Passive diffusion through nuclear pore complexes regulates levels of the yeast SAGA and SLIK coactivator complexes

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MS TITLE: Passive diffusion through nuclear pore complexes regulates levels of the yeast SAGA and SLIK coactivators complexes

AUTHORS: Tadashi Makio and Richard W. Wozniak
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers recognize that your study reports an original mechanism by which the nuclear pore complex influences gene expression. They suggest, however, additional experiments to further strengthen the proposed model. If you think that you can add the experiments suggested by the reviewers, particularly those indicated by reviewers#1 and #2, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

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

Reviewer 1

Advance summary and potential significance to field

In this study by Makio and Wozniak, the authors investigate the mechanism by which loss of the nucleoporin Nup170 may affect yeast gene expression. They show that among the 100 most affected genes in nup170D 38 are SAGA dominated genes. They provide very good biochemical evidence that loss of Nup170 decreases the ratio of SAGA/SLIK complex. They show that the cleavage of the C-terminal domain of the SAGA component Spt7 is increased in nup170D resulting in increased levels of the SLIK complex, which is unable to recruit Spt8 involved in the interaction with TBP. More importantly, they show that the increased cleavage of Spt7 in nup170D depends on the vacuolar peptidase Pep4. Furthermore, using well designed Spt7 cleavage reporters, they provide good evidence that loss of Nup170 favors import or diffusion of Pep4 into the nucleus. They also show that certain stresses such as H2O2 or Hexanediol treatment have the same effect on Spt7 cleavage as loss of Nup170, supporting that the effects of nup170D on passive diffusion rates through the NPC are mimicked by certain physiological conditions. Finally, to validate that loss of Nup170 reduces gene expression of SAGA dependent genes by favoring nuclear import of Pep4, they show that the reduced expression of the SAGA dominated HIS4 gene in nup170D is rescued in nup170Dpep4D.

Overall this study presents novel very good quality biochemical data and uses well designed tools, including a Spt7 cleavage reporter, to understand the mechanism by which loss of Nup170 modifies gene expression. The experiments and results are novel and convincing, the paper is clearly written and brings interesting new insights into the nucleo-cytoplasmic transport field. However, there is one or two points that should be addressed to make the conclusions even stronger.

Comments for the author

Major comments:

1. The authors present good evidence that the reduced expression of at least one SAGA dependent gene (*HIS4*) is rescued upon loss of Pep4 in nup170D (Figure 6); however the expression of the two other SAGA dependent genes analyzed in this experiment (*PHO5* and *PDR5*) remains low, suggesting that their reduced expression in nup170D is not linked to increased nuclear import of Pep4. This observations raises the question of the extent to which the relocalization of Pep4 and changes in the ratio of SLIK vs SAGA complex contribute to the reduced expression of SAGA-dominated genes in nup170D. The authors mention that “at least a subset of the transcriptional reductions observed in the nup170Δ mutant are linked to Pep4 cleavage of Spt7”. This weak point is not clearly addressed in the discussion.

To evaluate the relevance of the proposed mechanism, it appears important to define the fraction of genes that may be sensitive to Pep4. Is it just one geneout of 38 SAGA dominated genes, or is it more? Comparing the RNA seq of the nup170D and nup170Dpep4D strains to identify additional genes behaving like *HIS4* would greatly strengthen the paper.

2. The authors propose that certain physiological or stress conditions, such as H2O2 or Hexanediol treatment, may modify nuclear pore complex diffusion rates and increase nuclear import of Pep4. They claim that Pep4 is difficult to localize because HA- or GFP-tagged versions are unstable. Is this the case whether the tag is put at the N- or the C- terminus? If possible, it would be good to show that the distribution of Pep4 indeed becomes more nuclear in nup170D or specific physiological conditions.

Minor comments:

p.14: “leading us to conclude that Nup170 was functioning to upregulate Pep4-mediated cleavage of Spt7.”
The sentence should say that Nup170 is there to reduce Spt7 cleavage by Pep4 and not to upregulate Pep4 dependent cleavage.
The sentence should be “leading us to conclude that **loss of** Nup170 was functioning to upregulate

Pep4-mediated cleavage of Spt7".

Typos:

p. 5: "our previous analysis **of** cells lacking Nup170 showed"…

p. 5: "that representative genes within this group **(of)** show reduced levels

p. 6: "RNA Pol II density along two SAGA-dominated gene**s**, HIS4 and PDR5"…..
p. 6: By contrast, **(three) two** TFIID-dominated genes, ACT1 and RPL3, whose"…
p. 8: "as no peptides after the amino-acid residue 938 **(where) were** detected in this band"…

p. 17: "than previous**ly** considered. This is emphasized by recent work from Timney"..
p. 17: "diffuse across the NE, albeit with decrease**d** rates as their size increases." Is Spt7 fully nuclear in nup170D?

