

A novel strategy for the comprehensive analysis of the biomolecular composition of isolated plasma membranes

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

1st Editorial Decision

08 March 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the four referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. However, they raise substantial concerns on your work, which should be convincingly addressed in a major revision of the present work.

The major concerns raised by the reviewers refer to the following issues:

- Stronger and more rigorous evidence are required to demonstrate the purity of the PM preparations. The suggestions provided by reviewer #3 are particularly important in this regard.
- A much more detailed Materials & Methods section should be provided to describe rigorously your methodology.

We would also kindly ask you to provide the proteomic, lipidomic and glycomic datasets either in appropriate public database or, if such databases are not available, in supplementary information in a format that allows other to reanalyze the data and integrate it with other data types.

*** PLEASE NOTE *** As part of the EMBO Publications transparent editorial process initiative (see our Editorial at <http://www.nature.com/msb/journal/v6/n1/full/msb201072.html>), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the

scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office msb@embo.org.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favourable.

Yours sincerely,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

Summary: This is essentially the description of the development of a superparamagnetic bead system for purifying plasma membranes to be used for omics analyses. The method itself is well-described and well-developed. It is also an enormous improvement on all previous methods that I have ready about for this kind of analysis. I have a few concerns that I feel should be addressed, however:

1. The reported levels of contaminating internal membranes is very impressive, to the point where it is actually a little difficult to fathom! I would have expected (& this opinion is reflected in Fig. 1c) that uncoated parts of beads would stick to other membranes during lysis. At the very least the authors should discuss why this might not happen. I was going to ask that quantitation of additional ER markers in particular would help to convince me but based on the acronyms in X-axis of Fig. 1d I think the authors have already done that (e.g., HSP60, BiP, others). Those do not appear to be referenced anywhere in the manuscript or the legend so I would suggest stressing the point that the purities are backed up by multiple markers.
2. It is not at all clear from Fig. 4d that PSENdKO MEFs are more rounded-up than WT. Certainly there is fewer protrusions but the PSENdKO MEF shown doesn't look considerably different than a lot of WT MEFs I've seen. The point made with 4d is interesting but if there is no more convincing evidence of the rounded-up-ness then I suggest dropping that figure.
3. The glycoproteomics section is the weak point of the manuscript because it is not clear what value it adds, other than to show that it can be done. It does not appear to significantly contribute to the understanding of PSEN biology so it is imperative that this aspect be strengthened, possibly with follow-up experiments somehow linking the changes with PSEN.

Reviewer #2 (Remarks to the Author):

This manuscript reports what seems to be an improved method for obtaining highly purified plasma membranes. The new technology is applied to wild type and mutant mouse embryonic fibroblasts to compare the proteome, N-glycoproteome and lipidome. The descriptive nature of the paper makes me doubt whether this work falls within the scope of MSB.

The authors prepared SPMNPs to magnetically isolate plasmamembranes, as such an interesting concept and approach. However, it is impossible to judge the quality of the results, let alone to repeat the experiments, because the paper lacks an experimental section describing the experimental details of the preparation of the SPMNPs, the conditions of their application to cells, determination

and definitions of plasma membrane purity, etc. etc. The suppl. materials and methods section only describes the proteomics methods.

Did the authors verify that the enrichment of plasma membrane fractions was similar between the wild type and mutant cell lines used?

Moreover results are presented inadequately: e.g. error bars have not been defined and text is missing along the axes of graphs, SDs contain more decimals than the measured value. Other examples: Suppl. Fig. 1e (p.5 line 8) does not show that the particles are monodisperse and stable for weeks; Suppl. M&M states: "PMs were prepared as above", but where???, etc.

Reviewer #3 (Remarks to the Author):

Thimiri Govinda Raj et al. propose a strategy for the comprehensive analysis of plasma membrane (PM) constituents. The approach is based on isolating membrane fragments using a new type of magnetic nanoparticles and parallel proteomic, glycomic and lipidomic analysis.

Overall, the work reported in this manuscript is very interesting and promising. The authors validate the strategy by monitoring the abundance of putative intracellular vs. PM protein markers by western blot analysis and proteomic data. Their data indicate that the purity of the isolated PM preparations is in the range of 66-95%. To benchmark the strategy the authors present a comparative analysis of WT and PSENdKO mouse embryonic fibroblasts that reveal differences in the PM protein and lipid composition.

There are, however, several technical aspects of the work that are not well described (points identified below). Until these issues are resolved, the paper cannot be recommended for publication.

First, the manuscript does not present a material and methods section that would enable the reviewer (and the reader?) to evaluate and appreciate the technical aspects of the procedure. The authors provide a supplementary materials and methods which only outlines a subset of the applied methods.

The validation of the novel PM isolation strategy would benefit from a more systematic and quantitative evaluation of the PM isolation efficiency and, importantly, a comparison to established methods (e.g. used by Kalvodova et al. *J Virol.* 2009 or Zech et al. *EMBO J.* 2009). For example, is the novel method superior compared to other PM isolation procedures in terms of purity, yield and speed?

The authors state that the purity of the isolated PM is more than 95%. This estimate seems to be based primarily on the levels of soluble marker proteins. It would be more accurate to estimate the level contaminating organelles based on protein markers having transmembrane domains. The investigators present quantitative proteomic data which should allow a more accurate estimate of the PM purity. Without filtering for transmembrane marker proteins the purity appears to be 66% (fig 2a).

In addition, what controls have been implemented that demonstrate that the PM purity is independent of cell morphology. In figure 4 the authors demonstrate that the morphology of PSENdKO cells is different compared to wild-type. Does this difference affect the PM purity? Finally, it would be informative if the authors would explain the need for coating the SPMNPs with a PE-PEG-NH₂ instead of just using the nanoparticles.

The manuscript seeks to emphasize the efficacy of the novel procedure by profiling the PM lipidome. The authors compare the lipid composition of isolated PM to the postnuclear supernatant (PNS). This reviewer finds it more appropriate to make the comparison to total cell extracts (the total of all membranes and similar to other reports). Moreover, it is very surprising that the authors detect very low levels of cholesterol in both the PNS and PM isolates (supplementary table II). For example, the molar ratio between cholesterol and total PC is 0.0007 (= 4.4 pmol cholesterol / 6166 pmol PC) in wild-type PM. According to previous reports the PM cholesterol to PC ratio should be higher than 1 (Kalvodova et al. *J Virol.* 2009, Zech et al. *EMBO J.* 2009, Bruggger et al. *PNAS* 2006). The authors should resolve this discrepancy.

