Receptor-Type Protein Tyrosine Phosphatase Alpha (PTPα) mediates MMP14 localization and facilitates triple-negative breast cancer cell invasion.

Lisa Decotret, Brennan Wadsworth, Ling Vicky Li, Chinten Lim, Kevin Bennewith, and Catherine Pallen

Corresponding author(s): Catherine Pallen, University of British Columbia

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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 #E20-01-0060
TITLE: Receptor-Type Protein Tyrosine Phosphatase Alpha (PTPα) mediates triple-negative breast cancer cell invasion by controlling MMP14 dynamics.

Dear Dr. Decotret,

Two reviewers have evaluated your manuscript entitled "Receptor-Type Protein Tyrosine Phosphatase Alpha (PTPα) mediates triple-negative breast cancer cell invasion by controlling MMP14 dynamics". As you will see, while both reviewers found your findings interesting, they also brought up concerns that must be addressed. In particular, Reviewer 1 brought up issues with quantification and the inclusion of more relevant controls, and Reviewer 2 raised questions regarding the role of apoptosis in your findings.

I hope that you find the reviewers comments useful and that you will be able to provide a revised version of your work that addresses these points as well as the other minor issues raised.

I thank you for submitting your work to Molecular Biology of the Cell.

Sincerely,

Carole Parent
Monitoring Editor
Molecular Biology of the Cell

Dear Miss Decotret,

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.

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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.

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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):

Decotret et al "Receptor-type protein tyrosine phosphatase alpha mediates triple-negative breast cancer invasion by controlling MMP14 dynamics"

The authors report work that follows up on their previous work examining the effect of PTPalpha on focal adhesions. Here, they examine the potential role of PTPalpha in invasion. PTPalpha is knocked down with no effect on proliferation but an effect on ECM degradation. Transwell migration and invasion through Matrigel were found to be reduced. These results support the idea that invadopodia were affected. However, PTPalpha did not colocalize with the invadopodia marker TSK5. It did colocalize with the metalproteinase MMP14 and cortactin and the idea that PTPalpha may be in an endocytic structure was examined. PTPalpha colocalized with cav-1 and MAP14 in structures too large to be caveolae, so examined EEA, a marker of an early endocytic compartment and found colocalization. Then found that PTPalpha depletion reduced surface MMP14 and MMP14 in protrusions. In xenografts in mice, found reduced expression of PTPalpha led to decreased tumor size and loss of invasion. The paper is interesting but could be improved by more thoughtful analysis of trafficking, better quantification, additional controls and more precise language. Importantly, some results are overinterpreted.

Comments:

1. The authors do not test whether the effect of PTPalpha of reducing MMP14 in protrusions affects migration and invasion. It is reasonable assumption, but not tested in this paper.
2. The description and quantification of colocalization in endomembrane structures should be improved. The results as reported are difficult to interpret and, therefore, to draw conclusions from.
For instance, the authors report finding at least one puncta of colocalization in some percentage of cells. How many puncta of each were there, so what is the percent overlap? What is the pearson coefficient, or similar, to quantify colocalization. In another instance, state 39% colocalization of 3 markers over 13 cells. What is the variation/cell? What criterion for colocalization was used?

3. The idea that a defect in endocytic trafficking in the knockdown causes the reduced levels of MMP14 in the protrusions is not tested.

4. The effect of PTPalpha on surface expression of MMP14 was minor, tested for only one shRNA, and an experiment to determine if the effect could be rescued by expression with a shRNA resistant message was not performed, making the experiment difficult to interpret

5. Language should be more precise, especially in the title. PTPalpha promotes or facilitates invasion, it does not mediate invasion. Furthermore, it is not clear what MMP14 dynamics are. Do the authors mean membrane trafficking dynamics? That was not determined in this paper. Neither was the idea that the effects on MMP14 were linked to the effects on invasive behavior.

6. The conclusions would be strengthened by rescue experiments using sh resistant message where possible and, where not possible, using two different shs e.g. in the mouse xenograft studies.

Reviewer #2 (Remarks to the Author):

Results
KD of PTPA in 231 cells resulted in reduced matrix degradation. Motility was slightly reduced while invasion was more strongly inhibited. Transfection of tagged constructs showed some colocalization with cortactin, caveolin, EEA1 and MMP14 but not TKS5. KD of PTPA reduced surface MMP14, especially in protrusions into a membrane with .3 um diameter pores. Tumors formed from PTPA KD cells were had a reduced weight with slightly reduced Ki67 and more strongly increased cleaved caspase 3, indicating increase apoptosis. In addition there appeared to be less invasion into surrounding tissue in the PTPA KD.

