Regulated lipid synthesis and LEM2/CHMP7 jointly control nuclear envelope closure

Lauren Penfield, Raakhee Shankar, Erik Szentgyörgyi, Alyssa Laffitte, Michael Mauro, Anjon Audhya, Thomas Müller-Reichert, and Shirin Bahmanyar

Corresponding Author(s): Shirin Bahmanyar, Yale University

Review Timeline:

| Event                      | Date       |
|----------------------------|------------|
| Submission Date            | 2019-08-24 |
| Editorial Decision         | 2019-09-18 |
| Revision Received          | 2020-01-22 |
| Editorial Decision         | 2020-02-10 |
| Revision Received          | 2020-02-27 |

Monitoring Editor: Michael Rout

Scientific Editor: Marie Anne O'Donnell

Transaction Report:

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

DOI: https://doi.org/10.1083/jcb.201908179
September 19, 2019

Re: JCB manuscript #201908179

Prof. Shirin Bahmanyar
Yale University
Molecular Cellular and Developmental Biology
219 Prospect St.
Kline Biology Tower 800
New Haven, CT 06520

Dear Prof. Bahmanyar,

Thank you for submitting your manuscript entitled "Local regulation of lipid synthesis controls ER sheet insertion to close nuclear envelope holes" and thank you for your patience with the review process. The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers provided mixed critiques. Revs#1 and #2, who are closer to this topic than Rev#3, a broader nuclear envelope and organization expert, found the work of high quality and interesting. Rev#1 was most positive and only suggested minor changes prior to publication. Rev#2 asked for more clarity on the topological relationship between ER membranes and the NE and the mode of closure (point #1) as well as more evidence that these ER membranes do help the closure process (#2) and that CNEP-1 operates in NE closure independently of ESCRTs (#3 - 4, with requests for additional analyses of the localization of the machinery in cnep-1 mutant embryos). The referee additionally asked for controls for RNAi/CRISPR depletions (minor point #2). Rev#3 felt that the work was not yet accessible enough and their points could be used to guide revisions to streamline and clarify the interpretation and data presentation for non-experts. Rev#3's concerns about the degree of accessibility seemed important to us, coming from a broad expert with significant experience at the journal and in the field.

The reviewer response is divided, with two reviewers raising a significant number of serious issues that require revisions. However, if you feel you can fully address the points raised by Reviewers #2 and #3, we would be interested in considering a revised manuscript. The points they raise call into question some of the key conclusions of the Report, hence we feel that it will be important for these remarks to be addressed both experimentally and textually in full, as described. Please let us know if you anticipate any issues addressing these points. As well as addressing the experimental issues raised, and addressing Reviewer #3's request for "a careful and judicious evaluation of the data and their presentation", it is very important that significant effort be made to clarify the text throughout, as it is currently sufficiently abstruse as to exclude non-specialists; this would then make the work consistent with our Reports presenting novel results of high interest that are accessible to a broad cell biological audience. We would be happy to further discuss the revisions as needed.

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 prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

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.

Our typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. 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

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

Penfold et al., present a clear, concise and well written manuscript detailing the role of the CTDNEP1/LIPIN (CNEP-1/LPIN-1) in regulating phospholipid flux for the generation of ER-derived membranes that assist in the sealing of large gaps in the reforming nuclear envelope. They make
good use of the 1-cell c-elegans embryo to analyse events at the maternal pronucleus and later at the c-elegans mitotic nucleus. The data are convincing, are of high-quality, are well quantified and interpreted and make a compelling argument for how the NE is regenerated.

ESCRTs and the INM protein LEM2 have been implicated in this process in recent years, and importantly, whilst they observe ESCRT/LEM2 recruitment to the spindle-proximal NE of the maternal pronucleus, the rate of compartmentalisation acquisition was independent of ESCRTs, but dependent upon the generation of ER derived membranes. High resolution Cryo-ET approaches showed the presence of ER-sheets that acted to plug larger holes, and so narrow them through point-fusion. In the absence of Cnep-1, extensive ER-sheets penetrated the nuclear interior and compromised the ability of the nucleus to compartmentalised. Again, good usage of ET and genetic approaches to illuminate these phenotypes. These effects were not dependent upon ESCRTs, pointing to parallel hole-plugging and hole-closure activities. The hole-plugging activity of ER-derived membranes is novel, exciting and suitable for publication in the JCB.

I think the manuscript is of high quality; my only very minor critiques would be to include citation of Marakova et al., CB, 2016 in the discussion of hyperproliferation of ER membranes in the absence of Lipin/Nem1. Additionally, whilst Lipin dephosphorylates PA to make DAG, the text (and cartoon in Fig 4A) is written as though the switch is from one form of structural lipid to another (PI to PC/PS) and this makes it hard to understand where the excess membrane would come from. I may be wrong, but I thought an additional fate of Lipin-produced DAG was to produce storage lipids that no-longer contributed to membranes.

