JCB

Computational analyses show spatial relationships between nuclear pore complexes and specific lamins

Mark Kittisopikul, Takeshi Shimi, Meltem Tatli, Joseph Tran, Yixian Zheng, Ohad Medalia, Khuloud Jaqaman, Stephen Adam, and Robert Goldman

Corresponding Author(s): Mark Kittisopikul, Northwestern University and Robert Goldman,

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September 17, 2020

Re: JCB manuscript #202007082

Dr. Mark Kittisopikul
Northwestern University
Department of Cell and Developmental Biology
303 E Chicago Ave
Ward 11-145
Chicago, IL 60611-4296

Dear Dr. Kittisopikul,

Thank you for submitting your manuscript entitled "Computational analysis of lamin isoform interactions with nuclear pore complexes." The manuscript was assessed by the Editors and expert reviewers, whose comments are appended to this letter. Please note that editorially we felt your manuscript was a better fit in our Article format rather than Tools, as you originally submitted. Thus, we sent it out to review as an Article. Overall, the reviewers were enthusiastic about the study, although they raise a number of important points that must be addressed before we can proceed. Therefore, we invite you to submit a revision as an Article, if you can address the reviewers' key concerns, as outlined here.

You will also see that Reviewer 2 asks to test cell cycle effects on NPC assembly and Reviewer 3 asks to test effects of lamin B2 knockdown on NPC numbers. We believe these requests are reasonable and every effort should be made to address them with new data. Additionally, all reviewers request changes to text and figures for clarity as well as further details in methods and results. All of these should be addressed and we feel that questions regarding the implications of using knockout MEFs and long term siRNA on lamin/NPC organization, questions regarding affinities of antibodies used for EM, and the request for validation of the lamin C specific antibody are of particular importance.

Finally, we ask that you please add a reference to the study by Mahamid et al (doi.org/10.1126/science.aad8857) that is currently not cited. As that work examined the relationships between lamins and NPCs using cryo-EM we believe it is important to include this reference and you may of course discuss any differences between your methods and results.

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

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the

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

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

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

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

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

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

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

Sincerely,

Michael Rout, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

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

This manuscript reports an important computational advance to decipher and understand the ultrastructure of the nuclear envelope, a longstanding challenge in cell biology. The authors convincingly quantified large-scale spatial relationships between nuclear pore complexes (NPCs)

and filament networks formed by lamin A (LA), lamin C (LC), lamin B1 (LB1) and lamin B2 (LB2), to understand their potential inter-dependence in wildtype cells and in cells genetically-null for either LB1, LB2 or LA+LC. Significantly enhancing the impact of this manuscript, the authors also used siRNA-mediated downregulation to reduce protein levels of three nucleoporins known or positioned to bind lamins: ELYS (essential for NPC assembly after mitosis), Nup153 (essential for NPC assembly during interphase) or TPR (NPC basket; chromatin-free zones). The manuscript is overall very well-written.

(1) To this 'non-computational' cell biologist reviewer, nearly all of the Results are intelligible and logical, sometimes even understated (compared to the beautiful images), despite the unrelenting numerical density of the Results text. A lighter touch here, especially when differences are not significant, would improve the manuscript. Results text would also be significantly improved by adding 'plain English' conclusions (e.g., 'NPCs are offset from the center of LA fibers' = NPCs remain close to LA fibers).

(2) This manuscript would be significantly enhanced by a schematic summary of the main findings, and a model depicting potential roles played by ELYS, Nup153 and TPR. The data are all about 'distances', but this belies its impact on cell biology, which relates to potential molecular associations and/or connections between NPCs and each type of filament.

(3) Potentially flawed logic and conclusion? (lines 201-203 and elsewhere): The average numbers of NPCs per nucleus were calculated from the single focal plane closest to the coverslip, in Lmna-/- (loss of both LA and LC) or Lmnb1-/- cells. The 'suggestion' that LA [why not LC?] and LB1 are both involved in regulating NPC number fails to account for potential changes in nuclear surface area (hence NPC density) caused by loss of LA+LC, or loss of LB1.

(4) Graph and labelling- Where possible, switch to blue/yellow coloring (instead of red/green), or dark-vs-light gray (in all violin-graphs), to be interpretable when printed in black-and-white or when viewed by red/green colorblind people.

(5) Line 221 (first mention of siRNA downregulation)- Indicate how long the cells were downregulated, and estimate the fraction of cells that may have gone through mitosis immediately before analysis. Did the analysis (Figure 5, Figure S5, Table 2) account for potentially different outcomes in postmitotic cells, versus cells that stayed in interphase? These interesting possibilities are also brought up in the Discussion (lines 337-338).

(6) Lines 298-311 (NPC clustering phenotypes in Lmna-/- or Lmnb1-/- MEFs): This entire section related to Figure S10 needs revision for clarity and flow, and to account for potential differences based on the fraction of post-mitotic versus interphase cells. Or, consider moving results for each Nup to a relevant earlier section?

