PLP2 drives collective cell migration via ZO-1-mediated cytoskeletal remodeling at the leading edge in human colorectal cancer cells

Dipanjana Ghosh, Ankita Dutta, Anjali Kashyap, Neeraj Upmanyu and Sunando Datta
DOI: 10.1242/jcs.253468

Editor: Kathleen Green

Review timeline

Original submission:	3 September 2020
Editorial decision:	7 October 2020
First revision received:	11 February 2021
Editorial decision:	18 March 2021
Rebuttal received:	23 March 2021
Editorial decision:	6 April 2021
Second revision received:	2 July 2021
Editorial decision:	5 August 2021
Third revision received:	9 August 2021
Accepted:	11 August 2021

Original submission

First decision letter

MS ID#: JOCES/2020/253468

MS TITLE: Proteolipid protein 2 drives collective cell migration
via ZO-1 mediated cytoskeletal remodeling at the leading edge

AUTHORS: Dipanjana Ghosh, Ankita Dutta, Anjali Kashyap, Neeraj upmanyu, and SUNANDO DATTA
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage.

In particular, two of the referees are concerned that findings have been over interpreted, and that you failed to clearly demonstrate PLP2-dependent cytoskeletal rearrangements and PLP2-driven leading-edge dynamics. This, in turn, undermines the impact and novelty of the data, which fall short of significantly increasing our understanding of collective cell migration. If you think that you can deal thoroughly and satisfactorily with the criticisms on revision, I would be amenable to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so

within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Ghosh et al. studied the roles of PLP2 in collective migration of colon carcinoma cells. They characterize SW480 as a potential model to study collective cell migration. PLP2 is described to shuttle between CCJ and the free membrane regions. The authors also confirm previous observations that PLP2 can be secreted as part of exosomes. They propose that loss of PLP2 limited wound healing and this can be rescued by PLP2 expression or addition of PLP2 containing exosomes. Functionally, the propose that ZO-1 is recruited by PLP2 at the leading edged and CCJs. They map the key role of the C-terminal region of PLP2 to mediate this function.

Collectively, these data form an interesting and potentially important study. Uncovering the molecules that control CCM is a limiting step to understand this key process in metastasis. Once the authors revise their original submission, they may reveal PLP2 as such a novel player which will open up to further characterization of this molecule in CCM.

Comments for the author

Major Comments

1-The authors begin their manuscript by quantifying several parameters for SW480 and Caco-2 cells to define if they would be good models of collective cell migration. This section failed to completely convince this reviewer that the SW480 cells are a robust model of collective migration. Clear video imaging that this is occurring would strengthen the manuscript. This could be done in a wound healing assay or using a chemoattractant. In my opinion, it is unclear if what we learn, at the edge of the wounds, really represent collective migration.

2-The dynamic localization of PLP-2 at CCJ and at the free membrane edges is not completely convincing. A major worry is that the signal of GFP-PLP2 is proportional to membrane density. For example, in video 2, we see low signal in thin lamellipodial extensions, and the signal becomes stronger (almost like at the CCJ) when these retracts. The authors would need to convincingly demonstrate that there is a bona fide recruitment at the leading edge to support their claims of dynamic localizations. This limitation is present in figure 2-3.

3-The authors propose that PLP2 and ZO-1 colocalize at the wound edges. Just like stated above, there is a worry that this is non-specific and more a reflection of the membrane densities. For example, there are less ruffles in PLP2 KO cells which could contribute to a decreased ZO-1 staining.

4-Potentially more worrying is that fact that endogenous ZO-1 expression is decreased by PLP2 KO (very clear by WB). How do the authors take this into account when they compare staining intensities? WT and delta N PLP2 rescue the ZO-1 expression levels, but not the deltaC. What would be the localization of overexpressed ZO-1 in these conditions? Would that be a better and less biased readout? These comments are link to Figure 3-4.

5-One missing experiment is whether deletion of PLP2 blunts metastasis in vivo. Can SW480 cells (and rescued cells) be grafted orthopically and metastasis be tested? If this is not possible, this should be discussed. Many proteins are essential for metastasis in 2D models but not in vivo.

Minor Comments

1-The analyses of the number of the size of focal adhesions is not very well integrated in the manuscript. Live analyzes on sparse cells and wounded monolayers would provide much more insights into the role of PLP2 in adhesions dynamics. Otherwise, this data should be removed.

2-The discussion on Rac1 is interesting and appropriate, but Rac1 should be omitted from the model and the authors should focus on their data instead.

3-Movie S1. Adding arrows would facilitate the understanding of the phenotypes.

4-Movie S4. The GW4869 conditions appears out of focus. Also, the authors should tone down the statement “Drug treated cells showed much reduced CCNM…”. The difference remains minor in this video.

5-Methods: CRISPER should be corrected for “CRISPR”.

Reviewer 2

Advance summary and potential significance to field

Ghosh and colleagues describe a role for the transmembrane protein proteolipid protein 2 (PLP2) during collective cell migration. PLP2 was found to be enriched in cell membranes during migration as well as in secreted exosomes. PLP2 knock out cells showed decreased collective cell migration which could be rescued by expression of full-length of PLP2, an N-terminal deletion of PLP2 or the addition of exosomes. This rescue corresponded with the ability of PLP2 to recruit ZO-1 to the leading edge and cell-cell junctions during migration. While this is an interesting study potentially linking PLP2 to ZO-1, actin dynamics and cell migration, I have major concerns with the over interpretation of their findings and feel that they failed to clearly demonstrate PLP2-dependent cytoskeletal rearrangement and PLP2-driven leading-edge dynamics. I feel this paper in its current form fall short of making a significant and novel contribution to our understanding of collective cell migration.

General Comments:

Throughout the manuscript the authors use both bar graphs and scatter plots and seemingly switch back and forth between both presentations without justification. Bar graphs can obscure patterns in data and should be avoided when presenting data.

The authors choose to focus in SW480 cells over Caco-2 cells as the endogenous expression of PLP2 was higher in SW480 cells. As both the cells undergo collective cell migration in 2D, one could presume that some of the mechanisms that govern collective cell migration is the same. Results gathered in SW480 cells could be compared to those in Caco-2 cells that naturally express lower amounts of PLP2. For example, CRISPR-mediated KO of PLP2 resulted in loss of ZO-1 at the leading edge of migrating SW480 cells, do Caco-2 cells naturally have lower amounts of ZO-1 due to lower expression of PLP2? Can the PLP2-contain exosomes from SW480 cells affect the collective cell migration of Caco-2 cells? The comparison of these two cells lines would strengthen their findings.

One of the main claims of the manuscript is that PLP2 is recruiting ZO-1 to the leading edge during collective cell migration and this is resulting in cytoskeletal re-arrangement. This was demonstrated through fixed samples via staining with phalloidin or imaging of ZO-1. To better support this claim the authors should include analysis of other cytoskeletal markers such as the Arp2/3 complex, or cortactin. Furthermore, careful analysis of actin dynamics (rates of polymerization etc) could also be performed using live-cell imaging.

The inclusion of Rac into their model seems out of place as there was no empirical evidence for it and is speculation. The use of active Rac probes could be used here to back up the authors' claims, or in the very least staining for proteins know to be involved in Rac activation and recruitment (WASP, or N-WASP, WIP etc.).
Comments for the author

In Figure 1, PLP2 seems to be enriched in some belt around the cell not particularly at the leading edge. Dual live-imaging of actin or or some other leading edge marker would strengthen this claim as PLP2 is not classically considered a marker for the leading edge. The same is true for Figure 2.

In Figure 2. PIV analysis should be better explained, and the quality of the phase-contrast images presented could be improved. It is not clear to me what is being shown in graphs 2E and 2F. Are these individual cells or multiple cells? Why are there no error bars on these graphs. It also seems as though the addition of PLP2-GFP exosome increased directionality (and the knockout of PLP2 decreased directionality) this is an interesting aspect but was only briefly remarked upon. The authors should be careful when correlating focal adhesion size with kinetics in particular if their analysis relied only upon fix analysis which lacks dynamic behavior.

Reviewer 3

Advance summary and potential significance to field

The authors investigated the role of proteolipid protein 2 (PLP2) in collective cell migration (CCM) in colon cancer. Using PLP2 knockout (PLP2 KO) cells and fluorescent PLP2 overexpression constructs, they show that PLP2 localizes to both cell-cell junctions as well as free-edge membranes and that cells lacking PLP2 exhibit migration defects. Interestingly, the authors found that overexpression of PLP2-mCherry or addition of exosomes from PLP2-overexpressing cells rescued the migration defect of PLP2 KO cells. Next, the authors investigated the interaction between PLP2 and ZO-1. Using immunofluorescence and overexpression of PLP2 with either the N- or C-terminus deleted, the authors showed that the C-terminus of PLP2 is important for the proper localization of ZO-1 during CCM. The authors also showed the importance of the C-terminus of PLP2 in the speed and persistence of cells in CCM as well as the localization of actin to the leading edge. The authors conclude that the PLP2-ZO-1-actin axis is critical for leading-edge cell dynamics during CCM.

The authors present interesting data regarding the dynamics of PLP2 in CCM.
However, as it stands, the data presented do not clearly support the conclusions brought forward by the authors.

Comments for the author

Below is a list of some major concerns that need to be addressed.

1. To confirm that the CRISPR-mediated PLP2 KO cells do not exhibit off-target effects, experiments performed on a second stable clone should be included using a different guide RNA. In addition, to further support the migration phenotype observed in PLP2 KO cells, CCM should be measured in a cell line that has naturally low expression of PLP2 (e.g. CACO2 cells).

2. Although evidence is presented for the presence of PLP2-positive exosomes (Fig. 1G-1H) and that exosomes from PLP2-overexpressing cells can rescue the KO phenotype, it is important to include exosomes from SW480 and PLP2 KO cells as a negative control. Regarding the exosome preparations, the authors show that GW4869 treatment inhibits exosome production (Fr. S1I), yet in panel S1G, there is a minimal change in the presence of PLP2 in the exosome preparation. The IF images showing the distribution of PLP2-mCherry are also difficult to interpret. Overexpression of Rab27a could impact the extent of PLP2 in vesicles. The authors should show the distribution of endogenous PLP2, Rab27a, and CD63. How was the colocalization quantified?

3. While the small differences in speed and persistence of the cell migration are statistically significant (Fig. 2E-F, Fig. 4D-E'), it is not clear that they are biologically significant. This is

problematic as the high n can make minimal differences appear statistically significant. From how many different experiments are the individual points coming from?

4. The authors show robust data for an association between PLP2 and ZO-1; however, there is not enough evidence to say this is a direct interaction. Additionally, the evidence that this PLP2-ZO-1 complex works through ZO-1's association with actin is only correlative. The data in Fig. 4G demonstrate that PLP2 KO or removal of the C-terminus results in less actin at the leading edge but they do not demonstrate that ZO-1 is the connecting factor between PLP2 and actin.

5. In the pull-down assays (Fig. 3C-D, Fig. S3B) , the input/elution legend needs to be clearer. In the text, it is stated that ZO-1 is co-eluted with PLP2 but why is ZO-1 present in the input? Is ZO-1 the bait protein? How is GST eluted separately from GST-CBP? Although other MARVEL-domain-containing proteins have been shown to interact with ZO-1, pull-down assays alone are not enough to demonstrate a direct interaction between two proteins.

6. Please provide the number of times experiments were performed in legends.

7. The lack of a discussion section resulted in an abrupt ending to the paper. A discussion would be beneficial for analyzing the results and putting them into broader context.

First revision

Author response to reviewers' comments

We greatly appreciate the editor's and the reviewers' critical comments and insightful suggestions. We are thankful that they found a potential importance in our study, highlighting PLP2 as a vital player of collective cell migration (CCM) where PLP2 modulates leading edge actin cytoskeleton. Keeping this very essence, we have now revised the manuscript by addressing all the comments of the referees. We carried out several new experiments, extensively edited the document to keep the study more focused, and added appropriate controls to support our conclusions, wherever required.

We have now carried out extensive live cell imaging using time lapse confocal microscopy to investigate dynamics of PLP2 as well as its co-dynamics with the leading edge markers (Figure 2B and 6A, Movie S4 and S11) during CCM. We further validated PLP2 mediated cytoskeletal rearrangement at the leading edge by probing against leading edge marker, cortactin in control, PLP2 deficient, as well as the cells overexpressing the wild type PLP2 or the deletion mutants in the PLP2 knock-out background (Figure 5H, S5G).

To further strengthen our observation on PLP2 mediated cytoskeletal rearrangement at the leading edge, we have now performed set of experiments that demonstrate the involvement of Rac1, an established regulator for cytoskeletal remodelling during cell migration (Ridley et al., 1992). Both biochemical Rac1 activation assay as well as cell based imaging approaches were used to show that PLP2 mediated CCM involves active Rac1 in the cells (Figure 6, S6, Movie S11, S12).

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

Reviewer 1 Advance Summary and Potential Significance to Field:

In this manuscript, Ghosh et al. studied the roles of PLP2 in collective migration of colon carcinoma cells. They characterize SW480 as a potential model to study collective cell migration. PLP2 is described to shuttle between CCJ and the free membrane regions. The authors also confirm previous observations that PLP2 can be secreted as part of exosomes. They propose that loss of

PLP2 limited wound healing and this can be rescued by PLP2 expression or addition of PLP2 containing exosomes. Functionally, they propose that ZO-1 is recruited by PLP2 at the leading edged and CCJs. They map the key role of the C-terminal region of PLP2 to mediate this function. Collectively, these data form an interesting and potentially important study. Uncovering the molecules that control CCM is a limiting step to understand this key process in metastasis. Once the authors revise their original submission, they may reveal PLP2 as such a novel player which will open up to further characterization of this molecule in CCM.

Summary: We appreciate that the reviewer has found this study, highlighting the role of PLP2 in CCM, potentially important for the field. We thank the reviewer for his/her critical comments that helped to overall improve the quality of the manuscript.

Reviewer 1 Comments for the Author:
Major Comments

1-The authors begin their manuscript by quantifying several parameters for SW480 and Caco-2 cells to define if they would be good models of collective cell migration. This section failed to completely convince this reviewer that the SW480 cells are a robust model of collective migration. Clear video imaging that this is occurring would strengthen the manuscript. This could be done in a wound healing assay or using a chemoattractant. In my opinion, it is unclear if what we learn, at the edge of the wounds, really represent collective migration.

Response: We are thankful to the reviewer for pointing it out. As the focus of the study is to decipher the cellular role of PLP2 in the context of colorectal cancer (CRC) progression, we have selected SW480 cell line since it has better invasive potential (Kim et al., 2013; Warburton et al., 1992; Yoon et al., 2008) as well as higher endogenous expression of PLP2 compared to Caco-2 cells (Figure S1A, S1B).

SW480 cells are of epithelial origin where EMT has been triggered (Kim et al., 2013; Warburton et al., 1992; Yoon et al., 2008). Since EMT represents one of the hallmarks of metastasis and cancer progression (Nieto et al., 2016), this cell type with partial epithelial features, represents an appropriate model for studying CRC advancement (Yoon et al., 2008). Before proceeding for CCM studies, we characterized SW480 for its partial epithelial as well as migratory characteristics by probing for several markers for both the features (Figure S1C- S1E'). In addition, a video imaging of migrating wild type SW480 cells in response to the wound, has now been added in the revised manuscript (Movie S2) as recommended by the reviewer.

As per the suggestions from the other reviewers, we further monitored the collective behaviour exhibited by Caco-2 and SW480 cells and attached the data here for the reviewer's reference (Figure R1, Movie R1). Briefly, Caco-2 cells migrate with an average speed of ≈0.09 µm/min (Figure R1A-C) and shows highly directional movement as represented by the distribution of trajectory angles (Figure R1D, R1F) and persistence of the tracks (Figure R1E). These CCM features are closely associated with the reported values of polar epithelial MDCK cells (Petitjean et al., 2010). In contrast, SW480 cells migrate with an average speed of ≈0.05-0.06 µm/min (Figure R1A-C) and exhibit less directional migration compared to Caco-2 cells (Figure R1D-R1F). These features closely represent migration exhibited by fibroblast like NRK cells (Petitjean et al., 2010). However, the number of layers until which the horizontal component of the velocity remains largely unaltered, corresponds to ≈6-7 cell layers (≈110 µm away from the wound) (Figure S2Ja) in SW480 cells which is intermediate between MDCK and NRK cells (refer to 2nd paragraph of the Discussion section) (Petitjean et al., 2010). Hence SW480 cells represent a degree of collectiveness intermediate between the polar epithelial and the fibroblast like cell types which also corroborates with its partial epithelial characteristics as evident from the localization of cell-cell junction (CCJ) associated proteins, ZO-1 in 61% cells and occluding in 45% cells (Figure S1E-E').

From the above observations we propose that Caco-2 (Schreider et al., 2002) and SW480 (Faux, 2003), differing in their cell-cell adhesion properties (Collins and Nelson, 2015; Friedl and Mayor, 2017), could represent two autonomous models for studying CCM. CCM in Caco-2 closely represents "Moving sheets and clusters" (Friedl and Mayor, 2017) where cells within the moving sheet are tightly coupled to each other (Chapnick and Liu, 2014; Plutoni et al., 2016) and the

sheet displacement is driven by traction force generation via coordination between the leader and the follower cells (Bazellières et al., 2015; Brugués et al., 2014). Instead, CCM in SW480 closely represents “Moving cell networks” where cells move in a co-ordinated manner as loosely cohesive group with a variable tendency to individualize (Friedl and Mayor, 2017; Scarpa et al., 2015). Since CCM of “Moving cell networks” are closely related to cancer progression and metastasis (Haeger et al., 2014; Ilina et al., 2011), SW480 cells represent a more appropriate model for studying molecular and cellular mechanism of CCM during CRC advancement.
Figure R1 and Movie R1 provide a representation of the variable CCM types of these two cell lines.

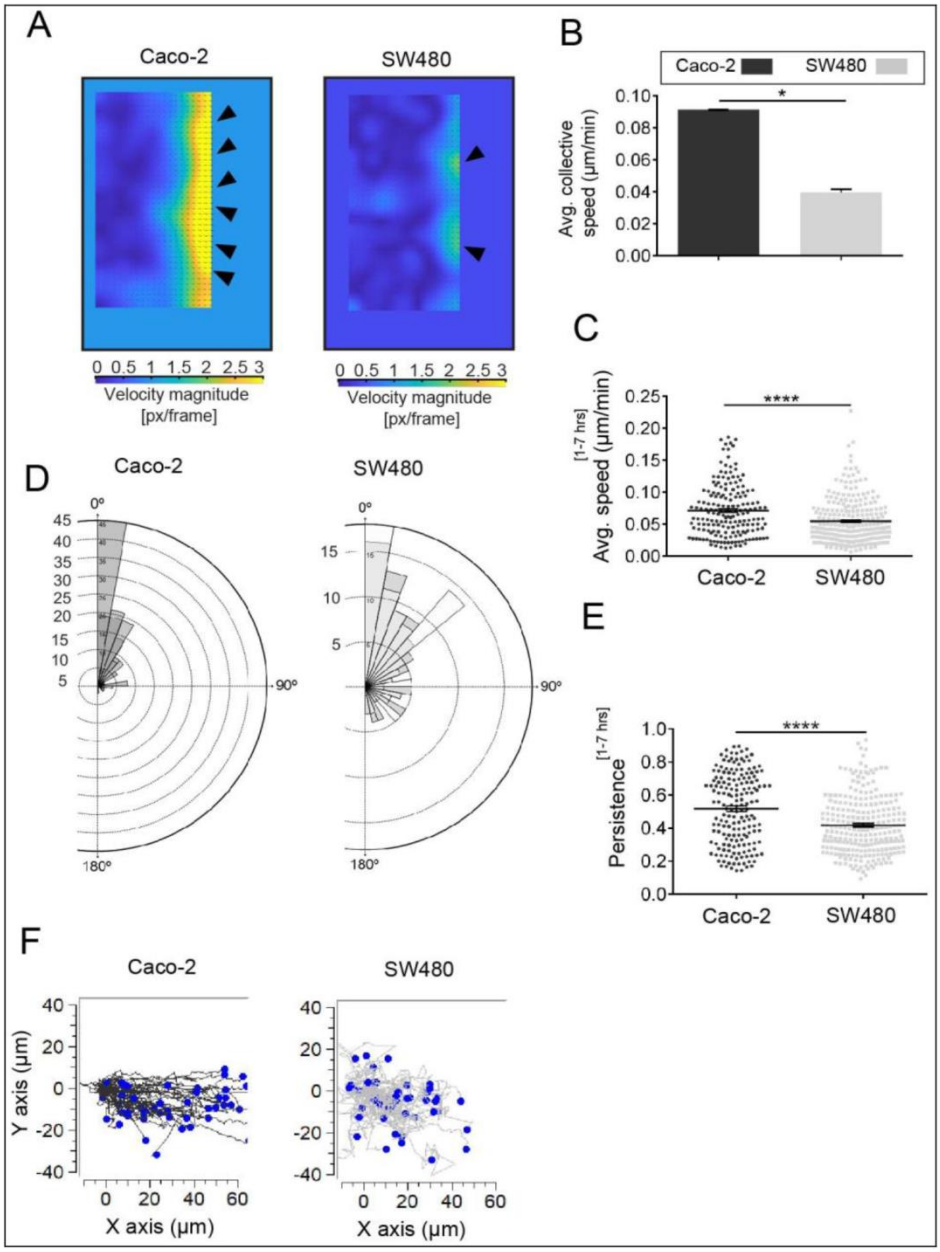


Figure R1: Collective behaviour of Caco-2 and SW480 cells. A) to F) Collective cell migration (CCM) measured for Caco-2 and SW480 cells. A) to B) PIV analysis. A) Velocity heatmaps (1 pixel = 0.586 µm; 1 frame= 15 mins; high-speed zones: yellow regions pointed with black arrows) and B) Average collective speed measured between 1 to 7 hrs of CCM from two independent experiments (N=2). C) to F) Track Analysis. Individual cells tracked from the first four layers of the progressing cell sheets. C) Average speed and E) persistence measured between 1 to 7 hrs of CCM. D) Rose plots of trajectory angles. The magnitude of each bar shows the fraction of cells with the indicated angle trajectory. For C) to E) n= 178 for Caco-2 (N=2); 255 for SW480 (N=2). F) Trajectories of 50 representative cells measured over 12 hrs. All panels: n= number of cells/tracks, N= number of

experiments; Data represented as mean±SEM (for B, C and E); Statistical significance was calculated using an unpaired two-tailed t-test. *, P<0.05; ****, P<0.0001. Ghosh et al., unpublished data.

2-The dynamic localization of PLP-2 at CCJ and at the free membrane edges is not completely convincing. A major worry is that the signal of GFP-PLP2 is proportional to membrane density. For example, in video 2, we see low signal in thin lamellipodial extensions, and the signal becomes stronger (almost like at the CCJ) when these retracts. The authors would need to convincingly demonstrate that there is a bona fide recruitment at the leading edge to support their claims of dynamic localizations. This limitation is present in figure 2-3.

Response: We sincerely thank the reviewer for pointing out this limitation. We have now added clear live cell confocal video imaging of SW480 cells co-expressing PLP2-GFP and mCherry-cortactin (Figure 2B, Movie S4). Cortactin is reported to bind to the cortical actin network at the leading edge lamellipodial structures during migration (Weed et al., 2000). The co-dynamics of PLP2-GFP with Cortactin-mCherry during CCM (for 32 minutes, captured at 4^{th} hr post wound) further demonstrate that there is a bona fide recruitment of PLP2 at the leading edge during collective migration.

3a)-The authors propose that PLP2 and ZO-1 colocalize at the wound edges. Just like stated above, there is a worry that this is non-specific and more a reflection of the membrane densities.
Response: We are thankful to the reviewer for pointing this out. To better support PLP2 and ZO-1 colocalization at the wound edge we have now incorporated the following in the revised version of the manuscript:

a) Data from live cell imaging using confocal microscopy further support the colocalization of PLP2-mCherry and mEmerald-ZO-1 (Movie S9) during collective migration as captured at 4^{th} hr post wound.
b) Montages corresponding to ZO-1 and PLP2 colocalization at 16 hr (Figure 4Ac) has been replaced with better representative images. Additional montages of fixed cell images representing PLP2 and ZO-1 colocalization at 0, 4 and 16 hrs during CCM are now been added in Figure S7A. Moreover, coincidence of line intensities of ZO-1 and PLP2 at the indicated line path (yellow) (Figure 4A, Figure S7A) on the wound edge further support their colocalization at the wound edge.

3b): For example, there are less ruffles in PLP2 KO cells which could contribute to a decreased ZO-1 staining.

Response: We agree with the reviewer that PLP2KO cells indeed develop less ruffles at the edge. However, we believe that the reduction in the ZO-1 level in PLP2KO cells is due to the loss of its association with PLP2.

Our data indicates that ZO-1 colocalizes with PLP2 at the cell periphery and shows its biochemical association with the cytosolic C-terminal end of PLP2. In absence of PLP2, ZO-1 could not localize to the cell periphery that could be rescued in PLP2KO cells overexpressing PLP2-mCherry and ΔNPLP2-mCherry but not mCherry-PLP2ΔC (Figure 4E, 5A). These data suggest that the absence of ZO-1 at the peripheral locations is the result of loss of PLP2 or loss of the association of ZO-1 with PLP2. The above discussion is added in the revised manuscript (Page No 8: line number 23 to 32 and page no 9: line number 1 to 7).

