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Dimerization and direct membrane interaction of Nup53 contribute to nuclear pore complex assembly

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1st Editorial Decision 16 May 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. While all three referees consider the study as interesting and referees 2 and 3 are clearly in favour of publication of the study here, referee 1 is considerably more critical and requires a substantial amount of additional data. On balance and given the strong support from the other two referees, we should be happy to consider a revised manuscript that addresses the referees' concerns in an adequate manner. Going through the comments raised by referee 1, I would suggest concentrating on points 3 and 4. It would certainly be good and strengthen the paper immensely to also look into his/her point 6. A summary scheme in the supplementary material listing all constructs used in the different experiments would be very helpful. Please do not hesitate to contact me at any time in case you would like to consult on any aspect of the revision further or to discuss any suggestions from your side during the revision process.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

We generally allow three months as standard revision time. As a matter of policy, competing

manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this paper, the authors characterize the role of Nup53 in nuclear pore complex assembly. While the paper contains novel and interesting aspects, at this point it remains rather preliminary, and many claims are not sufficiently supported by the data presented. Therefore, I cannot recommend publication in EMBO J at this time. In order to improve the paper, the authors need to more carefully dissect the biochemistry that underlies the Nup53 membrane interaction, provide more functional data, and present additional results in support of their models.

Below I will discuss each of the major findings described in the paper and discuss issues or problems with the data:

(1) Identification of the residues critical for xlNup53 dimerization
This part is very nicely done, and requires no attention.

(2) Identification of residues that are phosphorylated during mitosis
In the legend to Table S1, the authors claim that positions T100, T288 and S291 are specifically phosphorylated during mitosis. At the same time they use the S94/T100 mutants for their experiments to mimic the "mitotic" state of Nup53. Is S94 constitutively phosphorylated? If yes, what is the specific contribution of T100? The authors investigate the influence of mitotic phosphorylation on Nup53 membrane binding claiming that it is important for regulating membrane interactions. However, the question remains whether these phosphorylation(s) are significant in the context of FL protein? Also, this part is somewhat disconnected form the rest of the paper.

(3) Analysis of Nup53 truncations and point mutants in membrane flotation assays
The use of various deletions and point mutations in membrane flotation assays lead the authors to claim that 3 regions in xlNup53 contribute to membrane binding: (i) a rather broad N-terminal domain between amino acids 1-130, (ii) the RRM-like domain, and (iii) the most C-terminal part, previously implicated in NDC1 interaction. Usually, membrane binding domains are confined to rather small regions, e.g. regions of BAR-domain containing proteins that peripherally associate with membranes, or amphipathic helices integrated into the hydrophobic phase of the bilayer. In this study, the biochemical basis of how the different parts of Nup53 contribute to membrane interactions remain rather unclear. This raises concerns about the results and their interpretation. Specifically, it is not clear how the RRM domain contributes to membrane interaction? Does its ability to form dimers enhances the membrane-binding properties of the respective N- and C-terminal parts (e.g. by an avidity-type mechanism), does this domain per se show some weak membrane binding properties, or is this part required for proper protein folding?
Also, it is not clear why such a broad region at the N-terminus is required for membrane interaction? Does it display some weak membrane binding property by itself? How does this property change upon binding to large NPC scaffold elements (such as Nup93, Nup205)? The authors claim that certain basic residues (R105, K106) are important for the membrane binding because of charge interactions, yet they use non-charged lipids for their flotation assays questioning their interpretation? Could the phenotypes be explained by general folding problems? Can these mutants

still dimerize?
The cellular ER contains a large fraction of negatively charged lipids and does not contain NBD-PE. The types of lipids used may obviously influence the outcome of flotation experiments.
Regarding the C-terminus: does the C-terminus bind membranes by itself albeit weakly and what are the determinants? Is there some kind of C-terminal amphipathic helix (similar to yeast Nup53)?

(4) Analysis of various Nup53 truncations and point mutants in "post-mitotic" in vitro assembly assays.
Using 53-depleted extracts in in vitro nuclear assembly assays, the authors investigate the functionality of various Nup53 mutants in terms of NPC formation. They draw several conclusions: (i) the Nup53 RRM dimerization plays an essential role in NPC assembly by mediating membrane interaction. However, it is not clear whether this interpretation is correct. For example, the Nup53-312 C-terminal truncation binds membranes much weaker yet it fully supports NPC assembly. (ii) The authors claim that membrane binding of Nup53 is essential for NPC assembly. They base these conclusions on correlations between the membrane binding properties of the Nup53 mutants they have analyzed and their functionality in a nuclear assembly assay. A key issue is that the mutants used in the flotation assay are not the same as the ones used in the nuclear assembly reaction. Do the 1-319/ R105E/K106E and 1-319/ S94E/T100E variants fail to bind to membranes? Also, do these mutants act as dominant negatives? If the assembly defect is due to a specific deficiency in membrane binding, these variants should sequester their binding partners and are expected to act as dominant-negatives. Is this the case?

(5) Analysis of various Nup53 truncations and point mutants in "interphase" in vitro assembly assays
I am concerned about the interpretation of interphase NPC assembly results. Could it be that the 1-320 R105E/K106E and 1-320S94E/T100E variants make holes/NPC-precursors in the membrane instead on new NPCs? Detecting new NPCs could be done more safely using e.g. differentially labeled WGA.

(6) Assaying various liposome-bound truncations of Nup53 by negative staining EM.
There are multiple ways to deform liposomes on surfaces including osmotic stress, occasional liposome collapse, interactions with the grid surface, etc. The lipid "worms" like the ones shown on the images can sometimes be seen even without any proteins. Since this membrane-bending assay is prone to various artifacts, it is important that the authors provide additional evidence for the membrane bending capability of Nup53. For example, one could look at the specific enrichment of the bending protein on curved surfaces. This could be done either by immuno-EM (which may be technically challenging) or fluorescence microscopy with labeled protein(s) and lipids.

