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Peer Review File

DEAD-box RNA helicase 10 is required for 18S rRNA maturation by controlling the release of U3 snoRNA from pre-rRNA in embryonic stem cells

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Wang et al. presents a mechanism by which DDX10 promotes ribosomal biogenesis and regulates pre-rRNA processing in mESCs. Although the functions of helicases in ribosome biogenesis have been extensively studied in the past, including DDX10 (Turner et al, Mol Cell, 2009; Wild et al, Plos Bio, 2010; Zhang MCB 2011), this study by Wang et al. is conceptually and technically novel as it extends the significance to a developmental system, provides an interesting approach with the use of inducible degron, and performs a nicely designed time course analysis of DDX10 depletion to show that the crosstalk between U3 and DDX10 is essential for ribosome biogenesis. They then define the localisation of the protein in the nucleolus and show that it can undergo phase separation in vitro and in cells, relying primarily on its IDR3. The article is well written and has a clear and simple structure, and the methods are also described with great attention to detail. The authors have done an impressive job that is already paving the way to new important insights that are crucial for understanding the dynamics of rRNA rewiring in early development. However, some aspects - especially mechanistic ones - need to be further unraveled to make this work suitable for publication. I would like to suggest that the authors be given a chance to revise this paper, as it has the potential to develop into an interesting story. Therefore, I invite the authors to consider the following points in further improving the manuscript.

Major points:

The authors are encouraged to further decipher the mechanism of U3 snoRNA release, which is even emphasised in the title. The only reference to this in the results section is the conclusion: “Interestingly, we noted that DDX10 degradation led to an enrichment of U3 within larger complexes, roughly corresponding to preribosomes”. There are still some open questions here, at least some of which could be clarified in this revision step:

- How come the authors see the accumulation of U3 within fractions corresponding to 80S, given that loss of DDX10 blocks the biogenesis of the 40S ribosomal subunit and thus subsequent hindrance of 80S ribosome assembly? This would imply that the retention of U3 along nascent pre-rRNA did not impair 80S ribosomal assembly (e.g. 80S ribosome assembles despite increased U3 association)..

- What do the authors mean by preribosomes? Preribosomes are usually intermediates of early 90S biogenesis that are formed along the nascent pre-rRNA transcript?

- Can the authors comment on how this work goes mechanistically beyond the yeast study done on Dbp4 (a mammalian 4 homolog of DDX10)?

- Does depletion of IDR3 impact U3 release from pre-rRNA?

IDR3 impacts localisation and pre-rRNA processing outcome. There might be potential explanations to further disentangle the cooporactivity between U3/pre-rRNA processing/DDX10. Does IDR3 alters the binding of DDX10 to U3? Could the authors provide evidence of direct binding of DDX10 to U3?

- Loss of DDX10 leads to 34S accumulation, hence the maturation of 18S is expected to be impaired. Methylene blue staining of mature 18S in Figure 3C doesn't confirm prominent changes in 18S maturation (which would be in line with 34S accumulation and block of SSU processing). Could the authors comment this?

- CLIP results indicate DDX10 binding to small nucleolar RNAs (snoRNAs), including U22. This is an important mechanistic insight that should be presented in the figure and not only in the sup table.

Depletion of DDX10 impairs rRNA processing and ribosomal biogensis; two processes tightly linked with correct nucleolar organisation. The authors are encouraged to further investigate and quantify changes in FC/DFC/GC nucleolar organisation upon rapid DDX10 depletion (<24hours IAA) and their rescue experiments with DDX10 mutants.

Did the authors observe the formation of nucleolar caps that emerge as a result of disrupted vectorial flows of the molecules from pre-rRNA synthesis/early processing sites to the sites of pre-ribosomal subunits assembly.

Authors also highlight defects in ribosome biosynthesis upon DDX10 depletion. I would encourage the authors to quantify translational ribosome production and consequently protein synthesis following DDX10 depletion (and possibly also rescue/mutants). I find this crucial for the manuscript that relies on the identification of mechanism/phenotype of impaired ribosome biogenesis.

Fold change enrichment of 5'ETS isoforms 47S/45S should be quantified with northern blot using probes upstream of A' and A0 instead of RT-qPCR in order to conclude "Overall, these results underscore the necessity of DDX10 in facilitating the processing of cleavage sites within the 5'ETS and ITS1 regions, particularly at sites A0, 1, and 2b"

While the existence of compensatory mechanisms to sustain pluripotency in the absence of DDX10 is of course possible (and their understanding is beyond the scope of this paper), the authors should decipher how can cell fate remain unaffected despite blocking the SSU processing & even PCA-cluster along with WT in the wash-off experiment. Could this be abrogated upon blocking p53 induction (TP53 KO)? p53 upregulation is an anticipated outcome of ribosomal biogenesis block.

For further evaluation during the subsequent revision the authors should provide the token to access deposited data: GSE232096, CRA011147. In addition, reviewers should be able to access the code used in this paper.

Minor points:

- Authors should indicate enriched DDX10 interactors (Fig. 3D) on a volcano plot.

- Provide WB quantification of degrdon system?

- Further disentangle the effect on pluripotency, especially how come cells exhibit characteristic features of primed cells e.g. flattened colonies, reduced proliferation without impacting the pluripotency expression programme?. WB in Exp Data Fig 1d even indicates increased level of NANOG without impacting general pluripotency factors - could the expression of naïve pluripotency programme be more extensively evaluated over the existing IAA RNAseq timecourse?

- Can the authors further elucidate the binding preference to mature 18S as opposed to expected 5'ETS as one would expect based on protein enrichment in DFC/GC in mouse or even PDFC in human cells (3'ETS binding?)? Binding to 18S is overinterpreted to the effect on pre-rRNA processing. Could the author also provide CLIP motif analysis to decipher the birnding enrichment to (mature) 18S instead of 5'ETS/3/ETS/ITS or along complete 47S/34S?

Could the IAA-triggered impeded proliferation be attributed solely to increased apoptotic rate?

- Tir1 could be named as OsTir1 that is use in schematic presentation 1e? Easier to follow Fig 1f.

- Some minor typos: "As anticipated, 6 DDX10 showcased co-localized with Fibrillarin"; "To scrutinize the propensity of DDX10 proteins form condensates"

- HEK cells are repeatedly mentioned in the methods section however I do not see any results carried out using HEK cells.

- Methods could be further improved with additional details, such as, What is the Concentration of the probe solution used for Northern blots? More info (time/concentration/salts) on LLPS in vitro experiments.

Reviewer #2 (Remarks to the Author):

In this article by Wang et al, the researchers investigated the role of DDX10 in 18s rRNA maturation in mouse embryonic stem cells. They find that nucleolar localized DDX10 helps release U3 snoRNA from pre-rRNA. In addition, they studied how the ability of DDX10 to form condensates is linked to its function in rRNA processing. This study revealed important roles for DDX10 in regulating

ribosome biogenesis in mESCs. The study is interesting. There are a few issues that need to be addressed to strengthen the conclusions of the paper.

Major points:

1. The paper used mESC clone morphology as a proxy for mESC state. However, the expression of OCT4, NANOG, and SOX2 didn't change. Therefore, it is unclear what the smaller and flatter mESC clones mean if they are not more differentiated (also, the authors need to quantify these morphology changes in addition to showing the images in Fig. 1g, 4h, and 5c). Is it just a defect in mESC adhesion upon Ddx10 degradation? In short, to use mESC morphology as a readout of DDX2 function they authors need to provide more rationale for it.

2. The conclusion that DDX10 degradation decreases U3 release is not well supported. The only supporting evidence is in Fig. 3g, where at IAA 48 h, there is more U3 associated with 80S. Does DDX10 directly bind to U3? Which functional domain of DDX10 is related to U3 release? Are any of the IDRs involved? Without these deeper mechanistic insights the authors cannot comfortably make the conclusion that DDX10 promotes U3 release.

3. I find the experiments related to DDX10 phase separation not strong. Do the authors think IDRs help DDX10 incorporate into the nucleolus, or self-associate? The authors need to be clearer on that since Fig. 4c is testing self-association, and Fig. 4f is testing incorporation into the nucleolus and they may not be the same thing.

4. I also find it interesting that the authors didn't test the helicase domain of DDX10 to see if it affects 18S rRNA processing since it should most likely affect it.

5. In addition, it is unclear what it means to have different FRAP recovery rates in different mutants (Fig. 4g). I find it perplexing that the authors argue that faster recovery is not good for the cell, since in other studies, people may find faster recovery good for reactions.

Minor points:

1. I will find it helpful for the authors to introduce rRNA processing in more detail in the introduction. For example, which larger rRNA gets processed into which small rRNAs, and which snoRNAs are involved, etc. These will help with later understanding the data related to rRNA processing.

2. Inserting AID and eGFP to Ddx10 seem to degrade DDX10 (Fig. 1f) compared to WT. Why is that and does it matter for the experiments?

3. Page 5, lines 4-7: did the authors aggregate all the timepoints to find DEGs? It is unclear in the paper.

4. In Fig. 2c, the DDX10 seems to overlap more with DFC than GC. Why do the authors think DDX10 overlaps similarly to DFC and GC?

5. 20S signal is very dim in Fig. 3c so it is unclear why the authors say it decreases.

