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Torsin and NEP1R1-CTDNEP1 phosphatase affect interphase nuclear pore complex insertion by lipid-dependent and -independent mechanisms

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 9th Nov 2020

Thank you for submitting your manuscript entitled "Torsins inhibit the nuclear envelope NEP1R1-CTDNEP1/ Lipin lipid metabolism pathway for nuclear pore complex biogenesis" (EMBOJ-2020-106914) to The EMBO Journal. Please accept my apologies for the delay in getting back to you with our decision. Three referees were originally assigned to your manuscript, but one did not return his/her report. The study has now been assessed by two reviewers, whose reports are enclosed below for your information.

As you can see, the referees find your work potentially interesting, but also raise several issues - including the lack of controls and overstatements in the major conclusions - that need to be addressed before they can support publication in The EMBO Journal.

Given the overall interest of your study, we have decided to invite you to submit a new version of the manuscript revised according to the referees' requests. I should add that it is The EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in the revised version. Please note that strong support from the referees would also be needed for publication here.

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content is published elsewhere.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Before submitting your revised manuscript, deposit any primary datasets and computer code produced in this study in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). Please remember to provide a reviewer password, in case such datasets are not yet public. The accession numbers and database names should be listed in a formal "Data Availability" section (placed after Materials & Method). Provide a "Data availability" section even if there are no primary datasets produced in the study.

Feel free to contact me if you have any questions about the submission of the revised manuscript to The EMBO Journal. I thank you again for the opportunity to consider this work for publication and look forward to your revision.

Referee #1:

In their manuscript titled "Torsins inhibit the nuclear envelope NEP1R1-CTDNEP1/ Lipin lipid metabolism pathway for nuclear pore complex biogenesis," Jacquemyn et al. build upon prior work from their lab highlighting the role of Torsin ATPases in lipid metabolism. Namely, they link essent ial and disease-relevant Torsin ATPases to the Nep1r1/Ctdnep1/lipin pathway and nuclear pore

biogenesis, both areas of significant contemporary interest. The authors also add a nice structure-function element to their work and demonstrate that homotypic oligomerization of Torsins is essential in this context, while ATPase activity seems less critical. In sum, the observations are very interesting and the work advances our understanding of Torsin biology. The reported findings should be of significant interest across fields and spawn future investigation in this area. However, a number of revisions are recommended entailing some additional control experiments, downscaling of conclusions, and revising the text to make it more accessible to the broad readership of EMBO Journal.

Major points

1. On many occasions in this manuscript, correlations between experimental manipulation and observation seem somewhat overinterpreted with regards to function. For example, there is no data in this manuscript that show that Torsins regulate the lipin axis or even lipid metabolism. All you can say is that, for example, changes in lipid droplet size/lipin localization (or other readouts) can be reverted by genetic manipulation of lipid metabolism. Without further mechanistic follow-up analysis (arguably outside of the scope of this manuscript), this remains a correlation. Many functions/activities other than a bona fide regulatory role can be responsible for these effects and indirect effects are at least equally likely to account for the observed epistatic relationships. In other words, this is a classical chicken-and-egg scenario. Example: there is no evidence to support the idea that "homo-oligomerization is the event regulating lipid metabolism" (p9). It would be better to state "homo-oligomerization is important for..." and use similar neutral descriptive language in general while avoiding absolute statements. In general, the term "regulation" is used excessively throughout the text and should be removed in most instances. The reported observations are very interesting on their own right and represent important findings for the field. There is no need to jump to premature/universal conclusions.

2. Counter hypothesis: have the authors considered the possibility that genetic manipulation of lipid homeostasis could merely bypass/compensate for a specific defect in NPC assembly caused by Torsin mutation? For example, trafficking of lipid regulators could be compromised due to NPC defects resulting from Torsin deletion. Perhaps alternate possibilities could be elaborated on in the discussion to arrive at a more balanced interpretation while crystallizing points for future investigation. For example, the authors state on pg. 13 that "NEP1R1 and CTDNEP1 activity prevent INM/ONM fusion, and this explains why Torsins are required for NPC insertion at interphase". NE herniations could also result from redistribution of NEP1R1/CTDNEP1 and resulting changes in lipid availability/composition rather than a direct role of either protein in NPC biogenesis.
3. The NEP1R1/CTDNEP1 gain-of-function phenotype in dTorsinKO cells observed by the authors should result in altered intracellular lipid levels, which should return to WT levels upon RNAi treatment against CTDNEP1, NEP1R1, or lipin. The authors should quantify steady state abundances of membrane-forming phospholipids along with DAG/TAG in dTorsinKO and WT cells under the aforementioned RNAi conditions, noting that manipulation of lipid metabolism provokes numerous effects on nuclear envelope/ER morphology. Knocking down the CTDNEP1/NEP1R1/lipin pathway should cause similar effects/trends even in WT cells given that the authors suggest an inhibitory role for Torsins on the CTDNEP1/NEP1R1/lipin axis. This would nicely support the author's conclusions.
4. The authors show in Figure 3 that RNAi against CTDNEP1, NEP1R1, and lipin increase the ER density but decrease the lipid droplet density in dTorsinKO flies. It is not clear to this reviewer why decreasing lipin activity would result in more membrane proliferation but less TAG formation, as PA is a precursor for both TAGs and phospholipids. How can the authors explain this observation? Wouldn't a rate-limiting activity downstream of this bifurcation point have to be differentially

affected?
5. Related to the previous point, it is also unclear why this ER/lipid droplet modulation phenotype is not observed in WT flies. The only data the authors show in WT cells indicate a trend towards smaller cell size (Fig. S3B). While the authors state "...PA depletion suppresses membrane lipid synthesis" there is, to this reviewer's knowledge, little literature/data to back this up. The only support provided by the authors is a single publication reporting results from work in metabolically distinct plants. Do more supporting citations demonstrating that the presence of lipin negatively regulates membrane synthesis exist (e.g. via metabolic flux analysis)? Clarifying this point would make the manuscript more accessible to a broad readership.
6. On multiple occasions, the authors cite "Shin JY, et al. Nuclear envelope-localized torsinA-LAP1 complex regulates hepatic VLDL secretion and steatosis. The Journal of clinical investigation 130, 4885-4900 (2019)." The authors cite this paper after stating, "LAP1 regulates TAG" and "LAP1 also suppresses TAG deposition" as if to imply that LAP1 directly functions in lipid synthesis. Shin et al. specifically demonstrated in their paper that de novo lipid synthesis is unperturbed in cells devoid of LAP1 or TorsinA. Instead, their characterized lipid accumulation phenotypes result from defective secretion of VLDLs. It is therefore somewhat misleading to portray these data in a manner suggesting that LAP1/Torsin directly affect lipid synthesis.
7. The authors could provide evidence of changes in lipin phosphorylation status between WT and dTorsinKO flies. This would show that the lipin localization profile reflects a NEP1R1/CTDNEP1 pathway gain-of-function rather than alternative scenarios, i.e. defective nucleocytoplasmic trafficking due to Torsin dysfunction.
8. The authors should perform an immunofluorescence (IF) quantification of NE herniation using commonly employed K48-Ub as a marker to complement what they quantify by EM in figure 6. This will allow the authors to analyze more cells conveniently than can be done by EM (presently only from five nuclei). Counting the number of cells with K48-Ub-positive NE herniations for dTorsinKO cells and TorsinA/B KO MEFs under RNAi luciferase, CTDNEP1, and NEP1R1 will allow the authors to robustly characterize the ability for CTDNEP1/NEP1R1 knockdown to rescue the NE herniations arising in Torsin-deficient cells and demonstrate how conserved this rescue ability is.
9. The EM images in Fig. 7 and 8 are suboptimal and do not support the author's conclusion that the unusual structures or "channels" represent NPC intermediates. To support their conclusion, the authors need to demonstrate that Nups localize to these unusual structures via immunogold labeling/EM.

10. This reviewer does not feel the authors have provided sufficient evidence to conclude that "NEP1R1 and CTDNEP1 activity prevent INM/ONM fusion, and this explains why Torsins are required for NPC insertion at interphase (Fig. 6G)." It is not currently known at which point of interphase NPC biogenesis Torsins act, and the fusion step is but one possibility. Therefore, the authors should scale down their conclusions. Note also that no changes in phospholipid levels were detected in another experimental system with highly penetrant NPC defects upon combined Torsin deletion (Laudermilch et al., MBoC 2016). This argues against a straightforward causal connection, requiring a more balanced discussion of the findings.

Minor points
1. In the last paragraph on pg. 6 the authors state "Instead, oligomerization of the Torsin membrane domain removes the NEP1R1-CTDNEP1 phosphatase complex from the INM, in turn excluding Lipin from the INM and nucleus" however, Torsin oligomerization is not through the "membrane domain" but rather the intersubunit interface of the AAA domain. Please rephrase accordingly.
2. The authors conclude that ATP hydrolysis is unnecessary. This does not seem 100% accurate. While the effects are less pronounced than the interface mutation, the ATPase mutants are clearly compromised in their rescuing ability relative to WT (Fig. 2C, D). Still this is an interesting result as it

again argues against a processive activity of Torsins.
3. In the second paragraph of pg. 11, the authors state "The NE-localized NEP1R1mGFP and CTDNEP1mGFP signal in dTorsinKO cells exceeded that of Calnexin, suggesting it reflected protein mislocalization rather than altered ER/NE morphology (Fig. 4A - D, compare red and green signal)." This seems somewhat subjective as the authors do not perform a quantitative analysis. The sentence should therefore be rephrased.
4. Why is there so much nucleoplasmic CTDNEP1 in TorsinA/B KO cells (Fig. 4F)? This figure would benefit from a counterstain for NEs and merged images to show that CTDNEP1 puncta are at the NE.
5. It is not entirely clear from the images in Fig. 4I that NEP1R1V5- CTDNEP1myc co-overexpression produce Lipin1mGFP signal at the nuclear periphery.
6. In Fig. 5, please correct the label for panel Q' where this reviewer assumes you meant R.
7. The authors should reword the sentence in the second paragraph of the introduction stating "This DAG production is the penultimate step of triacylglyceride (TAG) synthesis" to reflect that DAG is also an intermediate for phospholipid synthesis.
8. Can the authors comment on the electron-dense particles resembling viral capsids in their EMs in Fig. 7 K (nucleoplasm)?

Referee #2:

Previous work from this group showed that Torsin regulates lipid metabolism and modulates the activity of Lipin, a key regulator of lipid metabolism. The first half of this study investigates the mechanism of Lipin regulation by Torsin. It finds Torsin controls the localization of the Lipin-regulating phosphatase complex NEP1R1-CTDNEP1 and that, remarkably, a number of phenotypes in flys lacking Torsin are reversed they also lack this complex. The remainder of the study investigates the consequences of Torsin regulation of the NEP1R1-CTDNEP1 complex and finds the complex is required for NPC insertion into the nuclear membrane. It argues this occurs because cells lacking Torsin have defects in fusion of the inner and outer membrane and cannot maintain a lipid composition in the nuclear envelope conducive to NPC insertion. These are a fascinating set of results that advance our understanding of how Torsin regulates lipid metabolism and NPC biogenesis. However, the findings are over interpreted.

1. It is not clear that Torsin directly regulates or interacts with the NEP1R1-CTDNEP1 complex. Some insight into how regulation occurs would significantly strengthen this study. How does Torsin regulate the localization the NEP1R1-CTDNEP1 complex? If the complex is re-localized to the inner nuclear membrane in cells lacking Torsin, is Lipin regulation restored? A complete answer to these questions is beyond the scope of this study but some indication of how regulation occurs would increase the significance of the paper. At a minimum, how Torsin could regulate NEP1R1-CTDNEP1 localization and how it could directly or indirectly affect it should be discussed.

