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Syntaxin 7 contributes to breast cancer cell invasion by promoting invadopodia formation

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MS TITLE: Syntaxin7 contributes to breast cancer cell invasion by regulating invadopodia associated activity

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers share enthusiasm for the study and also raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. Doing so will likely require a mix of new experiments, reanalysis, and critical rewriting of the text and figures. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.
I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript the authors investigate the role of SNARE proteins in the formation and functioning of invadopodia. The transport of lipid membrane and cargoes to the invadopodia is a pivotal step in the invasion process whose mechanisms remain poorly understood. Here, Parveen et al. report the implication of syntaxin7 (STX7) in regulating matrix-degradation mediated by invadopodia in human breast cancer cell lines. They made the interesting observation that STX7 knock-out in MDA-MB-231 cells leads to increased MT1-MMP transport and accumulation at the cell surface, yet reduces the number of invadopodia and the recruitment of MT1-MMP to invadopodia, which is proposed to account for the reduced degradative properties of the KO cells. Furthermore, the authors attempt to define the STX7-SNARE complex regulating the formation of Tks5-positive invadopodia. By genetic down-regulation, they found that VAMP3 and VAMP2 are required for invadopodia formation. The authors conclude that STX7 could contribute to the endosomal transport of the metalloprotease MT1-MMP to the cell surface and its concentration within invadopodia. The experiments are logically presented and well-controlled. However, major changes are necessary before I would advise publication. The authors have all the tools to perform the requested experiments. Also, the organization of the figures is quite confusing, especially 3 and 4, and should be rearranged more clearly.

Comments for the author

Major points Figure 1. First the authors have screened by siRNA a selection of SNARE proteins for their ability to inhibit gelatin degradation by MDA-MB-231 cells, followed by a confirmation in Matrigel Boyden chamber invasion assay. The authors focused on STX7 and confirmed, by using two different siRNA and a CRISPR KO clone, the inhibitory impact on gelatin degradation. The impact of down-regulating STX7 is further confirmed in two additional BC cell lines. The results are convincing but two points need to be clarified:
- The author should clearly state in the text that it is not a cell line but a clonal cell line, and some experiments (e.g. Fig1C and 3E) should be confirmed with another clone or a non-clonal cell-line.
- Figure 1D and Figure 2: what are the levels of expression of the exogenous STX7 constructs compared to the endogenous protein in control cells? The KO of STX7 inhibits gelatin degradation by 70% indicating that STX7 is not responsible for all gelatin degradation, yet its exogenous expression has a huge positive effect on gelatin degradation. This raises few questions: Does STX7 overexpression change its localization? Does it stimulate the formation of invadopodia, and does it promote/increase its co-localization with Tks5 in invadopodia? This would be important to properly analyse the data presented in Fig2, which except for panel A, were all obtained upon over-expression of STX7. The analyses of a Tks5 staining in control vs GFP-STX7 cells, and STX7 vs GFP-STX7 should be provided.
Figure 2. The authors present solid results (panels D,E,F) regarding the presence of STX7 within vesicles stained for EEA1 or carrying MT1-MMP. However, I have some caveats about the co-localization experiments (panels A,B,C) that need to be addressed.
The neat images presented in Fig2A show that the endogenous STX7 is not co-localized with Tks5, but they still suggest that STX7 could be present in the vicinity of invadopodia. STX7 does not have to be strictly co-localized with Tks5 or cortactin to play a role on invadopodia or MT1-MMP transport to invadopodia, yet the co-staining analyses shown in panels B and C do not support the conclusion that “STX7 is associated with invadopodia”. In panel B, arrows would help the reader to assess the co-localization that is otherwise not evident. Furthermore, the authors show a quantification that needs to be explained. What are “puncta”? Are they pixels or cortactin-positive invadopodia? Please precise the number of cells analysed and the number of independent repeats. Because cortactin is mostly not present in invadopodia and because its co-localization with the

spots of degraded gelatin is not strong (2C) one can hardly conclude that GFP-STX7 is seen together with cortactin in invadopodia. To be more convincing the authors should repeat the experiments presented in panels B and C staining for Tks5 rather than cortactin, and show the same kind of magnified images as presented in panel 2A.
Figure 3. The authors analyse the impact of the depletion of STX7 on the cell surface expression of MT1-MMP and observe an increased delivery (A) and steady state cell surface expression (B) of MT1-MMP. They also report a decreased number of invadopodia (E,F) and MT1-MMP concentration in invadopodia. Overall the data are convincing, but the authors should also measure the MT1-MMP cell surface endocytosis.
3A': It is unclear what the authors are counting and how they normalized the data. On the Figure panel and in the figure legend it is indicated "number of MT1-MMP vesicles". However, the pHluorin emits a signal when it is exposed to the extracellular milieu. Thus, it appears that the authors do not count vesicles but exocytic events at the cell surface. Also, the number (indicated as n on the figure) of "vesicles" should be normalized to the number of cells and to the time of observation. The authors should indicate how many cells they have analysed per condition and how many independent repeats.
3B: In order for the authors to conclude that there is no effect of STX7 on MT1-MMP endocytosis, they should measure it directly, for example by pulse-chase biotinylation or antibody internalization.
Figure 4: SNARE partners to STX7 were analysed by pull-down assays and immunofluorescence co-localization experiments Panels A,B,C: Why did the authors not perform their pull-down experiments in MDA-MB-231 cells? In addition, the authors did not use the same cell lines to assess the binding to all ligands. In the end, the three proteins expressed in HeLa cells showed a stronger binding signal to STX7 than the three others expressed in HEK cells. To rigorously compare the binding of STX7 from the cell lysate to all six exogenously expressed ligands it is necessary to perform these experiments using the same cell line.
The impact of VAMP2 and VAMP3 down-regulation on gelatin degradation should be shown.
Minor points Figure 2 The movies presented in panels E and F are more convincing and are indicative of the co-transport of MT1-MMP and GFP-STX7. Could the authors provide a quantitative analysis of the numbers of structures co-stained and moving together per cells?
Figure 3 3D, E, F: The quantification should indicate how many cells were counted and the number of independent repeats.
In the conclusion, the authors relate their observations to the phenomenon of MT1-MMP clearance from the cell surface. It seems contradictory with their conclusion that STX7 does not affect MT1-MMP endocytosis. Since the MT1-MMP that is delivered does not seem to go into invadopodia, could the authors explain why their results would be coherent with clearance.
Figure 4 Panel B: it should be noted VAMP7 and not VAMP2 on the figure?
What do the numbers on the panels A and B refer to? In the Legend it says "the number represents the quantified value". Is it the percentage of the total in WCL? It does not look like it.
It should be written in the Results and the legend that the data presented in panels D-G were obtained with MDA-MB-231 cells.
In the discussion, references are missing for the first hypothesis regarding the possible STX7-dependent transport of growth factors required for invadopodia formation (lane7-8).
The authors might discuss alternative hypotheses regarding the role of STX7: STX7 depletion might perturb the formation or maintenance of vesicles docking sites explaining the decreased incorporation of MT1-MMP into the invadopodia. Less likely but cannot be ruled out: invadopodia are also formed in response to stiffness, thus STX7 might participate to sense stiffness or signal the mechanical response.
In Fig4, the authors present data regarding STX7 incorporation into multiprotein complexes. The powerful stimulatory effect on gelatin degradation in response to its overexpression would suggest that STX7 is the limiting factor of these machineries. Could the authors elaborate on this in the context of current knowledge?