6. Fig. 3d, e: the authors only confirmed the interaction of DDX10 with some SSU processome components but not any of the pre-ribosome associated proteins, why? It is also unclear why the authors tested the association of DDX10 with Fibrillarin but not NPM1, which came up in the proteomics data.

7. Page 9, line 8: should be nucleolar not nuclear localization.

8. Fig. 4e is curiously missing data for DDX10ΔIDR3-NLS, while Fig. 4g is missing DDX10ΔIDR3.

9. Fig. 4i: Why would DDX10ΔIDR3-NLS not rescue 18S rRNA processing? The authors need to explain.

10. Page 13, lines 7-9: “we determined that the NUP98-DDX10 fusion protein failed to restore the impaired ribosome biogenesis resulting from AID-mediated DDX10 degradation. This observation suggests that NUP98-DDX10 fusion disrupts ribosome biogenesis”. Just because NUP98-DDX10 fails to restore ribosome biogenesis doesn’t mean it disrupts ribosome biogenesis. DDX10 degradation already disrupted it in the first place. The authors need to rewrite this part to make sure logic is sound.

11. It is unclear what the last sentence means: “therefore, we propose genomic targeting of DDX10 may hold great promise as a therapeutic approach for the treatment of NUP98-DDX10 fusion AML.” What exactly does genomic targeting mean? Knocking out? Or localizing DDX10 to nucleolus?

Reviewer #3 (Remarks to the Author):

In the manuscript entitled "DEAD-box RNA helicase 10 is required for 18S rRNA maturation by controlling the release of U3 snoRNA from pre-rRNA in embryonic stem cells", Wang et al used a AID approach to knock down DDX10 in mouse ESCs and found that Ddx10 is important for the ribosome biogenesis and proliferation of mouse ESCs. They further demonstrated that Ddx10 localized to DFC and GC of nucleoli and 18S rRNA biogenesis is significantly blocked, potentially due to the failure of cleavage near U3 snoRNA binding site. They found DDX10 can undergo phase separation in vitro and dissected the IDRs that are important for DDX10 phase separation and localization. These regions are also important for the function of DDX10 in regulating ribosome biogenesis and cell proliferation. Finally, they show that NUP98-DDX10 fusion of AML does not rescue the defect in ribosome biogenesis caused by the degradation of DDX10 in mouse ESCs. The study is well executed with proper controls and techniques. The conclusion is mostly justified by the data. Overall, the study is of broad interest for researchers in stem cell, cancer and ribosome biogenesis fields. Therefore I support the publication of this manuscript. However, concerns below should be addressed:

1. In NUP98-DDX10 fusion related malignancies, one of the DDX10 gene is intact, and therefore it is not likely that ribosome biogenesis is affected. At least there is no evidence presented here or by others to show that ribosome biogenesis is affected. For this reason, the claim that "this deficiency (pre-rRNA processing) could potentially contribute to the development and progression of AML" lacks evidence and should be removed or further toned down. Similarly, the discussion related to NUP98-DDX10 should also be toned down, especially for the speculation on therapy in the last paragraph.

2. Previous studies have found that p53 pathway is linked to the activation of 2-cell genes. Are 2-cell genes activated in Ddx10 knockdown ESCs? Likewise, p21 and various apoptotic genes were shown as the targets of ESC cell cycle regulating miRNAs (e.g. miR-290 family), do authors find miRNA biogenesis dysregulated in Ddx10 knockdown ESCs?

3. Does the deletion of IDRs affect the precise localization of DDX10 in nucleolus sub structures (GC, FC, DFC)?

4. The overexpression level of HA-tagged NUP98-DDX10 and DDX10 needs to be evaluated by western blotting, so that equal amount of proteins are expressed for both cell lines. In addition, a few previously identified targets of NUP98-DDX10 should be checked to make sure that sufficient level of NUP98-DDX10 is overexpressed.

5. The claim "the activation of p53 signaling pathway by DDX10 degradation may hinge on MDM2" is possibly right, but not because "DDX10 degradation results in an upregulation of Mdm2", likely due to the binding of free RPL11 or RPL5 to MDM2.

6. The details for washoff experiment should be described in methods.

Point-by-Point Response to the Reviewers' Comments

Response to reviewers' comments

We sincerely thank all reviewers for their insightful comments and constructive suggestions. Following the reviewers' comments and suggestions, we conducted additional experiments and data analyses to address the reviewers' concerns. Our point-by-point responses to the reviewers' concerns are listed below.

REVIEWER COMMENTS

Reviewer #1：

The manuscript by Wang et al. presents a mechanism by which DDX10 promotes ribosomal biogenesis and regulates pre-rRNA processing in mESCs. Although the functions of helicases in ribosome biogenesis have been extensively studied in the past, including DDX10 (Turner et al, Mol Cell, 2009; Wild et al, Plos Bio, 2010; Zhang MCB 2011), this study by Wang et al. is conceptually and technically novel as it extends the significance to a developmental system, provides an interesting approach with the use of inducible degron, and performs a nicely designed time course analysis of DDX10 depletion to show that the crosstalk between U3 and DDX10 is essential for ribosome biogenesis. They then define the localisation of the protein in the nucleolus and show that it can undergo phase separation in vitro and in cells, relying primarily on its IDR3. The article is well written and has a clear and simple structure, and the methods are also described with great attention to detail. The authors have done an impressive job that is already paving the way to new important insights that are crucial for understanding the dynamics of rRNA rewiring in early development. However, some aspects - especially mechanistic ones - need to be further unraveled to make this work suitable for publication. I would like to suggest that the authors be given a chance to revise this paper, as it has the potential to develop into an interesting story. Therefore, I invite the authors to consider the following points in further improving the manuscript.

Response: We thank Reviewer#1 for the positive comments on the importance of this work. By following the Reviewer#1's comments, we have performed more experiments and bioinformatic analysis to address the Reviewer's concerns.

Major points:

The authors are encouraged to further decipher the mechanism of U3 snoRNA release, which is even emphasised in the title. The only reference to this in the results section is the conclusion: "Interestingly, we noted that DDX10 degradation led to an enrichment of U3 within larger complexes, roughly corresponding to preribosomes". There are still some open questions here, at least some of which could be clarified in this revision step:

1. How come the authors see the accumulation of U3 within fractions corresponding to 80S, given that loss of DDX10 blocks the biogenesis of the 40S ribosomal subunit and thus subsequent hindrance of 80S ribosome assembly? This would imply that the retention of U3 along nascent pre-rRNA did not impair 80S ribosomal assembly (e.g. 80S ribosome assembles despite increased U3 association).

Response: From the experiments of the polysome profile in **Fig. 3a** and the Northern blot in **Fig. 3g** in origin manuscript, we observed that DDX10 degradation led to the accumulation of U3 in fractions around 60-80S, which corresponds to preribosomes (such as the 90S/SSU processome) according to the molecular mass[1], so we think that DDX10 degradation led to an accumulation of U3 in the preribosomes rather than 80S ribosome. In order to more intuitively reflect whether DDX10 degradation inhibits the release of U3 from preribosomes, we used a new experimental method to analyze the distribution of U3 in preribosomes. We isolated the nuclei and performed sucrose density gradient centrifugation to separate and collect the preribosome fractions, and then analyzed the distribution of U3 in each fraction by Northern blot. The results showed that DDX10 degradation led to increased enrichment of U3 in preribosomes. In addition, since the release of U3 from pre-rRNA is inhibited, the maturation of 18S rRNA is disrupted, resulting in a sharp decrease in the 40S subunit necessary for the formation of 80S ribosomes. Therefore, U3 does not directly bind to the 80S ribosome to affect its assembly, it reduces the assembly of 80S ribosome by affecting the generation of the 40S subunit. However, the obstruction of U3 release does not significantly impact the assemble of mature 40S and 60S subunits to form 80S ribosomes in cells.

2. What do the authors mean by preribosomes? Preribosomes are usually intermediates of early 90S biogenesis that are formed along the nascent pre-rRNA transcript?
Response: Preribosomes represent the 90S/SSU processome and the intermediates between 90S and pre-40S.

3. Can the authors comment on how this work goes mechanistically beyond the yeast study done on Dbp4 (a mammalian 4 homolog of DDX10)?
Response: In this study, we identified the RNA targets and subcellular localization of DDX10, elucidating that DDX10 mainly binds to pre-rRNA and localizes to the DFC and GC of the nucleolus. This provides direct evidence supporting the crucial role of DDX10 in ribosome biogenesis, which has not been studied in yeast. Loss of DDX10 (or Dbp4, a mammalian 4 homolog of DDX10) has different effects on ribosome biogenesis in mouse or yeast cells. In mESCs, loss of DDX10 led to a reduction in 40S subunits and an increase in free 60S subunits, consequently limiting 80S ribosome assembly. However, loss of Dbp4 in yeast resulted in a reduction of 40S subunits without affecting the level of 80S ribosomes and the mechanism still unexplained. In mESCs, DDX10 primarily regulates the processing and maturation of 18S rRNA by facilitating the release of U3 snoRNA from pre-rRNA, which is different in yeast showing that Dbp4 mainly regulates the release of U14 snoRNA with slight effect on U3 snoRNA.

