Distinct domains in Ndc1 mediates its interaction with the Nup84 complex and the nuclear membrane

Ingo Amm, Marion Weberruss, Andrea Hellwig, Johannes Schwarz, Marianna Tatarek-Nossol, Christian Lüchtenborg, Martina Kallas, Britta Brügger, Ed Hurt, and Wolfram Antonin

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Revision 0

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Ndc1 is a transmembrane nucleoporin, essential for insertion of the nuclear pore complex (NPC) and spindle pole body (SPB) into the nuclear envelope (NE). How NE-associated proteins contribute to the bending and fusion of membranes during NPC insertion has not been fully elucidated. Here, the authors report a number of loosely connected, interesting observations related to Ndc1 function. Their main findings are the following: (i) The N-terminal transmembrane domain of Ndc1 mediates the membrane recruitment of two Y-complex nucleoporins. Therefore, these interactions are likely to contribute to NPC biogenesis. (ii) Over-expression of a novel amphipathic helix (AH) in the non-essential C-terminus of Ndc1, and of a similar AH in the non-essential nucleoporin Nup53, alters the lipid composition and nuclear morphology of yeast cells, although the underlying mechanisms remain unknown. (iii) The essential function of Ndc1 can be suppressed by deleting the amphipathic helix from Nup53, or by deleting the transmembrane nucleoporin POM34. Surviving strains have altered nuclear morphology (NE expansions), and are sensitive to membrane-fluidizing drugs, suggesting that NPC assembly is somehow linked to lipid homeostasis.

Overall, the experiments are of high technical quality, are presented in a clear way, and the conclusions are well-supported by the data. I have some minor suggestions for clarifications, which can be addressed by textual changes or by additional experiments.

1. When overexpressed in budding yeast, the C-terminal domain of Ndc1 is toxic and induces membrane expansion with NPC-like openings, which the authors describe as enlarged ER membranes (Figure 2). Could these be NE expansions instead? ER and NE membranes are continuous but perhaps this issue could be addressed by examining the distribution of fluorescent markers specific for each compartment.

2. The essential function of Ndc1 can be suppressed by deleting the amphipathic helix from Nup53 or by deleting POM34. These experiments are done using a plasmid shuffle strategy, in which Ndc1 is temporarily expressed from a low copy plasmid. I wonder if surviving strains are stable, or whether they survive for a limited time only due to stabilisation of the Ndc1 protein in the absence of Nup53 or Pom34. Could the authors discard this possibility, for example by checking whether viable double mutants are recovered after backcrossing of the survivor strains?

3. Cells over-expressing Ndc1, and surviving ndc1-delta strains display ER and/or NE expansions. It would be interesting to discuss these observations in the context of nuclear morphology studies by the Cohen-Fix and Liakopoulos labs, among others, showing NE expansion is partially dependent on the coordination between lipid synthesis, cell growth rate,

and cell cycle progression (doi: 10.1091/mbc.E18-04-0204, 10.1091/mbc.e05-09-0839, 10.1016/j.cub.2012.04.022).

4. Related to the previous point: nuclear membrane expansions caused by metaphase arrest usually overlap with the nucleolus, and appear DAPI-negative. Did the authors examine nucleolar distribution relative to NE expansion in cells shown in figure 4C? Along the same lines, what is the cell cycle distribution of cells with ER/NE expansion? If they are delayed in mitosis, nuclear morphology defects may be a secondary consequence of cell cycle progression defects, themselves due to NPC and/or SPB insertion problems.

5. I suggest to rephrase the last sentence of the abstract: "nuclear membrane biogenesis dependent on a balanced ratio between amphipathic motifs in diverse nucleoporins is essential for interphase NPC biogenesis". This study does not directly assess NPC biogenesis and therefore, the interesting link between lipids and NPC biogenesis remains correlative.

6. It would be useful to include some information on the number of cells observed in the EM figures.

7. Results, first page: "Moreover, CtNup120 and CtNup133 did not associate with GUVs containing the unrelated inner nuclear membrane protein BC08/SCL1 (Fig. 1C)" should be Figure S1C.

8. P. 19: "Prompted by the finding that Ndc1 and Nup53/Nup59 amphipathic motifs may (modify?) the nuclear ... "

I am an expert in yeast genetics and cell cycle progression.

2. Significance:

Significance (Required)

Significance: This report describes novel functional motifs in the Ndc1 protein that may be important for NPC assembly, and intriguing genetic interactions between NPC assembly and lipid homeostasis pathways. Although the mechanisms linking Ndc1 motifs with NE expansion and lipid composition remain unclear, these observations will be interesting for researchers working on NPC biogenesis and nuclear morphology.

Reviewer Expertise: yeast genetics, cell cycle progression and NPCs.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

4. Review Commons values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at Publons; note that the content of your review will not be visible on Publons.

Reviewer Publons

Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Amm et al report on the role of new motifs and interactions between the essential and conserved integral nuclear pore membrane protein Ndc1 and other key components of the yeast nuclear pore complex. They show that members of the Y-subcomplex that coats the pore membrane bind directly to Ndc1 and identify an amphipathic helix at the C-terminus of Ndc1 that displays genetic interactions with other nucleoporins carrying analogous amphipathic helices. The authors find that cells can survive without Ndc1 when these related amphipathic helices from other nups are coincidentally deleted.

Despite significant recent advances in our structural understanding of the nuclear pore complex, how the NPC associates with the curved nuclear membrane remains poorly understood. Previous studies in yeast have uncovered significant redundancy in this association but again the basis for this remains unclear. Therefore, I find this study on the amphipathic helix of Ndc1 and its interaction with other membrane binding components of the NPC an important and timely contribution to the field. Technically, the paper is solid and I find that most of the authors' conclusions are well supported by the evidence they provide (but see below for few experimental issues). Overall, the paper is well written, and despite the use of several mutants and methodologies, it is easy to read. I think the paper's significance would improve if the authors could present some "larger picture" view on how the Ndc1 helix and/or domains they describe interact with the Nup84 complex and the pore membrane or other elements of the NPC. For example, the authors make the remarkable finding that removal of Nup53 makes ndc1 nulls able to survive. Would it be possible to use existing models of the yeast NPC and provide some

structural explanation of why that is? However, I would like to emphasize that this is not required to support the main claims of the paper and should only be considered if the authors wish to provide a more "molecular" view of their findings.

