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# Chm7 and Heh1 collaborate to link nuclear pore complex quality control with nuclear envelope sealing

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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 24 May 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are shown below.

As you will see, the referees appreciate your analyses. However, they both think that much more insight is required for further consideration in The EMBO Journal. If you can significantly extend your findings along the lines suggested by the referees, I will be happy to consider a revised version of your manuscript. However, I have to point out that we allow only a single round of revision. Given the competitive situation, I also won't be able to extend the revision time, and you would have to resubmit the revised work within three months. Therefore, please consider your options carefully. If you foresee that you won't be able to address the issues raised by the referees, you should seek publication elsewhere.

If you decide to thoroughly revise the manuscript for the EMBO Journal, please include a detailed point-by-point response to the referees' comments. Please bear in mind that this will form part of the 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.embo.org/embo-press

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.

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

 

revision.

REFEREE REPORTS

Referee #1:

Webster, Thaller and colleagues propose an interesting model for nuclear pore complex (NPC) quality control in which Chm7 and Heh1 form a nuclear envelope subdomain to delimit regions of assembling nuclear pores in yeast. This topic will certainly be of general interest for cell biology. The authors present detailed and complex work and support their conclusion with a variety of biochemical, cell biological and genetic experiments. Based on their results, it seems clear that Heh1 is essential to recruit Snf7 and Chm7 to storage compartments of defective NPCs (SINCs). Some of their findings, in particular the biochemical analysis of Heh1 / Snf7 / Chm7 interactions, need to be strengthen and a better defined physiological role of Chm7 should be assigned. Therefore we suggest to address the following points:

Major points:

1. Given the proposed role for Chm7, it remains quite puzzling that loss of Chm7 has no obvious phenotype (no growth defect or no loss of NE integrity). Deletion of Chm7 even suppresses the formation of SINCs in Vps4 mutants (Fig. 7). Yet, Chm7 accumulates in SINCs and appears to be required for the formation of SINCs (Fig 6B). One possible conclusion is that the accumulation of Chm7 (or Snf7) and hence the formation of a putative nuclear envelop subdomain causes toxicity in vps4, pom152 double mutants. How these findings support a critical role for Chm7 in NPC quality control remains unclear.

2. The binding experiments presented in Fig. 1 suggest direct binding of Heh2 to Chm7 and Snf7. Yet the data is not very convincing. This is particularly the case for Fig. 1F. A band with a molecular weight very similar to Heh2 (1-308) is detected in almost all lanes, even in lanes where Heh2(1-308) was not added. Fig. 1F should be completed with a western blot against anti-His6 to show specifically only the Heh2 levels. Also in the BiFC assay only moderate interaction between Heh2 and Chm7 is detected (Fig. 1G).

3. Several intracellular dots are detected for the Snf7-VC - Chm7-VN BiFC signal (Figure1 - Suppl. 2). Curiously, Chm7-GFP and the BiFC signal of Heh1-Snf7 (which depends on Chm7) or Heh1-Chm7 are detected as a single solitary dot on the NE as shown in Fig. 3B. Additionally Heh1 seems to be required for the recruitment of Chm7 to the NE. This raises several questions: Do the interactions of Chm7 and Snf7 occur only at the NE, or also on other organelles? Are the interactions of Chm7 and Snf7 dependent on Heh1?

4. Chm7, Snf7 and Heh1/2 are expressed in all cells. Yet the recruitment of ESCRT-III to the NE increases with the number of defective nuclear pore complexes, suggesting a tight regulation of the process. Are the protein levels of Chm7 or Heh1/2 up-regulated in response to defective nuclear pores? Is the increase in Chm7 foci dependent on Heh1 or Snf7 recruitment to SINCs?

5. Many experiments rely on GFP-tagged Chm7 or other ESCRT-III subunits with rather large tags. Most ESCRT-III subunits are not functional when fused GFP. Is Chm7-GFP a functional fusion protein and are the Snf7 -BiFC constructs still functional?

Minor points:

Figure 2D, there is a typo at the y-axis, which should say "% of cells with BiFC foci

Referee #2:

In a previous study, Webster et al. reported that in budding yeast the ESCRT-III Snf7 and Vps4 are involved in a surveillance pathway that prevents the formation of the "SINC" compartment, a NE subdomain containing aggregates of misassembled NPCs. They had further demonstrated that the

 

inner NE protein, Heh2, recruits the ESCRT-III subunit Snf7 to the NE and that Snf7 and the AAA-ATPase Vps4 were required to destabilize and clear these defective NPC assembly intermediates.

In this manuscript, the authors further investigate the contribution of S. cerevisiae Chmp7 in this process. This protein, that encompasses both ESCRT-II and ESCRT-III domains, was recently demonstrated to be required for the recruitment of other ESCRT-III components to NE holes in mammalian cells. However Chpm7 itself had not been localized to the NE.