4. Does depletion of IDR3 impact U3 release from pre-rRNA? IDR3 impacts localisation and pre-rRNA processing outcome. There might be potential explanations to further disentangle the cooporactivity between U3/pre-rRNA processing/DDX10. Does IDR3 alters the binding of DDX10 to U3? Could the authors provide evidence of direct binding of DDX10 to U3?
Response: By following the Reviewer#1's questions, we have performed more

experiments. We conducted RIP experiments to purify DDX10 bound RNAs, and Northern blot revealed that DDX10 indeed interacted with U3 snoRNA (**Fig. 4c**). However, since our DDX10 CLIP-seq could not detect U3, making it uncertain whether DDX10 could directly binds to U3 or not.

Furthermore, we investigated the impact of deleting IDR3 on the interaction between DDX10 and U3. Our results indicate a weakened interaction between DDX10 and U3 after deletion of IDR3 (**Fig. R1**). To investigate whether IDR3 deficiency affects the release of U3 from pre-rRNA, we isolated preribosomes and collected different fractions by sucrose density gradient centrifugation. Subsequently, RNAs were extracted from each fraction to detect the distribution of U3 in all fractions by Northern blot. The results showed that neither DDX10$_{\Delta IDR3}$ nor DDX10$_{\Delta IDR3}$-NLS could restore the blocked U3 release caused by endogenous DDX10 degradation (**Fig. 5j**), indicating that IDR3 is essential for DDX10 to regulate the release of U3 from pre-rRNA.

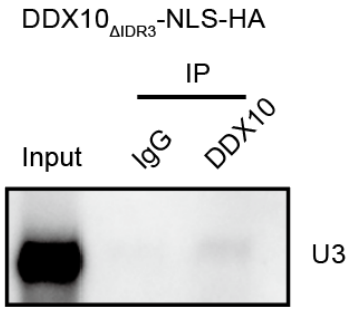

Fig. R1 Northern blot analysis of the interaction between DDX10$_{\Delta IDR3}$-NLS and U3 snoRNA.

5. Loss of DDX10 leads to 34S accumulation, hence the maturation of 18S is expected to be impaired. Methylene blue staining of mature 18S in Figure 3C doesn't confirm prominent changes in 18S maturation (which would be in line with 34S accumulation and block of SSU processing). Could the authors comment this?

Response: We think this Reviewer misunderstood these data. From our original data shown in the manuscript (also shown in **Fig. R2**), it is clear that mature 18S rRNA becomes progressively less prominent, with thinner and lighter bands during IAA treatment. These results clearly demonstrate that DDX10 degradation leads to a significant reduction in the level of mature 18S rRNA, which is consistent with the observed accumulation of 34S rRNA following DDX10 degradation.

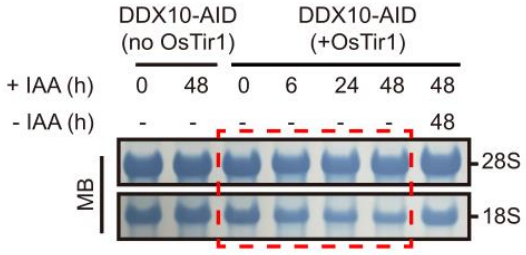

Fig. R2 Methylene blue staining showing the levels of both 28S and 18S rRNAs at different time points of IAA treatment.

6. CLIP results indicate DDX10 binding to small nucleolar RNAs (snoRNAs), including U22.

This is an important mechanistic insight that should be presented in the figure and not only in the sup table.

Response: We have followed the Reviewer#1's comments and marked DDX10-bound snoRNAs in **Fig. 2a** and presented the detailed information in **Supplementary table 2**.

7. Depletion of DDX10 impairs rRNA processing and ribosomal biogensis; two processes tightly linked with correct nucleolar organisation. The authors are encouraged to further investigate and quantify changes in FC/DFC/GC nucleolar organisation upon rapid DDX10 depletion (<24 hours IAA) and their rescue experiments with DDX10 mutants.

Response: By following the Reviewer#1's comments, we conducted transmission electron microscopy (TEM) experiments and observed that DDX10 degradation resulted in significant alterations in the nucleolar structure of FC/DFC/GC. Specifically, we noticed a marked loss of the characteristic structural components of FC, DFC, and GC at both 12 h and 24 h after IAA treatment (**Fig. 3d, e**). Furthermore, we also observed that overexpression of $DDX10_{FL}$ could restore the characteristic DFC/FC/GC structure of the nucleolus. but overexpression of either $DDX10_{\Delta IDR1}$, $DDX10_{\Delta IDR3}$ or $DDX10_{\Delta IDR3}$-NLS DDX10 could not (**Supplementary Fig. 4i. j**).

8. Did the authors observe the formation of nucleolar caps that emerge as a result of disrupted vectorial flows of the molecules from pre-rRNA synthesis/early processing sites to the sites of pre-ribosomal subunits assembly.

Response: We have followed the Reviewer#1's suggestion and further performed transmission electron microscopy (TEM) experiments. However, we did not observe the formation of nucleolar cap structures after DDX10 degradation (**Fig. 3d**).

9. Authors also highlight defects in ribosome biosynthesis upon DDX10 depletion. I would encourage the authors to quantify translational ribosome production and consequently protein synthesis following DDX10 depletion (and possibly also rescue/mutants). I find this crucial for the manuscript that relies on the identification of mechanism/phenotype of impaired ribosome biogenesis.

Response: We appreciate the Reviewer#1's valuable suggestion. Based on the reviewer's comments, we conducted the following experiments:

First, we quantified translating ribosomes after DDX10 degradation. By quantifying the peak area of polysomes representing active translating ribosomes, as well as polysomes and monosomes in polysome profiles, we found that DDX10 degradation resulted in a significant reduction in the proportion of these ribosomes. Overexpression of $DDX10_{FL}$ could rescue this defect, but $DDX10_{\Delta IDR3}$-NLS could not (**Fig. R3**).

Next, we assessed protein synthesis after DDX10 degradation by measuring the translational incorporation of HPG, an amino acid analog of methionine, into nascent protein. Our results showed that DDX10 degradation led to a severe impairment of protein synthesis, with nascent protein synthesis reduced by approximately 90% at 48 h after IAA treatment (**Fig. 3b, c**). Overexpression of $DDX10_{FL}$ could rescue the impaired protein synthesis, whereas neither $DDX10_{\Delta IDR1}$, $DDX10_{\Delta IDR3}$, nor $DDX10_{\Delta IDR3}$-NLS could not (**Supplementary Fig. 4g, h**).

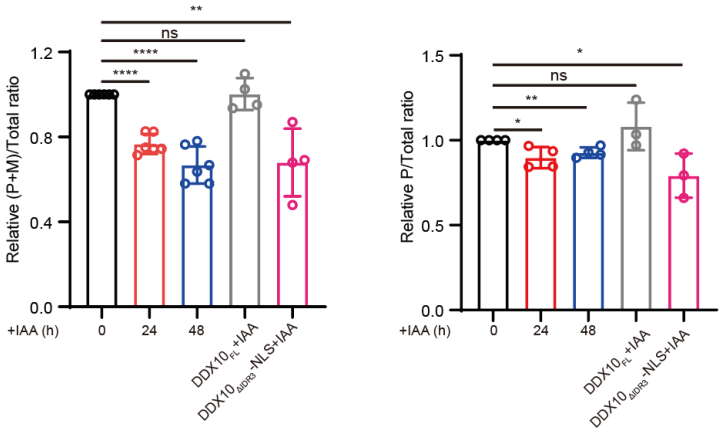

Fig. R3 Analysis of the changes in the ratio of the area under the curve (AUC) of polysomes and monomers to the total area ((P+M)/T) and the ratio of the AUC of polysomes to the total area (P/T) in polysome profiles.

10. Fold change enrichment of 5'ETS isoforms 47S/45S should be quantified with northern blot using probes upstream of A' and A0 instead of RT-qPCR in order to conclude "Overall, these results underscore the necessity of DDX10 in facilitating the processing of cleavage sites within the 5'ETS and ITS1 regions, particularly at sites A0, 1, and 2b"

Response: We have followed the Reviewer#1's suggestion and performed Northern blot experiments to quantify changes in 47S/45S rRNA using probes for the upstream of A' and A0 in the 5'ETS. Our findings showed that DDX10 degradation has no significant impact on 47S rRNA and 47S-45S rRNA (**Fig. 3f, g**). In addition, Northern blot results indicate that DDX10 degradation affects the processing of 34S rRNA to 18S rRNA, demonstrating that DDX10 is necessary for the cleavage of sites A0, 1, and 2b in the 5'ETS and ITS1, respectively. RT-qPCR experiments were conducted to validate the results obtained from our Northern blot experiments, further confirming that DDX10 degradation affects the cleavage sites within the 5'ETS and ITS1 regions.

11. While the existence of compensatory mechanisms to sustain pluripotency in the absence of DDX10 is of course possible (and their understanding is beyond the scope of this paper), the authors should decipher how can cell fate remain unaffected despite blocking the SSU processing & even PCA-cluster along with WT in the wash-off experiment. Could this be abrogated upon blocking p53 induction (TP53 KO)? p53 upregulation is an anticipated outcome of ribosomal biogenesis block.