Specific experimental issues and clarifications:

- A major part of the manuscript describes a detailed structure-function analysis of Ndc1. The link between the two domains of ScNdc1 studied and their effects on membrane proliferation could be better defined: specifically, can the authors exclude that the N-domain of Ndc1 that includes its transmembrane domain, is not also involved in the membrane proliferation phenotype shown in Fig2A and C? It also seems as if GAL-ProtA-ScNdc1 (1-260) also causes growth inhibition (Fig. 2F). How do cells with GAL-ProtA-ScNdc1 (1-260) look like? Finally, although the authors convincingly show that overexpression of 261-655 inhibits growth, from the EM it seems as its effects on membrane proliferation is not the same as that of the overexpression of full-length Ndc1 (compare Fig. 3D vs Fig. 2D).

- Figure 1A: Do the CtNups shown under "Input" represent 100% of what used in the binding reaction? If so, please indicate at the figure.

- CtNup120 and CtPom133 would migrate close to CtPom152, which could make visualization by Coomassie stain a bit tricky - if the authors could provide SDS PAGE gels with lower %, that would be helpful. Along similar lines, how do the authors know that CtNup120beta does not bind the CtNdc1 if these two appear to migrate at the same size (Fig. 1D)?

- Figure 1B, GUVs: Why do the authors use CtNup85 for the GUV experiment instead of CtNup84 that was used in Fig. 1A?

- Moreover, CtNup120 and CtNup133 ...BC08/SCL1 (Fig. 1C)" Don't see this in Fig. 1C

- The imaging of ProtA-AHNdc1-eGFP (Fig. 3C) is not great and the localization of the AH does not look very clear - can the authors provide better micrographs? Perhaps co-expression of a red ER reporter or similar reporter would also help.

- The ndc1 nup53 double mutant appears to display a striking cold-sensitive growth defect (Supplemental Figure 6A, compare 23 vs 30C). Can the authors comment on this?

2. Significance:

Significance (Required)

Despite significant recent advances in our structural understanding of the nuclear pore complex, how the NPC associates with the curved nuclear membrane remains poorly understood. Previous studies in yeast have uncovered significant redundancy in this association but again the basis for this remains unclear. Therefore, I find this study on the amphipathic helix of Ndc1 and its interaction with other membrane binding components of the NPC an important and timely contribution to the field. Technically, the paper is solid and I find that most of the authors'

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Audience: Mostly the following - Nuclear pore complex, nuclear envelope, and possibly some membrane biologists.

My field of expertise: Cell biology, Nuclear envelope.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

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Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In recent years, significant progress has been made in defining the molecular details of many structural features of the nuclear pore complex (NPC). However, one area that remains ill-defined is the interface between the core structures of the NPC and the pore membrane domain. This is an especially intriguing area when one considers that the NPC contains several integral proteins and numerous peripheral membrane proteins contain amphipathic helices whose functions and interactions with the membrane, as well as with one another, remain largely undefined.

In this manuscript by Amm et al., the authors have examined the functional role of the integral membrane Nup Ndc1 and its interactions with various peripheral membrane Nups, including members of the Nup84 complex (termed the Y-complex) and the linker Nups Nup53 and Nup59. The authors show that Ndc1 interacts with specific members of the Nup84 complex, namely Nup120 and Nup133, supporting the idea that Ndc1 functions, in part, to anchor this NPC substructure to the pore membrane. In addition, they identified an amphipathic helix (AH) within the C-terminal half of Ndc1, and they showed that it can directly bind to membranes. Importantly, they have used genetic assays to show that the Ndc1-AH functionally interacts with AHs present at the C-terminus of Nup53 and Nup59. Strikingly, they show that the lethal phenotype detected in strains lacking Ndc1 can be suppressed by the deletion of NUP53, but not NUP59, and, more specifically, only the loss of the C-terminal AH Nup53 was required to suppress the lethal phenotype of the ndc1 null mutation. Further ultrastructural analysis of these mutants revealed that, while these mutants were viable, they exhibited extensive NE expansion phenotypes.

Overall, the data presented in this manuscript are of high quality, and the experiments are well controlled. My specific comments are relatively minor and listed below.

Minor points

1) The authors state "Serial ultrathin sections of fixed yeast cells overexpressing ProtA-CtNdc1 revealed that these unusual extranuclear membrane proliferations exhibited pore-like structures with diameters similar to the diameter of NPCs within the nuclear membrane (Fig. 2C)." This is not entirely clear from the data. I suggest the authors provide direct measurements that support their statement.

2) The authors examined the total cellular lipid content following overexpression of Ndc1-AH-containing constructs, as well as ProtA-ScHmg1. There is little discussion of the significance of these results, which would provide a clear justification for including these data in the manuscript.

3) There are numerous typographical and grammatical errors throughout the manuscript that need to be addressed.

2. Significance:

Significance (Required)

The results presented in this manuscript provide further insight into the molecular interactions between Nups and the pore membrane. They suggest that AHs present in a subset of Nups perform linked functions and contribute, in part, to nuclear membrane biogenesis. As such, these results are an important advance in our knowledge of NPC structure and function. They will be of general interest to those studying the function of NPCs and, more generally, NE and organelle biogenesis.

Reviewer expertise: NPC structure and function, NE biogenesis, yeast model system.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

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Revision Plan

Manuscript number: RC-2022-01613
Corresponding author(s): Wolfram Antonin

1. General Statements [optional]

We thank the three reviewer for their helpful and positive evaluation. We will address the specific points as specified below.

2. Description of the planned revisions

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

Ndc1 is a transmembrane nucleoporin, essential for insertion of the nuclear pore complex (NPC) and spindle pole body (SPB) into the nuclear envelope (NE). How NE-associated proteins contribute to the bending and fusion of membranes during NPC insertion has not been fully elucidated. Here, the authors report a number of loosely connected, interesting observations related to Ndc1 function. Their main findings are the following: (i) The N-terminal transmembrane domain of Ndc1 mediates the membrane recruitment of two Y-complex nucleoporins. Therefore, these interactions are likely to contribute to NPC biogenesis. (ii) Over-expression of a novel amphipathic helix (AH) in the non-essential C-terminus of Ndc1, and of a similar AH in the non-essential nucleoporin Nup53, alters the lipid composition and nuclear morphology of yeast cells, although the underlying mechanisms remain unknown. (iii) The essential function of Ndc1 can be suppressed by deleting the amphipathic helix from Nup53, or by deleting the transmembrane nucleoporin POM34. Surviving strains have altered nuclear morphology (NE expansions), and are sensitive to membrane-fluidizing drugs, suggesting that NPC assembly is somehow linked to lipid homeostasis.

Overall, the experiments are of high technical quality, are presented in a clear way, and the conclusions are well-supported by the data. I have some minor suggestions for clarifications, which can be addressed by textual changes or by additional experiments.

> Our answer: We thank the reviewer for this positive evaluation. We will address the specific points as specified below.

