EMBO reports

Super-resolution microscopy reveals focal organization of ER-associated Y-complexes in mitosis

Rojapriyadharshini Gandhimathi, Dorothea Pinotsi, Mario Köhler, Jörg Mansfeld, Caroline Ashiono, Tatjana Kleele, Sumit Pawar, and Ulrike Kutay
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Dear Ulrike,

Thank you for submitting your research manuscript for consideration by EMBO reports. It has now been seen by three experts in the field, and I have already sent you a copy of their reports (included again below). I would also like to thank you for your point-by-point response to their comments and for your revision plan.

The referees are largely positive and acknowledge the novelty, significance, and high technical quality of the study, as well as the careful preparation of the manuscript. They also have a few suggestions for further improvement of the study and the manuscript. In particular, referee #2 points out that some insight into the functional significance of Y-complex association with the mitotic ER in NPC assembly would significantly enhance the advance of the study and suggests that this could be addressed by analyzing mutants of Nup160 and Nup133 lacking the ALPS motif. Although we agree that this would be an interesting and important goal, it will not be required for further consideration of your manuscript given the related technical limitations and the considerable amount of experimental work that this investigation would involve. Similarly, we understand the difficulties associated with disentangling the functional role of POM121 in NPC assembly from its potential function as binding partner of Y-complexes in mitosis (relevant to the other major point of referee #2), and we would therefore not require such experiments. However, we would encourage you to attempt the study of potential co-localization of mitotic Y-complexes with POM121 and Rtn4, which could provide some useful evidence in the direction of the suggestions of referees #2 and #3, respectively. Finally, all referees provide a number of additional suggestions for textual corrections or improvement of the manuscript, which should be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript along the lines you suggested in your revision plan, with the understanding that the referee concerns (as detailed above) must be taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we usually recommend a revision within 3 months (May 23rd). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

Please note that you can publish your study either as a Report or as an Article. For Reports, the manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For an Article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file. Please note that you could promote one or more of your current Expanded View figures to the main Figure status if you wish. More information on Expanded View figures follows below.

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See also <https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data availability statement is restricted to new primary data that are part of this study.

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter plots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
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- Please also include scale bars in all microscopy images.

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Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

NPCs mediate nucleocytoplasmic exchange. The Y-complex is the major scaffold component of the so-called outer rings of the NPC. This study by Gandhimathi et al investigates the fate of nuclear pore complexes during open mitosis, specifically why and in which form a pool of Y-complexes is retained at the ER subsequent to nuclear envelope breakdown.

Using a previously established in vitro assay, the authors first show that release of Y-complex Nups is slower as compared to central channel Nups and remains incomplete. Gradient fractionation and confocal microscopy underline mitotic ER membrane association. SRM did not identify any ring shaped structures that would have been expected if Y-complex oligomerization were entirely retained. Quantification suggested a reduced copy number of Y-complexes in the identified clusters.

This study extends our knowledge of the mitotic behavior of Y-complexes. It challenges the idea that the ring-shaped scaffolds are retained as seeds for re-assembly. It also provides very interesting quantitative SRM data about Y-complexes at kinetochores and ER.

I most enthusiastically recommend publication at EMBOR.

Minor comments:
- Although elsewhere in the paper the rationale for the experiments are very well explained, on first attempt this reviewer got a bit lost in figure 3. I assume you are removing the soluble pool of the GFP fusion proteins to assess if there is a membrane bound fraction? The fact that you also remove other cytosolic factors (which was important in Figure 1) is not important for this

experiment, correct? This could be explained better.
- On page seven you write 'post-mitotic disassembly'. This must be 'assembly'
- Can the authors speculate what 'a significant fraction of Y-complexes' means? This is an interesting question in order to hypothesize about their potential function in the future. You could provide an educated guess in the discussion: number of clusters * copy number of Y complexes, number of kinetochores * copy number of Y complexes, number of NPCs in u2os cells * 32. Would this be consistent with the gradient fractionation? Even if you just say it's a minor fraction, this is still interesting.

