nature portfolio

Peer Review File

Helicase-mediated mechanism of SSU processome maturation and disassembly

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1
(Remarks to the Author)
The manuscript by Buzovetsky et al. reports the results of an original study poised at deciphering the molecular mechanisms underlying the maturation and disassembly of the processome complex (from Saccharomyces cerevisiae), responsible for the biogenesis of the small ribosomal subunit (SSU, or 40S) particles. Through an ensemble of 16 intermediate states (solved by cryo-EM at various resolutions up to 2.7Å), the study reports the involvement of two helicases, Mtr4-exosome and Dhr1, in the regulation of the processome in order to drive the maturation of the SSU in an orderly and unidirectional fashion.

The manuscript conceptualizes a model for the unidirectional progression of the SSU maturation through the irreversible degradation of the 5' ETS RNA, via a complex synergetic interplay between the RNA exosome (including its associated helicase Mtr4), the maturing pre-40S particle and the processome leading ultimately to the release of the pre-40S and the disassembly of the processome, with the exosome playing the role of a molecular arbiter constantly assessing the RNA processing quality. In this model, Dhr1 is positioned and activated to unwind the U3 snoRNA in order to initiate the nucleolar release of the pre-40S.
The study applies a compelling set of methods including cryo-EM, genetics and in silico protein-protein interaction screen, to elucidated the RNA exosome mediated mechanism that explains both SSU processome maturation and disassembly.

The presented work is overwhelming and impressive and will be of great interest for the community. The manuscript is relatively clear, but some effort must be applied to clarify certain statements (see comments below), the figures are all important but insufficient to illustrate some of the key interpretations and conclusions of the presented work.
The reviewer recommends the publication of the submitted manuscript provided that the authors address the following major and minor points.

Major points:

Figure 1. Because of the wealth of structures, the different presented states are extremely difficult to compare! The first 9 states look identical at a first glance then after

a long game of "find the differences" one could start seeing some but others remain totally unfindable. For instance, between states 1 and 2 involving a "head rotation", even after investing a good 15 minutes, the reviewer still can't spot the differences and what does the "head rotation" refer to? The authors must find a way to highlight the essential differences between the different states sequentially (arrows, circles, etc…). Also, a small 3D model of the SSU mature rRNA domains colored similarly to the different sates will greatly help the non-experts in the SSU maturation to understand the progression of rRNA maturation and remodeling.

Most importantly, the elements colored in Figure 1 are confusing and it's totally unclear where is the 5' ETS? Also, the label "5' ETS becoming disordered/trimmed" is used at multiple places on multiple elements, sometimes in orange and sometimes in yellow… What is what? The authors should pick a color for each element (U3, 5'ETS and different domains of the 18S rRNA) and stick to it to the end. They can highlight the action at each element/domain in every state by a bright contour for example. The reviewer leaves this aesthetic choice to the discretion of the authors.

Figure 1: Between states B and C, there is the exosome binding. Where is it on the figure? What is it made of?

Figure 1, state G, Dim1 is nearly invisible.

The authors must revise the Figure 1 in such a way that all important elements can be easy to spot and the actions highlighted. In the current state, Figure 1 is of very limited contribution to the understanding of the described mechanisms.
Figure 3b: Is unfortunately unclear and confusing. It is difficult to suggest to the authors a specific solution. However, the reviewer can explain the underlying reasons of such unclarity. First, the transparent surfaces/densities are not enough transparent and mask significantly what's behind. Second, there are too many details that the authors wish to highlight on every panel. Third, similar to the reviewer's previous comment regarding the labels of Figure 2 (Some labels are hardly visible, use simple black font and point to the concerned residues with solid thin black lines. The current display in hard to read and in fact the numbers point nowhere, while it would be more useful and accurate to point to the concerned residues. In addition, the authors could leave out the name labels of different proteins in colored fonts (colored consistently with the protein name), but in this case they must use the exact same color and use bigger fonts. If the authors find

the colors to be pale, therefore the labels hard to read, then they should use brighter colors.)

Suggestion: Try more transparency or only color what's important and where the action is taking place, and leave everything else in very fade shades of the original colors.
Alternatively, try and remove the transparent densities all together.

Figure 5: This figure is supposed to present a model of the processome assembly, progression and disassembly in an intelligible manner. The presented panel b is useful, but it is not very intelligible and the reviewer recommends to the authors to display a simplified representation including only the large steps and discard as many fine details as possible, so not to dilute their important messages.

While the analysis of the presented structures is insightful, it is still unclear how the irreversible degradation of the 5' ETS is orchestrating this linear progression of the processome assembly and disassembly. The reader can connect certain dots and it is indeed reported that certain conformational changes due to the exosome activity on the 5' ETS trigger certain steps, some others don't seem to be related to this degradation, or perhaps they are but the authors failed to show the link. More importantly, what causes the exosome to pause so that discrete states can be captured? Finally, the link between the exosome and U3 removal isn't described in sufficient details, and more globally the removal process of U3 based on the presented structures. What have we learned from the presented structures about the U3 removal?

Minor points:

At no point in the manuscript the authors define what is the 5' ETS? This might be trivial for experts in the field; however, it would be appropriate to at least spell the abbreviation (external transcribed spacer) and explain in few words what this element is.

While impressive, the data processing scheme appears a bit inconsistent between Datasets 1, 2 and 3. Indeed, the processing schemes differ substantially between all datasets, as for each a different combination of image processing tools were used (Dataset 1: Relion 5 Beta, Dataset 2: Relion 5 Beta, crYOLO and CryoSparc, Dataset 3:

CryoSparc and pyEM), what justifies these choices? Could the authors explain the logic behind each "blend"?
Moreover, there are different interestingly shaped but ignored cryo-EM reconstructions after the first 3D sorting in Datasets 1 and 2, and only one or two classes were selected to pursue the data processing. Could the authors explain briefly the basis of their selection/exclusion?