Response: To answer this question, we have analyzed the RNA-seq data from primed (cultured in medium containing serum) and naive (cultured in medium containing 2i) mESCs[2], and integrated these data with our RNA-seq data. PCA results showed that mESCs following DDX10 degradation were neither close to the primed nor to the naive mESCs (**Supplementary Fig. 2a**). Additionally, we compared our RNA-seq data with the results from previously published 2-cell data[3] and observed that 2-cell specific genes, such as *Zscan4b* and *Zscan4d*, were significantly activated after 24 h of IAA treatment (**Supplementary Fig. 2e, f**). Together, these results indicate that DDX10 degradation promotes the transition of mESCs to 2CLCs. Therefore, we speculate that although the cell

state changes caused by short-term degradation of DDX10 are reversible, long-term degradation of DDX10 may lead to an irreversible transformation of mESCs into 2CLCs.

We knocked out *p53* in DDX10-AID (+OsTir1) mESCs and found that DDX10 degradation caused the cell clones to become smaller. However, they still maintained a convex clonal shape rather than becoming flattened, and the expression of pluripotency transcription factors (OCT4 and NANOG) did not change significantly after DDX10 degradation (**Fig. R4a-c**). Furthermore, we found that DDX10 degradation leads to significant activation of 2-cell specific gene expression, while this activation could be inhibited by *p53* knockout (**Fig. R4d**), suggesting that *p53* knockout can block the activation of 2-cell genes caused by DDX10 degradation.

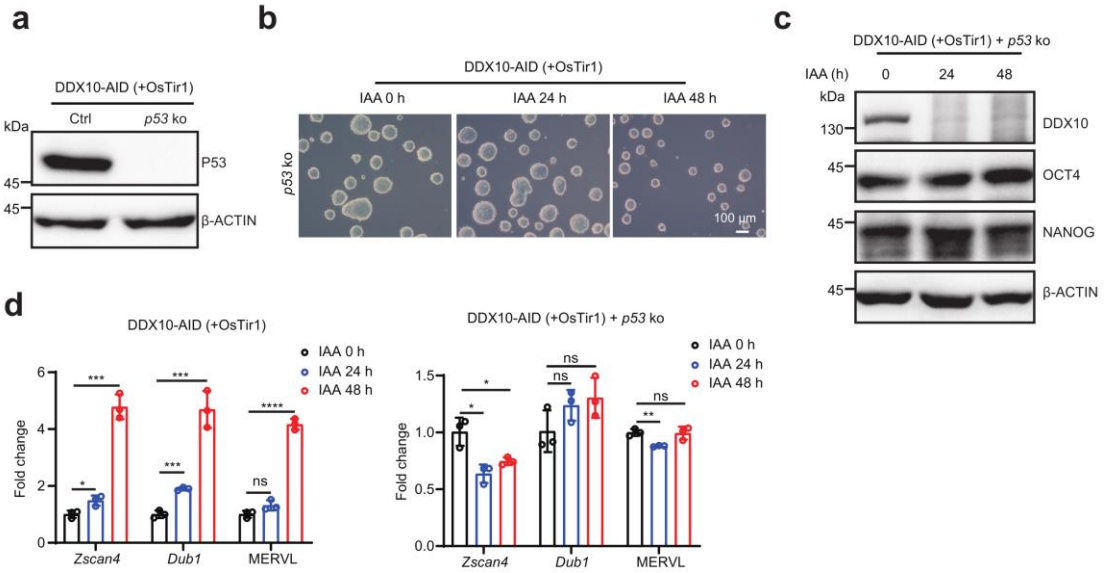

Fig. R4 *p53* knockout blocks the activation of 2-cell genes induced by DDX10 degradation. a, Western blot analysis of the knockout of P53. **b,** Bright-field images of *p53* knockout DDX10-AID (+OsTir1) mESCs treated with IAA for different time points (0 h, 24 h, and 48 h). Scale bar: 100 μm. **c,** Western blot showing the expression of pluripotency transcription factors OCT4 and NANOG in *p53* knockout DDX10-AID (+OsTir1) mESCs treated with IAA at different time points (0 h, 24 h, and 48 h). **d,** RT-qPCR analysis of the expression of 2 cell specific genes after DDX10 degradation in DDX10-AID (+OsTir1) and *p53* knockout DDX10-AID mESCs. Transcription levels were normalized against *Gapdh*. Data are shown as mean ± s.d., n=3. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

12. For further evaluation during the subsequent revision the authors should provide the token to access deposited data: GSE232096, CRA011147. In addition, reviewers should be able to access the code used in this paper.

Response: We have added the access token to the **Data availability** section by following the Reviewer#1's suggestion. Additionally, all code packages are publicly available.

Minor points:

1. Authors should indicate enriched DDX10 interactors (Fig. 3D) on a volcano plot.

Response: Since the DDX10-interactors identified by mass spectrometry are less important for this work, we decide to remove this data from the manuscript.

2. Provide WB quantification of degrdon system?
Response: We have conducted a quantitative analysis of the Western blot bands presented in **Fig.1e** by following the Reviewer#1's suggestion.

3. Further disentangle the effect on pluripotency, especially how come cells exhibit characteristic features of primed cells e.g. flattened colonies, reduced proliferation without impacting the pluripotency expression programme? WB in Exp Data Fig 1d even indicates increased level of NANOG without impacting general pluripotency factors - could the expression of naïve pluripotency programme be more extensively evaluated over the existing IAA RNAseq timecourse?
Response: Following the Reviewer#1's suggestion, we have analyzed the RNA-seq data from primed (cultured in medium containing serum) and naive (cultured in medium containing 2i) mESCs[2], and integrated these data with our RNA-seq data. PCA results showed that mESCs following DDX10 degradation were neither close to the primed nor to the naive mESCs (**Supplementary Fig. 2a**).

4. Can the authors further elucidate the binding preference to mature 18S as opposed to expected 5'ETS as one would expect based on protein enrichment in DFC/GC in mouse or even PDFC in human cells (3'ETS binding?)? Binding to 18S is overinterpreted to the effect on pre-rRNA processing. Could the author also provide CLIP motif analysis to decipher the binding enrichment to (mature) 18S instead of 5'ETS/3/ETS/ITS or along complete 47S/34S?
Response: Due to the very few peaks of DDX10 CLIP data, motif analysis failed to generate significant motifs. In fact, **Fig. 2b** clearly showed that DDX10 binds to 18S rRNA, instead of 5'ETS/3'ETS/ITS sequences.

As DDX10 located in the nucleolus, it is reasonable to conclude that DDX10 binds to 18S rRNA within the pre-rRNA, rather than the mature 18S rRNA, which is in the cytoplasm. Although DDX10 seems to not bind 5'ETS, our experimental results indicate that DDX10 plays a role in processing the cleavage site in the 5'ETS. DDX10 might regulates the processing and maturation of 18S rRNA through interacting with other complexes and U3.

5. Could the IAA-triggered impeded proliferation be attributed solely to increased apoptotic rate?
Response: Based on our data, DDX10 degradation arrested cell cycle at G1 (**Supplementary Fig. 1h, i**) and increased apoptosis (**Supplementary Fig. 1j, k**). Thus, we think that both arrested cell cycle and increased apoptosis are responsible for the impaired cell proliferation.

6. Tir1 could be named as OsTir1 that is use in schematic presentation 1e? Easier to follow Fig 1f.
Response: We have followed the Reviewer#1's suggestion and changed all "Tir1" to

"OsTir1" in the revised manuscript.

7. Some minor typos: “As anticipated, 6 DDX10 showcased co-localized with Fibrillarin”; “To scrutinize the propensity of DDX10 proteins form condensates”
Response: We have revised the sentences from "As anticipated, DDX10 showcased co-localized with Fibrillarin" to "As anticipated, DDX10 was co-localized with Fibrillarin" and replaced the sentence from "To scrutinize the propensity of DDX10 proteins form condensates" to "To scrutinize the propensity of DDX10 proteins to form condensates".

8. HEK cells are repeatedly mentioned in the methods section however I do not see any results carried out using HEK cells.
Response: We apologize for not clearly explaining the purpose of HEK293T cells in our original manuscript. HEK293T cells are primarily used as a tool to generate lentivirus supernatants. To avoid this confusion, we have added the following clarification to the **Plasmid Constructions** section of the **Methods** in the revised manuscript: "Lentivirus supernatants were generated with HEK293T cells. HEK293T cells were plated and cultured overnight. The medium of a 10 cm dish was replaced with 9 mL fresh culture medium, and then co-transfected with pSIN vector containing target genes together with the packaging plasmids pMD2.G and psPAX2 using polyethyleneimine (PEI; Polysciences, 24765–2). The culture medium was refreshed 12 h after transfection. After that, the supernatant from the transfected HEK293T cells was collected 48 h post-transfection using a syringe and filtered through a 0.45 μm filter.".