Minor corrections:
Line 17: Simplify or clarify (seems redundant; distinction is unclear): "the lamin filaments composing the fibers"

Line 31: Delete "highly"

Lines 85-86: Rephrase (add: ", respectively"?) to avoid suggesting that you visualized LA fibers in Lmna-/- MEF nuclei, and LB1 fibers in Lmnb1-/- nuclei.

Figure 2B,C,DE- The X-axis and Y-axis labels should be made larger.

Line 170 ("removal of either LA/C or LB1..")- Must state precisely which A-type lamin(s) were removed. E.g., change to "removal of either LB1, or both LA and LC". Ambiguous terms like "lamin A/C" can be misinterpreted as meaning that A=C.

Lines 188-191: "The results showed.." -- Which results? Revise this entire section for clarity.

Lines 192-200: Revise for clarity; lacks context and needs 'plain English' conclusions.

Lines 204 -201: Immunogold labeling-did the antibody recognize both LA+LC (lines 204, 209 and 210: "LA/C") or was it specific for LA (line 207, 208, 201)?

Line 210 and Figure 4 results: Missing caveat when counting LA/LC versus LB1 filaments- was this potentially influenced by the different affinities of the two antibodies used? Or were the individual filaments directly recognizable?

Line 220: Typo "tht"

Line 244-245: This sentence is uninterpretable. Change to plain English, and consider suggesting plausible molecular roles for Tpr? E.g., might TPR contribute to 'struts' or other structures that separate NPCs from LA fibers?

Line 207: Awkward phrasing: "hypothesized that this may shed additional insights on'

Lines 3657-363: Kudos on the excellent discussion of conflicting results from Xie et al (2016).

Line 374: Typo "assembly"

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

This manuscript by Kittisopikul et al. describes the application of 3D-SIM to comparatively study the distribution of NPCs and the lamina in MEFs. This work is complementary to, although not entirely consistent with, prior findings and does explore novel questions about the impact of specific basket nups in establishing spatial relationships of NPCs and the lamina. There is also evidence provided that the number of NPCs is regulated by specific lamin isoforms. Overall, this work does advance our understanding of NPC-lamina relationships and provides some new insights into how some NPC basket proteins help regulate NPC localization. There are some additional experiments, data analysis and/or discussion that would enhance the impact of these studies.

Specific comments:

Technically, are there limitations of 3D-SIM compared to other approaches resulting from the use of full-sized complexed antibodies for labeling? If so these should be discussed.

It might be worth mentioning the fusion of mEOS and not just exogenous re-expression could explain different outcomes of prior studies compared to the 3D-SIM results presented here.

The siRNA treatments appear to take place over 4 days between initial application of siRNA oligos and fixation/lysis. Presumably there is considerable cell division during this time so the observed relationships between NPCs and lamins would reflect envelope reassembly as well as interphase dynamics. Similarly, the lamin-deficient MEFs have obviously been lacking in the lamin proteins for even longer. Thus, what is being observed is a considerable downstream consequence of these proteins being depleted over many cell divisions and envelope reformations. That doesn't take away from the significance of the findings but it probably does bear further discussion as to the implications to the findings and their interpretation.

Given their differential mechanisms for incorporation into the NE, is there any evidence or reason to believe that post-mitotic and interphase assembled NPCs would be differentially localized in relationship to the lamins?

Does the variable decrease in pore number with lamin deficient cells reflect an inability for interphase assembly, or perhaps post-mitotic assembly? Perhaps a comparative early G1 versus a G2 count of NPC numbers help answer this question.

Some graphs in fig 2 lack the measurement scale (nm)

For the cryo-ET /immunogold experiments: Could the authors show and/or quantify the immunogold labeling of each antibody in non-NPC containing areas. If the Lamin-B1 antibody is simply less effective compared to the lamin-A/C antibody in labeling the NE by this method then the results could be unrelated to NPC proximity. Could a ratio of NPC-proximate vs NPC-distal be done to show preferential NPC association?

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

Kittisopikul, Shimi, et al. investigate the structural relationships between NPCs and each lamin isoform fiber meshwork through sub-pixel computational analysis of 3D-SIM images. Their analysis is based on imaging of immunostained MEFs, both wild-type and various lamin knockouts that have the advantage that the remaining lamin meshworks are less densely packed. They provide convincing evidence that NPCs tend to reside a defined distance from lamin A and lamin B1 fibers, suggesting they are structurally linked. A defined distance relationship is less evident between NPCs-lamin C and NPCs-lamin B2. The LA-NPC association does not significantly depend on LB1, while LA knockout did affect LB1-NPC distances. Overall NPC number was reduced in both LA and LB1 knockouts. Cryo-ET and immunogold labeling confirmed that the nucleoplasmic ring of NPCs associates with LA and LB1 filaments. ELYS knockdown led to NPC clustering, increased distance between NPCs and LA/LC/LB2 fibers, expansion of LA/LC/LB1 meshwork sizes with LB1 fibers protruding into NPC clusters, and decreased LB2 meshwork size. TPR knockdown increased the distance between NPCs and LA/LC fibers while leading to compaction of all lamin isoform meshworks. NUP153 knockdown also compacted all lamin isoform meshworks. These data indicate that nucleoplasmic Nups can affect NPC association with LA/LC fibers and lamin meshwork sizes.