Summary
This is an interesting paper that suggests a role for PTP alpha in MMP14 targeting to invadopodia, suggesting a possible role for PTP alpha in tumor cell invasion. An in vivo study suggests that knockdown of PTP alpha has more complex effects, involving apoptosis and overall tumor size. However there are some concerns as listed below which should be addressed.

1. Although it is suggested that in vitro there is no growth effect over 3 days, in vivo there is a two-fold difference in weight over 14 days with a significant increase in an apoptotic marker. Given the 2.5 day in vitro doubling rate in Figure S1, over 14 days a two-fold difference in cell number (corresponding to the 2 fold difference in tumor weight) would require only a 12% difference in doubling time, which might not be detectable. Thus it would be worthwhile to perform the CC3 staining to test if there is increased apoptosis in vitro as well as in vivo. If there is increased apoptosis in vitro as well, then the authors need to consider whether PTPalpha is working directly on MMP14 trafficking or perturbing trafficking due to induction of apoptosis. Evaluating the effect of an alternative method of inducing apoptosis on invasion and matrix degradation would be an appropriate control.

2. In addition, tumor invasion should be compared at the same primary tumor size - giving the PTPalpha KD line an extra doubling (say 2.5 days) compared the control should be test.

3. The localization of PTPA-mcherry in Figure 3A looks very different from the localization of PTPA-GFP in 3B -D. The localization of TKS5 seems odd in Figure 3A as well - as rings of dots as opposed
to isolated dots. I wonder if either or both constructs are creating localization artifacts. In addition, the localization of MMP14 in 3B is different from 3C (solid dot vs ring). These apparent discrepancies need to be clarified.

Minor point: The H&E images are the same for ctl and sha1 in Figure 5C.
Response to Reviewers:

We would like to express our thanks to the external reviewers for their insightful comments and suggestions, which have greatly helped to improve the quality of our manuscript. Overall, it appears that the reviewers share our enthusiasm for highlighting the role of a phosphatase in promoting breast cancer cell invasion. In the following, we address their concerns point-by-point. Note that all page numbers correspond to the updated version of the manuscript.

Reviewer 1 Comments:

The authors report work that follows up on their previous work examining the effect of PTPalpha on focal adhesions. Here, they examine the potential role of PTPalpha in invasion. PTPalpha is knocked down with no effect on proliferation but an effect on ECM degradation. Transwell migration and invasion through Matrigel were found to be reduced. These results support the idea that invadopodia were affected. However, PTPalpha did not colocalize with the invadopodia marker TSK5. It did colocalize with the metalproteinase MMP14 and cortactin and the idea that PTPalpha may be in an endocytic structure was examined. PTPalpha colocalized with cav-1 and MAP14 in structures too large to be caveolae, so examined EEA, a marker of an early endocytic compartment and found colocalization. Then found that PTPalpha depletion reduced surface MMP14 and MMP14 in protrusions. In xenografts in mice, found reduced expression of PTPalpha led to decreased tumor size and loss of invasion. The paper is interesting but could be improved by more thoughtful analysis of trafficking, better quantification, additional controls and more precise language. Importantly, some results are overinterpreted.

1. The authors do not test whether the effect of PTPalpha of reducing MMP14 in protrusions affects migration and invasion. It is reasonable assumption, but not tested in this paper.

We agree with the reviewer that this assumption was not tested in this paper, as this is extremely difficult, if not presently impossible, to test. Ideally, this would involve depleting PTPα while preventing the ensuing reduction of MMP14 in protrusions (the effect we identified) and at the same time not preventing any other potential effects of PTPα depletion, and then determining whether the prevention of only the specific PTPα-induced effect to reduce protrusion-localized MMP14 affected migration and invasion. Even if it were possible to somehow block the reduction in protrusion-localized MMP14, it would not be possible to draw any valid conclusions from such an experiment unless we were able to ascertain that all other potential effects of PTPα depletion are (a) identified and (b) independent of PTPα-regulated MMP14 localization.

