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Peer Review File

Seipin governs phosphatidic acid homeostasis at the inner nuclear membrane

Corresponding Author: Professor Alwin Koehler

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Seipin governs phosphatidic acid homeostasis at the inner nuclear membrane

This study elegantly characterizes lipid metabolism in the inner nuclear membrane and nucleus; an important and underexplored topic. The lipid composition of the INM differs from that of the ONM, but this is regulated and impacts nuclear envelope biology is largely unknowns. PA plays a versatile role in lipid metabolism and elevated PA levels at the INM lead either to NE proliferation or to nLD formation, depending on the metabolic branch that is active. The authors perform a genome-wide screen using a PA sensor to identify regulators of INM PA homeostasis. The studies are then focused on Seipin (Sei1) which was identified in this screen but which was also previously known to be involved in PA and nuclear lipid droplet (nLD) biogenesis. To further characterize the role of Sei1 on INM PA homeostasis and nuclear lipid droplet (nLD) biogenesis, they used microscopy techniques to localize a number of lipid sensors (PS, PA, DAG) and fluorescently tagged proteins involved in LD formation. The EM images of sei1Δstrains are very intriguing! The authors further investigate the specific function of Sei1, and its cooperative interplay with its co-factor Ldb16, by employing several Sei1 and Ldb16 mutants. Seipin selectively acts on PA and DAG lipids without affecting PS, and Sei1 and Ldb16 have a partially different effect on PA levels and nLD formation. The structural modeling and mutant analysis generate an interesting model predicting how the triacylglycerol site may be involved in LD formation. The main/key result of the paper is that Sei1 preserves NE membrane integrity by preventing ectopic membrane formation and NE deformation, which is likely associated with its role on PA homeostasis, while Ldb16 only affects LD formation.

The manuscript is very interesting and the data is overall solid. The work presents novel insights, but it also strongly builds on previous work from the lab and from others. In my opinion, the main point of improvement for this manuscript would be to point out more clearly what has been published previously and what is truly novel. Other specific minor points that need clarification are listed below.

Minor comments:

Related to the genome-wide screen for PA regulators. The screen is not discussed very extensively and this reader is left with several questions:
1) The screen identified a total of 26 factors that potentially regulate INM PA homeostasis. The 6 stated in figure 1 were all previously known to be involved in PA metabolism (if I am not mistaken). It makes me very curious what the other putative hits were and also what selection by manual inspection was based on and how it was done (the methods only reads “if the PA sensor localization was not uniformly nucleoplasmic in at least 25% of cells.”
2) As the screening was done in a strain containing Erg6-GFP tagged, it would be interesting to show which are the results related to its localization in the different mutants identified.
3) A discussion connecting the PA sensor localisation with the function of the different mutants in PA homeostasis and/or nLD biogenesis would be valuable. This includes a discussion why the confirmed hits display an increased in the PA sensor in the INM, while most of the not-confirmed (with the exception of Sei1) have PA foci or have an inhomogeneous distribution.
4) Some of the known factors to be involved in PA homeostasis and LD formation (cds1, pah1, among other) were not detected in the screening. It would be interesting to check whether they participate in INM PA homeostasis and nLD regulation as well.

Related to the data showing that Seipin preserves nuclear membrane architecture
5) The authors show that in the absence of Seipin, the PA-containing structures do not stain with BODIPY, and suggest that this is because nLDs did not undergo TAG-enrichment as would be expected in complete LD formation. However, TEM detected the presence of small lipid droplet-like structures in the nucleus (as known from previous research), as well as in the cytoplasm. Can the authors demonstrate/ explain why these discrepancies exist between nLDs and cLDs?
6) As the characterization of PA phenotypes is based on PA sensor localization, it would be informative to include exemplary images of the different phenotypes included in the quantifications.
7) Given the role of Sei1 in PA homeostasis, can the authors specify whether the NE deformation is related membrane growth or rather lipid storage? Also, do we know if this is only attributed to PA homeostasis in the INM or a more general deregulation in the whole endomembrane system? This should be discussed.

Related to the data showing that Seipin differentially alters lipid dynamics at the INM/ Lipid features of Seipin-induced nLDs
8) The authors show that Seipin selectively influences specific lipid species at the INM, affecting PA and DAG without impacting PS. How do the authors explain that the phenotypes with the DAG sensor are mild compared to the PA sensor phenotype? Does Sei1 affect DAG homeostasis or DAG distribution on the INM, and can the authors discuss or test how?
Related to the localization of cytoplasmic LD biogenesis factors to nLDs
9) The formation of nuclear lipid droplets is triggered by targeting Seipin to the INM (NLS-Sei). This is a trick previously developed and characterized, but as it is an important intervention also in this paper, it should be discussed how similar the droplets formed are compared to the rare droplets formed in wt conditions.
10) While I understand that for temporarily interactions between low abundant proteins the BiFC assay is sometimes the best option, it is very important to clarify its limitations as a proximity assay. Both when a signal is and is not observed.
11) The authors identified 12 nLD-associated factors that further complement the nLD proteome, and conclude that nLD and cLD composition are similar. The characterization of the cLD is not included in the manuscript however, and I am wondering how similar the assays and conditions in previous papers have been to justify the statement that they are similar. Also, how many of the 12 had previously been described to be in close proximity of nLDs? The paper would benefit from a more thorough discussion on how the localization of the different factors could contribute to LD biogenesis regulation.
12) I am surprised that the authors chose to express the 12 genes from overexpression plasmids. Why not genomically tag the endogenous genes avoiding overexpression artefacts? To the least such potential overexpression artifacts should be discussed.

Related to the Sei1 mutants important for INM PA homeostasis
13) In Figure 5f, how can the signals from the Sei1 construct tagged with GFP be discriminate from the BODIPY signal considering they are imaged in the same channel? A quantification of the phenotypes of the mutants characterized would be useful.

