.

5) The magnification of the images varies in all figures, which does not help the reader to fully appreciate the staining patterns. This is especially true when comparing the "RhoA-GTP

probe" (see major comment 7) and MyoIIA labeling in Fig3 D/F. Images of the same magnification as 3D need to be shown for 3F.

Response: We've now revised the figure (which becomes Figure 4) to include images for Myosin (Fig 4F) that are at the same magnification as in 4D.

6) Fig3D, the quality of the images is very poor especially for LARG KD, where no increase in RhoA staining is visible. At time 0, the image appears out of focus. New images need to be shown, as they do not reflect the quantification (i). (ii) Does LARG accumulate at migrating cell borders?

Responses:
i) The blurry quality of the images was because they were maximum-projection views from stacks of wide-field images. Furthermore, as LARG siRNA reduces junctional GTP- RhoA (AHPH; as we showed in Fig 4E), the signal/background ratio was also lower. Accordingly, we've replaced the maximum projection views with a single optical slice.

ii) Unfortunately, we don't have an antibody that immunostains for LARG.

7) Fig3D is entitled "GTP-RhoA", in the legend it says "GFP-RhoA (detecting GTP- RhoA)" and in the text only GTP-RhoA is mentioned. Did the authors really use GFP- RhoA as indicted in the figure legend or is it Anillin-GFP as in Fig2A (GFP-AHPH)? GFP- RhoA can not be used to label the GTP-bound form of RhoA, as GFP-RhoA is mainly GDP-bound. If it is not a labeling mistake, then the authors need to show staining with GFP-AHPH.

Response: Sorry, this was a typo in the legend: it should have been "GFP-AHPH" (now corrected).

8) Finally, there seems to be a discrepancy between the numbers shown in 4F and 4G for the KD samples. The KD0 and KD12 curves indicate that the plateau of recovery are much higher compared to the Ctrl0, yet the bar graph indicates a similar immobile fraction. The authors need to explain how they extracted the immobile fraction values from the Recovery curves?

Response: Sorry, this was an error in compiling the figure. We have now replaced the histograms with the correct data.

Minor comments:

1) Figure legend 1A,B indicates the use of Caco-2 cells, I believe the authors meant MCF-7 cells. Figure legend 1(E-J), "Myosin IIB (GI)" should be "Myosin IIB (GJ). Figure legend 3(J-K), it is written "Anillin increases AJ in migrating…". Should not it be "Anillin increases at AJ in migrating…"?

Response: All corrected. Many thanks. Note that the anillin data referred to by the reviewer is now to be found in Fig S4.

2) Fig1G, the authors show the accumulation of NMIIB together with that of F-actin and NMIIA at migrating cell borders and conclude that cortical actomyosin may contribute increased contractility during migration. Yet, in their previous work (Priya et al, 2015) with MCF7 cells, they showed that although NMIIB accumulates at ZA, its down-regulation did not affect RhoA or RhoA-GTP accumulation at cell junctions. The authors could consider analyzing the impact of the down-regulation of NMIIA or NMIIB on RhoA-GTP accumulation, or tune down their conclusion by referring to their previous observation.

Response: In the interests of clarity, we did not mean to suggest in this section that the increased levels of Myosin promoted tension by feeding-back to support RhoA signaling. Instead, our interpretation was simpler: that increased actomyosin-based at AJ might have contributed to the increased tension seen at AJ. Accordingly, we've modified the sentence to read:
"Overall, this local increase in actomyosin could potentially have contributed to

increasing tension at AJ during collective migration."

3) Fig3C, the WB of total p114 indicates that there is an increase in p114 levels at 6hrs as the loading control signal is much lower when compared to the control. The authors need to show a representative WB and the quantification of their replicates that match their conclusion.

Response: We have repeated the experiments to get more consistent loading. Quantitation confirms that there is no change in cellular levels of p114 RhoGEF. These new data are now shown in Fig 4C.

4) Fig S2C, the use of FRET reporter activity nicely confirmed the up-regulation of RhoA-GTP in migrating cells. However, in contrast with the Anillin-based probe, the FRET probe shows a strong accumulation of RhoA activation away from the cell junctions. (i) The authors should comment on the different patterns displayed by their two probes, and the possibility, or not, that activated RhoA away from cell junctions could play a role in the regulation of cortical mechanical forces in migrating cells. (ii) In light of the model proposed by the authors whereby RhoA activation stimulates E- cadherin stabilization and accumulation at migrating cell borders, could they comment on the sequential accumulation of the different molecular actors (E-cadh, p114, RhoA, Anillin, MyoIIA) upon migration, and the existence of positive feedback.