Finally, the authors should provide all lipidomic data in a supplementary excel sheet.

Other comments:

The authors state:

"... that allow one-step, rapid isolation of plasma membranes ready for..."

According to the text and figure 1c the isolation strategy requires at least 4 steps.

Moreover, the authors do not mention the time-scale of the rapid procedure.

In relation to figure 3;

the authors state:

"...and 41 down regulated proteins (filled circles)..."

Figure 3 shows an expression value higher than 1 for the proteins labeled with (filled circles). It would be more appropriate to display the ratio of PSENdKO to Wt to convey the point about down regulation.

the authors state:

"...using electron spray ionization (ESI)-MS."

It is called electrospray ionization. Please correct.

Please mention that cholesterol was measured by a fluorometric assay.

The authors state:

"This PNS is run over an LS column placed..."

What is a LS column? Please specify.

The authors state:

"After extensive washing, PMs are eluted by withdrawal of the magnet (4)"

It seems that one would obtain SPMNPs with attached plasma membrane fragments? It is unclear whether the plasma membrane fragments are separated from the SPMNPs prior to analysis of proteins, lipids and glycoproteins. Please specify.

The authors state:

"In WT MEFS close to 70% of the identified proteins..." and "..... the estimate of 70% is likely..."

Fig. 2a specifies 66%. Using 70% is inconsistent.

The authors state:

"... the cell surface or in functional complexes with the cell surface proteins"

Please add reference.

Supplementary figure 3a,b; please label the y-axis and the x-axis.

Supplementary table 2;

The authors state "SM species 16:1"

This reviewer believes that the authors intended to specify "SM 16:0".

Please correct?

Supplementary figure 5.

Please show all detected PS species (i.e. including PS 34:1, PS 36:1, etc.)

The abbreviation "DSPE" is redundant.

Did the authors consider using di-etherPE (e.g. 1,2-di-O-phytanyl-sn-glycero-3-phosphoethanolamine) or etherPC for coating the SPMNPs?

It could maybe be an alternative to the PE-PEG-NH₂ coating?

Reviewer #4 (Remarks to the Author):

In this manuscript, Annaert and colleagues described their work in the development and application of SPMNPs in analysis of proteomics, glycomics and lipidomics of the plasma membrane (PM).

First, they elegantly showed that the NH₂-terminated lipid-coated SPMNPs can be used as a magnetic chromatography to purify PM. Secondly, they illustrated that the SPMNP isolated PM is

suitable for profiling analysis of cell surface proteins, glycosylation, glycans and lipids. Finally, they showed that deficiency of presenilin could affect the composition of proteins, glycoproteins and lipids in the PM. The technology developed in this study will have a broad application in comparative analysis of proteomes, glycomes and lipidomes of PM. Obtaining of highly purified PM by a quick and easy method is a significant advance towards understanding the effect of biological and environmental factors on protein-protein and protein-lipids interaction network in PM.

Overall, this is an elegant series of experiments that add importantly to the field. There are a few issues that it would be helpful if the authors could address

1. In the analysis of qualitative and quantitative PM proteomics, authors applied post-metabolically labeling technique with ^{12}C or ^{13}C -propionyl-N-hydroxysuccinimide, Can these differences be confirmed by SILAC (stable isotope labeling with amino acids in cell culture) analysis. This would be straightforward experiments that allow to determine quantitatively differences between the presenilin expressing and efficient cells, and also to reduce the complexity of analysis.
2. Fig. 1 and Fig. 4 are difficult to read and should be fixed.

1st Revision - authors' response

06 June 2011

We are pleased to provide you with our revised manuscript MSB-11-2708 entitled "A novel strategy for the comprehensive analysis of the biomolecular composition of isolated plasmamembranes" by D.B.Thimiri Govina Raj et al.

Overall, we appreciate that all reviewers acknowledge that we established a superior method in isolating PMs ("enormous improvement" (ref 1); "broad application, significant advance" (ref 4)) and that it therefore holds a great promise for future subcellular systems biology on this particular membrane compartment. This strongly encouraged us to improve the manuscript over the past three months and to accommodate it to the criticisms and concerns raised by the four reviewers. As highlighted in your decision letter we have put most emphasis on the two major concerns raised by the reviewers and referring to more rigorous evidence to support the high quality purifications of our PM isolates and a more detailed Materials & Methods section.

With respect to the former, we have extended our qualitative analysis of the PM purity to all cell lines used in this study and not only for wild-type cells (as was the case in the initial submission). We included transmission EM data on these three cell lines as well as on the respective PM isolates to demonstrate that our SPMNPs can be broadly applied with highly similar or identical efficacies and yields. We also extended the quantitative westernblot analysis, not only to the different cell lines but also by including some more marker proteins. In addition, and with respect to the quantitative proteomics we extended the validation by westernblot analysis (new Fig. 4) to a similar quantitative approach: for up to 8 proteins (including PSEN1 and for which antibodies are available) out of 41 downregulated proteins we confirm their significant downregulation. In addition, other evidence underscoring the high purity of our PM isolates from different cell lines is also deductable from the lipid (selective increase of SMs and cholesterol, decrease for PIs as hallmarks for PM composition) and N-glycan analysis (a depletion of immature and a strong enrichment for mature N-glycan chains in PM versus total cells). Also these aspects scrutinize the quality of our PM isolates.

With respect to the lipidomics, and on request of reviewer#3, we have re-done the whole analysis this time comparing total cell extracts (instead of a postnuclear supernatant) to our PM isolates: this resulted in a satisfying new set of original data that allow comparison with published studies. All other smaller but relevant concerns of reviewer #3 with respect to the lipid analysis could be addressed as well. In addition we extensively commented and argued on the comparison of our method with established methods: in a nutshell, it remains difficult to provide a full comparison as no other studies are available that integrated different 'omics' analysis using one single isolation approach for PMs. However, we provide key information on the strengths and easiness of our method by comparing it with the current standard of cell surface biotinylation.

With respect to the second major concern, we have now included a very extensive and detailed

Material& Methods section that should allow colleague scientists to repeat our strategy. We have also included additional files and links to databases and all original datasets are available either as suppl information or via a link to PRIDE (www.ebi.ac.uk/pride/ using login "review43406" and password "n3mSbXfK"). This info as well as the specific project numbers in PRIDE are outlined in a specific section of the Materials & Methods and will guide the reader to these specific datasets.