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

Penfield et al. report new observations of the dynamics and morphology of the nuclear envelope (ER) during C. elegans embryogenesis. By synthesizing electron tomography with fluorescence imaging of living embryos and with genetic perturbations, the authors propose that sheets of the endoplasmic reticulum (ER) can patch holes in the nascent nuclear envelope during anaphase to promote early compartmentalization. As reported previously, the phosphatidic acid phosphatase, lipin, as well as lipin's activator, cnep-1, influence the biogenesis of these ER sheets. There are also notable genetic interactions between cnep-1 and components of the ESCRT pathway, which are critical for recruiting the microtubule severing enzymes like spastin that disassemble the mitotic/meiotic spindle and catalyze the final membrane fusion reaction that seals the nascent NE. Appropriate cnep-1 activity limits the growth of ER sheets, and cnep-1 loss of function leads to overgrowth of ER sheets. These "overgrown" membrane sheets sometimes invade the nucleoplasm and distort nuclear envelope morphology. The author's model builds on prior work concerning annulate lamellae and cnep-1 mutants, and proposes that-besides being a source for rapid NE expansion during embryogenesis-these membrane sheets have adapted to "invade" large gaps in the NE to limit mixing between the nucleoplasm and the cytoplasm. This idea warrants further study, but in its current form, I have some questions about both the experimental observations and alternative models.

Major:

1. In Figure 5B, the authors use a cartoon schematic to illustrate their model for the connectivity and topology of the ER sheets, which they propose help close the nucleus. This cartoon figure is reminiscent of the topological relationship between annulate lamellae and the NE, and it makes
sense to me. However, it's not clear to me that their data support or falsify this straightforward model. For the wild-type embryos studied by electron tomography in Figure 2B-D and the supplemental movies, the topology/connectivity of the "blue" membrane sheets is, at best, unclear. Rather than being topologically continuous with the ER, the putative membrane sheets in question (which we only see as false-colored objects because they are not apparent in the raw data) appear to be separated from the NE and overlay the NE gaps like "band-aids." Figure 2D is particularly hard to interpret in this regard. Can the authors improve the figures representing the raw data and the segmented tomograms to remove uncertainty about the topological relationship between the NE and these membrane sheets? I also found the phrasing "excess incorporation of ER membranes" to be too vague. What does "incorporation" mean in molecular terms? Applying a patch like a band-aid? Or lateral flow through sheet expansion (which is how I interpret the model in Fig. 5B)?

2. How can we be confident that the "blue" false-colored membrane sheets have a specific spatial and molecular relationship with the gaps in the NE? At this stage of anaphase, there are many holes in the NE, and ER sheets are abundant in proximity to the reforming nucleus, so it seems that we should expect some NE gaps will be close to ER sheets just by chance.

3. Some of these authors have shown in a prior study that loss of cnep-1 leads to profound distortions in overall ER/NE morphology (and this is again clear in Fig. 3A and a supplemental movie, which also suggests that combining loss of cnep-1 with loss of chmp7 aggravates the morphology of the NE and the ER). The observation that cdgs-1 loss-of-function rescues these phenotypes suggests alternative explanations for the defects in NE integrity seen with loss of cnep-1 +/- activity. As the authors wrote, loss of cnep-1 will lead to increased levels of the lipids PI and PIPs. In addition to the PIP2-binding and negative ESCRT-regulator, CC2D1B, many other membrane-binding and remodeling proteins will associate, inappropriately, with these over-abundant and PPIP-rich membranes. Thus, I am not yet persuaded by the author's argument, "our genetic interactions and localization studies indicate that the function of CNEP-1 in NE closure is independent of the ESCRT machinery." One way of interpreting an aggravated genetic interaction is the parallel but independent pathway model— but I think this is only justified when considering true null mutants that have not yet adapted to the loss of the gene in question (put another way, interpreting genetic interactions is rarely so straightforward).

4. Related to point 3, Figure S2 is far from definitive evidence that LEM2 localization is unaffected by loss of cnep-1. By contrast, LEM2-::GFP appears to be accumulating in malformed membranes—suggesting that loss of cnep-1 is directly distorting the localization and therefore the functioning of the LEM2-ESCRT pathway. In addition to a more quantitative description of LEM2 (mis)localization in cnep-1 mutants, I would also like to see where CC2D1B, CHMP7, and SPASTIN localize in cnep-1 mutant embryos. Finally, please assess nuclear entry of 70kDa dextran in lem2 and lem2xcnep-1 mutants in parallel with the chmp7 and chmp7xcnep-1 mutants.

Minor:

1. The authors repeatedly claim that lipid synthesis is "locally" regulated—without defining local. Given that membranes are 2D fluids within which lipids freely and rapidly diffuse, the need for local control is not clear to me. Moreover, the author show in Fig 3J that CNEP-1-GFP uniformly localizes around the NE. If by "local," the authors mean the entire NE, I would favor removing this descriptor from the manuscript. Especially considering how severely distorted the entire NE/ER system appears in cnep-1 and cnep-1 x chmp7 mutants.

