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The RNA helicase DHX9 establishes nucleolar heterochromatin and this activity is required for embryonic stem cell differentiation

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We would like to submit our work "*The RNA helicase DHX9 establishes nucleolar heterochromatin and this activity is required for embryonic stem cell differentiation*" for your consideration for publication in EMBO Reports.

POINT-BY-POINT RESPONSE
(see following pages)

Author responses to Reviewers

We thank the Reviewers for their useful comments and suggestions. Please find enclosed here below the detailed point-by-point responses to the comments. Modifications in the text were highlighted in red.

Table of modified Figures

	Previous version	Revised version	Reviewer
coIP endogenous TIP5 and DHX9	-----	Suppl. Fig. 1D	Ref. 2&3
in-vivo interaction of DHX9 with IGS-rRNA (RIP)	-----	Fig. 3F	Ref. 1
in-vivo interaction of DHX9 mutants with IGS-rRNA (RIP)	-----	Fig. 3G	Ref. 1
Expression of DHX9 mutants do not affect expression of endogenous TIP5 (western)	data not shown	Suppl. Fig. 3	Ref. 1
ESC differentiation with IGS-rRNA		Fig. 6K	Ref. 3
ESC differentiation with pRNA mutants that do not associate with TIP5		Fig. 6K	----
Molecular markers of differentiation		Suppl. Fig. 5C	Ref. 3
Quantification of cells with TIP5 or DHX9 nucleolar localization	Fig. 5C-E	Fig. 5C-E	Ref. 3

Referee #1:

Leone et al. provide evidence for the involvement of the RNA helicase DHX9 in processing of the IGS-rRNA into pRNA, which is involved in heterochromatinization of rDNA regions. In line with this role, the authors demonstrate a requirement for DHX9 for differentiation of embryonic stem cells (ESCs). In this study, DHX9 was first identified in a biochemical pull down approach as an IGS-rRNA binder. In support of their model, the authors show that knockdown of DHX9 interferes with IGS-rRNA processing from a reporter and leads to the accumulation of endogenous unprocessed IGS-rRNA. DHX9 physically interacts with TIP5 in an RNA-independent fashion and without the requirement of the RNAse activity of DHX9. Loss of DHX9 results in reduced TIP5 recruitment to rRNA genes and conversely DHX9 localization to the nucleolus relies on TIP5, establishing a mutual dependence of these two proteins and implicating them in pRNA production. Loss of DHX9 also resulted ultimately in reduced rDNA silencing. The authors then explore the role of DHX9 in ESC differentiation since IGS-rRNA processing is known to be suppressed in ESCs and is activated as cells differentiate. DHX9 is found both in ESC and differentiated cells, but it associates more readily with rDNA in differentiating cells. In line with a role for DHX9 in ESC differentiation, it is shown that differentiation is impaired in the absence of DHX9 and can be rescued by introduction of pRNA.

This is a sound study that identifies DHX9 as a protein involved in pRNA production and in ESC differentiation. The work is technically sound for the most part and the presented experiments are well designed and interpreted. The demonstration of a requirement for IGS-rRNA processing as a requirement for differentiation is of interest.

Although the study identifies DHX9 as a IGS-rRNA processing component, its weakness is the absence of a clear mechanism. While the link to TIP5 is robust, it is not clear that the recruitment of TIP5 is a critical step and/or the only function of DHX9, especially given the reciprocity of their interaction. Overall,

although DHX9 appears to clearly play a role in IGS-rRNA processing, I am not sure I know based on the presented data how the protein exerts its function in this process, lessening my enthusiasm.

Author. We thank the reviewer for the positive comments of our work. We have here discussed the points raised in this specific paragraph

1. "*absence of a clear mechanism."* We think that some parts of the manuscript were not clearly enough and have generated some confusion, and hence the criticisms for the "*absence of a clear mechanism."* Indeed, the mechanism of how only the mature pRNA guides TIP5 to rRNA genes with consequent establishment of rDNA heterochromatin has already been identified by previous work from our lab (Savic et al., 2014). These data showed that the association of TIP5 with mature pRNA allows the interaction with TTF1, a docking factor bound to the rDNA promoter. On the contrary, the association of TIP5 with unprocessed IGS-rRNA destroys the interaction with TTF1 and impairs TIP5 recruitment to rRNA genes. We have now included the description of these results in the manuscript (p. 4, lanes 3-6; p. 9, lanes 12-16), which clarifies the mechanisms how DHX9 through the production of mature pRNA induces recruitment of TIP5 to rRNA genes and establishment of rRNA heterochromatin. Thus, **the results shown in this manuscript unraveled the events upstream to TIP5 recruitment by showing that DHX9 is responsible for the processing of IGS-rRNA into pRNA and that this process is required for ESC differentiation.** The mechanistic and functional link between TIP5, DHX9 and pRNA is now further supported by new data (**Figure 6K**). These results showed that the defects in ESC differentiation observed upon DHX9-KD cannot be rescued by addition of the unprocessed transcript IGS-rRNA or a pRNA mutant with impaired ability to associate with TIP5. Since TIP5 associated with IGS-rRNA cannot bind rRNA genes and the association of TIP5 with pRNA is required for the interaction with rRNA genes (Savic et al., 2014, Mayer et al., 2006), these results further support a model where the activity of DHX9 to produce mature pRNA is required to recruit TIP5 to rRNA genes and establish heterochromatin at nucleolus, a process that is necessary for ESC differentiation.