Related to Ldb16 and Sei1 having distinguishable functions at the INM
14) The characterization of the Ldb16 6A mutant is very limited and poorly described. It seems that LD quantification (in h i) is done in cytoplasmic LD, not in nuclear LDs? See also point 16

Related to the Discussion:
15) The statement “Our screen successfully detected changes in INM PA levels due to precursor accumulation following the inhibition of phospholipid synthesis. This confirms that the INM senses local metabolic changes in the cell.” Does not reflect the main achievement of the paper in my opinion as the connection to metabolic changes are not prominent in the paper, nor does the screen play a very prominent role.
16) The authors state that, a key finding of this study is that a mutation in the TAG binding motif of Ldb16 specifically affects LD formation while leaving INM PA levels unchanged. The data that support this claim of the research is non exhaustive and confusing. The proper localization of PA sensor in this mutant (Figure 6 i) is the only data that support this claim, and the quantification (LD diameter Figure 6h) of LDs is done in the cytoplasm, as there is no nLDs.
17) A reference to the King et al and Meinema et al would be fitting related to the discussion of transport of membrane proteins to the inner nuclear membrane, and also in reference to the use of the linker and NLS region in NLS-Sei.
18) Please clarify the statement “possibly indicating localized lipid synthesis at INM””. In “nevertheless, such a scenario could prompt ….. localized lipid synthesis at INM.” Wasn’t this was established in previous work?
19) A discussion clarifying how the changes in the localization of the PA sensor, and factors involved in converting PA into DAG/TAG are associated to nLD maturation and biogenesis would help to put the findings into a more mechanistic perspective.

Reviewer #2

(Remarks to the Author)
Employing a genome-wide lipid biosensor screen in S. cerevisiae, the researchers identified Sei1 as crucial for maintaining PA homeostasis at the INM. The Sei1-null mutation perturbed only PA and DAG distribution at the INM without impacting PS, indicating that Sei1 specifically functions in neutral lipid homeostasis in the INM region. Additionally, this mutation resulted in small-sized nLDs and nuclear membrane deformation. The yeast analogue mutation of Sei1, associated with congenital lipodystrophy, also mimicked the null mutation by altering PA distribution in the INM and causing defective nLD formation. The manuscript reports that proteins associated with cytoplasmic LDs are also associated with nLDs when nLDs are induced by targeting Sei1 to the INM.
Overall, this is an interesting study that provides valuable insights. However, not all of the findings are novel. Despite this,

some of the findings are indeed significant and contribute meaningfully to the existing body of knowledge. In particular, the second half of the study, where the authors dissect the roles of Ldb16 and Sei1 in INM LD formation, is the most significant extension of the group's previous work. The data is sound, the methods are clear.
Find below some specific comments:
The screen is interesting, but it is unclear what impact it is adding to this specific study. The work later focuses on Seipin, and it is not surprising that Seipin is required for nLD formation and nuclear PA homeostasis, as this has been shown before (https://doi.org/10.1083/jcb.202005026). The screen also did not detect Ldb16, although specific experiments later on determined its involvement.
Can the authors comment on why Cds1 did not show up as a hit in the screen? Are those strains included in the DAmP library?
TEM in Figure and Supplementary Figure 1 are very hard to assess. Clustering of LDs is clear, but what the authors claim as defects in nER architecture is not sufficiently supported by the data provided. Higher resolution, better-quality images are needed to support this claim.
Enrichment of PA and DAG on nLDs is not new, as the authors have reported in their published work. The distribution of PS on the INM excluded from the nLD is interesting, but perhaps also not surprising. It is not clear what the functional implication or impact the authors are suggesting for this.
What do you mean by "proper" in this text: "Once more, it resembled sei1Δ cells and, unlike wild-type NLS-Sei1, could not form proper PA- and BODIPY-positive nLDs..."? In Figure 5D, NLS-Sei1 G225P has slightly more nLD + PA foci structures than NLS-Sei1. Also, the referenced Supplementary Figure 3E, F refers to "ØNLSSei1", which isn't clear from the text. Is this a control for the NLS target region?

Reviewer #3

(Remarks to the Author)
Mechanisms by which govern lipid membrane homeostasis at the nuclear membranes are fundamental questions and remain to be elucidated. This manuscript describes roles of yeast Seipin (Sei1) in lipid metabolism in the inner nuclear membrane and nuclear lipid droplet. It also describes that Seipin and its co-factors regulate phosphatidic acid homeostasis and nuclear lipid droplet formation. Experiments are well designed and carefully executed. Conclusions are supported by experimental data. I have no serious concerns but have only minor comments for readability.
1. The construct of NLS-Sei1 should be described more clearly as it plays an important role in the manuscript. On page 17, it is stated that "NLS-Sei1 contains the NLS and the linker of the INM transmembrane protein Heh2 (aa93-317) attached to Sei1". On page 6, it is stated that "appending the NLS of the INM protein Heh2 to Sei1 (abbreviated as NLS-Sei1)". NLS-Sei1 in Fig. 3d does not represent how NLS-Sei1 is constructed. In addition, Fig. 3d is not appropriately referred. Fig. 3d is cited at two places on page 7: "This targets Sei1 to the INM and leads to the formation of nLDs (Fig. 3d)", and "we induced nLD biogenesis via NLS-Sei1 (Fig. 3d)". These sentences have nothing to do with Fig. 3d.
2. Page 30, legend to Fig. 2a: The white dashed line is not explained throughout Fig. 2 to Fig. 6. Although it is described in Methods section, it would be nice to include "Cell contours are marked with a dashed white line" here at the first appearance in the figures.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I appreciate the extra experimental evidence, clarifications and discussion included in the manuscript and enthusiastically support publication of the manuscript.

Reviewer #3

(Remarks to the Author)
The comments have been appropriately addressed. I have no further concerns.

“Seipin governs phosphatidic acid homeostasis at the inner nuclear membrane”
Anete Romanauska, Edvinas Stankunas, Maya Schuldiner and Alwin Köhler

Point-by-point reply to the reviewers' comments

We appreciate the reviewers' positive feedback and valuable suggestions. Below, we have provided detailed explanations in response.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Seipin governs phosphatidic acid homeostasis at the inner nuclear membrane

This study elegantly characterizes lipid metabolism in the inner nuclear membrane and nucleus; an important and underexplored topic. The lipid composition of the INM differs from that of the ONM, but this is regulated and impacts nuclear envelope biology is largely unknowns. PA plays a versatile role in lipid metabolism and elevated PA levels at the INM lead either to NE proliferation or to nLD formation, depending on the metabolic branch that is active. The authors perform a genome-wide screen using a PA sensor to identify regulators of INM PA homeostasis. The studies are then focused on Seipin (Sei1) which was identified in this screen but which was also previously known to be involved in PA and nuclear lipid droplet (nLD) biogenesis. To further characterize the role of Sei1 on INM PA homeostasis and nuclear lipid droplet (nLD) biogenesis, they used microscopy techniques to localize a number of lipid sensors (PS, PA, DAG) and fluorescently tagged proteins involved in LD formation. The EM images of sei1Δstrains are very intriguing! The authors further investigate the specific function of Sei1, and its cooperative interplay with its co-factor Ldb16, by employing several Sei1 and Ldb16 mutants. Seipin selectively acts on PA and DAG lipids without affecting PS, and Sei1 and Ldb16 have a partially different effect on PA levels and nLD formation. The structural modeling and mutant analysis generate an interesting model predicting how the triacylglycerol site may be involved in LD formation. The main/key result of the paper is that Sei1 preserves NE membrane integrity by preventing ectopic membrane formation and NE deformation, which is likely associated with its role on PA homeostasis, while Ldb16 only affects LD formation.

