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PHOSPHATIDYLSERINE ENRICHMENT IN THE NUCLEAR MEMBRANE REGULATES KEY ENZYMES OF PHOSPHATIDYLCHOLINE SYNTHESIS

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Transaction Report:

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This manuscript was transferred to The EMBO JOURNAL following peer review at another journal.

Reviewer #1:

The authors have added several experiments that validate the sensors they used. I understand that this has taken a considerable amount of time, but such controls are a necessary foundation. Nevertheless, I remain very skeptical about the conclusions drawn from the existing data. I still find the assertion that phosphatidylserine (PS) is enriched at the inner nuclear membrane (INM) and the claim that PS at the INM regulates lipid droplet (LD) biogenesis via CCTa/Lipin1a to be lacking compelling evidence.

1) The authors have now provided some important controls for biosensor specificity for PS in the nucleus. The LactC2 mutant sensor appears to have less affinity than the wild-type version when expressed in cells, which is reassuring. But it remains difficult to determine the amount of sensor bound to the INM due to the higher concentration of mutant sensor in the nucleoplasm, even in cells with low expression levels. This makes it challenging to assess the sensor's distribution at the INM accurately. Photobleaching a region in the nucleoplasm does not provide convincing evidence, as it does not help evaluate sensor levels at the INM (see also point 5).

2) The authors conducted liposome binding assays indicating comparable binding affinities between the LactC2 and NLS-LactC2 sensors. While this finding is reassuring, it's regrettable that the recombinant variants of the mutant sensors weren't similarly assessed in this in vitro assay. Such analysis would have offered valuable insights into the extent of affinity loss.

3) I reiterate my major concern regarding the absence of EM/Immunogold labeling data to definitively pinpoint the localization of the sensors within the INM, the ONM, or both in normal cells and cells after hypotonic swelling. Thus, I remain skeptical of any assertions regarding the relative enrichment of PS at the INM over the ONM, which would have constituted the most important and novel finding of this study. This concern is heightened by the disparity with the findings of the Tsuji et al. study, which the authors now acknowledge.

EM/Immunogold labeling serves as an essential orthogonal technique in such investigations. Other research groups have already employed EM methods using either the GFP-LactC2 or a GST-LactC2 probes (Fairn et al., JCB, 2011), as well as the PH domain of evectin-2 (Tsuji et al., PNAS, 2019). These are the same sensor domains that were also utilized by the authors of this study. The arguments presented in the rebuttal to justify the absence of EM data are inadequate. The authors refer to one of their previous studies (Sohn et al, PNAS, 2016), however, this study also lacks EM data.

4) The authors have introduced TopFluor-PS as an alternative probe; however, this addition raises further questions. Firstly, this probe does not differentiate between the INM and outer nuclear membrane ONM, thus it does not substantiate the authors' claim of PS enrichment at the INM compared to the ONM/ER. Secondly, contrary to the authors' assertion that the probe does not stain the ER, I notice a distinct TopFluor-PS signal that partially coincides with the mCherry-Sec61beta ER signal. In the adjacent cell lacking a mCherry-Sec61beta signal, there is a reticulate TopFluor-PS signal in the cytoplasm, which may or may not overlap with the ER, though this is challenging to judge from individual cell data.

I attempted to locate the hypotonic swelling data referenced as Extended Fig. 4o and p in the rebuttal, but I could not find it in Extended Fig. 4, which concludes with Extended Fig. 4d.

5) In the rebuttal, the authors agree that a high level of nuclear NLS-mCherry-LactC2 sensor in cells expressing the yPSD1 in the cytoplasm, can mask membrane localization. To address this issue, they performed repeated photobleaching in a small area of the nucleus to reduce the fluorescent signal and conclude that that such repeated photobleaching shows lack of membrane signal when the cells express 2xNES-myc-yPSD1. I find this difficult to assess because the repeated photobleaching in a confined area of the nucleus evidently diminished the fluorescent signal across the entire nucleus, and may reduce any potential signal at the nuclear envelope (NE) if there is turnover of the sensor between the membrane-associating and free pool (which is very likely). My initial concerns about this experiment remain.

6) Regarding the question of whether PS regulates CCTa recruitment to the INM and LD biogenesis, the authors clarify in their rebuttal that "we do not claim that the PS increase (which is indeed modest after OA treatment) would govern the genesis of LDs via CCTa and Lipin1a. Indeed, even bigger PS increases caused by overexpression of PSS1Q353R does not move full-length CCTa to the inner nuclear membranes." confirms my previous critique that the claim of PS necessity at the INM for CCTa/Lipin1a-mediated LD formation lacks a convincing demonstration.

