The nucleoporin Nup50 activates the Ran guanyl-nucleotide exchange factor RCC1 to promote NPC assembly at the end of mitosis

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Transaction Report:

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Thank you for submitting your manuscript entitled "The nucleoporin Nup50 activates the Ran guanyl-nucleotide exchange factor RCC1 to promote mitotic NPC assembly" [EMBOJ-2021-1087788] to The EMBO Journal. I have now carefully read your study, the referees' reports from Review Commons, as well as your point-by-point rebuttal letter. This material has also been discussed with the other members of the editorial team.

We find your work potentially interesting. However, we concur with the referees that assessing the direct role of Nup50 in the regulation of RCC1 activity, as well as further substantiating the generality of the observations in mammalian cells would be essential for publication here.

Given the overall interest of your study, I am pleased to invite submission of a manuscript revised as indicated by the refeerees. I would like to point it out that addressing the points above and the other referees' points in a conclusive manner, as well as a strong support from the reviewers, would be essential for publication in The EMBO Journal. I should also add that it is our policy to allow only a single round of major revision. Therefore, acceptance of your manuscript will depend on the completeness of your responses in this revised version.

Review #1 -

In their manuscript „The nucleoporin Nup50 activates the Ran guanyl-nucleotide exchange factor RCC1 to promote mitotic NPC assembly", Holzer, Antonin and coworkers describe an unexpected function of Nup50 in assembly of the nuclear pore complex (NPC), likely based on its newly described activity as an activator of RCC1.

Nup50 is a component of the NPC with described functions in nucleocytoplasmic transport that are based on its interaction(s) with soluble transport factors and/or other nucleoporins. A role in NPC assembly has not been reported so far. Holzer et al. now use their established in vitro system to monitor NPC assembly in Xenopus egg extracts. Depletion of Nup50 from the extract resulted in strongly reduced NPC assembly, an effect that could be reversed by the addition of purified Nup50. Strikingly, binding of Nup50 to NPCs, membranes, importin alpha, importin beta, Ran or chromatin was not required for stimulation of NPC assembly. Instead, the authors convincingly show that a short fragment (aa 1-114) is sufficient for this effect. Using such a fragment in co-immunoprecipitation experiments, they then identify RCC1 as an interacting protein and characterize an unexpected activation of the nucleotide exchange activity of RCC1 by Nup50.

Major points:

It is a bit unfortunate that the addition of RCC1 or Ran is not a feasible option in the in vitro system "as appropriate titration of this system presents a significant challenge". The effects in the AL-assay (Fig. 8F) are interesting, but not directly comparable (here, the extract had not been depleted for Nup50). The arguments put forward ("Addition of RanQ69L is known to lead to ectopic NPC assembly in AL and block NPC assembly [44]. Furthermore, RCC1 addition might not be sufficient to substitute for the loss of Nup50 since RanBP1, which is highly abundant in egg extracts, blocks RCC1 activity") are not totally convincing, because i. the authors used the AL-assay and observed stimulation, not inhibition, by RanQ69L and ii. RanBP1 is probably present in all experiments performed here. The authors should consider testing additional conditions (e.g. using GTP and GTP analogues like GMP-PNP or GTP-gammaS; or RanT24N, an inhibitor of RCC1. Is RanGAP present in the extract? Its activity would counteract that of a stimulated RCC1-activity. Histones (which stimulate RCC1) and perhaps other small basic proteins (or fragments) are known to inhibit RanGAP (e.g. Nishijima et al., 2006, Mol. Biol. Cell). Although not absolutely required for publication, such experiments could potentially shed additional light on the Nup50-RCC1-Ran axis in NPC assembly.

Minor points

Fig. 5D: show the immobilized GST-proteins

Fig. 6A, B: Has the mutant Y145A been tested as well?

6D: mention Nup50 1-114 K3ER4DR38AR45D in the text

Fig. 7C: is labeling of the Y-axis („% of nuclei with ...") the same as in other figures? Use same labeling.

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Fig. 8E: show curves for the Nup-mutants; define "rate constant" in the methods section; also show individual data points in the quantification

Fig. S2: A control-IP using IgG should be performed. Molecular weight markers should be shown. S2B: rephrase the legend. Is the quantification for mitotic or interphase extract? S2D: Show individual data points as in other figures.

Fig. S4: This figure is partially redundant with Fig. 7D.

Fig. S5: The legend does not really fit to the figure (e.g. deltaNup50 not indicated).

Fig. S6: Briefly describe the assay in the legend; "data points" instead of "data point"

Fig. S7: the figure does not fit to the text. Imp alpha is not analyzed in A and mutants are not analyzed in B. What are the bands seen in S7A, input? Importantly, the FLAG-tagged proteins (input, IP) must be shown.

Lane 128: remove "in"

Lane 233: better explain why annulate lamellae are analyzed now

Lane 241: explain why those mutants were chosen

Suggestions

The authors could consider combining Figs. 1D,E with Fig. 2 (same approach in different species).

Moving Fig. 7 up might improve readability (in previous figures, the authors have already ruled out that NPC-binding is a major determinant. This becomes again a major point in Fig. 7).

Consider splitting Fig. 8 (e.g. F, G, H as a separate Fig. 9).

The authors mention in the discussion that they "attempted but failed to compensate for Nup50 depletion ...by addition of RCC1 or RanQ69L...". This is a very obvious experiment and the attempt should be mentioned earlier in the results section.

Together, this is an interesting manuscript, describing a novel function of Nup50. The experiments are very clear and of high technical quality. Although strictly it is not proven that RCC1-binding and -activation by Nup50 leads to an increase of (local) RanGTP-concentrations that promote post-mitotic NPC assembly, the demonstration of a role of Nup50 in the process is a significant and timely addition to the field. Thus, the manuscript could address a wider audience, in addition to the NPC biogenesis/nuclear transport field.

Review #2

The exchange of macromolecules between nucleoplasm and cytosol occurs through gigantic structures called Nuclear Pore Complexes (NPCs) that are embedded into nuclear envelope. Each NPC consists of >30 individual members, called nucleoporins, that are present in multiple copies following 8-fold symmetry. Although a lot of information is gathered regarding both the structure and function of the NPC, little is known regarding the function of each individual nucleoporin.

In this interesting manuscript Holzer and colleagues report that nucleoporin Nup50, which has been previously shown to influence nucleo-cytoplasmic transport, also have a surprising role in stimulating NPC formation. The authors show that depletion of Nup50 from either Xenopus egg extracts or from vertebrate culture cells results in significant reduction in amounts of NPCs on chromatin, suggesting that Nup50 drives formation of NPCs. The depletion/add-back experiments revealed that short N-terminal fragment of Nup50 is sufficient to recapitulate this particular Nup50 function. Interestingly, this fragment interacts with RCC1, a GEF factor for the RanGTPase, and the authors show that this fragment stimulates RCC1 activity two-fold. As high RanGTP levels are known to be required for NPC assembly, the authors thus conclude that such modest stimulation of Nup50-mediated RCC1 activity is sufficient to explain the observed Nup50-driven NPC assembly phenotype.

The manuscript is well-written, and the experiments are both interesting and convincing. All appropriate experiments are replicated, with adequate statistical analysis. However, the interpretation of the results remains ambiguous, and the manuscript requires major revision before publication.

Major points:

1. It is very surprising to see such a dramatic reduction of Mab414-positive signal after loss of Nup50 in vertebrate cells. To my knowledge, all previous data strongly indicate that there is minimal or no effect on the whole NPC structure after Nup50 depletion (see, for instance, PMID: 30084827, PMID: 25485891, PMID: 32917881 etc). The authors could reinforce their point by following alternative ways of inhibiting Nup50 function by using, for instance, commercial CRISPR KO and HDR plasmids.
2. I understand why the addition of RanQ69L may not have worked as expected, but why the addition of extra RCC1 to the Nup50-depleted extracts did not produce the desired outcome? If the proposed function of Nup50 is mediated through stimulation of the RCC1 activity, then surely titration of extra RCC1 should have worked. This is a straightforward experiment which has been successfully done before (for instance: PMID: 25458009). The presence of high doses of RanBP1 in XEE only inhibits cytosolic RCC1, and a significant fraction of extra RCC1 still associates with chromatin.
3. The authors focused on RCC1 but there are plenty of potential other targets, like Nup358 and Nup93, that the N-terminal domain of Nup50 seems to interact with. As Nup50G25F27 and Nup50K36R38 failed to rescue Nup50 depletion would it be more informative to compare pulldowns (or IP) between these mutants and the wt Nup50 followed by the mass-spec analysis? Will there be a specific nucleoporin in the mix? If yes - then the following scenario would also be plausible: chromatin-bound Nup50, with the help of ELYS, recruits NPC core components (like Nup93) before demembraned sperm is sealed with nuclear envelope.