4-Potentially more worrying is that fact that endogenous ZO-1 expression is decreased by PLP2 KO (very clear by WB). How do the authors take this into account when they compare staining intensities? WT and delta N PLP2 rescue the ZO-1 expression levels, but not the deltaC. What would be the localization of overexpressed ZO-1 in these conditions? Would that be a better and less biased readout? These comments are link to Figure 3-4.

Response: As noted by the reviewer, our results clearly suggest that protein level of ZO-1 is lower in PLP2 depleted cells (Figure S4D). So the loss of ZO-1 from cell periphery may be just an effect of reduction of its expression in the cell. Alternatively, as suggested by the in vitro biochemical interaction studies, ZO-1 could not localize to membrane in the absence of its association with

PLP2 (as observed in PLP2KO or PLP2KO+ mCherry-PLP2ΔC cells) (Figure 4E, 5A). To decipher which one of the above or a combination of both is causing the loss of membrane localization for ZO-1, we used fluorescence intensity based normalization while measuring ZO-1 intensity associated with the peripheral membrane locations. Following is a schematic of the intensity normalization approach (Figure R2).

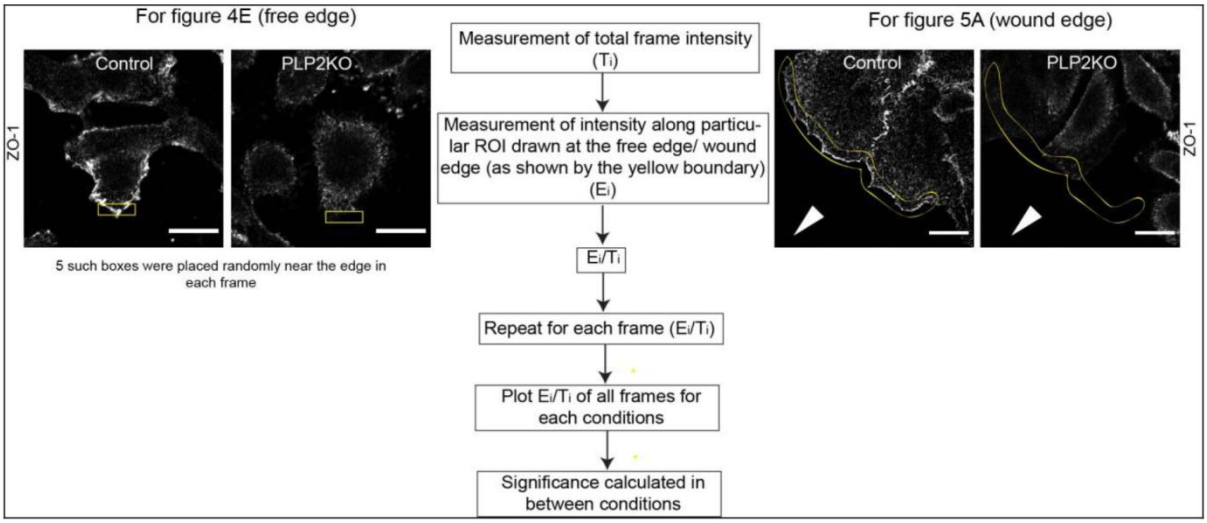


Figure R2: Schematic representation of intensity normalization at the cellular edge.

We have now added these results from the normalised fluorescence intensity based calculation in the revised MS (Figure S4D", S5A). We found that after normalization against total frame intensity, ZO-1 intensity at the edge shows significant reduction in PLP2KO and PLP2KO cells overexpressing mCherry-PLP2ΔC compared to the other conditions. Thus, our data suggests that the perturbation of peripheral ZO-1 localization is not exclusively an effect of reduced ZO-1 protein level rather it relies on ZO-1 association with PLP2 as well. The above discussion is now added to the revised version of the manuscript (Page No 8: line number 28 to 32).

In addition, as per the reviewer's suggestion, we have also tested the localization pattern of overexpressed ZO-1 in control and PLP2KO conditions and added the data here (Figure R3) for the reviewer's reference. It was observed that peripheral localization of mEmerald-ZO-1 is more pronounced in control cells compared to PLP2KO cells which further supports our claim.

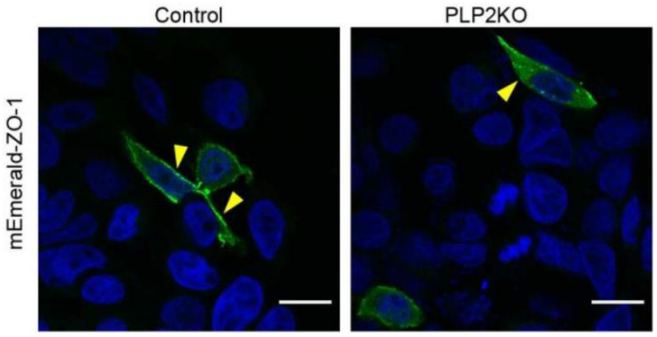


Figure R3: Localization of mEmerald-ZO-1 in control and PLP2KO cells. Yellow arrowhead indicates the peripheral localization. Ghosh et al., unpublished data.

5-One missing experiment is whether deletion of PLP2 blunts metastasis in vivo. Can SW480 cells (and rescued cells) be grafted orthopically and metastasis be tested? If this is not possible, this should be discussed. Many proteins are essential for metastasis in 2D models but not in vivo.

Response: We are thankful to the reviewer for the suggestion. Collective cell migration of cancer epithelium can lead to efficient metastasis (Cheung and Ewald, 2016) and PLP2 being a vital player of CCM in colon cancer epithelium, may thus contribute to the same. However, its

pathophysiological relevance should be further validated through in vivo studies using mouse xenograft models. Moreover, our results further showed the involvement of exosomal PLP2 in the regulation of CCM in colon cancer epithelium. Since exosomes are established well as a cell-cell communication device to support improved metastasis (Becker et al., 2016; Maia et al., 2018), our findings on exosomal PLP2 driving CCM in colon cancer epithelium is a novel addition to the list of exosomal metastatic factors.

We have now added the above discussion related to PLP2 in CRC metastasis in the discussion section of the revised manuscript (2nd last paragraph of Discussion section, page no 14, line no 21 to 33).

Minor Comments
1- The analyses of the number of the size of focal adhesions is not very well integrated in the manuscript. Live analyzes on sparse cells and wounded monolayers would provide much more insights into the role of PLP2 in adhesions dynamics. Otherwise, this data should be removed.

Response: We agree that our results on the focal adhesions are not well integrated in the manuscript and therefore as suggested by the reviewer, we have now omitted the results from the revised manuscript.

2- The discussion on Rac1 is interesting and appropriate, but Rac1 should be omitted from the model and the authors should focus on their data instead.

Response: We thank the reviewer for finding the discussion on Rac1 interesting and agree with him/her that without any experimental evidences we should not be adding Rac1 in our model. However, in the revised manuscript we have added results indicating that PLP2 mediated CCM and cytoskeletal changes at the leading edge involves Rac1 activation (Figure 6 and S6, Movie S11, S12).

The F-actin rich umbrella like structures formed at the migrating edges during CCM, resemble lamellipodia (Small et al., 1999) and formation of lamellipodial structures are known to depend on the activation of small GTPase Rac1 (Ridley et al., 1992). Hence, we proceeded to investigate whether Rac1 is involved in the PLP2 mediated collective migration of SW480 cells.

We began with asking whether the umbrella like structures formed during CCM at 4th hour post-wound, is positive for Rac1. Cells stably expressing PLP2-mCherry were transiently transfected with GFP-Rac1WT and were used to study CCM. Time lapse confocal microscopy revealed that PLP2-mCherry and GFP-Rac1WT indeed colocalize on the umbrella like structures during CCM (Figure 6A, Movie S11). The above finding suggests that these Rac1 positive structures may represent lamellipodia like extensions in our model (Ridley et al., 1992; Yamaguchi et al., 2015).

Since Rac1 activation is crucial for lamellipodia formation during single as well as collective cell migration (Ridley et al., 1992; Yamaguchi et al., 2015), we next assessed global Rac1 activation during 4th hour post-wound using biochemical Rac1 activation assay (Meriane et al., 2002). A marked reduction of global Rac1 activation was observed in PLP2KO cells as compared to the control cells (Figure 6B, S6B) which indicates that PLP2 is involved in the activation of Rac1 during CCM. In addition, PLP2KO cells overexpressing PLP2-mCherry or ΔNPLP2-mCherry showed notable recovery in the global Rac1 activation and overexpression of mCherry-PLP2ΔC also recovered substantial population of active Rac1 however marginally less than that of cells overexpressing PLP2-mCherry or ΔNPLP2-mCherry (Figure 6B', S6B'). Thus, perturbation of PLP2-ZO-1 association does not lead to complete loss of global Rac1 activation suggesting that, the association may not have any major role in global Rac1 activation. However, the perturbation of this association led to cytoskeletal alterations at the leading edge and successive abrogation of collective migration (Figure 5). Based on these results we propose that, association between ZO-1 and PLP2 may contribute to polarized activation of Rac1 that is important for directive cell migration during CCM. However, it would be interesting to unravel other intermediate molecular players that bridge between PLP2 and Rac1 during CCM.

To further investigate whether the Rac1 activity is important during PLP2 mediated CCM, we co-expressed dominant negative mutant of Rac1 (GFP-Rac1DN) (Foster et al., 1996; Nobes et al., 1998) with PLP2-mCherry in PLP2KO cells and studied the rescue in the number of the lamellipodia like structures. In GFP-Rac1DN background, PLP2-mCherry could not significantly rescue the number of F-actin rich lamellipodia like structures (Figure 6Cb, 6C' and S6Ab) compared to that of GFP/ GFP-Rac1WT (wild type Rac1) background (Figure 6Ca & 6Cc, 6C' and S6Aa, S6Ac). Next to address if the PLP2 mediated leading-edge cell dynamics during CCM is reliant on Rac1 activation, we carried out CCM in the PLP2KO cells co-expressing PLP2-mCherry and GFP/ GFP-Rac1DN/ GFP-Rac1WT (Figure 6D-H, S6C-F, Movie S12). PIV and track analysis revealed that the co-expression of PLP2-mCherry and GFP-Rac1DN failed to rescue the number of leading-edge high-speed zones (Figure 6D), average collective speed (Figure 6D', S6D), average track speed (Figure 6E, S6E), persistence (Figure 6F, S6F) and directionality (Figure 6G-H).
From the above results we conclude that Rac1 activation is involved in PLP2 mediated CCM and thus we have not omitted Rac1 from the model. The above results and related discussions are now added to the revised manuscript (Page no 10: line number 15-32, page no 11: line number 1-25, Figures 6, S6, Movies S11, S12).

3- Movie S1. Adding arrows would facilitate the understanding of the phenotypes.

Response: In the revised version, we have added arrows in Movie S1.

4- Movie S4. The GW4869 conditions appears out of focus. Also, the authors should tone down the statement "Drug treated cells showed much reduced CCNM…". The difference remains minor in this video.

Response: We sincerely apologize for the error and have now replaced the movie corresponding to GW4869 conditions (Movie S8).

We agree with the reviewer that the drug treated cells do not show a complete loss of CCM and accordingly reframed the results as "Drug treated cells showed partial abrogation of CCM". However, GW4869 being an established inhibitor of exosome biogenesis (Guo et al., 2015; Trajkovic et al., 2008), may serve as an appropriate control for demonstrating the implication of the exosomal PLP2 pool in CCM, and therefore we did not completely omit the results from the revised manuscript.

5- Methods: CRISPER should be corrected for "CRISPR".

Response: We sincerely apologize for the error and corrected the same in the revised version (Page no 17, line no 26).

Reviewer 2 Advance Summary and Potential Significance to Field:

Ghosh and colleagues describe a role for the transmembrane protein proteolipid protein 2 (PLP2) during collective cell migration. PLP2 was found to be enriched in cell membranes during migration as well as in secreted exosomes. PLP2 knock out cells showed decreased collective cell migration which could be rescued by expression of full-length of PLP2, an N- terminal deletion of PLP2 or the addition of exosomes. This rescue corresponded with the ability of PLP2 to recruit ZO-1 to the leading edge and cell-cell junctions during migration. While this is an interesting study potentially linking PLP2 to ZO-1, actin dynamics and cell migration, I have major concerns with the over interpretation of their findings and feel that they failed to clearly demonstrate PLP2-dependent cytoskeletal rearrangement and PLP2- driven leading-edge dynamics. I feel this paper in its current form fall short of making a significant and novel contribution to our understanding of collective cell migration.

Summary: We appreciate the reviewer for critically going through the manuscript and pointing out its limitations. We sincerely thank the reviewer for his/her insightful suggestions that have not only improved the data quality but also has substantially added to our current understanding of the molecular basis of PLP2 mediated cytoskeletal rearrangements at the leading edge during CCM. We have addressed below each of the issue raised and edited the manuscript accordingly.

General Comments:
Throughout the manuscript the authors use both bar graphs and scatter plots and seemingly switch back and forth between both presentations without justification. Bar graphs can obscure patterns in data and should be avoided when presenting data.

Response: We thank the reviewer for his/her suggestion. We have now used scattered plots throughout the revised manuscript.

The authors choose to focus in SW480 cells over Caco-2 cells as the endogenous expression of PLP2 was higher in SW480 cells. As both the cells undergo collective cell migration in 2D, one could presume that some of the mechanisms that govern collective cell migration is the same. Results gathered in SW480 cells could be compared to those in Caco-2 cells that naturally express lower amounts of PLP2. For example, CRISPR-mediated KO of PLP2 resulted in loss of ZO-1 at the leading edge of migrating SW480 cells, do Caco-2 cells naturally have lower amounts of ZO-1 due to lower expression of PLP2? Can the PLP2- contain exosomes from SW480 cells affect the collective cell migration of Caco-2 cells? The comparison of these two cells lines would strengthen their findings.

Response: We sincerely thank the reviewer for bringing up this discussion. Indeed, as pointed out by the reviewer, we used SW480 cells for studying the role of PLP2 in CRC progression, as the endogenous expression level of PLP2 was much higher in SW480 cells compared to that in Caco-2 cells. Although, both Caco-2 and SW480 cells undergo collective cell migration in 2D, the collective behaviour exhibited by these two cell types represent two variable CCM types as per our observation. We have attached the data here for the reference (Figure R1, movie R1). Briefly, Caco-2 cells migrate with an average speed of ≈0.09 μm/min (Figure R1A-C) and shows highly directional movement as represented by the distribution of trajectory angles (Figure R1D, R1F) and persistence of the tracks (Figure R1E). These CCM features are closely associated with the reported values of polar epithelial MDCK cells (Petitjean et al., 2010). In contrast, SW480 cells migrate with an average speed of ≈0.05μm/min (Figure R1A-C) and exhibit less directional migration compared to Caco-2 cells (Figure R1D-R1F). These features closely represent migration exhibited by “fibroblast like” NRK cells (Petitjean et al., 2010). However, the number of layers until which the horizontal component of the velocity remains largely unaltered, corresponds to ≈6-7 cell layers (≈110μm away from the wound) (Figure S2Ja) in SW480 cells which is intermediate between MDCK and NRK cells (refer to 2nd paragraph of Discussion section). Hence SW480 cells represent a degree of collectiveness intermediate between the polar epithelial and the fibroblast like cell types which also corroborates with its partial epithelial characteristics as evident from the localization of cell-cell junction (CCJ) associated proteins, ZO-1 in 61% cells and occludin in 45% cells (Figure S1C-E’).

From the above observations we propose that Caco-2 (Schreider et al., 2002) and SW480 (Faux, 2003), differing in their cell-cell adhesion properties (Collins and Nelson, 2015; Friedl and Mayor, 2017), could represent two autonomous models for studying CCM. CCM in Caco-2 closely represents “Moving sheets and clusters” (Friedl and Mayor, 2017) where cells within the moving sheet are tightly coupled to each other (Chapnick and Liu, 2014; Plutoni et al., 2016) and the sheet displacement is driven by traction force generation via coordination between the leader and the follower cells (Bazellières et al., 2015; Brugués et al., 2014). Instead, CCM in SW480 closely represents “Moving cell networks” where cells move in a co-ordinated manner as loosely cohesive group with a variable tendency to individualize (Friedl and Mayor, 2017; Scarpa et al., 2015). Since CCM of “Moving cell networks” are closely related to cancer progression and metastasis (Haeger et al., 2014; Ilina et al., 2011), SW480 cells represents a more appropriate model for studying molecular and cellular mechanism of CCM during CRC advancement.

Figure R1 and Movie R1 gives a representation of the variable CCM nature of these two cell lines.

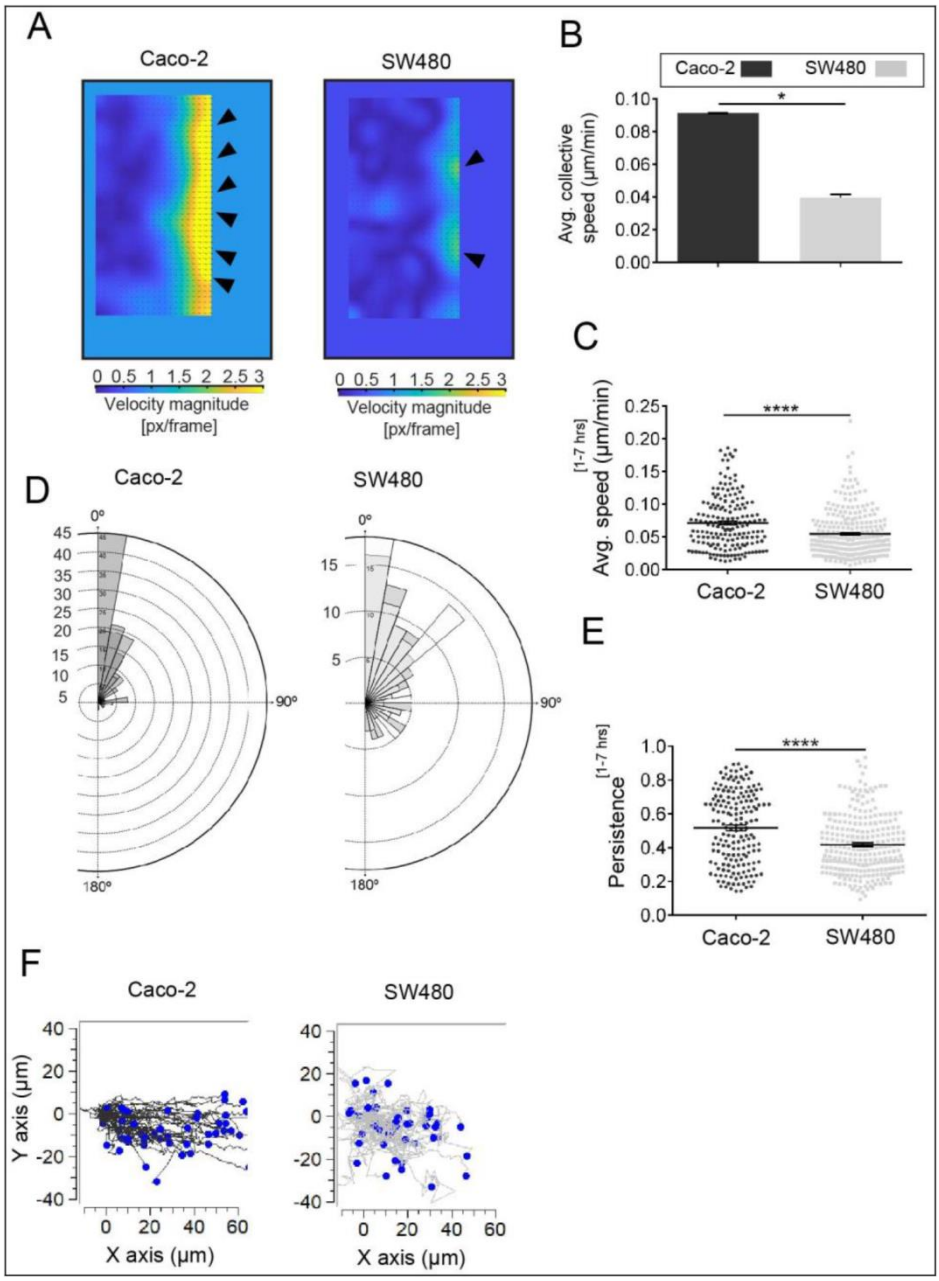


Figure R1: Collective behaviour of Caco-2 and SW480 cells. A) to F) Collective cell migration (CCM) measured for Caco-2 and SW480 cells. A) to B) PIV analysis. A) Velocity heatmaps (1 pixel = 0.586 µm; 1 frame= 15 mins; high-speed zones: yellow regions pointed with black arrows) and B) Average collective speed measured between 1 to 7 hrs of CCM from two independent experiments (N=2). C) to F) Track Analysis. Individual cells tracked from the first four layers of the progressing cell sheets. C) Average speed and E) persistence measured between 1 to 7 hrs of CCM. D) Rose plots of trajectory angles. The magnitude of each bar shows the fraction of cells with the indicated angle trajectory. For C) to E) n= 178 for Caco-2 (N=2); 255 for SW480 (N=2). F) Trajectories of 50 representative cells measured over 12 hrs. All panels: n= number of cells/tracks, N= number of experiments; Data represented as mean±SEM (for B, C and E); Statistical significance was calculated using an unpaired two-tailed t-test. *, $P<0.05$; ****, $P<0.0001$. Ghosh et al., unpublished data.

We further like to mention that our immunofluorescence study with paraformaldehyde fixed Caco-2 cells using ZO-1 specific antibody revealed that apparently there is no considerable difference in ZO-1 level compared to SW480 cells (Figure R4).

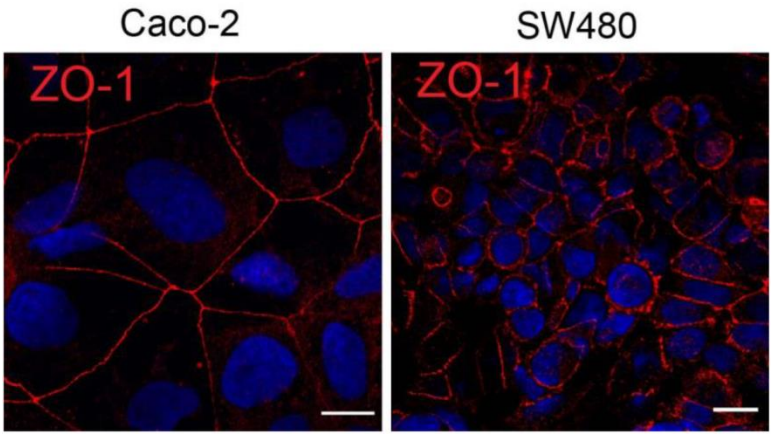


Figure R4: ZO-1 localization to the lateral cell-cell junctions in Caco-2 cells. Scale bar: 15 µm. Ghosh et al., unpublished data.

We appreciate the reviewer's suggestion to explore whether treatment of Caco-2 cells with PLP2 containing exosomes leads to any alteration in its collective migration profile, considering that PLP2 expression in the latter cells line being remarkably lower than that of SW480. Accordingly, we treated Caco-2 cells with PLP2-GFP containing exosomes but it did not show any measurable effect on the CCM (Figure R5, Movie R2). This may be due to the lack of any major role of PLP2 in collective migration of Caco-2 suggesting for a possible PLP2 independent mechanism underlying the CCM in Caco-2 cells. This interpretation further corroborates with the variable CCM type exhibited by Caco-2 cells as explained above. However, we cannot rule out the possibility of sub-optimal uptake of the exosomes by the Caco-2 cells, leading to no significant measurable alteration in the migration characteristics of the recipient cells.

Moreover the variations of these two cell lines at the genetic level (Ahmed et al., 2013) and degree of EMT (Epithelial Mesenchymal Transition) (Kim et al., 2013) created a limited scope for comparing their CCM characteristics.

Since the results from our exosome treatment experiments did not show any significant effect of PLP2 on CCM of Caco-2 cells, we have not included the data in the current manuscript. However, the results (Figure R5, Movie R2) are attached here for the reviewer's reference.

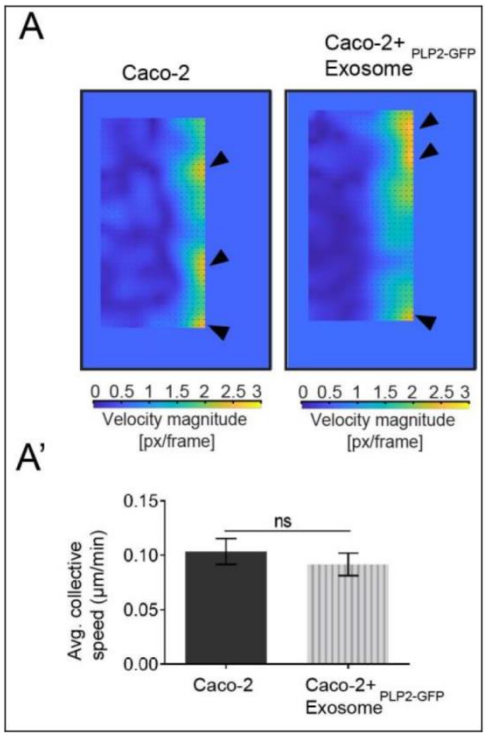


Figure R5: Effect of PLP2 containing exosomes on collective migration of Caco-2 cells. Collective cell migration (CCM) measured for Caco-2 cells with or without treatment with PLP2-GFP containing exosomes. A) to B) PIV analysis. A) Velocity heatmaps (1 pixel = 0.586 µm; 1 frame= 15 mins; high-speed zones: yellow regions pointed with black arrows) and B) Average collective speed measured between 1 to 7 hrs of CCM from two independent experiments (N=2). Data represented as mean±SEM; Statistical significance was calculated using an unpaired two-tailed t-test. ns= non-significant. Ghosh et al., unpublished data.