Line 122: What is site A1? Define it in few words.

Line 128: Rrp6 is barely visible, only as a very small density with the same color as the U3. For such a small density, it's preferable to use a brighter different color to highlight it and at least make it visible. Also, where is the 3' of the 5' ETS on the figure? And how does it relate to the Rrp6?

Line 130: Krr1 has the same color as the central domain of the 18S, very difficult to distinguishe it from the latter, especially on such a small panel in a giant figure.
Also, why do the authors believe that the restructuring of the 5'ETS allows the partial release of Krr1 and Faf1? These elements seem to be very far apart.

Lines-134-138: The authors write "the integration of helix 45 near the central domain results in the docking of the auto-inhibited

Dhr1 in State E. Surprisingly we find that A1 cleavage in State G is solely triggered by the incorporation of Dim1 as this protein couples the interrogation of helix 45 installation with the stabilization of U3 snoRNA- bound regions within the 18S rRNA in an upward conformation". The authors must rephrase and simplify, statements such as "…couples the interrogation of helix 45 installation with the stabilization…" are confusing. What does the "interrogation of the installation" mean? More importantly, panel G doesn't illustrate this statement.

Figure 2c: Some labels are hardly visible (rpS24, Pno1, utp20, Dhr1 anchor, the residues numbers, etc…). This is related to the reviewer's previous comment (suggests to the authors to use simple black font and point the concerned residues with solid thin black lines. The current display in hard to read and in fact the numbers point nowhere, while it would be more useful and accurate to point to the concerned residues. In addition, the authors could leave out the name labels of different proteins in colored fonts (colored consistently with the protein name), but in this case they must use the exact same color

and use bigger fonts). For instance, Utp20 is more orange than the ribbon of the actual protein, Pno1 is a bit green, while the actual ribbon is very light yellow… If the authors find the colors to be pale, therefore the labels hard to read, then they should use brighter colors.

Importantly, and this is not the first occurrence, the authors should use more contrasted colors, especially when highlighting key proteins/components (e.g., Utp14 and Dhr1, Dhr1 and Rrp6, Utp7 and Sof1, Mpp10 and Sas10, etc…).

Lines 205-207: The so-called "hook" in Utp14 is hardly visible on Figure 2c, because it's displayed poorly and the colors of Utp14 and Dhr1 are very similar.

Line 252: 5' ETS is nearly completely invisible. It is important to highlight this feature, the authors must find a way to illustrate that RNA exosome mediated processing of the 3' end of the 5' ETS alters the Sof1 binding site.

In Methods, Lines 617-618: Why did the authors prepare their samples at 18°C and not at 4°C, which could reduce the conformational heterogeneity of their structures, and perhaps in turn improve their resolutions?

In Methods, Lines 623-627: The authors describe the grids preparation protocol. The grids of each sample (Kre33, Dhr1 and Utp14…) have been prepared differently (variable in number of applications and blotting, time of blotting), why is that? Could the authors explain the process of optimization and how they came about these parameters? The reviewer is not questioning the quality of the experiments but it will be of use to the readers to understand this process. Even a brief description will be useful.

Referee #2

(Remarks to the Author)

Buzovetsky and Klinge report an impressive structural study that tackles the molecular mechanisms of the maturation and disassembly of the S.cerevisiae small subunit processome, leading to the formation of the pre-40S particle.

The SSU processome is a large ribonucleoprotein assembly that undergoes precise ribosomal RNA (rRNA) processing coupled to compositional changes of ribosomal proteins, but the sequel of events and mechanisms underpinning the unidirectionality and irreversibility of the process have been elusive. The authors used single-particle cryo-EM to obtain a series of structures from a large set of data (200.000 micrographs) that resulted in the determination of the structure of the major maturation and disassembly states of the SSU processome. Central to this process are two helicases, Mtr4 (that is connected to the nuclear exosome) and Dhr1.
By visualizing the structures of 16 native SSU processome intermediates, the authors show how the maturation of the 40S undergoes a gradual compaction over a single trajectory. The initial degradation of the 5' ETS triggers a set of compositional and conformational rearrangements in the SSU processome, with additional 5' ETS degradation triggering disassembly. This is followed by the repositioning and activation of Dhh1, which eventually triggers the release of the nucleolar pre-40S. Degradation by of the 5' ETS is known to be carried out by the exosome, although the reconstructions have limited density information for the nuclear exosome. The authors therefore use the identification of ribosomal assembly factors from the EM densities to carry out a computational interaction screen by AlphaFold Multimer, identifying four redundant pathways that tether exome subunits: Utp18-Mtr4, Utp14-Mtr4, Utp14-Rrp40 and an additional contact mediated by the nuclear exosome cofactor Rrp6. Using yeast genetics, the authors demonstrate that disrupting three of the four contacts is tolerated, whereas removal of the fourth is lethal to the cells, suggesting an inherent mechanism that imparts robustness to the maturation pathway.

Altogether, these findings establish a paradigm in which RNA degradation orchestrates compositional transitions and enzyme activation, ensuring unidirectional assembly and quality control of ribonucleoprotein complexes. The study is impressive for several reasons. First is the quality of the data. Second is the amount of structural information obtained on the large number of intermediates. In general, this is a tour de force study, made all the more impressive by the fact that it was conducted by just two authors.