Overall, the authors present a very complete dataset describing interactions between NPCs and different lamin isoforms. The analysis and documentation of their results are quite thorough. I suspect this manuscript will be of interest to many cell biologists. The specific data presented here will be of interest to investigators studying all aspects of nuclear structure and function, while the imaging and analysis approaches will be of more broad interest. A cohesive model is not presented

so it is somewhat difficult to understand how all of the results relate to each other. A variety of questions are raised: how do NPC-lamin interactions affect lamina meshwork sizes, NPC distributions/spacings/clustering, and NPC numbers?; why do different nucleoplasmic Nups seem to play different roles?; what are the cell cycle dependencies of these interactions? These NPC-lamin interactions are clearly complex and further studies will be necessary to work out all the details. That being said, I support publication of this manuscript in JCB with the following relatively minor comments.

1. I was initially confused by the discrepancy between the Lmnb1-/- LA data presented in Fig. 2C and Fig. 3B. I believe the authors' explanation is that Fig. 2 shows the analysis for a single nucleus, whereas Fig. 3 compiles data from multiple nuclei. For clarity, I wonder if showing only the data from multiple nuclei would be better, since these data presumably include the data from the single nucleus shown in Fig. 2. Also, it would be helpful to include the number of nuclei analyzed in each figure legend. Cell numbers are mentioned in the text and Methods but it is not always clear to which figures these numbers refer.

2. The legend to Fig. 3 indicates that NPCs are offset from the center of LA and LB1 fibers by 20-30 nm. It is not clear how they derived this 20-30 nm distance. It also doesn't seem to agree with the single nucleus analysis where the section heading reads: "Image analysis reveals enrichment of NPCs within 30 to 100 nm of LA fibers." Again, presenting only the data from multiple nuclei may be more straightforward.

3. I wonder if the authors can comment on whether the NPCs they detect upon Nup knockdown are fully formed, especially in the case of ELYS knockdown where NPCs cluster.

4. The observation that NPC numbers are reduced in LA and LB1 knockout cells is interesting. Understanding the mechanistic basis for this effect will be an interesting area for future investigation. For completeness, the authors should also test how lamin B2 knockdown affects NPC numbers. A related question, though perhaps not essential to this study, is if lamin overexpression affects NPC numbers.

5. There are several sentences in the Discussion that do not seem to accurately reflect the presented data and should be appropriately edited:

Lines 381-383: "In our experiments, we also observed a small, but statistically significant increase in NPC numbers after TPR knockdown in WT cells. When we depleted TPR in Lmna-/- and Lmnb1-/- cells, a similar small increase in NPCs was observed . . ." These statements are not consistent with the data presented in Fig. S10D.

Line 393-394: "Based on these results, it is tempting to speculate that the number of NPCs helps to determine lamin meshwork structure." This doesn't really make sense since Fig. S10D shows that TPR and NUP153 knockdowns did not affect NPC numbers.

6. I also feel that the Discussion could be streamlined a bit. I realize that the authors are not able to present a cohesive model at this time to account for all of their data, but a more concise Discussion with some kind of take-home message, however simple, would make the work more accessible to the general reader.

7. While likely beyond the scope of the current manuscript, it would be interesting to know if some of the key measurements reported here in MEFs are similar or different in other cell types.

8. Out of curiosity, how did the authors raise an antibody specific for lamin C for their immunostaining, one that does not also recognize lamin A? The reference to the antibody does not mention a lamin C antibody, at least that I could find.

Reviewer #1 Comments :

This manuscript reports an important computational advance to decipher and understand the ultrastructure of the nuclear envelope, a longstanding challenge in cell biology. The authors convincingly quantified large-scale spatial relationships between nuclear pore complexes (NPCs) and filament networks formed by lamin A (LA), lamin C (LC), lamin B1 (LB1) and lamin B2 (LB2), to understand their potential inter-dependence in wildtype cells and in cells genetically-null for either LB1, LB2 or LA+LC. Significantly enhancing the impact of this manuscript, the authors also used siRNA-mediated downregulation to reduce protein levels of three nucleoporins known or positioned to bind lamins: ELYS (essential for NPC assembly after mitosis), Nup153 (essential for NPC assembly during interphase) or TPR (NPC basket; chromatin-free zones). The manuscript is overall very well-written.