Essential points that need to be addressed:

-The authors need to more carefully dissect the biochemistry of Nup53 membrane binding in order to get a more coherent picture of this phenomenon.

-In order to be able to interpret correlations between membrane binding and protein functionality, the authors have to perform biochemical assays and functional (NPC assembly) assays with the same proteins. Additionally, since there is a huge gap in complexity between the liposome binding assay and the NPC assembly assay, the results need to be much more cautiously discussed and speculations that are not completely supported by data need to be moved to the discussion section.

-Both the interphase nuclear assembly and membrane curvature assays have to done more vigorously.

Referee #2

Despite multiples studies over the last years, the mechanisms contributing to NPC assembly are far from being fully understood. In particular, how nucleoporins interact and stabilize the pore membrane and to which extent post-mitotic NPC reassembly differs from NPC assembly during interphase remain debated.
Among the structural nucleoporins contributing to NPC assembly, Nup53 had previously been

demonstrated to be required, along with several other members of the Nup93 complex, for post-mitotic NPC assembly in vertebrates.

Here, the authors re-investigate the implication of Nup53 in this process. Interestingly, they demonstrate that Nup53 can bind to liposomes in vitro, that is, independently of any other protein. Using a mutant that impairs the in vitro dimerization of Nup53 (designed based on the structure of the central RRM domain of Nup53 - aa 162-250, Handa et al, 2006), they next show that dimerization contributes to its membrane binding and is critical for post-mitotic NPC assembly. They next identify two distinct membranes binding domains within Nup53, respectively within its N- and C-terminal domains, both of which further require dimerization of the RRM domain for efficient membrane binding:
- the N-terminal domain contains a pair of basic residues, whose mutation into acidic residues impairs membrane binding, without affecting the interaction of this domain with its previously know binding partners (Nup 93 and Nup205). Within this domain, they further identify two mitotic specific phosphorylation sites whose phosphorylation in vitro or mutation into negatively charged residues also affect membrane binding. This suggests that the N-terminal domain of Nup53 interacts with membranes via electrostatic interactions with negatively-charged lipid head groups.
- The C-terminal domain was previously suggested to contain an amphipatic helix that could serve as a hydrophobic module. Here the authors show that deletion of the last C-term tryptophane impairs Nup53 membrane binding without affecting Ndc1 interaction, whereas deletion of the last 8 amino acids abolishes in vitro liposome binding (and Ndc1 interaction as previously reported by others).
By using these mutations, alone or in combination, they show that either one of the two membrane binding regions of Nup53 is sufficient for postmitotic NPC assembly and stability, whereas mutants defective in both membrane binding sites no longer support post-mitotic NPC/NE reassembly. Finally, they show that solely removing the last C-terminal amino-acid of Nup53 prevents NPC assembly during interphase. Moreover, unlike the N-terminal domain, the C-terminal domain of Nup53 can induce tubulation of liposomes, a property lost upon deletion of the last tryptophane. The membrane deforming capacity of Nup53 is thus correlated to its critical role in interphase but no pos-mitotic NPC assembly.
Based on these data, they propose a model, that highlights the different contribution of Nup53 modes of interactions with membranes to post-mitotic and interphase NPC assembly.

Altogether, this is a very nice and impressive study, that provides an extensive dissection of the functional domains of Nup53 that contribute to NPC/NE assembly, and most importantly allows to build up a model that highlights the differences between post-mitotic versus interphase NPC assembly.
As detailed below, I however have a few comments that should be addressed before publication of this study.

Major comments

1) In the discussion p14, the authors indicate a clear discrepancy between their data, and a previous study demonstrating that " a fragment lacking the N-terminal region as well as the C-terminal 26 amino acids (that is hNup53 [167-300]) replaced endogenous Nup53 in nuclear assemblies in Xenopus egg extracts (Hawryluk-Gara et al, 2008), ... Indeed, this Nup53 fragment lacks both membrane binding regions identified in this study, whose combined deletion is reported here to affect post-mitotic NE/NPC reassembly.
The authors suggest that this discrepancy might be due to different Nup53 depletion (that is, presence of minor amounts of Nup53 in the experiments published by Hawryluk-Gara et al, 2008). They further indicate, " Using a number of different Nup53 fragments which lacked the Nup93 binding region we were not able to replace endogenous Nup53 in NPC assembly (unpublished observation)".
To avoid any subsequent controversy, these data should be provided (eventually as supplemental figure). At least, the closest mutant they have in hand (xNup53 162-312, used in Fig S4B, that only binds Nup155) should be tested for complementation in their Nup53-depleted nuclear assembly assay.
Indeed, one cannot formally exclude more complex hypotheses, such as a dominant phenotype induced by the replacement of positive with negative residues in the 1-312/1-319 R105EK106E mutants (or the addition of negative residues in the S94ET100E mutants).

2) SDS-PAGE and silver staining of all recombinant proteins used in this study should be provided (eventually as supplemental figure), as done in figure 1B for some of them. Indeed, in Figure 1, the fact that the bacterial 70 kD protein co-purifying with Nup53 does not co-purifies with the 162-320 domain (that nevertheless binds to liposomes) is important to rule out the contribution of this bacterial protein in indirectly mediating the binding of Nup53 to liposomes.
This control of equal purity is important for Figure 6 since (although this is unlikely) one cannot formally exclude that specific Nup53 domains or mutants may co-purify with bacterial protein that would in turn affect (positively or negatively) membrane tubulation.

3) Nup53 RRM/ dimerization mutant.
- Since this mutation maps within a domain know to interact with Nup155, the authors should analyze if introducing this mutation affects Nup155 binding (using GST pull-down on Nup53 [130-320 F172EW203E] construct used in Fig S3 or using a Nup53 [162-320 F172EW203E] as performed in fig S4 for the other mutants).

