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Nuclear lipid droplets form in the inner nuclear membrane in a seipin-independent manner

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Corresponding Author(s): Toyoshi Fujimoto, Juntendo University and Kamil Soltysik, University of Tokyo

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June 8, 2020

Re: JCB manuscript #202005026

Prof. Toyoshi Fujimoto
Juntendo University Graduate School of Medicine
Research Institute for Diseases of Old Age
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Nagoya, Tokyo 113-8421
Japan

Dear Prof. Fujimoto,

Thank you for submitting your manuscript entitled "Nuclear lipid droplets form in the inner nuclear membrane in a seipin-independent manner". 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 - and we agree - found the results interesting. We have discussed their remarks editorially and in our view, for publication in JCB, it will be important to clarify the localization, abundance, and role of lipin and address the shortcomings and interpretation of the Opi biosensor data. We and the referees are currently not convinced that the sensor is necessarily only binding PA; it might respond to other changes in membrane/surface properties. Both of Reviewer #3's major comments should be addressed and we suggest testing lipin-1 localization in seipin-deficient cells as per Rev#2. We completely agree with the referees that measuring PA (MS or TLC) would be really helpful to support the sensor data. Lastly, please consider in your model that release of seipin-bound PA may not make a difference in the overall pool of free PA (Rev#1 point #1). Please also address the reviewers' minor points to the best of your ability. Please do not hesitate to contact us with any questions. 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.

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***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.

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,

Tobias Walther, PhD
Monitoring Editor, Journal of Cell Biology

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

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

The biogenesis of nuclear LDs is an important subject in cell biology. These nLDs may regulate transcription (bacterial LDs can interact with DNA; LDs may also trap transcription factors), and therefore may profoundly impact cell function. The authors have made seminal discoveries of nLD formation in the past, primarily focusing on hepatocytes which generate lipoproteins. Here, the authors investigated the biogenesis of nLDs in non-hepatocytes. Using a series of genetic and cell biological techniques, the authors show that nLDs in U2OS cells can form in situ from INM through the actions of ACSL3, AGPAT2, GPAT3/GPAT4, and lipin-1 and DGAT1/DGAT2. Importantly, seipin is absent from INM, yet seipin deficiency promotes nLD formation. The authors further demonstrate that increased nuclear PA may contribute to nLD formation under seipin deficiency. The increased nuclear PA also help recruit lipin-1. Overall, the quality of data is very high, the paper is very well written, and the findings are of great general interest. There are only some minor concerns/suggestions.

1. Page 9, line 5 from bottom: The study that showed seipin binding to PA should be cited here (PMID: 30293840). However, it is not clear how much PA can be trapped by seipin complex since PA makes up at least 2% of ER phospholipids. The increase in PA under seipin deficiency is likely due to increased GPAT activity (see PMID: 27806294). To further support the role of PA in nLD formation, do nLDs increase when GPAT3/4 is overexpressed in normal U2OS cells? This would strengthen the paper but given the current Covid-19 situation, this experiment is preferred, not absolutely required. The possible involvement of GPAT under seipin deficiency should at least be discussed.
2. The authors used a well established PA sensor Opi1 and its PA binding mutant to detect PA. The data are solid and convincing. This could still be further strengthened by measuring PA through mass-spec or other biochemical methods in purified microsomes or ideally, INM (if it can be purified) (see PMID: 21829381). Again, this experiment is preferred but not absolutely required.
3. It would be great to detect endogenous seipin. However, there are no known antibodies that are good enough to detect seipin by IF or immunoEM.
4. Some discussion on how an increase in INM PA might stabilize INM-LD contact would be good.
5. Minor point: AGPAT3, but not AGAPT2 is known to be on LDs.

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

Soltysik et al. use U2OS cells to show the first evidence in human cells that nuclear LDs can form from the inner nuclear membrane (INM) - these events are rare because seipin restricts LD formation to the peripheral ER. Seipin does not localize to the INM and in the absence of seipin LD formation at the INM is increased. These data contradict a previous study in budding yeast showing that seipin is able to reach the INM to stabilize nuclear LDs and expands on other reported observations that nuclear LDs are increased in seipin-null yeast strains. Overexpression of GFP-lipin1b under conditions when mTOR is inhibited increases nuclear LD formation and this requires lipin1's PA phosphatase activity. Reducing seipin leads to increased mRNA and protein levels of lipin1 through an unknown mechanism. The increased number of nuclear LDs in seipin-deficient cells depends on lipin1 expression.

Overall, this is an interesting study and important in light of the significance of LDs to physiology and disease and the emerging role of the INM in lipid metabolism. The finding that seipin does not localize to the INM is important, and is supported by previous findings that seipin forms a large oligomer that would likely not be able to traverse the nuclear pore membrane to reach the INM. Most of the data in the manuscript is well presented although the labeling, quantitation and statistics could be improved (see minor point 3 below). The main weakness of the manuscript is the proposed mechanism for nuclear LD formation by the recruitment of lipin1 to PA accumulated at nuclear LDs. The authors should address the points below for the manuscript to be acceptable for publication in JCB.

Major issues:
1. From the evidence that is provided in the manuscript, it is difficult to interpret lipin1's role in nuclear LD formation in seipin-deficient cells. It seems the authors are drawing an analogy to the PA-dependent localization of Opi1 to nuclear LDs shown in budding yeast, however unlike Opi1 lipin1 is an enzyme that acts on PA. Moreover lipin1's catalytic activity towards PA is higher when it is in its dephosphorylated form (see Eaton et al. 2013 PMID: 23426360). The higher catalytic activity of nuclear-enriched lipin when it is in its more dephosphorylated (under torin1 or OA treatments, see Peterson et al. 2011 PMID: 21816276, Kim et al. 2007 PMID: 17420445) contradicts the authors'

findings that there is an increase in nuclear PA at LDs, as detected by the sensor. Perhaps lipin is recruited by PA to the surface of LDs by an unidentified protein or the lipid environment of LDs prevents lipin from dephosphorylating PA. However, currently there is not enough evidence in the manuscript to support this idea and so another more likely possibility is that lipin1 expression is increased in seipin-deficient cells to restore lipid homeostasis. Their conclusions require a more direct test of their model such as determining lipin's localization in seipin-deficient cells and how this localization changes when lipin1 is mutated so it is unable to bind or turnover PA would be necessary.