Reviewer 2

Advance summary and potential significance to field

The manuscript by Makio and Wozniak reports an original mechanism by which the nuclear pore complex (NPC) influences gene expression. Using a combination of molecular biology, biochemistry and microscopy approaches in budding yeast, the authors establish that the nucleoporin Nup170 is preferentially required for the transcriptional initiation of genes dependent on the SAGA coactivator. They further demonstrate that Nup170 controls the ratio of SAGA to SAGA-like (SLIK) complexes by impacting the nuclear localization of Pep4, a protease required for SLIK biogenesis. Their data support a model in which the absence of Nup170 stimulates the nuclear import of Pep4 by increasing its passive diffusion rather than regulating its facilitated transport. Stress situations in which NPC permeability is similarly increased also affect SAGA/SLIK levels, expanding the physiological relevance of these observations.
Overall, this report provides an accurate dissection of the multiple – direct and indirect – mechanisms by which nucleoporins can impact gene expression, and complements our current knowledge about the relationships between NPCs and nuclear metabolism. Additional experiments, as described below, should further strengthen the proposed model and determine its importance for SAGA-dependent gene regulation.

Comments for the author

1. To score Pep4 localization in the nucleus, the authors use a reporter system in which Pep4 cleavage activity is measured on nucleus- or cytoplasm-restricted substrates (Fig. 4A-D). They should also try to detect directly the increased nuclear localization of Pep4 in nup170Δ cells in order to validate their model.
- although Pep4-GFP fusions are non-functional for Spt7 cleavage and SLIK production, they were previously used to analyze the vacuolar targeting of Pep4 (PMID:31332264) or its vacuolar release in peroxyde-treated cells (Mason et al, 2005). The authors should take advantage of this tool to visualize Pep4 localization in nup170Δ cells.
- alternatively, Pep4 antibodies have been described (PMID:31332264) and could be used in immunofluorescence experiments.

2. It remains to be determined to which extent the described pathway accounts for the global changes in SAGA-dependent gene expression scored in the absence of Nup170. Preventing Pep4-dependent Spt7 cleavage in nup170Δ cells is shown to reverse the reduced expression of a single SAGA-dependent gene (out of three tested, Fig. 6). The authors should analyze in this experiment the expression of several other SAGA-dominated genes (from Fig. S1B) to provide a broader vision of the contribution of this mechanism to Nup170-dependent gene expression.

3. To test the physiological relevance of this pathway for SAGA regulation, the authors have measured Spt7 cleavage as a readout of SLIK production in different genetic and stress conditions known to impact NPC permeability (Fig. 4E and 5).

- Can the author control that the reported changes in Spt7 cleavage are dependent on Pep4 activity (by performing the same experiments in pep4 mutant cells)?
- Do these situations also affect SAGA-dependent gene expression? The authors should test mRNA levels for typical SAGA-dependent target genes (as in Fig. 1) in the nup188 mutant or upon peroxide / hexanediol treatment.

Minor comments:
- The 1st paragraph of the results section should indicate that 36% of the most downregulated genes are SAGA-dominated in the nup170Δ mutant (instead of 38%, see Fig. S1A).
- It would be helpful for the reader if the names of the proteins identified in mass spec experiments were indicated alongside the corresponding bands in Fig. 3A, and if a table summarizing the composition of SAGA/SLIK complexes was provided.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, the authors investigate their previously determined functional interaction between the nucleoporin Nup170 and the SAGA complex. QPCR and ChIP analysis demonstrates that deletion of NUP170 leads to changes in the transcript and PolII/SAGA occupancy of SAGA-specific genes. The authors then tie these transcriptional changes to the altered composition of SAGA/SLIK complexes, which is driven by a specific Pep4-dependent proteolytic cleavage of Spt7. By using a clever reporter assay, it is further established that the Pep4 cleavage likely occurs in the nucleus. Therefore, by drawing on prior work establishing that nup170Δ strains have a weaker diffusion barrier, a model is proposed where global changes in the diffusion barrier of the NPC may indirectly influence gene expression. Overall, the manuscript is presented with clarity and the data are of good quality. The major criticism of the work is that the authors are primarily investigating an indirect phenomenon whose physiological relevance remains unclear. Nonetheless, I think there is a valuable concept to be considered particularly in the context of the increasing number of functional links between disruptions in nuclear transport and nuclear envelope integrity (including breakdown of NPCs associated with aging).

Comments for the author

Some considerations to strengthen the manuscript:
1) The idea that Pep4 functions in the nucleus is conceptually challenging as it is (as pointed out by the authors) a vacuolar protease. While it is clear that the authors cannot tag Pep4, I suggest a crude subcellular fractionation followed by Western blot (as they have a Pep4 antibody) with the hope of showing that there is more Pep4 in the nucleus in the nup170Δ cells. In lieu of this (as it is likely a very small fraction of total Pep4 is released from the vacuole), some effort to exclude that Spt7 is degraded in the vacuole could help to solidify their conclusions. I suggest ruling out macroautophagy (with a atg8Δ strain) and also microautophagy
(using a nvj1Δ).
2) Pep4 is 43 kD. It remains unclear how much a protein of this size would even see the diffusion barrier in a way that would be significantly affected by loss of Nup170. The authors may want to consider that whether Pep4 acts in the nucleus or not may not really matter. Once the diffusion barrier is perturbed, the steady-state distribution of many proteins will be affected including Spt7. The NLS/NES reporters address this to some extent although there will be more of the NLS version in the cytosol (and NES version in the nucleus) in the nup170D due to increase leakage back through the pore (even if this isn't obvious by the images).

Additional minor point:

Figure 3. It would be helpful for the reader if the Spts could be labeled on the A-panel; a diagram of Spt7 with the location of the Pep4-cleavage site could also be helpful to interpret this figure.

