



Direct binding of ESCRT protein Chm7 to phosphatidic acid-rich membranes at nuclear envelope herniation

David Thaller, Danqing Tong, Christopher Marklew, Nicholas Ader, Philip Mannino, Sapan Borah, Megan King, Barbara Ciani, and C. Patrick Lusk

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May 27, 2020

Re: JCB manuscript #202004222

Dr. C. Patrick Lusk
Yale School of Medicine
Cell Biology
295 Congress Avenue
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New Haven, Connecticut 06520

Dear Patrick,

Thank you for submitting your manuscript entitled "Direct PA-binding by Chm7 is required for nuclear envelope surveillance at herniations". The manuscript has now been evaluated by three reviewers, whose reports are appended below. As you can see, all three reviewers found the work extremely interesting and groundbreaking, with many significant new insights. We, too, found the results interesting and exciting. However, there remain some key concerns, which must be addressed, as outlined below, so we would like to invite you to submit a revision if you can address the reviewers' points.

Most importantly, all three reviewers felt that key aspects of the model, in which it is proposed that Chm7 binds to PA and this influences its function, were not presently sufficiently supported. Co-localization experiments and PA binding in vitro show coincidence, but do not necessarily mean causation, and so the claims about the main finding of the paper - PA binding at the inner nuclear membrane - would need to be substantiated. The reviewers also were unsure about some of the genetic analyses and mutants used. They felt that some of the localization studies required higher resolution imaging (particularly use of CLEM at key issues, if possible). It is essential that these issues be fixed for the data to convince experts in the field and to move forward our understanding of nuclear envelope dynamics/surveillance and the role of lipids in this process.

The reviewers have detailed their queries, points, and requests, and we ask that you address each in detail. However, the reviewers also stressed that this is an interesting and potentially impactful piece of work, and they envisaged that any additional work and clarifications should be relatively straightforward and within your expertise. We'd be happy to discuss the revisions further as needed. Please let us know if you have any questions.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Reports may have up to 5 main text figures. To avoid delays in production, figures must be

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Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Michael Rout, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this study, Thaller et al carry out a structure-function analysis of Chm7, which has been previously shown to be involved in nuclear membrane repair and maintenance of NE integrity. They find that both putative NESs are needed for efficient export of the over expressed protein (although they favor a more significant role for one over the other), and that the H domain confers INM binding that was independent of Heh1. Note, however, that the membrane-binding activity associated with the H domain is specific to a Chm7 version that lacks its entire ESCRT III-like region,

which is half the protein; the full-length protein did not show general INM localization and had no membrane association (namely, in the form of foci) when Heh1 was missing. They then go on to show that Chm7 binds PA *in vitro*. However, the relevance of this to Chm7 function is not clear (see comment 1). They further show that a hyperactive form of Chm7, which localizes in foci at the INM, colocalizes with a PA sensor. However, an experiment showing that PA likely accumulates at nuclear membrane herniations (presumably independent of Chm7) led the authors to suggest that PA accumulation at Chm7 foci is likely due to the perturbation of the nuclear membrane, which leads to a local increase in PA levels. While this is interesting, it seems unrelated to Chm7's ability to bind PA. Since PA favors highly curved membranes, the association of PA with membrane herniations is not surprising (see papers by Kooijman et al and many others). In fact, the authors show that in an *apq12Δ* strain, PA foci are abundant but independent of Chm7. They further suggest that PA accumulates at sites of NPC assembly (or mis-assembly), but without CLEM, as they have done in their earlier study, this is pure conjecture. In sum, this study appears to make a number of interesting but largely unrelated observations that do not mesh together into a story that sheds light on the mechanism of Chm7 function and/or its relation to PA. It also does not expand on the possible cellular significance of PA accumulation at herniations, for example, in recruiting proteins involved in membrane repair or signaling membrane damage.

Other comments:

1. The physiological relevance of the ability of the N-terminal half of Chm7 not bind PA is not clear. In Figure 4A, in *pah1Δ* cells, PA levels are high at the plasma membrane (see Romanauska and Köhler, 2018). Chm7-GFP is mostly cytoplasmic, and yet it doesn't accumulate on the plasma membrane, only on the NE. This raises the possibility that Chm7's NE localization is related to NE integrity and not its ability to bind PA: does the NE accumulation of Chm7 in the *pah1Δ* strain depend on the H domain? If not, then the authors need to explicitly state that this localization of Chm7 is not related to PA binding. It also should be examined whether this localization is dependent on Heh1. If it is, that may further suggest that this localization is not related directly to PA binding.
2. All the panels with images also be quantified (namely, the fraction of cells showing the described phenotype)
3. Line 118: please explain and/or reference "MIM function"
4. Are the cells in Figure 4D wild type or *pah1Δ* (in particular, the cells in the upper panel, but not lower one, appear to have a *pah1Δ*-like nuclear extension).
5. A suggestion for future experiments (not this study): to minimize vacuole autofluorescence avoid using *ade2* strains. You can also try supplementing the media with extra adenine.

Reviewer #2 (Comments to the Authors (Required)):

Thaller et al: the manuscript focuses on the membrane-binding activity of the nuclear envelope-specific ESCRT, Chm7. The authors describe a putative amphipathic helix within the N-half of Chm7 which contributes to the binding to phosphatidate (PA)-enriched nuclear membranes. A PA reporter is shown to localize at/in proximity to an active form of Chm7 and nuclear pores at perinuclear foci under conditions that promote nuclear envelope herniations. The authors propose a model where PA accumulation at these sites has a physiological role in nuclear envelope remodeling/ surveillance.

There are many open questions on the remodeling of the double nuclear membrane and its coordination with other events at the nuclear envelope so I find this paper very interesting and of definite interest to the readership of JCB. Overall the experiments appear well executed and the data are described in a well-organized manner. However, a number of issues need to be addressed to substantiate the authors main conclusions:

1. The main novel finding of this study is that PA concentrates at herniations and that somehow Chm7 distribution responds to this. Lipid probes, including the one for PA used in this study, report only indirectly on lipid levels. I appreciate the methodological limitations of studying local lipid pools, but this conclusion should be strengthened, especially given the fact that the in vitro experiments in Fig. 3 show only partial effects on the PA - and helix -dependent specificity: (a) a different nuclear PA reporter (Spo20) should show a similar enrichment (b) increasing PA levels through deletion of PAH1 is an important experiment but the effects of this are not very clear, at least from what is shown in Fig. 4A: (i) if Chm7 associates with the INM PA, why there is no more prominent perinuclear localization for Chm7 under these conditions; (ii) quantification is needed to establish there is a significant difference when compared to the wild-type; the PA reporter could be used as a reference in this experiment (iii) how does the hydrophobic stretch mutant behaves in this assay? (iv) I would suggest changing statements like "Chm7 hyperactivation drives local PA accumulation" to "...drives local accumulation of the PA sensor".

