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Alu element-containing RNAs maintain nucleolar structure and function

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Anne Nielsen

1st Editorial Decision 23 April 2015

Thank you very much for providing us with an outline of the experiments you could include in a revised version of your manuscript. I have now had the chance to go through both reports and response, and the outcome is that we would be happy to invite a revised manuscript.

The inclusion of functional data for rRNA transcription and the demonstration of a direct interaction between Alu-containing RNAs and NPM/NCL are clearly important points for all three referees, as you also mention in the outline. It is obviously difficult to fully predict the outcome of these experiments at the current stage and the outcome of the proposed work will therefore be a factor in our final decision for the revised version.

Given the referees' positive recommendations, I would thus like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

Please feel free to contact me with any further questions. Given the extensive revision involved here we would be happy to grant an extension to the normal three months time-frame for revisions, if you would need it.

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Thanks again for giving us the chance to consider this work for The EMBO Journal, I look forward to receiving the revised manuscript in due time.

REFEREE COMMENTS

Referee #1:

The study by Caudron-Herger and colleagues proposes an intriguing involvement of Alu element containing RNAs in maintenance of nucleolar structure and function. While the model is novel and exciting, at this stage the experimental results do not fully support the bold conclusions. In particular, a potential impact of aluRNAs on nucleolar function is not demonstrated.
Specific comments:
1. Authors conclude that Pol II transcription is required for a functional nucleolar structure based on a 5 hour incubation with drugs that specifically target each of the three polymerases. However, drugs always have pleotropic, unspecific effects. What is the evidence to conclude that the drug treatments depicted in Figure 1A are not inhibiting Pol I activity? I consider that the results shown are not sufficient to convincingly state that "Pol II transcription is needed to maintain the structure of the nucleolus and the efficient production of pre-rRNA by Pol I" (pages 6/7).
2. Figure 1C shows that microinjection of nucleolar RNA into amanitin treated cells affects nucleolar structure, with larger and fewer nucleolar domains. However, these structures look very different from normal nucleoli. It is critical to assess whether the microinjected RNA rescues nucleolar function, namely, rRNA synthesis.
3. Enrichment for intron-encoded aluRNA sequences in the nucleolus is intriguing, considering that intronic sequences are normally degraded upon splicing.
4. The RNA-FISH experiments depicted in Figure 3A lack appropriate controls. The results confirm hybridization to RNA, but do not demonstrate specific hybridization to alu sequences. Given the high concentration of RNAs and RNA-binding proteins in nucleoli, it is critical to control for non-specific trapping of the FISH probes in the nucleolus. One possibility would be to introduce point mutations in the probes to specifically abolish hybridization with alu sequences.
5. In Figure 3B, the apparent "rescuing" of nucleolar morphology upon microinjection of aluRNAs should be confirmed for nucleolar function.
6. The aluRNA depletion experiment is very informative. However, it is not clear how the antisense oligos (ASO) result in aluRNA depletion. What mechanism triggers aluRNA degradation? Are ASOs targeting intronic aluRNA sequences co-transcriptionally? Are ASOs indirectly affecting Pol II transcription?
7. Upon overexpression of aluRNA, the size of nucleoli increases (Figure 4E, F), but how is nucleolar function affected?
8. The tethering experiments with MS2-aluRNA (Figures 6 and 7) are very nice. What control RNA was used? As discussed above, testing different control RNAs is important to rule out unspecific associations with alu sequences.
9. Finally, the proposed interaction between nucleolin and nucleophosmin and aluRNA should be confirmed with biochemical approaches.

Referee #2:

In the presented manuscript Caudron-Herger and colleagues analyze the role of highly abundant non-coding aluRNAs in the functional organization of the most prominent nuclear structure, the nucleolus. Using deep sequencing they discovered that transcripts synthesized from intronic Alu segments are enriched in the nucleolar RNA fraction. Interestingly, depletion of aluRNAs by anti-sense oligo treatment leads to the dispersion of the nucleolar structure and impairment of RNA polymerase I-driven transcription of ribosomal DNA genes.
This is an interesting manuscript which goes against the dogma that RNA polymease I transcription drives exclusively the nucleolar body formation. The experiments are generally well performed, data are novel, conclusions mostly follow the data, and will be of significant impact for the field of the nuclear organization and the nucleolar function.

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However, the following issues need to be addressed before this manuscript is further considered for publication in EMBO J:

The following major concerns need to be addressed:

The authors should show high resolution images of the intranucleolar localization of aluRNA detected by RNA FISH colocalized with markers for internal functional nucleolar subcompartments linked to steps of pre-ribosomal subunit biogenesis, such as UBF, fibrillarin and nucleophosmin. These essential data will provide the essential information where precisely aluRNA is present within the nucleolus and where is required to maintain the nucleolar structural integrity.

