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ALYREF links 3'-end processing to nuclear export of nonpolyadenylated mRNAs

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Transaction Report:

(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.)

1st Editorial Decision 19th July 2018

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees and their comments are shown below.

As you will see from the reports, the referees all express interest in the findings reported in your manuscript but they also raise a number of rather extensive experimental and conceptual concerns that you will have to address in full before they can support publication here. Most importantly, the referees are concerned that large parts of the current data rely on protein overexpression and reporter constructs and they stress that you will have to establish the endogenous (and therefore functional) relevance of the individual interactions and the overall model for histone mRNA export. In addition, the referees would like to see more evidence that ALY is responsible for U7 recruitment and a deeper understanding of the molecular interaction involved (ALYREF in isolation or in the context of TREX).

Should you be able to address these criticisms in full, we could consider a revised manuscript.

REFEREE REPORTS

Referee #1:

General summary and comments:

This manuscript addresses two longstanding questions in the field of histone mRNA biology: How are non-polyadenylated replication-dependent (RD) histone transcripts exported from the nucleus to the cytosol, and how does the stem-loop binding protein SLBP recruits U7-for 3'end processing as no physical interaction was identified so far between these factors.
The authors combine iCLIP with protein interaction studies and RNA FISH imaging experiments to test their hypothesis that the NXF1 export adapter ALYREF, a core component of the TREX

complex is the long sought factor that mediates and integrates 3'end processing and export of histone transcripts. Their conclusion that ALYREF facilitates proper histone mRNA 3'-end formation by ensuring efficient U7-snRNP recruitment and promotes histone mRNA export, is based on the following findings:

i) ALYREF shows enriched binding sites in a region ~50 nt downstream of the cleavage site of all RD histone mRNAs
ii) ALYREF interacts directly and RNA-independently with SLBP
iii) ALYREF interacts directly and RNA-independently with Lsm11
iv) ALYREF knockdown impairs histone mRNA 3'end processing
v) 3'end processing promotes ALYREF recruitment and histone mRNA export

The manuscript is interesting, includes several novel and important findings and is well written. However, most of the presented data are based on plasmid-based overexpression experiments, reporter constructs or in vitro translated proteins. Before publication, I require to include several control experiments for endogenous proteins and RNAs to better support the conclusions.

Major concerns:

It is not clear how the iCLIP data were normalized to derive enriched binding of ALYREF to histone mRNAs and how significance was calculated. Usually iCLIP shows a clear correlation between binding and the expression of the target RNAs. The authors should provide also ratios of abundance and binding from non-target transcripts.

Many other proteins, including UPF1 and SR proteins apparently crosslink to the very same region of RD histone mRNAs and it seems to be a hotspot for protein crosslinking (Anko, 2012; Brooks, 2015). There are several reasons for this. On the one hand there is a high number of almost identical histone genes from the same cluster. Only their 5'UTRs and 3'UTRs distinguish them as separate genes and yield uniquely mapping CLIP-Seq reads. On the other hand, many proteins bind to the histone stem loop and it is difficult for RNase I to generate RNA fragments from this densely coated region leaving only a small region of the histone mRNAs for the detection of binding sites. Therefore binding should be confirmed for selected targets by RIP.

The in vivo interaction between SLBP and ALYREF was only shown with a FLAG-tagged SLBP expressed at high levels from a plasmid. As SLBP is normally tightly regulated and exclusively expressed in S-phase, the authors should show that there is also an interaction between the endogenous SLBP and ALYREF at physiological concentrations.

The authors claim that ALYREF knockdown affects 3'end processing. This conclusion is not enough supported by data presented.
i) A 72 knockdown of an essential export factor such as ALYREF for 72 hours may decrease the levels of important histone 3'end processing factors. The authors need to show that those are unaffected by ALYREF depletion.
ii) The observed mis-processing effects appear minor. As a control to compare the magnitude of the observed effect, the authors should include a knockdown experiment of known histone 3'end processing factors, such as Ars2.

The in vivo interaction between LSM11 and ALYREF was only shown with a FLAG-tagged LSM11 expressed at high levels from a plasmid and nevertheless appear very low. The authors should include an experiment that confirms that there is an interaction between the endogenous LSM11 and ALYREF at physiological concentrations.

If ALYREF promotes export of histone mRNAs via NXF1, then we would expect that in the absence of ALYREF less NXF1 is recruited to histone mRNAs and vice versa overexpression of ALYREF should enhance NXF1 recruitment. The authors should show that this is the case, e.g. by RIP.

Even though the authors observe a minor effect of ALYREF knockdown on 3'end processing, ALYREF should be included in the export experiments to support the main conclusion of the paper.

The RNA FISH experiments for the endogenous histone mRNAs look not very convincing and need to be improved.

The data with the ribozyme cleaved histone mRNA reporters are not entirely convincing. Normally processed histone mRNAs are very rapidly exported to the cytoplasm and are not visible in the nucleus. Since ALYREF is a non-shuttling protein that is removed from the transcript when NXF1 joins the mRNP, its interaction with the histone mRNA must be very transient and hard to detect. The reporter construct with the ribozyme on the other hand is retained in the nucleus. Both reporter transcripts have a stem loop that is co-transcriptionally bound by SLBP and SLBP is sufficient to recruit ALYREF (in vitro data). The authors also showed that ALYREF facilitates recruitment of U7 snRNP.
So why would the Ribozyme construct bind less ALYREF? It has the chance to stay much longer associated with ALYREF, because ALYREF is not removed during export?
One possibility is that is the U7 snRNP recruitment to histone mRNAs that actually stabilizes the interaction to ALYREF in cells or it even recruits ALYREF only after successful 3'end processing. The authors should discuss this possibility and also test ALYREF binding experimentally by mutating the U7 binding site or depleting Lsm11.

Minor comments:

To avoid mapping artifacts, multimapping reads have to be handled in a similar manner for the RNA-Seq and CLIP-Seq data. Please indicate how multimappers were treated in both analyses.

Figure 5: What does FISH H.M. mean?