2. The authors verify that their RNAi knockdown of Vps32 (Snf7/CHMP4) reduces protein levels to
~<20% of wild type levels. Since some protein remains, there is a chance they are over-interpreting the apparent lack of a phenotype of this incomplete knockdown. It would be surprising, given the prior work on this problem, if the loss of Vps32 had no NE phenotype. The authors should also include data—even if reliable antibodies are not available for the C. elegans proteins—that addresses the effectiveness of their other RNAi and CRISPR-mediated genetic perturbations.

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

Penfield et al., use C. elegans as model to understand nuclear envelope closure. They report that CNEP-1 regulates the local control of glycerophospholipid synthesis to control ER sheet insertion into NE holes associated with microtubules. Further, recruitment of ESCRT-III by LEM-2 and CHMP7 mediates membrane closure.

The data presented in this report are overwhelming in abundance, and much of them appear peripheral to the main message of this Report. As a JCB Report, this work would benefit immensely from a careful and judicious evaluation of the data and their presentation. Moreover, the main message that CNEP-1 locally regulates LIPN is either lost in the presentation or is inconsistent with data presented in Figure 3J, which shows CNEP-1 distributed around the entire NE. LPIN-1 is not explicitly included in this study.

Additional points for the authors to consider:
"We report a role for CNEP-1 in biasing flux away from PI synthesis to promote closure of the NE..." - data are not supportive of this statement.
"short time between the end of meiosis II and entry into mitosis ... may not be sufficient..." statement is not supported or referenced.

Figure 1: Confusing - GFP should be labeled green and mCherry red (or magenta). This confuses the presentation. Arrowheads are not defined.
Figure 1F: not consistent with figure legend or text.
Figure 1H: what is x-axis? PC-regression not defined.

Figure 2: Seems overkill - multiple views of same image without orientation for the reader and warrants better explanation.
Figure 2E appears to be the same as Figure S2 C and D (yet in the latter the image is contiguous?) Is this a different slice?
Figure 3: Unclear what the point is of the major and minor phenotypes (3A).
Figure 3: Why are different NLS reporters presented and used? Where is the sperm nucleus in chmp-7(RNAi)?
Figure 3C-H: seem irrelevant, should be explained in text in context of overall hypothesis.
Figure 4A: Suggests the model is established, but elements are not actually tested directly.
Figure 5: Helpful model, not entirely clear the data support it, especially with respect to local lipid synthesis. Implies that CNEP-1 is localized to the rupture. Is it?
Reviewers: Penfold [Penfield] et al., present a clear, concise and well written manuscript detailing the role of the CTDNEP1/LIPIN (CNEP-1/LPIN-1) in regulating phospholipid flux for the generation of ER-derived membranes that assist in the sealing of large gaps in the reforming nuclear envelope. They make good use of the 1-cell c-elegans embryo to analyse events at the maternal pronucleus and later at the c-elegans mitotic nucleus. The data are convincing, are of high-quality, are well quantified and interpreted and make a compelling argument for how the NE is regenerated.

Reviewer #1: The hole-plugging activity of ER-derived membranes is novel, exciting and suitable for publication in the JCB.”

1. Reviewer #1. My only very minor critiques would be to include citation of Marakova et al., CB, 2016 in the discussion of hyperproliferation of ER membranes in the absence of Lipin/Nem1.

We thank the reviewer for bringing this to our attention. We have now included the reference in the introduction (new text on pg. 3).

2. Reviewer #1. Additionally, whilst Lipin dephosphorylates PA to make DAG, the text (and cartoon in Fig 4A) is written as though the switch is from one form of structural lipid to another (PI to PC/PS) and this makes it hard to understand where the excess membrane would come from. I may be wrong, but I thought an additional fate of Lipin-produced DAG was to produce storage lipids that no-longer contributed to membranes.

The reviewer is correct that an additional fate of lipin-produced DAG is to make storage lipids. Several lines of evidence suggest that the situation is slightly different in C. elegans embryos. First, in fungi structural phospholipids are synthesized through CDP-DAG, a branch that does not exist in metazoans and so the flux in the pathway is strictly between PI versus PC/PE and storage lipids (see new Fig. 5 A). Second, in C. elegans triglycerides are synthesized in the intestine and transferred to developing oocytes during reproduction (Watts and Ristow, 2017) and so we don’t expect that the flux of the pathway goes towards storage lipids during embryonic division. Third, our previous work (see Bahmanyar et al., 2014) provides biochemical evidence that cnepl-1 mutant worms contain higher levels of PI and RNAi-depletion of CDP-DAG synthase (cdgs-1), an enzyme required for one of the two steps to convert PA to PI, restores PI levels and ectopic ER sheets. In this manuscript, we provide genetic data (see new Fig. 5) that RNAi-depletion of cdgs-1 also rescues the leaky nuclear phenotypes of cnepl-1 mutants. We now provide data showing that RNAi-depletion of PI synthase (pisy-1), the other enzyme required for PI production, rescues the leaky nuclear phenotypes of cnepl-1 mutants as well (new panel Fig. S3 F). Together, these results suggest that high PI levels cause formation of ectopic ER sheets that interfere with nuclear sealing. Nevertheless, to help clarify that storage lipids are a part of the de novo synthesis pathway and that lipin-produced DAG is incorporated into storage lipids under certain situations, we have added new text to our introduction (see new text pg. 3-4) and also to the schematic in new Fig. 5 A.

