# Ras-mediated activation of mTORC2 promotes breast epithelial cell migration and invasion

Shannon Collins, Mollie Wiegand, Alyssa Werner, Isabella Brown, Isabelle Mundo, Douglas Swango, Ghassan Mouneimne, and Pascale Charest

*Corresponding author(s): Pascale Charest, University of Arizona*

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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 #E22-06-0236
TITLE: Ras-mediated activation of mTORC2 promotes breast epithelial cell migration and invasion

Dear Dr. Charest:

Dear Dr. Charest,
We have received the reviews from the referees and you will see that they feel your manuscript is of potential interest for publication but would like you to provide a bit more mechanistic insight, as described in the points they raise, as well as including more controls. Please resubmit your revised manuscript, once you have addressed these points.
Many thanks for your interest in MBoC.
Sincerely yours,
Jody

Sincerely,

Jody Rosenblatt
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Charest,

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 haveopted out of publishing the review history.

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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):

In this manuscript, the authors intend to show Ras-mTORC2 pathway plays an important role in promoting the migration/invasion of breast cancer cells. The authors used different cell lines to study cell migration but proper analysis of cell migration and controls are missing. Analysis of the impact of cell proliferation or apoptosis on cell migration dependent on TORC and RAS is crucial to validate their results. Also mechanisms or at least discussion about potential mechanisms are also missing.

Main comments:

- Analysis of the impact of inhibition of mTORC2 on cell migration using the "classical scratch test" need to be improved. It is known that mTORC and RAS are important for cell proliferation and apoptosis. For example, targeting of mTORC2 prevents cell migration and promotes apoptosis in breast cancer (Breast Cancer Res Treat . 2012 Aug;134(3):1057-66). Also, it is well known that RAS could affect cell proliferation. Here, the authors measured cell migration distances which were determined by analyzing the difference in wound sizes at T0 and T18h. An effect on cell proliferation or apoptosis will affect the data provide in Figure 1 and 2. Does inhibition of mTORC2 (Figure 1) or Ras (figure 2) affect cell proliferation / apoptosis? If proliferation is blocked by Mitomycin C, will inhibition of mTORC and RAS affect cell migration ? The authors should also measure the velocity of the cells when mTORC2 is inhibited? Does Ras-mTORC2 pathway inhibition affect cell persistence?
- For each shRNA used, at least for Raptor and Rictor shRNA, the effect of two independent shRNAs should be used or a rescue experiment should be performed in order to avoid any off-target effect. Torin 2 is not a specific inhibitor of mTORC2, it is also inhibiting mTORC1. the inhibition of both TORC could have a synergistic effect. The authors should moderate their statements when Torin2 effects is analysed.
- On Figure 4B, Rictor depletion looks efficient only in few cell looking at the immunostaining images (HRASca/MCF10A - shRictor). On Figure 3B, the effect of this cell line on cell migration is strong. Could the authors comments on this.
- Figure 4: what is observed in MCF10A overexpressing RAS looks more as a strong proliferation effect associated with a loss of cell polarity than an invasive effect. Could the authors comment on this and maybe perform and classical invasion assays in type I collagen (maybe also by blocking in parallel cell proliferation with Mitomycin C).
- Figure 5A/B/C/D: Which effects are significative ? In this line, for example, the effects on pAKT in HER2/MCF10A (Figure 5A) or in MCF10A+Pan-Ras siRNAs are low, are they significative ?

Minor points:

- Images of wound healing should be provided for the other cell lines used. Also, MCF10A and MCF7 cell migrations are collective whereas MDA-MB-231 are mesenchymal cells with no cell-cell contacts. Authors should comment on this.
- The authors should explain in the introduction and the results parts the specificity of the different substrates of mTORC1 and mTORC2 in order to facilitate the understanding of the manuscript.
- The authors should at least try to discuss how mTORC2 could control cell migration dependent on RAS.