Referee #2:

This paper is based on a previous study of the same group (Shi et al 2017 NAR), where the authors analyze the binding of ALY protein to mRNA by crosslinking and immunoprecipitation (iCLIP) experiments. In this new paper a deeper analysis of ALY/REF iCLIP data reveals that ALY binds to histone nonpolyadenylated RNAs, preferentially at the 3' UTR of histone mRNAs. The authors propose that ALY/REF plays a role connecting 3' processing and nuclear export of nonpolyadenylated mRNAs
Given the previously reported connection of ALY and its yeast ortholog protein Yra1 with the 3' end machinery and their role modulating mRNA 3'-end processing (Johnson et al 2011 Nat Struct Mol Biol), the role proposed for ALY in the processing and export of histone mRNAs in this paper is interesting, but expected. Moreover, the conclusions are not well supported by the data and there is an overinterpretation of the results. Then study is performed mainly with ALY, but conclusions all over the manuscript, even in the title, are extended to the so-called TREX complex. This is wrong and misleading. The authors cannot talk about TREX in Title, Abstract or along the manuscript; they can only refer to ALY/REF. Unless they confirm similar results with other components of TREX. It seems that authors are not really aware that TREX is a protein complex whose identity is unclear, provided that only the THO complex has been purified appropriately and part of the structure, whereas TREX is hardly seen as a purified complex, and Sub2/UAP56 and Yra1/ALY may only interact transiently with it.
Apart of this, the manuscript shows interaction of ALY/REF with a new protein that prompt them explore its function in non-polyA genes. However, the findings of the manuscript are incremental. It does not provide a major advance in understanding the role of ALY or transcription termination or 3'-end RNA processing including that of non-polyA RNAPII genes.

- The authors show that ALY/REF interacts with SLBP protein in vitro, but the interaction between the endogenous proteins in vivo is not demonstrated (see point 1). Authors claim that SLBP is required for efficient recruitment to histone mRNAs, as suggested by a reduction of ALY recruitment to histone genes, determined by RNA IPs in SLBP knock-down cells (figure 2G).

- (Figure2). The protein-protein interaction experiments are performed with tagged-protein (Co-IP

Flag tagged protein, and pull-down GST-fusion proteins, and in vitro translation experiments and pull down of MBP-tagged proteins). Co-immunoprecipitation assays with both endogenous proteins should be done in order to validate these protein interactions.
(Figure 4B) The complete image of western blot of the CoIP should be shown

- The authors propose that ALY has a role in 3' end processing of histone mRNAs. They show that ALY depletion leads to an increase in polyadenylated histone mRNAs (Figure 3). However, they do not observe an accumulation of these polyadenylated mRNAs after UAP56 or THOC2 depletion (Figure 3D), indeed they conclude that "...ALY participates in histone mRNA processing independent of other TREX components" (page 10). The molecular bases of this defect should be addressed.

Thus, in Figure 3, the defects on 3' end processing of histone mRNAs can be an indirect consequence of transcription elongation defects, as it has been recently suggested (Saldi et al Genes & Dev 2017 paper Bentley group). Authors should carry out ChIP RNAPII to test whether the increase of histone polyadenylated mRNAs in ALY KD cells is due to a slow Transcription elongation.

- The authors show that ALY recruitment to histone mRNAs were apparently reduced in SLBP KD cells as determined by RT-qPCR (Figure 2I). Nevertheless, these results can be also interpreted as an indirect consequence of a lower transcription elongation rate and recruitment of ALY.

- Using a ribozyme reporter system to compare precursor and processed transcripts, the authors demonstrate that the processing of histone mRNAs facilitate the mRNA export. They show that ALY binds preferentially to the ribozyme processed mRNA. Based on this observation they speculate that ALY could be necessary for transport of this processed mRNA, but they do not show in situ hybridization experiments in ALY knockdown cells; instead they use UAP56 in UAP56 KD cells, but not in ALY KD cells.
In Figure 6, to know the direct effect of ALY in mRNA histone export situ hybridization experiments should be performed in ALY knockdown cells. Given that the THOC5 subunit has been previously shown to affect polyadenylation choice of some genes, it would be also interesting to test also the mRNA export of histones genes in the absence of this factor.

- The model proposed (figure 7E), with two different export pathways for histone mRNA export should be tested. Test whether there is an increase of NXF1 binding to nonpolyadenylated histone RNAs in ALY knockdown cells. Binding of SR proteins and THOC5 subunit should also be tested.

Referee #3:

This manuscript from Cheng and colleagues nicely shows that the TREX component and adaptor for the mRNA export receptor is specifically recruited at the 3'end of the non-polyadenylated histone transcripts through its interaction with the Stem Loop-binding Protein and facilitates the recruitment of the U7-snRNP resulting in the pre-mRNA processing. In addition, such a recruitment of ALYREF coordinates 3' end processing and efficient nuclear export of these transcripts. This paper would in theory close the 15 years old controversy on this topic and definitely show the common pathways and machineries used by both polyadenylated and non-polyadenylated transcripts for coordinating their synthesis (including processing) to their nuclear export. However, some important conclusions need to be confirmed by additional control experiments, as outlined below:

1. In Figure 2D, the interaction between MBP-ALYREF and GST-SLBP is very weak as it can only be detected by WE and not by Coomassie staining. The same interaction also appears very weak in Figure 2C with an input corresponding to 3% of lysate used for IP. Could the authors comment on that? Would it suggest that the interaction is not direct but at least facilitated by a third partner, or a post-translational modification ?

2. The effect of SLBP knockdown on Alyref RIP as shown on Figure 2I is quite modest (with the exception of HIST1H3J). However, a negative control, such as a polyadelylated RNA is missing to correctly interpret this figure. Does ALYREF also bind at the 3'end of these histone mRNAs in the absence of SLBP? This important control is missing to analyze to which extent SLBP determines the ALYREF positioning on histone transcripts.

3. The authors affirm in the text as well as on the Figure7E that the TREX complex is recruited after 3'end processing in completed but it is not formally shown. In addition, the TREX complex is known to coordinate transcription to processing and export but not necessarily via RNA binding, as some components of the TREX complex associate with genes being transcribed rather than nascent transcripts. In this respect, it would be essential to analyze the efficiency and localization of NXF1 on histone transcripts after SLBP KD and ALYREF KD.

Minor comments
1. Why does exogenous expression of ALYREF only partially rescue the ALYREF KD effect in Figure 3H, as the expression of endogenous and ectopic ALYREF are similar (Figure 3G)?

2. Figure 4B is of very poor quality. Why is ALYREF so weak in the input. Please increase the amount of input loaded on the gel.

3. On p14, 2nd paragraph, line 9, please replace the processed H2AA3-SL by H2AA3-SLD. Otherwise, the text is in contradiction with data and title.