Finally, in the subsequent pages we provide an extensive point-by-point answer to all major and minor criticisms and questions for further explanation.

Point-by-point answer to the reviewers' comments

Reviewer #1 (Remarks to the Author):

Summary: This is essentially the description of the development of a superparamagnetic bead system for purifying plasma membranes to be used for omics analyses. The method itself is well-described and well-developed. It is also an enormous improvement on all previous methods that I have ready about for this kind of analysis. I have a few concerns that I feel should be addressed, however:

1. The reported levels of contaminating internal membranes are very impressive, to the point where it is actually a little difficult to fathom! I would have expected (& this opinion is reflected in Fig. 1c) that uncoated parts of beads would stick to other membranes during lysis. At the very least the authors should discuss why this might not happen. I was going to ask that quantitation of additional ER markers in particular would help to convince me but based on the acronyms in X-axis of Fig. 1d I think the authors have already done that (e.g., HSP60, BiP, others). Those do not appear to be referenced anywhere in the manuscript or the legend so I would suggest stressing the point that the purities are backed up by multiple markers.

In the first part of the remark, Reviewer#1 suggests that membrane contaminations could be indirectly generated by sticking to uncoated parts of beads after cell lysis. We did not observe this in any of the ultrastructural analysis that we performed on isolated plasma membranes. To strengthen this furthermore (and in response to a comment of Reviewer#2, see below) we have extended the transmission EM analysis to all three cell lines used in this study: in none of the sections analyzed we observed SPMNPs sticking nonspecifically to other membranes. The most likely explanation for us is that the isolation procedure is fast, particularly in the steps between applying nanoparticles to cells and loading the postnuclear supernatant on the column for magnetic isolation. After cell cracking, the total homogenate is immediately centrifuged to remove cell debris and nuclei, major resources for potential contaminations. In an immediate next step the postnuclear supernatant is poured over the magnetic column where nanoparticle-coated plasmamembranes are 'on line' separated from remaining non-coated contaminating membranes as they are not retained and flow through the column. These aspects are now included briefly in the Results, p. 5, lines 119 to 129. With respect to the second part, Reviewer#2 is clearly convinced of the high purity of our plasma membrane isolations and refers to the many markers proteins we have used to measure the contribution of contaminating organelles. In response to a comment of reviewer#2 and further comforting reviewer#1 we have extended this quantitative western blot analysis to all three cell lines and even included some more membrane marker proteins. The extensive use of multiple marker proteins for different compartments is more stressed in the legend to figure 1 and in the Results (p. 5, line 131 and following).

2. It is not at all clear from Fig. 4d that PSENdKO MEFs are more rounded-up than WT. Certainly there is fewer protrusions but the PSENdKO MEF shown doesn't look considerably different than a lot of WT MEFs I've seen. The point made with 4d is interesting but if there is no more convincing evidence of the rounded-up-ness then I suggest dropping that figure.

We do not fully agree with this opinion. PSENdKO MEFs have indeed a different morphology (more rounded) which is reflected in changes in the localization of proteins involved in adhesion/migration such as caveolin1, vinculin and actin (all depicted in different panels of figure 4. We have now adopted the text slightly by focusing more on these changed localizations. We have

replaced panel e (formerly panel d) in figure 4 therefore with better examples. We would like to point out that similar findings on morphology and adhesion have been reported for PSEN Δ KO (Waschb, sch et al., JBC, 284, 15, 10138-10149, 2009) which support our observations (ref is now included in the text). Moreover, both the morphological changes as well as the changes observed by quantitative proteomics are reverted by rescuing these KO cells with human presenilin 1 underscoring that both are related to presenilin deficiency.

3. The glycoproteomics section is the weak point of the manuscript because it is not clear what value it adds, other than to show that it can be done. It does not appear to significantly contribute to the understanding of PSEN biology so it is imperative that this aspect be strengthened, possibly with follow-up experiments somehow linking the changes with PSEN.

There were two major reasons why we included the glycoproteomics section. Firstly, as pointed out by reviewer#1, we indeed demonstrate that our new isolation procedure is compatible with such analysis. However, this is not the sole reason.

Glycosylation is a major posttranslational modification (a significant part of the human genome encodes enzymes and transporters involved in this process) that majorly defines the function of proteins and lipids. As such, it has significant impact on many biological processes, not in the least for those proteins/lipids being localized at the cell surface. To date there are a few methods to identify N-glycosylation sites but they are not compatible with other 'omics' analysis or give no or limited info on the carbohydrate structures. The integration of N-glycoproteomics with the possibilities to perform N-glycan profiling as well as proteomics and lipidomics on one and the same isolated plasmamembrane fraction is unprecedented and distinct our approach from all other existing strategies. Moreover, using the same isolation protocol independent from any subsequent analytical protocol has the additional advantage that the changes we observe (either with respect to glycosylation, proteins or lipids) cannot be attributed anymore to differences associated with different methods of isolation: this is likely the reason why thus far no integrated studies have been presented on the full range biomolecular composition of plasmamembranes. This is a distinctive feature of our work and for these reasons we find it important to keep these data included in the manuscript. We have highlighted the importance of integrating all omics analysis in one methodology in the Discussion section.

Reviewer #2 (Remarks to the Author):

This manuscript reports what seems to be an improved method for obtaining highly purified plasma membranes. The new technology is applied to wild type and mutant mouse embryonic fibroblasts to compare the proteome, N-glycoproteome and lipidome. The descriptive nature of the paper makes me doubt whether this work falls within the scope of MSB.

1. The authors prepared SPMNPs to magnetically isolate plasmamembranes, as such an interesting concept and approach. However, it is impossible to judge the quality of the results, let alone to repeat the experiments, because the paper lacks an experimental section describing the experimental details of the preparation of the SPMNPs, the conditions of their application to cells, determination and definitions of plasma membrane purity, etc. etc. The suppl. materials and methods section only describes the proteomics methods.

We fully agree with this major comment. A detailed description of the Methods was unintentionally overlooked when compiling the final draft for our first submission. We sincerely apologize for this but want to emphasize that this was purely a mistake. We have included the detailed materials and the methods section (Methods section; pages 19-24 and Supplemental Methods section: pages 2-3) with respect to the preparation of the SPMNPs, the isolation of plasma membranes and analytical approaches.

