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ER Membrane Contact Sites support endosomal small GTPase conversion for exosome secretion

Frederik Verweij, Maarten Bebelman, Anna George, Mickael Couty, Anaïs Bécot, Roberta Palmulli, Xavier Heiligenstein, Julia Sirés-Campos, Graça Raposo, Dirk Pegtel, and Guillaume van Niel

Corresponding Author(s): Frederik Verweij, Utrecht University

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January 10, 2022

Re: JCB manuscript #202112032

Dr. Frederik Johannes Verweij
Utrecht University
Padualaan 8
Utrecht 3584 CH
Netherlands

Dear Dr. Verweij,

Thank you for submitting your manuscript entitled "ER Membrane Contact Sites support endosomal small GTPase conversion for exosome secretion". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers find that your study provides potentially important insight into our understanding of the process of exosome secretion. However, they have made constructive suggestions of experiments to strengthen your conclusions as well as improvements to the presentation. We find all of their points valid and hope you will be able to completely address them in a revised study.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article 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.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

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As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened

to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

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

Sincerely,

Harald Stenmark, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The manuscript reports a study that focuses on defining the molecular events that generate secretory endosomes (releasing exosomes, CD63 as their marker), distinguishing them from degradative endo-lysosomes. The authors identify the compartment of origin of CD63 positive endosomes as a subclass of non-proteolytic endosomes at prelysosomal stage. They provide evidence for a GTPase cascade from Rab7a to Arl8b and finally Rab27a that eventually generates CD63(+) endosomes capable of fusing with the plasma membrane (PM). Importantly, they also show data suggesting that ER-endosomal membrane contact sites (MCS) mediated by the proteins ORP1L and Protrudin play an important role in modulating the generation of the CD63(+) endosomes fusing with the PM, by affecting late endosome motility, maturation and GTPase association. The research question is novel and previously unsolved - so the study is well motivated.
The work relies mainly on advanced live cell imaging (mainly TIRF microscopy) of transfected HeLa cells; The imaging data is appropriately quantified. The data is for the most part credible and convincing; However, I do see a number of major and minor issues that require further scrutiny:
(I find it difficult to comment details in the manuscript since page numbers are missing - the page numbering should absolutely always be there)

MAJOR
1. Throughout the manuscript the authors employ imaging of CD63(+) vesicle fusion events as a proxy for exosome secretion. They analyze by EV isolation and western analysis the actual release of exosomes only in Fig. 4F, where they study the effects of wt and mutant ORP1Ls on the release. In my opinion the authors should additionally validate also some other key findings of the study by measuring the actual release of exosomes by the cells, considering that even in the title of the paper they claim they have studied 'exosome secretion'. Such key data could be e.g. the effects of Rab27a, Rab7a or Arl8b manipulations.
2. The Rab7a-Arl8b-Rab27a cascade: The authors build the hypothesis on the cascade beginning with Rab7a based on the observation that Rab7a KD inhibited the fusion of CD63(+) endosomes with the PM and on a previous study (Jongsma et al, 2020). However, they do not show any evidence that the PM-fusing CD63(+) endosomes ever had Rab7a on their surface - the evidence is circumstantial. More direct evidence should be provided.
3. Protrudin overexpression effect is shown only for the fusion of CD63(+) vesicles, not LAMP1 vesicles, so it remains open if what the authors see is to any extent specific for the CD63(+) exosome releasing endosomes, or if protudin might have a general non-specific effect on any LE compartments. Is there a technical reason for the authors not to have measured the putative effect on LAMP1 endo-lysosome fusion?
4. If Arl8b precedes Rab27a in the GTPase cascade, why is the effect of siArl8b on the overlap between Rab27a and CD63 so weak in Sup Fig. 5F? The KD efficiency at the mRNA level seems quite good (Sup Fig. 5A). One would expect Arl8b KD to have a much stronger effect, if the authors' model were correct.
5. In the Methods section the authors specify that they employed in the siRNA knock-down experiments 5 micromolar siRNA concentrations. Is this really the final concentration they used? Commonly used siRNA concentrations are 5-30 nM, the highest I ever used myself in hard-to-transfect cells is 200 nM. High siRNA concentrations can easily saturate the RISC machinery and lead to unspecific effects by extensive disturbance of the cell's microRNA effector apparatus.
MINOR
1. The supplemental videos are in the text not referred to in numerical order: Suppl. Video 4 comes before number 2, and I could not find any reference to Suppl. Video 3.

2. Fig. 1D: The correlation plot looks strange, with a dark blue background. It is difficult to observe - please redo in another format.
3. End of paragarph 'Small GTPase decoration...' ....detected at the site of fusion (Fig. 2E-G; Sup. Fig. 2D) should be (Fig. 2F-G; Sup. Fig. 2D).
4. Paragraph 'ORP1L ER-LE MCS &...', 2nd page: ...2-fold compared to ORP1L-wt (Fig. 4G) should be (Fig. 4J).
5. Top of the next page: ....did not show any effect (Fig. 4E) should be (Fig. 4K).
6. Paragraph 'ORP1L ER-LE MCS & GTPase switching', 1st page, line 6 from the bottom: ...fusion activity (Fig. 5E, Sup Fig 5A) should be (Fig. 5D, Sup Fig. 5A)
7. Caption for Fig. 1: Bars: (a,c,f,h,i,j) should be in capital alphabet (A,C,F,H,I,J)
8. Suppl. Fig. 1: Here the authors report size distribution of fusion spots, but I cannot find a description of how this was measured. Please add this information in the Methods section.
9. Fig. 5F: How did the authors verify the overexpression of Rab5a, Rab7a and Arl8b in the imaged cells?

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

Summary:
In this manuscript Verweij et al use CD63-based quantitative dual-color live TIRF-microscopy to study the molecular identity of MVBs that fuse with the plasma membrane. They characterize this as a subpopulation of multivesicular organelles at a pre-lysosomal maturation stage that are not catalytically active. They show that whereas intracellular CD63 compartments are positive for the small GTPases RAB7A and ARL8B, the vesicles that fuse with the plasma membrane rather contain RAB27A/B. They suggest that the MVBs undergo a maturation that would prime them for fusion with the plasma membrane. Since RAB7 is involved in endosome maturation and motility through its various effector proteins and engagement in membrane contact sites, they set out to study the role of RAB7 and contact sites between the ER and late endosomes (ER-LE-MCS) in exocytosis of CD63 positive MVBs. They conclude that such exocytosis is inhibited by RAB7 depletion, and that it is regulated by ER-LE-MCS via the RAB7 effectors ORP1L and protrudin. Whereas protrudin mediated ER-LE-MCSs facilitate anterograde endosome transport, ORP1L is regulating retrograde translocation towards the microtubule organizing center (MTOC). They chose to continue to explore the role of ORP1L. By using mutants of ORP1L that affect its engagement with the ER or with its interaction with RILP/dynein, they conclude that CD63 positive MVBs are transported to the perinuclear region for efficient exocytosis. They go on to study the contribution from RAB7A, ARL8B and RAB27A in this process, and conclude that in order to be rendered fusion competent, the MVBs undergo a small GTPase cascade, switching from RAB7A to ARL8B to RAB27A, promoted by ORP1L.
This is a timely and interesting study that takes advantage of a recently developed TIRF-based dual-color live imaging approach to investigate the dynamic behavior of MVBs in exosome release. This approach gives the authors the possibility to study the involvement of MCSs and small GTPases in this process in a more dynamic way than previous work. Thus, the results from this study regarding the involvement of MCSs and small GTPases, represent an important advance in our understanding of exosome release, and the manuscript would be interesting to a broad readership.
The work is overall well conducted and presented in a logical way. However, some parts appear preliminary and poorly described. Before I can recommend publication, several issues need to be clarified and addressed:

Major points:

1. Measuring of fusion activity is an important readout throughout the manuscript. The authors need to explain in the methods how this is measured from the movies. Was it manually quantified or automatically segmented? Provide in the figure legends how many cells/movies/experiments that the data were collected from. In most graphs it is called fusion activity, in other graphs events per hour. Please define the measurement and be consistent. Some fusion activity data are shown as dot plots, or violin plots (I assume when the number of dots were too many), but also as bar diagrams. In the case of bar diagrams, please rather use violin or dot plots for transparency. The authors should also define what a dot in the diagram represents. Fusion events/hour/cell/movie, or something else? This point is relevant for Fig.1E, Fig.3A, C, F, Fig.4E, J, K, Fig.5 A, D, E,

2. The authors conclude that there is a subpopulation of MVBs that fuses with the plasma membrane for exosome release, by which I agree. Whereas the pHuji or pHluorin tagged CD63 are excellent markers for the population that undergo exosome fusion, they are less useful to study the intracellular population of MVBs, as they lose their fluorescence in acidic compartments (I assume that all the imaging using tagged proteins are captured from living cells, since otherwise is not stated). To emphasize their point that the fusion competent CD63 compartments define a specific subpopulation of MVBs, it would be very informative to compare the localization of the pHluorin tagged CD63 with a co-expressed globally localizing mCh-tagged CD63 (or a double tagged version). This could also be done in the context of the Magic Red CathepsinB in Fig.1E, comparing CD63-pHluorin with their eGFP tagged CD63, separately (which should be less affected by pH than the pHluorin version, and they claim give a more global distribution (Fig. 4H)).

3. A rather surprising finding in this work is the requirement for RILP-mediated perinuclear transport of MVBs prior to their fusion with the plasma membrane. Overexpression of RILP, accumulation of endosomal cholesterol by U18666A or the use of an ORP1L mutant that recruits dynein to endosomes (ORDPHDPHD), all lead to a perinuclear clustering of CD63 compartments

(as expected from the literature), and this unexpectedly facilitated fusion activity. Overexpression of SKIP, on the other hand did not increase fusion activity, despite is known role as an ARL8B effector that engages kinesin-1. There is little attempt in the present manuscript to explain this apparent paradox. Despite of that, the authors base their model on this observation, and suggest in the discussion that the perinuclear localization might facilitate the recruitment of ARL8B. If this is true, it would add important new knowledge to our mechanistic understanding of the switch from RAB7 to ARL8b.
SKIP and HOPS are mediating the switch from RAB7 to ARL8B, leading to kinesin-1 mediated translocation of ARL8B positive LEs to the cell periphery (Jongsma et al, 2020). RILP, on the other hand, has as far as I know not been implicated in this switch mechanism, but rather in the recruitment of dynein and HOPS leading to fusion and perinuclear localization of LEs, when LEs are rich in cholesterol. More experiments should be done to address the contribution from LE positioning in the GTPase switching. To that end, it would be important to uncouple the dynein-effect from the effect of MCS. What would happen with CD63 fusion activity in cells depleted for dynein (p150Glued)? Would this affect the appearance of RAB7, ARL8B or RAB27 in CD63 compartments, globally and in the cell periphery?
The authors try to mechanistically uncouple the dynein effect from the MCS by using a mutant of ORP1L, ORDFFATydaa. In the original publication where this mutant was used (Wijdeven et al., 2016) it is described as a mutant that cannot bind VAPA in the ER (loss of MCS), but it can induce RILP dependent perinuclear clustering. In the present manuscript, this mutant inhibits fusion, but there is no data showing how it affects the positioning of MVBs. Moreover, in the results section describing the use of this mutant (referring to Fig. 4G, but should be 4J), they claim that this mutant is not able to engage dynein. However, in the discussion, they state that this mutant can engage dynein. The authors need to clarify and correct this confusion, in order to be able to conclude about this mutant.
Jongsma et al 2020, show that ectopic expression of RILP leads to hyper fusion of LEs, perinuclear clustering and lack of canonical MVBs. Expression of SKIP in the background of RILP could rescue this phenotype. In the present manuscript, overexpression of RILP leads to perinuclear clustering and increased fusion of MVBs with the plasma membrane, whereas the overexpression of SKIP has no effect. How is the ultrastructure of endosomes under these conditions? Do the authors see canonical MVBs in RILP overexpressing cells? What would happen in cells co-expressing RILP and SKIP? Will there be a tug of war for LE localization, or will SKIP rather facilitate GTPase conversion in the presence of RILP, strengthening the notion that the perinuclear clustering is a prerequisite for fusion? How would this influence fusion activity?

4. A main conclusion in this manuscript, also reflected in the title, is that MCSs regulate GTPase conversion. This is mainly based on indirect evidence by characterizing the localization of the different small GTPases to CD63 positive compartments upon manipulation with ORP1L, or the different GTPases themselves. To make a stronger argument for their conclusion, the study would benefit from a more dynamic analysis of GTPase conversion. In Fig. 5B, C they use their dual-color live TIRF-microscopy to visualize the co-occurrence of RAB27A with either RAB7A or ARL8B. This type of imaging is a nice approach to learn more about such dynamic behavior. As it is difficult to judge the colocalization of the different GTPases in the still images in Fig. 5B, C, it would be important to track individual vesicles (should be possible with their imaging at 2 Hz) and present the transition of one GTPase to another as line plots. This could be done for some of the GTPases in combination with different types of manipulations like depletion or overexpression of ORP1L (wt or mutants) or upstream GTPases, RILP or SKIP over-expression, as well as in p150Glued depleted cells.

Specific points:
Figure 1
A) There is no EEA1 peaking at the site of CD63-pHuji fusion. How can we know that the EEA1-GFP reporter worked? Please provide an image showing the expression and localization of EEA1-GFP co-expressed with CD63-pHuji in the supplementary.
D) Collected from how many images/experiments? Please indicate in the Figure legend.
G) Show dot plots for transparency. Explain in the methods and/or in the figure legend how this was measured, how the dots were segmented and how overlapping fractions were determined (Manders?)
H) This is explained twice in slightly different wording in the figure legend. Please correct.
K) Please add label to the x-axis.

Figure 2
A, B) The authors claim in the text that this colocalizaiton analysis constitutes a global analysis of CD63 positive MVBs. How would the data look like using a pH stable tag for CD63 (see also major point 2)? Please define PCC. What is used as a control in the PCC analysis? How many cells/images were analyzed from how many experiments? Show dot plots for transparency. The PCC results in B) largely reflect the representative images shown in A). However, the high PCC value for Rab27a is not consistent with the rather low colocalization in the representative image.

Figure 3
B) myc or GFP-protrudin? Fixed or live imaging? Please describe in the figure legend.
F) The fusion activity (events/hour) for CD63 is very high (30-40) as compared to the other graphs where the mean fusion activity is typically below 10. Why?
G) Explain in the methods how this measurement was performed. Manual of automatic segmentation? Include in the figure legend from how many cells/movies/experiments these data are collected from.