1st Revision - authors' response 19th January 2019

Referee #1:

General summary and comments:
This manuscript addresses two longstanding questions in the field of histone mRNA biology: How are non-polyadenylated replication-dependent (RD) histone transcripts exported from the nucleus to the cytosol, and how does the stem-loop binding protein SLBP recruits U7-for 3'end processing as no physical interaction was identified so far between these factors.
The authors combine iCLIP with protein interaction studies and RNA FISH imaging experiments to test their hypothesis that the NXF1 export adapter ALYREF, a core component of the TREX complex is the long sought factor that mediates and integrates 3'end processing and export of histone transcripts. Their conclusion that ALYREF facilitates proper histone mRNA 3'-end formation by ensuring efficient U7-snRNP recruitment and promotes histone mRNA export, is based on the following findings:

i) ALYREF shows enriched binding sites in a region ~50 nt downstream of the cleavage site of all RD histone mRNAs
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iii) ALYREF interacts directly and RNA-independently with Lsm11
iv) ALYREF knockdown impairs histone mRNA 3'end processing
v) 3'end processing promotes ALYREF recruitment and histone mRNA export

The manuscript is interesting, includes several novel and important findings and is well written.
We appreciate the reviewer's enthusiasm and positive comments.

However, most of the presented data are based on plasmid-based overexpression experiments, reporter constructs or in vitro translated proteins. Before publication, I require to include several control experiments for endogenous proteins and RNAs to better support the conclusions.
We agree that the control experiments pointed out by the reviewer are very important. We have now validated the *in vivo* interaction of ALYREF with SLBP and Lsm11 using endogenous proteins (new **Figs 2A-B** and **4A-B**). We have also included three polyadenylated mRNAs as controls for the specificity of SLBP in regulating ALYREF binding on histone mRNAs (revised **Fig 2I**). The details are described in the point-to-point response in below.

Major concerns:
It is not clear how the iCLIP data were normalized to derive enriched binding of ALYREF to histone mRNAs and how significance was calculated. Usually iCLIP shows a clear correlation between binding and the expression of the target RNAs. The authors should provide also ratios of abundance and binding from non-target transcripts.
The ratio of histone iCLIP-seq read population to histone RNA-seq read population was calculated and shown, with the histone RNA-seq read population set as "1". The significance was calculated based on three iCLIP replicates. We have now included this information in the figure legend. As requested, we now show the ratios of ALYREF binding to abundance of long and short ncRNAs as well (new **Fig EV1A**). Importantly, ALYREF binding is not enriched on these RNAs, indicating the binding on histone mRNAs is specific.

Many other proteins, including UPF1 and SR proteins apparently crosslink to the very same region of RD histone mRNAs and it seems to be a hotspot for protein crosslinking (Anko, 2012; Brooks, 2015). There are several reasons for this. On the one hand there is a high number of almost identical histone genes from the same cluster. Only their 5'UTRs and 3'UTRs distinguish them as separate genes and yield uniquely mapping CLIP-Seq reads. On the other hand, many proteins bind to the histone stem loop and it is difficult for RNase I to generate RNA fragments from this densely coated region leaving only a small region of the histone mRNAs for the detection of binding sites. Therefore binding should be confirmed for selected targets by RIP.
We agree with the reviewer that it is important to carefully examine whether ALYREF specifically binds histone mRNAs at the SL region, considering that multiple proteins have been detected at this region.

1. As suggested, we have carried out ALYREF RIP-RT-qPCRs to examine the specificity of ALYREF binding on histone mRNAs. As shown in the new **Fig 1C**, ALYREF was significantly enriched on histone mRNAs, but not on a control tRNA.

2. To examine whether ALYREF enrichment at the SL region is due to inefficient RNase I digestion, we have optimized iCLIP conditions to ensure that RNase I digestion efficiency of the SL region is not generally lower than that of a 5' region of histone mRNAs (new **Appendix Fig S1**). Under this condition, in Cntl cells, ALYREF binding was still mostly enriched at the 3' region, although it was also partially detected at other regions (new **Fig 2J**). Also, KD of SLBP preferentially reduced ALYREF binding at the 3' region of histone mRNAs (new **Fig 2J**). In contrast, ALYREF distribution along the polyA+ mRNA was not apparently affected (new **Fig EV2C**). In addition, the new NXF1 iCLIP data demonstrate that NXF1 binding was mostly enriched at the 5' region, rather than the 3' region, on histone mRNAs (new **Fig EV5F**). These new data together support the idea that ALYREF binding enrichment at the SL region is specific.

The in vivo interaction between SLBP and ALYREF was only shown with a FLAG-tagged SLBP expressed at high levels from a plasmid. As SLBP is normally tightly regulated and exclusively expressed in S-phase, the authors should show that there is also an interaction between the endogenous SLBP and ALYREF at physiological concentrations.
We thank the reviewer for this suggestion. Following the suggestion, we have enriched S-phase HeLa cells and carried out IPs using ALYREF and SLBP antibodies. Consistent with the Flag-SLBP IP data (**Fig EV2A**), we found that endogenous ALYREF and SLBP proteins associate with each other in the absence of RNA (new **Fig 2A**, **B**), indicating that they interact at physiological concentrations.

The authors claim that ALYREF knockdown affects 3'end processing. This conclusion is not enough supported by data presented.
i) A 72 knockdown of an essential export factor such as ALYREF for 72 hours may decrease the levels of important histone 3'end processing factors. The authors need to show that those are unaffected by ALYREF depletion.

To address this concern of the reviewer, we have examined protein and/or mRNA levels of multiple histone 3' processing factors, including SLBP, Lsm11, ARS2, ZFP100, FLASH, CBP80, CFI-68, and CstF64, in Cntl and ALYREF KD cells. No apparent impact of ALYREF KD on either mRNA or protein levels of these factors was observed (new **Fig EV3B**, **C**), suggesting that 3' processing defect of histone pre-mRNAs in ALYREF KD cannot be ascribed to decreased levels of 3'

processing factors. Consistent with this possibility, KD of another key mRNA export factor, THOC2, for 72 hr did not result in similar histone mRNA 3' processing defects (**Fig 3D** and **EV3E**).

ii) The observed mis-processing effects appear minor. As a control to compare the magnitude of the observed effect, the authors should include a knockdown experiment of known histone 3'end processing factors, such as Ars2.
As suggested, we have now included ARS2 KD as a positive control for 3' processing defects. In general, the effect of ALYREF KD on histone mRNA processing was minor than that of ARS2 KD (revised **Fig 3E**, **F**). Considering the high abundance of ALYREF in the cells (5-fold of ARS2) (Biochem J. 2016 473:2911-35), it is possible that the leftover of ALYREF is enough for 3' processing of a significant fraction of histone pre-mRNAs in the KD cells.