Because PI is a low abundant lipid of the ER, the reviewer is correct that “it [is] hard to understand where the excess membrane would come from.” Understanding whether PI directly or indirectly
causes ectopic ER sheets to form is beyond the scope of this paper so we have added text to the discussion to provide potential explanations (see new text pg. 10).

Reviewer #2: The author's model builds on prior work concerning annulate lamellae and cnp-1 mutants, and proposes that besides being a source for rapid NE expansion during embryogenesis—these membrane sheets have adapted to "invade" large gaps in the NE to limit mixing between the nucleoplasm and the cytoplasm. This idea warrants further study, but in its current form, I have some questions about both the experimental observations and alternative models.

Reviewer #2: Major

1. Reviewer #2. In Figure 5B, the authors use a cartoon schematic to illustrate their model for the connectivity and topology of the ER sheets, which they propose help close the nucleus. This cartoon figure is reminiscent of the topological relationship between annulate lamellae and the NE, and it makes sense to me. However, it's not clear to me that their data support or falsify this straightforward model. For the wild-type embryos studied by electron tomography in Figure 2B-D and the supplemental movies, the topology/connectivity of the "blue" membrane sheets is, at best, unclear. Rather than being topologically continuous with the ER, the putative membrane sheets in question (which we only see as false-colored objects because they are not apparent in the raw data) appear to be separated from the NE and overlay the NE gaps like "band-aids." Figure 2D is particularly hard to interpret in this regard. Can the authors improve the figures representing the raw data and the segmented tomograms to remove uncertainty about the topological relationship between the NE and these membrane sheets?

We agree with the reviewer that this presentation of the tomography data was confusing. We have addressed this issue to reduce the confusion about the topological relationship between the NE and these membrane sheets in several ways.

1. We included more examples of membranes nearby holes to help clarify their topology (new Fig. 2 A and B). These examples are from a third tomography data set that more closely aligns with the timing of nuclear closure in the stage of meiosis II represented in our fluorescence imaging data (see Fig. S1 G, H and also see reviewer #2, point 3).

2. We trace and represent the membranes adjacent to the nuclear envelope in a single color to provide a less biased view of membrane topology (see new Fig. 2, new panel Fig. S1 F and Fig. S2 A).

3. To further clarify the relationship between the NE and membrane sheet-like structures, we show several more z-sections of the untraced 2D sections of the raw tomography data and include colored arrowheads to orient the reader (see 2D images in new Fig. 2 A and B and Fig. S2 A).

Thus, we believe the new examples and analysis strongly supports the schematic model (see new Fig. 5 G) that these membranes are feeding into NE holes through lateral flow of lipids. We have changed the wording of the manuscript to also represent this interpretation (see below).
2. Reviewer #2. I also found the phrasing "excess incorporation of ER membranes" to be too vague. What does "incorporation" mean in molecular terms? Applying a patch like a band-aid? Or lateral flow through sheet expansion (which is how I interpret the model in Fig. 5B)?

We thank the reviewer for this suggestion. We include new analysis, new representation of the tomography data, and a new tomography data set (see above, Reviewer #2 point 1) to provide further evidence in line with our interpretation that the cytoplasmic membrane structures are contiguous with the nuclear envelope, we have changed the wording "incorporation" to "lateral flow" or "feed into" when referring to these membranes (see new text throughout manuscript).

3. Reviewer #2. How can we be confident that the "blue" false-colored membrane sheets have a specific spatial and molecular relationship with the gaps in the NE? At this stage of anaphase, there are many holes in the NE, and ER sheets are abundant in proximity to the reforming nucleus, so it seems that we should expect some NE gaps will be close to ER sheets just by chance.

We thank the reviewer for this comment. To address this point we traced all membranes that contain a lumen in a ~2.5 x 3.5 x .15 micron region in the vicinity of the reforming nuclear envelope (see new Fig. 2 A and B). This analysis revealed that very few membrane structures are directly adjacent to the region of the NE that faces the meiotic spindle (see new Fig. 2 A and B). The number of ER membranes that contact the NE within this region is 14 in one electron tomogram(new Fig. S2 A and Video 3; also see new text pg. 6) and 15 in a different tomogram (new Fig. 2 A and B, Video 6 and 7; also see new text pg. 6) with 71% and 73% of them associated with NE holes, respectively (see new text pg. 6). These data indicate that cytoplasmic membranes are mostly excluded from the meiotic spindle region. The fact that the majority of the few membranes present in this region are directly adjacent to a NE hole suggests that this proximity is not just by chance.