9. Methods could be further improved with additional details, such as, What is the Concentration of the probe solution used for Northern blots? More info (time/concentration/salts) on LLPS in vitro experiments.
Response: Based on the Reviewer#1's opinions, we have incorporated the following experimental details into the Methods section: "The biotin-labelled oligonucleotide probe (**20-40 pmol**) was added to the hybridization solution, and the membranes were incubated at 50°C overnight." And "The indicated protein at a final concentration of **0.5 μM** was added to droplet formation buffer (5% (w/v) PEG8000, 50 mM Tris-HCl (pH 7.5), **150 mM NaCl**, 10% glycerol and 1mM DTT). The reaction mixture was incubated at RT for **1 min** to allow for droplet formation, and then loaded into a custom slide chamber made from glass coverslips mounted on two parallel strips of double-sided tape on slides. Reactions were incubated for **5 min** in the imaging vessel to allow droplets in solution to settle on the glass imaging surface.".

Reviewer #2:

In this article by Wang et al, the researchers investigated the role of DDX10 in 18s rRNA maturation in mouse embryonic stem cells. They find that nucleolar localized DDX10 helps release U3 snoRNA from pre-rRNA. In addition, they studied how the ability of DDX10 to form condensates is linked to its function in rRNA processing. This study revealed important roles for DDX10 in regulating ribosome biogenesis in mESCs. The study is interesting. There are a few issues that need to be addressed to strengthen the conclusions of the paper.

Response: We thank the Reviewer#2 for his or her comments on our manuscript. We have performed more experiments to address the Reviewer's questions and comments.

Major points:

1. The paper used mESC clone morphology as a proxy for mESC state. However, the expression of OCT4, NANOG, and SOX2 didn't change. Therefore, it is unclear what the smaller and flatter mESC clones mean if they are not more differentiated (also, the authors need to quantify these morphology changes in addition to showing the images in Fig. 1g, 4h, and 5c). Is it just a defect in mESC adhesion upon Ddx10 degradation? In short, to use mESC morphology as a readout of DDX2 function they authors need to provide more rationale for it.

Response: To further elucidate the effect of DDX10 degradation on cell state, we have analyzed the RNA-seq data from primed mESCs (cultured in medium containing serum) and naive mESCs (cultured in medium containing 2i)[2], and integrated these data with our RNA-seq data. PCA results showed that mESCs following DDX10 degradation were neither close to the primed nor to the naive mESCs (**Supplementary Fig. 2a**) Additionally, comparing our RNA-seq data with previously published 2-cell data[3] revealed significant activation of 2-cell specific genes, such as *Zscan4b* and *Zscan4d*, after IAA treatment (**Supplementary Fig. 2e, f**). These findings collectively suggest that DDX10 degradation promotes the transition of mESCs to 2CLCs. In addition, we quantified the clonal area of mESCs in **Fig. 1g, 4h, and 5c** of the original manuscript. Our results showed that DDX10 degradation significantly reduced the clonal area of mESCs. Overexpression of $DDX10_{FL}$ could reverse the clonal changes caused by DDX10 degradation, whereas DDX10 truncations and NUP98-DDX10 could not (**Supplementary Fig. 1d, 4f, and Fig. 6d**).

2. The conclusion that DDX10 degradation decreases U3 release is not well supported. The only supporting evidence is in Fig. 3g, where at IAA 48 h, there is more U3 associated with 80S. Does DDX10 directly bind to U3? Which functional domain of DDX10 is related to U3 release? Are any of the IDRs involved? Without these deeper mechanistic insights the authors cannot comfortably make the conclusion that DDX10 promotes U3 release.

Response: By following the Reviewer#2's suggestions, we performed more experiments to further support our conclusion. First, we conducted RIP experiments to isolate DDX10-bound RNAs, and subsequent Northern blot analysis revealed that DDX10 indeed interacted with U3 snoRNA (**Fig. 4c**). However, since our DDX10 CLIP-seq could not detect U3, making it uncertain whether DDX10 could directly binds to U3 or not.

To investigate which functional domain of DDX10 is involved in U3 release, we

generated and overexpressed HA-tagged $DDX10_{FL}$ (full-length DDX10), $DDX10_{\Delta HBD}$ (DDX10 lacking helicase ATP binding domain), and $DDX10_{\Delta HCD}$ (DDX10 lacking helicase C-terminal domain) in DDX10-AID mESCs. Then we extracted preribosomes from these cell lines at 0 h and 48 h after IAA treatment, and then separated and collected preribosome fractions by sucrose density gradient centrifugation. Subsequently, the distribution of U3 in each fraction were analyzed by Northern blot. Our findings indicated that $DDX10_{FL}$ could restore the blocked U3 release caused by DDX10 degradation, while $DDX10_{\Delta HBD}$ and $DDX10_{\Delta HCD}$ could not (**Fig. 4f**), suggesting that both helicase ATP binding domain and helicase C-terminal domain of DDX10 are crucial for regulating U3 release.

Furthermore, we also explored whether IDR1 and IDR3 of DDX10 are involved in regulating the release of U3. Our findings demonstrated that $DDX10_{\Delta IDR1}$, $DDX10_{\Delta IDR3}$, and $DDX10_{\Delta IDR3}$-NLS could not rescue the obstruction of U3 release from pre-rRNA caused by DDX10 degradation (**Fig. 5j**), indicating that IDR1 and IDR3 of DDX10 are involved in regulating the release of U3. In conclusion, these additional experiments provide robust support our conclusion that DDX10 degradation hinders the release of U3 from pre-rRNA.

3. I find the experiments related to DDX10 phase separation not strong. Do the authors think IDRs help DDX10 incorporate into the nucleolus, or self-associate? The authors need to be clearer on that since Fig. 4c is testing self-association, and Fig. 4f is testing incorporation into the nucleolus and they may not be the same thing.

Response: I think Reviewer#2 might be misunderstood our data. From our results, we think that nuclear localization signal (NLS) within DDX10, rather than the IDRs, assists DDX10 in entering the nucleolus. Specifically, the loss of IDR3 results in DDX10 diffusing into both cytoplasm and nucleolus, whereas the addition of an NLS enables DDX10 to relocate into the nucleolus, indicating that the NLS is crucial for DDX10's entry into the nucleolus. Regarding the *in vitro* experiments, these were designed to evaluate the ability of full-length DDX10 and various IDRs deletion truncations to form droplets, and the effect of different IDRs on the self-association of DDX10. Our data indicated that IDRs are required for DDX10 to form condensates in the nucleolus, thereby promoting phase separation.

4. I also find it interesting that the authors didn't test the helicase domain of DDX10 to see if it affects 18S rRNA processing since it should most likely affect it.

Response: We followed Reviewer#2's comments and overexpressed HA-tagged $DDX10_{FL}$, $DDX10_{\Delta HBD}$, $DDX10_{\Delta HCD}$, and $DDX10_{\Delta HD}$ (DDX10 lacking both helicase ATP binding and helicase C-terminal domains) in DDX10-AID mESCs (**Supplementary Fig. 3g, h**). Our data indicated that $DDX10_{FL}$ could successfully restore the pre-rRNA processing defect caused by endogenous DDX10 degradation, while $DDX10_{\Delta HBD}$, $DDX10_{\Delta HCD}$, and $DDX10_{\Delta HD}$ could not (**Fig. 3h**), indicating that the complete helicase domain of DDX10 is indispensable for the processing and maturation of 18S rRNA.

5. In addition, it is unclear what it means to have different FRAP recovery rates in different mutants (Fig. 4g). I find it perplexing that the authors argue that faster recovery is not good for the cell, since in other studies, people may find faster recovery good for reactions.

Response: The recovery rates represent the mobility of different truncated and full-length

DDX10 proteins. As the Reviewer#2's mentioned, in some studies, faster recovery rates are attributed to cellular responses, but there are exceptions. For example, the ALS-associated TDP-43 mutation Q331K disrupts the liquid-liquid phase separation (LLPS) of TDP-43 and exhibits faster mobility. This mutation results in a decreased ability of TDP-43 to autoregulate its own expression, leading to elevated protein levels and consequently affecting RNA splicing[4, 5]. Similarly, we believe that the loss of IDR1 and IDR3 disrupt DDX10's phase separation, leading to increased mobility and consequently having adverse effects on the cells.

Minor points:

1. I will find it helpful for the authors to introduce rRNA processing in more detail in the introduction. For example, which larger rRNA gets processed into which small rRNAs, and which snoRNAs are involved, etc. These will help with later understanding the data related to rRNA processing.

Response: We sincerely thank Reviewer#2 for the valuable suggestion. To facilitate understanding of our findings on pre-rRNA processing for reviewers and readers, we have provided a comprehensive introduction to pre-rRNA processing and the associated snoRNAs in the revised manuscript. We added the following sentences into the Introduction as below: "Ribosomal genes are transcribed by RNA polymerase I (Pol I) to produce the primary 47S rRNA precursor, which includes two external transcribed spacers (5'ETS and 3'ETS) and two internal transcribed spacers (ITS1 and ITS2) separating the mature 18S, 5.8S, and 28S rRNAs. To obtain these mature rRNAs, the transcribed spacers must be removed through a sequential series of endonucleolytic and exonucleolytic cleavages. In mouse cells, the 47S rRNA transcript is first cleaved at site A0, generating the 46S rRNA, and then at site 6, producing the 45S rRNA. The processing of mouse 45S rRNA occurs primarily through two pathways. In pathway 1, sites A0 and 1 in the 5'ETS are successively cleaved successively to produce 43S and 41S rRNA. Subsequently, site 2c in ITS1 is cleaved to produce 20S rRNA (precursor of 18S rRNA) and 36S rRNA (precursor of 28S and 5.8S rRNA). In pathway 2, site 2c in ITS1 is firstly cleaved to produce 34S rRNA and 36S rRNA. Subsequently, sites A0 and 1 in 34S rRNA are cleaved in sequence to produce 20S rRNA. These rRNA precursors are ultimately processed into mature rRNA." and "Some small nucleolar RNAs (snoRNAs) serve as scaffolds during snoRNPs formation and base pairing with pre-rRNA to guide the directional cleavage and folding of pre-rRNA, which is crucial for rRNA maturation. U3, U14, U22, U17/snR30, and snR83 affect the maturation of 18S rRNA, while U8 snoRNA is essential for the accumulation of mature 5.8S and 28S rRNAs."

