The Drosophila melanogaster Rab GAP RN-tre crosstalks with the Rho1 signaling pathway to regulate non-muscle myosin II localization and function.

Amy Platenkamp, Elizabeth Detmar, Liz Sepulveda, Anna Ritz, Stephen Rogers, and Derek Applewhite

Corresponding author(s): Derek Applewhite, Reed College

Review Timeline:

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- Editorial Decision: 2020-06-05
- Revision Received: 2020-07-17
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Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
June 5, 2020

1st Editorial Decision

RE: Manuscript #E20-03-0181
TITLE: The Drosophila melanogaster Rab GAP RN-tre crosstalks with the Rho1 signaling pathway to regulate non-muscle myosin II localization and function.

Dear Dr. Applewhite:

Your manuscript entitled "The Drosophila melanogaster Rab GAP RN-tre crosstalks with the Rho1 signaling pathway to regulate non-muscle myosin II localization and function" has been seen by two reviewers, whose verbatim comments are attached. Both referees felt that your findings, in principle, would be of interest to our MBC readership. However, both also felt that revisions, not requiring further experiments, would significantly improve the impact of your manuscript. Reviewer 1 noted only minor points, which I encourage you to read carefully and incorporate into the manuscript where appropriate. In particular this reviewer's first point regarding data presentation deserves careful consideration. Reviewer 2 had a number of significant concerns regarding data presentation and interpretation that you should also carefully consider in revising your manuscript. In particular that reviewer's point #2, regarding possible effects on the actin network, needs to be addressed or at least acknowledged in the manuscript as a possible alternative model.

In sum, we would be happy to consider a revised manuscript that satisfies the joint concerns of the referees. We look forward to receiving your revised manuscript, together with a letter indicating the changes you've made and your responses to the referees, in the near future.

Best regards,

Rick Fehon
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Applewhite,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is
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To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

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Reviewer #1 (Remarks to the Author):

Non-muscle myosin II (NMII) is crucial for myriad cellular processes, and its canonical upstream regulation by the small GTPase RhoA is well documented. However, novel inputs into this system of non-muscle cellular contractility are currently being uncovered. Here, Platenkamp et al. identified the GAP RN-tre as a regulator of non-muscle myosin. They then investigated the RN-tre's roles in the localization and function of NMII. RN-tre seems to partially act within the Rho1 GTPase pathway for this role, and not via its role in Rab signalling. Rather, its GAP activity partially functions within regulating NMII contractility via phosphorylation by Rok.

The manuscript is well written and easy to read. Platenkamp et al. address the gap in our current understanding about the players that regulate NMII contractility, and show an additional role of RN-tre regulation of NMII which is supported by the data. The conclusions are consistent with the data presented. The figures and tables mainly stand alone to explain the main themes of the paper.

There were no critical flaws with this manuscript. Therefore, we suggest it be accepted after the following minor points are addressed.
Minor Points:

1) Because the coalescence measurements quantified fluorescence intensity, it would be more appropriate to report those (normalized) intensity measurements rather than a binary score resulting from how the population scores. See Figures 7C and 8G.

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a) Abstract: The word "loss" generally implies the use of a genetic knock-out and/or the total absence of protein expression. Since RNAi is utilized, the word "depletion" would be more appropriate.

b) Figure S4: The figure title reads like a conclusion statement, and does not reflect the data presented.

c) Throughout text: The coalescence of NMII is not equivalent to the NMII assembly into fibers and filaments. NMII assembles into diffraction-limited bipolar filaments, which is not studied in this work. The authors need to clarify their characterizations of subcellular NMII organization and distribution.

3) The figures are mainly clear and the data nicely presented, but several minor improvements will further clarify data presentation:

Please label depletion conditions (i.e dsRNA name - RN-tre #1 or #2) clearly throughout.

An additional schematic showing Rho signalling with the GAPs, GEFs, etc. specific to this paper would make Lines 78-90 clearer, and would illustrate the proposed function of RN-tre.