2. *"...it is not clear that the recruitment of TIP5 is a critical step and/or the only function of DHX9.."* We have never stated in any part of the manuscript that the only function of DHX9 is to process IGS-rRNA. Since the aim of this work was to understand the function of IGS-rRNA processing in ESC differentiation, our study was focused on the function of DHX9 in processing IGS-rRNA. To exclude eventual roles of DHX9 not linked to IGS-rRNA processing, the specific contribution of DHX9-mediated IGS-rRNA processing in TIP5 association with rRNA genes and ESCs differentiation has been demonstrated by rescuing the phenotype of DHX9-KD through the transfection of mature pRNA, the final product DHX9-mediated IGS-rRNA processing (**Figs. 5&6**). Thus, this series of experiments demonstrates that it is the activity of DHX9 to process IGS-rRNA that is required for the recruitment of TIP5 to rRNA genes and ESC differentiation. The new results of **Figure 6K** further support these conclusions.

3. *"... especially given the reciprocity of their interaction".* We do not think that TIP5 and DHX9 reciprocity of their association with rRNA genes harms our conclusion. Indeed, these results further support the interaction analysis of DHX9 and TIP5 (**Fig. 4**). The experiments of **Figure 5** showed that in differentiated

cells in the absence of DHX9 TIP5 recruitment to rRNA genes is abrogated due to impairment of processing IGS-rRNA into pRNA. On the other hand, the exclusion of DHX9 from nucleoli and rRNA genes in TIP5-KD cells represents a strong indication that in differentiated cells (which produce pRNA) DHX9 requires the association of TIP5 with mature pRNA to bind to rRNA genes. We have clarified this point in the results (p. 10 from lanes 6-7).

Experimental points:

-It would be reassuring to demonstrate in-vivo interaction of DHX9 with IGS-rRNA rather than only in vitro binding in pull down experiments.

Author. We performed RNA immunoprecipitation analysis (RIP) from formaldehyde crosslinked NIH3T3 cells and showed that DHX9 associates with IGS-rRNA specific sequences but not with pRNA (**Figure 3F**). This result is consistent with the data showing that (1) DHX9 is implicated in IGS-rRNA processing (**Figures 2,5,6**), (2) DHX9 associates with IGS-rRNA (GRNA chromatography and EMSA assays, **Figs. 1&3**) and that (3) DHX9 was not detected in GRNA chromatography experiments using pRNA as bait (discussed data not shown discussed at p. 8, lanes 13-17).

-It would also be reassuring to show pull down experiments using mutants of DHX9 and mutants of IGS-rRNA to further demonstrate specificity of the interaction.

Author. We performed RIP analysis using DHX9 mutants and found that the RNA binding domains are required for the interaction with IGS-rRNA (**Figure 3G**).

-The authors state on p. 7 that "Knockdown of DHX9 also induced a consistent accumulation of endogenous unprocessed IGS-rRNA (Fig. 2B). Depletion of DHX9 did not affect the overall levels of IGS-rRNA and pRNA sequences, indicating that DHX9 is not implicated in the synthesis of IGS-rRNA itself but most likely acts on its processing (Supplemental Fig. 1D,E)". This seems a contradiction. Please clarify this statement.

Author. We edited the description of these results to clarify this point (p. 7, lanes 15-17; and Suppl. Figure legends 1E-G). The data shown in Figure S1E-G (previous S1D-F) represent the total amount of IGS-rRNA, pRNA and 45S pre-rRNA sequences and have been obtained by amplification of cDNA generated in reverse transcription reactions using random examers. In contrast, the data of Figure 2B represent the amounts of processed vs. unprocessed rRNA since they were obtained by amplification of cDNA generated in strand specific reverse transcription reactions (reverse oligo hybridizing -20/-1 rDNA sequences). Together, the results indicated that upon DHX9 KD it is the processing of IGS-rRNA into pRNA to be affected since the total levels of IGS-rRNA and pRNA sequences (they must not be in the same molecule) is not altered whereas 45S pre-rRNA is upregulated.

-The authors cite interaction data of endogenous DHX9 with TIP5, but do not show it. Please show it. This is a key piece of data.

Author. The association of TIP5 with endogenous DHX9 has been shown in **Fig. 1D.** Moreover, we included new data showing the interaction of endogenous TIP5 and endogenous DHX9 (**Supplemental Fig. 1D**).