The manuscript is very interesting and the data is overall solid. The work presents novel insights, but it also strongly builds on previous work from the lab and from others. In my opinion, the main point of improvement for this manuscript would be to point out more clearly what has been published previously and what is truly novel. Other specific minor points that need clarification are listed below.

We appreciate the positive feedback. In response to the suggested area for improvement, we have made every effort to be as explicit as possible in distinguishing our novel findings from existing research, and have included additional references. All other minor points are addressed below.

Minor comments:

Related to the genome-wide screen for PA regulators. The screen is not discussed very extensively and this reader is left with several questions:

1) The screen identified a total of 26 factors that potentially regulate INM PA homeostasis. The 6 stated in figure 1 were all previously known to be involved in PA metabolism (if I am not mistaken). It makes me very curious what the other putative hits were and also what selection by manual inspection was based on and how it was done (the methods only reads "if the PA sensor localization was not uniformly nucleoplasmic in at least 25% of cells."

We have expanded the list of initial positive hits to include those that did not pass the secondary validations. (Supplementary Figure 1a and b). A more detailed explanation of the selection criteria can be found in the methods section.

"Each strain was analyzed based on its PA sensor localization. A strain was considered a 'hit' if the PA sensor localization was not uniformly nucleoplasmic (e.g., foci, INM localization) in at least 25% of cells analyzed. For all identified hits, over 100 cells were examined, unless stated otherwise in Supplementary Fig. 1a. To validate the results, we first re-examined positively tested strains under exponential growth conditions in liquid media and then examined these strains in a different genetic background (BY4741) than the screening strain."

2) As the screening was done in a strain containing Erg6-GFP tagged, it would be interesting to show which are the results related to its localization in the different mutants identified.

We have now included experiments showing the localization of Erg6-mNeonGreen in the validated positive hits from the screen (Supplementary Fig. 1c). As previously reported, Erg6 localizes to the ER but becomes enriched on LDs as they begin to form (e.g. Jacquier et al., J Cell Sci, 2011). When the Erg6 fluorescence signal displays a circular pattern, it likely indicates association with spherical LDs.

3) A discussion connecting the PA sensor localisation with the function of the different mutants in PA homeostasis and/or nLD biogenesis would be valuable. This includes a discussion why the confirmed hits display an increased in the PA sensor in the INM, while most of the not-confirmed (with the exception of Sei1) have PA foci or have an inhomogeneous distribution.

Most factors that failed secondary validation did indeed display PA-positive foci, but we cannot provide a clear explanation for why their deletion results in PA foci rather than increased INM PA, or why the penetrance of the phenotype varies by strain background or growth conditions. Although these factors might influence the expression of lipid metabolism enzymes or Seipin to some extent, other possibilities exist, so we prefer to avoid speculation.

4) Some of the known factors to be involved in PA homeostasis and LD formation (cds1, pah1, among other) were not detected in the screening. It would be interesting to check whether they participate in INM PA homeostasis and nLD regulation as well.

It is common for screens to miss some theoretical candidates, and while the reasons for this are not always clear, we can address the two cases mentioned by the reviewer.

Upon further inspection, we found that the *pah1*Δ strain was not included in the screened libraries, likely due to technical difficulties during automated library preparation. Although the *cds1* allele was present in the DAmP library, the PA sensor in the screen was nucleoplasmic. In contrast, a different *cds1-ts* allele, which we characterized in a previous publication

(Romanauska & Koehler, Cell, 2018), showed a robust accumulation of the PA sensor at the INM and nLD formation.

The most likely explanation for this difference is the genotype of the mutants: The *ts* mutation may inactivate or destabilize the enzyme at 37°C, whereas the DAmP allele reduces expression of the (intact) enzyme by disrupting its 3′ UTR, lowering transcript levels sometimes by only twofold (Schuldiner et al., 2005). As a result, the *cds1-ts* allele may simply exhibit a stronger inactivation phenotype than the DAmP variant, explaining why we did not pick it up in the screen. We have now commented on this in the manuscript:

"Some expected hits were absent from our screen, likely due to technical issues during automated library preparation or strain propagation, such as the missing pah1Δ and ldb16Δ strains. Although a cds1 allele was included in the DAmP library, the PA sensor was nucleoplasmic. In contrast, a different cds1-ts allele that we previously studied[7] showed robust PA sensor accumulation at the INM and nLD formation. This discrepancy is likely because the cds1-ts allele has a stronger inactivation phenotype than the DAmP variant."

Other factors that theoretically increase cellular PA can go undetected if overexpression is required to elevate PA levels (e.g., Dgk1), a condition not tested in our screen.

Related to the data showing that Seipin preserves nuclear membrane architecture
5) The authors show that in the absence of Seipin, the PA-containing structures do not stain with BODIPY, and suggest that this is because nLDs did not undergo TAG-enrichment as would be expected in complete LD formation. However, TEM detected the presence of small lipid droplet-like structures in the nucleus (as known from previous research), as well as in the cytoplasm. Can the authors demonstrate/ explain why these discrepancies exist between nLDs and cLDs?

The question of whether nLDs and cLDs differ in lipid composition is intriguing, but we are cautious about speculating based on our current data. Importantly, our NLS-PA sensor is targeted specifically to the nucleus and does not monitor the cytoplasmic compartment, so we cannot directly compare the PA/TAG ratio of nLDs and cLDs.

Given that the nuclear droplet-like structures in *sei1*Δ cells are very small by TEM, it seems plausible why they stain poorly with the BODIPY dye. *sei1*Δ cells have been reported to contain a mix of supersized cytoplasmic LDs and very small, clustered LDs (Cartwright et al., MBoC, 2015; Fei et al., J Cell Biol, 2008; Fei et al., PLoS Genet, 2011; Szymanski et al., PNAS, 2007). While the supersized LDs stain brightly with BODIPY, the properties of the small cytoplasmic LDs are not well characterized; they might resemble the nuclear structures and also stain poorly with BODIPY.