2. In relation to the previous point, a more thorough analysis and validation of the PA sensor used in this study is necessary to interpret their results. The limitation of the Opi1 sensor should be discussed in reference to data showing that the PA binding by Opi1 is affected by the lipid environment (pmid: 28115519). In Fig. S3 A the authors use a mutant form of NLS-Opi1 Q2 that does not bind to PA. This is an important control, however the results are not quantified. Also, the magenta marker in the image in Fig S3A should be labeled - if it is marking LDs, why don't some of the puncta of NLS-Opi1 Q2 co-localize with nuclear LDs? From the image it seems the sensor may form aggregates in the nucleus that are not associated with LDs. If this is indeed the case, it is difficult to interpret the meaning behind "number of PA puncta in the nucleus" under the different conditions tested. How does RNAi of lipin1 or overexpression of lipin1 constructs under conditions tested in Fig. 3B or GPAT3/4 expression affect the PA sensor at nuclear LDs? Ideally, biochemical analysis of PA levels would be performed to more directly address these concerns.

Minor:

1. In figure 1E: the authors attempt to show the nuclear boundary by tracing the outline of the nucleus, but without a nuclear envelope/ER-membrane marker it is unclear if the LD is forming on the surface of the ER/ONM or indeed from the INM into the nucleus. Because of the lack of higher resolution images that show a connection between a forming LD and the INM, the authors can not definitively conclude that the LD is forming at the INM.

2. Also in relation to Figure 4F, it is not directly obvious why seipin knockdown would cause a greater proportion of nuclear LDs that remain attached to the INM. Please explain. Ideally, the authors would supplement these data with high resolution images of nuclear lipid droplets in seipin knockdown cells to show a greater proportion are directly associated with the INM.

3. Why are so few cells analyzed in Fig. 5C compared to the other conditions? Why are some plots shown as box and whisker plots (such as Fig. 3E) while most others are not? The number of times the experiments were performed is not specified in the figure legend. If only two experiments were performed for some figures (as indicated in the methods) then how were the statistics for significance performed? The authors should strongly consider replotting their data and performing their statistical analysis on the independent experimental repeats (see Lord et al. JCB 2020 PMID: 32346721).

4. In general, the figures require better labeling to represent the different conditions used. Some examples are: GFP should be included to clarify that the constructs used in Fig. 3B are the same as those used in Fig. 3D, it is unclear in Fig. 3E if lipin1 is RNAi-depleted under all of the conditions including cells expressing EGFP alone, OA + Torin should be included in Fig. 3D and E, labeling the protein that is immunolabeled in the EM image in Fig. 4E, labeling "% recovery GFP-ACLS3" in plots in Fig. 4F, proper labeling is required in the plot in Fig. 5B so that it is clear the values are normalized.

5. An immunoblot to show the efficiency of the double RNAi of lipin and seipin should be included. The seipin single RNAi on its own is not complete (Fig. 2B) but even so since lipin1 is the major PAP enzyme in TG synthesis, it is perhaps not surprising that its reduction would reduce LD formation. In addition to validation of knockdown by immunoblot, the authors should test if lipin1 RNAi-depletion alone reduces LD formation overall (in the cytoplasm and nucleus).

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

This study examines the mechanism and regulation of nuclear lipid droplet (nLD) formation in cells not derived from hepatocytes. It shows that nLDs bud from the inner nuclear membrane and enzymes necessary for TAG production are in the nucleus. There are two additional main findings: induction of nLDs is dependent on lipin-1 and seipin is not in the inner nuclear membrane and does not directly mediate nLD production. These are interesting results, but more work is necessary for this study to provide a substantial advance over previous work. There are number of concerns.

1. The study demonstrates that treating cells with oleic acid or Torin increases nLDs and this increase is dependent on lipin-1. It suggests lipin-1 must be in the nucleus but this has not been demonstrated. To show this, a version of lipin-1 that cannot enter the nucleus or that has a nuclear export signal should be used. There is a similar question about whether TAG production in the inner nuclear membrane is required for nLD formation. If DGAT-1 or one of the enzymes that produce TAG precursors is prevented from entering the nucleus, is nLD production decreased?

2. The implications of nLD production in the inner nuclear membrane in the absence of seipin have not been fully explored. Previous studies have shown that cytoplasmic LDs made in cells depleted of seipin are abnormally sized, often forming clusters of small LDs or large "supersized" LDs. Is the same true of nLDs? If possible, it would also be good to determine whether the number and size of nLDs changes when a fraction of seipin is relocalized to the inner nuclear membrane (since seipin determines the biogenesis site of LDs). Can the authors rule out that a small fraction of seipin is normally in the inner nuclear membrane and is response for the production nLDs?

Minor points

1. There should be a discussion of the limits of using a lipid sensor to measure PA levels in the inner nuclear membrane and on the surface of nLDs. Factors other than changes in PA levels could affect sensor binding to these surfaces. It would be better if PA levels were directly measured.

2. The text should more carefully explain what is meant by LDs being connected to the inner nuclear membrane (or the rest of the ER) and how this is measured. Has it been established that GFP-ACSL3 can only exchange between LDs and the ER by diffusing in the membrane? What do the authors think are the implications of changes in connectivity?

Point-by-point responses to the comments

Thank you for submitting your manuscript entitled "Nuclear lipid droplets form in the inner nuclear membrane in a seipin-independent manner". 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 - and we agree - found the results interesting. We have discussed their remarks editorially and in our view, for publication in JCB, it will be important to clarify the localization, abundance, and role of lipin and address the shortcomings and interpretation of the Opi biosensor data. We and the referees are currently not convinced that the sensor is necessarily only binding PA; it might respond to other changes in membrane/surface properties. Both of Reviewer #3's major comments should be addressed and we suggest testing lipin-1 localization in seipin-deficient cells as per Rev#2. We completely agree with the referees that measuring PA (MS or TLC) would be really helpful to support the sensor data. Lastly, please consider in your model that release of seipin-bound PA may not make a difference in the overall pool of free PA (Rev#1 point #1). Please also address the reviewers' minor points to the best of your ability. Please do not hesitate to contact us with any questions. We would be happy to further discuss the revisions as needed.

Thank you for your kind words. We are particularly pleased to learn that you and the reviewers found our study interesting. We conducted several new experiments in response to your comments and addressed other points raised by the reviewers. Please see the following pages for our answers to your individual comments and questions.