-The authors state that DHX9 mutants affect the expression of ectopic TIP5, but not of endogenous TIP5 (data not shown). Please explain this troubling discrepancy.

Author. We do not think that this is a troubling discrepancy since the expression of DHX9 mutants have been used only for interaction analyses (**Fig. 4C**). As stated in the text, we noted that *"co-expression of DHX9$_{\Delta DSRM}$ and DHX9$_{\Delta RGG}$ mutants negatively affects expression of ectopic Flag-HA-TIP5 (but not of the endogenous protein, data not shown*". We obtained similar results with other ectopically expressed proteins. We admit that this is an interesting phenotype, however, since it affects only ectopically expressed TIP5 we decided to draw our attention on pathways mediated by endogenous proteins. In this revised manuscript, we replaced "data not shown" with a western blot showing that TIP5 endogenous levels are not affected upon expression of DHX9$_{\Delta DSRM}$ and DHX9$_{\Delta RGG}$ mutants (**Supplementary Figure 3**).

-Please copyedit the manuscript. There are quite a few grammatical errors, missing words etc.

Author. The manuscript was extensively re-edited.

Referee #2:

In this manuscript, authors have used maintenance of ESC pluripotency as a model to study factors that regulate pRNA biogenesis, which has an influence on heterochromatinization of rRNA genes that ultimately is thought to be a key event in the course to successful differentiation. A GRNA-chromatography based approach is used to look for factors that specifically interact with IGS-rRNA, which revealed DHX9 as one of the top candidates. Authors show that DHX9 is required for proper pRNA processing which acts as guide to TIP5 that is necessary for heterochromatinization of rRNA genes.

Taking all the data presented into account, there is evidence linking DHX9 to rRNA production, either at the level of regulating pRNA biogenesis, or somehow directly rRNA transcription. However, despite many experiments to address it, the biochemical interaction of DHX9 and TIP5 is not solid. The authors did try various DHX9 (Fig. 4) or TIP5 (Fig. 1) constructs to look at this interaction more deeply, however it is still not clear whether N- or C- terminus of TIP5 interacts with N-, C- or middle part of DHX9. No mutation or deletion is able to disrupt this interaction, which might suggest that this an incredibly robust interaction and yet there is no evidence that shows that endogenously expressed TIP5 is able to interact with endogenously expressed DHX9.

Author.

Association of endogenous TIP5 and DHX9

We included new data showing the association of endogenous TIP5 with endogenous DHX9 (Supplementary **Figure 1D**), using an ESC line in which the endogenous TIP5 is expressed with a FLAG-HA tag at N-terminus. The recent establishment of this Crisp/Cas9 engineered cell line has overcome the use of TIP5 antibodies, which we have never managed to let them work for efficient co-IP experiments. The association of endogenous TIP5 and DHX9 in ESCs not only supports the solidity of the data showing the interaction of endogenous DHX9 with ectopically expressed TIP5 (**Figures 1D and 4**) but also it further supports the data showing that it is the processing of IGS-rRNA to be key for the recruitment of TIP5 to rRNA genes since in the absence of IGS-rRNA processing (in ESCs) the association of DHX9 and TIP5 is not sufficient for the recruitment of TIP5 or DHX9 to rRNA genes. We clarified this point in the result section (p. 10, lanes 24-26).

DHX9-TIP5 interaction.

We agree with the reviewer that TIP5-DHX9 is a robust interaction. The data shown in **Figure 1D** indicated that the N-terminus of TIP5 associates with endogenous DHX9 and that TIP5-DHX9 interaction does not depend on RNA (**Fig. 4**), as indicated by the results showing that (1) none of the DHX9 RNA binding domains mediates the association of TIP5, (2) this interaction is not affected by RNase A treatment and (3) TIP5 RNA binding deficient mutant still associates with DHX9. We think that not showing a *mutation or deletion able to disrupt this interaction* cannot harm the conclusion of this work demonstrating that ectopic or endogenous TIP5 and DHX9 interact and that endogenous DHX9 is necessary to process IGS-rRNA into pRNA, a reaction required for the recruitment of endogenous TIP5 to rRNA genes, rRNA gene silencing and ESC differentiation (**Fig. 4, 5 and 6**).

Fig2A: shows that unprocessed IGS-rRNA accumulate in DHX9-KD, both from a reporter and the endo. They could rescue this with GFP-DHX9 and GFP-DHX9(K417R) showing that helicase activity is not involved in this.

Author. Yes, this is exactly what the data showed.

p.17, Methods section. It is indicated twice that primer sequences are in Table S1, authors probably meant Table S4.

Author. We corrected the text.