The potential problem is the lack of evidence to show whether the interaction between nucleolin or nucleophosmin and aluRNA is direct or mediated by other factors. Nucleolin and nucleophosmin are highly abundant proteins well known for their ability to interact with multiple proteins. Therefore, it is essential that the authors should pull down aluRNA from the nucleolar RNA fraction and perform systematic mass spectrometry analysis of proteins associated with it. The size of aluRNA nucleolar transcripts, 90 nt and 300 nt, suggests that they fall into the size range of small nucleolar RNAs. Is there a possibility that they might share similar or overlapping protein component(s)? Furthermore, RNA immuniprecipitation using anti-nucleolin and nucleophosmin antibodies should be also performed and the presence of aluRNA should be validated to confirm the aluRNA-MS2 tethering experiment.

It is not clear to me why the authors performed double knockdown for nucleolin and nucleophosmin when tethering aluRNA-MS2 to the LacO array. The single knockdown for nucleolin and nucleophosmin should be done and evaluated whether aluRNA preferentially interact with one over the other. Is the expression of nucleolin or nucleophosmin affected by aluRNA siRNA treatment?

The authors should evaluate the genomic position of nucleolar-enriched aluRNA-containing clusters on chromosome 13 and 14 (Table S2) using specific BACs and DNA FISH microscopy. It is intriguing that these two are the only ribosomal DNA-containing chromosomes out of five (chr 13, 21 and 22) which might have RNA polymerase II transcriptionally active aluRNA clusters within the nucleolus. If so, this will require additional run on mapping of these RNA polymerase II-driven transcription sites within the nucleolar body. How well documented is the statement that the nucleolus "has no apparent RNA polymerase II activity" on page 6? Moreover, the authors must perform specific aluRNA DNA FISH to show whether wild type and dispersed nucleoli interact with endogenous aluRNA gene loci.

Authors claim that nucleoli are dispersed and smaller after aluRNA depletion. No proof for this, as the microscopic data presented might represent a total lack of nucleoli and redistribution of nucleolar components. If they are claiming this phenotype then they should show that these dispersed nucleoli colocalize with preserved pre-ribosomal RNAs detected by RNA FISH.

A few minor points:
Are aluRNAs sensitive to DRB or alpha-amanitin treatment?
- siRNA, alpha-aminitin etc treatment - please show that a few aluRNAs are specifically downregulated.
Table 1 and Figure 1B do not feel relevant to the story.
Provide proper controls
- Actin/GAPDH immunoblot for total cell fraction, lamin A/C for the nucleus, nucleophosmin/nucleolin for nucleoli
- siRNA B1 blot, PCR to prove knockdown was effective
- nucleophosmin/nucleolin staining for double knockdown
AluRNA and nucleophosmin/nucleolin siRNA treatment times are vastly different (14hr vs 48hr)
- Some of the bar graph values look really close to one another.
- Fig. 3B aluRNA reverse and forward are not that different.
- Fig. 4B right column of plates does not appear to be of the highest quality.
- Fig. 4D show + and - error bars in bar graph. This is the one case where there is a clear signal. Indicate p-values to be consistent with other figures.
- Fig. 4E - the difference does not look that great. Is there the subtle difference shown in the plates?

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Referee #3:

This is a very interesting study that implicates Alu-containing RNAs in nucleolar assembly/structure. Collectively the data provide a clear indication that there is an effect of Alu RNAs on the nucleolus. However I do have some concerns with individual experiments and their interpretation that are outlined below.

Major Points:

1. Fig. 1: Overall cellular physiology is changed fairly dramatically 5 hrs after shut off of Pol II transcription. Thus it is very difficult in my opinion to determine direct vs indirect effects on nucleoli under these conditions.
2. Single cells are shown in several key Figs. While the data shown by the single cell in these Figs is definitely compelling, it is not clear how reproducible or representative the selected cells are to the population as a whole.
3. Fig. 3B: It is not clear to me why the 'n' for each of these conditions was varied in this key experiment (as well as elsewhere in the manuscript). Given the relatively small differences, it would be cleaner from a statistical/experimental design perspective if the same number of samples were evaluated for each condition.
4. Fig. S4C: since there is significant background hybridization of the probe used in this northern blot to rRNA (and thus a high background in the blot), I do not find these data to provide compelling support for the conclusion that Pol II driven Alu containing RNAs are involved.
5. Fig. 6: Would it be straightforward for the authors to demonstrate a direct interaction between these RBPs and Alu-containing RNAs to corroborate the co-localization data?
6. Fig. 8: While structures consistent with nucleoli are formed, it is not clear to me that the nucleoli have been sufficiently demonstrated as 'functional'. Hence I would recommend removing this term from Fig. 8 as well as from the title of the manuscript.
7. Minor Points:
a. Pg. 6: I do not believe that NCL and NPM have been clearly defined for the general readership
b. Pg. 7, line 9: I would recommend changing the conclusion of 'devoid of contaminations' to 'devoid of substantial contamination' since there is clearly some background RNA associated with every fraction.
c. The Supplementary figs are discussed out of order in the manuscript (S4 is discussed before S2)
d. Pg. 7, line 9 from the bottom: extend = extent