2. The second half of the study is well done but the results are over interpreted. There is no evidence the structures seen in Fig. 5O are intermediates caused by a defection in membrane fusion, though they could be. There is also no evidence that the lipid composition of the nuclear envelope is altered or, if it is, how this how this could affect NPC insertion. The authors seem to assume that some biophysical property of nuclear envelope is altered cells lacking Torsin or overexpressing the NEP1R1-CTDNEP1 complex, but that has not been investigated and other explanations are possible. This is should be discussed.

3. The results shown in Figs 1 and 2 are well done but it is not clear how they are related to the rest of the study. The authors may want to consider removing them or else more clearly explaining the connection to what follows. It is notable that replacing the membrane-binding domain of Torsin with the TMD from Torip/dLAP1 is functional but the TMD from Sec61-beta is not. The Torip TMD could interact with the NEP1R1-CTDNEP1 complex, as the authors suggest, but there are other possibilities. A missing control for this experiment is a demonstration that the fusions are expressed at similar levels and oligomerize normally.

Referee #1:

In their manuscript titled "Torsins inhibit the nuclear envelope NEP1R1-CTDNEP1/ Lipin lipid metabolism pathway for nuclear pore complex biogenesis," Jacquemyn et al. build upon prior work from their lab highlighting the role of Torsin ATPases in lipid metabolism. Namely, they link essential and disease-relevant Torsin ATPases to the Nep1r1/Ctdnep1/lipin pathway and nuclear pore biogenesis, both areas of significant contemporary interest. The authors also add a nice structure-function element to their work and demonstrate that homotypic oligomerization of Torsins is essential in this context, while ATPase activity seems less critical. In sum, the observations are very interesting and the work advances our understanding of Torsin biology. The reported findings should be of significant interest across fields and spawn future investigation in this area.

However, a number of revisions are recommended entailing some additional control experiments, downscaling of conclusions, and revising the text to make it more accessible to the broad readership of EMBO Journal.

We want to thank the reviewer for their significant effort reviewing this manuscript and for stating that [our] “observations are very interesting and the work advances [ ] understanding of Torsin biology … findings should be of significant interest across fields”. We also thank the reviewer for helpful constructive critique of the manuscript. We have taken these in and believe they led to an improved study.

To first respond to the global comments of the reviewer. We agree that several conclusions were overstated and have downscaled these across the manuscript. However, we believe we also resolved this issue by adding substantial amounts of new data. This includes two new large series of lipidomic data that allowed us to revise and cement conclusions about the role of lipid metabolism in the biological processes associated with Torsin. We also took good note of the reviewer’s comment that the text should be more accessible to the broad readership of the EMBO Journal. We have introduced numerous revisions that better explain the existing literature, and more extensively reference the existing literature. We hope this also clarifies some occasions where it was not clear that we made a conclusion by integrating our new data with previously published data.

We respond point-by-point to the specific comments of the reviewer below.

Major points

1. On many occasions in this manuscript, correlations between experimental manipulation and observation seem somewhat overinterpreted with regards to function. For example, there is no data in this manuscript that show that Torsins regulate the lipin axis or even lipid metabolism. All you can say is that, for example, changes in lipid droplet size/lipin localization (or other readouts) can be reverted by genetic manipulation of lipid metabolism. Without further mechanistic follow-up analysis (arguably outside of the scope of this manuscript), this remains a correlation. Many functions/activities other than a bona fide regulatory role can be responsible for these effects and indirect effects are at least equally likely to account for the observed epistatic relationships. In other words, this is a classical chicken-and-egg scenario. Example: there is no evidence to support the idea that "homo-oligomerization is the event regulating lipid metabolism" (p9). It would be better to state "homo-oligomerization is important for..." and use

similar neutral descriptive language in general while avoiding absolute statements. In general, the term "regulation" is used excessively throughout the text and should be removed in most instances. The reported observations are very interesting on their own right and represent important findings for the field. There is no need to jump to premature/universal conclusions.

We thank the reviewer for their constructive and thoughtful critique. Firstly, we very much appreciate the fact the reviewer finds our data “interesting in their own right” and “represent important findings for the field”. We also appreciate that the reviewer recognises that a further mechanistic dissection is beyond the scope of feasible in a single study.

In general, we agree with the reviewer that some conclusions were not sufficiently supported. We have downscaled conclusions about how NEP1R1-CTDNEP1 interferes with INM/ONM fusion, as well as giving the impression that Torsin and NEP1R1-CTDNEP1 directly interact. In parallel, we added new data and thus some conclusions are now better supported than in the original manuscript. We hope the reviewer agrees that we provide more mechanistic insight and this alleviates some of the concerns of the reviewer.

- We added two sets of lipidomic profiling that show Torsin loss affects the lipidome via NEP1R1-CTDNEP1 and Lipin.
- We dissected the hierarchy between NPC defects and the lipidomic defects of Torsin loss, something that was previously suggested by existing literature.
- We provide new information about the origin of the channel-like structures.

1.1. *“there is no data in this manuscript that show that Torsins regulate the lipin axis or even lipid metabolism.”*

We had previously shown that Torsins regulate Lipin in two publications (Grillet *et al*, 2016) and (Cascalho *et al*, 2020). These existing data were the starting point for this study, to explain why this manuscript does not focus on establishing that Torsins regulate the Lipin axis.

We nevertheless agree with the reviewer that conclusions related to lipid metabolism should be supported by direct analyses of lipids (the original version of this manuscript only examined TAG (original **Figure 1J**)). We therefore added two new large sets of lipidomic data in the manuscript.

- **Figure 1K**. These data replicate our previously reported finding (Grillet *et al*, 2016) that the *dTor*^{KO} fat body has the lipidomic signature of Lipin hyperactivity (abnormally low PA levels and abnormally high DAG levels). We extend that previous data by parallel examination of *dLap1* deletion. *dLap1* loss reduced PI and PE, but did not significantly affect other lipids. Thus, although both dLap1 and dTor control tissue TAG levels, they differentially affect membrane lipid classes. This supports our original conclusion that Torsin and dLap1 affect lipid metabolism by non-identical mechanisms.

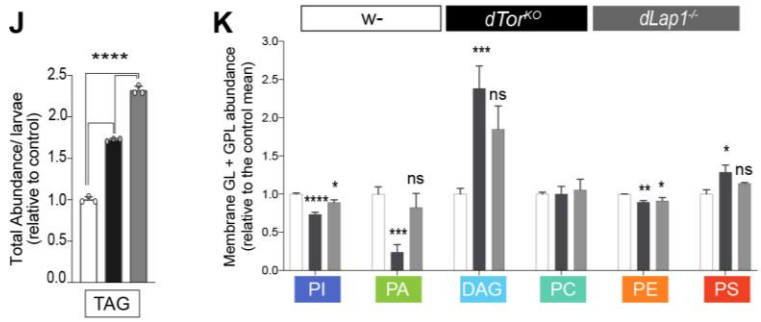


Figure 1. dTor and dLap1 differentially affect the fat body lipidome

J) TAG content of 3DO larvae measured by enzymatic assay. Bars show mean ± SEM measured in three sets of thirty larvae. (original data)

K) The abundance of individual lipid classes (mean ± SEM) within the set of six membrane GL and GPL extracted from 4DO fat body samples. * and ** indicate significant difference to control. w- n = 7, $dTor^{KO}$ n = 7, dLap1-/- n = 4.

- **Figure 3**. We examined the lipidome of *$dTor^{KO}$* animals expressing a control RNAi or *Nep1r1*, *Ctdnep1*, or *Lipin* RNAi. This shows that *Nep1r1*, *Ctdnep1* and *Lipin* RNAi revert the lipidomic defects of the *$dTor^{KO}$*. This again confirms the core finding of our previous publications (that Torsin affects the lipidome via Lipin hyperactivity), and extends this by placing *Nep1r1* and *Ctdnep1* in the genetic pathway between Torsin and Lipin.

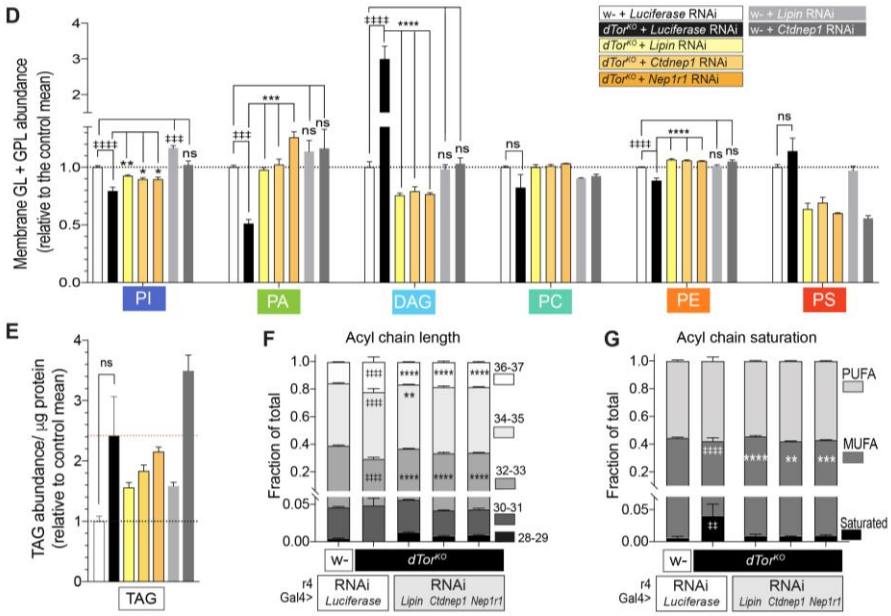


Figure 3: dTor affects the fat body lipidome via Nep1r1, Ctdnep1 and Lipin

D) The relative abundance of each lipid class (mean ± SEM) within the set of six membrane lipids. n = 3 MS analyses of 4DO fat body samples for each genotype. ‡ symbols indicate a significant difference compared to w- expressing Luciferase RNAi, * symbols indicate a significant difference compared to *$dTor^{KO}$* expressing luciferase RNAi. Note we only tested the effect of Lipin, Ctndep1, and Nep1r1 RNAi when a lipid class significantly differed between w- and *$dTor^{KO}$*.

E) As above except that TAG abundance was calculated relative to protein.

F and G) Bars show the fraction (mean ± SEM) of membrane GL and GPL lipids with each fatty acyl chain characteristic. MUFA, mono-unsaturated; PUFA, poly-unsaturated. ‡ indicate a significant difference between w- and *dTor*^{KO} expressing Luciferase RNAi, * indicate a significant difference compared to *dTor*^{KO} expressing Luciferase RNAi (Two-way ANOVA).

1.2. *All you can say is that, for example, changes in lipid droplet size/lipin localization (or other readouts) can be reverted by genetic manipulation of lipid metabolism.*

We hope that the new lipidomic data of **Figure 3** resolve the reviewers's concern about the evidence for altered lipid metabolism.

1.3. *It would be better to state "homo-oligomerization is important for..." and use similar neutral descriptive language in general while avoiding absolute statements.*

We have taken the reviewers advice and modified sub-title headings and figure titles throughout the paper including we separately state:

> This suggests that Torsin homo-oligomerization is required for its effects on TAG production and cell size.

and

> This suggests that specific information is encoded in the membrane domains of dTorsin and dLap1 and this is key to how both proteins affect cellular lipid metabolism.

