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tRNA processing defects induce replication stress and Chk2-dependent disruption of piRNA transcription

Anahi Molla-Herman, Ana Maria Vallés, Carine Ganem-Elbaz, Christophe Antoniewski and Jean-René Huynh

Corresponding author: Jean-René Huynh, Institut Curie

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

Editor: Anne Nielsen

1st Editorial Decision 26 February 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the slight delay in communicating our decision to you. Your study has now been seen by two referees whose comments are shown below. In addition, I have consulted with an external expert adviser given the slight discrepancy in the recommendations and requests from the two referees.

As you will see from the reports, both referees express interest in the findings reported in your manuscript, although they do also ask for additional data to be included before they can support publication in The EMBO Journal. Our expert advisor furthermore shared ref #1's concern that further genetic data would help distinguish direct and indirect effects of Chk2 activation. In addition, that person also brought up the following point:

'I would have liked to see more supporting evidence that tRNA processing activates the key upstream enzymes involved in replication stress; the data on claspin are nice and go into the right direction, but why not look directly at ATR - the 'master' of replication stress or its regulators such as TopBP1, ATRIP.... (I do realise that there might be genetic/experimental problems with these assays in Drosophilla such as viability; in this case an extended discussion might still be helpful)'

For the revised manuscript I would therefore ask you to focus your efforts on the following points:

-> Providing further insight on the mechanism of Chk2 activation upon defective tRNA processing (e.g. via the suggestions made by our advisor)

-> Elaborating on the outcome for piRNA transcription vs processing downstream of Chk2 (ref #1). For this point, I would like to add that I conducted cross-referee commenting on the reports for your manuscript and received the following from ref#2:

'I fail to see the necessity of an RNAseq on the double mutant though. This seems like a relatively big experiment for its purpose. Given that the piRNAs get back up, it seems inevitable that cluster transcripts do so as well. Still, it may be worth trying something along that line. A somewhat simpler version than RNAseq might be a simple qPCR on wild-type, single and double mutants, and/or a ChIP qPCR on these same samples (they did that for the rpp30 single mutant, that loses H3K9me at clusters; a repeat of this with a rpp30;chk2 double should be easy enough and informative).'

Including RNAseq data for the double mutant would certainly strengthen the manuscript overall, but if this should turn out not to be possible I would suggest you to perform the suggested single-locus assays in the double mutant instead.

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

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

In Drosophila, mutations that compromise germline genome integrity, including DNA repair and piRNA mutations, activate ATM/Chk2 signaling and lead to sterility. Molla-Herman et al. show that hypomorphic mutations in Rpp30, which encodes a subunit of the tRNA processing enzyme RNase P, lead to sterility that is largely due to Chk2 activation. Intriguingly, this does not appear to be due to a significant reduction in mature tRNA production, suggesting that the low levels of incompletely processed tRNAs that accumulate in the mutants may lead to damage. They go on to show that sterility is linked to defects in PCNA localization, suggesting a replication defect, and to increased transposon expression and loss of piRNAs. RNA sequencing indicates that some transposons are over expressed and piRNA precursor transcripts are reduced. Stellate is prepressed by piRNAs in the male germline, and Stellate crystals accumulate in mutant males. qPCR experiments indicate that this may be associated with reduced H3K9-me3 modification. Clusters of tRNA genes are located near piRNA clusters, and the authors propose that the Rpp30 mutations disrupt chomatin at the tRNA loci and that these defects spread to clusters, disrupts transcription and piRNA biogenesis.

The basic observations are very interesting and the genetic characterization is well done. However, the transposon silencing and piRNA biogenesis defects appear to be indirect consequences of Chk2 activation. The authors show that mnk/chk2 not suppresses the oogenesis defects and partially restores fertility. This is in striking contrast to mutations that disrupts the piRNA pathway, including genes that have been implicated in piRNA cluster transcriptions (i.e. rhino). With these mutations, mnk suppresses patterning defects, but 100% of the embryos die due to genome instability. More significantly, the authors show that mnk restores piRNA production. Based on this striking observation, they conclude that the Rpp30 mutation disrupts piRNA cluster transcription, but not processing. However, it seems much more likely that mnk stores expression of clusters and piRNA processing, and probably transposon silencing as well. This should be directly tested by RNAseq on the double mutants. Similarly, some of the other phenotypes, including loss of PCNA localization, could be downstream of Chk2 activation. Given restoration of fertility in the double mutants, this seems likely.

