

# Polarized human cholangiocytes release distinct populations of apical and basolateral small extracellular vesicles.

Brian Davies, Leslie Morton, John Jefferson, Cody Rozeveld, Luke Doskey, Nicholas F. LaRusso, and David Katzmann

*Corresponding author(s): David Katzmann, Mayo Clinic*

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*Editor-in-Chief: David Drubin*

## Transaction Report:

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

RE: E19-03-0133

TITLE: Polarized human cholangiocytes release distinct populations of apical and basolateral exosomes

Dear Dr. Katzman,

Thanks for submitting your paper to MBoC. The reviews are back on your paper, "Polarized human cholangiocytes release distinct populations of apical and basolateral exosomes". As you can read from their comments below, the reviewers found your paper interesting but felt that further work was necessary to clarify the origin of the EVs and other aspects of the study. If you are willing to deal with the reviewers' comments in a revised paper, I'd be happy to reconsider your work for publication in MBoC. Please send your revised paper back to our office as soon as possible.

Sincerely,  
Jennifer Lippincott-Schwartz

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Dear Dr. Katzmann:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office ([mboc@ascb.org](mailto:mboc@ascb.org)).

When submitting your revision online please use the link below, and include a cover letter that details, point-by-point, how the Monitoring Editor's and reviewers comments have been addressed. When entering the author names online, enter them exactly as they appear on the manuscript title page. Please send only the latest revised manuscript. DO NOT resend any previous versions. Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at [mboc@ascb.org](mailto:mboc@ascb.org).

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All images should be submitted at a minimum of 300dpi.

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1. crop out all extraneous white space
2. RGB color mode for color images, Grayscale for images not containing color
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**Locants and Labels.** Locants and labels can be between 1.5 and 2 mm high. Wherever possible, place locants and labels within the figures.

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To submit the cover letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): [Link Not Available](#)

Please contact us with any questions at [mboc@ascb.org](mailto:mboc@ascb.org).

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office

Reviewer #1 (Remarks to the Author):

The article by Davies et al uses a nice model of polarized in vitro culture to analyse extracellular vesicles (EVs) released by primary cholangiocytes in either the basolateral or apical sides. The authors observed interesting differences in the amount and protein and miRNA content of EVs released from either side of the cells. They show different sensitivities to treatments modulating intracellular molecules for secretion of these 2 types of EVs. Finally, they observe different signaling effects on target cells, with apical EVs affecting more strongly cholangiocytes, whereas basolateral EVs affect more a monocytic cell line.

These results are interesting although mainly descriptive (no molecular mechanisms proposed for the functional differences, nor explanation for the differential regulation of secretion by ALIX KO). They confirm previous observations by other groups that polarized cells release different EVs at their apical vs basolateral sides (van Niel et al Gastroenterology 2001; Tauro et al, Mol Cell proteomics 2013). They provide additional information, such as the differential presence of some miRNA species in one or the other type of EVs, and differential functionality of the apical and basolateral EVs (although the physiological relevance of the experimental settings of these functional tests is not really obvious to the non expert).

I have several technical concerns that should be addressed before considering this article for publication.

1) The authors do follow to some extent the guidelines on EV work of the International Society for EVs, MISEV2018, but not sufficiently in some experiments: the following article should be carefully read and used <https://www.tandfonline.com/doi/full/10.1080/20013078.2018.153575>.

In particular, the checklist provided at the end of the guideline article lists many issues to consider and report for any EV study.

Here in particular, the authors should refrain from using the term exosome for both types of EVs, since they never demonstrate the actual MVB origin of their vesicles. The final scheme of figure 7 is in fact misleading, and no observation reported here excludes that at least part of the EV analysed bud off the plasma membrane, rather than forming in MVBs. The higher amount of an ESCRT I protein (TSG101) in apical sEVs, and the counter-intuitive increased secretion of this protein upon depletion of the ESCRT-accessory protein ALIX does not allow to conclude on the actual intracellular origin of either type of EV. I would suggest to instead use the term small EV throughout, possibly with proposition that some of these EVs are indeed exosomes, but whether only exosomes are concerned by the reported observations cannot be concluded, since no other molecule than TSG101 (possibly considered a specific exosome marker) are analysed throughout the paper, to determine if they behave similarly or differently from TSG101.