In place of stating that ""homo-oligomerization is the event regulating lipid metabolism"

1.4. *this is a classical chicken-and-egg scenario*

We agree that, in isolation, the genetic rescue experiments did not establish the hierarchy between different cellular defects. We were insufficiently clear that some conclusions were made by considering our new data with the literature. We have used the reviewer's (broad) comments on this issue to improve the manuscript in a number of ways. Below, our responses to other comments further elaborate on this, but to summarise why we feel that the conclusions of the revised manuscript are justified:

- The efficiency that *Nep1r1* and *Ctdnep1* RNAi overcome the numerous cellular defects of *dTor*^{KO} are strong genetic evidence that these genes lie close together within a genetic pathway.
- We have added new data that provides direct evidence about the hierarchy between NPC and Lipin dysfunction. It shows that these phenomena are independent of each other but both result from NEPR1-CTDNEP1 dysfunction (explained below).
- We have added lipidomic data that shows the signature of Lipin hyperactivity (low PA and high DAG levels) in the *dTor*^{KO} is reverted by *Nep1r1*, *Ctdnep1*, or *Lipin* RNAi (new data in **Figure 3**).
- We agree that we do not present biochemical or structural data to establish a direct relationship between Torsins and NEP1R1-CTDNEP1. We added the following statements into a (highly) revised discussion where we make clear that Torsin may affect NEP1R1-CTDNEP1 by an intermediate.

Further, while we did not examine if Torsin directly binds NEP1R1 and/or CTDNEP1, we infer a relatively direct connection given that all three proteins are NE residents (Siniossoglou et al, 1998; Han et al, 2012; Bahmanyar et al, 2014; Goodchild & Dauer, 2004).

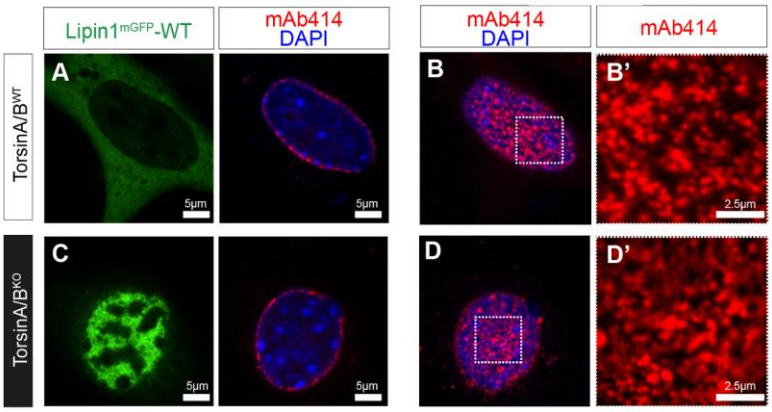
Additional work is needed to determine the structural basis of this, and given the importance of the Torsin/ dLap1 membrane-association domains, this should consider whether interactions occur within or are mediated by the lipid bilayer.

2. Counter hypothesis: have the authors considered the possibility that genetic manipulation of lipid homeostasis could merely bypass/compensate for a specific defect in NPC assembly caused by Torsin mutation? For example, trafficking of lipid regulators could be compromised due to NPC defects resulting from Torsin deletion. Perhaps alternate possibilities could be elaborated on in the discussion to arrive at a more balanced interpretation while crystallizing points for future investigation. For example, the authors state on pg. 13 that "NEP1R1 and CTDNEP1 activity prevent INM/ONM fusion, and this explains why Torsins are required for NPC insertion at interphase". NE herniations could also result from redistribution of NEP1R1/CTDNEP1 and resulting changes in lipid availability/composition rather than a direct role of either protein in NPC biogenesis.

2.1. For example, trafficking of lipid regulators could be compromised due to NPC defects resulting from Torsin deletion.

We have added new data to establish the hierarchy between the NPC and Lipin enzyme dysfunction. One key point is that Torsin loss from dividing cells only mildly affects the NPC unless the cell cycle is blocked in interphase (Rampello *et al*, 2020). This is relatively well-established and thus our original paper made the conclusion that the Lipin dysfunction occurred independently to NPC dysfunction by integrating the new data on Lipin protein mislocalization in fibroblasts lacking TorsinA/B, with our previously published data showing Lipin is hyperactive in MEFs lacking TorsinA/B (Cascalho *et al*, 2020), and published data on NPC levels in dividing cells.

1) We have revised the manuscript to confirm this conclusion by adding data on NPC levels in fibroblasts. As expected, there are abundant NPC in control and TorsinA/B fibroblast lines, including cells with verified mislocalized Lipin. We conclude from this that Torsin loss causes Lipin mislocalization even when the NPC is intact.



Appendix Figure S5

Related to Figure 6: Nep1r1 and Ctdnep1 downregulation restore NPC levels and prevent INM membrane herniations in dTor^{KO} cells

A - D) Confocal images of MEFs expressing Lipin^{mGFP} and labeled with mAb414 (red) and DAPI (blue). A & C optically bisect the nucleus, while B & D scan the nuclear surface. The density and distribution of mAb414 foci are similar in TorA/B^{WT} and TorA/B^{KO} nuclei.

2) **Figure 6**. We added new evidence that *Nep1r1* and *Ctdnep1* RNAi efficiently revert the dTor^{KO}-associated NPC deficit, to place these (genetically) between Torsin and the NPC defect. These data supplement and confirm the conclusion we made from electron microscopy data in the original manuscript.

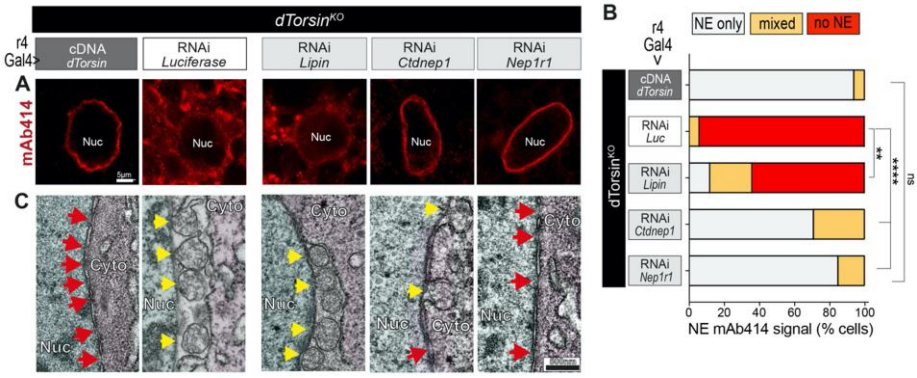


Figure 6: Nep1r1 and Ctdnep1 downregulation restore NPC levels and prevent INM membrane herniations in dTor^{KO} cells

A) mAb414 labeling of 5DO *dTor^{KO}* fat body cells expressing the dTor cDNA, Luciferase RNAi, or RNAi against *Nep1r1*, *Ctdnep1*, or *Lipin*.

B) The percentage of fat body cells with NE-localized mAb414. ** and **** indicate that *Lipin*, *Ctdnep1*, and *Nep1r1* RNAi significantly increased the number of *dTor^{KO}* cells with NE-specific mAb414 labeling compared to the Luciferase RNAi (Luc). Chi square test followed by individual post hoc Chi square tests.

C) NE ultrastructure in 5DO *dTor^{KO}* cells. Red arrows, nuclear pores; yellow arrows, abnormal INM herniations. (original data).

2.2. "NEP1R1 and CTDNEP1 activity prevent INM/ONM fusion, and this explains why Torsins are required for NPC insertion at interphase". NE herniations could also result from redistribution of

NEP1R1/CTDNEP1 and resulting changes in lipid availability/composition rather than a direct role of either protein in NPC biogenesis.

We agree completely with the reviewer's comment and apologise that the original manuscript gave the impression that (a) NEP1R1/CTDNEP1 directly participate in the production of membrane fusion defects/ impair NPC biogenesis, (b) it was certain that this was due to abnormal lipid metabolism.

1) We added text to the introduction and results that better explains why the literature has established the membrane defects of the *dTor*^{KO} are due to failed INM/ONM fusion during NPC biogenesis.

> Torsins also affect cellular events that have no clear relationship to lipid metabolism. This includes that Torsins have a poorly understood importance for NPC insertion into the NE of post-mitotic / interphase cells (Goodchild *et al*, 2005; Pappas *et al*, 2018; Liang *et al*, 2014; Laudermilch *et al*, 2016; VanGompel *et al*, 2015; Rampello *et al*, 2020). Interphase NPC biogenesis occurs by less well-understood mechanisms than its mitotic counterpart. It appears that it is initiated by membrane deforming nucleoporins (Nups) that curve the INM towards the ONM, followed by membrane fusion and the recruitment of the full complement of NPC subunits INM abnormalities (Naismith *et al*, 2004; Goodchild & Dauer, 2005; Kim *et al*, 2010; VanGompel *et al*, 2015; Tanabe *et al*, 2016; Pappas *et al*, 2018; Jokhi *et al*, 2013; Liang *et al*, 2014; Rampello *et al*, 2020), that are characteristic of failed INM/ONM fusion (Jokhi *et al*, 2013; Otsuka *et al*, 2016; Scarcelli *et al*, 2007; Allegretti *et al*, 2020), and appear to replace mature nuclear pores (Rampello *et al*, 2020; VanGompel *et al*, 2015; Laudermilch *et al*, 2016; Pappas *et al*, 2018).

2) We added new data that investigates the role of Lipin in the membrane defects/ NPC deficiency of the *dTor*^{KO} (**Figure 6**). In fact, Lipin RNAi is unable to revert the NPC defects even though it efficiently reverts the lipidomic defects of the *dTor*^{KO}. We revised the manuscript to include this new insight, including the title, abstract, graphical abstract and discussion now explain that NEP1R1-CTDNEP1 interfere with nuclear pore membrane fusion by an unknown mechanism that does not involve Lipin.

3. The NEP1R1/CTDNEP1 gain-of-function phenotype in dTorKO cells observed by the authors should result in altered intracellular lipid levels, which should return to WT levels upon RNAi treatment against CTDNEP1, NEP1R1, or lipin. The authors should quantify steady state abundances of membrane-forming phospholipids along with DAG/TAG in dTorKO and WT cells under the aforementioned RNAi conditions, noting that manipulation of lipid metabolism provokes numerous effects on nuclear envelope/ER morphology. Knocking down the CTDNEP1/NEP1R1/lipin pathway should cause similar effects/trends even in WT cells given that the authors suggest an inhibitory role for Torsins on the CTDNEP1/NEP1R1/lipin axis. This would nicely support the author's conclusions.

1) We performed this experiment and present the data in **Figure 3**. It shows the expected result; RNAi against *Nep1r1*, *Ctdnep1*, and *Lipin* corrected the membrane lipid defects of the *dTor*^{KO}. Unfortunately, the sample size was too low for conclusions on TAG. We nevertheless present the TAG data with the caveat that this particular analysis did not find statistically significant differences between control and *dTor*^{KO} animals (note that one published data set and **Figure 1** established this

is the case with a larger sample size). Given this experiment already delayed manuscript revision and resubmission, we hope the new data are sufficient to address the concerns of the reviewer.

2) **Figure 3** also presents how *Ctdnep1* and *Lipin* RNAi impact the lipidome of WT fat body cells. They had a relatively mild effect compared with their dramatic impact in the $dTor^{KO}$. This is consistent with the conclusion that there is a NEP1R1-CTDNEP1 / Lipin gain-of-function in the $dTor^{KO}$. To state another way, finding that these RNAi more strongly affect lipid levels in the $dTor^{KO}$ rules out the possibility that they reverse defects of the $dTor^{KO}$ simply because they have this effect under all circumstances.

