SNX27-retromer assembly recycles MT1-MMP to invadopodia and promotes breast cancer metastasis

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Re: JCB manuscript #201812098

Dr. Sunando Datta
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Dear Dr. Datta,

Thank you for submitting your manuscript entitled "SNX27-retromer assembly directs MT1-MMP trafficking to invadopodia and promotes breast cancer metastasis" to Journal of Cell Biology. The manuscript has now been assessed by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that while the reviewers find a potential link between SNX27 and MT1-MMP interesting, they have significant concerns that your model for SNX27 association with MT1-MMP is not currently sufficiently supported by the data. In addition to the need for further experimental proof for your model, such as a direct biochemical link between SNX27 and MT1-MMP, the reviewers have brought up serious concerns regarding the quality and presentation of your current data set, as well as a lack of novelty for the first half of your paper. I hope the reviewer suggestions to refocus your manuscript in addition to their experimental suggestions will be useful in improving both the data and presentation of your manuscript. Unfortunately I do not have the level of reviewer support that I would need to proceed further with the paper. I do realize that significant further work and expansion might convincingly address some of these issues, but I am hesitant to encourage you to work towards the aim of further consideration at JCB. The level of reviewer criticism makes it impossible for me to guarantee that we will be able to invite resubmission, even after revision. Therefore, it does seem that it will be best for you to consider another journal for this work. Our journal office will transfer your reviewer comments to another journal upon request.

I am sorry our decision is not more positive, but hope that you find the reviews constructive. Of course, this decision does not imply any lack of interest in your work and we look forward to future submissions from your lab.

Thank you for your interest in Journal of Cell Biology.

Sincerely,

Johanna Ivaska
Monitoring Editor

Andrea L. Marat
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In the present study, Sharma et al. identify an interaction between the SNX27-retromer supercomplex and the cell surface protease MT1-MMP. They demonstrate that SNX27 and retromer engage MT1-MMP (but not MT2-MMP) on discrete endosomal subdomains where this complex promotes the recycling of the protease to the cell surface. Depletion of SNX27 and/or retromer resulted in enhanced lysosomal turnover and in a loss of the protease from the cell surface. This loss of MT1-MMP also led to a reduction in matrix degrading capacity of the breast cancer cell line that was used for the study. The authors further demonstrate that SNX27 promotes metastasis in an in vivo model.

In my opinion, the study is of adequate quality and represents a significant scientific advance. While it is not really novel that SNX27 engages retromer and cargo to recycle cell surface cargo, the authors put a lot of effort into the understanding of the functional consequences of the loss of MT1-MMP recycling. SNX27 and retromer have not been convincingly linked to breast cancer and metastasis before, which makes this study a potentially important one for the field.

Overall, I am tentatively supportive of publication but I think that the authors need to improve several parts of their study to warrant acceptance at a high quality journal like JCB. Most importantly, the authors need to further investigate the biochemical link between SNX27 and MT1-MMP. It looks like the last three amino acids of MT1-MMP may be some kind of atypical PDZ ligand. The authors could purify the PDZ (and maybe also the FERM-like domain) domain as well as the cytosolic tail from bacteria and test the for direct interaction. If this does not bind, MT1-MMP would need to engage the core retromer trimer via its last three amino acids, which would be a real novelty and should be demonstrated more thoroughly. In addition, I have listed several points below that should be addressed before publication. Some of the problems/weaknesses that I spotted leave me somewhat worried about this study as they may be due to some "sloppiness" in the presentation and interpretation of data (Figure S3E, for example).

Major points:

1. Figure 2A: It is really surprising to see that knockdown of all these different endosomal complexes results in a complete and nearly identical loss of gelatin degradation. It would be great to have a negative control here, as the uniformity of the results leaves me slightly worried about this experiment. Alternatively, the authors could also perform a rescue experiment for at least one of these targets. As it is, it remains unclear whether the results for SNX27-retromer depletion are specific or whether ANY perturbation of the endocytic network leads to a loss of gelatin degradation.

2. Figure 3A: Why are the entire VPS26 and VPS35 KD cells grey, whereas the control cell is black with discrete and punctate patterns of pHluorin signal? How did the authors quantify this signal in the presence of so much (and bright!) grey background? Is it really only background or is it highly dispersed MT1-MMP?

3. Figure 3C and 5C: The MT1-MMP blots comparing scrambled and VPS26 KD conditions appear to be taken from separate gels. This is not best practice, the authors should run these assays on

the same gel to really be able to compare overall levels and recycling rates. There are only 10 lanes in total, which easily fits onto one gel and one membrane. As it is, it looks like knockdown of VPS26 boosts the levels of cell surface MT1-MMP (Figure 3C), which would be odd if there is less recycling. Also, I do not really see any difference in the band intensities between conditions in Fig. 5C, which makes the quantification a bit unconvincing. These assays should be repeated and loaded onto the same gel to strengthen the conclusions. It would also be good to show all blots that were used for the quantification in a supplementary Figure as the differences (especially Figure 5C) look really minor to this reviewer.

4. As mentioned above, the interaction between SNX27-retromer and the MT1-MMP tail needs to be mapped and analyzed more thoroughly, especially since knockdown of several unrelated complexes (see point 1) also reduced matrix degradation. A solid analysis of the interaction would really strengthen the claim that SNX27-retromer engage MT1-MMP via the DKV motif to promote recycling. I propose to use GST-MT1-MMP tail (and tail without the DKV motif) and recombinant SNX27 domains to test for a direct interaction. The fact that the retromer binding mutant SNX27 lost binding to MT1-MMP does not necessarily mean that MT1-MMP binds to the core retromer. Gallon et al. (2014) have shown that binding of SNX27 to VPS26 greatly enhances PDZ binding activity so that this mutant likely has far lower PDZ ligand affinity.

Figure 6E: The cropping of the blots is excessive and the result looks messy and is therefore unconvincing. The authors could try to improve this and maybe also repeat these IPs to obtain more convincing data.

Figure S3E: This blot is confusing. The input lanes for WASH1 indicate normal levels in VPS26 KD cells but the pellet and supernatant display much lower levels of WASH1. This makes no sense and the data is therefore inconclusive. I do not understand why the authors ignored such a striking loss of overall WASH1 without thinking about potential technical problems of this assay.

Minor points:
1. In the introduction, bottom page two, the authors state that retromer recycles an abundance of cargo to the TGN. They then cite the recycling of GLUT1 as an example for this. It should be noted that the majority of retromer based recycling is recycling from endosomes to the plasma membrane, and the authors should rewrite these sentences to make clear that GLUT1 is not recycled to the TGN.

2. Figure 2C: The authors have inserted very small numbers into the actin blot. I couldn't find a description of these numbers in the figure legend or in the text but I think they may be a ratio between MT1-MMP and actin. This should be clearly annotated, described in the legend and the numbers should be placed below the actin panel for better visibility.

General (minor comment): The authors often omitted necessary articles before nouns. Thus, the study would benefit from some minor editing by a native speaker.

Reviewer #2 (Comments to the Authors (Required)):

Sharma et al., "The SNX27-retromer assembly directs MT1-MMP trafficking to invadopodia and promotes breast cancer metastasis"

The study by Sharma et al. has investigated the trafficking of the matrix metaloprotease MT1-MMP and determined a role for SNX27 with retromer in localizing the MT1-MMP protein to the cell surface where its enzymatic action can promote invasion of surrounding tissue by metastatic cancer cells.

It is now well established that SNX27 with retromer and the WASH complex plays a key role in directing proteins from endosomes to the cell surface. There are numerous publications that have demonstrated this with various cargo molecules. It has also been established that MT1-MMP is important in cancer cell metastasis and elevated levels of MT1-MMP have been linked with poor prognosis for some cancers. Thus, much of the first part of the manuscript is lacking in novelty. It has also been shown that the WASH complex is important in transporting integrins to the cell surface and thereby promoting invasion by cancer cells (Zech et al., 2012) but this data is overlooked in the study by Sharma et al.

The most interesting aspect of this study by Sharma et al. is the finding that SNX27 can associate with MT1-MMP to direct its trafficking but does not appear to be important for MT2-MMP trafficking. These findings which are reported in the second half of the study by Sharma et al. could potentially form the basis for a much shorter but more clearly focussed manuscript detailing the selectivity of SNX27 for MT1-MMP over MT2-MMP.

Overall I found reading the manuscript to be somewhat frustrating as there are a great many mistakes and inconsistencies that result in a manuscript that appears to have been hurriedly assembled with insufficient time spent proof reading the paper prior to submission. There are also some significant deficiencies regarding the experimental approach relating to the use of siRNA knockdowns. Much is made of the fact that OnTarget Plus reagents were used to minimize off-target effects but these reagents are not infallible and can suffer from off-target effects similarly to other siRNA knockdown reagents. In order to rule out the possibility of off-target effects, the authors should use deconvolved (i.e. individual rather than pooled) siRNAs and/or rescue experiments to show that phenotypes observed are specific.

Set out below are some of the problems/issues I found whilst reading the manuscript - this is unlikely to be the entirety of the problems however.

1. In the summary, what does "monitor" mean? How exactly does SNX27 "monitor" the transport of MT-MMP1 to the cell surface? The use of the word "monitor" here is vague and uninformative.
2. Throughout the manuscript, there are inconsistencies in the text relating to the position of citations - sometimes a space to the text, sometimes not. There are also inconsistencies when describing GFP-tagged proteins - sometimes hyphenated, sometimes not. These issues may seem small but they create an impression of sloppiness.
3. The citations employed are often wrong or incomplete. For example, when describing the interaction of the WASH complex with retromer, the authors choose to cite the studies by Derivery et al., (2009) and McGough et al., (2014). The first report of the WASH complex was indeed the study by Derivery et al., but the first report of the interaction between retromer and the WASH complex was Harbour et al., 2010 (PMID: 20923837). Other studies detailed the interaction between retromer and the WASH complex including; Harbour et al., 2012; Jia et al., 2012 and Helfer et al., 2013. In the introduction, the Chen and Ritter citation lacks a capital letter for Chen. When discussing the role of the retromer cargo-selective complex, the authors should also now cite the recent study from Teasdale and colleagues recently published in JCB. Overall, the introduction is not a very good overview of the literature on retromer.
4. The authors need to specify which Vps26 protein is targeted in the siRNA experiments, Vps26a or Vps26b?
5. In determining the cell surface levels of MT1-MMP, could the authors employ a FACS-based analysis?

6. The role of VAMP7 in regulating MT1-MMP localization may be via retromer and VARP. The VARP protein associates with both VAMP7 and retromer and requires retromer for its membrane association. This possibility does not seem to have been considered by the authors.
7. In figure 3, the authors report that loss of retromer results in the WASH complex being displaced from the endosomal membrane. This observation is a glaring example of the lack of novelty in the first half of this manuscript. It has been reported in many independent studies that retromer is required for the endosomal localization of the WASH complex through Fam21 binding to Vps35 (see Harbour et al., 2010; Harbour et al., 2012; Jia et al., 2012; Helfer et al., 2013; Zavodszky et al., 2014; McGough et al., 2014).
8. Similarly, the authors report that SNX3 knockdown affects MT1-MMP trafficking and retromer localization. The role of SNX3 in promoting the recruitment of retromer to endosomes has been established previously - see Harterink et al., 2011; Vardarajan et al., 2012. This is another example of the lack of novelty.
9. In the methods section it was easy to find mistakes. For example, NaCl is shown as Nacl in the Co-immunoprecipitation paragraph. The penultimate sentence of the paragraph describing the Degradation experiment describes adding SDS but does not give an amount. These are just two examples of mistakes I found, I suspect that there are many more.
10. The figures are generally quite poorly presented and the figure legends lack necessary information relating to the experiment. For example, the arrows on figure 1A are not consistently and equally positioned three images with arrows. The figure legend makes no mention of the inset boxes and what is shown there. In figure 2B, there is a mistake, "TGFBRAP1 KD KD". In figure 3E, the spots inside the inset boxes are actually smaller than the corresponding spots on the actual image - something has clearly gone wrong with how that figure was produced. Figure 6 has some potentially useful data but is quite poorly presented (what are the marks on the blots shown in figure 6D? Why don't the panels line up?) and the images of some of the blots appear to be overexposed. Overall, the quality of the figures falls below the standard usual for JCB.

Reviewer #3 (Comments to the Authors (Required)):

The manuscript describes novel findings related to the regulation of MT1-MMP by retromer-SNX27 assembly and endosomal recycling pathway. In addition, association with invadosomes and function of less studied MT2-MMP is postulated in Matrigel invasion and degradative activities of fibronectin. Many of the presented experimental results are potentially important for cancer cell biology, and extensive collection of interesting data is presented. However, the manuscript is very diverse, and should focus to provide more convincing evidence and careful considerations of selected key questions and conclusions. The expression and possible functions of MT2-MMP would require a more thorough examination for solid conclusions. The results on MT1-MMP trafficking as well as SNX27 role in cancer metastasis in vivo, and the related conclusions also appear partially confusing and should be more clearly presented. Therefore, in the opinion of this reviewer, the study as presented is too speculative and premature for publication. Below listed are specific comments:

1. The link to previous literature and current knowledge could be more exact and up to date. Example concerns in the first paragraph of Introduction; a) basement membrane invasion is presented as the sole means of ECM degradation and metastasis, although the biologically well characterized MT1-MMP activity with relevance to metastasis is against interstitial collagen, in addition to basement membrane b) the references for other MT-MMPs in general are old, and c) previous MT2-MMP studies with mainly ectopic overexpression are referred to create a link to breast cancer, basement membrane invasion and metastasis.