Reviewer #1:

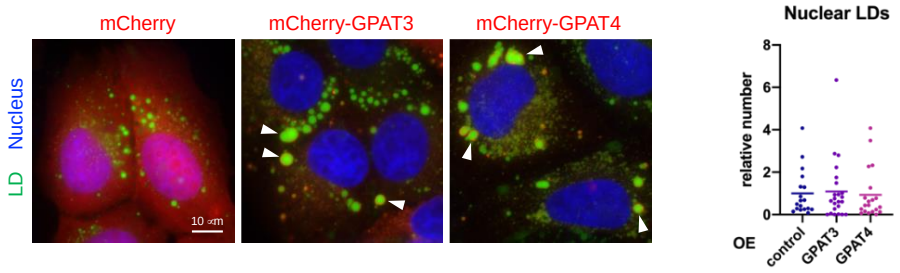
The biogenesis of nuclear LDs is an important subject in cell biology. These nLDs may regulate transcription (bacterial LDs can interact with DNA; LDs may also trap transcription factors), and therefore may profoundly impact cell function. The authors have made seminal discoveries of nLD formation in the past, primarily focusing on hepatocytes which generate lipoproteins. Here, the authors investigated the biogenesis of nLDs in non-hepatocytes. Using a series of genetic and cell biological techniques, the authors show that nLDs in U2OS cells can form in situ from INM through the actions of ACSL3, AGPAT2, GPAT3/GPAT4, and lipin-1 and DGAT1/DGAT2. Importantly, seipin is absent from INM, yet seipin deficiency promotes nLD formation. The authors further demonstrate that increased nuclear PA may contribute to nLD formation under seipin deficiency. The increased nuclear PA also help recruit lipin-1. Overall, the quality of data is very high, the paper is very well written, and the findings are of great general interest. There are only some minor concerns/suggestions.

We are very happy to know that you found our work interesting and our data of high quality. We also appreciate your careful reading of the manuscript and your suggestions to improve this paper. We have conducted new experiments according to your recommendations and revised the manuscript accordingly.

1. *Page 9, line 5 from bottom: The study that showed seipin binding to PA should be cited here (PMID: 30293840). However, it is not clear how much PA can be trapped by seipin complex since PA makes up at least 2% of ER phospholipids. The increase in PA under seipin deficiency is likely due to increased GPAT activity (see PMID: 27806294). To further support the role of PA in nLD formation, do nLDs increase when GPAT3/4 is overexpressed in normal U2OS cells? This would strengthen the paper but given the current Covid-19 situation, this experiment is preferred, not absolutely required. The possible involvement of GPAT under seipin deficiency should at least be discussed.*

Thank you for these important suggestions. We cited the two papers at the appropriate points in the paper and discussed the effect of seipin on GPAT activity (page 10, 2nd paragraph). We also examined the effects of GPAT3/4 overexpression on nuclear LDs in U2OS cells. As shown in the left figure below, mCherry-tagged GPAT3/4 was distributed only in the cytoplasm in most cells and induced enlarged cytoplasmic LDs, as reported previously in 3T3-L1 and Huh7 cells (Pagac et al., Cell Rep, 2016). This finding suggests that a large portion of PA produced by overexpressed GPAT3/4 may be converted to DAG and TAG locally, thereby forming large LDs, and may not significantly increase PA in other ER domains or the INM. On the other hand, for unknown reasons, the nuclear distribution of mCherry-GPAT3/4 was much less frequent than that of mCherry-tagged ACSL3 or DGAT2. Probably for these reasons,

overexpression of GPAT3/4 did not increase nuclear LDs (right graph). The effect of GPAT3/4 on nuclear PA was also examined, and our findings on this topic support the above interpretation. For our results regarding nuclear PA, please see our response to Reviewer #2 (Major comment #2).



2. *The authors used a well established PA sensor Opi1 and its PA binding mutant to detect PA. The data are solid and convincing. This could still be further strengthened by measuring PA through mass-spec or other biochemical methods in purified microsomes or ideally, INM (if it can be purified)(see PMID: 21829381). Again, this experiment is preferred but not absolutely required.*

According to this suggestion, we measured PA in isolated nuclei using an enzyme-coupled fluometric assay. We used the whole nuclear fraction rather than the INM preparation because most PA sensor signals were observed beside nuclear LDs that may not have been associated with the nuclear envelope. We found that the amount of PA normalized to that of PC in the nuclei increased upon seipin knockdown, confirming the results obtained using the fluorescent PA biosensor. The results of PA measurement in the nuclei were added in the new Fig. 5E.

3. *It would be great to detect endogenous seipin. However, there are no known antibodies that are good enough to detect seipin by IF or immunoEM.*

We had access to several anti-seipin antibodies, but none of them labeled endogenous seipin well. We hope that an antibody usable for immunolabeling will become available, so that the results obtained using tagged seipin expression can be verified.

4. *Some discussion on how an increase in INM PA might stabilize INM-LD contact would be good.*

The time course observed via live imaging indicates that LD formation in the INM is a slower process than that in the ER (deduced by comparison of Figs. 1D, E of our manuscript and the results reported by Kassan et al., JCB 203, 985, 2013 and Wang et

al., eLife 5, e16582, 2016). In seipin-deficient cells, the increase of PA in the INM is likely to make nuclear LD budding even more inefficient and stabilize the nuclear LD-INM membrane bridge, as suggested by an in vitro study (M'Barek et al, Dev Cell 41, 591, 2017) and a yeast study (Choudhary et al, Curr Biol 28, 915, 2018), respectively. Thus, many nuclear LDs are thought to be connected to the INM in seipin-deficient cells, causing the GFP-ACSL3 fluorescence recovery as observed in the FRAP experiment (Fig. 4F). A discussion of this issue was added to the revised manuscript (page 5, 3rd paragraph; page 10, 2nd paragraph).

5. *Minor point: AGPAT3, but not AGAPT2 is known to be on LDs.*

AGPAT2 has also been detected in LDs through proteomic analysis (e.g., Liu et al., JLR 58, 681, 2017), but it might be relatively minor in cytoplasmic LDs in comparison to AGPAT3. For nuclear LD formation, distribution of AGPAT2 (and AGPAT4) in the INM, but not in nuclear LDs per se, is thought to be sufficient.

