Cell ratcheting through the Sbf RabGEF directs force balancing and stepped apical constriction

Hui Miao, Timothy Vanderleest, Cayla Jewett, Dinah Loerke, and J. Todd Blankenship

Corresponding Author(s): J. Todd Blankenship, University of Denver

Review Timeline:
- Submission Date: 2019-05-11
- Editorial Decision: 2019-06-17
- Revision Received: 2019-08-05
- Editorial Decision: 2019-08-06
- Revision Received: 2019-08-15

Monitoring Editor: William Bement
Scientific Editor: Marie Anne O'Donnell

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.)

DOI: https://doi.org/10.1083/jcb.201905082
Dear Dr. Todd Blankenship,

Thank you for submitting your manuscript entitled "Cell ratcheting through the Sbf RabGEF directs force balancing and stepped apical constriction during Drosophila gastrulation". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Both reviewers found the study of considerable interest although both also highlighted a number of points that they would like to see addressed. We therefore invite you to submit a revised manuscript in which you address all of the major points raised by the reviewers. While there are several, many of them will apparently require no additional experiments. Specifically, for reviewer 1, points 2 and 3 and for reviewer 2 points 2, 3 and 4 the reviewers are asking for either textual changes or reanalysis of data in different ways. More importantly, addressing all of the reviewer points will strengthen your paper.

We look forward to seeing a revised version of this fascinating study.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

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

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, 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.
Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

William Bement, Ph.D.
Monitoring Editor

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

Journal of Cell Biology

-----------------------------------------------

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

Pulsatile contraction of actomyosin networks has been associated with the apical constriction of epithelial cells. However, the mechanisms that reorganize the plasma membrane during apical constriction are not well understood. In this manuscript, Miao and colleagues investigate the role of membrane trafficking during the invagination of the prospective mesoderm in Drosophila embryos. The authors find that Sbf, a potential GEF for Rab35, localizes to apical tubules in mesodermal precursors, where it colocalizes with Rab35. The DENN domain of Sbf can pull down Rab35 in a nucleotide dependent manner. In vitro, the DENN domain of Sbf seems to promote an increase in the GTP exchange rates of Rab35, suggesting that Sbf acts as a Rab35 GEF. Sbf shRNA and Rab35 shRNA disrupted apical constriction in the mesoderm, prevented changes in cell shape, and lead to the appearance of membrane blebs and defects in ventral furrow formation. In addition, in Sbf shRNA and Rab35 shRNA embryos, 15-30% of pulses of apical area reduction were followed by pulses of apical relaxation, suggesting that Sbf and Rab35 enforce the ratcheting of apical constriction. The role of Sbf and Rab35 was most pronounced in cells close to the ventral midline, which sustain the largest forces from their neighbors. Rab35 compartments preceded and eventually colocalized with myosin structures, and both Rab35 and Sbf were necessary for the correct accumulation of myosin on the apical surface of mesoderm precursors. Pharmacological inhibition of clathrin mediated endocytosis resulted in increased colocalization between Rab5 and Rab35 (suggesting that Rab5 associates with Rab35 compartments as they are trafficked into the cell) and a reduced size of Rab11-positive compartment (suggesting that Rab35 compartments are
delivered to recycling endosomes.

This a really nice paper, in which the authors carefully quantify the role of Rab35 and Sbf in ratcheting the contraction of mesodermal precursors. The molecular nature of the ratchet in processive constriction processes has remained elusive, so the work will be of broad interest. I have some questions about the interpretation of data (particularly situations in which the images do not reflect the results of the quants), a couple of problems with statistics (I don't think the use of Student's t-test is appropriate, and I find the use of bar graphs concerning), and smaller points that are just a matter of clarification. In particular, I suggest that the authors address the following points:

MAIN POINTS

1. Figure 2Q: How many times were the GTP exchange assays repeated? The error bars (which represent the standard error of the mean) are really large, particularly for the important experiment with MBP:Rab35 and no GMP-PNP. so I worry about what would happen if the authors added more data. Also, and as indicated below, the authors should show the individual data points that make up each bar.