To improve the manuscript and make it more accessible for a general audience, I would suggest the following text changes:

1. In general, I would suggest placing greater emphasis on the compositional rearrangements of the particles in the results

section and refer to the exosome (or rather exosome subunit x) only when the authors have experimental evidence. The reason is the limited density information on the exosome core or its nuclear cofactors. In contrast, the density information on Dhr1 is strong. While poorly defined density for the exosome in nucleolar steps of ribosome biogenesis has been reported before, the number of times the word 'exosome' appear in the results is somewhat disproportionate to the actual experimental information. I would suggest to keep most of the exosome discussion in the concluding parts. It would also be useful if the authors would include an explanatory sentence on the relative importance/involvement of Rrp44 and Rrp6 in this pathway.

2. The density for the C-terminus of Rrp6 appears rather small – were the authors able to unambiguously assign it, and if so, to which part of the so-called Rrp6 lasso does it correspond? One of the four redundant contacts involves a mutually exclusive interaction with the exosome cofactor Mpp6 – how do the authors envision this interaction to take place (is Mpp6 excluded from the nucleolus or from this pathway, is it a transient remodeling, or..).

3. I found the scheme in Figure 5A very interesting, as it suggests that rather than thinking of 'having completed particles to leave from the nucleolus', one may instead consider the edge of the nucleolus simply as the site where particle completion actually takes place. In the context of the current popularity of phase separation models of the nucleolus, which remain rather unsatisfying from a molecular perspective, I think the authors may have the opportunity to offer a more compelling and molecularly grounded perspective.

4. A major point of this manuscript is the disassembly of the SSU processome into a much smaller pre-40S particle. The authors describe this at a general level but there is no overview for the reader to highlight which states contains which proteins or protein complexes. A table containing this information would be helpful for clarity as it would also provide a direct link to the pdb coordinates and protein/RNA chains.

5. The authors have extensively processed their cryo-EM data to show that several post-translational modifications (phosphoserines) are visible in density maps shown in supplementary figures 3e and 27b-d. However, there is no mention in the text about the possible functions of these modifications. Here the authors should at least comment on the presence and/or potential roles of these modifications and for clarity prepare a figure to show their roles if possible.

6. Similarly, the high-resolution structure of State H should reveal the positions of modified nucleotides, which are also not mentioned in the text. Compared with the prior structure of the human SSU processome (Singh et al. 2021), are there significant new insights into the roles of these modifications?

Referee #3

(Remarks to the Author)
The authors report the purification and structural analysis of pre-ribosomal complexes on the 40S maturation pathway. These structures address a section of the ribosome synthesis pathway that has been quite extensively studied but for which key mechanistic understanding was lacking. Based on structure comparison, an detailed inferred chronology is proposed that seems reasonable and fits well with previous data. The structural data is supported by interaction predictions, applying Alphafold-Multimer, and in vivo testing of mutant proteins.

It has long been suspected that SSU processome disassembly would be substantially driven by exosome-mediated RNA degradation, but the support for this model and the mechanistic insights are very valuable.

The work represents a quite significant advance in understanding important features of a major biosynthetic pathway. Despite the excellent quality of the work, I would be concerned how digestible and informative the report will be for the very wide readership of Nature. For example, Figure 1 contains notable insights and summarizes a huge amount of data but, to follow it, a lot of background knowledge of the pathway is required. This problem is compounded by the, necessarily, highly compressed descriptions in the text and figure legends. As one of many possible examples, the phrase "With the processing of the 5' hinge," (line 147) will likely mystify all but the keenest ribosome aficionados. The term has not previously been mentioned, and the locations of the 5' hinge (and also the 3' hinge) are never described. I assume that this text refers to the 5' and 3' pre-rRNA interaction sites for the central, "hinge", region of the U3 snoRNA, but this inference needs a substantial understanding of the pathway (and may be incorrect).

Had it been submitted elsewhere, I would certainly recommend immediate acceptance. At NSMB, for example, the audience would generally be more receptive and a less compressed presentation would be possible. As it is, while I am reluctant to reject such fine work, I am not convinced that the Nature format is appropriate for publication of this MS.

Minor points
1) As space is very limiting, the start of the main text, and the list of examples of all the previously missing details, might be shortened. The space could be better used for more description of the different states, which are very briefly presented in the main text.

2) Figure 3A: What is meant by "ordered 5'ETS elements"? The 5'ETS contains a set of stem-loop structures, but far fewer than the elements indicated. The cartoon might be slightly easier to follow in a "conventional" 5'-3' orientation. Readers unfamiliar with the pathway will likely have difficulty in understanding the context. Including a larger pre-rRNA region in addition, for context, might be helpful.

3) Figure 5: As with Figure 1, the degree of detail is impressive, but following this will be challenging to non-experts. Perhaps the key components can be abstracted and highlighted in a simplified cartoon?
4) line 131; seems to be an extra “to”.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The reviewer appreciates the effort invested in the revision that improved the quality of presentation of the submitted manuscript.

The authors did attempt to address most of the reviewers' raised concerns. However, few minor points persist. It will be worthwhile to address the latter in order to improve further the quality and the clarity of the revised manuscript.

Remaining minor points:

Figure 1: If the authors don’t wish to display a panel of the SSU with its major domains annotated it’s fine, but they should at least annotate the rRNA domains of panel 16 (state O) along with some conventional land marks (Left foot, solvent side, head, body, platform, etc…)

Figure 5b: The presented model is still very dense in details.

Referee #2

(Remarks to the Author)
The authors have adequately addressed my comments, and those of the other Reviewers. It is certinly a complex paper, but that reflects the sheer amount of detailed mechanistic information. I find this all the more impressive considering that this is a two-author paper.

Referee #3

(Remarks to the Author)
The changes to the text and figures have made the results clearer and more accessible. I would support publication of the revised MS.

We appreciate the positive feedback of all reviewers and have implemented the requested changes in a carefully revised manuscript as outlined in the point-by-point response below.