(1) To this 'non-computational' cell biologist reviewer, nearly all of the Results are intelligible and logical, sometimes even understated (compared to the beautiful images), despite the unrelenting numerical density of the Results text. A lighter touch here, especially when differences are not significant, would improve the manuscript. Results text would also be significantly improved by adding 'plain English' conclusions (e.g., 'NPCs are offset from the center of LA fibers' = NPCs remain close to LA fibers).

Thank you for these comments. By providing both detailed images and numerical measurements we hope that our paper would be appealing to a wide audience. We are glad that the results are intelligible to a cell biologist reviewer.

In this revision we have included some plainer language conclusions although we still require some precision of language to accurately explain the observations. For example, saying that the "NPCs remain close to the LA fibers" does not describe the reduced frequency of NPCs directly colocalized with lamin fibers.

Examples:
Line 204: To summarize, the measured distances suggest the NPCs are positioned next to LB1 fibers and not within the fibers in both WT and Lmna-/- MEFs.
Line 261-263: These data suggested that the NPCs were less closely associated with LA fibers following TPR knockdown and tended to be in the middle of small faces of the LA meshwork. This suggests a structural role for TPR where it may contribute to a defined spacing between LA fibers and NPCs

(2) This manuscript would be significantly enhanced by a schematic summary of the main findings, and a model depicting potential roles played by ELYS, Nup153 and TPR. The data are all about 'distances', but this belies its impact on cell biology, which relates to potential molecular associations and/or connections between NPCs and each type of filament.

We have included a schematic (see Figure 9) detailing the significant findings with regard to lamin A , lamin B1 and the clustering phenotype induced by ELYS.

(3) Potentially flawed logic and conclusion? (lines 201-203 and elsewhere): The average numbers of NPCs per nucleus were calculated from the single focal plane closest to the coverslip, in Lmna-/- (loss of both LA and LC) or Lmnb1-/- cells. The 'suggestion' that LA [why not LC?] and LB1 are both involved in regulating NPC number fails to account for potential changes in nuclear surface area (hence NPC density) caused by loss of LA+LC, or loss of LB1.

We have now included NPC density for WT, Lmna-/-, Lmnb1-/-, and Lmnb2-/- MEFs in Figure S4. We have altered the text on lines 206-211to read as follows:

"The average number of NPCs per nucleus in a single focal plane closest to the coverslip was reduced to 1000 NPCs in Lmna-/- MEFs compared to 1200 in Lmnb1 -/- MEFs and 1500 in WT MEFs (Table 1, Figure S4A), suggesting that Lmna and Lmnb1 or their protein products (LA, LC, and LB1) are involved in regulating NPC number. Knocking out Lmna had a less pronounced effect on NPC density suggesting that change in NPC number may be related to changes in the size of the nucleus or cell cycle effects (Figure S4B). Lmnb2 -/- MEFs had comparable NPC numbers and density to WT MEFs implying that Lmnb2 has a minimal effect on NPC number and density"

(4) Graph and labelling- Where possible, switch to blue/yellow coloring (instead of red/green), or dark-vs-light gray (in all violin-graphs), to be interpretable when printed in black-and-white or when viewed by red/green colorblind people.

We are unable to identify the use of red/green in any of our figures (some of the authors are colorblind). We have stuck to a magenta (or purple) / green scheme in our fluorescent images and in our violin plots. We have adjusted the luminosity of some colors to make them more interpretable when printed in grayscale.

(5) Line 221 (first mention of siRNA downregulation)- Indicate how long the cells were downregulated, and estimate the fraction of cells that may have gone through mitosis immediately before analysis. Did the analysis (Figure 5, Figure S5, Table 2) account for potentially different outcomes in postmitotic cells, versus cells that stayed in interphase? These interesting possibilities are also brought up in the Discussion (lines 337-338).

This is a very interesting question that ultimately addresses the mechanisms of lamin interactions with NPCs. Because we are examining a population of cells with varying degrees of knockdown, it would be challenging to know the history of individual cells as the targeted protein decreases and the cells divide. Therefore, we do not explicitly categorize cells as postmitotic or interphase cells. Rather our analysis chose cells randomly and compared the observed distribution of NPCs to a simulated uniform distribution of NPCs in nuclei of the same size and shape.

(6) Lines 298-311 (NPC clustering phenotypes in Lmna-/- or Lmnb1-/- MEFs): This entire section related to Figure S10 needs revision for clarity and flow, and to account for potential

differences based on the fraction of post-mitotic versus interphase cells. Or, consider moving results for each Nup to a relevant earlier section?

In response to this comment, we rewrote the section. Please see the main text.