The authors propose a completely novel function of Nup50 that may change our views on how post-mitotic NPC assembly occurs. These results would be of considerable interest to those working in the fields of nuclear pore complexes, chromatin dynamics and cell cycle.

My fields of expertise include: Xenopus egg extracts, mitosis, nuclear pore architecture and cell cycle.

Review #3

The manuscript by Holzer et al describes a novel function of Nup50, which has been known as an auxiliary subunit at the nucleoplasmic side of the nuclear pore complex (NPC). Combining the cell-free Xenopus egg extract system and tissue culture cells, the authors report the N-terminal domain (aa. 1-114) of Nup50 can interact and stimulate the guanine nucleotide exchange activity of RCC1, a process critical for NPC formation. The authors also showed that this N-terminal domain is sufficient to rescue the NPC assembly defect caused by Nup50 depletion in egg extracts, while other known functional features of Nup50, such as localization of NPC (mediated by ELYS) and binding to DNA, liposomes, importins, Ran, Nu153, are dispensable for the requirement of Nup50 in NPC assembly.

Major comments:

1. One of the most exciting results of this paper is that Nup50 can stimulate guanine nucleotide exchange activity of RCC1 (Figure 8E). However, there are several technical concerns about this conclusion.

First, the authors should define the values and error bars reported in the bottom graph of Fig. 8E. Assuming that these are means and SEMs, there must be a large data variation in RCC1+Nup50 (and Nup50 only) data. The identical graph can be generated even if only one experiment showed an above average value. Thus, it is critical to show all data points for each condition.

Second, the large data variation is likely caused by extremely low concentrations of RCC1 (2 nM) and Nup50 (20 nM) used in this assay. Since concentrations of RCC1 and Nup50 are both ~150 nM in Xenopus eggs (PMID: 24954049), it is not obvious why these very low concentrations were used in this assay, given the assumption that local concentrations of RCC1 and Nup50 on chromatin should be much higher than those in the egg cytoplasm. Therefore, it would be important to repeat the experiment with higher RCC1 and Nup50 concentrations to rule out the possibility that the apparent Nup50 effect was caused by a few technical errors.

Third, based on Fig. S7A, the authors imply that Nup50 G25A, F27A and Nup50 K36A, R38A mutants failed to bind RCC1 "without affecting the importin α interaction" (line 240). However, the figure does not show any data related to importin α interaction. The figure also lacks a clear explanation of the presented data. Assuming that the data are western blotting of anti-RCC1 antibodies, it is not clear why there are two bands in input lanes, whereas there is only one band in IP lanes, and why their gel mobilities are different from bands in input

lanes. For a standard co-IP assay, it is essential to check the equal expression and pull-down of the bait proteins (FLAG-Nup50), but these data are missing. Therefore, the data fail to establish if these mutants are indeed defective in RCC1 binding. In addition, rationale behind choosing these mutations should be described. Also please clarify if amino acid residues mutated in human proteins and Xenopus proteins are identical (Fig. S7A and Figure 8).

Fourth, throughout the manuscript, quality of purified proteins is never assessed except for one gel showing recombinant wildtype Nup50 (Fig. S1C), although a variety of different versions of recombinant proteins are used in this manuscript. Based on Fig. S1C, I would assess that the purity of recombinant Nup50 is enough for egg extract complementation but may not be good enough for the guanine nucleotide exchange assay. A subtle difference in activity can be caused by a subtle technical error in protein concentration quantification (as point mutations could affect a way Coomassie dyes bind proteins, or a way nonspecific binding proteins are cofractionated due to a change in surface charge distribution) or by folding defects. This is especially important when a negative effect by mutations is assessed. Since the authors used proteins using one-step affinity purification (with obvious contaminating proteins), I would recommend them to apply additional purification steps (an ion exchange column and gel filtration). Gel filtration would be informative to assess existence of protein aggregation.

2. For Nup50 rescue experiments in Nup50 depleted egg extracts, it is important to disclose the concentration of added recombinant Nup50 proteins (Fig. 1A, Fig. 4, Fig. 6, and Fig. 7). The fact that the band intensity did not respond linearly to the amount of loaded extracts in the ΔNup50+Nup50 condition implies that excess Nup50 might have been added to see the rescue effect. As mentioned above, it is important to show the gel of purified proteins (especially those used for Figure 6).

3. For quantitative presentation of immunofluorescence data (Figures 1D, 2B, 6B, and 6D), I would recommend the authors to use the SuperPlot (c.f. Lord et al., JCB, 2020; PMID, 32346721) or an alternative visualization method to show the reproducibility of each independent experiment or biological replicate.

Minor comments:

1. The manuscript uses the term "mitotic NPC assembly" as an event in which the NPC is assembled at mitotic exit. Unfortunately, naïve readers would assume "mitotic NPC assembly" a process happening during mitosis. While one would argue that the NPC assembly indeed happens at the telophase stage of mitosis, the term may be confusing if it is used in the title. Since they used interphase extracts, the studied process is not "mitotic NPC assembly". While it is not constructive to have a semantic debate, it is important to clearly state the definition of the term and explain why the system indeed represents "mitotic NPC assembly".

2. The authors must describe more specific details of immunodepletion the procedure. In general, the method section should include more details of experimental procedures that involve specific reagents that are introduced for the first time in this manuscript.

3. At line 50-51, authors may refer Otsuka et al 2016 (PMID 27630123), which reported de novo NPC assembly in interphase through the membrane penetration.

4. . Data normalization procedure should be clearly described (e.g. Figure 1D, 2B, 5D, 7E etc).

5. Line 89, Figure 1B reports mock depleted extracts, but not untreated.

6. At line 93-94, "Consistent with the absence of NPCs, the nuclei are not competent for

nuclear import (Fig. S1B) and, hence, remain of small size." Size of the nuclei looks comparable between mock and ΔNup50 extracts in Fig. S1B.

7. Fig. S2A and B. It is a standard to show two negative controls in IP experiments; control IgG IP and western blotting of an abundant unrelated protein. Data distribution of Fig. S2B looks like random. Since molecular weights of Nup50 and IgG heavy chain are similar, I wonder if this large variation was in part caused by crossreaction of the secondary antibody to IgG heavy chain. In general, it is recommended to show positions of MW markers.

8. Figure 8A. ANP32A is not annotated.

9. Figure 4. Common artifactual negative data in egg extracts are derived from sporadic occurrence of "apoptosis" in a subset of tubes. Thus, it is important to have quality controls, such as maintenance of added recombinant proteins throughout the experiment by western blotting. Since neither data quantitation nor reproducibility is reported, this is a concerning point.

10. Line 256-258. Cite references to support the statement about Tpx2 or NuMA.

11. Please specify whether GST/GFPs are tagged at N-terminus or C-terminus of proteins in Materials & Methods.

12. Figure 8D. Intensities of titrated recombinant Nup50 proteins (1X - 30X) do not seem to linearly correlate with added protein amounts. Please define the detection methods for western blots. Although many figures have quantitation of western blots, it is not clear if these values are calibrated.

13. Figure S6B. Methods for liposome binding assay are missing. In figure legends, please describe how to read these data - it is not obvious what "floated protein" means.

Line 122-124 "although Nup50 depletion reduces the MEL28/ELYS levels by about 65% and MEL28/ELYS depletion reduces Nup50 by about 15%". Without serial dilution of samples and calibration of band intensities (to make sure that band intensities are linearly correlated with protein amounts), it is impossible to make this conclusion.