2. The authors state that (citation from last Introduction paragraph) "we demonstrated that MT2-MMP the other abundant protease associates with invadosomes and facilitate breast cancer cell invasion by preferentially degrading fibronectin."

It is not clear however, where the protease is abundant, since the authors fail to detect endogenous MT2-MMP protein, and only provide (in Fig. S1A) Real-time PCR results of the relative MT2-MMP mRNA levels in MCF-7 and MDA-MB-231 cells, normalized to the expression in MCF-10A cells. This all could still be marginal. Stronger expression data in breast cancer cells would be required to start considering the possible relevance of MT2-MMP activities in breast cancer, and regulation of it, for which a set of results are provided in this study regarding overexpression constructs.

Despite the conclusion, it also was not addressed, if MT2-MMP in MDA-MD-231 cells directly cleaved fibronectin, or which ECM proteins would MT2-MMP preferentially affect or degrade, since only gelatin and fibronectin were tested in the cell-based assay. In the used ECM degradation experiments, the effect could as well be indirect through, for example, affecting some of the fibronectin-associated proteins, or of the many another fibronectin-degrading proteases (or their inhibitors) expressed in these cells.

Further, the fibronectin degradation result was directly related to the result of Matrigel invasion assay, although Matrigel is a complex mixture of laminin, collagens and other proteins, and the relevance of possible fibronectin, laminin, collagen etc degradation in Matrigel invasion was not addressed.

3. Since the other presented MT2-MMP results are with overexpression, and largely negative findings compared to the MT1-MMP regulation and gelatin degradation, should this main study stand even better with MT1-MMP alone? Although interesting, the presented MT2-MMP results should deserve focused experimentation and much more careful considerations. Considering thes preliminary results, the localization and recycling of MT2-MMP could be better analyzed using fibronectin matrix as a cell adhesion substrate.

4. For conclusions of MT2-MMP in Figure 1, not only better expression data, but also comparable/more complete ECM degradation results should be provided. Laminin would be relevant addition to link the results to presented invasion results, as it is abundant in Matrigel.

Cannot find results for MT2-MMP KD in gelatin degradation, in spite of following statement in Results: "On the contrary, MT2-MMP depleted cells could degrade gelatin comparable to scrambled control cells, but depletion of MT1-MMP abolished gelatin degradation activity."

Figure S1B shows qualitative results (a single cell image) for gelatin degradation with MT1-MMP KD cells +/- GFP-MT2-MMP. The text concludes: "Interestingly, MT2 could completely rescue the loss of gelatin degradation activity in MT1-MMP KD cells"; for this the scramble/control and quantitative data is required.

5. For statistical analysis n (and N) numbers are mentioned for some but not all of the results in Figure legends. See e.g. Figure 1A', B, C'

6. Figure 1E: Only minority of MT1-MMP is detected in the selected assessed vesicular compartments in MCF10A and MCF7. Where is it localized in these cells?

7. Figure 2A: It is difficult to appreciate the gelatin degradation in the provided merged images. The red channel alone would be helpful. From current images, Vsp39 KD seems almost comparable with control. -In Figure S2A, mRNA KD results are only provided to 5/6 proteins in Figure 2A

8. Figure 2C: Quantitative data should be collected from multiple replicated experiments, and not from a single western blot. As provided in this and several other figures of this manuscript, the numbers are also too small to easily read the data.

9. Figure 2D: Is cortactin staining sufficient to conclude "Notably, we couldn't find any significant difference in the number of cells forming invadopodia upon knocking down the subunits of the MSCs (Fig. 2D, 2D')"? Atleast a more exact measure could be used to label the Y-axis in 2D', and to describe the results. This comment extends to many other results, and applies throughout the manuscript, where authors conclusions rather that the exact measured data seem to be presented as results.

10. Figure 3: Comparative detection of the decrease in total cell surface-labelled protein could allow conclusions between speculated effects of lysosomal degradation and defective recycling on the results.

11. Figure 5A and 6 for example: The western images should be cut further away from the protein bands, to show clearer results.

12. The conclusions from the in vivo xenograft experiment are highly speculative, and should be limited to those supported by the data. Additional time-point or real-time analysis would be required to solid conclusions about delayed metastasis.

13. Last Results paragraph: The data appear to be missing for the whole tail vein injection (also missing from the methods). As described, the results are more confusing than convincing for the drawn conclusions about the invasion and metastatic activities affected by SNX27. Solid data combining analysis of in vivo primary xenograft invasion and tail vein exit to lung tissue, for example, could be helpful, in addition to more relevant in vitro invasion assays.

We greatly appreciate the editors' and the reviewers' critical comments and insightful suggestions. We are thankful that they found a potential link in our study, highlighting SNX27 mediated MT1-MMP trafficking in breast cancer metastasis. Keeping this very essence, we have now revised the manuscript by addressing all the comments of the referees. We carried out several new experiments, extensively edited the document to keep the study more focused, and added appropriate controls to support our conclusions, wherever required.

We have now carried out several biochemical and biophysical assays to unravel the underlying mechanism behind retromer-SNX27 association with MT1-MMP. All the immunoprecipitation reaction are repeated using GFP binding protein instead of the anti-GFP antibody, to claim interactions observed in vivo strongly. As evident from Fig. 6 B, this approach provided better quality immunoblots. Molecular basis of these interactions was studied extensively, by carrying out GST pull down using the recombinant proteins followed up by isothermal titration calorimetry, where the associated thermodynamic parameters were analyzed (Fig. 6 D, D' and Table 1).
To rule out the off-target effect and provide better validation for the phenotypes reported in this manuscript, we have performed either rescue experiments (Fig. 4 B and Fig. 5 C) or carried out multiple individual-oligo based siRNA silencing (Fig. S2 B, C, D). We have provided either RNA based or protein based data to validate knock-down efficiency. To support the conclusions, better representative images are added with the detailed legends and appropriate axes labels. Also, we have added data from animal experiments, and the results section is edited as suggested by the reviewers.

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

Reviewer #1 (Comments to the Authors (Required)):

In the present study, Sharma et al. identify an interaction between the SNX27-retromer supercomplex and the cell surface protease MT1-MMP. They demonstrate that SNX27 and retromer engage MT1-MMP (but not MT2-MMP) on discrete endosomal subdomains where this complex promotes the recycling of the protease to the cell surface. Depletion of SNX27 and/or retromer resulted in enhanced lysosomal turnover and in a loss of the protease from the cell surface. This loss of MT1-MMP also led to a reduction in matrix degrading capacity of the breast cancer cell line that was used for the study. The authors further demonstrate that SNX27 promotes metastasis in an in vivo model.

In my opinion, the study is of adequate quality and represents a significant scientific advance. While it is not really novel that SNX27 engages retromer and cargo to recycle cell surface cargo, the authors put a lot of effort into the understanding of the functional consequences of the loss of MT1-MMP recycling. SNX27 and retromer have not been convincingly linked to breast cancer and metastasis before, which makes this study a potentially important one for the field.

Overall, I am tentatively supportive of publication but I think that the authors need to improve several parts of their study to warrant acceptance at a high quality journal like JCB. Most

importantly, the authors need to further investigate the biochemical link between SNX27 and MT1-MMP. It looks like the last three amino acids of MT1-MMP may be some kind of atypical PDZ ligand. The authors could purify the PDZ (and maybe also the FERM-like domain) domain as well as the cytosolic tail from bacteria and test the for direct interaction. If this does not bind, MT1-MMP would need to engage the core retromer trimer via its last three amino acids, which would be a real novelty and should be demonstrated more thoroughly. In addition, I have listed several points below that should be addressed before publication. Some of the problems/weaknesses that I spotted leave me somewhat worried about this study as they may be due to some "sloppiness" in the presentation and interpretation of data (Figure S3E, for example).

Summary: We appreciate that the reviewer has found this study, linking SNX27-retromer assembly and breast cancer metastasis, potentially important for the field. We thank the reviewer for his/her insightful suggestions that have not only improved the data quality but also has substantially added to our current understanding of the molecular basis of retromer-SNX27 mediated recycling of MT1-MMP. We have addressed below each of the issue raised and edited the manuscript following his/her suggestion.

Major points:

1. Figure 2A: It is really surprising to see that knockdown of all these different endosomal complexes results in a complete and nearly identical loss of gelatin degradation. It would be great to have a negative control here, as the uniformity of the results leaves me slightly worried about this experiment. Alternatively, the authors could also perform a rescue experiment for at least one of these targets. As it is, it remains unclear whether the results for SNX27-retromer depletion are specific or whether ANY perturbation of the endocytic network leads to a loss of gelatin degradation.

Response: We understand the reviewer’s concern for the sensitivity of the assay and has provided the additional controls. However, to make this study more focused now, we have addressed and elaborately studied the role of retromer and its associated sorting nexin, SNX27 in MT1-MMP recycling and breast cancer invasion.

Gelatin degradation and Matrigel invasion are the well-established classical assays for estimating the invasive potential of the metastatic cancer cell lines in vitro (Artym et al., 2006; Kleinman and Jacob, 2001; Donnelly et al., 2017; Qiang et al., 2019; MacDonald et al., 2018; Planchon et al., 2018). We want to add that the gelatin degradation assay is sensitive enough to discriminate the knockdown of Rab isotypes and their regulators. We could see that the cells depleted for individual Rab5A isoform could still degrade gelatin. On the contrary, knockdown of RABGEF1, (Guanine nucleotide exchange factor GEF for Rab5A/B/C) which activates all the three isotypes of Rab5, impaired the matrix degradation activity. We have now added the data in supporting material (Fig. S1 A).

However, we wanted to clarify that matrix degradation is the outcome of the complex series of events. A plethora of molecules engaged in diverse activities, i.e., cell adhesion, signaling,

mechanosensing, proteases secretion, etc. are involved (Murphy and Courtneidge, 2011; Linder and Scita, 2015). The signaling cues activate receptors on the cell surface that leads to the recruitment of these molecules to invadopodia. Intracellular trafficking exclusively governs this spatiotemporal delivery of the cargoes, crucial for matrix degradation. We disrupted CORVET, HOPS, TSG101 complexes in addition to retromer. Except for retromer, which is highly cargo-specific, the other multisubunit complexes are involved in endosomal tethering and fusion events, eventually leading to their maturation (Rink et al., 2005; Perini et al., 2014; Doyotte, 2005). These endosomes act as carriers and reservoirs for various soluble and transmembrane proteins. Thus disruption of their maturation will profoundly interfere with the fate of the cargoes present inside. This is in line with the prior argument where disruption of any of these multisubunit complexes is likely to perturb matrix degradation activity. Also, we downregulated retromer associated sorting nexins and found that cells with reduced expression of SNX1/2 (co-depletion) or SNX5/6 (co-depletion), known for Golgi retrieval of the cargo, could degrade gelatin similar to control cells (Fig. 3 A). On the contrary, SNX3 and SNX27 perturbation impaired matrix degradation activity. These SNXs are known to mediate cell surface transport of various transmembrane proteins. Hence, we hypothesized that these SNXs might be crucial for recycling invadopodia associated cargoes and are possibly required for its ECM degrading activity.

Additionally, we have now incorporated STX8 (Syntaxin8) as a negative control. STX8 is a member of the SNARE family (soluble N-ethylmaleimide sensitive factor attachment protein receptor), which are key regulators of membrane fusion thus mediate membrane trafficking and sorting events (Chen and Scheller, 2001; Jahn and Scheller, 2006; Risselada and Grubmüller, 2012). STX8 is reported to mediate homotypic (Antonin, 2002) and heterotypic fusion of late endosomes (Pryor et al., 2004). We checked the expression and siRNA mediated knockdown efficiency of STX8 in MDA-MB-231 cell line (Fig. 1 A). STX8 depleted cells could degrade gelatin and invade Matrigel similar to control cells (Fig. 1 B, C). Thus, suggesting that STX8 might not be involved in intracellular trafficking of cargoes which are required for invadosome mediated ECM degradation.