Minor corrections:
Line 17: Simplify or clarify (seems redundant; distinction is unclear): "the lamin filaments composing the fibers"

In this work, we observe lamins at two distinct resolution regimes with cryo-ET and structured illumination light microscopy. The individual molecular structures identified by cryo-ET are termed filaments. Individual filaments form bundles called fibers, which is the resolvable unit observed by light microscopy. We have explicitly clarified this point on Line 216-218: We use the term lamin "filaments" to describe the molecular structures of lamins observed using cryo-ET. In contrast, lamin "fibers" refers to a grouping or bundling of these filaments that are resolvable using light microscopy."

Line 31: Delete "highly"

Highly has been removed from line 31

Lines 85-86: Rephrase (add: ", respectively"?) to avoid suggesting that you visualized LA fibers in Lmna-/- MEF nuclei, and LB1 fibers in Lmnb1-/- nuclei.

Added "respectively"

Figure 2B,C,DE- The X-axis and Y-axis labels should be made larger.

The axis labels have been made larger.

Line 170 ("removal of either LA/C or LB1..")- Must state precisely which A-type lamin(s) were removed. E.g., change to "removal of either LB1, or both LA and LC". Ambiguous terms like "lamin A/C" can be misinterpreted as meaning that A=C.

Since we are using gene knockout and Lmna encodes both Lamin A and C through alternative splicing, knocking out Lmna results in removal of both LA and LC. We have added this explanation to the introduction and also expanded the text around Line 170.

Lines 188-191: "The results showed.." -- Which results? Revise this entire section for clarity.

We revised the section for clarity.

Lines 192-200: Revise for clarity; lacks context and needs 'plain English' conclusions.

We modified lines 194-197 as highlighted.

We added a sentence on Line 204: “To summarize, the measured distances suggest the NPCs are positioned next to LB1 fibers and not within the fibers in both WT and Lmna-/- MEFs.”

Lines 204 -201: Immunogold labeling-did the antibody recognize both LA+LC (lines 204, 209 and 210: "LA/C") or was it specific for LA (line 207, 208, 201)?
The antibody used recognizes both lamin A and lamin C. We have clarified this in the figure legend and text.

Line 210 and Figure 4 results: Missing caveat when counting LA/LC versus LB1 filaments- was this potentially influenced by the different affinities of the two antibodies used? Or were the individual filaments directly recognizable?

We clarified this in the text in the section on Cryo-electron tomography (Lines 214-226)

Line 220: Typo "tht"
Replaced with that

Line 244-245: This sentence is uninterpretable. Change to plain English, and consider suggesting plausible molecular roles for Tpr? E.g., might TPR contribute to 'struts' or other structures that separate NPCs from LA fibers?

Added plainer language (circa Line 261)

Line 207: Awkward phrasing: "hypothesized that this may shed additional insights on'

Rewrote this: “The higher resolution of cryo-ET over light microscopy allows us to assess the relative abundance of LA/C and LB1 filaments contacting the NPC at the molecular level.”

Lines 3657-363: Kudos on the excellent discussion of conflicting results from Xie et al (2016).

Thank you

Line 374: Typo "assembly"
Fixed

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

This manuscript by Kittisopikul et al. describes the application of 3D-SIM to comparatively study the distribution of NPCs and the lamina in MEFs. This work is complementary to, although not entirely consistent with, prior findings and does explore novel questions about the impact of specific basket nups in establishing spatial relationships of NPCs and the lamina. There is also evidence provided that the number of NPCs is regulated by specific lamin isoforms. Overall, this work does advance our understanding of NPC-lamina relationships and provides

some new insights into how some NPC basket proteins help regulate NPC localization. There are some additional experiments, data analysis and/or discussion that would enhance the impact of these studies.

Specific comments:

Technically, are there limitations of 3D-SIM compared to other approaches resulting from the use of full-sized complexed antibodies for labeling? If so these should be discussed.

We have addressed this on Lines 344-347: Our study uses indirect immunofluorescence to robustly label structures in situ with fluorescent labels and provides sufficient labeling accuracy for the resolution of 3D-SIM to localize the structures at the scale of interest. While smaller labeling complexes do exist (Carrington et al., 2019), these do not provide significant advantages for the resolution limits of 3D-SIM.

It might be worth mentioning the fusion of mEOS and not just exogenous re-expression could explain different outcomes of prior studies compared to the 3D-SIM results presented here.

We added a note in the introduction (circa line 56): “Super-resolution microscopy analysis of lamins and NPCs in Lmna-/- fibroblasts also found NPCs closely associated with exogenously expressed LA and LC in Xie et al. (2016), where an mEOS fluorescent protein tag was used in some experiments”

The siRNA treatments appear to take place over 4 days between initial application of siRNA oligos and fixation/lysis. Presumably there is considerable cell division during this time so the observed relationships between NPCs and lamins would reflect envelope reassembly as well as interphase dynamics. Similarly, the lamin-deficient MEFs have obviously been lacking in the lamin proteins for even longer. Thus, what is being observed is a considerable downstream consequence of these proteins being depleted over many cell divisions and envelope reformations. That doesn't take away from the significance of the findings but it probably does bear further discussion as to the implications to the findings and their interpretation.