- Assessing the effect of the RRM/dimerization mutant in both the interphase assembly assay (Fig 5., with the full -length Nup53 RRM mutant protein as in Fig 2B) and the membrane deformation assay (Figure 6, with the Nup53[130-320- F172EW203E] construct used in Fig S3) would have allowed to determine to which extent dimerization of the C-terminal domain is required for its function in interphase NPC assembly and liposome tubulation. Although not mandatory, this analysis could potentially have further strengthen their argument in the context of the current debate of the discrepancy (or not) between interphase and post-mitotic NPC assembly.

- As Nup53 dimerization was so far only demonstrated in vitro (Handa et al, 2006 and Fig 2A in this study), did the authors try to determine if this interaction takes place in vivo ?

4) The implication of reticulons, that were similarly shown to induce membrane tubulation and to contribute to de novo NPC assembly should be discussed (Dawson et al, J Cell Biol. 2009 and ref therein).

Minors comments

1) The relative liposome binding of the 162-320 domain (compared to full length) could be provided (this is the only construct for which this information is not given).

2) Page 7, end of 1rst chapter: "Together with the liposome-binding assay, this (lack of complementation of the dimerization mutant) suggests that Nup53 membrane binding is important for NPC assembly..."
At that stage of the manuscript, this is just a correlation, and this sentence should be corrected as " suggests that Nup53 membrane binding could be important" or "Nup53 membrane binding or oligomerization is important...."

3) Page 8, end of second chapter: dimerization via the RRM domain is necessary (for membrane binding). This is somehow overstated and should be rephrased since only a two-fold decrease is observed for the 130-230 FW mutant in Fig S3.

4) Page 14, first lines: "interaction with Ndc1 is not necessary for post-mitotic NPC assembly. ... possible explanation for this surprising finding might be that Nup53 can interact directly with membranes...." .
Since this was previously reported, this finding is not so surprising.
In addition, the alternative hypothesis, that is, the fact that this finding may reflect protein-protein interaction should be indicated (for instance in yeast, Nup170 also interacts with Pom152 - see Marelli et al., 2001). Indeed, in fig 4B, the interaction between Nup53 [162-312] and Nup155 is observed is a cytosolic fraction while interaction with Ndc1 was independently tested using Triton X100 solubilized membranes; thus, on cannot exclude that in the context of the NPC/NE assembly reaction, Nup53 [162-312] may indirectly interact with Ncd1 via Nup155.

5) How do the authors reconcile their data (2 membrane binding domains in the N/C-term domain of Nup53) with the linear arrangement of the Nups of this complex hypothesized by Amlacher et al.

(2011) for the yeast counterparts ? This might be briefly discussed.

6) The authors should mention somewhere in their manuscript that metazoan Nup53 is also termed Nup35 (nomenclature used by Handa et al., 2006; Rodenas 2009; Zuleger 2011...).

Referee #3

Here the authors provide evidence that direct membrane binding by Nup53 is required for NPC assembly. They analyze deletion and point mutants of recombinant Nup53 for the ability to bind lipid vesicles and to support NE assembly in vitro in Xenopus egg extracts depleted of the endogenous protein. The approaches implemented here to measure protein binding to lipid vesicles, which are widely used by this field, typically measure low affinity interactions and are not necessarily biologically specific. Nonetheless the authors have done the controls that are commonly used for this assay and have shown a strong correlation between the ability to bind polar lipid vesicles in vitro and to support NE formation. Overall his study is well conducted and provides significant new mechanistic insight on NPC formation. It should be published in EMBO J with minor modifications.

1) The title "... depends on a direct interaction with membranes" seems a bit too strong with the present data. Although physiological relevance of the in vitro binding of Nup53 to polar lipid vesicle is strongly suggested, it is not completely proven. For example, the R105E/K106E mutations could disrupt Nup53 folding important for NE assembly (not revealed in the nucleoporin binding assays), as well as the proposed electrostatic interaction of basic amino acid residues with acidic phospholipid head groups. More subtle alanine mutations, less prone to generating misfolded protein, were not tested. Moreover, the authors haven't tested whether the tryptophan residue in the C-terminal peptide sequence actually inserts in the bilayer milieu, although this is a reasonable proposal. The authors should consider softening the title somewhat, unless they can make a more compelling case for physiological relevant bilayer association of the indicated regions. However, I don't think more experimental work is needed to publish this study.

2) Since the insertion of the C-terminal tryptophan into the nuclear membrane bilayer would be expected to induce positive membrane curvature as shown in Fig. 7, it would be helpful for a discussion of how Nup53 by itself could give rise to the invaginated proposed intermediate structure with negative curvature.

3) The authors don't explain why they use E. coli polar lipids for their assay, instead of lipids from a higher eukaryotic source similar to Xenopus, although they hint at use of other lipids in the Discussion. Are tubules as in Fig. 6 obtained with vertebrate-like lipids?

Other minor points:

- More details of liposome preparation in Material and Methods would be helpful.

- Labeling of gel lanes in Fig. 1B could be improved, as it is a bit difficult to grasp the data at first glance because of the crowded display.