Ensure that all figures are called as they appear in the text. For example, Figure 8G is called before 8A-F.

Please show example images for all quantitation (ex. Adding Zipper and alpha-actinin to Figure 1; Addition of Rok1/2 to Figure 7)

The cells shown in Figure 2A-D are difficult to see at printed page size. Please mark all contracted cells in Figure 2A-D and show an enlarged single cell example +/- contraction.

Ensure that figure scale bars and labels are not missing (ex. Figure 4A-C kymographs)

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The statistical comparisons of Ssh RNAi with both control and the RN-tre depletion should be presented, where a single comparison is now shown.

Additional contextualization in either the results or discussion of why Dia and RhoGEF2 RNAi phenotypes seem more mild.

The RNAi of Rho pathway phenocopying RN-tre seems to logically be out-of-order with Fog experiments, since the Rho pathway is intermediate to NMII (Figure 1) and the Fog work. Consider re-ordering.
Ensure that images and their respective quantitations are in the same order (ex. Figure 5E-G, Figure 7D-E, Figure 8A-G).

Rearrange Figure 6 to show the control in the first column and add a space between live-cell conditions and fixed-cell conditions.

To highlight phospho-myosin positive cells more clearly in Figure 8A-F, consider inverting intensity on the image.
The blot in Figure S2F should be quantified and presented with the resulting graph.

4) It is not advisable to perturb Rho proteins by depletion since the shared GDI-as-chaperone mechanism means other Rho proteins' abundance changes in response (Boulter et al, Nat Cell Biol 2010). This is not a crucial experiment for points the paper makes, but rather, a positive control, so the authors could simply mention this potential caveat or if Rho inhibition by C3 treatment has already done, show this instead.

5) We noted the following typographical errors that need to be corrected:
   Line 53 - actinomyosin should be changed to actomyosin
   Line 279 - For "We observedThere," "We observed" should be omitted.
   Line 344 - The yellow bar between “Figure” and “6 E-H” should be removed.

Reviewer #2 (Remarks to the Author):

Platenkamp et al. developed a screen to identify other proteins involved in the regulation of NMII. To investigate this, they knocked down a curated set of proteins in drosophila S2 cell line expressing sqh-EGFP and mCherry Actin and looked for changes in NMII localization or dynamics. Somewhat surprisingly, the limited screen only yielded 1 hit, Rab GAP RN-tre, which they further characterized. The authors characterize the role of RN-tre as being required for robust activation of Rho, the loss of which leads to a disruption in NMII localization and decrease in contractility. This role is independent of its traditional role as a GAP for Rab-5. Also, this role is only partially dependent on RN-tre's GAP activity.

The authors nicely identify and characterize a novel role for RN-tre, a Rab-GAP, in regulating NMII assembly via regulation of Rho-A. They found that this activity is partially, but not exclusively, due to the RN-tre's GAP activity. Unfortunately, the impact of the paper is somewhat lessened by an only partial elucidation of the mechanism of RN-tre activity on Rho signaling. Additionally, although the authors mention that RN-tre represents a link between the secretion machinery and actomyosin contractility, there is little discussion or evidence of this crosstalk, and RN-tre appears to have a more 'moonlighting' role rather than represent cross-regulation of secretion and contraction.

Impact: Authors identify a new regulator of nmy2 in S2 cells, acting through the Rho pathway. The effect on myosin contractility is traced to an effect on Rho activity, possibly partly through RN-tre's GAP activity. The impact is somewhat lessened due to not completely determining the mechanism of RN-tre on Rho.
Major Points:

(1) The authors use Drosophila S2 cells, where NMII puncta undergo a stereotyped process of appearing first as puncta at the periphery, then undergoing retrograde flow toward the cell center-coalescing into higher ordered assemblies around the nucleus. The included movies very nicely show the formation of this higher-ordered assembly, and perhaps stills from these movies could be included as Figure 1 instead of the stills used, which give less information about the dynamic assembly of the NMII filaments. In order to quantify the difference between control and RNAi treated conditions, the authors used a metric of fluorescence intensity over entire cell/mean pixel intensity. While these results are consistent with the reported conclusions, namely that RN-tre and loss of zipper show a loss of coalescence this analysis loses some of the dynamic information in the assay itself. I wonder if an additional method of analysis could also be used, perhaps making use of kymographs?