Responses: i) This is a perspicuous point. We do see a pool of AHPH away from junctions: this is faintly evident in the "raw" AHPH images (Fig S2A) and think that this is likely to be in a medial-apical location. It is possible that this may have contributed to generating medial-apical forces that can be transmitted to AJ (as the Lecuit lab have suggested may pertain during Drosophila gastrulation, e.g. Rauzi et al., Nature 2010,

Munjal Nature 2015). (It should also be noted that a medial-apical pool may also pertain with GFP-AHPH, but this is much harder to separate from a cytosolic pool, making it more difficult to interpret.) However, we think that the junctional pool is dominant. We say this because of our anillin manipulation experiments, where α-Cat- AHPH, which selectively restores GTP-RhoA at AJ, substantially restored junctional tension to anillin KD cells. We now discuss this in the first paragraph of the Discussion.

ii) Response: To summarize, what we have observed is that migrating epithelial cells upregulate RhoA signaling at AJ. There is a baseline level of GTP-RhoA at these junctions in confluent monolayers, but it increases when they migrate. Based on our current data, we think that p114 RhoGEF is likely to be the trigger for this increase, potentially responding to tensile, tugging forces that locomoting cells exert on one another when they move. This is based on our recent evidence that p114 RhoGEF is part of a mechanosensitive apparatus AJ that senses tensile forces in epithelia to activate RhoA signaling at those junctions (Acharya et al., Dev Cell, 2018). The increase in junctional actomyosin and tension that we observe in migrating cells appear to arise in significant part from this pathway.

As the reviewer notes, there is the capacity for positive feedback to participate amongst these players. For example, we earlier found that Myosin II can feed-back to stabilize zones of RhoA itself (Priya et al., NCB 2015). Thus, what is initiated as a linear mechanotransduction pathway may engage positive feedback as the system evolves. We've now included a brief consideration of this in paragraph 1 of the Discussion.

(As well, it would be interesting to speculate whether anillin may itself play a role. Anillin can be recruited to the plasma membrane by GTP-RhoA and can also bind Myosin II. This increase in anillin could enhance the dwell time of GTP-RhoA by kinetic scaffolding (Budnar et al., Dev Cell 2019). However, these are currently more speculative than we'd like to incorporate in this manuscript.)

Reviewer 2

Comments for the Author:

The experiments seem to be performed carefully. However, some of the data analysis procedures or presentation need to be further clarified. Additionally, some figures definitely need to be remodeled, and the conclusion / discussion to be further developed.

General Response

We thank the reviewer for their thoughtful comments. We've endeavoured to address each of their points, with new data and analyses where appropriate. This includes an additional three new main figures.

Major comments:

1) Figure S1: It would be good to show lamellipodia in the context of the monolayer as well; it is difficult to be convinced only on the base of a close view on one cell. By the way, why such emphasis is made about cryptic lamellipodia (Figure S1, Movie 1) if they are not used, studied, at all in the core of the study ? Does the level of GTP-RhoA at cell contacts during MCF7 migration impact the formation or the dynamics of cryptic lamellipodia ?

Response: We noted the presence of cryptic lamellipodia because they are consistent with the notion that in this system we are dealing with a moving population that consists of individually-locomotilie cells connected together by AJ. (Rather than a situation where follower cells are being passively pulled along by the leader cells.) In truth, we don't regard this as a major aspect of the story, but rather introduce it as part of the basic characterization of the system. As such, we've not sought to examine whether GTP-RhoA levels at contacts correlate with the behaviour of the cryptic lamellipodia.

Accordingly, we have included an image which shows the cells around that displaying cryptic protrusions (Fig S1A). For this, we exploited low level expression of LifeAct in the surrounding cells to identify them.

2) Page 3, last paragraph: why discussing a data of the Figure 4D here ? Why not showing monolayers stained for E-cadherin at 0 and 12hr to start Figure 1?

Response: This reflected our desire to minimize the repetition of data in the manuscript (especially since we had submitted it as a JCS report). The point that we are trying to make is a peripheral one – that we are dealing with epithelial collective migration where the moving cells are connected by AJ.

Accordingly, in Fig S1B we now include images from another experiment that confirm the presence of E-cadherin between migrating cells.