TIP5 mass-spec: Why do the authors don't show this data. It is not sufficient to mention this information data not shown (p.5)

Author. InIn this manuscript, we listed several reasons that prompted us to analyze DHX9 among the six IGS-rRNA binding proteins identified from the GRNA chromatography assay. One of this was that we found DHX9 as TIP5-interacting protein in mass-spec analysis of TIP5 co-IP. Although we did not include

TIP5 mass spec data, we provide many data on the interaction of TIP5 and DHX9 (**Fig. 1D, Fig. 4 and new Supplementary Figure 1D**), supporting and demonstrating the mass-spec data we have not shown. There are two important reasons why we do not want to show TIP5 mass-spec data. First, the analysis of the whole TIP5 interactome is not an aim of this study. Second, TIP5 interactome is very complex and is currently under investigation in several projects of my lab. We hope that the reviewer will accept our reasons to not show these data.

Is there a particular reason why spliceosome might be implicated in pRNA processing? (p. 6)

Author. At p.6 (Result section), we described the results obtained from DHX9-interactome analyses of four cell lines (HEK293, NIH3T3, ESCs and differentiated cells). The top hit was the spliceosome and a similar result was obtained in the analysis of cellular component and pathways in each of the GRNA chromatography experiment. The discussion for the "*reason why spliceosome might be implicated in pRNA processing*" was placed in the Discussion section (p. 15). There, we discussed the following points: (1) the spliceosome as top hit in both IGS-rRNA pulled-down proteins and DHX9-associated factors, (2) the presence in the nucleolus of the identified DHX9 interacting spliceosome core components and (3) absence of ribonucleases among the identified IGS-rRNA and DHX9-interacting proteins. This latter point is important since splicing is achieved through transesterification. Moreover, we noted the absence of the canonical DExH/RHA helicases (i.e. PRPF 2, 16, 22 and 43 (Jarmoskaite and Russell, 2014) of the spliceosome complex among the DHX9 and IGS-rRNA interacting factors. Thus, it appears that DHX9 does not interact with the canonical spliceosome complex. We modified the text in both results and discussion section (pp. 6 and 15) to make clearer the reasons why we think that the spliceosome is likely to be implicated in pRNA processing.

Also, it is hard to understand the paragraph before the last (p.14). Why not finding Xrn2 helpful in understanding how spliceosome could be important for pRNA production? What careful analysis showed that Xrn2 is not involved in IGS-rRNA processing? Please explain.

Author. We would have loved to find Xrn2 as the exonuclease implicated in IGS-rRNA processing! However, as state in the Discussion, we performed experiments to test whether this was the case but we did not find any evidence supporting the role of Xrn2 in IGS-rRNA processing. We measured IGS-rRNA processing (endogenous and reporter assay) in cells depleted of Xrn2 by siRNA and did not observe defects in processing as the ones found upon DHX9 knockdown. We did not include these data since they are negative results. We modified the text to clarify how this analysis was performed (p. 15, lane 19-24).

Which recombinant DHX9 was used for EMSA (Fig.3)?

Author. We apologize with the reviewer. The information of TIP5 and DHX9 purification was inadvertently omitted in the previous version of the manuscript. EMSA assays were performed with purified recombinant

proteins. The details of the purification of DHX9 and TIP5 used in EMSA assay have now been included in Material and Method section (pp.- 23-24). Further details have been added in results and Figure legends.

DHX9 nucleoli localization (Fig5) Why not using GFP-DHX9, as in Fig.1C

Author. In this set of experiments (Fig. 5) we reasoned it was important to monitor endogenous DHX9 upon knockdown of endogenous TIP5.

Can they explain the experiment presented in Fig 2A a little bit better? It is not easy to follow the text.

Author. The text of results (p. 7), figure legend and Material and Method has been modified accordingly.

Minor points:

Figures depicting DHX9 domain organization contain a minor error for the DExH box helicase domain annotation, the domain is labeled as DEAD box.

Author. The Figure has been modified.

On Page 13 line 16 favour is misspelled as "favou"

Author. The text has been corrected.

Figure legend: Figure 3 (E) is called as (D), therefore there are two Figure 3 (D)s in the figure legends .Figure legend: Figure 4 (C) describes the mutants incorrectly, deltaDSRM is written as DDSRM and deltaRGG as DSRM referring to the left and right panels of the figure.

Author. The text has been corrected.

Referee #3:

The manuscript by Santoro and colleagues documents binding of the DHX9 helicase to rRNA, both the non processed and processed pRNA transcript, which the authors have previously shown to be required for H3K9me2 acquisition on rDNA genes upon ESC differentiation. The authors further show that siRNA for DHX9 impairs localisaiton of TIP5 on nucleolus in differentiated ESC, which they ascribe to deffective processing of the pRNA transcript. This is supported by supplementing siRNA DHX9 ES cells with the pRNA itself, which rescues the morphological phenotype of ES cell differentiation.

However, the authors do not rule out a possible differentiation rescue by the non processed transcript rDNA (controls used are 'RNA control' of the same size).

The direct, 'specific' phenotype claimed on DHX9 in regards to the processed pRNA transcript is not fully supported, which the authors should strength through either i) attempting rescuing the expeirment of ES cell differentiaton with the non processed transcript or ii) complementing with RNA binding mutants for

DHX9, compared to the wt DHX9 as described below..