As a further control we examined the impact of RNAi transgenes on the fat body lipidome of w- animals. There was no significant difference in the levels of PA, DAG or PE between w- animals expressing *Luciferase* RNAi versus *Lipin* or *Ctdnep1* RNAi (**Fig. 3D**).

4. The authors show in Figure 3 that RNAi against CTDNEP1, NEP1R1, and lipin increase the ER density but decrease the lipid droplet density in dTorKO flies. It is not clear to this reviewer why decreasing lipin activity would result in more membrane proliferation but less TAG formation, as PA is a precursor for both TAGs and phospholipids. How can the authors explain this observation? Wouldn't a rate-limiting activity downstream of this bifurcation point have to be differentially affected?

We address point 4 and point 5 together since these are closely related issues.

5. Related to the previous point, it is also unclear why this ER/lipid droplet modulation phenotype is not observed in WT flies. The only data the authors show in WT cells indicate a trend towards smaller cell size (Fig. S3B). While the authors state "...PA depletion suppresses membrane lipid synthesis" there is, to this reviewer's knowledge, little literature/data to back this up. The only support provided by the authors is a single publication reporting results from work in metabolically distinct plants. Do more supporting citations demonstrating that the presence of lipin negatively regulates membrane synthesis exist (e.g. via metabolic flux analysis)? Clarifying this point would make the manuscript more accessible to a broad readership.

We hope that the reviewer agrees that the addition of lipidomic data to manuscript **Figure 3** resolved the issue that we used cell size, ER density, and LD as read-outs of membrane GPL and TAG. We also provide more information about this below.

We decided to remove the data on ER density from the revised manuscript due to space constraints. This data is a relatively indirect read-out of membrane lipid levels; now addressed by directly measuring membrane lipids.

5.1. It is not clear to this reviewer why decreasing lipin activity would result in more membrane proliferation but less TAG formation, as PA is a precursor for both TAGs and phospholipids.

5.1.1. How can the authors explain this observation?

5.1.2. The only support provided by the authors is a single publication reporting results from work in metabolically distinct plants. Do more supporting citations demonstrating that the presence of lipin negatively regulates membrane synthesis exist (e.g. via metabolic flux analysis)?

We apologise that we did not better establish the basis of this key point for the reader. It is indeed true that DAG produced by Lipin is a precursor for both membrane GPL and TAG. We added this information into a new **Figure 1A** showing the network of GL and GPL enzymes.

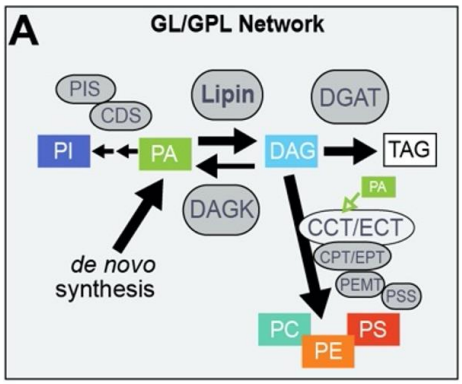


Figure 1: dTor and dLap1 differentially affect the fat body lipidome
A) GL and GPL and enzymes. Full abbreviations in Appendix Fig. S1A.

We explain this point again in the results so the broad readership of The EMBO Journal are aware of the mechanistic connection between different lipid classes.

We use these phenomena as read-outs since Lipin PAP activity promotes TAG and LD at the expense of membrane growth in yeast (Santos-Rosa et al, 2005; Han et al, 2007), plants (Craddock et al, 2015), Drosophila (Grillet et al, 2016) and mammals (Yang et al, 2019).

5.2. *it is also unclear why this ER/lipid droplet modulation phenotype is not observed in WT flies. The only data the authors show in WT cells indicate a trend towards smaller cell size (Fig. S3B).*

A Lipin mutant fly line has been shown to have larger cells and smaller LD than normal (Ugrankar *et al*, 2011). We did not observe this in our study, but believe this discrepancy is because *Lipin* RNAi more mildly reduces Lipin expression.

5.3. *To paraphrase: Why do the RNAi manipulations more dramatically affect the dTorKO than wild-type?*

We hope that we sufficiently addressed this issue in our response to comment 3, and the additional text we added to the results section. Briefly, it is expected that an RNAi more dramatically affects phenotypes if they are caused by a gain-of-function mechanism, than in control conditions where no gain-of-function is present.

6. On multiple occasions, the authors cite "Shin JY, et al. Nuclear envelope-localized torsinA-LAP1 complex regulates hepatic VLDL secretion and steatosis. The Journal of clinical investigation 130, 4885-4900 (2019)." The authors cite this paper after stating, "LAP1 regulates TAG" and "LAP1 also suppresses TAG deposition" as if to imply that LAP1 directly functions in lipid synthesis. Shin et al. specifically demonstrated in their paper that de novo lipid synthesis is unperturbed in cells devoid of

LAP1 or TorsinA. Instead, their characterized lipid accumulation phenotypes result from defective secretion of VLDLs. It is therefore somewhat misleading to portray these data in a manner suggesting that LAP1/Torsin directly affect lipid synthesis.

We believe that the data in Shin, *et al*, provides strong support that the mechanism we describe here is active in mouse liver. We understand the point of the reviewer that we reference data from this paper with a different mechanistic interpretation than the paper presents. We were attempting to discuss these data without directly contradicting the authors interpretation.

6.1. *… , their characterized lipid accumulation phenotypes result from defective secretion of VLDLs.*

Shin, *et al*, (2019) identifies excess TAG in the liver of LAP1^{KO} and TorsinA^{KO} mice. As a phenotype, this has clear parallels with what we describe in fat body (liver equivalent) of *dLap1*$^{-/-}$ and *dTor*^{KO} flies. Shin, *et al*, also find reduced VLDL secretion. However, Shin, *et al*, did not examine if reduced VLDL secretion causes the elevated tissue TAG: the conclusion that they are casually linked comes from this being the logical explanation based on the literature.

Notably: Lipin-1 overexpression in liver is known to suppress VLDL secretion, while Lipin-1 loss from liver stimulates VLDL secretion (Chen *et al*, 2008). Thus, Lipin hyperactivity is a unifying mechanism to account for the TAG and VLDL defects of the LAP1^{KO} and TorsinA^{KO} mouse liver. This was not considered by the authors of Shin, *et al*, even though we had previously established that fly *dTor* loss elevated fat body TAG through Lipin (Grillet *et al*, 2016).

We now more carefully explain that the phenotypes identified by Shin, *et al*, can be accounted for by altered Lipin activity, and include the references that support this, rather than giving the impression that this paper makes this conclusion.

Introduction

… and mammalian LAP1 affects TAG, LD, and lipid trafficking in a manner consistent with Lipin regulation (Grillet et al, 2016; Ugrankar et al, 2011; Schmitt et al, 2015; Shin et al, 2019).

6.2. *that loss of Tor1aip1/LAP1 or Tor1a/TorsinA from mouse liver elevates tissue TAG alongside a series of phenotypes that are closely connected with Lipin dysfunction (Shin et al, 2019; Ugrankar et al, 2011; Schweitzer et al, 2015; Chen et al, 2008). Shin et al. specifically demonstrated in their paper that de novo lipid synthesis is unperturbed in cells devoid of LAP1 or TorsinA.*

Shin, *et a*l, established that the rate of fatty acid incorporation into GL/GPL was unaltered by LAP1^{KO} or TorsinA^{KO}. They did not address metabolism within the GL/GPL enzyme network (after PA synthesis). Our data also suggests that LAP1 and TorsinA loss impacts lipid metabolism within the GPL/ GL network, rather than bulk *de novo* lipid synthesis.

7. The authors could provide evidence of changes in lipin phosphorylation status between WT and dTorKO flies. This would show that the lipin localization profile reflects a NEP1R1/CTDNEP1 pathway gain-of-function rather than alternative scenarios, i.e. defective nucleocytoplasmic trafficking due to Torsin dysfunction.

We made a major effort to address this using mass spectrometry. However, in repeated experiments, we insufficiently detected Lipin phosphorylation to draw conclusions about whether this was reduced by Torsin loss.

We hope that the new lipidomic data showing that *Nep1r1 and Ctdnep1 RNAi* revert the signature of Lipin hyperactivity in the *dTor*^{KO} fly (**Figure 3**) sufficiently complements the original data on Lipin localization, to demonstrate that a Nep1r1-Ctdnep1 gain-of-function is present upon Torsin loss and is responsible for Lipin mislocalization and hyperactivity.

8. The authors should perform an immunofluorescence (IF) quantification of NE herniation using commonly employed K48-Ub as a marker to complement what they quantify by EM in figure 6. This will allow the authors to analyze more cells conveniently than can be done by EM (presently only from five nuclei). Counting the number of cells with K48-Ub-positive NE herniations for dTorKO cells and TorsinA/B KO MEFs under RNAi luciferase, CTDNEP1, and NEP1R1 will allow the authors to robustly characterize the ability for CTDNEP1/NEP1R1 knockdown to rescue the NE herniations arising in Torsin-deficient cells and demonstrate how conserved this rescue ability is.

8.1. Counting the number of cells with K48-Ub-positive NE herniations for dTorKO cells .. [ ] … under RNAi luciferase, CTDNEP1, and NEP1R1 will allow the authors to robustly characterize the ability for CTDNEP1/NEP1R1 knockdown to rescue the NE herniations arising in Torsin-deficient cells

Unfortunately, after multiple attempts to optimize several ubiquitin antibodies (Anti-ubiquitin Lys48-Specific Clone Apu2 Millipore, Ubiquitin: mono-and-poly ubiquitinated conjugates and Anti-Ubiquitin Lys48-Specific Sigma) we failed to obtain specific immunostaining in the fly fat body.

We therefore used alternative approaches to robustly characterise how well Nep1r1, Ctdnep1 and Lipin rescue the NE herniations and NPC deficit.

1) We performed additional TEM (n ≥ 10 more nuclei per genotype) and quantified the percentage of NE with normal INM, ONM, and NPC morphology.

2) We used mAb414 to label the NPC of *dTor*^{KO} fat body cells expressing different transgenes, and quantified the percentage of cells where this was exclusively localized to the NE (the wild-type phenotype) **(Figure 6)**.

Taken together, these new data confirm that *Nep1r1* and *Ctdnep1* RNAi increase the density of nuclear pores in parallel with supressing the appearance of NE herniations. They also identified that *Lipin* RNAi is relatively ineffecitve. These new data are presented within a substantially expanded Figure 6.

8.2. Counting the number of cells with K48-Ub-positive NE herniations and TorsinA/B KO MEFs … [ ] under RNAi luciferase, CTDNEP1, and NEP1R1

We did not perform this experiment since it has previously been shown that robust numbers of NE herniations only occur when 3 or 4 Torsin genes are deleted from dividing mammalian cells, and cells are arrested by thymidine block to maximise their use of NPC insertion (Laudermilch *et al*, 2016; Rampello *et*

al, 2020). Here we have worked with TorsinA/B KO cells that have relatively normal NPC levels (see response to reviewer comment 2.

9. The EM images in Fig. 7 and 8 are suboptimal and do not support the author's conclusion that the unusual structures or "channels" represent NPC intermediates. To support their conclusion, the authors need to demonstrate that Nups localize to these unusual structures via immunogold labeling/EM.