1st Revision - authors' response 05 August 2015

Reviewer #1

The study by Caudron-Herger and colleagues proposes an intriguing involvement of Alu element containing RNAs in maintenance of nucleolar structure and function. While the model is novel and exciting, at this stage the experimental results do not fully support the bold conclusions. In particular, a potential impact of aluRNAs on nucleolar function is not demonstrated.

We thank the reviewer for appreciating the novel and exciting features of our story and are grateful for his/her specific comments. We have addressed them to strengthen the aspects on the functional impact of aluRNAs as described below.

Specific comments:
1. Authors conclude that Pol II transcription is required for a functional nucleolar structure based on a 5 hour incubation with drugs that specifically target each of the three polymerases. However, drugs always have pleotropic, unspecific effects. What is the evidence to conclude that the drug treatments depicted in Figure 1A are not inhibiting Pol I activity? I consider that the results shown are not sufficient to convincingly state that "Pol II transcription is needed to maintain the structure

of the nucleolus and the efficient production of pre-rRNA by Pol I" (pages 6/7).

It has been previously shown with an *in vitro* transcription system including isolated nuclei that Pol I is completely insensitive to alpha-amanitin concentrations up to several 100 μg/ml (Weinmann and Roeder 1974), which is now cited in the manuscript. Accordingly, we exclude a direct inhibition of Pol I by our alpha aminitin treatment with 50 μg/ml. Furthermore, the cycloheximide experiments exclude protein synthesis as a factor that inhibits Pol I on the 5 h time scale studied. At the same time, we show that inhibition of Pol II transcription by two well-characterized inhibitors of Pol II, namely alpha-amanitin and DRB leads to the same phenotype, i.e. disrupting the structure of the nucleolus and the efficient production of pre-rRNA by Pol I. Although not impossible, we consider it very unlikely that two inhibitors of Pol II with different molecular targets would have the same so far unknown effect on nucleolus structure and Pol I activity without involving a Pol II transcript. In the context of the first figure of our manuscript we have clarified our rational that Pol II transcription is linked to maintaining the structure of the nucleolus and to Pol I activity. It is noted that we find intronic *alu*RNAs that arise from Pol II activity as highly enriched in the nucleolus and exclude Pol III transcription as the source of these transcripts in the subsequent experiments.

2. Figure 1C shows that microinjection of nucleolar RNA into amanitin treated cells affects nucleolar structure, with larger and fewer nucleolar domains. However, these structures look very different from normal nucleoli. It is critical to assess whether the microinjected RNA rescues nucleolar function, namely, rRNA synthesis.

We agree that this would be an informative experiment. Accordingly, we have performed a microinjection experiment of *alu*RNA transcripts during Pol II inhibition coupled to a pulse labeling with EU to label nascent RNAs. The production of rRNA between untreated cells, alpha-amanitin-treated cell and *alu*RNA-rescued cells was compared. We found that microinjection on its own resulted in cellular stress that reduced the efficiency of rRNA transcription in untreated cells. The lack of a positive control makes the results difficult to interpret, and we have not included these experiments in the revised manuscript. However, we were able to successfully address the point raised by the reviewer in another set of experiments that are described below in response to point #7.

3. Enrichment for intron-encoded aluRNA sequences in the nucleolus is intriguing, considering that intronic sequences are normally degraded upon splicing.

This is indeed an intriguing and important finding from our study. We note in the manuscript that in addition to our experiments another study shows that specific intron-encoded and processed *Alu* sequences are retained in the nucleus (Jady et al. 2012).

4. The RNA-FISH experiments depicted in Figure 3A lack appropriate controls. The results confirm hybridization to RNA, but do not demonstrate specific hybridization to alu sequences. Given the high concentration of RNAs and RNA-binding proteins in nucleoli, it is critical to control for non-specific trapping of the FISH probes in the nucleolus. One possibility would be to introduce point mutations in the probes to specifically abolish hybridization with alu sequences.