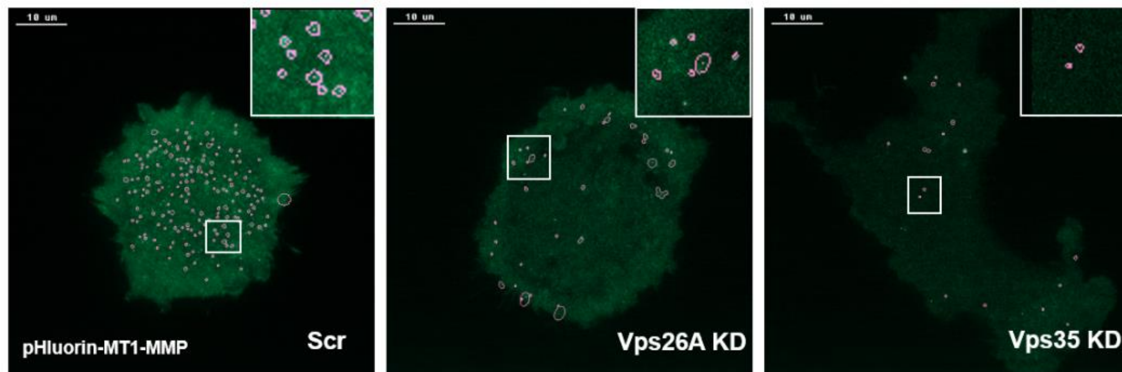
We agree with the reviewer that rescue is the best approach to authenticate gene specific phenotype. We had already carried out rescue experiments for SNX27 (please refer Fig. 6 A in the old figure). To highlight this, now a separate panel is added and mentioned in the text as well (Fig. 4 B) where the overexpression of the wild type SNX27 rescued the gelatin degradation activity in SNX27 depleted cells. In the next figure, quantification is provided for the same where rescue for SNX27WT and its domains is measured (Fig. 5 C). The individual domains showed varied rescue reflecting their relative involvement in the process.
However, we believe that performing rescue experiment for retromer is not the best option since it is a heterotrimeric complex that stays in 1:1:1 stoichiometry (Collins et al., 2005; Hierro et al., 2007). Its proper physiological functions will depend on the stoichiometry. Rescuing by overexpression of a single subunit would be challenging since this might act as a dominant negative by competing with the endogenous trimer complex. Alternatively, as suggested by the second reviewer, we have now validated the phenotype by multiple individual oligos. Among four, only two showed efficient knockdown of Vps26A (Fig. S1 B) and perturbed the gelatin degradation (Fig. S1 C) and Matrigel invasion activity (Fig. S1 D).

2. Figure 3A: Why are the entire VPS26 and VPS35 KD cells grey, whereas the control cell is black with discrete and punctate patterns of pHluorin signal? How did the authors quantify this signal in the presence of so much (and bright!) grey background? Is it really only background or is it highly dispersed MT1-MMP?

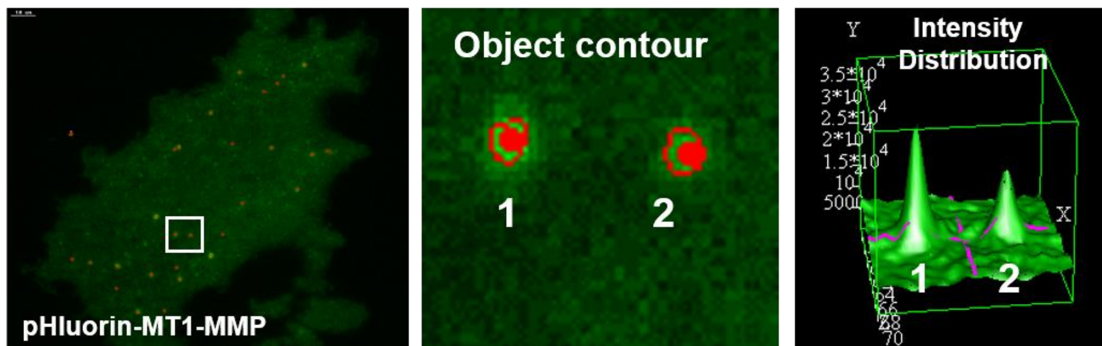
Response: Yes, we agree with the reviewer that in Vps26 and Vps35 KD cells, the background signal is much higher. We believe that this indeed shows a background noise because of the loss of the signal at the cell surface. As pointed out by the reviewer, it might also be due to the dispersion of the MT1-MMP in the cytoplasm upon knock-down. We have observed a similar trend in multiple sets of cells. However, for better representation, new images are added in Fig. 2 E and Fig. 4 C.

To quantify this signal in the high background was indeed a challenging task. However, the automated image analysis software, Motion Tracking (http://motiontracking.mpi-cbg.de), which was used for the analysis, has an optimized algorithm for detecting the inhomogeneous background of fluorescent cytoplasm. The objects are defined by probabilistic threshold and as such, are detected as additional intensity on top of the background. We identified the MT1-MMP puncta as an object and further quantified their physical properties such as size, intensity, and area. For better clarification, we have shown below a representative image with MT1-MMP objects depicted by object contour (A) and associated intensity distribution (B), as provided by Motion Tracking.

(A)



(B)



Legend: (A) pHluorin-MT1-MMP vesicles near cell surface (TIRF) for Scr and knockdown cells, identified as objects by motion tracking and their associated contour. (B) pHluorin-MT1MMP objects with their contour. Inset is showing the magnified boxed region with two objects (1 and 2) of different intensity. The representative intensity distribution of these two objects as analyzed by motion tracking.

3. Figure 3C and 5C: The MT1-MMP blots comparing scrambled and VPS26 KD conditions appear to be taken from separate gels. This is not best practice, the authors should run these assays on the same gel to really be able to compare overall levels and recycling rates. There are only 10 lanes in total, which easily fits onto one gel and one membrane. As it is, it looks like knockdown of VPS26 boosts the levels of cell surface MT1-MMP (Figure 3C), which would be odd if there is less recycling. Also, I do not really see any difference in the band intensities between conditions in Fig. 5C, which makes the quantification a bit unconvincing. These assays should be repeated and loaded onto the same gel to strengthen the conclusions. It would also be good to show all blots that were used for the quantification in a supplementary Figure as the differences (especially Figure 5C) look really minor to this reviewer.

Response: We sincerely apologize for not providing sufficient elaboration for the quantification. Here, what we measured is the amount of biotinylated MT1-MMP lost due to recycling at different time points as described previously (Remacle et al., 2003). We have used a membrane impermeable EZ-Link Sulfo-NHS-SS-Biotin, which is a thiol-cleavable biotin derivative. Proteins present at the cell surface were biotinylated, allowed to internalize and chased at different time points to ensure their recycling back to the cell surface. At each time point, cells were treated with MeSNa to cleave off the biotin from the recycled population. The signal intensity at the '0 min' time point corresponds to the total endocytosed population. Intensities at the further time points were normalized to the endocytosed population that represented the percentage recycling of the MT1-MMP (described in the equation below).

Calculation:

Time t=0, Intensity of the MT1-MMP band in the anti-MT1-MMP IB=A

$t=t_1$, Intensity of the MT1-MMP band in the anti-MT1-MMP IB =B

The amount of MT1-MMP recycled at time $t_1 = (\frac{A-B}{A}) \times 100$

In control cells, the MT1-MMP signal decreased with time. As pointed out by the reviewer, we wanted to clarify that this decreased signal corresponded to the increased recycling. On the contrary, the biotinylated MT1-MMP signal showed a much slower loss in intensity for the cells depleted for retromer, reflecting slow recycling. Thus, for scrambled or MT1-MMP knockdown condition, the recycling was measured with respect to the total endocytosed amount for that given condition. Hence, we could use two different gels/membranes for scrambled and MT1-MMP knockdown.

However, as per the reviewer's suggestion, we have repeated experiments by separating the scrambled and knockdown samples on the same gel and added to the figure as well (Fig. 2 G and Fig. 4E, other blots provided in Fig. S 5). Nevertheless, the differences in recycling kinetics between the scrambled and MT1-MMP knockdown is similar to our previous data.

Also, as the reviewer has pointed out, we agree that compared to retromer, the effect of SNX27 depletion on recycling kinetics of the MT1-MMP is marginal. This is also reflected by the quantification of the blots obtained from the Biotinylation assay (Fig. 4 E). Now, we have added biotinylation data where we measured the overall MT1-MMP population at the surface in the SNX27 downregulated cells using non-cleavable biotin (Steinberg et al., 2013; Qiang et al., 2019). Consistently, we found a marginal but significant difference in the cell surface population of MT1-MMP, whereas the total MT1-MMP levels were unaltered (Fig. 4 D). Moreover, we have investigated the effect of SNX27 on MT1-MMP recycling by multiple approaches. TIRF results showed that knockdown of SNX27 reduced MT1-MMP vesicles at the cell surface (Fig. 4 C). This effect could be rescued by overexpressing wild type SNX27, but not its retromer binding deficient mutant (Fig. 5 D). Similar results were obtained in live cell imaging (Fig. S4 D), antibody uptake assay (Fig. 4 F). Also, this defect in recycling led to MT1-MMP degradation in SNX27 depleted cells (Fig. S4 C). However, we speculate that biotinylation assay is less sensitive. Thus we found only minor differences in the MT1-MMP recycling kinetics.

All the above observations correlate well with the reduced gelatin degradation and Matrigel invasion upon SNX27 depletion (Fig. 3 A and Fig. 4 A). We should note that SNX27 phenocopied retromer in the proteases trafficking, selective association with MT1-MMP and perturbation of invasive potential of the tumor cells that reveals the critical role of SNX27-retromer assembly in the MT1-MMP trafficking and cellular invasion.

Now we have added the detailed description for the data interpretation in the method section and all the blots to support the quantification (Fig. S 5).

4. As mentioned above, the interaction between SNX27-retromer and the MT1-MMP tail needs to be mapped and analyzed more thoroughly, especially since knockdown of several unrelated complexes (see point 1) also reduced matrix degradation. A solid analysis of the interaction would really strengthen the claim that SNX27-retromer engage MT1-MMP via the DKV motif to promote recycling. I propose to use GST-MT1-MMP tail (and tail without the DKV motif) and recombinant SNX27 domains to test for direct interaction. The fact that the retromer binding mutant SNX27 lost binding to MT1-MMP does not necessarily mean that MT1-MMP binds to the core retromer. Gallon et al. (2014) have shown that binding of SNX27 to VPS26 greatly enhances PDZ binding activity so that this mutant likely has far lower PDZ ligand affinity.

Response: We thank the reviewer for this suggestion that has given better insight into the molecular mechanism underlying retromer-SNX27 mediated MT1-MMP recycling. As per his/her suggestion, we carried out GST pull down assay using GST-MT1-CT (Cytoplasmic tail of MT1-MMP). The purified His-tagged SNX27 and retromer subunits- Vps26, Vps29, Vps35 were used to examine the MT1-MMP direct interaction. Equimolar (0.5µM) of GST tag and GST-MT-CT

was used where GST tag was taken as a negative control. The result analyzed by Western blotting revealed that both Vps26 among retromer subunit and SNX27 could bind to MT1-MMP (Fig. 6 C). However, we could also detect a measurable amount of signal for SNX27 where only GST protein was used as a bait.

Further, to validate these results and measure the strength of the interaction, we went ahead to perform isothermal titration calorimetry (ITC). A 20 amino acid long peptide, corresponding to MT1-MMP tail residue (MT1-MMP-CT), was used as ligand. ITC was performed with the purified His-tagged SNX27 and Vps26 that showed binding in GST pull down. Interestingly, Vps26 showed binding in micromolar affinity, whereas SNX27 showed almost ten times higher affinity towards the peptide (Fig. 6 D, Table 1). Consistent with the pull down result, Vps29 subunit of the retromer failed to interact with the peptide.

MT1-MMP lacks classical PDZ ligand motif this encouraged us to investigate the mechanism behind its interaction with SNX27. We did ITC with the His-tagged PDZ and FERM-like domain of SNX27, to find which one is facilitating the direct binding. We could detect the binding with both the domains but the strength of interaction was 100 times weaker than the wild type (Fig. 6 D', Table 1). This suggested that both the domains are facilitating the MT1-MMP direct binding with the SNX27. As suggested by the reviewer, we can now draw conclusions that are in line with the study done by Gallon et al., (Gallon et al., 2014). Here, they have shown direct interaction of SNX27 with retromer and in a complex Vps26 greatly enhances the PDZ binding activity of SNX27. The observed direct interaction in our study suggests that both the molecules in a complex might enhance the MT1-MMP binding and thus more cargo enrichment on the sorting endosomes. Thus, explaining the SNX27-retromer assembly mediated MT1-MMP recycling.

Figure 6E: The cropping of the blots is excessive, and the result looks messy and is therefore unconvincing. The authors could try to improve this and maybe also repeat these IPs to obtain more convincing data.

Response: We appreciate the reviewer's suggestion for repeating IPs. However, we have now demonstrated the interaction by a different approach, i.e., GBP-trap pull down, wherein the GFP antibody is replaced with GFP Binding Protein (GBP). GBP is well reported to bind GFP strongly and can be used to efficiently purify GFP tagged protein similar to anti-GFP antibodies (Rothbauer et al., 2006, 2007). GBP was expressed, purified with an N-terminus GST tag and immobilized to sepharose beads. The lysate from the cells overexpressing GFP-tagged protein of interest was incubated with the GBP bound sepharose beads. Immunoblotting was performed, and much-improved quality data was obtained. We have added the result in Fig. 6 B and 6 E and uncropped blots are added to Fig. S 5.

Figure S3E: This blot is confusing. The input lanes for WASH1 indicate normal levels in VPS26 KD cells but the pellet and supernatant display much lower levels of WASH1. This makes no sense, and the data is therefore inconclusive. I do not understand why the authors ignored such a striking loss of overall WASH1 without thinking about the potential technical problems of this assay.

Response: We understand the reviewer's concern and apologize for the unclear data representation. The endosomal recruitment of the WASH complex by retromer is well studied (Helfer et al., 2013). We cross-examined the same in relevance to our study, elucidating retromer mediated MT1-MMP trafficking. First, we checked the effect of retromer depletion on the expression of WASH1, one of the subunits of the heteropentameric WASH complex. Immunoblotting was performed to measure WASH1 protein levels. WASH1 expression remained unaltered in the control and retromer depleted cells (Fig. S 2 F').

