EMBO reports

ALIX and ceramide differentially control polarized small extracellular vesicle release from epithelial cells

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1st Editorial Decision 22nd Sep 2020

Dear Prof. Fukuda

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, the referees also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed. It will be important to provide further quantification and all control experiments, to extend the analysis to other markers than CD9 and to discriminate whether the observed vesicles are indeed exosomes or rather small EVs budding from the plasma membrane.

Given the constructive comments from the referees, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be December 22nd in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further and also if you see a paper with related content published elsewhere.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).

Specifically, we would kindly ask you to provide public access to the following mass spectrometry dataset.

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

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- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n (biological, technical), P and the test applied.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

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Referee #1:

Matsui et al. studied the release of exosomes in polarized MDCK epithelial cells. They isolated extracellular vesicles that were shed from either the apical or basolateral domain followed by mass spec analysis to determine that there is some degree of differential release of cargos at either site. They then focused on CD9-positive exosomes and found that only those released into the apical medium contained GPRC5C. They further show that KD of Alix prevented apical but not basolateral release of CD9 exosomes. In contrast, inhibition of sphingomyelin synthesis with the inhibitor GW4869 decreased basolateral but not apical release of CD9 exosomes.

The study of differential exosome release from polarized cells is novel and of interest. However, the study needs further clarification. In my mind, the biggest caveat of the story is the relatively low percentage of CD9 exosomes released apically in comparison to overall apically released extracellular vesicles and also in comparison to CD9-positive exosomes released basolaterally. Can the authors rule out that apically released CD9-positive exosomes are not mistargeted? Why are only CD9-positive exosomes analyzed? Are there CD9-negative exosomes?

Major comments:

1) Figure 1C: The immuno-EM should be quantified. What is the percentage of CD9-positive EVs? It looks like less than 50% in the basolateral sample and maybe ~10% for the apical sample. Does that mean that exosomes are mainly secreted basolaterally? Are exosomes released at the apical membrane CD9 negative?

2) Figure EV2: A large portion of CD9 is localized at the basolateral membrane. This makes sense as CD9 has been described as a tetraspanin protein that is associated with integrins. Could the authors comment what this might mean with respect to CD9-positive exosomes? Are these exosomes released basolaterally because they contain CD9?

3) In the KD and inhibitor studies: How was cell polarity controlled? This is of concern, because MDCK cells that are not fully polarized may by default misdirect exosomes in non-specific ways.

4) Regarding the model:
a. Syndecan-1 is a protein that is targeted to the basolateral membrane by means of its PDZ domain [https://doi.org/10.1111/j.1600-0854.2008.00805.x]. How does its BL localization relate to a function in apical exosome release? This is the same question I had about the basolateral localization of CD9.
b. As far as to my knowledge, apical and basolateral MVBs have not been described. Although I am aware of biochemically distinct apical and basolateral early endosomes [DOI: 10.1091/mbc.01-07-

0320]. What is the reasoning for assuming that there are different populations of MVBs? Could this be demonstrated by immuno-EM? The currently presented data do not support this model.

Minor comments:

1) Please define and clarify the terms 'extracellular vesicle' and 'exosome' in the introduction including marker proteins on exosomes.

Referee #2:

The article by Matsui et al is a very interesting study showing different mechanisms of secretion of small extracellular vesicles (called here exosomes) from either the apical or the basolateral side of polarized cells (MDCK). The authors isolated EVs from MDCK cells cultured on inserts, separating the apical and basolateral sides of secretion. They analyzed the EV protein composition by Mass spectrometry, identified one novel protein specific of the apical EVs, GPRC5C, and analyzed the molecular mechanisms of EV release. They thus identify a specific effect of knocking down Alix on the apical side vesicles, and a specific effect of GW4869 on the basolateral EVs. It is a very interesting study, bringing novel insights into the heterogeneity of nature and biogenetic mechanisms of EV release. Other groups have previously compared EVs/exosomes from basolateral or apical side of polarized cells (see: Tauro et al 2013 #23230278; Van Niel et al 2001 #11487543, in addition to Banfer 2018 and Chen 2016 quoted here), but did not provide such mechanistic studies on the release machineries.
My main concern is about the interpretation that the vesicles analyzed are exosomes: the authors use mainly CD9 as a marker of these vesicles, however CD9 is mainly present at the plasma membrane, and very little in internal compartments, as indeed shown in figure EV2A. Therefore, one cannot exclude that some or even all the CD9+ EVs could be in fact small EVs budding from the plasma membrane (PM), which would be called ectosomes or microvesicles, rather than exosomes formed in MVBs. Consequently, the scheme of figure 4D is misleadingly suggesting that only MVB-derived exosomes are released by MDCK, both at the apical and basolateral sides. ESCRT and ALIX have both been shown to also be involved in budding of EVs from the plasma membrane (see review by Hurley EMBO J 2015: 26311197), and GW4869 also influences EV budding from the PM (Menck et al, JEV 2017 29184623). The authors should have used CD63, which is not present at the PM, as a more likely specific marker of exosomes, (in addition to the currently used CD9 and CD81 and the novel GPRC5C), to characterize the EVs released upon ESCRT or ALIX or nSMase2 knock down or GW4869 treatment. With the current data shown, the authors should a minima use more parcimonously the term exosomes, and instead talk about small EVs, and include in figure 4D small EVs budding from the PM both at the apical and basolateral sides, with possible involvement of ALIX vs ceramide in their release. Since the apical EVs bear more CD63, an interesting hypothesis could be that the apically released small EVs are bona fide exosomes, whereas the basolaterally released ones are PM-derived ectosomes, but this remains difficult to demonstrate.
My other concerns are technical:
1) The way CD9+ EVs are isolated and counted involves immuno-isolation followed by low pH treatment to release EVs from the isolating beads. This treatment may either destroy some fragile