7) With regards to the specificity of CCTa binding to PS, the authors now state that "PS binding is an important but not the sole contributor to the recruitment of CCTa to the INM." Furthermore, that "we are not arguing that this binding to PS is specific, likely other acidic lipids can substitute for PS." Unfortunately, the new experiments which use a fragment of recombinant CCTa in liposome binding assays, adds little to the paper. The authors found that this domain showed weak PS binding but only at high PS concentrations of 40% PS, consistent with the fact that this construct could not find PS in the cell without the added lipid modification. Technically, this experiment lacks a mutant control to evaluate the specificity of PS binding. The recruitment of CCTa to the NE remains unresolved.

As a whole, I fail to see how the initial discoveries, the updated data, and the tempered conclusions align with the revised title of the manuscript: "PHOSPHATIDYLSERINE ENRICHED IN THE NUCLEAR MEMBRANE REGULATES CRITICAL ENZYMES FOR PHOSPHATIDYLCHOLINE SYNTHESIS"

8) The authors have now included the Tsuji et al study in their references, which came to a different conclusion namely that the INM and ONM had equivalent amounts of PtdSer in mouse cells. These contradictions persist unresolved and several issues concerning the accurate citation of the literature remain. It is inaccurate to assert that "no studies have systematically investigated the distribution or regulatory roles of nuclear PS in mammalian cells" because Tsuji et al. have examined mouse embryonic fibroblasts, which are mammalian cells (again not cited in the Introduction).

Reviewer #2:

I consider that the authors have adequately addressed the points I raised when reviewing the initial version of the manuscript. They have fully characterized the constructs used to probe PS inside the nucleus and linked the data obtained in the first part of the Result section with those related to the recruitment of CCTa/Lipin1a inside the nucleus (therefore showing this process is PS-dependent). They also added some additional controls (cellular staining with recombinant C2Lact, another CCTa mutant, ..) and provided greater details on how they quantify the localization of PS probes to the INM. Overall, the authors have substantially strengthened their manuscript.

Minor comments.

I found that the experiments quantifying the extent of LactC2 localization to the nuclear membrane are more convincing (particularly when looking at microscopy pictures) when the myc-tagged PSD1 constructs were expressed without IRES2/EGFP.

It might be possibly interesting to swap Fig. 3d,e,i,j with Extended data Fig.3a,b,c,d (by the way, the titles of the x-axis of the graphs shown in Extended data Fig.3b & d look strange)

A schema summarizing the observations described in the Section ”Manipulation of PS in INM altered OA-induced flow” might be helpful.

Other comments

The authors should use the same acronyms over the text (LER or ILER?, INM or Inner NM ?...) and also directly call OLER “the cytoplasmic face of the ER membrane” (p8). Because the study is a lot about topology issues, this might greatly help.

Several figure panels are not called in the main text (e,g., Fig.1c, Fig.1e)

For clarity, the authors should not call Fig. 3b,c,g, and h simultaneously (especially as they call again Fig.3g and h a little later in the text)

p13, line 327 “Extended Data Figure 5e,f”. In fact, Extended Data Figure 6e,f

Extended Data Fig. 6a and b. The color code for “only in the nucleus” is slightly inconsistent between the two panels, creating confusion.

Sentence p9, line 231 ”Time lapse” sounds incomplete.

Reviewer #3:

The authors have addressed my concerns. Some EM analyses or identifying the proteins that control the PS gradient would have strengthen this study, but technical challenges do exist.

Dear Tamas,

Thank you for transferring the revised version of your manuscript to The EMBO Journal. Based on the positive input from two of the original referees, and since from the editorial side we find that electron microscopy analysis is not required for publication here, I will accept your manuscript after reformatting of the manuscript according to The EMBO Journal guidelines as listed below. I apologise for the rather long list.

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8. Please update the nomenclature for Expanded View figures in the manuscript (currently labelled as Extended Data figures).
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15. In Figure EV2C, there is a typo - should be "intra-nucleus".
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With best wishes,

Ieva

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Title:
Phosphatidylserine enrichment in the nuclear membrane regulates phosphatidylcholine synthesis

Blurb:
Phosphatidylserine in the nuclear inner membrane channels oleic acid away from lipid droplet formation by relocalising the phosphatidylcholine biosynthesis enzymes CCT and Lipin1 .

Synopsis:
Phosphatidylserine (PS) is a phospholipid mainly enriched in the inner leaflet of the plasma membrane; however, its role in the nucleus remains unclear. This study describes the presence of PS in the inner nuclear membrane and nuclear reticulum, where it promotes nuclear membrane association of two key enzymes of phosphatidylcholine (PC) biosynthesis in response to oleic acid treatment.
- PS biosensors show its enrichment in the inner nuclear membrane (INM) and the nuclear reticulum (NR).
- PS in the inner nuclear membrane is synthesized at the endoplasmic reticulum.
- Nuclear PS interacts with two PC biosynthetic enzymes, CCT and Lipin1 , promoting their translocation to the inner nuclear membrane.
- Nuclear PS directs oleic acid towards PC biosynthesis rather than lipid droplet formation.

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