Further, the immunofluorescence-based analysis showed a significant reduction in the number of WASH1 punctae (Fig. S2 F). This was in alignment with the previous reports where retromer depletion led to solubilization of the WASH complex. We carried out biochemical membrane fractionation experiment to measure its membrane recruitment. The control and siRNA transfected cells were snap frozen (Seaman et al., 2009). The lysate was centrifuged to prepare soluble (supernatant) and membrane (pellet) fractions, as explained in the method section. The purity of the fractions was shown by the Transferrin receptor (TfnR), a membrane protein (detected only in the pellet fraction). The amount of WASH1 on the membrane was analyzed by immunoblotting and quantified. While it was more evenly distributed between the pellet and the supernatant fraction prepared from the control cells; in the fractions prepared from retromer depleted cells, WASH1 was significantly reduced in the pellet compared to the respective supernatant.

We agree that WASH1 amount was different in the control and retromer depleted fractions that made the observation confusing. This observed difference could arise because of the difference in the amount of lysate used for the fractionation of control and Vps26A depleted cells. However, to analyze the distribution of WASH1, the ratio of supernatant or pellet to total protein (for each condition) was compared independently.

For better interpretation, now, we have added a new blot (Fig. S2 F') and also have provided a detailed description in the figure legend.

Minor points:

1. In the introduction, bottom page two, the authors state that retromer recycles an abundance of cargo to the TGN. They then cite the recycling of GLUT1 as an example of this. It should be noted that the majority of retromer based recycling is recycling from endosomes to the plasma membrane, and the authors should rewrite these sentences to make clear that GLUT1 is not recycled to the TGN.

Response: We apologize for not giving an appropriate example from the literature with the statement. We have now elaborated this in the revised version of the manuscript. The paragraph has been re-written as 'Retromer plays a vital role in recycling various transmembrane cargoes from endosomes along the retrograde pathway to TGN, *trans*-Golgi network, by recognizing several retrieval motifs in the associated cargoes (Vinet and Zhedanov, 2010; Seaman, 2005; Johannes and Popoff, 2008). For instance, retromer mediated TGN recycling of transmembrane proteins CI-M6PR, Wnt receptor Crumbs, and SorLA is required for lysosomal functioning, nutrient uptake, Drosophila wing development, maintaining apical polarity, and neuronal functions. The retromer driven cargo retrieval maintains crucial physiological functions, and thus,

the genetic defect leads to pathologies like metabolic myopathy, neurogenerative disorders (Wang and Bellen, 2015; Small and Petsko, 2015), etc. Further, a recent study has given more insights into its TGN retrieval mechanism. Cui et al. have shown that retromer engages with endosomal transport carriers to maintain retrograde trafficking of CI-M6PR (Cui et al., 2019).

Interestingly, retromer has also been identified as a central player in mediating endosome to plasma membrane recycling (Seaman et al., 2013; Chamberland and Ritter, 2017; Burd and Cullen, 2014). In Hela cells, a surface proteomic study revealed that more than 150 cargoes are dependent on retromer, and more than 80 require SNX27 for their cell surface recycling (Steinberg et al., 2013). Interestingly authors also found that some of them, including glucose transporter GLUT1 and copper transporter ATP7A, are recycled by both retromer and SNX27. Several reports have shown that GLUT1 and β-AR (Beta-adrenergic receptor) are directed to the plasma membrane by retromer and its associated SNX27, a PDZ domain-containing SNX protein (Steinberg et al., 2013; Temkin et al., 2011; Lauffer et al., 2010) where loss of SNX27 or retromer trimer resulted in cargo missorting and its lysosomal degradation.

2. Figure 2C: The authors have inserted very small numbers into the actin blot. I couldn't find a description of these numbers in the figure legend or in the text, but I think they may be a ratio between MT1-MMP and actin. This should be clearly annotated, described in the legend and the numbers should be placed below the actin panel for better visibility.

Response: We thank the reviewer for pointing it out. As this was also pointed out by the third reviewer, for clear representation, the numbers are added only to blots where the ratio between different conditions is calculated i.e. in the membrane fractionation blots, where they represent the ratio of supernatant or pellet fraction with the total protein (Fig. S2 F' and Fig. S3 B). Now, we have added the bigger font size along with the description in the legend. However, as suggested by the 2nd and 3rd reviewer to make the study more focused many figures are being rearranged and reorganized. Figure 2 C is not included in the modified manuscript.

General (minor comment): The authors often omitted necessary articles before nouns. Thus, the study would benefit from some minor editing by a native speaker.

Response: We thank the reviewer for this suggestion. We have now thoroughly edited the manuscript and added articles at the appropriate sites using well-sited software 'Grammarly' that have improved the manuscript presentation.

Reviewer #2 (Comments to the Authors (Required)):

Sharma et al., "The SNX27-retromer assembly directs MT1-MMP trafficking to invadopodia and promotes breast cancer metastasis"

The study by Sharma et al. has investigated the trafficking of the matrix metalloprotease MT1-MMP and determined a role for SNX27 with retromer in localizing the MT1-MMP protein to the

cell surface where its enzymatic action can promote invasion of surrounding tissue by metastatic cancer cells.

It is now well established that SNX27 with retromer and the WASH complex plays a key role in directing proteins from endosomes to the cell surface. There are numerous publications that have demonstrated this with various cargo molecules. It has also been established that MT1-MMP is important in cancer cell metastasis and elevated levels of MT1-MMP have been linked with poor prognosis for some cancers. Thus, much of the first part of the manuscript is lacking in novelty. It has also been shown that the WASH complex is important in transporting integrins to the cell surface and thereby promoting invasion by cancer cells (Zech et al., 2012) but this data is overlooked in the study by Sharma et al.
The most interesting aspect of this study by Sharma et al. is the finding that SNX27 can associate with MT1-MMP to direct its trafficking but does not appear to be important for MT2-MMP trafficking. These findings which are reported in the second half of the study by Sharma et al. could potentially form the basis for a much shorter but more clearly focussed manuscript detailing the selectivity of SNX27 for MT1-MMP over MT2-MMP.
Overall I found reading the manuscript to be somewhat frustrating as there are a great many mistakes and inconsistencies that result in a manuscript that appears to have been hurriedly assembled with insufficient time spent proofreading the paper prior to submission. There are also some significant deficiencies regarding the experimental approach relating to the use of siRNA knockdowns. Much of the fact that OnTarget Plus reagents were used to minimize off-target effects, but these reagents are not infallible and can suffer from off-target effects similarly to other siRNA knockdown reagents. In order to rule out the possibility of off-target effects, the authors should use deconvolved (i.e., individual rather than pooled) siRNAs and/or rescue experiments to show that phenotypes observed are specific.

Summary: We appreciate that the reviewer found our study interesting. We thank him/her for pointing out the weaknesses and strengths of the manuscript and directing us to highlight only the novel findings. Accordingly, we have revised the manuscript, which is more clear and focused now. However, some of the results, such as related to WASH and SNX3, which are well established in the literature, are kept. They served as controls in our model system, MDA-MB-231. We have accordingly edited the result sections in the revised manuscript.

Response: We thank the reviewer for the suggestion. It is true that there is always a possibility of off-target effects while using SMARTpool oligos. To rule out the off-target effect for the retromer specific phenotype, we have now used individual oligos designed against Vps26A isoform of the retromer subunit Vps26. Out of the four oligos, only oligo1 and oligo3 could efficiently suppress Vps26A expression (Fig. S1 B). Subsequently, only the cells transfected with these oligos could perturb the cellular ability to degrade gelatin and invade Matrigel (Fig. S1 C, D). We had already performed rescue experiments and reported in the initial submission, where we had measured the rescue of gelatin degradation activity by overexpressing GFP-SNX27WT and its deletion mutants and provided the quantification (please refer to Fig. 6 A of the previous manuscript, Fig. 5 C in the revised manuscript). However, to highlight this now, we have added a separate figure, and

same is added in the text (Fig. 4 B) where SNX27 depleted cells transfected with siRNA resistant GFP-SNX27 could show gelatin degradation activity in contrast to GFP vector control.

Set out below are some of the problems/issues I found whilst reading the manuscript - this is unlikely to be the entirety of the problems however.

1. In the summary, what does "monitor" mean? How exactly does SNX27 "monitor" the transport of MT1-MMP to the cell surface? The use of the word "monitor" here is vague and uninformative.

Response: We agree with the reviewer and thanks for pointing this out. Now, we have reframed the sentence, 'We show that SNX27-retromer, an endosomal sorting and recycling machinery, interacts with MT1-MMP, a major collagenase, and regulates its transport to the cell surface'. We would like to add that as suggested by the other reviewers and the editor; we have carried out GST pull down assay (Fig. 6 C) and isothermal titration calorimetry (Fig. 6 D, D') to confirm direct interaction of SNX27 or Vps26 with MT1-MMP.

2. Throughout the manuscript, there are inconsistencies in the text relating to the position of citations - sometimes space to the text, sometimes not. There are also inconsistencies when describing GFP-tagged proteins - sometimes hyphenated, sometimes not. These issues may seem small but they create an impression of sloppiness.

Response: We sincerely apologize for the typo and the inconsistencies. We have now proofread the entire manuscript and made sure the uniformity throughout the text.

3. The citations employed are often wrong or incomplete. For example, when describing the interaction of the WASH complex with retromer, the authors choose to cite the studies by Derivery et al., (2009) and McGough et al., (2014). The first report of the WASH complex was indeed the study by Derivery et al., but the first report of the interaction between retromer and the WASH complex was Harbour et al., 2010 (PMID: 20923837). Other studies detailed the interaction between retromer and the WASH complex including; Harbour et al., 2012; Jia et al., 2012 and Helfer et al., 2013. In the introduction, the Chen and Ritter citation lacks a capital letter for Chen. When discussing the role of the retromer cargo-selective complex, the authors should also now cite the recent study from Teasdale and colleagues recently published in JCB. Overall, the introduction is not a very good overview of the literature on retromer.

Response: We agree with the reviewer and apologize for the same. The appropriate references describing the interaction of WASH and retromer has now been added at the appropriate place. Moreover, we have now added more comprehensive literature citations to the introduction section. We have also cited the recent study from Teasdale and group describing the molecular mechanism of the retromer-mediated TGN retrieval of the cargo, published in JCB, which was published while our manuscript was in communication.

4. The authors need to specify which Vps26 protein is targeted in the siRNA experiments, Vps26a or Vps26b?

Response: We have now explicitly mentioned throughout the manuscript that we have used siRNA targeted against Vps26A isoform.

5. In determining the cell surface levels of MT1-MMP, could the authors employ a FACS-based analysis?

Response: We sincerely thank the reviewer for the suggestion. FACS can be used to measure surface levels to validate the result on a larger population of the cells. However, in our study, we have employed multiple well-established approaches to ensure that MT1-MMP cell surface level is reduced upon retromer or SNX27 depletion.

To confer that this observation is valid over a large population of cells, we have used a biochemical approach, surface biotinylation, which is a well-established assay to study cell surface recycling in the literature (Remacle et al., 2003; Steinberg et al., 2013; Qiang et al., 2019) (Fig. 2 G and Fig. 4 D, 4 E). Further, TIRF microscopy was used to determine cell surface MT1-MMP population in SNX27, and retromer depleted cells (Fig. 2 E and Fig. 4 C). Similarly, live cell imaging and antibody uptake assays were carried out to monitor the cell surface population of MT1-MMP using a laser scanning confocal microscopy (Fig. S4 D and Fig. 2 H, Fig. 4 F).
Nonetheless, we attempted to do FACS with the MT1-MMP antibody, cat. no. MAB 3328 that we have used throughout the study (in Western blot and immunofluorescence). Unfortunately, we could detect minimal MT1-MMP signal (only 1%) at the surface of the control cells. This might be due to sub-optimal antibody affinity for the epitope on MT1-MMP. Here, we would also like to add that endogenous MT1-MMP does not show prominent cell surface localization in MDA-MB-231 cells, as revealed by immunofluorescence microscopy using MAB 3328 (Fig. S2 C, F and Fig. S3 C). This indeed corroborates with the lack of detectable signal using FACS.

6. The role of VAMP7 in regulating MT1-MMP localization may be via retromer and VARP. The VARP protein associates with both VAMP7 and retromer and requires retromer for its membrane association. This possibility does not seem to have been considered by the authors.

Response: We thank the reviewer for bringing it up. The retromer mediated role of VAMP7 is now discussed in the revised manuscript:
"Additionally, retromer directly interacts with VARP (VPS9-ankyrin-repeat protein), a Rab32 effector, and recruit it on the endosomal membranes (Hesketh et al., 2014). This interaction governs the retromer, VARP, and VAMP7 mediated cell surface transport (Hesketh et al., 2014). VARP is also required for the directed movement of VAMP7 vesicles (Burgo et al., 2012). Moreover, the recycling of MT1-MMP from the late endosomes is known to be mediated by VAMP7 (Steffen et al., 2008; Williams and Coppolino, 2011; Williams et al., 2014). In light of these facts, there is a possibility that retromer mediated exocytosis of MT1-MMP involves WASH, VARP, and VAMP7".

