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Mitotic checkpoint protein Mad1 is required for early Nup153 recruitment to chromatin and nuclear envelope integrity

Ikram Mossaid, Guillaume Chatel, Valérie Martinelli, Marcela Vaz and Birthe Fahrenkrog
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MS TITLE: Mitotic checkpoint protein Mad1 is required for early Nup153 recruitment to chromatin and nuclear envelope integrity

AUTHORS: Ikram Mossaid, Guillaume Chatel, Valerie Martinelli, Marcela Vaz, and Birthe Fahrenkrog
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the two experts thought that the work was potentially quite interesting and significant but all also raised a number of concerns that must be dealt with before the manuscript can be reconsidered. Please address these issues as thoroughly as possible. I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

The spindle assembly checkpoint protein Mad1 interacts with the nuclear pore complex component (nucleoporin) Nup153 but the function of this interaction is unknown. Here, Mossaid et al show that Mad1 is required for timely recruitment of Nup153 to anaphase chromatin. Depletion of Nup153 or Mad1 enlarges the distance between the outer and inner nuclear membrane which together form the nuclear envelope. Surprisingly, Nup153 depletion displaces cytoplasmic nucleoporins from nuclear pore complexes and reduces the number of nuclear pore complexes assembled in interphase, which has been previously suggested from experiments in Xenopus egg extract system but not verified in cellular assays Together these data shed light on the Mad1-Nup153 interaction and show that Nup153 has distinct functions nuclear pore complex assembly at the end of mitosis and in interphase.

Comments for the author

Major points:
In the Figure 3A one might see a slight reduction of the Nup153 signal upon (including interphase cells). However, the manuscript says (line 174): “On the contrary, Nup153’s localisation in any other cell cycle phase was indistinguishable between control and Mad1-depleted cells (Fig. 3A)”. A quantitation of the IF signal would be helpful. The same applies to figure S3A/B. In general I miss intensity quantitations of the immunofluorescences and also information about the number of experiments and/or cells the data shown are derived from.
In the Figure a statistical analysis is missing. It would be of help to show, as supplementary material, the delay of telophase onset.
In the Figure S3C the authors should provide quantitation as in Figure 3C and a statistical analysis. In the trajectories shown is not clear to me that the Nup98 recruitment under siMad1 starts at min 9 (asterisk).
Figure &: In addition to a loss of rim staining for Nup214 and Nup358 upon Nup153 dwonregulation I also observe cytoplasmic aggregates, which disappear in the rescue. It would be worth to comment on this.

Minor points:
Line 770: it would be good to define GST-Nup153N again as aa 2-610
Line 776: “residues 1-240 (N120)” should read “residues 1-120 (N120)”
In the Figure 3B the authors need to indicate what they use as an indicator of beginning of telophase. I guess they use the first clue of the cleavage furrow, but would be of help to mention it. in the Figure legend the authors shoul change (line 807) “White arrows” by “ asterisk”.

Reviewer 2

Advance summary and potential significance to field

The present study provides deeper insights into the previously reported interaction of the spindle assembly checkpoint protein Mad1 and the basket nucleoporin Nup153. The idea that Mad1 is required for early recruitment of Nup153 to chromatin and potentially for NE integrity is very interesting and provocative.
The authors suggest that both Mad1 and Nup153 are necessary for proper post-mitotic nuclear envelope (NE) re-formation, because their depletion results in ultrastructural changes of the NE. The authors also show that depletion of Nup153 leads to defects in NPC assembly as well as a decrease in overall number of NPCs in HeLa cells upon G2/M arrest. They suggest two separable roles of Nup153: one in the reformation of the NE at the end of mitosis that is dependent on its interaction with Mad1, and another in interphase NPC assembly, which is Mad1-independent.
This study presents a number of interesting observations, however, the mechanistic links between Mad1, Nup153 and the nuclear envelope remain largely unclear.
Additionally, this paper lacks a number of important quantifications, which are essential to put the data on solid ground.
In summary, I would support its publication if the authors can address my concerns.