3) Page 4, first paragraph: does this increase in tension concern several cell rows ? Which ones ? In fact, it seems that increase in tension, as it is presented in 1A, occurs preferentially within the first rows of cells, close to monolayer margin, which is not consistent with the images of AHPH signal showed in 2A.

Response: Broadly speaking, we think that tension increases progressively over space in the migrating subpopulation of cells. This is something that we've seen with the BISM analysis, where tensile monolayer stress first increases in the first few rows of cells (which are the ones that first begin to translocate) and then spreads deeper into the cell population (again, consonant with an increased number of rows of migrating cells). We have now included a representative movie as Supplemental movie 2. (It should be noted that the BISM analysis doesn't allow us to identify the subcellular region where stress increases, but the destructive nature of the recoil measurements meant that these couldn't be used to evaluate the space and time evolution of AJ tension.)

We would note that the patterns of stress shown in Fig 1 can't be readily compared with those of AHPH shown in Fig 2. This is because the experiments in Fig 1 were performed on soft, PDMS substrata, whereas the data shown in Fig 2 were performed on glass. Therefore, in order to compare the spatial patterns of stress and RhoA more directly, we performed simultaneous BISM/TFM and GFP-AHPH imaging – new data included as Fig 3. It should be noted that GTP-RhoA (AHPH) levels at AJ were lower on the softer PDMS substrata than on glass. Nonetheless, cells migrated and quantifiably upregulated junctional GTP-RhoA on PDMS as they did on glass. With this approach we saw that the regions of enhanced monolayer tension mapped broadly onto the regions where junctional AHPH increased. So, this suggests that there is a spatial relationship between these two parameters.

(In the interests of discussion, we note that we would have liked to have directly compared

tension at AJ with RhoA signal levels at these sites, but this was technically unfeasible. We would have had to use a FRET-based α-catenin tension sensor – rather than the destructive measure of junctional recoil – and the available fluorophores were not compatible with our AHPH-GFP biosensor.)

4) Figure 1C: it would be nice to have images to visualize the recoil, in addition to the graph.

Response: These are now included in Fig S6, in the fuller context of anillin manipulation.

5) In the figure legend of Figure 1, why Caco2 cells are mentioned ? I didn't see any Caco2 cells in the manuscript nor figures.

Response: Sorry, this was a typo that is now corrected.

6) Figure 1D: it is difficult to distinguish between magenta and blue signals. Moreover, it would be good to show images of separate channels in addition to merged channels to better assess alpha18 increase. Does this increase in alpha 18 occur in the global monolayer, or in specific cell rows ?

Response: We've now included the separate channels in Fig S1C.

As is the case for other measures of epithelial tension, the increase in α-18 staining occurs primarily in the subpopulation of cells that become migratory. We've imaged and quantitated from the first 7 rows of cells as these were the ones that moved first. Indeed, unless otherwise stated, representative images and quantitation were performed from these first 7 rows of cells. We appreciate that this was not immediately apparent from the images, nor did we make this clear in the captions. So, we've included this in all the captions where appropriate.

7) Figure 1C-D: how many events or cells among three experiments have been used for these quantifications ?

Response: We have updated the figure captions to include the number of technical replicates as well as the biological replicates. In general, we analysed 18-22 technical replicates for each biological replicate.

8) Authors mention that the 3 NMII paralogs are expressed in MCF7 but only present the data for MyoIIAand MyoIIB; why not MyoIIC ?

Response: This is because we only have antibodies that reliably detect NMIIA and NMIIB in MCF7 cells. This is explained in the text (p 4, bottom para):

"MCF-7 cells express all three mammalian NMII paralogs (Smutny et al., 2010) and antibodies were available that effectively recognized NMIIA and NMIIB (Fig 1F,G, Fig S1D)."

9) Figure 1 F-G: does the increase of MyoIIA and IIB also occur at cell contacts in the monolayer margin?

Response: Indeed, it does. For clarity, we should note that the images shown in the Figure (and the quantitation) were taken from within the first 7 rows of cells, which are the ones that moved first and farthest in our experiments. We appreciate that the higher magnification views that are show may be misinterpreted as being from confluent monolayers.
Accordingly, we've included lower magnification views in Fig S1D.

10) Figure 1E-J: authors show here data about anillin-KD but they are not mentioned at all in the result section for Figure 1; why ??

Response: Again, this was done in order to limit the amount of data that was repeated

(albeit from separate experiments) in the manuscript. With respect, this order – although awkward – is something that we would prefer to keep.