EVs, or induce aggregation of other EVs, which could make them pellet at lower speed or be retained by filters, or release from the beads could be differently efficient for different EVs, all these aspects potentially leading to artefactual apparent decrease or increase of small EVs. A minima, the authors should take EVs isolated by differential centrifugation, treat them or not by low pH glycine followed by neutralization, and determine whether the number of EVs counted before or after this treatment is different. In addition, I would be curious to know if the total number of particles would be similarly or differently affected by all the treatments used in this paper (eg siRNAs against various molecules, GW4869 drug) as the number of CD9+-particles. Of course, I am not asking the authors to reperform all experiments, to get this comparative number of particles, but maybe they have done it before switching to the CD9-EV isolation, and they could give this info in the results or discussion.
2) another concern is that the Western blot experiments are shown as a single experiments, without quantification of the signals for EV markers in the different conditions, nor indication of the number of independent experiments performed, and thus the level of reproducibility of these experiments is difficult to evaluate. In my lab, siRNA-based experiments are tricky and give very variable results in terms of subsequent EV secretion.
3) The authors must indicate more clearly how they recover medium from the apical and basolateral sides of the culture insert, what volume of medium for how many cultured cells, and how then are the Western blots loaded (particles recovered from the whole conditioned medium, or given number of particles, or given amount of total proteins).
4) the authors must indicate more clearly the criteria for selection of proteins identified by mass spect: apparently 3 peptides is a must, but form how many individual replicates? In the supplementary tables, they must give the gene names for each protein, to facilitate future analysis of these results by readers.
5) p5 of results on EV2: the authors say hat CD63 and CD9 are not completely overlapping, but I would rather say that they overlap to a minor extent!
6) p7 the text should indicate that ESCRT family are not only regulating formation of ILVs in MVBs, but also budding of EVs from the plasma membrane
7) p9 indicates that results with KD of syntenin and syndecan as in figure EV3G, whereas these results are displayed in figure 4A-C

Referee #3:

This manuscript carefully and thoroughly documents distinct apical and basolateral pathways for the externalization of membrane vesicles that contain marker proteins found on exosomes. Moreover, the authors discovered a new marker GPCR5C characteristic of apically-destined EVs. It is a well-done study that reveals additional bases for EV heterogeneity and provides an interesting prelude for cell biological studies to further map the pathways involved. Comments are for potential revisions.

1. There has been a lot of controversy in exosome biology particularly relating to whether extracellular vesicles (EVs) are exosomes (ILVs secreted by MVB fusion) or vesicles budded from the plasma membrane. The latter was particularly true for CD9-containing vesicles. In the current article, the authors thoroughly characterize the apical and basolateral vesicles obtained from the medium of MDCK cells and, at one point indicate, "that vesicle size is inappropriate as a criterion for categorizing EVs, at least exosomes". This statement was a bit unclear as to what is intended. However, in the next paragraph the authors discuss using anti-CD9 antibody to enable the "capture of exosomes." This raises the question of what are the criteria the authors utilize to call membrane particles in the media exosomes as opposed to plasma membrane-derived budded

vesicles? Since membrane budding can also employ ALIX, this adds to the degree of complexity. These issues need clarification in the manuscript.

2. The authors clearly establish distinct pathways for the release of apical and basolateral particles that in turn have distinct compositions. This would contribute to the heterogeneity of EVs as stated in the abstract. However, is there any evidence that either apical and basolateral particles result from MVB exocytosis (as apposed to membrane budding)? Would immunocytochemistry with some of the marker proteins and localization to MVBs or to the plasma membrane help sort this out? The authors have methods to up or downregulate apical and basolateral pathways that may correlate with immunofluorescence. It would be of considerable interest to know whether MVBs of differing marker protein ratios showed any segregation in these polarized cells.

3. The authors indicate that cell lysates do not show dramatic changes in marker proteins under conditions where EV release has been up or downregulated likely (as the authors suggest) because secretory MVBs represent a minor subset of the MVB population. However, it would be of value to have a quantified estimate of how minor by comparing EV markers in the medium with total cell lysate values. It would even be more valuable if the authors were able to identify CD63/GPCR5C+ and CD81/CD9+ MVBs

4. It does seem in Fig. 3A that lysate amounts VPS4 and ALIX are altered by the knockdown of various ESCRT proteins without affecting apical or basolateral EV secretion.

5. Does PEG lead to fusion of EVs?

[Response to the editor]

1) A data availability section is missing.

We have added a data availability section in the revised manuscript (page 23).

2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

We have drawn error bars and performed statistical analyses based on at least three examinations.

[Response to the reviewer #1]

Matsui et al. studied the release of exosomes in polarized MDCK epithelial cells. They isolated extracellular vesicles that were shed from either the apical or basolateral domain followed by mass spec analysis to determine that there is some degree of differential release of cargos at either site. They then focused on CD9-positive exosomes and found that only those released into the apical medium contained GPRC5C. They further show that KD of Alix prevented apical but not basolateral release of CD9 exosomes. In contrast, inhibition of sphingomyelin synthesis with the inhibitor GW4869 decreased basolateral but not apical release of CD9 exosomes.

The study of differential exosome release from polarized cells is novel and of interest. However, the study needs further clarification. In my mind, the biggest caveat of the story is the relatively low percentage of CD9 exosomes released apically in comparison to overall apically released extracellular vesicles and also in comparison to CD9-positive exosomes released basolaterally. Can the authors rule out that apically released CD9-positive exosomes are not mistargeted? Why are only CD9-positive exosomes analyzed? Are there CD9-negative exosomes?