(2) The F-actin network with RN-tre RNAi looks significantly disrupted. While this would be expected if myosin activity is being affected such that myosin is unable to promote actin filament alignment away from the lamellar edge, it seems important to evaluate whether or not RN-tre is affecting actin assembly directly as this could, in turn, affect myosin filament assembly. However, I also understand that this may be beyond the scope of the current manuscript, particularly given difficulties in doing additional experiments at the moment.

(3) In the epistasis experiments establishing that Rho-1 activation is downstream of RN-tre, the experiments performed were supportive of this conclusion, but I found the order of presentation confusing. Figure 5 establishes, via RNAi, that Rho-1 and Rho-1 targets such as Rok, RhoGEF2 and Dia show similar effects on NMII coalescence as RNAi for RN-tre. This is suggestive that RN-tre is acting in the Rho pathway. In Figure 6, the authors convincingly rescue the RN-tre phenotype using constitutively active forms of Rho-1 and Rok. These results indicate that RN-tre is acting upstream of Rho-1. But then in Figure 7, the authors investigate the phosphorylation state of Sqh, a downstream target of Rho-1 and Rok, after already establishing that RN-tre is acting upstream of these factors. It might logically flow better if the phospho-staining of NMII is grouped with the other Rho-1 targets. If the experiment was placed there to group the phospho-myosin staining of Fig 7 and 8 together, more explanation as to why you would be examining a target two steps from what appears to be the established proximal target (Rho-1) is needed.

(4) The authors should address if the results presented for the RN-tre (WT) and RN-treR153A in H and I are consistent or if the expression levels vary in the same direction for each line. The data presented suggests that the RN-treR153A is highly overexpressed as assayed by WB and appears to be highly enriched at the cortex. Is this indicative of some kind of regulation or is the overexpression at times at the level of the RN-tre WT?

(5) In the discussion section, there are some sections that read as if the authors are proposing the proximal target of RN-tre is phosphorylation of RLC rather than Rho-1 (lines 466-467, and 469-470). I don't see that the data, as currently presented, supports this interpretation, rather the data seems to support RN-tre acting on Rho-1 (Fig 6 and 7D). If the authors are proposing phosphorylation of RLC is a direct target of RN-tre, the mechanism for this activity needs to clarified and discussed.

Minor points:
Typo/Editing at line 279
Figure 7, Panel C. The n.s. bar extends over RN-tre, Rok1, Rok2 and Rho1, but the Rho1 has some ** indicating significance and significance is indicated in the legend.
Dear Dr. Welch,

We would like to resubmit our revised manuscript entitled, “The *Drosophila melanogaster* Rab GAP RN-tre crosstalks with the Rho1 signaling pathway to regulate non-muscle myosin II localization and function” for publication in *Molecular Biology of the Cell*. We present here an examination, the Rab GAP RN-tre, a GAP traditionally associated with the regulation of vesicular transport. Our results suggest that RN-tre crosstalk with the Rho1 signaling pathway which in turn regulates non-muscle myosin II filament assembly. Furthermore, we find that in this role, RN-tre’s GAP activity is only partially required. Starting on the second page of this letter you will find the Reviewers' comments as well as our point-by-point response to these comments. We were delighted with the favorable initial review of the manuscript and found the Reviewers’ comments helpful in improving the clarity and precision of the manuscript. We feel that we have sufficiently addressed their concerns and are hopeful that we are now in a position to move forward.

We look forward to hearing from you in the near future.

