ARHGAP4-SEPT2-SEPT9 complex enables both up- and down-modulation of integrin-mediated focal adhesions, cell migration, and invasion

Na Kang, Tsubasa Matsui, Shiyou Liu, and Shinji Deguchi

Corresponding author(s): Shinji Deguchi, Osaka University

Review Timeline:

| Event                  | Date       |
|------------------------|------------|
| Submission Date:       | 2021-01-08 |
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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.)
RE: Manuscript #E21-01-0010
TITLE: ARHGAP4-SEPT2-SEPT9 complex enables both up- and down-modulation of integrin-mediated focal adhesions, cell migration, and invasion

Dear Dr. Deguchi,

Thank you for submitting your manuscript entitled ARHGAP4-SEPT2-SEPT9 complex enables both up- and down-modulation of integrin-mediated focal adhesions, cell migration, and invasion. Two reviewers with expertise on the topic assessed your manuscript and their comments are appended to this letter. Both reviewers agree that your findings are potentially interesting and that your data support the conclusions you make; however, they both raise concerns to address should you choose to submit a revision for further consideration.

Reviewer 1 indicates that impact would be strengthened with more direct evidence of an ARHGAP4-SEPT2-SEPT9 complex and suggests several ways to achieve this. Reviewer 1 also requests further evidence that ARHGAP-4 suppresses cell invasion. Reviewer 2 also indicates additional evidence for an ARHGAP4-SEPT2-SEPT9 complex, particularly using an approach that does not rely of heterologous expressed ARHGAP4, which I also believe if of fundamental importance. I also agree with comments on focal adhesion dynamics, which needs to be quantified to support your conclusion on ARHGAP4 and focal adhesion stability.

Reviewer 2 also has concerns on some of the figures as presented, which are important to resolve. As they indicate, volcano plots are standard to include for RNA-seq data to convey statistically significant differences.

Based on the reviewers’ critique, we invite you to submit a revision if you can address their key concerns. If you elect to prepare a revised manuscript, please include a cover letter addressing the reviewers’ comments point by point, and adhere to the MBoC guidelines for submissions.

Regards,
Diane Barber
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Deguchi,

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 accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available
Reviewer #1 (Remarks to the Author):

This is an interesting study that demonstrates that ARHGAP4, one of the Rho-GTPase-activating proteins, forms a complex with SEPT2 and SEPT9 via its Rho-GAP domain to regulate migration and invasion of epithelial cells in their microenvironment-dependent manner. Overall, the data are convincing and open up several new questions about ARHGAP4 biology. These findings are biologically important and interesting broadly for cell biologists. Experimental results are clear and the writing is easy to understand.

However, it may increase its impact to show direct evidence of ARHGAP4-SEPT2-SEPT9 complex formation in the MCF-710A cells (although this is not necessary). Thus, is it possible to perform some immunofluorescence assay against ARHGAP4 and SEPT2 or SEPT9 to demonstrate co-localization in MCF-10A cells?

To further demonstrate that ARHGAP-4 suppresses cell invasion under the negative regulation by SEPT2/9 the author could perform an MCF-10A spheroids /3D culture. In 3D, ECM cues promote vinculin recruitment to focal adhesions, projection formation, and cell invasion in MCF10A (doi: 10.1158/0008-5472.CAN-13-3698. PMID: 25183785; PMCID: PMC4191931)

Minor concerns:

1) In Figure 2 A, total lysate western blot against GFP are missed. Moreover IP: GFP, Blot :GFP does not show GFP expression in both conditions.

2) In Figure 2 D, Bottom, Blot :GFP is not indicated. It is not clear what this panel represents.

3) Fig. 3A, Fig 5A quality of some western blot must be improve.

4) In Figure 5A it should be FAK-Y397.

