nature portfolio

Peer Review File

The novel ribosome biogenesis inhibitor usnic acid blocks nucleolar pre-60S maturation

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Kofler, Grundmann et al. aims to characterize the 60S ribosomal biogenesis inhibitor usnic acid.

Major issues:
1. In their initial characterization of usnic acid (Awad et. al – Fig.3), nucleolar/nuclear accumulation of ribosomes is observed for both 40S and 60S ribosomal subunits. In addition, the accumulation of 20S rRNA is reported. In the current manuscript, the authors explore a possible inhibitory effect of usnic acid on 60S maturation, but there is no discussion of the previously reported effect on 40S. The authors must address this, as their title (and manuscript) implies a specific 60S-directed effect.
2. There are major discrepancies between the mass spec data and the cryo-EM analysis: The cryo-EM analysis reveals a dramatic enrichment of cluster 2 and an equally pronounced reduction in cluster 3. According to Table S3, the only compositional change between these two intermediates is the binding of Rpl23, Nog1, Ssf1 and Rrp15 to cluster 3. One therefore would expect that the mass spectrometry data would indicate an overall reduction in these factors after usnic acid treatment. In both Nop7-TAP and Nsa1-TAP purifications, however, Rrp15 and Ssf1 are among the most enriched factors while Nog1 is enriched in the Nsa1 sample and unchanged in the Nop7 sample. This discrepancy is left unexplained. Similarly, the authors state that "Usnic acid, in contrast, seems to directly interfere with the compaction of pre-60S particles in the nucleolus after Nog1, Rlp24 and Tif6 associate and prior to Dbp10 association" and "The stage of inhibition can be narrowed down to a step shortly after Nog1 incorporation but before Mak11 dissociates and Dbp10, Nug1 and Nsa2 bind at the transition from state B to C." However, according to their structural analysis (Table S3), Nog1 is not bound in the accumulated cluster 2, but only observed in cluster 3, i.e. in the depleted intermediate.
3. While the use of CryoDRGN has the benefit of being unbiased, in this case, using a global analysis of heterogeneity makes it hard to classify local differences in the region of interest (i.e. the domain VI region). One strategy would be to combine clusters 2,3,4 and then perform a skip align classification of the masked domain VI region to exhaustively define the heterogeneity in this region. This strategy would also reveal if a subset of particles in fact contains Mak11, the most enriched component upon usnic acid addition. The position of this protein is known (pdb 6C0F), so a skip align classification with a mask encompassing the known binding region should allow for a specific selection of Mak11-bound intermediates. If Mak11 is not observed, the authors should discuss why it is absent.

Minor issues:
1. The authors should cite and discuss published data on Mak11 accumulation after Nog1 depletion – Saveanu et al - doi: 10.1128/MCB.23.13.4449-4460.2003. At first glance, the enrichment pattern looks comparable to that observed upon usnic acid addition.
2. The authors make extensive use of the word "rebound" to describe the effect of 60S assembly blockage on earlier biogenesis steps. In a biological or medical context, however, the word rebound is used to describe the effect on a system when a specific treatment or drug is stopped. I.e. rebound would the term used to describe if/how cells recover after the removal of usnic acid. Such an analysis would in fact be very informative in understanding how usnic acid affects biogenesis, i.e. if assembly blockage is reversible. Some suggestions for more appropriate words and sentences to describe the "traffic jam" effect that the authors are describing would be: "bottleneck effect", "congestion collapse" or "retrograde assembly disruption".
3. Purification via Noc2-TAP is not ideal, because Noc2 engages the pre-60S twice: Early nucleolar 60S particles are bound by the Noc1/Noc2 heterodimer while Noc3/Noc2 heterodimers bind late nucleolar intermediates. This unnecessarily complicates proteomic and gel-based analysis in two ways: the purified intermediates represent two separate populations and usnic acid treatment is expected to

affect them in opposite ways.
4. According to the methods, usnic acid was not present in the purification buffers of the treated cells. While unlikely that it contributes to the structural integrity of cluster 2, it probably wouldn't hurt to have it around in case it does.

Overall, this manuscript as written has too many flaws and inconsistencies. The cryo-EM analysis is too superficial and does not perform a focused classification of the variability in the domain VI region, a possible reason why the factor composition within the clusters is inconsistent with the mass-spec data and no density is observed for the enriched Mak11 protein. Usnic acid is an intriguing compound that may prove an important tool in furthering our understanding of ribosome biogenesis. However, because the specific target for usnic acid-mediated inhibition of 60S assembly remains elusive and no data is shown to confirm or clarify previously published data indicating that usnic acid blocks assembly of both 40S and 60S, this paper does not offer enough insights to merit publication in Nature Communications.

Reviewer #2 (Remarks to the Author):

This manuscript from Helmut Bergler's group and their collaborators very nicely describes experiments to better understand when and how the small molecule usnic acid blocks large ribosomal subunit maturation in yeast. This is a very interesting and important problem (and manuscript) because usnic acid blocks biogenesis within minutes. This time frame is much faster than was previously possible using other approaches, such as depletion or inactivation of ribosomal proteins or assembly factors. Therefore, importantly, using such drugs may enable a more precise demarcation of events in the ribosome assembly pathway, and could eventually provide an entree into therapeutic approaches to deal with ribosomopathies. I suggest the following changes and additions to improve this manuscript:

(1) Because ribosome assembly is so very much integrated with regulation of cell growth and cell division, it is important to briefly explain whether there is any likelihood that the observed block is indirect. It does seem to me that the effects on subunit biogenesis are far too rapid to result from primary effects on growth or division propagated to assembly. Nevertheless, the authors might briefly comment whether or not such indirect effects have been ruled out.

(2) Page 3, lines 73-74: the authors mention that the pathway progresses rapidly. How rapidly? I recall that in a previous paper these authors might have said 15 minutes! Knowing this short time frame for assembly might help the reader even better appreciate the value of seeing effects of usnic acid within 2-5 minutes.

(3) Figure 1B: Can the authors describe in a little more detail how MICs were determined? Are the data shown the final OD to which cells grew? How long did this take to occur?

(4) Figure 2B and text, page 6: the "fragmented/dotted signal" for nucleolar reporters such as Nog1-GFP and Tif6-GFP is interesting and very important to begin to understand the relationship between nucleolar morphology and ribosome biosynthesis. Can the authors discuss it a little bit more? Has such fragmentation been observed under other different conditions that block ribosome assembly? Why do they speculate that particles are aggregating?

(5) Figure 3: Why were affinity purifications done from cells treated with 60 micromolar usnic acid rather than 40 micromolar used in experiments described in Figures 1 and 2? And, …does the drug work when cells are grown in rich media?

(6) Figures 3A and 4A: can the authors label the proteins in their gels, as they did in Figure 4B?

(7) Figure 3B: the northern blot experiments show that the 7S pre-rRNA is decreased and that 27SA2 pre-rRNA accumulates a little bit. However, these experiments do not directly assess effects on 27SB pre-rRNA. Yet, the proteomics data clearly indicate that assembly is blocked between "states B and C". These state B and C pre-ribosomes contain mostly 27SB pre-rRNA. Thus, it is important to show more clear assays for a block in 27SB pre-rRNA processing. This is best shown by primer extension assays, since it is often tricky to resolve 27SA from 27SB pre-rRNA on agarose gels used for northern blots.

(8) Figure 4E: why are effects shown for Nog2, Rsa4, and Bud20 using Nsa1-TAP as a bait for purification? Don't these factors assemble after the Nsa1 module exits? Do these data reflect some modest heterogeneity in the assembly pathway, i.e. that Nog2 etc. sometimes enter earlier, or that Nsa1 exits later? Or, might these data reflect contamination?

(9) Page 16, line 381: is this supposed to be state 1 or state 2?

(10)Figure 7: Nop2 is shown in dark blue. Shouldn't it decrease if it assembles after the usnic acid block?

(11) Understanding exactly where the usnic acid block occurs, and why, is potentially the most impactful part of the work in this manuscript. Therefore, could the authors maybe provide a little more detail in the Discussion? The authors rightly conclude that the usnic acid-induced block is after entry of Nog1, Tif6, and Rlp24, and immediately before entry of the assembly factor Dbp10. Only recently has Dbp10 been visualized by cryo-EM (in human and Chaetomium pre-ribosomes, but not in Saccharomyces). Therefore, exactly where Dbp10 fits into this assembly scheme had been somewhat unclear. Can the authors better clarify the distinction between intermediates 2, 3, and 4? They (rightly) state that their intermediates 2 and 4 resemble state B and state C particles from Kater et al.. Does their intermediate 3 resemble state 1 or state 2 from Sanghai et al. ? The text says state 1 but I think they mean state 2 see (9) above. . Would it be helpful to point out that the structures of these large subunit assembly intermediates (states A and B and state 2) from different investigators differ? For example, Kater et al. never visualize (or detect by SDS-PAGE)the Ssf1 module in their state A or B particles, while I recall that Sanghai et al. see these assembly factors in their state 2 particles. Is this state 2 (i.e., intermediate 3 of Dr. Bergler) otherwise identical to the B particles of Kater et al.?

(12) Continuing from (11) above: Can the authors speculate why they see a greater effect on assembly of Dbp10 than Nsa2 or Nug1? To what extent does entry of Dbp10 depend on Nsa2 or Nug1? Can the authors elaborate a little more about structural models to account for these different effects?

(13) Toward identifying the specific target of usnic acid...Does mass spec indicate whether usnic acid co-purifies with pre-ribosomes purified from usnic acid treated cells?

Sincerely,
John Woolford

Reviewer #3 (Remarks to the Author):

Kofler et al. perform a comprensive and exhaustive biochemical, structural, and cellular analysis of the novel compound usnic acid on pre-60S ribosome biogensis. Of significant interest, the authors find significant shifts in particle distributions between treated/untreated cells that provide insights into the blockage associated with usnic acid. Overall, the authors methodology is sound and supports their conclusions. The use of neural networks to cluster the particles and look at distributions is quite powerful and elegant. I recommend publication in Nature Communications, provided the authors address the concerns below.

1. I thank the authors for providing the maps for quality control and for their clarification about modeling: "We did not model identified clusters as they largely reconstitute previously published high resolution structures (Kater et al, Sanghai et al). We fitted these models in the reconstructions using flexible fitting and used them to color our maps in chimerax. A more detailed description can be found in the material and methods section."