4. Reviewer #2. Some of these authors have shown in a prior study that loss of cnep-1 leads to profound distortions in overall ER/NE morphology (and this is again clear in Fig. 3A and a supplemental movie, which also suggests that combining loss of cnep-1 with loss of chmp7 aggravates the morphology of the NE and the ER). The observation that cdgs-1 loss-of-function rescues these phenotypes suggests alternative explanations for the defects in NE integrity seen with loss of cnep-1 +/- activity. As the authors wrote, loss of cnep-1 will lead to increased levels of the lipids PI and PIPs. In addition to the PI2-binding and negative ESCRT-regulator, CC2D1B, many other membrane-binding and remodeling proteins will associate, inappropriately, with these over-abundant and PI/PIP-rich membranes. Thus, I am not yet persuaded by the author’s argument, "our genetic interactions and localization studies indicate that the function of CNEP-1 in NE closure is independent of the ESCRT machinery." One way of interpreting an aggravated genetic interaction is the parallel but independent pathway model-but I think this is only justified when considering true null mutants that have not yet adapted to the loss of the gene in question (put another way, interpreting genetic interactions is rarely so straightforward).

We agree with the reviewer that, based on the genetic evidence we obtained, we cannot be certain that the role of CNEP-1 in nuclear closure is completely independent of ESCRT components. We have changed this wording throughout the text to more directly describe our observations.
We fixed the abstract (pg. 1) to read:

“Loss of NE adaptors for ESCRT-III exacerbates ER invasion and nuclear permeability defects in cnep-1 mutants, suggesting that ESCRTs restrict excess ER membranes during NE closure.”

We fixed the conclusion paragraph (pg. 11) in the discussion to read:

“Together our data demonstrate that the CNEP-1-lipin pathway coordinates the production of ER membranes with the feeding of ER membranes into NE openings to promote nuclear closure. This process requires ESCRT-mediated membrane remodeling when membranes are in excess.”

5. Reviewer #2. Related to point 3, Figure S2 is far from definitive evidence that LEM2 localization is unaffected by loss of cnep-1. By contrast, LEM2::GFP appears to be accumulating in malformed membranes-suggesting that loss of cnep-1 is directly distorting the localization and therefore the functioning of the LEM2-ESCRT pathway. In addition to a more quantitative description of LEM2 (mis)-localization in cnep1- mutants, I would also like to see where CC2D1B, CHMP7, and SPASTIN localize in cnep-1 mutant embryos.

To address the reviewer’s comment for “a more quantitative description of LEM2 (mis)-localization in cnep1- mutants,” we quantified the dynamics of GFP::LEM2 localization at the nuclear rim in cnep-1 mutants using anaphase of meiosis II as a reference time point (see new Fig. S2 F). Our analysis revealed a slight delay of ~30 s in the first appearance of GFP::LEM2 to the reforming nuclear envelope, which led to a delay in subsequent events (plaque formation and dispersal) relative to anaphase II. These data support the reviewer’s argument that LEM-2 may be disrupted in its dynamics/localization in the cnep-1 mutant, which could in part contribute to the nuclear phenotypes in cnep-1 mutants. We have modified the text in the Results section (see new text pg. 8) to read:

“ESCRT components localized to the NE in cnep-1 mutants, although there was a mild delay in the onset of ESCRT-dependent events relative to anaphase II (Fig. S2 D and F-H).”

Also also added new text in the Discussion (pg. 10-11) that reads:

“PIP2 regulates the recruitment of ESCRTs to the reforming NE (Ventimiglia et al., 2018) and its misregulation may account for the mild delay we observed in the recruitment of ESCRT components in cnep-1 mutants.”

To address the comment “I would also like to see where CC2D1B, CHMP7, and SPASTIN localize in cnep-1 mutant embryos,” we did the following:

1. Used an antibody made against C. elegans CHMP-7 that showed CHMP-7 localizes to a plaque that co-localizes with GFP::LEM-2 in control and cnep-1 mutant embryos (new panels Fig. 1 B and Fig. S2 H).

2. Requested and used a published antibody made against C. elegans spastin from another lab, but this antibody did not work for immunofluorescence. As an alternative approach, we measured the time of detachment of the meiotic spindle from the reforming NE relative to anaphase II onset to determine if spastin function may be altered in cnep-1 mutant embryos (see new panel Fig. S2 G). To do this analysis we scored the time point at which the spindle and oocyte-derived pronucleus are no longer
adjacent to each other. We noticed a slight shift in the time of spindle detachment compared to the control (control average +/- SD, 390 s +/- 95 s; cnep-1 mutant average +/- SD, 446 s +/- 70 s) but this was not statistically significant, (p value: 0.19 Mann Whitney U test).

3. Unfortunately, we and others do not have the tools to study CC2D1B in C. elegans so we were unable to determine where it is localized in wild type and cnep-1 mutants. While we agree that it would be valuable to learn more about CC2D1B in our system, building the tools would take us several months and, given the evidence stated above, we do not think it will significantly change our interpretations and would require work outside the scope of this manuscript for full characterization.