Nonetheless, we take the point made by the reviewer, and thus have changed the title of the paper from “Receptor-Type Protein Tyrosine Phosphatase Alpha (PTPα) mediates triple-negative breast cancer cell invasion by controlling MMP14 dynamics” to “Receptor-Type Protein Tyrosine Phosphatase Alpha (PTPα) mediates MMP14 localization and facilitates triple-negative breast cancer cell invasion”. We believe the new title highlights two important findings of the paper while not assuming PTPα acts directly through MMP14 trafficking to facilitate breast cancer cell invasion.

2. The description and quantification of colocalization in endomembrane structures should be improved. The results as reported are difficult to interpret and, therefore, to draw conclusions from. For instance, the authors report finding at least one puncta of colocalization in some
percentage of cells. How many puncta of each were there, so what is the percent overlap? What is the pearson coefficient, or similar, to quantify colocalization. In another instance, state 39% colocalization of 3 markers over 13 cells. What is the variation/cell? What criterion for colocalization was used?

We have improved the description and method of quantifying colocalization of PTPα to endosomal structures in a revised Figure 3. Rather than quantifying the percentage of colocalization on a cell-to-cell basis, we have now presented the data as the percentage of GFP-PTPα-positive puncta that colocalize with each endosomal marker (Figure 3E). We now state the number of cells analyzed, the number of total puncta analyzed, and indicate the proportion of PTPα-positive puncta that 1) do not contain MMP14 or cortactin/Cav-1/EEA1, 2) contain MMP14 and not cortactin/Cav-1/EEA1, 3) contain cortactin/Cav-1/EEA1 without MMP14, or 4) contain MMP14 together with cortactin/Cav-1/EEA1. These data are now described in the newly revised Results section (pgs. 7-8).

We decided against using the Pearson correlation coefficient, as suggested by the reviewer, to measure the degree of colocalization since this quantification method is more appropriately used to measure overlap between two markers within the cell. The goal of these experiments was to determine the proportion of GFP-PTPα positive puncta that overlap with two other markers (i.e. 3 markers total), particularly focusing on co-localization within intracellular structures rather than cell-wide expression. Thus, analyzing each GFP-PTPα positive puncta individually provides more precise interpretations of our data.

3. The idea that a defect in endocytic trafficking in the knockdown causes the reduced levels of MMP14 in the protrusions is not tested.

We agree with the reviewer that we did not test the effect of PTPα depletion on endocytic trafficking, and therefore only raised the idea of this as a possibility (“Together, these data raise the possibility that PTPα may be involved in endocytic signaling mechanisms” – Discussion, page 13). At present, we cannot comment on all endocytic trafficking mechanisms, as we believe that such an investigation is beyond the scope of this manuscript. Here, we focused specifically on MMP14 localization to membrane protrusions since MMP14 is a key player in MDA-MB-231 cellular invasion. Therefore, we can only report on the observed reduction of MMP14 expression in membrane protrusions and associate these findings with the role of PTPα in facilitating invasion. In the revised Discussion, we discuss the potential molecular mechanisms by which PTPα might regulate MMP14 trafficking to protrusions (pg. 13). We have also added a statement to the Discussion section (pg. 13) to highlight a specific direction for future research airing from our findings – “The effect of PTPα knockdown on endocytic trafficking is an active area of research. Future work is required to elucidate the precise pathways linking PTPα, caveolin-1, and EEA1 to MMP14 trafficking in TNBC cells.”

4. The effect of PTPalpha on surface expression of MMP14 was minor, tested for only one shRNA, and an experiment to determine if the effect could be rescued by expression with a shRNA resistant message was not performed, making the experiment difficult to interpret.

We thank the reviewer for this comment and agree that the surface expression of MMP14 quantified by flow cytometry was low in the original Figure 4B. In retrospect, this was not particularly surprising as we
postulate a role for PTPα in MMP14 localization specifically to plasma membrane protrusions, which may not be consistently quantifiable when analyzing MMP14 on the entire plasma membrane by flow cytometry. In order to validate these original data, we repeated the flow cytometry experiments with a revised staining methodology for MMP14 and using intracellular Stat3 as a control. Consistent with the reviewer’s comment, we found that MMP14 on the membrane was not significantly affected by PTPα knockdown using two different shRNA constructs. We have therefore removed the original flow cytometry data from the manuscript and have added the following statement to the Results section (pg. 9) – “Using flow cytometry, we did not detect an altered level of membrane-bound MMP14 in PTPα knockdown versus control cells (data not shown)” (Figure R2R-1 in the figures accompanying this letter of response). Since the goal of these experiments was to determine the role of PTPα in MMP14 localization to plasma membrane protrusions rather than total MMP14 at the cell surface, we used confocal microscopy to precisely visualize MMP14 localization to plasma membrane protrusions in control and PTPα knockdown cells as a more relevant approach. To further support these data, we have added more representative z-stack images of plasma membrane protrusions to supplement the images shown in Figure 4C (revised Figure S3A).