Referee #2:

Gandhimathi and colleagues present a concise manuscript detailing the accumulation of Y shaped Nup160 Nup107 complexes at the ER during mitosis. This study builds upon work recently published by the Kirchhausen group (Chou et al. (2021) PMID: 34129835), adding a layer of biochemical validation, and establishing the stoichiometry and assembly state of ER associated Y-complexes. Overall, the manuscript is well constructed and easy to follow. However, prior to publication in EMBO reports additional experiments are necessary to provide a functional link to NPC assembly, which will elevate the beautiful data presented beyond an incremental advance.

Major comments:

(1) Figures 2 and 3. While the data presented are undoubtably gorgeous, these experiments warrant expansion to provide a mechanistic link between ER association and NPC assembly. In both figures the ALPS motifs present in Nup160 and Nup133 should be probed for their role in ER association via mutant variants lacking the ALPS motif. This could for example be achieved through the generation of Nup160 and Nup133 auxin-inducible degron cell lines and subsequent expression of Nup160 and Nup133 variants lacking the ALPS motif.

(2) Figure 3. POM121 has an established role during postmitotic NPC assembly: Antonin et al. (2005) PMID 15629719; Stavru et al. (2006) PMID 16702234; and Funakoshi et al. (2011) PMID 21289085. Thus, it is unexpected that POM121 localization during nuclear envelope breakdown is not examined, too. Mechanistically it would be intriguing to know whether ER localization is driven by local POM121 association with the Y-complex, or alternatively by direct association of the Y-complex with membranes via their ALPS motifs, or a combination of both.

Minor comments:

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(3) Figure 1. How were relative nuclear dextran-155 levels quantified, this information is missing from the methods. Additionally, an expanded materials figure that details representative examples of positive and negative dextran 155 transport would be welcomed.

(5) Discussion of ALPS motifs during Y-complex association with the ER. It would be appropriate to cite the following literature: Drin et al. (2007) PMID 17220896, which first proposed the ALPS motif; Berke et al. (2004) PMID:15557116; and Seo et al. (2009) PMID 19706512, who structurally resolved the ALPS domains within the N-terminal beta-propellers of Nup133 and Nup120, respectively.

(6) Discussion page 7. The functional commentary of AAA-ATPases upon nuclear envelope and NPC biogenesis would benefit from including the following citations, recent work from the Schlieker group; Laudermilch et al. (2016) PMID: 27798237; Rampello et al. (2020) PMID: 32342107; and Prophet et al. (2022) PMID: 36302970, along with work published within the Kutay group such as Luithle et al. (2020) PMID: 33320087, would be useful references.

Referee #3:

Summary

1. Does this manuscript report a single key finding? YES
The authors find that NPC outer rings show distinct disassembly kinetics, remain partially membrane-associated, form oligomeric assemblies but are importantly not present in an octameric ring-like structure during mitosis, which had been proposed by a previous report (Chou et al., 2021).

2. Is the reported work of significance? YES
In addition to the new findings, the work is highly significant and timely because it revises a model recently put forward by Chou et al.

3. Is it of general interest to the molecular biology community? YES
NPC structure and its mitotic dynamics are topics of active research that are connected to many fields such as cell cycle control, membrane-protein interactions and serve as paradigm for function and assembly/disassembly of macromolecular complexes.

4. Is the single major finding robustly documented using independent lines of experimental evidence? YES

Referee report
With their work, Gandhimathi et al., continue the Kutay lab's pioneering work on the fate of NPCs during mitosis. Using a range of methods, the authors show that at the onset of mitosis, the NPCs' cytoplasmic and nuclear rings are released with unique kinetics and that oligomeric assemblies of Y-complexes localize to the mitotic ER. Importantly, these oligomers are not present as octameric ring-like structures. Such octameric rings of Y-complexes have recently been suggested to persist in mitosis and to template NPC reassembly in late mitosis (Chou et al., 2021). However, the model of Chou et al. was based on rather indirect data. Gandhimathi et al. directly observed Y-complex assemblies during mitosis and are thus able to correct the previous model. Gandhimathi et al. find that mitotic Y-complex assemblies associate with the ER and thereby confirm one of the findings of Chou et al. However, Gandhimathi et al. provide more direct evidence also in this case with their biochemical fractionation experiment. These findings are critical for the NPC community to understand the dynamic architecture of NPC subcomplexes and also for researchers interested in mitotic cell organization and for investigators using super-resolution microscopy approaches.
The data are of high quality and concisely, clearly and appealingly presented. The conclusions are supported by the data and the authors are careful not to draw conclusions or to put forward a model not fully supported by the data. Previous findings are largely appropriately discussed. The possible reasons for the discrepancies to the findings of Chou et al. could be explained more so the reader can appreciate how the different conclusions arose.