Referees' comments:

Referee #1:
Remarks to the Author:

The manuscript by Buzovetsky et al. reports the results of an original study poised at deciphering the molecular mechanisms underlying the maturation and disassembly of the processome complex (from *Saccharomyces cerevisiae*), responsible for the biogenesis of the small ribosomal subunit (SSU, or 40S) particles. Through an ensemble of 16 intermediate states (solved by cryo-EM at various resolutions up to 2.7Å), the study reports the involvement of two helicases, Mtr4-exosome and Dhr1, in the regulation of the processome in order to drive the maturation of the SSU in an orderly and unidirectional fashion.
The manuscript conceptualizes a model for the unidirectional progression of the SSU maturation through the irreversible degradation of the 5' ETS RNA, via a complex synergetic interplay between the RNA exosome (including its associated helicase Mtr4), the maturing pre-40S particle and the processome leading ultimately to the release of the pre-40S and the disassembly of the processome, with the exosome playing the role of a molecular arbiter constantly assessing the RNA processing quality. In this model, Dhr1 is positioned and activated to unwind the U3 snoRNA in order to initiate the nucleolar release of the pre-40S.
The study applies a compelling set of methods including cryo-EM, genetics and *in silico* protein-protein interaction screen, to elucidated the RNA exosome mediated mechanism that explains both SSU processome maturation and disassembly.

The presented work is overwhelming and impressive and will be of great interest for the community. The manuscript is relatively clear, but some effort must be applied to clarify certain statements (see comments below), the figures are all important but insufficient to illustrate some of the key interpretations and conclusions of the presented work.
The reviewer recommends the publication of the submitted manuscript provided that the authors address the following major and minor points.

We thank reviewer 1 for the positive assessment of our work and the detailed constructive and helpful criticism, which we have addressed below.

Major points:

Figure 1. Because of the wealth of structures, the different presented states are extremely difficult to compare! The first 9 states look identical at a first glance then after a long game of "find the differences" one could start seeing some but others remain

totally unfindable. For instance, between states 1 and 2 involving a “head rotation”, even after investing a good 15 minutes, the reviewer still can’t spot the differences and what does the “head rotation” refer to? The authors must find a way to highlight the essential differences between the different states sequentially (arrows, circles, etc…). Also, a small 3D model of the SSU mature rRNA domains colored similarly to the different states will greatly help the non-experts in the SSU maturation to understand the progression of rRNA maturation and remodeling.

Most importantly, the elements colored in Figure 1 are confusing and it’s totally unclear where is the 5’ ETS? Also, the label “5’ ETS becoming disordered/trimmed” is used at multiple places on multiple elements, sometimes in orange and sometimes in yellow… What is what? The authors should pick a color for each element (U3, 5’ETS and different domains of the 18S rRNA) and stick to it to the end. They can highlight the action at each element/domain in every state by a bright contour for example. The reviewer leaves this aesthetic choice to the discretion of the authors.

We agree with reviewer 1 and have improved the clarity of figure 1 as follows:

1. **Movements of elements are indicated by arrows, which include protein/RNA movements as well as the head rotation.**
2. **Differences between states are highlighted by a thicker black outline**
3. **The 5’ETS has only one color and key processing steps are indicated in black.**
4. **Rrp6 is highlighted in states C-L in a distinct color**

Figure 1: Between states B and C, there is the exosome binding. Where is it on the figure? What is it made of?

We agree with reviewer 1 that this could be highlighted further and between states B and C we have indicated that Rrp6-exosome binding occurs so that the newly bound Rrp6 indicates exosome association. Rrp6 has a distinct color and is highlighted by a thicker black outline.

Figure 1, state G, Dim1 is nearly invisible.

We have addressed this by highlighting Dim1 more with a thicker black outline.

The authors must revise the Figure 1 in such a way that all important elements can be easy to spot and the actions highlighted. In the current state, Figure 1 is of very limited contribution to the understanding of the described mechanisms.

As indicated above, we have implemented the requested changes and believe that these changes have made Figure 1 much clearer.

Figure 3b: Is unfortunately unclear and confusing. It is difficult to suggest to the authors a specific solution. However, the reviewer can explain the underlying reasons of such unclarity. First, the transparent surfaces/densities are not enough transparent and mask significantly what's behind. Second, there are too many details that the authors wish to highlight on every panel. Third, similar to the reviewer's previous comment regarding the labels of Figure 2 (Some labels are hardly visible, use simple black font and point to the concerned residues with solid thin black lines. The current display in hard to read and in fact the numbers point nowhere, while it would be more useful and accurate to point to the concerned residues. In addition, the authors could leave out the name labels of different proteins in colored fonts (colored consistently with the protein name), but in this case they must use the exact same color and use bigger fonts. If the authors find the colors to be pale, therefore the labels hard to read, then they should use brighter colors.)

Suggestion: Try more transparency or only color what's important and where the action is taking place, and leave everything else in very fade shades of the original colors. Alternatively, try and remove the transparent densities all together.

We agree with reviewer 1 that this is a complex figure and have attempted to maximally increase clarity through the following means:

1. **As suggested by reviewer 1, we have maximally increased the level of transparency of surfaces while still allowing for a color distinction between transparent complexes such as UtpA and UtpB.**
2. **Where possible we have added additional labels and increased legibility.**
3. **Labels of proteins have been adjusted to the exact color of the protein.**
4. **Where possible, proteins are now displayed as surfaces (Rrp6, Utp24, and Pno1) to reduce the overall complexity of this figure.**

We note that cartoons are only shown for important elements that are changing during this disassembly series. Everything else is already removed and transparencies are used to provide an impression of depth.

Figure 5: This figure is supposed to present a model of the processome assembly, progression and disassembly in an intelligible manner. The presented panel b is useful, but it is not very intelligible and the reviewer recommends to the authors to display a simplified representation including only the large steps and discard as many fine details as possible, so not to dilute their important messages.