For a general reader, I think this short description or something similar would be helpful. It answers questions about these structures possibly being new states and how they are being identified/ordered. I also think that expanding on the rRNA conformations and/or degree of processing within these structures is warranted. It is a very cool result!

2. In general, I think the approach of pooling the particles from both treated and untreated cells is elegant and helps avoid certain biases, and it's exactly what I would do.

a. However, because the particles are pooled, how do you know that you aren't enriching for states that you might only find in one condition or missing some states that might exist in only one condition? This might be addressed by doing the same analysis on each set of particles and finding the same or similar structures as in the pooled dataset. Another approach would be to use the particles from each condition for each state, respectively, and perform a reconstruction.

b. I think technical replicates are important. I think biological replicates would be more insightful and important here, especially if you are going to calculate percentages of particles that make up each state.

c. I think describing briefly how the clustering is performed would help a general reader understand what you are doing in the results section. For instance, how does a neural network analysis help here? Where are you getting these clusters?

3. How can you be sure the tag used for pull down isn't influencing the states you are seeing? Similarly, could you comment on the linearity of the process? It seems to block maturation after a certain point, as you mention. Is it possible that it promotes disassembly of another state instead?

4. I think using this structural approach as a general strategy is a nice point from the manuscript. The other points about what was learned about ribosome biogensis and how this could be related to cancer properties of the compound did not immediately jump out at me after reading the manuscript. This could perhaps be made clearer in a revised manuscript.

David Taylor

Response to Reviewer #1:

We thank reviewer 1 for critically assessing our manuscript. Please find a point-to-point response to all queries below.

Major issues:

1. In their initial characterization of usnic acid (Awad et. al – Fig.3), nucleolar/nuclear accumulation of ribosomes is observed for both 40S and 60S ribosomal subunits. In addition, the accumulation of 20S rRNA is reported. In the current manuscript, the authors explore a possible inhibitory effect of usnic acid on 60S maturation, but there is no discussion of the previously reported effect on 40S. The authors must address this, as their title (and manuscript) implies a specific 60S-directed effect.

In our initial screen, we chose 60 minutes as incubation period for all inhibitors to do not miss subtle effects. This long incubation, however, does not allow discrimination between primary, secondary, or tertiary effects on ribosome biogenesis. In our current characterization we specifically aimed at the shortest (technically feasible) time frame, to identify the primary effects that occur upon usnic acid treatment. This revealed that 60S maturation defects are detectable almost immediately (within 2 min) while 40S defects take much longer to arise.

We now included additional microscopy data showing that accumulation of the 40S-reporter Rps9-GFP is only detected after longer incubation times (new Supplemental Fig. S2A). Consequently, we scored the 60S defects as the primary block and focused our analyses on large subunit maturation. We mention the outcome in the result section as suggested by the reviewer.

2. There are major discrepancies between the mass spec data and the cryo-EM analysis: The cryo-EM analysis reveals a dramatic enrichment of cluster 2 and an equally pronounced reduction in cluster 3. According to Table S3, the only compositional change between these two intermediates is the binding of Rpl23, Nog1, Ssf1 and Rrp15 to cluster 3. One therefore would expect that the mass spectrometry data would indicate an overall reduction in these factors after usnic acid treatment. In both Nop7-TAP and Nsa1-TAP purifications, however, Rrp15 and Ssf1 are among the most enriched factors while Nog1 is enriched in the Nsa1 sample and unchanged in the Nop7 sample. This discrepancy is left unexplained. Similarly, the authors state that "Usnic acid, in contrast, seems to directly interfere with the compaction of pre-60S particles in the nucleolus after Nog1, Rlp24 and Tif6 associate and prior to Dbp10 association" and "The stage of inhibition can be narrowed down to a step shortly after Nog1 incorporation but before Mak11 dissociates and Dbp10, Nug1 and Nsa2 bind at the transition from state B to C." However, according to their structural analysis (Table S3), Nog1 is not bound in the accumulated cluster 2, but only observed in cluster 3, i.e. in the depleted intermediate.

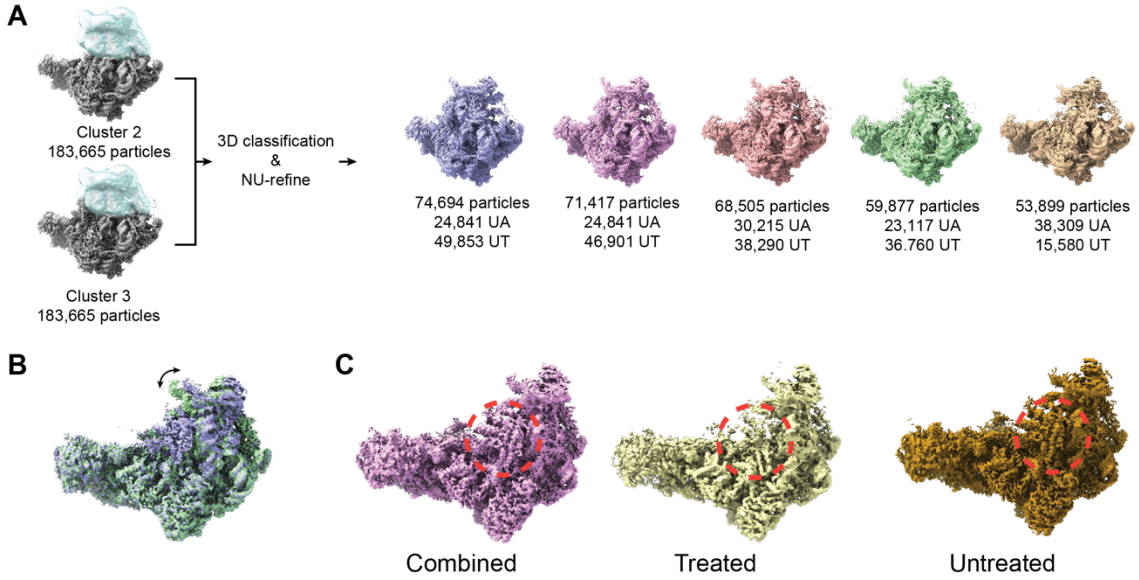
It is a well-known limitation of cryo-EM analyses that loosely bound and flexible components can in many cases not (fully) be resolved despite being associated with the particle (in the context of pre-ribosomes many of the transiently binding maturation factors). For proteins, qMS analysis is much more sensitive in the detection of even loosely bound factors but lacks the structural information about binding site and conformation. A well-documented example: As Rrp14 is already bound, Ssf1 and Rrp15 can bind, but are not stably incorporated yet as domain V is not fully folded. The Hurt lab, also detected Ssf1 in their purification (Figure S1 A), but could not resolve it in their structures. So Rrp15/Ssf1 are bound, but not stably incorporated and thus not resolvable. This explains discrepancies between structural and proteomics data.

Regarding Nog1 and its enrichment levels: A short helix of Nog1 is found near Rlp24 and Tif6 in reconstructions of cluster 2 (resembling state B). As it was not modeled in state B (PDB-6EM3) though being present in the cryo-EM map, we did miss this short helix. We are thankful to the reviewer for bringing this to our attention. Nog1 therefore is certainly present at this stage but not fully visualized

due to flexibility. Further parts of the middle region (approx. residue 347 to 448) of Nog1 are visible in cluster 3 (resembling State C). However, only upon full incorporation occurring in cluster 4 it is visualized by cryo-EM in its full entirety. Nop7, which is already present in the earliest, co-transcriptional pre-60S particles (Sanghai et al., NSMB 2023) and stays associated with Nog1 for a much longer time than Nsa1 (see Klinge and Woolford, 2019). This means that most of the Nop7-TAP particles are already associated with Nog1, which is only released in the cytoplasm. Since each pre-ribosome can contain only one copy of Nop7 and Nog1, the highest possible enrichment level (i.e. 100% of particles contain both proteins) of Nog1 is small. In contrast, Nsa1 particles associate with Nog1 for a shorter period of time, which means that fewer Nsa1-containing particles also contain Nog1. In that case, the maximum possible enrichment of Nog1 is higher. Therefore, the levels of enrichment are different for individual factors.

3. While the use of CryoDRGN has the benefit of being unbiased, in this case, using a global analysis of heterogeneity makes it hard to classify local differences in the region of interest (i.e. the domain VI region). One strategy would be to combine clusters 2,3,4 and then perform a skip align classification of the masked domain VI region to exhaustively define the heterogeneity in this region. This strategy would also reveal if a subset of particles in fact contains Mak11, the most enriched component upon usnic acid addition. The position of this protein is known (pdb 6C0F), so a skip align classification with a mask encompassing the known binding region should allow for a specific selection of Mak11-bound intermediates. If Mak11 is not observed, the authors should discuss why it is absent.