Minor questions/suggestions:

1. In addition to the ER-associated and kinetochore-associated Y-complex assemblies, are there also soluble cytosolic Y-complex assemblies present in mitosis?

2. In order to characterize the location of Y-complex assemblies on mitotic ER, the authors could try STED-based co-localization with RTN4, which is found on the edges of fenestrations in ER sheets (Schroeder ... Bahmanyar, JCB 2019).

We are grateful for the positive evaluation of our work and wish to thank the reviewers for their constructive suggestions to our manuscript.
Please find below our point-by-point responses to the reviewers' comments:

Referee #1

NPCs mediate nucleocytoplasmic exchange. The Y-complex is the major scaffold component of the so-called outer rings of the NPC. This study by Gandhimathi et al investigates the fate of nuclear pore complexes during open mitosis, specifically why and in which form a pool of Y-complexes is retained at the ER subsequent to nuclear envelope breakdown.

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I most enthusiastically recommend publication at EMBOR.

Minor comments:
- Although elsewhere in the paper the rationale for the experiments are very well explained, on first attempt this reviewer got a bit lost in figure 3. I assume you are removing the soluble pool of the GFP fusion proteins to assess if there is a membrane bound fraction? The fact that you also remove other cytosolic factors (which was important in Figure 1) is not important for this experiment, correct? This could be explained better.

In the revised version, we extended the explanation pertaining to the experiment presented in Figure 3. We changed the text, to read:

'Since a fraction of endogenous Y-complex Nups remains associated with membranes during mitosis, we presumed that this association must be discernible as ER association in semi-permeabilized cells that have lost their cytosolic content. To test this, HeLa cells were arrested at metaphase, a stage at which mitotic NPC disassembly should be completed. We then compared Y-complex Nup content and localization by immunofluorescence analyses of intact and semi-permeabilized cells using confocal microscopy (Fig 3A). We expected that Y-complexes stably bound to ER membranes should remain in the semi-permeabilized mitotic cells and not be lost with the extracted cytosol. Indeed, a fraction of the GFP-tagged Y-complex Nups Nup107 and Nup133 was retained in semi-permeabilized cells. They not only colocalized ….'.

- On page seven you write 'post-mitotic disassembly'. This must be 'assembly'.

Thanks for spotting this mistake. It has been corrected.

- Can the authors speculate what 'a significant fraction of Y-complexes' means? This is an interesting question in order to hypothesize about their potential function in the future. You could provide an educated guess in the discussion: number of clusters * copy number of Y complexes, number of kinetochores * copy number of Y complexes, number of NPCs in u2os cells * 32. Would this be consistent with the gradient fractionation? Even if you just say it's a minor fraction, this is still interesting.

We agree that 'significant' is not the precise wording in this context and have changed it to 'a considerable fraction, that is 10 to 20% of Y-complexes,''.

A back-of-the-envelope calculation would predict about 6'000 ER-associated Y-complex clusters if we assume that there are 5'000 NPCs per cell. (5'000 NPCs contain 160'000 Y-complexes as there are

32 Y-complexes per NPC. About 15% of those were found on mitotic ER membranes in our flotation assays. This equals 24'000 Y-complexes. With an average of about 4 Y-complexes per ER-associated NPC cluster, this brings it to about 6'000 clusters per cell.)

Referee #2

Gandhimathi and colleagues present a concise manuscript detailing the accumulation of Y shaped Nup160 Nup107 complexes at the ER during mitosis. This study builds upon work recently published by the Kirchhausen group (Chou et al. (2021) PMID: 34129835), adding a layer of biochemical validation, and establishing the stoichiometry and assembly state of ER associated Y-complexes. Overall, the manuscript is well constructed and easy to follow. However, prior to publication in EMBO reports additional experiments are necessary to provide a functional link to NPC assembly, which will elevate the beautiful data presented beyond an incremental advance.