2. Inserting AID and eGFP to Ddx10 seem to degrade DDX10 (Fig. 1f) compared to WT. Why is that and does it matter for the experiments?

Response: Regarding the decrease in DDX10 protein levels, we believe this may be due to mESC clonal heterogeneity or potentially as a consequence of the gene editing. Notably, despite the observed lower protein expression following AID-eGFP insertion, our comprehensive analysis revealed no significant effect on mESC clonal morphology, cell cycle, or small ribosomal subunit biogenesis (**Fig.R5**).

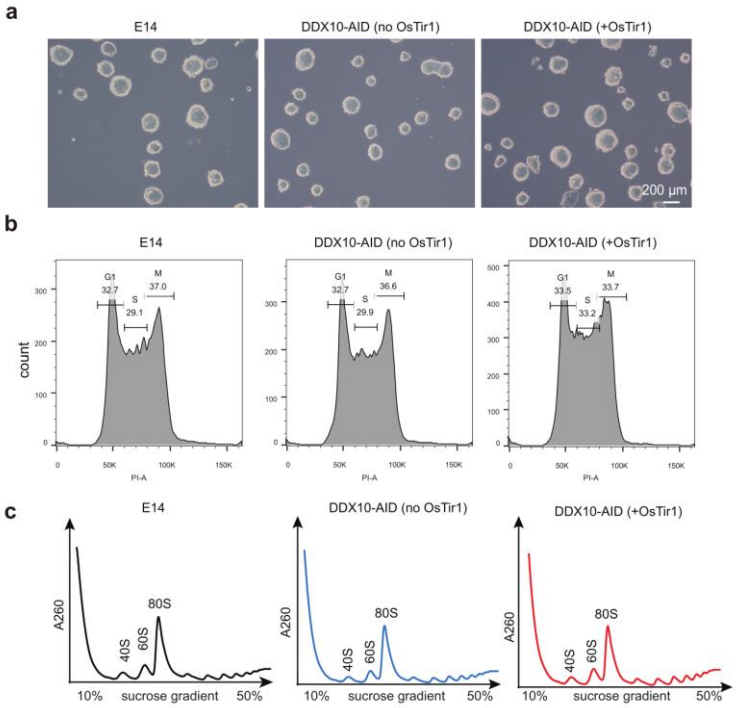

Fig. R5 a, Clonal morphology of wild-type E14 and DDX10-AID mESCs. Scale bar, 200 μm **b,** Cell cycle analysis of wild-type E14 and DDX10-AID mESCs. **c,** Polysome profiles analysis of wild-type E14 and DDX10-AID mESCs.

3. Page 5, lines 4-7: did the authors aggregate all the timepoints to find DEGs? It is unclear in the paper.

Response: We apologize for this confusion. In **Fig. 1h,** the DEGs were identified by comparing each time point of IAA treatment with the untreated cells. In **Fig. 1i**, we clustered the DEGs at all time points into 24 groups. To facilitate the understanding of readers and reviewers, we have revised the sentence from "we clustered these DEGs into 24 groups based on their expression patterns" to "we clustered the DEGs at all time points into 24 groups based on their expression patterns".

4. In Fig. 2c, the DDX10 seems to overlap more with DFC than GC. Why do the authors think DDX10 overlaps similarly to DFC and GC?

Response: We apologize for the inaccurate presentation of our results. We have revised the description of this result in the revised manuscript as follows: "Intriguingly, our data revealed that DDX10 overlaps more significantly with Fibrillarin than with NPM1, but not with RPA194".

5. 20S signal is very dim in Fig. 3c so it is unclear why the authors say it decreases.

Response: We are appreciated for the Reviewer's careful review. We apologize for the inaccurate presentation of our results. We agree with Reviewer#2's comment and changed the sentence from "coupled with a noteworthy reduction in both 20S and 18SE pre-rRNAs (other 18S rRNA precursors)" to "coupled with a noteworthy reduction in 18SE pre-rRNA (other 18S rRNA precursor)".

6. Fig. 3d, e: the authors only confirmed the interaction of DDX10 with some SSU processome components but not any of the pre-ribosome associated proteins, why? It is also unclear why the authors tested the association of DDX10 with Fibrillarin but not NPM1, which came up in the proteomics data.

Response: The SSU processome, also known as 90S preribosome, is an early assembly intermediate of the small ribosomal subunit. It undergoes structural changes to form the pre-40S ribosome, which is necessary for 18S rRNA maturation[6, 7]. Our results showed that DDX10 degradation disrupts 18S rRNA processing, thereby impairing 40S subunit biogenesis. Therefore, we focused on exploring the interaction between DDX10 and components of the SSU processome. Fibrillarin, a component of U3 snoRNPs, which is positioned at the center of the SSU processome to direct pre-rRNA folding and processing[8] and is required for the maturation of the 18S rRNA. Therefore, we examined the interaction of DDX10 and Fibrillarin.

7. Page 9, line 8: should be nucleolar not nuclear localization.

Response: We thank the Reviewer for pointing out this mistake. We have changed the "nuclear" to "nucleolar" in the revised manuscript.

8. Fig. 4e is curiously missing data for DDX10ΔIDR3-NLS, while Fig. 4g is missing DDX10ΔIDR3.

Response: Our data indicated that DDX10 could form droplets, and deletions of IDR1 and IDR3 weakened its droplet formation ability *in vitro*. Then, we further examined the effects of different IDR deletions on DDX10 in cells, and unexpectedly observed that deletion of IDR3 caused a significant alteration in the localization of DDX10 (diffused in both cytoplasm and nucleolus). This observation prompted us to speculated that IDR3 contains a key nuclear localization signal (NLS). To test this, we added a 7-amino acid NLS to the C-terminus of DDX10 lacking IDR3, and found that NLS could facilitate the relocation of DDX10 into the nucleolus.

Due to the fact that DDX10$_{\Delta IDR3}$-NLS only has 7 more amino acids than DDX10$_{\Delta IDR3}$, we did not conduct *in vitro* droplet formation experiments for DDX10$_{\Delta IDR3}$-NLS. Additionally, because FRAP experiments indicated that DDX10$_{\Delta IDR3}$ diffused in the cells, without forming distinct condensed areas, we did not show this data in the manuscript.

9. Fig. 4i: Why would DDX10ΔIDR3-NLS not rescue 18S rRNA processing? The authors need to explain.

Response: Although DDX10$_{\Delta IDR3}$-NLS, generated by adding an NLS to DDX10$_{\Delta IDR3}$, could relocate to the nucleolus, the loss of IDR3 significantly weakened the droplet formation ability of DDX10 *in vitro*. Therefore, we believe that DDX10$_{\Delta IDR3}$-NLS disrupts the phase separation of DDX10 and impairs DDX10 function. Consequently, DDX10$_{\Delta IDR3}$-NLS cannot restore the defect of 18S rRNA processing caused by endogenous DDX10 degradation.

10. Page 13, lines 7-9: “we determined that the NUP98-DDX10 fusion protein failed to restore the impaired ribosome biogenesis resulting from AID-mediated DDX10 degradation.

This observation suggests that NUP98-DDX10 fusion disrupts ribosome biogenesis". Just because NUP98-DDX10 fails to restore ribosome biogenesis doesn't mean it disrupts ribosome biogenesis. DDX10 degradation already disrupted it in the first place. The authors need to rewrite this part to make sure logic is sound.

Response: We apologize for the inaccurate statement in the original manuscript. In this revised version of manuscript, we have changed the sentence from "we determined that the NUP98-DDX10 fusion protein failed to restore the impaired ribosome biogenesis resulting from AID-mediated DDX10 degradation. This observation suggests that NUP98-DDX10 fusion disrupts ribosome biogenesis" to "we determined that NUP98-DDX10 fusion protein cannot participate in regulating ribosome biogenesis.".

11. It is unclear what the last sentence means: "therefore, we propose genomic targeting of DDX10 may hold great promise as a therapeutic approach for the treatment of NUP98-DDX10 fusion AML." What exactly does genomic targeting mean? Knocking out? Or localizing DDX10 to nucleolus?

Response: We apologize for this confusion. To better explain the above questions, we have rewritten this section as follows: "Therefore, targeted knockout or knockdown of *DDX10* in NUP98-DDX10 fusion AML cells may serve as a potential therapeutic approach for treating this type of AML".