We have re-rendered Figure 5 to provide better separation of the highlighted components and reduced labels where possible. In Figure 5 the original 16 states of disassembling SSU processomes have already been compressed to 8 representations that represent these transitions. Since each SSU processome contains approximately 70 components, we have only highlighted a few of the key

components that undergo conformational or compositional changes. Each of the selected components is already depicted as a low-pass filtered surface so that we have been unable to provide further abstraction.

While the analysis of the presented structures is insightful, it is still unclear how the irreversible degradation of the 5' ETS is orchestrating this linear progression of the processome assembly and disassembly. The reader can connect certain dots and it is indeed reported that certain conformational changes due to the exosome activity on the 5' ETS trigger certain steps, some others don't seem to be related to this degradation, or perhaps they are but the authors failed to show the link.

We have used Figure 3 to first highlight how the stepwise degradation of the 5'ETS triggers a series of disassembly states of the SSU processome. The systematic disassembly of the SSU processome is an essential requirement for both repositioning and activation of Dhr1 as shown in Figure 4.

More importantly, what causes the exosome to pause so that discrete states can be captured?

Our large dataset captures a wide range of disassembly states and we envision that the degradation of the 5'ETS involves key hurdles that need to be overcome by the RNA exosome. During stages where no conformational changes occur immediately there will be longer residence times for specific disassembly states. This rationale is consistent with the states depicted in Figure 3 a and b, which fall along this trajectory.

Finally, the link between the exosome and U3 removal isn't described in sufficient details, and more globally the removal process of U3 based on the presented structures.

The link between RNA exosome-mediated degradation of the 5'ETS, resulting compositional changes that allow for Dhr1 repositioning and activation to remove U3 is highlighted in Figures 3 and 4.

What have we learned from the presented structures about the U3 removal?

As Dhr1 is the bait for all our purified particles, we only obtain particles in which Dhr1 is still bound. Importantly we have discovered how Utp14 can activate Dhr1 in a state-specific manner towards the end of this disassembly pathway just before Dhr1 removes U3. As Dhr1 activity will result in the removal of U3 and hence the Dhr1 binding site itself (after State O), we do not observe the product of this reaction in our reconstructions.

Minor points:

At no point in the manuscript the authors define what is the 5' ETS? This might be trivial for experts in the field; however, it would be appropriate to at least spell the abbreviation (external transcribed spacer) and explain in few words what this element is.

We thank reviewer 1 for this comment and have added this definition in the introduction on page 3.

While impressive, the data processing scheme appears a bit inconsistent between Datasets 1, 2 and 3. Indeed, the processing schemes differ substantially between all datasets, as for each a different combination of image processing tools were used (Dataset 1: Relion 5 Beta, Dataset 2: Relion 5 Beta, crYOLO and CryoSparc, Dataset 3: CryoSparc and pyEM), what justifies these choices? Could the authors explain the logic behind each "blend"?

This was a multi-year project in which computational resources became a bottle neck. The collection of the different datasets is a reflection of this and a gradual transition occurred in which key strengths of each software were maximized. Specifically, earlier steps of the processing pipeline were taken over by Cryosparc, whereas intermediate steps, such as 3D classification without alignment were performed using RELION. Lastly, final steps, such as focused refinements, were again performed in CryoSparc.

Moreover, there are different interestingly shaped but ignored cryo-EM reconstructions after the first 3D sorting in Datasets 1 and 2, and only one or two classes were selected to pursue the data processing. Could the authors explain briefly the basis of their selection/exclusion?

Low resolution reconstructions were the main reason for excluding particles. For Dataset 1 the only higher resolution reconstruction excluded was State H for which a higher resolution reconstruction was obtained using Dataset 2. As indicated in the processing pipelines, during the earlier stages of processing, the few selected high-resolution reconstructions ended up being mixtures of several indicated states.

Line 122: What is site A1? Define it in few words.

We have defined site A1 on page 3 with *"cleavage of the mature rRNA 5' end (site A1)."*

Line 128: Rrp6 is barely visible, only as a very small density with the same color as the U3. For such a small density, it's preferable to use a brighter different color to highlight it

and at least make it visible. Also, where is the 3' of the 5' ETS on the figure? And how does it relate to the Rrp6?

This is an excellent point. We have revised figures to include a much thicker outline for Rrp6 as well as a distinct color. The initial observation of Rrp6 coincides with the removal of the outer helices of the 5'ETS. We have indicated the 3' end of the 5'ETS for states A* and B for reference.

Line 130: Krr1 has the same color as the central domain of the 18S, very difficult to distinguish it from the latter, especially on such a small panel in a giant figure.

We agree with reviewer 1 and have re-rendered Krr1 in a darker color.

Also, why do the authors believe that the restructuring of the 5'ETS allows the partial release of Krr1 and Faf1? These elements seem to be very far apart.

Krr1 and Faf1 are multi-domain RNA binding proteins with extensive contacts with the 5'ETS. In the revised form of Figure 1 these contacts near the 5'ETS are more clearly visible in State C. In Extended Data Fig. 3 (State C vs State D) this transition is illustrated in more detail.

Lines-134-138: The authors write "the integration of helix 45 near the central domain results in the docking of the auto-inhibited Dhr1 in State E. Surprisingly we find that A1 cleavage in State G is solely triggered by the incorporation of Dim1 as this protein couples the interrogation of helix 45 installation with the stabilization of U3 snoRNA-bound regions within the 18S rRNA in an upward conformation". The authors must rephrase and simplify, statements such as "…couples the interrogation of helix 45 installation with the stabilization…" are confusing. What does the "interrogation of the installation" mean? More importantly, panel G doesn't illustrate this statement.