2. If Sbf shRNA disrupts ventral furrow formation, how did the authors classify the stages of contractility (initial, early, mid, and late) when Sbf was disrupted? What are the provided time ranges measured with respect to?

3. Myosin levels when Rab35 or Sbf are knocked down: the authors measured the overall level of myosin II fluorescence upon Rab35 shRNA or Sbf shRNA and found no change with respect to controls (Figure 7E), but how about the mean fluorescence per cell? From Figure 7F-H, it seems like the mean myosin level per cell may decrease in Rab35 shRNA or Sbf shRNA? Also, the authors claim a greater variability in myosin levels per cell (lines 283-284), but could that be because the cell areas are more variable? The authors should compare mean myosin levels for cells of given sizes (e.g. by binning the distributions in Figures 7F-H into 50 micron^2 intervals).

4. Figure 8: The results obtained with chlorpromazine should be reproduced using a second treatment to ensure the specificity of the result. Using a second drug that blocks endocytosis (e.g. dynasore) or a genetic approach (e.g. the dynamin temperature-sensitive mutants that Drosophilists are fortunate to have available) would be a great way to demonstrate the specificity of these results.

5. Related to the previous question: is the effect of Rab35 and Sbf on apical constriction and mesoderm ingression through their role in membrane internalization or through their potential role in delivering membrane to the recycling endosome? The authors should examine apical constriction and mesoderm ingression in embryos with Rab11 loss of function to clarify this point.

STATISTICS

1. The authors use Student’s t-test throughout, which assumes that the data are normally distributed. They should either demonstrate that, or used the appropriate non-parametric tests (e.g. Mann-Whitney)

2. I'd encourage the authors to replace their bar graphs with box plots where the individual measurements can be observed, or with violin plots that reveal the distribution of the data.
ADDITIONAL POINTS

1. The authors should indicate the position of the anterior and ventral directions in the legend of each figure.

2. Lines 151-152: the authors argue that, during furrow ingression, both the number and size of Rab35+ compartments decrease. Is this in control embryos, or comparing controls to Sbf shRNA? From Figure 2L it seems that the number of compartments actually increases during gastrulation in control embryos. Please clarify.

3. Can the author explain (maybe in their discussion of Figure 3B or 6C) why does the Spider:GFP signal looks so grainy in Sbf shRNA or Rab35 shRNA embryos compared to controls? Is the signal weaker when trafficking is disrupted? If so, why? Wouldn't one naively expect that inhibiting membrane trafficking may lead to the accumulation of signal at the membrane?

4. Line 43: the acronym MSD is used in the summary before being defined.

5. Line 59: should "spatiotemporal" here be "increased temporal"?

6. Line 118: if the authors refer to the N-tagged construct (as suggested by Figure 1), this should be GFP:Sbf, not Sbf:GFP.

7. Lines 132-133: this is a strong conclusion at this point in the paper; what is the evidence for Sbf being a Rab35 GEF? Their similar localization? The authors should tone down this sentence or move to later in the paper, after they show the biochemical data to support this point.

8. Figure 2: are "late" and "8 min" equivalent? If so, please clarify in the figure legend (or maybe use just one of the two terms).

9. Figure 3C-D: how many embryos did the authors measure in each group?

10. Figure 4E: is this metric missing units (micrometers) on the Y axis?

11. Lines 251-254: this sentence should reference (Martin, Gelbart et al., 2010), which was the first publication to describe the anisotropic constriction of cells during mesoderm ingression.

12. Figure 6E: I don't think "S.T.D" is the best way to abbreviate standard deviation ... maybe use "St.D." or "std. dev.", or even the greek letter sigma?