Reviewer #2 (Remarks to the Author):

Ras controls multiple cellular processes, including cell migration and invasion. Likewise, mTORC2 is known to regulate the migration of cancer cells. The authors' group has previously found that mTORC2 is a downstream effector of Ras for controlling the directed migration of Dictyostelium. Other studies have also demonstrated that the Ras-mTORC2 axis is also present in mammalian cells. In this manuscript, Collins et al. combine all the available information together and delineate the role of the Ras-mTORC2 axis in breast epithelial cell migration and invasion. The authors have demonstrated that expression of RasCA increases pAKTS473 levels in MCF10A, indicating that mTORC2 activity is controlled by RasCA in breast epithelial cells. Moreover, disruption of mTORC2 attenuates the migration of MCF10A cells as well as a few breast epithelial cancer cell lines. Of note, the authors have shown that disruption of mTORC2 in RasCA-transformed MCF10A cells impairs invasiveness without affecting the uncontrolled proliferation phenotype, demonstrating the requirement of mTORC2 for invasiveness of RasCA-transformed breast epithelial cells. Most of the presented data clearly support the authors' claims and the main conclusion. I recommend the manuscript be accepted for publication if the authors address the following comments:

1. It is interesting that different wild-type and constitutively active Ras proteins promote the migration of MCF10A cells (Figure 3A and S3A). In general, RasCA proteins more effectively increase cell migration. Do authors think the migratory capacity of MCF10A cells expressing different Ras correlates with their mTORC2 activity levels? Given that Ras has a few downstream effectors for controlling cell migration, showing the correlation should strengthen the importance of the Ras-mTORC2 axis. It would be also helpful to see how expression of different Ras and RasCA proteins changes pERK levels.
2. The author claimed that pAKT levels are a little bit higher in MCF10A cells expressing HER2 compared to control MCF10A cells (Figure 5A). However, it is hard to tell without statistical analysis. Additionally, expression of HER2 is supposed to increase pERK levels; however, the blot doesn't show it. How do the authors explain this? Note that all the quantifications shown in Figure

5 do not include statistical analysis.
3. In Figure S2A, I suggest including quantification of pAKT/AKT.
4. In Figure S2B, how are pAKT and pERK levels changed when Ras proteins are depleted? Quantification of pAKT/AKT would be helpful.
5. In Figure 3C, the presented images do not appear to be representative.
6. In Figure 4A, protrusions and invasion events are not clearly seen in the presented images. Can protrusions and invasions be indicated in the images?

Dear Editor and Reviewers,

We have revised our manuscript entitled "Ras-mediated activation of mTORC2 promotes breast epithelial cell migration and invasion". We thank the Reviewers for their thorough reviewing of our manuscript and their suggestions for improvements. We believe that we have addressed all of the comments appropriately and that through this process, the quality of our manuscript has been enhanced. Please see below our point-by-point responses to the reviewers' comments:

Reviewer #1

In this manuscript, the authors intend to show Ras-mTORC2 pathway plays an important role in promoting the migration/invasion of breast cancer cells. The authors used different cell lines to study cell migration, but proper analysis of cell migration and controls are missing. Analysis of the impact of cell proliferation or apoptosis on cell migration dependent on TORC and RAS is crucial to validate their results. Also, mechanisms or at least discussion about potential mechanisms are also missing.

Main comments:

- Analysis of the impact of inhibition of mTORC2 on cell migration using the "classical scratch test" need to be improved. It is known that mTORC and RAS are important for cell proliferation and apoptosis. For example, targeting of mTORC2 prevents cell migration and promotes apoptosis in breast cancer (Breast Cancer Res Treat. 2012 Aug;134(3):1057-66). Also, it is well known that RAS could affect cell proliferation. Here, the authors measured cell migration distances which were determined by analyzing the difference in wound sizes at T0 and T18h. An effect on cell proliferation or apoptosis will affect the data provide in Figure 1 and 2. Does inhibition of mTORC2 (Figure 1) or Ras (figure 2) affect cell proliferation / apoptosis? If proliferation is blocked by Mitomycin C, will inhibition of mTORC and RAS affect cell migration?

*The Reviewer raises a point that has been discussed for many years in the cell migration research community, which is the question of the contribution of cell proliferation versus migration in the closure of wounds in these types of assays. In our study, though, we have good controls to monitor this: the inhibition of mTORC1 by Rapamycin as well as through the silencing of Raptor. Indeed, mTORC1 promotes cell growth and proliferation, and its inhibition by Rapamycin or through Raptor silencing effectively demonstrates the contribution of cell proliferation in the measured wound closures. Whereas we do observe some inhibition of wound closure upon mTORC1 inhibition, and thereby a contribution of cell proliferation, it is significantly less than inhibiting both mTORC1 and mTORC2 with Torin2 or inhibiting mTORC2 alone through Rictor silencing (Fig. 1). Consequently, this demonstrates that the effects of inhibiting mTORC2, as well as Ras, on wound closure are primarily due to inhibition of cell migration. This is now clarified in the Results section on pages 4 and 5 of the revised manuscript. Moreover, to better characterize cell proliferation during the wound healing assays and the effects of mTORC1 and mTORC1/2 inhibition, we*