It is worth noting that the authors made efforts to purify the vesicles by an additional density gradient step following ultracentrifugation, but the actual size and densities of EVs are not specific to exosomes and could also apply to plasma membrane-derived EVs. In addition, it would even have been more interesting to instead try to more exhaustively characterize the whole range of EVs secreted from the apical and basolateral sides, and whether different subtypes (of different sizes for instance, or different protein compositions) were differently secreted and affected by the reported treatments.

In general, characterization of the EVs could be more exhaustive, especially in terms of protein composition, in fig1C and upon inhibition of ALIX or treatment with GW4869: presence of transmembrane proteins (tetraspanins CD63 and CD9 or CD81, or integrins for instance) should be reported, as well as a generic cytosolic protein as "loading control" (actin, GAPDH, HSP70). Finally, the authors specify in several places that "equal numbers of EVs" were used (loaded on WB gels or used for functional assays), but what this means is unclear: equal number of particles, or of proteins, or EVs recovered from equal volumes of conditioned medium? Is it the same loading in fig3G and the WB of fig1C and 5? If yes, the lower amount of TSG101 in basolateral EVs whereas the overall protein content is equal or higher suggests that possibly the basolateral small EVs are possibly less of MVB origin, or at least less of TSG101-dependent biogenesis.

EM in Figure 1B is not satisfying: TSG101 is a cytosolic protein, so should not be detected by an antibody in the absence of permeabilisation. The santa cruz antibody used here is probably not specific of TSG101.

annexin V used in WB of figure 1C is misleadingly presented as a marker of apoptotic bodies: annexin V binds to phosphatidyl-serine, and is thus classically used as fusion to a fluorescent dye to detect PS exposed at the surface of unpermeabilised apoptotic cells, but its presence in cells or EVs is independent of an apoptotic status and does not provide any information.

all bar graphs must be replaced by graphs showing dots for individual biological replicates, to show actual variability of the experiments: Weissgerber TL, Milic NM, Winham SJ, Garovic VD. (2015) Beyond Bar and Line Graphs: Time for a New Data Presentation Paradigm. PLoS Biol 13(4): e1002128. In many graphs, in fact, the differences do not seem very strong, even if the authors show a p-value of 0.05.

the conditions of use of the inducible ALIX ko cells of figure 5 are not clear. In 5C, the WB shows 4 conditions (NHC cells and ALIX.19 cells each minus or plus doxycycline), whereas the particle quantification in the same panel only shows wt and kd cells: does wt correspond to ALIX.19 without doxo, or to NHC plus doxo? In any case, the full comparison of 4 conditions should be done, to exclude side effects of doxo on EV release at either apical or basolateral sides of NHC cells, and endogenous difference between clone ALIX.19 and NHC independent of ALIX deletion.

For the functional assays of figure 6, the authors claim that transfer to other cholangiocytes mimicks what happens at the apical side, whereas transfer to monocytes mimicks what happens at the basolateral side of a cholangiocyte layer. I am not sure I understand this statement: why would cholangiocytes not communicate with each others at the basolateral side too? If the authors wanted to mimick communication at these two separate sites, why did they not load the EVs on either the basolateral or the apical side of the culture?

Reviewer #2 (Remarks to the Author):

In this paper, the authors show that exosomes released from apical and basolateral sides of polarized cholangiocytes differ in their membrane composition and luminal miRNA cargo. The authors also conclude that the biogenesis of the exosomes occur from distinct membrane domains based on their observation that ALIX depletion reduced release of basolateral exosomes without affecting that of apical exosomes. The two sets of exosomes also elicited differential responses from target polarized NHC cells and human monocyte cell line. Based on these observations the authors draw the conclusion that distinct subsets of exosomes released from apical and

basolateral plasma membrane are used by the polarized cholangiocytes for intercellular communication. Extracellular vesicles (EVs, including exosomes and plasma membrane derived microvesicles) has recently emerged as an important mediator of intercellular communication in mammalian cells. However, the mechanisms regulating EV biogenesis, cargo selection within EVs and information transfer by EVs are still not entirely clear. In the present study, the authors put forward an interesting model regarding how polarized epithelial cells can utilize distinct sets of EVs for carrying out intercellular communication. However, as discussed below, the model proposed by the authors is not supported by the data in the paper and the authors need to do more experiments to clarify the origin of the EVs.