Reviewer #3 (Remarks to the Author):

In the manuscript entitled "DEAD-box RNA helicase 10 is required for 18S rRNA maturation by controlling the release of U3 snoRNA from pre-rRNA in embryonic stem cells", Wang et al used a AID approach to knock down DDX10 in mouse ESCs and found that Ddx10 is important for the ribosome biogenesis and proliferation of mouse ESCs. They further demonstrated that Ddx10 localized to DFC and GC of nucleoli and 18S rRNA biogenesis is significantly blocked, potentially due to the failure of cleavage near U3 snoRNA binding site. They found DDX10 can undergo phase separation in vitro and dissected the IDRs that are important for DDX10 phase separation and localization. These regions are also important for the function of DDX10 in regulating ribosome biogenesis and cell proliferation. Finally, they show that NUP98-DDX10 fusion of AML does not rescue the defect in ribosome biogenesis caused by the degradation of DDX10 in mouse ESCs. The study is well executed with proper controls and techniques. The conclusion is mostly justified by the data. Overall, the study is of broad interest for researchers in stem cell, cancer and ribosome biogenesis fields. Therefore I support the publication of this manuscript. However, concerns below should be addressed:

Response: We thank the Reviewer#3 for his or her positive comments on this work and valuable suggestions to improve the quality of our manuscript. We have performed more experiments to address the Reviewer#3' questions.

1. In NUP98-DDX10 fusion related malignancies, one of the DDX10 gene is intact, and therefore it is not likely that ribosome biogenesis is affected. At least there is no evidence presented here or by others to show that ribosome biogenesis is affected. For this reason, the claim that "this deficiency (pre-rRNA processing) could potentially contribute to the development and progression of AML" lacks evidence and should be removed or further toned down. Similarly, the discussion related to NUP98-DDX10 should also be toned down, especially for the speculation on therapy in the last paragraph

Response: We are very grateful for the Reviewer#3's valuable comments. By following the Reviewer#3's suggestion, we have deleted the sentence "This deficiency could potentially contribute to the development and progression of AML" from the original manuscript. Moreover, we revised the Discussion section to reduce the emphasis on NUP98-DDX10 in the last paragraph as follows: "In NUP98-DDX10 fusion-associated AML, only one allele of DDX10 gene is intact, resulting in a halved amount of DDX10 protein in these cells compared to normal cells. Consequently, tumor cells harboring this fusion are likely more sensitive to changes in DDX10 dosage compared to healthy cells. Therefore, targeted knockout or knockdown of DDX10 in NUP98-DDX10 fusion AML cells may serve as a potential therapeutic approach for treating this type of AML.".

2. Previous studies have found that p53 pathway is linked to the activation of 2-cell genes. Are 2-cell genes activated in Ddx10 knockdown ESCs? Likewise, p21 and various apoptotic genes were shown as the targets of ESC cell cycle regulating miRNAs (e.g. miR-290 family), do authors find miRNA biogenesis dysregulated in Ddx10 knockdown ESCs?

Response: By following the reviewer's comments. We compared our RNA-seq data with

the results from previously published 2-cell data[3] and observed that 2-cell specific genes, such as *Zscan4b* and *Zscan4d*, were significantly activated after 24 h of IAA treatment (**Supplementary Fig. 2e, f**). Together, these results indicated that DDX10 degradation promoted the transition of mESCs to 2CLCs.

In addition, we detected the expression level of miR-290 family members after DDX10 degradation. We found that DDX10 degradation had no significant impact on the level of miR-290a, miR-293, and miR-294, but DDX10 degradation significantly affected the levels of miR-295 and miR-292. Specifically, miR-295 expression significantly decreased, while miR-292 expression significantly increased after DDX10 degradation **(Fig. R6)**.

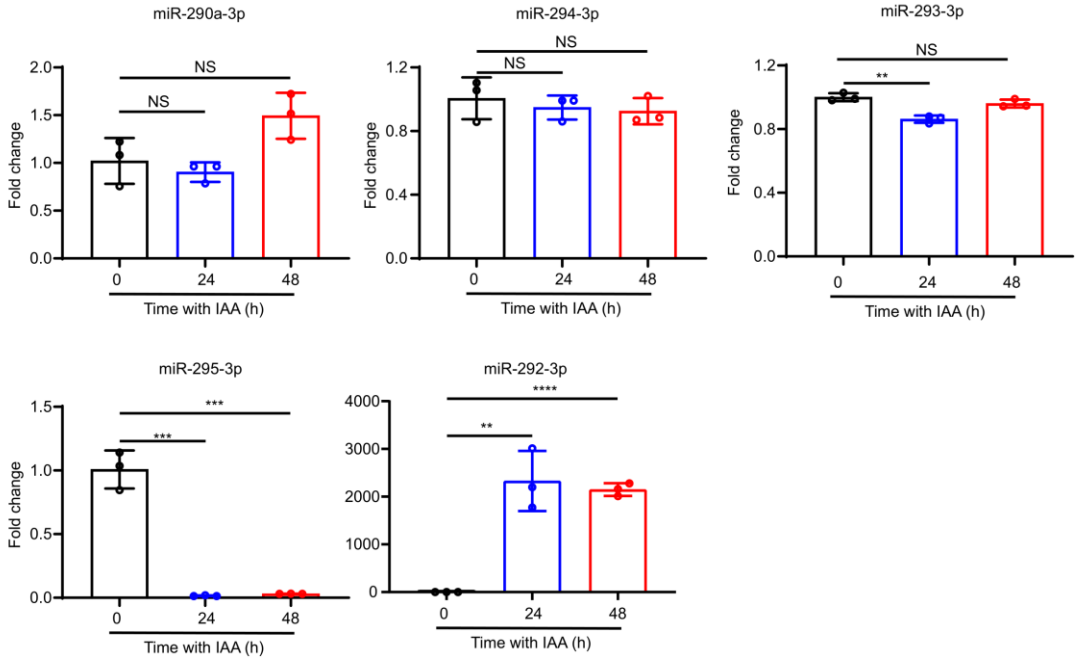

Fig. R6 RT-qPCR analysis of the expression levels of miR-290 family members after DDX10 degradation. Transcription levels were normalized against *U6*. Data are shown as mean ± s.d., n=3. **p < 0.01, ***p < 0.001, and ****p < 0.0001.

3. Does the deletion of IDRs affect the precise localization of DDX10 in nucleolus sub structures (GC, FC, DFC)?

Response: Following the Reviewer#3's suggestion, we performed immunofluorescence staining experiments followed by structured illumination microscopy (SIM) visualization. We stained cells overexpressing different IDRs deleted truncations of DDX10 using the following antibodies: HA (different HA-tagged DDX10 truncations), NPM1 (GC marker), Fibrillarin (DFC marker), and RPA194 (FC marker). We found that the localization of $DDX10_{\Delta IDR1}$, $DDX10_{\Delta IDR2}$ and $DDX10_{\Delta IDR3}$-NLS was similar to that of $DDX10_{FL}$, mainly localizing in the DFC and GC of the nucleolus. However, $DDX10_{\Delta IDR3}$ was present in both cytoplasm and nucleolus, with the nucleolar portion mainly localized in the DFC and GC of the nucleolus. (**Supplementary Fig. 4d**).

4. The overexpression level of HA-tagged NUP98-DDX10 and DDX10 needs to be evaluated by western blotting, so that equal amount of proteins are expressed for both cell lines. In addition, a few previously identified targets of NUP98-DDX10 should be checked

to make sure that sufficient level of NUP98-DDX10 is overexpressed.

Response: We showed the Western blot data in the original version of manuscript. And in the revised manuscript, we displayed the Western blot data for HA-tagged DDX10 and NUP98-DDX10 in **Fig. 6b.** Although the expression level of NUP98-DDX10 in the cell lines was slightly lower than that of DDX10, we conducted RT-qPCR to assess the expression of several target genes of NUP98-DDX10[9, 10], and the results indicated significant upregulation of these NUP98-DDX10 target genes in DDX10-AID mESCs overexpressing NUP98-DDX10, suggesting that the expression level of NUP98-DDX10 was adequate (**Fig. R7**).

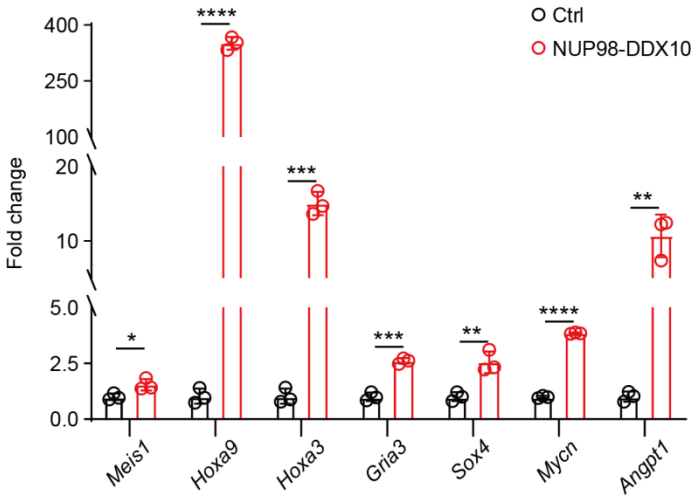

Fig. R7 RT-qPCR analysis of the expression levels of NUP98-DDX10 target genes. Transcription levels were normalized against *Gapdh*. Data are shown as mean ± s.d., n=3. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

5. The claim "the activation of p53 signaling pathway by DDX10 degradation may hinge on MDM2" is possibly right, but not because "DDX10 degradation results in an upregulation of Mdm2", likely due to the binding of free RPL11 or RPL5 to MDM2.

