Paxillin Promotes Breast Tumor Collective Cell Invasion through Maintenance of Adherens Junction Integrity

Christopher Turner, Weiyi Xu, Kyle Alpha, and Nicholas Zehrbach

Corresponding author(s): Christopher Turner, SUNY Upstate Medical University

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(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 #E21-09-0432
TITLE: Paxillin Promotes Breast Tumor Collective Cell Invasion through Maintenance of Adherens Junction Integrity

Dear Chris,

Thank you for sending us your manuscript for consideration. It has now been seen by two expert reviewers, whose comments follow below. As you can see, they are supportive but identify some areas for clarification that would strengthen the story.

Accordingly, I would ask you and your colleagues to address these issues in a revised manuscript. We look forward to seeing it soon.

Best wishes,

Alpha

Alpha Yap
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Turner,

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.

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

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

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

Sincerely,

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

Reviewer #1 (Remarks to the Author):

Xu et al use a newly generated PyMT-MMTV Paxilin null mouse to show paxillin regulates breast cancer tumor metastasis to the lung via a tumor-intrinsic mechanism. Breast cancer cells require paxillin for collective 2D and 3D invasion and maintenance of mature adherens junctions. This is regulated via several mechanisms including: paxillin's positive regulation of E-cadherin endocytosis, recycling, promotion of tubulin acetylation, and inhibition of Rho/activation of Rac. This is an interesting study, with a novel mouse model that sheds light on the role of Paxilin in tumor progression. Some clarifications are needed.

In vivo description of PyMT-MMTV knockout mouse: Some details are missing in the characterization that require clarification.
Figure 1A/C- clarify if n is animal # or tumors/mammary pads measured. Is there an effect on number and location of tumors?
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Figure 1G- it is unclear if the quantification is of surface level metastases or ones from the H&E staining. The authors state that the KO mice have 'fewer and lung metastases' but there is no quantification of metastases size. These should be quantified.
The methods say 'Lungs were evaluated when primary tumors reached 2 cm.' Is this one tumor reached 2cm? or total? The data in 1A suggests that tumor volume was max 1mm3. Is the limitation due to IACUC protocols? This is quite early in tumor progression to stop a PyMT-MMTV model, and it is possible that there could be a difference in tumor size if left longer.
The discussion claims paxillin does not affect proliferation based on the similar latency periods in WT and Paxillin KO tumor metastases, the authors should either show this with Ki67 staining.

Lack of quantification of in vivo staining: The data in figure 2 are very descriptive. The description in the text is clear with regards to the representative images, but some arrows might help to provide more information for the reader. The authors should quantify the frequency of single cell vs multi cell invasion along with E-cadherin intensity and subcellular localization in 2C. It is hard to say based on the representative images what is cytosolic vs membrane-bound protein. It is also important not overstate what the authors might be seeing unless it is clearly obvious or quantified for the reader.

Effect of Paxilin KO in collective vs. single cell invasion: In Figure 3, while there is a clear effect on circularity, it is not obvious that this shows that the tumor organoids lacking paxillin are "less invasive". Looking at these images, it can look like paxillin KO cells are possibly more invasive given that the organoid is much bigger and that there are an increased number of single cells invading individually, which suggests they might be more apt at single cell invasion. More quantification is required. Are the changes in cell shape and migration/invasion of the paxillin KO cells contact dependent? i.e. once a paxillin cell separates from its neighbors is it just as efficient (or more efficient) than a single WT cell? The single cells in the movie of Paxilin KO are invading at high speed.

The discussion could be much more focused, it is over 6 pages and raises many questions which make the data more confusing.