5 ) In Figure 6 A and C it must be FAK-Y397

Reviewer #2 (Remarks to the Author):

The proposal shows that siRNA-mediated ablation of the Rho GAP ARHGAP4 alters FA number. In addition, the authors show that ARHGAP4 binds to the Septins SEPT2 and 4 and that this interaction might regulate FA number and cell migration. Overall, I think that this is an interesting study that could add novel information to our understanding of Focal Adhesion (FA) and migration biology. However, I do have several concerns about the paper

Overarching Comments

1. My fundamental concern with this proposal is that the experiments only show that an ARHGAP4/SEPT2 or ARHGAP4/SEPT9 complex exists when ARHGAP4 is ectopically expressed. Some data (ip and images) looking at endogenous expression are important. In a similar vein, the data showing that SEPT2 /SEPT 9 have an antagonistic relationship with ARHGAP4 in the context of FA number is good but the assertion that the two function as a complex needs to be further examined: does the transfected ARHGAP4 plus endogenous SEPT2 or 9 interact at FAs or some other organelle? In addition, does interaction between ARHGAP4 and the Septins change in response to external stimuli know to increase or decrease FA number or size
2. Fig 4C shows that overexpressed ARHGAP4 is not specifically localized to FA but what about the endogenous protein?
2. Does ARHGAP4 ablation change the activation of FA signaling during FA disassembly in response to external stimuli (i.e FAK phosphorylation in response to osmotic stress).
3. The authors comment that ARHGAP4 ablation changes cell shape. Since FA number and size is related to cell shape, these cell shape changes should be quantified.

Major Experimental Comments
- I don't think the gene expression data in Fig 1 adds much to the story. It's likely better as supplementary data. Moreover, this data was obtained from another cell line instead of the HEK293 used in the remainder of the paper.
- The authors have not measured FA dynamics. To say that ARHGAP4 loss stabilizes FA number is incorrect. Rather, they should state that ARHGAP4 loss increases FA number.
- The data in showing that ARHGAP4 IPs with SEPT2 and 9 is convincing but is would be nice to see what co-ip looks like with endogenous ARHGAP4 and in a cell line other than HEK293.
- Fig 3C si-RNA control looks uninterpretable to me. I don't see a full cell there to compare with the other treatments. Also, the authors used manual tracing of FA to enumerate them. There are automated Fiji/Imaris plugins for this.
- I don't understand what is being performed in Fig 3F. Some images of the cells in question should be provided, not just the fluorescent intensity profiles.

Minor Experimental Comments
- Fig 1A is better expressed as a Volcano plot so that p value info can be included.
- The analysis in Fig 1B should not be expressed as total number of genes but as an enrichment fraction.
- Fig 2A should have the "Lysate" WB as part of the same gel as the IP to assess the fraction of SEPT2 or SEPT9 that is IPing with GFP-ARHGAP4.
- Fig 3A, Fig 4A and 5A should have untreated cells shown.
- Fig 3E and 5E are better presented as a Cumulative Distribution Function since 1.5um2 is an arbitrary cut-off value.
- Fig 3E should have the laminin and collagen data along with the fibronectin data.
- I am surprised that the GFP control in 5C doesn't show any paxillin staining. Surely these cells must have some FA? Fig 5D says they do.
- Nomenclature in Fig 7 should be consistent with the remainder of the paper, i.e. use SEPT2/9 instead of Septin2 or Spetin9.
We would like to express our sincere appreciation for the reviewers’ constructive comments concerning our manuscript titled “ARHGAP4-SEPT2-SEPT9 complex enables both up- and down-modulation of integrin-mediated focal adhesions, cell migration, and invasion” (manuscript ID: E21-01-0010). Thanks to their invaluable and professional feedbacks, we believe that the quality of our manuscript has now been significantly improved. We have made extensive modifications to our manuscript according to the reviewers’ comments to finally make our results more comprehensive and convincing. The reviewers’ comments are shown below in bold italic font, followed by our responses given in normal font. All the changes made to the manuscript are given in the highlight format within the revised manuscript.

Reviewer #1:

This is an interesting study that demonstrates that ARHGAP4, one of the Rho-GTPase-activating proteins, forms a complex with SEPT2 and SEPT9 via its Rho-GAP domain to regulate migration and invasion of epithelial cells in their microenvironment-dependent manner. Overall, the data are convincing and open up several new questions about ARHGAP4 biology. These findings are biologically important and interesting broadly for cell biologists. Experimental results are clear and the writing is easy to understand.