11) Figure 2A: the authors should use a color-coding of the AHPH signal to avoid confusion.

Response: We tried a number of colour coding schemes, but they all were visually quite ugly (because they clashed with the nuclear staining), so the current colour scheme seemed to be the clearest.

12) Page 5, second paragraph, and Figure 2A: do differences exist for AHPH junctional recruitment between cell rows from the monolayer margin? It seems to be the case from the images in 2A. In fact, quantifications according to cell rows are shown in Figure 2B but not described in the result section (i). Looking more carefully at the graph 2B, there are inconsistencies between graph2B and images presented in 2A: in 2A, it seems that cell rows 1 to 3-4 do not exhibit much junctional AHPH whereas cell rows 4-5 to 15 do. Is it the case? However, in the graph 2B, quantifications show a strongest junctional AHPH in cell rows 1 to 5. Could the authors comment on this? (ii)

Response:
(i) We have now described the regional changes in junctional GTP-RhoA in the Results.
(ii) Overall, we find that junctional GTP-RhoA are higher in the cells that are towards the migrating margin, than those further behind. However, there is some cell-to-cell variation in the junctional signal even within zones. We think that this is for a number of reasons: a) Expression of the transgene has some heterogeneity as a consequence of transduction; and b) not all cells were captured in the optical stacks after processing. This is the reason why we chose to perform our quantitation by zones which each contain 5 rows of cells.

13) By the way, could the authors add in this figure a scheme clearly explaining the cell row delimitations to avoid reader confusion.

Response: Done. It is now included in Fig S2C.

14) Figure 2A, time point 0h: why is there AHPH signal at “lamellae of leader cells”? As far as I understood,cells “were grown to confluence in silicon moulds that were then removed to allow the cells to migrate”; what exactly is the time 0h then?

Response: Time zero is when the first images can be taken after the silicon moulds have been removed. Typically, this is after a delay of 15-20 min, necessary to move the cells onto the microscope stage and then set it up for time-lapse imaging. This seems to be enough for lamellae to become apparent. (We do find that cryptic lamellae are apparent even in confluent monolayers.)

15) Figure S2B: an anillin-KD was used here but not described in the result section; why?

Response: In this case, the anillin KD refers to a point that we make later (namely that global anillin KD reduced GTP-RhoA at the leading edges of leader cells, p 8, top para last sentence). However, to minimize data repetition, it seems best to include it in Fig S2B, so that the changes with anillin KD can be directly compared with the controls.

16) Figure S2C: the authors should explain with in details the FRET experiments and data for the nonspecialist readers.

Response: We have endeavoured to expand this when we introduce the FRET sensor in the Results (p5). As FRET sensors are now quite extensively used, we have confined ourselves to noting that it is an intramolecular sensor where energy exchange increases when the RhoA moiety of the sensor is activated.

"The increase in junctional RhoA signaling was confirmed using an intramolecular FRET-based RhoA activity sensor (Fig S2D), where energy exchange increases upon activation of the RhoA moiety of the reporter. Quantitation of the junctional FRET signal in zone 1 (Fig S2C) showed that RhoA activation was increased in this region of the migrating monolayer (Fig S2D). The FRET reporter also revealed an increase in signal away from the junctions, that may correspond to a medial-apical pool of RhoA."

17) Figure 2D-E: the authors should detail more these data and explain what are the ZI and ZII zones for naive readers.

Response: We have now done so, both in the Results, in the figure itself and in the figure legend. These zones refer to regions that are in the marginal region of the ectoderm, moving towards the vegetal pole (zone I) and in the ectoderm closer to the animal pole (zone II).

18) Moreover, why does AHPH appear as "puncta at the medial-apical surface of the cells "in zebrafish, and not in MCF7 monolayers anytime? Could the authors comment on this? (i)
In addition, why is there an increase of junctional AHPH at the zebrafish monolayer margin ZI 8hpf and not in MCF7 monolayer margin 3h, 12h? In fact, it seems that there is an opposite tendency between MCF7 cells and zebrafish: in zebrafish, strong increase of junctional AHPH in ZI compared to ZII (significantly different in 2D), but in MCF7 cells (2A) no real change in the very first rows compared to the other (at least for the images in 2A).