2. Why removing the C-domain of Chm7 results only in nuclear rim-associated Chm7 accumulation? Since Chm7 lacks transmembrane domains, one would expect an increase also of its nucleoplasmic pool, especially under conditions that do not promote PA accumulation at the perinuclear spots (Fig. 2B).

3. In the genetics (Fig. 1) and in vitro binding (Fig. 3) the hydrophobic stretch point mutant is used, but in the chm7-N targeting experiments the deletion of the entire helix is used. Why? This makes the more difficult the interpretation of Fig 2B. This should be done with the W3AV1A mutant.

4. How do the authors conclude that the rim localization in Fig. 2B reflects inner and not outer nuclear membrane (l. 183)? The authors state that this "...association was likely with the INM as, were it the ONM, one would expect a broader distribution throughout the cortical ER as well"; this would not be the case for NPC-associated factors on the outer side of the nuclear envelope.

5. The authors state in the abstract (l22) as well as in the main text that PA binding is mediated through the hydrophobic stretch of the helix. Throughout the results, the data document binding to membranes, but no to PA itself, which is not the same (PA effectors bind first the membrane and then to PA via basic residues - electrostatic-hydrogen bond switch). Perhaps this should be clarified.

Other points:

6. Does the pah1delta mutant have a defect in nuclear import that could affect the NLS reporter distribution (Fig. 4C)?

7. Figure 1C; the authors should show the complementation of the helix mutant at 37C to be consistent with the NES mutants shown at the upper panel.

8. The changes reported on the localization of various Chm7 constructs in Fig. 1, are not always

apparent to me from the micrographs shown and some statements are a bit vague (i.e. "...qualitatively less focal and appeared more evenly distributed along the nuclear envelope"). Perhaps the authors could add some cartoons in the figure to illustrate what they refer to.

9. In general, lipid strip data are not very meaningful. I suggest moving the relevant results to supplementary information.

Reviewer #3 (Comments to the Authors (Required)):

Thaller and colleagues suggest that Chm7, a nuclear envelope (NE)-specific ESCRT protein, has an ability to bind phosphatidic acid (PA) at the inner nuclear membrane (INM). Chm7 has previously been implicated in NE surveillance, which appears to be linked to the occurrence of NE herniations. These NE herniations are suggested to contain higher amounts of PA. Hence, the authors conclude that PA at the INM could be important for NE surveillance.

Overall, this is an important subject of study because little is known about the lipid composition of the INM and the functional importance of INM lipids. How INM lipids might contribute to NPC biogenesis and NE surveillance is a very intriguing question and a new frontier in NE/NPC biology. In general, the data is of good quality, except for a number of cases, where important controls are missing. A major technical drawback is the lack of high-resolution data, such as TEM or CLEM. The biochemistry also needs to be improved. This would allow the authors to draw more robust conclusions. Overall, the manuscript is well written and easy to read.

I find the data potentially interesting, but in its present form the manuscript is premature and somewhat incomplete. The title is an overstatement because the authors do not link PA-binding of Chm7 to the surveillance of NE herniations in a convincing way. What is missing is more direct data that PA levels at the INM are mechanistically coupled to Chm7 function. The manuscript should be considered for publication if such mechanistic data can be provided together with convincing EM data.

Major criticisms:

- 1) I would like to know how increasing or decreasing PA levels in cells, either through nutrients or a number of mutant conditions, affects the formation of NE herniations. What is the number and morphology of these herniations when studied by EM? And how is Chm7 recruitment affected under these conditions. Although this data will be correlative, this could be a starting point for identifying how lipids and Chm7 cooperate for NE surveillance.
- 2) Figure 1B - are these foci indeed located at the INM rather than the ONM? Immunogold EM should be used to verify the localization of Chm7 mutants. The same criticism applies to Figure 2B.
- 3) The statement that "PA sensor recruitment is likely at nuclear envelope herniations " must be substantiated by immunogold EM or CLEM, because it is a central claim of the paper.
- 4) Figure 3D: several controls are missing, e.g. a control mixture of PC+PE was not used for full-length Chm7. A quantification across 3 independent experiments is required. I do not quite understand the rationale for using 75% PC. Why is this different from the liposome composition in Figure 3B? Finally, I would like to see the AH mutations inserted into the N-terminal Chm7 fragment

as a negative control.

5) Figure 3E,F: can the authors show that they have used equal amounts of lipids? I'd also like to see a) the Chm7 AH point mutant as a negative control, b) a known curvature-insensitive protein as a negative control and c) a well-characterized curvature-sensing protein as a positive control. Right now this data is not convincing.

Minor criticisms:

6) Mutant "chm7-(W3AV1A)" - I am confused by the nomenclature. Which amino acids are these?

7) The authors should use another method to show that the hydrophobic stretch in Chm7 indeed forms an amphipathic helix (AH) (e.g., recording CD spectra). That would also allow them to discern whether their AH mutants specifically disrupt the hydrophobic face while leaving the AH intact.

8) Figure 3A: a Chm7 mutant must be used as a negative control in the Lipid Strip assay.

9) Figure 3B: it is unclear to me why the authors add different amounts of PE to their liposomes given that PE shows no binding to Chm7 in the Lipid Strip. In doing so, they are not measuring the effect of PA alone but a mixed PA/PE effect. The experiment should be repeated with increasing PA conc. alone and a Chm7 mutant must be included as a negative control.

10) Figure 4A: as a control, the authors should use the Chm7 AH mutant to show that it does not bind PA-rich membranes in *pah1Δ* cells.

11) *chm7open* - please explain this mutant.

12) Figure 4D - wild-type Chm7 as a control is missing.

13) Figure 4F: *nup116Δ* cells have an impressive number of PA-positive foci than 4D. Is Chm7 recruited to all of these foci or only a subset?

14) The authors write: "Cumulatively then, we conclude that the changes in local PA distribution in *nup116Δ* and *apq12Δ* cells are not caused by Chm7, but instead by the upstream insult that leads to Chm7 recruitment." Chm7 recruitment in these cells was actually not shown.

15) Figure 5B - the authors stated that BRL1 was used (in the text and Figure) but in the figure legend it says that pGP564 contains a genomic fragment including BRL1, PIH1, YHR035W, PUT2, RRF1, MSC7, VMA10, BCD1, and SRB2. Are these other genes required? Why did they not use a plasmid that contains BRL1 alone?

16) The authors write in the summary: "...it may be possible to directly recruit additional PA (as seen in Fig. 4 D)", which is a contradiction, because Fig 4 D does not show that. Later in the results part, the authors state that PA recruitment to foci is independent of Chm7. This should be clarified.