In Fig. 3A and Appendix Fig. S5A-B, we show that endogenous intronic *Alu* element containing RNA fragments are enriched in the nucleolus. These transcripts, which we refer to as *alu*RNA, do not have a unique fully conserved sequence. Rather, they represent a group of *Alu* repeats that differ by point mutations in their sequences and arise from different intronic *loci*, consistent with the previously described *Alu* element sequence variations (Umylny et al. 2007). Accordingly, we were unable to design an *alu*RNA FISH probe with a single point mutation for which we could exclude hybridization to *Alu* sequences.
In order to control and verify that the RNA FISH probe is not trapped in the nucleoli in an unspecific manner, we have used in the manuscript a probe that targets L1-repeat containing RNA transcripts as control (Figure 3A, bottom images). As seen on the images, this probe did not accumulate in nucleoli. In addition, we have now included two additional controls, Met- and Ala-tRNA, which display only a weak residual nucleolar hybridization signal as compared to the *alu*RNA probe (Appendix Fig. S5A).

5. In Figure 3B, the apparent "rescuing" of nucleolar morphology upon microinjection of aluRNAs should be confirmed for nucleolar function.

See above, comments to point #2 and below, comment to point #7.

6. The aluRNA depletion experiment is very informative. However, it is not clear how the antisense oligos (ASO) result in aluRNA depletion. What mechanism triggers aluRNA degradation? Are ASOs targeting intronic aluRNA sequences co-transcriptionally? Are ASOs indirectly affecting Pol II transcription?

As described previously (Ideue et al. 2009), the ASOs for nuclear RNA knockdown are modified oligonucleotide sequences that contain a DNA sequence complementary to their target RNA sequence so that they are cleaved with endogenous RNase H in the nucleus. The ASOs contain a phosphorothioate backbone and flanking nucleotides 2'-O-methoxyethyl ribonucleotides to increase hybrid duplex stability and resistance towards degradation. This has been clarified in the manuscript (page 9, line 15).
To show that ASOs do not indirectly affect Pol II transcription we have evaluated the levels from house keeping genes in the sequencing data *e.g.* GAPDH, EMC7 etc., which remain essentially unchanged. This analysis is now included in the manuscript as Appendix Fig. S6C. In addition, data showing the decreased levels of target *Alu*-repeat containing transcripts have now been added to the manuscript (Appendix Fig. S6B).

7. Upon overexpression of aluRNA, the size of nucleoli increases (Figure 4E, F), but how is nucleolar function affected?

We agree with the reviewer's comment and have clarified this important point. We monitored via fluorescence microscopy the amount of nascent RNA transcripts in the nucleoli after a pulse labeling with EU. This provides an rRNA synthesis signal as most of active nucleolar transcription reflects the production of 47S rRNA. We find a significant increase in nucleoli activity after overexpression of *alu*RNA but not after overexpression of L1 repeat-containing transcripts. The new data are now included in the manuscript as Fig. 5C-D.

8. The tethering experiments with MS2-aluRNA (Figures 6 and 7) are very nice. What control RNA was used? As discussed above, testing different control RNAs is important to rule out unspecific associations with Alu sequences.

We have now clarified in the legend of Fig. 7 what control RNAs were used: A sequence containing only the MS2-and the MS2-CDV3, which is from the 3'UTR sequence of the CDV3 transcript and comprises 700 nucleotides. Three additional controls, namely MS2-RepA (a repeat-containing RNA derived from Xist (Zhao et al. 2008)) and two 3'UTRs from the STARD7 and CORO1C genes were additionally studied. None of these control sequences were as efficient as the MS2-*alu*RNA construct in terms of driving the localization of the *lac*O array into nucleoli (Fig. 7C).

9. Finally, the proposed interaction between nucleolin and nucleophosmin and aluRNA should be confirmed with biochemical approaches.

We have now included pull down experiments from nuclear extract using biotinylated RNA transcripts immobilized on streptavidin beads to the manuscripts. By western blot analysis of the bound protein fraction we now show that nucleolin from nuclear extracts binds *alu*RNA as well as *alu*RNA_L and *alu*RNA_R (Appendix Fig. S9E-F). Under the same experimental conditions, we do not detect an interaction of *alu*RNA with nucleophosmin (Appendix Fig. S9G). Accordingly, we modified our discussion, suggesting that NPM promotes indirect interactions with *alu*RNA in a nucleolar environment.

Reviewer #2
In the presented manuscript Caudron-Herger and colleagues analyze the role of highly abundant non-coding aluRNAs in the functional organization of the most prominent nuclear structure, the nucleolus. Using deep sequencing they discovered that transcripts synthesized from intronic Alu segments are enriched in the nucleolar RNA fraction. Interestingly, depletion of aluRNAs by anti-sense oligo treatment leads to the dispersion of the nucleolar structure and impairment of RNA

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polymerase I-driven transcription of ribosomal DNA genes.
This is an interesting manuscript which goes against the dogma that RNA polymease I transcription drives exclusively the nucleolar body formation. The experiments are generally well performed, data are novel, conclusions mostly follow the data, and will be of significant impact for the field of the nuclear organization and the nucleolar function.