One of the main claims of the manuscript is that PLP2 is recruiting ZO-1 to the leading edge during collective cell migration and this is resulting in cytoskeletal re-arrangement. This was demonstrated through fixed samples via staining with phalloidin or imaging of ZO-1. To better support this claim the authors should include analysis of other cytoskeletal markers such as the Arp2/3 complex, or cortactin. Furthermore, careful analysis of actin dynamics (rates of polymerization etc) could also be performed using live-cell imaging.

Response: We appreciate the reviewer for his/her suggestion. We have now included the results from fixed cell imaging showing cortactin distribution at the leading edge of control, PLP2KO and PLP2KO cells overexpressing PLP2-mCherry, ΔNPLP2-mCherry or mCherry- PLP2ΔC and quantified the number of cortactin rich 'umbrella structures' (Figure 5H, S5G). In addition, the co-dynamics of PLP2-GFP and mCherry-cortactin as observed through live cell confocal imaging experiments (Figure 2B, Movie S4) further supported our claim that PLP2 mediated collective migration involves cytoskeletal reorganization at the leading edge.

Further, we have also included the co-dynamics of Rac1 and PLP2 as measured by live cell confocal microscopy (Figure 6A, Movie S11) at 4th hr post wound. This result indicates that the PLP2 positive umbrella like extensions observed at the leading edge during CCM is also positive for Rac1 and thus resembling lamellipodia like structures (Ridley et al., 1992). This evidence further support the claim of PLP2 mediated cytoskeletal rearrangement at the leading edge since Rac1 activation is well known for actin dynamics via its effector, Arp2/3 (Sit and Manser, 2011).
Using the dominant negative mutant of Rac1 in the rescued PLP2KO cells, we have further demonstrated that PLP2 mediated CCM involves Rac1 dependent cytoskeletal remodelling (Figure 6, S6) at the leading edge. Some of these results are also discussed in detail below.

The inclusion of Rac into their model seems out of place as there was no empirical evidence for it and is speculation. The use of active Rac probes could be used here to back up the authors' claims, or in the very least staining for proteins know to be involved in Rac activation and

recruitment (WASP, or N-WASP, WIP etc.).

Response: We are thankful to the reviewer for pointing this out. In the revised manuscript, we have addressed this issue by including the results from our latest study which indicates that PLP2 mediated cytoskeletal rearrangement at the leading edge of collectively migrating cells involves Rac1 activation (Figure 6, S6, Movie S11, S12).

The F-actin rich umbrella like structures formed at the migrating edges during CCM, resemble lamellipodia (Small et al., 1999) and formation of lamellipodial structures are known to depend on the activation of small GTPase Rac1 (Ridley et al., 1992). Hence, we proceeded to investigate whether Rac1 is involved in the PLP2 mediated collective migration of SW480 cells.

We began with asking whether the umbrella like structures formed during CCM at 4^{th} hour post-wound, are positive for Rac1. Time lapse confocal microscopy revealed that PLP2- mCherry and GFP-Rac1WT indeed colocalize on the umbrella like structures during CCM (Figure 6A, Movie S11). The above finding suggests that these Rac1 positive structures may represent lamellipodia like extensions in our model (Ridley et al., 1992; Yamaguchi et al., 2015).

Since Rac1 activation is crucial for lamellipodia formation during single as well as collective cell migration (Ridley et al., 1992; Yamaguchi et al., 2015), we next assessed global Rac1 activation during 4^{th} hour post-wound using biochemical Rac1 activation assay (Meriane et al., 2002). A marked reduction of global Rac1 activation was observed in PLP2KO cells as compared to the control cells (Figure 6B, S6B) which indicates that PLP2 is involved in the activation of Rac1 during CCM.

In addition, PLP2KO cells overexpressing PLP2-mCherry or ΔNPLP2-mCherry showed notable recovery in the global Rac1 activation and overexpression of mCherry-PLP2ΔC also recovered substantial population of active Rac1 however marginally less than that of cells overexpressing PLP2-mCherry or ΔNPLP2-mCherry. Thus, perturbation of PLP2-ZO-1 association does not lead to complete loss of global Rac1 activation suggesting that, the association may not have any major role in global Rac1 activation. However, the perturbation of this association led to cytoskeletal alterations at the leading edge and successive abrogation of collective migration (Figure 5). Based on these results we propose that, association between ZO-1 and PLP2 may contribute to polarized activation of Rac1 that is important for directive cell migration during CCM. However, it would be interesting to unravel other intermediate molecular players that bridge between PLP2 and Rac1 during CCM.

To further investigate whether the Rac1 activity is important during PLP2 mediated CCM, we co-expressed dominant negative mutant of Rac1 (GFP-Rac1DN) (Foster et al., 1996; Nobes et al., 1998) with PLP2-mCherry in PLP2KO cells and studied the rescue in the number of the lamellipodia like structures. In GFP-Rac1DN background, PLP2-mCherry could not significantly rescue the number of F-actin rich lamellipodia like structures (Figure 6Cb, 6C', S6Ab) compared to that of GFP/ GFP-Rac1WT (wild type Rac1) background (Figure 6Ca, 6Cc, 6C', S6Aa, S6Ac).

Next to address if the PLP2 mediated leading-edge cell dynamics during CCM is reliant on Rac1 activation, we carried out CCM in the PLP2KO cells co-expressing PLP2-mCherry and GFP/ GFP-Rac1DN/ GFP-Rac1WT (Figure 6D-H, S6C-F, Movie S12). PIV and track analysis revealed that the co-expression of PLP2-mCherry and GFP-Rac1DN failed to rescue the number of leading-edge high-speed zones (Figure 6D), average collective speed (Figure 6D', S6D), average track speed (Figure 6E, S6E), persistence (Figure 6F, S6F) and directionality (Figure 6G-H).

The above results and the associated discussions are now added in the revised manuscript (Page no 10: line number 15-32, page no 11: line number 1-25, Figures 6, S6, Movies S11, S12). From these results we conclude that Rac1 activation is involved in PLP2 mediated CCM that supports inclusion of Rac1 in the model.

Reviewer 2 Comments for the Author:

In Figure 1, PLP2 seems to be enriched in some belt around the cell not particularly at the leading edge. Dual live-imaging of actin or some other leading edge marker would strengthen this claim as

PLP2 is not classically considered a marker for the leading edge. The same is true for Figure 2.

Response: We are thankful to the reviewer for the suggestion. In figure 1 representing fixed cell images, we have now incorporated cortactin along with F-actin as a leading edge marker, that showed marked colocalization with PLP2-GFP (Figure 1Ba). Cortactin is reported to bind to the cortical actin network at the leading edge lamellipodial structures during migration (Weed et al., 2000).

In Figure 2, we have now added time lapse images from live cell confocal microscopy on SW480 cells co-expressing PLP2-GFP and cortactin-mCherry (Figure 2B, Movie S4). The co-dynamics of PLP2-GFP with Cortactin-mCherry during CCM (for 32 minutes captured at 4 hrs post wound) further confirms the enrichment of PLP2 at the leading edge membrane structures.

In Figure 2. PIV analysis should be better explained, and the quality of the phase-contrast images presented could be improved.

Response: We are thankful to the reviewer for the suggestion. In the revised manuscript we have now explained that PIV analysis is a whole-field cross-correlation technique that provides local displacements in real-time for the entire cell monolayer (Page no 5, line no 2-4) (Petitjean et al., 2010; Vig et al., 2016). The detailed description of this analysis is also provided in the Materials and Methods section under “Particle Image Velocimetry (PIV)” (Page no 23).

We have also attempted to improve the quality of the phase contrast images for better representation (Figure 3A).

It is not clear to me what is being shown in graphs 2E and 2F. Are these individual cells or multiple cells? Why are there no error bars on these graphs.

Response: We apologise for the confusion. The results shown in Figure 3D and 3E (earlier Figure 2E and F) represents track speed (Figure 3D) or persistence (Figure 3E) plotted over 12 hrs time course. Tracks properties such as average speed, persistence at every time point were averaged for multiple cells within a single experiment. Averaged values for multiple experiments for each condition are plotted over time and error bars are now shown in these plots.

It also seems as though the addition of PLP2-GFP exosome increased directionality (and the knockout of PLP2 decreased directionality) this is an interesting aspect but was only briefly remarked upon.

Response: We are thankful to the reviewer for this critical comment.

Studies with exosomal pool of PLP2 investigated the effect of extracellular pool of the protein on CCM as well as establish PLP2 as a potential metastatic factor. Since exosomal PLP2 markedly rescued directional movement, we have discussed this aspect in the context of cell polarization effect as observed for other exosomal metastatic factors (Sung et al., 2015). The above discussion has now been added in the revised version of the manuscript (Discussion section, page no 12: line no 30-33, page no 13 line no 1-2).

The authors should be careful when correlating focal adhesion size with kinetics in particular if their analysis relied only upon fix analysis which lacks dynamic behavior.

Response: We agree with the reviewer in this point. As suggested by the other reviewer, we have now removed the results related to focal adhesion size, as it was not making any direct impact along the line of focus of the current manuscript.

Reviewer 3 Advance Summary and Potential Significance to Field:

The authors investigated the role of proteolipid protein 2 (PLP2) in collective cell migration (CCM) in colon cancer. Using PLP2 knockout (PLP2 KO) cells and fluorescent PLP2 overexpression constructs, they show that PLP2 localizes to both cell-cell junctions as well as free-edge membranes and that cells lacking PLP2 exhibit migration defects. Interestingly, the authors found

that overexpression of PLP2-mCherry or addition of exosomes from PLP2- overexpressing cells rescued the migration defect of PLP2 KO cells. Next, the authors investigated the interaction between PLP2 and ZO-1. Using immunofluorescence and overexpression of PLP2 with either the N- or C-terminus deleted, the authors showed that the C-terminus of PLP2 is important for the proper localization of ZO-1 during CCM. The authors also showed the importance of the C-terminus of PLP2 in the speed and persistence of cells in CCM as well as the localization of actin to the leading edge. The authors conclude that the PLP2-ZO-1-actin axis is critical for leading-edge cell dynamics during CCM.

The authors present interesting data regarding the dynamics of PLP2 in CCM. However, as it stands, the data presented do not clearly support the conclusions brought forward by the authors.

Summary: We appreciate that the reviewer found our study interesting. We thank him/her for pointing out the weaknesses and strengths of the manuscript and directing us to further reinforce the conclusion of our study. Accordingly, we have revised the manuscript, which is more clear and focused now.

Reviewer 3 Comments for the Author:

Below is a list of some major concerns that need to be addressed.

1a). To confirm that the CRISPR-mediated PLP2 KO cells do not exhibit off-target effects, experiments performed on a second stable clone should be included using a different guide RNA.

Response: We sincerely thank the reviewer for the suggestion. To rule out the off-target effects and provide better validation for the phenotypes reported in this manuscript, we have performed gene specific rescue experiments in PLP2KO cells for each phenotype observed (Figure 2, 4E, 5, 6) along with appropriate controls.

In addition, we have now used an alternative approach to deplete PLP2 using ON- TARGETplus SMARTpool SiRNA (Figure S2A-E). The use of low concentration of the pooled siRNA assures much less chance of off-target effect and hence adopted frequently in establishing gene specific phenotypes (Caffrey et al., 2011; Hannus et al., 2014). The efficiency of the KD was confirmed by Western blot using PLP2 specific antibody (Figure S2A-A'). Similar trends in the results (Figure S2B-E, Movie S5) using additional approach of silencing PLP2, emphasized the authenticity of the gene specific phenotypes. The results are included in the revised manuscript (Page no 4: line no 28-32, page no 5: 1-12, Figure S2A-E).

1b). In addition, to further support the migration phenotype observed in PLP2 KO cells, CCM should be measured in a cell line that has naturally low expression of PLP2 (e.g. CaCO2 cells).

Response: As suggested by the reviewer, we have carried out extensive studies on the collective behaviour of Caco-2 cells and analysed the results to quantify migration related parameters. Briefly, Caco-2 cells migrate with an average speed of ≈0.09 μm/min (Figure R1A-C) and shows highly directional movement as represented by the distribution of trajectory angles (Figure R1D, R1F) and persistence of the tracks (Figure R1E). These CCM features are closely associated with the reported values of polar epithelial MDCK cells (Petitjean et al., 2010). Thus we propose that CCM in Caco-2 closely represents "Moving sheets and clusters" (Friedl and Mayor, 2017) where cells within the moving sheet are tightly coupled to each other (Chapnick and Liu, 2014; Plutoni et al., 2016) and the sheet displacement is driven by traction force generation via coordination between the leader and the follower cells (Bazellières et al., 2015; Brugués et al., 2014). Our hypothesis further corroborates with the reported epithelial nature of this cell type (Ebnet et al., 2000; Hashimoto and Shimizu, 1993; Schreider et al., 2002).

In contrast, SW480 cells migrate with an average speed of ≈0.05 μm/min (Figure R1A-C) and exhibit less directional migration compared to Caco-2 (Figure R1D-F). These features closely represent migration exhibited by fibroblast like NRK cells (Petitjean et al., 2010). However, the number of layers until which the horizontal component of the velocity remains largely unaltered, corresponds to ≈6-7 cell layers (≈110 μm away from the wound) (Figure S2Ja) in SW480 cells

which is in-between MDCK and NRK cells (refer to 2nd paragraph of Discussion section). Hence SW480 cells represent a degree of collectiveness intermediate between the polar epithelial and the fibroblast like cell types which also corroborates with its partial epithelial characteristics as evident from the localization of cell-cell junction (CCJ) associated proteins, ZO-1 in 61% cells and occludin in 45% cells (Figure S1E-E'). CCM in SW480 thus closely represents "Moving cell networks" (Friedl and Mayor, 2017; Ilina et al., 2011) where cells move in a co-ordinated manner as loosely cohesive group with a variable tendency to individualize (Haeger et al., 2014; Scarpa et al., 2015).

From the above observations we propose that Caco-2 and SW480, differing in their cell-cell adhesion properties (Faux, 2003; Schreider et al., 2002), could represent two autonomous models for studying CCM (Friedl and Mayor, 2017). Hence we believe, comparing the migration phenotype observed in PLP2KO cells, with that of Caco-2 cells will not be appropriate.

Figure R1 and Movie R1 provide a representation of the variable CCM nature of these two cell lines.

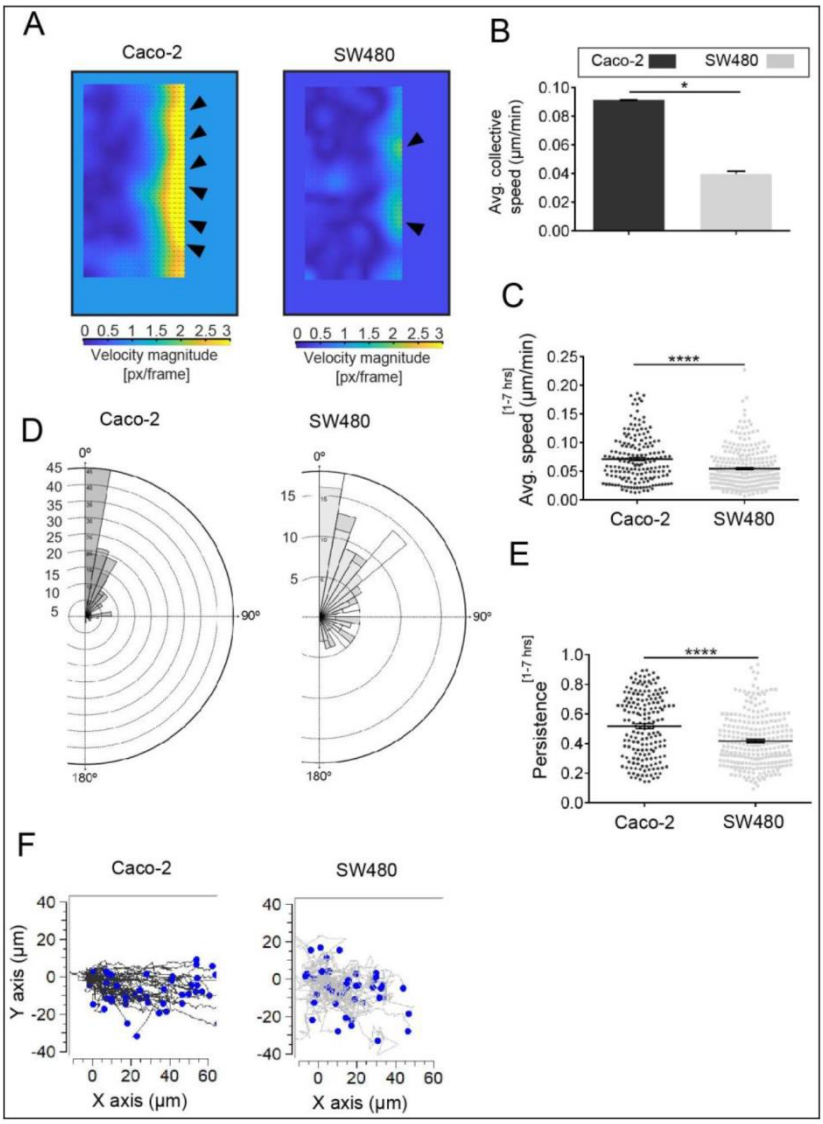


Figure R1: Collective behaviour of Caco-2 and SW480 cells. A) to F) Collective cell migration (CCM) measured for Caco-2 and SW480 cells. A) to B) PIV analysis. A) Velocity heatmaps (1 pixel = 0.586 µm; 1 frame= 15 mins; high-speed zones: yellow regions pointed with black arrows) and B) Average collective speed measured between 1 to 7 hrs of CCM from two independent experiments (N=2). C) to F) Track Analysis. Individual cells tracked from the first four layers of the progressing cell sheets. C) Average speed and E) persistence measured between 1 to 7 hrs of CCM. D) Rose plots of trajectory angles. The magnitude of each bar shows the fraction of cells with the indicated angle

trajectory. For C) to E) n= 178 for Caco-2 (N=2); 255 for SW480 (N=2). F) Trajectories of 50 representative cells measured over 12 hrs. All panels: n= number of cells/tracks, N= number of experiments; Data represented as mean±SEM (for B, C and E); Statistical significance was calculated using an unpaired two-tailed t-test. *, P<0.05; ****, P<0.0001. Ghosh et al., unpublished data.

2a). Although evidence is presented for the presence of PLP2-positive exosomes (Fig. S1G- 1H) and that exosomes from PLP2-overexpressing cells can rescue the KO phenotype, it is important to include exosomes from SW480 and PLP2 KO cells as a negative control.

Response: We appreciate that the reviewer suggested these control experiments. We have now purified exosomes from SW480 and PLP2KO cells using density gradient purification and named them exosome^{SW480} and exosome^{PLP2KO} respectively. These exosome pools were validated by Western blot for the presence of PLP2 (Figure S3C). PLP2KO cells were treated with these two pools of exosomes separately and CCM was measured in both conditions (Figure S3E-H, Movie S7). The results obtained from PIV as well as track analysis were compared with the corresponding results obtained for treatment of PLP2KO cells with exosomes purified from PLP2-overexpressing cells (exosome$^{PLP2\text{-}GFP}$) (Figure S3E-H, Movie S7). Migration parameters observed in PLP2KO cells treated with exosome^{PLP2KO} were comparable with that of the PLP2KO cells. Treatment of PLP2KO cells with exosome^{SW480} and exosome$^{PLP2\text{-}GFP}$ significantly enhanced the migration parameters including average collective speed and number of high speed zones at the wound edge obtained from PIV analysis (Figure S3E-F) and average track speed (Figure S3G) and persistence (Figure S3H) obtained from track analysis. When migration parameters were compared between treatment with exosome^{SW480} and exosome$^{PLP2\text{-}GFP}$, the latter showed much pronounced rescue than the former (Figure S3E-H, Movie S7). These results also further strengthened gene specific effect of exosomal PLP2 and are now been included in the revised manuscript.

2b). Regarding the exosome preparations, the authors show that GW4869 treatment inhibits exosome production (Fr. S1I), yet in panel S1G, there is a minimal change in the presence of PLP2 in the exosome preparation.

Response: We are thankful to the reviewer for this suggestion. GW4869 is a drug widely used as exosome biogenesis inhibitor that blocks neutral sphingomyelinase 2 (nSMase2) and therefore inhibits ceramide dependent exosome biogenesis in cells (Guo et al., 2015; Trajkovic et al., 2008). Our data showing marginal decrease of PLP2 abundance in the exosome pool (Figure S3A in revised manuscript) upon GW4869 treatment suggests that exosomal PLP2 is secreted through the ceramide based as well as non-ceramide based exosomes from SW480 cells. Hence after blocking with GW4869, PLP2 abundance is still detectable in the exosome pools and evidently this abundance represents PLP2 secreted through non-ceramide based exosome pools. This could also be due to a compensatory effect of PLP2 secretion through non-ceramide pools upon unavailability of the ceramide based pool (Palmulli and van Niel, 2018).

2c). The IF images showing the distribution of PLP2-mCherry are also difficult to interpret. Overexpression of Rab27a could impact the extent of PLP2 in vesicles. The authors should show the distribution of endogenous PLP2, Rab27a, and CD63. How was the colocalization quantified?

Response: We agree with the reviewer that, the exosomal localization of endogenous PLP2 should be determined using immunofluorescence (IF), however in spite of several trials anti- PLP2 antibody could not be effectively used for co-immunofluorescence studies. We also believe that the over-expression of Rab27 may impact vesicular population of PLP2. To circumvent the possible artifacts, we have carried out IF studies with a different strategy where we have used PLP2-GFP expressing cells to probe for endogenous CD63 (Figure 1Bc) and Rab27 (Figure S1F). Approximately 30% of the vesicular pool of PLP2-GFP colocalized with CD63, a marker for exosome (Andreu and María, 2014) and a small fraction (≈10%) of vesicular PLP2-GFP was also colocalized with Rab27a that regulates exosome secretion (Ostrowski et al., 2010). The above results are now included in the revised manuscript.

Object based colocalization analysis was performed by the automated image analysis program, MotionTracking (http://motiontracking.mpi-cbg.de; (Collinet et al., 2010; Rink et al., 2005)). The

cells were randomly selected for imaging in a given experimental setup. At least 15 images were acquired for each condition in a given experimental set up, and all the images were processed together for quantification. The objects were identified as vesicles in each channel based on their size, fluorescence intensity and other parameters by the MotionTracking software (Collinet et al., 2010; Rink et al., 2005). Objects detected in two different channels were considered as colocalized if the relative overlap of respective areas was above 35%. The apparent colocalization value was calculated as a ratio of integral intensities of colocalized objects to the integral intensities of all objects carrying the given marker and varies from 0.0 to 1.0. The colocalization-by-chance (random colocalization) was estimated by random permutation of object localization in different channels. The apparent colocalization was corrected for random colocalization.

3. While the small differences in speed and persistence of the cell migration are statistically significant (Fig. 2E-F, Fig. 4D-E'), it is not clear that they are biologically significant. This is problematic as the high n can make minimal differences appear statistically significant. From how many different experiments are the individual points coming from?
Response: We understand the reviewer's concern about the biological relevance for the observed phenotype. Here we would like to mention that the wound healing assay is a physiologically relevant set up which is well established to understand pathophysiology of the cancer cells (Foster et al., 2018; Grada et al., 2017; Rybinski et al., 2014). The results related to collective cell migration as acquired from the wound healing experiments performed in our study is a combination of PIV analysis and track analysis. In PIV analysis, measurements are performed at collective level on whole field basis whereas for the track analysis, individual cell properties are measured that grossly reflects their collective migration properties. In our study we observed at least two-fold reduction in average speed upon PLP2 deficiency both from the PIV (Figure 3C) as well as track analysis (Figure 3F, earlier figure 2E) and this corroborates with doubling in wound healing time (Figure S2I) which is physiologically relevant. Moreover, the horizontal component of the velocity ('u') that represent migration towards the wound is altered at least three-fold as evident from the PIV derived kymograph analysis and the associated plots (Figure S2Ja, S2J'a). Similar fold changes were observed for directional movement during CCM as reflected by track persistence (Figure 3G, earlier figure 2F) and polar order parameter (Figure S2Jd, S2J'b).
Results associated with PLP2-ZO-1 association (overexpression of ΔNPLP2-mCherry and mCherry-PLP2ΔC) also showed close to 1.5 fold alterations in the above mentioned parameters from track analysis (Figure 5D-E', earlier figure 4D-E') that were further supported by the data obtained from PIV analysis (Figure 5C, S5D-D'). Of note, similar fold change was reported for p-cadherin mediated CCM in myoblast cells (Plutoni et al., 2016).