7. In figure 3, the authors report that loss of retromer results in the WASH complex being displaced from the endosomal membrane. This observation is a glaring example of the lack of novelty in the first half of this manuscript. It has been reported in many independent studies that retromer is required for the endosomal localization of the WASH complex through Fam21 binding to Vps35

(see Harbour et al., 2010; Harbour et al., 2012; Jia et al., 2012; Helfer et al., 2013; Zavodszky et al., 2014; McGough et al., 2014).

Response: We agree with the reviewer that the loss of retromer in displacing WASH complex is well addressed previously in the literature (Harbour et al., 2010, 2012; Helfer et al., 2013). Here we have used this well-studied mechanism as a piece of evidence to conclude that retromer is regulating MT1-MMP recycling via endosomal recruitment of the WASH. Now, we have moved the results in the supplementary section (Fig. S2 F, F'). Accordingly, the following statement is added to the result section in the revised manuscript:
"WASH, a heteropentameric complex generates endosomal actin patches, and facilitate protein sorting (Derivery et al., 2009; Gomez and Billadeau, 2009). Besides, it is also known that retromer subunit Vps35 interacts with FAM21 subunit of the WASH complex thus facilitate its recruitment on the endosomal membrane (Harbour et al., 2010, 2012; McGough et al., 2014). In agreement with these studies, we found that retromer depletion led to a reduction in WASH1 punctae on the MT1-MMP endosomes in MDA-MB-231 cells (Fig. S2 F). Further, the biochemical assay also revealed lower WASH1 levels in the membrane fractions of Vps26A depleted cells (Fig. S2 F')".

8. Similarly, the authors report that SNX3 knockdown affects MT1-MMP trafficking and retromer localization. The role of SNX3 in promoting the recruitment of retromer to endosomes has been established previously - see Harterink et al., 2011; Vardarajan et al., 2012. This is another example of the lack of novelty.

Response: Yes, we agree with the reviewer that several studies have reported SNX3 mediated retromer recruitment (Harterink et al., 2011; Vardarajan et al., 2012). However, we would like to clarify that this is the first report in the breast cancer cell line. Among the retromer associated SNXs, depletion of SNX3 or SNX27 affected gelatin degradation activity (Fig. 3 A). Further, retromer displacement upon depletion of SNX3 or SNX27 supported the previous observation (Fig. S3 B, C). These findings also conveyed that the SNX3 mediated retromer recruitment is indeed a generalized mechanism. Now, we have modified the sentence stating:
"Further, in line with the previous reports, knock-down of SNX3 significantly affected the membrane association of retromer as revealed by the biochemical membrane fractionation assay (Harterink et al., 2011; Vardarajan et al., 2012) (Fig. S3 B)".
Corroboration of our results with the previously established observations on WASH recruitment and SNX3's role in retromer recruitment provided additional strength to the overall conduct for the current study.

9. In the methods section it was easy to find mistakes. For example, NaCl is shown as Nacl in the Co-immunoprecipitation paragraph. The penultimate sentence of the paragraph describing the Degradation experiment describes adding SDS but does not give an amount. These are just two examples of mistakes I found, I suspect that there are many more.

Response: We sincerely apologize for the mistakes in the methods section. Now, we have added more detailed information with correct annotations.

10. The figures are generally quite poorly presented and the figure legends lack necessary

information relating to the experiment. For example, the arrows on figure 1A are not consistently and equally positioned three images with arrows. The figure legend makes no mention of the inset boxes and what is shown there. In figure 2B, there is a mistake, "TGFBRAP1 KD KD". In figure 3E, the spots inside the inset boxes are actually smaller than the corresponding spots on the actual image - something has clearly gone wrong with how that figure was produced. Figure 6 has some potentially useful data but is quite poorly presented (what are the marks on the blots shown in figure 6D? Why don't the panels line up?), and the images of some of the blots appear to be overexposed. Overall, the quality of the figures falls below the standard usual for JCB.

Response: We sincerely apologize for the poor presentation of the results. The revised version of the manuscript has better representative images with detailed information in the figure legends. As per his/her and the 3rd reviewer's suggestion, the manuscript is modified to convey a clear and more focused story. The current version has lots of changes in the figure panels; however, we have made sure to address all the issues that were raised for the previous manuscript. The blot corresponding to Fig. 6 D is replaced and added to Fig. 6 B. The uncropped blot is shown in the supplementary Fig. S 5.

Summary:

Reviewer #3 (Comments to the Authors (Required)):

The manuscript describes novel findings related to the regulation of MT1-MMP by retromer-SNX27 assembly and endosomal recycling pathway. In addition, association with invadosomes and function of less studied MT2-MMP is postulated in Matrigel invasion and degradative activities of fibronectin. Many of the presented experimental results are potentially important for cancer cell biology, and extensive collection of interesting data is presented. However, the manuscript is very diverse, and should focus to provide more convincing evidence and careful considerations of selected key questions and conclusions. The expression and possible functions of MT2-MMP would require a more thorough examination for solid conclusions. The results on MT1-MMP trafficking as well as SNX27 role in cancer metastasis in vivo and the related conclusions also appear partially confusing and should be more clearly presented. Therefore, in the opinion of this reviewer, the study as presented is too speculative and premature for publication. Below listed are specific comments:

Summary: We are thankful to the reviewer for finding out the important and novel aspects of our study, which would be beneficial for the field of cancer cell biology. We also appreciate that the reviewer has very critically gone through the manuscript and pointed out its limitation. As per his/her suggestion, in the modified version, we have addressed the key problems extensively using multiple approaches. Several new experiments have been carried out as per the editor and all three reviewers' suggestion and the results are incorporated into the manuscript. The distinct trafficking route of these two proteases is one of the novel features of our findings, as pointed out by the reviewers. Hence, the current manuscript now mainly focuses on the role of SNX27-retromer in preferential MT1-MMP cell surface recycling. We have omitted the results related to the substrate specificity of MT2-MMP. However, relevant data is kept highlighting the localization, association,

and contribution to cancer cell invasion, for this protease. Additionally, more results are added from the mouse xenograft experiments to provide better clarification of the in vivo data.
Importantly, we have now added the protein expression data for MT2-MMP depicting its abundance in the breast cancer cell line. One of the major concern of the reviewer related to the functional relevance of MT2-MMP is addressed by citing the relevant literature.

1. The link to previous literature and current knowledge could be more exact and up to date. Example concerns in the first paragraph of Introduction; a) basement membrane invasion is presented as the sole means of ECM degradation and metastasis, although the biologically well characterized MT1-MMP activity with relevance to metastasis is against interstitial collagen, in addition to basement membrane b) the references for other MT-MMPs in general are old, and c) previous MT2-MMP studies with mainly ectopic overexpression are referred to create a link to breast cancer, basement membrane invasion, and metastasis.

Response: a) We are thankful to the reviewer for the suggestion. In the current version, we have now cited a study by Ouchi et al., 1997, where they have reported the dual role of MT1-MMP in ECM degradation by directly cleaving interstitial collagen and activating secretory protease MMP-2 (Ohuchi et al., 1997).

b) Also, we have referred to the recent studies highlighting involvement of other MT-MMPs in cancer metastasis (Wells et al., 2015; Tatti et al., 2011, 2015; Wu et al., 2017; Yip et al., 2017; Huang et al., 2015; Jiang et al., 2017; Wang et al., 2015) that has summarized recent advancements in understanding the role of MT-MMPs in cancer biology.

c) We would like to mention that we had included a study by Hotary et al., where authors showed that depletion of MT2-MMP in MDA-MB-231 cells inhibited basement membrane perforation and invasion (Hotary et al., 2006). Additionally, two other research groups have demonstrated different mechanisms involved in MT2-MMP mediated cancer cell invasion. Ota et al., had reported that Snail1, a transcription factor, induces aggressive phenotype in non-invasive breast carcinoma cell line MCF7, by regulating the expression levels of MT1 and MT2-MMPs. Also, they showed that depletion of endogenous MT1-MMP or MT2-MMP reduced the basement membrane invasive activity in the MDA-MB-231 cells, that expresses high levels of Snail1(Ota et al., 2009). The other study carried out in an ovarian carcinoma cell line showed that MT2-MMP degrades E-cadherin by its proteolytic activity leading to EMT and thereby facilitates cell invasion (Liu et al., 2016). These studies have elaborated the link of MT2-MMP with the cancer metastasis and are added in the revised manuscript.

2. The authors state that (citation from last Introduction paragraph) "we demonstrated that MT2-MMP the other abundant protease associates with invadosomes and facilitate breast cancer cell invasion by preferentially degrading fibronectin."

It is not clear however, where the protease is abundant, since the authors fail to detect endogenous MT2-MMP protein, and only provide (in Fig. S1A) Real-time PCR results of the relative MT2-MMP levels in MCF-7 and MDA-MB-231 cells, normalized to the expression in MCF-10A cells. This all could still be marginal. Stronger expression data in breast cancer cells would be required to start considering the possible relevance of MT2-MMP activities in breast

cancer, and regulation of it, for which a set of results are provided in this study regarding overexpression constructs.

Response: We understand the reviewer's concern and are thankful for the suggestion. To address the same, we have now added the protein expression data to the manuscript (Fig.2 A). Here the anti-MT2-MMP antibody is used to detect the endogenous protein levels. The comparative analysis among different cell line showed that the MT2-MMP is over-expressed in metastatic breast cancer cell line MDA-MB-231.

Despite the conclusion, it also was not addressed, if MT2-MMP in MDA-MD-231 cells directly cleaved fibronectin, or which ECM proteins would MT2-MMP preferentially affect or degrade, since only gelatin and fibronectin were tested in the cell-based assay. In the used ECM degradation experiments, the effect could as well be indirect through, for example, affecting some of the fibronectin-associated proteins, or of the many another fibronectin-degrading proteases (or their inhibitors) expressed in these cells.

Further, the fibronectin degradation result was directly related to the result of Matrigel invasion assay, although Matrigel is a complex mixture of laminin, collagens and other proteins, and the relevance of possible fibronectin, laminin, collagen etc degradation in Matrigel invasion was not addressed. With the current set of experiments. Conclusion is premature at this stage.

Response: We apologize for not linking the literature well with our findings that made an impression of premature conclusions for the substrate activity of MT2-MMP. However, now, we have referred to a study done by D'ortho et al., where direct substrate activity of MT1-MMP and MT2-MMP was addressed extensively (D'ortho et al., 1997). This was demonstrated by performing enzyme activity based assays, where authors used recombinant protease and measured its activity on various ECM components. They showed that MT2-MMP could cleave fibronectin, tenascin, laminin, and nidogen. Among these substrates, nidogen is unique in providing structural stability to basement membranes. Hence, these findings revealed a diverse catalytic activity of MT2-MMP that could promote ECM remodeling and destabilization of the basement membrane. The same is added in the revised version stating that:
"Previously, an in vitro assay based study has reported the broad spectrum of substrate activity for MT1 and MT2-MMP. They demonstrated that both the proteases cleaved fibronectin and tenascin, whereas only MT2-MMP could degrade laminin (D'ortho et al., 1997). Also, MT1-MMP is well characterized as an interstitial collagenase that could digest type I, II, and III collagens (Ohuchi et al., 1997). In cell line based assays, MT1-MMP, and MT2-MMP were shown to activate proMMP2 thereby contributing to the remodeling of the basement membrane (Morrison et al., 2001a; Nishida et al., 2008). Also, two independent studies showed that depletion of endogenous MT1-MMP or MT2-MMP reduced the basement membrane invasive activity in the MDA-MB-231 cell line (Hotary et al., 2006; Ota et al., 2009). Further, MT2-MMP is shown to degrade E-cadherin by its proteolytic activity, leading to EMT and thereby facilitates cell invasion (Liu et al., 2016). Consistent with these reports, we observed that depletion of MT2-MMP led to abrogation of Matrigel invasion activity (Fig. 2 B)".

Additionally, we have now referred to the study carried out by Morrison et al., where the authors have shown that MT2-MMP can activate proMMP-2, a fibronectinase (Morrison et al., 2001b).

We agree that directly linking fibronectin degradation with Matrigel invasion assay is not appropriate. However, the results related to the activity of MT2-MMP on fibronectin and gelatin are removed as per this reviewer's suggestion.

3. Since the other presented MT2-MMP results are with overexpression, and largely negative findings compared to the MT1-MMP regulation and gelatin degradation, should this main study stand even better with MT1-MMP alone? Although interesting, the presented MT2-MMP results should deserve focused experimentation and much more careful considerations. Considering these preliminary results, the localization and recycling of MT2-MMP could be better analyzed using fibronectin matrix as a cell adhesion substrate.

Response: We sincerely appreciate the reviewer's suggestion to focus on MT1-MMP. We agree that more focused experiments are required for MT2-MMP. However, the observation that in contrast to MT1-MMP, recycling of MT2-MMP is not mediated by retromer or SNX27 is of particular interest to the reviewers and the editor and therefore retained in the revised manuscript. Moreover, we have now carried out immunoblot to demonstrate overexpression of MT2-MMP in MDA-MB-231 cells and incorporated the data in the revised manuscript (Fig. 2 A).

4. For conclusions of MT2-MMP in Figure 1, not only better expression data, but also comparable/more complete ECM degradation results should be provided. Laminin would be relevant addition to link the results to presented invasion results, as it is abundant in Matrigel.

Cannot find results for MT2-MMP KD in gelatin degradation, in spite of following statement in Results: "On the contrary, MT2-MMP depleted cells could degrade gelatin comparable to scrambled control cells, but depletion of MT1-MMP abolished gelatin degradation activity."