One clear difference between the nucleus and cytoplasm is that we did not observe supersized LDs in any of the EM images of 310 *sei1*Δ cell nuclei examined. Whether this indicates a distinct nLD biogenesis mechanism or simply reflects the lower abundance of nuclear LDs, which do not fuse into larger structures, remains to be determined. We have added the following comment (underlined) in the results part:

"*Consistent with earlier EM data, we observed cytoplasmic LDs of heterogeneous size in sei1*Δ *cells, including clusters of small LDs and supersized LDs (Supplementary Fig. 2e, j, l)*[18,20-22]*. In contrast, no supersized LDs were observed in the nucleus of sei1*Δ *cells in any of the TEM images examined.*"

6) As the characterization of PA phenotypes is based on PA sensor localization, it would be informative to include exemplary images of the different phenotypes included in the quantifications.

We have included exemplary images of PA sensor localization in Supplementary Fig. 3a.

7) Given the role of Sei1 in PA homeostasis, can the authors specify whether the NE deformation is related membrane growth or rather lipid storage? Also, do we know if this is only attributed to PA homeostasis in the INM or a more general deregulation in the whole endomembrane system? This should be discussed.

Since PA is crucial to lipid metabolism and serves as a precursor for both membrane synthesis and lipid storage, we would expect to see effects on both of these pathways. However, it is challenging to mechanistically explain how these complex NE phenotypes develop. For instance, NE herniations, which are distinctive and observed in various conditions, still lack a unifying explanation for their origins (see Thaller & Lusk, Biochem Soc Trans, 2018). We cannot rule out potential perturbations of the endomembrane system, though TEM revealed no striking ER morphology changes in *sei1Δ* cells, except for ER membranes surrounding cLDs (Wolinski et al., Biochim Biophys Acta, 2011). However, our main finding is the NE's specific vulnerability to Seipin malfunction, which may potentially disrupt nuclear function.

We are covering these issues in the revised Discussion:

"Although we cannot determine the specific contribution of either the INM or ONM pool of Sei1 to these phenotypes, it appears likely that Sei1 deficiency affects the NE through its involvement in PA and TAG metabolism, possibly in combination. In bilayer membranes, TAG is soluble up to a concentration of about 3 mol%[77]. Beyond this concentration, oil lenses form spontaneously[64,78], which might disrupt the NE membrane and the function of NE resident proteins. The irregular LD budding processes themselves could potentially form weak points in the NE. The accumulation of PA as a non-bilayer lipid[4] may additionally destabilize the NE.

The unexpected appearance of ectopic intranuclear membranes suggests a potential increase in membrane production. Multiple studies across different model organisms have detected heightened cellular PA levels following Seipin depletion[21,69,79,80]. However, it remains uncertain whether this results from an inhibition of proper LD formation, causing a buildup of precursors (Fig. 1a). Regardless of the origin, increased cellular PA levels could prompt a diversion of PA toward PL synthesis, thereby boosting membrane production. The precise location where these membranes might form (bearing in mind that S. cerevisiae has a closed mitosis) poses an intriguing question, possibly indicating a misregulation of localized lipid synthesis at the INM. Regarding the NE herniations in sei1Δ cells, it is noteworthy that the ESCRT factor Chm7, which plays a role in NE membrane surveillance, specifically recognizes PA and is thought to repair these PA-rich NE defects[81]. If such nuclear irregularities occur in patients with Berardinelli-Seip lipodystrophy, this would offer intriguing new insights into the disease pathology."

Related to the data showing that Seipin differentially alters lipid dynamics at the INM/ Lipid features of Seipin-induced nLDs.

8) The authors show that Seipin selectively influences specific lipid species at the INM, affecting PA and DAG without impacting PS. How do the authors explain that the phenotypes with the DAG sensor are mild compared to the PA sensor phenotype? Does Sei1 affect DAG homeostasis or DAG distribution on the INM, and can the authors discuss or test how?

Yes, Sei1 is indeed necessary for the proper distribution of DAG in the INM. We do not characterize the effect as "mild"; rather, the intranuclear distribution of DAG is simply different from that of PA.

We state the following:

*"The DAG sensor labels the INM smoothly in wild-type cells (**Fig. 3a**). In contrast, in sei1Δ cells the DAG sensor exhibited non-homogenous staining and detected nuclear foci in ~70% of cells compared to ~14% in the wild type (**Fig. 3a, b**). Using Sec62 as an NE marker, we determined that ~ 10% of DAG foci are present in the nucleoplasm of sei1Δ cells, with the remainder localizing to the INM. In contrast, ~30% of PA foci in sei1Δ cells are nucleoplasmic (**Supplementary Fig. 3b, c**). Collectively this suggests that besides its role in maintaining nuclear PA homeostasis, Seipin is also required for a homogeneous distribution of DAG, the downstream metabolite of PA, at the INM."*

We conclude:

"These comparative assessments suggest that DAG is present on the surface of nLDs while still maintaining localization at the INM. Conversely, PA can be fully incorporated into the nLD lipid monolayer with minimal PA remaining at the INM."

Related to the localization of cytoplasmic LD biogenesis factors to nLDs
9) The formation of nuclear lipid droplets is triggered by targeting Seipin to the INM (NLS-Sei). This is a trick previously developed and characterized, but as it is an important intervention also in this paper, it should be discussed how similar the droplets formed are compared to the rare droplets formed in wt conditions.

Morphologically, we observe no significant differences between the nLDs induced by NLS-Sei1, those induced by oleic acid in wild-type cells, or the nLDs resulting from the deletion of *INO2/4* or *CDS1* inactivation. All nLDs stain positively with BODIPY and exhibit surface enrichment of PA and DAG. NLS-Sei1-induced nLDs frequently exhibit a widened perinuclear space next to the growing nLD when examined by TEM. It is currently unclear whether this is a distinctive feature or simply an intermediate state that becomes more apparent when Sei1 is concentrated at the INM. As demonstrated in this study, a number of factors are shared between nLDs and cLDs. We have updated the text address this issue (changes underlined):

"Because nLDs are rare in wild-type cells not overloaded with fatty acids, we targeted Seipin to the INM using a method developed earlier[8], which involves appending the NLS and linker region of the INM protein Heh2 (aa93-317) (Meinema et al., 2011) to Sei1 (abbreviated as NLS-Sei1). This targets Sei1 to the INM and leads to the formation of nLDs (Fig. 3d). These nLDs exhibit a notable enrichment in PA, forming a distinct outer shell around a BODIPY-positive core composed of neutral lipids (Fig. 3f). Based on these characteristics, they resemble the nLDs observed in wild-type cells after oleic acid supplementation or those induced by genetic modifications, such as INO4 deletion or CDS1 inactivation (Romanauska & Koehler, Cell, 2018)."