Reviewer 2

Advance summary and potential significance to field

In this four-figure MS, the authors identify Syntaxin7 as a SNARE protein involved in invadopodia-, protease-based matrix degradation and cancer cell invasion based on a systematic siRNA-based

SNARE screening approach. They provide evidence for a limited association of STX7 with cortactin (cttn), F-actin and TKS5-positive invadopodia structures and with MT1-MMP positive vesicles, the main trans-membrane metalloproteinase responsible for invadopodial matrix degradation by cancer cells. Interestingly, although STX7 loss-of-function can interfere with matrix degradation, it is correlated with an increase in surface MT1-MMP levels. Finally, STX7 is found to interact with several SNARE proteins some of which are already known to regulate MT1-MMP traffic and invadopodia activity. Overall, this is an interesting, well-executed and well-written MS.

Comments for the author

Point 1. The association of STX7-GFP with TKS5-positive structures is not particularly obvious in the selected image in Figure 2A. STX7-GFP distribution is very spread and only a minute fraction may be associated with TKS5-positive invadopodia. In addition, Fig. S1F shows a rather limited colocalization of STX7-GFP with CD63, while association is much stronger with the early endosomal markers (EE1, Rab4 and Rab11). According to the existing literature, early endocytic compartments are not the main localization for MT1-MMP that seems to recycle mostly from endolysomal compartments. The authors should comment on that.
Point 2. In general, it is not easy to understand what is quantified in Figure 3A based on TIRF-M imaging and the increase in STX7 KO condition is not obvious in the selected image. TIRF movies are rather low quality, and it is unclear whether the MT1-MMPpHLuorin signal represents surface-exposed MT1-MP or some undefined vesicles (must have neutral pH) in the TIRF field and which pool was actually quantified and is plotted as 'number of MT1-MMP vesicles'.
Point 3. The apparent discrepancy between the observed increase in surface MT1-MMP and decrease in invadopodia function upon loss of STX7 function is discussed in the context of a requirement for MT1-MMP endocytic clearance, which has been discussed in previous other studies. Although this may be a plausible explanation, the authors should also consider more recent work that inversely concluded that a decrease in MT1-MMP endocytic clearance leads to an increase in invadopodia activity (Colombero et al. Adv Sci; 2021, Lodillinsky et al. Oncogene 2021).
Point 4. Increased cttn phosphorylation in STX7 KO cells (Figure 3H) is unexpected as it correlates with a reduction in matrix degradation. A possible explanation is that tyrosine phosphorylated cttn (Tyr-482) can be targeted to degradation. As phosphorylation on Ser/Thr and on Tyr residues is known to affect cttn activity in complex and sometime opposite manner depending on which residues get modified, the authors should provide specific information regarding the antibodies that they used to detect phospho-cttn in the experiment reported in Figure 3H.
Point 5. STX7 is shown to interact strongly with VAMP2 in Fig. 4. The authors should address or at least comment on a potential role of VAMP2 in matrix degradation and invadopodia function.
Minor issue. The legend of Fig. 3C should indicate more clearly that the observed signal corresponds to internalized anti-MT1-MP antibodies.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Parveen et al. reports on the role of Syntaxin7 in invasion of breast cancer cells by contributing to the delivery of necessary cargo to the invadopodia. They identify a number of the SNARE proteins of the endocytic and recycling pathways with potential roles in invadopodia-mediated breast cancer cell invasion by a SMART pool siRNA based reverse genetics approach. The authors show that the STX7 as an important player in MDA-MB-231 cell invasion and co-traffics with MT1-MMP to invadopodia with microscopic and biochemical studies.
We are just beginning to understand the significant role of endolysosomal trafficking in cancer progression, which is why the topic of this study is very timely. The article is in line with the previous work from the same lab showing the role and importance of endosomal recycling in trafficking of MT1-MMP to invadopodia, and thereby promoting breast cancer metastasis (Sharma, Priyanka et al. Journal of Cell Biology 219.1 (2020)). It is a well-designed, executed and documented work.

Comments for the author

I have only some minor Comments for the authors.

- Rename GFPSTX7 to GFP-STX7, inserting a dash to the name, it is quite difficult to follow STX in the text.
- Figure 2D, shows 9.0±1.5% of vesicles positive for STX7 are positive for MT1-MMP and EEA1. Did the authors assess other (endolysosomal) markers for colocalization with STX7?
- In reported image analysis results, e.g. Figure 3C, it is not stated how many cells are analysed. Please explicitly state the sample volumes for image analysis.
- Figure 4D, the image chosen for VAMP3 has 2 adjacent cells, and the signal is concentrated on the cell-cell contact site. Can the authors comment on how representative this is?
- The authors comment on the hampered trafficking of signaling molecules or growth factors or an unknown cargo carried by STX7. Did they test this, at least trafficking of some well-characterized growth factor receptors, e.g. EGFR?
That would be a nice addition to the study.
- The authors suggests STX4 acts upstream of STX7 in trafficking of MT1-MMP, however this is not reflected in the schematic model. I would suggest to expand the schematic to recapitulate this, and also include other previously reported endolysosomal proteins with a function in MT1-MMP trafficking.

First revision

Author response to reviewers' comments

Response to the reviewers’

Author’s response to editor and reviewers' comments
We greatly appreciate the editor’s and the reviewers' critical comments and insightful suggestions. We are thankful that they shared the enthusiasm and found potential importance in our study, highlighting the importance of STX7 in invadopodia mediated matrix-degradation in human breast cancer cell lines. Keeping this very essence, we have now revised the manuscript by addressing all the referees' comments. We carried out several new experiments, reanalyzed the data, and extensively edited the document to support our conclusions, wherever required.

We have now performed the gelatin degradation and invadopodia formation experiments using non-clonal STX7 KO cell lines(STX7KO-NC) (Fig.1C, 4B). In an independent experiment, a gelatin degradation assay was performed to measure the impact of VAMP2 depletion (Fig. 6A). Further, we carried out immunoblot analysis to check the expression level of exogenous STX7 constructs compared to the endogenous protein in control cells (Fig. S1E, S1E’). The data depicted in panels 2B and 2C (now S2A, S2B) showing association of STX7 with cortactin is now repeated again using Tks5 as an invadopodia marker (Fig. 2B, 2C). To rule out if STX7 silencing has any effect on MT1-MMP endocytosis, pulse-chase biotinylation was carried out to measure MT1-MMP population upon endocytosis (Fig. 3D). Pull-down experiment in MDA-MB-231 cells was performed to reaffirm the interaction of STX7 (Fig. S4A, S4B). Further, to assess other endo-lysosomal compartment for STX7 and MT1-MMP positive vesicles we performed confocal based imaging to show the co-localization of STX7 and MT1-MMP positive vesicles with EEA1 or CD63 (Fig. 2D). As suggested by the reviewer we have now provided the quantitative analysis of the live cell imaging (Fig. 2E and 2F) to show the structures co-stained for GFP-STX7 and Cherry-MT1-MMP moving together per cell.

In the section below, we have addressed each of the issues raised by the reviewers and have revised the manuscript accordingly. We would like to mention that as per the reviewer’s suggestion, we have reorganized the previously presented data. Thus, in the revised manuscript, figure labels are changed. We have referred to the modified figure labels in the following section.