First revision

Author response to reviewers' comments

Response to Reviewer 1:

Comment 1: *"To evaluate the relevance of the proposed mechanism, it appears important to define the fraction of genes that may be sensitive to Pep4. Is it just one gene out of 38 SAGA dominated genes, or is it more? Comparing the RNA seq of the nup170D and nup170Dpep4D strains to identify additional genes behaving like HIS4 would greatly strengthen the paper."*

Response: We agree with the Reviewer that a broader view of the consequence of the altered SLIK:SAGA ratio in the various mutants, as it relates to the expression of SAGA-dominated genes, would strengthen the manuscript. Rather than performing RNA seq analysis (at significant cost), we expanded our RT-qPCR analysis to 10 genes downregulated in the *nup170Δ* strain (Fig. S1) and categorized as the SAGA-dominated genes. The results of this analysis in WT, *nup170Δ*, *pep4Δ* and *nup170Δ pep4Δ* mutants are summarized in a new version of Fig. 6 (also shown in **Fig. R1** below). The reduced expression of several of these genes in the *nup170Δ* mutant were suppressed, at least partially, by the additional *pep4Δ* mutation. Thus, we conclude that, of the SAGA-dominated genes downregulated in *nup170Δ* mutant, a subset is altered by Pep4 and changes in the SAGA/SLIK ratio. It is important to note, and we have mentioned this in the Discussion, that changes in the passive properties of NPC caused by the loss of Nup170 may also alter additional transcriptional events unlinked to Pep4 as highlighted by the Nup170-dependent expression of *PHO5*, *PDR5*, and *ADE17*.

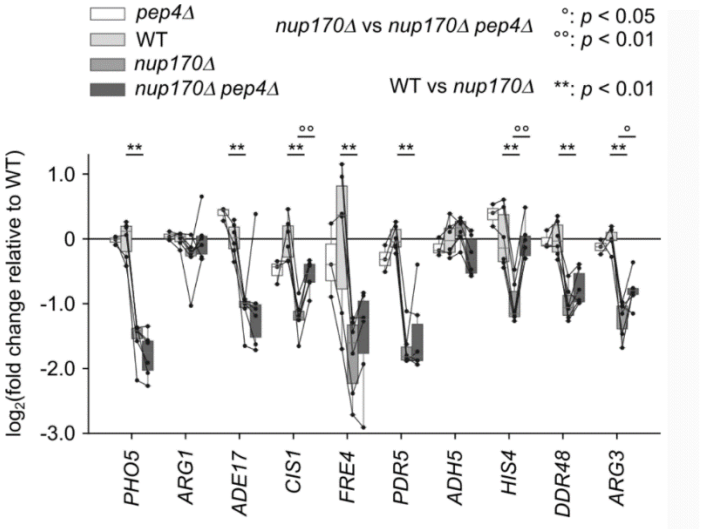

Figure R1. Pep4-dependent changes in expression of genes downregulated in the *nup170Δ* mutant.
Total RNA was isolated from WT, *nup170Δ*, *pep4Δ*, and *nup170Δ pep4Δ* mutant strains, and mRNA levels of the indicated genes were examined by the RT-qPCR. Levels of mRNA encoded by the indicated genes in each mutant were normalized to amounts in the WT strain. Fold changes in amounts detected in the mutants relative to WT cells were plotted. The individual biological replicates are plotted as filled circles (n = 3 for the *pep4Δ* strain, n = 6 for the WT, the *nup170Δ* and the *nup170Δ pep4Δ* strains), and the data points from samples analyzed in parallel are connected with lines. Boxes represent the first and third quartiles of the data points. Significant differences in mRNA levels between the WT and the *nup170Δ* strains, as determined by a two-tailed paired *t*-test, are indicated by asterisks (*). For the genes exhibiting reduced mRNA levels in the *nup170Δ* strain, we further compared their mRNA levels between the *nup170Δ* and the *nup170Δ pep4Δ* strains. Significant differences in mRNA levels between the *nup170Δ* and the *nup170Δ pep4Δ* strains, as determined by a two- tailed paired *t*-test, are indicated by circles (°).

Comment 2: *"The authors propose that certain physiological or stress conditions, such as H2O2 or Hexanediol treatment, may modify nuclear pore complex diffusion rates and increase nuclear import of Pep4. They claim that Pep4 is difficult to localize because HA- or GFP tagged versions are*

unstable. Is this the case whether the tag is put at the N- or the C terminus? If possible, it would be good to show that the distribution of Pep4 indeed becomes more nuclear in nup170D or specific physiological conditions."

Response: We agreed with the Reviewer that experiments allowing direct visualization of increased levels of Pep4 in nucleoplasm would provide additional supporting evidence for our Pep4 cleavage assay. In this regard, we used various approaches in an attempt to detect Pep4 outside of vacuoles including C-terminal GFP-tagging and epitope tagging (HA), the latter for the purpose of immunofluorescence and cell fractionation analysis. Anti-Pep4 antibodies were also examined using similar approaches. In all cases, we the results were not interpretable due to the instability of the fusion constructs or background produced by the anti-Pep4 antibody (See **Fig. R2**, and additional comments and data in response to Comment 2 of Reviewer 2). In addition, subcellular fractionation experiments are confounded by the damage that occurs to yeast vacuoles and the nuclear envelope membrane during isolation. Regarding the Reviewer's suggestion that we tag the N-terminus of Pep4, we considered this approach, but Pep4 contains an N- terminal ER-targeting sequence followed by a vacuolar-sorting sequence attached to the mature protein, which makes functional N-terminal tagging of the mature Pep4 problematic. It was with these various pitfalls in mind that we devised an in vivo assay to detect Pep4-specific cleavage activity using nuclear- and cytoplasmically-positioned reporters.