It is known that Nup50 localizes at the nucleoplasmic side of the NPC and plays an important role with Nup153 for the release of cargo proteins from the transport receptors such as importins. In contrast to such a canonical 'transport-related' role, the function of Nup50 in NPC assembly at the mitotic exit has not been characterized at all. In this manuscript, Holzer et al report a novel function of Nup50 in NPC assembly. The authors demonstrate that Nup50 depletion from Xenopus egg extracts inhibits NPC assembly, and that N-terminal domain (aa. 1-114) of Nup50 is necessary and sufficient to support this function. The authors also show that siRNA-mediated depletion of Nup50 in human cells or depletion of two forms of Nup50 (Nup50a and Nup50b) in mouse cells interfered with NPC assembly. Since Nup50b was previously thought to be a pseudogene, this is an important finding. Very interestingly, the authors report that this Nup50 N-terminal domain, which was previously known to interact with importin α, binds DNA as well as RCC1. The authors further report that the Nup50 stimulates guanine nucleotide exchange activity of RCC1. Since Nup50 mutations defective in RCC1 binding, but not in importin/DNA binding or NPC localization of Nup50, inhibit NPC assembly, the authors propose that Nup50 promotes NPC assembly via RCC1 activation. Consistent with this proposal, the authors show that excess Nup50 stimulates generation of annulate lamellae in Xenopus egg extracts. It has been known that RCC1 can be stimulated by histones H2A and H2B in vitro [PMID: 11375490], but this manuscript would provide a novel mechanism by which RCC1 activity is regulated. In addition, although the data imply that DNA-binding activity of Nup50 is not essential for NPC assembly, this paper presents the third chromatin-associated factor beside RCC1 and ELYS essential for NPC assembly at the exit from mitosis. Importantly, the new result also suggests that Nup50 may act as a platform to facilitate this process through clustering RCC1, ELYS and Ran on

chromatin. This is conceptually important for the nuclear biology field. Given the essential function of NPC in any kind of eukaryotic cellular processes, upon further confirmation of the conclusion by additional supporting data, the findings will have a major impact in biology.

The reviewer is an expert of Xenopus egg extract cell biology and biochemistry.

Referee Cross-commenting

In my opinion, I less care about reproducibility in mammalian cells; they may have to develop/introduce new experimental tools. However, I would like the authors to solidify their claim that Nup50 stimulates RCC1 activity. If this is the case, it is going to be a major finding by itself, though I'm not convinced by the current data. However, even without this result, the manuscript convincingly shows a new role of Nup50 in NPC assembly in Xenopus egg extracts.

Review #4

Here the authors carry out a detailed investigation of Nup50, a nucleoporin located on the nuclear side of the nuclear pore complex (NPC) that was previously linked to nucleocytoplasmic protein trafficking. They focus on a role of Nup50 in postmitotic NE reassembly, using an in vitro nuclear assembly system involving depleted/reconstituted Xenopus egg extracts. They also investigate Nup50 by RNAi in cultured HeLa and 3T3 cells. Their results reveal a previously unknown role for Nup50 in NPC assembly in the Xenopus system and suggest a similar function in mammalian cells. Their detailed mapping studies identify a 46 amino acid region of Nup50 involved in NPC targeting, which also is shown to dictate interaction (either direct or indirect) with MEL28/ELYS and Nup153 in Xenopus egg extracts. Unexpectedly, in vitro assembly don't require this region or the nuclear transport receptor and Ran binding functions of Nup50. Rather, the critical feature for NPC assembly is an N-terminal domain that the authors showed to bind and stimulate the activity of the RanGEF (guanine nucleotide exchange factor) RCC1.

Altogether this is a carefully conducted and clearly presented analysis that provides substantial new insight on Nup50. A strong experimental pipeline (depletion/reconstitution with the in vitro system), together with both point and deletion mutants, are deployed for the biochemical and functional analyses. The conclusions are generally well-supported, except for the one major
caveat noted below.

The authors make a clear-cut case for an essential role of Nup50 in postmitotic NPC assembly in the in vitro Xenopus system via the N-terminal RanGEF stimulatory region. They carry out this analysis with an impressive array of deletion and point mutants. The results with RNAi in mammalian cells are consistent with a postmitotic NPC assembly function for Nup50 in these cells, and make sense. However, the mammalian cells were examined at an endpoint of Nup50 depletion, and still retained ~33-50% of the FG-Nup staining. In this cell model it isn't clear whether the Nup50 loss seen was due to defects in postmitotic NPC assembly, interphase NPC assembly or stability, or all of these features. At least the postmitotic pathway should be accessible to live cell kinetic analysis in RNAi-

treated cells, e.g., using a GFP-reporter for nuclear import and fluorescently tagged Nup133 such as deployed by Dultz et al (2008), following the analysis in Fig. 8. In the absence of further direct experimentation, the authors should acknowledge that the role of Nup50 in postmitotic NPC assembly in mammalian cells is unresolved, even though they favor their stated interpretation.

Minor points:

1) The liposome binding experiments are difficult to interpret in terms of biological significance, since binding is not equivalent to functional validation. Please provide more information on the lipid content of the liposomes used in these experiments. The discussion implies that different lipid compositions (including phosphoinositides) were tested.

2) The binding of MEL28/ELYS and Nup153 to the 46 amino acid targeting region of Nup50 may be indirect, and mediated by another factor that binds both of these. I don't think this point was explicitly made.

3) The level of mab414 staining is a proxy for NPC number, which is likely correct and assumed in the field, but is not a direct measure of NPC numbers.

4) The authors state on p4: "Upon depletion of Nup50 a closed NE, indicated by the smooth membrane staining, forms..."
The NE is not strictly shown to be functionally intact here, though there clearly is continuous lipid staining.

5) "life cell imaging" should be stated as "live cell imaging"

The most significant aspect of this work is the demonstration that Nup50 has a role in postmitotic NPC assembly, very likely through the role of Nup50 in RanGEF stimulation that is clearly documented and mapped here. This expands the functional repertoire of Nup50 beyond its previously described role in nuclear transport. The postmitotic NPC assembly function is robustly demonstrated with the Xenopus in vitro assay, and may well be relevant in mammalian cells as well, though the latter is supported by circumstantial evidence. The work also expands understanding of the relevant interactions Nup50 in the NPC, by identification of a core region involved in MEL28/ELYS and Nup153 binding and NPC targeting. Finally, the work describes two paralogs of Nup50 in rodents but not humans that are functionally active. Altogether, these finding will be of considerable interest to scientists studying regulation of NPC assembly and functions, and Ran GTPase biology.

The expertise of this reviewer includes NE and membrane biology. Please note that the time needed for revisions depends on whether the authors are able to (or decide to) experimentally analyze postmitotic NE assemble in Nup50-depleted mammalian cells. Without further experimentation, revision time is very short.

Referee Cross-commenting

The assembly effect in Xenopus extracts is convincing to everyone. The sperm-supplemented extracts could contain a substantial excess of "soluble" RCC1, as well as RanBP1 and RanGEF. The exact composition of the unassembled Nup50/Elys/Nup153 complexes in the extract isn't known, but could well be in dynamic equilibria, and also, shift between assembly

incompetent and competent states. Thus, I'm not surprised that addition of the nontargeted N-term fragment of Nup50 stimulates assembly. The postmitotic assembly mechanism in mammalian cells is different due to lack of "soluble" RCC1, and Nup50 RCC1-stimulation may be more important in these cells for interphase transport functions (outlined as future important question in last paragraph of text) and not rate-limiting for postmitotic assembly. (Dultz et al report association of Nup133 with the reforming NE prior to Nup50 appearance). If the authors want to claim generality of the mechanism seen in Xenopus extracts, they should examine mammalian cell mitosis directly.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In their manuscript „The nucleoporin Nup50 activates the Ran guanyl-nucleotide exchange factor RCC1 to promote mitotic NPC assembly", Holzer, Antonin and coworkers describe an unexpected function of Nup50 in assembly of the nuclear pore complex (NPC), likely based on its newly described activity as an activator of RCC1.