1. When overexpressed in budding yeast, the C-terminal domain of Ndc1 is toxic and induces membrane expansion with NPC-like openings, which the authors describe as enlarged ER membranes (Figure 2). Could these be NE expansions instead? ER and NE membranes are continuous but perhaps this issue could be addressed by examining the distribution of fluorescent markers specific for each compartment.

Our answer: Membrane expansion is seen upon overexpression of full-length Ndc1. We will clarify this point in the manuscript. To address the question of an ER/NE membrane expansion phenotype we will employ Sec63-mCherry or HDEL-dsRed as an ER/NE marker in this experiment.

2. The essential function of Ndc1 can be suppressed by deleting the amphipathic helix from Nup53 or by deleting POM34. These experiments are done using a plasmid shuffle strategy, in which Ndc1 is temporarily expressed from a low copy plasmid. I wonder if surviving strains are stable, or whether they survive for a limited time only due to stabilisation of the Ndc1 protein in the absence of Nup53 or Pom34. Could the authors discard this possibility, for example by checking whether viable double mutants are recovered after backcrossing of the survivor strains?

Our answer: This is not a transient effect, but the strains are stable over a longer growth period and indeed continuously grow. We will mention this in the revised manuscript.

3. Cells over-expressing Ndc1, and surviving ndc1-delta strains display ER and/or NE expansions. It would be interesting to discuss these observations in the context of nuclear morphology studies by the Cohen-Fix and Liakopoulos labs, among others, showing NE expansion is partially dependent on the coordination between lipid synthesis, cell growth rate, and cell cycle progression (doi: 10.1091/mbc.E18-04-0204, 10.1091/mbc.e05-09-0839, 10.1016/j.cub.2012.04.022).

Our answer: The membrane phenotypes described in these publications are different from what we report here (see also point 4). We will clarify this point and include it in the discussion.

4. Related to the previous point: nuclear membrane expansions caused by metaphase arrest usually overlap with the nucleolus, and appear DAPI-negative. Did the authors examine nucleolar distribution relative to NE expansion in cells shown in figure 4C? Along the same lines, what is the cell cycle distribution of cells with ER/NE expansion? If they are delayed in mitosis, nuclear morphology defects may be a secondary consequence of cell cycle progression defects, themselves due to NPC and/or SPB insertion problems.

Our answer: The membrane phenotypes the reviewer is referring to are different from what we report here (see also point 3), although the membrane expansions are DAPI negative (see e.g. section II). We will mention this in the text, but will also set these nuclear membrane expansions in relation to the nucleolus as suggested using the Nop1 nucleolar marker (Nop1-mRFP, in combination with Pus1-eGFP as nuclear marker and eGFP-Nup49 as NPC marker). We will also check whether cell cycle progression is affected in this experiment.

5. I suggest to rephrase the last sentence of the abstract: "nuclear membrane biogenesis dependent on a balanced ratio between amphipathic motifs in diverse nucleoporins is essential for interphase NPC biogenesis". This study does not directly assess NPC biogenesis and therefore, the interesting link between lipids and NPC biogenesis remains correlative.

Our answer: We will rephrase this to: “Our data indicate that nuclear membrane and presumably NPC biogenesis depends on a balanced ratio between amphipathic motifs in diverse nucleoporins.

6. It would be useful to include some information on the number of cells observed in the EM figures.

Our answer: we will include this information

7. Results, first page: "Moreover, CtNup120 and CtNup133 did not associate with GUVs containing the unrelated inner nuclear membrane protein BC08/SCL1 (Fig. 1C)" should be Figure S1C.

Our answer: This will be corrected.

8. P. 19: "Prompted by the finding that Ndc1 and Nup53/Nup59 amphipathic motifs may (modify?) the nuclear ... "

Our answer: This will be corrected to “Prompted by the finding that Ndc1 and Nup53/Nup59 amphipathic motifs may affect nuclear membrane curvature at the NPC insertion sites in a coordinated fashion…”

Reviewer #1 (Significance (Required)):

Significance: This report describes novel functional motifs in the Ndc1 protein that may be important for NPC assembly, and intriguing genetic interactions between NPC assembly and lipid homeostasis pathways. Although the mechanisms linking Ndc1 motifs with NE expansion and lipid composition remain unclear, these observations will be interesting for researchers working on NPC biogenesis and nuclear morphology.

Revision Plan

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

Amm et al report on the role of new motifs and interactions between the essential and conserved integral nuclear pore membrane protein Ndc1 and other key components of the yeast nuclear pore complex. They show that members of the Y-subcomplex that coats the pore membrane bind directly to Ndc1 and identify an amphipathic helix at the C-terminus of Ndc1 that displays genetic interactions with other nucleoporins carrying analogous amphipathic helices. The authors find that cells can survive without Ndc1 when these related amphipathic helices from other nups are coincidentally deleted.
Despite significant recent advances in our structural understanding of the nuclear pore complex, how the NPC associates with the curved nuclear membrane remains poorly understood. Previous studies in yeast have uncovered significant redundancy in this association but again the basis for this remains unclear. Therefore, I find this study on the amphipathic helix of Ndc1 and its interaction with other membrane binding components of the NPC an important and timely contribution to the field. Technically, the paper is solid and I find that most of the authors' conclusions are well supported by the evidence they provide (but see below for few experimental issues). Overall, the paper is well written, and despite the use of several mutants and methodologies, it is easy to read. I think the paper's significance would improve if the authors could present some "larger picture" view on how the Ndc1 helix and/or domains they describe interact with the Nup84 complex and the pore membrane or other elements of the NPC. For example, the authors make the remarkable finding that removal of Nup53 makes ndc1 nulls able to survive. Would it be possible to use existing models of the yeast NPC and provide some structural explanation of why that is? However, I would like to emphasize that this is not required to support the main claims of the paper and should only be considered if the authors wish to provide a more "molecular" view of their findings.

> Our answer: We thank the reviewer for this positive evaluation. We will follow this suggestion and will include a more extensive discussion in the manuscript on how amphipathic helices in nucleoporins could contribute to NPC assembly and/or structure to present the “larger picture”. We will address the specific points as specified below.

Specific experimental issues and clarifications:

- A major part of the manuscript describes a detailed structure-function analysis of Ndc1. The link between the two domains of ScNdc1 studied and their effects on membrane proliferation could be better defined: specifically, can the authors exclude that the N-domain of Ndc1 that includes its transmembrane domain, is not also involved in the membrane proliferation phenotype shown in Fig2A and C? It also seems as if GAL-ProtA-ScNdc1 (1-260) also causes growth inhibition (Fig. 2F). How do cells with GAL-ProtA-ScNdc1 (1-260) look like? Finally, although the authors convincingly show that overexpression of 261-655 inhibits growth, from the EM it seems as its effects on membrane proliferation is not the same as that of the overexpression of full-length Ndc1 (compare Fig. 3D vs Fig. 2D).