The in vivo interaction between LSM11 and ALYREF was only shown with a FLAG-tagged LSM11 expressed at high levels from a plasmid and nevertheless appear very low. The authors should include an experiment that confirms that there is an interaction between the endogenous LSM11 and ALYREF at physiological concentrations.
We agree with the reviewer that examination of the interaction between endogenous Lsm11 and ALYREF is important. We have thus enriched S-phase cells and carried out co-IP experiments using antibodies to ALYREF and Lsm11 in the presence of RNase A. Consistent with the data obtained with overexpressed Flag-Lsm11 (revised **Fig EV4A**, **B**), endogenous ALYREF and Lsm11 co-precipitated each other in an RNA-independent manner (new **Fig 4A**, **B**). Thus, ALYREF indeed interacts with Lsm11 at physiological concentrations. The reviewer also raised a point that the *in vivo* interaction of ALYREF with Lsm11 seems weak. This is possibly due to that the *in vivo* ALYREF-Lsm11 interaction is transient, as histone mRNA processing occurs very rapidly.

If ALYREF promotes export of histone mRNAs via NXF1, then we would expect that in the absence of ALYREF less NXF1 is recruited to histone mRNAs and vice versa overexpression of ALYREF should enhance NXF1 recruitment. The authors should show that this is the case, e.g. by RIP.
To address this question of the reviewer, we have carried out NXF1 iCLIP in Cntl and ALYREF KD cells. We found that ALYREF KD indeed reduced NXF1 iCLIP read population on histone mRNAs (new **Fig 5F**), supporting the notion that ALYREF promotes histone mRNA export via NXF1. We think the reviewer's suggestion of examination of NXF1 binding in ALYREF overexpression cells is very good. However, we found that ALYREF overexpression did not promote histone mRNA export (new **Fig EV5D-E**), suggesting that ALYREF is not a limiting factor for histone mRNA export. In line with this notion, ALYREF is 7.5-fold more abundant than NXF1 (Biochem J. 2016 473:2911-35). We thus did not use ALYREF overexpression cells to further examine its role in NXF1 recruitment.

Even though the authors observe a minor effect of ALYREF knockdown on 3'end processing, ALYREF should be included in the export experiments to support the main conclusion of the paper.
We thank the reviewer for this suggestion. As requested, we have examined histone mRNA export in ALYREF KD. As shown in revised **Figs 5A-B** and **EV5A**, ALYREF KD resulted in increased nuclear/cytoplasmic ratios of both exogenous and endogenous histone mRNAs. This result lends a strong support to the role of ALYREF in nuclear export of histone mRNAs.

The RNA FISH experiments for the endogenous histone mRNAs look not very convincing and need to be improved.
As requested, we have improved the endogenous histone mRNA FISH experiments and now the data show that KD of ALYREF, THOC2, or UAP56 all resulted in increased nuclear signals and concomitantly reduced cytoplasmic signals of endogenous HIST2H2AA3 and HIST1H3H mRNAs (revised **Fig 5B** and **EV5A**).

The data with the ribozyme cleaved histone mRNA reporters are not entirely convincing. Normally processed histone mRNAs are very rapidly exported to the cytoplasm and are not visible in the nucleus. Since ALYREF is a non-shuttling protein that is removed from the transcript when NXF1 joins the mRNP, its interaction with the histone mRNA must be very transient and hard to detect. The reporter construct with the ribozyme on the other hand is retained in the nucleus. Both reporter transcripts have a stem loop that is co-transcriptionally bound by SLBP and SLBP is sufficient to recruit ALYREF (in vitro data). The authors also showed that ALYREF facilitates recruitment of U7 snRNP. So why would the Ribozyme construct bind less ALYREF? It has the chance to stay much

longer associated with ALYREF, because ALYREF is not removed during export? One possibility is that is the U7 snRNP recruitment to histone mRNAs that actually stabilizes the interaction to ALYREF in cells or it even recruits ALYREF only after successful 3'end processing. The authors should discuss this possibility and also test ALYREF binding experimentally by mutating the U7 binding site or depleting Lsm11.
We thank the reviewer for raising this point. Actually, to compare ALYREF association with U7-snRNP processed vs. ribozyme cleaved H1C mRNAs in an unbiased manner, we had blocked both of them in the nucleus by overexpressing the VSV M protein, which suppresses mRNA export by targeting nuclear pore proteins Nup98 and Rae1 (Mol Cell. 2005; 17: 93-102; Mol Cell. 2000; 6: 1243-1252). In the previous version of the manuscript, we had only mentioned this in the figure legend. We have now clearly explained that in the main text.

In addition, the reviewer raised a question why ribozyme cleaved histone mRNAs bind less ALYREF, considering that it also binds SLBP. We would like to point out that the reduced ALYREF binding on histone mRNAs in SLBP KD (revised **Fig 2I** and new **Fig 2J**) indicate SLBP is required for efficient ALYREF recruitment. If SLBP is also sufficient for this binding, one would expect mRNAs containing the SL structure, which co-transcriptionally binds SLBP, are exported efficiently. However, this is not the case. Export assays using reporter constructs demonstrate that only U7-snRNP processed histone mRNAs can be efficiently exported (**Figs 6A-C**), although all reporter mRNAs contain the SL structure, suggesting that SL/SLBP is not sufficient for ALYREF recruitment. Indeed, we found that ALYREF preferentially associates with U7-snRNP processed histone mRNAs, as compared to ribozyme cleaved ones (**Figs 6D-E**).

We thank the reviewer for raising the two possibilities for 3'-processing-dependent ALYREF recruitment. To test the possibility that U7-snRNP might stabilize ALYREF binding on histone pre-mRNAs, as suggested by the reviewer, we have mutated U7-snRNA binding sequence in the H1C-SLD-Rb reporter construct. This mutation significantly reduced ALYREF binding (new **Fig 6F**) and histone mRNA export (**Appendix Fig S5B**), supporting the view that U7-snRNP stabilizes ALYREF on histone mRNAs. The other possibility the reviewer raised is that ALYREF might be recruited after proper 3' processing. Our new RIP-RT-qPCR data showing that ALYREF associates with histone pre-mRNAs (new **Fig EV4C**) refute this possibility. Further, the data that ALYREF KD resulted in reduced U7-snRNP recruitment and defective histone mRNA processing are also inconsistent with this possibility. Thus, our data are more consistent with the former possibility, namely, U7-snRNP recruitment stabilizes ALYREF binding.