13. Figure 7: pixel units for areas or lengths are difficult to interpret. Please, convert to micron-based units.

14. Figure 8B: the authors claim no change in the size of Rab5-positive compartments upon chlorpromazine treatment, but based on Figure 8B, it looks like the compartments get larger. This is also apparent in the Rab5 panels in Figure 8F-G. Are these images not representative or does the result need to be re-evaluated?

Reviewer #2 (Comments to the Authors (Required)): 
In this manuscript the authors describe the identification of a new Rab-GEF for rab35 in Drosophila, the protein Sbf. Sbf appears to function with rab35 during the invagination of the mesoderm through apical constriction in the Drosophila embryo. The authors show colocalisation of rab35 and Sbf in vivo, in vitro binding of the two and stimulation of nucleotide exchange by Sbf. They go on to show that loss of rab 35 or Sbf leads to problems in apical constriction and mesoderm internalisation (this effect of interference with rab35 at least the authors had already previously shown in Jewett, C. E., Vanderleest, T. E., Miao, H., Xie, Y., Madhu, R., Loerke, D., & Blankenship, J. T. (2017). Planar polarized Rab35 functions as an oscillatory ratchet during cell intercalation in the Drosophila epithelium, 1-16). This seems to correlate with the appearance of large membrane blebs on the apical surface as well as problems in ratcheting behaviour, they stabilisation of a shrunken apical shape after constriction. The furthermore show that rab35 and Sbf compartments precede apical-medial myosin pulses, and that these compartments probably feed into rab11 recycling endosomes via rab5 intermediates.

This is a well executed study that builds on the authors already published finding during selective junction shrinkage in germband extension and cell intercalation that rab35 compartments play an important role in allowing cell shape change to occur (Jewett, C. E., Vanderleest, T. E., Miao, H., Xie, Y., Madhu, R., Loerke, D., & Blankenship, J. T. (2017). Planar polarized Rab35 functions as an oscillatory ratchet during cell intercalation in the Drosophila epithelium, 1-16). The data really point to a physical requirement for apical membrane removal, beautifully illustrated in the SEM images of control and loss of rab35 or loss of Sbf situation.

This study should be of wide interest in the field of trafficking, morphogenesis but also more widely for the cell biological community.

I have a few questions that I would like to see addressed:

1) As far as I can tell from the methods, all the Sbf localisation analysis is based on overexpression of UAS constructs with a maternal driver. Do the authors not worry that this might change compartments or their abundance or induce phenotypes? Have the authors analysed this through for instance comparison of rab11/rab5/rab35 (if there is an antibody) plus/minus the overexpression?

Along these lines, the panels for the deletion constructs in Fig. 1 C look weirdly homogeneous, it would be good to show a counterstaining channel, i.e. E-Cad or similar.

2) Figure 2: In order to be able to combine analysis of rab35 with the UAS-GFP-Sbf, they now use UAS-mCherry-rab35 (though this is not detailed in the figure legends when it should really be stated here): have the authors quantified the amount of different rab35 structures and their localisation in this overexpression of rab35 situation compared to the CRISPR-GFP-rab35 allele? In general, I would strongly suggest that genotypes for localisation analysis (i.e. is it a CRISPR allele or overexpression) should be clearly stated in the figure legend.

3) The authors can pull-down rab35 in vitro with Sbf GEF-DENN, why does this construct not localise at all in vivo when overexpressed? Isn't this a strange discrepancy? Could the authors discuss?

4) The one thing I find that is really lacking is an analysis of correlation and timing of the appearance can of rab35/Sbf compartments with active constriction/ apical area change. What is the temporal relationship between these?
This seems particularly relevant in the light of their findings that the rab35/Sbf endosomal structures prefigure where an apical-medial myosin pulse is about to appear. Wouldn't the authors have expected this to be the other way round? Again, it would be important to know what the time correlation is of myosin pulses, apical area change and rab35/Sbf compartments appearance and dynamics. Based on previous data by several labs, the appearance of rab35 prior to the myosin pulse would suggest that membrane removal begins prior to a constriction pulse? Can this be?

