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# Protrudin mediated ER-endosome contact sites promote MT1-MMP exocytosis and cell invasion

Nina Marie Pedersen, Eva Wenzel, Ling Wang, Sandra Antoine, Philippe Chavrier, Harald Stenmark, and Camilla Raiborg

*Corresponding Author(s): Camilla Raiborg, Oslo University Hospital*

## Review Timeline:

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| Submission Date: | 2020-03-10 |
| Editorial Decision: | 2020-04-06 |
| Revision Received: | 2020-05-04 |

*Monitoring Editor: Johanna Ivaska*

*Scientific Editor: Andrea Marat*

## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

**DOI: https://doi.org/10.1083/jcb.202003063**

April 6, 2020

RE: JCB Manuscript #202003063

Dr. Camilla Raiborg
Oslo University Hospital
Institute for Cancer Research, Dept. of Molecular Cell Biology
Montebello
Oslo N-0379
Norway

Dear Camilla and Harald,

Thank you for submitting your manuscript entitled "Protrudin-mediated ER-endosome contact sites promote MT1-MMP exocytosis and cell invasion" to Journal of Cell Biology. The manuscript was assessed by three expert reviewers, whose comments are appended to this letter.

As you can see, the reviews from these expert reviewers - all of them acknowledged leaders in the fields related to your study - are all enthusiastic about your study. They have relatively minor concerns and suggestions. In addition, we have outlined the necessary revisions below, taking into consideration that, given the current circumstances, these can be addressed from existing images and data without the need for new experiments. Consequently, we invite you to resubmit a revised version of this manuscript after resolving the points we have outlined below.

Reviewer #1 is wondering whether invadopodia number are altered in 3C and requests additional quantification related to the observations reported in Figure 5. In addition, further clarification to experimental set-up and labeling is requested for Figure 6A. Hopefully, you have data available that allows addressing these points without need for new experimentation. In addition, in case you have existing data investigating whether protruding silencing influences ER morphology please consider including those into the manuscript. Otherwise, it is not necessary to carry out new experiments to address this point.

Reviewer #2 would like to see additional quantification of the dimension of invadopodia for Fig 4A. Please provide these if possible. The other point raised, an additional ER marker used to verify that GFP-Protrudin is perhaps not necessary given the detailed analyses of the protruding localization provided in your earlier publication.

Reviewer#3 has specific points regarding the quantification of data in Figure 4. Please address those. In addition, please consider discussing the potential role of protruding splice isoforms, as suggested. We agree with the reviewer that it would be interesting to investigate which element/domain of protrudin is necessary in the rescue experiments (to define whether the pathway used by MT1-MMP is identical to the one regulating neurite outgrowth), however we don't think this is necessary for the current study.

Thank you very much for submitting this strong, carefully executed and valuable study to JCB.

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A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software

h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution.

Sincerely,

Johanna Ivaska
Monitoring Editor
Journal of Cell Biology

Andrea L. Marat
Scientific Editor
Journal of Cell Biology

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

The article "Protrudin mediated ER-endosome contact sites promote MT1-MMP exocytosis and cell invasion" shows that the protein protrudin, which is important for mediating ER membrane contact site formation, promotes invadopodia maturation and activity. The authors found that inhibition of protrudin causes impaired cancer cell invasion by preventing translocation of MT1-MMP-laden endosomes to the cell membrane. They also found that invadopodia expansion and elongation depends on endosome translocation which in turn depends on protrudin, FYCO1 and Rab7. Conversely, they find that overexpression of protrudin enhances formation of invadopodia-like protrusions, increased matrix degradation and invasion by non-cancerous cells.
These observations are very interesting and overall the studies have been carried out in a rigorous manner. With regard to the advance, the findings provide an important subcellular control point for maturation and activity of invadopodia and cancer cell invasion, via regulation of trafficking of MT1-MMP-containing endosomes to the plasma membrane where they can be docked. Thus ER-endosome contacts are important for the hand-off to kinesins to drive trafficking to the plasma membrane. This kind of molecular mechanistic insight is critical to provide support for the finding and the data are supportive of the conclusions. I have just a few points that would improve the manuscript before publication:

Specific comments:
1. In this study, you show that protrudin depletion causes inhibition of extracellular matrix degradation and invasion. Could authors show whether there is any effect on ER morphology or ER network formation in protrudin KO/KD cells?
2. In figure 3C, you mention that length of invadopodia is reduced. Are there any changes in number of invadopodia formed per cell?
3. More quantitation should be done for Fig 5, as follows:
a. In Figure 5A, Rab7 and MT1-MMP are colocalized in cells. Could you show the percentages?
b. In Figure 5D, the representative image shows MT1-MMP positive endosomes are perinuclear. If

you could quantitate this observation from multiple images and multiple independent experiments, it would strengthen the observation.
4. In Fig 6A, you show MT1-MMP exocytosis event per min per cell area in control and siProtrudin#1 and siProtrudin#2 cells to be different and it looks like siProtrudin#1 is actually a rescue experiment from the Western blot. If that is true, it is presented in a very confusing way - please do some relabeling and explanation. Also, please comment in the manuscript about the difference in the two conditions.

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

In the present manuscript, Pedersen et al. characterized a novel mechanism at the basis of invadopodia formation, extracellular matrix degradation and cell invasion. The authors found that the ER-resident protein Protrudin is required for the trafficking of Rab7-positive late endosomes/lysosomes to sites of invadopodia formation through the establishment ER-endosome contact sites. The fusion of endo-lyosomal compartment with the PM is then mediated by SYT7. They also confirmed the involvement of other players such as the kinesin-1 adaptor FYCO1: this same complex was previously shown by the authors to be involved in neurite outgrowth. The Protrudin-mediated transport provides membranes required for invadopodia maturation and is needed for MT1-MP metalloprotease relocalization to invadopodia.
While the involvement of Rab7 and endo-lysosome fusion in invadopodia formation was already known, the molecular mechanism involved in the delivery of lysosomes to invadopodia and their fusion with PM was not previously characterized. The manuscript describes a novel mechanism based on trafficking and communication among different organelles, critical for cancer cell invasion and with potential implications in cancer development and metastasis.
The message of the paper is relevant for the cell and cancer biology community. The experiments are technically well performed and carefully controlled. The flow is clear and logic, results are not overstated and the final model is supported by results. I therefore strongly recommend this manuscript for publication in JCB.

Minor issues:
1) In Fig. 4A, please provide here a quantitation of the dimension of the invadopodia in control and Protrudin OE cells, to support its role in invadopodia maturation.
2) In Fig. 5B, to support a role for contact sites between the ER and the endosome, please provide also another ER marker, in addition to GFP-Protrudin, to confirm that GFP-Protrudin is correctly localized in the ER and that it is exerting its function on MT1-MP from the ER compartment. A correlative EM analysis would also strengthen this result, to show proximity between Protrudin-enriched ER and MT1-MP-positive endosomes.

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

Cancer cells break tissue barriers with the help of actin-rich membrane protrusions called invadopodia. The manuscript focuses on the function of the ER-late endosome contact site component Protrudin, the role of which has earlier been characterized in neurite outgrowth, in invadopodia growth in the human adenocarcinoma cell line MDA-MB-231, as well as in the non-cancerous retinal pigment epithelial cell line RPE-1. The authors' working hypothesis is that, similar to neurite outgrowth, the growth of invadopodia, may depend on late endosome/lysosome transfer