Reviewer 1 Advance summary and potential significance to field
In this manuscript the authors investigate the role of SNARE proteins in the formation and functioning of invadopodia. The transport of lipid membrane and cargoes to the invadopodia is a pivotal step in the invasion process whose mechanisms remain poorly understood. Here, Parveen et al. report the implication of syntaxin7 (STX7) in regulating matrix-degradation mediated by invadopodia in human breast cancer cell lines. They made the interesting observation that STX7

knock-out in MDA-MB-231 cells leads to increased MT1-MMP transport and accumulation at the cell surface, yet reduces the number of invadopodia and the recruitment of MT1-MMP to invadopodia, which is proposed to account for the reduced degradative properties of the KO cells. Furthermore, the authors attempt to define the STX7-SNARE complex regulating the formation of Tks5-positive invadopodia. By genetic down-regulation, they found that VAMP3 and VAMP2 are required for invadopodia formation. The authors conclude that STX7 could contribute to the endosomal transport of the metalloprotease MT1-MMP to the cell surface and its concentration within invadopodia. The experiments are logically presented and well-controlled. However, major changes are necessary before I would advise publication. The authors have all the tools to perform the requested experiments. Also, the organization of the figures is quite confusing, especially 3 and 4, and should be rearranged more clearly.
Summary: We appreciate that the reviewer has found our study important that highlighted the role of STX7, in the formation and functioning of invadopodia. As suggested by the reviewer we have now changed the organization of the figure 3 and 4 to avoid any confusion. We thank the reviewer for his/her critical comments that helped to overall improve the quality of the manuscript.

Reviewer 1 Comments for the author

Major points

Figure1. First the authors have screened by siRNA a selection of SNARE proteins for their ability to inhibit gelatin degradation by MDA-MB-231 cells, followed by a confirmation in Matrigel Boyden chamber invasion assay. The authors focused on STX7 and confirmed, by using two different siRNA and a CRISPR KO clone, the inhibitory impact on gelatin degradation. The impact of down-regulating STX7 is further confirmed in two additional BC cell lines. The results are convincing but two points need to be clarified:

1. The author should clearly state in the text that it is not a cell line but a clonal cell line, and some experiments (e.g. Fig. 1C and 3E) should be confirmed with another clone or a non-clonal cell-line.
Response: We are thankful to the reviewer for providing an opportunity to further strengthens our observation using non-clonal cell-line and giving us an opportunity to clarify the use of clonal and non-clonal STX7 KO lines in this study. We have now performed the experiments Fig. 1C and 3E using STX7 KO non-clonal (STX7KO-NC) cell line. We have measured the gelatin degradation (Fig. 1C) and invadopodia formation (Fig. 4B, previously Fig. 3E) in control and the non-clonal (STX7KO-NC) cell line using confocal and fixed cells TIRF imaging respectively. The results are now added to the revised MS (Fig. 1C, 4B).

2a. Figure 1D and Figure 2: what are the levels of expression of the exogenous STX7 constructs compared to the endogenous protein in control cells?
Response: We sincerely thank the reviewer for pointing out this. To better understand the impact of STX7 overexpression, we have checked the levels of expression of exogenous STX7 constructs compared to the endogenous protein in control cells. It was observed that level of expression of exogenous STX7 (GFP-STX7) was found to be 1.0±0.1 that was comparable to the endogenous STX7 with expression of 1.1±0.0 (Fig. S1E, S1E'). The results are now added to the revised MS (Fig. S1E, S1E').

2b) The KO of STX7 inhibits gelatin degradation by 70% indicating that STX7 is not responsible for all gelatin degradation, yet its exogenous expression has a huge positive effect on gelatin degradation. This raises few questions: Does STX7 overexpression change its localization?
Response: We are thankful to the reviewer for raising this concern. To understand if STX7 overexpression changes its localization we checked the distribution of endogenous STX7 with EEA1 compartment in the presence of GFP-STX7 by immuno-fluorescence. At first, the % overlap of GFP-STX7 with STX7 and EEA1 were observed to be 56.0±6.4% and 52.4±9.3% respectively (Fig. S2E). Next, we measured the localization of STX7 with EEA1 in the presence of GFP-STX7 which was found to be 46.0±4.0% (Fig. S2E), similar to the UT condition (45.0±4.0%) (Fig. S1G'). Therefore, STX7 overexpression doesn't have any effect on its localization. The results are now added to the revised MS (Fig. S2E).

2c) Does it stimulate the formation of invadopodia, and does it promote/increase its co-localization with Tks5 in invadopodia? This would be important to properly analyse the data presented in Fig2, which except for panel A, were all obtained upon over-expression of STX7. The analyses of a Tks5 staining in control vs GFP-STX7 cells, and STX7 vs GFP-STX7 should be provided.
Response: We are thankful to the reviewer to raise this query, giving us an opportunity to better understand the underlying phenomenon upon STX7 overexpression. We observed that compared to GFP-Vector, the overexpression of STX7 (GFP-STX7) does not stimulate the invadopodia formation (Fig. S2C). However, the overexpression of STX7 does promote/increase STX7 co-localization with Tks5 in invadopodia (Fig. S2D); perhaps explains the positive effect with enhanced gelatin degradation upon GFP-STX7 overexpression. (Fig. 1D). The results are now added to the revised MS (Fig. S2C, S2D).

Figure 2. The authors present solid results (panels D,E,F) regarding the presence of STX7 within vesicles stained for EEA1 or carrying MT1-MMP. However, I have some caveats about the co-localization experiments (panels A,B,C) that need to be addressed.
The neat images presented in Fig2A show that the endogenous STX7 is not co-localized with Tks5, but they still suggest that STX7 could be present in the vicinity of invadopodia. STX7 does not have to be strictly co-localized with Tks5 or cortactin to play a role on invadopodia or MT1-MMP transport to invadopodia, yet the co-staining analyses shown in panels B and C do not support the conclusion that “STX7 is associated with invadopodia”.
Response: We are thankful to the reviewer for extending the support that STX7 does not have to be strictly co-localized with Tks5 or cortactin to play a role on invadopodia or MT1-MMP transport to invadopodia. Reviewer’s positive comment has given the strength to our observation.

3a) In panel B, arrows would help the reader to assess the co-localization that is otherwise not evident.
Response: As pointed out by the reviewer, to help the reader to assess the co-localization, we have now added the arrows in Fig. S2A (Previously Fig. 2B) showing the association of STX7 with cortactin and actin.

Furthermore, the authors show a quantification that needs to be explained. What are “puncta”? Are they pixels or cortactin-positive invadopodia?
Response: We have identified the punctae for each fluorescence channel as an object using an automated image analysis program, Motion Tracking (MT) (http://motiontracking.mpi-cbg.de). The objects were identified as vesicles in each channel based on their size, fluorescence intensity, and other parameters by Motion Tracking software {http://motiontracking.mpi-cbg.de (Rink et al., 2005; Collinet et al., 2010)}. Each object is indeed a cluster of pixels.

In Fig. S2A, the quantification shows the number of punctae (multicolour objects) that are positive for actin, cortactin and STX7 (Previously Fig. 2B). We followed following methods to identify these multicolour objects.

Objects detected in two different channels were considered to be colocalized if the relative overlap of respective areas was >35% (Rink et al., 2005; Collinet et al., 2010; Priya et al., 2015). The apparent co-localization value was calculated as the ratio of integral intensities of co-localized objects to the integral intensities of all objects carrying a given marker and varied from 0.0 to 1.0. The co-localization-by-chance (random co-localization) was estimated by random permutation of objects localization in different channels. The apparent co-localization was corrected for random co-localization. Based on this method of calculating co-localization, we thereafter identified multicolour object (Fig. R1).