6. Reviewer #2. Finally, please assess nuclear entry of 70kDa dextran in lem2 and lem2xcnep-1 mutants in parallel with the chmp7 and chmp7 x cnep-1 mutants.

We thank the reviewer for this suggestion. We added these data to the manuscript (see new panel Fig. 5 E).

Reviewer #2: Minor

1. Reviewer #2. The authors repeatedly claim that lipid synthesis is "locally" regulated-without defining local. Given that membranes are 2D fluids within which lipids freely and rapidly diffuse, the need for local control is not clear to me. Moreover, the author show in Fig 3J that CNEP-1-GFP uniformly localizes around the NE. If by "local," the authors mean the entire NE, I would favor removing this descriptor from the manuscript. Especially considering how severely distorted the entire NE/ER system appears in cnep-1 and cnep-1 x chmp7 mutants.

The reviewer makes a good point. We have removed "local" from the title and from the descriptions within the manuscript. We now include a panel that shows the localization of lipin to the ER and NE during meiosis II next to the panel that shows CNEP-1 enriched at the NE in staged matched embryos (see new panels Fig. 5 B and C) to clarify the spatial relationship of their localization and our interpretation that CNEP-1 is acting from the NE to regulate lipin and control nuclear and ER dynamics, as defined by our previous publication Bahmanyar et al., 2014.

2. Reviewer #2. The authors verify that their RNAi knockdown of Vps32 (Snf7/CHMP4) reduces protein levels to ~<20% of wild type levels. Since some protein remains, there is a chance they are over-interpreting the apparent lack of a phenotype of this incomplete knockdown. It would be surprising, given the prior work on this problem, if the loss of Vps32 had no NE phenotype. The authors should also include data—even if reliable antibodies are not available for the C. elegans proteins—that addresses the effectiveness of their other RNAi and CRISPR-mediated genetic perturbations.

To address the reviewer’s comment “Since some protein remains, there is a chance they are over-interpreting the apparent lack of a phenotype of this incomplete knockdown,” we include an immunoblot of VPS-32 RNAi-depletion with a dilution series to show that VPS-32 is depleted to <15% (see new panel Fig. S3 A). The reviewer is correct, however, that this level of knockdown is incomplete making it difficult to assess the consequence of loss of VPS-32 on NE sealing. To address
this issue, we show that VPS-32 fails to localize to the NE in the CHMP-7 deletion (see new panel in Fig. 1 C). This result supports our conclusion that NE-localized VPS-32 is not required to establish the nuclear permeability barrier in meiosis.

To our knowledge what the reviewer is referring to in the comment “it would be surprising, given the prior work on this problem, if the loss of Vps32 had no NE phenotype” was of RNAi-depletion of CHMP2A in mammalian cells, which causes the persistence of CHPMP4B foci and loss of spastin localization to the reforming NE (Olmos et al., 2015; Vietri et al., 2015). We believe that our data are in line with the results from these studies that show defects in the nuclear permeability barrier under are only observable with a more sensitive assay (see Vietri et al., 2015, Fig. 4) or after a prolonged time after NE formation (see Olmos et al., 2015, Fig. 4). Thus, an alternative explanation to why we do not see a NE phenotype in embryos RNAi-depleted for VPS-32 is that our reporter assays are limited in their sensitivity and there is a short time period between nuclear formation in meiosis II and nuclear envelope breakdown of the next mitosis in early embryos. To help clarify this discrepancy, we word our conclusion on VPS-32 RNAi-deletion (pg. 8) to read:

“Together, these data indicate that LEM-2/CHMP-7 contribute to nuclear closure, possibly independently of VPS-32, in cnep-1 mutants to restrict the free diffusion of large proteins across the NE.”

To address the reviewers comment: “The authors should also include data—even if reliable antibodies are not available for the C. elegans proteins—that addresses the effectiveness of their other RNAi and CRISPR-mediated genetic perturbations” we performed immunoblots of the strains with CRISPR-mediated genetic deletion of chmp-7 used in this study (see new panel in Fig. S1 A), we added a dilution series to address the levels of RNAi-depletion of vps-32 (see new panel Fig. S3 A), and we removed data in Fig. 1 in which RNAi-depletion of lem-2 was used and did not show a phenotype (see new panel Fig. 1 G). As an internal control to assess the partial RNAi-knockdown of lpin-1, we used the reported phenotype of ER clustering in the early embryos (see Fig. S3 C and Bahmanyar et al. 2014). Unfortunately, we do not have an antibody available for CDGS-1 (CDP-DAG synthase 1). To address this issue, we performed RNAi-depletion of pisy-1 (PI synthase 1), which is the enzyme acting downstream of CDGS-1 to produce PI, as an independent approach to test if reduced PI synthesis rescues the premature nuclear entry of GFP::α-tubulin that occurs in cnep-1 mutants (see new panel Fig. S3 F).