We apologise that we gave the impression that we concluded that the “channel-like” structures are NPC intermediates based on the EM images. We now better explain this and have added new data to support the conclusion.

1) We present a detailed investigation of which Nups are present vs. absent when the NE contains channels (**Figure 8**) rather than nuclear pore complexes. This finds multiple Nups that are very similarly distributed between the NE of control cells and cells with channels, thus associating channel-like structures with normal levels of basket, cytoplasmic filament and outer ring complex Nups.

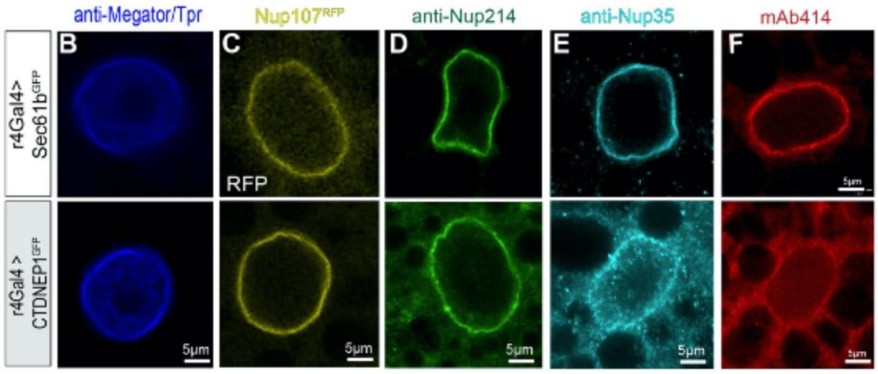
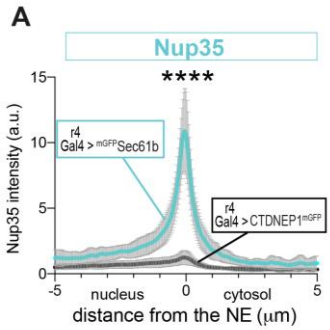


Figure 8: Impaired Nup35 recruitment NPC can explain the nuclear channels associated with excess PA to DAG conversion

B - F) Confocal imaging of 5DO fat body nuclei expressing UAS transgenes and labeled by anti-Megator/Tpr, Nup214, Nup35, or mAb414, or expressing Nup107^{RFP}.

2) We pursued the question of whether failed Nup35 recruitment was the reason why channels appeared; this hypothesis was raised in the discussion of the original manuscript. We addressed this by (1) quantifying Nup35 levels of cells where channels appear in place of NPC (**Appendix Figure S7A**), and (2) TEM on flies with mutations in *Nup35*, reasoning that this genetic insult would reduce how much Nup35 was available for NPC biogenesis (**Figure 8I-K**). This confirmed that Nup35 was poorly recruited to the NE when this contained channels in place of nuclear pores. More importantly, we establish a causal relationship because cells lacking Nup35 also contain these channel-like structures in the NE.



Appendix Figure S7

Related to Figure 8: Impaired Nup35 recruitment to nascent NPC can explain the nuclear membrane defects caused by excess PA to DAG conversion

A) Intensity of anti-Nup35 staining in fat body cells along a line bisecting the NE (defined by DAPI staining) expressing UAS transgenes. a.u., arbitrary units. Mean values for each genotype are indicated by solid lines, error bars show SD. **** indicates a significant difference between the NE-localized intensity of Nup35 labeling (values at 0µm distance from the NE).

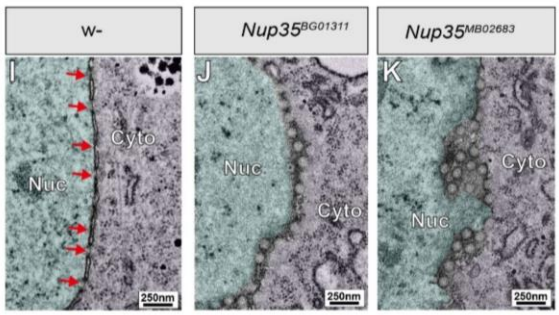


Figure 8: Impaired Nup35 recruitment NPC can explain the nuclear channels associated with excess PA to DAG conversion

I - K) NE ultrastructure of 5DO fat body cells from a (J) control animal, (K) homozygous *Nup35*^{BG01311} and (L) homozygous *Nup35*^{MB02683} animals.

We hope the reviewer agrees that these new data, especially that on the causal relationship between Nup35 loss and channels, is strong evidence that channels are indeed an intermediate of NPC assembly.

10. This reviewer does not feel the authors have provided sufficient evidence to conclude that "NEP1R1 and CTDNEP1 activity prevent INM/ONM fusion, and this explains why Torsins are required for NPC insertion at interphase (Fig. 6G)." It is not currently known at which point of interphase NPC biogenesis Torsins act, and the fusion step is but one possibility. Therefore, the authors should scale down their conclusions. Note also that no changes in phospholipid levels were detected in another experimental system with highly penetrant NPC defects upon combined Torsin deletion (Laudermilch et al., MBoC 2016). This argues against a straightforward causal connection, requiring a more balanced discussion of the findings.

10.1. *Note also that no changes in phospholipid levels were detected in another experimental system with highly penetrant NPC defects upon combined Torsin deletion (Laudermilch et al., MBoC 2016). This argues against a straightforward causal connection, requiring a more balanced discussion of the findings.*

While we appreciate the reviewer's overarching comment that some of our conclusions were overstated, there are two misconceptions about the work reported by Laudermilch, et al.

Firstly, this paper makes the following statement: "Of note, we did not observe material differences in the levels of PC, PE, PI, and PS in TorA/B/3A/2A KO and LULL1 KO cells compared with wild-type cells (Figure S3)." The paper does not contain data on PA or DAG that are the relevant lipid classes to assess whether cells have abnormal Lipin enzyme activity. While excess Lipin activity can affect other lipid classes, it varies considerably between experimental systems. Secondly, these cells do not have highly penetrant NPC defects unless the cell cycle is blocked to prevent mitotic NPC insertion.

We therefore had not considered that this particular work gave insight into whether there was a causal relationship between abnormal lipid metabolism and the NPC defects of Torsin loss.

Having said this, our new data of **Figure 3** and **Figure 6** show that the membrane defects of Torsin loss result from a non-lipid mediated mechanism. We have broadly adjusted the manuscript to reflect this, although do not refer to the Laudermilch, et al, study for the reasons above.

10.2. *It is not currently known at which point of interphase NPC biogenesis Torsins act, and the fusion step is but one possibility.*

A more recent publication from the same lab as the Laudermilch study determined that Torsin loss blocks the membrane fusion step of interphase NPC biogenesis. This is also supported by an earlier report by Jokhi, et al, who made the conclusion that Torsins promote INM/ONM fusion during a mysterious vesicular-trafficking event. While we are aware that membrane herniations have been associated with NPC degradation, fat body cells are rapidly inserting new NPC during nuclear growth and thus the mass appearance of NPC defects is inherently linked to biogenesis (also how it is considered in these previous papers).

As explained above, we have revised all parts to better explain the evidence that connects Torsins to INM/ONM fusion, and to reflect the new data of **Figure 3** and **Figure 6** that places NEP1R1-CTDNEP1, but not lipid metabolism, in the pathway between Torsin loss and NPC abnormalities.

Minor points

11. In the last paragraph on pg. 6 the authors state "Instead, oligomerization of the Torsin membrane domain removes the NEP1R1-CTDNEP1 phosphatase complex from the INM, in turn excluding Lipin from the INM and nucleus" however, Torsin oligomerization is not through the "membrane domain" but rather the intersubunit interface of the AAA domain. Please rephrase accordingly.

We want to thank the reviewer for pointing this out as it made us realise our sentence did not fully explain what we meant. We wanted to highlight two different things here 1) oligomerization is needed and 2) membrane binding is needed. The new manuscript has been revised to separately consider the needs for oligomerization and membrane-association.

Page 8

This suggests that Torsin homo-oligomerization is required for its effects on TAG production and cell size.

Page 9

This suggests that specific information is encoded in the membrane domains of dTorsin and dLap1 and this is key to how both proteins affect cellular lipid metabolism.

12. The authors conclude that ATP hydrolysis is unnecessary. This does not seem 100% accurate. While the effects are less pronounced than the interface mutation, the ATPase mutants are clearly compromised in their rescuing ability relative to WT (Fig. 2C, D). Still this is an interesting result as it again argues against a processive activity of Torsins.

We have made several revisions to address this concern.

Results:

This suggests that dTor continues to function even when its ATPase activity is severely impaired.

This suggests that Torsin homo-oligomerization is required for its effects on TAG production and cell size.

We also revised the discussion as it relates to the need for ATP hydrolysis.

However, our data also provide evidence that Torsin affects NEP1R1-CTDNEP1 by a non-canonical mechanism that diverges from classic AAA+ proteins that directly couple ATP hydrolysis to structural change. Firstly, the fat body role of dTor depends on a conserved residue that does not affect ATPase activity but is required for Torsin to homo-oligomerize into ATPase inactive structures that have membrane deforming properties (Demircioglu *et al*, 2019). Moreover, $dTor^{KO}$ more severely affects the fat body than $dLap1^{-/-}$, which is incompatible with a model where dTor solely operates within a Torsin-Activator ATPase complex. We therefore hypothesize that Torsin affects NEP1R1-CTDNEP1, and thus lipid metabolism and NPC insertion, as a homo-oligomer rather than as an active ATPase. Additional work

is needed to determine the structural basis of this, and given the importance of the Torsin/ dLap1 membrane-association domains, this should consider whether interactions occur within or are mediated by the lipid bilayer.

13. In the second paragraph of pg. 11, the authors state "The NE-localized NEP1R1mGFP and CTDNEP1mGFP signal in dTorKO cells exceeded that of Calnexin, suggesting it reflected protein mislocalization rather than altered ER/NE morphology (Fig. 4A - D, compare red and green signal)." This seems somewhat subjective as the authors do not perform a quantitative analysis. The sentence should therefore be rephrased.

We agree and rephrased this sentence to:

In contrast, the NE of *dTor*^{KO} cells was enriched in NEP1R1^{mGFP} (Fig. 4C; Appendix Fig. S4D & E) and especially CTDNEP1^{mGFP} (Fig. 4D; Appendix Fig. S4F & G) relative to the apperance of Calnexin (Fig. 4A – D, compare red and green signal).

14. Why is there so much nucleoplasmic CTDNEP1 in TorsinA/B KO cells (Fig. 4F)? This figure would benefit from a counterstain for NEs and merged images to show that CTDNEP1 puncta are at the NE.

We revised these data by imaging CTDNEP1^{Scarlet} in WT and TorA/B KO MEFs co-labeled with the INM marker, anti-Sun1 (**Figure 4E & F**).

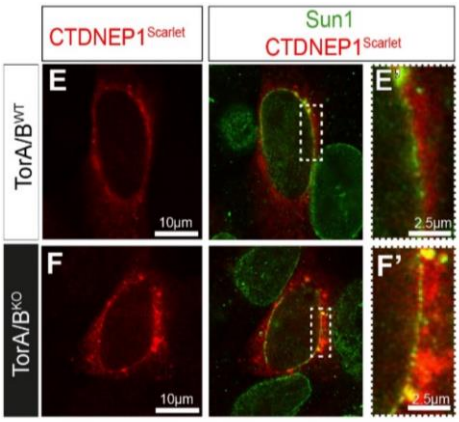
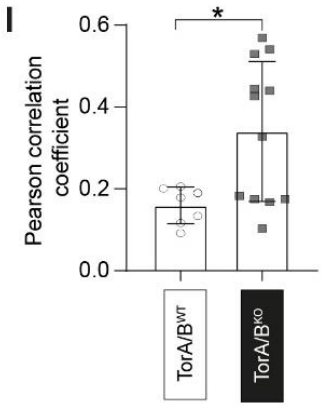


Figure 4: CTDNEP1 and Lipin concentrate at the NE of fly and mouse cells lacking Torsins
E & F) CTDNEP1^{Scarlet} and anti-Sun1 localization in MEFs.