The authors first show that the LEM domain of Heh2 binds directly to the "open" (ΔC) forms of the ESCRT-III domain of Chm7 and Snf7. Using BiFC, they visualize the interaction between Heh1/2 and Chm7 (and, consistent with their previous study, between Heh1/2 and Snf7) at one spot localized on the NE in some cells. Importantly, they show that GFP or mCherry-tagged Chm7 can be similarly form one spot on the NE (in only 25% of the cells) an that this spot colocalizes with the Heh1/Snf7 BiFC signal.
The next show that this localization of Chm7 is lost in heh1Δ cells. In contrast, mutations of several Nups/NPC assembly mutants (Pom152, Nup170, Nup133, apq12, ts nic96 and nup192), or overexpression of Nup53 increase the frequency of cell with at least one Chm7 foci and the number of foci per cell.

Finally, they show that Chmp7-GFP colocalizes with the SINC (visualized by Nup170-mCherry) in vps4Δ or vps4Δpom152Δ cells, and that CHM7 deletion prevents SINC formation and rescues both the impaired growth and the altered NLS-GFP nuclear accumulation in vps4Δpom152Δ cells.

This is a well-conducted and solid study, in which all the above-mentioned statements are convincingly demonstrated. However, the respective contribution of ESCRT-II versus-III domains of Chm7, and the crosstalk between Chm7 and Snf7 would deserved to be clarified to provide some novel mechanistic insights into the function of these ESCRT-III components in the NPC assembly/quality control pathway.

Major points:

1) In the introduction, the authors highlight the original organization of Chm7 as a chimera between ESCRT-II/III domains. However, besides the demonstration that the ESCRT-III domain mediates interaction with Heh2 in vitro, this key aspect is not further addressed in the manuscript ( i.e. the "compelling hypothesis that it might supplant the role of Vps25 and Vps20 at the NE" - page 6- is not addressed in the ms). The manuscript would thus be strengthened if the function of the ESCRT-II/III domain were tested using specific deletion/mutations of its N or C domains:
- Localization of N-term (ESCRT-II) /C-term (ESCRT-III) domain of Chm7
- Contribution of its ESCRT-II versus -III domains to Heh2/Snf7 interaction (BiFC) (Fig 2) and to the rescue of SINC formation in vps4Δpom152Δ cells (Fig 7).
- In vitro, may the ESCRT-II domain of Chm7 activate Snf7?

2) The crosstalk between Chm7 and Snf7 and their stepwise implication is not clarified.
- the number of cell with Chm7-GFP foci increases in vps4Δ cells. Does it also increase in snf7Δ cells? Conversely, does SNF7 deletion (that leads to an increased accumulation of Nups in SINC structure) stimulate or impair Heh1/2-Chm7 BiFC interaction?
- Since Heh2 directly binds to both Chm7-CΔC and Snf7ΔC in vitro, are these synergistic or competitive interactions?
- BiFC between these two ESCRT-III constituents is presented. Do these two proteins directly interact?

3) The Chm7 foci present in 25% of wt cells colocalize with the SINC present in vps4Δ and vps4Δpom152Δ cells.
In wild-type cells, is this foci retained in the mother cell as previously reported for the SINC?
Is the appearance of this NE foci cell cycle dependent?
Is this a transient or stable structure?

4) The final sentence of the abstract is not clear/well supported by the data: "quality control pathway whose regulation by Vps4 "and Pom152" prevents loss of nuclear compartmentalization by defective NPCs." The specific implication of Pom152 (as compared to other NPC constituents) in

 

this process is not so clear to me. Would nuclear integrity not be similarly altered for instance in vps4Δnup170Δ cell in a chm7-dependent manner?

5) The authors should test the prediction that "NPCs in chm7Δnup116Δ cells would fail to be sealed" (discussion p22); In particular, is the viability of nup116Δ cells altered upon chm7 deletion? They mention that similar sealed structures can be observed in apq12Δ cells (Scarcelli, 2007). As the apq12Δchm7Δ mutant is viable at 30{degree sign}C but lethal at 37{degree sign}C (Bauer 2015), EM analysis this strain after a short shift to 37{degree sign}C could possibly be used to test their prediction.

Minor points

1) While an increase in Chm7 foci is observed in all Nup mutants analyzed as well as in apq12Δ cells, there is also a clear increase depending on the temperature. As control, it would be important to determine if such an increase in foci frequency/number is also observed in other nup mutants (notably peripheral Nups that are not enriched in the SINC, i.e; nup60Δ, Mlp1/2Δ, ts nup82, or ts nup159 mutants) or under other stress conditions.