We have simplified this statement as follows:

"Surprisingly we find that A1 cleavage in State G is solely triggered by the incorporation of Dim1. Here Dim1 probes the placement of helix 45 close to the central domain and stabilizes U3 snoRNA-bound regions of the 18S rRNA in an upward conformation."

Comparing panels for States E and G in Extended Data Fig. 3 shows that the Dim1 binding site on the 18S rRNA is a composite of both helix 45 and helix 24 of the central domain. On the right side of Dim1 18S rRNA regions that are bound by U3 can be seen in an upward conformation. For an RNA-only view of these transitions, Extended Data Fig. 2b can also be used.

Figure 2c: Some labels are hardly visible (rpS24, Pno1, utp20, Dhr1 anchor, the residues numbers, etc…). This is related to the reviewer's previous comment (suggests to the authors to use simple black font and point the concerned residues with solid thin black lines. The current display in hard to read and in fact the numbers point nowhere, while it would be more useful and accurate to point to the concerned residues. In addition, the authors could leave out the name labels of different proteins in colored fonts (colored consistently with the protein name), but in this case they must use the exact same color and use bigger fonts). For instance, Utp20 is more orange than the ribbon of the actual protein, Pno1 is a bit green, while the actual ribbon is very light yellow… If the authors find the colors to be pale, therefore the labels hard to read, then they should use brighter colors.

We thank reviewer 1 for these suggestions and have revised Fig. 2c accordingly. Rrp6, Pno1 and Utp20 have been re-rendered as surfaces for clarity. To distinguish between Utp14 and Rrp6 numbering, we have maintained colored numbering, in each case indicating precise positions by black lines. Protein color labels have been matched precisely.

Importantly, and this is not the first occurrence, the authors should use more contrasted colors, especially when highlighting key proteins/components (e.g., Utp14 and Dhr1, Dhr1 and Rrp6, Utp7 and Sof1, Mpp10 and Sas10, etc…).

We have increased the contrast in colors between the indicated proteins for clarity.

Lines 205-207: The so-called "hook" in Utp14 is hardly visible on Figure 2c, because it's displayed poorly and the colors of Utp14 and Dhr1 are very similar.

We agree with reviewer 1 and have addressed the visibility by using a surface display for this small peptide motif in Figure 2c.

Line 252: 5' ETS is nearly completely invisible. It is important to highlight this feature, the authors must find a way to illustrate that RNA exosome mediated processing of the 3' end of the 5' ETS alters the Sof1 binding site.

The 5' hinge underneath Sof1-Utp7 is directly labelled with a black line in Fig. 3b.

In Methods, Lines 617-618: Why did the authors prepare their samples at 18°C and not at 4°C, which could reduce the conformational heterogeneity of their structures, and perhaps in turn improve their resolutions?

We agree with reviewer 1 that in a subsequent study sample preparation at 4°C could be attempted to reduce conformational heterogeneity.

In Methods, Lines 623-627: The authors describe the grids preparation protocol. The grids of each sample (Kre33, Dhr1 and Utp14…) have been prepared differently (variable in number of applications and blotting, time of blotting), why is that? Could the authors explain the process of optimization and how they came about these parameters? The reviewer is not questioning the quality of the experiments but it will be of use to the readers to understand this process. Even a brief description will be useful.

A common phenomenon of studies involving endogenous particles is the limiting amount of available sample. In this study the number of sample applications onto a given cryo-EM grid is inversely proportional to the amount of available sample. On page 32 of the revised manuscript, we have put this in context for the different datasets with the statement: *"The lower the concentration of particles of a given preparation, the higher the number of applications that were done on each grid."*

Referee #2:
Remarks to the Author:

Buzovetsky and Klinge report an impressive structural study that tackles the molecular mechanisms of the maturation and disassembly of the S.cerevisiae small subunit processome, leading to the formation of the pre-40S particle.

The SSU processome is a large ribonucleoprotein assembly that undergoes precise ribosomal RNA (rRNA) processing coupled to compositional changes of ribosomal proteins, but the sequel of events and mechanisms underpinning the unidirectionality and irreversibility of the process have been elusive. The authors used single-particle cryo-EM to obtain a series of structures from a large set of data (200.000 micrographs) that resulted in the determination of the structure of the major maturation and disassembly states of the SSU processome. Central to this process are two helicases, Mtr4 (that is connected to the nuclear exosome) and Dhr1.
By visualizing the structures of 16 native SSU processome intermediates, the authors show how the maturation of the 40S undergoes a gradual compaction over a single trajectory. The initial degradation of the 5' ETS triggers a set of compositional and conformational rearrangements in the SSU processome, with additional 5' ETS degradation triggering disassembly. This is followed by the repositioning and activation of Dhh1, which eventually triggers the release of the nucleolar pre-40S. Degradation by of the 5' ETS is known to be carried out by the exosome, although the reconstructions have limited density information for the nuclear exosome. The authors therefore use the identification of ribosomal assembly factors from the EM densities to carry out a computational interaction screen by AlphaFold Multimer, identifying four redundant pathways that tether exome subunits: Utp18-Mtr4, Utp14-Mtr4, Utp14-Rrp40 and an additional contact mediated by the nuclear exosome cofactor Rrp6. Using yeast

genetics, the authors demonstrate that disrupting three of the four contacts is tolerated, whereas removal of the fourth is lethal to the cells, suggesting an inherent mechanism that imparts robustness to the maturation pathway.

Altogether, these findings establish a paradigm in which RNA degradation orchestrates compositional transitions and enzyme activation, ensuring unidirectional assembly and quality control of ribonucleoprotein complexes. The study is impressive for several reasons. First is the quality of the data. Second is the amount of structural information obtained on the large number of intermediates. In general, this is a tour de force study, made all the more impressive by the fact that it was conducted by just two authors.

We thank reviewer 2 for the positive assessment of our work and the subsequent helpful comments aimed at improving this manuscript, which we have addressed below.