2. Did the authors verify that the enrichment of plasma membrane fractions was similar between the wild type and mutant cell lines used?

In our manuscript we have isolated plasma membrane from three independent cell lines. In the

original submission, we included only the quality control of the plasma membrane fraction derived from wild type cells. We have accommodated this concern of reviewer #2 by including the same quality control measurements for the remaining plasma membrane fractions of presenilin deficient and rescued cells. Using transmission EM data (Fig. 1, panel c) we now show for each cell line that SPMNPs selectively adhere to the surface of these cells. In addition TEM of isolated fractions reveal in all three cases long membranous sheets with associated SPMNPs and with an overall lack of contaminating organelles: the highly similar ultrastructural appearance and quality underscores highly similar enrichments/purities of the plasma membrane fractions in all three cell lines (Fig. 1, panel d). Similarly, we have extended the quantitative westernblot analysis for contaminating organelles (including ER, intermediate compartment, Golgi, mitochondria, peroxisomes, nuclear envelope, cytosol and cytoskeleton) as well as for two plasma membrane proteins (Na+K+ATPase and Fas) to all three cell lines. As can be concluded from Fig. 1 panel e, the (very low) level of contaminating organelles is essentially the same for all three cell lines. The same holds true for the very high recovery of both plasma membrane marker proteins (from 40% (Fas) up to about 50-60% (Na+K+ATPase). Hence, by using morphological and biochemical approaches we clearly underscore the similar high enrichments and yields of plasma membranes isolated from all three cell lines.

These new data have been added and changes in the text were made accordingly (page 6 lines 132 and following).

Finally, we would like to note that the similar or identical high purities can also be deduced from the quantitative proteomics and lipidomics analysis. With respect to the former we identified a limited number of proteins that are all downregulated in the plasma membrane of presenilin deficient cells while not different in the comparative analysis of wild-type versus presenilin rescued cells. It seems very unlikely that such subtle but defined changes (since they can be rescued) could be identified if plasma membrane fractions of all three cell lines were not of equal high purity. The same accounts for the lipid analysis: we identified selective decreases in the levels of PI and increases in the levels of SM and cholesterol in the plasma membrane fractions of both wild type and presenilin deficient cells: these changes are regarded as 'typical' for plasma membrane compositions and again can only be explained if the isolated fractions from different cell lines were of equal high purity. Particularly in FACE based glycome analysis, we observed about 5 times less high-mannose glycans in PMs compared to concentrated cell lysate. The PMs are on the other hand strongly enriched formature glycans. Thus the glycome analysis also confirms the high purity of plasmamembrane fractions.

3. *Moreover results are presented inadequately: e.g. error bars have not been defined and text is missing along the axes of graphs, SDs contains more decimals than the measured value. Other examples: Suppl. Fig. 1e (p.5 line 8) does not show that the particles are monodisperse and stable for weeks;*

Reviewer#2 is correct and we apologize that we neglected consistencies in presenting statistical data. We went carefully through the whole manuscript to accommodate for the errors in axis labeling and error bars/SDs. The

With respect to the comment on the stability and monodispersity of the particles we corrected this in the text:

In addition, we added a panel to Suppl. Figure 1 (panel a') displaying a picture of two vials with synthesized iron oxide SPMNPs functionalized with oleic acid (left) and phospholipids(right)stored for several weeks: each vial has two phases, a lower water- and upper solvent-phase. The NH₂-phospholipids-SPMNPs are water soluble and do not precipitate or clog after several weeks storage underscoring their stability.

4. *Suppl. M&M states: "PMs were prepared as above", but where???*; etc.

A complete Methods section has now been added to the manuscript (see point 1 of reviewer #2).

Reviewer #3 (Remarks to the Author):

Thimiri Govinda Raj et al. propose a strategy for the comprehensive analysis of plasma membrane (PM) constituents. The approach is based on isolating membrane fragments using a new type of magnetic nanoparticles and parallel proteomic, glycomic and lipidomic analysis.

Overall, the work reported in this manuscript is very interesting and promising. The authors validate the strategy by monitoring the abundance of putative intracellular vs. PM protein markers by western blot analysis and proteomic data. Their data indicate that the purity of the isolated PM preparations is in the range of 66-95%. To benchmark the strategy the authors present a comparative analysis of WT and PSENdKO mouse embryonic fibroblasts that reveal differences in the PM protein and lipid composition.

There are, however, several technical aspects of the work that are not well described (points identified below). Until these issues are resolved, the paper cannot be recommended for publication.

1. *First, the manuscript does not present a material and methods section that would enable the reviewer (and the reader?) to evaluate and appreciate the technical aspects of the procedure. The authors provide a supplementary materials and methods which only outlines a subset of the applied methods.*

In line with major comments of Reviewer#2, we again apologize here for the major gap in providing this detailed information. We refer to our answer to Reviewer #2 where we stated that we have now included a detailed Methods section.

2. *The validation of the novel PM isolation strategy would benefit from a more systematic and quantitative evaluation of the PM isolation efficiency and, importantly, a comparison to established methods (e.g. used by Kalvodova et al. J Virol. 2009 or Zech et al. EMBO J. 2009). For example, is the novel method superior compared to other PM isolation procedures in terms of purity, yield and speed?*

We would rather elaborate here on the one hand that we present a novel method that could ‘co-exist’ to some extent with existing other PM isolation procedures like cell surface biotinylation and antibody based magnetic isolation, to where Reviewer#3 refers to. On the other hand, our strategy holds major advantages with respect to the broad compatibility for (thusfar) any subsequent analysis (glycan profiling, proteins, lipids, active complexes (e.g. -secretase in this study)). No other method has demonstrated this and therefore a comparison over the full range of analyses with other methods is not possible. Most methods have individual applications and are adapted depending on the subsequent type of analysis.

Thusfar, biotinylation and antibody-based isolation are the only methods used for both proteomics as well as lipidomics. However, they have never been combined in one study. Indeed the group of Kai Simons has made significant progress in lipidomics analysis of isolated fractions and has optimized this rigorously for cell surface biotinylation. Particularly in the Kalvodova et al. J Virol. 2009-paper the authors elaborate and emphasize on the very extensive optimization steps to get the biotinylation to work for subsequent reliable lipidomics. We can only refer to their extensive comments and cautions in their methods/results sections which suggests that this approach is not easily transferrable to any other cell line. Moreover, the authors also state that this protocol is optimized for lipid analysis but not for proteome analysis, given the irreversible binding of proteins to biotin as they stated: "

PM fraction is irreversibly bound to the magnetic beads via the biotin-neutravidin interaction and hence is useful only for analysis that makes use of solubilization, harsh elution, or extraction of the bound material (solubilization by SDS-PAGE loading buffer or extraction with organic solvents). However, for our purpose, to analyze the lipidome after organic solvent extraction, the method was optimal."