Reviewer #2:

Soltysik et al. use U2OS cells to show the first evidence in human cells that nuclear LDs can form from the inner nuclear membrane (INM) - these events are rare because seipin restricts LD formation to the peripheral ER. Seipin does not localize to the INM and in the absence of seipin LD formation at the INM is increased. These data contradict a previous study in budding yeast showing that seipin is able to reach the INM to stabilize nuclear LDs and expands on other reported observations that nuclear LDs are increased in seipin-null yeast strains. Overexpression of GFP-lipin1b under conditions when mTOR is inhibited increases nuclear LD formation and this requires lipin1's PA phosphatase activity. Reducing seipin leads to increased mRNA and protein levels of lipin1 through an unknown mechanism. The increased number of nuclear LDs in seipin-deficient cells depends on lipin1 expression.

Overall, this is an interesting study and important in light of the significance of LDs to physiology and disease and the emerging role of the INM in lipid metabolism. The finding that seipin does not localize to the INM is important, and is supported by previous findings that seipin forms a large oligomer that would likely not be able to traverse the nuclear pore membrane to reach the INM. Most of the data in the manuscript is well presented although the labeling, quantitation and statistics could be improved (see minor point 3 below). The main weakness of the manuscript is the proposed mechanism for nuclear LD formation by the recruitment of lipin1 to PA accumulated at nuclear LDs. The authors should address the points below for the manuscript to be acceptable for publication in JCB.

Thank you for your kind words. We are really happy to learn that you found our study interesting and important. We also thank you for careful reading of the manuscript and for many constructive comments. We conducted several new experiments and revised the manuscript.

Major issues:

1. *From the evidence that is provided in the manuscript, it is difficult to interpret lipin1's role in nuclear LD formation in seipin-deficient cells. It seems the authors are drawing an analogy to the PA-dependent localization of Opi1 to nuclear LDs shown in budding yeast, however unlike Opi1 lipin1 is an enzyme that acts on PA. Moreover lipin1's catalytic activity towards PA is higher when it is in its dephosphorylated form (see Eaton et al. 2013 PMID: 23426360). The higher catalytic activity of nuclear-enriched lipin when it is in its more dephosphorylated (under torin1 or OA treatments, see Peterson et al. 2011 PMID: 21816276, Kim et al. 2007 PMID: 17420445) contradicts the authors' findings that there is an increase in nuclear PA at LDs, as detected by the sensor. Perhaps lipin is recruited by PA to the surface of LDs by an unidentified protein or the lipid environment of LDs prevents lipin from dephosphorylating PA. However, currently there is not enough evidence in the*

manuscript to support this idea and so another more likely possibility is that lipin1 expression is increased in seipin-deficient cells to restore lipid homeostasis. Their conclusions require a more direct test of their model such as determining lipin's localization in seipin-deficient cells and how this localization changes when lipin1 is mutated so it is unable to bind or turnover PA would be necessary.

Thank you for raising these important points. As far as we understand, lipin-1 is thought to function in two different modes (e.g., Kwiatek and Carman, J Lipid Res 61, 1232, 2020): in the first phase, lipin-1 hops onto the membrane, a movement facilitated by an interaction between dephosphorylated lipin-1 and PA, and converts PA to DAG; in the second phase, lipin-1 remains on the membrane, scoots and binds to another PA, and engages continuously in catalysis. Thus, despite its catalytic activity, lipin-1 is expected to remain in PA-containing membranes for a certain period of time, and the probability of finding lipin-1 in a membrane (or an LD) is expected to increase when the PA content is higher. Consistently, we found that the proportion of nuclear LDs showing GFP-lipin-1β accumulation is higher in seipin-deficient cells than in control cells. Moreover, the proportion of nuclear LDs showing lipin-1 accumulation was not significantly different between wild-type GFP-lipin-1β and the GFP-lipin-1β mutant (D714E) lacking PA phosphatase activity, indicating that active PA hydrolysis does not significantly affect the LD distribution of lipin-1. These data were added as Fig. 5F in the revised manuscript. The above results as well as the increase in PA in seipin-deficient cells suggest that lipin-1β is recruited to nuclear LDs through binding to PA. Nevertheless, the involvement of a non-PA mechanism in lipin-1 recruitment cannot be excluded, and thus this possibility was also mentioned in the revised manuscript (page 10, 1st paragraph).

2. *In relation to the previous point, a more thorough analysis and validation of the PA sensor used in this study is necessary to interpret their results.* *a)* *The limitation of the Opi1 sensor should be discussed in reference to data showing that the PA binding by Opi1 is affected by the lipid environment (pmid: 28115519).* *b)* *In Fig. S3 A the authors use a mutant form of NLS-Opi1 Q2 that does not bind to PA. This is an important control, however the results are not quantified.* *c)* *Also, the magenta marker in the image in Fig S3A should be labeled - if it is marking LDs, why don't some of the puncta of NLS-Opi1 Q2 co-localize with nuclear LDs? From the image it seems the sensor may form aggregates in the nucleus that are not associated with LDs. If this is indeed the case, it is difficult to interpret the meaning behind "number of PA puncta in the nucleus" under the different conditions tested.* *d)* *How does RNAi of lipin1 or overexpression of lipin1 constructs under conditions tested in Fig. 3B or GPAT3/4 expression affect the PA sensor at nuclear LDs?* *e)* *Ideally, biochemical analysis of PA levels would be performed to more directly address these concerns.*

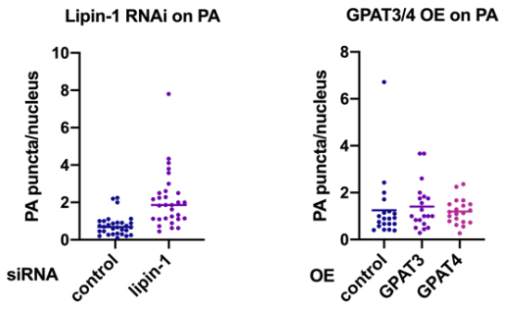
Thank you for pointing out these important issues with regard to the PA sensor. We have addressed them in the following ways.

a) We mentioned the potential problems with the PA sensor, citing the paper by Kassas et al. (J Biol Chem 292, 4266, 2017) (page 9, 3rd paragraph).

b) Our results using the mutant NLS-Opi1 Q2 were quantified and added to Fig S3D.