Response: We are grateful to the Reviewer#3 for pointing out this inaccurate description. We have rewritten this part in the revised manuscript from "Similarly, we determined that DDX10 degradation results in an upregulation of Mdm2, indicating that the activation of the p53 signaling pathway by DDX10 degradation may hinge on MDM2." to "Therefore, we speculate that impaired ribosome biogenesis caused by DDX10 degradation might result in the binding of free ribosomal proteins to MDM2, which prevent MDM2 from ubiquitinating P53, thereby activating the p53 signaling pathway.".

6. The details for washoff experiment should be described in methods.

Response: According to the reviewer's comments, we added the experimental details of the IAA wash off experiments to the **Methods** section as follows: "For the IAA wash off experiments, mESCs were initially treated with IAA for 2 days. Subsequently, the medium was replaced with culture medium without IAA, and the cells were cultured for an additional 2 days, with daily medium changes.".

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Overall, I am satisfied with the changes introduced in the new version of the draft, which addresses all my previous critiques. The authors have done a great job.

Reviewer #2 (Remarks to the Author):

The revised manuscript is improved over the previous version. I have two remaining questions that need addressing:

Major point #3: "I find the experiments related to DDX10 phase separation not strong. Do the authors think IDRs help DDX10 incorporate into the nucleolus, or self-associate?". This question is not fully answered. If the roles of the IDR1 and IDR3 are for DDX10 self association, as shown by the in vitro data, then in the in vivo data, we should see deleting IDR1 or IDR3 will not allow them to form the nucleolus. However we don't see these domains play a role in forming nucleolus, except in the case of IDR3 affecting nuclear localization. This raises the question of if condensate formation in vitro is related to condensate formation in vivo.

Major point #5: "In addition, it is unclear what it means to have different FRAP recovery rates in different mutants (Fig. 4g). I find it perplexing that the authors argue that faster recovery is not good for the cell, since in other studies, people may find faster recovery good for reactions."

This is also not answered well. Basically, the authors presented three pieces of data that are not consistent with each other logically: IDR1 and IDR3 deletions disrupt condensate formation in vitro (Figs. 5c-d) but not in vivo (Fig. 5f). In addition, IDR1 and 3 deletions result in faster FRAP recovery (Fig. 5g), which usually associate with better function. The authors need to provide better rationale in text about why they think the data makes sense. Can the roles of IDR1 and IDR3 are to increase stable interactions of DDX10 which is necessary for condensate formation and DDX10 function, which upon deletion, lead to more transient interactions which hamper function? The authors need to be more explicit in the main text when presenting these data. Otherwise, the conclusion of the paragraph: "Taken together, these findings underscore the pivotal roles played by the N-terminal IDR1 and the C-terminal IDR3 in driving DDX10 phase separation" is not supported.

In addition, it is ideal if the authors include some of the rebuttals explaining why they did certain experiments in the main text: Minor point #2 (inserting AID and eGFP), minor point #6 (interactions of DDX10 with others).

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my concerns. The manuscript is ready for publication. By the way, the expression changes for miR-295-3p and miR-292-3p (miR-292a-3p?) is very impressive (> 1000 fold). This degree of expression change is rarely seen especially when the expression level for other members of the same family does not vary much. If confirmed, this might be an important finding for the function of Ddx10 that are worth pursuing in the future.

Point-by-Point Response to the Reviewers' Comments

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Overall, I am satisfied with the changes introduced in the new version of the draft, which addresses all my previous critiques. The authors have done a great job.

Response: We thank Reviewer#1 for being satisfied with the changes of our last version of manuscript.

Reviewer #2 (Remarks to the Author):

The revised manuscript is improved over the previous version. I have two remaining questions that need addressing:

1. Major point #3: "I find the experiments related to DDX10 phase separation not strong. Do the authors think IDRs help DDX10 incorporate into the nucleolus, or self-associate?". This question is not fully answered. If the roles of the IDR1 and IDR3 are for DDX10 self association, as shown by the in vitro data, then in the in vivo data, we should see deleting IDR1 or IDR3 will not allow them to form the nucleolus. However we don't see these domains play a role in forming nucleolus, except in the case of IDR3 affecting nuclear localization. This raises the question of if condensate formation in vitro is related to condensate formation in vivo.

Response: We think that Reviewer#2 misunderstood our data. We apologize for the confusion caused by our insufficient description of the results, which led to Reviewer #2's misinterpretation. We have provided more details to clarify our findings.

The *in vivo* experiments showed that the loss of either IDR1 or IDR3 altered the condensate formation of DDX10. Specifically:

(1) $DDX10_{\Delta IDR1}$ exhibited predominantly nucleolar localization, accompanied by dispersed signals throughout the nucleoplasm. However, its aggregation in the nucleolus was significantly lower than that of $DDX10_{FL}$ and $DDX10_{\Delta IDR2}$, exhibiting a relatively diffused distribution (**Fig. 5f**).

(2) Although $DDX10_{\Delta IDR3}$ exhibited the diffused distribution in both the nucleolus and cytoplasm, the addition of a NLS to $DDX10_{\Delta IDR3}$ successfully redirected it to the nucleolus, while the diffused signal also observed in the nucleoplasm. Similar to $DDX10_{\Delta IDR1}$, the aggregation of $DDX10_{\Delta IDR3}$-NLS in the nucleolus was significantly lower than that of $DDX10_{FL}$ and $DDX10_{\Delta IDR2}$ (**Fig. 5f**).

Thus, IDR1 and IDR3 are crucial for condensate formation of DDX10 within the nucleolus, consistent with the impaired droplet formation capacity of DDX10 *in vitro* upon deletion of either IDR1 or IDR3 (**Fig. 5c-e**).

Disruption of DDX10 phase separation had no significant effect on nucleolus formation. Overall, deletion of both IDR1 and IDR3 weakens the ability of DDX10 in forming condensates both *in vivo* and *in vitro*, but could not significantly affect nucleolus formation.

2. Major point #5: "In addition, it is unclear what it means to have different FRAP recovery rates in different mutants (Fig. 4g). I find it perplexing that the authors argue that faster recovery is not good for the cell, since in other studies, people may find faster recovery good for reactions." This is also not answered well. Basically, the authors presented three pieces of data that are not consistent with each other logically: IDR1 and IDR3 deletions disrupt condensate formation in vitro (Figs. 5c-d) but not in vivo (Fig. 5f). In addition, IDR1 and 3 deletions result in faster FRAP recovery (Fig. 5g), which usually associate with better function. The authors need to provide better rationale in text about why they think the data makes sense. Can the roles of IDR1 and IDR3 are to increase stable interactions of DDX10 which is necessary for condensate

formation and DDX10 function, which upon deletion, lead to more transient interactions which hamper function? The authors need to be more explicit in the main text when presenting these data. Otherwise, the conclusion of the paragraph: "Taken together, these findings underscore the pivotal roles played by the N-terminal IDR1 and the C-terminal IDR3 in driving DDX10 phase separation" is not supported.

Response: As we responded in the above question: Results from both *in vivo* and *in vitro* experiments are consistent, demonstrating that deletion of either IDR1 or IDR3 weakens the ability of DDX10 in forming condensates.

Regarding the explanation that the deletion of either IDR1 or IDR3 accelerates recovery rates and impairs DDX10 function, we agree with this opinion raised by Reviewer #2. We have added the following description to the revised manuscript: "This suggests that both IDR1 and IDR3 contribute to stabilizing DDX10 interactions, thereby facilitating condensate formation, and their deletion may accelerate the dissociation of DDX10from RNA, leading to increased transient interactions that might affect its function.".

3. In addition, it is ideal if the authors include some of the rebuttals explaining why they did certain experiments in the main text: Minor point #2 (inserting AID and eGFP), minor point #6 (interactions of DDX10 with others).

Response: By following Reviewer #2's suggestions, we have provided explanations and descriptions of the relevant experiments in our revised manuscript.

For Minor point #2, we have included the following statement in the main text: "We observed that the protein level of DDX10 in DDX10-AID (+OsTir1) mESCs is lower compared to wild-type mESCs (**Fig. 1e**). However, this reduction had no significant effect on cell morphology, cell cycle, and apoptosis (**Supplementary Fig. 1d-f**) ".

For Minor Point #6, we have already added the rationale for investigating the interaction between DDX10 and SSU processome components in the previous revised manuscript: "The SSU processome, also known as 90S preribosome, is an early assembly intermediate of the small ribosomal subunit. It undergoes structural changes to form the pre-40S ribosome, which is necessary for 18S rRNA maturation".

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my concerns. The manuscript is ready for publication. By the way, the expression changes for miR-295-3p and miR-292-3p (miR-292a-3p?) is very impressive (> 1000 fold). This degree of expression change is rarely seen especially when the expression level for other members of the same family does not vary much. If confirmed, this might be an important finding for the function of Ddx10 that are worth pursuing in the future.

Response: We thank Reviewer #3 for agreeing to accept this manuscript for publication. In addition, we are grateful for Reviewer #3's valuable suggestions for pointing to the future direction.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The article is improved and ready for publication.