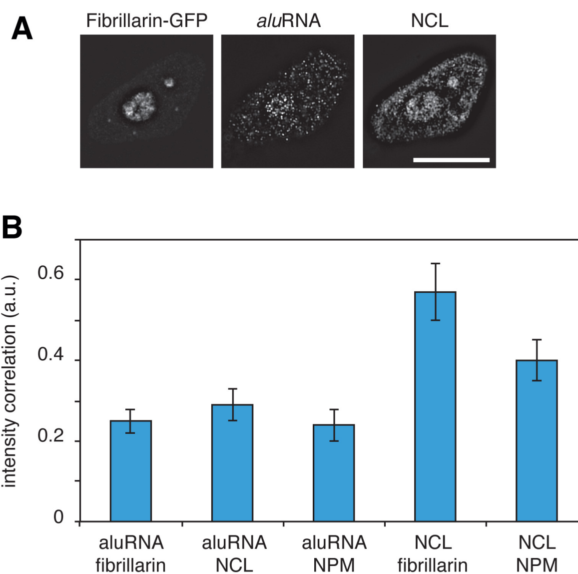
We thank the reviewer for acknowledging the high quality of our data and the significant impact of our study for the nuclear/nucleolar organization research field. We appreciate the detailed comments on aspects of our study that need to be strengthened and have addressed these as explained below.

However, the following issues need to be addressed before this manuscript is further considered for publication in EMBO J:

The following major concerns need to be addressed:

1. The authors should show high resolution images of the intranucleolar localization of aluRNA detected by RNA FISH colocalized with markers for internal functional nucleolar subcompartments linked to steps of pre-ribosomal subunit biogenesis, such as UBF, fibrillarin and nucleophosmin. These essential data will provide the essential information where precisely aluRNA is present within the nucleolus and where is required to maintain the nucleolar structural integrity.

We agree with the reviewer's comment that high-resolution images of the intranucleolar localization of *alu*RNA coupled to co-localization with markers for internal nucleolar subcompartments would be very informative. Our multiple color labeling system is not compatible with STED or localization microscopy and we therefore settled for structured illumination microscopy (SIM), which should give an about 2-fold higher resolution than confocal microscopy.
As shown below, we acquired images of GFP-fusion constructs of either fibrillarin or nucleophosmin, in addition to *alu*RNA (RNA FISH) and nucleolin (immunofluorescence). In these experiments, we detected no preferred localization of *alu*RNA in the nucleolus. We encountered some technical difficulties with bleaching of the fluorophores and found the resolution improvement over the confocal microscopy analysis insufficient to draw reliable conclusions. Accordingly, these data were not included into the revised manuscript.



SIM analysis of *alu*RNA nucleolar localization.
A. Images showing the distribution of fibrillarin-GFP, *alu*RNA (RNA FISH) and nucleolin (NCL, immunofluorescence). Scale bar, 10 µm.
B. Graph showing the pixel correlation intensity in the various channels. Error bars, +/- 95% CI (n = 15).

2. The potential problem is the lack of evidence to show whether the interaction between nucleolin or nucleophosmin and aluRNA is direct or mediated by other factors. Nucleolin and nucleophosmin are highly abundant proteins well known for their ability to interact with multiple proteins.

Therefore, it is essential that the authors should pull down aluRNA from the nucleolar RNA fraction and perform systematic mass spectrometry analysis of proteins associated with it. The size of aluRNA nucleolar transcripts, 90 nt and 300 nt, suggests that they fall into the size range of small nucleolar RNAs. Is there a possibility that they might share similar or overlapping protein component(s)?

This issue has been addressed as described above in our comment to reviewer #1 point #9.

3. Furthermore, RNA immunoprecipitation using anti-nucleolin and nucleophosmin antibodies should be also performed and the presence of aluRNA should be validated to confirm the aluRNA-MS2 tethering experiment.

These experiments have been conducted as described above (reviewer #1 point #9).

4. It is not clear to me why the authors performed double knockdown for nucleolin and nucleophosmin when tethering aluRNA-MS2 to the LacO array. The single knockdown for nucleolin and nucleophosmin should be done and evaluated whether aluRNA preferentially interact with one over the other. Is the expression of nucleolin or nucleophosmin affected by aluRNA siRNA treatment?

The rational for the double knockdown experiment is that the nucleolar dispersion phenotype was more frequent in the double than in the single knockdown samples. This has now been clarified in the text. The NCL/NPM siRNA experiment was made independently of *alu*RNA-MS2 recruitment to the *lacO* arrays and shows that the same nucleolar dispersion phenotype is observed upon (i) Pol II inhibition, (ii) *alu*RNA knockdown and (iii) double knockdown of NCL and NPM. This supports the idea that NCL but also NPM act together with *alu*RNAs to maintain nucleolar structure. Our results from the RNA affinity purification experiments corroborate the conclusion that NCL binds *alu*RNA. However, for NPM, the interaction seems to be weaker and is probably indirect (Appendix Fig. S9E-G). Accordingly, we revised our conclusions as described above.