Discussed circa line 395:
The knockdown occurred over the course of 96 hours, and thus a limitation of our study is that our observations are of cells that may have adapted to extended depletion of these nucleoporins.

Given their differential mechanisms for incorporation into the NE, is there any evidence or reason to believe that post-mitotic and interphase assembled NPCs would be differentially localized in relationship to the lamins?

Discussed circa line 362 and 432
362: NPCs assemble on chromatin during NE reassembly post-mitosis and new NPCs continue to be integrated into the NE throughout interphase
432: The lamins are also closely associated with chromatin at the nuclear periphery and it is likely that peripheral chromatin is also playing a role in mediating the association of lamins and

NPCs and their distribution in the NE, in particular, during post-mitotic NE assembly.

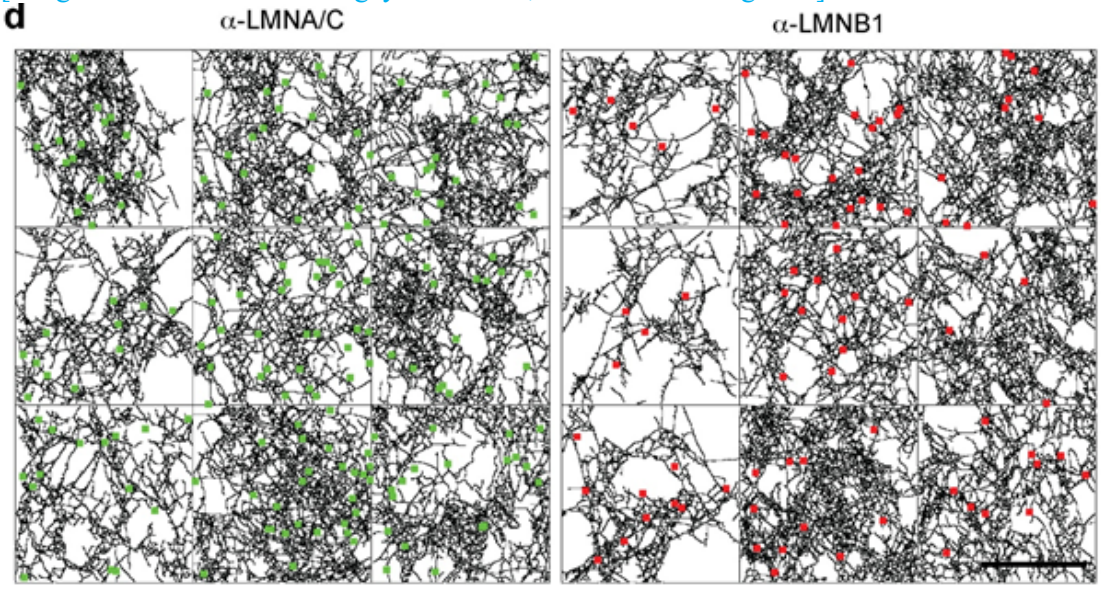
Does the variable decrease in pore number with lamin deficient cells reflect an inability for interphase assembly, or perhaps post-mitotic assembly? Perhaps a comparative early G1 versus a G2 count of NPC numbers help answer this question.
We have partially addressed this by using NPC density in S4 and discussed it on lines 208 -211.

Some graphs in fig 2 lack the measurement scale (nm)
We have added nm to the labels in Figure 2.

For the cryo-ET /immunogold experiments: Could the authors show and/or quantify the immunogold labeling of each antibody in non-NPC containing areas. If the Lamin-B1 antibody is simply less effective compared to the lamin-A/C antibody in labeling the NE by this method then the results could be unrelated to NPC proximity. Could a ratio of NPC-proximate vs NPC-distal be done to show preferential NPC association?

We have added this circa line 220:
In the 24 volumes around NPCs we observed 188 LA/C labels and only 28 LB1 labels. This results in a ratio of 6.7 LA/C labels for every LB1 label in volumes near NPCs. For comparison, in 9 randomly selected volumes with immunogold labeling without NPCs (Turgay et al., 2017), we observed 140 LA/C labels and 83 LB1 labels. This results in a ratio of 1.69 LA/C labels for every LB1 label in volumes not near NPCs. This suggests an enrichment of approximately four fold in the ratio of LA/C to LB1 filaments near NPCs versus volumes lacking NPCs. This enrichment suggests a bonafide preference for LA/C fibers over LB1 fibers contacting NPCs rather than one merely due to differences in the affinities of the antibodies used.