Minor comment:

5) Shouldn't abbreviations such as MSD and CME be explained when used in the Summary?
August 5, 2019

Dear Dr. Bement and Dr. O'Donnell,

We appreciate the opportunity to resubmit our manuscript, “Cell ratcheting through the Sbf RabGEF directs force balancing and stepped apical constriction”. A special thanks to the reviewers and editor for their constructive comments – these were some of the more helpful comments we’ve received. We have added new data and analysis as requested by the reviewers, and hope that our revised manuscript and resubmission comments will satisfactorily address the reviewer concerns.

Point-by-point responses to the reviewers’ comments are presented below. We hope that the manuscript is now suitable for publication in JCB and look forward to your decision.

Thank you for your time, best regards,

J. Todd Blankenship
Director, Molecular and Cellular Biophysics Program
Associate Professor of Biological Sciences
The University of Denver

*****************************
We thank the reviewers for their constructive comments – they were much appreciated. Thank you as well for taking the time to review the manuscript. We have added significant new data and analysis. We hope that the revised manuscript and resubmission comments will satisfactorily address many of the concerns were raised. Also, please note that Figure 2 has now been split into two figures due to its size, so some of the Figure panels referenced in the reviewer critiques will have different corresponding numbers in the new submission.

Reviewers’ comments:

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

Pulsatile contraction of actomyosin networks has been associated with the apical constriction of epithelial cells. However, the mechanisms that reorganize the plasma membrane during apical constriction are not well understood. In this manuscript, Miao and colleagues investigate the role of membrane trafficking during the invagination of the prospective mesoderm in Drosophila embryos. The authors find that Sbf, a potential GEF for Rab35, localizes to apical tubules in mesodermal precursors, where it colocalizes with Rab35. The DENN domain of Sbf can pull down Rab35 in a nucleotide dependent manner. In vitro, the DENN domain of Sbf seems to promote an increase in the GTP exchange rates of Rab35, suggesting that Sbf acts as a Rab35 GEF. Sbf shRNA and Rab35 shRNA disrupted apical constriction in the mesoderm, prevented changes in cell shape, and lead to the appearance of membrane blebs and defects in ventral furrow formation. In addition, in Sbf shRNA and Rab35 shRNA embryos, 15-30% of pulses of apical area reduction were followed by pulses of apical relaxation, suggesting that Sbf and Rab35 enforce the ratcheting of apical constriction. The role of Sbf and Rab35 was most pronounced in cells close to the ventral midline, which sustain the largest forces from their neighbors. Rab35 compartments preceded and eventually colocalized with myosin structures, and both Rab35 and Sbf were necessary for the correct accumulation of myosin on the apical surface of mesoderm precursors. Pharmacological inhibition of clathrin mediated endocytosis resulted in increased colocalization between Rab5 and Rab35 (suggesting that Rab5 associates with Rab35 compartments as they are trafficked into the cell) and a reduced size of Rab11-positive compartment (suggesting that Rab35 compartments are delivered to recycling endosomes.

This a really nice paper, in which the authors carefully quantify the role of Rab35 and Sbf in ratcheting the contraction of mesodermal precursors. The molecular nature of the ratchet in processive constriction processes has remained elusive, so the work will be of broad interest. I have some questions about the interpretation of data (particularly situations in which the images do not reflect the results of the quants), a couple of problems with statistics (I don't think the use of Student's t-test is appropriate, and I find the use of bar graphs concerning), and smaller points that are just a matter of clarification. In particular, I suggest that the authors address the following points:

MAIN POINTS

1. Figure 2Q: How many times were the GTP exchange assays repeated? The error bars (which represent the standard error of the mean) are really large, particularly for the important experiment with MBP:Rab35 and no GMP-PNP. so I worry about what would happen if the authors added more data. Also, and as indicated below, the authors should show the individual data points that make up each bar.