- It's a bit difficult to follow the mutant nomenclature (e.g. S94ET100E) and the authors should consider alternative presentations such as S94E/T100E or S94E,T100E

1st Revision - authors' response 14 August 2012

Point-By-Point Response

Referee #1

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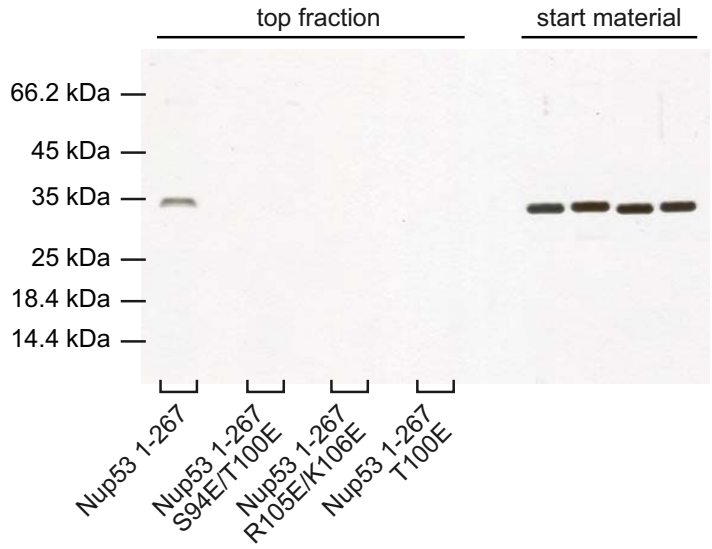
We detect phosphorylation of Serine 94 on Nup53 isolated both from mitotic and interphasic extracts. We therefore used the S94E/T100E mutant to mimic the mitotic state. In the 1-267 fragment, a T100E mutation significantly reduces membrane binding similar to the S94E/T100E or R105E/K106 mutant. We therefore focused in the following experiments on the S94E/T100E mutant.



As we have now also assayed as requested liposome binding in the context of full length Nup53 it is clear that the phosphorylation reduces membrane binding to approximately 40% (Figure 3C). However, because of the contribution of the C-terminal membrane binding site to NPC assembly we can only test the influence of the site in the context of the 1-319/1-312 truncations: In these instances the phosphorylation mimicking mutant is defective in NPC assembly (Figure 4A).

(3) Analysis of Nup53 truncations and point mutants in membrane flotation assays

The use of various deletions and point mutations in membrane flotation assays lead the authors to claim that 3 regions in xlNup53 contribute to membrane binding: (i) a rather broad N-terminal domain between amino acids 1-130,

In the N-terminus the critical region for membrane binding is located between aa 93 and 107 as indicated by a fourfold decrease in liposome binding upon removal of these 15 amino acids (Figure 3A). This is also supported by the point mutations located in this region which inactivate the N-terminal membrane binding site (Figure 3A and B) or reduce binding in the full-length context (Figure 3C).

(ii) the RRM-like domain, and (iii) the most C-terminal part, previously implicated in NDC1 interaction. Usually, membrane binding domains are confined to rather small regions, e.g. regions of BAR-domain containing proteins that peripherally associate with membranes, or amphipathic helices integrated into the hydrophobic phase of the bilayer. In this study, the biochemical basis of how the different parts of Nup53 contribute to membrane interactions remain rather unclear. This raises concerns about the results and their interpretation. Specifically, it is not clear how the RRM domain contributes to membrane interaction? Does its ability to form dimers enhances the membrane-binding properties of the respective N- and C-terminal parts (e.g. by an avidity-typemechanism), does this domain per se show some weak membrane binding properties,

We indeed favour the hypothesis that Nup53 dimerization enhances its otherwise weak membrane binding by an avidity-based mechanism. The RRM domain alone does not show membrane binding (Figure 1B, 3A)

or is this part required for proper protein folding?

We have no indication that the RRM domain is necessary for Nup53 folding: all RRM mutants run as proper monomer in size exclusion chromatography (Figure 2A & data not shown).

Also, it is not clear why such a broad region at the N-terminus is required for membrane interaction? Does it display some weak membrane binding property by itself?

Also for the function of the N-terminal binding site, the dimerization seems necessary, as the RRM mutant of an N-terminal fragment shows reduced binding (Figure S5) and the N-terminal fragment alone was not detected after flotation in the liposome containing fraction (Figure 1B fragment aa1-166).

How does this property change upon binding to large NPC scaffold elements (such as Nup93, Nup205) ?

Unfortunately, we cannot reconstitute these interactions in vitro in - for the NPC - relevant stoichiometry and geometry. In general, we don't know the contribution of other nucleoporins binding to Nup53's membrane interaction as mentioned in the discussion but we strengthen this point.

The authors claim that certain basic residues (R105, K106) are important for the membrane binding because of charge interactions, yet they use non-charged lipids for their flotation assays questioning their interpretation? Could the phenotypes be explained by general folding problems? Can these mutants still dimerize?

The liposomes used in the floatation assay are generated from E. coli polar lipids which contain 67% Phosphatidylethanolamine, 23,2% Phosphatidylglycerol (which is negatively charged) and 9,8% Cardiolipin and we therefore don't see any contradiction. The R105E/K106E mutants dimerize as judged by gel filtration (data not shown). In addition, they are functional in postmitotic and interphasic NPC assembly (Figure 3 and 5) for which Nup53 dimerization is mandatory (see Figure 2)

The cellular ER contains a large fraction of negatively charged lipids and does not contain NBD-PE. The types of lipids used may obviously influence the outcome of flotation experiments.

Certainly, the ER does not contain fluorescently labelled lipids such as NBD-PE. However, we observe identical effects using liposomes without NBD-PE that were labelled with the membrane dye DiIC18 (data not shown). To minimize the side effects of membrane labelling, necessary to allow for quantitation of membrane binding, we choose fatty acid labeled PE, to allow the interaction of proteins on the membrane surface. Certainly, the liposomes used in the floatation assay contain negatively charged lipids as specified above and we obtain

similar results when using Folch I lipids (data not shown, but mentioned in the discussion) or a lipid mixture resembling the ER/nuclear envelope (Figure S7).

Regarding the C-terminus: does the C-terminus bind membranes by itself albeit weakly and what are the determinants? Is there some kind of C-terminal amphipathic helix (similar to yeast Nup53)?