Response to Reviewers

Reviewer #1 Comments:

In this study, Thaller et al carry out a structure-function analysis of Chm7, which has been previously shown to be involved in nuclear membrane repair and maintenance of NE integrity. They find that both putative NESs are needed for efficient export of the over expressed protein (although they favor a more significant role for one over the other), and that the H domain confers INM binding that was independent of Heh1. Note, however, that the membrane-binding activity associated with the H domain is specific to a Chm7 version that lacks its entire ESCRT III-like region, which is half the protein; the full-length protein did not show general INM localization and had no membrane association (namely, in the form of foci) when Heh1 was missing.

Our response: We now provide several new data (in addition to the chm7-N localization) that demonstrate that the H domain can confer nuclear envelope binding outside of the context of Heh1. The first can be found in a completely revised Figure 3, which shows that in the absence *PAH1* and *HEH1*, full length Chm7 localizes to nuclear envelope membranes; this association depends on the hydrophobic residues in the H domain. Further, we also show that full length Chm7 in an *heh1Δ* strain treated with Leptomycin B (to inhibit Chm7 nuclear export) also localizes to the nuclear envelope. These data are now included in Figure S1 C.

*They then go on to show that Chm7 binds PA in vitro. However, the relevance of this to Chm7 function is not clear (see comment 1). They further show that a hyperactive form of Chm7, which localizes in foci at the INM, colocalizes with a PA sensor. However, an experiment showing that PA likely accumulates at nuclear membrane herniations (presumably independent of Chm7) led the authors to suggest that PA accumulation at Chm7 foci is likely due to the perturbation of the nuclear membrane, which leads to a local increase in PA levels. While this is interesting, it seems unrelated to Chm7's ability to bind PA. Since PA favors highly curved membranes, the association of PA with membrane herniations is not surprising (see papers by Kooijman et al and many others). In fact, the authors show that in an *apq12Δ* strain, PA foci are abundant but independent of Chm7. They further suggest that PA accumulates at sight of NPC assembly (or mis-assembly), but without CLEM, as they have done in their earlier study, this is pure conjecture. In sum, this study appears to make a number of interesting but largely unrelated observations that do not mesh together into a story that sheds light on the mechanism of Chm7 function and/or its relation to PA. It also does not expand on the possible cellular significance of PA accumulation at herniations, for example, in recruiting proteins involved in membrane repair or signaling membrane damage.*

Our response: We thank the reviewer for their thoughtful critique of our work and hope that our addition of new data and clarifications to the text will tell a more cohesive and impactful story. It is obvious that we did not draw sufficient distinction between the relationship of the PA-sensor distribution in the context of the chm7_{OPEN} construct and

the local PA-sensor accumulation that occurs in *apq12Δ* and *nup116Δ* cells. The former reflects a gain of function scenario in which $\text{chm7}_{\text{OPEN}}$ accumulates at the INM and drives changes to membrane morphology and proliferation (as detailed in our Thaller et al., *eLife*, 2019 paper). Thus, local accumulation of $\text{chm7}_{\text{OPEN}}$ at the INM is sufficient to drive the PA sensor to that site. These data ultimately reinforce the conclusion that Chm7 can directly bind to PA, but, as the reviewer points out, do not illuminate the function of Chm7's PA binding, *per se*.

In order to understand the function of Chm7's PA binding, we turned to *nup116Δ* and *apq12Δ* cells because these two genetic backgrounds have defects in NPC assembly and require Chm7 for viability. They thus provide the necessary backdrop to evaluate the role of Chm7 PA-binding. **Consistent with the idea that PA-binding is required for Chm7 function, we demonstrate that the *chm7-(W3AV1A)* allele is unable to complement the viability of *chm7Δapq12Δ* cells**, whereas the *chm7-(K121A-K124A)* allele can (Figure 1 B). These data clearly demonstrate (particularly in the context of the direct PA-binding data in Figure 2 and the more complete and revised localization data in Figure 1 C-F, Figure 3 A, and Figure S2 A) that Chm7's PA binding is required for Chm7 function in the context of NPC misassembly-induced nuclear envelope herniations.

The challenge is that one cannot easily connect PA accumulating in response to $\text{chm7}_{\text{OPEN}}$ and that which accumulates in the *nup116Δ* and *apq12Δ* contexts. Indeed, the latter is clearly a consequence of Chm7 building up at the INM whereas the former is a product of the nuclear envelope herniations themselves, which do not need Chm7 to form (see also Thaller et al. *eLife* 2019). First, it is worth pointing out that while PA-accumulation at membrane curvature may not be surprising to the reviewer, this is (to our knowledge) the first direct demonstration of such a change in local membrane composition at the INM, so it is a novel finding that will certainly be of interest to many in the nuclear envelope/nuclear transport fields. Nonetheless, to reinforce this point and to respond to the reviewer, we now include high resolution CLEM data that establishes that the PA sensor accumulates at nuclear envelope herniations in *nup116Δ* cells. These data are found in a new Figure 5 D and Figure S3 D-F. Second, with these data and the additional data provided in Figure 3 and Figure S2, we are more comfortable placing our findings within a model of Chm7 nuclear envelope surveillance that can explain all of our observations. This model is presented in Figure 5.

*Comment 1. The physiological relevance of the ability of the N-terminal half of Chm7 not bind PA is not clear. In Figure 4A, in *pah1Δ* cells, PA levels are high at the plasma membrane (see Romanauska and Köhler, 2018). Chm7-GFP is mostly cytoplasmic, and yet it doesn't accumulate on the plasma membrane, only on the NE. This raises the possibility that Chm7's NE localization is related to NE integrity and not its ability to bind PA: does the NE accumulation of Chm7 in the *pah1Δ* strain depend on the H domain? If not, then the authors need to explicitly state that this localization of Chm7 is not related to PA binding. It also should be examined whether this localization is dependent on Heh1. If it is, that may further suggest that this localization is not related directly to PA*

binding.

Our Response: We thank the reviewer for this suggestion. We have now included a substantial investigation into the Chm7's distribution in *pah1Δ* (and, as a control, *dgk1Δ* strains, which have lower PA levels) strains to address these concerns. As shown in a revised Figure 3 A and Figure S2A, Chm7-GFP specifically accumulates on proliferated nuclear envelope/ER membranes that (interestingly) seem to surround vacuoles. This localization occurs irrespective of the presence of Heh1 and requires the PA-binding motif in Chm7 (see new localization of chm7-(W3AV1A)-GFP). Thus, these data more fully reinforce the conclusion that Chm7 binds to PA-rich membranes and this association does not require Heh1. As to why Chm7 seems unable to bind to the plasma membrane in this scenario, we cannot fully explain. As the reviewer knows, there are other features of the plasma membrane that may inhibit Chm7 binding even in the presence of high levels of PA, for example differences in lipid packing (reviewed, for example by Bigay and Antonny, Dev. Cell, 2012). We also cannot rule out the possibility that there may be post-translational modifications of cytosolic Chm7 that might prevent PA binding specifically in the cytosol.