In summary, Molla-Herman et al. present a series of interesting observations indicating that mutations that disrupt tRNA biogenesis activate a DNA damage response, which appears to be distinct from defects in mature tRNA production. However, the mechanism of Chk2 activation is not addressed, and the piRNA defects appear to be downstream of Chk2 activation. To directly determine which defects are a direct consequence of the mutation and which are due to Chk2 activation, all phenotypic assays (RNAseq, PCNA localization, Aub localization, etc) should be done on the mnk double mutants. Control of the piRNA pathway by Chk2 is potentially very interesting, and certainly worth pursuing.

Referee #2:

The manuscript by Molla-Herman et al describes the thorough characterization of loss of Rpp30 on Drosophila gametogenesis. Following genetic characterization and rescue experiments, that firmly show that Rpp30 is required for proper processing of tRNAs, the authors demonstrate that DNA damage is a major cause of the phenotype. Importantly, while tRNA-processing intermediates accumulate, mature tRNA levels are normal, indicating that the observed phenotypes are not caused by dispersed defects due to lowered tRNA abundance. The authors continue to demonstrate that piRNA cluster transcription is disrupted, accompanied by loss of piRNAs and activation of transposons. Strikingly, in Rpp30;Chk2 doubles, piRNA levels get back up, strongly suggesting that the defect in Rpp30 lies in transcription of the piRNA clusters.

The presented data add up to a well-supported model, and as a whole represent an excellent piece of work. It was a pleasure to read. I only have a few relatively minor issues that could/should be addressed.

1) Is the transposon de-repression, and is the H3K9me3 marking of 1/42AB restored in Rpp30;Chk2 double mutants? This would be the only experiment I think that would still significantly add to the model.
2) It would be nice to compare the piRNA profiles of Rpp30;Chk2 doubles with published Aub;Chk2 double mutant piRNA data.

Minor issues:
-Page 17, 2nd paragraph, 3rd line: "...every single locus, it still held...". "It" in this sentence does not refer really to a statement. Possibly rephrase to for example: "...every single locus, the major piRNA clusters, accounting for the majority of germline piRNAs, are all close to tRNA genes."

-Page 18, 2nd line: "We can envisage at least three MUTUALLY non-exclusive..."

- Page 18, line 11: "...shown that CORRECT tRNA FOLDING is..."

1st Revision - authors' response 25 June 2015

We would like to thank the referees for their insightful suggestions and their encouraging comments. They have helped us to improve greatly our manuscript. We hope to have answered to all of their queries.

Editor's comments:

"As you will see from the reports, both referees express interest in the findings reported in your manuscript, although they do also ask for additional data to be included before they can support publication in The EMBO Journal. Our expert advisor furthermore shared ref #1's concern that further genetic data would help distinguish direct and indirect effects of Chk2 activation. In addition, that person also brought up the following point:

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upstream enzymes involved in replication stress; the data on claspin are nice and go into the right direction, but why not look directly at ATR - the 'master' of replication stress or its regulators such as TopBP1, ATRIP.... (I do realise that there might be genetic/experimental problems with these assays in Drosophilla such as viability; in this case an extended discussion might still be helpful)'

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Including RNAseq data for the double mutant would certainly strengthen the manuscript overall, but if this should turn out not to be possible I would suggest you to perform the suggested single-locus assays in the double mutant instead."

1) Genetic interactions

In the previous version of our manuscript, we showed that Rpp30 mutant phenotypes could be partially rescued by removing "general" DNA damage response genes such as *chk2* and *p53*. In addition, we showed that genes more specifically involved in replication stress, such as *claspin* and *squid*, were also able to rescue Rpp30 phenotypes (Figure 3).