Response:
(i) We have not explored the reason why AHPH identifies medial-apical puncta in these ectodermal cells. Similar patterns are seen during Drosophila gastrulation (e.g. Munjal et al., Nature 2015), which are thought to set up medial-apical zones of cortical contractility. Moreover, as noted by Reviewer 1, the RhoA FRET sensor suggests that there may be a medial-apical pool in MCF7 cells as well. This is harder to detect confidently using GFP-AHPH, because it is difficult to distinguish diffuse signals at the apical membrane (presumably marking active RhoA) from the cytoplasmic pool of AHPH (which is likely to simply reflect reporter that has not been recruited to GTP- RhoA at membranes).

(ii) We have found that the expression of AHPH is more uniform in our experiments with zebrafish than with cultured cells. This is probably because our zebrafish

experiments used a transgenic line, whereas in mammalian cells we expressed GFP- AHPH by lentiviral transduction and (as noted above) some cell-to-cell variability was always seen.

19) Page 5, end of the third paragraph: is there a correlation between AHPH junctional recruitment and enhancement of contractile tension at AJs? Could this be quantified somehow?

Response: We have endeavoured to do this from our simultaneous measurement of monolayer stress (BISM) and junctional AHPH by comparing the time-evolution of average values in the imaging fields. This new data, shown in Fig 3, shows that RhoA increases before the increase in monolayer stress. This supports the idea that the increase in junctional RhoA contributes to increasing monolayer stress (something that we then endeavour to test more directly by manipulating anillin).

(We also endeavoured to quantitatively compare spatial, as well as temporal, patterns of evolution, but this proved to be much more difficult. We feel that it is something that will require more time and resources than reasonable for a revision.)

20) Figure 3A: Could the authors show p114Rho-GEF staining on large monolayers as well, like in 2A? Where does this increase take place, according to the cell row

delimitations mentioned in the context of Figure 2 ?

Response: Unfortunately, the signal:background ratio is quite low making the low-magnification images poor quality.

Some p114 RhoGEF staining is seen in confluent monolayers, but it increases in the migratory subpopulation. Here the images are taken from within Rows 1-7. Again, we've clarified this in the Figure caption.

21) Figure 3D: the image of GTP-RhoA under p114 KD is astonishing; it looks like GTP- RhoA is totally excluded from cell contacts, or accumulated close to contacts; in addition, cytoplasmic GTP-RhoA signal seems to increase then. Is it the case ? Could the authors comment on these? (i) Authors mention Ect2 in the text; what happens for Ect2 in MCF7 monolayers ? (ii)

Response:

i) Indeed, we do find that the junctional RhoA signal is significantly reduced by p114 RhoGEF RNAi. However, the quality of the images shown was quite blurry (as noted by Reviewer 1 for LARG KD). This was because they were maximum-projection views from stacks of wide-field images. Accordingly, we've replaced the maximum projection views with a single optical slice.

Furthermore, the increase in cytoplasmic AHPH is what we would expect if cortical RhoA signaling was reduced. A point of clarification that we should emphasize: the AHPH sensor is really only interpretable when it marks discrete sites at membranes. This is because there is often a cytoplasmic pool of the sensor (as a consequence of exogenous expression). Moreover, the sensor is wholly cytoplasmic when we inhibit RhoA with C3-Transferase or when we mutate its ability to bind GTP- RhoA (Priya et al., NCB 2015). Thus, we would expect that inhibition (or a reduction in cortical GTP-RhoA) would result in the loss of AHPH from membranes and an increase in its level in the cytoplasm.

ii) Ect 2 is present in AJ at baseline but does not change on migration. This data is included now in Fig S3A.

22) Page 6, last paragraph: "depleting anillin by RNAi reduced junctional GTP-RhoA in premigratory monolayers and this was restored by expression of an RNAi-resistant transgene". Why there is no data showed for these results ? Why mentioning them in this part of result section then ?

Response: Our apologies. This was meant to refer to our earlier experiments published in Budnar et al. (Dev Cell, 2019), but the reference was omitted. This has now been corrected.

23) Figure 4C: anillin-KD cells do not upregulate GTP-RhoA at cell contacts in migrating cells, but a basal level of junctional GTP-RhoA remains; would anillin be required only during mechanical stimulation ?

Response: In our recent work we found that anillin was required to support junctional GTP-RhoA levels even in confluent monolayers (Budnar et al., Dev Cell 2019), thus its impact is not confined to situations of migration. It should be noted that in our experiments anillin KD does not abolish active RhoA at AJ. This may be because the knock-down is incomplete, but the major reason is likely to be because anillin prolongs the dwell time of active RhoA, but does not affect the prior activation step. So, we would expect RhoA to be activated even without the kinetic scaffolding of anillin, but its dwell time will be shorter.