Figure 4
A) ORP1L ORD should cause the attachment of LEs to the ER. This phenotype is apparent in Supplementary Fig. 4A, where

endogenous CD63 is co-localizing with ORP1L ORD and VAPA. In Fig.4A, however, CD63-pHluorin is not colocalizing with ORP1L ORD. It would be informative to explain the different behavior of CD63, which I assume is due to the pH sensitive tag (see also major point 2).

B) Please explain the red mask in the figure legend (I assume it is ER). The ultrastructure seems to be a bit differently preserved in the wt and ORD, or perhaps just differently contrasted. It would be nice to see a mask on ER in the wt image as well, to indicate that ER is equally well preserved in the two preparations.
C, D) This colocalization analysis with LAMP1 is based on CD63-pHluroin. Is this live of fixed? Endogenous of exogenous LAMP1? The colocalization between CD63 and LAMP1 would be different depending on this (see also major point 2). Please specify and comment. How would the co-localization be affected by ORP1L if using a global CD63 marker? Please describe how many images/cells/experiments this analysis was based on. Why do you prefer to use Manders for this analysis whereas you use PCC for others?
E) How can the authors explain that upon increased maturation to endolysosomes observed with the ORDPHDPHD mutant observed in C, D), that the CD63-pHluorin is more fusogenic? Since they argue before that it is the earlier subpopulation of CD63 MVBs that fuse. Please also correct the statistics in the graph. The wt/ ORDPHDPHD is presented with both two and four stars.
F, G) Remove lines between the three conditions in the graphs, and only show dot plots. Lines indicate a kinetic measurement. Statistics missing. Can the authors explain why CD63 is reduced but CD9 not in the EV fraction of ORD? What about other EV markers like CD81 or TSG101?
H) It is difficult to see the imotile and highly motile subpopulations in the supplemented movie. It would be helpful with some tracks indicating direction and velocity.
I) How was motility of CD63 compartments measured? Please describe in the methods.
K) This figure is not correctly cited in the results section (Says fig. 4E).

Figure 5
The letters of the figure legends to Fig. 5 do not correspond to the letters in the actual figure. This need to be corrected and double checked with the results section. The following comments rely on the numbering from the actual Figures:
B, C) This is a nice approach to visualize the co-occurrence of different GTPases. I assume that the white arrow heads indicate co-localizing dots (please describe the arrow heads in the legend). However, I find it difficult to be convinced that there is more co-occurrence with ARL8B in C) than with RAB7A in B). It would be important to quantify the co-localization from several images/cells/movies and experiments. This analysis would benefit from tracking the GTPase conversion of individual dots (see major point 4).
F) This figure (more examples in Supp Fig. 4E) shows that RAB27A is recruited to intracellular compartments upon over-expression of ARL8B, but not RAB5A or RAB7A. The images only show RAB27A. As an important control, the co-transfected GTPases should be visualized as well; in the same image as untransfected cells for directly comparison of RAB27A accumulation (ideally provided with a quantification of dot number and intensity from the different transfected vs untransfected cells).

Figure 6
Please refer to this figure in the text.

Supp Figure 1
B) A description of how the size (Bin Center, radius in nm) of the spots were measured is missing from the methods.
Supp Figure 2
A) Describe in the methods how the RT-PCR was conducted, including reagents.
B) Please provide a less contrasted WB, and describe WB in the methods.

Other points:

The dual-color TIRF microscopy developed to study exosome release in Bebelman 2020 and Verweij 2018 is a powerful method. In order for new readers to embrace this method as a reliable reporter system for exosome release, it would be important to provide a brief recapitulation of the method, the rationale behind the pHuji-tag and the use of NH4+. This can be done briefly in the results section, and more thoroughly in the methods section.

Explain the effect of U18666A

Explain better and correct the sentence in the results section regarding the use of NPY-pHluorin and VAMP2-pHluorin.

A reference to and discussion about the publication from Grinstein lab (Johnson et al., JCB 2016 PMID: 26975849), where they show that peripherally localizing endosomes are less acidic and have less degradative properties, should be relevant.

In the Methods and or Figure legends, please indicate which post hoc test was used in combination with ANOVA for multiple comparisons.

Which microscope was used for imaging of fixed cells? Please indicate which images/figures were based on Fixed cell imaging.

What does it mean that "Experiments were performed in RT (37oC)?

Which antibody was used to detect ORP1L by WB?

5 µM sounds like a very high concentration for siRNA, which is recommended to use in a concentration between 1 and 30 nM. Is this correct? For how long time was the siRNA transfection performed for the independent targets?

Rebuttal to the comments by the reviewers on manuscript "ER Membrane Contact Sites support endosomal small GTPase conversion for exosome secretion"

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

The manuscript reports a study that focuses on defining the molecular events that generate secretory endosomes (releasing exosomes, CD63 as their marker), distinguishing them from degradative endo-lysosomes. The authors identify the compartment of origin of CD63 positive endosomes as a subclass of non-proteolytic endosomes at prelysosomal stage. They provide evidence for a GTPase cascade from Rab7a to Arl8b and finally Rab27a that eventually generates CD63(+) endosomes capable of fusing with the plasma membrane (PM). Importantly, they also show data suggesting that ER-endosomal membrane contact sites (MCS) mediated by the proteins ORP1L and Protrudin play an important role in modulating the generation of the CD63(+) endosomes fusing with the PM, by affecting late endosome motility, maturation and GTPase association. The research question is novel and previously unsolved - so the study is well motivated.
The work relies mainly on advanced live cell imaging (mainly TIRF microscopy) of transfected HeLa cells; The imaging data is appropriately quantified. The data is for the most part credible and convincing; However, I do see a number of major and minor issues that require further scrutiny:
(I find it difficult to comment details in the manuscript since page numbers are missing - the page numbering should absolutely always be there)

MAJOR
1. Throughout the manuscript the authors employ imaging of CD63(+) vesicle fusion events as a proxy for exosome secretion. They analyze by EV isolation and western analysis the actual release of exosomes only in Fig. 4F, where they study the effects of wt and mutant ORP1Ls on the release. In my opinion the authors should additionally validate also some other key findings of the study by measuring the actual release of exosomes by the cells, considering that even in the title of the paper they claim they have studied 'exosome secretion'. Such key data could be e.g. the effects of Rab27a, Rab7a or Arl8b manipulations.

We thank the reviewer for his/her comment about the validation of our imaging approach by more conventional methods. We invite the reviewer to refer to our princeps paper where we have deeply investigated these aspects (Verweij et al JCB 2018). Conventional biochemical analysis of EVs with dUC/SEC followed by NTA or WB are well established approaches but have some limitations in that they measure 'bulk' sEVs of different subcellular origin(s) including exosomes. Moreover, the outcome measurement is the net-result of secretion minus re-uptake of EVs by the secreting cell, the extend of which might depend on various factors.

Exosome release quantification with CD63(-pHluorin) on the other hand is so far the only method able to specifically assess exosome release from internal compartments (ignoring EV release by budding from the PM) and therefore the best proxy. For the specific aim of elucidating MVB transport pathways to the PM, we therefore heavily rely on CD63-pHluorin/pHuji imaging, because in our opinion it is the most suitable approach as confirmed by several other independent studies (Messenger JCB 2018, Sung Nat Comm 2020).