In this novel version of our manuscript, we have added genetic interactions with several additional alleles of *claspin*. Previously, we used the null allele *claspin45* (complete deletion of the locus and one neighboring gene, (Lee EM, et al., DNA repair, 2012)) and one hypomorphic allele *claspinEY* (P-element insertion in the 3'end of *claspin*). *claspin45* was synthetic lethal with *Rpp30*, whereas *claspinEY* could rescue *Rpp3018.2*. These contrasting results may stem from the dual functions of Claspin, which is at the same time, part of the replication machinery, ie required for replication, and which can also signal to stop replication when activated by replication stress. We used 3 hypomorphic alleles of claspin generated by imprecise excision at the 3'end of the locus (same localization as *claspinEY*), *claspin279*, *claspinaq4* and *claspinaq5* (Lee EM, et al., DNA repair, 2012). We found that all three alleles can partially rescue Rpp30 phenotypes, which strongly support our conclusions with *claspinEY*. We further noticed an interesting and inverse correlation between the strength of the allele and the degree of rescue, with the weakest alleles rescuing the best (claspinaq4 < claspin279 < claspinaq5). These results have been added to Table 2 of our manuscript and are described p11 in part 4 of the Results section. It now reads: "*We confirmed these results by using three additional hypomorphic alleles of claspin, claspin279, claspinaq4 and claspinaq5, which could all partially rescue Rpp30 mutant phenotypes (Table 2) (Lee et al., 2012). We further noticed an inverse correlation between the strength of the allele and the degree of rescue of oogenesis, with the weakest alleles rescuing the best (claspinaq4 < claspin279 < claspinaq5).*"

In yeast, the helicase Rrm3 and the RNA-binding hnRNP Npl3 are required for replication of hard-to-replicate loci (tRNA and rRNA genes) by preventing R-loop mediated recombination (Santos-Pereira et al., Genes and Dev 2013, Herrera-Moyano, et al., Genes and Dev, 2014). In line with these results, we found that overexpression of Squid-S, aka Rrm3, could rescue partially *Rpp30* phenotypes (Figure 3). However, while revising our manuscript, we noticed that although Squid is referred to as Rrm3 in Flybase and several other databases, it is not the homologue of yeast Rrm3. Squid is a very abundant hnRNP of the same family as Npl3 (also referred to as Nplp3 and Np13 in the databases and literature…), and not a helicase like Rrm3. We believe that Squid is a functional homologue of Npl3 rather than Rrm3 in *Drosophila*. We thus changed the text describing our experiment on p11 in part 4 of the Results section. It now reads: "*Squid is an abundant shuttling hnRNP similar to yeast Npl3, and is required for fertility in flies (Norvell et al., 1999). The helicase Rrm3 and the hnRNP Npl3 were shown to promote DNA replication at difficult-to-replicate loci such as tRNA genes and rDNA in S. cerevisiae (Azvolinsky et al., 2009; Ivessa et al., 2003; Kelley, 1993; Santos-Pereira et al., Genes and Dev 2013, Herrera-Moyano, et al., Genes and Dev, 2014).*" Our conclusion remains identical and suggests that *Rpp30* mutations increase R-loop dependent blocks at transcription-replication conflict sites such as tRNA genes. These blocks can be alleviated by overexpressing hnRNP Squid or Npl3.

As suggested, we have also carried out additional genetic interactions by generating flies double mutant for *Rpp30* and strong alleles of DNA damage/replication stress checkpoint genes such as *mei41/ATR; ATRIP/mus304 and TopBP1/mus101*. Unfortunately, these experiments remained inconclusive for different reasons. We have used two different alleles of *mei-41*, and both alleles in homozygous or transheterozygous combination, induced strong oogenesis defects on their own. We did not see a rescue in *mei-41*; *Rpp30* mutant ovaries, but it is difficult to conclude whether it was due to the addition of oogenesis defects of both mutations or a lack of rescue. We also failed to detect a rescue by combining *Rpp30* mutations with *mus304D1* and *mus101D1/D2*, although *mus304* and *mus101* mutations do not induce oogenesis defects on their own. It is hard to conclude from the absence of interactions as it could be due to the nature of the alleles used (as seen with *claspin* above) or to a true lack of interaction between these genes. Testing all available allelic combinations is beyond the scope of this study in the time given for revisions (61 alleles for mei-41, 10 alleles for mus304 and 10 alleles for mus101). It remains possible that ATR, ATRIP and TopBP1 are activated in *Rpp30* mutant ovaries; we just could not detect it with the limitations of the available alleles we have used. Despite these results, the impressive rescues obtained with *claspin*, *squid*, as well as *chk2* and *p53* strongly support that the replication stress checkpoint is activated in Rpp30 mutant ovaries.