Our answer: The reviewer is right that overexpression of full-length Ndc1 and Ndc1 261-655 show different membrane proliferation phenotypes. And, indeed, overexpression of GAL-ProtA-ScNdc1 (1-260) shows a growth defect. We will clarify these points in the manuscript, and include EM pictures showing cells overexpressing the N-terminal part of Ndc1 that reveals proliferation of membranes typically observed upon overexpression of membrane proteins.

- Figure 1A: Do the CtNups shown under "Input" represent 100% of what used in the binding reaction? If so, please indicate at the figure.

Our answer: Will be indicated.

- CtNup120 and CtPom133 would migrate close to CtPom152, which could make visualization by Coomassie stain a bit tricky - if the authors could provide SDS PAGE gels with lower %, that would be helpful. Along similar lines, how do the authors know that CtNup120beta does not bind the CtNdc1 if these two appear to migrate at the same size (Fig. 1D)?

Our answer: We will include additional experiments aiming to resolve these points.

- Figure 1B, GUVs: Why do the authors use CtNup85 for the GUV experiment instead of CtNup84 that was used in Fig. 1A?

Our answer: When we started these experiments, we used CtNup85, which was available earlier than a corresponding CtNup84 construct, which accordingly served as negative control for this GUV experiment. Both Nup85 and Nup84 are part of the Y-complex and thus are both appropriate controls.

- Moreover, CtNup120 and CtNup133 ...BC08/SCL1 (Fig. 1C)" Don't see this in Fig. 1C

Our answer: This will be corrected and should read “Fig. S1C”

- The imaging of ProtA-AHNdc1-eGFP (Fig. 3C) is not great and the localization of the AH does not look very clear - can the authors provide better micrographs? Perhaps co-expression of a red ER reporter or similar reporter would also help.

Our answer: According to our experience in the yeast system, this are GFP fluorescence pictures of yeast cells, exhibiting a predominant cytoplasmic staining with vacuolar exclusion, or a non-cytoplasmic staining with patchy structures close to the plasma membrane, which appears to correspond to a cortical ER staining. We will include magnified insets to highlight the GFP marker distribution between these two strains.

- The ndc1 nup53 double mutant appears to display a striking cold-sensitive growth defect (Supplemental Figure 6A, compare 23 vs 30C). Can the authors comment on this?

Our answer: Thanks for pointing at this. The Ndc1/Nup53 double mutant shows indeed a cold-sensitive phenotype. These phenotypes are often observed in the case of defective assembly processes, which are in particular sensitive to low temperatures. We will include a comment in the manuscript.

Reviewer #2 (Significance (Required)):

Despite significant recent advances in our structural understanding of the nuclear pore complex, how the NPC associates with the curved nuclear membrane remains poorly understood. Previous studies in yeast have uncovered significant redundancy in this association but again the basis for this remains unclear. Therefore, I find this study on the amphipathic helix of Ndc1 and its interaction with other membrane binding components of the NPC an important and timely contribution to the field. Technically, the paper is solid and I find that most of the authors' conclusions are well supported by the evidence they provide (but see below for few experimental issues). Overall, the paper is well written, and despite the use of several mutants and methodologies, it is easy to read. I think the paper's significance would improve if the authors could present some "larger picture" view on how the Ndc1 helix and/or domains they describe interact with the Nup84 complex and the pore membrane or other elements of the NPC. For example, the authors make the remarkable finding that removal of Nup53 makes ndc1 nulls able to survive. Would it be possible to use existing models of the yeast NPC and provide some structural explanation of why that is? However, I would like to emphasize that this is not required to support the main claims of the paper and should only be considered if the authors wish to provide a more "molecular" view of their findings.

Our answer: As outlined above, we will follow the suggestion and include a more extensive discussion in the manuscript on how amphipathic helices in nucleoporins could contribute to NPC assembly and/or structure to present the “larger picture”.

Audience: Mostly the following - Nuclear pore complex, nuclear envelope, and possibly some membrane biologists.

Revision Plan

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

In recent years, significant progress has been made in defining the molecular details of many structural features of the nuclear pore complex (NPC). However, one area that remains ill-defined is the interface between the core structures of the NPC and the pore membrane domain. This is an especially intriguing area when one considers that the NPC contains several integral proteins and numerous peripheral membrane proteins contain amphipathic helices whose functions and interactions with the membrane, as well as with one another, remain largely undefined.

In this manuscript by Amm et al., the authors have examined the functional role of the integral membrane Nup Ndc1 and its interactions with various peripheral membrane Nups, including members of the Nup84 complex (termed the Y-complex) and the linker Nups Nup53 and Nup59. The authors show that Ndc1 interacts with specific members of the Nup84 complex, namely Nup120 and Nup133, supporting the idea that Ndc1 functions, in part, to anchor this NPC substructure to the pore membrane. In addition, they identified an amphipathic helix (AH) within the C-terminal half of Ndc1, and they showed that it can directly bind to membranes. Importantly, they have used genetic assays to show that the Ndc1-AH functionally interacts with AHs present at the C-terminus of Nup53 and Nup59. Strikingly, they show that the lethal phenotype detected in strains lacking Ndc1 can be suppressed by the deletion of NUP53, but not NUP59, and, more specifically, only the loss of the C-terminal AH Nup53 was required to suppress the lethal phenotype of the ndc1 null mutation. Further ultrastructural analysis of these mutants revealed that, while these mutants were viable, they exhibited extensive NE expansion phenotypes.

Overall, the data presented in this manuscript are of high quality, and the experiments are well controlled. My specific comments are relatively minor and listed below.

> Our Answer: We thank the reviewer for this positive evaluation. We will address the specific points as specified below.

Minor points.

1) The authors state "Serial ultrathin sections of fixed yeast cells overexpressing ProtA-CtNdc1 revealed that these unusual extranuclear membrane proliferations exhibited pore-like structures with diameters similar to the diameter of NPCs within the nuclear membrane (Fig. 2C)." This is not entirely clear from the data. I suggest the authors provide direct measurements that support their statement.

> Our answer: We have analyzed the pore diameters and will include these numbers in the manuscript (NPC diameter: 67.1 ± 2.3 nm (mean ± SD; n = 20), diameter of extranuclear pore like structures: 44.8 ± 3.4 nm (mean ± SD; n = 20)). Of note, the smaller diameter

of the pore-like structures likely reflects the fact that these structures do not contain the NPC membrane coat. Recent molecular dynamics simulations (DOI: 10.1126/science.abm9506) suggest that this membrane coat would widen the pore diameter, consistent with our data. We will discuss this in the manuscript.