Nevertheless, we validated some of our key findings with western blotting analysis on EVs for the various ORP1L constructs to consolidate the findings obtained with imaging. To further convince the reviewer, we have performed additional experiments including studying the effect of Rab7a (wt and DN) and Rab27a (wt and DN) on sEV secretion quantified by western blotting (Sup. Figs 3A, 5A).

2. The Rab7a-Arl8b-Rab27a cascade: The authors build the hypothesis on the cascade beginning with Rab7a based on the observation that Rab7a KD inhibited the fusion of CD63(+) endosomes with the PM and on a previous study (Jongsma et al, 2020). However, they do not show any evidence that the PM-fusing CD63(+) endosomes ever had Rab7a on their surface - the evidence is circumstantial. More direct evidence should be provided.

To provide evidence that PM-fusing CD63(+) endosomes had Rab7a on their surface prior to fusion we gathered multiple pieces of evidence that support this. Apart from Rab7a KD, we

1) show that Rab7a-wt and -DN overexpression directly impacts MVB/PM fusion; this is now also confirmed by western blot (Sup Fig 3A; see also our response to major point 1).
2) ORP1L is a direct effector of Rab7a, and indeed we see a strong overlap between the two (PCC ~0.8, Fig 5HI) in accordance with literature. While the effects of KD and (mutant) OE of Rab7a could potentially still be explained by indirect/secondary effects, the effects of ORP1L-dORD OE that immobilizes CD63/Rab7a(+/+) endosomes (Fig 4I) and strongly inhibits MVB/PM fusion and EV release (Fig 4EF) in our minds can only be explained if the population of these 'trapped' CD63/Rab7(+/+) endosomes include the 'precursors' of CD63(+) endosomes that would than later fuse with the PM.

Even though we made a serious effort in demonstrating the loss of Rab7a directly, this required complicated, advanced imaging: I) detecting a fusion event requires imaging with *at least* 1fps; II) tracking MVBs before fusion requires the addition of a second color to CD63-pHluorin/pHuji that is not pH-sensitive, with the GTPase tagged

in a third color; III) to span two subsequent GTPase-switches requires extended time-lapses (probably at least 15-20 minutes). This is still assuming that the 2 subsequent GTPase switching steps happens in the same imaging-plane as the fusion events. Otherwise, this longer-term, 3-color 1fps imaging would need to be done in volumes as well. For this reason, we have not tried this approach at the time of the first submission. In working on the revision, we generated emiRFP670-CD63-pHmScarlet that we expressed in Rab7-GFP HeLa cells. Unfortunately, our cells did not tolerate this modality of imaging for more than 3 minutes both on a TIRF as well as spinning disk set-up. This further confirms our previous concerns with this imaging approach.

We regret that the current state of art does not allow us to provide direct evidence in a timely manner and we now explain this more carefully in the text and use careful wording in that multiple lines of evidence suggest a model in which PM fusing CD63+ endosomes has lost Rab7a expression although we cannot formally rule out that a proportion of these compartments never carried Rab7a although we deem this scenario unlikely considering the evidence.

3. Protrudin overexpression effect is shown only for the fusion of CD63(+) vesicles, not LAMP1 vesicles, so it remains open if what the authors see is to any extent specific for the CD63(+) exosome releasing endosomes, or if protudin might have a general non-specific effect on any LE compartments. Is there a technical reason for the authors not to have measured the putative effect on LAMP1 endo-lysosome fusion?

The reviewer is right that we did not specifically look at LAMP1 vesicles in our Protrudin overexpression (OE) experiments. This is in part because LAMP1 is already present on CD63(+) MVBs fusing with the plasma membrane (Fig 1AB), albeit at seemingly low levels. LAMP1 presence would therefore not be the best proxy to determine if Protrudin has a general non-specific effect on any LE compartment. Because the point raised by the reviewer is nonetheless interesting, we reasoned that MagicRed, a marker for catalytic activity, would be more informative. We did not find MagicRed present at the site of fusion under steady-state conditions (Fig 1H,K), but if Protrudin OE would have a general effect on any LE compartments, one could expect to also see MagicRed to colocalize with (some) CD63(+) fusion events under Protrudin OE conditions. However, we obtained highly comparable results to steady-state conditions (Sup Fig 3C), showing absence of MagicRed signal at the site of fusion, while several MagicRed(+) vesicles could be observed near the PM. This suggests that the Protrudin OE effect on CD63(+) endosome fusion with the PM has a certain degree of selectivity, that is at least restricted to non-catalytically active endosomes.

4. If Arl8b precedes Rab27a in the GTPase cascade, why is the effect of siArl8b on the overlap between Rab27a and CD63 so weak in Sup Fig. 5F? The KD efficiency at the mRNA level seems quite good (Sup Fig. 5A). One would expect Arl8b KD to have a much stronger effect, if the authors' model were correct.

The reviewer is correct that the effect of siArl8b on Rab27a/CD63 overlap is modest. Yet, several lines of evidence implicate Arl8b in the process leading to MVB/PM fusion: 1) Arl8b KD reduces fusion activity; 2) Arl8b wt OE increases fusion activity; 3) Arl8b DN OE decreases fusion activity [new data included in revision]; 4) High ORP1L-dORDPHDPHD fusion activity is dampened by Arl8b DN OE [new data included in revision]; 5) overexpression of SKIP with its “known role as an ARL8B effector that engages kinesin-1” (reviewer 2) also shows a stimulatory effect on MVB/PM fusion. With respect to the precise mechanism, different explanations are possible: I) Switching from to Rab7a to Arl8b might be necessary for allowing the switch to Rab27a; II) Arl8b might facilitate MVB-PM fusion by promoting peripheral transport (kinesin), after which Rab27a can function (myosin) i.e. the effect is positional. Indeed, numerous reports have shown effects of Arl8b depletion on endolysosomal positioning in (e.g. Korolchuk et al., NCB 2011; Lu et al Sci Adv. 2020). The second mechanisms theoretically might not have immediate consequences on Rab27a/CD63 overlap upon siArl8b KD. Yet, they are not mutually exclusive, and we don’t have definitive proof to distinguish between or exclude one of these options.

5. In the Methods section the authors specify that they employed in the siRNA knock-down experiments 5 micromolar siRNA concentrations. Is this really the final concentration they used? Commonly used siRNA concentrations are 5-30 nM, the highest I ever used myself in hard-to-transfect cells is 200 nM. High siRNA concentrations can easily saturate the RISC machinery and lead to unspecific effects by extensive disturbance of the cell's microRNA effector apparatus.

We apologize for this mistake; indeed, we used a final concentration of 30 nM.

MINOR
1. The supplemental videos are in the text not referred to in numerical order: Suppl. Video 4 comes before number 2, and I could not find any reference to Suppl. Video 3.

Indeed, we missed this after an earlier rearrangement of the data, and we thank the reviewer for pointing this out.

2. Fig. 1D: The correlation plot looks strange, with a dark blue background. It is difficult to observe - please redo in another format.

The blue background was a heat-map to indicate density but indeed was not adding much; we therefore removed it in the current version of the manuscript.

3. End of paragarph 'Small GTPase decoration...' ....detected at the site of fusion (Fig. 2E-G; Sup. Fig. 2D) should be (Fig. 2F-G; Sup. Fig. 2D).

We corrected this reference in the text.