In the paper of Zech et al. EMBO J. 2009, the molecular lipid composition of TCR activation domains in the T-cell plasma membrane, not the whole plasmamembrane. Here, the method involves immunoisolation of plasma membrane fragments using CD3 and TfR antibody making it a limiting procedure with respect to the availability of antibodies. Moreover, such methods strongly depend on the abundances of the respective proteins at the cell surface and cell-type dependent variations in the amount of surface associated antigens may affect the yield and purity of isolated membranes. This hampers a broad applicability of antibody-based immunoisolation procedures, i.e. the same antibody for different cell lines.

Hence, with respect to purity, we agree that our method is comparable with other existing methods, if one considers only one analytical application. With respect to yield and speed we can answer this more directly. We have compared our method with a cell surface biotinylation approach combined with magnetic isolation using the same Dynabeads as in Kalvodova et al. A cell surface

biotinylation approach takes about 3hrs between applying biotin on the cells and having a washed bound dynabead fraction. Our approach is slightly faster by half an hour and explained by the fact that cells only need to be incubated once for 20min to bind SPMNPs and that immediately thereafter, cells are cracked, spun (10min) and loaded on a magnetic column. Importantly, the difference in speed becomes significantly increased to the benefit of SPMNPs when experiments are upscaled: isolating PMs from 5, 10 or 15 dishes (10cm \varnothing) hardly affects the procedure with respect to time, while this more strongly increases for biotinylation (because of the multiple steps in extensive washing, binding, quenching of biotin). Taking moreover into consideration that biotinylation, if used for lipidomics, requires extensive optimizations for each cell line, we challenge this approach by the easiness to use SPMNPs on different cell lines without noteworthy optimizations.

The increased efficiency of obtaining SPMNP-isolated PMs by upscaling the experiment underscores the easiness to obtain significantly higher yields per cell line as well. On average we isolate the equivalent of 70 to 120 μg of PM protein, independent of the cell line used and suitable to perform several analysis in parallel.

3. *The authors state that the purity of the isolated PM is more than 95%. This estimate seems to be based primarily on the levels of soluble marker proteins. It would be more accurate to estimate the level contaminating organelles based on protein markers having transmembrane domains. The investigators present quantitative proteomic data which should allow a more accurate estimate of the PM purity. Without filtering for transmembrane marker proteins the purity appears to be 66% (fig 2a).*

The estimate of 95% is indeed primarily based on the quantitative westernblot analysis for marker protein of PMs and contaminating organelles (Fig. 1d). However, in contrast to the remark of Reviewer#3, this was not solely based on soluble markers but included already membrane proteins like p58 and Rer1p (4 TMDs). We have extended the analysis with now two PM integral membrane proteins (Fas next to Na⁺K⁺-ATPase) and a peroxisomal membrane associated protein, Pex14p. Moreover, we have done the same extensive analysis in three independent cell lines with highly similar results underscoring that the enrichment and purity of PMs is the same independent of the cell line used (updated in a new panel d, figure 1). The estimate of 95% should not be compared or confused with the estimate of 66% from the proteomics analysis. This latter percentage is in the same range of other proteomics data obtained for isolated PMs. The reason why this percentage is lower compared to the 95% estimate is because this percentage is primarily generated from the existing gene ontology databases. It is therefore biased towards how proteins picked up in our analysis are annotated in these databases. It is obvious that such databases are far from complete or accurate and ignore the fact that a high number of proteins just have multiple locations in the cell or can change location depending on the status of the cell. Many proteins involved in actin rearrangements (or actin itself), or endosomal trafficking (like many Rabs) are also found partially at the cell surface: this information is not picked up from the current databases and if systematically corrected for this one would end up with far higher percentages. For instance components of the γ -secretase complex like nicastrin and presenilin are annotated as 'ER-localized' while nobody doubts that a portion is surface-localized, (we give more examples in the Results on page 7, line 172-179), etc. Furthermore, we have extended the quality control by including TEM of all the PM isolates used in this study and recovered in all cases large membranous sheets reminiscent of PMs without detectable or recognizable major contaminants. Taken all additional quality control data together we hope we have succeeded in comforting the reviewers about these concerns.

4. *In addition, what controls have been implemented that demonstrate that the PM purity is independent of cell morphology? In figure 4 the authors demonstrate that the morphology of PSENdKO cells is different compared to wild-type. Does this difference affect the PM purity?*

As mentioned in our comment to reviewer #2 (point 2) and above (point 3, reviewer#3) we have extended the quality control to all three cell lines with respect to western blot analysis, more marker proteins and TEM of cells and PM isolates: there are no apparent differences in the quality (and quantity) of the purified PM isolates underscoring that the morphology did not affect PM purity.

5. *Finally, it would be informative if the authors would explain the need for coating the SPMNPs with a PE-PEG-NH₂ instead of just using the nanoparticles.*

We established iron oxide (Fe₃O₄) SPMNPs (12-14nm) using thermal decomposition (see Materials and Methods, page 19, line 502-522) generating monocrystalline nanoparticles. They were synthesized in non-polar solvents and coated with non-polar surfactant (oleic acid) making them non-dispersible in water. In order to render them water-dispersible, we further functionalized SPMNPs using phospholipids. In this ligand addition approach, phospholipids were added on top of oleic acid coated SPMNPs. In this case the hydrophobic tail has van der Waals interaction with the oleic acid coating while the hydrophilic head groups (functionalized with NH₂-PEG makes the SPMNPs water dispersible and positively charged. This more detailed information is now added in the Methods section (page 19, line 512 to 522).