Figure S1B shows qualitative results (a single cell image) for gelatin degradation with MT1-MMP KD cells +/- GFP-MT2-MMP. The text concludes: "Interestingly, MT2 could completely rescue the loss of gelatin degradation activity in MT1-MMP KD cells"; for this the scramble/control and quantitative data is required.

Response: We appreciate the reviewer's suggestion. However, as per his/her and other reviewers suggestion, we have omitted the data related to the activity of MT2-MMP on different ECM components. Hence, this figure is removed from the modified manuscript.
We agree with the reviewer and understand his/her concern over linking invasion results for MT2-MMP. However, the activity of MT2-MMP on laminin is already reported in the literature. The authors have studied direct cleavage of laminin by MT2-MMP (D'ortho et al., 1997). They used the recombinant enzyme and laminin subunits at different molar ratios to detect its degradation and could demonstrate its ability to degrade α1, β1, and γ1 chains of the laminin. Further, cell-based assays should be carried out to confirm the physiological relevance of this in vitro observation.

5. For statistical analysis n (and N) numbers are mentioned for some but not all of the results in Figure legends. See e.g. Figure 1A', B, C'.

Response: We sincerely apologize for the inconsistencies in the figure legends. However, in the modified version, we have ensured to add all the relevant statistical parameters throughout the manuscript.

6. Figure 1E: Only minority of MT1-MMP is detected in the selected assessed vesicular compartments in MCF10A and MCF7. Where is it localized in these cells?

Response: Since we found only a small fraction of MT1-MMP in early and late endosomes, we used Golgi marker, and in MCF7, where MT1-MMP showed colocalization with TGN46. However, in the present study, to make it more focused, we have now decided to show the colocalization of the MT1-MMP protease only with retromer in MDA-MB-231 (Fig. 2 D).

7. Figure 2A: It is difficult to appreciate the gelatin degradation in the provided merged images. The red channel alone would be helpful. From current images, Vsp39 KD seems almost comparable with control. -In Figure S2A, mRNA KD results are only provided to 5/6 proteins in Figure 2A.

Response: We are thankful for the reviewer's suggestion. Now throughout the manuscript, red channel alone is shown for better representation of the gelatin degradation assay. Also, in the revised version we have assured to provide the results representing the efficacy of knockdown, at either protein or mRNA level, for all the molecules (Fig. 1 A, 2 B, 2 H, 3 A, 7 B and Fig. S1 A, B). As per the suggestion by the second reviewer, we have focused only on retromer and removed data for the other multi-subunit complexes.

8. Figure 2C: Quantitative data should be collected from multiple replicated experiments, and not from a single western blot. As provided in this and several other figures of this manuscript, the numbers are also too small to easily read the data.

Response: All these experiments are repeated at least 2-3 times (as mentioned in respective figure legends). The numbers are added in the western blots to show a better quantitative interpretation for the qualitative blot image. However, in the current version, the numbers are added only to the membrane fractionation blots where the ratio between different conditions is calculated i.e. the ratio of supernatant or pellet fraction with the total protein (Fig. S2 F' and Fig. S3 B). Now, we have added the bigger font size along with the description in the legend. As per the suggestion, additional blots are provided in supplementary (Fig. S 5).

9. Figure 2D: Is cortactin staining sufficient to conclude "Notably, we couldn't find any significant difference in the number of cells forming invadopodia upon knocking down the subunits of the MSCs (Fig. 2D, 2D')"? Atleast a more exact measure could be used to label the Y-axis in 2D', and to describe the results. This comment extends to many other results, and applies throughout the manuscript, where authors conclusions rather that the exact measured data seem to be presented as results.

Response: We sincerely apologize for mislabeling of the axis. In the revised version, all graphs are representing the exact measure of the plotted data.

Cortactin, an invadopodia marker, is found at the sites of dynamic actin assembly where it activates and stabilizes branched actin assembly and well used as invadopodia marker (Rosse et al., 2014; Artym et al., 2006; Oser et al., 2009). However, to further ensure our conclusions, we have now added immunofluorescence data for an additional invadosomal marker, Tks5 (Sharma et al., 2013; Lagoutte et al., 2016; Qiang et al., 2019). We immunostained retromer, SNX27, or MT1-MMP depleted MDA-MB-231 cells with anti-Tks5 antibody and counted the number of invadopodia (Fig. 1 E and Fig. 4 A'), thus ensuring that retromer and SNX27 are not involved in invadopodia assembly.

Additionally, a positive control, i.e., MT1-MMP knockdown, which is earlier shown to be crucial for invadopodia formation in MDA-MB-231 cell line (Steffen et al., 2008), has been added (Fig. 1 E and Fig. 4 A'). We also found that while the cells depleted for MT1-MMP showed reduced invadopodia formation, it remained unaltered in the cells with the reduced expression for retromer or SNX27.

10. Figure 3: Comparative detection of the decrease in total cell surface-labelled protein could allow conclusions between speculated effects of lysosomal degradation and defective recycling on the results.

Response: We agree and thank the reviewer for this suggestion. However, we had addressed the same by performing cycloheximide based lysosomal degradation experiments where MT1-MMP protein level was measured (please refer Fig. S5 C in the previous submission). We demonstrated that degradation kinetics of MT1-MMP was enhanced upon cycloheximide treatment, in retromer and SNX27 depleted cells (Fig. S4 C in the revised version). These findings led us to conclude that defect in the recycling of MT1-MMP results in its lysosomal mis-sorting and degradation.

11. Figure 5A and 6 for example The western images should be cut further away from the protein bands, to show clearer results.

Response: We have changed the western images as per the reviewer's suggestion (Fig. 3A and Fig. 6 B, E), and also uncropped images of the corresponding western images are added in the supplementary figure (Fig. S 5).

12. The conclusions from the in vivo xenograft experiment are highly speculative, and should be limited to those supported by the data. Additional time-point or real-time analysis would be required to solid conclusions about delayed metastasis.

Response: We understand the reviewer's concern for a possible over-interpretation of the data. In the modified manuscript, sentences are reframed so that the conclusions are limited only by the supported data. We agree that for stronger conclusions more exhaustive experiments are required. We have added this scope in the result section so that our claims do not seem to be over-interpreting the observed effect. However, in different studies, the expression of metastatic markers is related to the patient's survival and lifespan of xenograft mice model as well (Sprenger et al., 2015; Li et al., 2010; Seong et al., 2017). Our analysis on tumor datasets showed an increased lifespan, though marginal, of the breast cancer patients with lower expression of SNX27 (Fig. 3 E). Thus we

hypothesized that increased mice survival might be because of the delayed metastasis (Fig.7 D). This possible explanation is now added to the results stating:
“ This further correlates with our analysis for the breast cancer patients' survival index where patients with low SNX27 expression showed a marginally longer life span (Fig. 3 E). Thus, the extended survival in SNX27KO injected mice may suggest a correlation between survival and delayed onset of metastasis.”

13. Last Results paragraph: The data appear to be missing for the whole tail vein injection (also missing from the methods). As described, the results are more confusing than convincing for the drawn conclusions about the invasion and metastatic activities affected by SNX27. Solid data combining analysis of in vivo primary xenograft invasion and tail vein exit to lung tissue, for example, could be helpful, in addition to more relevant in vitro invasion assays.

Response: We sincerely apologize for not providing the tail vein data. We have now added the data to Fig.7 E. The detailed protocol for this experiment is also added to the method section in the revised manuscript. The short term colonization experiments, via tail vein injection, revealed that both the control and SNX27 knockout cell line could equally infiltrate the lung tissue. The number of tumors observed was comparable in the two groups. This observation indicates that SNX27 might not be facilitating tumor establishment on the new site. It is probably involved only during the initial phase of cancer cell invasion.

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August 26, 2019

Re: JCB manuscript #201812098R-A

Dr. Sunando Datta
Indian Institute of Science Education and Research Bhopal
Biological sciences
Bhopal Bypass Road
Bhopal 462066
India

Dear Dr. Datta,

Thank you for submitting your revised manuscript entitled "SNX27-retromer assembly directs MT1-MMP trafficking to invadopodia and promotes breast cancer metastasis". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that although reviewer #3 now recommends publication, reviewers #1 and #2 have voiced a number of lingering concerns which they feel need to be addressed before the paper would be ready for publication. Specifically, reviewer #1 is still concerned by the fact that MT1-MMP levels are not reduced at the cell surface after SNX27 depletion - this issue will need to be sufficiently addressed in the final revision. In addition, both reviewers feel that the evidence supporting the proposed direct interaction between the MT1-MMP tail and SNX27 is not sufficient - we concur with reviewer#1's suggestion that you remove this data and instead add further critical discussion of the potential complexity of the interaction, perhaps along the lines suggested by reviewer #1.
However, please note that while we appreciate reviewer #2's other points regarding conceptual novelty (pts #1 and 2) and incomplete depletion of SNX27 (pt#4), we do not agree that these issues need to be resolved in the final revision. However, please be sure to address this reviewer's two 'minor' issues regarding increasing the conciseness of the writing.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Johanna Ivaska, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Interregnum Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have performed a substantial amount of additional experimentation. The additional data have strengthened their conclusions. While I do think that the paper may be acceptable for publication, some concerns remain:

If SNX27 (and the core retromer) recycle MT1-MMP, why are MT1-MMP levels not reduced at the cell surface? This is really evident in the blots for Figure 2G and Figure 4E. The timepoint 0 without MesNa is basically a surface biotinylation. MT1-MMP surface levels are not affected at all by knockdown of SNX27 (they seem slightly increased!) and even seem to be strongly increased by knockdown of VPS26? If there is less MT1-MMP1 at the surface, why is the biotinylated (=internalized) amount before MesNa treatment not reduced? The same applies to MesNa treated cells at timepoint 0. Why is there not a reduction in internalized MT1-MMP before recycling has taken place?

The biochemical analysis of MT1-MMP cytotail with SNX27-retromer is confusing. How can a 20 amino acid peptide from the tail interact with BOTH the FERM like and the PDZ domain? And similarly, how can it interact both with SNX27 and the core retromer? Figure 6C should be removed as it is meaningless data. GST alone binds better to SNX27 than the GST-MT1 CT, indicating that there is no real binding between the purified proteins. If anything, this is neagtive data showing that they don't bind above background binding to GST alone. Maybe SNX27 needs to engage VPS26 in vivo to efficiently bind MT1-MMP? In light of Gallon et al, this would be conceivable. The authors could also leave Figure 6C in the manuscript and state that the individual proteins do not bind but this is hard to reconcile with the ITC data?

All of that said, the rescue experiments with the SNX27 mutants are very nice and convincing and in these assays, the SNX27 mutants behave like you would expect from previously published work. I do think that SNX27 really does promote MT1-MMP insertion into invadopodia, which makes this study important for the field.

I suspect that binding between the SNX27-retromer complex and MT1-MMP1 is there but more complex than a simple PDZ interaction. At the very least, the authors should critically discuss this and also try to explain why MT1 surface levels don't seem to be reduced by loss of SNX27 and or retromer. Since the study is focused on the biological effects of SNX27 in metastasis, which I find convincingly adressed, some weaknesses or ambiguities in the cell biological and biochemical data may be acceptable?

Reviewer #2 (Comments to the Authors (Required)):

The revised manuscript from Sharma et al., is improved from the first version but in my view the question of novelty remains, the manuscript is overly long and some of the data is unconvincing and contradictory.

Major points:
1. A key element of the study by Sharma et al., namely that SNX27 is important for the metastasis of MDA-MB-231 cells has recently been reported by Zhang et al., (see PMID:31182056) and therefore there is possibly less that is novel in the study by Sharma et al., than before.
2. The data relating to the role of SNX3 in recruiting retromer to the membrane remains in the manuscript despite the fact that it is not novel. Statements in the text relating to the role of sorting nexins in recruiting retromer are wrong. For example, the manuscript states, "Retromer is known to be recruited on the endosomes by SNX (Sorting nexin) family members..." There are more than 30 different sorting nexins but only SNX3 has a clear and unequivocal role in recruiting the retromer trimer onto endosomes. The Snx1 protein interacts only very weakly with the retromer trimer (see PMID:21629666) and does not perfectly colocalise with the trimer (see PMID:28935632) the role of Snx1 and Snx2 in recruiting retromer is questionable. I previously suggested that the data relating to SNX3 be removed from the manuscript but the authors appear not to have taken this advice on board.
3. The main addition to the manuscript - the interaction of the MT1-MMP cytoplasmic tail with SNX27 and VPS26 is unconvincing.
The lower panel of Figure 6a is a bit of a mess and it is not clear whether there is a band corresponding to the GFP-MT1-MMP in the GST-SNX27 lane. In Figure 6c, it appears that GST alone will pulldown His-SNX27 and His-Vps26 better than the GST-MT1-CT construct. Surely the authors are concerned about the specificity of the interactions they are reporting? The data in figure 6e is a bit better but it does appear that the pulldown assays suffer from worryingly high background/non specificity.
4.Why is there so much variability in SNX27 western blot signal in figure 7? There is a strong band in Figure 7a' (possibly saturated?) but the SNX27 band in Figure 7b is weak and I'm not totally convinced that there is a clean knock out of SNX27 in the cell lines highlighted (C4 and C24).