to the growing protrusion and there fusion with the plasma membrane to deliver (i) the membrane required and (ii) the matrix metalloproteinase MT1-MMP. Indeed, their data strongly suggests that the complete maturation of invadopodia depends on protrusion outgrowth and the targeted delivery of the matrix metalloproteinase MT1-MMP via endosomal transport to the growing protrusions. They present data that Protrudin orchestrates invadopodia maturation and function. Protrudin is shown to form ER contact sites with MT1-MMP positive endosomes that contain the RAB7-binding Kinesin-1 adaptor FYCO1. Depletion of RAB7, FYCO1 or Protrudin inhibits MT1-MMP dependent extracellular matrix degradation and cancer cell invasion by preventing anterograde translocation and exocytosis of MT1- MMP. Furthermore, the authors show that, when endosome translocation or exocytosis is inhibited by depletion of Protrudin or Synaptotagmin VII, respectively, invadopodia are unable to expand and elongate. In the reverse experiments, when Protrudin is overexpressed in the non-cancerous RPE-1 cells, these cells develop prominent invadopodia-like protrusions and show increased matrix degradation and invasion. In summary, the present data lends strong support to a model in which Protrudin-mediated ER-endosome contact sites promote cell invasion by facilitating translocation of MT1-MMP laden endosomes to the plasma membrane, enabling both MT1-MMP exocytosis and invadopodia outgrowth.
Even though it is previously known that the endocytic recycling of late endosomes/lysosomes plays an important role in invadopodia maturation and function, the function of the Protrudin pathway in this context is an absolutely novel observation. The work contains important implications for cancer therapy: Targeting the endosome transport/fusion process that delivers MT1-MMP to the plasma membrane for invadopodia growth may have potential as a new therapeutic approach.
The experimental work is exceptionally well designed and performed, and the imaging is of high quality. The authors' observations are robust and relevant from the point-of-view of innovating new approaches for cancer therapy. The manuscript is markedly well and professionally written, and the images clear and convincing. My very few criticisms towards this work are minor:

MINOR:
1. Fig. 4B: A western validating successful knock-down of SYT7 is missing. Please add it in the figure.
2. There are a number (at least 7) of Protrudin splice variants known. Do the authors know which one(s) are present in the breast cancer cell line they employed? Are different splice isoforms of Protrudin in different cell types (e.g. neurons vs. breast adenocarcinoma cells) known or expected to result in significant functional differences in the endo-lysosome transport mechanistics?
3. The protrudin overexpression data in Fig. 4A: The increase in the number of RPE-1 cells with invadopodia is modest, from 30 to 40%. Is this calculated per all cells in the fields subjected to observation, or per all transfected cells expressing the Protrudin construct? Please clarify.
4. In the experiments in which phenotypic rescue was perform by using an siRNA-resistant Protrudin expression construct, it would have been interesting to see which structural elements in Protrudin are necessary for the rescue capacity (such as the Kinesin-binding domain, the PI3P-binding FYVE domain, or the LCR mediating binding to Rab7). Did the authors test any of such mutant constructs in the function rescue experiments? If yes, did this support the interpretation that exactly the same Protrudin pathway is functional in invadopodia maturation and MT1-MMP transport as that described in neurite outgrowth?

TYPOS:
1. P. 8, line 2: ...at the tip of forming protrusions....
2. P. 9, line 16 from the bottom: ....degradation observed upon Protrudin depletion...
3. P. 11, line 14 from the bottom: ...outgrowth depends on Protrudin...
4. P. 13, line 11: ...are thought to be anchored...
5. P. 25, Plasmids: 'pCDNA-' should be 'pcDNA-

**Response to the reviewers:**

Reviewer #1

1. In this study, you show that protrudin depletion causes inhibition of extracellular matrix degradation and invasion. Could authors show whether there is any effect on ER morphology or ER network formation in protrudin KO/KD cells?