Major comments:

(1) Figures 2 and 3. While the data presented are undoubtably gorgeous, these experiments warrant expansion to provide a mechanistic link between ER association and NPC assembly. In both figures the ALPS motifs present in Nup160 and Nup133 should be probed for their role in ER association via mutant variants lacking the ALPS motif. This could for example be achieved through the generation of Nup160 and Nup133 auxin-inducible degron cell lines and subsequent expression of Nup160 and Nup133 variants lacking the ALPS motif.

We agree that it would be interesting to obtain insights into the functional importance of Y-complex association with the mitotic ER in the future. However, this question is very difficult to address experimentally for several reasons:

Firstly, and most importantly, based on published data (Doucet et al, Cell, 2010; Bolhy et al., JCB, 2011), interference with the ALPS motif of Nup133 or with POM121 (see point 2) is expected to affect NPC assembly in the preceding interphase. Therefore, if then changes in membrane association of Y-complexes during mitosis or NPC reassembly after mitosis were observed, it will be impossible to differentiate these defects from the prior NPC assembly defects.

Secondly, to our knowledge, the functionality of the ALPS motifs of Nup160 has not yet been experimentally addressed at all. It would present a study in itself to first characterize the role of the predicted ALPS motif in Nup160 in membrane association of Y-complexes, which we feel goes beyond the aim of our study.

Thirdly, the development of such auxin-inducible system for the simultaneous depletion of two Nups combined with the tunable re-expression of two Nups would present a tremendous effort. It should be noted that the lab of Mary Dasso has spent many years on establishing auxin-induced depletion of individual Nups. Their manuscript is now on BioRxiv (https://doi.org/10.1101/2020.11.13.381947). We hesitate to initiate such extensive experiments, in particular due to the caveat discussed above.

(2) Figure 3. POM121 has an established role during postmitotic NPC assembly: Antonin et al. (2005) PMID 15629719; Stavru et al. (2006) PMID 16702234; and Funakoshi et al. (2011) PMID 21289085. Thus, it is unexpected that POM121 localization during nuclear envelope breakdown is not examined, too. Mechanistically it would be intriguing to know whether ER localization is driven by local POM121 association with the Y-complex, or alternatively by direct association of the Y-complex with membranes via their ALPS motifs, or a combination of both.

As discussed in the reply to point 1 above, it will be impossible to disentangle the functional role of POM121 in NPC assembly from its potential function as binding partner of Y-complexes in mitosis.

To follow the suggestion of the reviewer, we have performed a co-localization experiment of GFP-Nup107 with POM121 (detected by immunofluorescence) in mitotic cells. This new data (EV2B) indeed suggests that a fraction of ER-associated Y-complexes colocalizes with POM121 on the ER of mitotic cells, consistent with its established role in post-mitotic NPC assembly, and we now also refer to this observation in the discussion:

‘Membrane attachment could involve the membrane-binding ALPS motifs of Nup133 (Amphipathic Lipid Packing Sensor) (Doucet et al., 2010) and possibly Nup160 (Hamed & Antonin, 2021), or association with other transmembrane Nups such as POM121, the latter supported by our co-localization experiment (EV2B). Previous work demonstrated a contribution of POM121 to NPC assembly during mitotic exit (Antonin et al., 2005), which could entail a function of POM121 in recruiting ER-associated Y-complexes to NPC assembly sites.’

Minor comments:

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We thank the reviewer for the suggestions and have inserted these references.

(2) Introduction page 2. When citing Bui et al. (2013) for their description of the Y-shaped Nup160 Nup107 complex architecture and arrangement it would also be appropriate to cite the crystal structure determined and docked into the human cryoET map in Stuwe et al. (2015) PMID 25745173, and the Y-ring model for Nup160-Nup107 complexes that was first proposed in Seo et al. (2009) PMID 19706512.

Many thanks. We have inserted these additional references.