6. *The manuscript seeks to emphasize the efficacy of the novel procedure by profiling the PM lipidome. The authors compare the lipid composition of isolated PM to the postnuclear supernatant (PNS). This reviewer finds it more appropriate to make the comparison to total cell extracts (the total of all membranes and similar to other reports). Moreover, it is very surprising that the authors detect very low levels of cholesterol in both the PNS and PM isolates (supplementary table II). For example, the molar ratio between cholesterol and total PC is 0.0007 (= 4.4 pmol cholesterol / 6166 pmol PC) in wild-type PM. According to previous reports the PM cholesterol to PC ratio should be higher than 1 (Kalvodova et al. J Virol. 2009, Zech et al. EMBO J. 2009, Bruggger et al. PNAS 2006). The authors should resolve this discrepancy.*

To accommodate to reviewer#3's request, we have redone all lipidomics analysis, this time by comparing total cell extracts with PM isolates. Overall this renewed analysis did not alter the final outcome, conclusions and interpretations. Merely some more subtle differences with respect to ratio's, as expected, were noticed. All new data are now incorporated in an updated Figure 6, as Supplementary Information Figs. 4, 5, and 6 and as Supplementary Information Table I and II). With respect to the specific comment on the cholesterol levels (Supplementary Information Table II), we can easily explain this discrepancy which is caused by the different representation of the levels of cholesterol versus those of other lipid species. Cholesterol levels are measured by a fluorometric assay and the data are represented in mg of cholesterol/ mg protein. In contrast, the MS-based analysis of the major lipid families including PC, PS, PI and PE are represented in nmol of lipids/mg protein. This was not mentioned in the table and the ratio as calculated by reviewer#3 is unexpectedly low and majorly discrepant with existing reports because of this. We corrected this in the new (Supplementary Information Table II) and present the cholesterol data with the same unit of nmol of lipids/mg protein. Hence the molar ratio between cholesterol and total PC is now 1.97 (=12138nmol cholesterol/6166 nmol PC) at the cell surface within the range of the values presented in the references indicated by reviewer#3.

7. *Finally, the authors should provide all lipidomic data in a supplementary excel sheet.*

As requested, we have provided all original lipidomics data in a supplementary excel sheet (Supplementary Information Table III in the revised version).

Other comments:

8. *The authors state:*

"... that allow one-step, rapid isolation of plasma membranes ready for..."

According to the text and figure 1c the isolation strategy requires at least 4 steps.

Moreover, the authors do not mention the time-scale of the rapid procedure.

Our statement on a 'one-step' procedure originated from our reasoning that several steps (applying an 'affinity' probe and cell fractionation) are common to isolation procedures. We agree that this raises some confusion and as suggested by the reviewers, we have omitted this statement. In the result section, we have included the time-scale for the isolation procedure and have mentioned the advantage of having a rapid procedure in the text (Results, page 5 line 122-126).

9. *In relation to figure 3:*

the authors state:

"...and 41 down regulated proteins (filled circles)..."

Figure 3 shows an expression value higher than 1 for the proteins labeled with (filled circles). It would be more appropriate to display the ratio of PSENΔKO to Wt to convey the point about down

regulation.

We agree with this comment and have 'inverted' the plot of Figure 3: we now display the ratio's of PSENdKO to WT.

10. *the authors state:*
"*...using electron spray ionization (ESI)-MS.*"
It is called electrospray ionization. Please correct.

This is corrected..

Please mention that cholesterol was measured by a fluorometric assay.

This is now added in the Methods section (page 23, line 637).

11. *The authors state:*
"*This PNS is run over an LS column placed...*"
What is a LS column? Please specify.

LS Column is a filtration column commercially available from Miltenyi and that constitutes an integral part of the magnetic isolation procedure using the SuperMACSII magnet . The LS column is packed with an optimized matrix to enhance the weak magnetism coming from the SPMNPs. This ensures an increase in the retainment of SPMNP-coated PMs by the magnet while non-SPMNP-coated membranes more easily flow through. This information is added in the Methods, page 21 line 551-560.

12. *The authors state:*
"*After extensive washing, PMs are eluted by withdrawal of the magnet (4)*"
It seems that one would obtain SPMNPs with attached plasma membrane fragments? It is unclear whether the plasma membrane fragments are separated from the SPMNPs prior to analysis of proteins, lipids and glycoproteins. Please specify.

This is indeed correct as stated: by withdrawal of the magnet we elute SPMNPs together with the associated plasma membranes. Once SPMNP-isolate plasma membranes are concentrated by centrifugation, pellets are resuspended in a minimal volume for protein determination and next directly subjected to the desired protocol for either protein, lipid or glycan analysis. The SPMNPs did not interfere with any of these subsequent processing steps (enzymatic digestions, solvent extractions etc) or analysis. We have added this detailed information in the Methods (page 21, line 562-564) as well as in the Discussion (page 11, line 295).

13. *The authors state:*
"*In WT MEFS close to 70% of the identified proteins...*" and "*..... the estimate of 70% is likely...*"
Fig. 2a specifies 66%. Using 70% is inconsistent.

We have corrected this in the text (Results, page 6 line 169-189).

14. *The authors state:*
"*... the cell surface or in functional complexes with the cell surface proteins*"
Please add reference.

We have included the references. (Results, page 7, line 175-179).

15. *Supplementary figure 3a, b; please label the y-axis and the x-axis.*
We have labeled the y- and x-axes (revised Supplementary figure 3a, b at Supplementary Information Page 6).

16. *Supplementary table 2;*
The authors state "SM species 16:1"

*This reviewer believes that the authors intended to specify "SM 16:0".
Please correct?*

We have corrected the supplementary Information table II for "SM species 16:1" to "SM 16:0".

17. *Supplementary figure 5.
Please show all detected PS species (i.e. including PS 34:1, PS 36:1, etc.)*

We have included the original lipidomics data including all the lipid species as a supplementary Information table III and also revised supplementary figure 5 at Supplementary Information Page 8.

18. *The abbreviation "DSPE" is redundant.*

The abbreviation "DSPE" is also used a couple of times in the Methods section (which is now extensively included). For that reason, the abbreviation becomes relevant and we maintained it in the text.

19. *Did the authors consider using di-etherPE (e.g. 1, 2-di-O-phytanyl-sn-glycero-3-phosphoethanolamine) or etherPC for coating the SPMNPs? It could maybe be an alternative to the PE-PEG-NH2 coating?*

Since SPMNPs (12-14nm) were synthesized in non-polar solvents and coated with non-polar surfactant (oleic acid) they are non-dispersible in water. In order to render them water-dispersible, we need to further functionalize SPMNPs using phospholipids that are stabilized using a water dispersant like PEG. In addition, by using PE-PEG-NH₂ we could generate positively charged SPMNPs necessary for plasmamembrane isolation. However using di-ether-PE, we wouldn't be able to generate +ve charged SPMNPs with high stability. Hence we preferred PE-PEG-NH₂ coating ahead of other lipids like di-ether-PE.