We would further like to mention here that the number of cells (n) is high for track analysis data since we followed maximum possible cells from each experiment to more accurately represent the collective behaviour of the moving sheet within a single experiment. Data from PIV analysis however represents single data point per experiment. Robust corroboration of migration parameters obtained from track analysis and PIV further authenticates the trend observed in collective behaviour during migration. Hence, we interpret that the statistically significant migration parameters presented in the manuscript will have extensive potential for biological significance.

Each experiment was carried out in minimum three biological replicates. The number of individual cells/ tracks and number of experiments are mentioned in detail in the captions of each related figures (Figure 3, 5, 6, S2, S3, S5, S6).

4. The authors show robust data for an association between PLP2 and ZO-1; however, there is not enough evidence to say this is a direct interaction. Additionally, the evidence that this PLP2-ZO-1 complex works through ZO-1's association with actin is only correlative. The data in Fig. 4G demonstrate that PLP2 KO or removal of the C-terminus results in less actin at the leading edge, but they do not demonstrate that ZO-1 is the connecting factor between PLP2 and actin.

Response: We appreciate that the reviewer found the interaction data robust. Our experiments using the GBP (GFP binding protein) and CBP (Cherry binding protein) pull down approach showed that ZO-1 associates with PLP2, however, it could only infer about their cellular association and not the possibility of direct interaction. Separate in vitro study with the recombinant ZO1- and PLP2 would be required to establish their direct interaction which is beyond the scope of the

current study. Accordingly, we have not claimed their direct interaction in the manuscript. The results from pull down experiments however enabled us to propose that they may be part of a complex in SW480 cells.

We went ahead and dissected out that the C terminal region of PLP2 is important for its association with ZO-1, thus deletion of the same results in perturbation of the association (Figure 4D). Hence failing to rescue the actin rich umbrella structures in PLP2KO cells expressing mCherry-PLP2ΔC indicates that PLP2-ZO-1 association is required for the rearrangement of actin cytoskeleton during CCM (Figure 5G). As suggested by the other reviewers, we have also extended the study with cortactin, a leading edge marker during cell migration. (Figure 5H). It is well established that ZO1 acts like an adaptor between tight junction proteins and actin (Fanning et al., 1998; Tornavaca et al., 2015). Furthermore, its role in integrin mediated single cell migration has also been demonstrated earlier (González- Tarragó et al., 2017; Taliana et al., 2005; Tuomi et al., 2009). Thus, ZO-1's involvement in cytoskeletal remodelling during CCM would be a plausible proposition which together with our rescue-based experiments prompted us to hypothesize that the association between PLP2 and ZO-1 plays a crucial role in cytoskeletal rearrangement at the leading edge.
To get further insight into the PLP2 mediated cytoskeletal rearrangement at the leading edge during CCM, we hypothesize that the umbrella like structures resemble lamellipodia like extensions and thus might involve Rac1 activity (Small et al., 1999; Ridley et al., 1992). The live cell co-dynamics of Rac1 and PLP2 at the umbrella structures (Figure 6A, Movie S11) indicated that these Rac1 positive structures closely represent lamellipodia (Ridley et al., 1992; Yamaguchi et al., 2015). Since Rac1 activation is crucial for lamellipodia formation during single as well as collective cell migration (Ridley et al., 1992; Yamaguchi et al., 2015), we next assessed global Rac1 activation during 4^{th} hour post-wound using biochemical Rac1 activation assay (Meriane et al., 2002). A marked reduction of global Rac1 activation was observed in PLP2KO cells as compared to the control cells (Figure 6B, S6B) which indicates that PLP2 is involved in the activation of Rac1 during CCM. In addition, PLP2KO cells overexpressing PLP2-mCherry or ΔNPLP2-mCherry showed notable recovery in the global Rac1 activation and overexpression of mCherry-PLP2ΔC also recovered substantial population of active Rac1 however marginally less than that of cells overexpressing PLP2- mCherry or ΔNPLP2-mCherry (Figure 6B', S6B'). Thus, perturbation of PLP2-ZO-1 association does not lead to complete loss of Rac1 activation suggesting that, the association may not have any major role in global Rac1 activation. However, the perturbation of this association led to cytoskeletal alterations at the leading edge and successive abrogation of collective migration (Figure 5). Based on these results we propose that, association between ZO-1 and PLP2 may contribute to polarized activation of Rac1 that is important for directive cell migration during CCM. However, our results do not completely rule out the possibility of the involvement of additional molecular players in this event. Moreover, in dominant negative Rac1 background, PLP2 failed to rescue the F-actin rich umbrella like structures as well as migration parameters during CCM of PLP2KO cells (Figure 6D-H, S6C-F, Movie S12).

Together, these results strengthened our claim that PLP2 plays a vital role in leading edge cytoskeletal rearrangement during CCM. The association between PLP2 and ZO-1 might serve as one of the underlying mechanisms of PLP2 mediated cytoskeletal alterations. However, unravelling other interacting partners of PLP2 or additional members of the PLP2- ZO-1 association will be a promising future scope of this study which will help to get further molecular insight into this event. The above discussion has now been included in the revised manuscript.

5. In the pull-down assays (Fig. 3C-D, Fig. S3B), the input/elution legend needs to be clearer. In the text, it is stated that ZO-1 is co-eluted with PLP2, but why is ZO-1 present in the input? Is ZO-1 the bait protein? How is GST eluted separately from GST-CBP? Although other MARVEL-domain-containing proteins have been shown to interact with ZO-1, pull- down assays alone are not enough to demonstrate a direct interaction between two proteins.

Response: We thank the reviewer for his/her suggestion. More clearer legends has been added in the revised version (Figure 4C-D, S4B and their legends; for legends of figure 4 please refer to page no 36-37 of manuscript file).

A brief description and schematic representation of the pull-down approach is added here for

better clarity (Figure R6).

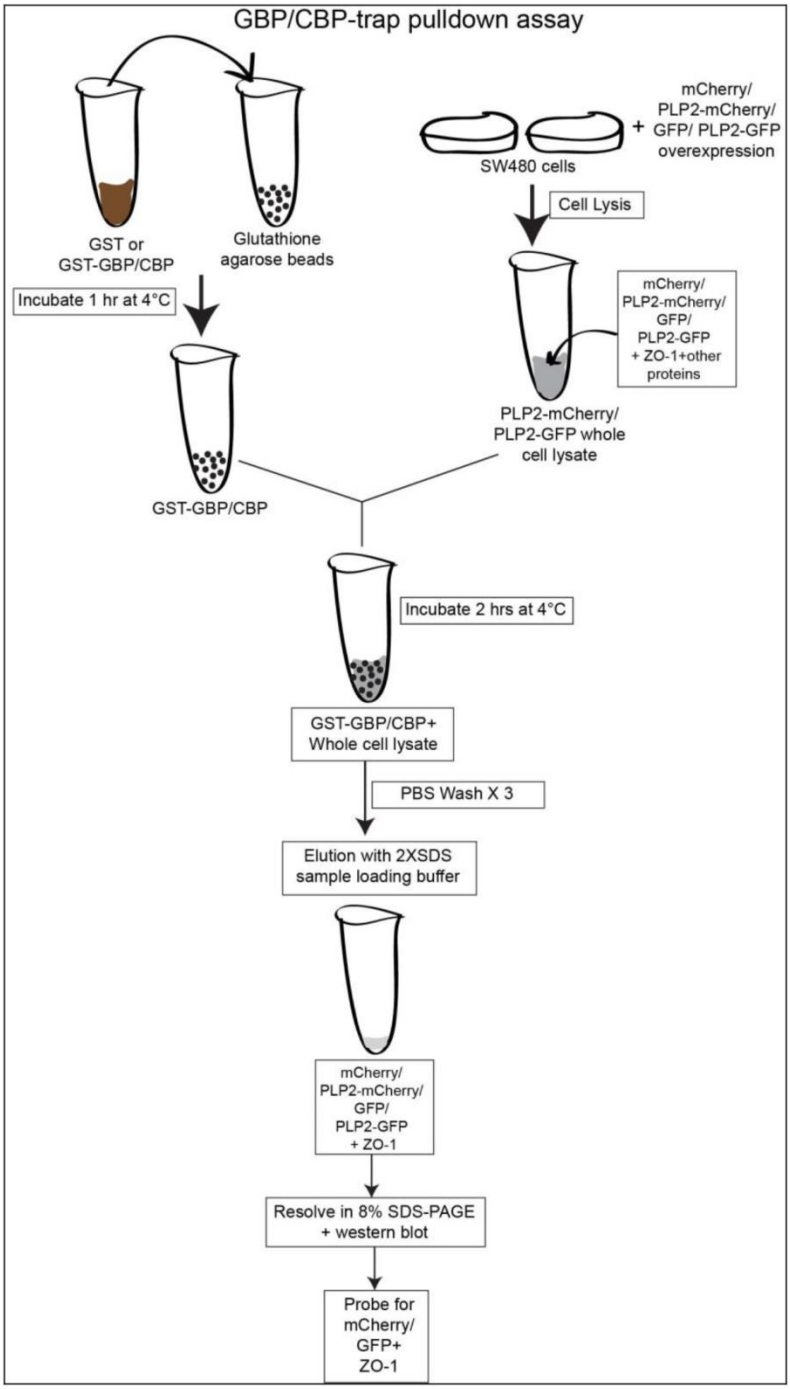


Figure R6: Schematic representation of GBP/CBP trap pull down assay

The GBP (GFP Binding Protein)/ CBP (Cherry Binding Protein)-trap pull down used in our study is a modified immunoprecipitation approach where antibody is being replaced by GBP or CBP in respective cases. The GBP or CBP was used here as the bait to pull down GFP or mCherry tagged PLP2 from the cell lysates along with the proteins that associate with PLP2 in the cellular context. GBP and CBP are well reported to bind strongly with GFP and mCherry respectively, and can be used to efficiently purify GFP/ mCherry tagged proteins (Rothbauer et al., 2006; Rothbauer et al., 2008). GBP/ CBP was expressed, purified with an N-terminus GST tag and immobilized to glutathione agarose beads. The lysate from the cells overexpressing GFP/ mCherry-tagged PLP2 was incubated with the GBP/ CBP bound agarose beads. The proteins that associate with PLP2 in the cellular context were also co-eluted with the bound GFP or mCherry tagged protein. The elution fraction was analyzed using western blot (Figure 4C-D, S4B).

10% of the total cell lysates overexpressing GFP or mCherry-tagged PLP2 were used as input. Hence ZO-1 protein present in the total cell lysate was detected in the input fraction. ZO-1 is not used here as a bait protein.

The band corresponding to 28 KDa in figure 4C and 4D is not representing GST rather mCherry. mCherry is pulled down from lysates of cells overexpressing mCherry and is eluted from the CBP bound glutathione agarose beads as CBP binds to mCherry tag.

We have correlated that MARVEL-domain-containing proteins have been shown to interact with ZO-1 (Furuse et al., 1994; Raleigh et al., 2010) to hypothesize for possible association between PLP2 and ZO-1. However, we agree to the point that pull-down assays alone are not enough to demonstrate a direct interaction between two proteins and accordingly did not claim the same between PLP2 and ZO-1. Our study indicates that PLP2 and ZO-1 coexist in a complex or associate with each other in the cellular context.

6. Please provide the number of times experiments were performed in legends.

Response: Each CCM experiment was performed minimum in three biological replicates. The detailed description of the number of cells/ tracks and number of individual experiments used for calculations is provided in the respective figure legends (Please ref to page no 35-39 of manuscript file for main figure legends and supplemental file for supplementary figure legends).

7. The lack of a discussion section resulted in an abrupt ending to the paper. A discussion would be beneficial for analyzing the results and putting them into broader context.

Response: In the revised version of the manuscript, we have added a discussion section where we discussed the collective behaviour of our model system followed by the analysis of altered migration parameters observed upon PLP2 deficient condition. We have discussed directional migration in the context of cell polarity and orientation. Additional discussion on the future scope of the study for unravelling the bridging molecules between PLP2 and Rac1 activation has also been added. A brief discussion in light of cancer progression and metastasis correlating the role of exosomal PLP2 has also been added (Page no 11-14, page no 15 line no 1-3).

References:

Ahmed, D., Eide, P. W., Eilertsen, I. A., Danielsen, S. A., Eknæs, M., Hektoen, M., Lind, G. E. and Lothe, R. A. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis* **2**,.

Andreu, Z. and María, Y.-M. (2014). Tetraspanins in Extracellular Vesicle Formation and Function. *Front. Immunol.* **5**, 1–12.

Bazellières, E., Conte, V., Elosegui-Artola, A., Serra-Picamal, X., Bintanel-Morcillo, M., Roca-Cusachs, P., Muñoz, J. J., Sales-Pardo, M., Guimerà, R. and Trepat, X. (2015). Control of cell–cell forces and collective cell dynamics by the intercellular adhesome. *Nat. Cell Biol.* **17**, 409–420.

Becker, A., Thakur, B. K., Weiss, J. M., Kim, H. S., Peinado, H. and Lyden, D. (2016). Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. *Cancer Cell* **30**, 836–848.

Brugués, A., Anon, E., Conte, V., Veldhuis, J. H., Gupta, M., Colombelli, J., Muñoz, J. J., Brodland, G. W., Ladoux, B. and Trepat, X. (2014). Forces driving epithelial wound healing. *Nat. Phys.* **10**, 683–690.

Caffrey, D. R., Zhao, J., Song, Z., Schaffer, M. E., Haney, S. A., Subramanian, R. R., Seymour, A. B. and Hughes, J. D. (2011). siRNA Off-Target Effects Can Be Reduced at Concentrations That Match Their Individual Potency. *PLoS One* **6**, e21503.

Chapnick, D. A. and Liu, X. (2014). Leader cell positioning drives wound-directed collective migration in TGFß-stimulated epithelial sheets. *Mol. Biol. Cell* **25**, 1586– 1593.

Cheung, K. J. and Ewald, A. J. (2016). A collective route to metastasis: Seeding by tumor cell clusters. *Science (80-.).* **352**, 167–169.

Collinet, C., Stöter, M., Bradshaw, C. R., Samusik, N., Rink, J. C., Kenski, D., Habermann, B., Buchholz, F., Henschel, R., Mueller, M. S., et al. (2010). Systems survey of endocytosis by multiparametric image analysis. *Nature* **464**, 243–249.

Collins, C. and Nelson, W. J. (2015). Running with neighbors: coordinating cell migration and cell–cell adhesion. *Curr. Opin. Cell Biol.* **36**, 62–70.

Ebnet, K., Schulz, C. U., Meyer Zu Brickwedde, M. K., Pendl, G. G. and Vestweber, D. (2000). Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J. Biol. Chem.* **275**, 27979–27988.

Fanning, A. S., Jameson, B. J., Jesaitis, L. A. and Anderson, J. M. (1998). The Tight Junction Protein ZO-1 Establishes a Link between the Transmembrane Protein Occludin and the Actin Cytoskeleton. *J. Biol. Chem.* **273**, 29745–29753.

Faux, M. C. (2003). Restoration of full-length adenomatous polyposis coli (APC) protein in a colon cancer cell line enhances cell adhesion. *J. Cell Sci.* **117**, 427–439.

Foster, R., Hu, K. Q., Lu, Y., Nolan, K. M., Thissen, J. and Settleman, J. (1996). Identification of a novel human Rho protein with unusual properties: GTPase deficiency and in vivo farnesylation. *Mol. Cell. Biol.* **16**, 2689–2699.

Foster, D. S., Jones, R. E., Ransom, R. C., Longaker, M. T. and Norton, J. A. (2018). The evolving relationship of wound healing and tumor stroma. *JCI Insight* **3**, 1–17.

Friedl, P. and Mayor, R. (2017). Tuning Collective Cell Migration by Cell–Cell Junction Regulation. *Cold Spring Harb. Perspect. Biol.* **9**, a029199.

Furuse, M., Itoh, M., Hirase, T., Nagaftichi, A., Yonemura, S., Tsukita, S. and Tsukita, S. **(1994).** Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J. Cell Biol.* **127,** 1617–1626.

González-Tarragó, V., Elosegui-Artola, A., Bazellières, E., Oria, R., Pérez-González, C. and Roca-Cusachs, P. (2017). Binding of ZO-1 to α5β1 integrins regulates the mechanical properties of α5β1-fibronectin links. *Mol. Biol. Cell* **28**, 1847–1852.

Grada, A., Otero-Vinas, M., Prieto-Castrillo, F., Obagi, Z. and Falanga, V. (2017). Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. *J. Invest. Dermatol.* **137**, e11–e16.

Guo, B. B., Bellingham, S. A. and Hill, A. F. (2015). The neutral sphingomyelinase pathway regulates packaging of the prion protein into exosomes. *J. Biol. Chem.* **290**, 3455–3467.

Haeger, A., Krause, M., Wolf, K. and Friedl, P. (2014). Cell jamming: Collective invasion of mesenchymal tumor cells imposed by tissue confinement. *Biochim. Biophys. Acta - Gen. Subj.* **1840**, 2386–2395.

Hannus, M., Beitzinger, M., Engelmann, J. C., Weickert, M.-T., Spang, R., Hannus, S. and Meister, G. (2014). siPools: highly complex but accurately defined siRNA pools eliminate off-target effects. *Nucleic Acids Res.* **42**, 8049–8061.

Hashimoto, K. and Shimizu, M. (1993). Epithelial properties of human intestinal Caco-2 cells cultured in a serum-free medium. *Cytotechnology* **13**, 175–184.

Ilina, O., Bakker, G.-J., Vasaturo, A., Hoffman, R. M. and Friedl, P. (2011). Two-photon laser-generated microtracks in 3D collagen lattices: principles of MMP-dependent and - independent collective cancer cell invasion. *Phys. Biol.* **8**, 029501–029501.

Kim, M. J., Cho, Y. B., Chun, H.-K., Lee, W. Y., Yun, S. H. and Kim, H. C. (2013). The expression of epithelial mesenchymal transition related factors in human colorectal cancer cell lines. *Korean J. Clin. Oncol.* **9**, 160–167.

Maia, J., Caja, S., Strano Moraes, M. C., Couto, N. and Costa-Silva, B. (2018). Exosome- based cell-cell communication in the tumor microenvironment. *Front. Cell Dev. Biol.* **6**, 1–19.

Meriane, M., Charrasse, S., Comunale, F., Méry, A., Fort, P., Roux, P. and Gauthier- Rouvière, C. (2002). Participation of small GTPases Rac1 and Cdc42Hs in myoblast transformation. *Oncogene* **21**, 2901–2907.

Nieto, M. A., Huang, R. Y.-J., Jackson, R. A. and Thiery, J. P. (2016). EMT: 2016. *Cell* **166**, 21–45.

Nobes, C. D., Lauritzen, I., Mattei, M. G., Paris, S., Hall, A. and Chardin, P. (1998). A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion. *J. Cell Biol.* **141**, 187–197.

Ostrowski, M., Carmo, N. B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., Moita, C. F., Schauer, K., Hume, A. N., Freitas, R. P., et al. (2010). Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat. Cell Biol.* **12**, 19–30.

Palmulli, R. and van Niel, G. (2018). To be or not to be... secreted as exosomes, a balance finely tuned by the mechanisms of biogenesis. *Essays Biochem.* **62**, 177–191.

Petitjean, L., Reffay, M., Grasland-Mongrain, E., Poujade, M., Ladoux, B., Buguin, A. and Silberzan, P. (2010). Velocity fields in a collectively migrating epithelium. *Biophys. J.* **98**, 1790–1800.

Plutoni, C., Bazellieres, E., Le Borgne-Rochet, M., Comunale, F., Brugues, A., Séveno, M., Planchon, D., Thuault, S., Morin, N., Bodin, S., et al. (2016). P-cadherin promotes collective

cell migration via a Cdc42-mediated increase in mechanical forces. *J. Cell Biol.* **212**, 199–217.
Raleigh, D. R., Marchiando, A. M., Zhang, Y., Shen, L., Sasaki, H., Wang, Y., Long, M. and Turner, J. R. (2010). Tight Junction–associated MARVEL Proteins MarvelD3, Tricellulin, and Occludin Have Distinct but Overlapping Functions. *Mol. Biol. Cell* **21**, 1200–1213.
Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401–410.
Rink, J., Ghigo, E., Kalaidzidis, Y. and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell* **122**, 735–749.
Rothbauer, U., Zolghadr, K., Tillib, S., Nowak, D., Schermelleh, L., Gahl, A., Backmann, N., Conrath, K., Muyldermans, S., Cardoso, M. C., et al. (2006). Targeting and tracing antigens in live cells with fluorescent nanobodies. *Nat. Methods* **3**, 887–889.
Rothbauer, U., Zolghadr, K., Muyldermans, S., Schepers, A., Cardoso, M. C. and Leonhardt, H. (2008). A Versatile Nanotrap for Biochemical and Functional Studies with Fluorescent Fusion Proteins. *Mol. Cell. Proteomics* **7**, 282–289.
Rybinski, B., Franco-Barraza, J. and Cukierman, E. (2014). The wound healing, chronic fibrosis, and cancer progression triad. *Physiol. Genomics* **46**, 223–244.
Scarpa, E., Szabó, A., Bibonne, A., Theveneau, E., Parsons, M. and Mayor, R. (2015). Cadherin Switch during EMT in Neural Crest Cells Leads to Contact Inhibition of Locomotion via Repolarization of Forces. *Dev. Cell* **34**, 421–434.
Schreider, C., Peignon, G., Thenet, S., Chambaz, J. and Pinçon-raymond, M. (2002). Integrin-mediated functional polarisation of Caco-2 cells through E-cadherin-actin complexes (Schreider et al., Journal Cell Science, 2002).pdf>.
Sit, S.-T. and Manser, E. (2011). Rho GTPases and their role in organizing the actin cytoskeleton. *J. Cell Sci.* **124**, 679–683.
Small, J. V., Rottner, K. and Kaverina, I. (1999). Functional design in the actin cytoskeleton. *Curr. Opin. Cell Biol.* **11**, 54–60.
Sung, B. H., Ketova, T., Hoshino, D., Zijlstra, A. and Weaver, A. M. (2015). Directional cell movement through tissues is controlled by exosome secretion. *Nat. Commun.* **6**, 7164.
Taliana, L., Benezra, M., Greenberg, R. S., Masur, S. K. and Bernstein, A. M. (2005). ZO-1: Lamellipodial localization in a corneal fibroblast wound model. *Investig. Ophthalmol. Vis. Sci.* **46**, 96–103.
Tornavaca, O., Chia, M., Dufton, N., Almagro, L. O., Conway, D. E., Randi, A. M., Schwartz, M. A., Matter, K. and Balda, M. S. (2015). ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation. *J. Cell Biol.* **208**, 821–838.
Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brügger, B. and Simons, M. (2008). Ceramide Triggers Budding of Exosome Vesicles into Multivesicular Endosomes. *Science (80-.).* **319**, 1244–1247.
Tuomi, S., Mai, A., Nevo, J., Laine, J. O., Vilkki, V., Öhman, T. J., Gahmberg, C. G., Parker, P. J. and Ivaska, J. (2009). PKCε regulation of an α5 integrin-ZO-1 complex controls lamellae formation in migrating cancer cells. *Sci. Signal.* **2**, 1–16.
Vig, D. K., Hamby, A. E. and Wolgemuth, C. W. (2016). On the Quantification of Cellular Velocity Fields. *Biophysical Journal* **110**, 1469–1475.
Warburton, D., Bernard Weinstein, I., Warburton, D., Kahn, S. M. and Bernard Weinstein, I. (1992). Isolation and Characterization of a Highly Malignant Variant of the SW480 Human Colon Cancer Cell Line. *Cancer Res.* **52**, 6840–6847.
Weed, S. A., Karginov, A. V., Schafer, D. A., Weaver, A. M., Kinley, A. W., Cooper, J. A. and Parsons, J. T. (2000). Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. *J. Cell Biol.* **151**, 29–40.
Yamaguchi, N., Mizutani, T., Kawabata, K. and Haga, H. (2015). Leader cells regulate collective cell migration via Rac activation in the downstream signaling of integrin β1 and PI3K. *Sci. Rep.* **5**, 7656.
Yoon, W.-H., Lee, S.-K., Song, K.-S., Kim, J.-S., Kim, T.-D., Li, G., Yun, E.-J., Heo, J.-Y., Jung, Y.-J., Park, J.-I., et al. (2008). The tumorigenic, invasive and metastatic potential of epithelial and round subpopulations of the SW480 human colon cancer cell line. *Mol. Med. Rep.* **1**, 763–768.

Second decision letter

MS ID#: JOCES/2020/253468

MS TITLE: Proteolipid protein 2 drives collective cell migration via ZO-1 mediated cytoskeletal remodeling at the leading-edge

AUTHORS: Dipanjana Ghosh, Ankita Dutta, Anjali Kashyap, Neeraj Upmanyu, and Sunando Datta
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see from their reports, while their opinions are divergent, all of the reviewers raised some issues, and in one case these were serious enough to prevent me from accepting your paper for publication. If this were in the first round of review we would give you an opportunity to address those concerns. But as we are far into the review process, I regret that we cannot invite another revised version due to the extensive nature of the concerns.

I will direct your attention to the reviews for details, but one of the major concerns is that the results presented in the manuscript are based on findings in one colon rectal cancer cell line. In addition, data implicating exosomes in the function of PLP2 are not clear as characterization of the exosomes does not follow the minimal criteria developed by the International Society of Extracellular Vesicles (Thery et al., J Extracellular Vesicles 2018). Other issues were outlined by this reviewer as well.