2) piRNA transcription and activation of Chk2

As suggested by reviewer 1, we have performed further characterization of *Rpp30, chk2* double mutant phenotype. To further distinguish between direct and indirect consequences of Chk2 activation, we have also activated Chk2 by other means (meiotic DSBs) and tested its effect on TEs

repression.

2a) Further characterization of *Rpp30, chk2* double mutant phenotype.
In the previous version of our manuscript, we showed that the global piRNA populations (Fig 5F), piRNAs from clusters 1 to 8 (unique mappers) (Fig 5E and EV3), Aubergine nuage staining (Fig 5G) and ping-pong signal (Fig 5H) were all significantly rescued in *Rpp30, chk2* double mutant ovaries. Although this rescue is substantial and some fertility is restored, the rescue is not complete, and as shown in Fig 5F the piRNA population ranges in 104 reads in *Rpp30, chk2* ovaries, while in wild type virgin ovaries, the range is around 105 reads (Figure 5A).

Despite this limitation, we found by RT-qPCR that transcription of the main piRNA clusters 1 and 2 were significantly increased in *Rpp30, chk2* double mutant as compared to *Rpp30* single mutant (Figure EV4A). Ratios over wild type (1 is the wild type ratio) went from 0.3 in *Rpp30* mutant ovaries to 0.6 in *Rpp30, chk2* double mutant for cluster 1; and from 0.1 to 0.5 for cluster 2. In addition, we found that the rescue of piRNA clusters transcription correlated well with a rescue of the H3K9me3 marks in *Rpp30, chk2* double mutant ovaries. We performed ChIP experiments for H3K9me3 marks and found for cluster 1 that in *Rpp30, chk2* double mutant ovaries H3K9me3 levels were around 60% of wild type as compared to 10% in *Rpp30* single mutant; and for cluster 2, H3K9me3 levels were back to around 90% of wild type as compared to 30% in *Rpp30* single mutant (Figure EV4C). We also found that PCNA staining in *Rpp30, chk2* nurse cell nuclei was similar to wild type (Figure EV4D). Finally, we also analyzed representative transposable elements (TEs) in *Rpp30, chk2* double mutant ovaries, and observed that H3K9me3 levels were back to 90% of wild type levels and transcription of TEs was silenced to wild type levels (Fig EV4B).

Overall, these novel results support our previous conclusions and indicate a rescue of piRNA cluster transcription, piRNA biogenesis and TEs silencing in *Rpp30, chk2* double mutant ovaries.

2b) Indirect vs direct effects of Chk2 activation.

To test whether mere Chk2 activation could directly inhibit piRNA transcription and lead to TEs transcription, we analyzed *spnA* mutant ovaries. SpnA is a Rad51 protein required to repair meiotic DSBs in *Drosophila* germ cells (Staeva-Vieira et al., EMBO Journal, 2003). *spnA* mutant ovaries show meiotic defects, abnormal shaped eggs and sterility. These phenotypes are due to the activation of Chk2, as these defects (except sterility) are rescued in *spnA*, *chk2* double mutant ovaries (Staeva-Vieira et al., EMBO Journal, 2003). As a control, we used *spnE* mutant flies, which were generated in the same genetic background as *spnA* but have well-characterized defects in piRNA biogenesis (Malone et al., Cell, 2009).

We performed RT-qPCR on representative TEs in *spnA* and *spnE* mutant ovaries, and found that TEs were not up-regulated in *spnA* mutant ovaries, whereas TEs were derepressed in *spnE* ovaries as published (Malone etal., Cell, 2009) (Figure EV5A). Aubergine staining in the nuage was also similar to wild type in *spnA* ovaries, while it was much reduced in *spnE* nurse cells (Figure EV5B, left). Finally, we found that PCNA staining was identical to wild type in both *spnA* and *spnE* mutant ovaries (Figure EV5B, right).

We concluded that activating Chk2 by other means (here unrepaired meiotic DSBs) is not sufficient to disrupt the nuage and the silencing of TEs. We have added a paragraph describing these results in part 7 of the Results section (p16) and in a novel supplementary figure (Figure EV5).