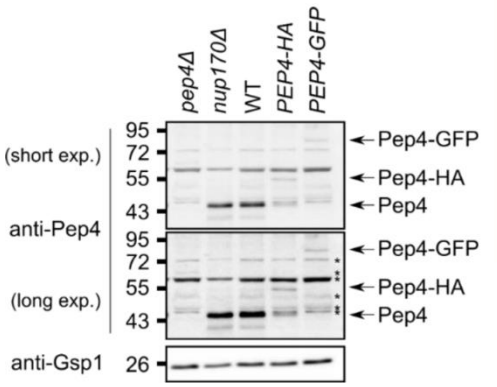

Figure R2. Pep4-GFP and Pep4-HA are labile. The whole cell extracts from the indicated strains were analyzed by western blot analysis using anti-Pep4 and anti- Gsp1 antibodies. For anti-Pep4 blot, images of two different exposure conditions are shown. The positions of mass markers are indicated in kilodaltons. Asterisks denote proteins non-specifically recognized by the anti-Pep4 antibodies, as they are present in protein samples from the *pep4Δ* strain.

Comment 3: *"p.14: "leading us to conclude that Nup170 was functioning to upregulate Pep4-mediated cleavage of Spt7." The sentence should say that Nup170 is there to reduce Spt7 cleavage by Pep4 and not to upregulate Pep4 dependent cleavage. The sentence should be "leading us to conclude that* ***loss of*** *Nup170 was functioning to upregulate Pep4-mediated cleavage of Spt7"."*

Response: Thank you for this correction. We have modified the text accordingly. We have also corrected the typos noted by the reviewer.

Comment 4: *"Is Spt7 fully nuclear in nup170D?"*

Response: Spt7-GFP localization to the nucleus in the *nup170Δ* mutant is indistinguishable from that observed in WT cells (see **Fig. R3 below**).

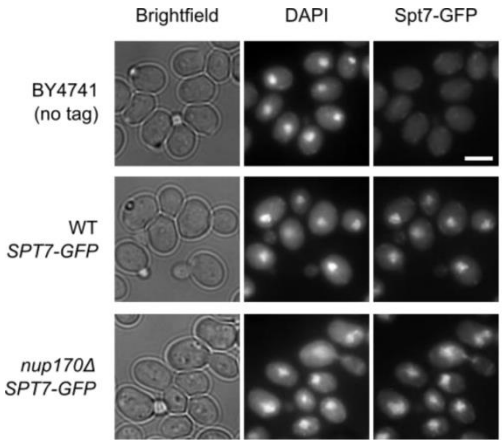

Figure R3. The *nup170Δ* mutation does not alter the localization of Spt7-GFP. The localization of SPT7-GFP was examined in WT and the *nup170Δ* mutant. The position of the nucleus was determined by DAPI staining. Untagged WT cells (BY4741) were also examined in order to assess the background autofluorescence signal. Bar: 5 µm.

Response to Reviewer 2:

Comment 1a: *"although Pep4-GFP fusions are non-functional for Spt7 cleavage and SLIK production, they were previously used to analyze the vacuolar targeting of Pep4 (PMID:31332264) or its vacuolar release in peroxyde-treated cells (Mason et al, 2005). The authors should take advantage of this tool to visualize Pep4 localization in nup170Δ cells."*

Response: Similar points were raised by Reviewer 1, comment 2. We direct Reviewer 2 to this response. In addition, we would add the following concerns to any use of Pep4-GFP fusions to assess the localization of Pep4. First, while the GFP signal in strains producing Pep4-GFP are largely vacuolar in previous publications (and in our hands, see **Fig. R4** below), our analysis suggests there is little full length Pep4-GFP in cells producing this fusion protein (see **Fig. R2**). Thus, much of the vacuolar GFP signal visible in these cells is likely arising from a truncation of Pep4-GFP or GFP alone. Second, while we thank the reviewer for pointing out the Mason et al., 2005 studies, our work has raised serious concerns regarding conclusions made by Mason and colleagues. We have performed similar experiments examining the effects of H_2O_2 on the localization of Pep4-GFP. Unfortunately, as shown in **Fig. R4**, treatment of cells lacking Pep4-GFP (i.e. no GFP tag) produced a cytoplasmic GFP channel fluorescence signal of similar intensity as that detected in strains expressing Pep4-GFP. This background autofluorescence signal prevents us from making any conclusions on the redistribution of Pep4-GFP. Thus, the data pointed out by the Reviewer as evidence for detecting release of Pep4-GFP from vacuoles was quite possibly an artefact.