*performed an additional experiment in which we counted the number of cells in the wells immediately after wounding and after 18 hrs., comparing between conditions where the cells were treated with 0.1% DMSO as control, with Rapamycin, and Torin2. As expected, we observed that Rapamycin treatment leads to a significant decrease in cell number compared to wells treated with only 0.1% DMSO, and that the effect of Torin2 treatment is the same as that of Rapamycin. Thus, this demonstrates that the additional inhibition of mTORC2 does not inhibit cell proliferation more than the inhibition of mTORC1 alone. Consequently, the contribution of cell proliferation to wound closure can be evaluated through the effect of Rapamycin, and we find this to be minimal compared to mTORC2 or Ras inhibition, thereby suggesting that these indeed promote migration. These data are now shown in a new Fig. S2D and referenced on pages 4 and 5 of the revised manuscript.*

*Further regarding the inhibition of Ras, it is indeed expected that the cell proliferation is inhibited and that, consequently, this contributes to the measured closure of wounds. However, in addition to our observations with Rapamycin that indicate that cell proliferation plays a minimal role in wound closure, as discussed above, the role of Ras in cell migration is further supported by the overexpression experiments and the transwell 3D migration/invasion assays presented in Fig. 3, which clearly indicate that Ras promotes the migration of breast epithelial cells.*

The authors should also measure the velocity of the cells when mTORC2 is inhibited? Does Ras-mTORC2 pathway inhibition affect cell persistence?

*It will be extremely interesting to study the different facets of the migration phenotypes and mechanism implicated in the Ras- and mTORC2-dependent migration of breast epithelial and cancer cells. The analyses are best measured using subconfluent cultures, in which migration of individual cells are characterized. Indeed, we are planning on performing these experiments; however, in the work presented here, we focus on characterizing the effect of Ras and mTORC2 on the global migratory phenotype of cell sheets using whole cell populations. Nonetheless, we think these are very important questions and we now added a discussion point about this on page 10 of the revised manuscript.*

- For each shRNA used, at least for Raptor and Rictor shRNA, the effect of two independent shRNAs should be used or a rescue experiment should be performed in order to avoid any off-target effect.

*We agree with the Reviewer that it is important to validate shRNAs. In fact, the Rictor and Raptor shRNAs constructs that we used in this study are gifts from Carole Parent and David Sabatini, respectively, who have previously described and rigorously validated them (Liu et al. Dev. Cell 2010; Sarbassov et al. Science 2005). We have clarified this in the text, in the Results section, on page 4 of the revised manuscript.*

Torin 2 is not a specific inhibitor of mTORC2, it is also inhibiting mTORC1. the inhibition of both TORC could have a synergistic effect. The authors should moderate their statements when Torin2 effects are analyzed.

*Torin2 is indeed an mTOR inhibitor and, thereby, inhibiting both mTORC1 and mTORC2. We clarified the usefulness of using Torin2 in our investigation of mTORC2 function in breast epithelial cell migration and the interpretation of the results on pages 4 and 5 of the revised manuscript.*

- On Figure 4B, Rictor depletion looks efficient only in few cells looking at the immunostaining images (HRASca/MCF10A -shRictor). On Figure 3B, the effect of this cell line on cell migration is strong. Could the authors comment on this.

*The effect of Rictor silencing on cell migration shown in Fig. 3B, and also on cell invasion shown in Fig. 4B and quantified in 4C, are very strong indeed, due to the efficient silencing of Rictor, as shown in Fig. S2C. Whereas the silencing of Rictor is heterogenous, which is normal and can be appreciated in the image presented in Fig. 4B, the expression is indeed relatively low in all the cells, just lower in some than others, and the image was obtained using a longer exposure time and higher gain, which were necessary to detect Rictor labeling and compare its expression levels. We have now clarified this in the legend to Figure 4 of the revised manuscript.*

- Figure 4: what is observed in MCF10A overexpressing RAS looks more as a strong proliferation effect associated with a loss of cell polarity than an invasive effect. Could the authors comment on this and maybe perform and classical invasion assays in type I collagen (maybe also by blocking in parallel cell proliferation with Mitomycin C).