While overall I find the manuscript thoughful, well written and novel, I suggest the authors renforce their model by doing the following three experiments (major comments below). I have in addition, a number of very small , mostly text corrections that should also be implemented in the revised manuscript (minor comments below). The title is a little bit out of 'scope' most of the manuscript deals with targeting Tip5 to the nucleolus, not to 'heterochromatin' establishment (actually only K9me2 is assessed as proxy and one ChIP showing rDNA binding of Tip5).

Author. We thank the reviewer for the positive comments of our work. The comments for the suggested experiments can be found in the following paragraphs. We do not think that the title is a little bit out of the scope of this work. This title does not only summarize the results of this work (DHX9 is required for IGS-rRNA processing and this activity is required for ESC differentiation) but also takes into account the well-described role of pRNA in establishing heterochromatin through recruitment of TIP5 to rRNA genes, which is key for the biological significance of this work.

Major comments.

1. The binding and specificity of the binding of DHX9 towards pRNA is not convincing at all. The gelshifts shown in figure 3 show a clear shift appearing with increasing amounts of DHX9 in both, pRNA, spacer and enhancer RNA (panel E), with a pretty similar intensity. It is unclear to me how and what has to be interpreted from the panel C, specially in comparison to the panel E (there is no shift, but a clear reduction in the labelled RNA, and more radioactivity 'stuck' at the top of the gel). The authors should a) include a positive control for binding in their panel C, and complement their gelshift in panel E with a supershift (e.g. incubation with anti-DHX9 antibody and the corresponding IgG as negative control) in addition to revise their conclusions on specific binding to pRNA and not to the non-processed transcripts: the non processed transcript full length may very well bind DHX9 (the authors only test pieces of the non processed transcript, some of which actually show binding to DHX9).

Author. We think that the description of this Figure was not clear enough and thus generated some confusion. Indeed, our conclusions are in line with the observation of this reviewer since we suggested that DHX9 has a preferential binding for IGS-rRNA specific sequences. Moreover, this conclusion is now further supported by new RIP experiments showing that DHX9 binds in vivo IGS-rRNA specific sequences but not pRNA (**Figures 3F,G**).

We clarified all the points concerning EMSA assay by modifying the text in result (pp. 7-8), figure legends and material and method sections.

The experiments shown in Figure 3 are EMSA competition assays, which have been used in the past to measure the affinity of TIP5 for a defined rRNA sequence (Mayer et al., 2006, Savic et al, 2014 etc). This assay is based on measurements of amounts of not-radiolabeled RNA sequences (in this work control RNA or rRNA) necessary to compete for the binding of recombinant TIP5 or DHX9 with an unspecific

radiolabelled RNA (RNA_{MCS}, a run-off transcripts from pBluescript). The efficiency of competition is monitored by the signal intensities of free RNA (RNA_{MCS}). Thus, in these experiments we did not alter the amounts of DHX9 or TIP5 but only the amounts of competitor RNA. The use of a defined amount of TIP5 and DHX9 moieties was determined by pilot titration experiments as the minimal amount of proteins necessary to obtain a shift of the radiolabelled RNA_{MCS} in the absence of competitor RNA (shown in the second lane of each EMSA competition experiment). We include this information in material and methods (pp. 23-24).

Figure 3B showed that TIP5 has a high affinity for pRNA since only pRNA can efficiently compete for the binding of TIP5 with radiolabelled RNA_{MCS} while control-RNA does not. This result is consistent with previous works. In Figure 3C&D, it is shown that DHX9 prefers to bind RNA (less moieties of competitor pRNA than rDNA are required to compete for DHX9 binding with radiolabelled RNA_{MCS}). However, the specificity of DHX9 for pRNA is less than the one observed for TIP5 since also the control-RNA can compete for the binding, although higher amounts of control-RNA than pRNA are required, which suggests that DHX9 has a modest higher specificity for pRNA than control-RNA (Figure 3C). Figure 4E compared the three different sequences of IGS-rRNA and reveled that less moieties of spacer and enhancer RNA than pRNA are necessary to compete for the binding with DHX9. We stated that this was a slight preferential binding of DHX9 for IGS-rRNA specific sequences. This result is consistent with the new RIP analyses from formaldehyde crosslinked NIH3T3 cells showing that DHX9 associates with IGS-rRNA but not with pRNA (**Fig. 3F,G**) and support the data obtained from the GRNA chromatography analysis that identified DHX9 as an IGS-rRNA-specific binding factor. Finally, the preferential association of DHX9 for IGS-rRNA sequences upstream of the pRNA element is also supported by the lack of DHX9 in GRNA chromatography experiments using pRNA as bait (discussed data not shown, p. 8, lanes 13-17).