1.1 ) However, it may increase its impact to show direct evidence of ARHGAP4-SEPT2-SEPT9 complex formation in the MCF-10A cells (although this is not necessary,). Thus, is it possible to perform some immunofluorescence assay against ARHGAP4 and SEPT2 or SEPT 9 to demonstrate co-localization in MCF-10A cells?

We thank the reviewer for the comment and suggestion. To convince the existence of the ARHGAP4-SEPT2-SEPT9 complexes, we performed the colocalization experiments in MCF10A cells as well as in HEK293 cells. We found ARHGAP4 is expressed mainly in the cytosol but not in the nucleus regardless of the cell types, whereas different SEPT2-SEPT9 expression patterns were observed between the two cell types. Specifically, SEPT2 and SEPT9 exhibit fiber-like patterns in MCF10A cells (See the figure below) but not in HEK293 cells (Fig. S4), although the mechanisms behind these differences remain unclear. We analyzed the colocalization among these three proteins along survey lines in the cytoplasm in HEK293 cell, in which a level of positive correlation is present (Fig. S4). Thus, the immunofluorescence suggests that these three proteins are more or less colocalized in the cytoplasm, supporting our conclusion drawn from the COIP experiments.
1.2) To further demonstrate that ARHGAP4 suppresses cell invasion under the negative regulation by SEPT2/9 the author could perform an MCF10A spheroids /3D culture. In 3D, ECM cues promote vinculin recruitment to focal adhesions, projection formation, and cell invasion in MCF10A (doi: 10.1158/0008-5472.CAN-13-3698. PMID: 25183785; PMCID: PMC4191931)

We thank the reviewer for the comment. In the suggested paper, BD PuraMatrix peptide hydrogel was used with fibronectin and growth medium. In our study using the invasion assay, the chamber was filled with Matrigel. Although there is a difference between the two studies in the ECM components used, we believe that the assay using Matrigel must be one of the standards to evaluate the behavior of cells in 3D conditions.

Minor concerns:

2.1) In Figure 2 A, total lysate western blot against GFP are missed. Moreover IP: GFP, Blot: GFP does not show GFP expression in both conditions.

We thank the reviewer for the comments. Regarding the second comment, we have replaced the figure with a new one that includes the expression of GFP. Regarding the first comment, the relative expression of GFP should be at the same level between the IP and total lysate, and we already showed that SEPT2 and SEPT9 are both obviously expressed in GFP as well as in GFP-GAP4. Therefore, we believe our data have provided convincing evidence for our conclusion.

2.2) In Figure 2 D, Bottom, Blot :GFP is not indicated. It is not clear what this panel represents.

We thank the reviewer for the comment. We have added the annotation and arrows in Figure 2D.

2.3) Fig. 3A, Fig 5A quality of some western blot must be improve.
We thank the reviewer for the comment. We performed the experiments again to get better quality and have replaced the two Figures with new ones.

2.4) In Figure 5A it should be FAK-Y397.

We thank the reviewer for bringing this misdescription to our attention. We have revised the figure.

2.5) In Figure 6A and C it must be FAK-Y397

We thank the reviewer for bringing this misdescription to our attention. We have revised the figure.

Reviewer #2 (Remarks to the Author):

The proposal shows that siRNA-mediated ablation of the Rho GAP ARHGAP4 alters FA number. In addition, the authors show that ARHGAP4 binds to the Septins SEPT2 and 4 and that this interaction might regulate FA number and cell migration. Overall, I think that this is an interesting study that could add novel information to our understanding of Focal Adhesion (FA) and migration biology. However, I do have several concerns about the paper.

Overarching Comments

1. My fundamental concern with this proposal is that the experiments only show that an ARHGAP4/SEPT2 or ARHGAP4/SEPT9 complex exists when ARHGAP4 is ectopically expressed. Some data (ip and images) looking at endogenous expression are important. In a similar vein, the data showing that SEPT2/SEPT9 have an antagonistic relationship with ARHGAP4 in the context of FA number is good but the assertion that the two function as a complex needs to be further examined: does the transfected ARHGAP4 plus endogenous SEPT2 or 9 interact at FAs or some other organelle? In addition, does interaction between ARHGAP4 and the Septins change in response to external stimuli know to increase or decrease FA number or size.