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

1. Reviewer #3. The data presented in this report are overwhelming in abundance, and much of them appear peripheral to the main message of this Report. As a JCB Report, this work would benefit immensely from a careful and judicious evaluation of the data and their presentation.

We thank the reviewer for feedback on the presentation of the data in our manuscript. To address this point, we did our best to omit information peripheral to the message, simplified figures and their representation by omitting data panels, placing some figure panels in the supplemental materials, and splitting some of the data into two figures (see new Fig. 3 and 4).

2. Reviewer #3. Moreover, the main message that CNEP-1 locally regulates LIPN is either lost in the presentation or is inconsistent with data presented in Figure 3J, which shows CNEP-1 distributed around the entire NE. LPIN-1 is not explicitly included in this study.
Thank you for this comment. We did not intend for “local” to mean that CNEP-1 is regulating lipin at the sealing site but rather from the nuclear envelope rather than from the contiguous ER. To clarify this, we have removed “local” from the title and throughout the manuscript. Because in early C. elegans embryos, lipin is localized throughout the ER and NE (see new Fig. 5 B and C, also see Bahmanyar et al., 2014), whereas CNEP-1 is localized to the NE, we suggest that CNEP-1 is regulating lipin from the NE. We have clarified this point throughout the text and in the title.

To address the point that “LPIN-1 is not explicitly included in this study” we show lipin’s localization (see new panel Fig. 5 C), we provide data that LPIN-1 is required for nuclear closure (see Fig. S3 C and D), and we include LPIN-1 in the schematic models (see Fig. 5 A) and more clearly highlight our results describing a role for lipin in nuclear closure (see new text throughout manuscript). Furthermore, it is well-established in yeast and C. elegans that CNEP-1/Nem1 is regulating a pool of lipin that is important for NE structure, which we reference in our introduction and throughout the manuscript.

Additional points for the authors to consider:

3. Reviewer #3. "We report a role for CNEP-1 in biasing flux away from PI synthesis to promote closure of the NE..." - data are not supportive of this statement.

Thank you for pointing this out. We omitted this statement. We also reference our previous work (Bahmanyar et al., 2014) throughout the text when we discuss CNEP-1’s role in biasing flux away from PI synthesis.

4. Reviewer #3. "short time between the end of meiosis II and entry into mitosis ... may not be sufficient..." statement is not supported or referenced.

We have omitted this statement.

5. Reviewer #3. Figure 1: Confusing - GFP should be labeled green and mCherry red (or magenta). This confuses the presentation. Arrowheads are not defined.

We intentionally labeled markers for the nuclear envelope in magenta and have kept the nuclear envelope in magenta consistently throughout the paper since we have markers both with a mCherry or GFP tag for the nuclear envelope. We have clearly labeled which marker is which color within the figures to minimize confusion. We have defined arrowheads in Fig. 1 legend.

6. Reviewer #3. Figure 1F: not consistent with figure legend or text.

We have changed the wording in the text that refers to this figure panel to be more explicit and it now reads (pg. 5): “the accumulation of the GFP::LEM-2 plaque correlates with loss of fluorescence signal of GFP::α-tubulin at the meiotic spindle (Fig. 1 F, compare 140 s to 220 s; Video 1).” We have simplified and shortened the figure legend and it is consistent with the figure panel.

7. Reviewer #3. Figure 1H: what is x-axis? PC-regression not defined.
We include a schematic and spell out pseudocleavage regression to clarify the fact that this stereotypical event is used a reference time point in the x-axis of the plot (see new Fig. 1 G). We also add new text in the figure legend for clarity and provide a reference of our previous paper that includes a detailed analysis of nuclear events relative to the reference time point used (see pg. 12).

8. Reviewer #3. Figure 2: Seems overkill- multiple views of same image without orientation for the reader and warrants better explanation.

We thank the reviewer for this comment. We have taken out most of the data from the old figure (see new panel Fig. S1 F and S2 A, B) and changed the main figure dramatically to clearly display examples from a new tomography data set of meiosis II that more closely aligns with the time point we analyze in the paper (see new Fig. 2). We have included arrowheads and description within the figure legend (see new text in Fig. 2 legend, pg. 12) and text to orient the reader of the models and tomogram z-slices (see new text pg. 6).

9. Reviewer #3. Figure 2E appears to be the same as Figure S2 C and D (yet in the latter the image is contiguous?) Is this a different slice?

We have removed these to avoid repetition as well as confusion.

10. Reviewer #3. Figure 3: Unclear what the point is of the major and minor phenotypes (3A).

We show examples and define “minor” and “major” in the text (see Fig. 3 B and text on pg. 7). To help clarify this point further, we have changed the color of our graph to more clearly represent the enhanced severity of the phenotype in the double mutant.

10. Reviewer #3. Figure 3: Why are different NLS reporters presented and used? Where is the sperm nucleus in chmp-7(RNAi)?