Comment 2. All the panels with images also be quantified (namely, the fraction of cells showing the described phenotype)

Our response: We have now thoroughly quantified all fluorescence images in the manuscript.

Comment 3. Line 118: please explain and/or reference "MIM function"

Our response: We apologize that this was vague. To simplify the manuscript, this has been removed along with the characterization of the point mutants within the NES domains, which detracted from the main point of the manuscript.

Comment 4. Are the cells in Figure 4D wild type of pah1Δ (in particular, the cells in the upper panel, but not lower one, appear to have a pah1Δ-like nuclear extension).

Our response: An interesting idea but we do not have strong evidence of any major change to overall nuclear morphology in this strain.

Comment 5. A suggestion for future experiments (not this study): to minimize vacuole autofluorescence avoid using ade2 strains. You can also try supplementing the media with extra adenine.

Our response: Thank you, as we describe in the methods section, we always add adenine to our media to limit vacuolar fluorescence.

Reviewer #2 (Comments to the Authors (Required)):

Thaller et al: the manuscript focuses on the membrane-binding activity of the nuclear envelope-specific ESCRT, Chm7. The authors describe a putative amphipathic helix within the N-half of Chm7 which contributes to the binding to phosphatidate (PA)-enriched nuclear membranes. A PA reporter is shown to localize at/in proximity to an active form of Chm7 and nuclear pores at perinuclear foci under conditions that promote nuclear envelope herniations. The authors propose a model where PA accumulation at these sites has a physiological role in nuclear envelope remodeling/ surveillance.

There are many open questions on the remodeling of the double nuclear membrane and its coordination with other events at the nuclear envelope so I find this paper very interesting and of definite interest to the readership of JCB. Overall the experiments appear well executed and the data are described in a well-organized manner. However, a number of issues need to be addressed to substantiate the authors main conclusions:

Our response: Thank you for the positive summary of our work.

1. The main novel finding of this study is that PA concentrates at herniations and that somehow Chm7 distribution responds to this. Lipid probes, including the one for PA used in this study, report only indirectly on lipid levels. I appreciate the methodological limitations of studying local lipid pools, but this conclusion should be strengthened, especially given the fact that the in vitro experiments in Fig. 3 show only partial effects on the PA - and helix -dependent specificity: (a) a different nuclear PA reporter (Spo20) should show a similar enrichment (b) increasing PA levels through deletion of PAH1 is an important experiment but the effects of this are not very clear, at least from what is shown in Fig. 4A: (i) if Chm7 associates with the INM PA, why there is no more prominent perinuclear localization for Chm7 under these conditions

Our response: We have completely revised Figure 4 (now Figure 3, and see also Figure S2A) and have more thoroughly investigated the consequences of elevating total cellular levels (in *pah1Δ* cells) and decreasing (in *dgk1Δ* cells) PA levels on Chm7 distribution. First, we observe a remarkable accumulation of Chm7 on nuclear envelope/ER membranes in *pah1Δ* cells. This localization is independent of Heh1, which supports the conclusion that Chm7 can bind to membranes without its established protein binding partner. Further, it requires the PA-binding stretch as *chm7-(W3AV1A)-GFP* does not exhibit any obvious membrane enrichment in the *pah1Δ* cells.

(ii) quantification is needed to establish there is a significant difference when compared to the wild-type; the PA reporter could be used as a reference in this experiment

Our response: We have now quantified all fluorescence images in the study.

(iii) how does the hydrophobic stretch mutant behaves in this assay?

Our response: This was a great suggestion and as shown in the revised Figure 3 and S2A, it fails to accumulate on any membranes in *pah1Δ* cells.

(iv) I would suggest changing statements like "Chm7 hyperactivation drives local PA accumulation" to "...drives local accumulation of the PA sensor".

Our response: We agree - this has been changed throughout.

2. Why removing the C-domain of Chm7 results only in nuclear rim-associated Chm7 accumulation? Since Chm7 lacks transmembrane domains, one would expect an increase also of its nucleoplasmic pool, especially under conditions that do not promote PA accumulation at the perinuclear spots (Fig. 2B).

Our response: We do in fact observe an increase in a nucleoplasmic pool of chm7-N as we elevate its levels. Indeed, as this truncation lacks the NESs (and does not have an NLS) overexpression results in a pan-cell distribution. Nonetheless, we found the perinuclear localization of great interest as it suggests that there are sufficient levels of PA at (likely) the INM to help with chm7-N membrane recruitment (and see response to point 3).

3. In the genetics (Fig. 1) and in vitro binding (Fig. 3) the hydrophobic stretch point mutant is used, but in the chm7-N targeting experiments the deletion of the entire helix is used. Why? This makes the more difficult the interpretation of Fig 2B. This should be done with the W3AV1A mutant.

Our response: We completely agree and have now included these data demonstrating that the residual nuclear periphery localization of chm7-N is abolished upon making the W3AV1A mutations (see revised Figure 1 F). We simply ran out of time before the COVID lockdown to perform this experiment properly.

4. How do the authors conclude that the rim localization in Fig. 2B reflects inner and not outer nuclear membrane (l. 183)? The authors state that this "...association was likely with the INM as, were it the ONM, one would expect a broader distribution throughout the cortical ER as well"; this would not be the case for NPC-associated factors on the outer side of the nuclear envelope.

Our response: The reviewer is correct, we cannot rule out an association with NPCs, although the distribution of chm7-N does not resemble the classic punctate NPC localization that we would expect for such an association. Nonetheless, we have re-stated this interpretation to include this caveat.

5. The authors state in the abstract (l22) as well as in the main text that PA binding is mediated through the hydrophobic stretch of the helix. Throughout the results, the data document binding to membranes, but no to PA itself, which is not the same (PA effectors bind first the membrane and then to PA via basic residues - electrostatic-hydrogen bond switch). Perhaps this should be clarified.

Our response: Thank you to the reviewer, we are now more precise with our language.

Other points:

6. Does the *pah1delta* mutant have a defect in nuclear import that could affect the NLS reporter distribution (Fig. 4C)?

Our response: We cannot fully exclude that there is a defect in nuclear import in these strains and have included this caveat in the results.

7. Figure 1C; the authors should show the complementation of the *helix* mutant at 37°C to be consistent with the NES mutants shown at the upper panel.