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Comments for the author

1. In Figure 1, it is not clear whether the same amount of GST and GST-Nup153-N were used. “Materials and methods” only indicates 500µl of GST and 200µl of GST-Nup153-N, which does not say much. This is important because an excess of GST can coat the beads and reduce unspecific binding, whereas a lesser amount of GST-Nup153-N could promote false-positive binding of Mad1. An input gel of GST and GST-Nup153-N needs to be included and this should show the actual amount/quality of protein that was used. I would also like to see a binding assay, which shows that a Mad1 fragment comprising residues 552 to 596 is sufficient to bind to NUP153 N. Have the authors tried the reciprocal experiment with immobilized Mad1 fragments (binding site 1 and 2) and free Nup153?

2. It could be very interesting that Mad1 has two binding sites for Nup153, but what does it functionally mean? What is the stoichiometry of the Nup153-Mad1 interaction? Are the two sites on Mad1 independent of each other such that one Mad1 molecule binds two Nup153 molecules, or is this a composite binding site for Nup153? Where do the two Mad1 sites bind Nup153? Are there also two distinct Mad1 binding sites on Nup153? The authors need to clarify this biochemically.

3. What are the phenotypes of deleting the two Nup153 binding sites of Mad1 (individually and in combination)?

4. Please label the Nup153 binding sites of Mad1 in the cartoon (Fig. 1a)

5. Figure 2 shows that interaction between Nup153 and Mad1 is cell cycle-dependent. The authors claim that presence of the nuclear envelope is a prerequisite for this interaction. What does that actually mean? Does it reflect the necessity of NPCs or membrane-binding of Nup153? “Nup153 and Mad1 interact exclusively in the presence of the NE” is an overstatement, since the authors present only correlative data. Are Mad1 and Nup153 levels constant across the cell-cycle?.

6. Figure 2 also suggests that the Mad1/Nup153 interaction is established around telophase. How do the authors explain recruitment of Nup153 through Mad1 in anaphase (Fig.3A), before the Mad1/Nup153 interaction is established (based on the PLA signal)? In general, it would be useful to use Mad1 mutants, in which the two Nup153 binding sites were deleted (Fig. 1) to characterize the recruitment/interaction dynamics.

7. In Figure 4, the authors observed that NE ultrastructure is altered upon depletion of both Nup153 and Mad1. This observation is interesting, but the quantification should be improved. The widening of the perinuclear space in Nup153- and Mad1-depleted space is highly irregular. How do the authors choose where to measure the distance? “10 nuclei were analysed per condition and NE width was measure at 10 different points per nucleus.”

8. The 3D-SIM experiment in Figure 4D needs to be supported by a proper quantification.

9. In the discussion (line 321), the authors suggest that defects in NE curvature at the NPC are probably caused by impaired binding of Nup153 to the NE. Can the authors test this assumption, for example by deleting the Nup153 amphipathic helix and analysis of NE ultrastructure? What would happen if the Nup153 amphipathic helix was deleted together with the Mad1 interaction site? This experiment could be very informative.

10. In one of their earlier publications (“Structural characterization of altered nucleoporin Nup153 expression in human cells by thin-section electron microscopy”, Duheron et al, 2014) this group has depleted Nup153 in HeLa cells by RNAi (similarly as here) and analyzed the NE by TEM. How do the authors explain that the current analysis of the NE phenotype differs from the previously published one?

11. The EM data in the Supplement requires a proper quantification (Figure S4 - siNup50 and siTpr seem to have some NE effect, too).

12. What happens when both Nup153 and Mad1 are depleted? Is the NE phenotype enhanced?

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13. Figure 5 needs a proper quantification.

14. Figure 6 needs a proper quantification. I have some doubts about the rescue of cytoplasmic Nup localization upon expression of GFP-Nup153. The cytoplasmic foci still seem to be present.

15. Line 337 I don't understand this statement. Mislocalization?

16. Line 148: Having confirmed the interaction between Nup153 and Mad1 at the NE….
Since no direct interaction is shown by PLA, this should be called an "association".

First revision

Author response to reviewers' comments

Response to Reviewers

Reviewer 1
Major points:
In the Figure 3A one might see a slight reduction of the Nup153 signal upon (including interphase cells). However, the manuscript says (line 174): "On the contrary, Nup153's localisation in any other cell cycle phase was indistinguishable between control and Mad1-depleted cells (Fig. 3A)". A quantitation of the IF signal would be helpful. The same applies to figure S3A/B. In general I miss intensity quantitations of the immunofluorescences and also information about the number of experiments and/or cells the data shown are derived from.