Sincerely,

DEREK A. APPLEWHITE, PhD
Biology Department
3203 SE Woodstock Boulevard, Portland, Oregon 97202-8199
phone: 503-517-5017 email: applewhd@reed.edu
We thank the reviewers for their thoughtful comments and have revised the manuscript accordingly. Please see the details of these revisions below. For clarification, the reviewers comments as we received them are in bold.

Reviewer #1 (Remarks to the Author):

Non-muscle myosin II (NMII) is crucial for myriad cellular processes, and its canonical upstream regulation by the small GTPase RhoA is well documented. However, novel inputs into this system of non-muscle cellular contractility are currently being uncovered. Here, Platenkamp et al. identified the GAP RN-tre as a regulator of non-muscle myosin. They then investigated the RN-tre’s roles in the localization and function of NMII. RN-tre seems to partially act within the Rho1 GTPase pathway for this role, and not via its role in Rab signalling. Rather, its GAP activity partially functions within regulating NMII contractility via phosphorylation by Rok.

The manuscript is well written and easy to read. Platenkamp et al. address the gap in our current understanding about the players that regulate NMII contractility, and show an additional role of RN-tre regulation of NMII which is supported by the data. The conclusions are consistent with the data presented. The figures and tables mainly stand alone to explain the main themes of the paper.

There were no critical flaws with this manuscript. Therefore, we suggest it be accepted after the following minor points are addressed.

Minor Points:

1) Because the coalescence measurements quantified fluorescence intensity, it would be more appropriate to report those (normalized) intensity measurements rather than a binary score resulting from how the population scores. See Figures 7C and 8G.

   We thank the Reviewer for bringing this to our attention. Rather than quantifying fluorescence intensity, cells with clear phospho-myosin staining were tallied as phospho-myosin positive and those without were considered negative in both the previous and current versions of the manuscript. The last lines of the methods under the Phospho-Myosin staining section were amended to read, “Cells were imaged by TIRF and tallied as ‘phospho-myosin- positive’ if the staining was clear or ‘-negative’ staining if it was not. This was expressed as a fraction, with the number of phospho-myosin positive cells over the total number of cells counted.”

2) While the paper was well written, there were a few instances where the terminology could be more precise.

   a) Abstract: The word "loss" generally implies the use of a genetic knock-out and/or the total absence of protein expression. Since RNAi is utilized, the word "depletion" would be more appropriate.

   We agree with the Reviewer and have replaced the word “loss” (with regards to protein) knock-down to “depletion” in the abstract as recommended.
b) Figure S4: The figure title reads like a conclusion statement, and does not reflect the data presented.

We agree with the Reviewer and report that the figure title has been changed to reflect the data presented rather than state a conclusion (see Figure S4 title).

c) Throughout text: The coalescence of NMII is not equivalent to the NMII assembly into fibers and filaments. NMII assembles into diffraction-limited bipolar filaments, which is not studied in this work. The authors need to clarify their characterizations of subcellular NMII organization and distribution.

We thank the reviewers for pointing out this critical distinction. We have addressed this in the results section and hope that the caveat we gave is sufficient.

3) The figures are mainly clear and the data nicely presented, but several minor improvements will further clarify data presentation:

Please label depletion conditions (i.e. dsRNA name - RN-tre #1 or #2) clearly throughout.

The RN-tre target number is now labelled clearly in each figure legend.

An additional schematic showing Rho signalling with the GAPs, GEFs, etc. specific to this paper would make Lines 78-90 clearer, and would illustrate the proposed function of RN-tre.

We appreciate the Reviewer's comments regarding signaling from GEFs and GAPs in the Rho pathway and have included and model as Figure 9 at the end of our manuscript. We also address this model in the discussion.

Ensure that all figures are called as they appear in the text. For example, Figure 8G is called before 8A-F.

All figures are now called in the order as they appear in the text we are sorry for the confusion we may have caused.