5. Language should be more precise, especially in the title. PTPalpha promotes or facilitates invasion, it does not mediate invasion. Furthermore, it is not clear what MMP14 dynamics are. Do the authors mean membrane trafficking dynamics? That was not determined in this paper. Neither was the idea that the effects on MMP14 were linked to the effects on invasive behavior.

We have modified the title of the manuscript to more precisely reflect our experimental findings and have revised text throughout the manuscript to provide more precise interpretations of our data.

6. The conclusions would be strengthened by rescue experiments using sh resistant message where possible and, where not possible, using two different shs e.g. in the mouse xenograft studies.

We thank the reviewer for this important comment and have repeated the in vivo orthotopic xenograft mammary fat pad invasion assay with a second shRNA targeting PTPα (shα2 cells). Note that the shα2 construct was also used for the in vitro experiments shown in Figure 2 and Figure 4. Our new results are shown in the revised Figure 5. We found that PTPα-knockdown tumours (shα2) recapitulated the data using shα1-expressing cells form the original submission, providing stronger support for our conclusion that PTPα promotes breast cancer cell invasion.

Reviewer #2 (Remarks to the Author):

KD of PTPA in 231 cells resulted in reduced matrix degradation. Motility was slightly reduced while invasion was more strongly inhibited. Transfection of tagged constructs showed some colocalization with cortactin, caveolin, EEA1 and MMP14 but not TKS5. KD of PTPA reduced surface MMP14, especially in protrusions into a membrane with .3 um diameter pores. Tumors formed from PTPA KD cells were had a reduced weight with slightly reduced Ki67 and more strongly increased cleaved caspase 3, indicating increased apoptosis. In addition, there appeared to be less invasion into surrounding tissue in the PTPA KD. This is an interesting paper that suggests a role for PTP alpha in MMP14 targeting to invadopodia, suggesting a possible role for PTP alpha in tumor cell invasion. An in vivo study suggests that
knockdown of PTP alpha has more complex effects, involving apoptosis and overall tumor size. However, there are some concerns as listed below which should be addressed.

1. Although it is suggested that in vitro there is no growth effect over 3 days, in vivo there is a two-fold difference in weight over 14 days with a significant increase in an apoptotic marker. Given the 2.5 day in vitro doubling rate in Figure S1, over 14 days a two-fold difference in cell number (corresponding to the 2 fold difference in tumor weight) would require only a 12% difference in doubling time, which might not be detectable. Thus, it would be worthwhile to perform the CC3 staining to test if there is increased apoptosis in vitro as well as in vivo. If there is increased apoptosis in vitro as well, then the authors need to consider whether PTPalpha is working directly on MMP14 trafficking or perturbing trafficking due to induction of apoptosis. Evaluating the effect of an alternative method of inducing apoptosis on invasion and matrix degradation would be an appropriate control.

We thank the reviewer for their well thought out comment. To test the role of PTPalpha on apoptosis in vitro, lysates of MDA-MB-231 cells stably depleted of PTPalpha (shCtl, sho1, sho2 cells) were immunoblotted for poly (ADP-ribose) polymerase (PARP), a stress response protein that is proteolytically cleaved by caspases upon the induction of apoptosis. For a positive control, parental MDA-MB-231 cells were treated with 25 nM staurosporine (STS), an agent that inhibits cellular growth by inducing intrinsic apoptotic signaling pathways. We found that stably depleting PTPalpha in MDA-MB-231 cells does not induce apoptosis in vitro (new Figure S4). These data suggest that PTPalpha does not protect cells from apoptosis in vitro and have included these important data in a new Figure S4. Thus, our finding that PTPalpha knockdown resulted in increased CC3 expression in vivo indicates a tumor microenvironment specific effect. The role of PTPalpha in protecting tumour cells from apoptosis in vivo is an area of research beyond the scope of this present study.