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Reviewer #2 (Remarks to the Author):

The Manuscript by Xu et al., explores the role of the focal adhesion protein paxillin in collective cell invasion of breast cancer- a key step in metastasis. Their findings suggest a role for paxillin in maintaining cell-cell junctions, a requirement for collective invasion. They show that paxillin regulates cell-cell adhesion- to facilitate collective cell migration away from the primary tumor into the surrounding stroma. The loss of paxillin results in a decrease in metastatic dissemination to distal sites- with a decrease in lung metastases. This work builds on two publications- work identifying a role for paxillin in breast cancer cell invasion in 3D (MDA-MB-231; Deakin and Turner, JCB 2014) and a recent publication investigating the role of paxillin in apical-basal polarity of mammary glands (Xu et al., Development 2019). Their observations on the importance of cell-cell adhesion in metastatic seeding are complementary to recent findings by the Ewald lab who identified that breast cancers need E-cadherin and cell-cell adhesion for the early steps of metastasis. E-cadherin supports the survival of tumor cells - during the initial speeding phases of breast cancer metastasis. The findings will be of interest to cell, cancer, cytoskeletal and cell adhesion biologists. Future studies to identify the mechanistic link between paxillin, cadherin recycling and Rho GTPases will build on our knowledge of the crosstalk between cell-cell and cell-matrix adhesions.
Whilst I recommend the manuscript for publication there are a several points of clarification and addition quantitation that are required to support their claims.
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b. Figure 4, 2D wound assays. Is there an alteration in the levels of vinculin? Is there more at cell-matrix or cell-cell junctions in figure 4D. (please display vinculin as individual greyscale images)

c. It looks like vinculin localization at the periphery of cells is increased, and cell-matrix adhesions are larger. Are there defects in adhesion turnover and size in paxillin KO cells?
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4) Re figure 3. The organoids show in the figure are convincing- however, the morphology of the WT organoid in the supplementary movie was less convincing. I'm guessing there is a significant amount of heterogeneity within the system. The addition of a panel to the supplementary data showing a range of WT and KO organoid morphologies would strengthen this data. The KO organoid movie- I don't see any cells actively leaving the organoid during the time period displayed. If possible- it would be ideal to include a movie of a cell actively leaving the organoid. I agree- there are a lot of mesenchymal like cells invading around the organoid but would like to see the cell actively leaving the organoid as a single cell. The cells migrating around in the matrix- that have left the organoids- are of interest, as highlighted by the Asterix in figure 3B- is it is possible to go back through the data and add some frames of these cells to the figure?
5) "Following tubacin treatment, the paxillin KO tumor organoids exhibited hyper-MT acetylation and a significant increase of invasiveness, as evaluated by their degree of circularity". I agree that tubacin may change the morphology of the organoids (circularity is a read out of this) however, more evidence - i.e., timelapse analysis of invasion from organoids would be required to support this statement of a decrease in invasiveness.

Pg 16 "The paxillin KO tumor mouse developed fewer lung metastases, without a change of tumor size or latency (Figure 1), indicating that paxillin does not significantly impact tumor cell survival or proliferation". As paxillin KO is altering E-cadherin trafficking (endocytosis/recycling) - it would be worth adding to the discussion how this compares this to studies looking at cadherin KO (i.e. Padmanaban et al., Nature 2019).

Minor:
Figure 1A: Please use different colors for the categories (WT & KO) i.e. the colors used in 1C.
Figure 1C: add "n.s" with a line between categories
Statistics- parametric tests (student's T test) are used throughout the study. What test of normality was used?
Figure 2C: would benefit from the addition of a line marking the tumor stroma interface in the WT images (similar to what has been done in the Paxillin KO images). This might not be done as what is displayed is entirely stroma with invading tumor cells? Although it appears to be stroma on the far left of "2C tumor edge, WT"
Figure 2: Each panel in the figure should have its own letter. i.e. (A) (hyperplasia/Adenoma (B) Carcinoma center (C) carcinoma edge (D) tumor edge (E) tumor center.
Re: "Recent studies have shown that in highly invasive breast cancer, cells accomplish collective invasion through maintaining cell-cell contacts (Friedl and Gilmour, 2009; Cheung and Ewald, 2016)." I would add the more recent Ewald reference that addresses exactly this https://doi.org/10.1038/s41586-019-1526-3
Figure 4D actin is written in cyan, but displayed in blue. Please display in cyan.
Figure 7. 50 uM Y drug and blebbistatin are incredibly high concentrations, 5-10X higher than what is needed. These drugs have decreased specificity at higher concentrations. Please add a zoom of the control conditions. It is hard to see in the images presented.
Figure S5. Please no error bars are on the graphs. Please display the mean of each independent repeat as a single data point. (similar to 6D- there are 3 data points and a box & whisker plot over the top)
Pg 18 Although we did not observe any significant change in phospho-FAK levels in the paxillin KO tumor cells versus the WT cells (data not shown)- please add to supplementary data.