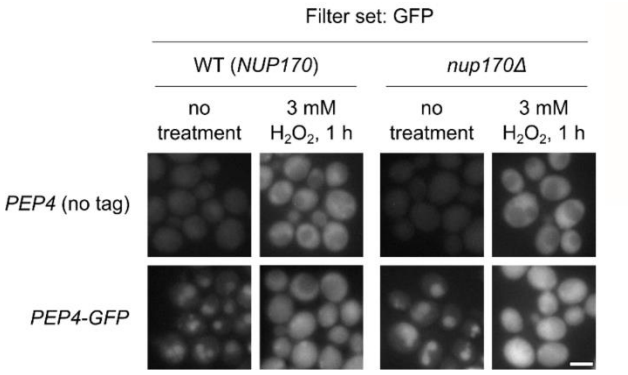

Figure R4. Analysis of fluorescent signal arising from cells treated with H2O2. Strains expressing *PEP4* or *PEP4-GFP* were subjected to the fluorescent microscopy analysis. The indicated samples were treated with 3 mM H_2O_2 for 1 h before the examination. Bar: 5 µm.

Comment 1b: *"- alternatively, Pep4 antibodies have been described (PMID:31332264) and could be used in immunofluorescence experiments."*

Response: This point was also addressed in our response to Reviewer 1, comment 2 above. To expand on this discussion, our attempts to use anti-Pep4 antibodies for immunofluorescence microscopy revealed significant background signal that was not appreciably reduced by pretreatment of the antiserum with cell extracts from *pep4Δ* mutant cells (**Fig. R5**). Thus, the results of these experiments were not interpretable.

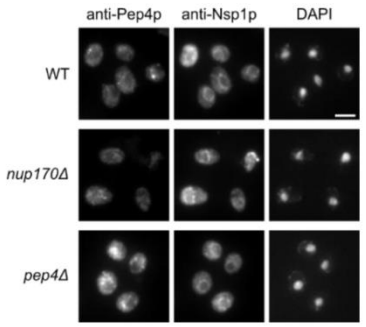

Figure R5. Immunofluorescence experiment using anti-Pep4 antibody. The strains with indicated genotypes were fixed with formaldehyde and subjected to the immunofluorescence analysis using an anti-Pep4 (precleared with *pep4Δ* mutant cell extracts and anti-Nsp1 (an NPC protein) antibodies. Note that the signal from the anti- Pep4 antibody observed in the *pep4Δ* cells. Bar: 5 μm

Comment 2: *"The authors should analyze in this experiment the expression of several other SAGA-dominated genes (from Fig. S1B) to provide a broader vision of the contribution of this mechanism to Nup170-dependent gene expression."*

Response: This point was addressed in our response to Reviewer 1, comment 1 above.

Comment 3a: *"To test the physiological relevance of this pathway for SAGA regulation, the authors have measured Spt7 cleavage as a readout of SLIK production in different genetic and stress conditions known to impact NPC permeability (Fig. 4E and 5).*

- Can the author control that the reported changes in Spt7 cleavage are dependent on Pep4 activity (by performing the same experiments in pep4 mutant cells)?"

Response: As shown in Fig. 5A of the manuscript, we have now included data showing that the enhanced cleavage of Spt7-TAP after treatment of WT cells with H_2O_2 or 1, 6-hexanediol was dependent on Pep4.

Comment 3b: *"- Do these situations also affect SAGA-dependent gene expression? The authors should test mRNA levels for typical SAGA-dependent target genes (as in Fig. 1) in the nup188 mutant or upon peroxide*
/ hexanediol treatment."

Response: As suggested by the Reviewer, we have examined the change in mRNA levels of the 10 genes examined in the *nup170Δ* and the *pep4Δ nup170Δ* mutants (see **Fig. R1** and manuscript Fig. 6) in the *nup188Δ* and the *pep4Δ nup188Δ* mutants. Overall, as in the *nup170Δ* mutant, the *nup188Δ* mutant showed a decreased or no change in the expression of the genes examined. The decreases in expression detected in the *nup188Δ* mutant were uniformly less than observed in the *nup170Δ* mutant. In addition, three genes (*HIS4*, *DDR48*, and *FRE4*) showing little or no change in expression in the *nup188Δ* mutant where among those showing a Pep4-dependent reduction in the *nup170Δ* mutant (**Fig. R6** and manuscript Fig. S3). We interpret these results to suggest that the smaller

changes in passive diffusion barrier observed in the *nup188Δ* mutant (Shulga et al., 2000), and the corresponding reduced impact on the SAGA/SLIK ratio (see manuscript Fig. 4E), reduces the effect of the *nup188Δ* mutant on expression of the examined SAGA- dependent genes as compared to the Pep4-dependent effects of the *nup170Δ* mutant on genes including *HIS4* and *CIS1*. This is now addressed in the Discussion.

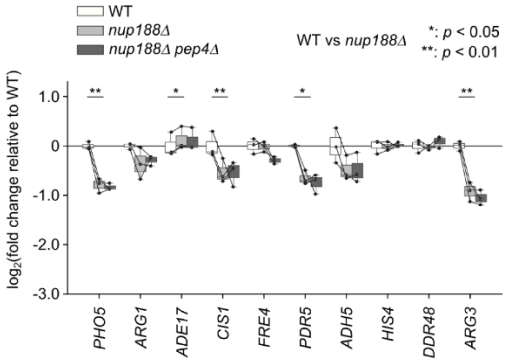

Figure R6. Change in mRNA levels of various genes in the *nup188Δ* and *nup188Δ pep4Δ* mutants. Total RNA was isolated from WT, *nup188Δ* and *pep4Δ nup188Δ* mutant strains, and the mRNA levels of the indicated genes were examined by the RT-qPCR. Levels of mRNA encoded by the indicated genes in each mutant were normalized to amounts in the WT strain. Changes in mRNA level detected in the mutants relative to WT cells were plotted. The individual biological replicates are plotted as filled circles (n = 3), and the data points from samples analyzed in parallel are connected with lines. Boxes represent the first and third quartiles of the data points. Significant differences in mRNA levels between the WT and the *nup188Δ* strains, as determined by a two-tailed paired *t*-test, are indicated by asterisks.