Our response: We agree but we have removed the NES mutant data to try to simplify and focus the manuscript. Nonetheless, we observe the growth defect at room temperature, 30°C and 37°C.

8. The changes reported on the localization of various *Chm7* constructs in Fig. 1, are not always apparent to me from the micrographs shown and some statements are a bit vague (i.e. "...qualitatively less focal and appeared more evenly distributed along the nuclear envelope"). Perhaps the authors could add some cartoons in the figure to illustrate what they refer to.

Our response: Yes, this figure is very busy. We thank the reviewer for the suggestion and now provide a schematic with more extensive quantification to facilitate navigating these data presented in Figure 1 F and G.

9. In general, lipid strip data are not very meaningful. I suggest moving the relevant results to supplementary information.

Our response: We tend to agree but there is clear specificity in the lipid strip data and it provides an orthogonal approach, which further shores up the conclusion that *Chm7* binds to PA-rich membranes.

Reviewer #3

Thaller and colleagues suggest that Chm7, a nuclear envelope (NE)-specific ESCRT protein, has an ability to bind phosphatidic acid (PA) at the inner nuclear membrane (INM). Chm7 has previously been implicated in NE surveillance, which appears to be linked to the occurrence of NE herniations. These NE herniations are suggested to contain higher amounts of PA. Hence, the authors conclude that PA at the INM could be important for NE surveillance.

Overall, this is an important subject of study because little is known about the lipid composition of the INM and the functional importance of INM lipids. How INM lipids might contribute to NPC biogenesis and NE surveillance is a very intriguing question and a new frontier in NE/NPC biology. In general, the data is of good quality, except for a number of cases, where important controls are missing. A major technical drawback is the lack of high-resolution data, such as TEM or CLEM. The biochemistry also needs to be improved. This would allow the authors to draw more robust conclusions. Overall, the manuscript is well written and easy to read.

*Our response: We thank the reviewer for recognizing the potential importance of the work. We have considerably revised the manuscript with several new data presented in all Figures but most notably in completely new Figure 3 and Figure 5. The latter includes CLEM of the PA sensor in *nup116Δ* cells as requested. We have not, however, substantially improved the biochemistry as this was not a major concern for the other reviewers. Further, performing these experiments is extremely challenging under normal circumstances. With the lab running at less than 50%, these experiments were simply not feasible in a reasonable timeframe. We have nonetheless attempted to address the reviewers concerns in the manuscript text and below; we hope that we have moved the bar sufficiently to gain their support.*

I find the data potentially interesting, but in its present form the manuscript is premature and somewhat incomplete. The title is an overstatement because the authors do not link PA-binding of Chm7 to the surveillance of NE herniations in a convincing way. What is missing is more direct data that PA levels at the INM are mechanistically coupled to Chm7 function. The manuscript should be considered for publication if such mechanistic data can be provided together with convincing EM data.

Major criticisms:

1) I would like to know how increasing or decreasing PA levels in cells, either through nutrients or a number of mutant conditions, affects the formation of NE herniations. What is the number and morphology of these herniations when studied by EM? And how is Chm7 recruitment affected under these conditions. Although this data will be correlative, this could be a starting point for identifying how lipids and Chm7 cooperate for NE surveillance.

Our response: We would also like to have this information but, as the reviewer states, the resulting data would ultimately only be correlative in nature and would require a massive effort to provide. For example, to examine herniations in every genetic background would require months of effort exploring ultrastructure alone. Therefore, we felt our efforts would be better placed addressing other concerns.

2) Figure 1B - are these foci indeed located at the INM rather than the ONM? Immunogold EM should be used to verify the localization of Chm7 mutants. The same criticism applies to Figure 2B.

Our response: As we more thoroughly describe in our prior work (Thaller et al., *eLife*, 2019), Chm7 is constitutively cytosolic and therefore always has access to the ONM. It becomes focal upon LMB treatment or upon mutation of its NES, which allow nuclear entry and binding to Heh1 at the INM. CLEM confirms that these foci are at the INM in our prior work. In Figure 2 B (now revised as Figure 1 E), we acknowledge we cannot unequivocally establish that this nuclear peripheral localization is at the INM. We have therefore more clearly articulated this caveat in the results.

3) The statement that "PA sensor recruitment is likely at nuclear envelope herniations " must be substantiated by immunogold EM or CLEM, because it is a central claim of the paper.

Our response: We completely agree and simply ran out of time before the COVID lockdown to perform these experiments. We now include in a completely revised Figure 5 and Figure S3, CLEM data demonstrating that the PA-sensor localizes to nuclear envelope herniations.

4) Figure 3D: several controls are missing, e.g. a control mixture of PC+PE was not used for full-length Chm7. A quantification across 3 independent experiments is required. I do not quite understand the rationale for using 75% PC. Why is this different from the liposome composition in Figure 3B? Finally, I would like to see the AH mutations inserted into the N-terminal Chm7 fragment as a negative control.

Our response: All of the in vitro binding experiments have been reproduced and quantification of replicates is provided in a revised Figure 2 C, E and G. The control of the PC+PE mixture for full length Chm7 was provided in Figure 3 B. We used PC simply because it is a neutral lipid. There was no change in lipid composition between 3B and 3D as we also tested 75% PC and 25% PE in Figure 3 B. The rationale for testing PE is that, like PA, it can introduce small imperfections in the bilayer. Thus, it was a control to ensure that we observed specificity for PA over similarly cone-shaped lipids. This has been more clearly articulated in the text. We chose not to introduce the AH mutations into the N-terminus of Chm7 as these experiments are not trivial and we prioritized other experiments.

5) Figure 3E,F: can the authors show that they have used equal amounts of lipids? I'd also like to see a) the Chm7 AH point mutant as a negative control, b) a known curvature-insensitive protein as a negative control and c) a well-characterized curvature-sensing protein as a positive control. Right now this data is not convincing.

Our response: As described in the methods, the molar protein:lipid ratio is kept constant with each experiment. Further, these experiments were performed in triplicate and the quantification is shown in Figure 2 G. We are also fairly circumspect about these data and they will not be fundamentally altered by the inclusion of the suggested controls. As

they are not part of any major conclusion of the work, we again chose to prioritize other experiments.

Minor criticisms:

6) Mutant "chm7-(W3AV1A)" - I am confused by the nomenclature. Which amino acids are these?

Our response: We apologize for the confusion, the mutations are described in Figure 1 A and the specific residues are also now introduced in the text.

7) The authors should use another method to show that the hydrophobic stretch in Chm7 indeed forms an amphipathic helix (AH) (e.g., recording CD spectra). That would also allow them to discern whether their AH mutants specifically disrupt the hydrophobic face while leaving the AH intact.