Please show example images for all quantitation (ex. Adding Zipper and alpha-actinin to Figure 1; Addition of Rok1/2 to Figure 7)

Thank you for pointing out this oversight. We have included Zipper and Alpha-Actinin images in Figure 1 and Rok1/2 and Rho1 images in Figure 7.

The cells shown in Figure 2A-D are difficult to see at printed page size. Please mark all contracted cells in Figure 2A-D and show an enlarged single cell example +/- contraction.

All constricted cells in A-D were demarcated with a white arrow. Panels E and F were added to show enlarged examples of a constricted and relaxed cell.

Ensure that figure scale bars and labels are not missing (ex. Figure 4A-C kymographs)

Scale bars are now included.
In Figure 7D right, Rho1-GTP should probably be GTPγS

The Figure 7D label was changed to “GTPγS.”

The statistical comparisons of Ssh RNAi with both control and the RN-tre depletion should be presented, where a single comparison is now shown.

We thank the Reviewer for catching this omission and we have corrected this error.

Additional contextualization in either the results or discussion of why Dia and RhoGEF2 RNAi phenotypes seem more mild.

We appreciate the reviewer’s comment regarding RN-tre’s phenocopying proteins in the Rho1 pathway. Statistically, the depletion of RN-tre was no different than depletion of Dia, RhoGEF2, Rok, or Rho1 as quantified by our coalescence assay. We have therefore replaced these images with those that are more representative of the phenotype. We have also addressed this in the discussion section.

The RNAi of Rho pathway phenocopying RN-tre seems to logically be out-of-order with Fog experiments, since the Rho pathway is intermediate to NMII (Figure 1) and the Fog work. Consider re-ordering.

We agree with the Reviewer and have reordered the figures so that the figures relevant to the Rho pathway (currently Figures 2, 3, and 4) come before the Fog figures (now Figures 5 and 6).

Ensure that images and their respective quantitations are in the same order (ex. Figure 5E-G, Figure 7D-E, Figure 8A-G).

The images in Figure 5E-G, Figure 7D-E, and Figure 8A-G are now in the correct order.

Rearrange Figure 6 to show the control in the first column and add a space between live-cell conditions and fixed-cell conditions.

The controls for both the live cells and the fixed cells are in their respective left columns and there is now a space between the live and fixed conditions.

To highlight phospho-myosin positive cells more clearly in Figure 8A-F, consider inverting intensity on the image.
The blot in Figure S2F should be quantified and presented with the resulting graph.

We appreciate the Reviewer’s comments and fully agree. The images in Figure 8A-F are now inverted as suggested. We also agree with the comments and have quantified the blot presented in Figure S2F and is now labelled as Figure S2G.

4) It is not advisable to perturb Rho proteins by depletion since the shared GDI-as-chaperone mechanism means other Rho proteins’ abundance changes in response (Boulter et al, Nat Cell Biol 2010). This is not a crucial experiment for points the paper makes, but rather, a positive control, so the authors could simply mention this potential caveat or if Rho inhibition by C3 treatment has already done, show this instead.
We thank the reviewers for pointing this out and have included a caveat regarding Rho1 depletion in the results section of the manuscript.

5) We noted the following typographical errors that need to be corrected:
Line 53 - actinomyosin should be changed to actomyosin
Corrected.

Line 279 - For "We observed There," "We observed" should be omitted.
Omitted.

Line 344 - The yellow bar between "Figure" and "6 E-H" should be removed.
Removed.

Reviewer #2 (Remarks to the Author):

Platenkamp et al. developed a screen to identify other proteins involved in the regulation of NMII. To investigate this, they knocked down a curated set of proteins in drosophila S2 cell line expressing sqh-EGFP and mCherry Actin and looked for changes in NMII localization or dynamics. Somewhat surprisingly, the limited screen only yielded 1 hit, Rab GAP RN-tre, which they further characterized. The authors characterize the role of RN-tre as being required for robust activation of Rho, the loss of which leads to a disruption in NMII localization and decrease in contractility. This role is independent of its traditional role as a GAP for Rab-5. Also, this role is only partially dependent on RN-tre's GAP activity.