Comment 4: *"- The 1st paragraph of the results section should indicate that 36% of the most downregulated genes are SAGA-dominated in the nup170Δ mutant (instead of 38%, see Fig. S1A)."*

Response: The numbers in Fig. S1A were wrong (38% is correct). We have now corrected Fig. S1A.

Comment 5: *"- It would be helpful for the reader if the names of the proteins identified in mass spec experiments were indicated alongside the corresponding bands in Fig. 3A, and if a table summarizing the composition of SAGA/SLIK complexes was provided. "*

Response: We have added the extra elements in Fig. 3A. We have also summarized all known SAGA/SLIK components in Table S1.

Response to Reviewer 3:

Comment 1a: *"The idea that Pep4 functions in the nucleus is conceptually challenging as it is (as pointed out by the authors) a vacuolar protease. While it is clear that the authors cannot tag Pep4, I suggest a crude subcellular fractionation followed by Western blot (as they have a Pep4 antibody) with the hope of showing that there is more Pep4 in the nucleus in the nup170Δ cells."*

Response: We direct Reviewer 2 to our response to comment 2 of Reviewer 1. To reiterate, the subcellular fractionation is accompanied by some lysis of vacuoles and damage to the NE of isolated nuclei, as well as diffuse in and out of the nucleoplasm. Thus, it is not reasonable to drawn conclusion on the cytoplasmic versus nucleoplasmic distribution of Pep4.

Comment 1b: *"In lieu of this (as it is likely a very small fraction of total Pep4 is released from the vacuole), some effort to exclude that Spt7 is degraded in the vacuole could help to solidify their*

conclusions. I suggest ruling out macroautophagy (with a atg8Δ strain) and also microautophagy (using a nvj1Δ).”

Response: We agree with the reviewer, and we have examined the effects of two mutations that inhibit autophagy on Spt7 cleavage: *atg8Δ* (abrogating autophagy in general) and *nvj1Δ* (abrogating piecemeal microautophagy of the nucleus) mutations. As shown in **Fig. R7**, analysis of Spt7 cleavage based on levels of the CT-Spt7-TAP revealed neither of the autophagy mutants affected Spt7 cleavage, suggesting Spt7 is cleaved outside of vacuoles. This result is mentioned in the newer version of the manuscript (Fig. S2B).

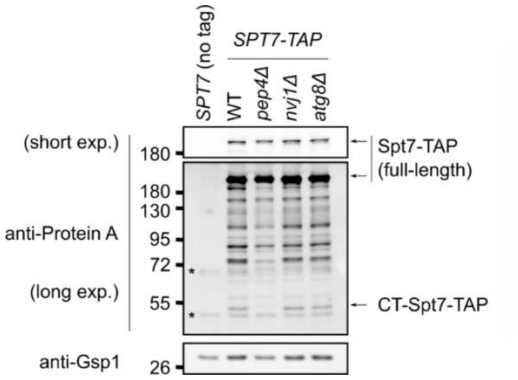

Figure R7. The Spt7 cleavage is unaffected in autophagy mutants. The whole cell extracts were prepared from the indicated mutant cells producing Spt7-TAP, and extracts were analyzed by western blot using anti- Protein A and anti-Gsp1 (loading control) antibodies. The protein extracts from strains without the TAP tag to the *SPT7* gene (no tag) were also analyzed to reveal background signals (asterisks). Indicated are full-length Spt7-TAP (two exposures are shown) and CT-Spt7-TAP (C-terminal fragment). The positions of mass markers are shown in kD.

We also examined the cleavage of the cytoplasmic reporter protein (GFP-NES-tSpt7-GST) in the *atg8Δ* mutant. This reporter protein was still cleaved in the *atg8Δ* mutant background, supporting our conclusion that Pep4-dependent cleavage of the reporter protein takes place in the cytoplasm. These data are provided solely for the Reviewers in **Fig. R8** below.

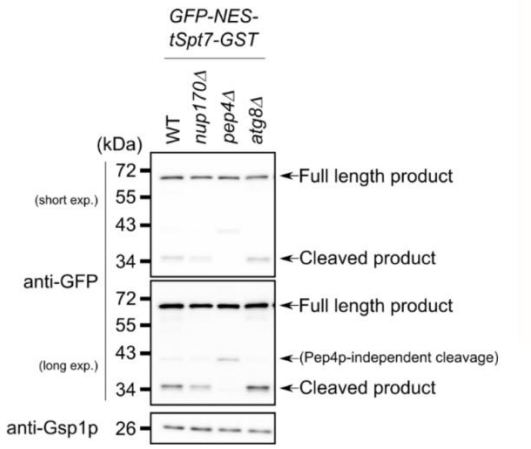

Figure R8. Pep4-dependent cleavage of the reporter protein in the cytoplasm. Cell lysates from WT, *nup170Δ*, *pep4Δ,* and *atg8Δ* mutant strains producing the GFP-NES-tSpt7-GST reporter gene construct analyzed by western blotting using the indicated antibodies to detect the full length and the cleaved NES reporter proteins (anti-GFP), and a loading control (Gsp1). Two exposures are shown of the anti-GFP immunoblots. The positions of mass markers are shown in kD.