We thank the reviewer's critical assessment of our paper and helpful suggestions. Since CD9 was not enriched in apical exosomes (or small extracellular vesicles; sEVs), we have also analyzed CD63-positive exosomes (i.e., CD63 was enriched in apical exosomes) and confirmed our original findings. Our new data showed that purified CD63-positive apical exosomes (or sEVs) also contained CD9 albeit to a lesser extent (new Fig. 1C), suggesting that apically released CD9-positive exosomes may not be caused by mistargeting. Moreover, we have quantified the percentages of CD9-positive sEVs in both apical and basolateral samples (new Fig. EV1F and G). Relatively low percentages of CD9-positive sEVs by immuno-EM may

be attributable to the detection limit or efficiency (see response to #1 for details).

Major comments:

1) Figure 1C: The immuno-EM should be quantified. What is the percentage of CD9-positive EVs? It looks like less than 50% in the basolateral sample and maybe ~10% for the apical sample. Does that mean that exosomes are mainly secreted basolaterally? Are exosomes released at the apical membrane CD9 negative?

As suggested, we have quantified the percentages of CD9-positive sEVs in both apical and basolateral samples and the results have been shown in new Fig. EV1G. Although the amount of CD9-positive apical sEVs was two times higher than that of CD9-positive basolateral exosomes ($P<0.01$; two-sided Student's unpaired *t* test), their relative amounts (10-20%) in total sEVs were not so high under our experimental conditions. We think that a small amount of CD9 in sEVs may not be efficiently detected by immunonegative staining, and thereby our results may be underestimated.

2) Figure EV2: A large portion of CD9 is localized at the basolateral membrane. This makes sense as CD9 has been described as a tetraspanin protein that is associated with integrins. Could the authors comment what this might mean with respect to CD9-positive exosomes? Are these exosomes released basolaterally because they contain CD9?

We agreed that CD9 was mostly present in the basolateral membrane, and such localization may ensure the enrichment of CD9 in basolateral exosomes (or sEVs). In contrast, however, CD63 was enriched in apical exosomes, even though CD63 was not preset in the apical membrane (or plasma membrane), suggesting that sorting of CD9 (or CD63) into intraluminal vesicles in MVBs may not be so simple. At any rate, we have described the intracellular distribution of CD9 and CD63 in polarized MDCK cells and discuss this possibility in the revised manuscript (page 7, lines 4-15).

3) In the KD and inhibitor studies: How was cell polarity controlled? This is of concern, because MDCK cells that are not fully polarized may by default misdirect exosomes in non-specific ways.

To address the reviewer's concern, we have checked the polarity formation of ALIX-KD and GW4869-treated MDCK cells. The results showed that the normal polarity was formed even in the ALIX-KD and GW4869-treated cells (Reviewers only Fig. 1A and B).

4) Regarding the model:
a. Syndecan-1 is a protein that is targeted to the basolateral membrane by means of its PDZ domain [https://doi.org/10.1111/j.1600-0854.2008.00805.x]. How does its BL localization relate to a function in apical exosome release? This is the same question I had about the basolateral localization of CD9.

As pointed out by the reviewer, Syndecan1 was mainly present at the basolateral membrane. However, it also present in punctate structures, which partially overlapped with CD63 dots (arrowheads in Reviewers only Fig. 2). We think that such punctate structures (CD63- and Syndecan1-positive structures) would be precursors of exosomes.

b. As far as to my knowledge, apical and basolateral MVBs have not been described. Although I am aware of biochemically distinct apical and basolateral early endosomes [DOI: 10.1091/mbc.01-07-0320]. What is the reasoning for assuming that there are different populations of MVBs? Could this be demonstrated by immuno-EM? The currently presented data do not support this model.

As pointed out by the reviewer, apical and basolateral MVBs have not yet been described previously. They are postulated in our "working model" shown in Fig. 4E. If ALIX-mediated and ceramide-mediated intraluminal vesicle formation occur in the same MVBs, ALIX-KD or GW4869 should affect both apical and basolateral exosome release. However, our results showed that ALIX-KD and GW4869 specifically inhibited apical and basolateral exosome release, respectively. We thus postulated the presence of two different MVBs. Since CD9 and CD63 were present in both apical and basolateral sEVs, it is extremely difficult to show the existence of apical and basolateral MVBs even by immuno-EM. We would like to investigate this in our future study.

Minor comments:

1) Please define and clarify the terms 'extracellular vesicle' and 'exosome' in the introduction including marker proteins on exosomes.

As suggested by the reviewer, we have defined and clarified small extracellular vesicle (sEV), microvesicle, and exosome together with their marker proteins in the Introduction (pages 3-4) and the first paragraph in the Results section (page 5).

[Response to the reviewer #2]

The article by Matsui et al is a very interesting study showing different mechanisms of secretion of small extracellular vesicles (called here exosomes) from either the apical or the basolateral side of polarized cells (MDCK). The authors isolated EVs from MDCK cells cultured on inserts, separating the apical and basolateral sides of secretion. They analyzed the EV protein composition by Mass spectrometry, identified one novel protein specific of the apical EVs, GPRC5C, and analyzed the molecular mechanisms of EV release. They thus identify a specific effect of knocking down Alix on the apical side vesicles, and a specific effect of GW4869 on the basolateral EVs. It is a very interesting study, bringing novel insights into the heterogeneity of nature and biogenetic mechanisms of EV release. Other groups have previously compared EVs/exosomes from basolateral or apical side of polarized cells (see: Tauro et al 2013 #23230278; Van Niel et al 2001 #11487543, in addition to Banfer 2018 and Chen 2016 quoted here), but did not provide such mechanistic studies on the release machineries.