2) The authors examined the total cellular lipid content following overexpression of Ndc1-AH-containing constructs, as well as ProtA-ScHmg1. There is little discussion of the significance of these results, which would provide a clear justification for including these data in the manuscript.

Our answer: We will discuss this more thoroughly. As expected, the lipid profiles correlate nicely with membrane proliferation phenotype.

3) There are numerous typographical and grammatical errors throughout the manuscript that need to be addressed.

Our answer: We will ask a native speaker to check our finally revised manuscript.

Reviewer #3 (Significance (Required)):

The results presented in this manuscript provide further insight into the molecular interactions between Nups and the pore membrane. They suggest that AHs present in a subset of Nups perform linked functions and contribute, in part, to nuclear membrane biogenesis. As such, these results are an important advance in our knowledge of NPC structure and function. They will be of general interest to those studying the function of NPCs and, more generally, NE and organelle biogenesis.

Reviewer expertise: NPC structure and function, NE biogenesis, yeast model system.

3. Description of the revisions that have already been incorporated in the transferred manuscript

We have not yet changed the manuscript.

4. Description of analyses that authors prefer not to carry out

Does not apply

October 24, 2022

Re: JCB manuscript #202210059T

Prof. Wolfram Antonin
RWTH University
Institute for Biochemistry and Molecular Cell Biology
Pauwelsstraße 30
Aachen 52074
Germany

Dear Prof. Antonin,

Thank you for submitting your manuscript entitled "New motifs in Ndc1 mediating interaction with the Nup84 complex and nuclear membranes". We have assessed your manuscript, the reviews from Review Commons, and your revision plan. We think that your work is interesting and would like to invite you to submit a revision if you can address the reviewers' key concerns, as outlined in your revision plan.

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

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Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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

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

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

Sincerely,

Michael Rout
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

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

Ndc1 is a transmembrane nucleoporin, essential for insertion of the nuclear pore complex (NPC) and spindle pole body (SPB) into the nuclear envelope (NE). How NE-associated proteins contribute to the bending and fusion of membranes during NPC insertion has not been fully elucidated. Here, the authors report a number of loosely connected, interesting observations related to Ndc1 function. Their main findings are the following: (i) The N-terminal transmembrane domain of Ndc1 mediates the membrane recruitment of two Y-complex nucleoporins. Therefore, these interactions are likely to contribute to NPC biogenesis. (ii) Over-expression of a novel amphipathic helix (AH) in the non-essential C-terminus of Ndc1, and of a similar AH in the non-essential nucleoporin Nup53, alters the lipid composition and nuclear morphology of yeast cells, although the underlying mechanisms remain unknown. (iii) The essential function of Ndc1 can be suppressed by deleting the amphipathic helix from Nup53, or by deleting the transmembrane nucleoporin POM34. Surviving strains have altered nuclear morphology (NE expansions), and are sensitive to membrane-fluidizing drugs, suggesting that NPC assembly is somehow linked to lipid homeostasis.

Overall, the experiments are of high technical quality, are presented in a clear way, and the conclusions are well-supported by the data. I have some minor suggestions for clarifications, which can be addressed by textual changes or by additional experiments.

> Our answer: We thank the reviewer for this positive evaluation. We have addressed the specific points as specified below.

1. When overexpressed in budding yeast, the C-terminal domain of Ndc1 is toxic and induces membrane expansion with NPC-like openings, which the authors describe as enlarged ER membranes (Figure 2). Could these be NE expansions instead? ER and NE membranes are continuous but perhaps this issue could be addressed by examining the distribution of fluorescent markers specific for each compartment.

> Our answer: In Figure 2 and now also Figure 3, membrane expansion is seen upon overexpression of full-length Ndc1. We clarified this point in the manuscript. To address the question of an ER/NE membrane expansion phenotype upon overexpression of the C-terminal domain of Ndc1 but also full-length Ndc1 we have employed DsRed-HDEL in the experiments presented in Fig. 3, 4, and S3.

2. The essential function of Ndc1 can be suppressed by deleting the amphipathic helix from Nup53 or by deleting POM34. These experiments are done using a plasmid shuffle strategy, in which Ndc1 is temporarily expressed from a low copy plasmid. I wonder if surviving strains are stable, or whether they survive for a limited time only due to stabilisation of the Ndc1 protein in the absence of Nup53 or Pom34. Could the authors discard this possibility, for example by checking whether viable double mutants are recovered after backcrossing of the survivor strains?

> Our answer: This is not a transient effect. The strains are stable over a longer growth period and can be maintained by continual re-streaking on YPD plates or FOA media. We have included this information in the revised manuscript.

3. Cells over-expressing Ndc1, and surviving ndc1-delta strains display ER and/or NE expansions. It would be interesting to discuss these observations in the context of nuclear morphology studies by the Cohen-Fix and Liakopoulos labs, among others, showing NE expansion is partially dependent on the coordination between lipid synthesis, cell growth rate, and cell cycle progression (doi: 10.1091/mbc.E18-04-0204, 10.1091/mbc.e05-09-0839, 10.1016/j.cub.2012.04.022).

Our answer: This is a very valid point. Indeed, the NE expansions resemble "flares" as seen upon deletion of SPO7, NEM1 or genes linked to cell cycle progression. As suggested in point 4 we have examined nucleolar distribution relative to NE expansion using Nop1-mRFP as a nucleolar marker in combination with Pus1-eGFP as a nuclear marker. These data are now included in Figure Fig. 6D and S5B.

4. Related to the previous point: nuclear membrane expansions caused by metaphase arrest usually overlap with the nucleolus, and appear DAPI-negative. Did the authors examine nucleolar distribution relative to NE expansion in cells shown in figure 4C? Along the same lines, what is the cell cycle distribution of cells with ER/NE expansion? If they are delayed in mitosis, nuclear morphology defects may be a secondary consequence of cell cycle progression defects, themselves due to NPC and/or SPB insertion problems.

Our answer: As indicated above we have examined nucleolar distribution relative to NE expansion using Nop1-mRFP as a nucleolar marker in combination with Pus1-eGFP as a nuclear marker. These data are now included in Figure Fig. 6D and S5B and accordingly discussed.

5. I suggest to rephrase the last sentence of the abstract: "nuclear membrane biogenesis dependent on a balanced ratio between amphipathic motifs in diverse nucleoporins is essential for interphase NPC biogenesis". This study does not directly assess NPC biogenesis and therefore, the interesting link between lipids and NPC biogenesis remains correlative.