I am very sorry to give you such disappointing news, but we are currently under great pressure for space and it takes a very enthusiastic recommendation by the referees for a manuscript to be accepted.

I do hope you find the comments of the reviewers helpful in allowing you to revise the manuscript for submission elsewhere, and many thanks for sending your work to Journal of Cell Science.

Reviewer 1

Advance summary and potential significance to field

The authors have conducted several additional experiments to improve their studies. I am satisfied with their thoughtful answers to my initial comments and by the quality of the new data added to the paper. Globally this study is significantly improved.

Comments for the author

The new text additions make the paper a bit "patchy". I would consider attempting to increase the fluidity of the text and revise the English.

Reviewer 2

Advance summary and potential significance to field

Ghosh and colleagues characterize the relationship between PLP2, ZO-1, and Rac during collective cell migration and find that knock-out of PLP2 decreases the rate of cell migratioN, inhibits the lamellipodial recruitment of ZO-1, and leads to a decrease in the amount of active Rac. These phenotypes were rescued by full-length PLP2 but not a PLP2 construct that is missing the C-

terminus. Collectively, these results suggest that PLP2 stimulates cell migration through recruitment of ZO-1 to leading edge and activation of Rac.

Comments for the author

I appreciate that Ghosh and colleagues address many of my major concerns in this revised version. I do have just a few minor comments I feel need to be addressed.

Figure 1. I appreciate the quantification of the co-localization of PLP2 and Cortactin. However, with out proper positive and negative controls the Pearson's coefficient really does not tell us whether for example 0.5 represent high co-localization in this system or poor co-localization. In the very least they could have compared the co-localization of Cortactin with of actin since these two proteins are known to co-localize at the leading edge for the sake of comparison. Similarly, it is not clear what %Colocalization means with regards to the co-localization of CD63.

Figure 6. The introduction of GFP-tagged Rac will inevitable stimulate lamellipodia formation so it is hard to interpret these results. Staining for endogenous Rac rather than transfecting in GFP Rac would have been more impactful. That being said the authors show an increase in active Rac upon rescue wit PLP2 which is a better demonstration of the relationship between PLP2 and Rac.

It is also puzzling that mCherry-PLP2deltaC was not able to rescue the PLP2 KO cell migration phenotype, but was able to rescue the loss of active Rac. The authors do comment on this, but may be this is case where some sort of quantification of the amount of active Rac will be useful here so that the reader can have an understanding marginally as it not entirely clear from the blot presented.

Reviewer 3

Advance summary and potential significance to field

This is a revised version of a manuscript previously reviewed. While the authors attempted to answer the issues raised, some of the concerns originally brought up remain.

Comments for the author

1. The fact that the results presented in the manuscript are based on findings in one colon rectal cancer cell line is worrisome and suggests that the proposed role for PLP2 in regulating collective cell migration is not generally applicable.

2. The data presented to implicate exosomes in the function of PLP2 are not clear. The characterization of the exosomes does not follow the minimal criteria developed by the International Society of Extracellular Vesicles (Thery et al., J Extracellular Vesicles 2018). Ultracentrifugation fractionation with positive and negative markers, electron microscopy, and NTA analyses that show the size and homogeneity of the exosome population, should be presented. The PLP2/CD63 co-localization data the authors now provide are perplexing. What do each point represent in the quantification graph presented? The figure legend is not clear about this (20 frames from 3 independent experiments; how many cells were analyzed?). Why are the colocalized puncta mostly perinuclear? What is the nature of the PLP2-positive and CD63-negative puncta, which represent the majority of the signal? It would be expected that cells have more CD63 positive vesicles compared to PLP2. Is this an artefact of PLP2-GFP overexpression? Images with staining of the endogenous PLP2 would help. The effect of GW4869 treatment on PLP2 exosomes (Fig. S3A&B) and collective cell migration (Fig. S3I-M) appears modest. The authors mention that secretion of PLP2 through non-ceramide exosomes in the presence of GW4869 is a possible explanation for the discrepancy between the amount of PLP2 present and the number of exosomes (Fig. S3A&B). However, the western blot (S3A) shows a modest decrease in PLP2, whereas the number of exosomes decreases more than 50% (S3B). This would indicate that the compensation occurs through packaging more PLP2 into non-ceramide exosomes rather than the production of more non-ceramide exosomes. Finally, it is not clear how exosomal PLP2 regulates collective cell migration. In the cartoon presented in Fig. 7, the topography of PLP2 in exosomes is not properly drawn.

Author rebuttal letter

We are thankful to the editor's and the reviewers' critical comments on the revised manuscript. We understand that Journal of Cell Science, like many similar journals does not allow any second major revision and therefore based on a major concern the editor chose to reject the manuscript. However, we find that the editor's decision taken on an extensively reviewed and revised manuscript could have been more favourable for acceptance particularly since two out of three reviewers have expressed their acceptance with minor revisions. Interestingly, the third reviewer has raised a completely new concern which we could have already addressed during the 1st revision, if raised earlier. Unfortunately, the editor has taken a negative decision solely based on this reviewer.

Moreover, the major concern as mentioned by the editor after the 1st review process was the lack of enough experimental evidences to support the major claim of the manuscript focusing on PLP2's role in the cytoskeletal remodeling at the leading edge during collective cell migration. We worked extensively on this part and addressed each of the comments with strong experimental support. Accordingly, both the first and the second reviewer have expressed their confidence with the revised manuscript with a very few minor concerns.

Hence, we would greatly appreciate if the editor provides a scope for minor revision to amend the manuscript as per the comments raised, particularly when the manuscript has been extensively revised as per all the reviewers' comments at this stage and has strengthen the major claim of the manuscript.

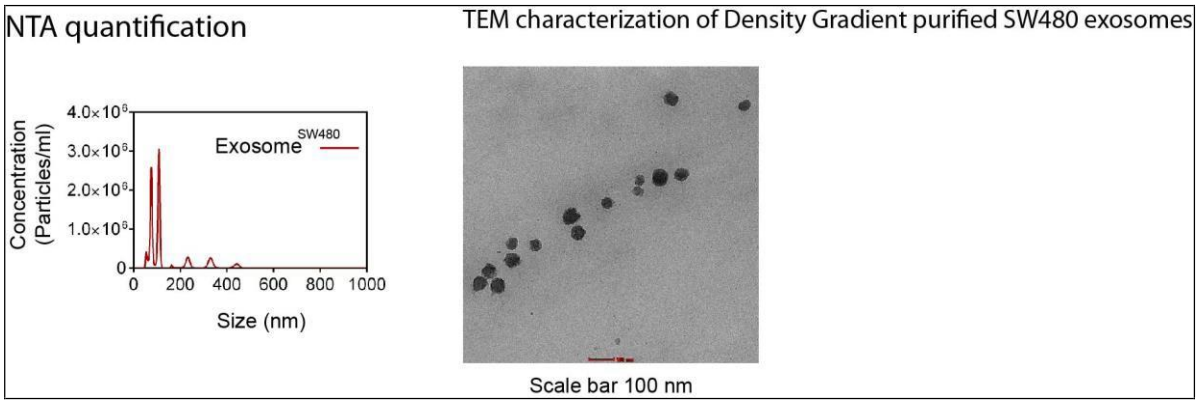
In the current review process, two major concerns were addressed by the editor and these are totally associated with the comments of reviewer 3. In the following sections we are addressing these concerns point by point.

1. One of the two major concerns of the editor is that "the results presented in the manuscript are based on findings in one colon rectal cancer cell line". We agree that our data is focused on a particular colorectal cancer (CRC) cell line SW480, which represent a typical stage of progressive CRC where cells have undergone EMT while keeping their partial epithelial characteristics.
   The current study is the very first report where PLP2's function in CRC progression is studied with mechanistic details at a cellular level. The study demonstrates that PLP2 drives the CCM of SW480 cells by modulating cytoskeletal organization at the leading edge. The expression of PLP2 in corroboration with its role in CCM of SW480 cell line is projecting the molecule as a potential target for controlling CRC progression at a stage where cells have undergone EMT however keeping partial epithelial nature. We believe that this could also be in line with the 'tailor made' treatment strategy currently followed in healthcare sector.
   Also, it is not justified to expect that the same set of upstream machineries will control the CCM in different CRC cell lines of variable epithelial nature. This is based on the literature that cancer cells of variable epithelial characteristics exhibit different types of CCM such as the 'moving sheet' and 'moving cell networks' type (Friedl and Mayor, 2017). The same has also been very clearly demonstrated by our experimental evidences on the CCM characteristics of CaCO2 and SW480 which we have provided in the earlier response letter against the comments addressed by all three reviewers (Response to the reviewers file: reviewer 1 major comments point 1; page no 1 to 3; and reviewer 2 general comments point 1; page no 9 to 11; reviewer 3 point 1b; page no 16 to 18; Figure R1 and movie R1). Notably both the first and the second reviewer found this data and explanation satisfactory. We have discussed this point very clearly in the discussion part of the revised manuscript (Page no 11: line no 27 to 32 and page no 12: line no 1 to 25 of revised manuscript).

   PLP2 being demonstrated as one of the very few upstream regulators of leading-edge actin cytoskeleton during CCM, the current study, would substantially advance our current understanding of CCM in cancer progression, although the study is being focused in a single

CRC cell line.

2. The second major concern of the editor is that "the characterization of the exosomes does not follow the minimal criteria developed by the International Society of Extracellular Vesicles (Thery et al., J Extracellular Vesicles 2018)". Interestingly this issue has been newly raised by the reviewer 3, despite the very fact that exosome data were always being a part of the manuscript since the first submission. Instead, reviewer 3 has earlier recommended to include two more control experiments using exosomes purified from SW480 cells as well as PLP2KO cells to compare with the results obtained from the treatment of PLP-GFP exosomes to the PLP2KO cells. We have performed these extensive control experiments as per the suggestion of reviewer 3 and included the results in the revised manuscript (Figure S3) and included that in the response letter, accordingly (Response to the reviewers file, page no 18, point 2a). However, we do understand that the ultracentrifugation fractionation with positive and negative markers, electron microscopy, and NTA analyses are three criteria for firm characterization of exosomes. We would like to state here that, we have already reported the ultracentrifugation fractionation with positive and negative markers (Figures S1G-H, S3A, S3C) and the concentrations measured by NTA analyses (Figure S3B) (mentioned in methods section: page no 18-19 in revised manuscript). In addition to that, we do have the TEM characterization of purified SW480 exosomes that we could have included during the first revision itself, if this issue was raised during the first review. However, we can certainly provide the data given a chance of minor revision for the manuscript. Here we have attached the size distribution obtained from NTA quantification as well as the TEM characterization of the purified SW480 exosomes for your reference.



Alternatively, upon the editor's recommendation, we could also exclude the exosome related results from the manuscript as we believe that the functional role of PLP2 is well demonstrated without the same. The main focus of the manuscript is on the role of PLP2 in regulating CCM of a stage specific CRC line where it modulates the leading-edge actin cytoskeleton via its association with ZO-1. Function of exosomal PLP2 to the CCM is an additional information provided.

3. Needless to mention that reviewer 1 and 2 have raised minor comments associated with newly included data which are absolutely usual during a peer review process. Both of them have also clearly mentioned about their satisfactory opinion regarding the responses given against their comments raised and the comments included in the second review process are minor. Hence the editor's comment on "all of the reviewers raised some issues" is disappointing.
All the other points raised by the reviewers are minor issues and could be addressed within a short time frame given a scope for minor revision for this manuscript.
Taken together, may we further request the editor to reconsider the manuscript for minor revision?

Reference:

Friedl, P. and Mayor, R. (2017). Tuning Collective Cell Migration by Cell–Cell Junction Regulation. *Cold Spring Harb. Perspect. Biol.* **9**, a029199.

Rebuttal response letter

MS ID#: JOCES/2020/253468

MS TITLE: Proteolipid protein 2 drives collective cell migration via ZO-1 mediated cytoskeletal remodeling at the leading-edge

AUTHORS: Dipanjana Ghosh, Ankita Dutta, Anjali Kashyap, Neeraj Upmanyu, and Sunando Datta
ARTICLE TYPE: Research Article

I apologize for not having gotten back to you right away regarding your rebuttal for JOCES/2020/253468. I was out of town and unable to thoroughly consider the case until now.

I understand your disappointment after having gone to considerable lengths to respond to the referees concerns in a thorough fashion during the previous round of review. That being said, in my opinion referee #3 did have legitimate concerns that I think we need to carefully consider: the fact that the paper is based on only one line, and questions regarding the analysis of extracellular vesicles. I will elaborate on these points below, as well as another issue regarding statistics. In my opinion the decision to reject was not out of line, based on the concerns raised by referee #3. However, I would be amenable to considering a revised version that takes into account these concerns and answers all of the other issues raised by the other referees.

Issue 1: With respect to cell lines, you carried out extensive experimental analysis to support the idea that Caco2 cells are not a good comparator to the SW480 line. While this may be true, this leaves you with the formal possibility that you are looking at biology that holds only in vitro and for one line. Showing a particular phenomenon in more than one cell line (if using an immortalized line) is generally expected for all JCS articles I handle. I think the issue of personalized medicine you raise is an interesting point, but because this is all done with a line in vitro it is unclear whether this would ever apply in vivo to a real patient. To be fair to referee #3, they did bring up the issue of comparing the KOs to the lower expressing Caco line when they said " to further support the migration phenotype observed in PLP2 KO cells, CCM should be measured in a cell line that has naturally low expression of PLP2 (e.g. CaCO2 cells)." I think if this could have been a useful experiment it would have helped addressed this issue for them. While it would have been ideal if you were to have included another line with attributes similar to SW480 and/or shown some sort of in vivo correlate (if only with minimal analysis), at this stage I expect this is not something you are in a position to do. (If you are in a position to include such data, please consider doing so). At a minimum, I would expect you include wording in the discussion that makes it clear the caveats of showing this biology in one line only.

Issue 2: You state that referee #3 brought up the issue of the minimal criteria developed by the International Society of Extracellular Vesicles for the first time in the second review. In your rebuttal you respond to this part of their comments (which I think would be helpful to include in an official response); but in my opinion the crux of their concerns was spelled out in the questions that followed this statement, which I would expect you to address in a revised version of the paper, as follows:

"The PLP2/CD63 co- localization data the authors now provide are perplexing. What do each point represent in the quantification graph presented? The figure legend is not clear about this (20 frames from 3 independent experiments; how many cells were analyzed?). Why are the colocalized puncta mostly perinuclear? What is the nature of the PLP2-positive and CD63-negative puncta, which represent the majority of the signal? It would be expected that cells have more CD63 positive vesicles compared to PLP2. Is this an artefact of PLP2-GFP overexpression? Images with staining of the endogenous PLP2 would help. The effect of GW4869 treatment on PLP2 exosomes (Fig. S3A&B) and collective cell migration (Fig. S3I-M) appears modest. The authors mention that secretion of PLP2 through non-ceramide exosomes in the presence of GW4869 is a possible explanation for the discrepancy between the amount of PLP2 present and the number of exosomes (Fig. S3A&B). However, the western blot (S3A) shows a modest decrease in PLP2, whereas the number of exosomes decreases more than 50% (S3B). This would indicate that the compensation occurs

through packaging more PLP2 into non-ceramide exosomes rather than the production of more non-ceramide exosomes. Finally, it is not clear how exosomal PLP2 regulates collective cell migration. In the cartoon presented in Fig. 7, the topography of PLP2 in exosomes is not properly drawn."

Issue 3: An issue brought up in the first and second rounds related to lack of clarity regarding number of cells analyzed and number of independent experiments performed. While you did carry out 3 independent experiments, it appears that in most cases you combined the "n" from all three experiments together, which will give you a much more significant p value than if you compare averages from the three independent experiments. There is a very useful piece published recently in the Journal of Cell Biology you may want to look at if you haven't already. You can find it at the following link:
https://rupress.org/jcb/article/219/6/e202001064/151717/SuperPlots-Communicating-reproducibility-and
I would refer you to the comment that "Problematic plots treat n as the number of cells, resulting in tiny error bars and P values. These plots also conceal any systematic run-to-run error, mixing it with cell-to-cell variability."
I highly recommend you take a look at your data again with these considerations in mind.

Again, I you feel you can address these issues in another revised version of the paper, I would be amenable reconsidering it for publication in the JCS.

Second revision

Author response to reviewers' comments

Response to the editor and the reviewers

We greatly appreciate the editor's decision of allowing to revise the manuscript. We are also thankful for the editor's and reviewer's critical comments and insightful suggestions. Keeping the main focus of the manuscript as PLP2's role in collective cell migration by impacting the leading-edge actin cytoskeleton, we have now revised the manuscript as per the suggestion from the reviewers and the editor. We carried out a couple of new experiments and added the results to strengthen our observations. In the section below, we have addressed each of the issues raised by the editor and the reviewers and provided a point-by-point response. While incorporating the suggestions in the revised manuscript, figure labels are changed at few places. We have referred to the modified figure labels in the following section. The response to the reviewers' files have been referred in the following sections as "response to the reviewers_1" and "Response to the editor & reviewers" for the responses against the first review (submitted in response to the first review process) and the second review process, respectively.

Editor's comment
Issue 1: With respect to cell lines, you carried out extensive experimental analysis to support the idea that Caco2 cells are not a good comparator to the SW480 line. While this may be true, this leaves you with the formal possibility that you are looking at biology that holds only in vitro and for one line. Showing a particular phenomenon in more than one cell line (if using an immortalized line) is generally expected for all JCS articles I handle. I think the issue of personalized medicine you raise is an interesting point, but because this is all done with a line in vitro it is unclear whether this would ever apply in vivo to a real patient. To be fair to referee #3, they did bring up the issue of comparing the KOs to the lower expressing Caco line when they said "to further support the migration phenotype observed in PLP2 KO cells, CCM should be measured in a cell line that has naturally low expression of PLP2 (e.g. CaCO2 cells)." I think if this could have been a useful experiment it would have helped addressed this issue for them. While it would have been ideal if you were to have included another line with attributes similar to SW480 and/or shown some sort of in vivo correlate (if only with minimal analysis), at this stage I expect this is not something you are in a position to do. (If you are in a position to include such data, please consider doing so). At a minimum, I would expect you include wording in the discussion that

makes it clear the caveats of showing this biology in one line only.

Response: We are thankful to the editor for the insightful suggestion. As per the editor's suggestion, we have now incorporated relevant discussion in the revised manuscript (From page no 14, line no 34 to page no 15, line no 4). We have stated that, "However, to establish that the observed role of PLP2 in collective cell migration is not only limited to SW480 cells, the study can further be extended to additional cell lines with similar collective features as SW480. Exploring the function in an *in vivo* model would add up an even higher impact on the findings.".

Issue 2: You state that referee #3 brought up the issue of the minimal criteria developed by the International Society of Extracellular Vesicles for the first time in the second review. In your rebuttal you respond to this part of their comments (which I think would be helpful to include in an official response);

Response: We are thankful to the editor for the suggestion. While reviewing the revised manuscript (MS# JOCES/2020/253468), the reviewer 3 has brought up an issue stating "the characterization of the exosomes does not follow the minimal criteria developed by the International Society of Extracellular Vesicles (Thery *et al.*, J Extracellular Vesicles 2018)". Interestingly this issue has been newly raised by the reviewer 3, despite the fact that exosome data were always being a part of the manuscript since the first submission (MS# JOCES/2020/253468). The reviewer has earlier recommended to include two more control experiments using exosomes purified from SW480 cells as well as PLP2KO cells to compare with the results obtained from the treatment of PLP-GFP exosomes to the PLP2KO cells. We have already performed these control experiments and included the results in the revised manuscript (Fig. S3; response to the reviewers_1, page no 18, point 2a).

However, we do understand that the ultracentrifugation fractionation with positive and negative markers, electron microscopy (EM), and nanoparticle tracking analysis (NTA) are three criteria for firm characterization of exosomes. We would like to state here that, we have already reported the ultracentrifugation fractionation with positive and negative markers (Fig.S1G,H, S3A, S3C) and the concentrations measured by NTA analyses (Fig. S3B) (mentioned in methods section: page no 20 in the manuscript). The size distribution of exosomes purified from SW480 cells as measured by NTA is indeed a part of the analysis and we do have the characterization which we have now included in the revised manuscript (Figure S1G'). In addition, at the initiation of our exosome preparation from SW480 cells we had also performed the TEM (Transmission electron microscopy) characterization of purified SW480 exosomes to ensure the shape and size of our exosome preparation. It is also now included in the revised manuscript (Figure S1G"). The relevant descriptions have also been included in the revised version of the manuscript (page no 4, line no 19-21; page no 20, line no 25-31.). For the editor's reference we are attaching the NTA and TEM data for SW480 cells, below as well.

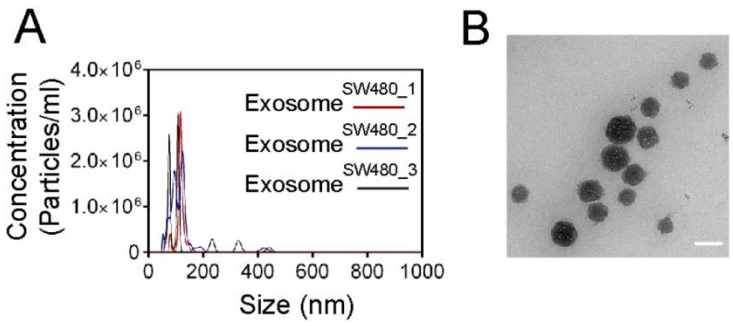


Figure R1: Characterization of size distribution and shape of the exosomes purified from SW480 cells. A) Nanoparticle tracking analysis. B) Transmission electron microscopy, scale bar=100 nm.

Editor's comment (issue 2 continued):
but in my opinion the crux of their concerns was spelled out in the questions that followed this statement, which I would expect you to address in a revised version of the paper, as follows:

Response: We sincerely appreciate the editor's effort to point out the major concerns of the third reviewer. We have addressed all of them during the current revision and provided a point by point response below.

a) "The PLP2/CD63 co- localization data the authors now provide are perplexing. What do each point represent in the quantification graph presented? The figure legend is not clear about this (20 frames from 3 independent experiments; how many cells were analyzed?).

Response: We apologise that the scatter representation of the colocalization values was not clarified in the figure legend.

In the revised version of the manuscript, we have now replaced the previous colocalization data with the colocalization of endogenous PLP2 and endogenous CD63 (Fig. 1D). The colocalization was quantified using object based colocalization analysis using automated image analysis program, MotionTracking (http://motiontracking.mpi-cbg.de; (Collinet et al., 2010; Rink et al., 2005)). The cells were randomly selected for imaging in a given experimental setup. For this experiment, total 2349 cells (559+701+1089) from three independent experiments were analyzed for quantification. The intracellular puncta or the vesicles were identified as objects in each channel based on their size, fluorescence intensity and image background by the software (Collinet et al., 2010; Rink et al., 2005). Objects detected in two different channels were considered as colocalized if the relative overlap of respective areas was above 35%. The apparent colocalization value was calculated as a ratio of integral intensities of colocalized objects to the integral intensities of all objects carrying the given marker (In this case PLP2) and varies from 0.0 to 1.0. The colocalization-by-chance (random colocalization) was estimated by random permutation of object localization in different channels. The apparent colocalization was corrected for random colocalization. The obtained colocalization value was converted into the percentage values by multiplying with 100 and this represents the percentage population of PLP2 that colocalizes with CD63. The above description is incorporated in the method section (confocal image analysis) of the revised manuscript (Page no 23, line no 9-24).

Hence each point in the presented quantification graph (Fig 1D' in the revised manuscript) represents the percentage colocalization of PLP2 with CD63 obtained from each experiment. In the figure legend of the revised manuscript, we have now clearly mentioned that n=2349 and N=3 where 'n' represents number of cells and 'N' represents number of independent experiments (manuscript page no 33, line no 22-25; figure 1 legend).

We have also provided the results obtained from colocalization of PLP2-GFP and CD63 in the supplementary figure (Fig. S1F and legends).

Additionally, a gallery of images (Figure R2 in next section) related to the colocalization of PLP2/PLP2-GFP with CD63 are provided below for the editor's and reviewers' reference. In all related images (Fig. 1D, S1F and R2) the zoomed panel of the merged channels clearly pointed towards colocalized puncta by yellow arrowhead.

b) Why are the colocalized puncta mostly perinuclear?

Response: We agree that the colocalized puncta of PLP2 and CD63 or PLP2-GFP and CD63 has a predominant perinuclear pattern (Fig. 1D, S1F and R2 zoomed insets). However, they are not only limited to this location as colocalized puncta away from the perinuclear region are also observed and represented in the zoomed insets of Fig. 1D and S1F of the revised manuscript. Additional montages are also provided here for the editor's and reviewer's reference (R2 zoomed insets).

Nevertheless, the predominant perinuclearly located colocalized puncta of PLP2/ PLP2-GFP and CD63, may possibly represent the mature endocytotic compartments, part of which will eventually fuse with plasma membrane to release exosomes (Bobrie et al., 2011; Colombo et al., 2014; Hurwitz et al., 2017; Park et al., 2018; Verweij et al., 2011). The following detailed discussion with supporting literature will provide better clarity.

