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Peer Review File

The SPATA5-SPATA5L1-CINP-C1ORF109 complex is essential for cytoplasmic pre-60S ribosome maturation

Corresponding Author: Professor Chunaram Choudhary

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Communications Biology.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I still fundamentally disagree with the authors' interpretation of "added complexity" in metazoan ribosome biogenesis and therefore ask them again to revise both the abstract and the introduction accordingly. The most comprehensive current catalog of ribosome biogenesis factors was compiled by the Kutay laboratory (PMID: 36762427) and demonstrates a high degree of conservation across eukaryotes. The available evidence strongly supports a shared core biogenesis machinery, with lineage-specific differences more plausibly reflecting additional regulatory features or "bells and whistles" (as exemplified by Drg1/SPATA5), rather than wholesale deviations from a conserved mechanism.
To be clear, I did not ask the authors to provide validating data for every newly proposed factor. However, I also object to the indiscriminate labeling of a broad set of proteins as metazoan-specific ribosome biogenesis factors simply because they co-elute with ribosomal particles, are poorly annotated, or because the authors have not engaged with the extensive proteomic literature on yeast ribosome biogenesis, much of which is not cited. In fact, inspection of the list in the Kutay review reveals numerous bona fide ribosome biogenesis factors that have been well characterized in yeast but do not have clear human homologs (arguably more than the converse, at least at present). By the authors' logic, this would imply that yeast ribosome biogenesis is more complex, which is not a defensible conclusion. I am nevertheless surprised that the proteomic data have been removed entirely from the main figures rather than being more carefully curated and contextualized. Proper integration of these datasets would strengthen, rather than weaken, the manuscript.
That said, beyond these conceptual concerns, the manuscript does provide some incremental insights into SPATA protein function that may be of interest to the field, and on that basis publication could be warranted, provided the authors substantially revise their framing and interpretation of metazoan ribosome biogenesis.

Reviewer #2

(Remarks to the Author)
The authors have addressed all of my concerns and questions. I find the revisions in the manuscript and the explanations provided in the rebuttal letter to be satisfactory. I have no further comments.

Reviewer #3

(Remarks to the Author)
The manuscript entitled "The SPATA5-SPATA5L1-CINP-C1ORF109 complex is essential for cytoplasmic pre-60S ribosome maturation" by Walter et al. combines mass spectrometry, fluorescence microscopy, cell-based assays, x-ray crystallography and cryoEM to evaluate the role of the 55LCC complex in human ribosome maturation. The authors have carried out an impressive amount of work which included countless mass spectrometry experiments, the generation of numerous cell lines and the laborious optimization of expression/purification procedures for the 55LCC complex.

I was asked by the editor to especially comment on the author response to reviewer 3:

Point 1: I agree with the reviewer that the mass spectrometry analysis of pre-ribosomal particles carried out by the authors does not allow conclusions about direct interactions between proteins. The authors in response to the reviewer claim that they discovered that the 55LCC complex is a GTPBP4 interactor (although I didn't find such an explicit statement in their manuscript). However, in the manuscript itself they don't provide results to really demonstrate this (in-vitro pulldown, crosslinking mass spectrometry etc.) I'm also confused by the statement of the authors that there is currently no cryoEM structure of the 55LCC complex. In the manuscript they refer to published 55LCC cryoEM structures, even in complex with pre-ribosomal particles.

Point 2: I agree with the authors that they validated their tagged bait proteins by analyzing cell proliferation. However, they did not comment on the reviewer's criticism concerning the SPATA5/SAPTA5L co-purification with small subunit as well as early and late large subunit precursors.

Point 3: The authors do not hide the fact that they provide low resolution structures of the 55LCC complex. In my opinion, they do not overinterpret their maps and provide complementary insights to the already published structures. Their functional characterization of the 55LCC complex includes cell confluence assays, SILAC mass spec, cellular location of 55LCC substrates and analysis of global protein synthesis levels, which I find rather complementary to Ni et al.. Naturally some of the results overlap, but I think this is actually beneficial for the field and should not be hold against the authors. I my version of the manuscript I cannot find the revised paragraph the authors refer to (pages 7–8, lines 273–283, in my manuscript version there is a paragraph about the crystal structure of the ΔCINP-C1ORF109 complex)

I think its justified to compare 55LCC with respect to p97/VCP.

The discussion of the 55LCC mechanism does include references to the published cryoEM structures, but I cannot find the paragraph the authors refer to (pages 17–18, lines 675–690, in my manuscript version that is already material and methods)

I agree with the statement of the authors that ATP binding seems to stabilize 55LCC complex formation. Figure 5A-B demonstrates that they can get stoichiometric, well defined complexes in the presence of non-hydrolysable/slowly hydrolysable ATP analogues (AMPNP/ATPγS). However, as pointed out by the reviewer, it is then indeed strange why ATPase deficient mutants should interfere with complex formation. The authors refer to supplementary figure 3C to illustrate this point. Unfortunately, the figure does not specify the kind of mutation. From the overall context, I assume the authors refer to the Walker-B E Q mutation. The Walker-B E Q mutation is supposed to impair ATPase activity, so in the presence of ATP it should stabilize the ATP bound state. One would expect to see similar effects on 55LCC complex stability like for AMPNP/ATPγS in the case of the wild type proteins.
In response to the reviewer's comments on stability of the ATPase deficient mutants, the authors state that they have revised the section. However, in my manuscript version the relevant section (page 16, lines 554 – 565, which is different from the page/line numbers in the rebuttal letter) still includes the sentence: "In agreement with these observations, SPATA5/SPATA5L1 ATPase-deficient mutants fail to yield stable complexes in our purifications (Supplementary Figure S3C)", which does not seem to be significantly different from the original version.