Our answer: We had to shorten the abstract to meet the JCB requirements. The relevant sentence now reads "Our data indicate that nuclear membrane and presumably NPC biogenesis depends on a balanced ratio between amphipathic motifs in diverse nucleoporins."

6. It would be useful to include some information on the number of cells observed in the EM figures.

Our answer: We have detected all the described membrane changes in several micrographs and, importantly, not in control cells. However, we prefer not to include these data into the manuscript as they are as often in EM analysis not a quantitation from a truly non-random recording of cells but might be misinterpreted as this. We are happy to provide the data for the reviewers:

Fig. 2A: OE ProtA-*Ct*Ndc1	**146 (of 166) cells: cytoplasmic membrane clusters.**
Fig. 3A: ProtA-*Sc*Ndc1	**50 (of 54) cells: cytoplasmic membrane cluster.**
Fig. 3C: ProtA-*Sc*Hmg1	**92 (of 101) cells: karmellae.**
Fig. 3D: ProtA-*Sc*Ndc1 N-ter.	**37 (of 70) cells: membrane cluster.**

	32 (of 70) cells: ER whorls.
CONTROL: ProtA	**no comparable structures (36 cells).**
Fig. 4D: ProtA-*Sc*Ndc1 C-ter.	**27 (of 30) cells: cytoplasmic vesicular membranes.**
CONTROL: ProtA-*Sc*Ndc1 C-ter. L461D	**no comparable structures (59 cells).**
Fig. S4B: ProtA-*Sc*Nup53	**84 (of 99) cells: INM associated membranes and tubules.**
CONTROL: ProtA-*Sc*Nup53ΔAH	**no comparable structures (20 cells)-**
Fig. S4D: ProtA-NLS-*Sc*Ndc1 C-ter.	**57 (of 65) cells: intranuclear vesicular membranes.**
Fig. 6E: *ndc1*Δ*nup53*Δ	**26 (of 34) cells: abnormal nuclear shape, cytoplasm. protrusions, which partially fuse back, often increased nuclear size.**
Fig. S5C: *ndc1*Δ*nup53*Δ*nup59*Δ	**48 (of 66) of cells: abnormal nuclear shape, cytoplasm. protrusions, which partially fuse back, often increased nuclear size.**
Fig. S5D: *ndc1*Δ*pom34*Δ	**66 (of 83) cells: abnormal nuclear shape, cytoplasm. protrusions, which partially fuse back, often increased nuclear size.**
Fig. 8D: *ndc1*Δ*nup59*Δ + pNdc1ΔAH	**105 (of 145) of cells: abnormal nuclear shape, cytoplasm. protrusions, which partially fuse back.**
CONTROL: *ndc1*Δ + pNdc1ΔAH	**no comparable structures (33 cells).**

7. Results, first page: "Moreover, CtNup120 and CtNup133 did not associate with GUVs containing the unrelated inner nuclear membrane protein BC08/SCL1 (Fig. 1C)" should be Figure S1C.

Our answer: This has been corrected.

8. P. 19: "Prompted by the finding that Ndc1 and Nup53/Nup59 amphipathic motifs may (modify?) the nuclear ... "

Our answer: This is corrected to "Prompted by the finding that Ndc1 and Nup53/Nup59 amphipathic motifs may affect nuclear membrane curvature at the NPC insertion sites in a coordinated fashion…"

Reviewer #1 (Significance (Required)):

Significance: This report describes novel functional motifs in the Ndc1 protein that may be important for NPC assembly, and intriguing genetic interactions between NPC assembly and lipid homeostasis pathways. Although the mechanisms linking Ndc1 motifs with NE expansion and lipid composition remain unclear, these observations will be interesting for researchers working on NPC biogenesis and nuclear morphology.

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

Amm et al report on the role of new motifs and interactions between the essential and conserved integral nuclear pore membrane protein Ndc1 and other key components of the yeast nuclear pore complex. They show that members of the Y-subcomplex that coats the pore membrane bind directly to Ndc1 and identify an amphipathic helix at the C-terminus of Ndc1 that displays genetic interactions with other nucleoporins carrying analogous amphipathic helices. The authors find that cells can survive without Ndc1 when these related amphipathic helices from other nups are coincidentally deleted.
Despite significant recent advances in our structural understanding of the nuclear pore complex, how the NPC associates with the curved nuclear membrane remains poorly understood. Previous studies in yeast have uncovered significant redundancy in this association but again the basis for this remains unclear. Therefore, I find this study on the amphipathic helix of Ndc1 and its interaction with other membrane binding components of the NPC an important and timely contribution to the field. Technically, the paper is solid and I find that most of the authors' conclusions are well supported by the evidence they provide (but see below for few experimental issues). Overall, the paper is well written, and despite the use of several mutants and methodologies, it is easy to read. I think the paper's significance would improve if the authors could present some "larger picture" view on how the Ndc1 helix and/or domains they describe interact with the Nup84 complex and the pore membrane or other elements of the NPC. For example, the authors make the remarkable finding that removal of Nup53 makes ndc1 nulls able to survive. Would it be possible to use existing models of the yeast NPC and provide some structural explanation of why that is? However, I would like to emphasize that this is not required to support the main claims of the paper and should only be considered if the authors wish to provide a more "molecular" view of their findings.

> Our answer: We thank the reviewer for this positive evaluation. We have followed this suggestion and included a more extensive discussion in the manuscript on how amphipathic helices in nucleoporins could contribute to NPC assembly. Moreover, we address the specific points as described below.

Specific experimental issues and clarifications:

- A major part of the manuscript describes a detailed structure-function analysis of Ndc1. The link between the two domains of ScNdc1 studied and their effects on membrane proliferation could be better defined: specifically, can the authors exclude that the N-domain of Ndc1 that includes its transmembrane domain, is not also involved in the membrane proliferation phenotype shown in Fig2A and C? It also seems as if GAL-ProtA-ScNdc1 (1-260) also causes growth inhibition (Fig. 2F). How do cells with GAL-ProtA-ScNdc1 (1-260) look like? Finally, although the authors convincingly show that overexpression of 261-655 inhibits growth, from the EM it seems as its effects on membrane proliferation is not the same as that of the overexpression of full-length Ndc1 (compare Fig. 3D vs Fig. 2D).