10) While I understand that for temporarily interactions between low abundant proteins the BiFC assay is sometimes the best option, it is very important to clarify its limitations as a proximity assay. Both when a signal is and is not observed.

We agree. This is why we began with standard LD-protein co-localization experiments (Fig. 4a), followed by BiFC to capture more transient interactions (Fig. 4b), reasoning that using two orthogonal techniques is superior to relying on just one. The text states clearly where both techniques produce the same results and where they differ. We have now also added a reference to direct the reader to an in-depth review that addresses the advantages and pitfalls of BiFC (Kerppola, Annu Rev Biophys., 2008).

11) The authors identified 12 nLD-associated factors that further complement the nLD proteome, and conclude that nLD and cLD composition are similar. The characterization of the cLD is not included in the manuscript however, and I am wondering how similar the assays and conditions in previous papers have been to justify the statement that they are similar. Also, how many of the 12 had previously been described to be in close proximity of nLDs? The paper would benefit from a more thorough discussion on how the localization of the different factors could contribute to LD biogenesis regulation.

We agree that the question of how similar or different nLDs and cLDs are is a fascinating one. However, we are hesitant to speculate too much on this matter and suggest leaving it for a follow-up paper. Our main point is that many components of the cLD biogenesis machinery can be present in the nucleus, which is an important finding. We currently take no stance on the stoichiometry of these components, and indeed, variations in the concentration of LD-related factors may speed up or slow down steps in LD formation, influencing how many LDs of a given size are produced. Additionally, differences in lipid metabolism enzymes could lead to variations in the lipid composition of the nLD monolayer, potentially resulting in distinct lipid-protein interactions that are optimized for nuclear rather than cytoplasmic proteins.

We have now included a discussion of how differences in LD factor stoichiometry could regulate nLD vs. cLD production. It reads:

"Our study suggests that the core machinery of Seipin-dependent LD formation is shared between nLDs and cLDs. However, the stoichiometry and abundance of these components may vary, as NPCs might hinder or restrict access to the INM for some factors. This could influence the biogenesis kinetics, number, and morphology of LDs formed in each compartment. Consequently, variations in the stoichiometry and abundance of lipid metabolism enzymes and other LD-associated factors in the nucleus could result in a distinct nLD monolayer composition and protein inventory, potentially leading to functional specializations of nLDs that are tailored to the nuclear environment."

In response to the reviewer's comment, we have replaced the images for Nem1, Dga1, Pet10, Pdr16 and Tgl1 in Fig. 4a with ones that display both nLDs and cLDs in the same field of view. Additionally, we have labeled cLDs in the images for Erg6, Ldo45, and Tgl5 to facilitate comparison between nLDs and cLDs and we highlight similarities and differences in the text. While these are clear-cut results for factors that associate strongly with nLDs and cLDs, transient interactions will require alternative methods for a comparative assessment.

To highlight the factors previously known to be associated with NLS-Sei1-induced nLDs, as well as those that are newly identified, we have included the following statement in the manuscript:

"*For NLS-Sei1 induced nLDs, only two factors (Dga1 and Pet10) were previously identified as associated with nLDs (Romanauska & Koehler, DevCell, 2021), while this study has identified ten additional factors (Nem1, Spo7, Pah1, Pex30, Lro1, Erg6, Pdr16, Ldo45, Tgl1, Tgl5).*"

We have now also updated the last paragraph in that section (underlined):

"*Collectively, these findings indicate that many factors involved in the formation of cLDs can reach the INM and associate with nLDs (Fig. 4c). For NLS-Sei1-induced nLDs, only two factors (Dga1 and Pet10) were previously identified as associated with nLDs8, while this study has identified ten additional factors (Nem1, Spo7, Pah1, Pex30, Lro1, Erg6, Pdr16, Ldo45, Tgl1, Tgl5). The presence of PA metabolic enzymes and TAG synthase is attributed tocan mediate*

nLD growth, while lipases catalyze nLD degradation and TAG mobilization51, indicating both nLD formation and turnover in the nucleus. Consequently, the processes governing nLD and cLD formation in yeast share the same core molecular machinery, despite occurring in distinct cellular compartments. However, differences in enzyme abundance and other yet-to-be-identified factors may create compositional differences between nLDs and cLDs."

12) I am surprised that the authors chose to express the 12 genes from overexpression plasmids. Why not genomically tag the endogenous genes avoiding overexpression artefacts? To the least such potential overexpression artifacts should be discussed.

To ensure the detection of all factors tagged with mGFP, we used stronger promoters (e.g., the *GPD* promoter). Upon repeating the experiments with the factors that exhibit the strongest association with nLDs, we observed a clear association with nLDs for Dga1, Nem1, Tgl1, Tgl5, Erg6, and Pdr16, even when these proteins were expressed from their endogenous promoters (see new Supplementary Figure 4a). Pet10 in Fig. 4a was already expressed from its endogenous promoter. We have now specified the promoters used for the constructs also in the figure legends.

Related to the Sei1 mutants important for INM PA homeostasis
13) In Figure 5f, how can the signals from the Sei1 construct tagged with GFP be discriminate from the BODIPY signal considering they are imaged in the same channel? A quantification of the phenotypes of the mutants characterized would be useful.

This is correct and was intentional. The BODIPY fluorescence signal of LDs is significantly brighter and distinctive (appearing as filled green spheres) compared to the weakly expressed *SEI1* construct (ER/NE puncta), so there was no risk of confusing LDs with Sei1. We also prioritized using the same *SEI1* construct to simplify comparisons rather than creating different versions with and without GFP. We now explain the rationale for this approach when it is first used for Sei1 in the relevant figure legend 5c and similarly for Ldb16-mGFP in 6h:

"Note that even though cells contain mGFP-Sei1, the green BODIPY fluorescence signal is significantly brighter, hence Sei1 fluorescence remains undetectable when the settings for BODIPY imaging are applied."