The Nup53 C-terminus lacking the RRM domain (aa 254-320) does not bind liposomes (see Figure 1B) and the same is true for shorter fragments (data not shown). We cannot exclude that Xenopus Nup53 contains a C-terminal amphipathic helix, but without structural data this can be at best a prediction similar as for yeast Nup53.

(4) Analysis of various Nup53 truncations and point mutants in "post-mitotic" in vitro assembly assays.
Using 53-depleted extracts in in vitro nuclear assembly assays, the authors investigate the functionality of various Nup53 mutants in terms of NPC formation. They draw several conclusions: (i) the Nup53 RRM dimerization plays an essential role in NPC assembly by mediating membrane interaction. However, it is not clear whether this interpretation is correct. For example, the Nup53-312 C-terminal truncation binds membranes much weaker yet it fully supports NPC assembly.

This is not correct because Nup53 aa1-312 is able to bind membranes (see Figure 3C) as it possesses the N-terminal binding site.

(ii) The authors claim that membrane binding of Nup53 is essential for NPC assembly. They base these conclusions on correlations between the membrane binding properties of the Nup53 mutants they have analyzed and their functionality in a nuclear assembly assay. A key issue is that the mutants used in the flotation assay are not the same as the ones used in the nuclear assembly reaction. Do the 1-319/ R105E/K106E and 1-319/ S94E/T100E variants fail to bind to membranes?

We have added these data as Figure 3C and they fully support our interpretation.

Also, do these mutants act as dominantnegatives? If the assembly defect is due to a specific deficiency in membrane binding, these variants should sequester their binding partners and are expected to act as dominant-negatives. Is this the case?

We have tested the prediction and found indeed an inhibitory effect for some of the mutants (e.g. 1-312). However, for inhibition we need to add rather high concentrations of the respective proteins in order to avoid significant dilution of the assembly reactions, which would also block nuclear reassembly. Therefore we cannot systematically analyse the effect and prefer not to present these data.

(5) Analysis of various Nup53 truncations and point mutants in "interphase" in vitro assembly assays
I am concerned about the interpretation of interphase NPC assembly results. Could it be that the 1-320 R105E/K106E and 1-320S94E/T100E variants make holes/NPC-precursors in the membrane instead on new NPCs? Detecting new NPCs could be done more safely using e.g. differentially labeled WGA.

This is a valid point. However, the assay for measuring interphasic NPC assembly using differentially labeled WGA established by the Hetzer lab (D'Angelo et al, 2006) requires considerable amounts of extracts, even if downscaled to 2ml per reaction. This is for depleted extracts not feasible. However, we have counted NPC numbers in wildype, the R105E/K106E mutant and the 1-319 truncation with and without inhibition of interphasic NPC assembly (Figure 6C). These newly included data fully support our conclusion that interphasic NPC assembly for the 1-319 truncation is blocked. Please note the similar level of inhibition as compared to importinβ addition, which is known to inhibit this mode of pore assembly. Importantly, as NPCs in this assay are identified by mAB4141 staining which recognizes FG-repeat containing nucleoporins this shows that indeed NPCs and not just empty holes are formed.

(6) Assaying various liposome-bound truncations of Nup53 by negative staining EM.
There are multiple ways to deform liposomes on surfaces including osmotic stress, occasional liposome collapse, interactions with the grid surface, etc. The lipid "worms" like the ones shown on the images can sometimes be seen even without any proteins.

Since this membrane-bending assay is prone to various artifacts, it is important that the authors provide additional evidence for the membrane bending capability of Nup53. For example, one could look at the specific enrichment of the bending protein on curved surfaces. This could be done either by immuno-EM (which may be technically challenging) or fluorescence microscopy with labeled protein(s) and lipids.

We are aware of this problem and have carefully avoided osmotic stress also in the washing procedures. Importantly, we included proper positive (EHD2) and negative (PBS, Nup133) controls and would not report this tubulation as a phenotype if seen in samples without any protein. We have now included more proteins which show a reduced membrane binding (93-320 RRM mutant and 130-320 RRM mutant) which consistently do not induce membrane tubulation.
We have not been able to specifically visualize Nup53 enrichment on the curved surface by negative staining probably because it does not form highly oligomeric and ordered structures as e.g. dynamin. Both immuno-EM on liposomes as well as visualizing liposome tubulation by fluorescence microscopy is technically extremely challenging as admitted by the reviewer and we have not succeeded in these experiments. Nevertheless, as the negative controls and all Nup53 constructs, which lack the C-terminal tryptophan or show reduced membrane interaction, do conclusively not show the tubulation phenotype, we are confident that this observation is not an artefact of the procedure.

Essential points that need to be addressed:

-The authors need to more carefully dissect the biochemistry of Nup53 membrane binding in order to get a more coherent picture of this phenomenon.

-In order to be able to interpret correlations between membrane binding and protein functionality, the authors have to perform biochemical assays and functional (NPC assembly) assays with the same proteins. Additionally, since there is a huge gap in complexity between the liposome binding assay and the NPC assembly assay, the results need to be much more cautiously discussed and speculations that are not completely supported by data need to be moved to the discussion section.

-Both the interphase nuclear assembly and membrane curvature assays have to done more vigorously.

We have clarified these points in the above detailed response.

Referee #2

Despite multiples studies over the last years, the mechanisms contributing to NPC assembly are far from being fully understood. In particular, how nucleoporins interact and stabilize the pore membrane and to which extent post-mitotic NPC reassembly differs from NPC assembly during interphase remain debated.
Among the structural nucleoporins contributing to NPC assembly, Nup53 had previously been demonstrated to be required, along with several other members of the Nup93 complex, for post-mitotic NPC assembly in vertebrates.