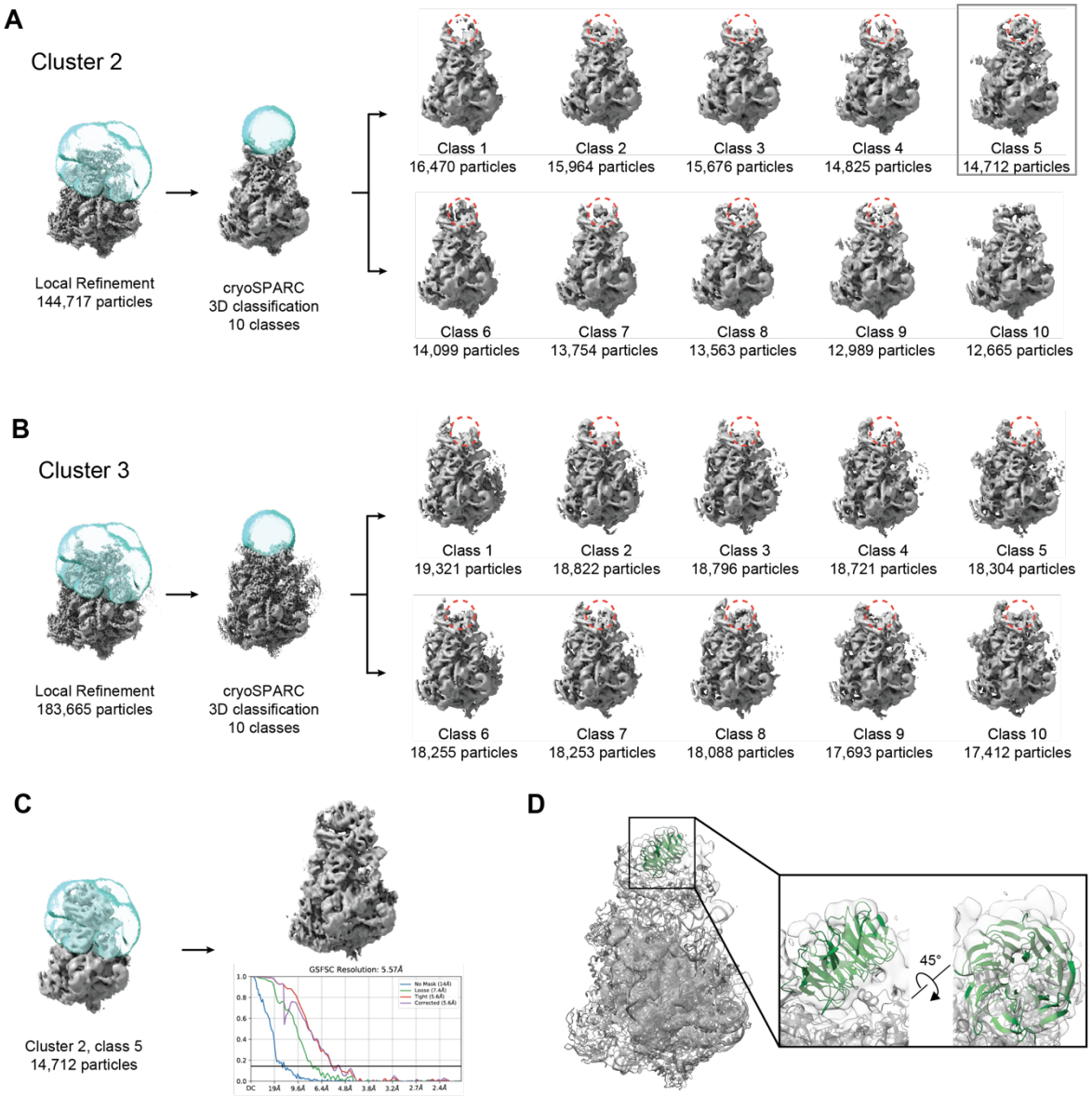
We agree with the reviewer, that classical focused refinements could allow to classify further structural heterogeneity within domain VI and identify Mak11 binding. As our manuscript focuses on the effect of usnic acid, we combined cluster 2 and 3, enriched in the treated and untreated dataset, respectively for one round focused 3D classification masking around Domain 6 (Revision figure 1A). The resulting classes were highly similar in regard to the compositional heterogeneity in the domain 6. Moreover, our classes showed conformational heterogeneity, caused by flexible movement of the domain 6 (Revision Figure 1 B). We observed density for the Rrp15-Ssf1 for the combined classes 1 to 4. Therefore, we separated these classes into treated and untreated particles for refinement. Strikingly, the separated classes recapitulated cluster 2 and 3 presented in the current manuscript. In summary, we observed no further compositional heterogeneity in cluster 2 and 3 than already described by our cryoDRGN approach. The observed conformational heterogeneity in domain 6, does not grant further insights into the inhibitory mechanism on pre-60S biogenesis by usnic acid. Consequently, we decided to omit this data from our manuscript.



Revision Figure 1: 3D classification focused on domain 6, identifies further conformational heterogeneity in the combined clusters 2 and 3. *(**A**) Cluster 2 and 3 were combined for a focused classification in cryoSPARC without image alignment (Target resolution 8Å). The resulting 5 classes were refined using Non-uniform (NU)-refinement. The combined number of particles and for the unsic acid treated (UA) and untreated (UT) particle numbers are*

*indicated under the classes. (**B**) Overlay of class 1 and 4, demonstrating the different conformations classified. (**C**) Class 2 was separated into treated and untreated particles and refined. The combined and untreated refinement show density corresponding to Rrp15-Ssf1, which is missing the treated cryo-EM map.*

As suggested by the reviewer, we performed 3D classifications without alignment, focused on the Mak11 binding site, separately on the particles of cluster 2 and cluster 3. Consistent with our biochemical results, we only detect Mak11 in a subpopulation of Cluster 2 from the treated strain, but not in cluster 3, that is enriched in the untreated particles (Revision Figure 2). However, due to flexibility and the low number of particles in this reconstruction, the density is not well resolved. We therefore refrain from including it in the manuscript. Further, we would like to point out that the resolution for Mak11 on the state 2 cryo-EM density (EMD-7324) also did not allow the building of an atomic model. Mak11 was therefore modelled without sidechain and sequence information in PDB-6C0F. Additionally, Sanghai et al. purified ribosomes from starved yeast cells. Hence, it is likely that the Mak11-bound state 2 does not represent a stable pre-60S intermediate in cells during log-phase growth.



Revision Figure 2: Mak11 is found in the usnic acid enriched cluster 2 but not on cluster 3. *The particles of cluster 2 and cluster 3 (**A, B** respectively) were locally refined using a mask encompassing Domain 6. The corresponding reconstructions were used for focused 3D classifications without alignment in cryoSPARC (10 classes, force hard classification), masking around the reported binding site of Mak11. **(A)** Class 5 showed a defined density at the Mak11 binding interface, indicated by the dashed red circle. **(B)** No 3D class of cluster 3 had a density at the Mak11 binding interface. **(C)** Cluster 2, class 5 particles were locally refined to a resolution of 5.6 Å. **(D)** PDB-6EM4 fitted as described in the methods of the manuscript, and Mak11 from from PDB-6C0F fitted as a rigid body.*

Minor issues:

1. The authors should cite and discuss published data on Mak11 accumulation after Nog1 depletion – Saveanu et al - doi: 10.1128/MCB.23.13.4449-4460.2003. At first glance, the enrichment pattern looks comparable to that observed upon usnic acid addition.

We thank the reviewer for drawing our attention to the fact that we forgot to mention Cosmins paper. We discussed and included it as a reference in the revised manuscript.

The overall changes in nucleolar particles isolated via Nop7-TAP were indeed highly similar to effects described for depletion of the so-called "B-factors" (e.g., Rlp24, Nsa2, Nog1, Nog2, Spb4, Dbp10 and Mak11). Depletion of Nsa2, for example showed overall a very similar phenotype (Biedka et al., J. Cell Biol. 2018), but there were also significant differences to usnic acid treatment. While depletion of Nsa2, had hardly any effect on Mak11 (slight decrease) on Nop7-TAP particles (Biedka et al., J. Cell Biol. 2018), usnic acid caused a strong accumulation. This shows that the inhibitor mediated block is different to depletion of Nsa2 and indicates that a step upstream of Nsa2 association is impaired. Nog1 is clearly present in the MS and Western blot analysis and therefore the block occurs after its binding. As we still find Nog1 on our Nsa1-TAP particles after usnic acid treatment, the accumulation of Mak11 cannot be explained by the lack of Nog1.

We added a short paragraph in the discussion section to make this clearer.

2. The authors make extensive use of the word "rebound" to describe the effect of 60S assembly blockage on earlier biogenesis steps. In a biological or medical context, however, the word rebound is used to describe the effect on a system when a specific treatment or drug is stopped. I.e. rebound would the term used to describe if/how cells recover after the removal of usnic acid. Such an analysis would in fact be very informative in understanding how usnic acid affects biogenesis, i.e. if assembly blockage is reversible. Some suggestions for more appropriate words and sentences to describe the "traffic jam" effect that the authors are describing would be: "bottleneck effect", "congestion collapse" or "retrograde assembly disruption".

The term "rebound" has indeed various meanings in different (medical) disciplines. We use the term as defined in Kofler et al., (2020, IJMS) in the context of ribosome biogenesis and not in a medical context. We thoroughly considered the suggested alternatives, but concluded that they do not adequately describe the observed effects. Thus, we decided to stay with "rebound effect". To avoid misunderstandings, we describe the definition of this term more clearly in the introduction and discussion and also refer the reader to our previously published description of this effect.

3. Purification via Noc2-TAP is not ideal, because Noc2 engages the pre-60S twice: Early nucleolar 60S particles are bound by the Noc1/Noc2 heterodimer while Noc3/Noc2 heterodimers bind late nucleolar intermediates. This unnecessarily complicates proteomic and gel-based analysis in two ways: the purified intermediates represent two separate populations and usnic acid treatment is expected to affect them in opposite ways.

We did choose Noc2 as bait protein for initial characterization, as it already binds co-transcriptionally to the nascent pre-60S particle together with Noc1 (Sanghai et al., NSMB 2023). This allows purification of the earliest pre-60S ribosomes which would not be possible with other well-established bait proteins. While it is true that Noc2 also binds at a later stage together with Noc3, we can distinguish the two complexes in our purifications by monitoring the presence of Noc1 and Noc3 by western blotting, as shown in Figures 4A and B. These analyses demonstrate a decrease of Noc2/3 and an increase of Noc1/2, in line with the 27SA$_2$ pre-rRNA enrichment within the total 27S pre-rRNA pool (Fig 4C). We included a statement in the results section to make it clearer why we used Noc2 as a bait.

4. According to the methods, usnic acid was not present in the purification buffers of the treated cells. While unlikely that it contributes to the structural integrity of cluster 2, it probably wouldn't hurt to have it around in case it does.

Ribosome biogenesis is driven by complex assembly and disassembly reactions of ~250 maturation factors and takes place in several different compartments of the cell. Once the cells are disrupted, ex vivo continuation of these complex processes during the purification procedure is not very likely. Therefore, we are in line with the reviewer and regard it as unlikely that the presence of the drug during the purification procedure affects the structural integrity of cluster2 or influences the particle composition. This was the reason why we decided not to add the compound during purification.

Overall, this manuscript as written has too many flaws and inconsistencies. The cryo-EM analysis is too superficial and does not perform a focused classification of the variability in the domain VI region, a possible reason why the factor composition within the clusters is inconsistent with the mass-spec data and no density is observed for the enriched Mak11 protein. Usnic acid is an intriguing compound that may prove an important tool in furthering our understanding of ribosome biogenesis. However, because the specific target for usnic acid-mediated inhibition of 60S assembly remains elusive and no data is shown to confirm or clarify previously published data indicating that usnic acid blocks assembly of both 40S and 60S, this paper does not offer enough insights to merit publication in Nature Communications.

We take the comments of the reviewer as indication that certain aspects of our manuscript were not presented clearly enough. In the revised manuscript, we tried to improve this wherever possible by additional analyses, comprehensive refinement and validation of our cryo-EM approach as well as by clarifying statements throughout the text.