Point 4. Out of curiosity, I checked the following statement: "This arrangement supports our hypothesis that ATP-driven conformational changes within the 55LCC complex could promote coordinated release of GTPBP4 and RSL24D1 maturation factors, which have been proposed to dissociate together during cytoplasmic pre-60S remodeling (18,32)" (page 17, lines 592 – 595 in my manuscript version). Although I admittedly did not read reference 32 thoroughly (Kappel et al., JCB, 2012), a quick look seems to suggests that yeast Drg1 (human 55LCC) removes Rpl24 (human RSL24D1) but not Nog1 (human GTPBP4) from pre-60S particles (Figure 7 and last paragraph of discussion). That seems to be quite the opposite of the statement above.

Version 1:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors addressed all my comments/concerns

Responses to reviewers' comments

We thank all three reviewers for evaluating our work and providing constructive feedback. We have carefully revised the manuscript text in response to the referees´ feedback.

We have also revised the Discussion to include the mention that 55LCC likely evolved in early metazoans, and physical interaction between SPATA5, SPATA5L1, and CINP has also been found in recent large-scale interactomes in Drosophila.

Beyond ribosome biogenesis, 55LCC has also been implicated in nuclear processes. We have expanded the Discussion to discuss these prior findings in the context of our work. We believe that this open discussion will be useful to readers in further clarifying the roles of 55LCC.

Reviewer #1

Walter, Weisser, Masternak et al. present a study aimed at characterizing ribosome biogenesis factors in mammalian cells, with further analysis of the essential 55LCC complex.

We thank the reviewer for evaluating the manuscript and for providing critical feedback. Below, we respond to their specific points.

One of the stated motivations of the study is to identify mammalian-specific ribosome biogenesis factors. However, this analysis appears to rely almost entirely on database annotations, with the result that well-established RBFs are described as novel in this context (e.g., SURF6/Rrp14, DDX21, NOP14/Nop14, URB1/Urb1, among several others). The resulting candidate list also contains many proteins that are unlikely to represent genuine discoveries and should have been annotated as such. Without careful manual curation, the dataset as presented is both misleading and of limited usefulness to the field. A critical question is whether, beyond the 55LCC components, there are any truly novel factors that emerge from this study and withstand careful scrutiny. The authors cite studies (both over 10 years old) that proposed specific lists of potential mammalian-specific RBFs, but never discuss whether these factors are also recovered in the current dataset. Structural studies of ribosome biogenesis consistently support the view that the core ribosomal assembly pathway is highly conserved between yeast and metazoans. Therefore, the hypothesis that there remain numerous essential, mammalian-specific RBFs must be rigorously tested, rather than simply stated without supporting evidence, as done in the opening line of the abstract.

We appreciate the referee's point that ribosome biogenesis is highly conserved between yeast and mammals, and we agree that the number of factors specific to metazoan ribosome biogenesis may be limited. Nonetheless, several higher metazoan–specific factors have been reported, and among the four members of the 55LCC complex, three (SPATA5L1, CINP, C1ORF109) lack direct yeast orthologs.

AP-MS analyses provide a useful first step for identifying putative interactors, but additional validation is required to confirm these interactions. This is clarified in the revised text by stating that: "To avoid ambiguity, we would clarify that proteins enriched in our AP–MS analyses represent putative interactors, and additional validation will be required to confirm these interactions."

In this study, we rigorously validated interactions, subcellular localization, and functions for four enriched factors (SPATA5, SPATA5L1, CINP, and C1ORF109). However, it is not feasible to experimentally confirm every interaction or to annotate each protein's potential role. Even if one assumes that no additional novel ribosome-biogenesis factors are present among the enriched

interactors, our central finding remains unchanged: 55LCC is an essential regulator of pre-60S ribosome biogenesis. This conclusion both confirms and extends recent studies of this complex.

Because we are not aware of a single comprehensive database cataloguing all known ribosome-biogenesis factors, we relied on multiple databases complemented by manual annotation. This is a standard practice in the field.

As the authors note, several structure-function studies of the 55CC complex have been reported since its initial characterization in 2022. While its functional divergence from Drg1 is indeed interesting and represents a genuine metazoan specialization, the characterization of SPATA5/5L1 in this manuscript offers only incremental insights and does not resolve outstanding questions regarding its role in ribosome biogenesis or its putative role in DNA replication. The cryo-EM analysis is particularly underwhelming, as 4.5 and 3.5 Å structures of the complex are already available.

We acknowledge that higher-resolution structures (4.5 Å and 3.5 Å) of the complex have been previously reported. While we are unable to improve the resolution of our current dataset, we believe our structure provides valuable complementary insights. Specifically, our data were obtained from wild-type complexes expressed in mammalian cells, which allows us to capture the conformational heterogeneity present in the physiological context. This inherent native flexibility and dynamic nature of the complex likely contributed to the lower resolution observed in our maps. We have elaborated on these points in the manuscript (pages 10–11, lines 395–402). In addition, we have rephrased the text, including mentions of the higher resolution of the previous structures compared to ours.