We think that the description of these experiments generated further confusion as evident by the request to perform a supershift assay. Indeed, the use of antibodies in EMSA assay is well reasoned in case of nuclear extracts to prove that a defined protein binds to the radiolabeled nucleic acid. However, this is not the case of our experimental setup since we used purified recombinant DHX9 and performed EMSA competition assays. Moreover, a supershift cannot be technically detected in these experiments since DHX9-RNA complex accumulates on the top of the gel. Finally, the affinity of DHX9 for IGS-rRNA has been demonstrated with the GRNA chromatography (**Fig. 1, Supplemental Fig. 1 and Supplemental Table 1**) and now also with the RIP analyses (**Fig. 3F,G**).

2. Along the same lines, to substantiate their claim that 'DHX9-dependent processing of pRNA" is important for ESC differentiation, the authours should repeat experimetn shown in Figure 6H-J with the non processed transcript as well. Their differentiation claims could be strengthen with addition of some molecular markers (e.g. not only with brighfield pictures of ES cells colonies). The same colonies could e.g. be stained with specific markers, or the expression of the same genes shown in the panel F could be assessed.

Author.

Rescue with IGS-rRNA. We included this important experiment, which showed that differentiation defects observed upon DHX9 knockdown cannot be rescued with the transfection of the unprocessed transcript IGS-rRNA (**Fig. 6K**). This result supports our conclusion that DHX9 mediated processing of IGS-rRNA is required for ESC differentiation. Moreover, we included new data (**Fig. 6K**) showing that pRNA mutants with impaired ability to associate with TIP5 cannot rescue the differentiation defects upon DHX9-KD. This is an important result that supports the essential role of mature pRNA in recruiting TIP5 to rRNA genes and establishing nucleolar heterochromatin to allow cells to enter into differentiation.

Molecular markers of differentiation.

We included measurements of the expression of pluripotent molecular markers (*Nanog* and *Rex1*) in ESCs+LIF and differentiated cells obtained upon withdrawal of LIF (ESCs -LIF) (**Supplemental Fig. 5C)**. As expected, expression of *Nanog* and *Rex1* decreases upon withdraw of LIF, indicating that cells entered into a differentiation state.

3. Additionally, it is not clear to me, that the differentiation defect seen in DHX9 siRNA ES cells, is due to the RNA binding activity of DHX9 (which would suggest that this is the 'direct' mechanism). While my points 1 and 2 above are key, if the authors could use their vectors to rescue the differentiation phenotype, that would strengthen their conclusions (e.g. complement with wt DHX9, with the ATPase mutant that they use in the earlier figure, and with a mutant unable to bind RNA).

Author. To determine whether the RNA binding domain of DHX9 is required for ESCs differentiation, we followed the suggestion of the reviewer using DHX9 mutants unable to bind RNA in siRNA-DHX9 ESCs. However, we have encountered several technical issues that did not allow to perform this experiment. Indeed, after the transfection with siRNA, ESCs become extremely resistant for the transfection of plasmids expressing GFP-DHX9 (monitored by GFP). The efficiency was very low (ca. 10-20%) and we were unable to assess whether cells transfected with DHX9 plasmids were also transfected with siRNA. Since this experiment cannot be controlled, we feel that we cannot draw any conclusion. However, we want to make clear that the use of DHX9 mutants will not add any further insights into the requirement of DHX9-mediated IGS-rRNA processing for differentiation, which is the main conclusion of this work. Indeed, the rescue experiments with pRNA (**Figures 5 and 6**) are the key experiments that support the role of DHX9 in rRNA gene heterochromatin whereas the use of DHX9 mutants would not have been conclusive since we cannot exclude that DHX9 is implicated in other processes.

Minor comments.

1. The scale of the y axis on the right graph of figure 2B and the graph of figure 2C are rather different even though it is suppossedly the same experiment. T2he % of non processed iGS transcripts in both control and siRNA for DHX9 is VERY different between the two experiments, why is that? This casts some

doubts on the quantitative nature of these analysis, if there is so much variability : can the authors assess ratios of processed versus non processed transcript through Northern Blot It would certainly be more convencing as one can directly appreciate both transcripts on the same lane.

Author. Figure 2B and 2C are not the same experiment. Figure 2B analyzed the endogenous IGS-rRNA while Fig. 2A and 2C analyzed the IGS-rRNA reporter (and the values are very similar). In the past, extensive efforts from our and other labs failed to visualize pRNA and IGS-rRNA by Northern blot. Thus, we cannot measure IGS-rRNA processing by Northern blot.

2. Figure 4 is exclusively done with ectopically expressed proteins, which is not ideal as non specific binding may result from this. While I understand that with the mutants this is the only way forward, they could use an antibody for the endogenous respective partner for WB (e.g. TIP5 in panel B and DHX9 in panels C and D)

Author. We have now included a co-IP experiment showing the interaction between endogenous TIP5 and DHX9 (**Supplemental Fig. 1D**).