We also quantified the colocalization between CTDNEP1^{Scarlet} and Sun1 (**Appendix Figure S4I**).



Appendix Figure S4

Related to Figure 4: CTDNEP1 and Lipin concentrate at the NE of fly and mouse cells lacking Torsins

I) The Pearson correlation coefficient (mean ± SD) between CTDNEP1^{Scarlet} and Sun1 signal in the NE of individual cells.

15. It is not entirely clear from the images in Fig. 4I that NEP1R1V5- CTDNEP1myc co-overexpression produce Lipin1mGFP signal at the nuclear periphery.

We have revised these data by adding a higher-power image of the nuclear periphery and quantifying the relative intensity of Lipin1^{mGFP} signal at the nuclear periphery (**Figure 4G'**).

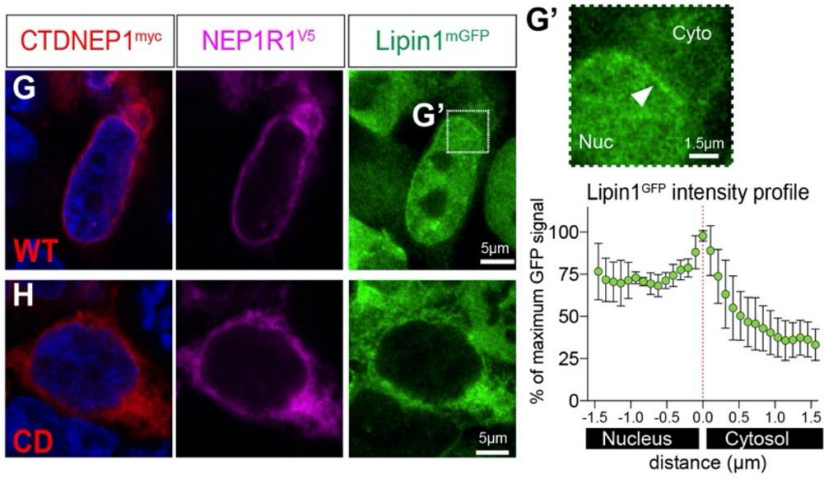


Figure 4: CTDNEP1 and Lipin concentrate at the NE of fly and mouse cells lacking Torsins

G & H) Lipin1^{mGFP} localization in HEK293T cells transfected with a plasmid that co-expresses CTDNEP1^{myc}-WT or -CD (red) and NEP1R1^{V5} (magenta). (G') Upper: enlargement of panel G, white arrow highlights NE enrichment. Lower: plot showing the intensity of Lipin1^{mGFP} signal (mean ± SD, n = 5 cells) along a 3 µm profile that transects the NE at 0 µm.

16. In Fig. 5, please correct the label for panel Q' where this reviewer assumes you meant R.

We apologise and this has been corrected.

17. The authors should reword the sentence in the second paragraph of the introduction stating "This DAG production is the penultimate step of triacylglyceride (TAG) synthesis" to reflect that DAG is also an intermediate for phospholipid synthesis.

    This comment is addressed above. We added a scheme showing GL/GPL synthesis as **Figure 1A** (and **Appendix Figure S1A**) and refer to this in the introduction. The text and new **Figure 1A** show that DAG is the precursor of PC, PE and PS lipids, but also emphasize that membrane lipid production is highly influenced by PA levels.

18. Can the authors comment on the electron-dense particles resembling viral capsids in their EMs in Fig. 7 K (nucleoplasm)?

    We have not investigated these structures but hypothesize they are stress granules (ribonucleoprotein particles) that accumulate in the nucleus due to NPC trafficking defects.

Referee#2:

Previous work from this group showed that Torsin regulates lipid metabolism and modulates the activity of Lipin, a key regulator of lipid metabolism. The first half of this study investigates the mechanism of Lipin regulation by Torsin. It finds Torsin controls the localization of the Lipin-regulating phosphatase complex NEP1R1-CTDNEP1 and that, remarkably, a number of phenotypes in flys lacking Torsin are reversed they also lack this complex. The remainder of the study investigates the consequences of Torsin regulation of the NEP1R1-CTDNEP1 complex and finds the complex is required for NPC insertion into the nuclear membrane. It argues this occurs because cells lacking Torsin have defects in fusion of the inner and outer membrane and cannot maintain a lipid composition in the nuclear envelope conducive to NPC insertion. These are a fascinating set of results that advance our understanding of how Torsin regulates lipid metabolism and NPC biogenesis. However, the findings are over interpreted.

We wish to thank the reviewer for their effort to review the manuscript and their thoughtful and supportive comments about the importance of the results.

1. It is not clear that Torsin directly regulates or interacts with the NEP1R1-CTDNEP1 complex. Some insight into how regulation occurs would significantly strengthen this study. How does Torsin regulate the localization the NEP1R1-CTDNEP1 complex? If the complex is re-localized to the inner nuclear membrane in cells lacking Torsin, is Lipin regulation restored? A complete answer to these questions is beyond the scope of this study but some indication of how regulation occurs would increase the significance of the paper. At a minimum, how Torsin could regulate NEP1R1-CTDNEP1 localization and how it could directly or indirectly affect it should be discussed.

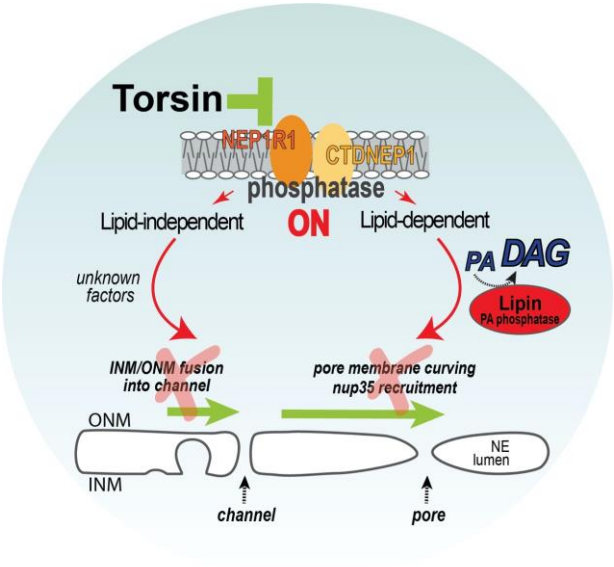
   We have revised the discussion to better address this issue.

   This study also provides new information on the structural basis of Torsin function. Torsins are members of the AAA+ ATPase family which has many members that dissassmble otherwise stable protein complexes (Hanson & Whiteheart, 2005). Torsin loss is associated with more NE-localized CTDNEP1, which might be explained if Torsins act to dissociate the NE-localized NEP1R1-CTDNEP1 complex since this, in turn, would induce CTDNEP1 turnover. Further, while we did not examine if Torsin directly binds NEP1R1 and/or CTDNEP1, we infer a relatively direct connection given that all three proteins are NE residents (Siniossoglou *et al*, 1998; Han *et al*, 2012; Bahmanyar *et al*, 2014; Goodchild & Dauer, 2004). However, our data also provide evidence that Torsin affects NEP1R1-CTDNEP1 by a non-canonical mechanism that diverges from classic AAA+ proteins that directly couple ATP hydrolysis to structural change. Firstly, the fat body role of dTor depends on a conserved residue that does not affect ATPase activity but is required for Torsin to homo-oligomerize into ATPase inactive structures that have membrane deforming properties (Demircioglu *et al*, 2019). Moreover, $dTor^{KO}$ more severely affects the fat body than $dLap1^{-/-}$, which is incompatible with a model where dTor solely operates within a Torsin-Activator ATPase complex. We therefore hypothesize that Torsin affects NEP1R1-CTDNEP1, and thus lipid metabolism and NPC insertion, as a homo-oligomer rather than as an active ATPase. Additional work is needed to determine the structural basis of this, and given the importance of the Torsin/ dLap1 membrane-association domains, this should consider whether interactions occur within or are mediated by the lipid bilayer.

2. The second half of the study is well done but the results are over interpreted. There is no evidence the structures seen in Fig. 5O are intermediates caused by a defection in membrane fusion, though they could be. There is also no evidence that the lipid composition of the nuclear envelope is altered or, if it is, how this how this could affect NPC insertion. The authors seem to assume that some biophysical property of nuclear envelope is altered cells lacking Torsin or overexpressing the NEP1R1-CTDNEP1 complex, but that has not been investigated and other explanations are possible. This is should be discussed.

We have made major revisions that we hope resolve the concerns of both reviewers about the mechanism(s) and stage(s) that Torsin loss, NEP1R1-CTDNEP1 +/- Lipin interfere with NPC biogenesis.

1) The original manuscript identified two distinct membrane remodeling events during NPC biogenesis; an event that (when blocked) produces "vesicle-like" structures, and an event that (when blocked) results in elongated channels. We more clearly emphasize there are two events in the revised manuscript and included a synopsis figure that highlights these two events.



Synopsis figure

2) We added text to the introduction and results that better explains the existing data about the connection between the membrane defects of the *dTor*^{KO} and INM/ONM fusion during NPC biogenesis. We agree that the data of our study does not establish this, but that it is reasonable to state given the literature on Torsin and NPC biogenesis.

> This includes that Torsins have a poorly understood importance for NPC insertion into the NE of post-mitotic / interphase cells (Goodchild *et al*, 2005; Pappas *et al*, 2018; Liang *et al*, 2014; Laudermilch *et al*, 2016; VanGompel *et al*, 2015; Rampello *et al*, 2020). Interphase NPC biogenesis occurs by less well-understood mechanisms than its mitotic counterpart. It appears that it is initiated by membrane deforming nucleoporins (Nups) that curve the INM towards the ONM, followed by membrane fusion and the recruitment of the full complement of NPC subunits INM abnormalities (Naismith *et al*, 2004; Goodchild & Dauer, 2005; Kim *et al*, 2010; VanGompel *et al*, 2015; Tanabe *et al*, 2016; Pappas *et al*, 2018; Jokhi *et al*, 2013; Liang *et al*, 2014; Rampello *et al*, 2020), that are characteristic of failed INM/ONM

fusion (Jokhi *et al*, 2013; Otsuka *et al*, 2016; Scarcelli *et al*, 2007; Allegretti *et al*, 2020), and appear to replace mature nuclear pores (Rampello *et al*, 2020; VanGompel *et al*, 2015; Laudermilch *et al*, 2016; Pappas *et al*, 2018).

3) The channel-like structures are, to our knowledge, newly identified. The original manuscript provided evidence that excess PA to DAG lipid metabolism is necessary and sufficient for their appearance, namely they are produced by Lipin overexpression and are removed by DAG kinase overexpression. We now added new data showing that they likely result because abnormal PA metabolism impairs the recruitment of the membrane-remodeling Nup35 subunit of the NPC (known as Nup53 in other species). This includes data showing that Nup35 is absent from the NE containing channels, and that Nup35 genetic deletion is sufficient to induce similarly appearing channels (**Figure 8G & J-L**).