Fig. R1 Method for calculation of co-localization by MotionTracking. (A). The green vesicle is co-localized with red if more than 35% of its area is covered by red vesicle, shown in yellow. (B) Similarly three vesicles are considered to be co-localized when area shared by them is > 35%, shown in white. This co-localization could be asymmetric depending upon area overlap of vesicle with each other. For example, if A = 50%, then green is co-localized with red, but if B= 25% then red is not co-localized with green. It means that co-localization is asymmetric.
Here, X = Area of overlap,
A= Area of green vesicle overlapping with red vesicle,
B= Area of red vesicle overlapping with green vesicle.

Number of such overlapping vesicles are searched in motion tracking program and termed as multi-color object.
Please precise the number of cells analysed and the number of independent repeats.
Response: Each experiment was performed minimum in three biological replicates. The detailed description of the number of cells (n) and number of individual experiments (N) used for calculations is provided in the respective figure legends (3 different color code for each replicate, where N=3, n=77).
(Fig. S2A).

3b) Because cortactin is mostly not present in invadopodia and because its co-localization with the spots of degraded gelatin is not strong (2C) one can hardly conclude that GFP-STX7 is seen together with cortactin in invadopodia. To be more convincing the authors should repeat the experiments presented in panels B and C staining for Tks5 rather than cortactin, and show the same kind of magnified images as presented in panel 2A.
Response: We are thankful to the reviewer for providing us an opportunity to further cross-validate and strengthen our observation using different invadopodia marker. The results shown in panels 2B and 2C that is association of GFP-STX7 with invadopodia (marked by cortactin) and availability of GFP-STX7 near the labelled gelatin degradation area (marked by cortactin) respectively, is now repeated again that showed STX7 association with Tks5, as an invadopodia marker (Fig. 2B, 2C). Also, we have shown the same kind of magnified images as presented in panel 2A for association of STX7 with Tks5 near the TIRF surface. Now, we have shifted our previous observed image panel using cortactin marker into supplementary file (Fig. S2A, S2B). The results are now added to the revised MS (Fig. 2B, 2C).

Figure 3. The authors analyse the impact of the depletion of STX7 on the cell surface expression of MT1-MMP and observe an increased delivery (A) and steady state cell surface expression (B) of MT1-MMP. They also report a decreased number of invadopodia (E,F) and MT1-MMP concentration in invadopodia. Overall the data are convincing, but the authors should also measure the MT1-MMP cell surface endocytosis.
Response: We are sincerely thankful to the reviewer for finding the data convincing. We have now measured the MT1-MMP endocytosis following surface biotinylation. And, we did not find any significant difference in the endocytosis of MT1-MMP in clonal STX7KO cells compared to the control (Fig. 3D). The results are now added to the revised MS (Fig. 3D).

4a) 3A': It is unclear what the authors are counting and how they normalized the data.
Response: We sincerely apologize for not providing sufficient elaboration for the quantification. In Fig. 3A' we have quantified the number of MT1-MMP vesicles that are available in the TIRF plane and could form a track spanning minimum 4 consecutive frames in live cell TIRF video captured over 1 minute duration.

- Please find below the detailed step involved in quantification

- Step1 Object identification: Object based analysis was performed by the automated image analysis program, MotionTracking {(http://motiontracking.mpi-cbg.de; (Rink et al., 2005; Collinet et al., 2010)}. The core of Motion Tracking is finding the position of objects in the acquired frames of the movie, such as fluorescent molecule. Once found, the software assigns an x-y position and quantifies the intensity profiles of the objects. Each object is basically a group of pixels. The number of pixels combined with the scale will lead to "Object Size" and "Area". Each pixel has its own brightness called "intensity". The vesicles are identified as objects by setting parameters (Fig. R2A, R2B).

Fig R2: (A) The image shows the identified pHluorin-MT1-MMP punctae or vesicle near the cell surface (TIRF), identified as object by motion tracking and their associated contour. Scale bar 3µm. (B) The representative intensity distribution of the objects enclosed in a box as analysed by motion tracking.

- Step 2 Identifying the Tracks: Once Motion Tracking has successfully localized every object in the movie, the objects in consecutive frames can be linked into trajectories, or tracks. A track is the location of an object over time. Tracks have properties such as speed, direction, maximum

displacement, intensity, Area that is adjusted to identify tracks. The Relative Weights can take any positive values from 0 to 1.

- Step 3 Track break thresholds: Adjust the Track break thresholds, present in the same dialogue. These are: Total score threshold, Area score threshold, Intensity score threshold, Integral score threshold. These thresholds are critical as it will command the program to select a range of the variation in properties of the object that it can tolerate while building a track.

- Step 4 Searching Tracks in entire movie (Fig. R3).

Fig. R3 Image represent the formation of track formed by identified objects in a movie. Scale bar 3µm.

- Step 5 Quantify number of vesicles near cell surface: This is the number of tracks detected in a 1 minute movie. Each of the tracks identified using above method are considered as a MT1- MP vesicles near TIRF surface.

On the Figure panel and in the figure legend it is indicated “number of MT1-MMP vesicles”. However, the pHluorin emits a signal when it is exposed to the extracellular milieu. Thus, it appears that the authors do not count vesicles but exocytic events at the cell surface.
Response: We apologise to reviewer for inadvertently providing incomplete information. We have now changed the label of graph axis with number of MT1-MMP vesicles/cell/min. In our data we have quantified the number of MT1-MMP vesicles that have entered into the TIRF field and it could be observed over a minimum of 4 consecutive frames. It was further normalised with respect to the number of cell and the duration of the captured video. In the revised manuscript we have now modified the graph axis and the figure legend (Fig. 3A’).

Also, the number (indicated as n on the figure) of “vesicles” should be normalized to the number of cells and to the time of observation.
Response: We have now changed the label of graph axis to this “number of MT1-MMP vesicles/cell/min” to reflect the normalization (Fig. 3A’).

The authors should indicate how many cells they have analysed per condition and how many independent repeats.
Response: Each experiment was performed minimum in three biological replicates. The detailed description of the number of cells (n) is written over the graph (Fig. 3A’) and number of individual experiments (N) used for calculations is provided in the respective figure legends {3 different color code for each replicate, where N=3, n=77 (Control), n=83 (STX7KO), n=51 (SHC002), n=47 (shSTX4 C3)}. Please ref to page no. 32, line no. 1017-1021 of figure legends 3A’.

4b) 3B: In order for the authors to conclude that there is no effect of STX7 on MT1-MMP endocytosis, they should measure it directly, for example by pulse-chase biotinylation or antibody internalization.
Response: We are thankful to the reviewer for the valuable suggestion. To rule out that STX7 depletion doesn’t have any effect on endocytosis, we have now performed the pulse chase biotinylation assay. In comparison to control cells, the clonal STX7 KO cells didn’t show any significant difference in the population of MT1-MMP post-endocytosis (Fig. 3D). The results are now added to the revised MS (Fig. 3D).

5) Figure 4: SNARE partners to STX7 were analysed by pull-down assays and immunofluorescence co-localization experiments

Panels A,B,C: Why did the authors not perform their pull-down experiments in MDA-MB-231 cells? In addition, the authors did not use the same cell lines to assess the binding to all ligands. In the end, the three proteins expressed in HeLa cells showed a stronger binding signal to STX7 than the three others expressed in HEK cells. To rigorously compare the binding of STX7 from the cell lysate to all six exogenously expressed ligands it is necessary to perform these experiments using the same cell line.
Response: Carrying out interaction studies in HeLa or HEK cells is routinely reported in relevant literature (Sakurai-Yageta et al., 2008; Monteiro et al., 2013; Kajiho et al., 2016). This is largely

due to the fact that both HeLa and HEK cell lines are excellent systems for exogenous protein expression and for analysis of protein-protein interactions. By contrast, equivalent protein expression in MDA-MB 231 cells is heavily resource driven due to lower transfection efficiency, even with commercially optimised transfection reagents. Moreover, since earlier studies have reported that the proteins we are studying are endogenously expressed in HeLa and HEK cells (Gordon et al., 2009; He and Linder, 2009; Kubo et al., 2015), it is biologically relevant to carry out the interaction studies in these cell lines. Also, the comparison of binding strengths from pull down/immuno-precipitation studies is largely qualitative and derived by normalization with respect to the precipitated protein and the expression of the binding partners. We have provided the quantification of the protein bands and based on this we have estimated the binding strength of STX7 interaction with various other SNAREs.