*We agree that HRasCA expression has indeed a strong proliferation effect on MCF10A cell, and that this proliferation is a very important aspect of the phenotype. However, there are also specific characteristics of these 3D cell cultures that clearly indicate invasion, as compared to oncogenes that only promote cell proliferation, as reported in previous studies including of HRasCA-transformed MCF10A cells (Basolo et al., Mol Carcino. 1991; Moon et al., Int. J. cancer 2000; Muthuswamy et al., Nat. Cell Biol. 2001; Debnath et al., Cell 2002; Debnath et al., Methods 2003). Indeed, the expression of some oncogenes such as ErbB2/HER2 in MCF10A cells elicits a multiacinar phenotype indicative of excessive proliferation (as well as protection from apoptosis) but not invasion of the surrounding matrix (Muthuswamy et al., Nat. Cell Biol. 2001; Debnath et al., Cell 2002). On the other hand, HRasCA-transformed MCF10A cells have been shown to have an invasive phenotype in more "classical" transwell invasion assays (Basolo et al., Mol Carcino. 1991; Moon et al., Int. J. cancer 2000), which we have also performed in our study and showed in Fig. 3C. In the 3D cell cultures used as Matrigel invasion assays, the HRasCA-transformed MCF10A cells clearly display invasive projections (now more clearly shown in the revised manuscript) as well as migration through the acini's basement membranes (Fig. 4A). Furthermore, the presence of many single cells in the matrix surrounding the HRasCA/MCF10A cell colonies (not acinar structures anymore) further indicates that cells have migrated away from the colonies, a defining feature of invasion. A new image more clearly showing invasive projections of HRasCA/MCF10A cells has been included in a revised Figure 4A and the description of the invasive phenotype has been clarified on page 7 of the revised manuscript.*

*Regarding the use of Mitomycin C here, because the 3D cell culture/acini invasion assay is performed over 12 days, this experiment is not amenable for use of this strong drug that would cause senescence and then cell death before the cells have time to form any cell clusters or acinar structures.*

- Figure 5A/B/C/D: Which effects are significative? In this line, for example, the effects on pAKT in HER2/MCF10A (Figure 5A) or in MCF10A+Pan-Ras siRNAs are low, are they significative?

*As part of our revisions to Fig. 5, we have now added the statistical analyses of the immunoblot data using an appropriate nonparametric, unpaired one-tailed Mann-Whitney test (for population < 5 and normalized data). The results of these analyses now allow to better appreciate the differences between the data sets and their significance. We have then changed the wording in the results section accordingly, on page 8 of the revised manuscript.*

*Also note that, upon revision, we found mistakes in the graphs shown in 5B and 5D, which we have corrected in the revised Figure 5, in addition to the modification added to 5A.*

Minor points:

- Images of wound healing should be provided for the other cell lines used.

*We have added a new supplementary Figure S1 showing examples of MCF7, MDAMB231, MDAMB453, and HER2/MCF10A cells would healing assays.*

Also, MCF10A and MCF7 cell migrations are collective whereas MDA-MB-231 are mesenchymal cells with no cell-cell contacts. Authors should comment on this.

*This is a good point and we have added a comment on this in the Discussion section on page 8 of the revised manuscript.*

- The authors should explain in the introduction and the results parts the specificity of the different substrates of mTORC1 and mTORC2 in order to facilitate the understanding of the manuscript.

*We thank the reviewer for this suggestion, which indeed will help readers keep track of the differences between mTORC1 and mTORC2 and how these are considered in our study. As suggested, we have added a section in the Introduction section outlining the similarities and differences between mTORC1 and mTORC2 on page 3 of the revised manuscript, and we have clarified the use of AKT as an mTORC2 specific substrate in the Results section on page 7 of the revised manuscript. The substrate specificity as it relates to mTORC1 versus mTORC2 is clearly indicated in the legend to Figure S2 where the mTORC1 substrates S6K1 is used.*

- The authors should at least try to discuss how mTORC2 could control cell migration dependent on RAS.

*As suggested, we have expanded our discussion of the potential mechanisms through which mTORC2 could control the migration of breast epithelial cells on page 10 of the revised manuscript.*