Typos
10 minuets, should be minutes
phase contract should be phase contrast.
"Movies 1-2. Tumor organoids in 3D collagen gels demonstrating reduced collective cell invasion in the paxillin KO organoids. Paxillin WT (movie 1) and KO tumor organoids (movie 2) were cultured for 2 days and then subjected to phase contract movies. Images were taken every 10 minuets for total 8 hours."

Professor Christopher E. Turner, Ph.D.
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29 October 2021

RE: E21-09-0432

Dr. Matt Welch and Editorial Staff
MBoC

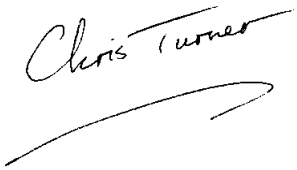
Dear Dr. Welch,

Please find enclosed a revised version of the manuscript by Xu et al. entitled **Paxillin Promotes Breast Tumor Collective Cell Invasion through Maintenance of Adherens Junction Integrity** for publication in MBoC.

We would like to thank the reviewers for their support of our manuscript and their helpful suggestions for improvement. In the revised manuscript we have included several additional datasets and quantitation, where feasible, as requested by the reviewers and also addressed their other queries regarding data analysis and interpretation. A detailed point-by-point response to the reviewer's comments is provided below. We have also checked that all the text and image files conform to MBoC guidelines a requested.

We are hopeful that the manuscript is now acceptable for publication in MBoC.

Sincerely,



Christopher E. Turner, Ph.D.
SUNY Distinguished Professor, Cell and Developmental Biology

Reviewer #1 (Remarks to the Author):

Xu et al use a newly generated PyMT-MMTV Paxilin null mouse to show paxillin regulates breast cancer tumor metastasis to the lung via a tumor-intrinsic mechanism. Breast cancer cells require paxillin for collective 2D and 3D invasion and maintenance of mature adherens junctions. This is regulated via several mechanisms including: paxillin's positive regulation of E-cadherin endocytosis, recycling, promotion of tubulin acetylation, and inhibition of Rho/activation of Rac. This is an interesting study, with a novel mouse model that sheds light on the role of Paxilin in tumor progression. Some clarifications are needed.

In vivo description of PyMT-MMTV knockout mouse: Some details are missing in the characterization that require clarification.
Figure 1A/C- clarify if n is animal # or tumors/mammary pads measured. Is there an effect on number and location of tumors?

In Figure 1A, n = the number of animals analyzed. We have now included more complete details of the number of animals evaluated at each of the time points as indicated in red and blue on the graph. These data represent the total tumor burden in each mouse. Typically, all animals developed a similar number of tumors, occurring in all of the mammary fat pads, regardless of genotype.

Importantly, we have also now included a new graph in Figure S1A, detailing the size of the primary tumor in these animals, which follows a similar trend. Please note, that our IACUC is very thorough in helping us assess the health and well-being of the animals and the reduced numbers of animals at later time points reflects the need to terminate any animals that were in poor health, regardless of tumor load.

Figure 1B - what is the difference between tumor and tumor cell lysate? This is not explained.

The whole/bulk tumor sample represents a lysate of homogenized primary tumor and therefore contains both tumor and stromal cells (hence some residual pxn signal), while the tumor cell lysate was obtained from isolated primary tumor cells (without stromal cells). This has been clarified in the Methods section.