Reviewer 1 comments:

"The basic observations are very interesting and the genetic characterization is well done. However, the transposon silencing and piRNA biogenesis defects appear to be indirect consequences of Chk2 activation. The authors show that mnk/chk2 not suppresses the oogenesis defects and partially restores fertility. This is in striking contrast to mutations that disrupts the piRNA pathway, including genes that have been implicated in piRNA cluster transcriptions (i.e. rhino). With these mutations, mnk suppresses patterning defects, but 100% of the embryos die due to genome instability. More significantly, the authors show that mnk restores piRNA production. Based on this striking observation, they conclude that the Rpp30 mutation disrupts piRNA cluster transcription, but not processing. However, it seems much more likely that mnk stores expression of

clusters and piRNA processing, and probably transposon silencing as well. This should be directly tested by RNAseq on the double mutants.
Similarly, some of the other phenotypes, including loss of PCNA localization, could be downstream of Chk2 activation. Given restoration of fertility in the double mutants, this seems likely.
In summary, Molla-Herman et al. present a series of interesting observations indicating that mutations that disrupt tRNA biogenesis activate a DNA damage response, which appears to be distinct from defects in mature tRNA production. However, the mechanism of Chk2 activation is not addressed, and the piRNA defects appear to be downstream of Chk2 activation. To directly determine which defects are a direct consequence of the mutation and which are due to Chk2 activation, all phenotypic assays (RNAseq, PCNA localization, Aub localization, etc) should be done on the mnk double mutants. Control of the piRNA pathway by Chk2 is potentially very interesting, and certainly worth pursuing."

As asked by reviewer 1, we have carried out all phenotypic assays in *Rpp30, chk2* double mutant background (see answer 2a to the editor's comments). As suggested by the editor, we chose to perform RT-qPCR on piRNA cluster using 3 different pairs of primers for cluster 1 and 1 pair for cluster 2, as a faster and simpler experiment than genomewide RNAseq. Overall, our novel results presented on Figure EV4 support our previous conclusions and indicate a rescue of piRNA cluster 1 and 2 transcription, piRNA biogenesis and TEs silencing in *Rpp30, chk2* double mutant ovaries.

In addition, we show that TEs were not activated in *spnA* mutant flies despite the activation of Chk2 (see answer 2b to the editor's comments). This result suggests that activation of Chk2 is not sufficient to disrupt the piRNA pathway. However, it remains possible that Chk2 activation can have local and direct effects on transcription of neighboring genes (including piRNA clusters) in *Rpp30* mutant ovaries. This would not be the case in *spnA* mutant ovaries as DSBs have little chance to be close to piRNA clusters. Distinguishing local vs global effects of Chk2 activation (if it exists) are future exciting lines of research.

Reviewer 2 comments:

"The presented data add up to a well-supported model, and as a whole represent an excellent piece of work. It was a pleasure to read. I only have a few relatively minor issues that could/should be addressed.

We thank this referee for these encouraging comments.

1) Is the transposon de-repression, and is the H3K9me3 marking of 1/42AB restored in Rpp30;Chk2 double mutants? This would be the only experiment I think that would still significantly add to the model.

We performed ChIP experiments for H3K9me3 marks and found for cluster 1/42AB that in *Rpp30, chk2* double mutant ovaries H3K9me3 levels were around 60% of wild type as compared to 10% in *Rpp30* single mutant; and for cluster 2, H3K9me3 levels were back to around 90% of wild type as compared to 30% in *Rpp30* single mutant (Figure EV4C). We also observed that H3K9me3 levels were back to 90% of wild type levels on a representative set of TEs (Figure EV4C). Accordingly, we measured by RT-qPCR that this same set of TEs were silenced transcriptionally in *Rpp30, chk2* double mutant ovaries (Figure EV4B).

2) It would be nice to compare the piRNA profiles of Rpp30;Chk2 doubles with published Aub;Chk2 double mutant piRNA data.

We agree entirely with the referee's suggestion however we could not find *aubergine; chk2* small RNAs datasets in any public databases. In addition, we are not aware of the publication of small RNA datasets for such double mutant combinations (with *aubergine* or other piRNA pathway members).

Minor issues: -Page 17, 2nd paragraph, 3rd line: "...every single locus, it still held...". "It" in this sentence does not refer really to a statement. Possibly rephrase to for example: "...every single locus, the major piRNA clusters, accounting for the majority of germline piRNAs, are all close to tRNA genes."