Response 1.1: We performed this assay 3 times in the original submission – we have since added two more trials for an n=5 , and this is now indicated in the figure legend. The results are consistent with our earlier data. We have also switched the data to a box-and-whisker plot to better show the result and variability (this is now Figure 3 as we split the previous Figure 2 into two figure due to its size).
2. If Sbf shRNA disrupts ventral furrow formation, how did the authors classify the stages of contractility (initial, early, mid, and late) when Sbf was disrupted? What are the provided time ranges measured with respect to?

Response 1.2: Constriction rates are very similar between wild-type and Sbf embryos in the first 120 seconds of furrow formation, which is consistent with a requirement for Rab35 in the ratcheting of the actomyosin network. Additionally, there is a small DV displacement in the epithelium when the ventral furrow first initiates which is still perceptible in Sbf embryos and appears to have a similar timing post-cellularization as in wild-type embryos, so we have used this first inflection in the constriction rate as our t=0. We have added this brief description to the Methods.

3. Myosin levels when Rab35 or Sbf are knocked down: the authors measured the overall level of myosin II fluorescence upon Rab35 shRNA or Sbf shRNA and found no change with respect to controls (Figure 7E), but how about the mean fluorescence per cell? From Figure 7F-H, it seems like the mean myosin level per cell may decrease in Rab35 shRNA or Sbf shRNA? Also, the authors claim a greater variability in myosin levels per cell (lines 283-284), but could that be because the cell areas are more variable? The authors should compare mean myosin levels for cells of given sizes (e.g. by binning the distributions in Figures 7F-H into 50 micron^2 intervals).

Response 1.3: Yes, a good point – while Rab35 has near wild-type levels of Myosin II (11% of wild-type) when comparable cell sizes (20-40µm^2) are measured (new data in Fig. 8L), Sbf is more disrupted with a 43% decrease in Myosin II. It is also interesting to note that the fractional standard deviation (37% in Rab35 and 31% in Sbf versus 25% in wild-type) is greater in this measurement of comparable cell sizes. We note these points in new lines in the Results and Discussion (lines 283-285; 345-348).

4. Figure 8: The results obtained with chlorpromazine should be reproduced using a second treatment to ensure the specificity of the result. Using a second drug that blocks endocytosis (e.g. dynasore) or a genetic approach (e.g. the dynamin temperature-sensitive mutants that Drosophilists are fortunate to have available) would be a great way to demonstrate the specificity of these results.

Response 1.4: Yes, we agree – we have repeated the experiment with PitStop2, a second endocytosis inhibitor that is chemically distinct from chlorpromazine and works well in Drosophila (Jewett et al., NatComms, 2017). The PitStop2 results are very similar to the chlorpromazine results and are presented in Fig. S5.

5. Related to the previous question: is the effect of Rab35 and Sbf on apical constriction and mesoderm ingression through their role in membrane internalization or through their potential role in delivering membrane to the recycling endosome? The authors should examine apical constriction and mesoderm ingestion in embryos with Rab11 loss of function to clarify this point.

Response 1.5: Another good point – it appears that Rab35 and Sbf have a primary effect on apical constriction through their function on membrane internalization. Disruption of Rab11 by the same version of shRNA as used for Rab35 and Sbf has no effect on ventral furrow formation (lines 311-314; Fig. S5J).

STATISTICS

1. The authors use Student’s t-test throughout, which assumes that the data are normally distributed.
They should either demonstrate that, or used the appropriate non-parametric tests (e.g. Mann-Whitney)

**Response 1.2.1:** Thank you for pushing us on the statistical analyses – it is appreciated. We have gone through all data and examined it for normal distributions and, where appropriate, have performed the appropriate non-parametric or parametric analyses. The statistical tests performed are listed at the end of each figure legend. The overall significances reported in the first submission did not change, although we have updated P-values where appropriate.

2. I’d encourage the authors to replace their bar graphs with box plots where the individual measurements can be observed, or with violin plots that reveal the distribution of the data.