My main concern is about the interpretation that the vesicles analyzed are exosomes: the authors use mainly CD9 as a marker of these vesicles, however CD9 is mainly present at the plasma membrane, and very little in internal compartments, as indeed shown in figure EV2A. Therefore, one cannot exclude that some or even all the CD9+ EVs could be in fact small EVs budding from the plasma membrane (PM), which would be called ectosomes or microvesicles, rather than exosomes formed in MVBs. Consequently, the scheme of figure 4D is misleadingly suggesting that only MVB-derived exosomes are released by MDCK, both at the apical and basolateral sides. ESCRT and ALIX have both been shown to also be involved in budding of EVs from the plasma membrane (see review by Hurley EMBO J 2015: 26311197), and GW4869 also influences EV budding from the PM (Menck et al, JEV 2017 29184623). The authors should have used CD63, which is not present at the PM, as a more likely specific marker of exosomes, (in addition to the currently used CD9 and CD81 and the novel GPRC5C), to characterize the EVs released upon ESCRT or ALIX or nSMase2 knock down or GW4869 treatment. With the current data shown, the authors should a minima use more parcimonously the term exosomes, and instead talk about small EVs, and include in figure 4D small EVs budding from the PM both at the apical and basolateral sides, with possible involvement of ALIX vs ceramide in their release. Since the apical EVs bear more CD63, an interesting hypothesis could be that the apically released small EVs are bona fide exosomes, whereas the basolaterally released ones are PM-derived ectosomes, but this remains difficult to demonstrate.

We thank the reviewer's positive comments and helpful suggestions. We missed several key publications regarding exosomes and extracellular vesicles (EVs) and most of them have now been cited in the revised manuscript. As for CD9, we also agreed that it was mainly localized at the plasma membrane, suggesting that CD9-positive small EVs (sEVs) may be derived from the plasma membrane rather than

MVBs. However, both the apical and basolateral CD9- (or CD63-) positive sEVs did not contain any Annexin I, a known microvesicle marker (new Fig. 1C and F, bottom panels). Moreover, we have analyzed CD63-positive sEVs (i.e., CD63 was enriched in apical exosomes, but not present in the plasma membrane) and obtained essentially the same results as CD9-positive sEVs (e.g., new Figs. 1C-E, 2F, and EV3A-C). We thus think that CD9/63-positive and Annexin I-negative apical and basolateral sEVs are likely to be exosomes. However, we cannot completely rule out the possibility that CD9/63-positive and Annexin I-negative sEVs are formed directly form the plasma membrane (PM). Thus, we have also added PM-derived sEVs in our working model (new Fig. 4E).

My other concerns are technical:

1) The way CD9+ EVs are isolated and counted involves immuno-isolation followed by low pH treatment to release EVs from the isolating beads. This treatment may either destroy some fragile EVs, or induce aggregation of other EVs, which could make them pellet at lower speed or be retained by filters, or release from the beads could be differently efficient for different EVs, all these aspects potentially leading to artefactual apparent decrease or increase of small EVs. A minima, the authors should take EVs isolated by differential centrifugation, treat them or not by low pH glycine followed by neutralization, and determine whether the number of EVs counted before or after this treatment is different. In addition, I would be curious to know if the total number of particles would be similarly or differently affected by all the treatments used in this paper (eg siRNAs against various molecules, GW4869 drug) as the number of CD9+-particles. Of course, I am not asking the authors to reperform all experiments, to get this comparative number of particles, but maybe they have done it before switching to the CD9-EV isolation, and they could give this info in the results or discussion.

We understand this concern. As suggested, we have counted the number of sEVs obtained by PEG precipitation with or without treatment with low pH. As shown in Reviewers only Fig. 3, the number of apical sEVs was clearly reduced by low pH treatment, whereas the number of basolateral sEVs was not significantly changed by low pH treatment. Although some portions of apical sEVs were lost by low-pH treatment, our original finding that apical and basolateral sEV marker (CD9 and CD63) release are independently regulated by ALIX and ceramide, respectively, were also confirmed by using “total sEV (P100)” samples (Figure EV3A and B). We thus think that our NTA data on CD9- or CD63-positive sEVs are not artificial results. Currently,

we are not sure the nature of low-pH-sensitive apical sEVs, but given that total sEVs also contain CD9/63-negative sEVs, it is tempting to speculate that such sEVs may be sensitive to low pH. We would like to investigate the nature of these vesicles in our future study.

As suggested, we have also investigated the effect of ALIX-KD and GW4869 on total number of sEVs and the results have been included in Fig. EV3C and D (bottom). The same as the CD9/63-positive sEVs, ALIX-KD specifically inhibited apical release of total sEVs, but had no significant effect on basolateral release of total sEVs (although slightly reduced). Interestingly, however, GW4869 treatment also reduced apical release of total sEVs in addition to basolateral release of total sEVs. However, since the amount of CD9 and CD63 proteins in the apical total sEVs was not reduced even after the GW4869 treatment (Fig. EV3A and B), GW4869-sensitive EVs detected by NTA are likely to be CD9/CD63-negative. Since we did not focus on the CD9/CD63-negative sEVs in the present manuscript, we will investigate the nature of such GW4869-sensitive and CD9/CD63-negative sEVs in our future study.

2) another concern is that the Western blot experiments are shown as a single experiments, without quantification of the signals for EV markers in the different conditions, nor indication of the number of independent experiments performed, and thus the level of reproducibility of these experiments is difficult to evaluate. In my lab, siRNA-based experiments are tricky and give very variable results in terms of subsequent EV secretion.

We have performed at least three independent experiments, and one representative data are shown. As suggested, we have quantified the intensity of the immunoreactive bands and quantitative analyses were performed (e.g., Figs. 3B, 4B, EV2B and I, EV3B, EV4B, EV5B and F).

3) The authors must indicate more clearly how they recover medium from the apical and basolateral sides of the culture insert, what volume of medium for how many cultured cells, and how then are the Western blots loaded (particles recovered from the whole conditioned medium, or given number of particles, or given amount of total proteins).

As suggested, we have described such information in more detail in the Methods section.

4) the authors must indicate more clearly the criteria for selection of proteins identified

by mass spect: apparently 3 peptides is a must, but form how many individual replicates? In the supplementary tables, they must give the gene names for each protein, to facilitate future analysis of these results by readers.