Minor comments:
To avoid mapping artifacts, multimapping reads have to be handled in a similar manner for the RNA-Seq and CLIP-Seq data. Please indicate how multimappers were treated in both analyses.
We only analyzed uniquely mapped reads for both RNA-seq and iCLIP-seq data. We have now made this clear in the method.

Figure 5: What does FISH H.M. mean?
H.M. meant high magnification. Note that in the revised manuscript, higher magnification has been removed.

Referee #2:
This paper is based on a previous study of the same group (Shi et al 2017 NAR), where the authors analyze the binding of ALY protein to mRNA by crosslinking and immunoprecipitation (iCLIP) experiments. In this new paper a deeper analysis of ALY/REF iCLIP data reveals that ALY binds to histone nonpolyadenylated RNAs, preferentially at the 3' UTR of histone mRNAs. The authors propose that ALY/REF plays a role connecting 3' processing and nuclear export of nonpolyadenylated mRNAs.
Given the previously reported connection of ALY and its yeast ortholog protein Yra1 with the 3' end machinery and their role modulating mRNA 3'-end processing (Johnson et al 2011 Nat Struct Mol Biol), the role proposed for ALY in the processing and export of histone mRNAs in this paper is interesting, but expected.
We thank the reviewer for agreeing that our findings of ALYREF promoting histone mRNA processing and export are interesting. ALYREF is well-known for its roles in nuclear export of polyadenylated mRNAs. Further, ALYREF, and its yeast counterpart Yra1, are physically and/or functionally linked to 3' processing of polyadenylated mRNAs (Mol Cell. 2009; 33:215-26; Nat

Struct Mol Biol. 2011; 18:1164-71). However, it is unknown whether ALYREF or any other TREX component is involved in regulating RNAs beyond polyadenylated mRNAs. Further, the current view is that processing and nuclear export machineries for polyadenylated and nonpolyadenylated mRNAs are largely distinct. Thus, we had not anticipated a role of ALYREF in histone mRNA metabolism before we observed its prevalent binding on these mRNAs. Based on the comment of the reviewer, we have now modified our expression. In the revised manuscript, we emphasize that these roles are important, rather than unexpected.

Moreover, the conclusions are not well supported by the data and there is an overinterpretation of the results. Then study is performed mainly with ALY, but conclusions all over the manuscript, even in the title, are extended to the so-called TREX complex. This is wrong and misleading. The authors cannot talk about TREX in Title, Abstract or along the manuscript; they can only refer to ALY/REF. Unless they confirm similar results with other components of TREX. It seems that authors are not really aware that TREX is a protein complex whose identity is unclear, provided that only the THO complex has been purified appropriately and part of the structure, whereas TREX is hardly seen as a purified complex, and Sub2/UAP56 and Yra1/ALY may only interact transiently with it.
We apologize for confusing the roles of ALYREF and the TREX complex in histone mRNA metabolism. We agree with the reviewer for histone mRNA processing, only ALYREF is involved and have thus removed the TREX complex from the title, abstract and related text. However, we would like to point out that our data support the view that TREX components function in histone mRNA export as an integrated complex. First, KD of ALYREF, UAP56, and THO all inhibited histone mRNA export (revised **Figs 5A-D** and **EV5A**). Second, ALYREF KD reduced THOC2 binding on most histone mRNAs we examined (new **Fig EV5B-C**). In line with this view, although THO interactions with ALYREF and UAP56 are dynamic, in the mammalian system, the interaction and recruitment of ALYREF, THO and UAP56 are inter-dependent (Nucleic Acids Res. 2013; 41:1294-306).

Apart of this, the manuscript shows interaction of ALY/REF with a new protein that prompt them explore its function in non-polyA genes. However, the findings of the manuscript are incremental. It does not provide a major advance in understanding the role of ALY or transcription termination or 3'-end RNA processing including that of non-polyA RNAPII genes.
The reviewer raised a concern that our work does not provide a major advance in understanding the role of ALYREF in histone mRNA 3' processing. Following the suggestions of the reviewer (details in below), we have now carried out extensive experiments to further investigate this role and to understand the molecular basis.
Together with the new data, our work reveals that ALYREF is prevalently recruited to a region near the SL structure on histone pre-mRNAs (**Figs 1** and **EV1**), and SLBP plays a determinant role in this recruitment (**Fig 2I** and new **Fig 2J**). ALYREF, in the context of TREX, associates with U7 snRNP and histone pre-mRNAs (new **Fig EV4C**). Interestingly, ALYREF, but not UAP56, makes direct contact with both SLBP and the U7-snRNP component, Lsm11 (**Figs 2D**, **4C**; new **Figs EV2B** and **EV4D**). This suggests that ALYREF might be the long-sought mediator of SLBP and U7-snRNP. In support of this view, ALYREF KD resulted in reduced U7-snRNP recruitment and widespread 3' processing defect of histone pre-mRNAs (**Figs 4D-E** and **Fig 3**). Further, our study also reveals that ALYREF, together with other TREX component, functions in histone mRNA export as an integrated complex (revised **Figs 5A-B** and **EV5A-C**). Importantly, we demonstrate that 3' processing promotes ALYREF recruitment and histone mRNA export (**Figs 6A-E**), and provide evidence that U7-snRNP is important for this promotion (new **Fig 6F**). Now, we hope that the reviewer agrees that our study reveals the important aspects of histone mRNA metabolism.

- The authors show that ALY/REF interacts with SLBP protein in vitro, but the interaction between the endogenous proteins in vivo is not demonstrated (see point 1). Authors claim that SLBP is required for efficient recruitment to histone mRNAs, as suggested by a reduction of ALY recruitment to histone genes, determined by RNA IPs in SLBP knock-down cells (figure 2G).
We agree with the reviewer that examination of endogenous ALYREF and SLBP protein interaction is important to support the main conclusion of the work. We have thus carried out co-IP experiments using S-phase cells and antibodies to ALYREF and SLBP. Consistent with the data obtained with Flag-SLBP (**Fig EV2A**), endogenous SLBP and ALYREF were co-precipitated with each other in the absence of RNAs (new **Figs 2A**, **B**).