Our response: We are quite careful about how we describe this stretch of amino acids. As we demonstrate that the hydrophobic residues are important for binding PA-rich membranes in vitro and in vivo, and are functionally relevant, whether this stretch is actually an amphipathic helix is not a central part of our conclusions. There is also uncertainty as to whether CD would provide conclusive data since to be rigorous, we would need to examine the folding of this region in the context of the full length protein and in the presence of liposomes, which would complicate straightforward conclusions.

8) *Figure 3A: a Chm7 mutant must be used as a negative control in the Lipid Strip assay.*

Our response: Lipid strip data is notoriously ambiguous, which is why we chose to perform the direct liposome binding experiments with proper controls.

9) *Figure 3B: it is unclear to me why the authors add different amounts of PE to their liposomes given that PE shows no binding to Chm7 in the Lipid Strip. In doing so, they are not measuring the effect of PA alone but a mixed PA/PE effect. The experiment should be repeated with increasing PA conc. alone and a Chm7 mutant must be included as a negative control.*

Our response: First, as we alluded to above, the lipid strip data can only be used as a guide and is not as strong of an approach as the liposome experiments. Second, a PA/PE mixture is more physiological as a PA-only mixture would only be relevant to a scenario in which there is a PA-enriched microdomain. Third, PE introduces similar imperfections in the membrane as PA, which may contribute to Chm7 binding; in fact there are data to support that PE may actually enhance PA binding of some proteins (see Putta et al., BBA, 2016). Therefore, the reviewer is correct, there is likely a contribution from membrane imperfections (which likely also underlies the curvature sensitivity) in addition to specificity to PA. We have tried to make this more clear in the

text and thank the reviewer for bringing up these points.

10) *Figure 4A: as a control, the authors should use the Chm7 AH mutant to show that it does not bind PA-rich membranes in pah1Δ cells.*

Our response: This was an excellent suggestion and these data are now provided in a completely revised Figure 3 A and B and Figure S2 A.

11) *chm7open - please explain this mutant.*

Our response: This is a historic name (from our Webster et al., *EMBO J*, 2016 paper) that reflects that the deletion of the C-termini of most ESCRT-III's is thought to mimic the "open" active form of the proteins. We have more clearly defined this in the revised text and added a cartoon (Figure 4 A) as a visual aid.

12) *Figure 4D - wild-type Chm7 as a control is missing.*

Our response: We apologize for the confusion as this was a result of our labeling. The strain pictured in the now labeled "wildtype" micrograph (and explained in the figure legend as "no GFP") possesses the wildtype *CHM7* gene.

13) *Figure 4F: nup116Δ cells have an impressive number of PA-positive foci than 4D. Is Chm7 recruited to all of these foci or only a subset?*

Our response: This is a difficult proposition as Chm7-GFP recruitment in this context is transient as ESCRT-mediated fusion/fission events occur on the order of minutes to seconds. In contrast, the PA accumulation is more stable as we now show it accumulates within the herniations (see CLEM, Figure 5).

14) *The authors write: "Cumulatively then, we conclude that the changes in local PA distribution in nup116Δ and apq12Δ cells are not caused by Chm7, but instead by the upstream insult that leads to Chm7 recruitment. " Chm7 recruitment in these cells was actually not shown.*

Our response: We showed this in Webster et al., *EMBO J*, 2016. We have now been more explicit about describing this in the text.

15) *Figure 5B - the authors stated that BRL1 was used (in the text and Figure) but in the figure legend it says that pGP564 contains a genomic fragment including BRL1, PIH1, YHR035W, PUT2, RRF1, MSC7, VMA10, BCD1, and SRB2. Are these other genes required? Why did they not use a plasmid that contains BRL1 alone?*

Our response: This was a quick experiment that we managed to squeeze in before the lockdown and simply took advantage of a plasmid in our possession from a genomic

library. The purpose of the experiment was to shore up evidence that the PA sensor accumulated at the nuclear envelope herniations as it had previously been shown that the herniations could be resolved by expressing *BRL1*. As we now show that the PA sensor accumulates at herniations with the CLEM in Figure 5 and Figure S3, we removed this data altogether as it was ultimately distracting.

16) *The authors write in the summary: "...it may be possible to directly recruit additional PA (as seen in Fig. 4 D)", which is a contradiction, because Fig 4 D does not show that. Later in the results part, the authors state that PA recruitment to foci is independent of Chm7. This should be clarified.*

Our response: We did not do a good job at differentiating between the expression of *chm7_{OPEN}*, which accumulates at the INM and drives local membrane remodeling/expansion and the herniations observed in the *nup116Δ* cells. See a lengthier discussion in our response to reviewer 1 and also the revised text.

December 1, 2020

RE: JCB Manuscript #202004222R

Dr. C. Patrick Lusk
Yale School of Medicine
Cell Biology
295 Congress Avenue
BCMM 254 B
New Haven, Connecticut 06520

Dear Dr. Lusk,

Thank you for submitting your revised manuscript entitled "Direct binding of Chm7 to PA-rich membranes at nuclear envelope herniations" to JCB and for your patience with the re-review process. You will see that the reviewers remain excited by the discovery of Chm7 binding to PA. Two referees now recommend publication, whereas Reviewer #1 has a few remaining concerns. The referee feels that a NE marker is needed for you to state that Chm7 relocates to that compartment in pah1-null cells. We agree that the addition of this marker would be valuable. The reviewer additionally requests more colocalization studies between Chm7 and the PA sensor, and we have discussed these requests editorially. In our view, the consensus from the reviewers is that, even if there's some uncertainty into how the Chm7-PA relationship works, the results are novel and provocative and expand our understanding of NE dynamics/NPC assembly control and surveillance and the roles of local lipid dynamics in these processes. Therefore, we feel that the results are a great fit for the JCB Report format and we will not require the additional colocalization studies for publication as they would not clarify the functional interplay between PA and Chm7. If you have such data, please feel free to include them to strengthen the claim that Chm7 targets to PA membranes in cells; on the other hand, if these experiments are not already performed, we will be happy to publish the paper pending final edits to meet our formatting requirements (see below) and pending the addition of the NE marker as suggested by Rev#1 as well as responses to the other reviewer points. Please include a point-by-point response to the reviewer comments with your final files.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: Direct binding of ESCRT protein Chm7 to phosphatidic acid-rich membranes at nuclear envelope herniations

Running title (50 characters max, including spaces):
ESCRT Chm7 directly binds to phosphatidic acid

eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the

present tense and refer to the work in the third person.

- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.

2) Figure formatting:

Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

Please be sure to include unit labels (for instance, they are missing in 2BDF)

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

- For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features *even if described in other published work or gifted to you by other investigators*

- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.

- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.

- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:

a. Make and model of microscope

b. Type, magnification, and numerical aperture of the objective lenses

c. Temperature

d. imaging medium

e. Fluorochromes

f. Camera make and model

g. Acquisition software

h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

5) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

- Please check our preprint citation guidelines and update the ref list/in-text citations as needed:

<https://rupress.org/jcb/pages/reference-guidelines>

6) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

7) Author contributions: A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by

their full names. We encourage use of the CRediT nomenclature.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, <https://jcb.rupress.org/submission-guidelines#revised>. ****Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.****

B. FINAL FILES:

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-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, <https://jcb.rupress.org/fig-vid-guidelines>.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Michael Rout, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD

Reviewer #1 (Comments to the Authors (Required)):

This is a much-improved manuscript with better focus than the original version, although there are still two somewhat loosely related parts. Overall, the experiments are elegantly designed, carefully executed and nicely quantified. In the first part the authors show that Chm7, an ESCRT-III component, can associate with phosphatidic acid (PA) in vitro (the association with PA in vivo is less convincing; see comments 1 and 2 below). In the second, the authors show that PA accumulates in sites of likely NE damage such when nuclear pore complex assembly is defective (the *nup160* and *apq12* mutants), at nuclear membrane herniation or when Chm7 is constitutively active. In the latter case the authors suggest that PA is either recruited directly by Chm7 or that Chm7 alters PA metabolism, resulting in a local accumulation (lines 255-257), but later they show that PA accumulation is independent on Chm7 and propose that PA accumulation is upstream of Chm7 (line 301). While they try to rationalize this apparent contradiction, this underscores the fact that the relationship between PA and Chm7 is not really understood: the order of events at sites of NE damage and the nature of relationship between PA and Chm7 remains unknown: does Chm7 affect PA levels/metabolism, or does PA recruit Chm7, or perhaps both, leading to a positive feedback loop, perhaps driving herniation? The authors really want to present a unified model that links Chm7, PA and Heh1 in membrane repair, but the data tying it all together is not yet at hand.

That said, as far as I can tell the accumulation PA at NE herniations is novel and could potentially lead to insights as to how the cell deals with this type of insult.

Specific comments:

1. Figure 3A: The authors show that in the *pah1Δ* mutant, which exhibits ER proliferation as determined by EM (see earlier work by several labs), Chm7 accumulates as a band, which they interpret as ER/NE association due to enrichment for PA. First, to conclude that this band reflects NE association the authors must use a NE-specific marker. Second, the authors should use the PA-specific sensor based on Opi1 that was used later in the study to determine if the band formed by Chm7 is specific to a PA rich-membrane. This is important because part of the key conclusion of this study is that Chm7 is attracted to PA, and if these bands do not co-localize with PA-rich membrane then the authors need to rethink their conclusion.
2. Figure 4E: another case where the authors could have examined the co-localization of Chm7 and PA is in the *nup116* mutants (and to a lesser extent in the *apq12Δ* mutants), where clear PA sensor foci are seen. Does Chm7 also localize to these foci, and if so- to what fraction of foci? If the localization is not complete, could Chm7 act in a "catalytic" manner, creating a localized PA focus before moving to the next spot? And if there is little or no co-localization, how do the authors explain that?
3. In the CLEM analysis, the authors note that 11 of 12 PA sensor foci localized to regions of NE herniation. What about the converse? Namely, where there herniations that did not co-localize with PA sensor foci? If so, what would be the explanation?

Reviewer #2 (Comments to the Authors (Required)):

The authors addressed most of the issues I raised. Together with their responses to the other reviewers, their paper has now significantly improved - in particular the revised Fig. 3 and 5 - and in

my view the paper is now appropriate for publication. I have only two (minor) comments: (a) L. 238 "Chm7 hyperactivation drives local PA accumulation at the INM" I still think it would be better to specify that these conclusions are based on the distribution of a PA sensor rather than actual PA levels and (b) revised Fig. 3A and S2A: have the authors confirmed that the W3AV1A-GFP is expressed at similar levels as the wild-type protein in the pah1 mutant?

Reviewer #3 (Comments to the Authors (Required)):

Thaller et al. have improved the manuscript. In principle, I agree to its publication.

In particular, they have addressed my criticism about the ultrastructural data. The new CLEM experiments are impressive. The biochemistry has not substantially improved and this is still a weak part of the paper, but it should not prevent us from moving ahead.

An issue remains to be addressed.

I still don't think that the Fig.2G quantification matches with the actual data that they show. In Fig.2B, the labeling of the last lane for GST-Chm7 indicates 25% PI and nothing else- what are those other 75% of lipids?

Response to Editors and Reviewers regarding MS# **202004222R**

Editors

Dear Dr. Lusk,

*Thank you for submitting your revised manuscript entitled "Direct binding of Chm7 to PA-rich membranes at nuclear envelope herniations" to JCB and for your patience with the re-review process. You will see that the reviewers remain excited by the discovery of Chm7 binding to PA. Two referees now recommend publication, whereas Reviewer #1 has a few remaining concerns. The referee feels that a NE marker is needed for you to state that Chm7 relocates to that compartment in *pah1*-null cells. We agree that the addition of this marker would be valuable.*

Our response: First, it is worth reiterating that the purpose of this experiment was to demonstrate that Chm7 recruitment to membranes in *pah1Δ* cells (where PA levels are elevated) was not a result of triggering nuclear envelope surveillance (i.e. independent of Heh1). Consistent with this, we demonstrated in the revised Fig. 3 that Chm7 still localized to these membranes in *heh1Δpah1Δ* cells. To address the reviewer's remaining concerns, we present co-localization with a dsRed-HDEL (a soluble luminal NE/ER protein) in **Figure S2**. We are confident in concluding, based on these data, that Chm7-GFP is recruited to NE/ER membranes as stated.

The reviewer additionally requests more colocalization studies between Chm7 and the PA sensor, and we have discussed these requests editorially. In our view, the consensus from the reviewers is that, even if there's some uncertainty into how the Chm7-PA relationship works, the results are novel and provocative and expand our understanding of NE dynamics/NPC assembly control and surveillance and the roles of local lipid dynamics in these processes. Therefore, we feel that the results are a great fit for the JCB Report format and we will not require the additional colocalization studies for publication as they would not clarify the functional interplay between PA and Chm7.

Our response: We agree with your assessment, thank you.

If you have such data, please feel free to include them to strengthen the claim that Chm7 targets to PA membranes in cells; on the other hand, if these experiments are not already performed, we will be happy to publish the paper pending final edits to meet our formatting requirements (see below) and pending the addition of the NE marker as suggested by Rev#1 as well as responses to the other reviewer points. Please include a point-by-point response to the reviewer comments with your final files.