> Our answer: The reviewer is right that overexpression of full-length Ndc1 and Ndc1 261-655 show different membrane proliferation phenotypes. We have clarified this point in the manuscript. We included EM pictures showing cells overexpressing the N-terminal part of Ndc1 is similar to the overexpression of full-length Ndc1 and reveals proliferation of membranes typically observed upon overexpression of membrane proteins (included in

Figure 3D). And, indeed, overexpression of GAL-ProtA-ScNdc1 (1-260) shows a growth defect, which is, however, less pronounced than for Ndc1 261-655. In the case of *Chaetomium thermophilum* Ndc1, overexpression of full-length protein is more toxic as the N-domain while overexpression of the C-domain is most harmful.

- Figure 1A: Do the CtNups shown under "Input" represent 100% of what used in the binding reaction? If so, please indicate at the figure.

Our answer: This information has now been included in the figure legend.

- CtNup120 and CtPom133 would migrate close to CtPom152, which could make visualization by Coomassie stain a bit tricky - if the authors could provide SDS PAGE gels with lower %, that would be helpful. Along similar lines, how do the authors know that CtNup120beta does not bind the CtNdc1 if these two appear to migrate at the same size (Fig. 1D)?

Our answer: Carful inspection of these and additional experiments did not indicate the binding of CtNup120 and CtPom133 to CtPom152, which is, admittedly, difficult to evaluate as especially CtPom133 migrates close to CtPom152. Also other gels did not separate these proteins better. However, we have included the CtPom152 negative controls of the pulldown experiments shown in Fig 1C indicating that Nup133 fragments (and Nup133, albeit again close to Pom152) do not interact with Pom152. A similar problem indeed applies for CtNup120beta and CtNdc1 but we have now included a GUV binding assay showing that CtNup120beta does not detectably interact with CtNdc1 (Fig S1G).

- Figure 1B, GUVs: Why do the authors use CtNup85 for the GUV experiment instead of CtNup84 that was used in Fig. 1A?

Our answer: When we started these experiments, we used CtNup85, which was available earlier than a corresponding CtNup84 construct, which accordingly served as a negative control for this GUV experiment. Both Nup85 and Nup84 are part of the Y-complex and thus are appropriate controls.

- Moreover, CtNup120 and CtNup133 ...BC08/SCL1 (Fig. 1C)" Don't see this in Fig. 1C

Our answer: This should read “Fig. S1C” and is now corrected.

- The imaging of ProtA-AHNdc1-eGFP (Fig. 3C) is not great and the localization of the AH does not look very clear - can the authors provide better micrographs? Perhaps co-expression of a red ER reporter or similar reporter would also help.

Our answer: Fig. 3C is now replaced by better micrographs and includes now as suggested a red ER reporter (DsRed-HDEL)

- The ndc1 nup53 double mutant appears to display a striking cold-sensitive growth defect (Supplemental Figure 6A, compare 23 vs 30C). Can the authors comment on this?

Our answer: Thanks for pointing at this. The Ndc1/Nup53 double mutant shows indeed a cold-sensitive phenotype. These phenotypes are often observed in the case of defective

assembly processes, which are in particular sensitive to low temperatures. We have discussed the cold-sensitive phenotype in greater detail in the revised manuscript.

Reviewer #2 (Significance (Required)):

Despite significant recent advances in our structural understanding of the nuclear pore complex, how the NPC associates with the curved nuclear membrane remains poorly understood. Previous studies in yeast have uncovered significant redundancy in this association but again the basis for this remains unclear. Therefore, I find this study on the amphipathic helix of Ndc1 and its interaction with other membrane binding components of the NPC an important and timely contribution to the field. Technically, the paper is solid and I find that most of the authors' conclusions are well supported by the evidence they provide (but see below for few experimental issues). Overall, the paper is well written, and despite the use of several mutants and methodologies, it is easy to read. I think the paper's significance would improve if the authors could present some "larger picture" view on how the Ndc1 helix and/or domains they describe interact with the Nup84 complex and the pore membrane or other elements of the NPC. For example, the authors make the remarkable finding that removal of Nup53 makes ndc1 nulls able to survive. Would it be possible to use existing models of the yeast NPC and provide some structural explanation of why that is? However, I would like to emphasize that this is not required to support the main claims of the paper and should only be considered if the authors wish to provide a more "molecular" view of their findings.

Our answer: As outlined above, we followed the suggestion and included a more extensive discussion in the manuscript on how amphipathic helices in nucleoporins could contribute to NPC assembly in the context of a “larger picture”.

Audience: Mostly the following - Nuclear pore complex, nuclear envelope, and possibly some membrane biologists.

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

In recent years, significant progress has been made in defining the molecular details of many structural features of the nuclear pore complex (NPC). However, one area that remains ill-defined is the interface between the core structures of the NPC and the pore membrane domain. This is an especially intriguing area when one considers that the NPC contains several integral proteins and numerous peripheral membrane proteins contain amphipathic helices whose functions and interactions with the membrane, as well as with one another, remain largely undefined.

In this manuscript by Amm et al., the authors have examined the functional role of the integral membrane Nup Ndc1 and its interactions with various peripheral membrane Nups, including members of the Nup84 complex (termed the Y-complex) and the linker Nups Nup53 and Nup59. The authors show that Ndc1 interacts with specific members of the Nup84 complex, namely Nup120 and Nup133, supporting the idea that Ndc1 functions, in part, to anchor this NPC substructure to the pore membrane. In addition, they identified an amphipathic helix (AH) within the C-terminal half of Ndc1, and they showed that it can directly bind to membranes. Importantly, they have used genetic assays to show that the Ndc1-AH functionally interacts with AHs present at the C-terminus of Nup53 and Nup59. Strikingly, they show that the lethal phenotype detected in strains lacking Ndc1 can be suppressed by the deletion of NUP53, but not NUP59, and, more specifically, only the loss of the C-terminal AH Nup53 was required to suppress the lethal phenotype of the ndc1 null mutation. Further ultrastructural analysis of these mutants revealed that, while these mutants were viable, they exhibited extensive NE expansion phenotypes.

Overall, the data presented in this manuscript are of high quality, and the experiments are well controlled. My specific comments are relatively minor and listed below.

> Our Answer: We thank the reviewer for this positive evaluation. We have addressed the specific points as described below.

Minor points.

1) The authors state "Serial ultrathin sections of fixed yeast cells overexpressing ProtA-CtNdc1 revealed that these unusual extranuclear membrane proliferations exhibited pore-like structures with diameters similar to the diameter of NPCs within the nuclear membrane (Fig. 2C)." This is not entirely clear from the data. I suggest the authors provide direct measurements that support their statement.

> Our answer: We have analyzed the pore diameters and included these numbers in the manuscript (NPC diameter: 67.1 ± 2.3 nm (mean ± SD; n = 20), the diameter of the extranuclear pore-like structures: 44.8 ± 3.4 nm (mean ± SD; n = 20)). Of note, the smaller diameter of the pore-like structures likely reflects the fact that these structures do not contain the NPC membrane coat. Recent molecular dynamics simulations (DOI: 10.1126/science.abm9506) suggested that this membrane coat would widen the pore diameter, consistent with our data. We have discussed this in the manuscript.