Figure 1G- it is unclear if the quantification is of surface level metastases or ones from the H&E staining. The authors state that the KO mice have 'fewer and lung metastases' but there is no quantification of metastases size. These should be quantified.

The quantification of metastasis number is from the H&E staining. This has been clarified in the text and we have also swapped the two lung metastasis panels around so that the quantitation is adjacent to the H&E panel. We believe that “number of mets” is the most typical way of presenting these data and we removed the statement about the size of lung metastasis--“smaller lung metastases”

The methods say 'Lungs were evaluated when primary tumors reached 2 cm.' Is this one tumor reached 2cm? or total? The data in 1A suggests that tumor volume was max 1mm3. Is the limitation due to IACUC protocols? This is quite early in tumor progression to stop a PyMT-MMTV model, and it is possible that there could be a difference in tumor size if left longer.

IACUC requires that animals are sacced when any one tumor approaches 2cm in diameter, or if two adjacent tumors “merge” to create the impression of a 2cm tumor. Unfortunately, we also had to terminate a significant number of animals for other health reasons, including ulceration and thus in the original manuscript did not provide tumor volume beyond 5 weeks. However, we have now included the data for week 6 (Figures 1A and S1A), which shows a simar trend (this is also true at week 7 but we only had one KO mouse to measure at this time point). Given the minimal differences throughout tumor progression and the time and numbers involved in breeding substantially larger numbers of KO animals, we feel that this dataset adequately represents the phenotype. The number of animals used is consistent with our previous work on Hic-5 (Goreczny et al., 2017).

The discussion claims paxillin does not affect proliferation based on the similar latency periods in WT and Paxillin KO tumor metastases, the authors should either show this with Ki67 staining.

Thank you for pointing out this oversight. As requested, we have now included the Ki67 staining in Figure S1, demonstrating no significant difference in tumor cell proliferation.

Lack of quantification of in vivo staining: The data in figure 2 are very descriptive. The description in the text is clear with regards to the representative images, but some arrows might help to provide more information for the reader. The authors should quantify the frequency of single cell vs multi cell invasion along with E-cadherin intensity and subcellular localization in 2C. It is hard to say based on the representative images what is cytosolic vs membrane-bound protein. It is also important not overstate what the authors might be seeing unless it is clearly obvious or quantified for the reader.

As requested, we have now included quantification of multi-cell vs. single cell invasion into the stroma in vivo in the original Fig 2C, now relabeled 2E. These data are presented in a new Figure 2F. Quantification was based on EpCAM or E-cadherin staining on tissue sections from the data we already acquired. We agree that we overstated the phenotype and the statement regarding the subcellular distribution of E-cadherin has been modified.

Effect of Paxilin KO in collective vs. single cell invasion: In Figure 3, while there is a clear effect on circularity, it is not obvious that this shows that the tumor organoids lacking paxillin are "less invasive". Looking at these images, it can look like paxillin KO cells are possibly more invasive given that the organoid is much bigger and that there

are an increased number of single cells invading individually, which suggests they might be more apt at single cell invasion.

The assessment of organoid circularity as a measure of tumor collective invasiveness was modeled on previous work by the Ewald lab (Padmanaban et al., 2020). As a result of the isolation procedure, there is substantial variation in the size of resulting organoids, from either the WT or KO tumors. We have added a series of representative organoid "masks" in Figure S3A to show this heterogeneity of size and phenotype in both paxillin WT and paxillin KO tumor organoids. We did not see any evidence that organoid size correlates with decreased/increased circularity/invasiveness.

Importantly, we do not see a significant difference in single cell invasion "events" in the primary tumor sections suggesting that this is not a major mechanism of invasion in the mice, even if the number of single cell invasions events is slightly increased in the KO organoids.

More quantification is required. Are the changes in cell shape and migration/invasion of the paxillin KO cells contact dependent? i.e. once a paxillin cell separates from its neighbors is it just as efficient (or more efficient) than a single WT cell? The single cells in the movie of Paxilin KO are invading at high speed.