We have performed a MASS analysis once and focused on proteins detected with minimum three "independent" peptides. To avoid confusion, we have corrected the sentence in the revised manuscript. We have also described gene names in supplemental tables.

5) p5 of results on EV2: the authors say hat CD63 and CD9 are not completely overlapping, but I would rather say that they overlap to a minor extent!

We agreed with the reviewer. In the revised manuscript, we have described that "some of the CD9-positive dots overlapped CD63-positive dots" (page 5, lines 14-17 and new Fig. EV1B).

6) p7 the text should indicate that ESCRT family are not only regulating formation of ILVs in MVBs, but also budding of EVs from the plasma membrane

We have described this in the revised manuscript (page 8, lines 19-21).

7) p9 indicates that results with KD of syntenin and syndecan as in figure EV3G, whereas these results are displayed in figure 4A-C.

We have now displayed these results in Fig. EV4 (page 10).

[Response to the reviewer #3]

This manuscript carefully and thoroughly documents distinct apical and basolateral pathways for the externalization of membrane vesicles that contain marker proteins found on exosomes. Moreover, the authors discovered a new marker GPCR5C characteristic of apically-destined EVs. It is a well-done study that reveals additional bases for EV heterogeneity and provides an interesting prelude for cell biological studies to further map the pathways involved. Comments are for potential revisions.

We appreciate the reviewer's very positive comments and helpful suggestions.

Major comments:

1. There has been a lot of controversy in exosome biology particularly relating to whether extracellular vesicles (EVs) are exosomes (ILVs secreted by MVB fusion) or

vesicles budded from the plasma membrane. The latter was particularly true for CD9-containing vesicles. In the current article, the authors thoroughly characterize the apical and basolateral vesicles obtained from the medium of MDCK cells and, at one point indicate, "that vesicle size is inappropriate as a criterion for categorizing EVs, at least exosomes". This statement was a bit unclear as to what is intended. However, in the next paragraph the authors discuss using anti-CD9 antibody to enable the "capture of exosomes." This raises the question of what are the criteria the authors utilize to call membrane particles in the media exosomes as opposed to plasma membrane-derived budded vesicles? Since membrane budding can also employ ALIX, this adds to the degree of complexity. These issues need clarification in the manuscript.

We agreed with the reviewer's comments. We have removed these unclear statements from the revised manuscript. Since CD9 was mainly present at the plasma membrane, it is possible that CD9-positve small extracellular vesicles (sEVs) are formed from the plasma membrane. However, we have also analyzed CD63-positive sEVs (i.e., CD63 was enriched in apical exosomes, but not present in the plasma membrane) and obtained essentially the same results as CD9-positive sEVs (e.g., new Figs. 1C-E, 2F, and EV3). Moreover, both the apical and basolateral CD9-positive sEVs did not contain any Annexin I, a known microvesicle marker (new Fig. 1C and F, bottom panels). We thus think that CD9/63-positive and Annexin I-negative apical and basolateral sEVs are likely to be exosomes. However, since we cannot completely rule out the possibility that CD9/63-positive and Annexin I-negative sEVs are formed directly form the plasma membrane (PM), we have also added PM-derived sEVs in our model (new Fig. 4E).

2. The authors clearly establish distinct pathways for the release of apical and basolateral particles that in turn have distinct compositions. This would contribute to the heterogeneity of EVs as stated in the abstract. However, is there any evidence that either apical and basolateral particles result from MVB exocytosis (as apposed to membrane budding)? Would immunocytochemistry with some of the marker proteins and localization to MVBs or to the plasma membrane help sort this out? The authors have methods to up or downregulate apical and basolateral pathways that may correlate with immunofluorescence. It would be of considerable interest to know whether MVBs of differing marker protein ratios showed any segregation in these polarized cells.

We also think that this is an interesting point. However, an immunofluorescence analysis showed that no apparent change was observed for CD9 and CD63 signals or distribution even in ALIX-KD or GW4869-treated cells

(Reviewers only Fig. 4A). This was probably because released sEVs contain "less than 1/100" of total EV markers (CD9, CD63, CD81, and GPRC5C) in the cells (Reviewers only Fig. 4B).

3. The authors indicate that cell lysates do not show dramatic changes in marker proteins under conditions where EV release has been up or downregulated likely (as the authors suggest) because secretory MVBs represent a minor subset of the MVB population. However, it would be of value to have a quantified estimate of how minor by comparing EV markers in the medium with total cell lysate values. It would even be more valuable if the authors were able to identify CD63/GPCR5C+ and CD81/CD9+ MVBs.