As outlined in detail in our point-to-point responses, the differences between cryo-EM and qMS reflect technological limitations and are not flaws or inconsistencies in our paper. It is true, that we could not yet identified the target of usnic acid. Target identification of inhibitors remains one of the biggest challenges in modern drug discovery. Nevertheless, we are convinced that our data provide valuable and hitherto unachievable insights for ribosome biogenesis research.

Ribosome biogenesis is currently regarded as one of the most promising target pathways for the development of novel anti-cancer drugs (e.g., Burger et al. 2010; Brighenti, Treré, and Derenzini 2015; Bywater et al. 2012; Catez et al. 2019; M. Derenzini, Montanaro, and Trerè 2017; E. Derenzini, Rossi, and Treré 2018; Elhamamsy et al. 2022). Thus, it is crucial to understand what happens upon perturbation of this pathway by low molecular weight inhibitors like usnic acid, which has proven anti-tumor activity (e.g., Geng et al. 2018; Guzow-Krzemińska, Guzow, and Herman-Antosiewicz 2019; Pyrczak-Felczykowska et al. 2019; 2022)). The inhibition of ribosome biogenesis by usnic acid, as described in our manuscript provides an explanation for the anti-proliferative activity of usnic acid and must definitely be considered to evaluate the therapeutic potential of this compound.

Moreover, ribosome biogenesis is a highly complex and dynamic pathway that involves ~300 transacting factors that manage to assemble the ribosomal subunits within about 15 to 20 minutes. The consequences of perturbations, i.e. the enrichment or depletion of certain factors at distinct stages provide important insights into the order and coordination of the different maturation steps. Previous strategies for the investigation of ribosome biogenesis used for example genetic manipulations, depletion or targeted degradation of assembly factors. All these approaches, however, work at time scales larger than the time required for the transition of the pre-ribosomes through the whole pathway. A fast, dynamic, and interconnected pathway like ribosome biogenesis cannot be fully understood without approaches that can dissect individual events on a timescale that reflects the pace of the

pathway. As a consequence, it was impossible to dissect primary, secondary and tertiary effects that happen on a scale of a few minutes.

The approach presented here, using a fast-acting low molecular weight inhibitor, allows us to monitor changes after only 2 minutes which was not possible with other methods. With the combination of structural and biochemical analyses, this allows us for the first time to order still controversially discussed binding and dissociation orders of assembly factors (Nsa2, Mak11, Nug1, Dbp10). Moreover, we detect in near real-time how perturbations in the pathway rapidly build up and are step by step transmitted to the very first steps of ribosome formation. Therefore, our manuscript helps to pave the ground for understanding the dynamics of the pathway which is particularly important to prevent of factors misassignments to distinct or multiple maturation steps based on scoring secondary or tertiary effects upon their depletion. Our manuscript thus clearly provides hitherto unachievable insights into the dynamics of the ribosome biogenesis pathway.

Despite the negative comments of this reviewer, we are therefore convinced that our manuscript will be of great interest for the broad readership of Nature communications.

Reviewer #2 (Remarks to the Author):

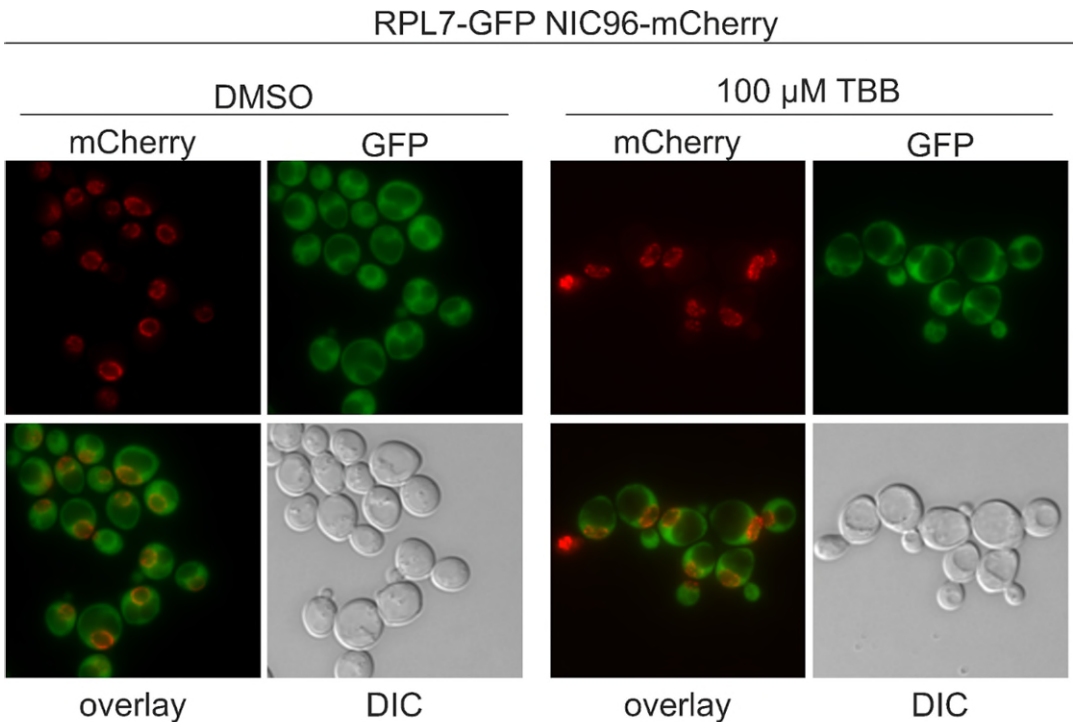
We are thankful for John Woolford`s suggestion to improve our manuscript, please find a point-to-point response below.

(1) Because ribosome assembly is so very much integrated with regulation of cell growth and cell division, it is important to briefly explain whether there is any likelihood that the observed block is indirect. It does seem to me that the effects on subunit biogenesis are far too rapid to result from primary effects on growth or division propagated to assembly. Nevertheless, the authors might briefly comment whether or not such indirect effects have been ruled out.

As pointed out by John Woolford, TORC1 (and downstream the CK2 Kinase) is one of the master regulators of cell growth and proliferation in response to nutrient availability and is thus also involved in regulating ribosome biogenesis (Kos-Braun et al., 2017; Talkish et al., 2010 and 2012, Powers and Walter, 1999, Honma et al., 2006). Honma and coworkers (2006, EMBO J) for example showed that late nucleoplasmic steps of ribosome biogenesis are affected by the TORC1 inhibitor rapamycin.

To compare the effects of rapamycin and usnic acid on ribosome biogenesis, we monitored the localization of Rpl7-mCherry and Nog1-GFP in the presence of the inhibitors. As now also shown in the manuscript ***(Supplemental Fig. S2B)****, effects of usnic acid are clearly visible within the first 5 minutes indicated by nucleolar accumulation of both Rpl7-mCherry and Nog1-GFP. As described by Homna and coworkers, nucleolar accumulation of Nog1-GFP is also observed upon Rapamycin treatment (Honma et al., 2006), but at a much slower timeframe as with usnic acid. Most importantly, the Rpl7-mCherry reporter (red) stays in the cytoplasm after rapamycin treatment, while it accumulates in the nucleolus after usnic acid treatment. Furthermore, rapamycin treatment causes different rRNA processing defects than usnic acid treatment (Kos-Braun et al., 2017, Awad et al., 2019,). Taken together, this shows that the effect of usnic acid on ribosome biogenesis cannot be caused by TORC1 inhibition.*

To evaluate a possible involvement of the CK2 kinase, we tested whether the specific CK2 inhibitor TBB leads to similar effects as usnic acid. We used the same conditions previously shown to affect ribosome biogenesis (Kos-Braun et al., 2017) and observed no nuclear accumulation of Rpl7-GFP even after 45 min incubation with 100 µM TBB ***(Revision Figure 3****).*



Revision Figure 3: The CK2 kinase inhibitor TBB does not result in nucleolar accumulation of the Rpl7A-GFP reporter. *The Nic96-mCherry Rpl7A-GFP strain was treated with 100 µM TBB for 45 minutes and inspected by fluorescence microscopy. As shown in this figure, TBB treatment does not lead to accumulation of Rpl7A-GFP in the nucleus, while usnic acid does (compare to Figure 1).*

Taken together, these results show that the effects of usnic acid on ribosome biogenesis are neither transmitted through TORC1 nor through the CK2 kinase. However, as ribosome biogenesis is embedded in multiple regulatory networks in mammalian and yeast cells due to its central importance, we cannot fully rule out effects from (yet unknown) regulators.

(2) Page 3, lines 73-74: the authors mention that the pathway progresses rapidly. How rapidly? I recall that in a previous paper these authors might have said 15 minutes! Knowing this short time frame for assembly might help the reader even better appreciate the value of seeing effects of usnic acid within 2-5 minutes.

The reviewer correctly recalls our earlier published findings (Zisser et al., 2018). In this publication we mentioned that in the presence of diazaborine 50% of Nog1 are transferred from the nucle(ol)us into the cytoplasm within 15 minutes and the majority of Nog1 associated pre-rRNAs are converted into their mature forms in this timeframe. When we add another few minutes by considering the final cytoplasmic maturation events, we expect that the large ribosomal subunit is fully assembled within 15 to 20 minutes. We now state this rough estimate in the introduction of the revised introduction section as recommended by the reviewer.

(3) Figure 1B: Can the authors describe in a little more detail how MICs were determined? Are the data shown the final OD to which cells grew? How long did this take to occur?

MIC measurements were performed with C303 (an ADE2 derivative of W303, which is the parent strain for all strains used in this study) in SDC + all medium in microtiter plates using a Bioscreen device. The strain was inoculated to an OD_{600} of 0.01 and incubated for 48h in a volume of 300 µl at 30°C with shaking and the OD_{600} was automatically measured every 30 minutes throughout the experiment. In accordance with common practice ((Kowalska-Krochmal and Dudek-Wicher, 2021), the MIC was defined as the lowest inhibitor concentration that prevented growth after 24 hours of incubation. The

reached OD_{600} of the untreated control is characteristic for growth curves recorded with the Bioscreen device. We revised the respective method section and the figure legend for more clarity.