Comment 2: *“Pep4 is 43 kD. It remains unclear how much a protein of this size would even see the diffusion barrier in a way that would be significantly affected by loss of Nup170. The authors may*

want to consider that whether Pep4 acts in the nucleus or not may not really matter. Once the diffusion barrier is perturbed, the steady-state distribution of many proteins will be affected including Spt7. The NLS/NES reporters address this to some extent although there will be more of the NLS version in the cytosol (and NES version in the nucleus) in the nup170D due to increase leakage back through the pore (even if this isn't obvious by the images)."

Response: Recent evidence (Timney et al., 2016) has shown that inert molecules in the size range of Pep4 shown significantly higher (>2-fold) measured NPC permeability coefficients (molecules/sec/NPC/μM) in a *nup170Δ* mutant than in WT cells, suggesting, even in its monomeric state, Pep4 passive diffusion would be altered in a *nup170Δ* mutant. By contrast, the large sizes of Spt7 and cleaved Spt7 (200 kD and 180 kD), coupled with their presence in the SAGE/SLIK complexes, make it very unlikely are they could diffuse through NPCs in either the *nup170Δ* mutant or WT cells. Moreover, as shown in Fig. S2A and **Fig. R3**, SAGA/SLIK components Spt20-GFP and Spt7-GFP are nuclear in both the *nup170Δ* mutant and WT cells. Finally, regarding the diffusion of the NLS reporter out of the nucleus in the *nup170Δ* mutant, the best we can do is to examine its localization by imaging, and we show it similarly concentrates in the nucleoplasm of WT cells and the *nup170Δ* mutant.

Comment 3: *"Figure 3. It would be helpful for the reader if the Spts could be labeled on the A-panel; a diagram of Spt7 with the location of the Pep4-cleavage site could also be helpful to interpret this figure."*

Response: We have added the extra elements in Fig. 3A.

References

Mason, D.A., N. Shulga, S. Undavai, E. Ferrando-May, M.F. Rexach, and D.S. Goldfarb. 2005. Increased nuclear envelope permeability and Pep4p-dependent degradation of nucleoporins during hydrogen peroxide-induced cell death. *FEMS Yeast Res.* 5:1237–51. doi:10.1016/j.femsyr.2005.07.008.
Shulga, N., N. Mosammaparast, R. Wozniak, and D.S. Goldfarb. 2000. Yeast Nucleoporins Involved in Passive Nuclear Envelope Permeability. *J. Cell Biol.* 149:1027–1038. doi:10.1083/jcb.149.5.1027.
Timney, B.L., B. Raveh, R. Mironska, J.M. Trivedi, S.J. Kim, D. Russel, S.R. Wente, A. Sali, and M.P. Rout. 2016. Simple rules for passive diffusion through the nuclear pore complex. *J. Cell Biol.* 215:57–76. doi:10.1083/jcb.201601004.

Second decision letter

MS ID#: JOCES/2019/237156

MS TITLE: Passive diffusion through nuclear pore complexes regulates levels of the yeast SAGA and SLIK coactivator complexes

AUTHORS: Tadashi Makio and Richard W. Wozniak
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers recognize that most of their initial criticisms have been addressed in your revised manuscript. However, reviewer #1 still raised issues that will require amendments to

your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

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

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

Reviewer 1

Advance summary and potential significance to field

The summary of the advance made in this paper and its potential significance is part of my initial review of this manuscript.

Comments for the author

In the revised version of the paper by Makio and Wozniak, the authors have addressed most issues raised by the reviewers by making new experiments, adding data and modifying the text accordingly, as well as by convincingly explaining why certain proposed experiments are not feasible. The paper should now be acceptable for publication considering the few comments below:

New Figure 5: if the cleavage of Spt7-TAP is indeed reduced in the pep4D mutant, one would expect to observe higher levels of Spt7-TAP full length in this strain compared to WT, which does not seem to be the case. It would be good to comment on that and/or to include a non-tagged WT and pep4D strain in these Western blot analyses to confirm that the 55kDa protein really corresponds to CT-Spt7-TAP.

New Figure 6: in this new experiment the authors quantified the levels of few additional SAGA dependent transcripts in the nup170D single and nup170Dpep4D double mutants and show that besides HIS4, the expression of few other genes, but not all, is substantially decreased in nup170 and rescued upon loss of Pep4, indicating that at least a subset of the transcriptional reductions are linked to Spt7 cleavage by Pep4.
The RNA quantifications have been repeated several times and the results and statistical analyses included in the new figure. However the presented box plots are very hard to read. It is certainly informative and perhaps more convincing to link the points belonging to the same experiment, but the overall picture becomes quite incomprehensible; same comment for Figures 2B and Figure S3, although these are easier to read. In the case of Figure 6, wouldn’t it be sufficient to show just the boxes without the lines and to include the version with the lines as a supplementary figure?

Reviewer 2

Advance summary and potential significance to field

The authors have carefully addressed my previous comments by performing additional experiments and providing new pieces of discussion in the text. Their data now convincingly support their conclusions.

Comments for the author

The manuscript is suitable for publication as such, without further modifications.