2. In addition, tumor invasion should be compared at the same primary tumor size - giving the PTPalpha KD line an extra doubling (say 2.5 days) compared the control should be test.

We respectfully disagree with the reviewer, as providing additional time for only the PTPalpha knockdown tumours to grow would also allow additional time for the PTPalpha knockdown cells to (potentially) migrate and invade, which would invalidate the in vivo invasion assay. Importantly however, our data indicate that MDA-MB-231 tumour cell invasion in vivo is independent of the size of the primary tumour. Firstly, it should be noted that we repeated the in vivo experiments using a second shRNA construct (also used for in vitro experiments) and another cohort of shCtl tumours. These experiments produced shCtl tumours with a range of weights and a mean that was not significantly different from the previous shCtl or sho1 tumours (Figure 5B), although the total tumour area in the mammary fat pad was higher than the previous experiments for both the shCtl and the shRNA tumours (Figure 5G). Notably, despite the differences in total tumour area between the experiments, both cohorts of shCtl tumours had significantly higher invasive tumour area compared to tumours grown from either sho1 or sho2 cell lines (Figure 5H).

Secondly, we plotted solid tumour area vs invasive tumour area for all shCtl and shRNA lines, and have included these data as a new Supplemental Figure 5. We found that even small shCtl tumours had high invasive tumour areas, and that comparatively large sho1 and sho2 tumours had very low invasive tumour areas. Our data therefore indicate that MDA-MB-231 invasion is not associated with tumour volume, and reinforce our conclusion that PTPalpha inhibition decreases tumour cell invasion in vivo.
3. The localization of PTPA-mcherry in Figure 3A looks very different from the localization of PTPA-GFP in 3B-D. The localization of TKS5 seems odd in Figure 3A as well - as rings of dots as opposed to isolated dots. I wonder if either or both constructs are creating localization artifacts. In addition, the localization of MMP14 in 3B is different from 3C (solid dot vs ring). These apparent discrepancies need to be clarified.

To assess mCherry-PTPα and GFP-PTPα localization, we co-transfected MDA-MB-231 cells with both mCherry- and GFP-tagged PTPα constructs (Figure new S2). This method of validation is necessary since there are no reliable immunofluorescent PTPα antibodies commercially available. These data show a high degree of colocalization between the GFP-tagged and mCherry-tagged PTPα constructs with PTPα being primarily expressed on the cell membrane. Upon further analysis of the image shown in the original Figure 3A, we found that cell was slightly out of focus and have now improved the quality of the representative image.

To confirm that the GFP-PTPα construct was not inducing localization artifacts, both wild-type and GFP-PTPα-expressing MDA-MB-231 cells were stained for MMP14, Cav-1, or EEA1 alone to analyze staining patterns in the presence or absence of expressed GFP-PTPα (Figure R2R-2 in the figures accompanying this letter of response). Here we show that MMP14, Cav-1, and EEA1 staining is similar in parental and GFP-PTPα-expressing cells, indicating that the introduction of GFP-tagged PTPα does not create artificial interactions between proteins. Based on these data, we are confident that the puncta analyzed in Figure 3 of the manuscript are in fact intracellular structures and not artificial aggregates.

To validate the MDA-MB-231 cell line expressing GFP-TKS5 (Figure 3A), the cells were co-stained with three different TKS5 antibodies (Figure R2R-3 in the figures accompanying this letter of response). We found that GFP-TKS5 expressing MDA-MB-231 cells formed both ring-like and punctate-like structures that colocalized with all three antibodies, suggesting TKS5 can accumulate in cellular structures of differing shape. As detailed further below, these shapes are entirely consistent with 2-dimensional images of cylindrical 3-dimensional cellular protrusions.

To clarify the apparent discrepancies in MMP14 localization within and between cells, particularly the ring-like structures versus the solid puncta observed, we took z-stack images of MDA-MB-231 cells stained for anti-MMP14 (Figure R2R-4 in the figures accompanying this letter of response). We found that depending on the plane imaged, MMP14 may appear in a ring-like structure or a dot-like structure which can be attributed to the fact that plasma membrane protrusions are cylindrical structures. Furthermore, MMP14 is a membrane-bound protein found in multiple cellular structures including endosomes so the ring-like structures observed in Figure 3C could be MMP14 bound to the membrane of caveolin-1 positive endosomes. In a report by Monteiro and colleagues, MMP14-positive endosomes, as determined by treating cells with FITC-dextran, appeared as both ring-like and dot-like structures (see Figure 4A in referenced manuscript) [1]. MMP14 staining may therefore be a combination of both dot-like and ring-like structures. Nonetheless, we appreciate the reviewers’ comments and have changed the image displayed in Figure 3C such that the imaging plane of MMP14 matches the plane of view of the other cells in Figures 3B-D.