(4) Figure 2B and text, page 6: the "fragmented/dotted signal" for nucleolar reporters such as Nog1-GFP and Tif6-GFP is interesting and very important to begin to understand the relationship between nucleolar morphology and ribosome biosynthesis. Can the authors discuss it a little bit more? Has such fragmentation been observed under other different conditions that block ribosome assembly? Why do they speculate that particles are aggregating?

We observed such phenotypes with other ribosome biogenesis inhibitors (Awad et al., 2019; Pertschy et al., 2004)). This most likely reflects nucleolar fragmentation/disruption, which is frequently observed upon nucleolar stress also in human cells (Lafita-Navarro and Conacci-Sorrell 2023; Lafontaine et al. 2021; Yang, Yang, and Yi 2018). We mention this possibility in the revised version of the manuscript as proposed by the reviewer.

We hypothesized that this could be related to aggregating particles, as we know that assembly factors that should be incorporated into the pre-ribosome after the inhibited step can accumulate in free form, aggregate and precipitate. Furthermore, the Shore and the Churchman Labs showed precipitation of ribosomal proteins after inhibition of ribosome biogenesis (Tye et al., 2019, Albert et al., 2019). However, we do not have evidence for pre-ribosomal particle aggregation at this stage and therefore removed that part of the sentence.

(5) Figure 3: Why were affinity purifications done from cells treated with 60 micromolar usnic acid rather than 40 micromolar used in experiments described in Figures 1 and 2? And, …does the drug work when cells are grown in rich media?

While 40 µM, the determined MIC for usnic acid, was used for the small-scale microscopy experiment, we increased the concentration for the biochemical experiments to ensure complete inhibition. This is also based on our experience with the low molecular weight inhibitor diazaborine, which showed that cells grown in baffled flasks with very high aeration can tolerate higher inhibitor concentrations. We added a sentence in the methods section to explain this difference. Usnic acid does not act in rich media in water soluble concentrations. For comparison, diazaborine also shows an about 5 fold lower MIC in SDC-medium than in complete medium.

(6) Figures 3A and 4A: can the authors label the proteins in their gels, as they did in Figure 4B?

We analyzed the samples shown in Figure 3A and 4A by SDS-PAGE, qMS and western blots but did not cut out and analyze individual bands in these gels. We therefore cannot label the bands in the shown gel. However, since the used precast gels run very reproducibly, the reader can compare the pattern of the gels in 3A and 4A with that shown in 4B. Most importantly, all conclusions from these experiments were drawn from the western blots and the qMS data.

(7) Figure 3B: the northern blot experiments show that the 7S pre-rRNA is decreased and that 27SA2 pre-rRNA accumulates a little bit. However, these experiments do not directly assess effects on 27SB pre-rRNA. Yet, the proteomics data clearly indicate that assembly is blocked between "states B and C". These state B and C pre-ribosomes contain mostly 27SB pre-rRNA. Thus, it is important to show more clear assays for a block in 27SB pre-rRNA processing. This is best shown by primer extension assays, since it is often tricky to resolve 27SA from 27SB pre-rRNA on agarose gels used for northern blots.

We analyzed changes upon usnic acid treatment of Nop7-TAP particles on various levels, which led us to the conclusion that the particle population is stalled at the 27SB pre-rRNA level, with rebound effects on earlier stages associated with $27SA_2$, 32S and 35S pre-rRNA. The strong reduction of 7S pre-rRNA in

the Northern Blot from the Nop7-TAP purification (Fig. 3B) demonstrates that 27SB pre-rRNA cannot be processed to downstream products.

Moreover, the fact that Arx1 cannot associate with the pre-ribosomes, shows that the usnic acid induced block occurs at an earlier stage of 27SB maturation, prior full assembly of the PET platform which is required for Arx1 joining (Biedka et al., 2018 J. Cell Biol.). This is in line with our structural investigations. Nevertheless, these are still 27SB containing pre-ribosomes as the reviewer correctly states. We are therefore convinced that primer extension does not lead to additional information on 27SB pre-rRNA as the block occurs within the 27SB pre-ribosome containing population. 27SB pre-rRNA is by far the most prominent pre-rRNA present in the Nop7-TAP purification. We underline this in the revised version of the manuscript with an additional Supplementary figure (Supplemental Figure S5A), where we show exposures of northern blots with the A2A3 or EC2 probes exposed to similar intensities for the 35S pre-rRNA in the usnic acid treated sample. This allows better comparison of the pre-rRNA species present in the purification.

The possible enrichment of $27SA_2$ upon perturbations in the pathway is limited, because it rapidly rebounds on the progenitor 32S and/or 35S rRNAs (as already seen after 2 min in the usnic acid treated sample).

(8) Figure 4E: why are effects shown for Nog2, Rsa4, and Bud20 using Nsa1-TAP as a bait for purification? Don't these factors assemble after the Nsa1 module exits? Do these data reflect some modest heterogeneity in the assembly pathway, i.e. that Nog2 etc. sometimes enter earlier, or that Nsa1 exits later? Or, might these data reflect contamination?

Thank you for bringing this up. First, we selected factors for the Nop7-TAP qMS (Figure 3D) to show in the bar chart. As this is a bait protein covering a broader range of pre-ribosome intermediates it also contains later factors like Bud20 and Arx1. When we then selected assembly factors for the bar chart of the Nsa1-TAP qMS (Figure 4E), we wanted to show the same factors as for the Nop7-TAP for better comparison. Therefore, we included Bud20 and Arx1, that are - as obvious from Supplementary Table S8 - present on the Nsa1-TAP particle in very low abundance compared to the bait protein (i.e., 0.05% for Bud20). While the scatter plot (Figure 4D) visualizes the abundance of the individual proteins relative to the bait, the bar chart does not (Figure 4E), but makes it easier to identify the most prominent de- and increasing assembly factors. To make things clearer we now excluded factors with an abundance below 1% relative to the bait. Therefore, Bud20 and Arx1 are not included in the Nsa1-TAP bar chart anymore; still the raw data can be found in Supplementary Table S8, and the scatter plot remains unchanged.

(9) Page 16, line 381: is this supposed to be state 1 or state 2?

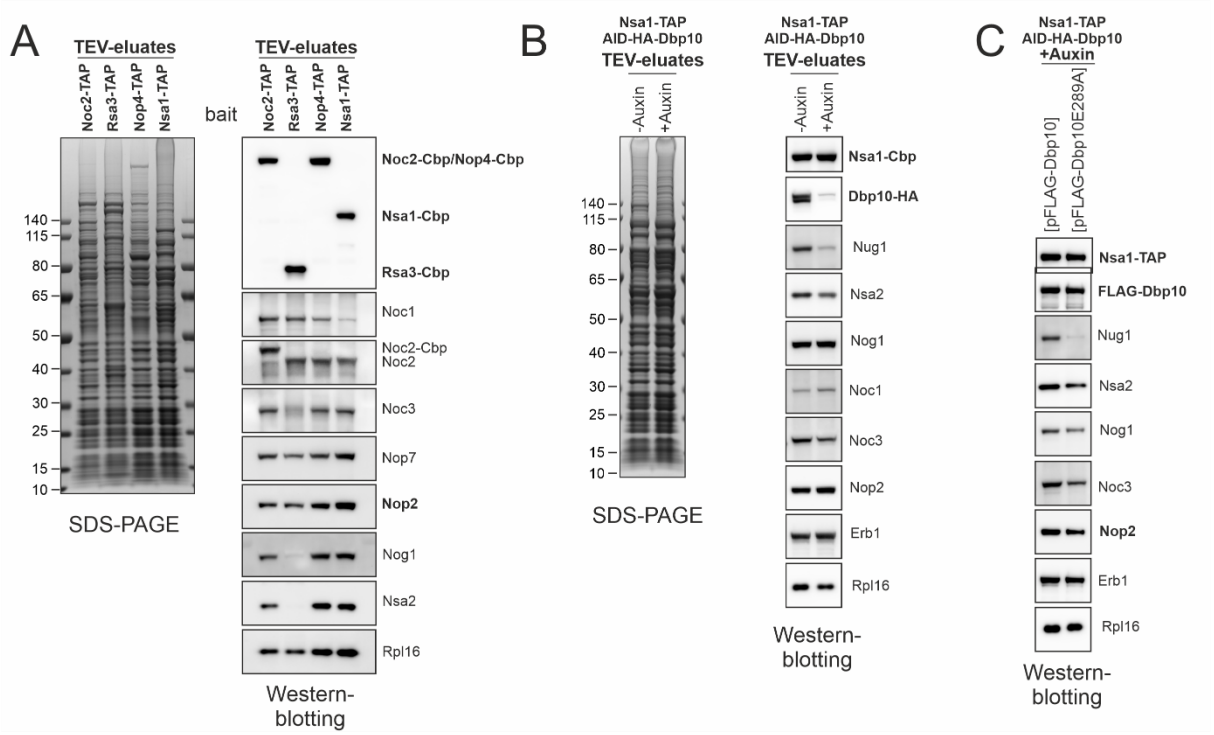
Thank you for highlighting this typo, it is state 2!

(10)Figure 7: Nop2 is shown in dark blue. Shouldn't it decrease if it assembles after the usnic acid block?

Nop2 shows a slight increase on the qMS of the Nsa1-TAP particle, hence it was marked dark blue. It is more or less unchanged on Nop7-TAP. On human pre-ribosomes it behaves similarly as Dbp10 and VandenBroek and Klinge (2023) hypothesize that Dbp10 recruits Nop2/Nip7. In contrast, in yeast depletion of Dbp10 does not lead to reduced levels of Nop2 (Talkish et al., 2012). In Chaetomium thermophilum, Nop2 is detected in the earliest purification (Mak16 as bait proteins) together with Dbp10, but both have not been resolved on the very first particle yet. This suggests that Nop2 might be present on the particle already before the usnic introduced blockage.

To clarify the situation with Nop2, we performed TAP-purifications using early nucleolar bait proteins. As shown in Revision Figure 4A, Nop2 is already detectable on pre-60S particles purified with Rsa3-TAP

as bait protein, while Nog1 and Nsa2 are not. This shows that Nop2 is already bound to very early nucleolar pre-60S particles, albeit it only becomes visible in cryo-EM structures after Nsa2 incorporation. This explains why we did not detect a decrease of Nop2 upon usnic acid treatment. Since Nop2 obviously binds prior to the usnic acid induced blockage and stays associated long after the blockage, it is not relevant for the topic discussed in this manuscript. We therefore removed Nop2 from the scheme in Fig. 7.