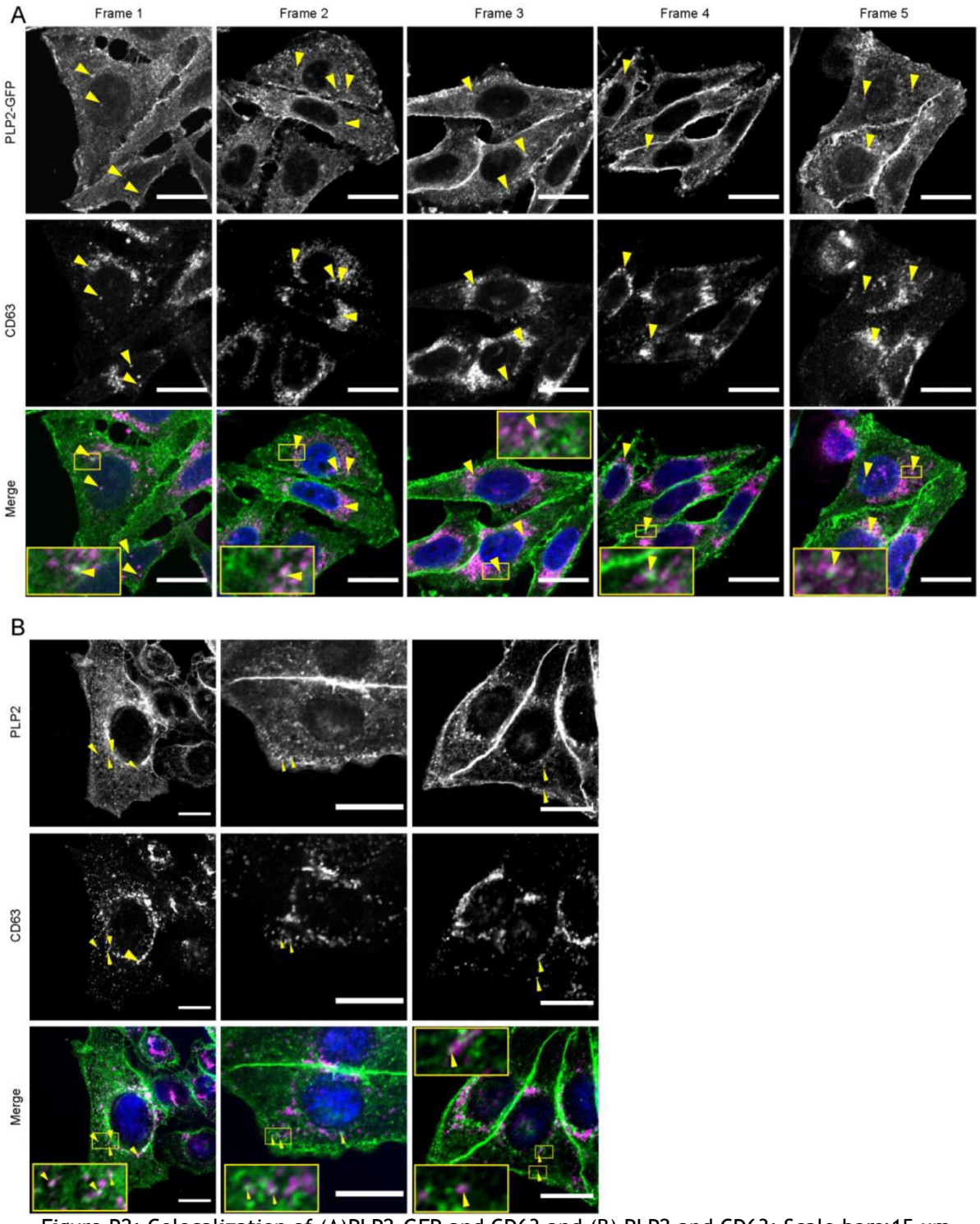


Figure R2: Colocalization of (A)PLP2-GFP and CD63 and (B) PLP2 and CD63; Scale bars:15 μm

The tetraspanin family protein CD63 is one of the well-studied membrane associated exosomal proteins (Gurung et al., 2021). It is selectively enriched on the intraluminal vesicles (ILVs) of the multivesicular endosomes (MVE or MVBs) that are destined to be secreted as exosomes (Escola et al., 1998). CD63 is commonly present in exosomes secreted from all cell types (Hessvik and Llorente, 2018) including the colorectal cancer cells (Hon et al., 2017; Ji et al., 2013) and appropriately, can be found in databases for the molecular composition of the exosomes (Kalra et al., 2012; Kim et al., 2013). A substantial portion of CD63 positive late endosomal compartments (Kobayashi et al., 2000; van Niel et al., 2011) are reported to be clustered at perinuclear regions (Hurwitz et al., 2017; Park et al., 2018; Verweij et al., 2011) from where they either proceed for fusion with lysosome for degradation or with plasma membrane (PM) for releasing the ILVs as

exosomes (Bobrie et al., 2011; Colombo et al., 2014). The MVBs that fuse with PM for exosome release are also reported as "secretory lysosomes" (Buratta et al., 2020; GRIFFITHS, 1996; Heijnen et al., 1998; Nieuwenhuis et al., 1987; Rodríguez et al., 1997). The perinuclear localization of MVBs destined for exosomal secretion can also be supported by the perinuclear clustering of the major population of Rab27a that are reported for docking the MVBs towards PM and thereby contributing to exosome secretion (Hume et al., 2001; Ostrowski et al., 2010).

Moreover, in SW480 cell line, the endogenous localization of CD63 is mostly perinuclear as observed in our extensive imaging studies of paraformaldehyde fixed cells (refer to the image gallery below, Figure R3). Of note, some population do exist in the regions outside the perinuclear zone. Similar localization pattern is recently reported for CD63-GFP in SW480 cells by Eng *et al* (Eng et al., 2021).

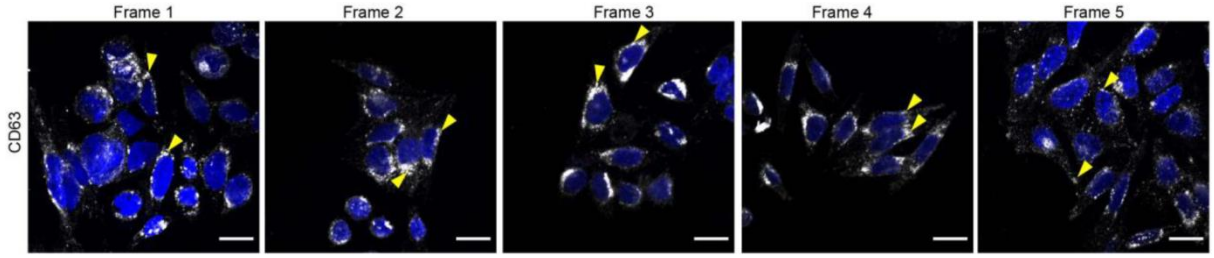


Figure R3: Localization of endogenous CD63 in SW480 cells; Scale bars:15 µm

In our study with the CRC cell line, SW480, endogenous staining of PLP2 showed distinct localization on the plasma membrane as well as on some intracellular puncta that are distributed throughout the cells including some in the perinuclear region (Figure 1Aa and d, pink arrows). We have also observed similar localization pattern upon expression of PLP2 with C terminal GFP or mCherry tag (Figure 1Ab,c,e,f). Notably this localization pattern is in agreement with the previously reported studies (Son et al., 2004; Timms et al., 2013; Wang et al., 2003).
Since the major population of CD63 positive compartments localizes perinuclearly in SW480 cells, the intracellular puncta of PLP2 that are located in the perinuclear region, showed colocalization. We propose that these perinuclear pool of colocalized PLP2 and CD63 vesicles represent a population of MVBs part of which eventually fuse with the PM and release the ILVs as exosomes (Bobrie et al., 2011; Colombo et al., 2014; Hurwitz et al., 2017; Park et al., 2018; Verweij et al., 2011).
The above discussion has now been included in the revised version of the manuscript (Page no 4, line no 7-13).

c) What is the nature of the PLP2-positive and CD63-negative puncta, which represent the majority of the signal? It would be expected that cells have more CD63 positive vesicles compared to PLP2. Is this an artefact of PLP2-GFP overexpression? Images with staining of the endogenous PLP2 would help.

Response: We appreciate the reviewer's comment related to the localization of vesicular pool of PLP2 which is poorly explored till date (Son et al., 2004; Timms et al., 2013; Wang et al., 2003).
We agree with the reviewer that a certain portion of PLP2 positive vesicles are devoid of CD63. Since exosomes are derived from the MVBs which are part of the endocytotic population (Colombo et al., 2014; Harding et al., 1983; Pan et al., 1985), we proposed that the CD63 negative PLP2 vesicles represents the endosomal population which will eventually mature into MVBs. An earlier study also reported PLP2 as an endosomal protein (Timms et al., 2013). We have tested this hypothesis by probing for early endosomal marker EEA1 along with CD63 in PLP2-GFP expressing cells and observed a certain proportion of PLP2-GFP vesicles that are devoid of CD63 and positive for EEA1 (Fig S1F, F'). However, PLP2's sub-cellular localization and its intracellular trafficking is beyond the scope of current study's objectives, where we had focussed on studying the functional contribution of PLP2 in collective migration of colon cancer cells. It will be therefore interesting to carry out a thorough investigation to study PLP2's trafficking pathways and delineate the machineries involved in the same in a separate study.

Perhaps the reviewer's expectation that cells will have more CD63 positive vesicles compared to

PLP2, is related to the fact that CD63 is established as a machinery of endosomal trafficking whereas PLP2 is being introduced as a cargo molecule here. However, it should be taken into consideration that PLP2 is a very less explored molecule till date which leaves immense scope for exploring its role in endosomal trafficking (Son et al., 2004; Timms et al., 2013; Wang et al., 2003). It will further help in explaining the relative abundance of PLP2 containing vesicles with that of vesicles containing intracellular trafficking machineries such as CD63.

We understand the reviewers concern on the artefactual localization of overexpressed PLP2- GFP as it might contribute to the increased abundance of PLP2 containing vesicles within the cell. However, in the revised manuscript, we have tested the colocalization of endogenous PLP2 and CD63 which worked under methanol fixation condition (Fig. 1D). The abundance of endogenous PLP2 containing vesicles (Fig. 1Aa,d, 1D, R2B) were comparable with that of the cells expressing PLP2-GFP or PLP2-mCherry as observed from confocal microscopic studies (Fig. 1Ab,c,e,f, S1F, movie S1, S3, S4, S9, S11, R2A). Moreover, to avoid artefactual abundance of PLP2-GFP or PLP2-mCherry containing vesicles, we have carefully chosen moderately expressing cells for imaging and further analysis.

Based on the above results and discussion, we have now revised the manuscript (page no 3, line no 31-32, page no 4, line no 1-13, Fig 1D, S1F).

d) The effect of GW4869 treatment on PLP2 exosomes (Fig. S3A&B) and collective cell migration (Fig. S3I-M) appears modest. The authors mention that secretion of PLP2 through non-ceramide exosomes in the presence of GW4869 is a possible explanation for the discrepancy between the amount of PLP2 present and the number of exosomes (Fig. S3A&B). However, the western blot (S3A) shows a modest decrease in PLP2, whereas the number of exosomes decreases more than 50% (S3B). This would indicate that the compensation occurs through packaging more PLP2 into non-ceramide exosomes rather than the production of more non-ceramide exosomes.

Response: We do agree with the reviewer that the compensation could occur through packaging more PLP2 into non-ceramide exosomes upon unavailability of ceramide dependent exosomes (under GW4869 treatment) rather than the production of more non-ceramide exosomes. This would indicate that PLP2 might not be exclusively secreted through the ceramide dependent exosomes. Indeed, the possibility of production of more non-ceramide exosome under GW4869 treatment is not in accord with the exosome quantification data (Fig S3B).

We have now added the above discussion in the revised version of the manuscript (page no 6, line no 14-20).

Of note, the above discussion was already included in the previous response (response to the reviewers_1 page no 18, point 2b).

e) Finally, it is not clear how exosomal PLP2 regulates collective cell migration.

Response: We understand the reviewer's concern however, the focus of the manuscript was to decipher the role of a poorly explored molecule, PLP2 in collective cell migration. Through a systematic series of studies, we demonstrated that PLP2 functions at the leading edge of the migrating cells via its association with of ZO-1 and activation of Rac1 GTPase. During this study we have observed that in addition to the plasma membrane, PLP2 also localizes to intracellular puncta, with some of them colocalizing with exosomal markers, CD63 and Rab27a. PLP2 was identified in the purified exosomes. We further explored the functional relevance of the exosomal pool of PLP2, by conducting rescue experiments using PLP2 carrying exosomes. We agree that the detailed mechanistic investigation on how the exosomal PLP2 contributes to the CCM measured in the target cell is beyond the scope of this investigation and discussed the same in the revised manuscript (page no 6, line no 22-23). Notably, this issue was also not raised during the first review although the exosome data were always being a part of the manuscript. Indeed, as per the reviewer's suggestion during the first review, we have already included the results from the control experiments using exosomes purified from SW480 and PLP2KO cells to compare with the treatment of exosomes purified from PLP2-GFP expressing cells (Fig. S3).

However, we strongly believe that the current study will open up new avenues for mechanistic

investigation on the role of exosomal PLP2 in CCM, since this is the first report describing the functional relevance of exosomal PLP2. We would like to add that in the recent past, only a limited number of studies have been reported demonstrating the role of exosomal cargo in directed cell migration (Sung and Weaver, 2018; Sung et al., 2020). To the best of our knowledge, this is the first report on an exosomal cargo which contributes to CCM. Our study will therefore invite broad readership and open new avenues of cancer research.

Additionally, in the revised manuscript, we have now discussed how future studies deciphering the mechanistic details of exosomal PLP2 mediated CCM in the target cells could be employed to reveal the pathophysiological relevance of PLP2 in colon cancer progression (page no 15, line no 26-28).

f) In the cartoon presented in Fig. 7, the topography of PLP2 in exosomes is not properly drawn."

Response: We are thankful to the reviewer for pointing this out and sincerely apologise for the error. We have now corrected this topology in the revised version of the manuscript (Figure 7).

PLP2 is an integral membrane protein with a topology of its N and C terminal towards the cytosol. Hence after endocytosis, in the limiting membrane of the endosomes (MVBs in the cartoon), PLP2 topology will be flipped facing the N and C terminal outside the MVB lumen or towards the cytosol of the cell. We have now corrected this topology in the revised version of the manuscript (Figure 7).

However, exosomes represent the ILVs that are derived from the inward budding of the limiting membrane of the MVBs. Therefore, in ILVs and exosomes, the N and C terminus of PLP2 will be facing towards the exosomal lumen. This has been correctly drawn in the previous as well as revised version of the manuscript (Figure 7).

Issue 3: An issue brought up in the first and second rounds related to lack of clarity regarding number of cells analyzed and number of independent experiments performed. While you did carry out 3 independent experiments, it appears that in most cases you combined the "n" from all three experiments together, which will give you a much more significant p value than if you compare averages from the three independent experiments. There is a very useful piece published recently in the Journal of Cell Biology you may want to look at if you haven't already. You can find it at the following link:
https://rupress.org/jcb/article/219/6/e202001064/151717/SuperPlots-Communicating-reproducibility-and

I would refer you to the comment that "Problematic plots treat n as the number of cells, resulting in tiny error bars and P values. These plots also conceal any systematic run-to-run error, mixing it with cell-to-cell variability."

I highly recommend you take a look at your data again with these considerations in mind.

Response: We are thankful to the editor for the suggestion. Although we were earlier following another article published in JCB (Plutoni et al., 2016) for such representation, we have now gone through the recommended article and agreed to the fact that comparing averages from independent experiments can better represent variability in cell biology experiments.

Thus, we have reanalysed our data and presented them in figures as averages from independent experiments and compared the same for error bar and significance calculations. This has been done in all figures related to colocalization calculations (Fig. 1B", 1C", 1D', S1F, S4A"), intensity based image analyses (Fig. 4E'a,b, 6C', S1C', S1E', S4D", S5A, S5F, S5G) and speed and persistence calculations for track analysis (Fig. 3F, 3G, 5D, 5D', 5E, 5E', 6E, 6F, S2D, S2E, S3G, S3H, S3K, S3K', S6E, S6F). Corresponding figure legends are being modified accordingly where we have clearly mentioned total number of cells used (n) for the analysis and number of independent experiments (N) performed in each cases.

Reviewer 1 Advance Summary and Potential Significance to Field:
The authors have conducted several additional experiments to improve their studies. I am satisfied with their thoughtful answers to my initial comments and by the quality of the new data

added to the paper. Globally, this study is significantly improved.

Response: We appreciate that the reviewer has found this study significantly improved and satisfactory and further highlighted our responses as thoughtful.

Reviewer 1 Comments for the Author:

The new text additions make the paper a bit "patchy". I would consider attempting to increase the fluidity of the text and revise the English.

Response: We are thankful to the reviewer for the comment. We have revised the manuscript as per the comments from the editor and the other reviewers and therefore added some new information to the revised version. We have ensured that the newly added text did not disrupt the continuity of the entire text and made some changes wherever required for maintaining the flow. Some of the examples include:

1. Page no 3, line no 18-19
2. Page no 4, line no 32 to page no 5, line no 1 ("Therefore, we proceeded to investigate the possible involvement of PLP2 in CCM. At first, we monitored…")

We have also carefully read and scrutinized the language and the grammar in the revised manuscript using the commercially available application "Grammarly".

Reviewer 2 Advance Summary and Potential Significance to Field:
Ghosh and colleagues characterize the relationship between PLP2, ZO-1, and Rac during collective cell migration and find that knock-out of PLP2 decreases the rate of cell migration, inhibits the lamellipodial recruitment of ZO-1, and leads to a decrease in the amount of active Rac. These phenotypes were rescued by full-length PLP2 but not a PLP2 construct that is missing the C-terminus. Collectively, these results suggest that PLP2 stimulates cell migration through recruitment of ZO-1 to leading edge and activation of Rac.

Reviewer 2 Comments for the Author:

I appreciate that Ghosh and colleagues address many of my major concerns in this revised version. I do have just a few minor comments I feel need to be addressed.

Response: We appreciate that the reviewer has addressed that the major concerns raised were being answered and the minor comments mentioned below has raised from the newly added data and being answered point by point below.

Figure 1. I appreciate the quantification of the co-localization of PLP2 and Cortactin. However, without proper positive and negative controls the Pearson's coefficient really does not tell us whether for example 0.5 represent high co-localization in this system or poor co-localization. In the very least they could have compared the co-localization of Cortactin with of actin since these two proteins are known to co-localize at the leading edge for the sake of comparison.

Similarly, it is not clear what %Colocalization means with regards to the co-localization of CD63.

Response: We are thankful to the reviewer for the suggestion. We have now carried out the colocalization studies of cortactin and F-actin in the cells expressing PLP2-GFP. The measured colocalization value for PLP2-GFP:cortactin as well as PLP2-GFP:F-actin (phalloidin) was comparable with that of cortactin:actin (Fig. 1B, B', B"). These results are added in the revised version of the manuscript (Fig. 1B, B', B", manuscript page no 3, line no 29-30; figure legend of Fig. 1B-B" page no 33, line no 15-19).

The percentage colocalization of PLP2-GFP with that of CD63 was calculated using object based colocalization method (method section in manuscript page no 23 line no 9-24) where vesicular pool of PLP2-GFP and CD63 has been identified as separate objects and the percentage of PLP2-GFP vesicles that colocalized with CD63 vesicles were reported. Object- based colocalization analysis was performed by the automated image analysis program, MotionTracking (http://motiontracking.mpi-cbg.de; (Collinet et al., 2010; Rink et al., 2005)). The cells were randomly selected for imaging in a given experimental setup. The intracellular puncta or the

vesicles were identified as objects in each channel based on their size, fluorescence intensity and image background by the MotionTracking software (Collinet et al., 2010; Rink et al., 2005). Objects detected in two different channels were considered as colocalized if the relative overlap of respective areas was above 35%. The apparent colocalization value was calculated as a ratio of integral intensities of colocalized objects to the integral intensities of all objects carrying the given marker (in this case PLP2) and varies from 0.0 to 1.0. The colocalization-by-chance (random colocalization) was estimated by random permutation of object localization in different channels. The apparent colocalization was corrected for random colocalization. The obtained colocalization value was converted into the percentage values by multiplying with 100 and this represents the percentage population of PLP2 that colocalizes with CD63. The reference to the method section is mentioned in the figure legends of all related images (Fig. 1D, S1F) in the revised manuscript.

Figure 6. The introduction of GFP-tagged Rac will inevitable stimulate lamellipodia formation so it is hard to interpret these results. Staining for endogenous Rac rather than transfecting in GFP Rac would have been more impactful. That being said the authors show an increase in active Rac upon rescue with PLP2 which is a better demonstration of the relationship between PLP2 and Rac.

Response: We agree with the reviewer that expression of GFP-Rac1WT would enhance lamellipodia formation. However, it was used for the following two purposes in the study where this effect does not impact interpretation of the results.

1. To investigate the co-dynamics of Rac1 with PLP2-mCherry during CCM in live cell imaging studies.

2. To investigate the functional involvement of Rac1 GTPase activity in PLP2 mediated CCM. Here, GFP-tagged dominant negative Rac1 (GFP-Rac1DN) was co-expressed with PLP2- mCherry in PLP2KO background to demonstrate involvement of Rac1 activation in PLP2 mediated CCM as well as umbrella like protrusions formation. The umbrella like structures were stained with phalloidin in GFP/GFP-Rac1DN/GFP-Rac1WT background of PLP2KO cells (Fig 6C-C', S6A). Expression of GFP-Rac1WT was used as a control condition, to compare the results obtained in Rac1DN background (mentioned in manuscript page no 12, line no 2-3). However, keeping in mind that expressing GFP-Rac1WT might impact the number of lamellipodia formation, it was not used as the only control. Instead, expression of GFP vector was also used as the control (Fig 6C-C', S6A, manuscript page no 12, line no 2-3). Alternatively, we could have also depleted Rac1 to address these objectives, however, the use of Rac1DN instead of silencing approach provides direct evidence for importance of the GTP/GDP cycle in the process in addition to minimize off-target effect.

It is also puzzling that mCherry-PLP2 deltaC was not able to rescue the PLP2 KO cell migration phenotype, but was able to rescue the loss of active Rac. The authors do comment on this, but may be this is case where some sort of quantification of the amount of active Rac will be useful here so that the reader can have an understanding marginally as it not entirely clear from the blot presented.

Response: We are thankful to the reviewer for the suggestion. We have indeed calculated the ratio of active Rac1 and the total Rac1 from the intensities of the appropriate bands in the western blot and provided the ratio for each of the conditions in the figure 6B". We have now given reference of the quantitation table within the text of the revised manuscript (page no 11, line no 27). The quantification was based on duplicate experiments. The blots from the duplicate experiments are provided in the Fig. S1B". The same has been explained in the figure legends of Fig. 6B" the revised manuscript (page no 36, line no 10-11).

We would also like to mention here that the disparity in the fact that mCherry-PLP2ΔC was not able to rescue the PLP2KO cell migration phenotype, but was able to rescue the loss of active Rac1, was explained in the discussion section (manuscript page no 14, line no 21-29) as follows:
"Interestingly, the perturbation of PLP2-ZO-1 association did not markedly reduce the active Rac1 population (Fig. 6B'-B", S6B'-B") suggesting that the association may not have any major role in global Rac1 activation. However, the perturbation of this association led to cytoskeletal alterations at the leading edge and successive abrogation of collective migration (Figs 5B-H, S5). Based on these results, we propose that the association between ZO-1 and PLP2 may contribute to polarized activation of Rac1 that is important for directive cell migration during CCM. However, it

would be interesting to unravel other intermediate molecular players that bridge between PLP2-ZO-1 association and Rac1 during CCM."

Reviewer 3 Advance Summary and Potential Significance to Field:
This is a revised version of a manuscript previously reviewed. While the authors attempted to answer the issues raised, some of the concerns originally brought up remain.

Reviewer 3 Comments for the Author:
1. The fact that the results presented in the manuscript are based on findings in one colon rectal cancer cell line is worrisome and suggests that the proposed role for PLP2 in regulating collective cell migration is not generally applicable.

Response: We understand the reviewer's concern that our findings are focused on a particular colorectal cancer (CRC) cell line, SW480. We agree to the fact that PLP2's role in CCM might not be claimed to be generalised in all other CRC cell lines. As suggested by the editor, we have now mentioned this limitation in the revised version of the manuscript and further discussed the possibility of extending this study to other cell lines with similar collective features as SW480 (page no 14, line no 34 to page no 15, line no 3).

However, cell lines with equivalent collective nature should be carefully selected for comparison since cancer cells of variable epithelial characteristics exhibit different types of CCM such as the 'moving sheet' and 'moving cell networks' type as described in literature (Friedl and Mayor, 2017). The same has also been very clearly demonstrated by our experimental evidences on the CCM characteristics of CaCO2 and SW480 which we have provided in the earlier response letter against the comments addressed by all three reviewers (response to the reviewers_1: reviewer 1 major comments point 1; page no 1 to 3; and reviewer 2 general comments point 1; page no 9 to 11; reviewer 3 point 1b; page no 16 to 18; Figure R1 and movie R1). Therefore, it is not justified to expect that the same set of upstream machineries will control the CCM in different CRC cell lines of variable epithelial nature.

PLP2 being demonstrated as one of the very few upstream regulators of leading-edge actin cytoskeleton during CCM, the current study, would substantially advance our current understanding of CCM in cancer progression, although the study is being focused in a single CRC cell line. In the manuscript, we have also discussed the scope for extending the investigation for its pathophysiological relevance in an *in vivo* xenograft model (manuscript page no 15, line no 3-4 and line no16-17).