24) Page 7, first paragraph: I don't understand why data 1E-J are presented in Figure 1 since they are not used at the beginning of the result section but only page 7. Same remark for S2B. This is really disagreeable for the readers; remodeling of these figures is absolutely required.

Response: Sorry. As noted above, this was to minimize the repetition of results and data.

25) The authors should explain in more details in the text why AHDM does not allow rescue of junctional GTP-RhoA.

Response: Done! The AH^{DM} construct lacks the ability to bind GTP-RhoA, something that we showed was a precondition for anillin to act as a kinetic scaffold that prolongs the lifetime of active RhoA. We've modified the sentence to read:

> "Indeed, expression of AH-α-catenin restored the capacity for migrating KD cells to upregulate GTP-RhoA at their junctions, despite anillin being otherwise depleted in the cells (Fig 4B,C), but this did not occur with an AH^{DM}-α-catenin mutant that cannot stabilize GTP-RhoA, because it lacks key residues needed to bind GTP-RhoA (Fig 4B,C; (Budnar et al., 2019)."

26) Figure 4D: under anillin-KD, there is a decrease of E-cadherin at 0 and 12hr but no gross impact on theMCF7 monolayer; why? In fact, it looks more like a global decrease of E-cadherin level when anillin expression is inhibited: there is not accumulation of E-cadherin at cell contacts neither in the cytoplasm; did the authors check the E- cadherin level in anillin-KD cells ?

Response: Indeed, total cellular levels of E-cadherin do not change with anillin KD (data now in Fig S4F). The impact on "apparent" levels of cadherin at junctions is an astute point that is consistent with our current understanding of RhoA action at the zonula adherens. In brief, in our earlier work we found that RhoA at junctions serves to concentrate surface E-cadherin to form the apically-located zonula adherens (Ratheesh et al., NCB 2011), a process that is mediated by non-muscle myosin II (Smutny et al, 2010). More recently, we found that anillin functions at adherens junctions as a promoter of RhoA signaling (Budnar et al., Dev Cell, 2019), an effect that is expected to affect the surface concentration of E-cadherin into the ZA "ring". Of note, the impact of RhoA and actomyosin to concentrate and stabilize E-cadherin in the ZA is post-translational and does not affect the total cellular levels of E-cadherin (Smutny et al., 2010; Priya et al., Differentiation, 2013). Thus, we think that apparent decrease in E-cadherin at cell-cell contacts reflects a reduction in the ability of cells to concentrate surface cadherin to form a ZA (something that is accentuated by when imaged in the apical region of the cells). Cadherin instead distributes more broadly throughout the contact zones between cells when this mechanism is compromised.

27) Conclusion / Discussion should be more developed.

Response: Done!

Minor points:

i) Figure S1: why the "A" annotation ? There is no other item in this Figure.
Response: Corrected.

ii) 2° Page 12: is plasmocin from Invitrogen or Invivogen ? I didn't find it on the Invitrogen (ThermoFisher) website.
Response: Our mistake: it is InvivoGen. Corrected now.

iii) Figure legend 2: (GFP-AHPH_ should be I guess (GFP-AHPH).
Response: Corrected.

iv) Page 5 third paragraph: "… where it subsequently increased essed": what is "essed" for?
Response: It was an edit that failed to be edited out. Now gone.

Second decision letter

MS ID#: JOCES/2021/258767

MS TITLE: Enhanced RhoA signaling stabilizes E-cadherin in migrating epithelial monolayers.

AUTHORS: Shafali Gupta, Kinga Duszyc, Suzie Verma, Srikanth Budnar, Xuan Liang, Guillermo Gomez, Philippe Marcq, Ivar Noordstra, and Alpha Yap

ARTICLE TYPE: Research Article

Congratulations! 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 authors have provided additional experiments that strengthen their conclusions.

Comments for the author

The authors have appropriately responded to all my critics and comments. The revised manuscript is now suitable for publication in Journal of Cell Science.

Reviewer 2

Advance summary and potential significance to field

I have reviewed the revisions made by the authors and am satisfied with the extra data presented and the replies to my queries. This has turned out to be a nice paper and I recommend publication.

Comments for the author

I have reviewed the revisions made by the authors and am satisfied with the extra data presented and the replies to my queries. This has turned out to be a nice paper and I recommend publication.