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Major points:

It is a bit unfortunate that the addition of RCC1 or Ran is not a feasible option in the in vitro system "as appropriate titration of this system presents a significant challenge". The effects in the AL-assay (Fig. 8F) are interesting, but not directly comparable (here, the extract had not been depleted for Nup50). The arguments put forward ("Addition of RanQ69L is known to lead to ectopic NPC assembly in AL and block NPC assembly [44]. Furthermore, RCC1 addition might not be sufficient to substitute for the loss of Nup50 since RanBP1, which is highly abundant in egg extracts, blocks RCC1 activity") are not totally convincing, because i. the authors used the AL-assay and observed stimulation, not inhibition, by RanQ69L and ii. RanBP1 is probably present in all experiments performed here. The authors should consider testing additional conditions (e.g. using GTP and GTP analogues like GMP-PNP or GTP-gammaS; or RanT24N, an inhibitor of RCC1. Is RanGAP present in the extract? Its activity would counteract that of a stimulated RCC1-activity. Histones (which stimulate RCC1) and perhaps other small basic proteins (or fragments) are known to inhibit RanGAP (e.g. Nishijima et al., 2006, Mol. Biol. Cell). Although not absolutely required for publication, such experiments could potentially shed additional light on the Nup50-RCC1-Ran axis in NPC assembly.

> For RCC1, we have now included experiments showing that a 10-fold or even better 30-fold excess of RCC1 (based on the 40nM RCC1 concentration in egg extracts reported in PMID: 25458009) compensates the loss of Nup50 in NPC assembly in vitro (now Fig. 8H). We think that because of the reportedly high inhibitory RanBP1 concentrations in egg extracts the high excess of RCC1 is required to rescue the Nup50 depletion phenotype.

> We understand that we have not been clear enough explaining why the loss of RCC1 activation is difficult to compensate with RanQ69L, the constitutive active form of Ran. RanQ69L induces ectopic NPC formation, which <u>are</u> annulate lamellae (AL). Thus, the RanQ69L dependent stimulation of AL formation is not contradicting our statement that it blocks NPC assembly (on the chromatin). Indeed, it is thought that AL formation, i.e.

massive formation of NPCs into ER, limits the availability of nucleoporins for NPC assembly on chromatin (PMID: 12894213). GMP-PNP or GTP-gammaS have the same effect as RanQ69L because they both block Ran in its active state, induce AL formation and block NPC assembly on the chromatin (e.g. PMID: 10911995). Ran T24N also inhibits NPC assembly as an RCC1 inhibitor NPC assembly, by preventing RCC1 mediated Ran activation (PMID: 10911995). RanT24N is thus not expected to overcome the loss of Nup50. In essence, Ran is required both at the correct location (in proximity to the chromatin) as well as in its GTP-state for NPC formation on the chromatin. Interfering with this system blocks NPC assembly on chromatin.

Minor points

Fig. 5D: show the immobilized GST-proteins

The equal input of the GST-baits for the experiment presented in Fig. 5D is now documented Appendix Figure S1A.

Fig. 6A, B: Has the mutant Y145A been tested as well?

Has not been tested since it is expected to behave as the D178A and Y179A mutant.

6D: mention Nup50 1-114 K3ER4DR38AR45D in the text

Has been added as "Also in the context of this minimal fragment, the mutation compromising importin α binding does not interfere with NPC formation."

Fig. 7C: is labeling of the Y-axis („% of nuclei with ...") the same as in other figures? Use same labeling.

This inconsistency has been corrected.

Fig. 8A: What are the blue dots? Better/briefly explain approach in the legend.

The previously blue dots (now in black) show a 4-fold-change compared to the control and $p < 0.01$, red dots were in addition confirmed by Western blotting. The information is now included in the figure legend.

Fig. 8E: show curves for the Nup-mutants; define "rate constant" in the methods section; also show individual data points in the quantification

The data from Nup50 mutants is now included. We now show in the lower panel the rate constant with the unit [min^{-1}]. A description of the calculation of the rate constant is added to the method section. Individual data points are also shown. Please note that a novel set of data is shown as compared to the original submission as we followed the recommendation to use further (size exclusion chromatography) purified proteins.

Appendix Figure S2: A control-IP using IgG should be performed. Molecular weight markers should be shown. S2B: rephrase the legend. Is the quantification for mitotic or interphase extract? S2D: Show individual data points as in other figures.

> This now refers to Appendix Figure S3: we have removed the data set previously presented in Appendix Figure S2A and B because it is redundant with other data presented (e.g. Fig 5 and 7). For the previous Appendix Figure S2C and D molecular weight markers have been added and individual data points are shown (now Appendix Figure S3A and B).

Appendix Figure S4: This figure is partially redundant with Fig. 7D.

> Refers now to Appendix Figure S5: True, but it shows a conservation within this region also outside of vertebrates to support the idea that this region could have a similar function in Nup2 of yeast species. In contrast, Fig. 7D is intended to highlight that certain amino acids are highly conserved in vertebrates and mutation of these often interferes with NPC localization. It also rationalizes the Q177D mutation we introduced (Fig. 7F).

Appendix Figure S5: The legend does not really fit to the figure (e.g. deltaNup50 not indicated).

> Refers now to Appendix Figure S6: Has been corrected.

Appendix Figure S6: Briefly describe the assay in the legend; "data points" instead of "data point".

> This refers to the membrane binding assays showing direct membrane association of Nup50. The description falls indeed a bit short. We suggest removing this set of data as it, in the current state, does not add much to the manuscript. It might rather distract from the main message.

Appendix Figure S7: the figure does not fit to the text. Imp alpha is not analyzed in A and mutants are not analyzed in B. What are the bands seen in S7A, input? Importantly, the FLAG-tagged proteins (input, IP) must be shown.

> We have replaced the Appendix Figure S7A by a more extensive western blot analysis which now includes importin alpha, RCC1, actin (as negative control) and FLAG (showing the efficiency of the FLAG pulldown (now Appendix Figure S7B). The figure now supports the statement that the two RCC1 binding mutants do not prevent importin

alpha binding. The reference to Appendix Figure S7B in the result section has now been corrected in Fig. 8F.

Lane 128: remove "in"

Has been done.

Lane 233: better explain why annulate lamellae are analyzed now

Has been done: We included the sentence "Thus, AL formation in Xenopus egg extracts can serve as a sensitive readout for perturbations of the Ran system".

Lane 241: explain why those mutants were chosen

These mutants were produced by generating pairs of mutations of highly conserved amino acids with the N-terminal region. The mutants are still interfering with RCC1 but not important alpha binding were further used. This information has now been included in the text.

Suggestions

The authors could consider combining Figs. 1D,E with Fig. 2 (same approach in different species).

Moving Fig. 7 up might improve readability (in previous figures, the authors have already ruled out that NPC-binding is a major determinant. This becomes again a major point in Fig. 7).

Consider splitting Fig. 8 (e.g. F, G, H as a separate Fig. 9).

Combining Fig. 1 and Fig. 2 might generate a huge figure difficult to read. Figure 7 should, in our eyes, not be moved up because the Q177D exchange is rationalized on findings presented in Fig. 5 and 6. We will be happy to split Fig. 8 into two separate ones if the editors allow.

The authors mention in the discussion that they "attempted but failed to compensate for Nup50 depletion ...by addition of RCC1 or RanQ69L...". This is a very obvious experiment and the attempt should be mentioned earlier in the results section.

As we now include data showing that excess of RCC1 compensates the loss of Nup50 we restructured this part of the discussion.

Reviewer #1 (Significance (Required)):

Together, this is an interesting manuscript, describing a novel function of Nup50. The experiments are very clear and of high technical quality. Although strictly it is not proven that RCC1-binding and -activation by Nup50 leads to an increase of (local) RanGTP-concentrations that promote post-mitotic NPC assembly, the demonstration of a role of Nup50 in the process is a significant and timely addition to the field. Thus, the manuscript could address a wider audience, in addition to the NPC biogenesis/nuclear transport field.