In light of the different functions of Prorudin in ER shaping and endosome positioning, the reviewer raises an important question. Protrudin is a transmembrane ER protein and has a role in ER shaping (Chang et al., 2013; Hashimoto et al., 2014). The role of Protrudin in ER shaping was shown to be independent of its FYVE domain, indicating that this function is independent of ER endosome contact sites and endosome positioning. In the present study, we did not perform experiments to further validate these findings in MDA-MB-231 cells or RPE-1 cells. Importantly, we have shown before that Protrudin depletion does not induce ER stress in RPE-1 cells (Hong et al., 2017). Although the data presented in the present manuscript point to the role of Protrudin in endosome positioning, we cannot rule out that changes in ER morphology could play an additional part in invadopodia maturation. Since a clear link between ER shaping and invadopodia is missing in the literature and due to space limitations, we suggest to not including this point in the discussion.

2. In figure 3C, you mention that length of invadopodia is reduced. Are there any changes in number of invadopodia formed per cell?

We agree that it is important to quantify the number of invadopodia in Protrudin knock out cells. We observed no changes in the number of invadopodia between MDA-MB-231 parental and Protrudin KO cells, supporting our data from the siRNA treated cells, that Protrudin is dispensable for invadopodia initiation. The graph is shown in new Fig. S3B, and is referred to in the manuscript on page 7.

3. More quantitation should be done for Fig 5, as follows:

a. In Figure 5A, Rab7 and MT1-MMP are colocalized in cells. Could you show the percentages?

By analyzing confocal images using Mander's overlap coefficient, we find that a significant amount of MT1-MMP positive pixels overlap with Rab7 positive pixels. The percentages of overlap are now included in the figure legend to Fig. 5A.

b. In Figure 5D, the representative image shows MT1-MMP positive endosomes are perinuclear. If you could quantitate this observation from multiple images and multiple independent experiments, it would strengthen the observation.

We agree with the reviewer that such quantification will indeed strengthen the observation. We have now quantified the degree of perinuclear clustering of MT1-MMP positive endosomes in MDA-MB-231 parental and Protrudin KO cells from three independent experiments (new Fig. 5E). These data support the image in Fig. 5D, showing that MT1-MMP endosomes have a perinuclear localization in Protrudin KO cells. The new Fig.5E is referred to in the manuscript on page 8.

4. In Fig 6A, you show MT1-MMP exocytosis event per min per cell area in control and siProtrudin#1 and siProtrudin#2 cells to be different and it looks like siProtrudin#1 is actually a rescue experiment from the Western blot. If that is true, it is presented in a very confusing way - please do some relabeling and explanation. Also, please comment in the manuscript about the difference in the two conditions.

We apologize for any confusion regarding the experimental setup in Figure 6A. We have now added an explanation of the rescue setup in the legend to Figure 6 and in addition relabeled the figure to indicate which images/lanes are rescued.

Reviewer #2

1) In Fig. 4A, please provide here a quantitation of the dimension of the invadopodia in control and Protrudin OE cells, to support its role in invadopodia maturation.

We thank the reviewer for this excellent suggestion. We have quantified the dimension of invadopodia, which increases in RPE-1 cells stably overexpressing Protrudin. The graph is included in Fig. 4A.

2) In Fig. 5B, to support a role for contact sites between the ER and the endosome, please provide also another ER marker, in addition to GFP-Protrudin, to confirm that GFP-Protrudin is correctly localized in the ER and that it is exerting its function on MT1-MP from the ER compartment. A correlative EM analysis would also strengthen this result, to show proximity between Protrudin-enriched ER and MT1-MP-positive endosomes.

Protrudin has been shown by us and others to be a transmembrane ER resident protein which localizes in VAP-A positive areas of the ER. To emphasize this fact, we have included a sentence on page 8 in the manuscript followed by the proper references.

Reviewer #3

1. Fig. 4B: A western validating successful knock-down of SYT7 is missing. Please add it in the figure.

Due to the lack of a functioning antibody against SYT7 (we have tested several), we chose to show the knock down efficiency by qPCR in “old” Fig. S2C (referred to on page 8). We hope that this is sufficient, and suggest that perhaps these data was overlooked by the reviewer.

2. There are a number (at least 7) of Protrudin splice variants known. Do the authors know which one(s) are present in the breast cancer cell line they employed? Are different splice isoforms of Protrudin in different cell types (e.g. neurons vs. breast adenocarcinoma cells) known or expected to result in significant functional differences in the endo-lysosome transport mechanistics?