2) The authors show that when nuclear transport is impaired (by treatment with hexanediol or 2-deoxyglucose -Fig 4 C,D), there is no increase in the frequency of these Chm7 foci. Here however, acute ( 10 - 45 min treatments) are used. Could this explain the lack of phenotype? In other words, how rapidly are these structures expected to assemble (on this line, the authors should also indicate in the fig legend how long the ts Nup96-1 and Nup192-5 cells were shifted to 37{degree sign}C).

3) p16: overexpression of Nup53 that induced mb stacks, leads to incremental accumulation of Chm7-GFP on "these" mb (p16) (Fig 5, C,D). Formal proof that these are the "same" mb would require colocalization with Pom152 or Ndc1, or EM analyses

4) The fact that a genetic interaction between Apq12 and Chm7 was previously described (Bauer, genetics 2015) should be indicated when the apq12Δ mutant is introduced (p 14). The publication from Bauer et al should also be better referred to in the introduction (notably the notion that Chm7 was proposed to "perform a novel function at the ER as part of an alternative ESCRT-III complex").

5) Page 11: ..." despite the protein being produced at levels "comparable" to wild type cells".
In fact, it seems that the level of Vps20-VC is the lowest in the vps25Δ background in which no interaction is seen by BiFC. Since the aim of this figure was to provide a technical validation of the approach, and is not really key to this study, I would remove panel A and the corresponding quantification from the main manuscript and combine it with the western blot in a Supplemental figure (using it more to show that Chm7 deletion does not affect interaction of Snf7 with other partners like Vps20).
The authors should also state that since the Heh1/2-VN protein levels were below the detection sensitivity (fig 2 Suppl 1), they cannot formally exclude that their expression or stability could be altered in chm7Δ cells.

1st Revision - authors' response 04 September 2016

**Point-by-point response to referees:**

***Referee #1***

***1.*** *Given the proposed role for Chm7, it remains quite puzzling that loss of Chm7 has no obvious phenotype (no growth defect or no loss of NE integrity).*

We were puzzled by this as well, although it bears mentioning that even *VPS4* and *SNF7* are not essential and their deletion results in few growth abnormalities in budding yeast. However, in a more extensive analysis of the localization of a NLS-GFP reporter, new data are presented in **Figure 9** showing (at 37$^{o}$C) that some *chm7Δ* cells no longer accumulate NLS-GFP in the nucleus. This effect is more dramatic in *chm7Δapq12Δ* cells consistent with a loss of nuclear compartmentalization being responsible for the synthetic growth delays of this strain.

 

***1. con't:*** *Deletion of Chm7 even suppresses the formation of SINCs in Vps4 mutants (Fig. 7). Yet, Chm7 accumulates in SINCs and appears to be required for the formation of SINCs (Fig 6B). One possible conclusion is that the accumulation of Chm7 (or Snf7) and hence the formation of a putative nuclear envelop subdomain causes toxicity in vps4, pom152 double mutants. How these findings support a critical role for Chm7 in NPC quality control remains unclear.*

We completely agree with the conclusion that the accumulation of Chm7 in the SINC (in the absence of *POM152* and *VPS4*) reflects a toxic gain-of-function. We elaborate on this more in the discussion of the paper but, in brief, we suggest that Chm7's role in NPC quality control is misregulated without *POM152* and *VPS4* leading to the inappropriate recognition and sealing of 'normal' forming NPCs.

***2.*** *The binding experiments presented in Fig. 1 suggest direct binding of Heh2 to Chm7 and Snf7. Yet the data is not very convincing. This is particularly the case for Fig. 1F. A band with a molecular weight very similar to Heh2 (1-308) is detected in almost all lanes, even in lanes where Heh2(1-308) was not added. Fig. 1F should be completed with a western blot against anti-His6 to show specifically only the Heh2 levels.*

As requested, we have included a Western blot (**Figure 3C**) that demonstrates the specific binding of heh2(1-308) to the Chm7 truncation. It is clear from these experiments (and additional experiments with Snf7, see **Figure 3F**), that the binding of Heh2 and Snf7 to chm7-$CTD_{OPEN}$ is sub-stoichiometric. We interpret this to suggest that there are other factors (for example lipids or post-translational modifications) missing from the *in vitro* preparations that stabilize these interactions. Nonetheless, we emphasize that the specificity of these interactions is reproducible and mirrored by our *in vivo* analyses.

***2. con't:*** *Also in the BiFC assay only moderate interaction between Heh2 and Chm7 is detected (Fig. 1G).*

The relationship between Heh1, Heh2 and Chm7 is very challenging to dissect genetically, biochemically or functionally. This is best exemplified by our observations (presented in **Figure 2**) that while the recruitment of Chm7 to the NE is uniquely dependent on Heh1, it is in fact increased in *heh2Δ* cells. While these data suggest a role for Heh2 in antagonizing Chm7 recruitment, the expression of either *HEH1* or *HEH2* rescue its recruitment in *heh1Δ* cells. Thus, depending on the context, Heh1 and Heh2 can act antagonistically or redundantly.