Referee Cross-commenting

The four reviews complement each other very well.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

The exchange of macromolecules between nucleoplasm and cytosol occurs through gigantic structures called Nuclear Pore Complexes (NPCs) that are embedded into nuclear envelope. Each NPC consists of >30 individual members, called nucleoporins, that are present in multiple copies following 8-fold symmetry. Although a lot of information is gathered regarding both the structure and function of the NPC, little is known regarding the function of each individual nucleoporin.

In this interesting manuscript Holzer and colleagues report that nucleoporin Nup50, which has been previously shown to influence nucleo-cytoplasmic transport, also have a surprising role in stimulating NPC formation. The authors show that depletion of Nup50 from either Xenopus egg extracts or from vertebrate culture cells results in significant reduction in amounts of NPCs on chromatin, suggesting that Nup50 drives formation of NPCs. The depletion/add-back experiments revealed that short N-terminal fragment of Nup50 is sufficient to recapitulate this particular Nup50 function. Interestingly, this fragment interacts with RCC1, a GEF factor for the RanGTPase, and the authors show that this fragment stimulates RCC1 activity two-fold. As high RanGTP levels are known to be required for NPC assembly, the authors thus conclude that such modest stimulation of Nup50-mediated RCC1 activity is sufficient to explain the observed Nup50-driven NPC assembly phenotype.

The manuscript is well-written, and the experiments are both interesting and convincing. All appropriate experiments are replicated, with adequate statistical analysis. However, the interpretation of the results remains ambiguous, and the manuscript requires major revision before publication.

Major points:

1. It is very surprising to see such a dramatic reduction of Mab414-positive signal after loss of Nup50 in vertebrate cells. To my knowledge, all previous data strongly indicate that there is minimal or no effect on the whole NPC structure after Nup50 depletion (see, for instance, PMID: 30084827, PMID: 25485891, PMID: 32917881 etc). The authors could reinforce their point by following alternative ways of inhibiting Nup50 function by using, for instance, commercial CRISPR KO and HDR plasmids.

Given that all reviewers agree on the importance of the biochemical and cell free analysis of the Nup50-RCC1 interplay we will focus on this. It should be, however, noted that we use tissue culture cells from human and mice yielding similar conclusions. In addition, in published work the Nup50 knockdown efficiency is often hard to judge as e.g. in PMID 25485891.

2. I understand why the addition of RanQ69L may not have worked as expected, but why the addition of extra RCC1 to the Nup50-depleted extracts did not produce the desired outcome? If the proposed function of Nup50 is mediated through stimulation of the RCC1 activity, then surely titration of extra RCC1 should have worked. This is a straightforward experiment which has been successfully done before (for instance: PMID: 25458009). The presence of high doses of RanBP1 in XEE only inhibits cytosolic RCC1, and a significant fraction of extra RCC1 still associates with chromatin.

We have now included, also in response to reviewer 1, data showing that excess of RCC1 can compensate the loss of Nup50 with regard to NPC assembly. This data is now included as Fig. 8H.

3. The authors focused on RCC1 but there are plenty of potential other targets, like Nup358 and Nup93, that the N-terminal domain of Nup50 seems to interact with. As Nup50G25F27 and Nup50K36R38 failed to rescue Nup50 depletion would it be more informative to compare pulldowns (or IP) between these mutants and the wt Nup50 followed by the mass-spec analysis? Will there be a specific nucleoporin in the mix? If yes - then the following scenario would also be plausible: chromatin-bound Nup50, with the help of ELYS, recruits NPC core components (like Nup93) before demembraned sperm is sealed with nuclear envelope.

Using GST-pulldown experiments we analyzed the effect of the G25F27 and K36R38 mutations on the Nup50-Nup93 and Nup50-RanBP2 interactions in Xenopus egg extracts. These data are now presented as Appendix Figure S7C. We see a weak interaction with RanPB2 and Nup93 to Nup50 N-terminus in the context of the wild type and the two mutant sequences. We do not think that the reduced RanBP2 binding to the mutant can account for the observed phenotype, i.e. a failure to support NPC assembly as RanBP2 is reportedly not required for NPC assembly in the egg extract system (PMID: 12105182). Similarly, the reduced Nup93 binding should not account for the NPC assembly defect given that Nup93 needs to be depleted to nearly zero levels to observe defects in NPC assembly (PMID: 22171326). In addition, the fact that excess RCC1 compensates the loss of Nup50 (now included as Fig. 8H) rather supports the idea that it is the Nup50-RCC1 axis, which is crucial for Nup50 effect on NPC assembly.

Reviewer #2 (Significance (Required)):

The authors propose a completely novel function of Nup50 that may change our views on how post-mitotic NPC assembly occurs. These results would be of considerable interest to those working in the fields of nuclear pore complexes, chromatin dynamics and cell cycle.

My fields of expertise include: Xenopus egg extracts, mitosis, nuclear pore architecture and cell

cycle.

Referee Cross-commenting

The focus of the manuscript could be shifted exclusively to the results obtained from XEE. But in this case, the authors really need to extend their data regarding potential activation of RCC1 by Nup50. I agree that when measuring RCC1 activity it would be more relevant to adhere to the endogenous XEE concentrations of the components (40 nM RCC1, 1-3 mM Ran) and titrate Nup50. Also, it would be extremely interesting to see whether Nup50 could further increase activity of "activated" H2A/H2B-bound RCC1.

> We could not perform the GEF assays with higher protein concentrations as specified in the answer for reviewer 3, who raised this point (see below) but confirmed our data with proteins further purified by size exclusion chromatography as requested. We could also not realize the idea to test the combined action of H2A/B and Nup50 due to time constrains. It should be, however, noted that a two-fold activation of RCC1's GEF activity is only seen when 333 nM H2A/B was added to 0.2 nM RCC1 while other H2A/B-RCC1 ratios result in much smaller stimulations. We suspect that it will require extensive testing to find the optimal relative protein concentrations to observe an effect and will attempt this in a future project.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

The manuscript by Holzer et al describes a novel function of Nup50, which has been known as an auxiliary subunit at the nucleoplasmic side of the nuclear pore complex (NPC). Combining the cell-free Xenopus egg extract system and tissue culture cells, the authors report the N-terminal domain (aa. 1-114) of Nup50 can interact and stimulate the guanine nucleotide exchange activity of RCC1, a process critical for NPC formation. The authors also showed that this N-terminal domain is sufficient to rescue the NPC assembly defect caused by Nup50 depletion in egg extracts, while other known functional features of Nup50, such as localization of NPC (mediated by ELYS) and binding to DNA, liposomes, importins, Ran, Nu153, are dispensable for the requirement of Nup50 in NPC assembly.

Major comments:

1. One of the most exciting results of this paper is that Nup50 can stimulate guanine nucleotide exchange activity of RCC1 (Figure 8E). However, there are several technical concerns about this conclusion.

First, the authors should define the values and error bars reported in the bottom graph of Fig. 8E. Assuming that these are means and SEMs, there must be a large data variation in RCC1+Nup50 (and Nup50 only) data. The identical graph can be generated even if only one experiment showed an above average value. Thus, it is critical to show all data points for each condition.

We show in now the lower panel the rate constant with the unit [min^{-1}]. Individual data points are now shown. Please note that a novel set of data is shown as compared to the original submission as we followed the recommendation to use further (size exclusion chromatography) purified proteins.

Second, the large data variation is likely caused by extremely low concentrations of RCC1 (2 nM) and Nup50 (20 nM) used in this assay. Since concentrations of RCC1 and Nup50 are both ~150 nM in Xenopus eggs (PMID: 24954049), it is not obvious why these very low concentrations were used in this assay, given the assumption that local concentrations of RCC1 and Nup50 on chromatin should be much higher than those in the egg cytoplasm. Therefore, it would be important to repeat the experiment with higher RCC1 and Nup50 concentrations to rule out the possibility that the apparent Nup50 effect was caused by a few technical errors.

We tried but we could not run the assay at higher protein concentrations because we could not further concentrate the purified Nup50 proteins. It should be, however, noted that exchange assays are usually run at the GEF concentrations employed here, see PMID: 29951569, which we follow and cite as ref. 72 for a detailed description and discussion. Indeed, exchange assays employing RCC1 used e.g. RCC1 concentrations in the range 0.2-2.2 nM or a fixed concentration of 0.9 nM (PMID: 11375490). Please note that in this publication addition of 333 nM Histone H2A/H2B stimulated RCC1 activity about twofold (PMID: 11375490, Fig. 2C). Similarly, the classical characterization of RCC1 as ran GEF employs RCC1 concentrations of 0.012 nM to 0.12 nM (PMID: 1944575).