2) The authors examined the total cellular lipid content following overexpression of Ndc1-AH-containing constructs, as well as ProtA-ScHmg1. There is little discussion of the significance of these results, which would provide a clear justification for including these data in the manuscript.

Our answer: We have moved the data from the Supplementary material to Figure 5 and discuss this more thoroughly. The data indicate a severe perturbation in lipid homeostasis.

3) There are numerous typographical and grammatical errors throughout the manuscript that need to be addressed.

Our answer: We carefully checked our revised manuscript and corrected errors.

Reviewer #3 (Significance (Required)):

The results presented in this manuscript provide further insight into the molecular interactions between Nups and the pore membrane. They suggest that AHs present in a subset of Nups perform linked functions and contribute, in part, to nuclear membrane biogenesis. As such, these results are an important advance in our knowledge of NPC structure and function. They will be of general interest to those studying the function of NPCs and, more generally, NE and organelle biogenesis.

Reviewer expertise: NPC structure and function, NE biogenesis, yeast model system.

March 7, 2023

RE: JCB Manuscript #202210059R

Prof. Wolfram Antonin
RWTH University
Institute for Biochemistry and Molecular Cell Biology
Pauwelsstraße 30
Aachen 52074
Germany

Dear Prof. Antonin:

Thank you for submitting your revised manuscript entitled "New motifs in Ndc1 mediating interaction with the Nup84 complex and nuclear membranes". The original reviewers have now assessed your revised manuscript and, as you can see, they are satisfied with the revisions. However, we have noticed that relevant yeast NPCs structures are missing from your citations. Thus, we would like to kindly request that you cite these studies wherever appropriate in your text. We would be happy to publish your paper in JCB pending final revisions to address this minor editorial point. In your final revision, please ensure that you comply with our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully. Please go through all the formatting points paying special attention to those marked with asterisks.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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

2) Figures limits: Articles and Tools may have up to 10 main text figures.

Please note that main text figures should be provided as individual, editable files.

3) Figure formatting:
*** Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add MW markers to Figs 1A and 1C-D.

Scale bars must be present on all microscopy images, including inset magnifications.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

4) Statistical analysis:
Error bars on graphic representations of numerical data must be clearly described in the figure legend.

The number of independent data points (n) represented in a graph must be indicated in the legend. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments).

If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please, see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

Statistical methods should be explained in full in the materials and methods in a separate section.

For figures presenting pooled data the statistical measure should be defined in the figure legends.

Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.).

If you used parametric tests in your study (i.e. t-tests), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title:
The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience.

*** The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership. To convey the advance more clearly, we suggest the following title: "Distinct domains in Ndc1 mediates its interaction with the Nup84 complex and the nuclear membrane."

6) Materials and methods:
Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. The text should not refer to methods "...as previously described."

Also, the materials and methods should be included in the main manuscript text and not in the supplementary materials.

7) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate).

Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods.

You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary, and the system used to collect the signal from the antibodies. If antibodies are not commercial, please add a reference citation if possible.

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

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

10) Supplemental materials:
There are strict limits on the allowable amount of supplemental data. Articles and Tools may have up to 5 supplemental figures. There is no limit for supplemental tables.

Please note that supplemental figures and tables should be provided as individual, editable files.

A summary of all supplemental material should appear at the end of the Materials and Methods section (please see any recent JCB paper for an example of this summary).

11) Video legends:
Video legends should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments, concentration and duration, or transfection), the imaging method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.

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

13) Conflict of interest statement:
JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests."

14) Author contribution:
A separate author contribution section is required following the Acknowledgments in all research manuscripts.

*** All authors should be mentioned and designated by their first and middle initials and full surnames and the CRediT nomenclature is encouraged (https://casrai.org/credit/).

15) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

16) Materials and data sharing:
All animal and human studies must be conducted in compliance with relevant local guidelines, such as the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals or MRC guidelines, and must be approved by the authors' Institutional Review Board(s). A statement to this effect with the name of the approving IRB(s) must be included in the Materials and Methods section.

*** Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: https://rupress.org/jcb/pages/data-deposition), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database.

17) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. The Source Data files will be directly linked to specific figures in the published article.

As your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size

standards should be labeled wherever possible.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

B. FINAL FILES:

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Michael Rout
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

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

Ndc1 is a transmembrane nucleoporin, essential for insertion of the nuclear pore complex (NPC) and spindle pole body (SPB) into the nuclear envelope (NE). How NE-associated proteins contribute to the bending and fusion of membranes during NPC insertion has not been fully elucidated. Here, the authors report a number of observations related to Ndc1 function. Their main findings are the following: (i) The N-terminal transmembrane domain of Ndc1 mediates the membrane recruitment of two Y-complex nucleoporins. Therefore, these interactions are likely to contribute to NPC biogenesis. (ii) Over-expression of a novel

amphipathic helix (AH) in the non-essential C-terminus of Ndc1 alters the lipid composition and triggers massive membrane proliferation, although the underlying mechanisms remain unknown. (iii) The essential function of Ndc1 can be suppressed by deleting the amphipathic helix from Nup53, or by deleting the transmembrane nucleoporin POM34. Surviving strains have NE expansions ("flares") different from those observed in Ndc1 over-expression, but reminiscent of those observed in cells with defects in either phospholipid biosynthesis or mitotic progression.

The authors have satisfactorily addressed all of the comments I made on their Review Commons submission. In particular, they include new images of membrane expansions labelled with the ER marker DsRed-HDEL, and further characterize the NE/ER morphological defects of cells either over-expressing or lacking Ndc1. These new results provide a more nuanced, if complicated, view: Ndc1 over-expression vs deletion have distinct consequences on membrane properties (over-proliferation of endomembranes vs. mitotic delay and NE "flares"). In both cases, there is evidence of lipid homeostasis defects (lipid analysis vs sensitivity to benzyl alcohol, respectively). It is likely that the specific lipid defects will be different in each case. In the latter case, the primary defect are hard to disentangle - are lipid/NE alterations a cause or a consequence of the mitotic delay? However, the main conclusion stands, that the AH of Ndc1 is important for the regulation of membrane properties and thus, is likely important for NPC and/or SPB integration into the NE.

The authors also made some changes in their introduction and discussion sections, and the new manuscript is much easier to read as a result.

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

The authors addressed my comments.

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

The points raised in my review of the previous manuscript have been sufficiently addressed in the revised version.