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Major Points:

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which give less information about the dynamic assembly of the NMII filaments. In order to quantify the difference between control and RNAi treated conditions, the authors used a metric of fluorescence intensity over entire cell/mean pixel intensity. While these results are consistent with the reported conclusions, namely that RN-tre and loss of zipper show a loss of coalescence this analysis loses some of the dynamic information in the assay itself. I wonder if an additional method of analysis could also be used, perhaps making use of kymographs?

We agree with Reviewer’s two observations regarding the quantification of NMII localization and dynamics and after exploring other methods (kymographs, integrated fluorescence intensity) we found that these methods were either noisy or did not fully capture the full extent of the phenotype. In particular kymographs were subjective when selecting which portion of the cell to make the kymograph. Our coalescence assay takes into account the entire cells’ imaged area which eliminates some of the subjectivity, and gives the most consistent and reliable results. We have replaced Figure 1A-B for stills from similar time series to the supplemental movie now labelled Figure 1A-D, including representative cells for each RNAi condition.

(2) The F-actin network with RN-tre RNAi looks significantly disrupted. While this would be expected if myosin activity is being affected such that myosin is unable to promote actin filament alignment away from the lamellar edge, it seems important to evaluate whether or not RN-tre is affecting actin assembly directly as this could, in turn, affect myosin filament assembly. However, I also understand that this may be beyond the scope of the current manuscript, particularly given difficulties in doing additional experiments at the moment.

We appreciate the Reviewer’s comments regarding the actin organization following RN-tre depletion. I would like to point the reviewer’s attention to Figure 7, where we have quantified actin retrograde flow. These results suggest that actin cytoskeleton is not only intact but the rates of polymerization (as measured by retrograde flow rates) is increased in RN-tre depleted cells as compared to control cells ($5.8 \pm 0.3$ vs $3.7 \pm 0.1 \mu m^*min.$). This increase represents a 1.5X increase in actin retrograde flow which is inline with the previously reported increase in protrusion when NMII is pharmacologically inhibited or knocked-out genetically (Cai et al., 2006; Even-Ram et al., 2007; Vicente-Manzanares et al., 2007). In addition, inhibition of NMII by blebbistatin left the lamellipodia fully intact in endothelial cells (Ponti et al., 2004). In light of this, we have replaced the previous RN-tre depleted with another cell in Figure 1, and have provided several more examples of NMII and actin following control and RN-tre depletion in a new supplement figure (Supplementary Figure 4). We have also addressed this critical point in the results section of the manuscript.

(3) In the epistasis experiments establishing that Rho-1 activation is downstream of RN-tre, the experiments performed were supportive of this conclusion, but I found the order of presentation confusing. Figure 5 establishes, via RNAi, that Rho-1 and Rho-1 targets such as Rok, RhoGEF2 and Dia show similar effects on NMII coalescence as RNAi for RN-tre. This is suggestive that RN-tre is acting in the Rho pathway. In Figure 6, the authors convincingly rescue the RN-tre phenotype using constitutively active forms of Rho-1 and Rok. These results indicate that RN-tre is acting upstream of Rho-1. But then in Figure 7, the authors investigate the phosphorylation state of Sqh, a downstream target of Rho-1 and Rok, after already establishing that RN-tre is acting upstream of these factors. It might logically flow better if the phospho-staining of NMII is grouped with the other Rho-1 targets. If the experiment was placed there to group the phospho-myoosin staining of Fig 7
and 8 together, more explanation as to why you would be examining a target two steps from what appears to be the established proximal target (Rho-1) is needed.

We thank the reviewer for this suggestion. We have reordered the figures so that the Rho-relevant experiments come first (now Figures 2, 3 and 4) and the Fog and actin retrograde flow experiments come afterwards (now Figures 5, 6, and 7). We have left the Phospho-myosin experiment in Figure 8 where it was originally, because after having established that RN-tre knockdown results in fewer Phospho-myosin positive cells in Figure 4, we are able to use the Phospho-myosin test to interrogate whether RN-tre R153 is necessary for the effect on Myosin that we observe.