Reviewer #4 (Remarks to the Author):

In this manuscript, Annaert and colleagues described their work in the development and application of SPMNPs in analysis of proteomics, glycomics and lipidomics of the plasma membrane (PM). First, they elegantly showed that the NH₂-terminated lipid-coated SPMNPs can be used as a magnetic chromatography to purify PM. Secondly, they illustrated that the SPMNP isolated PM is suitable for profiling analysis of cell surface proteins, glycosylation, glycans and lipids. Finally, they showed that deficiency of presenilin could affect the composition of proteins, glycoproteins and lipids in the PM. The technology developed in this study will have a broad application in comparative analysis of proteomes, glycomes and lipidomes of PM. Obtaining of highly purified PM by a quick and easy method is a significant advance towards understanding the effect of biological and environmental factors on protein-protein and protein-lipids interaction network in PM. Overall, this is an elegant series of experiments that add importantly to the field. There are a few issues that it would be helpful if the authors could address

1. In the analysis of qualitative and quantitative PM Proteomics, authors applied post-metabolically labeling technique with ¹²[C₃] or ¹³[C₃]-propiony-N-hydroxysuccinimide, Can these differences be confirmed by SILAC (stable isotope labeling with amino acids in cell culture) analysis. This would be straightforward experiments that allow to determine quantitatively differences between the presenilin expressing and efficient cells, and also to reduce the complexity of analysis.

We have preferred the postmetabolic labeling technique to obtain quantitative proteomics data enabling to compare subtle changes between the plasma membrane composition of cells that only differ the presence or absence of presenilin expression. Not only the fact that we can identify such changes at the cell surface but demonstrate that these changes are not detected when re-introducing

human presenilin 1 underscore that this approach works.

We did not ignore the more broadly used SILAC approach but couldn't apply it successfully in our experiments. The major reason for this is that yields of plasma membrane isolates greatly varied between the different cell lines in the same experiment. Our experience is that for SILAC labeling cells need to be grown for an extended period in mildly starvation conditions and that this challenges the normal growth conditions of each cell line differently to the magnitude that it compromises subsequent plasma membrane isolation. From our experience, we predict that these effects are more pronounced in specific cell lines, such as in knock-out cell lines, in which the normal vesicular transport (and hence the balance with cell surface delivery) is expected to be compromised. This is indeed the case in presenilin deficient cells that present with constitutively induced autophagy and the accumulation of degradative organelles and autophagosomes (Wilson et al., 2004; Lee et al., 2010; Neeley et al., 2011). As the normal metabolism is significantly affected it is not surprising that an additional challenge of their growth and transport balance by culturing them in mildly starvation conditions (which is inherently to the SILAC approach), would exacerbate the differences with wild-type cells. Subsequent proteome analysis of subcellular compartments such as the plasma membrane would reveal differences that are not necessarily physiological relevant. On the contrary, when using post-metabolic labeling, cell lines can be kept in the most optimal conditions and the proteome analysis of the plasma membrane in this case would reflect with more confidence the normal physiological state of the cell at the moment the plasma membranes were isolated. Our case with presenilin deficient cells does not stand alone and we anticipate that for many other diseases including neurodegenerative diseases and particularly the many lysosomal storage diseases, this strategy might be required as well.

In addition, although we appreciate that SILAC labeling is currently more widely used; reviewer#4 can agree with us that both approaches allow quantitative proteomics. Therefore it seems redundant to us to validate one MS-based quantitative approach with another MS-based quantitative approach. To our opinion, and without ignoring the power of SILAC, the better validation for observed changes (in our case we identified 41 proteins) is to test individual proteins through an independent quantitative approach. We have done this through quantitative western blotting for 7-8 proteins (hence, a coverage of almost 20% of the downregulated hits) and confirmed their downregulation at the cell surface to be statistically significant. Further validation comes from confocal imaging demonstrating that the same proteins are indeed mislocalized in PSENdKO. Hence, the data presented in Figure 4 must be regarded as an independent valorization of the quantitative proteomics. Obviously, this approach is always limited to the availability of specific antibodies.

Opposed to the experience or opinion of reviewer#4, post-metabolic labeling did not affect the complexity of the analysis and turned out to be more straightforward. The post-metabolic labeling using 12[C3] or 13[C3]-propiony-N-hydroxysuccinimide is a more recent approach but already validated by several publications of our collaborating experts at the University of Ghent (Ghesqui'ere et al, 2009 and Ghesqui'ere et al, 2011). The labeling method is however related to iTRAQ which is also post-metabolic and uses amine-specific reagents as well (e.g. Maqharious et al., 2011; Gouw et al., 2011). Although SILAC labeling has many advantages, post-metabolic labeling may gain some field/attention in selective cases, like we demonstrated and argued here. Moreover, and whereas SILAC is mostly restricted to cell culture, post-metabolic labeling has the advantage that it can be applied to in vivo animal models. It is worth mentioning in this context that the research team led by my colleague Prof. Kris Gevaert dedicates most of their time in developing improved methods for the quantification of proteomics data, and is making major contributions to quantifying not only proteins but increasingly posttranslational modifications. Their established and expanding track record may increase the confidence of reviewer #4 that we seek for the best collaborations to obtain the most forefront strategies for quantitative proteomics, taking into account the features and limitations imposed by the cell lines under investigation. We hope we have provided a solid argumentation for our choice and hope to have satisfied adequately this particular concern of reviewer#4.

2. Fig. 1 and Fig. 4 are difficult to read and should be fixed.

Both figures (and respective legends) have been revised and adopted to the comments of the different reviewers. Besides adding quantitative data in Figure 4 (now panel b) we have provided better examples on the distinct morphology of the PSENdKO MEFs (new panel c) and simplified panel d by showing only a dual labeling of CD47 and Caveolin1. We hope that these changes make

the interpretation of the figure and data more satisfying.

2nd Editorial Decision

24 July 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who accepted to evaluate the revised study. As you will see, the referees find the topic of your study of potential interest and are now more supportive. They raise however a series of remaining concerns and make several suggestions for several important clarifications, which need to be convincingly addressed in a final round of revision of the present work. We would also urge you to make sure that the complete data is available in supplementary information (see comment of reviewer #3) and appropriately annotated so that interested readers can re-use and re-analyze your datasets.