4. Paragraph 'ORP1L ER-LE MCS &...', 2nd page: ...2-fold compared to ORP1L-wt (Fig. 4G) should be (Fig. 4J).

We corrected this mistake.

5. Top of the next page: ....did not show any effect (Fig. 4E) should be (Fig. 4K).

We corrected this mistake.

6. Paragraph 'ORP1L ER-LE MCS & GTPase switching', 1st page, line 6 from the bottom: ...fusion activity (Fig. 5E, Sup Fig 5A) should be (Fig. 5D, Sup Fig. 5A)

We corrected this mistake.

7. Caption for Fig. 1: Bars: (a,c,f,h,i,j) should be in capital alphabet (A,C,F,H,I,J)

We corrected this mistake.

8. Suppl. Fig. 1: Here the authors report size distribution of fusion spots, but I cannot find a description of how this was measured. Please add this information in the Methods section.

We have now added this description in the methods section (page 23), and we thank the reviewer for pointing this out.

9. Fig. 5F: How did the authors verify the overexpression of Rab5a, Rab7a and Arl8b in the imaged cells?

All cells shown overexpressed the respective small-GTPases, as verified by the (GTPase-)RFP signal. The red channel is left out here, as it allows to appreciate the Rab27a localization better. We have now provided the RFP signals for the small GTPases (see data to the reviewers).

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

Summary:
In this manuscript Verweij et al use CD63-based quantitative dual-color live TIRF-microscopy to study the molecular identity of MVBs that fuse with the plasma membrane. They characterize this as a subpopulation of multivesicular organelles at a pre-lysosomal maturation stage that are not catalytically active. They show that whereas intracellular CD63 compartments are positive for the small GTPases RAB7A and ARL8B, the vesicles that fuse with the plasma membrane rather contain RAB27A/B. They suggest that the MVBs undergo a maturation that would prime them for fusion with the plasma membrane. Since RAB7 is involved in endosome maturation and motility through its various effector proteins and engagement in membrane contact sites, they set out to study the role of RAB7 and contact sites between the ER and late endosomes (ER-LE-MCS) in exocytosis of CD63 positive MVBs. They conclude that such exocytosis is inhibited by RAB7 depletion, and that it is regulated by ER-LE-MCS via the RAB7 effectors ORP1L and protrudin. Whereas protrudin mediated ER-LE-MCSs facilitate anterograde endosome transport, ORP1L is regulating retrograde translocation towards the microtubule organizing center (MTOC). They chose to continue to explore the role of ORP1L. By using mutants of ORP1L that affect its engagement with the ER or with its interaction with RILP/dynein, they conclude that CD63 positive MVBs are transported to the perinuclear region for efficient exocytosis. They go on to study the contribution from RAB7A, ARL8B and RAB27A in this process, and conclude that in order to be rendered fusion competent, the MVBs undergo a small GTPase cascade, switching from RAB7A to ARL8B to RAB27A, promoted by ORP1L.
This is a timely and interesting study that takes advantage of a recently developed TIRF-based dual-color live imaging approach to investigate the dynamic behavior of MVBs in exosome release. This approach gives the authors the possibility to study the involvement of MCSs and small GTPases in this process in a more dynamic way than previous work. Thus, the results from this study regarding the involvement of MCSs and small GTPases, represent an important advance in our understanding of exosome release, and the manuscript would be interesting to a broad readership.
The work is overall well conducted and presented in a logical way. However, some parts appear preliminary and poorly described. Before I can recommend publication, several issues need to be clarified and addressed:

Major points:

1. Measuring of fusion activity is an important readout throughout the manuscript. The authors need to explain in the methods how this is measured from the movies. Was it manually quantified or automatically segmented? Provide in the figure legends how many cells/movies/experiments that the data were collected from. In most graphs it is called fusion activity, in other graphs events per hour. Please define the measurement and be consistent. Some fusion activity data are shown as dot plots, or violin plots (I assume when the number of dots were too many), but also as bar diagrams. In the case of bar diagrams, please rather use violin or dot plots for transparency. The authors should also define what a dot in the diagram represents. Fusion events/hour/cell/movie, or something else? This point is relevant for Fig.1E, Fig.3A, C, F, Fig.4E, J, K, Fig.5 A, D, E,

We have made all graphs referring to fusion activity consistent by showing them as violin plots and equalizing the measurement unit ("fusion activity") which is now also defined in the methods section. Events were quantified manually and validated using the AMvBE Fiji/ImageJ macro (Bebelman et al. 2020) for quality control.

2. The authors conclude that there is a subpopulation of MVBs that fuses with the plasma membrane for exosome release, by which I agree. Whereas the pHuji or pHluorin tagged CD63 are excellent markers for the population that undergo exosome fusion, they are less useful to study the intracellular population of MVBs, as they lose their fluorescence in acidic compartments (I assume that all the imaging using tagged proteins are captured from living cells, since otherwise is not stated). To emphasize their point that the fusion competent CD63 compartments define a specific subpopulation of MVBs, it would be very informative to compare the localization of the pHluorin tagged CD63 with a co-expressed globally localizing mCh-tagged CD63 (or a double tagged version). This could also be done in the context of the Magic Red CathepsinB in Fig.1E, comparing CD63-pHluorin with their eGFP tagged CD63, separately (which should be less affected by pH than the pHluorin version, and they claim give a more global distribution (Fig. 4H)).

This analysis was in fact done on fixed cells, meaning that the late-endosomal low-pH quenching effect on pHluorin as expected in living cells is absent. We apologize to the reviewer for not stating this more clearly in the methods, which we have now corrected. With that, the suggestions of the reviewer are in fact already incorporated in the first version of the manuscript. Indeed, the PM-fusing MVBs represent a subpopulation of late endosomes. With CD63-pHluorin, we do not only label the PM-fusing compartments but also a subpopulation of catalytically active endo-lysosomes, a notion we also explicitly state now in the manuscript on page 3. However, we could not find any evidence that this latter population is also fusing with the plasma membrane.

3. A rather surprising finding in this work is the requirement for RILP-mediated perinuclear transport of MVBs prior to their fusion with the plasma membrane. Overexpression of RILP, accumulation of endosomal cholesterol by U18666A or the use of an ORP1L mutant that recruits dynein to endosomes (ΔORDPHDPHD), all lead to a perinuclear clustering of CD63 compartments (as expected from the literature), and this unexpectedly facilitated fusion activity. Overexpression of SKIP, on the other hand did not increase fusion activity, despite is known role as an ARL8B effector that engages kinesin-1. There is little attempt in the present manuscript to explain this apparent paradox.

We thank the reviewer for highlighting this puzzling discrepancy. As we were also puzzled by the absence of an effect of SKIP overexpression, we decided to perform additional stainings and observed that the plasmid did not always lead to consistent expression levels of SKIP. After resolving these issues, we could detect a consistent stimulatory effect of SKIP overexpression on fusion activity. We rectified this in the current manuscript and further developed the concept of a two steps process that requires first a retrograde transport of MVBs and then an anterograde transport to mature and acquire a secretory capacity.

Despite of that, the authors base their model on this observation, and suggest in the discussion that the perinuclear localization might facilitate the recruitment of ARL8B. If this is true, it would add important new knowledge to our mechanistic understanding of the switch from RAB7 to ARL8b.