Third, based on Appendix Figure S7A, the authors imply that Nup50 G25A, F27A and Nup50 K36A, R38A mutants failed to bind RCC1 "without affecting the importin α interaction" (line 240). However, the figure does not show any data related to importin α interaction. The figure also lacks a clear explanation of the presented data. Assuming that the data are western blotting of anti-RCC1 antibodies, it is not clear why there are two bands in input lanes, whereas there is only one band in IP lanes, and why their gel mobilities are different from bands in input lanes. For a standard co-IP assay, it is essential to check the equal expression and pull-down of the bait proteins (FLAG-Nup50), but these data are missing. Therefore, the data fail to establish if these mutants are indeed defective in RCC1 binding. In addition, rationale behind choosing these mutations should be described. Also please clarify if amino acid residues mutated in human proteins and Xenopus proteins are identical (Appendix Figure S7A and Figure 8).

We have replaced the figure by a more extensive western blot analysis which now includes importin alpha, RCC1, actin (as negative control) and FLAG (showing the efficiency of the FLAG pulldown. The figure now supports the statement that the two RCC1 binding mutants are not affected with respect to importin alpha binding (Appendix Figure S7B). For generating RCC1 binding mutant highly conserved amino acids were pairwise exchanged and the respective proteins analyzed. We have clarified this is the main text. The amino acid position exchanged are identical between human and Xenopus. We state now in each figure legend whether Xenopus, human or mouse Nup50 proteins are used.

Fourth, throughout the manuscript, quality of purified proteins is never assessed except for one gel showing recombinant wildtype Nup50 (Appendix Figure S1C), although a variety of different versions of recombinant proteins are used in this manuscript. Based on Appendix Figure S1C, I would assess that the purity of recombinant Nup50 is enough for egg extract complementation but may not be good enough for the guanine nucleotide exchange assay. A subtle difference in activity can be caused by a subtle technical error in protein concentration quantification (as point mutations could affect a way Coomassie dyes bind proteins, or a way nonspecific binding proteins are cofractionated due to a change in surface charge distribution) or by folding defects. This is especially important when a negative effect by mutations is assessed. Since the authors used proteins using one-step affinity purification (with obvious contaminating proteins), I would recommend them to apply additional purification steps (an ion exchange column and gel filtration). Gel filtration would be informative to assess existence of protein aggregation.

> We confirm in each addback experiment by western blotting that the recombinant Nup50 is added to approximately endogenous concentrations. This is now stated in the method section. The purification of recombinant Nup50 includes a Ni-NTA affinity chromatography to isolate His6-SUMO-tagged Nup50 fusions, which is followed by TEV cleavage and an additional passage over the affinity matrix to remove the His6-SUMO part from the Nup50 protein. This is now better described in the method section. We have now used, as requested, for the proteins employed in the GEF assay an additional purification step (size exclusion chromatography on a Sephadex 200 column). The purity of all employed proteins is now shown in Appendix Figure S1C and S7D.

2. For Nup50 rescue experiments in Nup50 depleted egg extracts, it is important to disclose the concentration of added recombinant Nup50 proteins (Fig. 1A, Fig. 4, Fig. 6, and Fig. 7). The fact that the band intensity did not respond linearly to the amount of loaded extracts in the ΔNup50+Nup50 condition implies that excess Nup50 might have been added to see the rescue effect. As mentioned above, it is important to show the gel of purified proteins (especially those used for Figure 6).

> We confirm in each addback experiment by western blotting that the recombinant Nup50 is added to approximately endogenous concentrations. This is now stated in the method section. The purified proteins are now shown in Appendix Figure S1C.

3. For quantitative presentation of immunofluorescence data (Figures 1D, 2B, 6B, and 6D), I would recommend the authors to use the SuperPlot (c.f. Lord et al., JCB, 2020; PMID, 32346721) or an alternative visualization method to show the reproducibility of each independent experiment or biological replicate.

> This has been done for Figures 1D and 2B as suggested because experiments consists of numerous data points (i.e. nuclei to be analyzed). For Fig. 6B and 6D each data point represents a single experiment and a presentation as suggested is not possible. We have

used the way of data presentation as in Fig 6B and 6D in the past (PMID: 26051542, PMID: 29150488).

Minor comments:

1. The manuscript uses the term "mitotic NPC assembly" as an event in which the NPC is assembled at mitotic exit. Unfortunately, naïve readers would assume "mitotic NPC assembly" a process happening during mitosis. While one would argue that the NPC assembly indeed happens at the telophase stage of mitosis, the term may be confusing if it is used in the title. Since they used interphase extracts, the studied process is not "mitotic NPC assembly". While it is not constructive to have a semantic debate, it is important to clearly state the definition of the term and explain why the system indeed represents "mitotic NPC assembly".

> We have changed the term to NPC assembly at the end of mitosis throughout the manuscript to avoid confusion. I would nevertheless like to comment on this: As the corresponding author, I have previously and for a long time used the term postmitotic NPC assembly. However, I have learnt that scientists working outside of the NPC field often understand this term as NPC assembly occurring in postmitotic, i.e. non-dividing, cells. NPC assembly in postmitotic cells is the assembly mode we usually refer to as "interphase assembly", i.e. exactly what we aim to distinguish from "postmitotic/mitotic". In addition, telophase, when "postmitotic/mitotic" NPC assembly occurs is in my eyes still part of mitosis. I therefore avoid the term "postmitotic NPC assembly" wherever possible.

2. The authors must describe more specific details of immunodepletion the procedure. In general, the method section should include more details of experimental procedures that involve specific reagents that are introduced for the first time in this manuscript.

> A full description in of the immunodepletion procedure is now included in the method section. The novel Xenopus Nup50 antibody is described and characterized (e.g. Fig 1A, 1B, Appendix Figure S1A). A list of constructs is included as Dataset EV1.

3. At line 50-51, authors may refer Otsuka et al 2016 (PMID 27630123), which reported de novo NPC assembly in interphase through the membrane penetration.

> Reference has been added.

4. . Data normalization procedure should be clearly described (e.g. Figure 1D, 2B, 5D, 7E etc).

> Normalization methods are now better described in the respective figure legends, figures 1D and 2B now show non-normalized data.

5. Line 89, Figure 1B reports mock depleted extracts, but not untreated.

Has been corrected.

6. At line 93-94, "Consistent with the absence of NPCs, the nuclei are not competent for nuclear import (Appendix Figure S1B) and, hence, remain of small size." Size of the nuclei looks comparable between mock and ΔNup50 extracts in Appendix Figure S1B.

We have now changed the figure and included a more representative picture of a nucleus assembled in Nup50 depleted extracts.

7. Appendix Figure S2A and B. It is a standard to show two negative controls in IP experiments; control IgG IP and western blotting of an abundant unrelated protein. Data distribution of Appendix Figure S2B looks like random. Since molecular weights of Nup50 and IgG heavy chain are similar, I wonder if this large variation was in part caused by cross-reaction of the secondary antibody to IgG heavy chain. In general, it is recommended to show positions of MW markers.

In western blots analyzing IP experiments we use Protein-A-HRP as secondary antibody, which does not recognize the heavy and light chains of IgGs on Western blots but only the primary antibodies. This has been now specified in the method section. In addition, antisera cross-linked with dimethyl pimelimidate to Protein-A Sepharose was employed in the experiments to avoid protein dephosphorylation in the egg extracts by phosphatases abundant in antisera. We have nevertheless removed this figure part because it is largely redundant with data presented in the main figures (e.g. Fig 5 and 7).

8. Figure 8A. ANP32A is not annotated.

ANP32A is now annotated.

9. Figure 4. Common artifactual negative data in egg extracts are derived from sporadic occurrence of "apoptosis" in a subset of tubes. Thus, it is important to have quality controls, such as maintenance of added recombinant proteins throughout the experiment by western blotting. Since neither data quantitation nor reproducibility is reported, this is a concerning point.