Overall, the study would benefit from a more careful and informative curation of the MS dataset, which could yield a useful resource for the field. As currently presented, however, the work does not provide sufficient novelty or depth of insight to merit publication in Nature Communications. A more specialized journal may be a more appropriate venue for these findings.

While we independently identified and characterized 55LCC, we acknowledge that recent publications have reduced the novelty of its discovery. We view these studies as providing valuable confirmation and complementary perspectives. For instance, to our knowledge, our work is the only one that demonstrates immunofluorescence-based subcellular localization of endogenously expressed, GFP-tagged 55LCC. Also, this is the only effort that has used unbiased AP-MS analyses of RLP28, RLP10A, and GTPBP4, after the depletion of 55LCC constituents, to demonstrate enrichment of pre-60S associated factors on the ribosome in an unbiased manner. Moreover, by precisely ablating all four complex members and performing complementation with multiple mutants, we establish the functional relevance of several key interactions.

We hope the reviewer finds the revised manuscript suitable for publication in *Communications Biology*.

Reviewer #2

This manuscript reports the results of a comprehensive and multifaceted study on the SPATA5-SPATA5L1-CINP-C1ORF109 (55LCC) complex. The authors combine advanced proteomics, cellular engineering, and structural biology to present strong evidence that this complex plays a crucial role in pre-60S maturation. Overall, the experiments are very well-controlled, and the data is of high quality. The deep mechanistic analysis of the 55LCC complex's function presented in this paper appears to contain many unique insights.

However, the current structure of the manuscript does not adequately consider prior research and requires major revision. This study enters a very rapidly advancing field, and three other major studies on the same factors have already been published (References 8-10). Although the authors indicate their awareness of this situation by citing these papers, the manuscript's framing as an "independent discovery" is not appropriate. The central challenge for the authors in their revision will be to more sharply define and emphasize the unique and novel contributions of their study.

We thank the reviewer for positive and constructive comments. We appreciate their feedback. We are encouraged that the Reviewer found “the experiments are very well-controlled, and the data is of high quality.” Below, we respond to specific comments.

Specific Comments

1. Throughout the manuscript, the positioning of this work relative to the three major related studies (References 8-10) is not well described. For example, in the abstract and introduction, the authors frame their findings as an "independent discovery" and avoid comparing their results with the achievements of prior studies. While independent discoveries are certainly important in science, this claim by itself adds little to the paper's value. Furthermore, failing to frame the manuscript based on the established findings of previous research raises issues of fairness. The authors should revise the manuscript to clearly define what was already known from prior studies and what remained unknown, and then frame their work to highlight its unique and novel contributions.

We wish to clarify that we identified the 55LCC complex about a decade ago, at a time when nothing was known about the roles of SPATA5L1, CINP, or C1ORF109. However, substantial technical challenges—particularly difficulties in obtaining the structure—delayed publication. During this period, other groups (cited in our paper) independently reported the 55LCC complex and its involvement in ribosome biogenesis. Accordingly, we present our work as an independent discovery that both validates and extends those earlier findings. We wish to highlight that:

1. Our study is the only one to analyse interactions of all endogenously expressed 55LCC subunits.
2. This is also the only work that examined the function of 55LCC after acute depletion of each of the constituents.
3. Our unbiased AP-MS analyses show accumulation of specific pre-60S-bound biogenesis factors on the ribosome after acute depletion of 55LCC constituents.
4. This is the only study that has used the complementation-based approach to deplete endogenous 55LCC constituents and conditionally express ectopic versions to show functional requirement of SPATA5 and SPATA5L1 ATPase activities, as well as the importance of the identified SPATA5 and CINP interaction regions.
5. For the first time, this study shows sub-cellular localization of all four 55LCC constituents using endogenous GFP-fusion proteins.

We also wish to mention that several other studies have reported interactions between 55LCC constituents and proteins involved in cell-cycle regulation, DNA damage responses, replication, and transcription. However, neither we nor others have detected reproducible interactions with these nuclear factors, and we find no credible evidence for nuclear localization of the complex. This underscores the need for independent validation: our data support a role for 55LCC in ribosome biogenesis, but not in chromatin-associated processes.

2. Negative controls are lacking in several experiments. In Fig. 3 and 4, experiments should be performed on a strain lacking the FKBP12F36V degron tag to confirm that the experimental procedure for depletion does not itself affect the arrest in cell proliferation and the protein enrichment.

We would like to clarify that dTAG-V1 has been shown not to affect cell proliferation (PMID: 32948771) and has been used in dozens of studies to degrade FKBP12F36V-fused proteins. Importantly, in cells expressing endogenous FKBP12F36V-tagged 55LCC components, ectopic expression of Halo-tagged versions of these components confers complete resistance to dTAG-V1. This demonstrates that the observed phenotypes arise from depletion of the intended targets (Supplementary Fig. 11).

Reviewer #3

Evaluation of the manuscript „The SPATA5-SPATA5L1-CINP-C1ORF109 complex is essential for cytoplasmic pre-60S ribosome maturation" by Jonas Walter et al.
The paper describes an MS based protein-protein interaction screen with ribosomal proteins and ribosome assembly factors in human cells. This screen led to identification of the SPATA5–SPATA5L1–CINPC1ORF109 (55LCC) complex that seems to play a key regulatory function in ribosome biogenesis. Further structural analysis by cryo-EM and X-ray crystallography addressed the structure of this complex. Functional studies showed that the 55LCC complex is essential and might be important for the release of RSL24D1, EIF6 and GTPBP4 in the cytoplasm.