c) The magenta marker in the original Supplementary Fig. 3A indicates LDs. We apologize for not having specified this in the legend. Some NLS-Opi1 Q2 puncta in the original figure appeared not to be associated with nuclear LDs, but this was only because small LDs were not clearly observed. By extending the exposure time, we were able to observe that virtually all NLS-Opi1 Q2 puncta were associated with LDs (Fig. S3C). Additionally, in other instances, NLS-Opi1 Q2 puncta that appeared to be solitary in one focal plane were actually associated with LDs in a neighboring focal plane (either above or below).

d) We performed the suggested experiments. First, lipin-1 RNAi increased the number of NLS-Opi1 Q2 puncta in cells treated with OA and Torin1 (left graph). This result is thought to be caused by a decrease in PA phosphatase activity of lipin-1. Second, NLS-Opi1 Q2-GFP and mCherry-GPAT3/4 showed colocalization, supporting the functionality of NLS-Opi1 Q2-GFP as a PA biosensor (Fig. S3B). However, the nuclear distribution of mCherry-GPAT3/4 occurred only infrequently. Moreover, mCherry-tagged GPAT3/4 in the cytoplasm induced enlarged cytoplasmic LDs, as previously reported in 3T3-L1 and Huh7 cells (Pagac et al., Cell Rep, 2016), suggesting that a large portion of the PA produced by overexpressed GPAT3/4 may be converted to DAG and TAG locally. Probably for these reasons, GPAT3/4 overexpression did not increase nuclear PA puncta significantly (right graph). For the effect of GPAT3/4 on nuclear LDs, please see our response to Reviewer #1-Comment #2.



e) According to your suggestion, we measured PA in isolated nuclei using an enzyme-coupled fluometric assay. We found that the amount of PA normalized to that of PC in the nuclei increased upon seipin knockdown, confirming the result that we obtained using the fluorescent PA biosensor. The results of our PA measurement in the nuclei were

added as Fig. 5E.

Minor:

1. *In figure 1E: the authors attempt to show the nuclear boundary by tracing the outline of the nucleus, but without a nuclear envelope/ER-membrane marker it is unclear if the LD is forming on the surface of the ER/ONM or indeed from the INM into the nucleus. Because of the lack of higher resolution images that show a connection between a forming LD and the INM, the authors can not definitively conclude that the LD is forming at the INM.*

   To address the problem raised by the referee, we used Lap2β as an INM marker and showed by confocal microscopy that a new LD forms as a punctum at the INM and grows toward the nucleoplasm. The connection between forming LDs and the INM cannot be resolved by this method, but the result supports our conclusion that nuclear LDs form at the INM. These data were added as the new Video 2 and Fig. 1E.

2. *a) Also in relation to Figure 4F, it is not directly obvious why seipin knockdown would cause a greater proportion of nuclear LDs that remain attached to the INM. Please explain. b) Ideally, the authors would supplement these data with high resolution images of nuclear lipid droplets in seipin knockdown cells to show a greater proportion are directly associated with the INM.*

   a) The time course observed by live imaging indicates that LD formation in the INM is a slower process than that in the ER (deduced through a comparison of Figs. 1D, E of our manuscript and the results reported by Kassan et al., JCB 203, 985, 2013 and Wang et al., eLife 5, e16582, 2016). In seipin-deficient cells, the increase in PA in the INM is likely to make nuclear LD budding even more inefficient and stabilize the nuclear LD-INM membrane bridge, as suggested by an in vitro study (M'Barek et al, Dev Cell 41, 591, 2017) and a yeast study (Choudhary et al, Curr Biol 28, 915, 2018), respectively. Thus, many nuclear LDs are thought to be connected to the INM in seipin-deficient cells, causing the GFP-ACSL3 fluorescence recovery observed in the FRAP experiment (Fig. 4F). A discussion of this issue was added to the revised manuscript (page 5, 3rd paragraph; page 10, 2nd paragraph).
   b) We used super-resolution microscopy of NLSx3-HPos to examine whether seipin knockdown induces a larger number of nuclear LDs to be associated with the INM. Our results showed that a close association between nuclear LDs and the INM occurs more frequently in seipin-deficient cells than in control cells (Fig. 4G).

3. *a) Why are so few cells analyzed in Fig. 5C compared to the other conditions? b) Why are some plots shown as box and whisker plots (such as Fig. 3E) while most others are not? c) The number of times the experiments were performed is not specified in the figure*

legend. If only two experiments were performed for some figures (as indicated in the methods) then how were the statistics for significance performed? The authors should strongly consider replotting their data and performing their statistical analysis on the independent experimental repeats (see Lord et al. JCB 2020 PMID: 32346721).

We apologize for any insufficient evidence or ambiguous points in the original manuscript.

a) We increased the number of cells analyzed for the experiment depicted in Fig. 5C and revised the figure accordingly.

b) We used box and whisker plots for graphs that had too many data points (> 40) to be shown in simple scatter plots. All experiments were repeated two or three times, and, if essentially the same result was obtained, the result of one experiment was shown in the figure as a representative result. This practice is commonly seen in many studies published these days. These points are now stated in the "Statistical analysis and plot" subsection of the Methods section. Lord et al. (J Cell Biol, e202001064, 2020) have proposed an excellent method for presenting full data sets to readers, but we think that the conventional method is sufficient to show the reproducibility of our results.

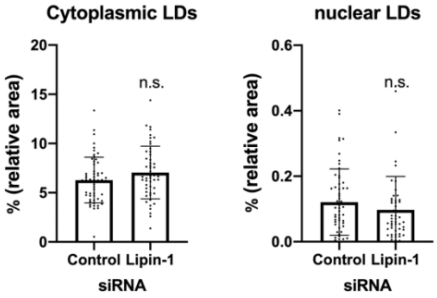
4. *In general, the figures require better labeling to represent the different conditions used. Some examples are: GFP should be included to clarify that the constructs used in Fig. 3B are the same as those used in Fig. 3D, it is unclear in Fig. 3E if lipin1 is RNAi-depleted under all of the conditions including cells expressing EGFP alone, OA + Torin should be included in Fig. 3D and E, labeling the protein that is immunolabeled in the EM image in Fig. 4E, labeling "% recovery GFP-ACLS3" in plots in Fig. 4F, proper labeling is required in the plot in Fig. 5B so that it is clear the values are normalized.*

We apologize for the inconvenience caused by insufficient labeling in the figures. We have added labels to all of our figures as suggested by the reviewer. For some figures, the legend was also revised to make the experimental conditions clearer.