This sentence has been changed to (page 18, second paragraph): "*Although we did not find a perfect correlation between tRNA genes and piRNA cluster proximity for every single locus, the major piRNA clusters, accounting for the majority of germline piRNAs, are all close to tRNA genes.*"

-*Page 18, 2nd line: "We can envisage at least three MUTUALLY non-exclusive..."*
corrected

- *Page 18, line 11: "...shown that CORRECT tRNA FOLDING is..."*
corrected

2nd Editorial Decision 03 August 2015

Thank you again for submitting your revised manuscript to The EMBO Journal and my apologies for the slight delay in the re-review process here. We have now heard back from the two original referees and as you will see from the comments included below they are both satisfied with the experiments you have included in the revised version. They are therefore both happy to support publication, pending a few issues with rephrasing the abstract and title as pointed out by ref #1.

Before we can proceed to officially accept your study for publication I would also ask you to address the editorial points listed below (I discussed some of these per email with Anahi a few weeks back so she is informed about them as well):

-> Please include a completed author checklist (as described in our guide to authors http://emboj.embopress.org/authorguide)

-> Please provide a new version of figure 5 containing scale bars for size in panel D and G

-> Please also include a new version of figure 3 where the middle panel in fig 3A depicts an intact, unmodified image as captured by the microscope rather than the current composite image. I appreciate that the raw data included as source data illustrates the full capture but as described in our author guidelines we do not allow this type of composite image unless the partial images are clearly marked by separating lines. Looking at the source data file I would encourage you to instead crop the image to display the upper section as a single image.

-> Please make sure that all database accession codes for RNA seq data are included in the main text.

-> Could I also ask you to refer to 'Appendix table S1' and (and not 'Supplemental data and material') at the end of the materials and methods section?

Given the positive recommendation from the referees, I would invite you to submit a final version of the manuscript, incorporating the points listed above.

Please feel free to contact me with any questions about this and thank you again for the opportunity to consider this work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

Molla-Herman et al. have done an excellent job responding to comments on the initial submission, performing a number of important experiments that help clarify the relationship between the Rpp30 mutations, DNA damage signaling through Chk2 and the piRNA pathway. As detailed in the response to the previous reviews and in the revised manuscript, they now very convincingly show that essentially all of the piRNA pathway defects are suppressed by null mutations in mnk, which encodes the Drosophila Chk2 homologue. This includes total piRNA production, ping pong

amplification, and piRNA cluster transcription (Fig. EV4). Nonetheless, the authors conclude that Rpp30 is required for piRNA cluster transcription. It seems to me that the data rule this out a direct role for Rpp30 in the piRNA pathway. In summary, the current version of the manuscript is experimentally very sound and documents a novel function of Rpp30 in genome maintenance. However, the title, abstract and summary model are inconsistent with the experimental data, which indicate that Rpp30 mutations lead to genome instability and Chk2 activation, and that defects in the piRNA pathway are downstream of Chk2. A more appropriate title might be "tRNA processing defects are associated with replication stress, genome instability and Chk2-dependent disruption of the piRNA pathway".

Referee #2:

The manuscript has been improved and important data has been added. Potential concerns have been addressed well. I have no further comments on this manuscript.

2nd Revision - authors' response 01 September 2015

We are happy to submit the revised version of our manuscript (EMBOJ-2015-91006R), which includes all the changes that we discussed by email and the suggestions made by referee 1.

1) The title has been changed to "*tRNA processing defects induce replication stress and Chk2-dependent disruption of piRNA transcription*" to underline that piRNA transcription defects are downstream of chk2 activation as proposed by referee1.

2) We have also included all the changes that you suggested for the abstract.

3) We have also changed the order of the steps in our summary model (Figure 7) to highlight the fact that activation of checkpoint proteins (Claspin, Chk2) is upstream of piRNA transcription defects. Activation of checkpoints is now number 3. Changes have been made in the corresponding figure legends.

4) -> Please include a completed author checklist (as described in our guide to authors http://emboj.embopress.org/authorguide)
DONE

5) Please provide a new version of figure 5 containing scale bars for size in panel D and G
DONE

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DONE

7) Please make sure that all database accession codes for RNA seq data are included in the main text.

DONE (included in the material and methods "RNA sequencing" and "small RNA sequencing" section)

8) Could I also ask you to refer to 'Appendix table S1' and (and not 'Supplemental data and material') at the end of the materials and methods section?
DONE