Reviewer #2

Ras controls multiple cellular processes, including cell migration and invasion. Likewise, mTORC2 is known to regulate the migration of cancer cells. The authors' group has previously found that mTORC2 is a downstream effector of Ras for controlling the directed migration of Dictyostelium. Other studies have also demonstrated that the Ras-mTORC2 axis is also present in mammalian cells. In this manuscript, Collins et al. combine all the available information together and delineate the role of the Ras-mTORC2 axis in breast epithelial cell migration and invasion. The authors have demonstrated that expression of RasCA increases pAKTS473 levels in MCF10A, indicating that mTORC2 activity is controlled by RasCA in breast epithelial cells. Moreover, disruption of mTORC2 attenuates the migration of MCF10A cells as well as a few breast epithelial cancer cell lines. Of note, the authors have shown that disruption of mTORC2 in RasCA-transformed MCF10A cells impairs invasiveness without affecting the uncontrolled proliferation phenotype, demonstrating the requirement of mTORC2 for invasiveness of RasCA-transformed breast epithelial cells. Most of the presented data clearly support the authors' claims and the main conclusion. I recommend the manuscript be accepted for publication if the authors address the following comments:

1. It is interesting that different wild-type and constitutively active Ras proteins promote the migration of MCF10A cells (Figure 3A and S3A). In general, RasCA proteins more effectively increase cell migration. Do authors think the migratory capacity of MCF10A cells expressing different Ras correlates with their mTORC2 activity levels? Given that Ras has a few downstream effectors for controlling cell migration, showing the correlation should strengthen the importance of the Ras-mTORC2 axis. It would be also helpful to see how expression of different Ras and RasCA proteins changes pERK levels.

*As the reviewer suggested, we compared side-by-side the effect of wild-type Ras versus RasCA expression on mTORC2 activity to assess whether this correlates with the level to which they each increase cell migration. Using MCF10Acels exogenously expressing HRas and HRasCA, we observed that HRas increases mTORC2 activity (as measured by AKT phosphorylation at S473) by an average 1.6-fold over MCF10A control cells whereas HRasCA induced a 3-fold average increase. Interestingly, the effects of HRas and HRasCA on mTORC2 activity indeed correlate to their effect on MCF10A cell migration, which was increased by 1.6- and 2.6-fold on average, respectively. The new data with the effect of HRas on mTORC2 activity compared to that induced by HRasCA are now included in an updated Fig. 5A, described on p. 7-8 and discussed on p. 10 of the revised manuscript.*

*As a control, we also assessed the effect of HRas and HRasCA expression on ERK activity (as measured by ERK phosphorylation; pERK), and we observed that they both similarly increased pERK compared to MCF10A cells. These data are included in the updated Fig. 5A.*

2. The author claimed that pAKT levels are a little bit higher in MCF10A cells expressing HER2 compared to control MCF10A cells (Figure 5A). However, it is hard to tell without statistical analysis. Additionally, expression of HER2 is supposed to increase pERK levels; however, the blot doesn't show it. How do the authors explain this? Note that all the quantifications shown in Figure 5 do not include statistical analysis.

*Regarding the statistical analysis of data presented in Fig. 5, please see our response to Reviewer 1's fifth main comment.*

*Regarding pERK, we indeed do not detect an increase in pERK in HER2/MCF10A compared to MCF10A, but only detect a reproducible increase in pAKT. Sometimes, we did detect higher pERK but that is always accompanied by increased ERK expression. Moreover, this has also been observed in a previous study (Yang et al. Oncotarget 2016), which we have now referenced, and we have also added a comment addressing this observation on page 7 of the revised manuscript.*

3. In Figure S2A, I suggest including quantification of pAKT/AKT.

*The data shown in this figure (now Fig. S3A) accompany those in Fig. 5B where this quantification is included.*

4. In Figure S2B, how are pAKT and pERK levels changed when Ras proteins are depleted? Quantification of pAKT/AKT would be helpful.

*The data shown in this figure (now Fig. S3B) accompany those in Fig. 5C, where pAKT and pERK levels as well as the pAKT/AKT quantifications are shown.*

5. In Figure 3C, the presented images do not appear to be representative.

*The images represent one field of view/area in which cells were counted, out of 13 areas to cover the whole well. We have now clarified this in the legend to Fig. 3C as well as by indicating in the graphs that they represent the number of <u>total</u> migrated cells.*

6. In Figure 4A, protrusions and invasion events are not clearly seen in the presented images. Can protrusions and invasions be indicated in the images?

*As suggested by the reviewer, we have now indicated where invasive processes are observed in the images provided in Fig. 4A as well as 4B with explanation in the figure legend, including migration through the basement membrane, cells migrating away from cell clusters, and cellular protrusions. For the latter, we have now included a better picture to show these.*

RE: Manuscript #E22-06-0236R
TITLE: "Ras-mediated activation of mTORC2 promotes breast epithelial cell migration and invasion"

Dear Dr. Charest:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Have a happy rest of 2022!

Sincerely,
Jody Rosenblatt
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Charest:

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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