5. *a) An immunoblot to show the efficiency of the double RNAi of lipin and seipin should be included. b) The seipin single RNAi on its own is not complete (Fig. 2B) but even so since lipin1 is the major PAP enzyme in TG synthesis, it is perhaps not surprising that its reduction would reduce LD formation. In addition to validation of knockdown by immunoblot, the authors should test if lipin1 RNAi-depletion alone reduces LD formation overall (in the cytoplasm and nucleus).*

a) Western blotting data showing the effect of double knockdown of lipin-1 and seipin were added as Fig. S3A in the revised manuscript.

b) The effect of lipin-1 knockdown on cytoplasmic and nuclear LDs was examined. As shown in the graphs below, lipin-1 knockdown did not significantly influence either cytoplasmic LDs or nuclear LDs. This result is somewhat counter-intuitive, although, in mouse liver, for example, triglyceride synthesis and LD biogenesis were shown to persist even in the absence of lipin-1 (Schweitzer et al., J Lipid Res 56, 848, 2015). We speculate that SREBP activation induced by lipin-1 deficiency (Peterson et al., Cell 146, 408, 2011) and the resultant upregulation of lipid synthesis, especially an increase of cholesterol ester synthesis, may also be involved. Although the detailed mechanism is not yet clear, we can conclude that lipin-1 expression is not directly correlated with the level of LD formation.



Reviewer #3:

This study examines the mechanism and regulation of nuclear lipid droplet (nLD) formation in cells not derived from hepatocytes. It shows that nLDs bud from the inner nuclear membrane and enzymes necessary for TAG production are in the nucleus. There are two additional main findings: induction of nLDs is dependent on lipin-1 and seipin is not in the inner nuclear membrane and does not directly mediate nLD production. These are interesting results, but more work is necessary for this study to provide a substantial advance over previous work. There are number of concerns.

We are happy to know that you found our results interesting. We also thank you for providing us with important comments. We conducted several new experiments and revised the manuscript accordingly.

1. *The study demonstrates that treating cells with oleic acid or Torin increases nLDs and this increase is dependent on lipin-1. It suggests lipin-1 must be in the nucleus but this has not been demonstrated. a) To show this, a version of lipin-1 that cannot enter the nucleus or that has a nuclear export signal should be used. b) There is a similar question about whether TAG production in the inner nuclear membrane is required for nLD formation. If DGAT-1 or one of the enzymes that produce TAG precursors is prevented from entering the nucleus, is nLD production decreased?*

Thank you for asking this essential question.

a) With regard to lipin-1, we compared the effect of wild-type lipin-1 with that of nuclear export signal (NES)-tagged lipin-1, which we confirmed to be excluded from the nucleus (Fig. S2B). In cells depleted of endogenous lipin-1, the expression of NES-lipin-1β induced significantly fewer nuclear LDs than the expression of wild-type lipin-1β did (Fig. S2B). This result indicates that the nuclear translocation of lipin-1β is essential to an increase in nuclear LDs.

b) For TAG production in the INM, we manipulated DGAT2 rather than DGAT1 because knockdown of DGAT2 was found to be more effective than knockdown of DGAT1 at reducing nuclear LDs. A truncated version of chicken muscle pyruvate kinase (PK; ca. 50 kDa) was tagged to GFP-DGAT2 to preclude its access to the INM (Soullam and Worman, J Cell Biol 130, 15, 1995) and the effect of its expression on nuclear LD formation was compared with that of GFP-DGAT2 in cells depleted of endogenous DGAT2. As expected, GFP-DGAT2-PK cannot enter the nucleus (Fig. S1H), and cells expressing GFP-DGAT2-PK had significantly fewer nuclear LDs than cells expressing GFP-DGAT2 had (Fig. 2E). This result supports the conclusion that TAG synthesis in the INM is crucial for nuclear LD formation.

2. *The implications of nLD production in the inner nuclear membrane in the absence of seipin have not been fully explored. Previous studies have shown that cytoplasmic LDs made in cells depleted of seipin are abnormally sized, often forming clusters of small LDs or large "supersized" LDs. Is the same true of nLDs? If possible, it would also be good to determine whether the number and size of nLDs changes when a fraction of seipin is relocalized to the inner nuclear membrane (since seipin determines the biogenesis site of LDs). Can the authors rule out that a small fraction of seipin is normally in the inner nuclear membrane and is response for the production nLDs?*

Both bimolecular fluorescence complementation and immunoelectron microscopy detected no seipin in the INM, but even these results cannot rule out the possibility that a very small amount of seipin exists and is involved in LD formation in the INM. We consider this possibility unlikely, however, because the overexpression of seipin, which we would expect to increase seipin in the INM as well, did not increase but rather decreased the number of nuclear LDs. An experiment artificially relocalizing seipin to the INM may be interesting, but considering that proper oligomer formation is necessary for seipin functionality, this may not be an easy experiment.

Minor points

1. *There should be a discussion of the limits of using a lipid sensor to measure PA levels in the inner nuclear membrane and on the surface of nLDs. Factors other than changes in PA levels could affect sensor binding to these surfaces. It would be better if PA levels were directly measured.*

The limitations of using our fluorescence PA biosensor are discussed in the revised manuscript, citing an article (Kassas et al., JBC, 2017) that examines the effect of the lipid environment on several PA biosensors. We measured PA in isolated nuclei using an enzyme-coupled fluometric assay and found that the amount of PA normalized to that of PC in the nuclei increased upon seipin knockdown, confirming the result obtained using the fluorescent PA biosensor. The results of our PA measurement in nuclei were added as Fig. 5E.