We thank the reviewer for the comments. We performed the experiments using the Bxpc3 cell line, a cell line with a high endogenous expression of ARHGAP4. The COIP result shows that endogenous ARHGAP4 are associated with SEPT2 and SEPT9 (Fig. S3A). In addition, immunofluorescence assay shows that there is a positive colocalization between ARHGAP4 and SEPT2/SEPT9 (Fig. S3B). These results suggest that endogenous ARHGAP4 indeed interacts with SEPT2/SEPT9.

Next, because it was difficult to stain all the proteins with different fluorescence
colors with the current technology, we analyzed the level of the colocalization between ARHGAP4 and paxillin (one of the representative proteins associated with focal adhesions) in Bxpc3 cells. The results attached below show that there is no obvious colocalization between them, suggesting that ARHGAP4 is not necessarily localized at focal adhesions but instead may work on the regulation of focal adhesions by mediating other focal adhesion-associated proteins.

Finally, to the best of our knowledge, it is still difficult to control and examine the binding capacity between ARHGAP4 and SEPT2/9. Thus, while we think the reviewer has raised an interesting point, we hope that he/she understands this situation that we have not accomplished this very challenging experiment and therefore keep it for the subject of further future investigation.

1.2. **Fig 4C shows that overexpressed ARHGAP4 is not specifically localized to FA but what about the endogenous protein?**

We thank the reviewer for the question. We have checked the expression of ARHGAP4 in Bxpc3 cells as well because these cells were found to exhibit higher expressions of endogenous ARHGAP4 according to our separate experiments. We found that endogenous ARHGAP4 is not specifically localized at focal adhesions as shown in the above image.

1.3. **Does ARHGAP4 ablation change the activation of FA signaling during FA disassembly in response to external stimuli (i.e FAK phosphorylation in response to osmotic stress).**

We thank the reviewer for the question. Indeed, the reviewer has raised an interesting point. With this question, we reviewed relevant articles. In a paper (Kuo et al., Nat Cell Biol 13(4), 383-393. doi:10.1038/ncb2216, 2011) in which comprehensive analysis on focal adhesions was performed, the ratio of average fluorescence density of paxillin, vinculin, zyxin, talin, phospho-tyrosine, and VASP immunostaining signal within segmented focal adhesions in hypnotically shocked cells relative to that in intact cells or the ratio of that in isolated focal adhesions relative to that in intact cells have no significant difference. Thus, we thought it seems unlikely that we can easily
detect osmotic stress-related changes associated with ARHGAP4. In addition, the relevance to osmotic stress is beyond the scope of our current study, so that we would like to keep such challenges on such external stimuli for future investigation.

1.4. The authors comment that ARHGAP4 ablation changes cell shape. Since FA number and size is related to cell shape, these cell shape changes should be quantified.

We thank the reviewer for this suggestion. We have quantified the alteration in cell shape caused by ARHGAP4 silencing in Fig. S5B.

Major Experimental Comments

2.1-I don’t think the gene expression data in Fig 1 adds much to the story. It’s likely better as supplementary data. Moreover, this data was obtained from another cell line instead of the HEK293 used in the remainder of the paper.

We thank the reviewer for the suggestion. We have transferred Fig. 1C to Fig. S1A. Regarding the difference in the cell lines used, MCF10A and HEK293 cells are both often categorized as epithelial cells, and importantly the immunoblots on the response of integrin molecules to ARHGAP4 exhibit similar trends between the two cell lines (Fig. S1B). In addition, in the cell images, MCF10A cells exhibit relatively stable focal adhesions, and thus it was easier to find the tendency of the change in focal adhesions caused by siRNA-GAP4 using HEK293 cells rather than using MCF10A cells. Thus, we used HEK293 cells for the experiments specifically aimed at focal adhesions.

2.2-The authors have not measured FA dynamics. To say that ARHGAP4 loss stabilizes FA number is incorrect. Rather, they should state that ARHGAP4 loss increases FA number.

We thank the reviewer for the comment. We believe the FRAP experiments are typically regarded as a method to evaluate the dynamics of proteins. In our study, the steady state value of the recovered fluorescence intensity (stated here as Ymax) is quantified, as often done in other studies as well, to measure the stability or maturity of focal adhesions. We have revised the manuscript to more clearly describe the point of this experiment.