To improve the manuscript and make it more accessible for a general audience, I would suggest the following text changes:

1. In general, I would suggest placing greater emphasis on the compositional rearrangements of the particles in the results section and refer to the exosome (or rather exosome subunit x) only when the authors have experimental evidence. The reason is the limited density information on the exosome core or its nuclear cofactors. In contrast, the density information on Dhr1 is strong. While poorly defined density for the exosome in nucleolar steps of ribosome biogenesis has been reported before, the number of times the word ‘exosome’ appear in the results is somewhat disproportionate to the actual experimental information. I would suggest to keep most of the exosome discussion in the concluding parts. It would also be useful if the authors would include an explanatory sentence on the relative importance/involvement of Rrp44 and Rrp6 in this pathway.

We agree with reviewer 2 that only Rrp6 can be unambiguously identified in our cryo-EM maps (see comments regarding point 2 below) and that the presence of Rrp6 is used as a proxy for the RNA exosome complex. We have now clarified this further on page 6, where we state: *“The presence of the Rrp6 C-terminus in States C-L highlights the tethered presence of the RNA exosome, which is mediating transitions between states.”* At the figure level we have highlighted the presence of Rrp6 in states C-L with a thicker black outline in Figure 1. Separately, on page 3 we have added a sentence in the introduction that introduces the roles of Rrp6 and Rrp44 more properly.

2. The density for the C-terminus of Rrp6 appears rather small – were the authors able to unambiguously assign it, and if so, to which part of the so-called Rrp6 lasso does it correspond? One of the four redundant contacts involves a mutually exclusive interaction with the exosome cofactor Mpp6 – how do the authors envision this interaction to take place (is Mpp6 excluded from the nucleolus or from this pathway, is it a transient remodeling, or..).

We can unambiguously identify the C-terminus of Rrp6 in our structures, especially in State H, which at a resolution of 2.7 Ångstroms provides sufficient detail for this assignment. The presence of Rrp6 is further in agreement with our prior structural data in the human system (*Singh et al. 2021*). In Extended Data Fig. 5a we have annotated the regions of the Rrp6 lasso where mutations were introduced, which correspond to areas that we observe in our structures. Extended Data Fig. 6a further shows which of these segments are visible as a function of the different states, thereby complementing the general overview of Figure 1.

We agree with reviewer 2 that the positioning of Mpp6 and the ERM1 and ERM2 peptides of Utp14 result in mutual exclusivity of these proteins on the RNA exosome. Therefore we envision exclusion as a mechanism to separate Mpp6- and Utp14-dependent pathways.

3. I found the scheme in Figure 5A very interesting, as it suggests that rather than thinking of 'having completed particles to leave from the nucleolus', one may instead consider the edge of the nucleolus simply as the site where particle completion actually takes place. In the context of the current popularity of phase separation models of the nucleolus, which remain rather unsatisfying from a molecular perspective, I think the authors may have the opportunity to offer a more compelling and molecularly grounded perspective.

We thank reviewer 2 for this comment and have attempted to address this precise point in our conclusion where we have previously used molecular tethering to describe very specific interactions within particles (intra-SSU processome), between particles (SSU processome- RNA exosome), and within the entire organelle (nucleolus). We have expanded this point and on page 15 we state:

"These data suggest that rather than the loss of non-specific interactions with a biomolecular condensate, it is the loss of specific protein-protein and protein-RNA interactions that enables the release of pre-ribosomal particles from the nucleolus."

4. A major point of this manuscript is the disassembly of the SSU processome into a much smaller pre-40S particle. The authors describe this at a general level but there is

no overview for the reader to highlight which states contains which proteins or protein complexes. A table containing this information would be helpful for clarity as it would also provide a direct link to the pdb coordinates and protein/RNA chains.

We thank reviewer 2 for the constructive comment and have added a supplementary table (Extended Data Table 2), which provides the requested overview.

5. The authors have extensively processed their cryo-EM data to show that several post-translational modifications (phosphoserines) are visible in density maps shown in supplementary figures 3e and 27b-d. However, there is no mention in the text about the possible functions of these modifications. Here the authors should at least comment on the presence and/or potential roles of these modifications and for clarity prepare a figure to show their roles if possible.

We thank reviewer 2 for making this point and have added both a sentence on page 6 in the manuscript and added an additional figure (Supplementary Fig. 28).

6. Similarly, the high-resolution structure of State H should reveal the positions of modified nucleotides, which are also not mentioned in the text. Compared with the prior structure of the human SSU processome (Singh et al. 2021), are there significant new insights into the roles of these modifications?

We indeed see modified nucleotides in State H but as this has already been discussed in our prior work in the human SSU processome (*Singh et al. 2021*), we decided not to cover this in more detail in this manuscript.

Referee #3:
Remarks to the Author:
The authors report the purification and structural analysis of pre-ribosomal complexes on the 40S maturation pathway. These structures address a section of the ribosome synthesis pathway that has been quite extensively studied but for which key mechanistic understanding was lacking. Based on structure comparison, an detailed inferred chronology is proposed that seems reasonable and fits well with previous data. The structural data is supported by interaction predictions, applying Alphafold-Multimer, and in vivo testing of mutant proteins.

It has long been suspected that SSU processome disassembly would be substantially driven by exosome-mediated RNA degradation, but the support for this model and the mechanistic insights are very valuable.

The work represents a quite significant advance in understanding important features of a major biosynthetic pathway. Despite the excellent quality of the work, I would be concerned how digestible and informative the report will be for the very wide readership of Nature. For example, Figure 1 contains notable insights and summarizes a huge

amount of data but, to follow it, a lot of background knowledge of the pathway is required. This problem is compounded by the, necessarily, highly compressed descriptions in the text and figure legends.