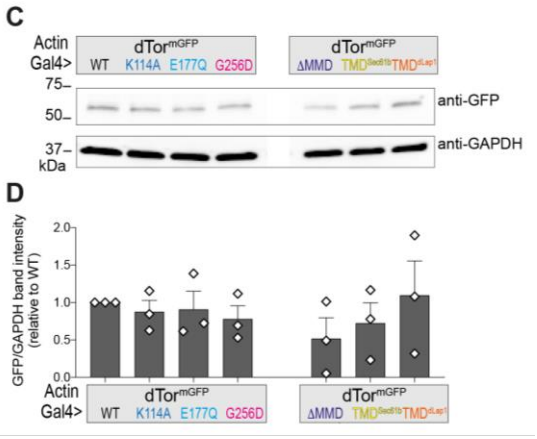
4) The original manuscript did not explicitly address whether the INM defects of the $dTor^{KO}$ (defined as the result of failed INM/ONM fusion) were due to abnormal lipid metabolism downstream of NEP1R1-CTDNEP1 activity. We have now explored this in detail using lipidomics (**Figure 3**), TEM (**Figure 6**), and immunofluorescent detection of the NPC in the $dTor^{KO}$ expressing Lipin RNAi (**Figure 6**). We conclude that these INM herniations occur because NEP1R1-CTDNEP1 block early nuclear pore membrane remodelling by an unknown mechanism that does not involve lipids.

We have extensively revised the abstract, graphical abstract and discussion with the aim to take the reader step wise through the two distinct membrane remodeling events and what we have uncovered about the underlying mechanism why they are blocked by Torsin/ NEP1R1-CTDNEP1 and/or Lipin.

3. The results shown in Figs 1 and 2 are well done but it is not clear how they are related to the rest of the study. The authors may want to consider removing them or else more clearly explaining the connection to what follows. It is notable that replacing the membrane-binding domain of Torsin with the TMD from Torip/dLAP1 is functional but the TMD from Sec61-beta is not. The Torip TMD could interact with the NEP1R1-CTDNEP1 complex, as the authors suggest, but there are other possibilities. A missing control for this experiment is a demonstration that the fusions are expressed at similar levels and oligomerize normally.

We have kept these data in the revised manuscript but introduced new lipidomic data (**Figure 1**) to better establish why they are important to the second part of the manuscript on lipid metabolism. We believe that the data showing that Torsin and dLap1 have different effects on lipid metabolism is important for the reader to (later) understand that it is possible that *dTor* loss induces INM-herniations associated with impaired INM/ONM fusion, while *dLap1* loss induces channel-like structures that we associated with loss of membrane curvature. This is because it has been generally thought Torsin operates as a AAA+ type protein from within a Torsin-LAP1 complex.

We have added Western blotting (**Appendix Figure S2C & D**) showing the relative expression of each dTor cDNA that shows this cannot explain for their varied activity. Unfortunately, due to the small size of *Drosophila* larvae, it is not technically feasible to biochemically examine dTor oligomerization in this system. We have more clearly explained how the point mutations we introduced into fly dTor correspond to previously studied residues of mammalian Torsins.



Appendix Figure S2

Related to Figure 2: The dTor membrane domain and homo-oligomerization motif are important for dTor function in the fat body

C) Representative Western blot showing anti-GFP and anti-GAPDH (loading control) signal from fat body lysates prepared from 5DO larvae expressing UAS transgenes.

D) Western blotting anti-GFP band intensity (mean ± SEM; a.u.) in lysates prepared from 5DO fat bodies expressing the indicated UAS transgenes. Values are relative to GAPDH and are expressed as a fraction of the mean intensity of $dTor^{mGFP}$-WT control samples.

1st Revision - Editorial Decision 26th Apr 2021

Thank you for submitting your revised study. The manuscript has now been sent back to the original referees, whose comments are appended below.

As you will see, referee #1 finds that his/her criticisms have been adequately addressed. Referee #2 stresses that the main findings are fascinating but continue to be significantly overinterpreted. Therefore, I would ask you to tone down the key conclusions and discuss also alternative models as suggested by referee #2. Please modify the text as indicated and return it with track changes activated.

In addition, there are few editorial issues concerning the text and the figures that I need you to address before we can officially accept your manuscript.

Referee #1:

In their updated manuscript titled "Torsin and NEP1R1-CTDNEP1 affect interphase NPC insertion by lipid-dependent and -independent mechanisms," Jacquemyn et al. make great improvements to their previously reviewed submission. We thank the authors for their extremely careful and thorough responses to our review. With the addition of the lipidomic datasets, the more detailed investigation

of Lipin's relevance to membrane curvature events, and the identification of failed Nup35 recruitment to the channel-like NPC structures, the conclusions of this manuscript are greatly improved. Furthermore, the authors have successfully clarified points in the text that were previously confusing and used more neutral wording to avoid overstating conclusions. Given these major improvements, we now recommend this manuscript for publication in EMBO Journal. A few final text edits are recommended:
Minor points
1. There are a few instances where the text should be revised (italicized).
a. "This includes it is dephosphorylated by an evolutionarily conserved transmembrane complex formed between the C-Terminal Domain Nuclear Envelope Phosphatase 1 (CTDNEP1) and its regulatory subunit 1 (NEP1R1)." omitted "that"?
b. "It appears that it is initiated by membrane deforming nucleoporins (Nups) that curve the INM towards the ONM, followed by membrane fusion and the recruitment of the full complement of NPC subunits INM abnormalities, that are characteristic of failed INM/ONM fusion, and appear to replace mature pores" (citations removed from quote for clarity). The italicized half of this sentence seems out of place or mistakenly inserted.
c. "This very suggests that membrane curving is inhibited by excess Lipin-mediated PA to DAG conversion." The word "very" should be removed.

2. A little more clarification is warranted concerning the two distinct mechanisms by which the authors describe the CTDNEP1-NEP1R1 axis affects NE membrane deformation. On the one hand, CTDNEP1-NEP1R1 but not Lipin RNAi suppress the NE herniations arising upon dTor depletion. On the other hand, the authors report that membrane channels form upon excess Lipin PA metabolism because this prevents normal Nup35 recruitment. These are vastly different situations that the authors do not necessarily compare, but they could perhaps more clearly state the independence of the two situations in the discussion.

Referee #2:

While this study is improved, my major concerns remain. As I said in my previous review, there are many fascinating results here, but they continue to be significantly overinterpreted.

1. The finding that some phenotypes of Torsin knockout animals are reversed by knockdown of NEP1R1-CTDNEP1 is fascinating but there is no mechanistic insight into how this genetic bypass occurs. There is a hint it could involve changes in NEP1R1-CTDNEP1 localization, and the revision now includes a lipidomic analysis consistent with Torsin regulating the NEP1R1-CTDNEP1 complex, but this is not demonstrated. I had hoped the revised version would have more mechanistic insight, but it could at least have had a balanced discussion of what could account for the genetic interaction of Torin and NEP1R1-CTDNEP1. Instead, there is now more a detailed justification of the authors' preferred model. This is disappointing.

2. There is a similar issue with the discussion of the aberrant nuclear structures. The suggested model is interesting and consistent with the findings but there is not strong evidence for it. The authors seem to have misunderstood what I was requesting. It was not further description or justification of their model but a discussion of other possibilities and what it will take distinguish between them.

Referee #1:

> In their updated manuscript titled "Torsin and NEP1R1-CTDNEP1 affect interphase NPC insertion by lipid-dependent and -independent mechanisms," Jacquemyn et al. make great improvements to their previously reviewed submission. We thank the authors for their extremely careful and thorough responses to our review. With the addition of the lipidomic datasets, the more detailed investigation of Lipin's relevance to membrane curvature events, and the identification of failed Nup35 recruitment to the channel-like NPC structures, the conclusions of this manuscript are greatly improved. Furthermore, the authors have successfully clarified points in the text that were previously confusing and used more neutral wording to avoid overstating conclusions. Given these major improvements, we now recommend this manuscript for publication in EMBO Journal. A few final text edits are recommended:

We want to thank the reviewer for their kind comments and are pleased that they find the manuscript is greatly improved.

> Minor points
> There are a few instances where the text should be revised (italicized).

> a. "This includes it is dephosphorylated by an evolutionarily conserved transmembrane complex formed between the C-Terminal Domain Nuclear Envelope Phosphatase 1 (CTDNEP1) and its regulatory subunit 1 (NEP1R1)." omitted "that"?

We added “that” into this sentence.

> b. "It appears that it is initiated by membrane deforming nucleoporins (Nups) that curve the INM towards the ONM, followed by membrane fusion and the recruitment of the full complement of NPC subunits INM abnormalities, that are characteristic of failed INM/ONM fusion, and appear to replace mature pores" (citations removed from quote for clarity). The italicized half of this sentence seems out of place or mistakenly inserted.

We examined this sentence and saw that we (in error) had inappropriately fused two concepts. It has been adjusted to:

> *It appears that it is initiated by membrane deforming nucleoporins (Nups) that curve the INM towards the ONM, followed by membrane fusion and the recruitment of the full complement of NPC subunits (Otsuka et al, 2016; Otsuka & Ellenberg, 2018). Torsin loss from worm, fly and mammalian cells induces characteristic nuclear membrane herniations that are believed to result from failed INM/ONM fusion (Naismith et al, 2004; Goodchild & Dauer, 2005; Kim et al, 2010; Jokhi et al, 2013; Liang et al, 2014; Tanabe et al, 2016; Pappas et al, 2018; Rampello et al, 2020) and these cells have correspondingly fewer mature nuclear pores (VanGompel et al, 2015; Laudermilch et al, 2016; Pappas et al, 2018; Rampello et al, 2020).*

> c. "This very suggests that membrane curving is inhibited by excess Lipin-mediated PA to DAG conversion." The word "very" should be removed.

This sentence was revised while addressing the other comments from both reviewers.

2. A little more clarification is warranted concerning the two distinct mechanisms by which the authors describe the CTDNEP1-NEP1R1 axis affects NE membrane deformation. On the one hand, CTDNEP1-NEP1R1 but not Lipin RNAi suppress the NE herniations arising upon dTor depletion. On the other hand, the authors report that membrane channels form upon excess Lipin PA metabolism because this prevents normal Nup35 recruitment. These are vastly different situations that the authors do not necessarily compare, but they could perhaps more clearly state the independence of the two situations in the discussion.

We have modified the discussion to emphasize that distinct mechanisms cause membrane herniations versus membrane channels.

The data also define that Torsin/Nep1r1/Ctdnep1, but not Lipin, are a genetic network that interferes with INM/ONM fusion during NPC insertion. Finally, although unconnected with membrane fusion, excess Lipin-mediated PA metabolism impairs NPC membrane curving and maturation.

The analyses of NPC biogenesis led to the unexpected discovery that independent molecular events drive the membrane fusion versus the membrane curving of interphase NPC insertion. This includes that, while we found no evidence that Lipin affected fusion, NPC membrane curving was impaired in cells overexpressing Lipin.

Referee #2:

> While this study is improved, my major concerns remain. As I said in my previous review, there are many fascinating results here, but they continue to be significantly overinterpreted.
>
> 1. The finding that some phenotypes of Torsin knockout animals are reversed by knockdown of NEP1R1-CTDNEP1 is fascinating but there is no mechanistic insight into how this genetic bypass occurs. There is a hint it could involve changes in NEP1R1-CTDNEP1 localization, and the revision now includes a lipidomic analysis consistent with Torsin regulating the NEP1R1-CTDNEP1 complex, but this is not demonstrated. I had hoped the revised version would have more mechanistic insight, but it could at least have had a balanced discussion of what could account for the genetic interaction of Torin and NEP1R1-CTDNEP1. Instead, there is now more a detailed justification of the authors' preferred model. This is disappointing.
>
> 2. There is a similar issue with the discussion of the aberrant nuclear structures. The suggested model is interesting and consistent with the findings but there is not strong evidence for it. The authors seem to have misunderstood what I was requesting. It was not further description or justification of their model but a discussion of other possibilities and what it will take distinguish between them.