[Image below and is from Turgay et al. 2017,Extended Data Figure 3]



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

Kittisopikul, Shimi, et al. investigate the structural relationships between NPCs and each lamin isoform fiber meshwork through sub-pixel computational analysis of 3D-SIM images. Their analysis is based on imaging of immunostained MEFs, both wild-type and various lamin knockouts that have the advantage that the remaining lamin meshworks are less densely packed. They provide convincing evidence that NPCs tend to reside a defined distance from lamin A and lamin B1 fibers, suggesting they are structurally linked. A defined distance relationship is less evident between NPCs-lamin C and NPCs-lamin B2. The LA-NPC association does not significantly depend on LB1, while LA knockout did affect LB1-NPC distances. Overall NPC number was reduced in both LA and LB1 knockouts. Cryo-ET and immunogold labeling confirmed that the nucleoplasmic ring of NPCs associates with LA and LB1 filaments. ELYS knockdown led to NPC clustering, increased distance between NPCs and LA/LC/LB2 fibers, expansion of LA/LC/LB1 meshwork sizes with LB1 fibers protruding into NPC clusters, and decreased LB2 meshwork size. TPR knockdown increased the distance between NPCs and LA/LC fibers while leading to compaction of all lamin isoform meshworks. NUP153 knockdown also compacted all lamin isoform meshworks. These data indicate that nucleoplasmic Nups can affect NPC association with LA/LC fibers and lamin meshwork sizes.

Overall, the authors present a very complete dataset describing interactions between NPCs and different lamin isoforms. The analysis and documentation of their results are quite thorough. I suspect this manuscript will be of interest to many cell biologists. The specific data presented here will be of interest to investigators studying all aspects of nuclear structure and function, while the imaging and analysis approaches will be of more broad interest. A cohesive model is not presented so it is somewhat difficult to understand how all of the results relate to each other. A variety of questions are raised: how do NPC-lamin interactions affect lamina meshwork sizes, NPC distributions/spacings/clustering, and NPC numbers?; why do different nucleoplasmic Nups seem to play different roles?; what are the cell cycle dependencies of these interactions? These NPC-lamin interactions are clearly complex and further studies will be necessary to work out all the details. That being said, I support publication of this manuscript in JCB with the following relatively minor comments.

Thank you for these comments. We agree that there are a number of important questions in the field regarding NPCs and lamins, and we hope that our study begins to answer some of these questions and inspires further studies.

1. I was initially confused by the discrepancy between the Lmnb1-/- LA data presented in Fig. 2C and Fig. 3B. I believe the authors' explanation is that Fig. 2 shows the analysis for a single nucleus, whereas Fig. 3 compiles data from multiple nuclei. For clarity, I wonder if showing only the data from multiple nuclei would be better, since these data presumably include the data from the single nucleus shown in Fig. 2. Also, it would be helpful to include the number of nuclei analyzed in each figure legend. Cell numbers are mentioned in the text and Methods but it is not always clear to which figures these numbers refer.

Thank you for pointing this out. We used the individual nuclei in Figure 2 to help illustrate the technique. We have added a note to the figure legend to clarify the purpose of Figure 2. We have

also added cell numbers to the figure legends.

2. The legend to Fig. 3 indicates that NPCs are offset from the center of LA and LB1 fibers by 20-30 nm. It is not clear how they derived this 20-30 nm distance. It also doesn't seem to agree with the single nucleus analysis where the section heading reads: "Image analysis reveals enrichment of NPCs within 30 to 100 nm of LA fibers." Again, presenting only the data from multiple nuclei may be more straightforward.

For distances up to 30 nm from the center of the fibers we generally observe fewer NPCs than we would expect from a uniform distribution of NPCs. This is indicated by the green “valleys” in Figure 2 Panel C and Figure 3 Panel B. Note that the extent of the x-axis extends to 600 nm in Figure 2C and to 150 nm in Figure 3B. Our interpretation of this is that NPCs tend not to be directly colocalized and are not embedded in LA or LB1 fibers.

For distances greater than 30 nm and less than 100 nm we observe _more_ NPCs than we would expect from a uniform distribution of NPCs in Figure 2C and Figure 3B as shown by the purple “hills” in the violin plot.

The distance where our observed frequency switches from being less than the expected frequency to more than the expected frequency is ~30 nm.

3. I wonder if the authors can comment on whether the NPCs they detect upon Nup knockdown are fully formed, especially in the case of ELYS knockdown where NPCs cluster.

We commented on NPC assembly in the discussion circa line 405: “The clustering of NPCs after ELYS knockdown is likely due to the failure of NPCs to correctly assemble on chromatin following mitosis suggesting that, at least for NPCs formed at NE reformation, their association with lamins occurs at that time.” We cannot resolve nucleoporins via light microscopy where we observed this phenomena. This is a topic for further investigation with higher resolution methods.