**Response 1.2.2:** Again, thank you for pushing us on this front as well. We have shifted to box-and-whisker plots where possible. In some figures, the results are from an n=3 embryos (although representing averages from ~50-100 cells per embryo), which makes the quartile bounds of a box-and-whisker plot problematic.

**ADDITIONAL POINTS**

1. The authors should indicate the position of the anterior and ventral directions in the legend of each figure.

**Response 1.3.1:** This is now indicated at the end of each figure legend.

2. Lines 151-152: the authors argue that, during furrow ingression, both the number and size of Rab35+ compartments decrease. Is this in control embryos, or comparing controls to Sbf shRNA? From Figure 2L it seems that the number of compartments actually increases during gastrulation in control embryos. Please clarify.

**Response 1.3.2:** Yes, this in reference to Rab35 compartments in Sbf disrupted embryos (comparing WT to Sbf Rab35 compartment number at 8 min). We have edited these sentences for clarity.

3. Can the author explain (maybe in their discussion of Figure 3B or 6C) why does the Spider:GFP signal looks so grainy in Sbf shRNA or Rab35 shRNA embryos compared to controls? Is the signal weaker when trafficking is disrupted? If so, why? Wouldn’t one naively expect that inhibiting membrane trafficking may lead to the accumulation of signal at the membrane?

**Response 1.3.3:** This is because the Spider:GFP signal is heterozygous in these crosses and is an effect of the lower dosage. From our results, it does not appear that trafficking affects Spider:GFP or Resille:GFP signal (both are peripheral, and not transmembrane, proteins). We have noted the heterozygosity in the Fig. 7 legend.

4. Line 43: the acronym MSD is used in the summary before being defined.

**Response 1.3.4:** Yes, apologies, JCB has a small abstract word-limit, we define it when it is used for the first time in the main text.

5. Line 59: should "spatiotemporal" here be "increased temporal"?
Response 1.3.5: Careful reading :-) – we agree, and have changed the text.

6. Line 118: if the authors refer to the N-tagged construct (as suggested by Figure 1), this should be GFP:Sbf, not Sbf:GFP.

Response 1.3.6: Thank you for catching this – it has been changed.

7. Lines 132-133: this is a strong conclusion at this point in the paper; what is the evidence for Sbf being a Rab35 GEF? Their similar localization? The authors should tone down this sentence or move to later in the paper, after they show the biochemical data to support this point.

Response 1.3.7: Agreed – we have edited the sentence to describe Sbf as a “candidate” GEF.

8. Figure 2: are "late" and "8 min" equivalent? If so, please clarify in the figure legend (or maybe use just one of the two terms).

Response 1.3.8: We have clarified this in the figure legend.

9. Figure 3C-D: how many embryos did the authors measure in each group?

Response 1.3.9: We have added this information to the figure legend.

10. Figure 4E: is this metric missing units (micrometers) on the Y axis?

Response 1.3.10: We changed the Y-axis label and figure legend to clarify this – the y-axis is a convolution metric (measured surface area length/linear length).

11. Lines 251-254: this sentence should reference (Martin, Gelbart et al., 2010), which was the first publication to describe the anisotropic constriction of cells during mesoderm ingression.

Response 1.3.11: We have inserted this reference.

12. Figure 6E: I don't think "S.T.D" is the best way to abbreviate standard deviation ... maybe use "St.D." or "std. dev.", or even the greek letter sigma?

Response 1.3.12: We have changed this to Std. Dev.

13. Figure 7: pixel units for areas or lengths are difficult to interpret. Please, convert to micron-based units.

Response 1.3.13: We have changed this to microns.

14. Figure 8B: the authors claim no change in the size of Rab5-positive compartments upon chlorpromazine treatment, but based on Figure 8B, it looks like the compartments get larger. This is also apparent in the Rab5 panels in Figure 8F-G. Are these images not representative or does the result need to be re-evaluated?
Response 1.3.14: Thank you for noticing – we have replaced the image panels with more representative images.