Reviewer 3

Advance summary and potential significance to field

This is an interesting paper exploring how changes to the diffusion barrier may specifically impact some gene expression programs. The revised version is improved over the original and I support publication.

Comments for the author

The authors have satisfactorily addressed all of my concerns.

Second revision

Author response to reviewers' comments

Response to Reviewer 1:

Comment 1: "*New Figure 5: if the cleavage of Spt7-TAP is indeed reduced in the pep4D mutant, one would expect to observe higher levels of Spt7-TAP full length in this strain compared to WT, which does not seem to be the case. It would be good to comment on that and/or to include a non-tagged WT and pep4D strain in these Western blot analyses to confirm that the 55kDa protein really corresponds to CT-Spt7-TAP.*"

Response: Although the comment is directed to Fig. 5, the identification of the 55 kD protein species was essentially made by the data presented in Fig. 3. As shown in Fig. 3B, when cleavage of Spt7-TAP is blocked in the *pep4Δ* mutant, differences in levels of the 55 kD protein species (CT- Spt7-TAP) in this mutant and WT cells is clearly visible as essentially none of the 55 kD protein species is seen in the *pep4Δ* mutant. Furthermore, as the amount of the CT-Spt7-TAP cleavage product is relatively small compared to full-length Spt7-TAP (compare full-length and the cleavage product in WT fractions in the long exposure panel), differences in levels of the full- length product in the WT versus the *pep4Δ* mutant are more difficult to see in these western blots exposures. This would also apply to the results shown Fig. 5. By comparison, differences in full-length Spt7-TAP are more apparent when comparing the *nup170Δ* mutant to the *nup170Δ pep4Δ* double deletion mutant (Fig. 3B, lanes 4 and 5). This is also the case for the 55 kD protein species (CT-Spt7-TAP), which is markedly enhanced in the *nup170Δ* mutant (Fig. 3B and C). We have added a comment on this in the Results section (line 222-225) and in the legend for Fig. 5A (line 680-681) to address the Reviewer's concern.

Comment 2: "*The RNA quantifications have been repeated several times and the results and statistical analyses included in the new figure. However the presented box plots are very hard to read. It is certainly informative and perhaps more convincing to link the points belonging to the same experiment, but the overall picture becomes quite incomprehensible; same comment for Figures 2B and Figure S3, although these are easier to read. In the case of Figure 6, wouldn't it be sufficient to show just the boxes without the lines and to include the version with the lines as a supplementary figure?*"

Response: For clarity purposes, we have followed the suggestion of the reviewer. We have prepared the newer version of Fig. 6, which only contains box-and-whisker plots (also shown in the next page). The previous version of the figure (containing lines) is now Supplementary Figure S3. We have changed the figure number accordingly in the text.

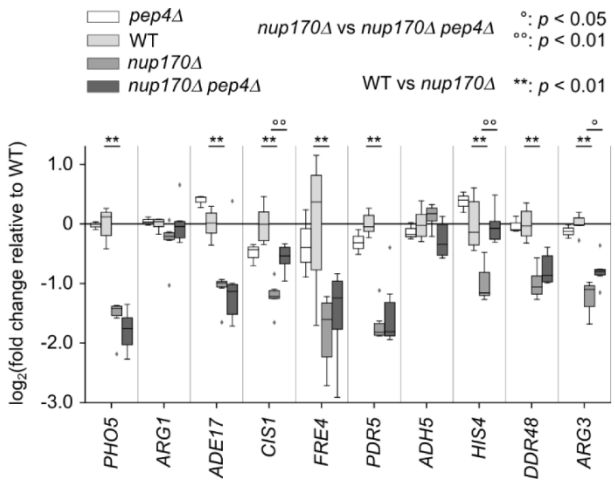

Figure 6. Reduced expression of several genes in the *nup170*Δ mutant is Pep4-dependent. Total RNA was isolated from WT, *nup170Δ*, *pep4Δ* and *nup170Δ pep4Δ* mutant strains, and the mRNA levels of the indicated genes were examined by the RT-qPCR. Levels of mRNA encoded by the indicated genes in each mutant were normalized to amounts in the WT strain. Fold changes in amounts detected in the mutants relative to WT cells were plotted on a log2 scale with a box- and-whisker plot (*n* = 3 for *pep4Δ*, *n* = 6 for WT, *nup170Δ*, and *nup170Δ pep4Δ*). Boxes represent the first and third quartiles of the data points, and horizontal lines in the boxes represent the median. The whiskers extend over all the data points except outliers. The outliers are plotted as diamonds. Significant differences in mRNA levels between WT and the *nup170Δ* strain, as determined by a two-tailed paired *t*-test, are indicated by asterisks (*). For the genes exhibiting reduced mRNA levels in the *nup170Δ* strain, we further tested their mRNA levels between the *nup170Δ* and the *nup170Δ pep4Δ* strains. Significant differences (two-tailed paired *t*-test) in mRNA levels between the *nup170Δ* and the *nup170Δ pep4Δ* strains are indicated by open circles (°). All the observed data are plotted in Fig. S3.

Third decision letter

MS ID#: JOCES/2019/237156

MS TITLE: Passive diffusion through nuclear pore complexes regulates levels of the yeast SAGA and SLIK coactivator complexes

AUTHORS: Tadashi Makio and Richard W. Wozniak
ARTICLE TYPE: Research Article

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