We carefully sort out activated eggs during egg extract preparation (described in the cited reference). Using apoptotic egg extracts is easily detectable because of the disintegrated appearance of the chromatin. However, we observe this very seldom, probably three times in the last 10 years and certainly discard these experiments. In all depletion-addback experiments, full-length Nup50 is added to depleted extracts as positive control. If this reaction does not rescue the NPC assembly phenotype to 50% or the mock depletion did not yield more than 70% of chromatin templates with full rim NPC staining the experiment is now discarded. We have specified this now in the method section.

10. Line 256-258. Cite references to support the statement about Tpx2 or NuMA.

      References have been added.

11. Please specify whether GST/GFPs are tagged at N-terminus or C-terminus of proteins in Materials & Methods.

      GST tags are at the N-Terminus. We used N- and C-terminal EGFP fusions, indicated as EGFP-Nup50xx or Nup50xx-EGFP. This has been now clarified in the method section and corrected in Figure 4 and 7F. We also follow this for the EGFP-Nup107 and EGFP-Nup133-fusions newly presented in Appendix Figure S2.

12. Figure 8D. Intensities of titrated recombinant Nup50 proteins (1X - 30X) do not seem to linearly correlate with added protein amounts. Please define the detection methods for western blots. Although many figures have quantitation of western blots, it is not clear if these values are calibrated.

      We use ECL detection throughout the manuscript, which is now specified in the method section. Recombinant Nup50 is added here in huge excess over the endogenous Nup50 concentration. Hence, for higher concentrations the signal is nonlinear. The Nup50 western blot is intended to show depletion and addback (1xfold Nup50) to nearly endogenous levels, which is well achieved (Nup50 signal is 36,588,716 a.u. in mock vs 34,685,663 a.u. in DNup50-1xNup50 or Nup50 signal is 25,667,257 a.u. in mock vs 29,506,551 a.u. in DNup50-1xNup50. We included now a better description of the western blots quantitations in the method section.

13. Appendix Figure S6B. Methods for liposome binding assay are missing. In figure legends, please describe how to read these data - it is not obvious what "floated protein" means.

      We suggest removing this set of data as it, in the current state, does not add much to the manuscript. It might rather distract from the main message.

Line 122-124 "although Nup50 depletion reduces the MEL28/ELYS levels by about 65% and MEL28/ELYS depletion reduces Nup50 by about 15%". Without serial dilution of samples and calibration of band intensities (to make sure that band intensities are linearly correlated with protein amounts), it is impossible to make this conclusion.

      The western blot presented in Figure 1A shows a nearly linear dose-signal response for Nup50 in the concentration range used. We validate all our antibodies in this way. In our eyes it is hence appropriate in indicate these approximate levels. We have nevertheless phrased this sentence more carefully.

Reviewer #3 (Significance (Required)):

It is known that Nup50 localizes at the nucleoplasmic side of the NPC and plays an important role with Nup153 for the release of cargo proteins from the transport receptors such as importins. In contrast to such a canonical 'transport-related' role, the function of Nup50 in NPC assembly at the mitotic exit has not been characterized at all. In this manuscript, Holzer et al report a novel function of Nup50 in NPC assembly. The authors demonstrate that Nup50 depletion from Xenopus egg extracts inhibits NPC assembly, and that N-terminal domain (aa. 1-114) of Nup50 is necessary and sufficient to support this function. The authors also show that siRNA-mediated depletion of Nup50 in human cells or depletion of two forms of Nup50 (Nup50a and Nup50b) in mouse cells interfered with NPC assembly. Since Nup50b was previously thought to be a pseudogene, this is an important finding. Very interestingly, the authors report that this Nup50 N-terminal domain, which was previously known to interact with importin α, binds DNA as well as RCC1. The authors further report that the Nup50 stimulates guanine nucleotide exchange activity of RCC1. Since Nup50 mutations defective in RCC1 binding, but not in importin/DNA binding or NPC localization of Nup50, inhibit NPC assembly, the authors propose that Nup50 promotes NPC assembly via RCC1 activation. Consistent with this proposal, the authors show that excess Nup50 stimulates generation of annulate lamellae in Xenopus egg extracts. It has been known that RCC1 can be stimulated by histones H2A and H2B in vitro [PMID: 11375490], but this manuscript would provide a novel mechanism by which RCC1 activity is regulated. In addition, although the data imply that DNA-binding activity of Nup50 is not essential for NPC assembly, this paper presents the third chromatin-associated factor beside RCC1 and ELYS essential for NPC assembly at the exit from mitosis. Importantly, the new result also suggests that Nup50 may act as a platform to facilitate this process through clustering RCC1, ELYS and Ran on chromatin. This is conceptually important for the nuclear biology field. Given the essential function of NPC in any kind of eukaryotic cellular processes, upon further confirmation of the conclusion by additional supporting data, the findings will have a major impact in biology.

The reviewer is an expert of Xenopus egg extract cell biology and biochemistry.

Referee Cross-commenting

In my opinion, I less care about reproducibility in mammalian cells; they may have to develop/introduce new experimental tools. However, I would like the authors to solidify their claim that Nup50 stimulates RCC1 activity. If this is the case, it is going to be a major finding by itself, though I'm not convinced by the current data. However, even without this result, the manuscript convincingly shows a new role of Nup50 in NPC assembly in Xenopus egg extracts.

> We have followed this advice by putting an additional effort in ensuring purity of recombinant Ran, Nup50 and RCC1 to better control the GEF assays. In addition, the data showing that excess of RCC1 can compensate the loss of Nup50 fully supports or hypothesis that the Nup50-RCC1 axis is crucial for Nup50 effect on NPC assembly.

Reviewer #4 (Evidence, reproducibility and clarity (Required)):

Here the authors carry out a detailed investigation of Nup50, a nucleoporin located on the nuclear side of the nuclear pore complex (NPC) that was previously linked to nucleocytoplasmic protein trafficking. They focus on a role of Nup50 in postmitotic NE reassembly, using an in vitro nuclear assembly system involving depleted/reconstituted Xenopus egg extracts. They also investigate Nup50 by RNAi in cultured HeLa and 3T3 cells. Their results reveal a previously unknown role for Nup50 in NPC assembly in the Xenopus system and suggest a similar function in mammalian cells. Their detailed mapping studies identify a 46 amino acid region of Nup50 involved in NPC targeting, which also is shown to dictate interaction (either direct or indirect) with MEL28/ELYS and Nup153 in Xenopus egg extracts. Unexpectedly, in vitro assembly don't require this region or the nuclear transport receptor and Ran binding functions of Nup50. Rather, the critical feature for NPC assembly is an N-terminal domain that the authors showed to bind and stimulate the activity of the RanGEF (guanine nucleotide exchange factor) RCC1.

Altogether this is a carefully conducted and clearly presented analysis that provides substantial new insight on Nup50. A strong experimental pipeline (depletion/reconstitution with the in vitro system), together with both point and deletion mutants, are deployed for the biochemical and functional analyses. The conclusions are generally well-supported, except for the one major caveat noted below.

The authors make a clear-cut case for an essential role of Nup50 in postmitotic NPC assembly in the in vitro Xenopus system via the N-terminal RanGEF stimulatory region. They carry out this analysis with an impressive array of deletion and point mutants. The results with RNAi in mammalian cells are consistent with a postmitotic NPC assembly function for Nup50 in these cells, and make sense. However, the mammalian cells were examined at an endpoint of Nup50 depletion, and still retained ~33-50% of the FG-Nup staining. In this cell model it isn't clear whether the Nup50 loss seen was due to defects in postmitotic NPC assembly, interphase NPC assembly or stability, or all of these features. At least the postmitotic pathway should be accessible to live cell kinetic analysis in RNAi-treated cells, e.g., using a GFP-reporter for nuclear import and fluorescently tagged Nup133 such as deployed by Dultz et al (2008), following the analysis in Fig. 8. In the absence of further direct experimentation, the authors should acknowledge that the role of Nup50 in postmitotic NPC assembly in mammalian cells is unresolved, even though they favor their stated interpretation.