Indeed, the link between perinuclear localization and Rab7-Arl8b switching is intriguing, and further supported by our new data showing that Arl8b-DN overexpression inhibits the stimulatory effect of ORP1l-ΔORDPHDPHD on MVB-PM fusion. The reviewer is right in that exploring this would bring important new insights to our understanding of the Rab7a-Arl8b switch. We thank the reviewer for stressing this point and added this notion to the discussion.

SKIP and HOPS are mediating the switch from RAB7 to ARL8B, leading to kinesin-1 mediated translocation of ARL8B positive LEs to the cell periphery (Jongsma et al, 2020). RILP, on the other hand, has as far as I know not been implicated in this switch mechanism, but rather in the recruitment of dynein and HOPS leading to fusion and perinuclear localization of LEs, when LEs are rich in cholesterol. More experiments should be done to address the contribution from LE positioning in the GTPase switching. To that end, it would be important to uncouple the

dynein-effect from the effect of MCS. What would happen with CD63 fusion activity in cells depleted for dynein (p150Glued)? Would this affect the appearance of RAB7, ARL8B or RAB27 in CD63 compartments, globally and in the cell periphery?

We have uncoupled ORP1L's MCS- from its dynein-effect by using the different mutants, notably the ORP1L-FFAT mutant that harbors specific point-mutations (ydaa) preventing its binding to VAP-A, and showing less fusion activity compared to control. The ORP1L-ΔORDPHDPHD mutant on the other hand showed higher fusion activity and was associated most strongly with Arl8b. In addition, we overexpressed RILP which stimulated fusion activity. Since depletion of dynein is lethal to cells, we took a slightly different approach by overexpressing RILP-ΔN and p50/dynamityn. This data is now added to the manuscript. Overexpression the p50 fragment acts as a dominant negative to $p150^{Glued}$ (Jacqot et al., JBC 2010) and reduced MVB-PM fusion activity. RILP-ΔN can still associate to endosomes, but cannot recruit any of its effectors, thereby functioning as a dominant negative. As expected, RILP-ΔN overexpression reduced fusion activity. The effects of various of these constructs on endosomal positioning are in full accordance with current literature, and are included in the current version of the manuscript, in Sup Fig 4E.

In summary, to further implicate RILP in fusion activity and location-based switching, we find:
1) RILP-ΔN (no dynein recruitment, less perinuclear clustering), decrease in fusion activity
2) p50 subunit overexpression (DN on dynein activity, endosomal scattering) decreases fusion activity
3) High ORP1L-dORDPHDPHD fusion activity is decreased by Arl8b DN OE.

The authors try to mechanistically uncouple the dynein effect from the MCS by using a mutant of ORP1L, ΔORDFFATydaa. In the original publication where this mutant was used (Wijdeven et al., 2016) it is described as a mutant that cannot bind VAPA in the ER (loss of MCS), but it can induce RILP dependent perinuclear clustering. In the present manuscript, this mutant inhibits fusion, but there is no data showing how it affects the positioning of MVBs.

Indeed, it is definitely of interest to couple the ΔORDFFATydaa effect to MVB positioning. We compared CD63 localization under ΔORDFFATydaa- to ΔORD- and ΔORDPHDPHD-ORP1L overexpression, that is now included in Sup Fig 4D. We observed a moderate centripetal movement for CD63 compartments under ΔORDFFATydaa overexpression conditions, compared to ΔORD, but much less pronounced than ΔORDPHDPHD. Wijdeven et al., 2016 indeed showed that combination with RILP OE shows endosomal accumulation around the nucleus, confirming that ΔORDFFATydaa can still interact with dynein. Taken together with the ΔORD effect on fusion activity, this indicates that apart from the dynein effect, (dynamic) ER-LE MCS are likewise required for maturation into PM-fusion competent MVBs.

Moreover, in the results section describing the use of this mutant (referring to Fig. 4G, but should be 4J), they claim that this mutant is not able to engage dynein. However, in the discussion, they state that this mutant can engage dynein. The authors need to clarify and correct this confusion, in order to be able to conclude about this mutant.

The statement in the discussion, i.e. that the mutant can engage dynein is correct, see cited references. We apologize for the confusion as we stated in the results that the mutant is not *constitutively* engaging dynein. This as opposed to dORDPHPHD (that does *constitutively* engage dynein) and dORD (that cannot engage dynein). We realize this was not clear from the text and have now clarified this aspect.

Jongsma et al 2020, show that ectopic expression of RILP leads to hyper fusion of LEs, perinuclear clustering and lack of canonical MVBs. Expression of SKIP in the background of RILP could rescue this phenotype. In the present manuscript, overexpression of RILP leads to perinuclear clustering and increased fusion of MVBs with the plasma membrane, whereas the overexpression of SKIP has no effect. How is the ultrastructure of endosomes under these conditions? Do the authors see canonical MVBs in RILP overexpressing cells? What would happen in cells co-expressing RILP and SKIP? Will there be a tug of war for LE localization, or will SKIP rather facilitate GTPase conversion in the presence of RILP, strengthening the notion that the perinuclear clustering is a prerequisite for fusion? How would this influence fusion activity?

We now realized this (lack) of effect of ectopic SKIP expression was due to expression issues (see also our response to comment Rev 1). The ultrastructure of endosomes under both conditions was demonstrated by Jongsma et al 2020; we now refer to this study in the discussion of our manuscript also with reference to the ultrastructural features. It should be noted that at the most rudimentary level our study assesses MVB/PM fusion per se. Effects on maturation (ILV generation and/or degradation) may not immediately affect the fusion activity, whereas dUC and western-blotting analysis might be impacted by this.
In the revised manuscript, we added a number of (control) experiments that further strengthen the notion that centripetal movement / perinuclear clustering is a prerequisite for fusion, including overexpression of RILP-ΔN and p50/dynamityn (Sup. Fig. 4G). In addition, we demonstrated that the stimulatory effect of ORP1L-dORDPHDPHD, that correlates with perinuclear clustering and increased overlap/switching to Arl8b, could be nullified by co-expression of Arl8b-DN (Fig. 5J). We did do some preliminary tests with SKIP/RILP co-expression,

but on average we could not demonstrate a significant effect on fusion activity. This might indeed be the results of a tug of war as the reviewer suggests interpretation of these experiments is challenging. Since these results remain inconclusive, we decided not to include them in the manuscript as these negative findings do not change the main conclusion of this manuscript.

4. A main conclusion in this manuscript, also reflected in the title, is that MCSs regulate GTPase conversion. This is mainly based on indirect evidence by characterizing the localization of the different small GTPases to CD63 positive compartments upon manipulation with ORP1L, or the different GTPases themselves. To make a stronger argument for their conclusion, the study would benefit from a more dynamic analysis of GTPase conversion. In Fig. 5B, C they use their dual-color live TIRF-microscopy to visualize the co-occurrence of RAB27A with either RAB7A or ARL8B.
This type of imaging is a nice approach to learn more about such dynamic behavior. As it is difficult to judge the colocalization of the different GTPases in the still images in Fig. 5B, C, it would be important to track individual vesicles (should be possible with their imaging at 2 Hz) and present the transition of one GTPase to another as line plots. This could be done for some of the GTPases in combination with different types of manipulations like depletion or overexpression of ORP1L (wt or mutants) or upstream GTPases, RILP or SKIP over-expression, as well as in p150Glued depleted cells.