Revision Figure 4: TAP purifications using early nucleolar proteins as bait proteins *(response to q10 and q12). The SDS-PAGE and western-blots with antibodies directed to selected maturation factors are shown.* ***A: Nop2 associates already very early with the pre-60S particle.*** *Various bait proteins were used to purify early nucleolar pre-ribosomal particles to gain insights into joining of Nop2, Nog1 and Nsa2. Nop2 is detectable already in very early nucleolar pre-ribosomes purified with Rsa3 as bait protein and thus binds at a very early stage during pre-60S maturation, prior to Nog1 and Nsa2.* ***B: Depletion of Dbp10 using the AID system affects Nug1 incorporation.*** *As shown in this figure, absence of Dbp10 results in strong depletion of Nug1.* ***C: Binding of Nug1 but not Nsa2 to the pre-ribosome requires the ATPase activity of Dbp10.*** *The AID-HA-Dbp10 strain carrying plasmids expressing FLAG-tagged wild-type Dbp10 or the FLAG-tagged ATP hydrolysis deficient Dbp10E289A variant was grown to early log phase. After addition of Auxin for 120 minutes, cells were harvested and pre-ribosomes purified on anti-FLAG resin.*

(11) Understanding exactly where the usnic acid block occurs, and why, is potentially the most impactful part of the work in this manuscript. Therefore, could the authors maybe provide a little more detail in the Discussion? The authors rightly conclude that the usnic acid-induced block is after entry of Nog1, Tif6, and Rlp24, and immediately before entry of the assembly factor Dbp10. Only recently has Dbp10 been visualized by cryo-EM (in human and Chaetomium pre-ribosomes, but not in Saccharomyces). Therefore, exactly where Dbp10 fits into this assembly scheme had been somewhat unclear. Can the authors better clarify the distinction between intermediates 2, 3, and 4? They (rightly) state that their intermediates 2 and 4 resemble state B and state C particles from Kater et al.. Does their intermediate 3 resemble state 1 or state 2 from Sanghai et al. ? The text says state 1 but I think they mean state 2 see (9) above. . Would it be helpful to point out that the structures of these large subunit assembly intermediates (states A and B and state 2) from different investigators differ? For example, Kater et al. never visualize (or detect by SDS-PAGE)the Ssf1 module in their state A or B particles, while I recall that Sanghai et al. see these assembly factors in their state 2 particles. Is this state 2 (i.e., intermediate 3 of Dr. Bergler) otherwise identical to the B particles of Kater et al.?

We now provide more detail on the usnic acid induced block in the maturation pathway in the revised version of the manuscript as suggested by the reviewer.

Cluster 3 indeed resembles state 2 from Sanghai et al., 2018. We corrected this typo in the text!

We agree that the reconstruction of the same states from different labs can show subtle variances that can reflect experimental or processing differences. The Ssf1 module is a good example, where factors can be detected in the SDS-Gel (also in Kater et al., 2017, Fig. S1A), but cannot be visualized on the particles. In our opinion, the mode of purification (i.e. magnetic beads vs conventional resins), Mg^{2+} concentration, ionic strength and detergent concentration are the main parameters affecting the purification of pre-ribosomal particles and copurification of not fully incorporated assembly factors. We included a short paragraph on this topic in the discussion section as suggested by the reviewer.

Regarding Dbp10 please see our answer to query (12)

(12) Continuing from (11) above: Can the authors speculate why they see a greater effect on assembly of Dbp10 than Nsa2 or Nug1? To what extent does entry of Dbp10 depend on Nsa2 or Nug1? Can the authors elaborate a little more about structural models to account for these different effects?

Very recently published studies showed that Nug1 binding is reduced on nucleolar pre-60S particles (e.g. Nsa1-TAP), when Dbp10 is either mutated or depleted and also provided structural insights into the mode of action of the RNA helicase (Mitterer et al., 2023, doi: 10.1093/nar/gkad1206; Cruz et al 2024, doi: 10.1038/s41467-024-47616-7).

In response to the reviewer's query, we also addressed this question. Our data also show that Nug1 recruitment requires ATP hydrolysis in Dbp10 while the sole presence of Dbp10 is not sufficient (Dbp10E289A; Revision Figure 4 B and C). This suggests that Dbp10 dependent RNA remodeling is required for Nug1 binding to the pre-ribosome. Cruz et al., showed that Dbp10 remodels the region around the PTC (H90, H91, H92; Cruz et al., 2024).

*For Nsa2, this is different, because the levels of Nsa2 in the ATP hydrolysis deficient dbp10E289A mutant are similar to that of the previously incorporated Nog1 and Nop2 (**Revision Figure 2** C). Thus, the presence of Dbp10 results in stabilization of Nsa2 although Dbp10 is not capable for ATP hydrolysis (Dbp10E289A; Revision Figure 4C). We propose that Nsa2 is binding shortly prior to Dbp10 but might only be stably incorporated once Dbp10 has joined. This view is in line with extensive interactions between Dbp10 and Nsa2 (Cruz et al., 2024) and with the stronger reduction of Nsa2 on the Nsa1-TAP particle after usnic acid treatment compared to that after Dbp10 depletion (**Revision Figure 4** B and C; Figure 4). As Nsa2 is longer present on the particle, it will also be affected differently than Dbp10. In general, the depletion should be stronger the closer the block is to the stage of entry/exit and the shorter the time a protein is present on the particle.*

We included a short paragraph into the revised version of the manuscript to expand on this point.

(13) Toward identifying the specific target of usnic acid...Does mass spec indicate whether usnic acid co-purifies with pre-ribosomes purified from usnic acid treated cells?

We did try various approaches but could not detect usnic acid in pre-ribosomes from treated cells. We propose, that the relatively low affinity (MIC in mid µM range) is the cause for our failure to identify the target.

Sincerely,

John Woolford

We thank John Woolford for his very constructive and helpful comments!

Reviewer #3 (Remarks to the Author):

We thank David Taylor for his helpful comments on our manuscript, please find a detailed response to als points raised below.

1. I thank the authors for providing the maps for quality control and for their clarification about modeling: "We did not model identified clusters as they largely reconstitute previously published high resolution structures (Kater et al, Sanghai et al). We fitted these models in the reconstructions using flexible fitting and used them to color our maps in chimerax. A more detailed description can be found in the material and methods section."
For a general reader, I think this short description or something similar would be helpful. It answers questions about these structures possibly being new states and how they are being identified/ordered. I also think that expanding on the rRNA conformations and/or degree of processing within these structures is warranted. It is a very cool result!

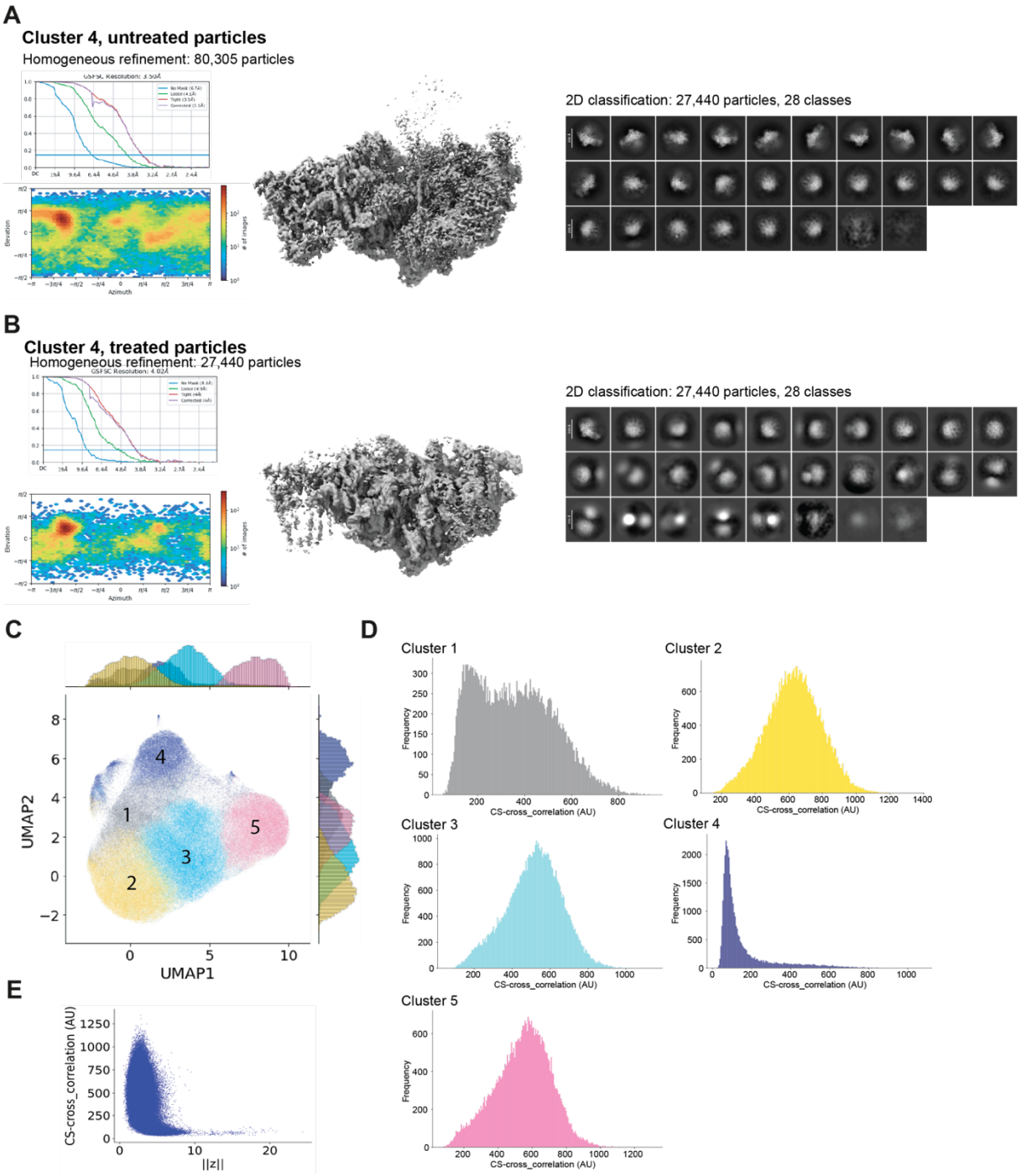
We included a statement in the results (page 16 of the manuscript) and expanded our description on the rRNA conformations as recommended by the reviewer.