One of the main concerns is that it is not clear from the data presented in the paper whether the apically and basolaterally isolated EVs are exosomes or microvesicles shed from the plasma membrane. The apical plasma membrane has different lipid and protein composition compared to the basolateral membrane (apical membrane generally has more cholesterol). Instead of the EVs being generated in distinct domains of MVBs inside the cell as the authors propose, it is possible that either one or both of the EV subsets are actually released by budding of the plasma membrane. The authors need to do further experiments (preferably imaging based assays) to provide some direct evidence about the site of biogenesis of the two subsets of EVs. Further more, the authors should perform experiments that can interfere with fusion of MVBs with plasma membrane to support the notion that both sets of the EVs are actually exosomes. In absence of such data, the model proposed by the authors is at best a speculative interpretation of the data presented in the paper. I would not recommend publication of the paper before these additional information are included in the study.



200 First Street SW  
Rochester, Minnesota 55905  
507-284-2511

August 7, 2020

**David J. Katzmann, Ph.D.**  
Biochemistry and Molecular Biology  
507-266-5264 Fax 507-284-2053

katzmann.david@mayo.edu

Dear Dr. Lippincott-Schwartz,

We are pleased to resubmit this manuscript (Polarized human cholangiocytes release distinct populations of apical and basolateral extracellular vesicles) to *Molecular Biology of the Cell*. This work has been previously submitted and reviewed as “E19-03-0133.” We thank the reviewers for their interest and constructive comments regarding the initial submission. A number of important issues were raised, and we feel the manuscript has been improved through addressing those. The primary issue was whether exosome was the relevant term for the particles being isolated and characterized. While this could have been addressed through language (i.e. “extracellular vesicles” as opposed to “exosomes”), we felt that a better understanding of the extracellular vesicle population would strengthen the work. Further analysis has clarified that while exosomes contribute to our EV fractions, small microvesicles are also present, and that a change in microvesicle and exosome contributions to the apical small EV (sEV) fraction occur with ALIX depletion. The manuscript has been altered to more accurately refer to our EV fractions as “sEVs” and have revised the model to depict these complexities. Due to extensive changes throughout the document we have not tracked these alterations. While it has been some time since the initial review, a lack of funding for this project and the necessity to develop additional approaches and reagents took substantially more time than anticipated. We are hopeful that your willingness to reconsider this work remains and it is acceptable for publication in *Molecular Biology of the Cell*.

Sincerely,



David J. Katzmann



### Responses to reviewer 1 comments:

- 1. The authors do follow to some extent the guidelines on EV work of the International Society for EVs, MISEV2018, but not sufficiently in some experiments. The checklist provided at the end of the guideline article lists many issues to consider and report for any EV study. Here in particular, the authors should refrain from using the term exosome for both types of EVs, since they never demonstrate the actual MVB origin of their vesicles. The final scheme of figure 7 is in fact misleading, and no observation reported here excludes that at least part of the EV analysed bud off the plasma membrane, rather than forming in MVBs. The higher amount of an ESCRTI protein (TSG101) in apical sEVs, and the counter-intuitive increased secretion of this protein upon depletion of the ESCRT-accessory protein ALIX does not allow to conclude on the actual intracellular origin of either type of EV. I would suggest to instead use the term **small EV** throughout, possibly with proposition that some of these EVs are indeed exosomes, but whether only exosomes are concerned by the reported observations cannot be concluded, since no other molecule than TSG101 (possibly considered a specific exosome marker) are analysed throughout the paper, to determine if they behave similarly or differently from TSG101. It is worth noting that the authors made efforts to purify the vesicles by an additional density gradient step following ultracentrifugation, but the actual size and densities of EVs are not specific to exosomes and could also apply to plasma membrane-derived EVs.*

Multiple measures have been implemented to address this concern. We have expanded our analysis of the particles and surmise that microvesicles and exosomes contribute to the small EV fraction we have studied (please see response to Reviewer 2, point 2 for additional details), and we have shifted to use “sEV” throughout the manuscript as recommended.

We have altered the model in the discussion to highlight that both microvesicles and exosomes contribute to the sEV populations. We have illustrated that ALIX depletion alters the sEVs in a manner distinct from inhibition of the ceramide pathway. The increase in Tsg101 in apical sEVs with ALIX depletion correlated with increases in AnnexinA1 and CD9, suggesting that increased microvesicle release is contributing to a change in apical sEVs in this context. While we agree that additional experiments are needed to address the specifics of how these different pathways alter the sEV fraction to change signaling, we feel this demonstration extends our understanding of the central issue of ESCRT contributions to EV communication.