5. The authors should evaluate the genomic position of nucleolar-enriched aluRNA-containing clusters on chromosome 13 and 14 (Table S2) using specific BACs and DNA FISH microscopy. It is intriguing that these two are the only ribosomal DNA-containing chromosomes out of five (chr 13, 21 and 22) which might have RNA polymerase II transcriptionally active aluRNA clusters within the nucleolus. If so, this will require additional run on mapping of these RNA polymerase II-driven transcription sites within the nucleolar body. How well documented is the statement that the nucleolus "has no apparent RNA polymerase II activity" on page 6? Moreover, the authors must perform specific aluRNA DNA FISH to show whether wild type and dispersed nucleoli interact with endogenous aluRNA gene loci.

We agree that some Pol II activity in the nucleolus has been documented in previous studies, which was estimated to be about 20-fold less than Pol I (Weinmann and Roeder 1974; Bierhoff et al. 2014). However, based on our deep-sequencing analysis of the nucleolar RNA content, we find no evidence that the *alu*RNAs enriched originate from a small number of specific nucleolar enriched cluster (e.g. those on chromosome 13 and 14 mentioned by the reviewer). Rather, we find that the intronic *alu*RNAs enriched in the nucleolus originate from more than a thousand intronic regions with most of them not being associated with the nucleolar periphery according to the genomic map derived in a previous study (Németh et al. 2010). Thus, we conclude that the *alu*RNAs are mostly produced elsewhere in the nucleus and subsequently accumulate in the nucleolus. Consistent with this view, we find that the plasmid derived MS2 tagged ectopic *alu*RNAs enrich in the nucleolus, too (Figure 7A).

6. Authors claim that nucleoli are dispersed and smaller after aluRNA depletion. No proof for this, as the microscopic data presented might represent a total lack of nucleoli and redistribution of nucleolar components. If they are claiming this phenotype then they should show that these dispersed nucleoli colocalize with preserved pre-ribosomal RNAs detected by RNA FISH.

We have now defined more clearly in the text on page 6 what we call “nucleolar dispersion or dispersed nucleoli”.

A few minor points:
Are aluRNAs sensitive to DRB or alpha-amanitin treatment?
- siRNA, alpha-aminitin etc treatment - please show that a few aluRNAs are specifically downregulated.

We have now added this data in the manuscript as Appendix Fig. A-B for ASO treatment and Appendix Fig. D-E for a-amanitin treatment.

Table 1 and Figure 1B do not feel relevant to the story.
Those data have been moved to the Appendix file as Appendix Table S1 and Appendix Fig. S2 respectively. While we agree that they are not central to the study we believe that Table S1 is helpful to give an overview of the nucleolar RNA content/enrichment and that Fig. S2 provides an important control that demonstrates the high quality of our nucleoli preparation.

Provide proper controls
- Actin/GAPDH immunoblot for total cell fraction, lamin A/C for the nucleus, nucleophosmin/nucleolin for nucleoli

We think that the quality control for the fractionation should be based on the distribution of RNAs as these are in the focus of our study. This is provided in Appendix Fig. S2 (former Fig. 1A) as mentioned above.

- siRNA B1 blot, PCR to prove knockdown was effective

It is difficult to measure the levels of B1 RNAs by RT-qPCR due to the repetitive nature of these sequence. We have used primer pairs that simultaneously target 6022 or 3890 B1 elements, respectively. These, however, represent only a subset of the total of 20411 or 12666 potential targets of the B1 ASO-1 and B1 ASO-2 probes, respectively. Having this caveat in mind, we still measured a significant reduction by ~30% of the B1 levels after ASO treatment. This finding is now stated in the text (page 12).

- nucleophosmin/nucleolin staining for double knockdown

Those images are presented in Appendix Fig. S9H. It is shown that the nucleoplasmic staining for both proteins is largely reduced and residual staining is found in the dispersed nucleolar structures.

AluRNA and nucleophosmin/nucleolin siRNA treatment times are vastly different (14hr vs 48hr)

Targeting the nuclear RNA transcript directly by ASO treatment degradation is a relatively fast process. In contrast, the siRNA-mediated knockdown of a protein will only display a phenotype once also the existing pool of corresponding protein has been depleted. Accordingly, we used the different treatment incubation periods.

- Some of the bar graph values look really close to one another.

We have checked that the bar graphs consistently display the error of the mean value or 95% confidence interval and that statistically significant differences at the $p = 0.05$ and $p = 0.01$ levels are indicated by one or two stars, respectively.