We have now stained the organoids for paxillin and vinculin to determine if there is evidence of cell-ECM contacts. Accordingly, we have added a new Figure S3B that shows their enrichment in puncta at the front of the leader cells of collectively invading paxillin WT tumor cells, consistent with the formation of 3D cell-ECM adhesions. The paxillin KO tumor cells also appear to form small cell-ECM contacts as demonstrated by punctate vinculin staining at the edge of the organoids. This is now documented in the Results and Discussion. Obviously, extensive additional analysis would be required to confirm these interactions with the ECM. The primary focus on the current study is the effect of paxillin expression on cell-cell interactions

We have also added a new panel in Figure S3C highlighting examples of single cells migrating out of the paxillin KO tumor organoids. We have not observed sufficient numbers of single cells invading from paxillin WT tumor organoids to perform a meaningful comparison of the single cell speed. However, paxillin has been previously shown to affect single cell migration speed in 3D CDMs and collagen plugs, and this was acknowledged in the Discussion section. (eg. Deakin et al., 2011).

The discussion could be much more focused, it is over 6 pages and raises many questions which make the data more confusing.

We have streamlined the Discussion, as requested.

For statistical analysis- it should be clarified in the one-way ANOVAs which secondary test was performed to calculate differences between conditions (i.e. Tukeys multiple comparison) (Section I MBoC Checklist)

Yes, the multiple comparison was done using a Tukey's multiple comparison test. This, and other requested details regarding the statistical analysis have been added to the Methods.

Reviewer #2 (Remarks to the Author):

The Manuscript by Xu et al., explores the role of the focal adhesion protein paxillin in collective cell invasion of breast cancer- a key step in metastasis. Their findings suggest a role for paxillin in maintaining cell-cell junctions, a requirement for collective invasion. They show that paxillin regulates cell-cell adhesion- to facilitate collective cell migration away from the primary tumor into the surrounding stroma. The loss of paxillin results in a decrease in metastatic dissemination to distal sites- with a decrease in lung metastases. This work builds on two publications- work identifying a role for paxillin in breast cancer cell invasion in 3D (MDA-MB-231; Deakin and Turner, JCB 2014) and a recent publication investigating the role of paxillin in apical-basal polarity of mammary glands (Xu et al., Development 2019). Their observations on the importance of cell-cell adhesion in metastatic seeding are complementary to recent findings by the Ewald lab who identified that breast cancers need E-cadherin and cell-cell adhesion for the early steps of metastasis. E-cadherin supports the survival of tumor cells - during the initial speeding phases of breast cancer metastasis. The findings will be of interest to cell, cancer, cytoskeletal and cell adhesion biologists. Future studies to identify the mechanistic link between paxillin, cadherin recycling and Rho GTPases will build on our knowledge of the crosstalk between cell-cell and cell-matrix adhesions.

Whilst I recommend the manuscript for publication there are a several points of clarification and addition quantitation that are required to support their claims.
1) Vinculin plays a role in the cross talk between cell-cell and cell-matrix adhesion. Throughout the manuscript this is never addressed- although vinculin is used as a marker of cell-matrix adhesion. Is there an effect on vinculin?

We apologize for this oversight. We have now included a western blot for vinculin (Figure S4 C and D), which indicates that the vinculin level is not affected by paxillin KO. The role of vinculin, as a paxillin binding partner and its role as mediator in cell-ECM cell-cell crosstalk is also clearly documented in the Discussion.

a. Figure 3 B- where is vinculin? Do WT organoid cells have cell-matrix attachments? (i.e., stain organoids for vinculin). Where does paxillin localized in the WT organoids? Is it at cell-matrix attachments?

Thank you for this suggestion. We have now included tumor organoids stained for vinculin and paxillin in Figure S3B. Both paxillin WT and KO tumor organoids showed potential vinculin-rich 3D matrix adhesions.

b. Figure 4, 2D wound assays. Is there an alteration in the levels of vinculin? Is there

more at cell-matrix or cell-cell junctions in figure 4D. (please display vinculin as individual greyscale images)

Due to space limitations in Figure 4, we have provided all of the grayscale images for this figure in Figure S4 A and B and the distribution of vinculin is documented in the text.

c. It looks like vinculin localization at the periphery of cells is increased, and cell-matrix adhesions are larger. Are there defects in adhesion turnover and size in paxillin KO cells?