Here, the authors re-investigate the implication of Nup53 in this process. Interestingly, they demonstrate that Nup53 can bind to liposomes in vitro, that is, independently of any other protein. Using a mutant that impairs the in vitro dimerization of Nup53 (designed based on the structure of the central RRM domain of Nup53 - aa 162-250, Handa et al, 2006), they next show that dimerization contributes to its membrane binding and is critical for post-mitotic NPC assembly.
They next identify two distinct membranes binding domains within Nup53, respectively within its N- and C-terminal domains, both of which further require dimerization of the RRM domain for efficient membrane binding:
- the N-terminal domain contains a pair of basic residues, whose mutation into acidic residues impairs membrane binding, without affecting the interaction of this domain with its previously know binding partners (Nup 93 and Nup205). Within this domain, they further identify two mitotic specific phosphorylation sites whose phosphorylation in vitro or mutation into negatively charged residues also affect membrane binding. This

suggests that the N-terminal domain of Nup53 interacts with membranes via electrostatic interactions with negatively-charged lipid head groups.
- The C-terminal domain was previously suggested to contain an amphipatic helix that could serve as a hydrophobic module. Here the authors show that deletion of the last C-term tryptophane impairs Nup53 membrane binding without affecting Ndc1 interaction, whereas deletion of the last 8 amino acids abolishes in vitro liposome binding (and Ndc1 interaction as previously reported by others).
By using these mutations, alone or in combination, they show that either one of the two membrane binding regions of Nup53 is sufficient for postmitotic NPC assembly and stability, whereas mutants defective in both membrane binding sites no longer support post-mitotic NPC/NE reassembly.
Finally, they show that solely removing the last C-terminal amino-acid of Nup53 prevents NPC assembly during interphase. Moreover, unlike the N-terminal domain, the C-terminal domain of Nup53 can induce tubulation of liposomes, a property lost upon deletion of the last tryptophane. The membrane deforming capacity of Nup53 is thus correlated to its critical role in interphase but no pos-mitotic NPC assembly.
Based on these data, they propose a model, that highlights the different contribution of Nup53 modes of interactions with membranes to post-mitotic and interphase NPC assembly.

Altogether, this is a very nice and impressive study, that provides an extensive dissection of the functional domains of Nup53 that contribute to NPC/NE assembly, and most importantly allows to build up a model that highlights the differences between post-mitotic versus interphase NPC assembly.
As detailed below, I however have a few comments that should be addressed before publication of this study.

Major comments
1) In the discussion p14, the authors indicate a clear discrepancy between their data, and a previous study demonstrating that " a fragment lacking the N-terminal region as well as the C-terminal 26 amino acids (that is hNup53 [167-300]) replaced endogenous Nup53 in nuclear assemblies in Xenopus egg extracts (Hawryluk-Gara et al, 2008), ... Indeed, this Nup53 fragment lacks both membrane binding regions identified in this study, whose combined deletion is reported here to affect post-mitotic NE/NPC reassembly.
The authors suggest that this discrepancy might be due to different Nup53 depletion (that is, presence of minor amounts of Nup53 in the experiments published by Hawryluk-Gara et al, 2008).
They further indicate, " Using a number of different Nup53 fragments which lacked the Nup93 binding region we were not able to replace endogenous Nup53 in NPC assembly (unpublished observation)".
To avoid any subsequent controversy, these data should be provided (eventually as supplemental figure). At least, the closest mutant they have in hand (xNup53 162-312, used in Fig S4B, that only binds Nup155) should be tested for complementation in their Nup53-depleted nuclear assembly assay. Indeed, one cannot formally exclude more complex hypotheses, such as a dominant phenotype induced by the replacement of positive with negative residues in the 1-312/1-319 R105EK106E mutants (or the addition of negative residues in the S94ET100E mutants).

We have included the data mentioned in the manuscript including the Nup53 162-312 fragment as a new supplementary Figure S6 suggesting that the Nup53-Nup93 is important for postmitotic NPC assembly. However, we agree that we cannot formally exclude more complicated hypotheses and therefore prefer not to overemphasize this set of data.

2) SDS-PAGE and silver staining of all recombinant proteins used in this study should be provided (eventually as supplemental figure), as done in figure 1B for some of them. Indeed, in Figure 1, the fact that the bacterial 70 kD protein co-purifying with Nup53 does not co-purifies with the 162-320 domain (that nevertheless binds to liposomes) is important to rule out the contribution of this bacterial protein in indirectly mediating the binding of Nup53 to liposomes.
This control of equal purity is important for Figure 6 since (although this is unlikely) one cannot formally exclude that specific Nup53 domains or mutants may co-purify with bacterial protein that would in turn affect (positively or negatively) membrane tubulation.

We have included the requested data as Figure S2 showing the purity of all recombinant proteins used in this study

3) Nup53 RRM/ dimerization mutant.

- Since this mutation maps within a domain know to interact with Nup155, the authors should analyze if introducing this mutation affects Nup155 binding (using GST pull-down on Nup53 [130-320 F172EW203E] construct used in Fig S3 or using a Nup53 [162-320 F172EW203E] as performed in fig S4 for the other mutants).

We have included the requested data as new supplementary Figure S1. Interestingly, although the RRM domain alone is not sufficient for binding Nup205, Nup93, Nup155 or NDC1, introducing the RRM dimerization mutation weakens the interaction to Nup205, Nup93 and Nup155 but not NDC1. We have therefore rephrased relevant passages in the manuscript.

- Assessing the effect of the RRM/dimerization mutant in both the interphase assembly assay (Fig 5., with the full -length Nup53 RRM mutant protein as in Fig 2B) and the membrane deformation assay (Figure 6, with the Nup53[130-320- F172EW203E] construct used in Fig S3) would have allowed to determine to which extent dimerization of the C-terminal domain is required for its function in interphase NPC assembly and liposome tubulation. Although not mandatory, this analysis could potentially have further strengthen their argument in the context of the current debate of the discrepancy (or not) between interphase and post-mitotic NPC assembly.