We thank the reviewer for their time and effort in evaluating our work and providing constructive feedback. Please see below our responses to specific comments.

I have several problems with this paper, that are listed below
1. The strategy to tag ribosomal proteins and maturation factors and perform pulldowns from cell extracts for analysis by mass spectrometry is not really a straight forward approach for ribosome biogenesis factors. The tagged proteins are all part of larger assemblies, i.e. ribosomes or precursors thereof, each consisting of several pre-rRNAs and many different proteins (ribosomal proteins and assembly factors). It is clear, that this is not a protein-protein interaction screen as claimed by the authors, because most of the identified proteins will not show direct interaction, although they are part of the same macromolecular structure. I strongly question the significance for such an approach in times where we already have high resolution cryo-EM structures of both, human nucleolar and nucleoplasmic pre-ribosomal particles (e.g. from the Klinge and Gao labs).

We fully acknowledge that advances in cryo-EM have allows obtaining high-resolution insights into macromolecular structures, including ribosomes. However, cryo-EM does not replace the value of AP-MS–based interaction mapping. AP-MS has repeatedly proven useful for identifying protein interactions involved in ribosome biogenesis, and our own discovery of 55LCC as a GTPBP4 interactor and pre-60S maturation factor reinforces this point. Although we and others show that 55LCC plays a key role in pre-60S biogenesis, no cryo-EM structure is currently available for this complex. We believe that cryo-EM and AP-MS complement each other, rather than one replacing the other.

2. According Figure 1, SPATA5 and SPATA5L1 pop up in the GTPBP4 purification, the TSR1 purification and the NOP56 purifications. TSR1 associates with the nascent small subunit at a later stage and accompanies the small subunit precursor into the cytoplasm, while Nop56 is an U3 snoRNP component that is associated with very early assembly intermediates (SSU processome) localized to the nucleolus. In contrast GTPBP4 is a large subunit specific assembly factors associated with early nucleolar to early cytoplasmic pre-60S particles. These are three very different pre-ribosomal particles. The copurification of the 55LCC complex with all three bait proteins is not explained by the authors and likely points to specificity problems in the purifications.
In addition, GTPBP4 only copurifies with Rpl3 as sole large subunit ribosomal protein, which is not consistent with the function of GTPBP4 in large ribosomal subunit assembly. This suggests that the tagged protein is not fully functional, which might explain the prominent copurification of the 55LCC

complex. The authors should be aware, that the C-terminal tail of GTPBP4 is exposed into the poly-peptide exit tunnel (PET). The used C-terminal 3xFLAG tag for the IP is likely to interfere with the insertion of the C-terminal helix of the GTPase into the PET which means that the obtained results are likely not reflecting the natural situation.

We acknowledge that ribosome biogenesis is a complex, multi-step process. AP-MS provides an initial means of identifying putative interactors, but additional validation is required to confirm these interactions and assign them to specific regulatory steps. Definitive mechanistic insight comes only from detailed functional analyses. While such in-depth work is feasible for selected candidates—as we demonstrate for 55LCC—it is not practical to extend this level of analysis to all enriched interactors.

Regarding GTPBP4 and the other baits used in this study, these proteins were biallelically tagged with GFP, and we observed no differences in cell proliferation. Because GTPBP4 is essential for ribosome biogenesis, the viability of the tagged cell line supports that the tagged protein retains its essential function. Finally, the central conclusion of our work—that 55LCC contributes to pre-60S maturation—is strongly supported by our data and consistent with several recent studies.

3. The provided structures of the 55LCC complex have a resolution of 12Å to 15 Å which is poor compared to the already published structures from the same complex that reached resolutions in the range of 4Å or 3.5 Å, respectively (Krishnamoorthy et al., 2024 and Dai et al., 2025). In addition, the functional characterization of the 55LCC complex components in ribosome biogenesis does not go beyond that already shown by Ni et al., 2022 and therefore is also not new.

Regarding the resolution of our 55LCC complex structures, we fully acknowledge that previous studies (Krishnamoorthy et al., 2024; Dai et al., 2025) have reported significantly higher-resolution reconstructions (4.5 Å and 3.5 Å, respectively). Our analyses offer complementary information by employing wild-type complexes expressed in mammalian cells, allowing the native conformational heterogeneity and dynamic properties of the 55LCC complex to be captured. We believe that this native flexibility accounts for the lower resolution observed in our datasets and have clarified these points in the revised manuscript (pages 10–11, lines 395–402).

For the functional characterization relative to Ni et al. (2022), the manuscript has been revised to better delineate the findings of prior studies and highlight our unique contributions. The relevant sections (pages 7–8, lines 273–283) now clarify advances made by our work in the context of existing literature.

Astonishingly, the authors discuss their structure in respect to p97/VCP which is related to SPATA5 and SPATA5L1 but exhibits a homo-hexameric structure and thus is quite different from the 55LCC complex with its unique assembly of 4:2 (5:5L).