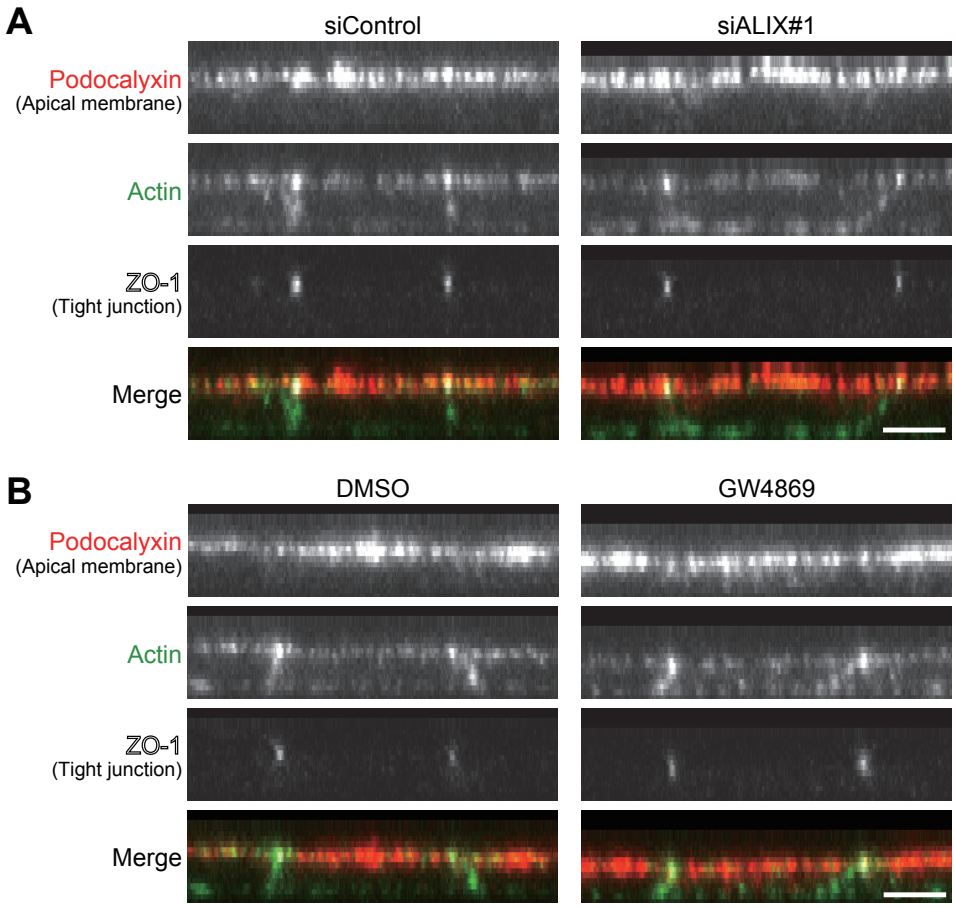
As described above, the amount of EV markers in sEVs was "less than 1/100" of that in the cell lysates (Reviewers only Fig. 4B). Unfortunately, antibodies against GPRC5C and CD81 used in this study did not work for immunofluorescence. However, our new biochemical data showed that CD63-positive sEVs also contained GPRC5C (new Fig. 2F).

4. It does seem in Fig. 3A that lysate amounts VPS4 and ALIX are altered by the knockdown of various ESCRT proteins without affecting apical or basolateral EV secretion.

We do not fully understand this comment, because knockdown of ESCRT components except for ALIX drastically increased both apical and basolateral EV marker release (Fig. 3A and B). As pointed out by the reviewer, the lysate amounts of VPS4 and ALIX were moderately altered by other ESCRTs-KD (we are not sure the exact reason currently), but such changes were much smaller than those by VPS4-KD and ALIX-KD itself. So, we speculate that increase of sEV release by VPS4-KD is much higher than decrease of sEV release by partial decrease of ALIX protein induced by VPS4-KD.

5. Does PEG lead to fusion of EVs?

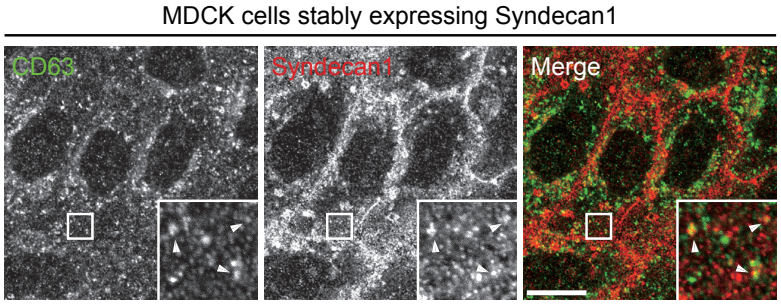
No. We have checked the number and size of sEVs before and after PEG precipitation (Reviewers only Fig. 3B and C), but their numbers were not changed even after PEG treatment, indicating that PEG is unlikely to promote fusion of sEVs.



Reviewers only Figure 1. Normal cell polarity formation in ALIX-KD and GW4869-treated MDCK cells.