***3.*** *Several intracellular dots are detected for the Snf7-VC - Chm7-VN BiFC signal (Figure1 - Suppl. 2). Curiously, Chm7-GFP and the BiFC signal of Heh1-Snf7 (which depends on Chm7) or Heh1-Chm7 are detected as a single solitary dot on the NE as shown in Fig. 3B. Additionally Heh1 seems to be required for the recruitment of Chm7 to the NE. This raises several questions:*

*Do the interactions of Chm7 and Snf7 occur only at the NE, or also on other organelles? Are the interactions of Chm7 and Snf7 dependent on Heh1?*

We include new data presented in **Figure EV4A and C** showing BiFC between Chm7 and Snf7 at structures throughout the cell suggesting that these interactions are not exclusive to the nuclear envelope. Moreover, these data suggest that Chm7 and Snf7 can interact without Heh1 or Heh2. Consistent with this assertion, BiFC between Chm7 and Snf7 is not affected in *heh1Δ* or *heh2Δ* cells (**Figure EV4B**), which is further supported by new direct binding data in **Figure 3F** where we show that Chm7 can directly bind Snf7.

***4****. Chm7, Snf7 and Heh1/2 are expressed in all cells. Yet the recruitment of ESCRT-III to the NE increases with the number of defective nuclear pore complexes, suggesting a tight regulation of the process.*

*Are the protein levels of Chm7 or Heh1/2 up-regulated in response to defective nuclear pores?*

This is a very interesting idea and we assessed whether Chm7 levels increase upon imposition of NPC assembly blocks. However, in new data presented in **Figure EV1D** and **EV5C**, we are unable

 

to observe any detectable changes to Chm7 levels. We suggest that there is a sufficient non-NE pool of Chm7 capable of being recruited to the NE when needed. Consistent with this idea, in our new domain analysis, the chm7-NTD and chm7$_{OPEN}$ (which mimics a potentially active form) constructs are expressed at similar levels (see Western blots, **Figure EV1C**), however, there is a dramatic accumulation of the 'activated' chm7$_{OPEN}$ at the NE suggesting that there is a large cytosolic pool that is competent for a regulated NE recruitment (**Figure 1B and C**).

*Is the increase in Chm7 foci dependent on Heh1 or Snf7 recruitment to SINCs?*

We have now addressed the Heh1-dependent recruitment of Chm7 in several contexts including in *vps4Δ* and *snf7Δ* cells (**Figure EV4D**) where NE foci are specifically perturbed, and in nup knockouts like *nup170Δ* where the increased NE recruitment due to NPC assembly delays still requires Heh1(**Figure EV5G**).

***5.*** *Many experiments rely on GFP-tagged Chm7 or other ESCRT-III subunits with rather large tags. Most ESCRT-III subunits are not functional when fused GFP. Is Chm7-GFP a functional fusion protein and are the Snf7 -BiFC constructs still functional?*

Several data point to the functionality of the Chm7-GFP protein. First, there are SINCs in Chm7-GFP-containing strains that are absent from *chm7Δ* cells. Second, we now show data where Chm7-GFP in *apq12Δ* cells does not lead to synthetic sickness **(Figure EV1A)**. While we can't completely rule out that Snf7-VC is not fully functional, our BiFC data supports that at least the Snf7-Vps20 interaction depends on *VPS25* suggesting that it retains its biochemical interactions.

*Minor points:*

*Figure 2D, there is a typo at the y-axis, which should say "% of cells with BiFC foci*

***Referee #2:***

*1) In the introduction, the authors highlight the original organization of Chm7 as a chimera between ESCRT-II/III domains. However, besides the demonstration that the ESCRT-III domain mediates interaction with Heh2 in vitro, this key aspect is not further addressed in the manuscript ( i.e. the "compelling hypothesis that it might supplant the role of Vps25 and Vps20 at the NE" - page 6- is not addressed in the ms). The manuscript would thus be strengthened if the function of the ESCRT-II/III domain were tested using specific deletion/mutations of its N or C domains:*

*- Localization of N-term (ESCRT-II) /C-term (ESCRT-III) domain of Chm7*

We have followed the reviewer's excellent suggestion and now include an extensive domain analysis of Chm7 presented in a completely revised **Figure 1** and **Figure 2**. These data show that the ESCRT-II domain (chm7-NTD) is sufficient for nuclear envelope targeting. Moreover, there is a remarkable accumulation of a Chm7 truncation that would mimic the activated polymerized form of other ESCRT-III's (chm7$_{OPEN}$) that is suggestive that Chm7 might itself be capable of forming higher order assemblies.