However, we have attempted to perform the pull-down using the same cell line i.e MDA-MB-231 and the blots for the same are provided in supplementary file (Fig. S4A, S4B).

6) The impact of VAMP2 and VAMP3 down-regulation on gelatin degradation should be shown.
Response: As suggested by the reviewer, we have now performed the experiment showing the impact of VAMP2 depletion on gelatin degradation and it was observed that there was 57% reduction in gelatin degradation activity (Fig. 6A). The results are now added to the revised MS (Fig. 6A).

We have already shown the effect of VAMP3 down-regulation on gelatin degradation where VAMP3 was one of the candidates in our initial screen (Fig. 1A and S1B).

Minor points:
1) Figure 2
The movies presented in panels E and F are more convincing and are indicative of the co-transport of MT1-MMP and GFP-STX7. Could the authors provide a quantitative analysis of the numbers of structures co-stained and moving together per cells?
Response: As suggested by the reviewer we have now analysed the videos and provided the quantitative analysis of the numbers of structures co-stained and moving together per cells (Fig. 2E, 2F) in the revised manuscript.

2) Figure 3
3D, E, F: The quantification should indicate how many cells were counted and the number of independent repeats.
Response: Each experiment was performed minimum in three biological replicates. The detailed description of the number of cells (n) is written over the graph of Fig. 4A, 4B, 4C (Previously Fig. 3D, 3E, 3F) and number of individual experiments (N) used for calculations is provided in the respective figure legends (3 different color code for each replicate where
For Fig. 4A: N=3, n=118(Control), n=145 (STX7KO);
For Fig. 4B: N=3, n=157(Control), n=159(STX7KO);
: N=3, n=108(Control), n=122(STX7KO-NC);
For Fig. 4C: N=3, n=136(Control), n=132(STX7KO);
(Please ref to page no 33, line no. 1053-1065 of figure legend 4A, 4B, 4C).

In the conclusion, the authors relate their observations to the phenomenon of MT1-MMP clearance from the cell surface. It seems contradictory with their conclusion that STX7 does not affect MT1-MMP endocytosis. Since the MT1-MMP that is delivered does not seem to go into invadopodia, could the authors explain why their results would be coherent with clearance.
Response 1: We are thankful to the reviewer for pointing this out. Based on our initial observation that STX7 depletion enhanced the surface MT1-MMP level, we too were surprised to note that in spite of increase in surface population of MT1-MMP (Fig. 3A, 3B, 3C) there is reduction in gelatin degradation activity (Fig. 1A, 1C). To validate our observation, we came across the literature that suggested that disruption of MT1-MMP's endocytic trafficking significantly decreases its ability to promote ECM degradation and invasion (Uekita et al., 2001; Williams and Coppolino, 2011; Frittoli et al., 2014). However, we did not observe any disruption in endocytosis (Fig. 3D). Next, we observed that STX7 depletion abrogated invadopodia formation (Fig. 4B) and hence MT1-MMP delivery to the invadopodia (Fig. 4A). This observation provided us an insight that because

invadopodia itself is not forming so even if MT1-MMP is abundant at the surface it is not able to perform its activity.

Therefore, based on our observation and as pointed by reviewer we agree that our conclusion is not going in line with the observation that STX7 does not affect MT1-MMP endocytosis. Thus, we will modify the conclusion. We believe loss in gelatin degradation activity is due to reduced invadopodia formation rather than defect in surface clearance. In the revised manuscript we have now removed it from our conclusion and discussion.

3) Figure 4
Panel B: it should be noted VAMP7 and not VAMP2 on the figure?
Response: We would like to mention that it is VAMP2 only. For VAMP7, blot is provided in supplementary file (Fig. S4C).

What do the numbers on the panels A and B refer to? In the Legend it says “the number represents the quantified value”. Is it the percentage of the total in WCL? It does not look like it.
Response: In our pull-down study the binding strengths is derived by normalization of prey protein (STX7) with respect to the precipitated protein (GFP-tagged protein). This normalisation of the individual bands is written in figure legend as “the number represents the quantified value”. In our current manuscript we have now modified the figure legend to “The number represents the normalized value of prey protein (STX7) with respect to the precipitated protein (GFP-tagged protein)” (Fig. 5A, 5B, 5C, S4A, S4B, S4C).

It should be written in the Results and the legend that the data presented in panels D-G were obtained with MDA-MB-231 cells.
Response: As suggested by the reviewer we have now written in the results and the legend that the data presented in panels 5D, 6B, 6C, 6D (Previously, Fig. 4D-G) were obtained with MDA-MB-231 cells (Please refer to page no. 34, line no. 1090,1103, 1108, 1114 of figure legend 5 and 6 (Fig. 5 and Fig.6).

In the discussion, references are missing for the first hypothesis regarding the possible STX7-dependent transport of growth factors required for invadopodia formation (lane7-8).
Response: We have now added the missing reference (Please refer to page no. 9, line no. 258 of main manuscript.

The authors might discuss alternative hypotheses regarding the role of STX7: STX7 depletion might perturb the formation or maintenance of vesicles docking sites explaining the decreased incorporation of MT1-MMP into the invadopodia. Less likely but cannot be ruled out: invadopodia are also formed in response to stiffness, thus STX7 might participate to sense stiffness or signal the mechanical response. Response: We are thankful to the reviewer for suggesting an alternative hypothesis regarding the role of STX7 in invadopodia formation. As reported earlier, in comparison to soft polyacrylamide gels, breast carcinoma cells cultured over hard polyacrylamide gels produced more invadopodia contributing to more ECM degradation (Alexander et al., 2008). This observation was further validated in a very recent computational study through simulations that has demonstrated enhanced MT1-MMP secretion at the invadopodia in a stiff ECM (Kim et al., 2022). In an independent study, it was also found that during membrane docking and fusion, the tension generated at the membrane due to the stiff environment regulates the fusion pore dynamics and release of the vesicular content (Wang and Galli, 2018). It has also been observed that substrate rigidity from 1.5kPa to 28kPa promoted the exocytosis of VAMP7 vesicles in fibroblasts whereas VAMP2 exocytosis was insensitive to rigidity (Wang et al., 2018). Therefore, there is a possibility that increased matrix stiffness may trigger some mechanical response that in turn prompts STX7 to transport cargo molecules to initiate invadopodia formation. The cargo molecule delivered at the surface initiate the formation of contact-site, that involves incorporation of MT1-MMP promoting invadopodia formation (Zagryazhskaya-Masson et al., 2020). This could not be achieved when STX7 mediated cargo trafficking is hampered. In our revised manuscript we have now incorporated this alternative hypothesis regarding the role of STX7 in invadopodia formation. Please refer to page no. 9-10, line no. 280-297 of main manuscript).