- 2. In addition, it would even have been more interesting to instead try to more exhaustively characterize the whole range of EVs secreted from the apical and basolateral sides, and whether different subtypes (of different sizes for instance, or different protein compositions) were differently secreted and affected by the reported treatments.*

We agree that further characterization of the full range of EVs secreted apically and basolaterally will be interesting to explore using this polarized cholangiocyte system. The current work is an initial investigation supporting the hypothesis that polarized cholangiocytes release distinct apical and basolateral sEVs that mediate distinct signaling, thereby justifying the characterization described. However, additional resources will be required to undertake the suggested studies. Publication of the current studies will provide a foundation on which to build.

- 3. In general, characterization of the EVs could be more exhaustive, especially in terms of protein composition, in fig1C and upon inhibition of ALIX or treatment with GW4869: presence of transmembrane proteins (tetraspanins CD63 and CD9 or CD81, or integrins for instance) should be reported, as well as a generic cytosolic protein as "loading control" (actin, GAPDH, HSP70).*

We have expanded our analysis of these particles using ALIX, HRS, and AnnexinA1 and tetraspanins, including CD63 (Figures 2A, 2H, 5G) and CD9 (Figure 2H, 5G). These tetraspanins were difficult to detect, and CD81 was below detection. GM130 was employed as a negative control and was not detected in our floated sEV fraction.

4. *Finally, the authors specify in several places that "equal numbers of EVs" were used (loaded on WB gels or used for functional assays), but what this means is unclear: equal number of particles, or of proteins, or EVs recovered from equal volumes of conditioned medium? Is it the same loading in fig3G and the WB of fig1C and 5? If yes, the lower amount of TSG101 in basolateral EVs whereas the overall protein content is equal or higher suggests that possibly the basolateral small EVs are possibly less of MVB origin, or at least less of TSG101-dependent biogenesis.*

We have clarified where samples were normalized for equal sEV particle numbers - such as the silver staining (Figure 3G), RNA analysis (Figures 3B, 4B), and Pathways Scan analysis of signaling (Figure 6A, B) - versus normalized to equal volume of the sEV floated fraction to maximize detection in western blots and other analyses (Figures 2A, 2D, 2H, 5B, 5C, 5G, 5H, and 6C). Relative contributions of microvesicles and exosomes to the sEV fraction is an interesting issue but not something we currently address. This issue would be further complicated by the ability of Tsg101 to enter both pathways. We have acknowledged the contributions of both exosomes and microvesicles to our EV fractions by using the sEV term as recommended.

5. *EM in Figure 1B is not satisfying: TSG101 is a cytosolic protein, so should not be detected by an antibody in the absence of permeabilisation. The santa cruz antibody used here is probably not specific of TSG101.*

We surmise that some degree of permeabilization must have occurred during processing to permit detection of Tsg101 in the EVs; however, as the point is made by the anti-CD63 immuno EM we have removed the TSG101 data from this figure to avoid any confusion.

6. *annexin V used in WB of figure 1C is misleadingly presented as a marker of apoptotic bodies: annexin V binds to phosphatidyl-serine, and is thus classically used as fusion to a fluorescent dye to detect PS exposed at the surface of unpermeabilised apoptotic cells, but its presence in cells or EVs is independent of an apoptotic status and does not provide any information.*

We appreciate the reviewer's comment and have removed the Annexin V western blot (which did not reveal detectable levels of AnnexinV) and focused on the GM130 and Arf6 westerns (Figure 2D) as negative controls.

7. *all bar graphs must be replaced by graphs showing dots for individual biological replicates, to show actual variability of the experiments: Weissgerber TL, Milic NM, Winham SJ, Garovic VD. (2015) Beyond Bar and Line Graphs: Time for a New Data Presentation Paradigm. PLoS Biol 13(4): e1002128. In many graphs, in fact, the differences do not seem very strong, even if the authors show a p-value of 0.05.*

The reviewer commented that the differences do not seem "very strong" in many graphs. We are limited by the complexity of this model system and have applied statistical analyses to the data generated therein to support conclusions pertaining to observed differences. While differences within a given experiment appear more robust, we felt the most accurate way to present the findings would be to pool the data even though this resulted in larger deviation (that remained statistically significant). We have also included the individual data points to support our analyses, as requested. However, in Figure 3A, we had a greater sample size (24 pairs of Ap and Ba sEV analyzed) such that we used the bar graph with SEM; the plotting of individual data points in this context unduly complicated the presentation. In Figures 4A, 6A and 6B, we also excluded the individual data points as the presentation of trends over the miRNA panel (Figure 4A) or signaling array (Figure 6A, B) was the focus of analyses rather than the individual component magnitude. For the miRNA panel, we supported this analysis with Figure 4B where individual replicates are presented.