We have included the Nup53 130-320 F172E/W203E as well as the Nup53 93-320 F172E/W203E protein in the liposome tubulation assay. The RRM muationslargly reduces lipsome binding of both proteins (Figure S5) and we consistently do not detect membrane tubulation. Certainly, it would be interesting to monitor the effect of the RRM mutant for interphasic assembly but the experimental setup does not allow doing this. The replacement of endogenous Nup53 with this mutant blocks postmitotic nuclear reassembly and nuclear reformation. Therefore, interphasic NPC assembly does not occur on these chromatin templates as they do not possess a closed nuclear envelope.

- As Nup53 dimerization was so far only demonstrated in vitro (Handa et al, 2006 and Fig 2A in this study), did the authors try to determine if this interaction takes place in vivo?

We have performed transfection experiments in HeLa cells which show that wildtypeXenopus Nup53 is able to dimerize (or oligomerize as we cannot exclude higher oligemerization states) whereas the RRM mutant is defective in this. This data has been included as new panel B of Figure 2.

4) The implication of reticulons, that were similarly shown to induce membrane tubulation and to contribute to de novo NPC assembly should be discussed (Dawson et al, J Cell Biol. 2009 and ref therein).

We have included a discussion of this point.

Minors comments

1) The relative liposome binding of the 162-320 domain (compared to full length) could be provided (this is the only construct for which this information is not given).

We apologize for this mistake. We have included the data in Figure 3A.

2) Page 7, end of 1rst chapter: "Together with the liposome-binding assay, this (lack of complementation of the dimerization mutant) suggests that Nup53 membrane binding is important for NPC assembly..."
At that stage of the manuscript, this is just a correlation, and this sentence should be corrected as " suggests that Nup53 membrane binding could be important" or "Nup53 membrane binding or oligomerization is important...."

This is a valid point. We have changed the text accordingly.

3) Page 8, end of second chapter: dimerization via the RRM domain is necessary (for membrane binding). This is somehow overstated and should be rephrased since only a two-fold decrease is observed for the 130-230 FW mutant in Fig S3.

We have changed the sentence accordingly.

4) Page 14, first lines: "interaction with Ndc1 is not necessary for post-mitotic NPC assembly. ... possible explanation for this surprising finding might be that Nup53 can interact directly with membranes...." .
Since this was previously reported, this finding is not so surprising. In addition, the alternative hypothesis, that is, the fact that this finding may reflect protein-protein interaction should be indicated (for instance in yeast, Nup170 also interacts with Pom152 - see Marelli et al., 2001). Indeed, in fig 4B, the interaction between Nup53 [162-312] and Nup155 is observed is a cytosolic fraction while interaction with Ndc1 was independently tested using Triton X100 solubilized membranes; thus, on cannot exclude that in the context of the NPC/NE assembly reaction, Nup53 [162-312] may indirectly interact with Ncd1 via Nup155.

These are valid points. We have changed the paragraph accordingly.

5) How do the authors reconcile their data (2 membrane binding domains in the N/C-term domain of Nup53) with the linear arrangement of the Nups of this complex hypothesized by Amlacher et al. (2011) for the yeast counterparts ? This might be briefly discussed.

We don't think that the idea of two membrane binding domains in Nup53 is in conflict with the proposed linear arrangement of Nups in the Nic96/Nup93 complex. Indeed, our pulldown data (Figure S1) together with a number other studies (Hawryluk-Gara et al, 2008; Hawryluk-Gara et al, 2005; Lusk et al, 2002; Mansfeld et al, 2006; Sachdev et al, 2012) are consistent with the hypothesis of a linear arrangement of Nups. Both ideas can go well together: First, membrane binding might not interfere with any of the Nup interactions. Second, not all Nup53 proteins in the complex might show the same interaction pattern (e.g. a fraction of Nup53 might interact with Nup93/Nup205 whereas another fraction might bind NDC1 and/or the pore membrane). As we have not done experiments supporting any of these scenarios we prefer not to speculate about these.

6) The authors should mention somewhere in their manuscript that metazoan Nup53 is also termed Nup35 (nomenclature used by Handa et al., 2006; Rodenas 2009; Zuleger 2011...).

Thanks for this comment; we have included a note in the introduction.

Referee #3

Here the authors provide evidence that direct membrane binding by Nup53 is required for NPC assembly. They analyze deletion and point mutants of recombinant Nup53 for the ability to bind lipid vesicles and to support NE assembly in vitro in Xenopus egg extracts depleted of the endogenous protein. The approaches implemented here to measure protein binding to lipid vesicles, which are widely used by this field, typically measure low affinity interactions and are not necessarily biologically specific. Nonetheless the authors have done the controls that are commonly used for this assay and have shown a strong correlation between the ability to bind polar lipid vesicles in vitro and to support NE formation. Overall his study is well conducted and provides significant new mechanistic insight on NPC formation. It should be published in EMBO J with minor modifications.

1)The title "... depends on a direct interaction with membranes" seems a bit too strong with the present data. Although physiological relevance of the in vitro binding of Nup53 to polar lipid vesicle is strongly suggested, it is not completely proven. For example, the R105E/K106E mutations could disrupt Nup53 folding important for NE assembly (not revealed in the nucleoporin binding assays), as well as the proposed electrostatic interaction of basic amino acid residues with acidic phospholipid head groups. More subtle alanine mutations, less prone to generating misfolded protein, were not tested. Moreover, the authors haven't tested whether the tryptophan residue in the C-terminal peptide sequence actually inserts in the bilayer milieu, although this is a reasonable proposal.