2. *a) The text should more carefully explain what is meant by LDs being connected to the inner nuclear membrane (or the rest of the ER) and how this is measured. b) Has it been established that GFP-ACSL3 can only exchange between LDs and the ER by diffusing in the membrane? c) What do the authors think are the implications of changes in connectivity?*

Thank you for pointing out these important issues.

a) For a discussion of the connectivity issue, please see c). We used super-resolution

microscopy of NLSx3-HPos to examine whether seipin knockdown induces a larger number of nuclear LDs to be associated with the INM. Our results showed that a close association between nuclear LDs and the INM occurs more frequently in seipin-deficient cells than in control cells (Fig. 4G).

b) ACSL3 is anchored to LDs and the ER through the N-terminal hydrophobic domain (Poppelreuther et al., J Lipid Res 53, 888, 2012) and is not detected in the cytosolic fraction (Kimura et al., J Lipid Res 59, 805, 2018). Thus, the FRAP results regarding GFP-ACSL3 are thought to reflect the connectivity between the INM and nuclear LDs.

c) The time course observed in live imaging indicates that LD formation in the INM is a slower process than that in the ER (deduced through a comparison of Figs. 1D, E of our manuscript and the results reported by Kassan et al., JCB 203, 985, 2013 and Wang et al., eLife 5, e16582, 2016). In seipin-deficient cells, the increase in PA in the INM is likely to make nuclear LD budding even more inefficient and stabilize the nuclear LD-INM membrane bridge, as suggested by an in vitro study (M'Barek et al, Dev Cell 41, 591, 2017) and a yeast study (Choudhary et al, Curr Biol 28, 915, 2018), respectively. Thus, many nuclear LDs are thought to be connected to the INM in seipin-deficient cells, causing the GFP-ACSL3 fluorescence recovery seen in the FRAP experiment (Fig. 4F). A discussion of this issue was added to the revised manuscript (page 5, 3rd paragraph; page 10, 2nd paragraph).

October 20, 2020

RE: JCB Manuscript #202005026R

Prof. Toyoshi Fujimoto
Juntendo University
Graduate School of Medicine
2-1-1 Hongo, Bunkyo
Nagoya, Tokyo 113-8421
Japan

Dear Prof. Fujimoto,

Thank you for submitting your revised manuscript entitled "Nuclear lipid droplets form in the inner nuclear membrane in a seipin-independent manner". The manuscript was assessed by the original reviewers, who appreciated the changes made in revision. However, they also shared comments that require your attention. As you know, JCB limits all manuscripts to one round of major experimental revision. We have discussed the reviewer comments editorially, and because they only require minor changes at this stage, we are open to one final round of revision. We would not require new experimentation to address Reviewer #3 points #2 or #4 but instead recommend that you dedicate efforts to amend the manuscript as follows:

i) Please provide the controls for expression of NES-lipin1beta and GFP-DGAT2-PK (Ref #3, pt #1)

ii) Please re-consider the discussion as per Reviewers #2 and #3. We feel that more discussion would benefit readers and would be interesting. We can accommodate reasonable extensions to the character count if this was a concern.

iii) Please include more rigorous descriptions of repeats/reproducibility as per JCB policy. Each figure should state the number of repeats and stats applied. You can see also below our general guidelines and can find more information on data presentation in this article: J Cell Biol (2020) 219 (6): e202001064 https://doi.org/10.1083/jcb.202001064

We would be happy to publish your paper in JCB pending changes as described above and final revisions necessary to meet our formatting guidelines (see details below). Please also provide a response to the reviewers' comments with your resubmission.

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

Running title (50 characters max, including spaces): Nuclear lipid droplets form without seipin (We strongly recommend avoiding acronyms to increase the accessibility of the work)

eTOC summary:
- Please include it 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: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 2F (insets), S1B (magnifications), S2E (magnifications) Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add molecular weight with unit labels on the following panels: 5A (please add unit labels), S1C (unit labels), S2CF (unit labels), S3AG (unit labels)

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.
Please indicate n/sample size/how many experiments the data are representative of for each panel representing pooled data.

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*
- 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.
- Refs in the citation list should not be numbered. For more on our style, please check this link: https://rupress.org/jcb/pages/reference-guidelines

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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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.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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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,

Tobias Walther, PhD
Monitoring Editor, Journal of Cell Biology

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

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

This paper has been thoroughly revised. The authors did a great job. The quality of the data is very high. It should be accepted in its current form.

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

The authors convincingly show that seipin restricts LD formation to the cytoplasm and away from the INM. When seipin levels are low, lipin1 is involved in LD formation at the INM, although the

mechanism is unclear. The authors suggest that an increase in PA at the surface of nuclear LDs recruits lipin1 to nLDs.

The authors have addressed my concerns, and do a nice job showing the role of seipin in restricting LDs to the cytoplasm and the involvement of lipin1 in formation/stability of nLDs. However, their conclusions about the role of PA remains the major weakness of their paper despite the new biochemical evidence that is shown in Fig. 5E. While I appreciate that this is a challenging experiment, it is difficult to assess the data as shown because there are overlapping values between the control and RNAi for PA levels in the nucleus. The authors should use different symbols for each trial so the results from each trial can be directly compared. Overall, I think the authors should consider deemphasizing the role of PA in their abstract and model because of the questions that have been raised. Although it hints at mechanism, it is not an essential aspect of the nice story they have put together.

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

1. The experiments with NES-lipin-1beta and GFP-DGAT2-PK are well done, but there are no controls showing that the proteins are expressed at levels similar to the proteins lacking the NES or PK.

2. With regard to the role of seipin in nLD production, is it possible to compare the size of nLDs in cells that lack seipin with cells that express seipin? Cytoplasmic LD size is altered in cells lacking seipin and it would be interesting to know whether this is also true of nLDs. A finding that there is no size difference would support the idea that seipin plays no role in nLD biogenesis.

3. The idea that seipin sequesters PA and therefore regulates PA distribution in the ER seems implausible. PA is probably much more abundant than seipin and each seipin would have to bind multiple PA molecules. There are other ways seipin could suppresses nLD production. The authors might want to discuss them.

4. The issue of LD-INM connections is still somewhat unclear. The manuscript says the connections are transient, but this has not been quantified. Are broken connections ever restored and, if so, how often does that happen?

Point-by-point responses to the comments

Thank you for submitting your revised manuscript entitled "Nuclear lipid droplets form in the inner nuclear membrane in a seipin-independent manner". The manuscript was assessed by the original reviewers, who appreciated the changes made in revision. However, they also shared comments that require your attention. As you know, JCB limits all manuscripts to one round of major experimental revision. We have discussed the reviewer comments editorially, and because they only require minor changes at this stage, we are open to one final round of revision. We would not require new experimentation to address Reviewer #3 points #2 or #4 but instead recommend that you dedicate efforts to amend the manuscript as follows:

i) Please provide the controls for expression of NES-lipin1beta and GFP-DGAT2-PK (Ref #3, pt #1)

We compared GFP-NES-lipin-1β and GFP-PK-DGAT2 expression with that of the respective controls, GFP-lipin-1β and GFP-DGAT2, using Western blotting. The results indicate that both GFP-NES-lipin-1β and GFP-PK-DGAT2 are expressed in comparable amounts with the controls. The results were added to the manuscript as Figs. S1H (GFP-PK-DGAT2) and S2B (GFP-NES-lipin-1β).

ii) Please re-consider the discussion as per Reviewers #2 and #3. We feel that more discussion would benefit readers and would be interesting. We can accommodate reasonable extensions to the character count if this was a concern.