We only use one NLS reporter throughout the manuscript. The figure the reviewer is referring to is now simplified and some of the data is placed in a new figure (see new Fig. 3 and Fig. 4). We do point out that the differences in the size of the NLS reporter versus the GFP::α-tubulin reporter provides us with valuable information on the degree of severity of the nuclear leakiness phenotype in different genetic backgrounds (see pg. 8: “While a small NLS reporter leaks out of the NE in cnep-1 mutants, cnep-1Δ pronuclei were able to exclude larger macromolecules since GFP::α-tubulin, which forms a ~125 kDa heterodimer with β-tubulin and is excluded from the nucleus” and pg. 8 “nuclear GFP::α-tubulin was observed at significantly earlier time points, and exclusively in the oocyte-derived pronucleus, in cnep-1Δ embryos also RNAi-depleted for chmp-7 or lem-2 (Fig. 4 A and B and Video 9”).

In response to the reviewer’s comment “where is the sperm nucleus in chmp-7(RNAi)” we are showing a single z-slice of the embryo and the sperm-derived pronucleus is not in the same z-slice. We have added a note to the figure legend for clarification.
11. Reviewer #3. Figure 3C-H: seem irrelevant, should be explained in text in context of overall hypothesis.

We address this issue by distributing the data in old figure 3 into two figures (see new Fig. 3 and Fig. 4) to clarify the point of the data in the figure panels the reviewer is referring to in the context of the overall hypothesis (see text pg. 8-9).

11. Reviewer #3. Figure 4A: Suggests the model is established, but elements are not actually tested directly.

The reviewer is referring to new Fig. 5A, which provides background of the well-established pathway of de novo glycerolipid synthesis in metazoans. We clarify that the pathway represents the glycerophospholipid synthesis pathway in metazoans (see new text in Fig. 5A legend, pg. 13). We also edited the schematic to provide a more complete representation of the lipid synthesis pathway in metazoans (new Fig. 5A).

Figure 5: Helpful model, not entirely clear the data support it, especially with respect to local lipid synthesis. Implies that CNEP-1 is localized to the rupture. Is it?

Thank you for pointing this out to us. We have simplified aspects of the schematic to not include the role of proteins but instead to represent our interpretation of the tomography data as well as to summarize the phenotypic outcomes on nuclear sealing in the different genetic backgrounds we include in this study (see new Fig. 5G).

Rebuttal References
Bahmanyar, S. et al. (2014) ‘Spatial control of phospholipid flux restricts endoplasmic reticulum sheet formation to allow nuclear envelope breakdown’, Genes & development, 28(2), pp. 121–126.

Olmos, Y. et al. (2015) ‘ESCRT-III controls nuclear envelope reformation.’, Nature, 522(7555), pp. 236–9. doi: 10.1038/nature14503.

Ventimiglia, L. N. et al. (2018) ‘CC2D1B Coordinates ESCRT-III Activity during the Mitotic Reformation of the Nuclear Envelope’, Developmental Cell, 47(5), p. 547–563.e6. doi: 10.1016/j.devcel.2018.11.012.

Vietri, M. et al. (2015) ‘Spastin and ESCRT-III coordinate mitotic spindle disassembly and nuclear envelope sealing.’, Nature, 522(7555), pp. 231–5. doi: 10.1038/nature14408.

Watts, J. L. and Ristow, M. (2017) ‘Lipid and Carbohydrate Metabolism in Caenorhabditis elegans, Genetics, 207(2), p. 413 LP-446. doi: 10.1534/genetics.117.300106.
February 10, 2020

RE: JCB Manuscript #201908179R

Prof. Shirin Bahmanyar
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Dear Prof. Bahmanyar:

Thank you for submitting your revised manuscript entitled "Regulation of ER membrane biogenesis from the nuclear envelope mediates nuclear closure". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

- We agree that the reviewer's suggestion for a more nuanced title is appropriate
- Provide main and supplementary text as separate, editable .doc or .docx files
- Provide figures as separate, editable files according to the instructions for authors on JCB's website, paying particular attention to the guidelines for preparing images at sufficient resolution for screening and production
- Add scale bars to figures 5B
- Provide tables as excel files - key resources should be included in Materials and Methods, not supplementary materials
- Add a paragraph after the Materials and Methods section briefly summarizing the online supplementary materials - i.e. use figure titles
- Add conflict of interest statement to Acknowledgements section

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Sincerely,

Michael Rout, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

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

I think this is a cool story and good revisions - happy to recommend publication!

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

The authors have satisfied my technical requests with additional experiments and reformulated their model accordingly. On balance, I support publication of their revised model and data--but in my opinion the title and abstract overemphasize the role of regulated lipid synthesis in sealing the nucleus. Put another way, I am convinced that CNEP-1 is important for determining the lipid content of the ER and associated NE, and that these lipid species cooperate with LEM2 and ESCRTs to remodel the nuclear membrane. I suggest a title that reflects this nuance, something more like,
"Regulated lipid synthesis cooperates with the LEM2-ESCRT pathway to mediate nuclear envelope closure."