(4) The authors should address if the results presented for the RN-tre (WT) and RN-treR153A in H and I are consistent or if the expression levels vary in the same direction for each line. The data presented suggests that the RN-treR153A is highly overexpressed as assayed by WB and appears to be highly enriched at the cortex. Is this indicative of some kind of regulation or is the overexpression at times at the level of the RN-tre WT?

Thank you for this opportunity to clarify. The cells used for the experiments shown in Figure 8A-H (including the WB) were stable cell lines expressing refractory Myc-tagged RN-tre WT and RN-tre R153A. The cells used in Figure 8G had transient transfections with constructs containing non-refractory RN-tre with fluorescent tags and expression levels were not determined as the transfection rates can be variable from cell to cell. The expression of RN-tre R153A refractory line was consistently higher than that of the RN-tre WT refractory levels. The constructs are genetically identical, including the promoters, with the exception of the single amino acid substitution. We don't know at this time whether the differences in expression we observe are due to differential regulation of the WT GAP and its mutant, differential toxicity to their over-expression, or some other artifact of the stable cell lines we produced. Because of the differences in expression levels, we reported the normalized values as shown in the original and current versions of the manuscript. As for whether fluorescently-tagged RN-tre R153A appears to be enriched at the cortex, it's possible that the RabGAP-dead version is trafficked at a lower rate to the inner parts of the cell or it's possible that this appearance is due to greater copy number of the fluorescently-tagged RN-tre R153A construct. We have amended the Figure 8 legend to emphasize that the fractions in 8G are normalized and that the variance in expression level observed in the WB in 8H were consistent throughout experimentation.

(5) In the discussion section, there are some sections that read as if the authors are proposing the proximal target of RN-tre is phosphorylation of RLC rather than Rho-1 (lines 466-467, and 469-470). I don't see that the data, as currently presented, supports this interpretation, rather the data seems to support RN-tre acting on Rho-1 (Fig 6 and 7D). If the authors are proposing phosphorylation of RLC is a direct target of RN-tre, the mechanism for this activity needs to clarified and discussed.

We appreciate the Reviewer pointing this out and we have clarified this in our discussion section.

Minor points:
Typo/Editing at line 279
Corrected.
Figure 7, Panel C. The n.s. bar extends over RN-tre, Rok1, Rok2 and Rho1, but the Rho1 has some ** indicating significance and significance is indicated in the legend.

Corrected.

References

Cai, Y. et al. (2006). Nonmuscle myosin IIA-dependent force inhibits cell spreading and drives F-actin flow. Biophys. J. 91, 3907–3920.

Even-Ram, S., Doyle, A. D., Conti, M. A., Matsumoto, K., Adelstein, R. S., and Yamada, K. M. (2007). Myosin IIA regulates cell motility and actomyosin-microtubule crosstalk. Nat. Cell Biol. 9, 299–309.

Ponti, A., Machacek, M., Gupton, S. L., Waterman-Storer, C. M., and Danuser, G. (2004). Two distinct actin networks drive the protrusion of migrating cells. Sci. 305, 1782–1786.

Vicente-Manzanares, M., Zareno, J., Whitmore, L., Choi, C. K., and Horwitz, A. F. (2007). Regulation of protrusion, adhesion dynamics, and polarity by myosins IIA and IIB in migrating cells. The Journal of Cell Biology 176.
Dear Dr. Applewhite,

Thank you for revising your manuscript in response to the referees' recommendations. I have now had a chance to read through the revised manuscript carefully along with your responses to the referees. It's clear that you have addressed their major concerns and I'm happy to inform you that we are pleased to publish your manuscript in MBoC. Congratulations to you and your colleagues on this important paper!

Best,

Rick Fehon

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Applewhite:

Congratulations on the acceptance of your manuscript.

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