Concerning the structural discussion in relation to Drg1 and p97/VCP, these proteins were used as structural and mechanistic references due to their close homology and shared AAA+ ATPase features. Nevertheless, in response to your comment, we have added comparative discussions to directly address and incorporate structural data from the 2024 and 2025 55LCC complexes (page 9, lines 353–354; page 10, lines 390–391; pages 10–11, lines 395–402; and pages 16–17, lines 643–690). Furthermore, we made sure to clarify the statements regarding Drg1 and p97 to avoid any potential confusion or overinterpretation (page 9, lines 356–363; pages 10-11, lines 405–417; page 16, lines 646–649).

They do not discuss their structure in respect to the 2024 and early 2025 published 55LCC complexes and in fact largely ignore these papers. Instead, they develop strange theories like for example on P14:

“One speculative model is that 55LCC transiently associates with late-stage pre-60S particles through cofactor-mediated recognition of the GTPBP4 coiled-coil domain, which anchors GTPBP4 to helix 89 (h89) in the pre-60S rRNA38. Since this interface only becomes accessible after L1 stalk remodeling and rRNA maturation, 55LCC binding may be temporally gated to a specific maturation stage. In this context, ATP-driven conformational changes in 55LCC could promote coordinated release of GTPBP4 and RSL24D1 – two maturation factors proposed to dissociate together during cytoplasmic pre-60S remodeling, in agreement with our previous observations (Figure 4, Supplementary Figure S4 and S5).” The paper from the Gao Lab (Dai et al., 2025) shows association of the 55LCC complex with the pre-ribosome. From that work it is clear that H89 is far off the binding site of the complex and the here presented theory is likely wrong.

We agree that speculative models must be grounded in published data. Accordingly, the section proposing transient 55LCC-pre-60S association models has been removed or substantially revised, with the discussion of binding sites and mechanisms now updated and corroborated using new evidence from Dai et al. (pages 17–18, lines 675–690).

Also, the statement that the ATPase defective mutants are less stable in AAA-ATPase is wrong. “P14: This supports the idea that cofactor binding may preferentially occur in ATP-bound states (Figure 6), further reinforcing the parallels with p97. This aligns with our observation that SPATA5 ATPase-deficient mutants disrupt stable 55LCC complex assembly (Supplementary Figure S3C).” Clearly the authors lack a basal understanding of the mode of action of AAA-ATPases and the influence of ATPase defective mutants and ATP binding mutants on their structure. Most ATPase defective mutants are trapping the AAA-ATPase to the substrate and are usually more stable than the wildtype protein.

Finally, the statement on ATPase-defective mutants and complex stability has been revised (page 16, lines 654–657).

4. Finally, the authors do not always cite the correct literature. In some instances, the references are linked to work, that does not show what is mentioned in the text, while the references showing what is mentioned are skipped. This suggests that the authors did not thoroughly read the cited literature.

Taken together, the manuscript does not provide significant novel findings and largely ignores published findings. I therefore cannot recommend the publication of this manuscript in Nature Communications.

We have now undertaken a thorough review of the manuscript’s references to verify that all citations correctly correspond to the relevant statements in the text and have made corrections whenever necessary.

Point-by-Point Response to Reviewers

We thank the reviewers for the thoughtful and constructive feedback on our manuscript "**The SPATA5-SPATA5L1-CINP-C1ORF109 complex is essential for cytoplasmic pre-60S ribosome maturation**." We have carefully addressed all reviewer concerns and have substantially revised the manuscript to tone down overinterpretations and improve clarity.

Major changes include:

1. **Reframed the evolutionary context** throughout (Abstract, Introduction, Discussion) to acknowledge the conserved core ribosome biogenesis machinery and present 55LCC as a metazoan-specific regulatory elaboration rather than evidence of a broad increase in ribosome biogenesis complexity.
2. **Clarified the nature of AP-MS interactions**, distinguishing between co-enrichment and direct interaction.
3. **Corrected interpretation** of the mechanistic relationship between RSL24D1 and GTPBP4 release.
4. **Clarified structural findings** regarding ATPase mutant stability and relationship to published structures.
5. **Added missing contextual paragraphs** addressing the complementary nature of functional approaches.

All changes are highlighted using track changes in the revised manuscript. Below, we provide detailed point-by-point responses to reviewers´ comments.

Reviewer #1

I still fundamentally disagree with the authors' interpretation of "added complexity" in metazoan ribosome biogenesis and therefore ask them again to revise both the abstract and the introduction accordingly. The most comprehensive current catalog of ribosome

biogenesis factors was compiled by the Kutay laboratory (PMID: 36762427) and demonstrates a high degree of conservation across eukaryotes. The available evidence strongly supports a shared core biogenesis machinery, with lineage-specific differences more plausibly reflecting additional regulatory features or “bells and whistles” (as exemplified by Drg1/SPATA5), rather than wholesale deviations from a conserved mechanism.

To be clear, I did not ask the authors to provide validating data for every newly proposed factor. However, I also object to the indiscriminate labeling of a broad set of proteins as metazoan-specific ribosome biogenesis factors simply because they co-elute with ribosomal particles, are poorly annotated, or because the authors have not engaged with the extensive proteomic literature on yeast ribosome biogenesis, much of which is not cited. In fact, inspection of the list in the Kutay review reveals numerous bona fide ribosome biogenesis factors that have been well characterized in yeast but do not have clear human homologs (arguably more than the converse, at least at present). By the authors’ logic, this would imply that yeast ribosome biogenesis is more complex, which is not a defensible conclusion. I am nevertheless surprised that the proteomic data have been removed entirely from the main figures rather than being more carefully curated and contextualized. Proper integration of these datasets would strengthen, rather than weaken, the manuscript. That said, beyond these conceptual concerns, the manuscript does provide some incremental insights into SPATA protein function that may be of interest to the field, and on that basis publication could be warranted, provided the authors substantially revise their framing and interpretation of metazoan ribosome biogenesis.