We agree that dynamic analysis would be of added value. However as discussed in our response to reviewer one (major point 2), visualizing these GTPase cascades leading up to MVB-PM fusion with our live imaging proved extremely challenging. We could imagine a follow-up study by generating endogenous CRISPR-CAS knock-in cell lines for Rab7 and Arl8b. Though this might provide more definite proof, generating such lines, characterization and the imaging will take a serious effort and we feel we cannot provide such results in a timely fashion.

Specific points:
Figure 1
A) There is no EEA1 peaking at the site of CD63-pHuji fusion. How can we know that the EEA1-GFP reporter worked? Please provide an image showing the expression and localization of EEA1-GFP co-expressed with CD63-pHuji in the supplementary.

We always select double transfected cells before doing dual-color TIRF acquisitions. We have now included a representative image in Sup Fig 1A.

D) Collected from how many images/experiments? Please indicate in the Figure legend.

In the resubmission we have added all relevant statistical details to the methods section and/or legends now, according to the JCB resubmission guidelines.

G) Show dot plots for transparency. Explain in the methods and/or in the figure legend how this was measured, how the dots were segmented and how overlapping fractions were determined (Manders?)

We have added the measurement details to the material and methods section. Because of the different morphological appearance of CD63 and LAMP1 (dot vs donut shape) we did not opt for conventional colocalization analysis with MagicRed, as this would not be a solid comparison.

H) This is explained twice in slightly different wording in the figure legend. Please correct.

We have corrected this.

K) Please add label to the x-axis.

The label was accidentally cropped, we have now corrected this.

Figure 2
A, B) The authors claim in the text that this colocalizaiton analysis constitutes a global analysis of CD63 positive MVBs. How would the data look like using a pH stable tag for CD63 (see also major point 2)?

Fixation neutralizes the pH, rendering all CD63-pHluorin fluorescent. Therefore, there is no reason to assume a pH-stable tag would render different results.

Please define PCC. What is used as a control in the PCC analysis? How many cells/images were analyzed from how many experiments? Show dot plots for transparency. The PCC results in B) largely reflect the representative images shown in A). However, the high PCC value for Rab27a is not consistent with the rather low colocalization in the representative image.

PCC was analyzed using the JACoP plugin in Fiji, using Costes' automated thresholding. As control we used the Rab5 data flipped in Y and Z. In the resubmission we have added all relevant analysis details to the methods section. We showed dot plots. Regarding the PCC value for Rab27a, this overlap is consistent throughout experiments, and close inspection of the zoom in (1st submission) shows that roughly half of the CD63 compartments (perhaps the less bright/smaller ones) are associated to Rab27a. In the current manuscript, we have nevertheless replaced this figure for one that shows this phenotype more clearly.

Figure 3
B) myc or GFP-protrudin? Fixed or live imaging? Please describe in the figure legend.

We have mentioned this in the figure and the figure legend.

F) The fusion activity (events/hour) for CD63 is very high (30-40) as compared to the other graphs where the mean fusion activity is typically below 10. Why?
This is due to the different unit, i.e. fusion events/hour. We have now homogenized this by using the same units for fusion activity as for all other experiments using this read-out.

G) Explain in the methods how this measurement was performed. Manual of automatic segmentation? Include in the figure legend from how many cells/movies/experiments these data are collected from.

These vesicles are manually counted upon superfusion of the cells with a NH4+ solution using a barrel pipette. This is now explained in the material and methods section.

Figure 4
A) ORP1L ΔORD should cause the attachment of LEs to the ER. This phenotype is apparent in Supplementary Fig. 4A, where endogenous CD63 is co-localizing with ORP1L ΔORD and VAPA. In Fig.4A, however, CD63-pHluorin is not colocalizing with ORP1L ΔORD. It would be informative to explain the different behavior of CD63, which I assume is due to the pH sensitive tag (see also major point 2).

We now provide the single channel images in gray-scale with inverted LUT for the pictures of Fig.4A in Sup Fig 1B. These images make it easier to appreciate the overlap of ORP1L ΔORD with CD63(-pHluorin), which is in fact rather good, and does not differ significant from endogenous CD63 (Sup Fig 1A). It is true that individual fluorescent intensity levels fluctuate more with the ORP1L ΔORD/CD63 and ORP1L wt /CD63, but we observe the same phenomenon for endogenous CD63. In addition, and as mentioned above, fixation neutralizes the pH, which should render all CD63-pHluorin fluorescent.

B) Please explain the red mask in the figure legend (I assume it is ER). The ultrastructure seems to be a bit differently preserved in the wt and ΔORD, or perhaps just differently contrasted. It would be nice to see a mask on ER in the wt image as well, to indicate that ER is equally well preserved in the two preparations.

The mask indeed indicates the ER, we have now added this to the figure legend. The reviewer is right, there appears to be a slightly different contrasting efficiency in the two conditions. We indicated the ER now also in the wt image and changed the figure slightly so that everything can be better appreciated.

C, D) This colocalization analysis with LAMP1 is based on CD63-pHluroin. Is this live of fixed? Endogenous of exogenous LAMP1? The colocalization between CD63 and LAMP1 would be different depending on this (see also major point 2). Please specify and comment. How would the co-localization be affected by ORP1L if using a global CD63 marker? Please describe how many images/cells/experiments this analysis was based on. Why do you prefer to use Manders for this analysis whereas you use PCC for others?

This analysis was done cells with labelling for endogenous LAMP1 and CD63-pHluorin co-expression as indicated in the figure legend. This IF labelling is always done on fixed cells, where PFA fixation unquenches the fluorescence of CD63-pHluorin. This will therefore not influence our analysis (see also our comment on major point 2). This analysis was done approximately 6 years ago on a dataset acquired on a wide-field set-up, which is why the chosen analysis is sometimes different. In the resubmission we have added all relevant statistical details to the methods section and/or legends now, according to the JCB resubmission guidelines.

E) How can the authors explain that upon increased maturation to endolysosomes observed with the ΔORDPHDPHD mutant observed in C, D), that the CD63-pHluorin is more fusogenic? Since they argue before that it is the earlier subpopulation of CD63 MVBs that fuse. Please also correct the statistics in the graph. The wt/ΔORDPHDPHD is presented with both two and four stars.

We have now corrected the mislabeling of the statistical analysis. We do indeed think that maturation is increased with the ΔORDPHDPHD mutant, including maturation into endolysosomes. Regarding this point, we want to make three remarks:

1) It is not said that the “majority phenotype” of late-endosomes (in this case showing increased LAMP1 overlap) is identical to the population that actually fuses with the plasma membrane. In fact, Figures 1 and 2 combined amply demonstrate that it is the Rab27 sub-population that is fusing with the plasma membrane, whereas the two dominant/main LE markers (Rab7a and Arl8b) are absent from the site of fusion with the plasma membrane.
2) That being said, we do show that MVBs fusing with the PM do have LAMP1 present on their limiting membrane, even though they do not harbor catalytic activity. This could mean that the ΔORDPHDPHD mutant as opposed to the ΔORD mutant provides enough maturation for these MVBs to become fusogenic with either the plasma membrane or lysosomes.
3) We therefore think that ΔORDPHDPHD could be stimulating the fusion of MVBs with lysosomes *as well as* with the plasma membrane (the latter is what we demonstrate). Thus, these two alternative fates for MVBs can be simultaneously stimulated, i.e. the ‘turnover’ of MVBs could be increased.