It is not clear to us how a WB for TIP5 (Fig. 4B, FLAG-HA-TIP5) and DHX9 (Figs. C and D, GFP-DHX9) might serve to evaluate whether TIP5-DHX9 association is specific. The experiments showing the association with TIP5 with DHX9 (Figures 1 and 4) contained all the controls that support that this interaction is specific. The specificity of TIP5 and the endogenous DHX9 interaction (Figure 1D) is indicated by the lack of Pol II signal in TIP5 immunoprecipitated proteins. The coIPs of Figure 4 showed an optimal control, which is the IP with HA antibodies from nuclear extracts of cell transfected only with GFP-DHX9-expression plasmids.

We realized that during the assembly of Figure 4, Figure 4D was incorrectly labeled: the interaction of wt and mutant TIP5 was measured for endogenous DHX9. We corrected this panel.

3. Figure 5, panel C : what are the 'blobs' remaining of Tip5 accumulation in the siRNA-DHX9 condition?

Author. We ignored what are the "blobs" of TIP5, which do not colocalize within nucleoli of both siRNA-control and siRNA-DHX9 treated cells.

4. Figure 5, panel E, does siRNA affect global DHX9 levels? (e.g. the lack of accumulation in the nucleolus could be do to global reduction in DHX9 levels rather than specific lack of 'retention' in the nucleolus.

Author. Measurements of DHX9 levels in siRNA-TIP5 cells have been shown in **Supplemental Figure 3** (**now 4**). The data indicated that DHX9 levels are not affected in cells depleted of TIP5. We modified the text to clarify this point (p. 10, lanes 3).

5. Throughout all the figures showing immunofluorescence data, please provide the number of cells analysed and in how many the described phenotype is observed. This is particularly important since the

authors do siRNA transfections, and therefore the efficiency of transfection is not 100% of the cells.

Author. We added this information in the corresponding Figures. We invested a lot of time and energy to optimize all siRNA transfections in order to obtain a 80-90 % transfection efficiency.

6. Figure 6, panels C and D: as above, please provide numbers of cells analysed and how many showed this phenotype.

Author. The phenotype of DHX9 localization in ESCs and differentiated cells was observed in all cells. We added this information in the text (p. 11, lanes 11-12).

7. Figure 6D - do the authors have a co-staining with UBF?

Author. Figure 6D is a life cell image and therefore we cannot detect UBF. This experiment has been done on purpose since we wanted to avoid criticisms for eventual fixation artifacts. In life cell image nucleoli can be easily visualized by phase contrast. The results from this life cell image support the IF data of UBF and DHX9 of fixed cells (**Figures 1 and 5**) showing the nucleolar localization of DHX9 in differentiated cells (e.g. NIH 3T3 and U2OS).

8. Page 7, line 11, the reference to the respective figure is missing.

Author. We added the reference of the Figure.

9. Why are there so few peptides in the mass spec data in Figure 1B, specially considering the quite large size of the identified proteins?

Author. We never stated to have identified a large number of peptides. The number of peptides showed in Figure 1B corresponds to the data of each GRNA chromatography experiment, which can be found in Supplemental Table 1.

10. Page 25, figure 3 legend, the second 'D' should be 'E'

Author. We corrected the text.

11. DNA Methylation is described in the methods, but I saw no figure using DNA Methylation data?

Author. The DNA methylation data can be found in **Figure 5G**.

1st Editorial Decision 19 April 2017

Thank you for the transfer of your revised manuscript to EMBO reports. We have now received the enclosed comments from referee 1 (former referee 3) who was asked to assess it. This referee still has one more suggestion that I would like you to incorporate before we can proceed with the official acceptance of your manuscript. Former Referee #1 already felt that the revised study would be suitable for publication in EMBO reports.

Regarding referee 1's comment, please provide the source data for figure 1D. Please upload the full gels including (handdrawn) size markers and band labels. The current "original blots" are not full gels. Source data files also need to be uploaded as separate files per figure or figure panel. They will be linked to the main figure legend online.

Figures 3F, G, 5B and 6I,J show statistical analyses that cannot be calculated since n=2. Please either repeat the experiment one more time or remove the error bars and p-values. If n=2 all datapoints from both experiments can be shown in the figure along with their mean. Figures 6K, SF4 and SF5C do not specify "n" nor the error bars. Please add this information.

Please include scale bars in all microscopy images.

The supplemental tables S1-3 should be called Datasets instead. The supplementary table S4 can either be a regular table in the methods section, or an EV table (table EV1). EV tables also need to be uplaoded as individual files.

The EMBO reports reference style is numbered and part of EndNote, please correct.