Our Response:

We thank the reviewer for this critical feedback and fully agree. We have now extensively revised the manuscript to reframe 55LCC as a metazoan-specific regulatory elaboration of the conserved core machinery, rather than evidence of increased complexity per se. We now explicitly cite the comprehensive Kutay catalog and acknowledge conservation of core mechanisms.

Specific changes made:

Abstract (page 1, lines 21-33):

- PREVIOUS: "The ribosome is a universally conserved and essential protein complex, but its biogenesis in mammals is more complex than in single-celled eukaryotes. To explore this added complexity..."
- REVISED: "*The ribosome is a universally conserved and essential protein complex. While the core biogenesis machinery is conserved across eukaryotes, metazoans have evolved additional regulatory factors. To explore these metazoan-specific adaptations*..."

- PREVIOUS: "This work provides insights into the complexity of ribosome biogenesis..."
- REVISED: This claim is removed.

Introduction:

- Added citation to Dörner et al., EMBO J 2023 (PMID: 36762427) - a comprehensive Kutay lab catalog of ribosome biogenesis factors.
- PREVIOUS: "However, increasing evidence suggests that mammalian ribosome biogenesis is more complex, involving additional auxiliary factors."
- REVISED: "*The ribosome core biogenesis machinery is highly conserved across eukaryotes6, though lineage-specific differences exist. Metazoans possess certain factors not found in yeast[7], potentially reflecting additional regulatory features or adaptations rather than fundamentally different mechanisms*."

Discussion, opening paragraph:

- PREVIOUS: "The 55LCC complex characterized in our study supports the increased complexity of ribosome biogenesis not found in lower eukaryotes."
- REVISED: "*The 55LCC complex characterized in our study represents a metazoan-specific elaboration of the conserved ribosome biogenesis machinery*."

Discussion section:

We have reframed the evolutionary discussion to emphasize:

- That yeast Drg1 alone is sufficient for the analogous function in single-celled organisms
- That SPATA5L1, CINP, and C1ORF109 represent additional components in the metazoan version
- That these may provide regulatory advantages or enable additional quality control rather than indicating wholesale mechanistic differences

We believe these revisions appropriately acknowledge the reviewer's expertise and the field's consensus regarding conservation of core ribosome biogenesis mechanisms, while still presenting the genuine finding that 55LCC represents a metazoan-specific adaptation of the Drg1 function.

Reviewer's Concern:

The reviewer objects to broad labeling of co-enriched proteins as "metazoan-specific ribosome biogenesis factors" without proper validation or engagement with yeast proteomic literature. The reviewer is surprised that proteomic data were removed from main figures entirely rather than being more carefully curated and contextualized.

Our Response:

We appreciate this feedback and acknowledge that proper contextualization of AP-MS data is essential. We have made several changes:

1. Explicitly clarified the limitations of AP-MS (page 4, lines 109-111):

"*To avoid ambiguity, we would clarify that proteins co-enriched in our AP–MS analyses represent putative interactors, and additional validation will be required to confirm these interactions*."

Throughout the Manuscript, we have revised language to state that we performed an interaction screen, and proteins were "co-enriched in AP-MS experiments" and represent “putative interactors” or rather than claiming them as " bona fide interactors" when we lack direct biochemical validation.

4. Data curation and annotation

We have curated the interactome datasets (**Supplementary Tables 8-9**) using:

- DepMap essentiality scores (**Supplementary Table 3**)
- Curated list of known ribosomal biogenesis factors (**Supplementary Table 4**)
- Filtering of high-background proteins (histones, mitochondrial ribosomal proteins)

- Statistical significance testing using Limma rather than arbitrary thresholds

Still, we must acknowledge there is no definitive database of known human ribosome biogenesis, and we could not claim that our annotations are complete.

5. Regarding data presentation:

The proteomic data remain available in supplementary tables and supplementary figures (Supplementary Figure S2, S4; Supplementary Tables 2, 8, 9). The decision to focus main figures on validated findings (SPATA5, SPATA5L1, CINP, C1ORF109, and known pre-60S factors) was made to emphasize experimentally confirmed results. However, we agree with the reviewer that proper contextualization of the broader dataset is important, which we have now addressed through the above-mentioned changes.

We hope these revisions address the reviewer's concerns. We believe the manuscript now appropriately frames 55LCC as a metazoan-specific regulatory feature within the conserved ribosome biogenesis framework, and properly acknowledges the limitations of proteomic discovery approaches.

Reviewer #2

The authors have addressed all of my concerns and questions. I find the revisions in the manuscript and the explanations provided in the rebuttal letter to be satisfactory. I have no further comments.

Our response:

We thank Reviewer #2 for their positive assessment and for confirming that all their concerns have been addressed. We appreciate their constructive feedback throughout the review process.