*- Contribution of its ESCRT-II versus -III domains to Heh2/Snf7 interaction (BiFC) (Fig 2) and to the rescue of SINC formation in vps4Δpom152Δ cells (Fig 7).*

We show several new data supporting that only full length Chm7 is capable of carrying out its function at the nuclear envelope including complementation of *apq12Δchm7Δ* synthetic growth delays (**Figure 1E**) and the rescuing of the Heh1-Snf7 BiFC in *chm7Δ* cells (**Figure 4F and G**). We felt this was sufficient and did not pursue complementing the SINC.

*- In vitro, may the ESCRT-II domain of Chm7 activate Snf7?*

We present new data in **Figure 3F** that show Snf7 directly binds Chm7. It was our hope that these data might also be suggestive a direct role for activation of Snf7 but they were inconclusive. It is

 

likely that additional components are required to stimulate Snf7 polymerization. As Chm7 recruitment to the nuclear envelope is triggered by defects in NPC assembly, we wonder whether there are additional factors like post-translational modifications that are required for its local activation at the nuclear envelope.

*2) The crosstalk between Chm7 and Snf7 and their stepwise implication is not clarified.*

We absolutely agree that this needed to be clarified and we present several new Chm7-GFP localization data and BiFC data (discussed below) that allow us to present the step-wide model in **Figure 9D**.

*- the number of cell with Chm7-GFP foci increases in vps4Δ cells. Does it also increase in snf7Δ cells?*

Yes, these data are presented in **Figure 5A** and support that (like Vps4) Snf7 acts downstream of Chm7.

*Conversely, does SNF7 deletion (that leads to an increased accumulation of Nups in SINC structure) stimulate or impair Heh1/2-Chm7 BiFC interaction?*

In *snf7Δ* cells, Heh1/2-Chm7 BiFC is more robust supporting that Snf7 acts downstream of the Chm7-Heh1 interaction. These new data are presented in **Figure 4D** and **EV3D**.

*- Since Heh2 directly binds to both Chm7-CΔC and Snf7ΔC in vitro, are these synergistic or competitive interactions?*

This is an excellent question and the data are presented in **Figure EV2**. As shown, the binding of Heh2 and Snf7 are unchanged by co-incubation of stoichiometric amounts with the GST-chm7-$CTD_{OPEN}$ construct. As the individual proteins bind in sub stoichiometric amounts, we cannot be definitive as to whether they act competitively or cooperatively. As we suggest above, we believe that there are additional factors (other proteins, post-translational modifications, membranes?) that are required to stabilize these interactions *in vivo*.

*- BiFC between these two ESCRT-III constituents is presented. Do these two proteins directly interact?*

Yes, we now show direct binding between Chm7 and Snf7 in **Figure 3F** and **Figure EV2**.

*3) The Chm7 foci present in 25% of wt cells colocalize with the SINC present in vps4Δ and vps4Δpom152Δ cells.*

*In wild-type cells, is this foci retained in the mother cell as previously reported for the SINC?*

No, we have extensively investigated the providence and dynamics of the Chm7 focus using timelapse imaging and we capture the transmission of an NE foci into daughter cells (see **Movie EV1**). A key distinction, however, is that the SINC is caused by a toxic gain-of-function of Chm7, which might inappropriately stabilize its association with assembling NPCs, whereas in wild type cells the Chm7-GFP accumulation would be predicted to be functional and perhaps more dynamic.

*Is the appearance of this NE foci cell cycle dependent?*
*Is this a transient or stable structure?*

We were unable to discern any cell cycle dependence of the NE foci but, as shown in **Movie EV1**, they are dynamic structures that move along the NE.

*4) The final sentence of the abstract is not clear/well supported by the data: "quality control pathway whose regulation by Vps4 "and Pom152" prevents loss of nuclear compartmentalization by defective NPCs." The specific implication of Pom152 (as compared to other NPC constituents) in this process is not so clear to me. Would nuclear integrity not be similarly altered for instance in*

*vps4Δnup170Δ cell in a chm7-dependent manner?*

We agree with all of the reviewer's comments and have omitted this sentence while trying to be more clear in a revised abstract that also incorporates the findings from our extensive new data and analysis.