In Fig4, the authors present data regarding STX7 incorporation into multiprotein complexes. The powerful stimulatory effect on gelatin degradation in response to its overexpression would suggest

that STX7 is the limiting factor of these machineries. Could the authors elaborate on this in the context of current knowledge?
Response: We sincerely thank the reviewer for bringing up this discussion. SNARE proteins always work by forming trans-SNARE complex having one v-SNARE and three t-SNARE protein. Each of these SNAREs may form multiple other trans-SNARE complexes in a system (Südhof and Rothman, 2009). The molecules which we have identified in our current study as an interacting partner of STX7 were VAMP2, VAMP3, VAMP7, STX4 and SNAP23. Each of these interacting partners when depleted, had similar impact on loss of gelatin degradation activity as observed from our experimental data (Fig. 1A, S1B) or from others study (Williams, McNeilly and Coppolino, 2014). VAMP2, VAMP3 and STX4 have also resulted reduced invadopodia formation when depleted (Fig. 6B, 6C, 6D), therefore phenocopying STX7. When STX7, one of the SNARE partners is depleted, others were still able to form trans-SNARE complex, albeit none of the partners could degrade gelatin owing to reduced invadopodia formation. This same phenotype could also be observed upon depletion of other SNARE partner. Therefore, we will refrain from identifying/reporting STX7 as the limiting factor of the machineries.

Reviewer 2 Advance summary and potential significance to field
In this four-figure MS, the authors identify Syntaxin7 as a SNARE protein involved in invadopodia-, protease-based matrix degradation and cancer cell invasion based on a systematic siRNA-based SNARE screening approach. They provide evidence for a limited association of STX7 with cortactin (cttn), F-actin and TKS5-positive invadopodia structures and with MT1-MMP positive vesicles, the main trans-membrane metalloproteinase responsible for invadopodial matrix degradation by cancer cells. Interestingly, although STX7 loss-of-function can interfere with matrix degradation, it is correlated with an increase in surface MT1-MMP levels. Finally, STX7 is found to interact with several SNARE proteins some of which are already known to regulate MT1-MMP traffic and invadopodia activity. Overall, this is an interesting, well-executed and well-written MS.
Summary: We are thankful to the reviewer for finding this study interesting and well-executed. We also appreciate that the reviewer has very critically gone through the manuscript and we thank him/her for directing us to further reinforce the conclusion of our study. Following the reviewer's suggestion we have addressed below each of the issue raised and edited the manuscript following the suggestion.

Reviewer 2 Comments for the author
1a) Point 1. The association of STX7-GFP with TKS5-positive structures is not particularly obvious in the selected image in Figure 2A. STX7-GFP distribution is very spread and only a minute fraction may be associated with TKS5-positive invadopodia.
Response: We are thankful to the reviewer for raising this concern and giving us an opportunity to clarify our observation. We would like to draw reviewer's attention, that at the TIRF plane, endogenous staining was used for STX7 rather than GFP-STX7 (Fig. 2A). Further, we agree with the reviewer that in Fig. 2A, STX7 is not perfectly co-localized with Tks5. Our observation is in line with the study where authors have shown that Exo84 puncta was detected partially overlapping with WASH and cortactin-positive subdomains on MT1-MMP–containing endosomes (Monteiro et al., 2013). Of note, this has also been raised and discussed by Reviewer 1 that STX7 does not need to be perfectly co-localized with Tks5 or cortactin to play a role in cargo transport to invadopodia.

1b) In addition, Fig. S1F shows a rather limited co-localization of STX7-GFP with CD63, while association is much stronger with the early endosomal markers (EEA1, Rab4 and Rab11). According to the existing literature, early endocytic compartments are not the main localization for MT1-MMP that seems to recycle mostly from endolysomal compartments. The authors should comment on that.
Response: We agree with the reviewer that in most of the reports MT1-MMP recycling has been reported from late endosomes (Steffen et al., 2008; Williams, McNeilly and Coppolino, 2014). But, there are also reports that showed MT1-MMP recycles from Rab5-Rab4 positive compartments or via retromer (Frittoli et al., 2014; Sharma et al., 2020). RAB5a/RAB4a route is preferentially used when rapid delivery and activation of MT1-MMP is required in response to stimulus. Further, unlike multiple other cell line like CHO, MDCK, A431 and HeLa where endosomal compartments are distinct from one another (Ullrich et al., 1996; Sheff et al., 1999; Sönnichsen et al., 2000; Wilcke et al., 2000), the MDA-MB-231 doesn't have well characterized trafficking pathway. It has mostly bulky endosomes which majorly referred as sorting endosomes (Sharma et al., 2020).

We now carried out co-localization experiments using fixed cell confocal imaging and observed that 30.0±3.0% of STX7 endosomes are positive for MT1-MMP and EEA1, however only 16.8±1.9% of STX7 are positive for MT1-MMP and CD63 (Fig. 2D). We are not ruling out that MT1-MMP doesn't recycle from endolysosomal compartments. But, we wish to emphasize that STX7 positive MT1-MMP populations is definitely less from CD63 positive compartment. The above observation is now added in the revised manuscript (Fig. 2D).

2a) Point 2. In general, it is not easy to understand what is quantified in Figure 3A based on TIRF-M imaging
Response: We sincerely apologize for not providing sufficient elaboration for the quantification. In Fig. 3A we have quantified the number of MT1-MMP vesicles that are available in the TIRF plane and could form a track spanning minimum 4 consecutive frames in live cell TIRF video captured over 1 minute duration.
- Please find below the detailed step involved in quantification

- Step1 Object identification: Object based analysis was performed by the automated image analysis program, MotionTracking {(http://motiontracking.mpi-cbg.de; (Rink et al., 2005; Collinet et al., 2010)}. The core of Motion Tracking is finding the position of objects in the acquired frames of the movie, such as fluorescent molecule. Once found, the software assigns an x-y position and quantifies the intensity profiles of the objects. Each object is basically a group of pixels. The number of pixels combined with the scale will lead to "Object Size" and "Area". Each pixel has its own brightness called "intensity". The vesicles are identified as objects by setting parameters (Fig. R2A, R2B).

Fig R2: (A) The image shows the identified pHluorin-MT1-MMP punctae or vesicle near the cell surface (TIRF), identified as object by motion tracking and their associated contour. Scale bar 3µm. (B) The representative intensity distribution of the objects enclosed in a box as analysed by motion tracking.

- Step 2 Identifying the Tracks: Once Motion Tracking has successfully localized every object in the movie, the objects in consecutive frames can be linked into trajectories, or tracks. A track is the location of an object over time. Tracks have properties such as speed, direction, maximum displacement, intensity, Area that is adjusted to identify tracks. The Relative Weights can take any positive values from 0 to 1.

- Step 3 Track break thresholds: Adjust the Track break thresholds, present in the same dialogue. These are: Total score threshold, Area score threshold, Intensity score threshold, Integral score threshold. These thresholds are critical as it will command the program to select a range of the variation in properties of the object that it can tolerate while building a track.

- Step 4 Searching Tracks in entire movie (Fig. R3).

Fig. R3 Image represent the formation of track formed by identified objects in a movie. Scale bar 3µm.

- Step 5 Quantify number of vesicles near cell surface: This is the number of tracks detected in a 1 minute movie. Each of the tracks identified using above method are considered as a MT1-MMP vesicles near TIRF surface.

2b) and the increase in STX7 KO condition is not obvious in the selected image. TIRF movies are rather low quality, and it is unclear whether the MT1-MMPpHLuorin signal represents surface-exposed MT1-MP or some undefined vesicles (must have neutral pH) in the TIRF field and which pool was actually quantified and is plotted as 'number of MT1-MMP vesicles'.
Response: We sincerely thank the reviewer for pointing out this and we apologize for the poor selection and presentation of the video and representative image. In the revised version of the manuscript, we have provided better representative image from the TIRF video for control and STX7 KO cells (Fig. 3A and Video 3).