As suggested, a quantification of 5f is now included in Supplementary Figure 5h. This confirmed our original statement in the manuscript:

"The combined mutant (Patches1+2) exhibited an abnormal PA distribution in the nucleus and could not form nLDs when targeted to the INM with an NLS (Fig. 5f and Supplementary Fig. 5g, h). This defect could not be compensated by overexpressing the mutant from the strong GPD promoter (Fig. 5f and Supplementary Fig. 5h, i).

Related to Ldb16 and Sei1 having distinguishable functions at the INM
14) The characterization of the Ldb16 6A mutant is very limited and poorly described. It seems that LD quantification (in h i) is done in cytoplasmic LD, not in nuclear LDs? See also point 16

We have characterized the Ldb16 6A mutant with respect to its 1) localization, 2) impact on PA metabolism at the INM, and 3) effects on cellular LDs.

The reviewer is correct that we quantified the total LD content in this mutant, which consists mostly of cLDs. This is because the Ldb16 6A mutant behaves similarly to wild-type Ldb16 and produces few, if any, LDs. Following the reviewer's suggestion, we conducted additional

experiments where we targeted NLS-Sei1 to the INM in a Ldb16 6A mutant background to be able to assess the effect on nLDs. The new results are now included in the manuscript:

*"To explore the effect of the hydroxyl residues of Ldb16 specifically on nLD formation, we induced nLDs via NLS-Sei1 in the Ldb16 6A mutant. Interestingly, this led to a reduced number of nLDs, which were significantly larger compared to those generated in Ldb16 wild-type cells (**Fig. 6j, k**)."*

Related to the Discussion:
15) The statement "Our screen successfully detected changes in INM PA levels due to precursor accumulation following the inhibition of phospholipid synthesis. This confirms that the INM senses local metabolic changes in the cell." Does not reflect the main achievement of the paper in my opinion as the connection to metabolic changes are not prominent in the paper, nor does the screen play a very prominent role.

We have followed the reviewer's suggestion and revised the text accordingly (changes underlined):

"The close proximity of the INM and ONM makes it difficult to isolate them into pure fractions suitable for lipidomics[68]. To identify INM regulators of the key precursor lipid PA, we conducted what we believe to be the first genome-wide screen to detect changes in INM lipid dynamics. Our screen successfully detected changes in INM PA levels, with Seipin emerging as a key regulator. We found that Sei1 is essential for maintaining nuclear envelope integrity and controlling nLD biogenesis, and identified specific residues, including those analogous to human lipodystrophy mutations, as critical for INM PA homeostasis. We also examined Seipin's co-factor Ldb16, demonstrating that TAG enrichment and INM PA regulation are distinct functions. Our mapping of nLD-associated factors reveals that nLDs and cLDs share core machinery for biogenesis and turnover. However, variations in some factors may indicate differences in the lipid and protein composition between nLDs and cLDs."

16) The authors state that, a key finding of this study is that a mutation in the TAG binding motif of Ldb16 specifically affects LD formation while leaving INM PA levels unchanged. The data that support this claim of the research is non exhaustive and confusing. The proper localization of PA sensor in this mutant (Figure 6 i) is the only data that support this claim, and the quantification (LD diameter Figure 6h) of LDs is done in the cytoplasm, as there is no nLDs.

The normal localization of the PA sensor in the Ldb16 mutant is a surprising and significant result that aligns with our statement. Since cLDs make up the majority of LDs, our characterization focused on total LDs.

To further advance the analysis, we conducted an experiment to examine the effect of the Ldb16 6A mutation specifically on nLDs. We induced nLD formation using NLS-Sei1 and compared the characteristics of nLDs between cells expressing the Ldb16 6A mutant and those with wild-type Ldb16. Notably, the Ldb16 6A mutation resulted in fewer nLDs, which were significantly larger than those found in wild-type cells (new Fig. 6j, k). This finding is important as it reveals that the Ldb16 6A mutation negatively impacts nLDs, despite normal INM PA levels. It supports the idea that TAG delivery into nLDs and INM PA homeostasis can be experimentally separated. We have simplified and adjusted the model in Fig. 7b accordingly.

"*To explore the effect of the hydroxyl residues of Ldb16 specifically on nLD formation, we induced nLDs via NLS-Sei1 in the Ldb16 6A mutant. Interestingly, this led to a reduced*

number of nLDs, which were significantly larger compared to those generated in Ldb16 wild-type cells (Fig. 6j, k).”

17) A reference to the King et al and Meinema et al would be fitting related to the discussion of transport of membrane proteins to the inner nuclear membrane, and also in reference to the use of the linker and NLS region in NLS-Sei.

Thank you for the suggestion, we have included these references.

18) Please clarify the statement “possibly indicating localized lipid synthesis at INM””. In “nevertheless, such a scenario could prompt ….. localized lipid synthesis at INM.” Wasn’t this was established in previous work?

Our previous research primarily centered on lipid storage metabolism and the biogenesis of nLDs, rather than *de novo* membrane synthesis. Our new findings reveal the formation of intranuclear membranes, which likely result from dysregulated membrane synthesis at the INM. This suggests that normal Seipin function prevents ectopic intranuclear membrane formation.

We rewrote the statement to make it more clear:

“Regardless of the origin, increased cellular PA levels could prompt a diversion of PA toward PL synthesis, thereby boosting membrane production. The precise location where these membranes might form (bearing in mind that S. cerevisiae has a closed mitosis) poses an intriguing question, possibly indicating a misregulation of localized lipid synthesis at the INM.”

19) A discussion clarifying how the changes in the localization of the PA sensor, and factors involved in converting PA into DAG/TAG are associated to nLD maturation and biogenesis would help to put the findings into a more mechanistic perspective.

We have added the following paragraph to the Discussion:

“Our study suggests that the core machinery of Seipin-dependent LD formation is shared between nLDs and cLDs. However, the stoichiometry and abundance of these components may vary, as NPCs might hinder or restrict access to the INM for some factors. This could influence the biogenesis kinetics, number, and morphology of LDs formed in each compartment. Consequently, variations in the stoichiometry and abundance of lipid metabolism enzymes and other LD-associated factors in the nucleus could result in a distinct nLD monolayer composition and protein inventory, potentially leading to functional specializations of nLDs that are tailored to the nuclear environment.”

We are hesitant to provide any further speculation beyond what was stated earlier.