- Fig. 3B aluRNA reverse and forward are not that different.

According to the Student's t-test evaluation, *alu*RNA forward and reverse are significantly different with a p-value < 0.01.

- Fig. 4B right column of plates does not appear to be of the highest quality.

In order to facilitate comparison of the three different color channels, we have replaced the merged picture by the NCL channel alone in Fig. 3B.

- Fig. 4D show + and - error bars in bar graph. This is the one case where there is a clear signal.

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Indicate p-values to be consistent with other figures.

This has been modified accordingly to reviewer's comment.

- Fig. 4E - the difference does not look that great. Is there the subtle difference shown in the plates?

The former Fig. 4E is now displayed as Fig. 5A with newly added data (Fig. 5C-D) on the effect of *alu*RNA overexpression. Based on measurements of 20 cells, the change of the nucleolar volume fraction by about 30% is clearly statistically significant ($p < 0.01$). We disagree with the notion that 30% would be only a "subtle difference" as compared to the wild type conditions. With the newly added data, we now show that the nucleoli size increase is accompanied by a corresponding increase of RNA production as determined by the EU pulse label (Fig. 5C-D). This, reinforces the conclusions that *alu*RNA are involved in both structural and functional maintenance of nucleoli.

Reviewer #3

This is a very interesting study that implicates Alu-containing RNAs in nucleolar assembly/structure. Collectively the data provide a clear indication that there is an effect of Alu RNAs on the nucleolus. However I do have some concerns with individual experiments and their interpretation that are outlined below.

We thank the reviewer for acknowledging that our study demonstrates the relevance of Alu-containing RNAs for the nucleolus and for communicating the concerns that refer to some of our experiments. These were addressed in the revised manuscript as described below.

Major Points:

1. Fig. 1: Overall cellular physiology is changed fairly dramatically 5 hrs after shut off of Pol II transcription. Thus it is very difficult in my opinion to determine direct vs indirect effects on nucleoli under these conditions.

See above, comments to Reviewer #1, point #1.

2. Single cells are shown in several key Figs. While the data shown by the single cell in these Figs is definitely compelling, it is not clear how reproducible or representative the selected cells are to the population as a whole.

In general, the images shown in our work are representative for the results of a given experiment and are accompanied with an image analysis to quantitate the results given either in the figure legend (Fig. 3A, Fig. 4A) or in form of a graph (Fig. 1B, Fig. 3B, Fig. 5, Fig. 7, Fig.8). In case of Fig. 1A, the phenotypes have been reported in other studies and in other instance different images are shown in various Figure panels as for example in the case of nucleoli dispersion after *alu*RNA ASO treatment (Fig. 4A-B, Appendix Fig. S7B-C, Appendix Fig. S8A-B)

3. Fig. 3B: It is not clear to me why the 'n' for each of these conditions was varied in this key experiment (as well as elsewhere in the manuscript). Given the relatively small differences, it would be cleaner from a statistical/experimental design perspective if the same number of samples were evaluated for each condition.

Additional microinjection experiments were conducted to increase the sample numbers to similar values for all conditions in one experiment. That being said, comparing samples with different sample sizes is technically no problem as this is accounted for in the statistical test used to evaluate the significance of the observed differences.

4. Fig. S4C: since there is significant background hybridization of the probe used in this northern blot to rRNA (and thus a high background in the blot), I do not find these data to provide compelling support for the conclusion that Pol II driven Alu containing RNAs are involved.

We agree with the comment of this reviewer that there is a high background in the blot but did not get better results with additional probes tested. We would like to note, however, that the purpose of

the blot is simply to demonstrate that stable *Alu*-repeat containing transcripts exist, either within large primary transcripts (long transcripts in the blot), as full-length *Alu* elements (300 nt) or as processed right arm transcript (90 nt). We have now included an additional lane in the Appendix Fig. S5D that shows the same size *Alu* RNAs, with the RNA sample being treated with the Ribo-Zero-rRNA removal protocol to deplete the ribosomal RNA.

5. Fig. 6: Would it be straightforward for the authors to demonstrate a direct interaction between these RBPs and Alu-containing RNAs to corroborate the co-localization data?

This experiment has been included, see Reviewer #1 point #9.

6. Fig. 8: While structures consistent with nucleoli are formed, it is not clear to me that the nucleoli have been sufficiently demonstrated as 'functional'. Hence I would recommend removing this term from Fig. 8 as well as from the title of the manuscript.

We agree that this point was only based on the resulting decreased Pol I activity observed upon *alu*RNA knockdown. As described above in the comments to Reviewer #1, point #7, we have conducted additional experiments. We now show that both depletion (Fig. 4) and overexpression (Fig. 5) of *alu*RNA leads to changes of the amount of rRNA produced. Therefore, we consider it justified to concluded that also rRNA production is affected. Furthermore, we have modified the former Fig. 8 (now included as Fig. 9) to link it better to our experiments.