Please resubmit your revised manuscript online, with a covering letter listing amendments and responses to each point raised by the referees. Please resubmit the paper ****within one month**** and ideally as soon as possible. If we do not receive the revised manuscript within this time period, the file might be closed and any subsequent resubmission would be treated as a new manuscript. Please use the Manuscript Number (above) in all correspondence. Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed all of my concerns.

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed my previous concerns, except for one important issue: the definition of plasma membrane purity. On page 6 line 155, the preparation is estimated to be 95% pure.

How was this estimate calculated based on the enrichment factors, recoveries, and contaminations with other organelles listed on p.6? How is the percentage of purity defined?

To be able to judge the quality of the new method it is essential to compare the purity of the preparation to that of plasma membrane preparations obtained with other methods. This issue was raised by reviewer 3 (point 2) and has not been addressed. The authors should simply compare their factors of enrichment of plasma membrane markers and/or factors of depletion of contaminating cellular fractions, and yield with numbers from the literature.

Minor points:

Fig. 1e: insert (%) along the y-axis

Fig. 6a: change text along y-axis to: fold PM/total cell extract

Tables in suppl. info: decimal point instead of comma!

Reviewer #3 (Remarks to the Author):

Thimiri Govinda Raj et al. have addressed majority of my questions. After carefully reading the revised manuscript, there are still issues that need to be resolved:

The authors emphasize the enrichment of SM 18:1 in the PSENdKO plasma membrane as compared to WT plasma membrane. The provided data also indicates that SM 18:1, SM 18:0 and SM 24:1 are enriched in plasma membrane fractions as compared to total cell lysates (see suppl. xls file). Given that the authors use a low mass resolution instrument (4000 QTRAP) for the lipid analysis, it is difficult to ascertain whether the enrichment of SM species is genuine or attributed to detector saturation and isotope effects from neighboring and more abundant PC species (e.g. PC 32:2 m/z 730.54 vs SM 18:1 m/z 731.61). The authors should independently prove the enrichment of the SM species by showing precursor ion m/z 184.07 spectra of WT cells, PSENdKO cells, PM from WT, and PM from PSENdKO. This approach was elegantly applied by Brügger et al. in "The HIV lipidome: A raft with an unusual composition, figure 2, PNAS 2006" to show the enrichment of a specific SM species.

Regarding the quantitative lipidomic data in supplementary table I and II, which the authors seem to use for displaying relative differences in Fig. 6.

The molar ratio of total SM to total PC is different when using values for mol% and nmol/mg protein. According to mathematics, the SM to PC ratio should be independent of the normalization. For example, in "TOTAL Wild-type", the authors obtain 89 nmol SM/mg protein and 2247 nmol PC/mg protein which yield a molar ratio of SM/PC = 0.04. In "TOTAL Wild-type", the authors also obtain 3.98 mol% SM and 51.8 mol% PC which yield a molar ratio of SM/PC = 0.08. That is, twice as much SM in one data format as compared to the other data format? This inconsistency pertains to all samples and also the molar ratio of PI to PC.

The authors should correct this issue and potentially revise figure 6.

Moreover, there are few inconsistencies between supplementary table II and figure 6b:

- i) the ratio of "Total SM" for "TOTAL" is 1.5 in the table and approx. 2 in the figure?
- ii) the ratio of "SM 18:0" for "TOTAL" is 1.1 in the table and more than 2 in the figure?

The authors should address these inconsistencies.

All lipidomic data is not present in the supplementary excel file despite the authors stated in the rebuttal: "As requested, we have provided all original lipidomics data in a supplementary excel sheet".

Data from supplementary table I and II are missing, which makes it hard and time-consuming for independent investigators to access the data for comparative purposes.

Access to all lipidomics data is important since it can become a resource for future studies of PM dynamics.

According to the details on the lipid MS analysis in the new material and method section, the authors have monitored PC, SM, PE, PI and PS species by multiple reaction monitoring (MRM) on a 4000 QTRAP. In this mode of analysis, the lipid species are typically quantified based on the intensity of lipid class-specific fragment ions, and NOT the intensity of intact lipid precursor ions as recorded in survey scans.

The authors state that "Data were corrected for ¹³C isotope effects using the following formula: ...". The formula is, in part, only valid for correcting C-13 isotope effects (also termed deisotoping) in survey scans of INTACT lipid precursor ions, and NOT for processing data obtained by MRM or precursor ion scanning as done by the authors.

Moreover, the authors do not employ an isotope correction to correct for differences in the isotope distribution of lipid precursor ions having relative low number of atoms (e.g. PC 28:0) and high number of atoms (e.g. PC 38:5).

The authors should employ appropriate deisotoping and isotope correction routines to avoid bias of the estimated amounts of monitored lipid species.

The authors state:

"... that allow one-step, rapid isolation of plasma membranes ready..."

According to the text and figure 1c the isolation strategy requires at least 4 steps.

In the rebuttal, the authors stated "We agree that this raises some confusion and as suggested by the reviewers, we have omitted this statement."

Apparently not.

Fig. 1e:

Please change the y-axis to logarithmic scale.

This will communicate the reduction of potential PM contaminants more effectively. At the present, the reader can only observe and appreciate the enrichment of the Na-K-ATPase and Fas (Fatty acid synthase?).

Could the authors please add an (*) to the marker proteins having transmembrane domains.

Fig. 1c and d:

It is difficult to read the dimension of the scale-bar in all micrographs.

Please increase the font-size.

Fig. 1a (insert): "Coomassie Brilliant Blue staining of PNS, unbound (UB) and bound (B) fractions obtained from WT MEFs."

Reads like the size marker and the PNS are mixed. Please correct.

The authors state:

Line 245 "Qualitative and Quantitative PM Lipidomics"

The term "qualitative" is redundant since the authors only present quantitative data.

The authors state:

Line 248 "We extracted lipids from total cells and isolated PMs (see Material & Methods) and analyzed them using electrospray ionization (ESI)-MS."

Please add that that cholesterol was measured by fluorometry, not by MS.

According to the available material and method section the authors in fact applied nanoelectrospray ionization.

The authors state:

line 110 "... renders these particles monodispersible and stable for several weeks (Supplementary Fig. 1a')."

The legend for fig 1a' does not communicate anything about stability, but only that the authors decided to show a picture of "nanoparticles in water-organic solvent interface".

Please revise the text.