*5) The authors should test the prediction that "NPCs in chm7Δnup116Δ cells would fail to be sealed" (discussion p22); In particular, is the viability of nup116Δ cells altered upon chm7 deletion? They mention that similar sealed structures can be observed in apq12Δ cells (Scarcelli, 2007). As the apq12Δchm7Δ mutant is viable at 30{degree sign}C but lethal at 37{degree sign}C (Bauer 2015), EM analysis this strain after a short shift to 37{degree sign}C could possibly be used to test their prediction.*

We appreciate this excellent suggestion. First, we now show new data of a genetic interaction between *CHM7* and *NUP116* in **Figure 8D**. These data are consistent with the interpretation that Chm7 acts to protect the viability of cells with malformed NPCs. We were unable, however, to acquire electron micrographs of *nup116Δchm7Δ* NPCs (but could of *nup116Δ* alone, see **Figure 8C**) because these strains are effectively dead (**Figure 8D**). In lieu of this, we provide remarkable data that at 37$^{o}$C there is a dramatic loss of nuclear compartmentalization in *apq12Δchm7Δ* cells that is at least consistent with the interpretation of a lack of NE seals in these strains (**Figure 9A-C**). We also acknowledge that we cannot discern between a lack of NPC sealing and a nuclear envelope rupture. Regardless, our data are consistent with Chm7 playing a protective role at the nuclear envelope barrier.

**Minor points**

*1) While an increase in Chm7 foci is observed in all Nup mutants analyzed as well as in apq12Δ cells, there is also a clear increase depending on the temperature. As control, it would be important to determine if such an increase in foci frequency/number is also observed in other nup mutants (notably peripheral Nups that are not enriched in the SINC, i.e; nup60Δ, Mlp1/2Δ, ts nup82, or ts nup159 mutants) or under other stress conditions.*

Absolutely. We have further investigated several additional nup deletion strains that show clear specificity in the properties of Chm7-GFP recruitment to the nuclear envelope - these data are presented in a revised **Figure 7A and B**. To evaluate whether Chm7 localization responded more generally to stress, we have also assessed the impact of high levels of salt and reactive oxygen species (by treatment with menadione, **Figure EV5D and E**). Neither of these treatments impacted Chm7 distribution.

*2) The authors show that when nuclear transport is impaired (by treatment with hexanediol or 2-deoxyglucose -Fig 4 C,D), there is no increase in the frequency of these Chm7 foci. Here however, acute ( 10 - 45 min treatments) are used. Could this explain the lack of phenotype? In other words, how rapidly are these structures expected to assemble (on this line, the authors should also indicate in the fig legend how long the ts Nup96-1 and Nup192-5 cells were shifted to 37{degree sign}C).*

We agree that we were not explicit enough about the timing of temperature shifts and this has been amended in the revised figure legends. We also show new data where there is a specific increase in Chm7-GFP foci in *apq12Δ* cells within the 45 minutes of the hexanediol (and other) treatments supporting that, in principle, Chm7 could accumulate within this timeframe. These data are shown in **Figure EV5D and E.**

*3) p16: overexpression of Nup53 that induced mb stacks, leads to incremental accumulation of Chm7-GFP on "these" mb (p16) (Fig 5, C,D). Formal proof that these are the "same" mb would require colocalization with Pom152 or Ndc1, or EM analyses*

We completely agree. We have chosen to omit these data because ultimately they did not provide any new insight into Chm7 function. We have focused more on *nup116Δ* cells where we can correlate a dramatic increase in Chm7-GFP recruitment to ultrastructure showing nuclear envelope herniations and double membrane seals over NPCs. We also show a genetic requirement for *CHM7* to protect the viability of the *nup116Δ* strain. All of these data are presented in a new **Figure 8**.

 

*4) The fact that a genetic interaction between Apq12 and Chm7 was previously described (Bauer, genetics 2015) should be indicated when the apq12Δ mutant is introduced (p 14). The publication from Bauer et al should also be better referred to in the introduction (notably the notion that Chm7 was proposed to "perform a novel function at the ER as part of an alternative ESCRT-III complex").*

Yes, thank you for bringing this to our attention as we regret that we were not sufficiently explicit about the contributions of Bauer et al.; we have amended this oversight in the revised manuscript. We have also reproduced the *chm7Δapq12Δ* data and it was instrumental in allowing us to probe the function of Chm7 as *chm7Δapq12Δ* cells showed clear losses of nuclear compartmentalization at 37$^{o}$C. These new data are presented in **Figure 9**.

*5) Page 11: ..." despite the protein being produced at levels "comparable" to wild type cells". In fact, it seems that the level of Vps20-VC is the lowest in the vps25Δ background in which no interaction is seen by BiFC. Since the aim of this figure was to provide a technical validation of the approach, and is not really key to this study, I would remove panel A and the corresponding quantification from the main manuscript and combine it with the western blot in a Supplemental figure (using it more to show that Chm7 deletion does not affect interaction of Snf7 with other partners like Vps20).*
*The authors should also state that since the Heh1/2-VN protein levels were below the detection sensitivity (fig 2 Suppl 1), they cannot formally exclude that their expression or stability could be altered in chm7Δ cells.*

We have followed the reviewer's suggestions regarding these points.

Thank you for your thoughtful appraisal of our work.

2nd Editorial Decision 17 September 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the original referees whose comments are enclosed. As you will see, they are now both broadly in favour of publication, pending satisfactory minor revision.