We still need a completed author checklist that can be found at
http://embor.embopress.org/authorguide#revision

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I would like to suggest a few changes to the abstract:

Long noncoding RNAs (lncRNAs) have been implicated in the regulation of chromatin conformation and epigenetic patterns. lncRNA expression levels are widely taken as an indicator for functional properties. However, the role of RNA processing in modulating distinct features of the same lncRNA is less understood. The establishment of heterochromatin at rRNA genes depends on the processing of IGS-rRNA into pRNA, a reaction that is impaired in embryonic stem cells (ESCs) and activated only upon differentiation. The production of mature pRNA is essential since it guides the repressor TIP5 to rRNA genes, and IGS-rRNA abolishes this process. Through screening for IGS-rRNA-binding proteins we here identify the RNA helicase DHX9 as a regulator of rRNA processing. DHX9 binds to rRNA genes only upon ESC differentiation and its activity guides TIP5 to rRNA genes and establishes heterochromatin. Remarkably, ESCs depleted of DHX9 are unable to differentiate and this phenotype is reverted by the addition of pRNA, whereas providing IGS-rRNA and pRNA mutants deficient for TIP5 binding are not sufficient. Our results reveal insights into lncRNA biogenesis during development and support a model in which the state of rRNA gene chromatin is part of the regulatory network that controls exit from pluripotency and initiation of differentiation pathways.

Please let me know whether you agree with these changes.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

The authors have satisfactorily addressed all the comments that I raised in my original review.

Further comments:

I have looked at the response of the authors to Reviewer's 2 points. I believe that the main concern for endogenous interaction is partly/mostly covered by the new data presented in Figure 1D (and actually also by the experiments the authors had done to address some of the points that I had raised myself). So, from this perspective, I believe authors have addressed comments by reviewer 2, also including extra discussion, explanation and text corrections.

My only request would be, after carefully looking at Figure 1D, it seems that the gels come from different experiments (the lanes are cut differently), so I would request, for the sake of transparency, that the authors make this point explicit and remove their 'boxing' around the gels in figure 1d, and present full scans of their blots in the supplements.

Other than that, the manuscript would be suitable for publication in my opinion.

1st Revision - authors' response 22 April 2017

Please find here enclosed the revised version of our manuscript "The RNA helicase DHX9 establishes nucleolar heterochromatin and this activity is required for embryonic stem cell differentiation" for your consideration for publication in *EMBO reports*.

We were very happy to see that also the last reviewer was satisfied of all the changes of our revised work. We have modified text, figures and style according to your and reviewer' suggestions.

1. We agree with your changes in the abstract and replaced the old abstract with this new version with only one small correction to add clarity ("pRNA processing" instead of "rRNA processing").

2. We corrected the statistical analysis and included, where missed, the number of performed experiments.

3. We provided the original scans of all western blots with size markers (now in Source data), which showed that all immunoblots derive from the same gel.

4. Following the reviewer's suggestion, we modified **Figure 1D** by removing the box around the gel and separating the slices. This figure was obtained by combining together splices from the same membrane of the same gel and by removing one sample that was irrelevant for this study. The previous version of this manuscript had already included the original membranes of Fig. 1D since we wanted to clarify how this Figure was build. We have now provided the full scan of this blot which shows that this experiment come from the same gel.

5. As in the case of Figure 1D, **Figure 4B** was obtained by combining together splices from the same membrane and removing samples that were irrelevant for this study. The previous version of this manuscript had already included the original membranes of Fig. 4B since we wanted to clarify how this Figure was build. We have now provided the full scan of this blot which shows that this experiment come from the same gel.

6. As you will notice, all immunoblots of **Figure 4** originate from membranes, which were cut in two slices. Indeed, since TIP5 is a large protein (ca. 250 KDa) and can be well separated from DHX9 (140 KDa), we have always cut the membranes to process them at the same time with the different antibodies and get the complete result in shorter time instead to do sequential incubation with antibodies using the same membrane. We provided the original scans for all the immunoblots of Figure 4, which showed that all the membranes derive from the same gel. All images were obtained with the Odissey Imaging System (Li-COR Bioscience) and therefore there is not handwriting as done in the past for X-ray films.

7. We provided the original scan of the immunoblot of **Fig S5B** (now EV5B). In this experiment we measured the protein levels of DHX9 in ESCs and NPCs. Since it was hard to normalize the protein levels of these two cell types, we used Pol I signal (which is known to not change) to normalize sample loading. This result is supported by qRT-PCR (EV5A). This result does not represent a major point of this work since the increase expression of DHX9 in differentiated cell is minor (1.5 fold) and per se does not explain the recruitment of DHX9 to rRNA genes.

8. We included the missing scale bars of the IF of **Figures 6C** and **6D** and corrected the "flying" bar of **5C.**

9. **Figures 6G, J and K** are images taken with a microscope that does not give scale bars. However, we want to point out that the readout of these images is to visualize the difference in cell numbers (further quantified in 6I and 6K panels) and not the size of the cells.

I wanted to thank you for all the help we received and hope that this work can be considered for publication in *EMBO reports.*

2nd Editorial Decision 25 April 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Raffaella Santoro
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2017-44330V2

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Abs were listed in the Method section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines of this study are routinely tested for mycoplasma contamination and are negative.

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D- Animal Models

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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not relevant for this work
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not relevant for this work
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Not relevant for this work
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G- Dual use research of concern

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