Minor points:
1. The introduction is too long and wordy and doesn't seem to have a focus.
2. The summary is poorly worded in places - for example describing SNX27 as "highly altered" in patients does not provide much actual meaning, altered in what way?

Reviewer #3 (Comments to the Authors (Required)):

The manuscript describes a set of interesting findings related to the regulation of MT1-MMP, but not MT2-MMP, by retromer-SNX27 assembly and endosomal recycling.

In this new submission of their revised manuscript, the authors have added an extensive amount of new data, and modified the data presentation in figures to address the reviewer's extensive

comments. They have also added new literature references to support their statements and conclusions, as well as revised the text.
I am in general satisfied on the major revision and careful responses to my previous comments and concerns. The manuscript text, although improved markedly, would still benefit from editing for grammar and clarity.

2nd Revision - Authors' Response to Reviewers: September 26, 2019

Johanna Ivaska, Ph.D.
Monitoring Editor
Journal of Cell Biology
Rockefeller University Press

26th Sept. 2019

Dear Dr. Ivaska,

Thank you for giving us the opportunity to revise our manuscript (201812098R-A) entitled 'SNX27-retromer assembly directs MT1-MMP trafficking to invadopodia and promotes breast cancer metastasis'. We have uploaded the revised manuscript, modified figures and supplementary material on the JCB submission portal. **Please find below the point-by-point response to the editors' and reviewers' comments.**

We are hopeful that you will find the revised version satisfactory and suitable for publication in JCB.

Looking forward to hearing from you.

Dr. Sunando Datta,

Associate Professor,

Department of Biological Sciences,

IISER, Bhopal.

A Point-by-point response to the reviewers:

We sincerely thank the editors' and the reviewers' for their critical comments and insightful suggestions that have markedly improved the manuscript. We are glad that they have found the revised manuscript focused, potentially highlighting the role of SNX27 in promoting breast cancer metastasis by mediating the recycling of MT1-MMP. However, there are still a couple of concerns raised by the reviewers, which includes MT1-MMP surface levels and its interaction with retromer or SNX27. To address these, we have now measured the surface levels of MT1-MMP by biotinylation using non-cleavable biotin and carried out the interaction studies with a modified experimental protocol. The data obtained from the newly conducted experiments have been added to the revised manuscript. In the following section, we have referred to the modified figure labels, as reported in the current version of the manuscript. As per journal's policy, to restrict Title/abstract within the mentioned characters/word limit we have now modified the Title and the abstract accordingly. Also, we apologize for uploading Video 4 labeled as Video 2 mistakenly, in the previous submission. We have now added the correct version of the Video files.

In the surface biotinylation experiment, we found a significant reduction in the levels of MT1-MMP upon depletion of Vps26 or SNX27. Moreover, the newly carried out in vitro pull-down assay with modified protocols demonstrate the interaction of MT1-cytoplasmic tail with recombinant Vps26 and SNX27. For both SNX27 and Vps26, the binding to GST protein is almost insignificant compared to that for GST-MT1-tail providing the authenticity of the interaction. This is further supported by our ITC studies. Therefore, we have decided to report these results. We believe that this is an important piece of information which will further help the community to explain the molecular basis of recycling of the protease by the retromer-SNX27 assembly. However, following the suggestion of 1st reviewer, we have now explained the mode of binding in a more careful manner with a possibility of multiple potential binding mechanisms. Also, we have omitted the ITC data related to the individual domains of SNX27.

The second reviewer has suggested making the introduction section more focused. We have now modified the text wherever required as per his suggestion. In the section below, we have addressed each of the issues raised by the reviewers.

Reviewer #1 (Comments to the Authors (Required)):
The authors have performed a substantial amount of additional experimentation. The additional data have strengthened their conclusions. While I do think that the paper may be acceptable for publication, some concerns remain:
If SNX27 (and the core retromer) recycle MT1-MMP, why are MT1-MMP levels not reduced at the cell surface? This is really evident in the blots for Figure 2G and Figure 4E. The timepoint 0 without MesNa is basically a surface biotinylation. MT1-MMP surface levels are not affected at all by knockdown of SNX27 (they seem slightly increased!) and even seem to be strongly increased by knockdown of VPS26? If there is less MT1-MMP1 at the surface, why is the biotinylated (=internalized) amount before MesNa treatment not reduced? The same applies to MesNa treated cells at timepoint 0. Why is there not a reduction in internalized MT1-MMP before recycling has taken place?

Response: We understand the reviewer's concern and thanks to him/her for pointing this out. However, we wanted to clarify that this assay has been used in literature to study the recycling kinetics of the transmembrane proteins at the cell surface (Huang et al., 2016; Remacle et al., 2003; Macpherson et al., 2014). The surface proteins are labeled with the **cleavable form of biotin** at 4°C. After labeling, the cells are shifted to 37°C for 30min to allow endocytosis. Cell lysate collected at this time point is referred to as the '0' min time point. Multiple parameters would influence the biotinylated surface population at this time point; therefore, we believe that the '0' min could not be used as the readout for the absolute surface levels of MT1-MMP. We had performed these assays in order to address if perturbation of retromer or SNX27 had any effect on the recycling kinetics of MT1-MMP at the cell surface. Indeed Fig. 2G and Fig. 4E shows that compared to that of control, the Vps26A or SNX27 siRNA treated cells showed **slower** recycling of the protease. The unaltered surface levels of MT1-MMP at '0' min, is consistent with the previous observation made by Huang et al., where they have studied the recycling kinetics of APP upon SNX27 or SORLA depletion, please refer to Fig. 5 D (Huang et al., 2016).

To precisely measure the surface population, **non-cleavable biotin** has been used in various studies (Steinberg et al., 2013; Qiang et al., 2019). We had performed the same to study the role of SNX27 in controlling the surface population of MT1-MMP and reported the results in the submitted manuscript (Fig. 4D). Depletion of SNX27 subtly but significantly reduced the surface population of MT1-MMP. Now, we have carried out the same experiment for retromer where the cells with the reduced expression for Vps26A showed a significant reduction in the surface levels of MT1-MMP compared to the control cells (Fig. 2G). Additional blots are given in the supplementary Fig. S5. The data showing the recycling kinetics of MT1-MMP upon Vps26A or SNX27 depletion has been shifted to the supplementary figure, i.e. Fig. S2 D and Fig. S3 D.

We believe that data obtained from the biotinylation assay is one of the key findings of our study which provides the evidence that retromer or SNX27 plays a vital role in the recycling of MT1-MMP to the plasma membrane and thereby contributing to cancer cell invasion. To better interpret the results from this biochemical assay, the experiments were performed at least twice independently along with technical repeats. The intensity of the biotinylated MT1-MMP is quantified for scrambled and siRNA treated samples for Vps26A **(Figure 1)** and SNX27 **(Figure 2),** using ImageJ software (http://rsb.info.nih.gov/ij/index.html). The scanned western blot image was converted to grayscale for its densiometric analysis. Using the square selection tool, a box was plotted on the lanes that are representing MT1-MMP bands and another box of the exactly same dimension is plotted to measure the background for the corresponding lanes.

Normalized intensity of MT1-MMP in Scr = $Scr_{MT1\text{-}MMP\ intensity}$-$Scr_{background}$

Normalized intensity of MT1-MMP in Vps26A KD = Vps26A $KD_{MT1\text{-}MMP\ intensity}$- Vps26A $KD_{background}$

Normalized intensity of MT1-MMP in SNX27 KD = SNX27 $KD_{MT1\text{-}MMP\ intensity}$- SNX27 $KD_{background}$

The normalized intensities of MT1-MMP in Scr are compared to that of Vps26A KD or SNX27 KD. These details are added in the methods section now.

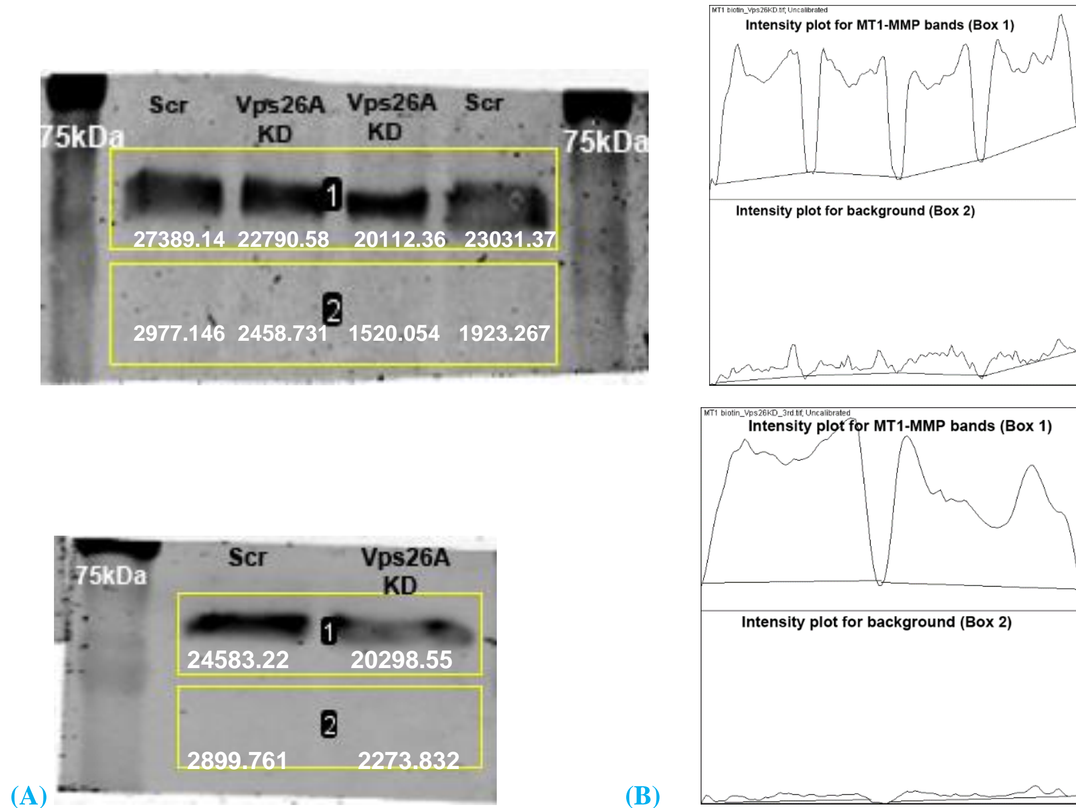

Figure1: **(A)** The square box next to the ladder on the scanned image represents the selection area to measure the band intensity (1) and intensity of the background (2) for Scr and Vps26A KD samples. The numbers are representing the intensity obtained from the plots of intensity. **(B)** The intensity of the plots from the square box selection. The upper plots are for the intensity of MT1-MMP bands, whereas lower plots represent the background for the corresponding lanes.

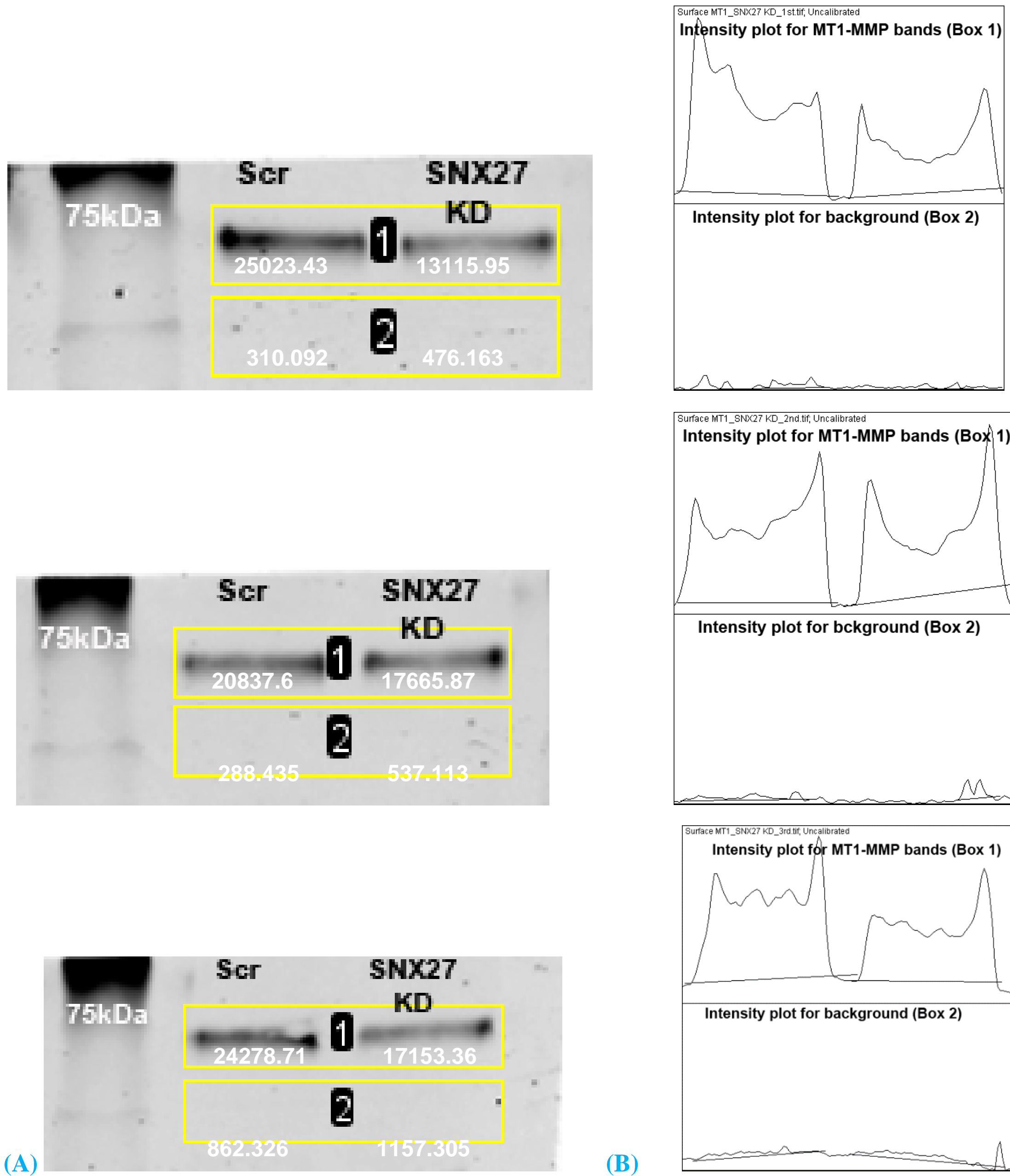

Figure 2: **(A)** The square box next to the ladder on the scanned image represents the selection area to measure the band intensity (1) and intensity of the background (2) for Scr and SNX27 KD samples. The numbers are representing the intensity obtained from the plots of intensity. **(B)** The intensity of the plots from the square box selection. The upper plots are for the intensity of MT1-MMP bands, whereas lower plots represent the background for the corresponding lanes.