2.3-the data in showing that ARHGAP4 IPs with SEPT2 and 9 is convincing but is would be nice to see what co-ip looks like with endogenous ARHGAP4 and in a cell line other than HEK293.

We thank the reviewer for the suggestion. We checked the expression of ARHGAP4 in Bxpc3 cells, which exhibit a higher expression of endogenous ARHGAP4, and found that the trend was similar with HEK293 cells (Fig. S3).
2.4-Fig 3C si-RNA control looks uninterpretable to me. I don’t see a full cell there to compare with the other treatments. Also, the authors used manual tracing of FA to enumerate them. There are automated Fiji/Imaris plugins for this.

We thank the reviewer for the comment. To distinguish between individual cells, nuclear staining was performed (Fig. S5A). Regarding the automation of the analysis, indeed there are some software like ImageJ to enable us to automatically calculate the parameters on focal adhesions. However, for some populations of focal adhesions, it was really difficult in our pilot study to separate them by the software intelligently as some dots irrelevant to focal adhesions are also grouped into the categories of focal adhesions. Therefore, we did our best to use such software but actually gave up using them, and instead we traced them by ourselves with the biggest care. With the careful analysis, we believe the results are more accurate than the case using the software. Actually, we have drawn all the focal adhesions that are visible to the naked eyes and added more detailed descriptions on the method section.

2.5-I don’t understand what is being performed in Fig 3F. Some images of the cells in question should be provided, not just the fluorescent intensity profiles

We thank the reviewer for the comment. In the original Fig. 3F, the results of the FRAP experiments are described, in which the temporal evolutions of the fluorescence intensity are shown. We believe that our approach is consistent with ones typically taken in conventional FRAP analyses. Here, to measure the stability or maturity of the individual focal adhesions in these experiments, the steady state value of the recovered fluorescence intensity (stated here as Ymax) was quantified. We have added associated images in supplemental Fig. S6 in accordance with the reviewer’s suggestion.

Minor Experimental Comments
3.1-Fig 1A is better expressed as a Volcano plot so that p value info can be included -The analysis in Fig 1B should not be expressed as total number of genes but as an enrichment fraction

We thank the reviewer for the comment. In our experiments, to reduce expenditures for the microarray assay, three separately obtained samples (comprised of two groups, i.e., GFP-overexpression and ARHGAP4-overexpression) were mixed and then sequenced. Therefore, it is difficult to draw the Volcano plot. To increase the credibility of the analysis, however, all the DEGs that involve our follow-up experiments were one by one verified at the protein level (Fig. S1B). The purpose of the bioinformatics technology here is to provide us with a research direction that allowed us to notice the impact of ARHGAP4 on the focal adhesions. Regarding Fig. 1B, we have changed the description according to the reviewer’s suggestion.
3.2-Fig 2A should have the "Lysate" WB as part of the same gel as the IP to assess the fraction of SEPT2 or SEPT9 that is IPing with GFP-ARHGAP4

We thank the reviewer for the comment. For the lysate, endogenous expressions of SEPT2 and SEPT9 are shown in the bottom two bands, which belong to a part of the lysate for COIP. While the percentage of ARHGAP4-binding SEPT2/SEPT9 expression in the total proteins is knowable according to the loading concentration, it is difficult to enrich all the proteins (obtained from 60-cm diameter dishes) in one lane to be as lysate bands.

3.3-Fig3A, Fig 4A and 5A should have untreated cells shown

We thank the reviewer for the suggestion. “GFP” is the transfection control, and thus to the best of our knowledge, and it is enough to make our conclusion.