We revised the discussion on the PA issue by incorporating the results of GPAT3/4 overexpression that we previously presented only to the reviewers. The experiment showed that mCherry-GPAT3/4, overexpressed by transient cDNA transfection, were mostly distributed in the cytoplasm and induced large cytoplasmic LDs as reported before (Pagac et al, Cell Rep, 2016), but it did not increase nuclear LDs or nuclear PA puncta. This result suggests that, in the presence of seipin, PA produced by GPAT3/4 is used efficiently for LD biogenesis in the ER and scarcely increases PA in the INM. Diffusion of PA to the INM may also be restricted by binding to seipin (Yan et al, Dev Cell, 2018). Moreover, in the normal setting, seipin down-regulates GPAT3 and GPAT4 (Pagac et al, Cell Rep, 2016). When these effects are lost by seipin depletion, freely-diffusible PA is thought to increase in the ER and reach the INM.

We incorporated the result of the GPAT3/4 overexpression to the manuscript as Fig. S3H, and revised the related discussion. Possible involvement of a non-PA mechanism was also mentioned in the discussion (page 10, 2nd paragraph). We also modified the abstract and the introduction.

iii) Please include more rigorous descriptions of repeats/reproducibility as per JCB policy. Each figure should state the number of repeats and stats applied. You can see also below our general guidelines and can find more information on data presentation in this article: J Cell Biol (2020) 219 (6): e202001064 https://doi.org/10.1083/jcb.202001064

We would be happy to publish your paper in JCB pending changes as described above and final revisions necessary to meet our formatting guidelines (see details below). Please also provide a response to the reviewers' comments with your resubmission.

Please see below for our answers to the respective comments from the editors and the reviewers.

Editorial comments

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

Running title (50 characters max, including spaces): Nuclear lipid droplets form without seipin (We strongly recommend avoiding acronyms to increase the accessibility of the work)

The running title was revised as suggested.

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

The eTOC Summary, which begins with Soltysik et al., was added to the title page.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 2F (insets), S1B (magnifications), S2E (magnifications) Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add molecular weight with unit labels on the following panels: 5A (please add unit labels), S1C (unit labels), S2CF (unit labels), S3AG (unit labels)

Scale bars and molecular weight markers were added to the respective figures.

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.
Please indicate n/sample size/how many experiments the data are representative of for each panel representing pooled data.

The number of samples, the number of experiments, and other necessary information were added to each figure legend.

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*- 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**

Detailed information on vectors was added to the Materials and Methods section.

- 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.).

All the requested information was added to the Materials and Methods section.

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.
- Refs in the citation list should not be numbered. For more on our style, please check this link: https://rupress.org/jcb/pages/reference-guidelines

The Reference section was revised in accordance with the guideline.

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

This paper has been thoroughly revised. The authors did a great job. The quality of the data is very high. It should be accepted in its current form.

Thank you again for your insightful comments and questions. We believe that the manuscript was improved significantly by incorporating the changes that you suggested.

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The authors convincingly show that seipin restricts LD formation to the cytoplasm and away from the INM. When seipin levels are low, lipin1 is involved in LD formation at the INM, although the mechanism is unclear. The authors suggest that an increase in PA at the surface of nuclear LDs recruits lipin1 to nLDs.

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The authors have addressed my concerns, and do a nice job showing the role of seipin in restricting LDs to the cytoplasm and the involvement of lipin1 in formation/stability of nLDs. However, their conclusions about the role of PA remains the major weakness of their paper despite the new biochemical evidence that is shown in Fig. 5E. While I appreciate that this is a challenging experiment, it is difficult to assess the data as shown because there are overlapping values between the control and RNAi for PA levels in the nucleus. The authors should use different symbols for each trial so the results from each trial can be directly compared. Overall, I think the authors should consider deemphasizing the role of PA in their abstract and model because of the questions that have been raised. Although it hints at mechanism, it is not an essential aspect of the nice story they have put together.

For Fig. 5E, the three independent samples were prepared for both control RNAi and seipin RNAi, and the PA/PC ratio was quantified. The relatively minor difference between control and seipin RNAi in this measurement is likely caused because the nuclear fraction inevitably contains the outer nuclear membrane and some ER membranes. We think that the increase in PA in the nucleus is a major factor causing the increase of nuclear LDs in seipin-deficient cells, but it does not exclude the possibility that other factors are also involved. We mentioned this possibility in the revised manuscript (page 10, 2nd paragraph), and also modified the abstract and the introduction.

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

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We did not compare the results quantitatively, but the size of nuclear LDs in seipin-deficient cells does not appear to be significantly different from that in control cells that express seipin. Super-sized LDs and a cluster of small-sized LDs, which are known characteristics of cytoplasmic LDs in seipin-deficient cells, were not observed for nuclear LDs.

3. The idea that seipin sequesters PA and therefore regulates PA distribution in the ER seems implausible. PA is probably much more abundant than seipin and each seipin would have to bind multiple PA molecules. There are other ways seipin could suppresses nLD production. The authors might want to discuss them.

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We incorporated the results of the GPAT3/4 overexpression to the manuscript as Fig. S3H and revised the related discussion (page 10, 2nd paragraph).

4. The issue of LD-INM connections is still somewhat unclear. The manuscript says the connections are transient, but this has not been quantified. Are broken connections ever restored and, if so, how often does that happen?

We think that the nuclear LD–INM connection is transient, because the fluorescence recovery of GFP-DGAT2 and GFP-ACSL3 after photobleaching is observed only rarely in normal cells, although those proteins reach nuclear LDs through the INM. The increase in the ratio of the GFP-ACSL3 fluorescence recovery in seipin-deficient cells (Fig. 4E) indicates that the nuclear LD–INM connection may be more stable than in normal cells, but even in this condition, recovery occurs in only about half of the cases. Currently, there is no reliable method that can quantify the LD–membrane connection, nor a method to examine whether the connection can be restored after separation. We share these questions with the reviewer, and hope that they will be addressed in the near future.