7. Minor Points:
a. Pg. 6: I do not believe that NCL and NPM have been clearly defined for the general readership

We have added some information and reference on NCL in the introduction (pages 4/5).

b. Pg. 7, line 9: I would recommend changing the conclusion of 'devoid of contaminations' to 'devoid of substantial contamination' since there is clearly some background RNA associated with every fraction.

This has been changed in the text (page 7).

c. The Supplementary figs are discussed out of order in the manuscript (S4 is discussed before S2)

Figure S2, now Appendix Fig. S3 is discussed page 9, line 9, and Figure S4, now Appendix Fig. S5 is discussed page 9, line 16.

d. Pg. 7, line 9 from the bottom: extend = extent

This has been changed in the text (page 7, line 23).

References

Bierhoff H, Dammert MA, Brocks D, Dambacher S, Schotta G, Grummt I. 2014. Quiescence-Induced LncRNAs Trigger H4K20 Trimethylation and Transcriptional Silencing. *Mol Cell* 54(4): 675-682.

Ideue T, Hino K, Kitao S, Yokoi T, Hirose T. 2009. Efficient oligonucleotide-mediated degradation of nuclear noncoding RNAs in mammalian cultured cells. *RNA* 15(8): 1578-1587.

Jady BE, Ketele A, Kiss T. 2012. Human intron-encoded Alu RNAs are processed and packaged into Wdr79-associated nucleoplasmic box H/ACA RNPs. *Genes Dev* 26(17): 1897-1910.

Németh A, Conesa A, Santoyo-Lopez J, Medina I, Montaner D, Péterfia B, Solovei I, Cremer T, Dopazo J, Längst G. 2010. Initial genomics of the human nucleolus. *PLoS Genet* 6(3): e1000889.

Umylny B, Presting G, Efird JT, Klimovitsky BI, Ward WS. 2007. Most human Alu and Murine B1 repeats are unique. *J Cell Biochem* 102(1): 110-121.

Weinmann R, Roeder RG. 1974. Role of DNA-dependent RNA polymerase 3 in the transcription of the tRNA and 5S RNA genes. *Proc Natl Acad Sci U S A* 71(5): 1790-1794.
Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT. 2008. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science* 322(5902): 750-756.

2nd Editorial Decision 21 August 2015

Thank you for submitting the revised version of your manuscript toThe EMBO Journal. It has now been seen by all three original referees and their comments are shown below.

As you will see, they all find that the original criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can proceed to officially accept your manuscript there are a few minor editorial issues concerning text and figures that I need you to address:

-> We noticed that the image presented in fig 4D seems to contain non-continuous lanes and while this is in principle ok we do require that this is indicated with a line separator. In addition, we would ask you to provide the full gel image as source data for this figure.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your final revision.

2nd revision 25 August 2015

I have submitted the revised filed, which include:
- the Source Data file for Figure 4D
- modification of Figure 4 (Figure 4D now includes clear line separators)
- the image for the synopsis

We also went through another round of careful reading of the manuscript and appendix and made some corrections (typos, sentence construction, references format etc) but nothing that would change the findings and the conclusions. For you to follow the changes, Please find enclosed the “track changes” word files.

In addition, we have modified some Figures:
- Figure 2A was changed to improve clarity.
- Figure 9, I removed the black frame.

- Figure S5C was changes to improve clarity.
- Figure S9F, I added a line separator (as already mentioned in the legend, 2 unused lanes were removed).

REFEREE COMMENTS

Referee #1:

This revised version of the manuscript has been significantly strengthened by inclusion of additional critical experiments. Although I remain skeptical about a direct involvement of Pol II in nucleolar structure and the ability of microinjected nucleolar RNA to rescue the alterations induced in the nucleolus by Pol II inhibitors, I think this study will attract much attention and will certainly stimulate further investigations by the community of cell and molecular biologists interested in nuclear structure and function.

Referee #2:

In my opinion the current form of the manuscript is suitable for publishing in the EMBO J.

Referee #3:

The authors have done a reasonable job in responding to the points raised in the original round of critiques. The manuscript is improved, convincing and very interesting.

2nd Revision - authors' response 25 August 2015

I have submitted the revised filed, which include:
- the Source Data file for Figure 4D
- modification of Figure 4 (Figure 4D now includes clear line separators)
- the image for the synopsis

We also went through another round of careful reading of the manuscript and appendix and made some corrections (typos, sentence construction, references format etc) but nothing that would change the findings and the conclusions. For you to follow the changes, Please find enclosed the "track changes" word files.

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