I would thus like to invite you to incorporate the text changes proposed by referee #2 and to submit a revised version of the manuscript.
Additionally, please:

- provide author contribution and conflict of interest statement
- remove the movie legends from the main text and zip it together with the movie files as a .txt file
- provide the legend to appendix figure S1. The appendix figures should be combined as single PDF file including a TOC
- source data for figure EV1C's actin blot - in our routine figure check this blot shows some artifacts/splice site
- please suggest (in a cover letter) a one-sentence summary 'blurb' of your paper, as well as 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper; this will form the basis for an editor-drafted 'synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.
- as you might know, we encourage our authors to provide original source data, particularly uncropped/-processed electrophoretic blots for the main figures of your manuscript. If you would like to add source data, we would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

 

REFEREE REPORTS:

Referee #1:

The authors have fully and convincingly addressed all our original concerns. Overall the manuscript has really improved and the proposed model makes quite a bit of sense.

minor point:

on page 14: vps25 instead vps24 (which is listed twice)

Referee #2:

In their revised version and rebuttal letter, Webster et al have extensively addressed all points I had previously raised. The revised manuscript includes a lot of novel data that strengthen it.
I have only minor comments/ suggestions that could be addressed by modifying the text.

1) Page 12: "we observed specific binding of heh2(1-308) to GST-snf7OPEN, but not to full length Snf7". In fact, there is a very faint band detected on the western blot with GST-Snf7 and not GST. The statement might thus be toned down= "not reproducibly, not efficiently....

2) Page 12-13: Fig 3F and EV2
Since it is only (or mainly see above) Snf7 "open" and not Snf7 that interacts with Heh2 (Fig 3C,D), it is not clear why the authors did not test a MBP-Snf "open" form rather than MBP-Snf7 for interaction with Chm7-open alone (fig 3E)or in the presence of Heh2 (Fig EV2).

3) page 12-13: The authors indicate that "All interactions, including Heh2 with Snf7 open are *substoichiometric...* "
I'm not sure this is solely reflecting the lack of additional partners, rather than the fact that Chm7 may preferentially interacts with Heh1.

4) On the blots (Figure 3), are the eluates loaded at 1x equivalent compared to inputs? This should be indicated in the figure legend or at least in the methods.

5) Page13:
"To assess whether direct biochemical interactions between Heh1, Heh2, Chm7
and Snf7 occurred at the NE, we turned to the BiFC".
This statement should be corrected.
I agree that BiFC can provide an "in vivo" validation of the interaction, and further help to assess the interaction in various backgrounds. However, since the interaction between VN and VC is extremely stable, and as such non-physiological, it could take place anywhere in the cell and the stabilized dimer be subsequentially brought to the NE thanks to one of the two partners. The title of this section should thus also be corrected.

6) Page 14:
"we failed to observe substantial BiFC signal at endosomes between Vps20-VN and Snf7-VC in the absence of VPS25 (Fig EV3A, B),despite the fusion proteins being produced at levels comparable to wildtype cells (Fig EV3C)."

This was my previous minor point 5:
Well, unless stated, in the blot that is presented, it is obvious that the levels of Vps20-VN and Snf7-VC are lower in the vps25Δ compared to wt or other mutants. As such, the decrease in BiFC fluorescence could be unspecific (despite making sense!!). I suggested in my previous review that this dataset could be kept to indicate that Chm7deletion does not affect the interaction between Vps20 and Snf7, BUT that no clear conclusion could be drown regarding vps25Δ (if the authors want to keep this data and interpretation, another more convincing blot should be presented....). So

 

please tone down this statement.

7) Page 15:
"only the full length Chm7 (and not the isolated N or C-terminal domains) was capable of restoring BiFC between Heh1-VN and Snf7-VC (Fig 4F, G)".
On figure 4G, it is indicated chm7-NTD "open". I assume this is a mistake and should be chm7-NTD. However, in view of this mistake, it should be clarified if the CTD used is in the open or full-length form.

2nd Revision - authors' response 21 September 2016

**Response to Reviewers MS# EMBOJ-2016-94574R**

**Referee #1:**

*on page 14: vps25 instead vps24 (which is listed twice)*

-This has been corrected.

**Referee #2:**

*1) Page 12: "we observed specific binding of heh2(1-308) to GST-snf7OPEN, but not to full length Snf7". In fact, there is a very faint band detected on the western blot with GST-Snf7 and not GST. The statement might thus be toned down= "not reproducibly, not efficiently....*

We have changed this statement to more accurately reflect our data to "As shown in Fig 3C, heh2(1-308) bound to GST-snf7$_{OPEN}$ preferentially to full length Snf7."