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

In this manuscript the authors describe the identification of a new Rab-GEF for rab35 in Drosophila, the protein Sbf. Sbf appears to function with rab35 during the invagination of the mesoderm through apical constriction in the Drosophila embryo. The authors show colocalisation of rab35 and Sbf in vivo, in vitro binding of the two and stimulation of nucleotide exchange by Sbf. They go on to show that loss of rab 35 or Sbf leads to problems in apical constriction and mesoderm internalisation (this effect of interference with rab35 at least the authors had already previously shown in Jewett, C. E., Vanderleest, T. E., Miao, H., Xie, Y., Madhu, R., Loerke, D., & Blankenship, J. T. (2017). Planar polarized Rab35 functions as an oscillatory ratchet during cell intercalation in the Drosophila epithelium, 1-16). This seems to correlate with the appearance of large membrane blebs on the apical surface as well as problems in ratcheting behaviour, they stabilisation of a shrunken apical shape after constriction. The furthermore show that rab35 and Sbf compartments precede apical-medial myosin pulses, and that these compartments probably feed into rab11 recycling endosomes via rab5 intermediates.

This is a well executed study that builds on the authors already published finding during selective junction shrinkage in germband extension and cell intercalation that rab35 compartments play an important role in allowing cell shape change to occur ( Jewett, C. E., Vanderleest, T. E., Miao, H., Xie, Y., Madhu, R., Loerke, D., & Blankenship, J. T. (2017). Planar polarized Rab35 functions as an oscillatory ratchet during cell intercalation in the Drosophila epithelium, 1-16). The data really point to a physical requirement for apical membrane removal, beautifully illustrated in the SEM images of control and loss of rab35 or loss of Sbf situation.

This study should be of wide interest in the field of trafficking, morphogenesis but also more widely for the cell biological community.

I have a few questions that I would like to see addressed:

1) As far as I can tell from the methods, all the Sbf localisation analysis is based on overexpression of UAS constructs with a maternal driver. Do the authors not worry that this might change compartments or their abundance or induce phenotypes? Have the authors analysed this through for instance comparison of rab11/rab5/rab35 (if there is an antibody) plus/minus the overexpression>

Along these lines, the panels for the deletion constructs in Fig. 1 C look weirdly homogeneous, it would be good to show a counterstaining channel, i.e. E-Cad or similar.

Response 2.1: Yes, a good concern – to examine this more closely, we quantified the number and size of Rab5 and Rab11 compartments either in the presence or absence of Rab35:mCh (new data in Fig. S5). In total, mCh:Rab35 expression did not change the behavior of these compartments, although there is a very slight increase in Rab11 compartment size (which would be consistent with the decrease in Rab11 size after Rab35 disruption). We also compared Rab35 compartments from the endogenous CRISPR GFP:Rab35 to mCh:Rab35 (new data in Fig. S1B,C). The behavior of these compartments is approximately the same, although we do detect slightly fewer mCh:Rab35 compartments than the endogenous GFP:Rab35, consistent with the dimmer signal we observe when using mCh fluorophores versus GFP. We have also added images of counter-stained Sbf deletion constructs – due to the number of image panels we have added this data in a supplementary figure (Fig. S1A).

2) Figure 2: In order to be able to combine analysis of rab35 with the UAS-GFP-Sbf, they now use UAS-mCherry-rab35 (though this is not detailed in the figure legends when it should really be stated
have the authors quantified the amount of different rab35 structures and their localisation in this overexpression of rab35 situation compared to the CRISPR-GFP-rab35 allele?

In general, I would strongly suggest that genotypes for localisation analysis (i.e. is it a CRISPR allele or overexpression) should be clearly stated in the figure legend.

**Response 2.2:** We agree, and this has been clarified in each figure legend.

3) The authors can pull-down rab35 in vitro with Sbf GEF-DENN, why does this construct not localise at all in vivo when overexpressed? Isn't this a strange discrepancy? Could the authors discuss?