We want to thank the reviewer for continuing to appreciate that the study contains fascinating results. We have revised the manuscript upon considering their new comments and re-considering comments in the first review.

We have carefully revised the discussion in response to the reviewer's comments. This includes we now separated how the discussion handles the three questions in our manuscript: (1) how does Torsin affect the lipidome? (2) how does Torsin loss block NPC insertion? and (3) how does the Lipin pathway affect NPC insertion? We feel that our data on questions (1) and (3) provide mechanistic answers (go beyond defining a genetic interaction), albeit that the structural basis of Torsin function remains unclear. We have therefore expanded the discussion to explain the lines of evidence that support these conclusions. On the other hand, the reviewer is correct that data pertaining to Question (2) show a genetic interaction. We have revised the manuscript to make this limitation clearer.

On question (1), we explain that the data indicating that NEP1R1-CTDNEP1 connect Torsin to Lipin come from mammalian and fly cells. The conclusion also considers findings from many papers in the literature, which have shown that NEP1R1-CTDNEP1 regulate Lipin phosphorylation state and this alters Lipin activity and localization. This information is provided in a revised discussion paragraph (paragraph two).

> *CTDNEP1 is a transmembrane ER/NE protein with a catalytic phosphatase domain facing the cytosol or nucleus. NEP1R1 is its transmembrane binding partner that stabilizes CTDNEP1 against degradation (Han et al, 2012). The NEP1R1-CTDNEP1 complex is known to dephosphorylate Lipin to, in turn, target Lipin to the nucleus and activate Lipin PA phosphatase activity (Kim et al, 2007; Han et al, 2012; Santos-Rosa et al, 2005; Bahmanyar et al, 2014). Our data now extend this knowledge by indicating that NEP1R1-CTDNEP1 connects Torsins of the NE-lumen to Lipin. The evidence includes that Torsin loss led to elevated NE-localized levels of CTDNEP1 and/or NEP1R1 in*

mammalian and fly fat body cells, that NEP1R1-CTDNEP1 overexpression in mammalian cells mimicked how Torsin loss affects Lipin localization, and that NEP1R1-CTDNEP1 expression was required for mammalian Torsins to affect Lipin localization. Further, Nep1r1 or Ctdnep1 RNAi in fly almost completely reversed the lipidomic defects assocated with dTor loss, thus indicating that Torsin only affects Lipin PA phosphatase activity and the cellular lipidome when NEP1R1 and CTDNEP1 are also present.

On question (2), we have made clear that our data only establish a genetic interaction and we have not defined the mechanism by which Torsin/NEP1R1-CTDNEP1 prevent INM/ONM fusion during NPC insertion.

Paragraph one of the discussion:

The data also define that Torsin/Nep1r1/Ctdnep1, but not Lipin, are a genetic network that interferes with INM/ONM fusion during NPC insertion.

Paragraph three of the discussion:

The genetic analyses showed that NEP1R1-CTDNEP1 are key to why Torsin loss blocks INM/ONM fusion during NPC insertion. […] Additional work is needed to define why NEP1R1-CTDNEP1 removal allows INM/ONM fusion to proceed in the absence of Torsins. There are many outstanding questions, including whether Nep1r1 and Ctdnep1 RNAi act by reducing NEP1R1-CTDNEP1 phosphatase activity or by releasing proteins that interact with NEP1R1 and/or CTDNEP1. Further, while our data define that dTor acts upstream of Nep1r1 and Ctdnep1 within a genetic network that interferes with INM/ONM fusion during NPC insertion, the mechanism could be indirect whereby none of these three proteins directly affect the NPC.

On question (3), we make it clear that pore membrane curving is distinct to the membrane remodeling considered in question (2). We explain the data that indicate that excess PA to DAG conversion blocks pore membrane curving because it inhibits Nup35/53 recruitment.

The analyses of NPC biogenesis led to the unexpected discovery that independent molecular events drive the membrane fusion versus the membrane curving of interphase NPC insertion. This includes that, while we found no evidence that Lipin affected fusion, NPC membrane curving was impaired in cells overexpressing Lipin. Moreover, PA metabolism appears responsible given that co-overexpression of DAG kinase restored the normal highly-curved morphology of mature pores. We also present evidence that Nup35/53 recruitment is the key driver of membrane curving, which is consistent with its known roles in membrane deformation and anchoring the inner ring complex / FG-Nups to the NPC (Otsuka et al, 2016; Vollmer et al, 2012).

We also revised the discussion to consider the following points from reviewer two.

It is not clear that Torsin directly regulates or interacts with the NEP1R1-CTDNEP1 complex. **Some insight into how regulation occurs would significantly strengthen this study. How does Torsin regulate the localization the NEP1R1-CTDNEP1 complex?** If the complex is re-localized to the inner nuclear membrane in cells lacking Torsin, is Lipin regulation restored? A complete answer to these questions is beyond the scope of this study but some indication of how regulation occurs would increase the significance of the paper. **At a minimum, how Torsin could regulate NEP1R1-CTDNEP1 localization and how it could directly or indirectly affect it should be discussed.**

We discuss the evidence that Torsin homo-oligomerization is the important event for Torsin function in the fat body.

> *The AAA+ ATPase superfamily has numerous members that assemble into oligomeric assemblies and use the energy of ATP hydrolysis to dissociate otherwise stable protein complexes (Hanson & Whiteheart, 2005). However, torsins are atypical members that form at least two different structures; a Torsin-Activator complex that has ATPase activity, or Torsin homo-oligomers that lack ATPase activity but have membrane deforming properties (Chase et al, 2017; Demircioglu et al, 2019). Here we find that $dTor^{KO}$ and $dLap1^{-/-}$ differentially affect fat body lipid homeostasis and NPC insertion, which suggests that dTor retains at least some function when its ATPase activity is lost. This is further supported by the fact a dTor residue involved in homo-oligomerization is essential for fat body development (Chase et al, 2017; Demircioglu et al, 2019). We therefore hypothesize that homo-oligomer formation is key to dTor function in the developing fat body.*

We expanded the discussion section considering how Torsin might regulate NEP1R1-CTDNEP1 localization and levels. We explain the evidence in favour, including that excess NEP1R1-CTDNEP1 mimics the effect of Torsin loss on Lipin localization, but make clear that CTDNEP1 mislocalization is not necessarily the *Nep1r1/Ctdnep1 gain-of-function* event that underlies how Torsin loss affects the cell.

> *Additional work is required to understand the mechanistic and structural basis by which Torsin homo-oligomers suppress NEP1R1-CTDNEP1 activity. One possiblity is that they dissociate NEP1R1 binding to CTDNEP1 since this would, in turn, trigger CTDNEP1 turnover. This mechanism is suggested by the data showing that NEP1R1-CTDNEP1 overexpression mimic the effect of Torsin loss on Lipin localization, and that cells lacking Torsins have elevated levels of NE-localized CTDNEP1. However, altered CTDNEP1 protein levels may not be the only Nep1r1/Ctdnep1 gain-of-function mechanism in cells lacking Torsins. It is also unclear whether Torsins directly bind NEP1R1 or CTDNEP1 and, if so, which domains are responsible. It is alternatively feasbile that the interaction is indirect and, given the importance of dTor membrane association, involves structural changes within or via the nuclear membrane. Finally, it is unlikely that all Torsin functions are mediated by its homo-oligomeric structures. There is substantial evidence that Torsin-Activator ATPase activity modulates NE-localized LINC complexes (Van der Heyden et al, 2009; Nery et al, 2008; Saunders et al, 2017; Jungwirth et al, 2011; Dominiguez Gonzalez et al, 2018; Chalfant et al, 2019) and modifies LAP1 chromatin binding (Luithle et al, 2020). It therefore appears that Torsin function varies by cellular context and it will be interesting to discover how the cell controls whether Torsin homo-oligomer or Torsin-Activator complexes predominate.*

2nd Revision - Editorial Decision 28th Jun 2021

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dr. Rose E. Goodchild
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-106914R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size to examine whether Nep1r1/Ctdnep1/Lipin reverted abnormalities in the dTorKO was based on the sample size required to detect statistically significant differences between control and dTorKO flies, including that found in a previous study (Grillet, et al, 2016). The sample size used to examine whether TorsinA/B or NEP1R1/CTDNEP1/Lipin1 affected protein localization was based on finding a sample size where at least one manipulation had a statistically significant effect (compared to control/ wild-type).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample size that examined whether Nep1r1/Ctdnep1/Lipin RNAi reverted abnormalities in the dTorKO was based on that which detected statistically significant differences between control and dTorKO flies in this study or a previous study (Grillet, et al, 2016).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All samples/ animals were included in analyses except where specified (protein localization was not examined in mammalian cells with grossly abnormal morphology).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Cell lines treated with control or Nep1r1/Ctdnep1 RNAi plasmids were plated in triplicate and processed in triplicate. Other experiments did not have treated vs. untreated animals/ samples.
For animal studies, include a statement about randomization even if no randomization was used.	Developmentally-staged-larvae were collected (randomized) 3, 4, or 5 days after placing male and female flies in fresh vials for a 12-hour period.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Image acquisition and analysis were assessed blind to genotype and/or transfected plasmid.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Fat body cells were imaged in 5DO animals, unless otherwise stated, by a researcher blind to genotype. Fly and cell line image analysis was performed using FIJI unless otherwise stated, by a researcher blinded to animal genotype and/or plasmid transfection.
5. For every figure, are statistical tests justified as appropriate?	Statistical analyses were justified and performed using GraphPad Prism 8.2.1 software. Differences between two groups were assessed using a two-tailed T-Test, differences between three or more groups were assessed by One-way ANOVA, with Bonferroni's post hoc test, unless otherwise stated. The criteria for significance are: ns (not significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution to test parametric or non-parametric was justified using GraphPad Prism 8.2.1 .

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	All data is presented with a SD (if individual data points derive from individual animals), or SEM for experiments where an individal data point is a technical replicate (from multiple animals/ repeated experiments on a single cell line)..
Is the variance similar between the groups that are being statistically compared?	Variance was frequently greater for groups of mutant animals compared to controls.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies are listed in Appendix table S3. This table includes vendor information and/or a reference.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T were from Invitrogen. Lipin1-mGFP expression was verified by fluorescent microscopy with reference to previous studies describing this for wild-type cells (Peterson, et al, 2011). TorA WT and KO MEF cell lines were previously authenticated by PCR genotyping (Cascalho, et al, 2020. Tor1b disruption was authenticated by sanger sequencing Tor1b exon1 and by anti-TorsinB western blotting. All cell lines used in this study were tested for mycoplasma. dLAP1 gene knock-out (fly) was authenticated by sanger sequencing, qRT-PCR, and dTor-mGFP localization. RNAi transgene expression (in flies) was authenticated by qRT-PCR.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	The source, genetic background and modifications contained by individual Drosophila lines are listed in Appendix Table S1. Fly lines were maintained using standard protocols and fed a standard diet consisting of cornmeal, agar, yeast, sucrose, and dextrose. Experimental crosses were kept at 25°C. This information is provided in the methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	no