We have no indication that the R105E/K106E (or other) mutations disrupt the folding of Nup53. As shown in Figure S1 the interaction partners Nup93 and Nup205 are still able to bind Nup53. The recombinant fragments and full length R105E/K106E mutants (1-312; 1-319; 1-320; 93-276; 93-320) showed no difference in size exclusion chromatography compared to their wild type counterparts. In addition, the full length and fragments of Nup53 containing the N-terminal mutations were able to substitute for endogenous Nup53 in postmitotic (Figure 4) and interphasic (Figure 5) NPC assembly.

The authors should consider softening the title somewhat, unless they can make a more compelling case for physiological relevant bilayer association of the indicated regions. However, I don't think more experimental work is needed to publish this study.

We have changed and “softened” the title

2) Since the insertion of the C-terminal tryptophan into the nuclear membrane bilayer would be expected to induce positive membrane curvature as shown in Fig. 7, it would be helpful for a discussion of how Nup53 by itself could give rise to the invaginated proposed intermediate structure with negative curvature.

We have included a discussion of this topic in the manuscript.

3) The authors don't explain why they use E. coli polar lipids for their assay, instead of lipids from a higher eukaryotic source similar to Xenopus, although they hint at use of other lipids in the Discussion. Are tubules as in Fig. 6 obtained with vertebrate-like lipids?

The experiments shown in Figure 6 were done with liposomes made of Folch fraction I lipids which are derived from bovine brain extracts. The membrane binding of Nup53 was also tested with liposomes prepared of Folchfraction I lipids or a mixture of lipids resembling the ER/NE lipid composition (Figure S7) and no difference was found.

Other minor points:

- More details of liposome preparation in Material and Methods would be helpful.

We have provided more detailed information in the Material and Methods section and in the Supplementary Material.

- Labeling of gel lanes in Fig. 1B could be improved, as it is a bit difficult to grasp the data at first glance because of the crowded display.

We have rearranged these and other parts of Figure 1.

- It's a bit difficult to follow the mutant nomenclature (e.g. S94ET100E) and the authors should consider alternative presentations such as S94E/T100E or S94E,T100E

We have changed the nomenclature in the manuscript

2nd Editorial Decision 17 August 2012

Thank you for sending us your revised manuscript. Referee 2 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner. You may wish to follow the suggestions he/she puts forward in the light of the new data (please see below).

Furthermore, prior to formal acceptance, there are two editorial issues that need further attention:

* You mention that figure 2C is based on n=2. In the light of this low sample number, we need to ask you to either show one representative experiment and to indicate this in the figure legend or to use an alternative presentation for the data that shows the two individual values.

Thank you for your kind cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

In this revised version, the authors have done an impressive job and have answered to all my previous concerns by performing multiple additional experiments. In addition, they also answers to what I considered to be the most relevant comments of the other reviewers (except perhaps the title, see reviewer 3 and comments below). I thus consider that this manuscript thus now deserves to be published without any further delay.

My only minor comment would be that in view of the new data, the authors should highlight the contribution of Nup53 dimerization in NPC assembly (best in the title, and at least in the abstract). A potential title better reflecting this study might be:
"Dimerization and direct interaction of Nup53 with membranes contribute to nuclear pore (complex) assembly"

In the abstract (that could be a bit longer, and include dimerization data), the sentence " Most NPC proteins do not contain integral membrane domains and thus it is largely unclear how NPCs are embedded in the nuclear envelope." appears to be a bit out of the topic of this manuscript, which is mainly related to NPC assembly.

Last sentence of the abstract: The vertebrate protein comprises two membrane binding sites, of which the Cterminal one has membrane deforming capabilities "which are" crucial for de novo NPC assembly and insertion into intact nuclear envelopes during interphase.
As there is no direct proof that it is indeed the membrane deforming capability of Nup53 that contributes to de novo NPC assembly, "which are" should be replaced by "and is".

p15, the sentence "could be a possible mechanism how Nup53 is linked to the pore membrane which...." is complicated and could be replaced by " could be a possible mechanism linking to the pore membrane which...".

2nd Revision - authors' response 20 August 2012

Please find enclosed our modified revised manuscript “Dimerization and direct membrane interaction of Nup53 contribute to nuclear pore complex assembly” for resubmission as an article to The EMBO Journal.

Following our discussion we show now one representative data point in Figure 2C (the one that corresponds to the presented gel) and mention in the legend that this is one of two experiments.

Response to the 2nd referee

My only minor comment would be that in view of the new data, the authors should highlight the contribution of Nup53 dimerization in NPC assembly (best in the title, and at least in the abstract).A potential title better reflecting this study might be:"Dimerization and direct interaction of Nup53 with membranes contribute to nuclear pore (complex) assembly"

We have done so, but included a “the” according to a native speaker’s advice.

In the abstract (that could be a bit longer, and include dimerization data),

We have done this.

the sentence " Most NPC proteins do not contain integral membrane domains and thus it is largely unclear how NPCs are embedded in the nuclear envelope." appears to be a bit out of the topic of this manuscript, which is mainly related to NPC assembly.

We regard this as an important point as the transmembrane protein - nucleoporin interactions are not sufficient to explain how NPCs are linked to the membranes (see also the Introduction of the manuscript). We hope our point is clarified by adding “and anchored”.

Last sentence of the abstract: The vertebrate protein comprises two membrane binding sites, of which the Cterminal one has membrane deforming capabilities "which are" crucial for de novo NPC assembly and insertion into intact nuclear envelopes during interphase. As there is no direct proof that it is indeed the membrane deforming capability of Nup53 that contributes to de novo NPC assembly, "which are" should be replaced by "and is".

The sentence is changed accordingly.

p15, the sentence "could be a possible mechanism how Nup53 is linked to the pore membrane which...." is complicated and could be replaced by " could be a possible mechanism linking to the pore membrane which...".

The sentence is changed accordingly.