F, G) Remove lines between the three conditions in the graphs, and only show dot plots. Lines indicate a kinetic measurement. Statistics missing. Can the authors explain why CD63 is reduced but CD9 not in the EV fraction of ΔORD? What about other EV markers like CD81 or TSG101?

With the dashed lines we linked the individual experiments as this provides a more detailed insight into the trends. After reading the reviewers comment, we understand this could nevertheless also cause confusion for some readers, which is what we aim to minimize. We have now changed the graph type to a column bar graph type. Regarding the reviewer’s comment on the reduction of CD63 but not CD9 in the EV fraction: CD9 is more enriched in ectosomes budding from the plasma membrane whereas CD63 is relatively more enriched in/on late endosomes and endosome derived exosomes (Mathieu et al Nat Comm 2021). Since ORP1L acts specifically on LE/MVBs and not on the plasma membrane, this result is as one would expect. We thank the reviewer for pointing this out, and have mentioned this now in the revised manuscript. We did not test CD81, but we have now included Alix as additional EV marker.

H) It is difficult to see the imotile and highly motile subpopulations in the supplemented movie. It would be helpful with some tracks indicating direction and velocity.

In the new version of the manuscript, we now also visualized the only ORP1L(+),the CD63/ORP1L(+/+), and the only CD63(+), that are shown in red, yellow and green next to the composite. We hope this helps the reviewer to appreciate the motile subpopulations of only CD63(+).

I) How was motility of CD63 compartments measured? Please describe in the methods.

The motility was measured using TrackMate plugin in Fiji. We have now mentioned this in the methods section and thank the reviewer for pointing this out.

K) This figure is not correctly cited in the results section (Says fig. 4E).

We have corrected this mis-citation in the current version of the manuscript.

Figure 5
The letters of the figure legends to Fig. 5 do not correspond to the letters in the actual figure. This need to be corrected and double checked with the results section.

We have now corrected the legends for figure 5 and thank the reviewer for pointing this out.

The following comments rely on the numbering from the actual Figures:
B, C) This is a nice approach to visualize the co-occurrence of different GTPases. I assume that the white arrow heads indicate co-localizing dots (please describe the arrow heads in the legend). However, I find it difficult to be convinced that there is more co-occurrence with ARL8B in C) than with RAB7A in B). It would be important to quantify the co-localization from several images/cells/movies and experiments. This analysis would benefit from tracking the GTPase conversion of individual dots (see major point 4).

Indeed, the reviewer is correct in assuming that the white arrowheads indicate co-localizing/co-occurrence of the respective small GTPases. This was indeed missing in the legend and we have now corrected this. We have now included a quantification in Sup Fig 5A.

F) This figure (more examples in Supp Fig. 4E) shows that RAB27A is recruited to intracellular compartments upon over-expression of ARL8B, but not RAB5A or RAB7A. The images only show RAB27A. As an important control, the co-transfected GTPases should be visualized as well; in the same image as untransfected cells for directly comparison of RAB27A accumulation accumulation (ideally provided with a quantification of dot number and intensity from the different transfected vs untransfected cells).

We kindly refer the reviewer to our comment to Reviewer 1 point 9. In our opinion comparing different small GTPases to each other – as we did - provides the best control. We do agree with the reviewer that a small

GTPase non-transfected but Rab27a transfected cell next to a double transfected cell could indeed serve as an additional, internal control. However, we regularly achieve >90% co-transfection rates, and therefore, this exercise would unfortunately be the proverbial searching for a needle in a haystack. To still accommodate the reviewer's request, we included Rab27a expression data in control cells (See Sup Fig 5BC), that shows little recruitment of Rab27a to compartments, consistent with Hannah et al., 2003. In addition, we also added the quantification of Rab27a endosome number and -intensity (as fold-change over background) in Sup Fig. 5D as per reviewer's request.

Figure 6
Please refer to this figure in the text.

We now refer to figure 6 in the text.

Supp Figure 1
B) A description of how the size (Bin Center, radius in nm) of the spots were measured is missing from the methods.

We have now added this description in the methods section (page 23), and we thank the reviewer for pointing this out.

Supp Figure 2
A) Describe in the methods how the RT-PCR was conducted, including reagents.

We have now added the RT-PCR description in the methods section (page 22), and we thank the reviewer for pointing this out.

B) Please provide a less contrasted WB, and describe WB in the methods.

Since Supp Figure 2B does not contain a WB but Supp Figure 3B does, we assume this is the panel the reviewer is referring to. We have updated these panels with less contrasted images.

Other points:

The dual-color TIRF microscopy developed to study exosome release in Bebelman 2020 and Verweij 2018 is a powerful method. In order for new readers to embrace this method as a reliable reporter system for exosome release, it would be important to provide a brief recapitulation of the method, the rationale behind the pHuji-tag and the use of NH4+. This can be done briefly in the results section, and more thoroughly in the methods section.

We thank the reviewer for this appreciation of our live-cell exosome release reporter and following these suggestions we have briefly explained the dual-color TIRF approach in the results section. The NH4+ approach is explained now briefly in the results as well as in the methods section.

Explain the effect of U18666A

We have now briefly explained the effect of U18666A treatment in the results section.

Explain better and correct the sentence in the results section regarding the use of NPY-pHluorin and VAMP2-pHluorin.

We have now better corrected and better explained the result section concerning the use of NPY- and VAMP2-pHluorin

A reference to and discussion about the publication from Grinstein lab (Johnson et al., JCB 2016 PMID: 26975849), where they show that peripherally localizing endosomes are less acidic and have less degradative properties, should be relevant.

We thank the reviewer for bringing this relevant study to our attention, we now have indeed referred to and discussed this study.

In the Methods and or Figure legends, please indicate which post hoc test was used in combination with ANOVA for multiple comparisons.

For ANOVA for multiple comparisons, we used Tukey post hoc tests.

Which microscope was used for imaging of fixed cells? Please indicate which images/figures were based on

Fixed cell imaging.

For imaging fixed cells, we used confocal and wide-field microscopy. We have indicated this in the text now.

What does it mean that "Experiments were performed in RT (37oC)?
This was supposed to read “experiments were performed at 37oC (…)”, and we have adapted the text accordingly.

Which antibody was used to detect ORP1L by WB?

The ORP1L antibody uses is a Rabbit monoclonal Ab132265; we added the missing information to the M&M section.

5 µM sounds like a very high concentration for siRNA, which is recommended to use in a concentration between 1 and 30 nM. Is this correct? For how long time was the siRNA transfection performed for the independent targets?

We apologize for this mistake; indeed, we used a final concentration of 30 nM. The siRNA/RT-PCR experiments are now described in the methods section.

August 22, 2022

RE: JCB Manuscript #202112032R

Dr. Frederik Johannes Verweij
Utrecht University
Padualaan 8
Utrecht 3584 CH
Netherlands

Dear Dr. Verweij:

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e. Fluorochromes
f. Camera make and model
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We agree that overexpression of Rab27a-wt does not show a clear increase in CD63 levels compared to control, even though the HSP70 levels do show an increase. The decrease for Rab27a-DN on the other hand is clearly visible. We focused mostly on the latter but agree with the reviewer that the increase for the Rab27a-wt overexpression is not as striking as one would expect. This could be due to a more general increase of LRO/PM fusion, and/or Rab27a-wt overexpression does not promote maturation as measured by ILV formation. Rab27a-wt overexpression would in that case lead to pre-mature fusion of (CD63+) MVBs with the PM.
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