We agree with the Reviewer that the image shown for Nup153 staining in interphase in the Mad1 depleted cell, appeared slightly weaker than in the control example. We have replaced the image to avoid this impression and have quantified the IF signal as suggested (shown in Fig. 3 B and C). We have quantified the signal for cells in interphase, anaphase, and telophase/cytokinesis. The number of analysed cells for each cell cycle state is indicated in the graphs. The results are described in the corresponding Results section (line 179-186). We have furthermore quantified importin-ß and Tpr intensities, now shown in Fig. S4.

In the Figure a statistical analysis is missing. It would be of help to show, as supplementary material, the delay of telophase onset.

A statistical analysis is now included as well as a quantification of anaphase duration (new Fig. S5A).

In the Figure S3C the authors should provide quantitation as in Figure 3C and a statistical analysis. In the trajectories shown is not clear to me that the Nup98 recruitment under siMad1 starts at min 9 (asterisk).

We agree with the reviewer that the recruitment sites for Nup98 were not easy to identify. We have now marked the sites by arrows (now Fig. S5B). A quantification of Nup98 recruitment is not possible, because we saw in none of our movies a delay in the recruitment, hence all values are zero. We have included a quantification of anaphase duration, which is now shown Fig. S5A.

Figure &: In addition to a loss of rim staining for Nup214 and Nup358 upon Nup153 dwonregulation I also observe cytoplasmic aggregates, which disappear in the rescue. It would be worth to comment on this.

Thank you for pointing this out. We are now commenting this, as suggested (line 259-261).

Minor points:
Line 770: it would be good to define GST-Nup153N again as aa 2-610

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Done as suggested.

Line 776: “residues 1-240 (N120)” should read “residues 1-120 (N120)”
This has been corrected.

In the Figure 3B the authors need to indicate what they use as an indicator of beginning of telophase. I guess they use the first clue of the cleavage furrow, but would be of help to mention it. in the Figure legend the authors shoul change (line 807) “White arrows” by “ asterisk”.
As suggested, a definition for the beginning of telophase in now mentioned in the figure legend and the text has been corrected.

Reviewer 2
1. In Figure 1, it is not clear whether the same amount of GST and GST-Nup153-N were used. “Materials and methods” only indicates 500µl of GST and 200µl of GST-Nup153-N, which does not say much. This is important because an excess of GST can coat the beads and reduce unspecific binding, whereas a lesser amount of GST-Nup153-N could promote false-positive binding of Mad1. An input gel of GST and GST-Nup153-N needs to be included and this should show the actual amount/quality of protein that was used. I would also like to see a binding assay, which shows that a Mad1 fragment comprising residues 552 to 596 is sufficient to bind to NUP153 N.
Have the authors tried the reciprocal experiment with immobilized Mad1 fragments (binding site 1 and 2) and free Nup153?

We have now included Coomassie gels showing the input for the pull-down assays (new Figure S1). We agree with the Reviewer that it would be nice to see a binding assay for the Mad1 fragment comprising 552-596. We were for unclear reasons unable to express this fragment in vitro and can unfortunately not include this. We have attempted to do reciprocal binding assays, but observed unspecific binding of GST to the FLAG agarose beads that remained even after washing with high salt concentrations (up to 500 mM NaCl). However, we have previously shown by co-immunoprecipitation assays that Nup153 and Mad1 co-purify in both directions (Lussi et al., 2010, Nucleus).

2. It could be very interesting that Mad1 has two binding sites for Nup153, but what does it functionally mean? What is the stoichiometry of the Nup153-Mad1 interaction? Are the two sites on Mad1 independent of each other such that one Mad1 molecule binds two Nup153 molecules, or is this a composite binding site for Nup153? Where do the two Mad1 sites bind Nup153? Are there also two distinct Mad1 binding sites on Nup153? The authors need to clarify this biochemically.

Thank you for pointing out all these highly interesting questions. We perfectly agree that further biochemical and biophysical experiments are required to clarify those. This however is in our opinion a story by itself and will be subject for future studies.

3. What are the phenotypes of deleting the two Nup153 binding sites of Mad1 (individually and in combination)?
Thank you for putting out this next highly interesting question. To address it, Nup153 needs to be modified by genome-editing techniques and several stable cell lines need to be established and characterised in depth. This will take several months, if not years, and goes beyond a realistic timeframe for the revision of the current manuscript.