2. In general, I think the approach of pooling the particles from both treated and untreated cells is elegant and helps avoid certain biases, and it's exactly what I would do.

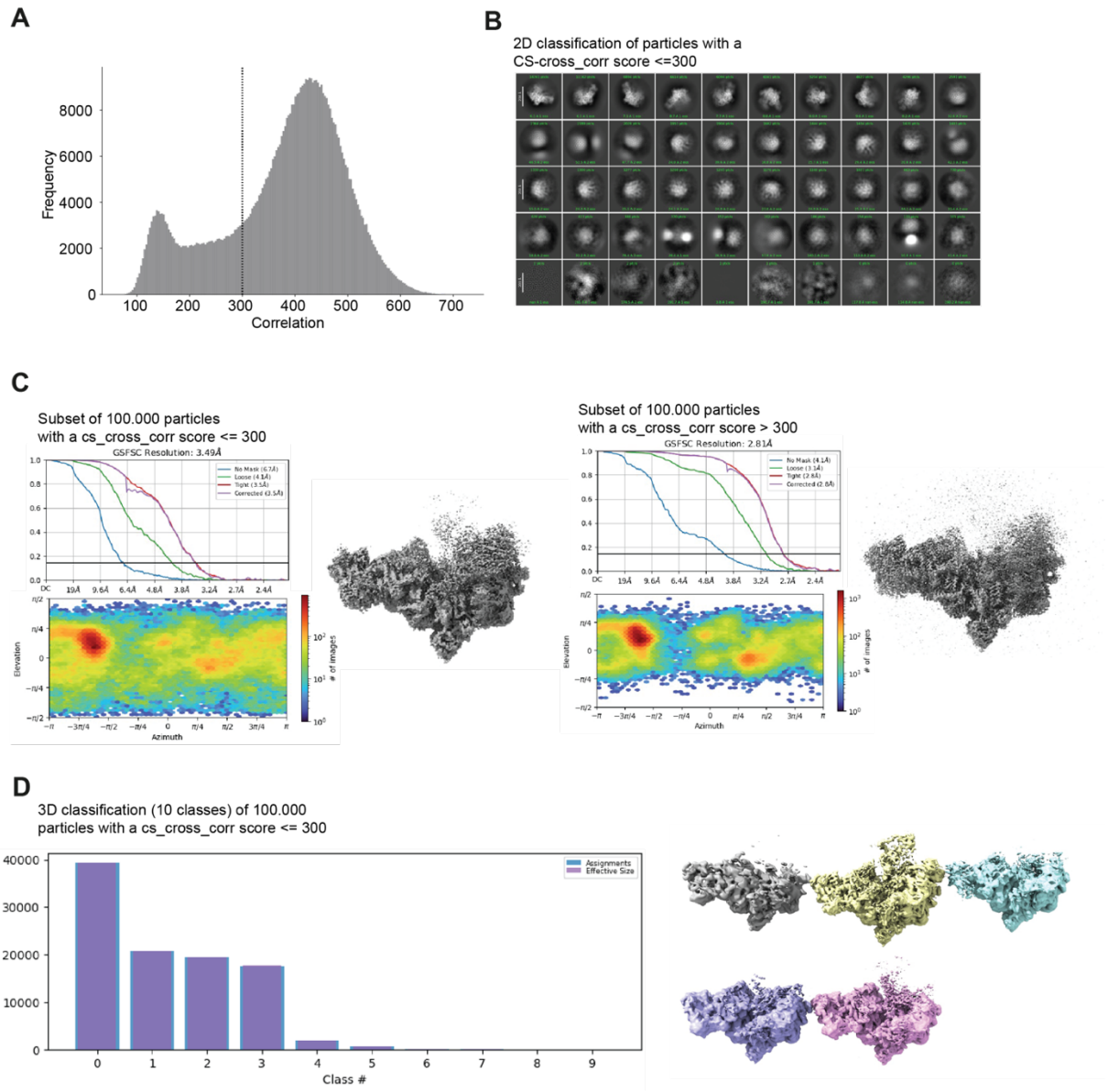
a. However, because the particles are pooled, how do you know that you aren't enriching for states that you might only find in one condition or missing some states that might exist in only one condition? This might be addressed by doing the same analysis on each set of particles and finding the same or similar structures as in the pooled dataset. Another approach would be to use the particles from each condition for each state, respectively, and perform a reconstruction.

This is a very important point and we thank the reviewer for bringing this up. Pooling is essential for this analysis as the algorithms used have stochastic components. The data in the dataset influences the results by, for instance, directly affecting the training trajectory of the cryoDRGN analysis. As your concern is still very valid, we performed individual reconstructions of the individual datasets. To our concern, in the case of cluster 4 the reconstructions indeed did not fully match the average maps representing the average cluster (Revision Figure 5 A,B). This prompted us to critically revisit our analysis scheme and reanalyse the dataset. We found that the issue is a small fraction of remaining non-particle outliers (Revision Figure 5 A,B) in the dataset. As the differences to the real particles were so large, these non-particle outliers influenced the VAE in cryoDRGN so that some of the differences in the real particles were neglected. Especially clusters 1 and 4 contained significant amounts of outliers. We tested multiple strategies to remove these outliers and found a correlation cut-off based approach to be the most effective strategy. In Revision Figure 4 D you can see that there are significant amounts of badly correlating particles within the named clusters. In a first step, we analysed all low correlation particles in the overall dataset and found that while containing a small fraction of good particles (Revision Figure 6 A,B, C), the majority of these particles are noise. (Revision Figure 6 A,B and D). We ran correlation based thresholding over several iterations (Revision Figure 7) to yield a cleaner dataset but without loosing a significantly large fraction of real particles. With this we could bring down the outliers to such a minimum that cryoDRGN's results were not influenced anymore in a noticeable fashion and the reconstructions of the individual clusters of the separated datasets now match the maps of the cluster centers. While this did only change the results numerically, but not the

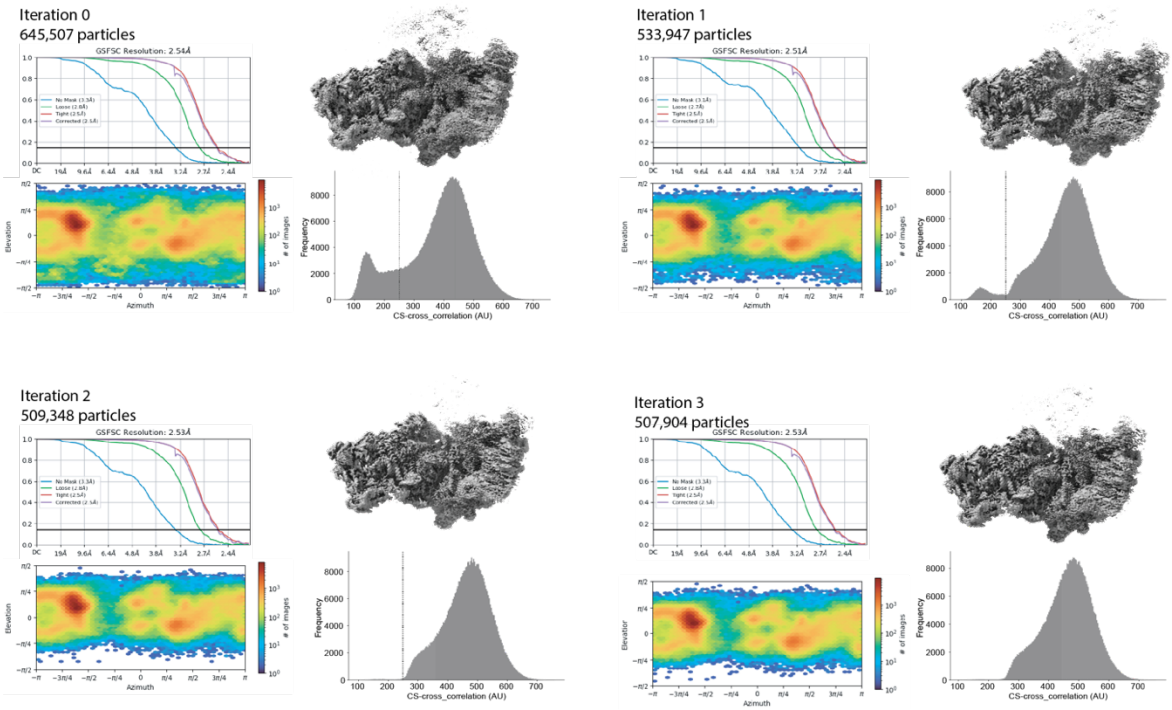
interpretation, we are very thankful for bringing us to this realization as this significantly improved the manuscript.



Revision Figure 5: Cluster 4 after the initial clustering contains particles with a low CryoSparc (CS)-cross_correlation score, that do not align well in 2D. *Particles of cluster 4 were separated into* ***(A)*** *treated and* (B) *untreated particles and separately homogenously refined and 2D classified. Both reconstructions and 2D classes indicate noise and dirt in the respective particle stacks.* ***(C)*** *Example UMAP of batch 1.* ***(D)*** *particles with a low CS-cross_correlation values are clustered into cluster 1 and 4.* ***(E)*** *Low CS-cross_correlation values correlate with low Eigenvektor values (||z||).*



Revision Figure 6: Particles with a low CS-cross_correlation score are structurally heterogeneous and do not resolve well in 3D and 2D. (A) *All particles that went into the previous cryoDRGN analysis were filtered into two particle stacks with a CS-cross_correlation £300 or >300.* ***(B)*** *Particles with a cross_correlation £300 do not align properly in a 2D classification (cryoSparc).* ***(C)*** *1*10ˆ5 particles with a CS-cross_correlation £300 and >300 were homogeneously refined.* ***(D)*** *3D classification (cryoSparc, default settings) of particles with a CS-cross_correlation £300 classified into multiple structurally divergent classes.*



Revision Figure 7: Iterative exclusion of particles with a CS-cross_correlation score £ 250*. Included particles (CS-cross_correlation score >250) were refined and filtered once more. The corresponding particle stack was used for the new cryoDRGN analysis after further refinement.*

b. I think technical replicates are important. I think biological replicates would be more insightful and important here, especially if you are going to calculate percentages of particles that make up each state.