We have not observed any obvious and consistent change in the distribution or size of vinculin adhesions. Since the primary focus of the paper is the effect of paxillin on AJs, we respectfully suggest that, while potentially interesting, further analysis of vinculin FAs is beyond the scope of the current manuscript. Importantly, the primary tumor cells are difficult to transfect and they change their morphology upon extended culture (more than 1-2 passages), so analysis of FA dynamics in these cells would be extremely challenging.

2) Quantitation of data
a. Figure 2C - I would recommend quantitating the cell invasion into the stroma in WT vs KO

Quantification of multi-cell vs single-cell invasion has now been documented in Figure 2F.

3) Clarification of bridge adhesions vs. retraction fibers. Bridge adhesions are representative of "nascent" adhesions- and imply a failure to zipper/reinforce the junction. (A process that is dependent on Arp2/3-dependent actin polymerization). In the context of collective cell migration (and organoids) the junctions are already formed, albeit weaker in the paxillin KO. The data presented does not rule out that these adhesions may be retraction fibers rather than "bridge AJ", and could be due to a reinforce the junction rather than form one?

We agree that the use of “bridge junction” was misleading and inaccurate in the context of the wound assays. Accordingly, we have eliminated the use of the term “bridge AJ” and replaced it with the more general term “disorganized AJ” throughout the manuscript. The schematic depicting mature vs bridge junctions has also been removed from the manuscript.

In figure 4D- the junctions visible in Fig 4D paxillin KO panel look like they are failing to resist the pulling of cells at the leading edge- stretching the junctions. Which makes more sense to me that the cells are more easily able to "let go" and invade into the surrounding matrix if their cell-cell junctions are weaker. The addition of the timelapse movies analyzed in Figure 4A would facilitate the interpretation of this data.

Again, we agree with this assessment, and have clarified the text accordingly. We have also added the 2D cell migration time-lapse movies as movie 3 and 4.

The authors should differentiate between bridge adhesions and retraction fibers throughout the manuscript as these are mechanistically distinct processes. Note, the calcium switch experiments suggest there is an effect on cell-cell junction zippering- which would fit under the description of "bridge adhesion".

Agreed. The term “bridge AJ” is changed to “disorganized AJ” throughout the MS.

4) Re figure 3. The organoids show in the figure are convincing- however, the morphology of the WT organoid in the supplementary movie was less convincing. I'm guessing there is a significant amount of heterogeneity within the system. The addition of a panel to the supplementary data showing a range of WT and KO organoid morphologies would strengthen this data.

We have added a new figure, S3A of representative organoid “masks” to demonstrate the range of size and phenotype of the organoids.

The KO organoid movie- I don't see any cells actively leaving the organoid during the time period displayed. If possible- it would be ideal to include a movie of a cell actively leaving the organoid. I agree- there are a lot of mesenchymal like cells invading around the organoid but would like to see the cell actively leaving the organoid as a single cell. The cells migrating around in the matrix- that have left the organoids- are of interest, as highlighted by the Asterix in figure 3B- is it is possible to go back through the data and add some frames of these cells to the figure?

We have added a new Figure, S3C comprising individual frames from two movies (one of which is from movie 2) to show examples of individual cells migrating out from paxillin KO tumor organoids.

5) "Following tubacin treatment, the paxillin KO tumor organoids exhibited hyper-MT acetylation and a significant increase of invasiveness, as evaluated by their degree of circularity". I agree that tubacin may change the morphology of the organoids (circularity is a read out of this) however, more evidence - i.e., timelapse analysis of invasion from organoids would be required to support this statement of a decrease in invasiveness.

We have softened the statement about the increase of invasiveness and changed it to “a significant decrease in circularity, more indicative of increased invasiveness”.