2. a) The data presented to implicate exosomes in the function of PLP2 are not clear. The characterization of the exosomes does not follow the minimal criteria developed by the International Society of Extracellular Vesicles (Thery et al., J Extracellular Vesicles 2018). Ultracentrifugation fractionation with positive and negative markers, electron microscopy, and NTA analyses that show the size and homogeneity of the exosome population, should be presented.

Response: While reviewing the revised manuscript (MS# JOCES/2020/253468), the reviewer has brought up an issue stating "the characterization of the exosomes does not follow the minimal criteria developed by the International Society of Extracellular Vesicles (Thery *et al.*, J Extracellular Vesicles 2018)". Interestingly this issue has been newly raised by the reviewer 3, despite the fact that exosome data were always being a part of the manuscript since the first submission (MS# JOCES/2020/253468). The reviewer has earlier recommended to include two more control experiments using exosomes purified from SW480 cells as well as PLP2KO cells to compare with the results obtained from the treatment of PLP-GFP exosomes to the PLP2KO cells. We have already performed these control experiments and included the results in the revised manuscript (Fig. S3; response to the reviewers_1, page no 18, point 2a).
However, we do understand that the ultracentrifugation fractionation with positive and negative markers, electron microscopy (EM), and nanoparticle tracking analysis (NTA) are three criteria for firm characterization of exosomes. We would like to state here that, we have already reported the ultracentrifugation fractionation with positive and negative markers (Fig.S1G-H, S3A, S3C) and the concentrations measured by NTA analyses (Fig. S3B) (mentioned in methods section: page no 20 in revised manuscript). The size distribution of exosomes purified from SW480 cells as measured by NTA is indeed a part of the analysis and we do have the characterization which we have now

included in the revised manuscript (Figure S1G'). In addition, at the initiation of our exosome preparation from SW480 cells we had also performed the TEM (Transmission electron microscopy) characterization of purified SW480 exosomes to ensure the shape and size of our exosome preparation. It is also now included in the revised manuscript (Figure S1G"). The relevant descriptions have also been included in the revised version of the manuscript (page no 4, line no 19-21; page no 20, line no 25-31.). For the reviewer's reference we are attaching the NTA and TEM data for SW480 cells, below as well.

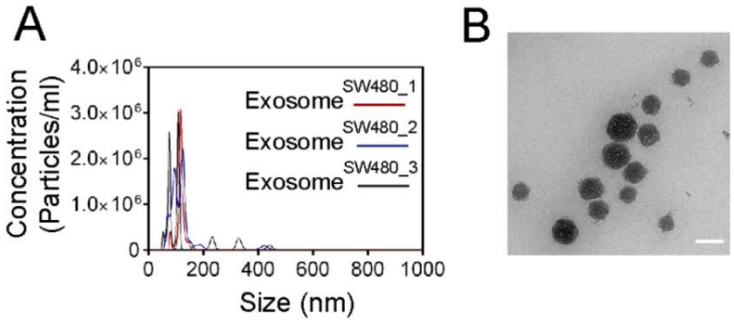


Figure R1: Characterization of size distribution and shape of the exosomes purified from SW480 cells. A) Nanoparticle tracking analysis. B) Transmission electron microscopy, scale bar=100 nm.

2b) The PLP2/CD63 co-localization data the authors now provide are perplexing. What do each point represent in the quantification graph presented? The figure legend is not clear about this (20 frames from 3 independent experiments; how many cells were analyzed?).

Response: We apologise that the scatter representation of the colocalization values was not clarified in the figure legend.

In the revised version of the manuscript, we have now replaced the previous colocalization data with the colocalization of endogenous PLP2 and endogenous CD63 (Fig. 1D). The colocalization was quantified using object based colocalization analysis using automated image analysis program, MotionTracking (http://motiontracking.mpi-cbg.de; (Collinet et al., 2010; Rink et al., 2005)). The cells were randomly selected for imaging in a given experimental setup. For this experiment, total 2349 cells (559+701+1089) from three independent experiments were analyzed for quantification. The intracellular puncta or the vesicles were identified as objects in each channel based on their size, fluorescence intensity and image background by the software (Collinet et al., 2010; Rink et al., 2005). Objects detected in two different channels were considered as colocalized if the relative overlap of respective areas was above 35%. The apparent colocalization value was calculated as a ratio of integral intensities of colocalized objects to the integral intensities of all objects carrying the given marker (In this case PLP2) and varies from 0.0 to 1.0. The colocalization-by-chance (random colocalization) was estimated by random permutation of object localization in different channels. The apparent colocalization was corrected for random colocalization. The obtained colocalization value was converted into the percentage values by multiplying with 100 and this represents the percentage population of PLP2 that colocalizes with CD63. The above description is incorporated in the method section (confocal image analysis) of the revised manuscript (Page no 23, line no 9-24).

Hence each point in the presented quantification graph (Fig 1D' in the revised manuscript) represents the percentage colocalization of PLP2 with CD63 obtained from each experiment. In the figure legend of the revised manuscript, we have now clearly mentioned that n=2349 and N=3 where 'n' represents number of cells and 'N' represents number of independent experiments (manuscript page no 33, line no 22-25; figure 1 legend).

We have also provided the results obtained from colocalization of PLP2-GFP and CD63 in the supplementary figure (Fig. S1F and legends).

Additionally, a gallery of images (Figure R2 in next section) related to the colocalization of PLP2/PLP2-GFP with CD63 are provided below for the editor's and reviewers' reference. In all

related images (Fig. 1D, S1F and R2) the zoomed panel of the merged channels clearly pointed towards colocalized puncta by yellow arrowhead.

b) Why are the colocalized puncta mostly perinuclear?

Response: We agree that the colocalized puncta of PLP2 and CD63 or PLP2-GFP and CD63 has a predominant perinuclear pattern (Fig. 1D, S1F and R2 zoomed insets). However, they are not only limited to this location as colocalized puncta away from the perinuclear region are also observed and represented in the zoomed insets of Fig. 1D and S1F of the revised manuscript. Additional montages are also provided here for the editor's and reviewer's reference (R2 zoomed insets).

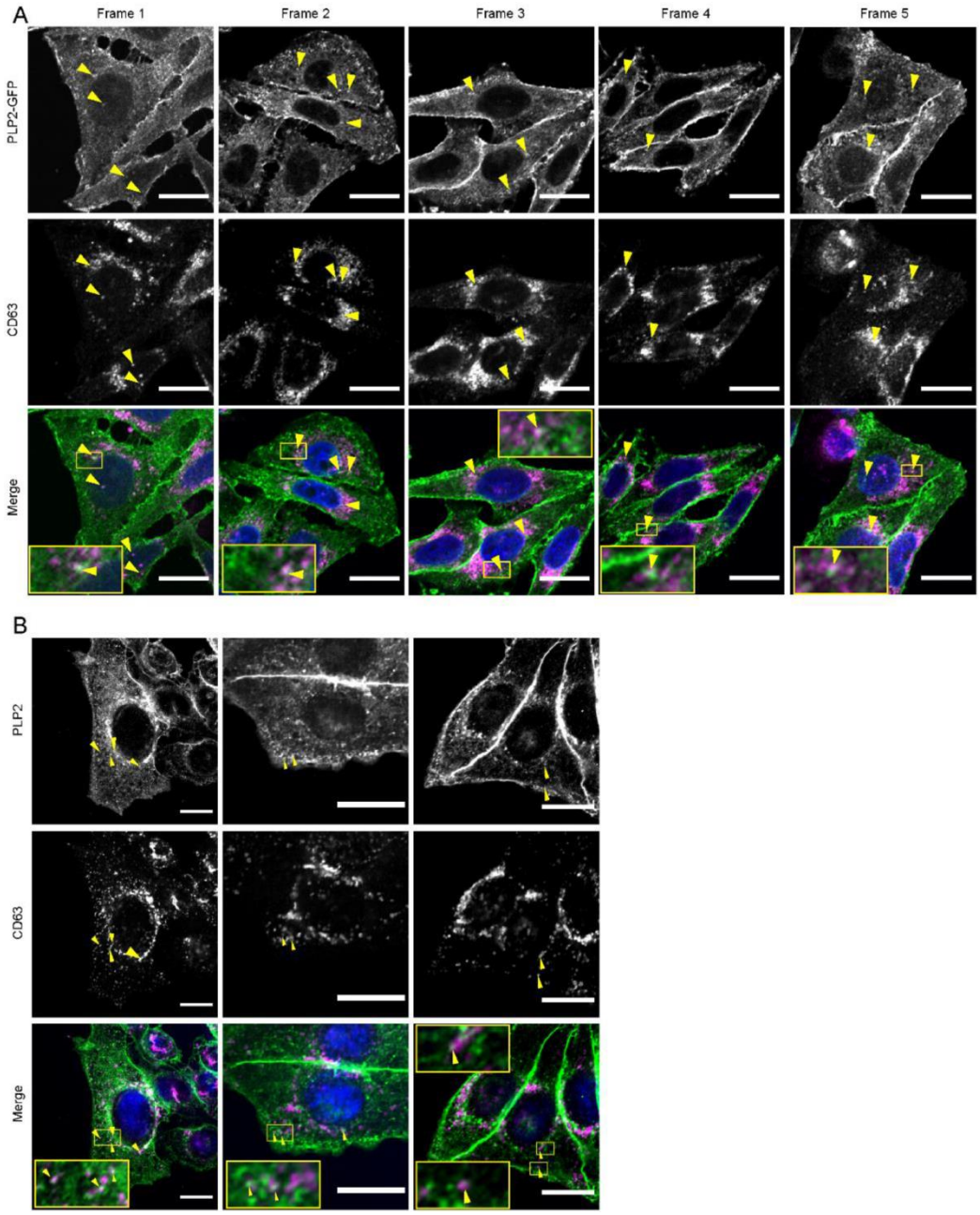


Figure R2: Colocalization of (A)PLP2-GFP and CD63 and (B) PLP2 and CD63; Scale bars:15 µm

Nevertheless, the predominant perinuclearly located colocalized puncta of PLP2/ PLP2-GFP and CD63, may possibly represent the mature endocytotic compartments, part of which will eventually fuse with plasma membrane to release exosomes (Bobrie et al., 2011; Colombo et al., 2014; Hurwitz et al., 2017; Park et al., 2018; Verweij et al., 2011). The following detailed discussion with supporting literature will provide better clarity.

The tetraspanin family protein CD63 is one of the well-studied membrane associated exosomal proteins (Gurung et al., 2021). It is selectively enriched on the intraluminal vesicles (ILVs) of the multivesicular endosomes (MVE or MVBs) that are destined to be secreted as exosomes (Escola et al., 1998). CD63 is commonly present in exosomes secreted from all cell types (Hessvik and Llorente, 2018) including the colorectal cancer cells (Hon et al., 2017; Ji et al., 2013) and appropriately, can be found in databases for the molecular composition of the exosomes (Kalra et al., 2012; Kim et al., 2013). A substantial portion of CD63 positive late endosomal compartments (Kobayashi et al., 2000; van Niel et al., 2011) are reported to be clustered at perinuclear regions (Hurwitz et al., 2017; Park et al., 2018; Verweij et al., 2011) from where they either proceed for fusion with lysosome for degradation or with plasma membrane (PM) for releasing the ILVs as exosomes (Bobrie et al., 2011; Colombo et al., 2014). The MVBs that fuse with PM for exosome release are also reported as "secretory lysosomes" (Buratta et al., 2020; GRIFFITHS, 1996; Heijnen et al., 1998; Nieuwenhuis et al., 1987; Rodríguez et al., 1997). The perinuclear localization of MVBs destined for exosomal secretion can also be supported by the perinuclear clustering of the major population of Rab27a that are reported for docking the MVBs towards PM and thereby contributing to exosome secretion (Hume et al., 2001; Ostrowski et al., 2010).

Moreover, in SW480 cell line, the endogenous localization of CD63 is mostly perinuclear as observed in our extensive imaging studies of paraformaldehyde fixed cells (refer to the image gallery below, Figure R3). Of note, some population do exist in the regions outside the perinuclear zone. Similar localization pattern is recently reported for CD63-GFP in SW480 cells by Eng *et al* (Eng et al., 2021).

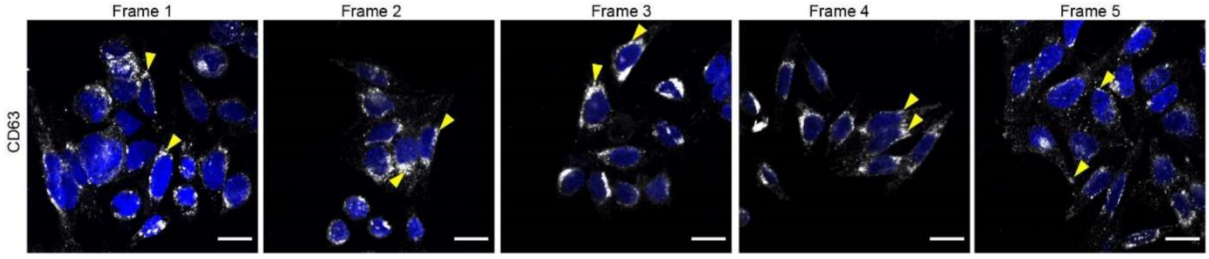


Figure R3: Localization of endogenous CD63 in SW480 cells; Scale bars:15 µm

In our study with the CRC cell line, SW480, endogenous staining of PLP2 showed distinct localization on the plasma membrane as well as on some intracellular puncta that are distributed throughout the cells including some in the perinuclear region (Figure 1Aa and d, pink arrows). We have also observed similar localization pattern upon expression of PLP2 with C terminal GFP or mCherry tag (Figure 1Ab,c,e,f). Notably this localization pattern is in agreement with the previously reported studies (Son et al., 2004; Timms et al., 2013; Wang et al., 2003).

Since the major population of CD63 positive compartments localizes perinuclearly in SW480 cells, the intracellular puncta of PLP2 that are located in the perinuclear region, showed colocalization. We propose that these perinuclear pool of colocalized PLP2 and CD63 vesicles represent a population of MVBs part of which eventually fuse with the PM and release the ILVs as exosomes (Bobrie et al., 2011; Colombo et al., 2014; Hurwitz et al., 2017; Park et al., 2018; Verweij et al., 2011).

The above discussion has now been included in the revised version of the manuscript (Page no 4, line no 7-13).

c) What is the nature of the PLP2-positive and CD63-negative puncta, which represent the majority of the signal? It would be expected that cells have more CD63 positive vesicles compared to PLP2. Is this an artefact of PLP2-GFP overexpression? Images with staining of the endogenous PLP2 would help.

Response: We appreciate the reviewer's comment related to the localization of vesicular pool of PLP2 which is poorly explored till date (Son et al., 2004; Timms et al., 2013; Wang et al., 2003). We agree with the reviewer that a certain portion of PLP2 positive vesicles are devoid of CD63. Since

exosomes are derived from the MVBs which are part of the endocytotic population (Colombo et al., 2014; Harding et al., 1983; Pan et al., 1985), we proposed that the CD63 negative PLP2 vesicles represents the endosomal population which will eventually mature into MVBs. An earlier study also reported PLP2 as an endosomal protein (Timms et al., 2013). We have tested this hypothesis by probing for early endosomal marker EEA1 along with CD63 in PLP2-GFP expressing cells and observed a certain proportion of PLP2-GFP vesicles that are devoid of CD63 and positive for EEA1 (Fig S1F, F'). However, PLP2's sub-cellular localization and its intracellular trafficking is beyond the scope of current study's objectives, where we had focussed on studying the functional contribution of PLP2 in collective migration of colon cancer cells. It will be therefore interesting to carry out a thorough investigation to study PLP2's trafficking pathways and delineate the machineries involved in the same in a separate study.

Perhaps the reviewer's expectation that cells will have more CD63 positive vesicles compared to PLP2, is related to the fact that CD63 is established as a machinery of endosomal trafficking whereas PLP2 is being introduced as a cargo molecule here. However, it should be taken into consideration that PLP2 is a very less explored molecule till date which leaves immense scope for exploring its role in endosomal trafficking (Son et al., 2004; Timms et al., 2013; Wang et al., 2003). It will further help in explaining the relative abundance of PLP2 containing vesicles with that of vesicles containing intracellular trafficking machineries such as CD63.

We understand the reviewers concern on the artefactual localization of overexpressed PLP2- GFP as it might contribute to the increased abundance of PLP2 containing vesicles within the cell. However, in the revised manuscript, we have tested the colocalization of endogenous PLP2 and CD63 which worked under methanol fixation condition (Fig. 1D). The abundance of endogenous PLP2 containing vesicles (Fig. 1Aa,d, 1D, R2B) were comparable with that of the cells expressing PLP2-GFP or PLP2-mCherry as observed from confocal microscopic studies (Fig. 1Ab,c,e,f, S1F, movie S1, S3, S4, S9, S11, R2A). Moreover, to avoid artefactual abundance of PLP2-GFP or PLP2-mCherry containing vesicles, we have carefully chosen moderately expressing cells for imaging and further analysis.

Based on the above results and discussion, we have now revised the manuscript (page no 3, line no 31-32, page no 4, line no 1-13, Fig 1D, S1F).

d) The effect of GW4869 treatment on PLP2 exosomes (Fig. S3A&B) and collective cell migration (Fig. S3I-M) appears modest. The authors mention that secretion of PLP2 through non-ceramide exosomes in the presence of GW4869 is a possible explanation for the discrepancy between the amount of PLP2 present and the number of exosomes (Fig. S3A&B). However, the western blot (S3A) shows a modest decrease in PLP2, whereas the number of exosomes decreases more than 50% (S3B). This would indicate that the compensation occurs through packaging more PLP2 into non-ceramide exosomes rather than the production of more non-ceramide exosomes.

Response: We do agree with the reviewer that the compensation could occur through packaging more PLP2 into non-ceramide exosomes upon unavailability of ceramide dependent exosomes (under GW4869 treatment) rather than the production of more non-ceramide exosomes. This would indicate that PLP2 might not be exclusively secreted through the ceramide dependent exosomes. Indeed, the possibility of production of more non-ceramide exosome under GW4869 treatment is not in accord with the exosome quantification data (Fig S3B).

We have now added the above discussion in the revised version of the manuscript (page no 6, line no 14-20).

Of note, the above discussion was already included in the previous response (response to the reviewers_1 page no 18, point 2b).

e) Finally, it is not clear how exosomal PLP2 regulates collective cell migration.

Response: We understand the reviewer's concern however, the focus of the manuscript was to decipher the role of a poorly explored molecule, PLP2 in collective cell migration. Through a systematic series of studies, we demonstrated that PLP2 functions at the leading edge of the migrating cells via its association with of ZO-1 and activation of Rac1 GTPase. During this study we

have observed that in addition to the plasma membrane, PLP2 also localizes to intracellular puncta, with some of them colocalizing with exosomal markers, CD63 and Rab27a. PLP2 was identified in the purified exosomes. We further explored the functional relevance of the exosomal pool of PLP2, by conducting rescue experiments using PLP2 carrying exosomes. We agree that the detailed mechanistic investigation on how the exosomal PLP2 contributes to the CCM measured in the target cell is beyond the scope of this investigation and discussed the same in the revised manuscript (page no 6, line no 22-23). Notably, this issue was also not raised during the first review although the exosome data were always being a part of the manuscript. Indeed, as per the reviewer's suggestion during the first review, we have already included the results from the control experiments using exosomes purified from SW480 and PLP2KO cells to compare with the treatment of exosomes purified from PLP2-GFP expressing cells (Fig. S3).

However, we strongly believe that the current study will open up new avenues for mechanistic investigation on the role of exosomal PLP2 in CCM, since this is the first report describing the functional relevance of exosomal PLP2. We would like to add that in the recent past, only a limited number of studies have been reported demonstrating the role of exosomal cargo in directed cell migration (Sung and Weaver, 2018; Sung et al., 2020). To the best of our knowledge, this is the first report on an exosomal cargo which contributes to CCM. Our study will therefore invite broad readership and open new avenues of cancer research.
Additionally, in the revised manuscript, we have now discussed how future studies deciphering the mechanistic details of exosomal PLP2 mediated CCM in the target cells could be employed to reveal the pathophysiological relevance of PLP2 in colon cancer progression (page no 15, line no 26-28).

f) In the cartoon presented in Fig. 7, the topography of PLP2 in exosomes is not properly drawn."

Response: We are thankful to the reviewer for pointing this out and sincerely apologise for the error. We have now corrected this topology in the revised version of the manuscript (Figure 7).

PLP2 is an integral membrane protein with a topology of its N and C terminal towards the cytosol. Hence after endocytosis, in the limiting membrane of the endosomes (MVBs in the cartoon), PLP2 topology will be flipped facing the N and C terminal outside the MVB lumen or towards the cytosol of the cell. We have now corrected this topology in the revised version of the manuscript (Figure 7).

However, exosomes represent the ILVs that are derived from the inward budding of the limiting membrane of the MVBs. Therefore, in ILVs and exosomes, the N and C terminus of PLP2 will be facing towards the exosomal lumen. This has been correctly drawn in the previous as well as revised version of the manuscript (Figure 7).

References:

Bobrie, A., Colombo, M., Raposo, G. and Théry, C. (2011). Exosome Secretion: Molecular Mechanisms and Roles in Immune Responses. *Traffic* **12**, 1659–1668.

Buratta, S., Tancini, B., Sagini, K., Delo, F., Chiaradia, E., Urbanelli, L. and Emiliani, C. (2020). Lysosomal Exocytosis, Exosome Release and Secretory Autophagy: The Autophagic- and Endo- Lysosomal Systems Go Extracellular. *International Journal of Molecular Sciences* **21**, 2576.

Collinet, C., Stöter, M., Bradshaw, C. R., Samusik, N., Rink, J. C., Kenski, D., Habermann, B., Buchholz, F., Henschel, R., Mueller, M. S., et al. (2010). Systems survey of endocytosis by multiparametric image analysis. *Nature* **464**, 243–249.

Colombo, M., Raposo, G. and Théry, C. (2014). Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. *Annual Review of Cell and Developmental Biology* **30**, 255–289.

Eng, S.-K., Imtiaz, I. R., Goh, B.-H., Ming, L. C., Lim, Y.-C. and Lee, W.-L. (2021). Does KRAS Play a Role in the Regulation of Colon Cancer Cells-Derived Exosomes? *Biology* **10**, 58.

Escola, J. M., Kleijmeer, M. J., Stoorvogel, W., Griffith, J. M., Yoshie, O. and Geuze, H. J. (1998). Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *Journal of Biological Chemistry* **273**, 20121–20127.

Friedl, P. and Mayor, R. (2017). Tuning Collective Cell Migration by Cell–Cell Junction Regulation. *Cold Spring Harbor Perspectives in Biology* **9**, a029199.

GRIFFITHS, G. (1996). Secretory lysosomes — a special mechanism of regulated secretion in haemopoietic cells. *Trends in Cell Biology* **6**, 329–332.
Gurung, S., Perocheau, D., Touramanidou, L. and Baruteau, J. (2021). The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Communication and Signaling* **19**, 47.
Harding, C., Heuser, J. and Stahl, P. (1983). Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *Journal of Cell Biology* **97**, 329–339.
Heijnen, H. F. G., Debili, N., Vainchencker, W., Breton-Gorius, J., Geuze, H. J. and Sixma, J. J. (1998). Multivesicular Bodies Are an Intermediate Stage in the Formation of Platelet α-Granules. *Blood* **91**, 2313–2325.
Hessvik, N. P. and Llorente, A. (2018). Current knowledge on exosome biogenesis and release. *Cellular and Molecular Life Sciences* **75**, 193–208.
Hon, K. W., Abu, N., Ab Mutalib, N.-S. and Jamal, R. (2017). Exosomes As Potential Biomarkers and Targeted Therapy in Colorectal Cancer: A Mini-Review. *Frontiers in Pharmacology* **8**, 583.
Hume, A. N., Collinson, L. M., Rapak, A., Gomes, A. Q., Hopkins, C. R. and Seabra, M. C. (2001). Rab27a Regulates the Peripheral Distribution of Melanosomes in Melanocytes. *Journal of Cell Biology* **152**, 795–808.
Hurwitz, S. N., Nkosi, D., Conlon, M. M., York, S. B., Liu, X., Tremblay, D. C. and Meckes, D. G. (2017). CD63 Regulates Epstein-Barr Virus LMP1 Exosomal Packaging, Enhancement of Vesicle Production, and Noncanonical NF-κB Signaling. *Journal of Virology* **91**, 1–19.
Ji, H., Greening, D. W., Barnes, T. W., Lim, J. W., Tauro, B. J., Rai, A., Xu, R., Adda, C., Mathivanan, S., Zhao, W., et al. (2013). Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components. *PROTEOMICS* **13**, 1672–1686.
Kalra, H., Simpson, R. J., Ji, H., Aikawa, E., Altevogt, P., Askenase, P., Bond, V. C., Borràs, F. E., Breakefield, X., Budnik, V., et al. (2012). Vesiclepedia: A Compendium for Extracellular Vesicles with Continuous Community Annotation. *PLoS Biology* **10**, e1001450.
Kim, D.-K., Kang, B., Kim, O. Y., Choi, D., Lee, J., Kim, S. R., Go, G., Yoon, Y. J., Kim, J. H., Jang, S. C., et al. (2013). EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles. *Journal of Extracellular Vesicles* **2**, 20384.
Kobayashi, T., Vischer, U. M., Rosnoblet, C., Lebrand, C., Lindsay, M., Parton, R. G., Kruithof, E. K. O. and Gruenberg, J. (2000). The Tetraspanin CD63/lamp3 Cycles between Endocytic and Secretory Compartments in Human Endothelial Cells. *Molecular Biology of the Cell* **11**, 1829–1843.
Nieuwenhuis, H., van Oosterhout, J., Rozemuller, E., van Iwaarden, F. and Sixma, J. (1987). Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000- molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood* **70**, 838–845.
Ostrowski, M., Carmo, N. B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., Moita, C. F., Schauer, K., Hume, A. N., Freitas, R. P., et al. (2010). Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nature Cell Biology* **12**, 19–30.
Pan, B. T., Teng, K., Wu, C., Adam, M. and Johnstone, R. M. (1985). Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *The Journal of Cell Biology* **101**, 942–948.
Park, S. J., Kim, J. M., Kim, J., Hur, J., Park, S., Kim, K., Shin, H.-J. and Chwae, Y.-J. (2018). Molecular mechanisms of biogenesis of apoptotic exosome-like vesicles and their roles as damage-associated molecular patterns. *Proceedings of the National Academy of Sciences* **115**, E11721–E11730.
Plutoni, C., Bazellieres, E., le Borgne-Rochet, M., Comunale, F., Brugues, A., Séveno, M., Planchon, D., Thuault, S., Morin, N., Bodin, S., et al. (2016). P-cadherin promotes collective cell migration via a Cdc42-mediated increase in mechanical forces. *Journal of Cell Biology* **212**, 199–217.
Rink, J., Ghigo, E., Kalaidzidis, Y. and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell* **122**, 735–749.
Rodríguez, A., Webster, P., Ortego, J. and Andrews, N. W. (1997). Lysosomes Behave as Ca2+-regulated Exocytic Vesicles in Fibroblasts and Epithelial Cells. *Journal of Cell Biology* **137**, 93–104.
Son, K., Hwang, J., Shin, Y., Kim, H., Lee, C., Ko, J., Sun, D., Kwon, B. S. and Kim, J. (2004). PLP2/A4 interacts with CCR1 and stimulates migration of CCR1-expressing HOS cells.