4. The observation that NPC numbers are reduced in LA and LB1 knockout cells is interesting. Understanding the mechanistic basis for this effect will be an interesting area for future investigation. For completeness, the authors should also test how lamin B2 knockdown affects NPC numbers. A related question, though perhaps not essential to this study, is if lamin overexpression affects NPC numbers.

In response ,we have now included the effect of knocking out Lamin B2 on NPC numbers and density in Figure S4.

5. There are several sentences in the Discussion that do not seem to accurately reflect the presented data and should be appropriately edited:

Lines 381-383: "In our experiments, we also observed a small, but statistically significant increase in NPC numbers after TPR knockdown in WT cells. When we depleted TPR in Lmna-/-

and Lmnb1-/- cells, a similar small increase in NPCs was observed . . ." These statements are not consistent with the data presented in Fig. S10D.

We have corrected the statement to state that both observed increases are statistically insignificant.

Line 393-394: "Based on these results, it is tempting to speculate that the number of NPCs helps to determine lamin meshwork structure." This doesn't really make sense since Fig. S10D shows that TPR and NUP153 knockdowns did not affect NPC numbers.

We changed this to state “number of and structural composition of NPCs” on lines 426 and 427.

6. I also feel that the Discussion could be streamlined a bit. I realize that the authors are not able to present a cohesive model at this time to account for all of their data, but a more concise Discussion with some kind of take-home message, however simple, would make the work more accessible to the general reader.

We added a take-home message and a cartoon (S9) illustrating it circa Line 441: “Specifically, among other findings, NPCs are positioned next to LA and LB1 fibers in an ELYS dependent manner and removing any of these components in whole or in part changes how the remaining components are distributed”

7. While likely beyond the scope of the current manuscript, it would be interesting to know if some of the key measurements reported here in MEFs are similar or different in other cell types.

Unfortunately, this is beyond the scope of the current manuscript. Our study also suggests that some of these findings may be contingent on the relative abundance of the lamin isoforms in distinct cell types. This is supported by previous studies involving mouse Embryonic Stem Cells (ESCs) and ESC-derived fibroblast-like cells (EDFCs) (see Guo Y, Kim Y, Shimi T, Goldman RD, Zheng Y. Concentration-dependent lamin assembly and its roles in the localization of other nuclear proteins. Molecular Biology of the Cell. 2014 Apr; 25(8):1287–1297. https://doi.org/10.1091/mbc.e13-11-0644, doi: 10.1091/mbc.e13-11-0644; and Guo Y, Zheng Y. Lamins position the nuclear pores and centrosomes by modulating dynein. Molecular Biology of the Cell. 2015 oct; 26(19):3379–3389. https://doi.org/10.1091%2Fmbc.e15-07-0482, doi: 10.1091/mbc.e15-07-0482. References are included).

8. Out of curiosity, how did the authors raise an antibody specific for lamin C for their immunostaining, one that does not also recognize lamin A? The reference to the antibody does not mention a lamin C antibody, at least that I could find.

This has been added to the Materials and Methods: “The lamin C antibody (321) was made in rabbits with a synthetic peptide (CHHVSGSRR) conjugated to Keyhole Limpet hemocyanin.”

November 20, 2020

RE: JCB Manuscript #202007082R

Dr. Mark Kittisopikul
Northwestern University
Department of Cell and Developmental Biology
303 E Chicago Ave
Ward 11-145
Chicago, IL 60611-4296

Dear Dr. Kittisopikul,

Thank you for submitting your revised manuscript entitled "Computational analysis of lamin isoform interactions with nuclear pore complexes." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). Thank you also for providing the additional antibody validation figure.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

1) Text limits: Character count for Articles < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The summary should be no longer than 160 words and should communicate

the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

While your current title will be appreciated by the specialists, we do not feel that it will be accessible to a broader cell biology audience and the usage of the word 'isoform' may be confusing. Therefore we suggest the following title: "Computational analyses of spatial relationships between nuclear pore complexes and lamin family proteins."

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

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

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

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures.
Please also note that tables, like figures, should be provided as individual, editable files. A summary description of all supplemental material should appear at the end of the Materials and methods section. As discussed previously we will be able to give you the extra space for your additional supplemental figures. If at all possible please try to add the new antibody validation figure to an existing supplemental figure.

11) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

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are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

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Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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

Sincerely,

Michael Rout, Ph.D.

Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

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

The authors have fully addressed my previous concerns. The revised manuscript is rigorous, data-rich and comprehensive, and the new conceptual schematic is perfect.

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

This revised manuscript has address all of my prior concerns and I have not identified any new ones. This should prove a valuable addition to our understanding of the relationships between the nuclear lamina and nuclear pore complexes.

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

In their revised manuscript, Kittisopikul, Shimi, et al. have satisfactorily addressed my minor comments. I support publication of this interesting manuscript in JCB.