“Earlier reports suggested that PA plays a role in the formation of cytoplasmic LDs[21], however, the precise mechanism is still unclear. PA, being a cone-shaped lipid, promotes negative membrane curvature due to its small anionic phosphomonoester head group lying relatively close to the lipid bilayer’s hydrophobic interior. PA can therefore trigger various membrane fusion and fission events, possibly because of its capacity to create non-bilayer phases[4]. At the INM, PA might play a role in nLD biogenesis by inducing nascent nLD membrane curvature and bud neck remodelling. The extent to which PA at the INM acts as a precursor for localized TAG synthesis also requires further clarification. Enzymes involved in converting PA into TAG,

such as Pah1, its regulators Nem1 and Spo7, and the TAG-synthase Dga1 are in proximity to nLDs, as shown in this study. While Seipin has been reported to interact with Lipin (the mammalian ortholog of Pah1) in adipocytes[69], this connection has yet to be validated in yeast."

Reviewer #2 (Remarks to the Author):

Employing a genome-wide lipid biosensor screen in S. cerevisiae, the researchers identified Sei1 as crucial for maintaining PA homeostasis at the INM. The Sei1-null mutation perturbed only PA and DAG distribution at the INM without impacting PS, indicating that Sei1 specifically functions in neutral lipid homeostasis in the INM region. Additionally, this mutation resulted in small-sized nLDs and nuclear membrane deformation. The yeast analogue mutation of Sei1, associated with congenital lipodystrophy, also mimicked the null mutation by altering PA distribution in the INM and causing defective nLD formation. The manuscript reports that proteins associated with cytoplasmic LDs are also associated with nLDs when nLDs are induced by targeting Sei1 to the INM.
Overall, this is an interesting study that provides valuable insights. However, not all of the findings are novel. Despite this, some of the findings are indeed significant and contribute meaningfully to the existing body of knowledge. In particular, the second half of the study, where the authors dissect the roles of Ldb16 and Sei1 in INM LD formation, is the most significant extension of the group's previous work. The data is sound, the methods are clear.

We appreciate the reviewer's positive feedback. Below, we address the points that were raised:

Find below some specific comments:
The screen is interesting, but it is unclear what impact it is adding to this specific study. The work later focuses on Seipin, and it is not surprising that Seipin is required for nLD formation and nuclear PA homeostasis, as this has been shown before (https://doi.org/10.1083/jcb.202005026)

We think the value and innovation of this screen lie in its unbiased, genome-wide approach, which enables the investigation of the distribution and regulation of critical lipids at the INM. To our knowledge, this approach is unprecedented. Seipin emerged as a significant hit, making it a compelling target for further investigation - not only to showcase the screen's utility but also because of its crucial role in LD biogenesis.

In response to the reviewer's reference to the study by Soltysik et al. (JCB, 2020) from the Fujimoto lab, we would argue that the role of Seipin in nLD biogenesis is less definitive than commonly perceived, partly due to the generalizations made in that study.

While Soltysik et al. conclude that nLD formation is independent of Seipin based on their findings and techniques used in a single human cell line, our data directly challenge this generalization. Both our current and previous studies demonstrate that Seipin plays an essential role in the proper biogenesis of nLDs (Romanauska & Koehler, Cell, 2018; Romanauska & Koehler, DevCell, 2021).

Specifically, Soltysik et al. assert that nuclear LDs form in the INM independently of Seipin based on their studies of an osteosarcoma cell line (U2OS). Their key evidence for this model is the apparent lack of immunogold labeling of Seipin at the INM. However, a plausible alternative explanation is that Seipin might be present at the INM in such low abundance that it was not detected. The absence of evidence does not equate to evidence of absence.

Overall, while it is known that "droplet-like structures" can form in the absence of Seipin, these LDs often exhibit abnormal properties. For instance, in yeast, *SEI1* deletion results in clusters of irregularly sized lipid droplets (Szymanski et al., PNAS, 2007), with some localizing to the nucleus (Cartwright et al., MBoC, 2015). Thus, not all droplet-like structures seem properly matured, which is a critical consideration when studying Seipin deletions or mutations.

We have addressed these issues in the revised Discussion section:

"Studying Seipin function specifically at the INM poses experimental challenges due to the proximity of the INM and ONM. This is further exacerbated because Seipin is estimated to be expressed at low levels (~850 molecules in yeast[70]), which translates to only about 85 decameric complexes in the entire yeast ER/NE network. This low abundance makes immunogold EM, which has low sensitivity, unreliable to confirm Seipin localization to the INM. In contrast, techniques such as BiFC[7] and split-GFP[71] detect Seipin at the INM. A previous study, using a human osteosarcoma cell line, arrived at the conclusion that nLD formation does not depend on Seipin[72]. This conclusion was based on the inability to detect Seipin at the INM through immunogold EM and on the assumption that uncontrolled phase separation of TAG from membranes in the absence of Seipin equates to properly matured LDs[72]. However, without Seipin, a conserved factor from yeast to human, the phase transition of neutral lipids becomes irregular, resulting in formation of many small and few supersized LDs[20,22]. Therefore, the presence of LD-like structures alone does not indicate that LD formation is independent of Seipin. Although TAG phase separation can occur without Seipin, proper LD biogenesis requires it. Seipin is essential for the coordinated transfer of lipids and proteins onto LDs[51]. An imbalance of phospholipid surfactants, an increase in fusogenic lipids such as PA and ripening defects might cause LDs to merge into larger supersized LDs[21,73,74] or prevent the accumulation of sufficient TAG into an nLD, as shown in this study."

The screen also did not detect Ldb16, although specific experiments later on determined its involvement.

Yes, that is correct. Due to technical issues during automated strain crossings and propagation, this deletion strain must have been lost. The text reads:

"In our high-throughput PA screen, the ldb16Δ well contained no viable cells, preventing us from assessing the impact of Ldb16 on INM PA."

Can the authors comment on why Cds1 did not show up as a hit in the screen? Are those strains included in the DAmP library?

Although the *cds1* allele was present in the DAmP library, the PA sensor in the screen was nucleoplasmic. In contrast, a different *cds1-ts* allele, which we characterized in a previous publication (Romanauska and Koehler, Cell, 2018), showed a robust accumulation of the PA sensor at the INM and nLD formation. We comment on this in the materials and methods section:

"Some expected hits were absent from our screen, likely due to technical issues during automated library preparation or strain propagation, such as the missing pah1Δ and ldb16Δ strains. Although a cds1 allele was included in the DAmP library, the PA sensor was nucleoplasmic. In contrast, a different cds1-ts allele that we previously studied[7] showed robust PA sensor accumulation at the INM and nLD formation. This discrepancy is likely because the cds1-ts allele has a stronger inactivation phenotype than the DAmP variant."