(3) Figure 1. How were relative nuclear dextran-155 levels quantified, this information is missing from the methods. Additionally, an expanded materials figure that details representative examples of positive and negative dextran 155 transport would be welcomed.

As suggested by the reviewer, we have expanded the method section to better describe the quantification of dextran influx, to read:

‘Time-lapse confocal microscopy (Zeiss LSM 880, 40X water immersion objective) was used to monitor the progression of NPC disassembly at 30°C. Z-stacks were acquired at the indicated time intervals and quantified using a MATLAB-based custom application, which performed drift correction of the image sequences, detection of cell nuclei exploiting automated Otsu thresholding and measurements of the mean fluorescence intensities in the nuclear interior (for TRITC-dextran measurements) and at the nuclear NE (for GFP-Nup intensity at NE measurements) over time (Marino et al., 2014).’

We also note that we had previously published an extensive methods chapter, referred to in our manuscript, containing example pictures of dextran-positive and negative nuclei *(Marino J, Champion L, Wandke C, Horvath P, Mayr MI, Kutay U (2014). An in vitro system to study nuclear envelope breakdown. Methods Cell Biol 122: 255-76).* This chapter gives very detailled decscription of the method including the quantification.

(5) Discussion of ALPS motifs during Y-complex association with the ER. It would be appropriate to cite the following literature: Drin et al. (2007) PMID 17220896, which first proposed the ALPS motif; Berke et al. (2004) PMID:15557116; and Seo et al. (2009) PMID 19706512, who structurally resolved the ALPS domains within the N-terminal beta-propellers of Nup133 and Nup120, respectively.

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These references refer to luminal Torsin AAA-ATPases, which we don’t consider candidates for Y-ring disintegration.

Referee #3

Referee report
With their work, Gandhimathi et al., continue the Kutay lab's pioneering work on the fate of NPCs during mitosis. Using a range of methods, the authors show that at the onset of mitosis, the NPCs' cytoplasmic and nuclear rings are released with unique kinetics and that oligomeric assemblies of Y-complexes localize to the mitotic ER. Importantly, these oligomers are not present as octameric ring-like structures. Such octameric rings of Y-complexes have recently been suggested to persist in mitosis and to template NPC reassembly in late mitosis (Chou et al., 2021). However, the model of Chou et al. was based on rather indirect data. Gandhimathi et al. directly observed Y-complex assemblies during mitosis and are thus able to correct the previous model. Gandhimathi et al. find that mitotic Y-complex assemblies associate with the ER and thereby confirm one of the findings of Chou et al. However, Gandhimathi et al. provide more direct evidence also in this case with their biochemical fractionation experiment. These findings are critical for the NPC community to understand the dynamic architecture of NPC subcomplexes and also for researchers interested in mitotic cell organization and for investigators using super-resolution microscopy approaches.
The data are of high quality and concisely, clearly and appealingly presented. The conclusions are supported by the data and the authors are careful not to draw conclusions or to put forward a model not fully supported by the data. Previous findings are largely appropriately discussed. The possible reasons for the discrepancies to the findings of Chou et al. could be explained more so the reader can appreciate how the different conclusions arose.

The major shortcoming of the study by Chou et al. is that they did not directly study the configuration of ER-associated Y-complexes (as already discussed in the manuscript). We agree with their conclusion on ER membrane association of Y-complexes during mitosis. We note that the number of Y-complexes per ER-associated cluster is in a similar range in both studies, with our estimate being somewhat smaller than what was reported by Chou et al. This is now also explicitly mentioned in the text, to read:

The copy number of Y-complexes in the ER-associated clusters that we determined is in a similar range but somewhat smaller than what was previously reported based on a different microscopic approach. Based on calibrated lattice light sheet microscopy, it was concluded that these foci contain 8 to 10 Y-complexes (Chou et al., 2021), but using sequential bleaching as an alternative approach to infer copy number, the number of bleaching steps that was needed to erase the signal from these spots was fitted with a maximum at 4 to 6 steps (Chou et al., 2021). In contrast, in our study, we used a quantification method based on single molecule localization microscopy (Thevathasan et al., 2019), which yielded an estimated number of 2 to 6 (4 ± 2) copies of Y-complexes per ER-associated cluster (see Fig 5).