Our response: A point-by-point response is included.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: Direct binding of ESCRT protein Chm7 to phosphatidic acid-rich membranes at nuclear envelope herniations

Our response: We have changed the title as suggested.

Running title (50 characters max, including spaces):
ESCRT Chm7 directly binds to phosphatidic acid

Our response: We have changed the running title as suggested.

eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.

Our response: Thaller et al. demonstrate that direct binding between phosphatidic acid (PA) and the ESCRT Chm7 is required for nuclear envelope surveillance; PA also accumulates at nuclear envelope herniations. Thus, tight control of PA metabolism is required for nuclear envelope homeostasis.

2) Figure formatting:

Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

Please be sure to include unit labels (for instance, they are missing in 2BDF)

Our response: These have all been added.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

Our response: Done.

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

Our response: Done.

- For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features *even if described in other published work or gifted to you by other investigators*

Our response: Done.

- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.

Our response: Done.

- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.

Our response: Done.

- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:

- a. Make and model of microscope
- b. Type, magnification, and numerical aperture of the objective lenses
- c. Temperature
- d. imaging medium
- e. Fluorochromes
- f. Camera make and model
- g. Acquisition software
- h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

Our response: Done.

5) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

- Please check our preprint citation guidelines and update the ref list/in-text citations as needed: <https://rupress.org/jcb/pages/reference-guidelines>

Our response: Done.

6) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

Our response: Done.

7) Author contributions: A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

Our response: Done.

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Our response: I think we are ok.

Reviewer #1 (Comments to the Authors (Required)):

This is a much-improved manuscript with better focus than the original version, although there are still two somewhat loosely related parts. Overall, the experiments are elegantly designed, carefully executed and nicely quantified. In the first part the authors show that Chm7, an ESCRT-III component, can associate with phosphatidic acid (PA) in vitro (the association with PA in vivo is less convincing; see comments 1 and 2 below). In the second, the authors show that PA accumulates in sites of likely NE damage such when nuclear pore complex assembly is defective (the nup160 and apq12 mutants), at nuclear membrane herniation or when Chm7 is constitutively active. In the latter case the authors suggest that PA is either recruited directly by Chm7 or that Chm7 alters PA metabolism, resulting in a local accumulation (lines 255-257), but later they show that PA accumulation is independent on Chm7 and propose that PA accumulation is upstream of Chm7 (line 301). While they try to rationalize this apparent contradiction, this underscores the fact that the relationship between PA and Chm7 is not really understood: the order of events at sites of NE damage and the nature of relationship between PA and Chm7 remains unknown: does Chm7 affect PA levels/metabolism, or does PA recruit Chm7, or perhaps both, leading to a positive feedback loop, perhaps driving herniation? The authors really want to present a unified model that links Chm7, PA and Heh1 in membrane repair, but the data tying it all together is not yet at hand.

Our response. This is a fair point.

That said, as far as I can tell the accumulation PA at NE herniations is novel and could potentially lead to insights as to how the cell deals with this type of insult.

Our response. We agree.

Specific comments:

1. Figure 3A: The authors show that in the *pah1* Δ mutant, which exhibits ER proliferation as determined by EM (see earlier work by several labs), Chm7 accumulates as a band, which they interpret as ER/NE association due to enrichment for PA. First, to conclude that this band reflects NE association the authors must use a NE-specific marker. Second, the authors should use the PA-specific sensor based on Opi1 that was used later in the study to determine if the band formed by Chm7 is specific to a PA rich-membrane. This is important because part of the key conclusion of this study is that Chm7 is attracted to PA, and if these bands do not co-localize with PA-rich membrane then the authors need to rethink their conclusion.

Our response. We disagree about the importance of this experiment to informing our model as by far the strongest data demonstrating that Chm7 binds directly to PA is found in the liposome experiments in Figure 2. We have, nonetheless, included data showing co-localization of Chm7-GFP in *pah1* Δ cells with a NE/ER marker (dsRED-HDEL). These data are presented in **Figure S2**.

2. Figure 4E: another case where the authors could have examined the co-localization of Chm7 and PA is in the *nup116* mutants (and to a lesser extent in the *apq12* Δ mutants), where clear PA sensor foci are seen. Does Chm7 also localize to these foci, and if so- to what fraction of foci? If the localization is not complete, could Chm7 act in a "catalytic" manner, creating a localized PA focus before moving to the next spot? And if there is little or no co-localization, how do the authors explain that?

Our response. This point was brought up in the initial review and was addressed in the first rebuttal.

3. In the CLEM analysis, the authors note that 11 of 12 PA sensor foci localized to regions of NE herniation. What about the converse? Namely, where there herniations that did not co-localize with PA sensor foci? If so, what would be the explanation?

Our response. A limitation of CLEM is that substantial fluorescence is lost during the freeze substitution and embedding process. Therefore, one cannot make any conclusions about the absence of fluorescence in a cell (indeed, many cells simply don't have any fluorescence left). Conversely, the protocol ensures that we are only examining areas where there are high levels of fluorescence, or in this case, of the PA sensor. As these locations were essentially always associated with herniations, we are confident in our conclusions.

Reviewer #2 (Comments to the Authors (Required)):

The authors addressed most of the issues I raised. Together with their responses to the other reviewers, their paper has now significantly improved - in particular the revised Fig. 3 and 5 - and in my view the paper is now appropriate for publication. I have only two (minor) comments: (a) L. 238 "Chm7 hyperactivation drives local PA accumulation at the INM" I still think it would be better to specify that these conclusions are based on the distribution of a PA sensor rather than actual PA levels and (b) revised Fig. 3A and S2A: have the authors confirmed that the W3AV1A-GFP is expressed at similar levels as the wild-type protein in the pah1 mutant?

Our response: We have amended all the text where we indicated direct binding to PA. Further, while we do not have western blots, we examined all constructs at many different levels of expression (as assessed by total fluorescence) and did our best to normalize within experiments.

Reviewer #3 (Comments to the Authors (Required)):

Thaller et al. have improved the manuscript. In principle, I agree to its publication.

In particular, they have addressed my criticism about the ultrastructural data. The new CLEM experiments are impressive. The biochemistry has not substantially improved and this is still a weak part of the paper, but it should not prevent us from moving ahead.

An issue remains to be addressed.

I still don't think that the Fig.2G quantification matches with the actual data that they show.

Our response: We apologize that we missed this concern of the reviewer. We agree that, for the replicate shown, that the quantification did not seem to match well with the raw data. We have replaced that panel with a more representative experiment.

In Fig.2B, the labeling of the last lane for GST-Chm7 indicates 25% PI and nothing else- what are those other 75% of lipids?

Our response: this has been fixed.