> We have now performed live cell imaging following the fate of Nup107 and Nup133, which are both part of the Nup107-160-complex within NPCs using HeLa cell lines stably expressing EGFP-Nup107 or EGFP-Nup133 (the latter containing a triple EGFP fused of mouse Nup133). We observe a delay of Nup107 and Nup133 chromatin recruitment upon Nup50 downregulation. These data are now included as Appendix Figure S2. We did not use an EGFP-reporter for nuclear import because any change in nuclear import rate/restart of nuclear import upon Nup50 downregulation cannot be unambiguously assigned to Nup50 function in NPC assembly or (more directly) to its auxiliary role in nuclear import (as a platform to support the Ran-mediated dissociation of importin-cargo complexes (see introduction of the MS) and/or probably the now reported stimulatory effect on RCC1).

Minor points:

1) The liposome binding experiments are difficult to interpret in terms of biological significance, since binding is not equivalent to functional validation. Please provide more information on the lipid content of the liposomes used in these experiments. The discussion implies that different lipid compositions (including phosphoinositides) were tested.

> We suggest removing this set of data as it, in the current state, does not add much to the manuscript. It might rather distract from the main message.

2) The binding of MEL28/ELYS and Nup153 to the 46 amino acid targeting region of Nup50 may be indirect, and mediated by another factor that binds both of these. I don't think this point was explicitly made.

> Has been now included in the discussion: "…although we currently cannot ascertain whether Nup153 and/or MEL28/ELYS binding is direct or indirect"

3) The level of mab414 staining is a proxy for NPC number, which is likely correct and assumed in the field, but is not a direct measure of NPC numbers.

> Valid point, but we do not find a specific sentence/figure where we made this mistake. We will be more than happy to correct this sloppiness. We have nevertheless modified the line 93-95 to "mAB414… serves as a robust marker for intact NPCs and as proxy for NPC numbers" to avoid any misunderstanding.

4) The authors state on p4: "Upon depletion of Nup50 a closed NE, indicated by the smooth membrane staining, forms..."
The NE is not strictly shown to be functionally intact here, though there clearly is continuous lipid staining.

> Indeed, we avoid here term "functional intact" in this sentence. We now also refer to Appendix Figure S1B in the previous sentence where we speak of "a nucleus with a functional NE and NPCs is formed" to explain the egg extract system. Because the nuclei show nuclear import and export activity it is, in our eyes, safe to state that NE and NPCs are functional.

5) "life cell imaging" should be stated as "live cell imaging"

> Has been changed.

Reviewer #4 (Significance (Required)):

The most significant aspect of this work is the demonstration that Nup50 has a role in postmitotic NPC assembly, very likely through the role of Nup50 in RanGEF stimulation that is clearly documented and mapped here. This expands the functional repertoire of Nup50 beyond its previously described role in nuclear transport. The postmitotic NPC assembly function is robustly demonstrated with the Xenopus in vitro assay, and may well be relevant in mammalian cells as well, though the latter is supported by circumstantial evidence. The work also expands understanding of the relevant interactions Nup50 in the NPC, by identification of a core region involved in MEL28/ELYS and Nup153 binding and NPC targeting. Finally, the work describes two paralogs of Nup50 in rodents but not humans that are functionally active. Altogether, these finding will be of considerable interest to scientists studying regulation of NPC assembly and functions, and Ran GTPase biology.

The expertise of this reviewer includes NE and membrane biology. Please note that the time needed for revisions depends on whether the authors are able to (or decide to) experimentally analyze postmitotic NE assemble in Nup50-depleted mammalian cells. Without further experimentation, revision time is very short.

Referee Cross-commenting

The assembly effect in Xenopus extracts is convincing to everyone. The sperm-supplemented extracts could contain a substantial excess of "soluble" RCC1, as well as RanBP1 and RanGEF. The exact composition of the unassembled Nup50/Elys/Nup153 complexes in the extract isn't known, but could well be in dynamic equilibria, and also, shift between assembly incompetent and competent states. Thus, I'm not surprised that addition of the nontargeted N-term fragment of Nup50 stimulates assembly. The postmitotic assembly mechanism in mammalian cells is different due to lack of "soluble" RCC1, and Nup50 RCC1-stimulation may be more important in these cells for interphase transport functions (outlined as future important question in last paragraph of text) and not rate-limiting for postmitotic assembly. (Dultz et al report association of Nup133 with the reforming NE prior to Nup50 appearance). If the authors want to claim generality of the mechanism seen in Xenopus extracts, they should examine mammalian cell mitosis directly.

> As indicated above a new set of experiments following Nup107 and Nup133 at the end of mitosis points to a function of Nup50 also in mammalian NPC assembly. It should be noted that in the Dultz el al manuscript Nup50 is detected about 2 min after Nup133 on the chromatin and thus much earlier compared to most other nucleoporins (Fig. 5C in Dultz et al). In this manuscript, the authors thus suggest that the early recruited pool of Nup50 together with the Nup107-160 complex and Nup153 acts as starting point for NPC assembly, at that time referred to a "prepore" on the chromatin (Fig. 5F and beginning of the discussion in Dultz et al).

Thank you for submitting your revised manuscript to The EMBO Journal. We have now heard back from three of original Review Commons referees, and I am pleased to say that they all found the previously-raised points satisfactorily addressed. We shall therefore be happy to accept the study for publication in our journal, pending incorporation of the following editorial points:

REFEREE REPORTS

Referee #1:

In their revised manuscript „The nucleoporin Nup50 activates the Ran guanyl-nucleotide exchange factor RCC1 to promote mitotic NPC assembly" the authors addressed most of the major and minor points that were raised by this (and the other) reviewer(s). The demonstration of Nup50 as an activator of RCC1 is unexpected and important. Likewise, the combined role of Nup50 and RCC1 in postmitotic NPC assembly is very interesting and merits publication in the EMBO Journal.

Referee #2:

In the revised manuscript Holzer and colleagues addressed most of my concerns. Importantly, they provided the essential evidence that directly link RCC1 and Nup50 for nuclear pore assembly during telophase. I support publication.

Referee #3:

The authors did a great job in addressing criticisms raised in their original submission. They now show compelling evidence to support the novel concept that the most critical function of Nup50 for nuclear pore complex assembly is to stimulate GEF activity of RCC1.

The authors have made all requested editorial changes.

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ⬇

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

| Corresponding Author Name: Wolfram Antonin |
| Journal Submitted to: EMBO Journal |
| Manuscript Number: EMBOJ-2021-108788 |

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 justified
➜ 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 χ2 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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Cell experiment: sample size of at least 80 cells were taken. When individual cells were followed over time a minimum of 10 cells were acquired. For egg extract and western blotting a minimum of n=3 independent experiment were performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was at minimal n=3 in our egg extract experiments. Individual datapoint are shown on our graphs. Note that in this study, animals were used to obtain egg but no were not the subjects of the experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The following criteria was established for data exclusion: if an egg extract experiment displayed a percentage of positive (closed) mAB414 staining below 50% in the mock or control rescue condition, the experiment was discarded.
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.	Such step were not taken as in this study, animals were used to obtain egg but no were not the subjects of the experiments.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Statistical test are described when performed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution of were tested using GraphPad statistical tool and statistical test were performed accordingly.
Is there an estimate of variation within each group of data?	Yes as each individual data point is displayed in our draph.

Is the variance similar between the groups that are being statistically compared?	No

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).	Reference numbers of all commercially available antobodies are written in the method section. Reference are provided when home-made antibodies were used. This is described in the Method section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Our cells are routinely tested for mycoplsama contamination.

* 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.	For the cell free experiments female Xenopus laevis frogs (not gentically modified) for obtaining eggs were used to generate egg extracts. Frogs were mantained in an aquqrium system and experiemnts performed according to the German law pertaining to the use of vertebrate animals in research (Tierschutzgesetz).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Included
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.	We comply to the German law pertaining to the use of vertebrate animals in research (Tierschutzgesetz).

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	The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Perez-Riverol et al., 2019) with the dataset identifier PXD028701.
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).	Dataset is provided as Table S2
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.	NA