The biochemical analysis of MT1-MMP cytotail with SNX27-retromer is confusing. How can a 20 amino acid peptide from the tail interact with BOTH the FERM like and the PDZ domain? And similarly, how can it interact both with SNX27 and the core retromer? Figure 6C should be removed as it is meaningless data. GST alone binds better to SNX27 than the GST-MT1 CT, indicating that there is no real binding between the purified proteins. If anything, this is negative data showing that they don't bind above background binding to GST alone. Maybe SNX27 needs to engage VPS26 in vivo to efficiently bind MT1-MMP? In light of Gallon et al, this would be conceivable. The authors could also leave Figure 6C in the manuscript and state that the individual proteins do not bind but this is hard to reconcile with the ITC data?

Response: We sincerely apologize for not providing sufficient elaboration for the biochemical analysis done via ITC. Here, we have used commercially synthesized peptide mimicking 20 amino acids long cytoplasmic tail of MT1-MMP. To measure the strength of interaction with the peptide, the following proteins were recombinantly expressed and purified from E. coli: His-SNX27, His-PDZ domain, His-FERM-like domain, His-Vps26, and His-Vps29. Each ITC run was performed using the peptide as a ligand, and the purified proteins were independently tested for the interaction. The experiments were repeated at least thrice with the same concentration of protein and ligand.

We agree with the reviewers' that the data from GST pull-down (Fig. 6 C) was not appropriate to draw any conclusion supporting the direct binding of MT1-tail with SNX27 or Vps26, particularly because, the negative control, GST protein itself also showed significant binding with the proteins. We believe that this might have occurred because of the non-specific binding between the purified proteins or the use of an anti-His antibody in immunoblotting. To ensure if there is genuine interaction between GST-MT1 tail and Vps26 or SNX27, we have re-performed the pull-down assay with a modified protocol. 0.5% BSA was added in the binding buffer to block non-specific binding, and immunoblotting was done with the protein-specific antibodies. These modifications in the assay have tremendously improved the quality of the blot where a clear binding is seen for both Vps26 and SNX27 compared to GST protein (negative control). The new results are added in the revised manuscript (Fig. 6 C). However, the existing blot clearly shows that Vps29 and Vps35 subunits of retromer do not bind to GST or GST-MT1-tail; therefore, we have decided to keep this blot as a supplementary figure (Fig. S5 B) to demonstrate the specificity of binding for Vps26.

These results along with our earlier observation have further strengthened our findings from the ITC studies, where purified His-tagged SNX27 or Vps26 were checked independently for their direct interaction with the 20 amino acid long synthetic peptide mimicking the cytoplasmic tail of the MT1-MMP (Fig. 6 D). In literature, the binding affinity of transmembrane cargoes that directly interact with SNX or retromer is often measured by using the synthetic peptides representing the cytoplasmic tail of these transmembrane proteins (Gallon et al., 2014; Ghai et al., 2013). Our results from the ITC showed that recombinantly expressed and purified SNX27, its domains: PDZ, FERM-like domain or Vps26 subunit of retromer could individually bind to the MT1-tail peptide with variable binding parameters whereas Vps29 could not (Fig. 6 D, D' of the previous manuscript), highlighting the specificity of the interactions. The detailed thermodynamic parameters that govern the binding were also provided (Fig. 6 D').

However, as suggested by the reviewers and to keep our focus on the relevant results, we have now removed the ITC data for the individual domains of SNX27.

All of that said, the rescue experiments with the SNX27 mutants are very nice and convincing and in these assays, the SNX27 mutants behave like you would expect from previously published work. I do

think that SNX27 really does promote MT1-MMP insertion into invadopodia, which makes this study important for the field.

I suspect that binding between the SNX27-retromer complex and MT1-MMP1 is there but more complex than a simple PDZ interaction. At the very least, the authors should critically discuss this and also try to explain why MT1 surface levels don't seem to be reduced by loss of SNX27 and or retromer. Since the study is focused on the biological effects of SNX27 in metastasis, which I find convincingly adressed, some weaknesses or ambiguities in the cell biological and biochemical data may be acceptable?

Response: We thank the reviewer for his/her suggestion. As mentioned above, the newly carried out pull-down experiments demonstrated that MT1-tail directly binds to SNX27 and specifically to Vps26 subunit of retromer (Fig. 6 C). Moreover, these observations further corroborated with our results from ITC based studies where Vps26 or SNX27 could independently bind to the MT1-tail peptide with a distinct binding affinity (Fig. 6 D, D'). Importantly, ITC studies have provided the thermodynamic parameters governing these interactions.

However, the analysis of the results from the rescue based functional assays and TIRF microscopy revealed that the cells expressing retromer binding deficient SNX27 mutant, i.e., SNX27Δ67-77 could not regain the gelatin degradation activity (Fig. 5 C) and showed a reduced population of MT1-MMP at the cell surface (Fig. 5 D), respectively. Thus, although SNX27 or Vps26 could bind to MT1-MMP tail independently, in vitro, their association might be playing a crucial role in vivo to recognize the protease for its efficient recycling.

As pointed out by the reviewer, we would like to add that earlier Gallon et al., measured and demonstrated that the binding affinity of SNX27 for its cognate cargoes was allosterically increased when it was bound to Vps26 (Gallon et al., 2014). They also found that the SNX27 mutant, deficient in binding to retromer, showed abrogation of this observed cooperative effect. In line with this, our results suggest that MT1-MMP might be actually recognized by SNX27; however, the binding of Vps26 to SNX27 may further strengthen the latter's interaction with the protease.

Alternatively, another possibility that MT1-MMP might bind at the interface between Vps26 and SNX27 cannot be ruled out. This mode of binding of retromer, SNX proteins, to the cargo is already known in the literature (Lucas et al., 2016). In a structure-based study, authors have shown that the binding of the recycling motif of DMT1-II, a retrograde cargo, required its coincident interaction with retromer and SNX3 (Lucas et al., 2016).

Additionally, it would be important to identify the specific motif (s) in MT1-MMP tail which is recognized by the retromer-SNX27 complex. SNX27, the only member of SNX family with a PDZ domain binds to PDZbm (PDZ binding motif) present in the C-terminal region of the PDZ ligands such as β2AR, Kir3 (potassium channel inwardly rectifying), SDC2 (Syndecan2) (Cao et al., 1999; Lauffer et al., 2010; Lunn et al., 2007; Steinberg et al., 2013). Nonetheless, the cytoplasmic tail of MT1-MMP does not harbor any of these PDZbm. However, it is interesting to note that 'DKV' motif in the protease tail possesses the features of ClassIII PDZbm, i.e., X[DE]Xϕ (Nourry et al., 2003). The results from our GBP pull-down studies revealed that the DKV motif is vital for the association of the protease with retromer-SNX27 in MDA-MB-231 cells (Fig. 6 E). Further direct interaction studies need to be carried out to investigate if this motif is recognized by retromer or SNX27 or both.

Further delineation of the molecular mode of binding of MT1-MMP with retromer-SNX27 assembly would require extensive biophysical and structural studies.

The above discussion has been incorporated in the revised manuscript.

Reviewer #2 (Comments to the Authors (Required)):
The revised manuscript from Sharma et al., is improved from the first version but in my view the question of novelty remains, the manuscript is overly long and some of the data is unconvincing and contradictory.
Major points:
1. A key element of the study by Sharma et al., namely that SNX27 is important for the metastasis of MDA-MB-231 cells has recently been reported by Zhang et al., (see PMID:31182056) and therefore there is possibly less that is novel in the study by Sharma et al., than before.
2. The data relating to the role of SNX3 in recruiting retromer to the membrane remains in the manuscript despite the fact that it is not novel. Statements in the text relating to the role of sorting nexins in recruiting retromer are wrong. For example, the manuscript states, "Retromer is known to be recruited on the endosomes by SNX (Sorting nexin) family members..." There are more than 30 different sorting nexins but only SNX3 has a clear and unequivocal role in recruiting the retromer trimer onto endosomes. The Snx1 protein interacts only very weakly with the retromer trimer (see PMID:21629666) and does not perfectly colocalise with the trimer (see PMID:28935632) the role of Snx1 and Snx2 in recruiting retromer is questionable. I previously suggested that the data relating to SNX3 be removed from the manuscript but the authors appear not to have taken this advice on board.

Response: We apologize for the inappropriate statements generalizing the role of sorting nexins in the endosomal recruitment of retromer. We agree that there are only a few SNXs which are known to associate with retromer i.e., SNX3, SNX12, SNX27, SNX1 and SNX2, SNX5 and SNX6. Among these, SNX3, SNX12, SNX1, and SNX2 have been demonstrated to recruit retromer on the membrane. Therefore, in the revised manuscript, we have replaced the statement 'Retromer is known to be recruited on the endosomes by SNX family members' with 'The Vps trimer of core retromer lacks intrinsic ability to bind to the endosomal membrane. Its recruitment is rather facilitated by late endosomal GTPase Rab7A (Rojas et al., 2008; Seaman et al., 2009; Priya et al., 2015) and a few selected members of SNX family such as SNX3, SNX12, SNX1 and SNX2 (Rojas et al., 2007; Wassmer et al., 2009; Harterink et al., 2011; Priya et al., 2017; Harrison et al., 2014)'.

3. The main addition to the manuscript - the interaction of the MT1-MMP cytoplasmic tail with SNX27 and VPS26 is unconvincing.

Response: We understand the reviewer's concern. As per his/her and the 1st reviewer's suggestion, we have now addressed the direct interaction by re-performing the pull-down with a modified protocol. The previous blot is replaced with that of the new one (Fig. 6 C).

The lower panel of Figure 6a is a bit of a mess and it is not clear whether there is a band corresponding to the GFP-MT1-MMP in the GST-SNX27 lane. In Figure 6c, it appears that GST alone will pulldown His-SNX27 and His-Vps26 better than the GST MT1-CT construct. Surely the authors are concerned about the specificity of the interactions they are reporting? The data in Figure 6e is a bit better but it does appear that the pulldown assays suffer from worryingly high background/non specificity.
4.Why is there so much variability in SNX27 western blot signal in Figure 7? There is a strong band in Figure 7a' (possibly saturated?) but the SNX27 band in Figure 7b is weak and I'm not totally convinced that there is a clean knock out of SNX27 in the cell lines highlighted (C4 and C24).

Minor points:
1. The introduction is too long and wordy and doesn't seem to have a focus.

Response: We thank the reviewer for pointing this out. In the current version, the introduction section has been modified to make it concise and focused.

2. The summary is poorly worded in places - for example describing SNX27 as "highly altered" in patients does not provide much actual meaning, altered in what way?

Response: We apologize for the unclear statements in summary. We have now modified the statement as 'Analysis from the publically available database showed SNX27 to be overexpressed or frequently altered in the patients having invasive breast cancer.'

Reviewer #3 (Comments to the Authors (Required)):
The manuscript describes a set of interesting findings related to the regulation of MT1-MMP, but not MT2-MMP, by retromer-SNX27 assembly and endosomal recycling. In this new submission of their revised manuscript, the authors have added an extensive amount of new data, and modified the data presentation in Figures to address the reviewer's extensive comments. They have also added new literature references to support their statements and conclusions, as well as revised the text. I am in general satisfied on the major revision and careful responses to my previous comments and concerns. The manuscript text, although improved markedly, would still benefit from editing for grammar and clarity.

Response: We thank the reviewer for finding the revised manuscript improved along with the better presentation of the data. His/her suggestions were very helpful for presenting our findings with a clear interpretation of data and updated literature.

Further for better clarity, now we have carefully edited the manuscript using 'Grammarly' software (www.grammarly.com).

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October 7, 2019

RE: JCB Manuscript #201812098RR

Dr. Sunando Datta
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Dear Dr. Datta:

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