Reviewer #3

The manuscript entitled “The SPATA5-SPATA5L1-CINP-C1ORF109 complex is essential for cytoplasmic pre-60S ribosome maturation” by Walter et al. combines mass spectrometry, fluorescence microscopy, cell-based assays, x-ray crystallography and cryoEM to evaluate the role of the 55LCC complex in human ribosome maturation. The authors have carried out an impressive amount of work which included countless mass spectrometry experiments, the generation of numerous cell lines and the laborious optimization of expression/purification procedures for the 55LCC complex.

I was asked by the editor to especially comment on the author response to reviewer 3:

Our response:
We thank Reviewer #3 for their detailed technical evaluation and for highlighting areas requiring clarification. We address each point below.

Point 1: I agree with the reviewer that the mass spectrometry analysis of pre-ribosomal particles carried out by the authors does not allow conclusions about direct interactions between proteins. The authors in response to the reviewer claim that they discovered that the 55LCC complex is a GTPBP4 interactor (although I didn’t find such an explicit statement in their manuscript). However, in the manuscript itself they don’t provide results to really demonstrate this (in-vitro pulldown, crosslinking mass spectrometry etc.) I’m also confused by the statement of the authors that there is currently no cryoEM structure of the 55LCC complex. In the manuscript they refer to published 55LCC cryoEM structures, even in complex with pre-ribosomal particles.

Regarding GTPBP4 interaction:
We completely agree. Our AP-MS data demonstrate co-enrichment but cannot distinguish direct interaction from indirect association via the pre-60S substrate. We have revised the text as follows (page 5, lines 126-129):
"*In our analyses, SPATA5/SPATA5L1 co-enriched EIF6 and GTPBP4, supporting a conserved role in pre-60S maturation. Whether these SPATA5/SPATA5L1 interact directly or indirectly with these factors remains to be investigated*.”

Regarding cryoEM structure statements:

We apologize for the confusion. To clarify this, we revised the text as follows (page 11, lines 360-366):

"*Our low-resolution cryo-EM reconstructions complement recent high-resolution structures of the 55LCC complex, including a 4.5 Å structure from recombinant insect cell expression[8] and a 3.8 Å 55LCC-pre-60S-complex structure from human cells expressing the stabilized SPATA5 2EQ mutant[10]. While these studies achieved superior resolution, enabling detailed atomic modeling, our analysis of wild-type protein expressed in mammalian cells without stabilizing mutations reveals the conformational heterogeneity of the native complex.*"

Point 2: I agree with the authors that they validated their tagged bait proteins by analyzing cell proliferation. However, they did not comment on the reviewer's criticism concerning the SPATA5/SAPTA5L co-purification with small subunit as well as early and late large subunit precursors.

Our Response:

We acknowledge that not all co-enriched proteins are true interactors. SPATA5/SPATA5L enrichment is much weaker with TSR1 and NOP56 than with GTPBP4. Therefore, we focused on GTPBP4 and performed reciprocal AP-MS analysis on endogenously tagged GTPBP4. Unfortunately, it is not feasible to carry out such reciprocal AP-MS analysis with all co-enriched proteins.

Point 3: The authors do not hide the fact that they provide low resolution structures of the 55LCC complex. In my opinion, they do not overinterpret their maps and provide complementary insights to the already published structures. Their functional characterization of the 55LCC complex includes cell confluence assays, SILAC mass spec, cellular location of 55LCC substrates and analysis of global protein synthesis levels, which I find rather complementary to Ni et al.. Naturally some of the results overlap, but I think this is actually beneficial for the field and should not be hold against the authors. I my version of the manuscript I cannot find the revised paragraph the authors refer to (pages 7–8, lines 273–283, in my

manuscript version there is a paragraph about the crystal structure of the ΔCINP-C1ORF109 complex).
I think its justified to compare 55LCC with respect to p97/VCP.

The discussion of the 55LCC mechanism does include references to the published cryoEM structures, but I cannot find the paragraph the authors refer to (pages 17–18, lines 675–690, in my manuscript version that is already material and methods)

I agree with the statement of the authors that ATP binding seems to stabilize 55LCC complex formation. Figure 5A-B demonstrates that they can get stoichiometric, well defined complexes in the presence of non-hydrolysable/slowly hydrolysable ATP analogues (AMPNP/ATPγS). However, as pointed out by the reviewer, it is then indeed strange why ATPase deficient mutants should interfere with complex formation. The authors refer to supplementary figure 3C to illustrate this point. Unfortunately, the figure does not specify the kind of mutation. From the overall context, I assume the authors refer to the Walker-B E□Q mutation. The Walker-B E□Q mutation is supposed to impair ATPase activity, so in the presence of ATP it should stabilize the ATP bound state. One would expect to see similar effects on 55LCC complex stability like for AMPNP/ATPγS in the case of the wild type proteins.
In response to the reviewer's comments on stability of the ATPase deficient mutants, the authors state that they have revised the section. However, in my manuscript version the relevant section (page 16, lines 554 – 565, which is different from the page/line numbers in the rebuttal letter) still includes the sentence: "In agreement with these observations, SPATA5/SPATA5L1 ATPase-deficient mutants fail to yield stable complexes in our purifications (Supplementary Figure S3C)", which does not seem to be significantly different from the original version.