ALYREF RIP-RT-qPCR data obtained from Cntl and SLBP KD cells indicated that SLBP is required for efficient ALYREF binding on histone mRNAs (**Fig 2I**). To determine how SLBP impacts ALYREF distribution along the histone mRNA, we have now carried out ALYREF iCLIP in Cntl and SLBP KD cells. Significantly, SLBP KD reproducibly resulted in a preferential reduction in ALYREF binding at the 3' region of histone mRNAs (new **Fig 2J**). These data lend a strong support to the view that SLBP plays a determinant role in ALYREF binding at the 3' region of histone mRNAs.

- (Figure2). The protein-protein interaction experiments are performed with tagged-protein (Co-IP Flag tagged protein, and pull-down GST-fusion proteins, and in vitro translation experiments and pull down of MBP-tagged proteins). Co-immunoprecipitation assays with both endogenous proteins should be done in order to validate these protein interactions.
(Figure 4B) The complete image of western blot of the CoIP should be shown.

We thank the reviewer for this suggestion. As described above, we have now validated the interaction of endogenous proteins of ALYREF and SLBP (new **Fig 2A**, **B**). We have also improved the co-IP experiment and replaced the data in the previous Figure 4B. Now we show that complete image of western blot (revised **Fig EV4B**).

- The authors propose that ALY has a role in 3' end processing of histone mRNAs. They show that ALY depletion leads to an increase in polyadenylated histone mRNAs (Figure 3). However, they do not observe an accumulation of these polyadenylated mRNAs after UAP56 or THOC2 depletion (Figure 3D), indeed they conclude that "...ALY participates in histone mRNA processing independent of other TREX components" (page 10). The molecular bases of this defect should be addressed.
We thank the reviewer for raising this important question. To understand the molecular basis for the role of ALYREF in promoting histone mRNA processing, we have first examined the association of other TREX components with U7-snRNP. We found that similar to ALYREF, UAP56 and THO also associate with Lsm11 *in vivo* (revised **Fig EV4A**). Further, our new RIP-RT-qPCR data showed that ALYREF, UAP56 and THO are all detected on histone pre-mRNAs (new **Fig EV4C**). These data together suggest that ALYREF interacts with U7-snRNP on histone pre-mRNAs in the context of TREX. We then asked whether, similar to ALYREF, other TREX components also directly interact with SLBP and U7-snRNP. We note that it is hard to obtain the purified THO, as it is a 6-subunit complex with ~500 kD molecular weight. We thus used UAP56 to answer this question. We found that unlike ALYREF, UAP56 did not physically interact with either SLBP or Lsm11 (new **Figs EV2B** and **EV4D**). These results suggest that although the whole TREX complex associates with U7-snRNP on histone pre-mRNAs, only ALYREF makes direct contact with SLBP and U7-snRNP. This provides an explanation for specific role of ALYREF in histone mRNA 3' processing. We have modified our model based on these new results (revised **Fig 7D**).

Thus, in Figure 3, the defects on 3' end processing of histone mRNAs can be an indirect consequence of transcription elongation defects, as it has been recently suggested (Saldi et al Genes & Dev 2017 paper Bentley group). Authors should carry out ChIP RNAPII to test whether the increase of histone polyadenylated mRNAs in ALY KD cells is due to a slow Transcription elongation.
The reviewer raised the possibility that histone mRNA processing defects in ALYREF KD could be due to slow transcription elongation. To examine this possibility, as requested, we have carried out RNAP II ChIP-PCRs to examine its distribution along three histone genes exhibiting significant processing defects in ALYREF KD. As shown in the new **Fig EV3D**, no apparent RNAP II distribution change along these genes was observed in ALYREF KD vs Cntl cells, suggesting that 3' processing defects cannot be mainly ascribed to slow transcription elongation.

- The authors show that ALY recruitment to histone mRNAs were apparently reduced in SLBP KD cells as determined by RT-qPCR (Figure 2I). Nevertheless, these results can be also interpreted as an indirect consequence of a lower transcription elongation rate and recruitment of ALY.
The reviewer raised the possibility that SLBP KD might reduce ALYREF recruitment through altering RNAP II transcription elongation. Similar to ALYREF KD, SLBP KD did not apparently impact RNAP II distribution along histone genes we examined (new **Fig EV3D**), suggesting that SLBP KD did not apparently slow down transcription elongation. In line with this notion, studies so far do not support a role of SLBP in transcription elongation of histone genes. First, SLBP was not detected in the immunoprecipitate of either phosphorylated or nonphosphorylated forms of RNAP II

(Cell Rep. 2017; 20:1173-1186). Second, SLBP is specifically bound at the SL region of histone genes (Genes Dev. 2018; 32:297-308), consistent with the idea that SLBP recruitment is mediated by binding to the SL structure of nascent histone transcripts, rather than through recruitment to the RNAP II.

- Using a ribozyme reporter system to compare precursor and processed transcripts, the authors demonstrate that the processing of histone mRNAs facilitate the mRNA export. They show that ALY binds preferentially to the ribozyme processed mRNA. Based on this observation they speculate that ALY could be necessary for transport of this processed mRNA, but they do not show in situ hybridization experiments in ALY knockdown cells; instead they use UAP56 in UAP56 KD cells, but not in ALY KD cells. In Figure 6, to know the direct effect of ALY in mRNA histone export situ hybridization experiments should be performed in ALY knockdown cells. Given that the THOC5 subunit has been previously shown to affect polyadenylation choice of some genes, it would be also interesting to test also the mRNA export of histones genes in the absence of this factor.
We would like to clarify that ALYREF preferentially binds with U7-snRNP-processed histone mRNAs, as compared to ribozyme cleaved ones (Fig **6D**, **E**).
As suggested, we have now included ALYREF KD cells for histone mRNA export experiments. Consistent with the view that ALYREF coordinates processing and nuclear export of histone mRNAs, ALYREF KD impaired nuclear export of both exogenous and endogenous histone mRNAs (revised **Figs 5A-B** and **EV5A**). The reviewer also requested to examine the impact of THOC5 KD on histone mRNA export. The KD efficiencies of THOC5 siRNAs (including the one used in Nucleic Acids Res. 2013; 41:7060-72) were very low in our hands. Considering that THOC2 KD results in co-KD of other THO subunits including THOC5 (Nucleic Acids Res. 2013; 41:1294-306) and impacts alternative polyadenylation (**Reviewer Figure 1**), we used THOC2 siRNA instead. In line with previous findings with polyadenylated mRNAs, KD of THO also impaired nuclear export of histone mRNAs (revised **Figs 5A-B** and **EV5A**), indicating that the whole TREX complex is involved in histone mRNA export.