Minor questions/suggestions:

1. In addition to the ER-associated and kinetochore-associated Y-complex assemblies, are there also soluble cytosolic Y-complex assemblies present in mitosis?

It is already an established fact that mitotic Y-complexes are found as soluble components in mitotic cell extracts, as mentioned in the introduction: 'The human Y-complex contains nine core members (Nup160, Nup133, Nup107, Nup96, Nup75, Nup43, Nup37, Seh1, Sec13) and is released from NPCs into the mitotic cytosol as an intact entity (Heusel et al., 2020, Orjalo et al., 2006, Walther et al., 2003).' Figure 1 of our manuscript most directly demonstrates the release of Y-complexes from the membrane into the surrounding buffer.

However, we do not have much information on the copy number of soluble Y-complexes in mitosis. Analysis by size exclusion chromatography coupled to SWATH-MS, previously performed in collaboration with the Aebersold group (Heusel et al., Cell Systems, 2020), had revealed co-elution of all Y-complex Nups in a peak of about 2.8 MDa, which might indeed reflect higher order complex formation.

2. In order to characterize the location of Y-complex assemblies on mitotic ER, the authors could try STED-based co-localization with RTN4, which is found on the edges of fenestrations in ER sheets (Schroeder ... Bahmanyar, JCB 2019).

We have attempted these colocalization experiments as suggested, initially using different super-resolution set-ups available to us, including lattice SIM and STED. However, a major limitation in our STED was photobleaching of the dispersed GFP-Nup signal in mitotic cells, due to the high laser intensities used for depletion and excitation. Additionally, the required deconvolution steps resulted in obvious artefacts, due to the low signal to background ratio. Similarly, our attempt to use lattice SIM did not yield better results, again due low signal-to-noise ratios which lead to noise amplification during the necessary post-processing steps.

Still, to address the point of the reviewer, we have performed diffraction-limited co-localization experiments of GFP-Nup107 with either CLIMP63 or RTN4 (new Figure EV2A). These images confirm the colocalization of GFP-Nup107 with the mitotic ER, but do not allow to conclude on any preferential co-localization of Y-complexes with tubules or sheets.

Dear Ulrike,

Thank you for the submission of your revised manuscript to EMBO reports and for your patience during peer review. We have now received the full set of reports from the three referees that were asked to re-evaluate your study (please find their comments below). As you will see, all referees mention that their previous concerns have been successfully addressed, and they all support publication of the manuscript in EMBO reports.

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- Please update your data availability statement to "Our study includes no data deposited in public repositories." (under the heading "Data availability").

- Please correct "Material and Methods" to "Materials and Methods".

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- We noticed that callouts for individual panels of Fig. EV1, Fig. EV3 and Fig. EV4 are missing; please make sure that all figure panels are called out individually in your revised manuscript.

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Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

The authors have addressed all of my points.

The results are striking. I do not think additional experiments are within the scope of this study.

I most enthusiastically recommend publication in EMBOR.

Referee #2:

I would like to congratulate Gandhimathi and colleagues on addressing the majority of my concerns with this timely revision. I am still of the view that directly probing the role of the ALPS motifs present in Nup160 and Nup133 is a necessary to fully unravel the role of these ER associated Y-complexes during NPC assembly, and believe this could be the basis of a high impact study in its own right. However, in light of the other reviewers positive comments, and the revisions presented having addressed all my remaining comments I'm disinclined to delay publication of this manuscript. Thus, this beautifully produced manuscript can be published in EMBO reports as is. In particular I'd like to congratulate the authors on the impact boosting colocalization experiment presented in EV2B, which shows that ER associated Y-complexes and POM121 are associated with one another.

Referee #3:

My concerns were adequately addressed during revision and I strongly support publication of the article in EMBO reports.

The authors have addressed all minor editorial requests.

Prof. Ulrike Kutay
ETH Zurich
Institute of Biochemistry
ETH Hönggerberg
Otto-Stern-Weg 3
Zurich 8093
Switzerland

Dear Ulrike,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Best regards,

Ioannis

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

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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