324, 768–772.
Sung, B. H. and Weaver, A. M. (2018). Directed migration: Cells navigate by extracellular vesicles. *Journal of Cell Biology* **217**, 2613–2614.
Sung, B. H., von Lersner, A., Guerrero, J., Krystofiak, E. S., Inman, D., Pelletier, R., Zijlstra, A., Ponik, S. M. and Weaver, A. M. (2020). A live cell reporter of exosome secretion and uptake reveals pathfinding behavior of migrating cells. *Nature Communications* **11**, 2092.
Timms, R. T., Duncan, L. M., Tchasovnikarova, I. A., Antrobus, R., Smith, D. L., Dougan, G., Weekes, M. P. and Lehner, P. J. (2013). Haploid Genetic Screens Identify an Essential Role for PLP2 in the Downregulation of Novel Plasma Membrane Targets by Viral E3 Ubiquitin Ligases. *PLoS Pathogens* **9**, e1003772.
van Niel, G., Charrin, S., Simoes, S., Romao, M., Rochin, L., Saftig, P., Marks, M. S., Rubinstein, E. and Raposo, G. (2011). The Tetraspanin CD63 Regulates ESCRT-Independent and -Dependent Endosomal Sorting during Melanogenesis. *Developmental Cell* **21**, 708–721.
Verweij, F. J., van Eijndhoven, M. A. J., Hopmans, E. S., Vendrig, T., Wurdinger, T., Cahir-McFarland, E., Kieff, E., Geerts, D., van der Kant, R., Neefjes, J., et al. (2011). LMP1 association with CD63 in endosomes and secretion via exosomes limits constitutive NF-κB activation. *The EMBO Journal* **30**, 2115–2129.
Wang, B., Nguyen, M., Breckenridge, D. G., Stojanovic, M., Clemons, P. A., Kuppig, S. and Shore, G. C. (2003). Uncleaved BAP31 in Association with A4 Protein at the Endoplasmic Reticulum Is an Inhibitor of Fas-initiated Release of Cytochrome c from Mitochondria *. **278**, 14461–14468.

Third decision letter

MS ID#: JOCES/2020/253468

MS TITLE: Proteolipid protein 2 drives collective cell migration via ZO-1 mediated cytoskeletal remodeling at the leading-edge

AUTHORS: Dipanjana Ghosh, Ankita Dutta, Anjali Kashyap, Neeraj Upmanyu, and Sunando Datta
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but one raised a critical point that will require amendments to your manuscript. The comment related to your mention of the study being limited by the fact that only one cell line is used. I agree with reviewer #3 that as this comment stands, it doesn't really add much value or insight to the paper. I recommend that you revised this section to add more substantive discussion along the line suggested by the referee.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In my opinion, the study is exciting, but has limitations. Most important being the single cell line used as highlighted by the editor and reviewers. But I feel the cell biology is well conducted and provides advances that are exciting. I remain supportive of publication but given that the 2 other reviewers have made valid and insightful comments, will leave them assess the rebuttal in details.

In my opinion, the comments by the editor/reviewers are generally well addressed - there are obvious limitations as to what the authors could do experimentally during COVID etc. More data would have been better, but I am trying to be understanding.

Comments for the author

See above

Reviewer 2

Advance summary and potential significance to field

Ghosh and colleagues characterize the relationship between PLP2, ZO-1, and Rac during collective cell migration and find that knock-out of PLP2 decreases the rate of cell migration, inhibits the lamellipodial recruitment of ZO-1, and leads to a decrease in the amount of Rac. These phenotypes were rescued by full-length PLP2 but not a PLP2 construct that is missing the C-terminus. Collectively, these results suggest that PLP2 stimulates cell migration through recruitment of ZO-1 to the leading edge of and activation of Rac.

Comments for the author

My minor comments were adequately addressed in this latest version of the manuscript.

Reviewer 3

Advance summary and potential significance to field

This is a re-revise manuscript that was previously reviewed.

Comments for the author

The authors provided in depth answers to the remaining issues raised. I would have liked to see at least some of their key findings reproduced in a different cell line. While they now included a comment regarding this in the discussion, that aspect of the work remains problematic. Perhaps a more insightful comment that discusses the implications of this work on cell lines with different levels of PLP2 expression would be more enlightening.

Third revision

Author response to reviewers' comments

We are thankful to the editor and the reviewers for their critical comments and insightful suggestions throughout the review process. As suggested by the reviewer and the editor, we have now amended the 'discussion' section of the manuscript, highlighting the relevance of the current study on multiple cell lines and with different levels of PLP2 expression.
Below are the point-to-point responses to the reviewers' comments. The page numbers referred in the response letter indicates the ones from the latest revised version of the manuscript.

Editorial comments:
As you will see, the reviewers gave favourable reports but one raised a critical point that will require amendments to your manuscript. The comment related to your mention of the study being limited by the fact that only one cell line is used. I agree with reviewer #3 that as this comment stands, it doesn't really add much value or insight to the paper. I recommend that you revised this section to add more substantive discussion along the line suggested by the referee.

Response: Thank you for the suggestion. We have now revised the 'discussion' as per the suggestion by reviewer 3 (Revised manuscript page no: 15, line no: 3-18).

Reviewer 1 Advance Summary and Potential Significance to Field:
In my opinion, the study is exciting, but has limitations. Most important being the single cell line used as highlighted by the editor and reviewers. But I feel the cell biology is well conducted and provides advances that are exciting. I remain supportive of publication but given that the 2 other reviewers have made valid and insightful comments, will leave them assess the rebuttal in details. In my opinion, the comments by the editor/reviewers are generally well addressed - there are obvious limitations as to what the authors could do experimentally during COVID etc. More data would have been better, but I am trying to be understanding.

Reviewer 1 Comments for the Author:

See above

Response: We are thankful to the reviewer for being supportive throughout the revision process and help to improve the manuscript through his/her critical comments.

Reviewer 2 Advance Summary and Potential Significance to Field:
Ghosh and colleagues characterize the relationship between PLP2, ZO-1, and Rac during collective cell migration and find that knock-out of PLP2 decreases the rate of cell migration, inhibits the lamellipodial recruitment of ZO-1, and leads to a decrease in the amount of Rac. These phenotypes were rescued by full-length PLP2 but not a PLP2 construct that is missing the C-terminus. Collectively, these results suggest that PLP2 stimulates cell migration through recruitment of ZO-1 to the leading edge of and activation of Rac.

Reviewer 2 Comments for the Author:

My minor comments were adequately addressed in this latest version of the manuscript.

Response: We are happy to note that the reviewer has found our responses satisfactory and we would like to thank him/her for the critical comments throughout the revision process.

Reviewer 3 Advance Summary and Potential Significance to Field: This is a re-revise manuscript that was previously reviewed.

Reviewer 3 Comments for the Author:
The authors provided in depth answers to the remaining issues raised. I would have liked to see at least some of their key findings reproduced in a different cell line. While they now included a comment regarding this in the discussion, that aspect of the work remains problematic. Perhaps a more insightful comment that discusses the implications of this work on cell lines with different levels of PLP2 expression would be more enlightening.

Response: We are thankful to the reviewer for being critical. As suggested by the reviewer, we have now substantiated the discussion, highlighting the relevance of the current study in multiple

cell lines. We have further discussed the scope of alternative approaches to be used for cell lines with different levels of PLP2 expression to establish the protein as a regulator of CCM in cancer epithelia. The following discussion has been added in the revised version of the manuscript (Revised manuscript page no: 15, line no: 3-18):

'However, before PLP2 could be established as a common CCM regulator, one needs to extend the study beyond a specific CRC cell line. Here, we utilized the loss-of-function approach in a cell line adequately expressing PLP2. A gain-of-function approach in cell line(s) like Caco-2 (Fig. S1A-B) or HT-29 (Oliva et al., 1993) which scarcely express the protein, will complement the current study. An earlier study demonstrating P-cadherin induced intercellular mechanotransduction during CCM, used similar approach where C2C12 myoblasts with no endogenous expression of P-cadherin was utilized (Plutoni et al., 2016). Another degree of complexity that inevitably arise while studying CCM in multiple cell lines, is the variable CCM types, exhibited by different types of cancer epithelial cells. Complete epithelial CRC cells like Caco2, HT29, HCT116 are likely to exhibit CCM of "moving sheet" (Karagiannis et al., 2014; Ozawa et al., 2020; Stadler et al., 2018), whereas lymph node metastatic CRC cells SW620 (Leibovitz et al., 1976) and LoVo (Aznavoorian et al., 1990) with partial epithelial features may exhibit CCM of "moving cell network" (Bozzuto et al., 2015; Stadler et al., 2018). It will be therefore interesting to investigate whether PLP2's function is limited to the CCM sub-type observed here or it plays diverse role in multiple CCM types.'

References:

Aznavoorian, S., Liotta, L. A. and Kupchik, H. Z. (1990). Characteristics of Invasive and Noninvasive Human Colorectal Adenocarcinoma Cells. *JNCI: Journal of the National Cancer Institute* **82**, 1485–1492.

Bozzuto, G., Condello, M. and Molinari, A. (2015). Migratory behaviour of tumour cells: a scanning electron microscopy study. *Annali dell'Istituto superiore di sanita* **51**, 139–147.

Karagiannis, G. S., Schaeffer, D. F., Cho, C.-K. J., Musrap, N., Saraon, P., Batruch, I., Grin, A., Mitrovic, B., Kirsch, R., Riddell, R. H., et al. (2014). Collective migration of cancer-associated fibroblasts is enhanced by overexpression of tight junction-associated proteins claudin-11 and occludin. *Molecular Oncology* **8**, 178–195.

Leibovitz, A., Stinson, J. C., McCombs, W. B. 3rd, McCoy, C. E., Mazur, K. C. and Mabry, N. D. (1976). Classification of human colorectal adenocarcinoma cell lines. *Cancer research* **36**, 4562– 4569.

Oliva, M. M., Wu, T. C. and Yang, V. W. (1993). Isolation and characterization of a differentiation- dependent gene in the human colonic cell line HT29-18. *Archives of biochemistry and biophysics* **302**, 183–192.

Ozawa, M., Hiver, S., Yamamoto, T., Shibata, T., Upadhyayula, S., Mimori-Kiyosue, Y. and Takeichi, M. (2020). Adherens junction regulates cryptic lamellipodia formation for epithelial cell migration. *Journal of Cell Biology* **219**,.

Plutoni, C., Bazellieres, E., le Borgne-Rochet, M., Comunale, F., Brugues, A., Séveno, M., Planchon, D., Thuault, S., Morin, N., Bodin, S., et al. (2016). P-cadherin promotes collective cell migration via a Cdc42-mediated increase in mechanical forces. *Journal of Cell Biology* **212**, 199–217.

Stadler, M., Scherzer, M., Walter, S., Holzner, S., Pudelko, K., Riedl, A., Unger, C., Kramer, N., Weil, B., Neesen, J., et al. (2018). Exclusion from spheroid formation identifies loss of essential cell-cell adhesion molecules in colon cancer cells. *Scientific Reports* **8**, 1151.

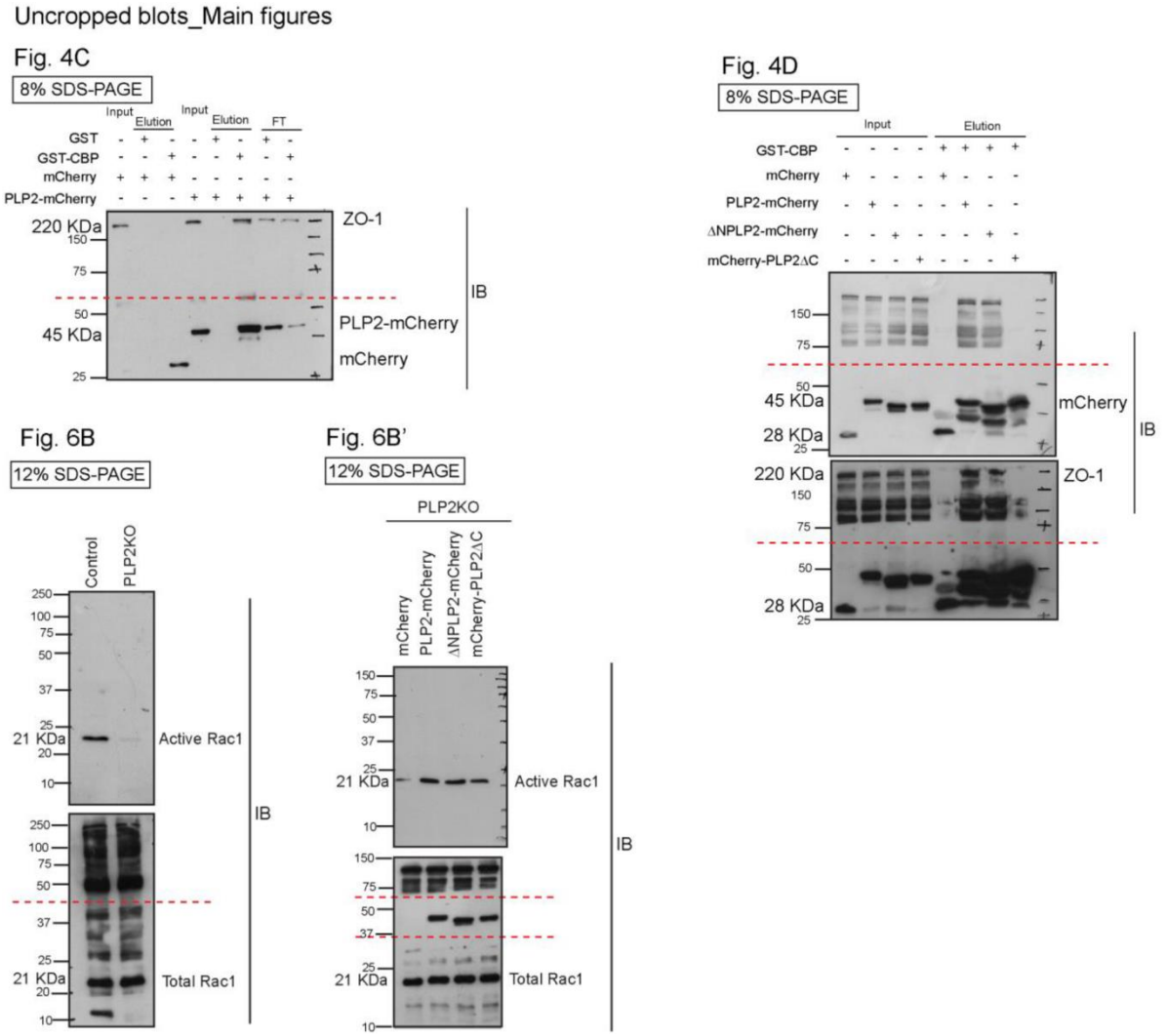


Figure A1. Uncropped images of blots against respective main figures. All panels: red dotted line indicates region of excision for probing with different antibodies, FT: Flow through. Respective figure numbers are mentioned on top of each panel.

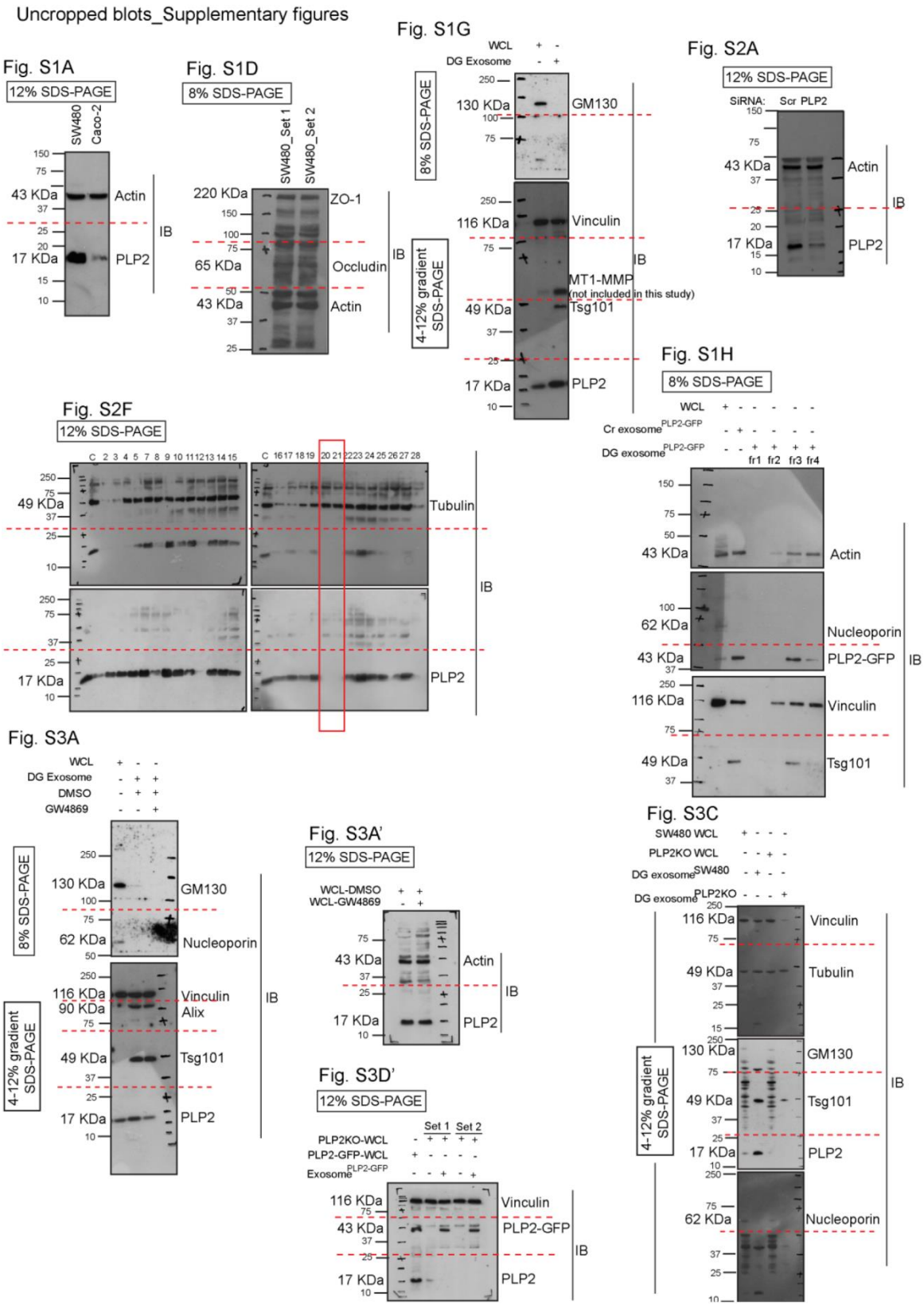


Figure A2. Uncropped images of blots against supplementary figures. All panels: red dotted line indicates region of excision for probing with different antibodies. Respective figure numbers are mentioned on top of each panel.

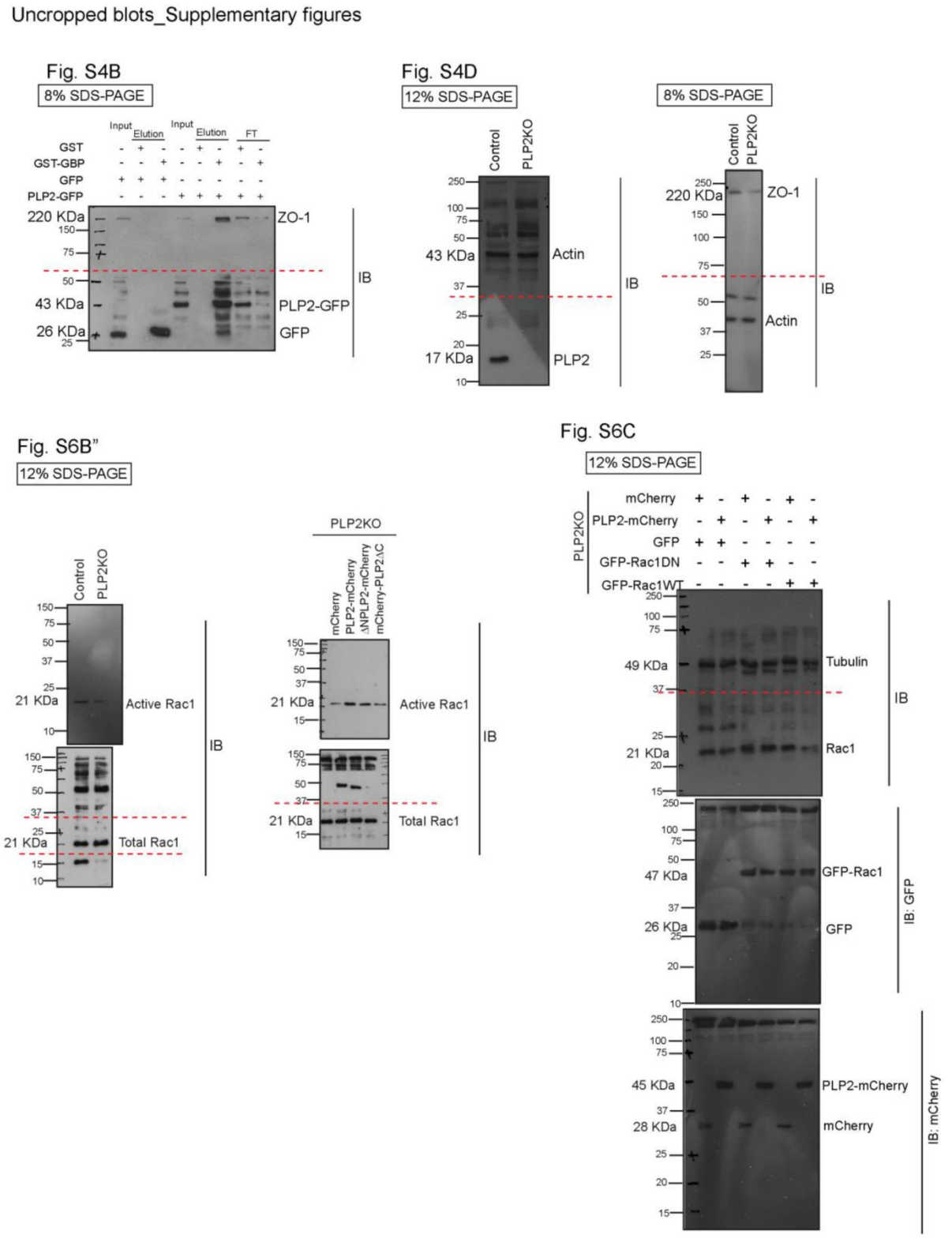


Figure A3. Uncropped images of blots against respective supplementary figures. All panels: red dotted line indicates region of excision for probing with different antibodies, FT: Flow through. Respective figure numbers are mentioned on top of each panel.

Fourth decision letter

MS ID#: JOCES/2020/253468

MS TITLE: Proteolipid protein 2 drives collective cell migration via ZO-1 mediated cytoskeletal remodeling at the leading-edge

AUTHORS: Dipanjana Ghosh, Ankita Dutta, Anjali Kashyap, Neeraj Upmanyu, and Sunando Datta
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.