We thank reviewer 3 for appreciating the quality of our work and for the constructive criticism. We agree that a lot of interesting specifics have not been described in detail in the text to ensure that we both reach the broad readership of *Nature* while at the same time providing information that is also useful for experts in the field. However, we believe that this manuscript (with the implemented changes based on all three reviewers), is now accessible to the broad readership of *Nature*.

As one of many possible examples, the phrase “With the processing of the 5’ hinge,” (line 147) will likely mystify all but the keenest ribosome aficionados. The term has not previously been mentioned, and the locations of the 5’ hinge (and also the 3‘ hinge) are never described. I assume that this text refers to the 5’ and 3’ pre-rRNA interaction sites for the central, “hinge”, region of the U3 snoRNA, but this inference needs a substantial understanding of the pathway (and may be incorrect).

We thank reviewer 3 for this comment and have edited the main text on page 3 to include the following statement: *“By serving as the architectural blueprint for the SSU processome, the chaperone U3 snoRNA base-pairs with parts of the SSU pre-rRNA (via its box A and box A’ segments) as well as the 5’ETS (via its 3’ and 5’ hinges).”* We believe that this text together with the indicated locations of these segments in Figures 1 and 3 will provide sufficient background for a general audience.

Had it been submitted elsewhere, I would certainly recommend immediate acceptance. At NSMB, for example, the audience would generally be more receptive and a less compressed presentation would be possible. As it is, while I am reluctant to reject such fine work, I am not convinced that the Nature format is appropriate for publication of this MS.

While we agree that a relatively brief format presents its own challenges for the wealth of data we are presenting, we believe that the requested revisions (see above) and the addressed minor points (see below) have significantly improved this manuscript and have made it more accessible for the broad readership of *Nature*.

Minor points
1) As space is very limiting, the start of the main text, and the list of examples of all the previously missing details, might be shortened. The space could be better used for

more description of the different states, which are very briefly presented in the main text.

We thank reviewer 3 for this suggestion and will shorten the introduction if we are limited by space. In this manuscript we have chosen to focus on the mechanism of SSU processome disassembly (States H-O) for the broad readership of *Nature.* Since the mechanism of cleavage at site A1 (States A-G) will be specifically of interest to a more specialized audience that is already familiar with prior literature in this area (*Cheng et al. 2020, Singh et al. 2021*), we have decided to move the corresponding figures into Extended Data Figs. 2 & 3. We believe that the result of this organization is a manuscript that is accessible for a general audience while simultaneously providing deeper insights for experts in the field as well.

2) Figure 3A: What is meant by "ordered 5'ETS elements"? The 5'ETS contains a set of stem-loop structures, but far fewer than the elements indicated. The cartoon might be slightly easier to follow in a "conventional" 5'-3' orientation. Readers unfamiliar with the pathway will likely have difficulty in understanding the context. Including a larger pre-rRNA region in addition, for context, might be helpful.

We thank reviewer 3 for this comment and have re-rendered this figure for clarity. The entire 5'ETS is represented by a yellow bar with visible parts of the 5'ETS highlighted in orange. Since the RNA exosome travels in a 3' to 5' direction, we wanted to make the transition of the exosome, and hence the transitions from state H to O legible from left to right.

3) Figure 5: As with Figure 1, the degree of detail is impressive, but following this will be challenging to non-experts. Perhaps the key components can be abstracted and highlighted in a simplified cartoon?

The model in Figure 5a provides a non-expert overview of where this pathway takes place, whereas panel b already condenses 16 structures down to 8 representations of the observed dynamics. We have chosen to highlight a limited number of factors to explain the key transitions guided by the RNA exosome and Dhr1 to focus the readers' attention on key insights that are unique to this study. In addition, we have included arrows, numbering and key words to guide the reader through the pathway.

4) line 131; seems to be an extra "to".

We thank reviewer 3 for this suggestion and have changed this sentence to: *"This leads to the 3' end of the 18SrRNA precursor (helix 45) being chaperoned by the assembly factor Pno1 towards the interior of the particle, thus allowing an N-terminal element of the DEAH-box helicase Dhr1 to bind."*

We appreciate the positive feedback of all reviewers as outlined in the point-by-point response below.

Referees' comments:

Referee #1 (Remarks to the Author):

The reviewer appreciates the effort invested in the revision that improved the quality of presentation of the submitted manuscript.

The authors did attempt to address most of the reviewers' raised concerns. However, few minor points persist. It will be worthwhile to address the latter in order to improve further the quality and the clarity of the revised manuscript.

We thank reviewer 1 for the positive assessment and constructive criticism our work.

Remaining minor points:

Figure 1: If the authors don't wish to display a panel of the SSU with its major domains annotated it's fine, but they should at least annotate the rRNA domains of panel 16 (state O) along with some conventional land marks (Left foot, solvent side, head, body, platform, etc…)

We thank reviewer 1 for this suggestion and have added landmarks for visible elements of State O.

Figure 5b: The presented model is still very dense in details.

We agree with reviewer 1 that the complexity of eukaryotic ribosome assembly is apparent in figure 5. While we have reduced the number of displayed components to an absolute minimum, as stated by reviewer 2, it *"reflects the sheer amount of detailed mechanistic information"* we have uncovered.

Referee #2 (Remarks to the Author):

The authors have adequately addressed my comments, and those of the other Reviewers. It is certainly a complex paper, but that reflects the sheer amount of detailed mechanistic information. I find this all the more impressive considering that this is a two- author paper.

We thank reviewer 2 for the positive assessment and constructive criticism our work.

Referee #3 (Remarks to the Author):

The changes to the text and figures have made the results clearer and more accessible. I would support publication of the revised MS.

We thank reviewer 3 for the positive assessment and constructive criticism our work.