- 8. the conditions of use of the inducible ALIX ko cells of figure 5 are not clear. In 5C, the WB shows 4 conditions (NHC cells and ALIX.19 cells each minus or plus doxycycline), whereas the particle quantification in the same panel only shows wt and kd cells: does wt correspond to ALIX.19 without doxo, or to NHC plus doxo? In any case, the full comparison of 4 conditions should be done, to exclude side effects of doxo on EV release at either apical or basolateral sides of NHC cells, and endogenous difference between clone ALIX.19 and NHC independent of ALIX deletion.*

We have included the comparable doxycycline treatment of the parental NHC cells in Figure 5E to demonstrate that doxycycline itself does not alter sEV release as was observed with doxycycline treatment of the ALIX.19 clone with inducible ALIX depletion (Figure 5F-H). While the ratios of Ap:Ba sEV release are similar, the release of sEVs by the ALIX.19 prior to induced depletion of ALIX is greater than sEV release by the NHC parental line. The comparison of EV release prior or subsequent to ALIX depletion – using the ALIX.19 clone - is our focus.

- 9. For the functional assays of figure 6, the authors claim that transfer to other cholangiocytes mimicks what happens at the apical side, whereas transfer to monocytes mimicks what happens at the basolateral side of a cholangiocyte layer. I am not sure I understand this statement: why would cholangiocytes not communicate with each others at the basolateral side too? If the authors wanted to mimick communication at these two separate sites, why did they not load the EVs on either the basolateral or the apical side of the culture?*

The use of the transwell system as a target for examination of apical or basolateral induced signaling by sEVs has not yet been reproducible in our hands, although it is a system we would like to explore more in the future. We believe this difficulty is due to technical difficulties of the in vitro experimental system. Use of the transwell culture system is also more costly, further complicating optimization. The two systems we described – use of NHC grown on standard culture dishes or the THP-1 cells grown in suspension – afforded more reproducible analyses and were thus used. The THP-1 cells were used to model cholangiocyte signaling to liver resident macrophages, as the basolateral surface would border intrahepatic milieu. While cholangiocyte-to-cholangiocyte signaling via the basolateral surface is reasonable to presume, this issue has been left to future studies.

#### Responses to reviewer 2 comments:

- 1. One of the main concerns is that it is not clear from the data presented in the paper whether the apically and basolaterally isolated EVs are exosomes or microvesicles shed from the plasma membrane. The apical plasma membrane has different lipid and protein composition compared to the basolateral membrane (apical membrane generally has more cholesterol). Instead of the EVs being generated in distinct domains of MVBs inside the cell as the authors propose, it is possible that either one or both of the EV subsets are actually released by budding of the plasma membrane. The authors need to do further experiments (preferably imaging based assays) to provide some direct evidence about the site of biogenesis of the two subsets of EVs. Further more, the authors should perform experiments that can interfere with fusion of MVBs with plasma membrane to support the notion that both sets of the EVs are actually exosomes.*

We generated WT and dominant negative Rab27A (dnRab27A) overexpression constructs to interfere with docking and fusion of MVBs in the NHC transwell cultures. Overexpression of dnRab27A reduced apical and basolateral EV release (Figure 2E, F, G, H), indicating that exosomes contribute to the sEV fraction. Given that the reduction in EVs was incomplete and there were microvesicle markers present in EV fractions of dnRab27A we have adjusted our interpretations accordingly. We have altered the model in the discussion to highlight that both microvesicles and exosomes contribute to the sEV populations. As mentioned in our response to reviewer 1, the appreciation of small microvesicles within our sEV pool helps to clarify the change in apical sEVs upon ALIX depletion.

RE: Manuscript #E19-03-0133R

TITLE: "Polarized human cholangiocytes release distinct populations of apical and basolateral small extracellular vesicles."

Dear Dr. Katzmann:

I am pleased to accept your manuscript for publication in *Molecular Biology of the Cell*.

Sincerely,  
Jennifer Lippincott-Schwartz  
Monitoring Editor  
*Molecular Biology of the Cell*

-----  
Dear Dr. Katzmann:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at [www.molbiolcell.org/toc/mboc/0/0](http://www.molbiolcell.org/toc/mboc/0/0) is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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