Here we have quantified the number of MT1-MMP vesicles that have entered into the TIRF field and it could be observed over a minimum of 4 consecutive frames. It was further normalised with respect to the number of cell and the duration of the captured video (Fig. 3A').

In most of the study's authors do calculate exocytic event per minute (Kajiho et al., 2016; Planchon et al., 2018) however, calculating the vesicle count at the TIRF surface is also not uncommon (Macpherson et al., 2014; Sharma et al., 2020). In our study we have quantified the number of surface MT1-MMP vesicles. In the revised manuscript we have now modified the graph axis and the figure legend (Fig. 3A').

3) Point 3. The apparent discrepancy between the observed increase in surface MT1-MMP and decrease in invadopodia function upon loss of STX7 function is discussed in the context of a requirement for MT1-MMP endocytic clearance, which has been discussed in previous other studies. Although this may be a plausible explanation, the authors should also consider more recent work that inversely concluded that a decrease in MT1-MMP endocytic clearance leads to an increase in invadopodia activity (Colombero et al. Adv Sci; 2021, Lodillinsky et al. Oncogene 2021).
Response: We are thankful to the reviewer for pointing out this. The reduced invadopodia function i.e reduced gelatin degradation activity (Fig. 1A, 1C) is due to reduced invadopodia formation (Fig. 4B, 4C). Since Invadopodia, a matrix degrading device itself is not forming; therefore even if abundant MT1-MMP is available at the surface it is not able to perform collagenolytic activity. Therefore, we have now modified our conclusion and discussion section. We conclude that reduced gelatin degradation activity is due to reduced invadopodia formation rather than surface clearance. In the revised manuscript we have now removed it from our conclusion and discussion.

4) Point 4. Increased cttn phosphorylation in STX7 KO cells (Figure 3H) is unexpected as it correlates with a reduction in matrix degradation. A possible explanation is that tyrosine phosphorylated cttn (Tyr-482) can be targeted to degradation. As phosphorylation on Ser/Thr and on Tyr residues is known to affect cttn activity in complex and sometime opposite manner depending on which residues get modified, the authors should provide specific information regarding the antibodies that they used to detect phospho-cttn in the experiment reported in Figure 3H.
Response: Antibody used for detecting phospho-cortactin in the experiment is α-mouse Phospho-tyrosine (Millipore, 05-1050). The phospho-antibody used in the study does not recognise specific phosphorylated residue of Cortactin, therefore, we could not provide specific information regarding it.

5) Point 5. STX7 is shown to interact strongly with VAMP2 in Fig. 4. The authors should address or at least comment on a potential role of VAMP2 in matrix degradation and invadopodia function.
Response: We are thankful to the reviewer for raising this query. We have now performed the experiment showing the potential role of VAMP2 depletion on gelatin degradation. It was observed that there was 57% reduction in gelatin degradation activity upon VAMP2 depletion (Fig. 6A). Further we would like mention that in Fig. 6D (previously Fig. 4H) we have shown that VAMP2 depletion led to reduced invadopodia formation and MT1-MMP trafficking to invadopodia demonstrating the invadopodia function. The result is now added in the revised manuscript (Fig. 6A).

Minor issue. The legend of Fig. 3C should indicate more clearly that the observed signal corresponds to internalized anti-MT1-MP antibodies.
Response: We are thankful to the reviewer to pointing this out. As suggested by reviewer we have now modified the legend of Fig. 3C. (Please refer to page no. 32 and line no. 1031-1032 of manuscript for figure legend 3C).

Reviewer 3 Advance summary and potential significance to field
The manuscript by Parveen et al. reports on the role of Syntaxin7 in invasion of breast cancer cells by contributing to the delivery of necessary cargo to the invadopodia. They identify a number of the SNARE proteins of the endocytic and recycling pathways with potential roles in invadopodia-mediated breast cancer cell invasion by a SMART pool siRNA based reverse genetics approach. The authors show that the STX7 as an important player in MDA-MB-231 cell invasion and co-traffics with MT1-MMP to invadopodia with microscopic and biochemical studies.

We are just beginning to understand the significant role of endolysosomal trafficking in cancer progression, which is why the topic of this study is very timely. The article is in line with the previous work from the same lab showing the role and importance of endosomal recycling in trafficking of MT1-MMP to invadopodia, and thereby promoting breast cancer metastasis (Sharma, Priyanka, et al. Journal of Cell Biology 219.1 (2020)). It is a well-designed, executed and documented work.
Summary: We are highly thankful to the reviewer for finding our study significant and timely, which would be beneficial for the field studying the role of endolysosomal trafficking in cancer progression. As per the reviewers suggestion we have now performed the experiments and edited the manuscript, wherever required.

Reviewer 3 Comments for the author
I have only some minor Comments for the authors.

1) -Rename GFPSTX7 to GFP-STX7, inserting a dash to the name, it is quite difficult to follow STX in the text.
Response: We apologise for the inconvenience caused to the reviewer and sincerely thank the reviewer for pointing out this. We have now renamed the GFPSTX7 to GFP-STX7 throughout the manuscript.

2) -Figure 2D, shows 9.0±1.5% of vesicles positive for STX7 are positive for MT1-MMP and EEA1. Did the authors assess other (endolysosomal) markers for co-localization with STX7?
Response: We are thankful to the reviewer for bringing our attention towards it. As pointed out by the reviewer, we have now conducted new experiments and analysed the data. We have observed that 30.0±3.0% of STX7 vesicles are positive for MT1-MMP and EEA1, while only 16.8±1.9% of STX7 vesicles are positive for MT1-MMP and CD63 (Fig. 2D). The result is now added in the revised manuscript (Fig. 2D).

3) -In reported image analysis results, e.g. Figure 3C, it is not stated how many cells are analysed. Please explicitly state the sample volumes for image analysis.
Response: Each experiment was performed minimum in three biological replicates. The detailed description of the number of cells (n) and number of individual experiments (N) used for calculations is provided in the respective figure legends where N=3, n ≥ 300. (Please ref to page no 32 and line no 1031 of figure legend 3C).

4) -Figure 4D, the image chosen for VAMP3 has 2 adjacent cells, and the signal is concentrated on the cell-cell contact site. Can the authors comment on how representative this is?
Response: We are thankful to the reviewer for drawing our attention towards it. We would like to clarify that often, MDA-MB-231 cells were found to be multinucleated under fixed cell imaging apart from population that has single nucleus. The representative image in Fig. 5D (Previously Fig. 4D) is a solitary cell only with two nuclei and not two adjacent cells. The VAMP3 and STX7 were observed to be accumulated in between the nuclei; however, it is not the case in every cell.

5) -The authors comment on the hampered trafficking of signaling molecules or growth factors or an unknown cargo carried by STX7. Did they test this, at least trafficking of some well-characterized growth factor receptors, e.g. EGFR?
That would be a nice addition to the study.
Response: We are thankful to the reviewer for this query that served as nice addition to the study. We did surface biotinylation to measure EGFR level and we didn't see significant difference in the surface level. We have quantified the blot and the result is now added in the revised manuscript (Fig. 3B).