Figures for Referees not shown.

We would like to point out that based on our data, we reason that ALYREF KD might lead to decreased NXF1 binding on nonpolyadenylated histone mRNAs. We have now carried out NXF1 iCLIP to examine this possibility. As shown in the new **Fig 5F**, ALYREF KD indeed led to reduced NXF1 iCLIP read population on histone mRNAs. As suggested, we have carried out RIPs to examine how ALYREF KD impacts THO binding on histone mRNAs. Using the THOC2 antibody, which co-precipitated the whole THO complex (Nucleic Acids Res. 2013; 41:1294-306; Genes Dev. 2005; 19:1512-1517), we found that ALYREF KD reduced the binding of THO on 5 out of 6 histone mRNAs we examined (new **Fig EV5B-C**), supporting the notion that TREX components promotes histone mRNA export as an integrated complex.

The reviewer also suggested to examine how SR protein binding on histone mRNAs is impacted by ALYREF KD. To do this, we have tried several commercial available SR antibodies (e.g. SRSF3, Thermo #334200 and SRSF2, Sigma #S4045). However, none of them worked for RIP in our hands. We would like to point out that sequence-dependent binding of SR proteins on histone mRNAs has been well established (Mol Cell. 2001; 7:899-905). Further, no physical or functional interaction of SR proteins with TREX proteins has been identified. Thus, we speculate that SR binding on histone mRNAs might not be affected by ALYREF. Considering that this is not a main point of this work and we do not have experimental evidence, we have removed SR protein part from the model and more focused on our findings with ALYREF.

Referee #3:
This manuscript from Cheng and colleagues nicely shows that the TREX component and adaptor for the mRNA export receptor is specifically recruited at the 3'end of the non-polyadenylated histone transcripts through its interaction with the Stem Loop-binding Protein and facilitates the recruitment of the U7-snRNP resulting in the pre-mRNA processing. In addition, such a recruitment of ALYREF coordinates 3' end processing and efficient nuclear export of these transcripts. This paper would in theory close the 15 years old controversy on this topic and definitely show the common pathways and machineries used by both polyadenylated and non-polyadenylated transcripts for coordinating their synthesis (including processing) to their nuclear export. However,

some important conclusions need to be confirmed by additional control experiments, as outlined below:
We thank the reviewer for the highly positive comments on our work.

1. In Figure 2D, the interaction between MBP-ALYREF and GST-SLBP is very weak as it can only be detected by WE and not by Coomassie staining. The same interaction also appears very weak in Figure 2C with an input corresponding to 3% of lysate used for IP. Could the authors comment on that? Would it suggest that the interaction is not direct but at least facilitated by a third partner, or a post-translational modification?
The reviewer raised a concern that the interaction of purified ALYREF with SLBP is weak and can only be detected by western blotting. We have improved the pull down experiment and now the interaction can be detected by coomassie staining (revised **Fig 2D**). We would like to note that in the pull-down experiment of *in vitro* translated SLBP, around 10% of input was pulled down, indicating that the interaction is meaningful. Despite of this, we agree with the reviewer on the possibility that modifications of SLBP and ALYREF could enhance their interaction. In line with this, previous studies have shown that phosphorylation and ubiquitination of SLBP facilitates its RNA binding and 3' processing activities, respectively (Proc Natl Acad Sci U S A. 2006; 103:3094-9; Mol Cell. 2016; 62:627-35). Similarly, methylation and phosphorylation of ALYREF also impact its functions (Nucleic Acids Res. 2010; 38:3351-61; Proc Natl Acad Sci U S A. 2008; 105:8649-54). We have included these discussions in the revised manuscript.

2. The effect of SLBP knockdown on Alyref RIP as shown on Figure 2I is quite modest (with the exception of HIST1H3J). However, a negative control, such as a polyadenylated RNA is missing to correctly interpret this figure. Does ALYREF also bind at the 3'end of these histone mRNAs in the absence of SLBP? This important control is missing to analyze to which extent SLBP determines the ALYREF positioning on histone transcripts.
The reviewer pointed out that the effect of SLBP KD on ALYREF RIP is modest. Our new ALYREF iCLIP data demonstrate that in Cntl cells, except for the most prominent binding at the 3' region, ALYREF could also be partly detected at the 5' and the middle regions, and SLBP KD preferentially reduced ALYREF binding at the 3' region (new **Fig 2J**). Thus, the modest effect of SLBP KD might be due to the specific reduction of ALYREF binding at the SL region, but not other regions.

As suggested, we have now included three polyadenylated mRNAs to examine the specificity of SLBP KD impact on ALYREF binding with histone mRNAs. As shown in revised **Fig 2I**, SLBP KD did not apparently affect ALYREF association with these polyadenylated mRNAs, supporting the notion that SLBP specifically affects ALYREF binding on histone mRNAs. In line with this, the new ALYREF iCLIP data demonstrate that SLBP KD preferentially reduced ALYREF binding at the 3' region of histone mRNAs, but did not apparently affect its distribution along the polyA+ mRNA (new **Figs 2J** and **EV2C**).

3. The authors affirm in the text as well as on the Figure7E that the TREX complex is recruited after 3'end processing in completed but it is not formally shown. In addition, the TREX complex is known to coordinate transcription to processing and export but not necessarily via RNA binding, as some components of the TREX complex associate with genes being transcribed rather than nascent transcripts. In this respect, it would be essential to analyze the efficiency and localization of NXF1 on histone transcripts after SLBP KD and ALYREF KD.
We thank the reviewer for raising this point. To examine whether the TREX complex is recruited after 3' processing is completed, we have carried out ALYREF, UAP56 and THO RIPs. RT-qPCR data revealed that similar to ALYREF, UAP56 and THO also associate with histone pre-mRNAs (new **Fig EV4C**), indicating that the TREX complex is recruited before 3' processing is accomplished. In agreement with this view, UAP56 and THO also associate with Lsm11 (revised **Fig EV4A**). However, unlike ALYREF, UAP56 did not physically interact with either SLBP or Lsm11 (new **Figs EV2B** and **EV4D**). This new result, together with the finding that ALYREF, but not UAP56 and THO, promotes histone pre-mRNA processing, suggests that probably only ALYREF functions in mediating the interaction between SLBP and U7-snRNP. Based on these new data, we have fixed our model. In the new model, ALYREF, in the context of TREX, is recruited to histone pre-mRNAs by SLBP, on which only ALYREF directly interacts with U7-snRNP and facilitates its recruitment.