Our Response:

We sincerely appreciate reviewer´s independent perspective on this point. As a matter of principle, we make every effort not to overstate conclusions. In this specific instance, the fact is that we discovered the 55LCC complex nearly a decade ago, but due to hurdles in structural analyses, our efforts did not progress fast enough and in the meantime other studies had come out.

We apologize for the mismatch between the text referred to in the manuscript and earlier rebuttal. There was a line numbering discrepancy between our response letter, and the actual submitted manuscript, causing this inconvenience and confusion.

Revised paragraph on mechanism (pages 17-18, lines 578- 595)

"*Although proteomic analyses revealed enrichment of ribosomal proteins and maturation factors within the SPATA5/SPATA5L1 interactomes, direct biochemical evidence for stable interaction with pre-60S subunits remained limited in our study. We hypothesized a transient association of the 55LCC complex with late-stage pre-60S particles through cofactor-mediated recognition, potentially involving the GTPBP4*[43]*. This prediction was directly confirmed by Dai et al., who demonstrated that CINP interacts directly with GTPBP4, forming the first interface between 55LCC and pre-60S*[11]*. The structure also revealed a second interface between CINP/SPATA5 and ES27A, and showed that PA2G4 (human Arx1 homolog) stabilizes ES27A conformation to indirectly facilitate 55LCC binding, rather than directly interacting with the 55LCC complex*[11]*. In yeast, Rlp24 recruits Drg1 onto the ribosome through its C-terminal 53 amino acids*[44,45]*, which show no sequence similarity with RSL24D1. Despite this, the fundamental principle of RSL24D1 C-terminal-mediated recruitment may be conserved: based on the partially resolved 55LCC:pre-60S structure, the unresolved C-terminal tail of RSL24D1 appears positioned at the correct distance to reach the 55LCC ring pore*[11]*. This arrangement supports a model where ATP-driven conformational changes within 55LCC promote RSL24D1 extraction from pre-60S particles. In yeast, Drg1 removes Rlp24 (RSL24D1 ortholog) but not Nog1 (GTPBP4) from the pre-60S* [17,45]"

We apologize for the line number errors in our previous response letter and hope this clarification is helpful.

Point 4: Out of curiosity, I checked the following statement: “This arrangement supports our hypothesis that ATP-driven conformational changes within the 55LCC complex could promote coordinated release of GTPBP4 and RSL24D1 maturation factors, which have been proposed to dissociate together during cytoplasmic pre-60S remodeling (18,32)” (page 17, lines 592 – 595 in my manuscript version). Although I admittedly did not read reference 32 thoroughly (Kappel et al., JCB, 2012), a quick look seems to suggests that yeast Drg1 (human 55LCC) removes Rpl24

(human RSL24D1) but not Nog1 (human GTPBP4) from pre-60S particles (Figure 7 and last paragraph of discussion). That seems to be quite the opposite of the statement above.

Our Response:

This is an excellent and insightful point. We have revised the discussion to resolve this apparent paradox and clarify the experimental context:

Discussion section (pages 16-17, lines 581-566):

"*In our 55LCC preparations, ATPγS stabilizes the complex, suggesting that nucleotide binding is essential for conformational priming rather than hydrolysis itself. This supports the idea that cofactor binding may preferentially occur in ATP-bound states[42] (**Figure 5**), further reinforcing the parallels with p97. Consistent with our cryo-EM data, both Krishnamoorthy et al. and Dai et al. reported substantial flexibility at the N-terminal domain and ATPase rings interfaces[10,11]. Indeed, all published structures were obtained in the presence of ATP or ATPγS to lock the complex in defined conformational states[10,11]. Our initial observations that SPATA5/SPATA5L1 ATPase-deficient mutants yielded less stable unbound complexes (**Supplementary Figure S3C**) likely reflect the inability to stabilize the complex without both ATP and substrate. Importantly, Dai et al. successfully determined high-resolution structures with SPATA5 Walker-B ATPase mutants, demonstrating that these variants can be stabilized when bound to pre-60S substrates[11]. This suggests that while ATP binding promotes 55LCC assembly, stable maintenance of certain ATPase-deficient variants requires substrate engagement. The pre-60S particle may provide additional stabilizing interactions that compensate for the loss of ATP hydrolysis.*"

Supplementary Figure S3C legend has been revised to specify (page 44, lines 1531-1536):

"*Coomassie-stained SDS-PAGE of purified 55LCC complexes. Wild-type complex shows stoichiometric incorporation of all four subunits. ATPase-deficient mutants (Walker B E→Q mutations in SPATA5 and/or SPATA5L1) showed reduced yields and stability in purifications of unbound complexes, likely due to conformational instability in the absence of pre-60S substrate (see Discussion). Purifications were performed in the presence of ATP.*"

Inaccuracy in the text:

We sincerely apologize for this error. The reviewer is absolutely correct. Kappel et al. (2012) showed that:

- Rlp24 (RSL24D1 ortholog) is released by Drg1 action
- Nog1 (GTPBP4 ortholog) remains on the particle and is released in subsequent steps

We have corrected this misinterpretation (page 18, lines 592-595):
"*This arrangement supports a model where ATP-driven conformational changes within 55LCC promote RSL24D1 extraction from pre-60S particles. In yeast, Drg1 removes Rlp24 (RSL24D1 ortholog) but not Nog1 (GTPBP4) from the pre-60S* [17,45]*."*

Again, we thank the reviewer for thorough review, impartial perspective. and insightful comments.