Pg 16 "The paxillin KO tumor mouse developed fewer lung metastases, without a change of tumor size or latency (Figure 1), indicating that paxillin does not significantly impact tumor cell survival or proliferation". As paxillin KO is altering E-cadherin trafficking (endocytosis/recycling) - it would be worth adding to the discussion how this compares this to studies looking at cadherin KO (i.e. Padmanaban et al., Nature 2019).

We have now incorporated this reference into the discussion.

Minor:
Figure 1A: Please use different colors for the categories (WT & KO) i.e. the colors used in 1C.

Done

Figure 1C: add "n.s" with a line between categories

Done

Statistics- parametric tests (student's T test) are used throughout the study. What test of normality was used?

A Shapiro-wilk test for normality was performed. We have added additional details regarding statistical analysis to the Methods section.

Figure 2C: would benefit from the addition of a line marking the tumor stroma interface in the WT images (similar to what has been done in the Paxillin KO images). This might not be done as what is displayed is entirely stroma with invading tumor cells? Although it appears to be stroma on the far left of "2C tumor edge, WT"

A dashed line interface was added in upper panel in Figure 2E, as requested. As the reviewer noted, the image is showing mostly stroma and the tumor edge is at the right corner.

Figure 2: Each panel in the figure should have its own letter. i.e. (A) (hyperplasia/Adenoma (B) Carcinoma center (C) carcinoma edge (D) tumor edge (E) tumor center.

Done

Re: "Recent studies have shown that in highly invasive breast cancer, cells accomplish collective invasion through maintaining cell-cell contacts (Friedl and Gilmour, 2009; Cheung and Ewald, 2016)." I would add the more recent Ewald reference that addresses exactly this https://doi.org/10.1038/s41586-019-1526-3

We have added this reference.

Figure 4D actin is written in cyan, but displayed in blue. Please display in cyan.

We changed the actin text to blue to make it consistent with the figure.

Figure 7. 50 uM Y drug and blebbistatin are incredibly high concentrations, 5-10X higher

than what is needed. These drugs have decreased specificity at higher concentrations. Please add a zoom of the control conditions. It is hard to see in the images presented.

We originally used lower concentrations on the WT cells, but saw very limited effect on the AJs. Accordingly, we performed a titration with both drugs and determined the lowest concentration when we started to see substantial perturbation of AJs. A zoomed image of the control conditions has been added in Figure 7 as requested.

Figure S5. Please no error bars are on the graphs. Please display the mean of each independent repeat as a single data point. (similar to 6D- there are 3 data points and a box & whisker plot over the top)

We have added individual data points to the graphs in Figure S8. We have added error bars to the Rac1 data (n = 3), but not the Rho analysis (n = 2).

Pg 18 Although we did not observe any significant change in phospho-FAK levels in the paxillin KO tumor cells versus the WT cells (data not shown)- please add to supplementary data.

The total FAK and phospho-FAK blots, along with quantitation, have been added in Figure S9.

Typos
10 minuets, should be minutes
phase contract should be phase contrast.
"Movies 1-2. Tumor organoids in 3D collagen gels demonstrating reduced collective cell invasion in the paxillin KO organoids. Paxillin WT (movie 1) and KO tumor organoids (movie 2) were cultured for 2 days and then subjected to phase contract movies. Images were taken every 10 minuets for total 8 hours."

We have hopefully now corrected all typos-thank you.

RE: Manuscript #E21-09-0432R
TITLE: "Paxillin Promotes Breast Tumor Collective Cell Invasion through Maintenance of Adherens Junction Integrity"

Dear Chris,

Thank you for sending us your revised MS. As you can see, all now seems in order. So, I am happy to accept it for publication in MBoC.

Best wishes,

Alpha

Alpha Yap
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Turner:

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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We are pleased that you chose to publish your work in MBoC.

Sincerely,

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

Reviewer #1 (Remarks to the Author):

The authors have answered my queries.

Reviewer #2 (Remarks to the Author):

All of my suggestions have been incorporated- I am happy with the changes.