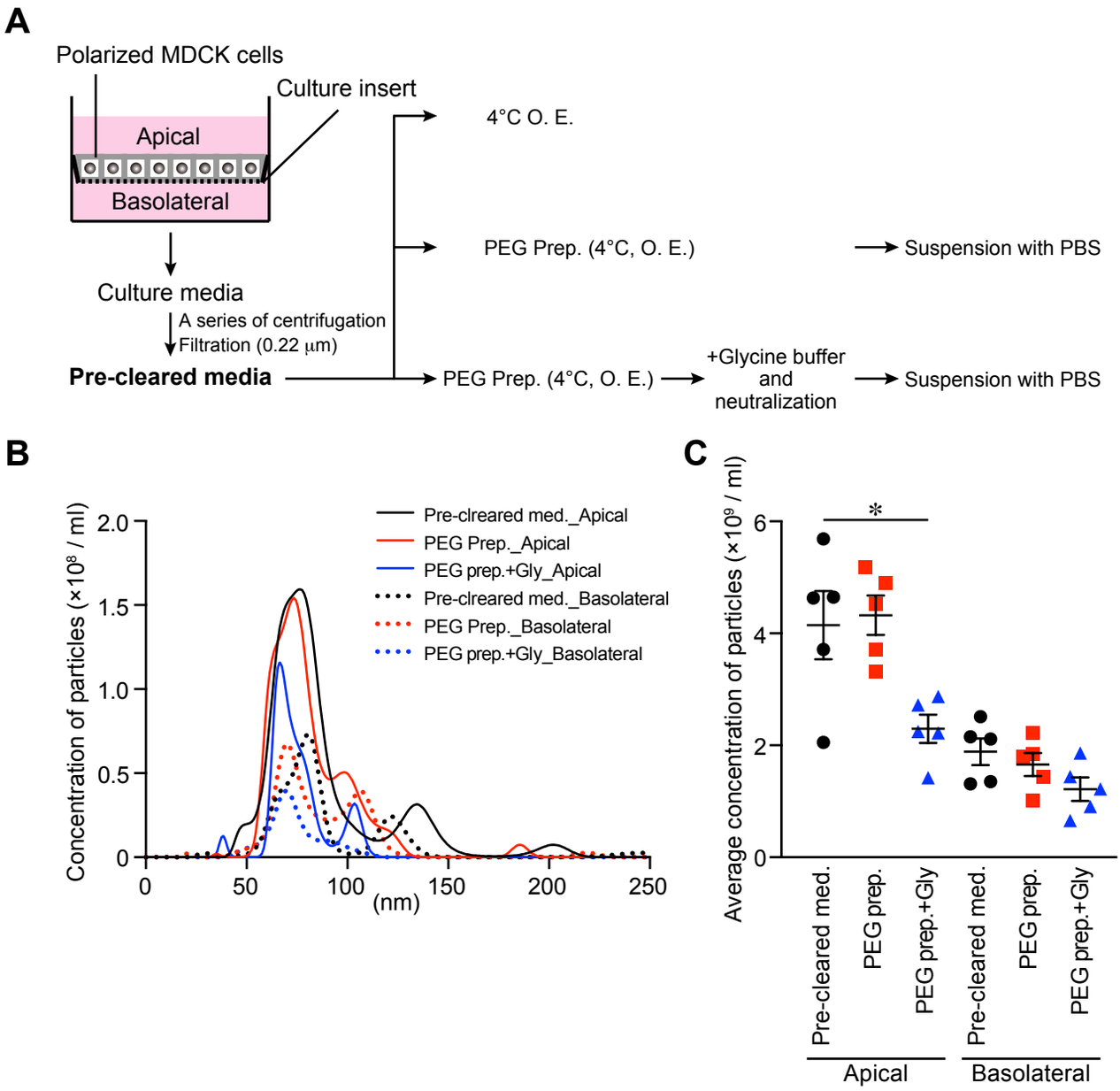
A. MDCK cells were transfected with siControl or siALIX, and the cells were cultured on coverslips for 3 days. The cells were then fixed and immunostained with the antibodies indicated. The confocal XZ sections were shown.

B. MDCK cells were cultured on coverslips for 3 days. On the last day, the culture medium was changed to a medium containing DMSO or 10 nM GW4869. The cells were analyzed as in (A). Scale bars, 5 μm.



Reviewers only Figure 2. Syndecan1 partially colocalizes with CD63.

MDCK cells stably expressing Syndecan1 were cultured on coverslips for 3 days. The cells were fixed and immunostained with the antibodies indicated. Note that some of the Syndecan1 colocalized with CD63, although it mainly localized at the plasma membrane. Scale bars, 20 μm.

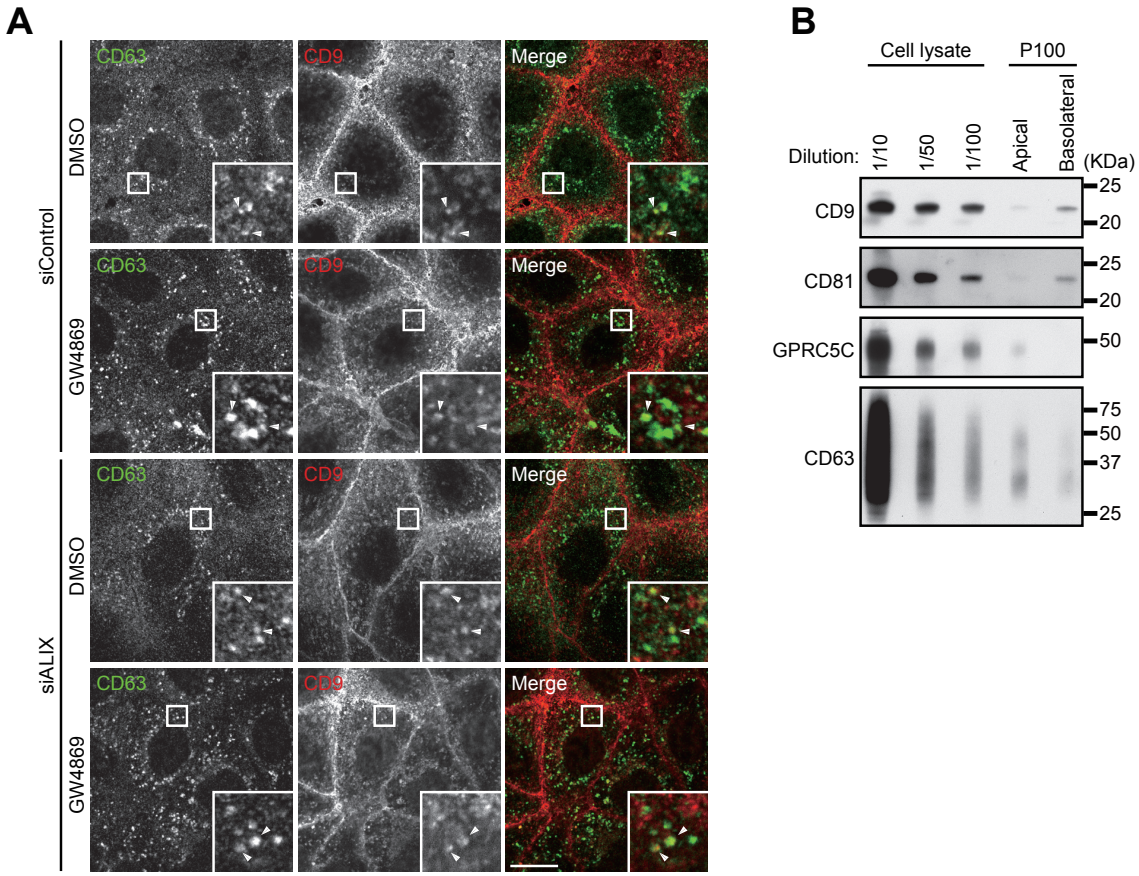


Reviewers only Figure 3. Effect of a glycine buffer on the number of sEVs.