4. Please label the Nup153 binding sites of Mad1 in the cartoon (Fig. 1a)

The binding sites for Nup153 are now indicated in Fig. 1a.

5. Figure 2 shows that interaction between Nup153 and Mad1 is cell cycle-dependent. The authors claim that presence of the nuclear envelope is a prerequisite for this interaction. What does that actually mean? Does it reflect the necessity of NPCs or membrane-binding of Nup153? “Nup153 and Mad1 interact exclusively in the presence of the NE” is an overstatement, since

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the authors present only correlative data. Are Mad1 and Nup153 levels constant across the cell-cycle?.
We do not fully agree that "Nup153 and Mad1 interact exclusively in the presence of the NE" is an overstatement, because we have shown a direct interaction between the two proteins in Figure 1 (and previously). It is in our opinion therefore justified to call it an interaction, although PLA assays only show proximities, but not interactions. We have nevertheless rephrased the text.
Concerning the expression levels of Nup153 and Mad1: Nup153 levels are similar during interphase and mitosis, as can be seen in Fig. S3A. We ourselves have not monitored Mad1 levels throughout the cell cycle, but also Mad1 levels are constant, as shown, for example, in Lee et al., 2008, Genes&Dev. 22:2926.
Whether the interaction between Nup153 and Mad1 requires NPCs or membrane-binding of Nup153: difficult to answer. In our opinion more likely NPCs, or at least Nup153 at NPCs. While Nup153 can bind membranes without any doubts, it has not been shown that it binds membranes throughout interphase. Given its high dynamics and turn-over, it is difficult to imagine that Nup153 is membrane-bound throughout. It might also be only a minor pool of Nup153 that binds membrane, just as only a minor pool of Nup153 is recruited to chromatin in anaphase. There are certainly more studies necessary to fully understand how and when Nup153 exactly binds membranes.

6. Figure 2 also suggests that the Mad1/Nup153 interaction is established around telophase. How do the authors explain recruitment of Nup153 through Mad1 in anaphase (Fig.3A), before the Mad1/Nup153 interaction is established (based on the PLA signal)? In general, it would be useful to use Mad1 mutants, in which the two Nup153 binding sites were deleted (Fig. 1) to characterize the recruitment/interaction dynamics.

Actually, we agree that it appears a bit contradictory that Mad1 is required for Nup153 recruitment to chromatin, when the interaction between them is only established when the NE reforms in telophase. However, as stated in the Discussion (line 302 onwards), Mad1 might directly target Nup153, but, more likely, there is one (or more) bridging factor involved, which needs to be identified. To characterise the recruitment/interaction dynamics in the presence of Mad1 mutants, is an excellent suggestion, but would again need genome-editing techniques to modify the endogenous Mad1 and this again goes beyond a realistic timeframe for the revision of the current manuscript.

7. In Figure 4, the authors observed that NE ultrastructure is altered upon depletion of both Nup153 and Mad1. This observation is interesting, but the quantification should be improved. The widening of the perinuclear space in Nup153- and Mad1-depleted space is highly irregular. How do the authors choose where to measure the distance? “10 nuclei were analysed per condition and NE width was measure at 10 different points per nucleus.”

We agree with the reviewer that the widening of the perinuclear space in Nup153- and Mad1-depleted cells is highly irregular. We have indeed randomly chosen sites along the NE to cover the range of width that can be detected. We have now re-examined the EM micrographs and measured the width in a more systematic way, exclusively adjacent to NPCs. The data are now presented as scatter plot with median, so that the individual values are distinguishable.

8. The 3D-SIM experiment in Figure 4D needs to be supported by a proper quantification.

While we agree with the reviewer that a quantification of the 3D-SIM data would be beneficial, this is in our opinion for practical reasons not possible. To detect a widening of the PNS, the distance between the ONM and the INM needs to be larger than 100 nm, the optical resolution of SIM. Such a spacing occurs, but only occasionally, while the average distance in Nup153- and Mad1-depleted cells is 80-100 nm, based on the quantification of the EM images (Fig. 4C). Therefore, it is unfortunately impossible to provide a meaningful quantification of the 3D-SIM images.