The reviewer correctly points out that there are at least 7 splice variants of human Protrudin, ranging from 31.6 to 46.3 kDa. Whereas isoforms 1-3 (as annotated by Uniprot) will have all the domains required for the generation of ER-endosome contact sites, the remaining shorter isoforms are lacking parts of their N-terminus (encoding transmembrane domains) and are likely not localizing properly in the ER. No studies exist which address the possible different functions of the individual splice variants in humans. However, studies from mice have shown that there are two main isoforms of Protrudin; a neuro specific L-form and a shorter S-form which is expressed in all tissues (Ohnishi et al., 2013). Both isoforms have the same domain structure, but the L-form has an insertion of 7 amino acids which increases its binding affinity for VAP-A, and makes it more potent in neurite outgrowth. In our view, both versions will be able to mediate ER-endosome contact sites and regulate endosome positioning. Interestingly, the canonical human isoform 1 is similar to the mouse L-version.

We do not know which of the splice variants are expressed in MDA-MB-231 cells. By Western blot analysis using an antibody raised against the Protrudin C-terminus (which should detect all possible variants), we observe a broad band around 50 kDa. This band, which could represent the isoforms 1-3, disappears upon treatment with Protrudin siRNA and in Protrudin KO cells. Since we do not observe any prominent bands of lower molecular weight, we assume that the lower splice forms are not expressed. For overexpression or rescue experiments we have used the canonical human isoform 1 of Protrudin consisting of 411 amino acids (cDNA clone BC030621 from ImaGene), which is now specifically annotated in the methods on page 22.

Due to the lack of proper data regarding the differential expression and function of the different human Protrudin isoforms, we suggest to omit this discussion in the current manuscript. However, we think that the reviewer raises and important question that will be interesting to study in more detail in the future.

3. The protrudin overexpression data in Fig. 4A: The increase in the number of RPE-1 cells with invadopodia is modest, from 30 to 40%. Is this calculated per all cells in the fields subjected to observation, or per all transfected cells expressing the Protrudin construct? Please clarify.

We apologize for the insufficient labeling of this experiment. In the legend to Figure 4A, we have now clarified the experimental setup showing that the number of invadopodia positive cells increases in Protrudin overexpressing cells. These experiments were done in RPE-1 cells with or without stable overexpression of Protrudin. The reviewer points out that the increase from 30% to 40 % is modest. We think that these data are now strengthened by the additional quantification of the increased invadopoda dimension in Protrudin overexpressing cells, which we have included in Figure 4A.

4. In the experiments in which phenotypic rescue was perform by using an siRNA-resistant Protrudin expression construct, it would have been interesting to see which structural elements in Protrudin are necessary for the rescue capacity (such as the Kinesin-binding domain, the PI3P-binding FYVE domain, or the LCR mediating binding to Rab7). Did the authors test any of such mutant constructs in the function rescue experiments? If yes, did this support the interpretation that exactly the same Protrudin pathway is functional in invadopodia maturation and MT1-MMP transport as that described in neurite outgrowth?

We agree that it would be interesting to test the different structural domains of Protrudin in a rescue setup; to characterize the importance of the different domains regarding invadopodia maturation, but also to compare if there are different requirements for invadopodia maturation versus neurite outgrowth. Unfortunately we have only used full length Protrudin in our rescue experiments, and we hope to address this in our future work.

TYPOS:
1. P. 8, line 2: ...at the tip of forming protrusions....
2. P. 9, line 16 from the bottom: ....degradation observed upon Protrudin depletion...
3. P. 11, line 14 from the bottom: ...outgrowth depends on Protrudin...
4. P. 13, line 11: ...are thought to be anchored...
5. P. 25, Plasmids: 'pCDNA-' should be 'pcDNA-

The typos pointed out by the reviewer have been corrected.