6) -The authors suggests STX4 acts upstream of STX7 in trafficking of MT1-MMP, however this is not reflected in the schematic model. I would suggest to expand the schematic to recapitulate this, and also include other previously reported endolysosomal proteins with a function in MT1-MMP trafficking.
Response: We appreciate the suggestion made by the reviewer. However, we would like to mention that our model is a phenotype-driven model wherein we have tried to capture invadopodia-associated function under different conditions, such as depletion of some SNAREs. Based on the observed phenotypes, we suggested that STX4 may work upstream of STX7; however, due to lack of

clarity in terms of definition of specific endosomal compartments in MDA-MB-231 cells and due to inherent limitation of our study itself, we avoided attributing SNARES to any specific compartments. We propose to modify the discussion section to avoid inclusion of any hierarchy in terms of the functions of the SNAREs without diminishing the uniqueness shown by STX7 compared to STX4/VAMP2 or VAMP3.
We also agree that the inclusion of the endo-lysosomal proteins that are involved in MT1-MMP trafficking will be very much informative. This will add quite a few members including, Exocyst and WASH complex (Monteiro et al., 2013), SNX27- Retromer (Sharma et al., 2020), RabGTPases (Bravo-Cordero et al., 2007; Linder, 2015; Kajiho et al., 2016) and CLIC3 (Macpherson et al., 2014) to our model. However, our current model is solely based on the observations we made in the current study involving STX4, VAMP2, and VAMP3 in addition to STX7. In fact, all reported SNAREs have not been included in the model (Miyagawa et al., 2019; West et al., 2021). We would like to refrain from adding any additional molecules to our current model to avoid unnecessary complexity (Please refer to page no. 8 and line no. 247-251 of main manuscript).

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Second decision letter

MS ID#: JOCES/2021/259576

MS TITLE: Syntaxin7 contributes to breast cancer cell invasion by regulating invadopodia associated activity

AUTHORS: Sameena Parveen, Amrita Khamari, Jyothikamala R, Marc G Coppolino, and Sunando Datta
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers are satisfied with the revisions you have made. They suggest you write a more specific title and that you should plot all cells in the indicated figures. I agree with both points. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have improved their manuscript with new experiments and textual revisions that have strengthened the study. Significant new data have been added that address my concerns about quantitation. The authors have responded satifactorily to all my comments, I thus recommend publication of the manuscript.

Comments for the author

The authors have improved their manuscript with new experiments and textual revisions that have strengthened the study. Significant new data have been added that address my concerns about quantitation. The authors have responded satifactorily to all my comments, I thus recommend publication of the manuscript.

Reviewer 2

Advance summary and potential significance to field

In general, the revised manuscript addressed all points to my satisfaction, and I would therefore recommend publication at this stage.

Comments for the author

I would recommend that the author rephrase the title of the manuscript, which is vague and does not convey sufficient useful information regarding the main message of their study.

Reviewer 3

Advance summary and potential significance to field

I thank the authors for having sufficiently addressed the comments raised by this reviewer. The paper is a useful and interesting contribution to understanding the recruitment of MT1-MMP to invadopodia during cellular invasion.

Comments for the author

As a final note, I recommend replotting some experiments (e.g. Figure 2d, 3d 5d)to improve data visibility. I suggest the authors to plot for each cell, rather than only the average within three experimental replicates. Currently it is not clear how representative and accurate the final averages are, in particular for the co-localization analysis.

Second revision

Author response to reviewers' comments

Response to the reviewers'

Author's response to editor and reviewers' comments
We are glad that the editor and the reviewers shared the enthusiasm to consider the study for publication following minor revision. We have now revised the manuscript by addressing all the reviewers' minor comments.

Reviewer 1 Advance summary and potential significance to field
The authors have improved their manuscript with new experiments and textual revisions that have strengthened the study. Significant new data have been added that address my concerns about quantitation. The authors have responded satisfactorily to all my comments, I thus recommend publication of the manuscript.

Reviewer 1 Comments for the author
The authors have improved their manuscript with new experiments and textual revisions that have strengthened the study. Significant new data have been added that address my concerns about quantitation. The authors have responded satifactorily to all my comments, I thus recommend publication of the manuscript.
Response: We are thankful to the reviewer for finding the response satisfactory and recommend the study for publication. We sincerely thank the reviewer for the critical comments throughout the revision process that improvised the study.

Reviewer 2 Advance summary and potential significance to field
In general, the revised manuscript addressed all points to my satisfaction, and I would therefore recommend publication at this stage.

Reviewer 2 Comments for the author
I would recommend that the author rephrase the title of the manuscript, which is vague and does not convey sufficient useful information regarding the main message of their study.
Response: We are happy to note that the reviewer has found the responses satisfactory and considered the study for publication. We are thankful to the reviewer for the critical comments. We have now rephrased the title of the manuscript from "Syntaxin7 contributes to breast cancer cell invasion by regulating invadopodia associated activity" to "Syntaxin7 contributes to breast cancer cell invasion by promoting invadopodia formation".Please refer to page number 1 and line number 1-2 of the revised manuscript. We believe that the rephrased title will carry the main message of the study.

Reviewer 3 Advance summary and potential significance to field
I thank the authors for having sufficiently addressed the comments raised by this reviewer. The paper is a useful and interesting contribution to understanding the recruitment of MT1-MMP to invadopodia during cellular invasion.

Reviewer 3 Comments for the author
As a final note, I recommend replotting some experiments (e.g. Figure 2d, 3d, 5d)to improve data visibility. I suggest the authors to plot for each cell, rather than only the average within three experimental replicates. Currently it is not clear how representative and accurate the final averages are, in particular for the co-localization analysis.
Response: We are thankful to the reviewer for finding this study useful and an interesting contribution to the field. As suggested by the reviewer, we have now re-plotted the data from the experiments; Main figures 2, 4, 5; supplementary figures S1, S2 ,S4 and accordingly revised the associated figure legend. The data are displayed using SuperPlots where each biological replicate is distinctly color-coded and each dot represents percentage colocalization in a field of view (frame). One frame is comprised of 1-3 cells, contributing to a total number of cells (n) across the total number of frames acquired. The frame-wise values were separately pooled for each biological

replicate to calculate the mean in the SuperPlots and the standard deviation represents the deviation of the data from these three means (Lord et al., 2020) . Please refer to page number 20 and line number 633-640.

Please refer to the following page number and line number for the modified figure legends in the main text
For Figure 2: Page number-31-32; Line number-1015-1018
For Figure 4: Page number-33-34; Line number-1084-1087
For Figure 5: Page number-34; Line number-1106-1109

Please refer to the following page number and line number for the modified figure legends in the supplementary file
For Figure S1: Page number-1; Line number-25-28
For Figure S2: Page number-2; Line number-57-60
For Figure S4: Page number-4; Line number-117-120.

We would further like to add that the data for figure 3D is from a biochemical experiment i.e. biotinylation to show that STX7 depletion doesn't perturb endocytosis. Each data point shown in the figure represents an individual biological replicate comprised of an average $3*10^5$ cells. Hence, We keep this figure unaltered.

Reference:
Lord, S. J. et al. (2020) 'SuperPlots: Communicating reproducibility and variability in cell biology', Journal of Cell Biology, 219(6). doi: 10.1083/JCB.202001064.

Third decision letter

MS ID#: JOCES/2021/259576

MS TITLE: Syntaxin7 contributes to breast cancer cell invasion by promoting invadopodia formation

AUTHORS: Sameena Parveen, Amrita Khamari, Jyothikamala R, Marc G Coppolino, and Sunando Datta
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 2

Advance summary and potential significance to field

Recommendation is: to be published

Comments for the author

Recommendation is: to be published

Reviewer 3

Advance summary and potential significance to field

The authors have addressed all the raised points and suggestions in the revised manuscript , and I would therefore recommend publication in the current form.

Comments for the author

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