4. Minor point: The H&E images are the same for ctl and sha1 in Figure 5C.

We appreciate the reviewer mentioning this important point. This mistake has been fixed.
References

1. Monteiro P, Rossé C, Castro-Castro A, et al (2013) Endosomal WASH and exocyst complexes control exocytosis of MT1-MMP at invadopodia. Journal of Cell Biology 203:1063–1079. https://doi.org/10.1083/jcb.201306162
RE: Manuscript #E20-01-0060R
TITLE: Receptor-Type Protein Tyrosine Phosphatase Alpha (PTPα) mediates MMP14 localization and facilitates triple-negative breast cancer cell invasion.

Dear Dr. Pallen,

Thank you for providing the revisions to your manuscript. The two reviewers have re-evaluated your work, and, as you will see, while Reviewer 1 is satisfied with the revised version, Reviewer 2 brings up a concern regarding the PTPα and TKS5 fusion images presented in Fig. 3A. I agree that there seems to be a discrepancy in the localization of PTPα in those images.

I look forward to a revise version of your manuscript that addresses this concern.

Sincerely,

Carole Parent
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Pallen,

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.

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

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In preparing your revised manuscript, please follow the instruction in the Information for Authors.
Reviewer #1 (Remarks to the Author):

The authors thoroughly and satisfactorily addressed concerns.

Reviewer #2 (Remarks to the Author):

KD of PTPα reduced matrix degradation. It reduced migration slightly and invasion more significantly. PTPα does not seem to colocalize with TKS5 but does show some colocalization with MMP14 and cortactin, caveolin and EEA1. PTPα KD reduced the amount of MMP14 in protrusions through .3 um diameter pores. PTPα KD led to reduced tumor size, with increased apoptotic markers. Interestingly, in vitro growth and apoptosis were not increased by PTPα KD, suggesting an in vivo specific effect. Local invasion in vivo was also reduced by PTPα KD.

Figure 3A remains completely confusing to me. In the top row PTPα-GFP is in rings and TKS5 is diffuse while in the bottom row TKS5-GFP is in rings and PTPα is diffuse/in broadly staining clumps! Supplementary figure 2 does not show any PTPα-GFP in rings and seems to be above the plane of the coverslip and looking at side views of the plasma membrane. I am very suspicious of the TKS5-GFP and mcherry PTPα result. I think it is important to do a western on these lines for mcherry and GFP to test whether most of the fluorescent protein remains as a fusion protein and is not cleaved. The other studies in Figure 3 seem to use the PTPα GFP construct.
**Response to Reviewers:**

We would like to express our thanks to the external reviewers for their insightful comments and suggestions. In the following text, we have addressed comments made in the second round of revisions.

**Reviewer 1 Comments:**

The authors thoroughly and satisfactorily addressed concerns.

Thank you for your comment.

**Reviewer 2 Comments:**

Figure 3A remains completely confusing to me. In the top row PTPα-GFP is in rings and TKS5 is diffuse while in the bottom row TKS5-GFP is in rings and PTPα is diffuse/in broadly staining clumps! Supplementary figure 2 does not show any PTPα-GFP in rings and seems to be above the plane of the coverslip and looking at side views of the plasma membrane. I am very suspicious of the TKS5-GFP and mcherry PTPα result. I think it is important to do a western on these lines for mcherry and GFP to test whether most of the fluorescent protein remains as a fusion protein and is not cleaved. The other studies in Figure 3 seem to use the PTPα GFP construct.

Thank you for this important comment and thorough review of the immunofluorescent images.