A. Scheme of the sample preparation.

B. sEVs prepared as in (A) were analyzed by NTA. Representative NTA traces were shown.

C. Quantification of the NTA data obtained in five independent experiments. *$P < 0.01$ (one-way ANOVA and Tukey's test). Mean ± s.e.m. was shown.



Reviewers only Figure 4. Effect of ALIX-KD and GW4869 treatment on intracellular EV marker signals.

A. MDCK cells were transfected with siControl or siALIX, and the cells were cultured on coverslips for 3 days. On the last day, the culture medium was changed to a medium containing DMSO or 5 nM GW4869. The cells were then fixed and immunostained with the antibodies indicated.

B. MDCK cells were cultured on cell culture insert for 4 days. On the last day, the culture medium was replaced with EV-depleted medium. Cell lysates were collected from the cell culture inserts with an SDS sample buffer without reducing agent and dispersed through a 25-gauge needle. sEVs released from the apical and basolateral sides of MDCK cells were purified by ultracentrifugation. Diluted cell lysates and sEV samples were analyzed by immunoblotting with the antibodies indicated.

1st Revision - Editorial Decision 5th Feb 2021

Dear Prof. Fukuda

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the delay in handling your manuscript. We have now received the reports from former referee 1 and 2, while referee 3 was unfortunately not available anymore.

As you will see, both referees are very positive about the study and request only minor revisions to clarify and more clearly present some of your data. Please address the remaining concerns from referee 1 and 2.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Please move the reagent table (Appendix table S1) as table to the main manuscript file.

- Please add all funding information listed in the manuscript to the relevant section in our online submission system.

- Please note that all 'omics' primary datasets produced in your study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Specifically, we would kindly ask you to provide public access to the the mass spec datasets and to list the accession numbers and database in the "Data Availability" section. Please also update the Author Checklist accordingly (section F).

- Please remove the EV and Appendix table information from the Article (the list of Table EV1-3 and Appendix Table S1).

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission. I have also taken the liberty to make some changes to the Abstract. Could you please review it?

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We look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

I congratulate the authors for the extensive revisions and believe the manuscript is now ready to be published after a few minor corrections:

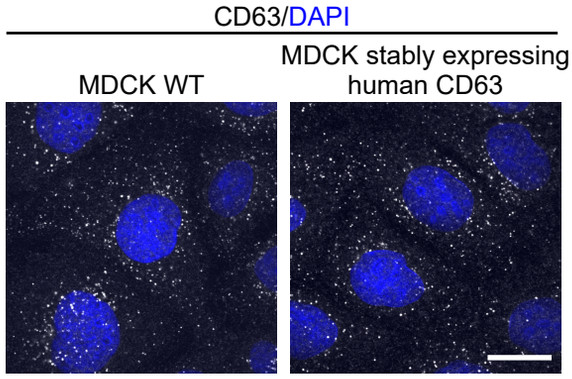
- page 7, line 3: I believe it should read only (Fig 1D) and not (Fig 1D and E)
- page 7, line 10: I believe it should read (Fig 1F and G) and not (Fig 1F-H)
- page 11, line 18: I believe it should read (Fig EV5E-G) and not (Fig EV5E-H)

Referee #2:

The revised version of the article by Matsui et al addressed properly my previous questions. However, I noticed two experimental details that should be more clearly presented when describing the results, ie involving minor editorial changes:
1) The authors have included in many of the previous Western blot figures additional panels showing either Annexin I or CD63. However, given the time between the previous version and this one, it seems unlikely that these new panels were obtained by re-incubating the previous WB membranes of the rest of the figure with new antibodies. Can the authors clarify? If new blots have been generated to make these new panels, the authors must clearly indicate it in the figure legend, and display the new panel(s) separated from the others.
2) The authors now isolate CD63+ EVs by IP, however, to do so, they apparently had to generate new MDCK cells expressing the human CD63, probably because the anti-CD63 they used for WB (which recognized canine CD63) did not allow IP? The authors must clarify this choice in the M&M and in the figure legend. Indeed, overexpression of CD63 may change its actual behavior, in terms of intracellular localization and release in EVs, as compared to the release of the endogenous CD63. Thus, although I do not want to ask the authors to perform an exhaustive comparative characterization of the EV release of endogenous canine versus overexpressed human CD63, they must at least mention this experimental situation clearly. Ideally, at least describing the localization of hCD63 in the stable MDCK cells, and whether it is now expressed in the basolateral and/or apical membrane would have been appreciated.

The authors have addressed all minor editorial requests.

Reviewers only Figure



Reviewers only Figure. Subcellular localization of CD63 in WT and human CD63-expressing MDCK cells. MDCK WT and human CD63 stably expressing cells were cultured on coverslips for 3 days. The cells were fixed and immunostained with anti-CD63 antibody, which can recognize both canine and human CD63. Note that CD63 localization seems not to be affected by the expression of human CD63. Scale bar, 20 μm.

2nd Revision - Editorial Decision 8th Feb 2021

Prof. Mitsunori Fukuda
Tohoku University, Graduate School of Life Sciences
Department of Integrative Life Sciences
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Sendai, Miyagi 980-8578
Japan

Dear Prof. Fukuda,

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
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Corresponding Author Name: Mitsunori Fukuda
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51475V3

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	At least three times independent experiments
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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Is there an estimate of variation within each group of data?	Yes

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes, we provided in apropriate imfomation.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Our MDCK cells have not been authenticated, but they form normal epitherium in vitro. We have checked DAPI staining and cells are probably mycoplasma negative.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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E- Human Subjects

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12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Mass spectrometry data have been deposited in PRIDE (https://www.ebi.ac.uk/pride/). The accession number is PXD024031.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No