3.4-Fig 3E and 5E are better presented as a Cumulative Distribution Function since 1.5um² is an arbitrary cut-off value

As we described in our response at 2.4, the size of individual focal adhesions is overall rather small. In this situation, please note that there must include some levels of unavoidable errors regardless of the use of automatic identification (using ImageJ software) or manual analysis that we employed. Therefore, we do not think that cumulative distribution function is a good choice because it requires a markedly high level of preciseness. Nevertheless, our analysis that has separated the size according to a specific threshold (set here as 1.5 um²) should provide more reliable evaluation to determine how the size of focal adhesions is affected because the grouping (i.e., whether it is larger or not than 1.5 um²) and the judgement of how the separated populations change are made with higher reliability (compared with the case of the absolute determination based on the cumulative distribution function). While the choice of 1.5um² is to some extent arbitrary, this value is within the characteristic size of individual focal adhesions, and indeed similar approaches have been done in previous studies (e.g., Angely et al., PLOS ONE 15(5), doi.org/10.1371/journal.pone.0228606, 2020).

3.5-Fig 3E should have the laminin and collagen data along with the fibronectin data

We thank the reviewer for the suggestion. We have added new data on laminin and collagen in Fig. 3E.

3.6-I am surprised that the GFP control in 5C doesn't show any paxillin staining. Surely these cells must have some FA? Fig 5D says they do.

We thank the reviewer for the question. Indeed, it does exist so that, to show it more
clearly, we have replaced the images with other clearer ones.

3.7-Nomenclature in Fig 7 should be consistent with the remainder of the paper, i.e. use SEPT2/9 instead or Septin2 or Spetin9

We thank the reviewer for bringing this inconsistency to our attention. We have fixed all the descriptions to be consistent throughout the manuscript.
Dear Dr. Deguchi,

Thank you for submitting your revised manuscript entitled ARHGAP4-SEPT2-SEPT9 complex enables both up- and down-modulation of integrin-mediated focal adhesions, cell migration, and invasion. Both previous reviewers indicate that your revision mostly addresses the concerns they previously raised. Reviewer 2, however, requests two additional changes, which require minimal effort on your part and no additional data.

1) Include a higher magnification for images in S3A and discuss the modest colocalization in the results and/or discussion section.
2) Include justification of the 1.5 micron cutoff in 3E and 5E as they have done in the rebuttal. Hence, we welcome a revision that includes these minor changes.

Regards,
Diane Barber
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Deguchi,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) 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 accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,
Reviewer #1 (Remarks to the Author):

This is an interesting study that demonstrates that ARHGAP4 forms a complex with SEPT2 and SEPT9 to regulate migration and invasion of epithelial cells. Overall, the data are convincing and open up several new questions about ARHGAP4 biology. These findings are biologically important and interesting broadly for cell biologists. Experimental results are now clear and the writing is easy to understand. The data now support all the conclusions. The authors have done a careful and complete response to my comments and concerns and have included new and significant data that strengthen and clarify their conclusions.

Reviewer #2 (Remarks to the Author):

Overall the paper is much improved and the authors have addressed the majority of my comments. However, I would like the authors to revise the paper as follows:

- Include higher magnification images of s3A and to discuss the modest colocalization in the results and/or discussion section
- Include in the revised paper the rebuttal figure of ARHGAP4 and Paxillin colocalization and discuss this in the results and/or discussion section
- Include justification of the 1.5 micron cutoff in 3E and 5E as they have done in the rebuttal
We would like to thank the reviewers for the comments. Reviewer #2’s comments are shown below in italic font, followed by our responses in normal font. All the changes made to the manuscript are given in red font color within the revised manuscript.

Reviewer #2 (Remarks to the Author):

*Overall the paper is much improved and the authors have addressed the majority of my comments. However, I would like the authors to revise the paper as follows:*

- Include higher magnification images of s3A and to discuss the modest colocalization in the results and/or discussion section
- Include in the revised paper the rebuttal figure of ARHGAP4 and Paxillin colocalization and discuss this in the results and/or discussion section
- Include justification of the 1.5 micron cutoff in 3E and 5E as they have done in the rebuttal

We thank the reviewer for the comments. We have revised the manuscript according to the reviewer’s suggestions.
Dear Dr. Deguchi,

Thank you for submitting your revised manuscript entitled ARHGAP4-SEPT2-SEPT9 complex enables both up- and down-modulation of integrin-mediated focal adhesions, cell migration, and invasion. We believe your revision adequately addresses the remaining concerns of your previous revision and is acceptable for publication. Congratulations and thank you for submitting your work to MBoC.

Sincerely,
Diane Barber
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Deguchi:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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Sincerely,
Eric Baker
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