To address your comment and confirm whether the mCherry and GFP fluorescent tags remain fused to PTPα, MDA-MB-231 cells were either untransfected (-), transfected with mCherry or GFP alone (+) or with 0.5 μg, 1.0 μg, or 5.0 μg of mCherry-tagged or GFP-tagged PTPα DNA. As suggested by the reviewer, cell lysates were immunoblotted for mCherry or GFP, PTPα, and actin (Figure R2R-1). PTPα is a transmembrane protein with a glycosylated extracellular domain. N-glycosylated PTPα is ~100 kDa, and this is processed to a mature form of N- and O-glycosylated PTPα that has a molecular weight of ~130 kDa [1]. Immunoblotting for mCherry revealed a band at ~25 - 30 kDa in the mCherry-positive control lane and three distinct bands at ~130 kDa (mCherry-tagged N-glycosylated PTPα), ~140 kDa (mCherry-tagged N- and O-[partially] glycosylated PTPα) and ~160 - 170 kDa (mCherry-tagged N- and O-[fully] glycosylated PTPα) upon transfection with mCherry-PTPα (Figure R2R-1A). Immunoblotting for GFP revealed a band at ~25 kDa in the GFP-only positive control lane as well as two distinct bands at ~130 kDa (GFP-tagged N-glycosylated PTPα), and ~160 kDa (GFP-tagged N- and O-glycosylated PTPα) upon transfection with GFP-PTPα (Figure R2R-1B). Importantly, these bands are also visible when probing the western blots for PTPα, confirming that the fluorescent tags are conjugated to PTPα. In summary, in cells transfected with either mCherry-PTPα or GFP-PTPα, we do not observe any bands between 20 – 30 kDa that would indicate cleavage of these forms of tagged PTPα. This analysis confirms that the fluorescent proteins remain fused to PTPα and have not been cleaved.

To address the reviewer’s comment about TKS5 staining in original versions of Figure 3A and Supplemental Figure 2, TKS5 appears diffuse in wild-type MDA-MB-231 cells or in MDA-MB-231 cells transfected with GFP-PTPα because TKS5 is a cytoplasmic protein that only forms puncta upon the formation of invadopodia [2]. We found that MDA-MB-231 cells form very few invadopodia in our hands (new Figure 3A), but the transfection of cells with a GFP-TKS5 construct resulted in increased
TKS5-positive puncta formation (new Figure 3D), which may appear dot-like or ring-like. We have also found that untagged TKS5 and PTPα may appear as distinct puncta, rings, or as “clumps” as indicated by the reviewer. We have now provided evidence that the appearance of “clumps” in the images is due to unintentional overexposure of the image or when multiple structures (i.e. invadopodia, endosomes, etc.) form in close proximity to each other, making the resolution of these structures difficult to distinguish. Importantly, we have updated Figure 3 by splitting Figure 3A into a new Figure 3A – D to more clearly demonstrate that (a) PTPα does not localize to endogenous TKS5 puncta, (b) MDA-MB-231 cells transfected with GFP-TKS5 form TKS5-positive invadopodia confirmed by co-staining with a TKS5 antibody, (c) mCherry-PTPα and GFP-PTPα fluorescent tags both colocalize to intracellular puncta, and (d) PTPα-positive puncta do not express TKS5 (Figure 3). The new Figure 3D is a less over-exposed version of the original Figure 3A, and the “clumps” identified by the reviewer are no longer obscuring the image. The data presented in the revised Figure 3 strongly support our conclusion that PTPα and TKS5 do not colocalize and that PTPα is not a component of TKS5-positive invadopodia.

References:
1. Daum G, Regenass S, Sap J, et al (1994) Multiple forms of the human tyrosine phosphatase RPTPα. Isozymes and differences in glycosylation. Journal of Biological Chemistry 269:10524–10528. https://doi.org/10.1016/s0021-9258(17)34091-7
2. Saini P, Courtneidge SA (2018) Tks adaptor proteins at a glance. Journal of Cell Science 131:jcs203661–6. https://doi.org/10.1242/jcs.203661
Figure 1 for Reviewers

A

Figure R2R-1. MDA-MB-231 cells were either transfected with mCherry/GFP alone (+), untransfected (-), or with 0.5 μg, 1.0 μg, or 5.0 μg of mCherry-tagged/GFP-tagged PTPα DNA. Cell lysates (5 – 20 μg protein as indicated below each figure) were run on a NuPAGE 4-12% bis-tris gradient gel. (A) Lysates were probed for mCherry, PTPα, or actin. Red arrows indicate PTPα tagged with mCherry. (B) Lysates were probed for GFP, PTPα, and actin expression. Green arrows indicate PTPα tagged with GFP.
RE: Manuscript #E20-01-0060RR
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