TEM in Figure and Supplementary Figure 1 are very hard to assess. Clustering of LDs is clear, but what the authors claim as defects in nER architecture is not sufficiently supported by the data provided. Higher resolution, better-quality images are needed to support this claim.

We believe that the quality of our TEM data is on par with previous TEM data from our lab (Romanauska & Köhler, Cell, 2018; Romanauska & Köhler, DevCell, 2021; Romanauska & Köhler, NCB, 2023). We apologize if the labeling of the figures caused confusion; we have now optimized the labeling to clarify the continuity of the NE. We are unsure of another way to classify these intranuclear membranes beyond describing them as “ectopic intranuclear membranes” and “omega-shaped NE herniations,” which closely resemble other reported examples (e.g., Thaller & Lusk, Biochem Soc Trans, 2018). Having screened 310 cell nuclei by TEM - a substantial number for such studies - we hope the improved labeling resolves the issue.

Enrichment of PA and DAG on nLDs is not new, as the authors have reported in their published work. The distribution of PS on the INM excluded from the nLD is interesting, but perhaps also not surprising. It is not clear what the functional implication or impact the authors are suggesting for this.

From a technological standpoint, the simultaneous study of nLD lipids has value by revealing both similarities and differences in lipid behavior. We did not have preconceived notions about how PS would behave, but were intrigued by its distinct behavior compared to PA and DAG as it highlights the differential dynamics of lipids during nLD biogenesis. We also believe that our sensor will be a valuable asset to the community.

What do you mean by "proper" in this text: "Once more, it resembled sei1Δ cells and, unlike wild-type NLS-Sei1, could not form proper PA- and BODIPY-positive nLDs..."?

Thank you for highlighting this ambiguity. By "proper," we referred to nLDs that are positive for both PA and BODIPY, as observed in wild-type cells fed with oleic acid, rather than the "aberrant" membrane structures that are PA-positive but not reactive to BODIPY, and may lack proper TAG accumulation. We have revised the sentence accordingly (changes underlined):

“About 50% of sei1Δ cells showed foci that were positive for PA but did not stain with BODIPY; ~10% displayed a mixed phenotype with both PA-positive/BODIPY-negative foci and PA-positive/BODIPY-positive foci. In contrast, only a very small fraction of cells (~2%) contained PA-positive/BODIPY-positive structures <u>which resemble properly matured LDs</u> (Fig. 2b and Supplementary Fig. 3a).”

Also, we rewrote the sentence:

“*Once more, it resembled sei1Δ cells and, unlike wild-type NLS-Sei1, <u>was unable to form properly matured nLDs which are characterized by PA- and BODIPY-positive staining</u> (Fig. 5c, d and Supplementary Fig. 5e, f).”*

In Figure 5D, NLS-Sei1 G225P has slightly more nLD + PA foci structures than NLS-Sei1.

This is correct. Our focus, though, was on the fact that the mutant lacks mature nLDs that are BODIPY-positive and have a PA shell. The “nLD+PA” foci category may reflect biogenesis intermediates which, however, do not ripen further into mature nLDs. To better

understand these PA phenotypes, we have now included representative images in Supplementary Figure 3a (as also asked by Reviewer 1, point 6).

Also, the referenced Supplementary Figure 3E, F refers to "ØNLSSei1", which isn't clear from the text. Is this a control for the NLS target region?

We are sorry for not having explained the abbreviation well enough. Yes, this indicates the deletion of the Heh2 NLS in the NLS-Sei1 construct. This control demonstrates that the Heh2 NLS is necessary for the import into the nucleus and formation of nLDs. Without it, Sei1 is not targeted to the INM and has the same PA phenotype as wild-type Sei1.

We have described it in the corresponding Supplementary Figure legend and have now included an expanded description to make it clearer for the reader in Supplementary Figure legend 5b:

"NLS-Sei1 contains the nuclear localization sequence (NLS) and the linker of the INM protein Heh2, whereas the ØNLS-Sei1 lacks the NLS and contains only the linker of the INM transmembrane protein Heh2 (aa138-317) attached to Sei1."

and Supplementary Figure legend 5f:

"ØNLS-Sei1 lacks the NLS and contains only the linker of the INM transmembrane protein Heh2 (aa138-317) attached to Sei1."

Reviewer #3 (Remarks to the Author):

Mechanisms by which govern lipid membrane homeostasis at the nuclear membranes are fundamental questions and remain to be elucidated. This manuscript describes roles of yeast Seipin (Sei1) in lipid metabolism in the inner nuclear membrane and nuclear lipid droplet. It also describes that Seipin and its co-factors regulate phosphatidic acid homeostasis and nuclear lipid droplet formation. Experiments are well designed and carefully executed. Conclusions are supported by experimental data. I have no serious concerns but have only minor comments for readability.

We appreciate the reviewer's positive feedback. The minor comments are addressed below:

1. The construct of NLS-Sei1 should be described more clearly as it plays an important role in the manuscript. On page 17, it is stated that "NLS-Sei1 contains the NLS and the linker of the INM transmembrane protein Heh2 (aa93-317) attached to Sei1". On page 6, it is stated that "appending the NLS of the INM protein Heh2 to Sei1 (abbreviated as NLS-Sei1)". NLS-Sei1 in Fig. 3d does not represent how NLS-Sei1 is constructed. In addition, Fig. 3d is not appropriately referred. Fig. 3d is cited at two places on page 7: "This targets Sei1 to the INM and leads to the formation of nLDs (Fig. 3d)", and "we induced nLD biogenesis via NLS-Sei1 (Fig. 3d)". These sentences have nothing to do with Fig. 3d.

We are sorry for having caused confusion. The same NLS-Sei1 construct has been consistently used throughout the paper. We have now provided a more detailed description of this construct when it is first introduced:

"…we targeted Seipin to the INM using a method developed earlier[8], which involves appending the NLS and the linker region of the INM protein Heh2 (aa93-317) (Meinema et al., 2011) to Sei1 (abbreviated as NLS-Sei1)."

We have optimized Figure 3d to include an additional illustration that depicts the NLS-Sei construct, specifying the exact amino acid boundaries of Heh2.

2. Page 30, legend to Fig. 2a: The white dashed line is not explained throughout Fig. 2 to Fig. 6. Although it is described in Methods section, it would be nice to include “Cell contours are marked with a dashed white line” here at the first appearance in the figures.

This has been corrected. We have now included the description of the white dashed line in the Figure 2 legend: *"Cell contours were marked with a dashed white line based on brightfield imaging."*