**Response 2.3:** We believe this makes sense – the Sbf DENN domain can interact with Rab35 in vitro and cause a higher rate of Rab35 GTP exchange, but it lacks the PH, CC, and GRAM domains that appear necessary to localize it to the plasma membrane (we are currently working on a manuscript which shows that PIP3 lipids are required to localize Sbf and Rab35 to the apical surface, and that the PH domain of Sbf directly binds PIP3). Therefore, these constructs lacking PH, CC, and/or GRAM domains could still interact with Rab35 in vivo, but they would likely be cytoplasmic in their distributions which would lead to a near absence of signal. Consistent with this, Rab35 is missing from the cell surface when Sbf is disrupted, but it does have this odd phenotype of re-localizing strongly to small cytoplasmic puncta. We discuss this in the Discussion (lines 371-284) and suggest this may imply that there is a different, endosomal and non-Sbf GEF for Rab35.

4) The one thing I find that is really lacking is an analysis of correlation and timing of the appearance of rab35/Sbf compartments with active constriction/ apical area change. What is the temporal relationship between these?

This seems particularly relevant in the light of their findings that the rab35/Sbf endosomal structures prefigure where an apical-medial myosin pulse is about to appear. Wouldn't the authors have expected this to be the other way round? Again, it would be important to know what the time correlation is of myosin pulses, apical area change and rab35/Sbf compartments appearance and dynamics. Based on previous data by several labs, the appearance of rab35 prior to the myosin pulse would suggest that membrane removal begins prior to a constriction pulse? Can this be?

**Response 2.4:** We agree – we have added new data showing that Rab35 precedes apical constriction (Fig. S3). We also agree that Rab35 preceding Myosin II localization is a somewhat surprising result and had expected that Rab35 might load on to the apical membrane after Myosin II, however the results suggest that there may be a higher order coordination between endocytic and actomyosin pathways. It appears that the signal that initiates apical constriction (perhaps the PIP3 mentioned above?) begins recruiting Sbf and Rab35 to the apical surface in advance of Myosin II. Therefore, we theorize that Rab35 could have 2 possible functions – a signaling function where it directly recruits Myosin II activating machinery, or a physical function in which Sbf/Rab35 are needed to remodel the apical surface by removing infoldings of the apical membrane that permit the assembly of contiguous and contractile actomyosin arrays. The SEM and TEM data is consistent with the physical model, while work from the Lecuit lab (Jha et al., Curr Biol, 2018) suggests that tubular infoldings can act as signaling platforms for serpentine G protein signaling that in turn leads to the activation of Myosin II. We discuss these potential interpretations in the Discussion (lines 350-363), but it will take additional work to differentiate among them.

Minor comment:

5) Shouldn't abbreviations such as MSD and CME be explained when used in the Summary?

**Response 2.5:** Yes, apologies, JCB has a small abstract word-limit, so we define them when they are used for the first time in the main text. This can be changed if necessary.
August 6, 2019

RE: JCB Manuscript #201905082R

Dr. J. Todd Blankenship
University of Denver
2101 E Wesley Ave Rm 301
Denver, CO 80210

Dear Dr. Blankenship:

Thank you for submitting your revised manuscript entitled "Cell ratcheting through the Sbf RabGEF directs force balancing and stepped apical constriction". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

- Provide main and supplementary text as separate, editable .doc or .docx files
- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website *paying particular attention to the guidelines for preparing images and blots at sufficient resolution for screening and production*
- Add conflict of interest statement to Acknowledgements section
- Add a paragraph after the Materials and Methods section briefly summarizing the online supplementary materials
- Add scale bar Fig 2A, 6A,
- Fig 3A splices should be indicated, blot resolution 3B looks too poor for screening and production

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

B. FINAL FILES:

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

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

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.
-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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

**The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.**

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

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,

William Bement, Ph.D.
Monitoring Editor

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

Journal of Cell Biology