*2) Page 12-13: Fig 3F and EV2*
*Since it is only (or mainly see above) Snf7 "open" and not Snf7 that interacts with Heh2 (Fig 3C,D), it is not clear why the authors did not test a MBP-Snf "open" form rather than MBP-Snf7 for interaction with Chm7-open alone (fig 3E)or in the presence of Heh2 (Fig EV2).*

We would like to perform this experiment in the future.

*3) page 12-13: The authors indicate that "All interactions, including Heh2 with Snf7 open are substoichiometric... "*
*I'm not sure this is solely reflecting the lack of additional partners, rather than the fact that Chm7 may preferentially interacts with Heh1.*

This is also a possibility, but think it is encompassed by the rather broad "additional binding partners."

*4) On the blots (Figure 3), are the eluates loaded at 1x equivalent compared to inputs? This should be indicated in the figure legend or at least in the methods.*

Yes, they are loaded to allow for a direct comparison; this has been addressed in the methods.

*5) Page13:*
*"To assess whether direct biochemical interactions between Heh1, Heh2, Chm7*
*and Snf7 occurred at the NE, we turned to the BiFC".*
*This statement should be corrected.*
*I agree that BiFC can provide an "in vivo" validation of the interaction, and further help to assess the interaction in various backgrounds. However, since the interaction between VN and VC is extremely stable, and as such non-physiological, it could take place anywhere in the cell and the stabilized dimer be subsequentially brought to the NE thanks to one of the two partners. The title of*

 

*this section should thus also be corrected.*

We agree and have toned down the language to “can” and “could” occur at the NE.

*6) Page 14:*
*"we failed to observe substantial BiFC signal at endosomes between Vps20-VN and Snf7-VC in the absence of VPS25 (Fig EV3A, B), despite the fusion proteins being produced at levels comparable to wildtype cells (Fig EV3C)."*

*This was my previous minor point 5:*
*Well, unless stated, in the blot that is presented, it is obvious that the levels of Vps20-VN and Snf7-VC are lower in the vps25Δ compared to wt or other mutants. As such, the decrease in BiFC fluorescence could be unspecific (despite making sense!!). I suggested in my previous review that this dataset could be kept to indicate that Chm7deletion does not affect the interaction between Vps20 and Snf7, BUT that no clear conclusion could be drown regarding vps25Δ (if the authors want to keep this data and interpretation, another more convincing blot should be presented....). So please tone down this statement.*

We toned down “levels comparable” to “levels similar”. We also note that this experiment is effectively a reproduction of published work and that the modest reduction of the VN and VC fusion levels seen in the Westerns cannot explain the significant loss of BiFC signal observed specifically in the *vps25*Δ strain.

*7) Page 15:*
*"only the full length Chm7 (and not the isolated N or C-terminal domains) was capable of restoring BiFC between Heh1-VN and Snf7-VC (Fig 4F, G)".*
*On figure 4G, it is indicated chm7-NTD "open". I assume this is a mistake and should be chm7-NTD. However, in view of this mistake, it should be clarified if the CTD used is in the open or full-length form.*

We are grateful that the reviewer caught this error and it has been corrected.

3rd Editorial Decision 22 September 2016

Thank you for submitting your revised manuscript to us. I appreciate the introduced changes and I am happy to accept your manuscript for publication in The EMBO Journal.
Congratulations!

 

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

| |
|---|
| Corresponding Author Name: C. Patrick Lusk |
| Journal Submitted to: EMBO Journal |
| Manuscript Number: EMBOJ-2016-94574R |

**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

### 2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

## B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

| | |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | All experiments were conducted with three, independent biological replicates. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | All samples were included. |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | Yes, all experimental data were independently analyzed by at least two, if not three authors. |
| For animal studies, include a statement about randomization even if no randomization was used. | NA |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | Yes, all experimental data were independently analyzed by at least two, if not three authors. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA |
| 5. For every figure, are statistical tests justified as appropriate? | Yes, statistical analyses were performed with Prism (Graph Pad). Unpaired student's T-tests were used in all figures except Figures 6B and 7B, where two-way ANOVA tests were used. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | The distrubution of the data was verifiied using D'Agostino-Pearson omnibus test. For non-gaussian data, Mann-Whitney tests were performed to assess statistical significance. |
| Is there an estimate of variation within each group of data? | Yes. F-tests were used to calculate sample variance. |
| Is the variance similar between the groups that are being statistically compared? | If variance between two comparison groups was dissimilar, a Student's T-test with Welch's correction was performed. |

## C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | Antibodies were only used in western blots, which include controls for specificity |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | NA |

* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | NA |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | NA |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | NA |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | NA |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | NA |

## F- Data Accessibility

| | |
|---|---|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | NA |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | NA |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | NA |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | NA |
| 22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | NA |

## G- Dual use research of concern

| | |
|---|---|
| 23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | NA |