9. In the discussion (line 321), the authors suggest that defects in NE curvature at the NPC are probably caused by impaired binding of Nup153 to the NE. Can the authors test this assumption, for example by deleting the Nup153 amphipathic helix and analysis of NE ultrastructure? What would happen if the Nup153 amphipathic helix was deleted together with the Mad1 interaction site? This experiment could be very informative.

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This is indeed another interesting question. To address it, however, Nup153 would again needed to be modified by genome-editing techniques and new cell lines need to be established and characterised. This would go beyond a realistic timeframe for the revision of the current manuscript.

10. In one of their earlier publications ("Structural characterization of altered nucleoporin Nup153 expression in human cells by thin-section electron microscopy", Duheron et al, 2014) this group has depleted Nup153 in HeLa cells by RNAi (similarly as here) and analyzed the NE by TEM. How do the authors explain that the current analysis of the NE phenotype differs from the previously published one?
In the previous study, we have not analysed the NE after Nup153 depletion, but exclusively NPCs. The focus of the previous study was on the architecture of the nuclear basket and the importance of Nup153 for nuclear basket integrity. From the images shown in Fig. 2 in the previous study, the only figure with EM images of Nup153-depleted cells, only few selected NPCs are shown, which do not allow any conclusion about the integrity of the NE in these cells. Therefore, we do not see any contradiction in our two studies as they addressed two completely different questions/aspects. We have rephrased the figure legend accordingly.

11. The EM data in the Supplement requires a proper quantification (Figure S4 - siNup50 and siTpr seem to have some NE effect, too).
We have quantified the EM data as requested. The EM micrographs and the quantification are now presented in Fig. S6, A and B. There is no significant difference in the widening of the perinuclear space in Nup50-depleted cells, whereas indeed depletion of Tpr has an effect. This, however, could be an indirect effect, because in the absence of Tpr, Mad1 recruitment to NPCs might be impaired. We now point this out in the text (line 214-221).

12. What happens when both Nup153 and Mad1 are depleted? Is the NE phenotype enhanced?
We have not tried a co-depletion of Nup153 and Mad1. If viable, we would expect severe cell cycle defects, at different stages of the cell cycle, given the respective role that both proteins play in cell cycle regulation. This gives rise to the question how meaningful the experiment would be. It would certainly require an in-depth characterisation of the double-depleted cells, before analysing the NE phenotype on EM level.

13. Figure 5 needs a proper quantification.
14. Figure 6 needs a proper quantification. I have some doubts about the rescue of cytoplasmic Nup localization upon expression of GFP-Nup153. The cytoplasmic foci still seem to be present.
Concerning point 13 and 14, we respectfully disagree. In Figure 6, we show in each panel that cells expressing RNAi-resistant GFP-Nup153 in Nup153-depleted cells show normal Nup214 and Nup358 localisation, respectively. Cytoplasmic foci of Nup214 and Nup358 can only be observed in neighbouring cells that do not express the RNAi-resistant GFP-Nup153. For clarification, we point this out in the revised manuscript (line 259-61). Given the unambiguousness of the results shown in Figure 5 and 6, it is unclear to us, what could be quantified in the two cases.

15. Line 337 I don't understand this statement. Mislocalization?
Thank you for pointing this out, this was indeed a mistake. We have corrected the sentence, starting now with "Partial mis-localisation…".

16. Line 148: Having confirmed the interaction between Nup153 and Mad1 at the NE…. Since no direct interaction is shown by PLA, this should be called an "association".
We have corrected this, as suggested.

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Second decision letter

MS ID#: JOCES/2020/249243

MS TITLE: Mitotic checkpoint protein Mad1 is required for early Nup153 recruitment to chromatin and nuclear envelope integrity

AUTHORS: Ikram Mossaid, Guillaume Chatel, Valerie Martinelli, Marcela Vaz, and Birthe Fahrenkrog
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed all raised questions. Some of the suggested experiments could not be performed within the time frame of a resubmission. I agree with these statements. From my point of view the manuscript is now acceptable for publication.

Comments for the author

The authors use sometimes the greek beta, sometimes the German ß (ss) for naming importin beta. Might be worth to check for consistenty, also in the labeling of Fig S4

Reviewer 2

Advance summary and potential significance to field

The authors have addressed my concerns and have significantly improved the manuscript.

Comments for the author

I fully support its publication in JCS.

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