To address the question whether ALYREF binding indeed impacts NXF1 recruitment, we have carried out NXF1 iCLIP in cells treated with siCntl, siALYREF or siSLBP. We found that KD of ALYREF and SLBP both reduced NXF1 iCLIP read population on histone mRNAs (new **Fig 5F**), suggesting that they are required for efficient NXF1 recruitment. Further, SLBP KD resulted in a preferential reduction in NXF1 binding at the 3' region of histone mRNAs (new **Fig 5G** and **EV5F**), indicative of a determinant role SLBP in NXF1 binding at the 3' region. In contrast, ALYREF KD did not demonstrate such trend (new **Fig 5G** and **EV5F**). This might be due to the enhancement of ALYREF in NXF1 binding along the full-length of histone mRNA, but not restricted to the 3' region. Also, except for ALYREF, other proteins might also contribute to NXF1 binding at the 3' region. In line with this, SR proteins that also serve as NXF1 adaptors bind at the 3' region of histone mRNAs (Genome Biol. 2012; 13:R17). We have discussed these possibilities in the revised manuscript.

Minor comments

1. Why does exogenous expression of ALYREF only partially rescue the ALYREF KD effect in Figure 3H, as the expression of endogenous and ectopic ALYREF are similar (Figure 3G)?

The reviewer raised a concern that exogenous expression of ALYREF only partially restored the ALYREF KD effect on histone mRNA processing. We note that for some unknown reason, exogenously expressed ALYREF is partly mis-localized to the cytoplasm (**Reviewer Figure 2**). Thus, the partial rescue effect might be due to this mis-localization. We would like to point out the reproducible rescue effect, together with the observation that two different ALYREF siRNAs led to similar 3' processing defect, indicate that this defect is unlikely due to siRNA off-target effect.

Figures for Referees not shown.

2. Figure 4B is of very poor quality. Why is ALYREF so weak in the input. Please increase the amount of input loaded on the gel.

As requested, we have improved the experiments and replaced the data (revised **Fig EV4B**).

3. On p14, 2nd paragraph, line 9, please replace the processed H2AA3-SL by H2AA3-SLD. Otherwise, the text is in contradiction with data and title.

As requested, we have replaced the processed H2AA3-SL by H2AA3-SLD.

Accepted 14th February 2019

Thank you for submitting your revised manuscript to The EMBO Journal. I have taken over its handling from my colleague Anne Nielsen, who has recently left our journal. Your manuscript has now been re-reviewed by the original referees, and I am pleased to inform you that all of them consider the study significantly improved and the earlier concerns adequately addressed. Referee 1 raises an issue related to the generation/presentation of the new data in Figure EV4, which I notice is easily clarified through the Figure Source Data containing images of full gels and blots, which you kindly already uploaded with the revised version. However, I realize that a clear explanation in the legend of Fig EV4 (as well as in all other figures where irrelevant gel lanes have been removed is missing.

Therefore, before we can proceed with formal acceptance of the paper, I would kindly ask you to go through all main/EV/Appendix figure legends and include a brief explanation wherever irrelevant lanes have been removed, as well as a reference to the available Source Data files. Furthermore, while the assembly sites are well visible in most panels, please make sure to also introduce clearly visible (black?) dividing lines in the blots shown in Fig EV4D.

Referee #1 (Report for Author)

The revised manuscript by Fan et al., has been significantly improved through the inclusion of many additional experiments and controls that I required. Most of my concerns have been sufficiently addressed. I only have one minor request:

The authors now validated the in vivo interactions between endogenous ALYREF and SLBP and Lsm11 using Co-IP experiments. The results seem clear, but the blots are spliced and it is unclear why. Nothing is mentioned in the figure legend. Did the authors need to use two different exposures to visualize the input and IP'ed proteins? Was there too much space between the lines? Is this picture even from the same gel? In the first case, I would suggest to use simply less input material (1%) in these cases and show blots and exposures from the same gel without splice. Alternatively, two individual exposures can be shown.

Referee #2 (Report for Author)

The authors have significantly improved their manuscript by providing new experiments and addressing most of my original concerns. In particular, they have performed novel CoIP experiments that now demonstrate the in vivo interaction between ALY/REF and SLBP proteins, an important data to support one of the main conclusions of the work. The authors have also included new in situ hybridization experiments to show that depletion of ALY factor impairs nuclear export of both exogenous and endogenous histone mRNAs. This was an important issue, also addressed by other referees. Now the proposed model is more solid.
Importantly, the authors has also changed the title, (TREX has been removed from the title as asked), thus the message is now more accurate and is in agreement with data (new and revised figures) that support an specific role of ALY and its contribution to SLBP-mediated histone mRNA processing and export.
Other points have been addressed: new ChIP RNAPII experiments seem to indicate that histone mRNA processing defects in ALYREF KD are not due to transcription elongation defects; iCLIP experiments show that NXF1 levels on histone mRNAs are reduced in the absence of ALY. This last data is also important, since it suggests that ALY/REF could promote histone mRNA export via NXF1.
In summary, the revised manuscript has clearly been strengthened and improved, and the manuscript seems appropriate for publication. No further suggestions.
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Referee #3 (Report for Author)

In the revised version of their manuscript, Cheng and colleagues answered very precisely and seriously to each referee's comments and include many appropriate additional data. To my opinion, they perfectly addressed all concerns and this article now deserves publication in the EMBO 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: Hong Cheng
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99910

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.
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- 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 $\chi 2$ 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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5. For every figure, are statistical tests justified as appropriate?	For every panel we reported the stadistical tests that was used in the figure legend
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C- Reagents

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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).	Antibody information were described in section of Plasmids and antibodies.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell line was described in section of Cell Culture and Transfections

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	The RNA-Seq and iCLIP-Seq are deposited at the Gene Expression Omnibus .
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	we have deposited our Dataset EV1-2 in the manuscript as a Supplementary Doucument.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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G- Dual use research of concern

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