We concur with the reviewer that incorporating biological replicates would enhance the analysis by adding statistical robustness. Nevertheless, conducting these experiments is currently unfeasible for us, primarily due to the significant costs involved. To acquire three biological replicates, it would necessitate recording six Krios datasets across multiple days. The cryoDRGN analysis relies on the consistency of each particle's contrast and signal-to-noise ratio, necessitating data collection on the same microscope and, ideally, on successive days. This approach is essential to prevent artifacts that may arise due to the differing optical characteristics of various microscopes. It also seems beyond the standards of the field at the moment where not even technical replicates are used routinely.

c. I think describing briefly how the clustering is performed would help a general reader understand what you are doing in the results section. For instance, how does a neural network analysis help here? Where are you getting these clusters?

Indeed, including a short description right before going into the results of the cryoDRGN analysis would help the reader to understand the data shown. As suggested we added a short overview of the workflow in the results section.

3. How can you be sure the tag used for pull down isn't influencing the states you are seeing? Similarly, could you comment on the linearity of the process? It seems to block maturation after a certain point, as you mention. Is it possible that it promotes disassembly of another state instead?

Affinity purification via tagged versions of assembly factors is currently the only possible approach to get hold on pre-ribosomal particles that are only present in very small amounts in the cell. We are

thereby limited by the available maturation factors that can effectively be used as bait proteins to purify pre-ribosomes from different stages. If tagging of a maturation factor causes strong problems during maturation, this will also result in a growth defect of the strain and these factors can be avoided. The TAP-tags on Nsa1 and Nop7 are well-established and frequently used in ribosome biogenesis research. Tagging of Nsa1 and Nop7 does not affect growth of the modified strains, indicating that it does not severely affect the function of the proteins. Nsa1-TAP particles were for example used in the study for nucleolar pre-60S particles from Kater et al., 2017. We can of course never fully exclude (subtle) changes due to the addition of a tag, but this is an intrinsic problem of affinity-tag purification.

Thank you for bringing up the interesting possibility of disassembly of a state instead of accumulation. We agree that it is possible that this could happen upon inhibitor treatment. However, in general defective/blocked pre-ribosomal particles that cannot mature further are actively disassembled in a controlled manner including activity of the exosome among others. Thus, although we cannot rule out the possibility that one specific intermediate state is disassembled upon usnic acid treatment, the fact that we do not see partially degraded (pre-)rRNAs in the total RNA isolates as well as RNA isolated from the pull-downs of Nsa1, Noc2 or Nop7, argues against it. Further enriched levels of exosome specific factors would be expected to be seen in the qMS analysis. We included a statement in the discussion section to address this point.

4. I think using this structural approach as a general strategy is a nice point from the manuscript. The other points about what was learned about ribosome biogenesis and how this could be related to cancer properties of the compound did not immediately jump out at me after reading the manuscript. This could perhaps be made clearer in a revised manuscript.

Thank you for pointing out that these aspects were not sufficiently addressed in the discussion. We expanded the discussion by emphasizing the learnings about ribosome biogenesis and implications for its anti-cancer drug characteristics. In general ribosome biogenesis pathway inhibition is currently explored as potential cancer treatment as a new route for chemotherapy. Currently mainly rRNA synthesis inhibitors are used however later acting inhibitors could also have potential.

David Taylor

We thank David Taylor for his very constructive and helpful comments!

References

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Major issues:
Point 1: Addressed.

Point 2: Dynamic regions that are less well resolved and have higher local resolution are indeed a hallmark of cryo-EM reconstructions but can typically be visualized in low-pass filtered maps. Indeed, for 3EM4 (state B in Kater et al), some occupancy for Ssf1 can be observed in the low-pass filtered maps. Similarly, recent (and much better) reconstructions of Chaetomium and human pre-60S particles show incorporated Rrp15/Ssf1 in intermediates equivalent to the Kater State B (i.e. State 5S RNP from Lau et al and state A from Vanden Broeck at al). However, for a factor to be enriched in qMS but entirely absent in its defined binding site requires a different explanation, typically the presence of a secondary binding interaction that "tethers" the factor to the pre-60S before the primary binding interaction is established. These interactions do frequently occur and indeed appears to be the explanation for the presence of Nog1 in clusters 2 and 3 before the N-terminal portion of the protein binds the pre-60S. Given the identification of the C-terminal helix of Nog1 in cluster 2, it is now important for the authors to distinguish between these two binding modes, especially since binding of Nsa2 and Dbp10 (along with other factors) requires the presence of the N-terminal module of Nog1. Indeed, the authors should consider the hypothesis that it is the transition from initial, tethered engagement to full binding by Nog1 that is disrupted by usnic acid, as it would seem to fit the observed data.

Point 3: The low occupancy of Mak11 in cluster 2 despite the observed qMS enrichment still suggests that there could be an issue with particle integrity during grid preparation. While the argument that factors are present but "invisible" in the reconstructions is convenient, it has also been well established that many nucleoprotein complexes are destabilized by interactions with the carbon support or with the air-water interface. The newly added section in the discussion effectively discusses this concern.

Minor issues:
Point 1: Here it is again important to distinguish between the initial Nog1 binding via the CTD from the later, more extensive engagement via the NTD. Perhaps it is this transition that leads to Mak11 release. Again, how Nog1 binds is more important than when it binds.
In this context, preventing full engagement of Nog1 could block the same step as Nog1 depletion, explaining the enrichment of Mak11 in both cases.

Point 2: Rebound is a pharmacological term and it just seems bizarre to use in a different context in a paper describing the effect of a small molecule inhibitor. Self-referencing their earlier papers just highlights the fact that the term is not generally used in the ribosome biogenesis field. However, ultimately the authors need to decide this for themselves. I can only point out that the term is not accurate.

Point 3: I could not find the statement that clarifies the use of Noc2 as a bait in the results. My point was just that the choice of tag increased the "background" in the experiments, something that could possibly have been avoided by choosing a different tag.

The revised manuscript clarifies the most obvious inconsistencies in the data. My final recommendation before publication is that the authors make a clearer distinction between the two binding modes of Nog1 as outlined above.

Reviewer #2 (Remarks to the Author):

I have carefully studied the revised manuscript and the responses to all three reviewers.
I think that the authors have adequately addressed all concerns of all three reviewers.

Reviewer #3 (Remarks to the Author):

The authors have for the most part satisfied the concerns of this reviewer. I recommend publication in Nature Communications.

David Taylor

Reviewer #1 (Remarks to the Author):

Major issues:
Point 1: Addressed.

Point 2: Dynamic regions that are less well resolved and have higher local resolution are indeed a hallmark of cryo-EM reconstructions but can typically be visualized in low-pass filtered maps. Indeed, for 3EM4 (state B in Kater et al), some occupancy for Ssf1 can be observed in the low-pass filtered maps. Similarly, recent (and much better) reconstructions of Chaetomium and human pre-60S particles show incorporated Rrp15/Ssf1 in intermediates equivalent to the Kater State B (i.e. State 5S RNP from Lau et al and state A from Vanden Broeck at al). However, for a factor to be enriched in qMS but entirely absent in its defined binding site requires a different explanation, typically the presence of a secondary binding interaction that "tethers" the factor to the pre-60S before the primary binding interaction is established. These interactions do frequently occur and indeed appears to be the explanation for the presence of Nog1 in clusters 2 and 3 before the N-terminal portion of the protein binds the pre-60S. Given the identification of the C-terminal helix of Nog1 in cluster 2, it is now important for the authors to distinguish between these two binding modes, especially since binding of Nsa2 and Dbp10 (along with other factors) requires the presence of the N-terminal module of Nog1. Indeed, the authors should consider the hypothesis that it is the transition from initial, tethered engagement to full binding by Nog1 that is disrupted by usnic acid, as it would seem to fit the observed data.

As suggested by the reviewer, we added a statement in the discussion section highlighting the sequential N-terminal incorporation of Nog1. A disruption of the full coordination of Nog1 by unsic acid would indeed fit our observations. This hypothesis, however, lacks clear experimental evidence. We discuss this accordingly in the added section to the discussion.

Point 3: The low occupancy of Mak11 in cluster 2 despite the observed qMS enrichment still suggests that there could be an issue with particle integrity during grid preparation. While the argument that factors are present but "invisible" in the reconstructions is convenient, it has also been well established that many nucleoprotein complexes are destabilized by interactions with the carbon support or with the air-water interface. The newly added section in the discussion effectively discusses this concern.

We are glad to hear that we could address the concerns of the reviewer appropriately.

Minor issues:
Point 1: Here it is again important to distinguish between the initial Nog1 binding via the CTD from the later, more extensive engagement via the NTD. Perhaps it is this transition that leads to Mak11 release. Again, how Nog1 binds is more important than when it binds.
In this context, preventing full engagement of Nog1 could block the same step as Nog1 depletion, explaining the enrichment of Mak11 in both cases.

We now mention this possibility in the discussion section together with the statement mentioned above as suggested by the reviewer.

Point 2: Rebound is a pharmacological term and it just seems bizarre to use in a different context in a paper describing the effect of a small molecule inhibitor. Self-referencing their earlier papers just

highlights the fact that the term is not generally used in the ribosome biogenesis field. However, ultimately the authors need to decide this for themselves. I can only point out that the term is not accurate.

We are aware that the term is not optimal, but still consider it the best available option to describe this concept.

Point 3: I could not find the statement that clarifies the use of Noc2 as a bait in the results. My point was just that the choice of tag increased the "background" in the experiments, something that could possibly have been avoided by choosing a different tag.

Unfortunately, we forgot to add the explanation into the final version of the revised manuscript. We apologize and thank the reviewer for drawing our attention to this mistake. We now included the statement in the results section when we introduce Noc2-TAP.

The revised manuscript clarifies the most obvious inconsistencies in the data. My final recommendation before publication is that the authors make a clearer distinction between the two binding modes of Nog1 as outlined above.

We followed the recommendations of the reviewer and added clarifying statements regarding the Nog1 binding modes (i.e. partial engagement only with residues 448 to 466 in cluster 2 vs full engagement with the N-domain in cluster 4) in the discussion section as also mentioned above.

We thank reviewer 1 for his efforts to improve our manuscript.

Reviewer #2 (Remarks to the Author):

I have carefully studied the revised manuscript and the responses to all three reviewers.
I think that the authors have adequately addressed all concerns of all three reviewers.

We thank John Woolford for his very constructive suggestions during the review process.

Reviewer #3 (Remarks to the Author):

The authors have for the most part satisfied the concerns of this reviewer. I recommend publication in Nature Communications.

We thank David Taylor for his very helpful recommendations during the review process.