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Red1 Promotes the Elimination of Meiosis-Specific mRNAs in Vegetatively Growing Fission Yeast

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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 13 October 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. All three reviewers find your identification of a novel factor involved in the selective elimination of meiosis-specific mRNAs interesting and thus potentially suitable for publication in The EMBO Journal, pending adequate revision of a number of specific issues. As you will see, while the majority of these points pertain to aspects of presentation and discussion, there are however also some concerns where the referees request additional experiments to add controls as well as some further insight. With regard to the last point, this concerns mostly the incomplete characterization of Red1 as an RNA-binding protein criticized by both reviewers 1 and 2.

Should you be able to adequately address these various points in a revised version of the manuscript, then we shall be happy to consider the study further for publication. I should however remind you that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the experimental and editorial points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Recent studies by others have revealed roles for the canonical nuclear poly(A) polymerase Pla1 and RNA-binding protein Mmi1 in the constitutive degradation of meiosis-specific mRNAs in vegetatively growing fission yeast. The authors outline a role for the previously uncharacterized CCCH zinc finger protein Red1, in this mRNA turnover pathway. The authors identified Red1 as a potential component of this system on the basis of its intranuclear localization, as revealed by an earlier ORFeome-wide CFP-tagging study by another group. The data are of a high overall quality and the manuscript is generally well written (some minor errors of grammar and spelling will easily be picked up at the copy editing stage).
Overall, the authors make a strong case for the involvement of Red1 in the clearance of meiotic mRNAs that contain DSR elements from vegetative cells, though the nature of this involvement at the biochemical level has not been addressed, beyond the demonstration that an intact zinc finger motif is required for Red1 function. The authors speculate, as they are entitled to in the discussion section, about the potential relevance of their findings to RNA turnover in other systems, but some of these links are quite tenuous at this stage. In any event, the following points should be addressed before publication:

1 The authors state that disassembly of Red1 dots "apparently inactivates Red1 function" (p4), but the only evidence to support this claim is correlative, rather than indicating a clear cause and effect. The molecular function of Red1 is in any case not defined beyond its broad involvement in DSR-RNA turnover, so it seems a little premature to be discussing its inactivation.

2 The authors note that about half of the mRNAs that decrease in abundance in the red1 deletion strain are encoded by sub-telomeric genes. This observation would seem to warrant more attention in the discussion - can the authors suggest a possible explanation?

3 Red1 is described as being "homologous to human CCDC131" (p6), but in the following sentence it is pointed out that the similarity between the two proteins is limited to the zinc finger. Unless there is further evidence of conservation of function, it seems very premature to suggest that the proteins are homologous.

4 It is not clear from the Materials and Methods section whether the epitope-tag alleles used to generate the data of Figure 2D retain their authentic 3' UTRs (and hence their DSR elements). In fact, if the method of Bahler et al was followed, as suggested, the modified alleles should lack their original UTRs. The authors should clarify this point and, if appropriate, suggest why the Red1 pathway operates on mRNAs that lack DSR elements.

5 The sentence beginning "These results suggest the following interpretations ..." (p11) lists three interpretations, only the second of which is suggested by the data described in the section of which the sentence forms a part.

6 It might be unreasonable to expect a full biochemical characterization of Red1, but it would at least be interesting to know whether its zinc finger does indeed possess RNA-binding activity, and whether any of the co-immunoprecipitation data described reflect interactions mediated via RNA (are they sensitive to incubation with RNAses?).

7 The text (p10) indicates that Red1 co-localized with Pla1, but the data presented (Fig. 4A) suggest that the co-localization is at best partial, despite the co-immunoprecipitation of the two proteins. As both proteins are nuclear, some level of coincidence of signal would be expected, considering the small dimensions of the yeast nucleus. How have the authors quantified the extent of co-localization?

Referee #2 (Remarks to the Author):

Manuscript # EMBOJ-2010-76103
Sugiyama & Sugioka-Sugiyama

In this manuscript, Sugiyama & Sugioka-Sugiyama characterize Red1, which is a new protein involved in the mitotic degradation of meiotically expressed genes. They show that Red1 works in the same pathway than the previously characterized Mmi1 degrading mRNAs that contain the DSR signal. Like Mmi1, cells that lack Red1 accumulate DSR-mRNAs in mitotic cells. They also show that Red1 interacts with Mmi1 by Co-IP and with other components of the machinery involved in the degradation of the DSR-mRNAs (like Pla1, Rrp6 and Pcf1).
This is very well presented piece of work, nicely written and easy to follow, even for readers outside of the field. The data is clear and convincing. Nevertheless, the manuscript could benefit from a few clarifying experiments and the authors should address some minor problems with the text and references.

Major issues:
1- A mmi1 deletion is lethal, mainly due to the "overexpression" of Mei4 which triggers the expression of a large set of meiotic genes. That is why in a mei4 mutant background is possible to delete mmi1, as the authors point out in the manuscript (Fig 3D). Interestingly, when red1 is deleted there is more mei4 mRNA and Mei4 protein than in mmi1 mutant cells, but delta-red1 cells are viable. The authors should find an explanation for this observation.
2- Figure 6 and the Red1 Zinc-finger domain: in panel E, the authors should show the control of delta-red1 cells, so it can be compared the accumulation of the meiotic mRNAs in delta-red1 cells with Red1-H637I cells (without that is impossible to deduce that the H637I mutant has a completely inactive Red1).
3- Also, in this section, the authors conclude that the Red1 zinc-finger domain has an RNA-binding activity, but they do not show any conclusive experiment in that direction; a simple RNA-IP comparing wt and H637I Red1 should be shown.
4- The authors argument that Red1 is not degraded during meiosis, based only on a snapshot western blot of meiotic cells (Figure 7B), but is impossible to observe any fluorescence of the Red1-tomato cells during conjugation or before meiosis I (Figure 7A). Although their hypothesis is probably correct, a western blot of a complete meiosis time-course should be shown; alternatively, they could show a western blot of a time-course of nitrogen starvation in the lys1::matPc cells (that they use in Figure 7D).

Minor issues:
1- There are several typos in the text that need to be corrected (i.e. exosom in page 10, exsosome in page 16).
2- p.3, second paragraph. The reference Hariyaga & Yamamoto is not appropriate as a general revision, since it is a specific revision of pombe meiosis.
3- Supplementary Fig S3D, second cluster (from NCRNA.70 to AC977.02): the drawing is reversed, with the complementary strand-encoded genes drawn on top.

Referee #3 (Remarks to the Author):

Fission yeast cells have a mechanism to eliminate meiotic RNAs in vegetative cells, which is based on the targeting of specific RNAs to the exosome in a pathway that involves the mmi1 protein and the nuclear poly(A)-binding protein. This pathway serves as a paradigm for how posttranscriptional mechanisms are used to eliminate RNAs resulting from 'residual' transcription and is therefore of general interest. A second feature of interest is the specific and dynamic sub-nuclear localisations of the components involved in this process.

This manuscript describes the identification and characterisation of a novel component of this pathway (Red1). Red1 is deficient in the degradation of meiotic RNAs in vegetative cells, interacts with components of the nuclear exosome and polyadenylation machinery and is localised to a specific subnuclear compartment.

The manuscript is well-written, and the experiments are generally well controlled and documented. I

think the presentation of the microarray data and their analysis could be improved, and I enclose a few suggestions below:

[1] It would be useful to have a spreadsheet with the microarray data as supplementary data accompanying the manuscript (indicating the expression value in each of the two experiments, the common name and the systematic name of each of the genes). I am aware that the authors have deposited the data in a public database, but this would make the data more accessible.
[2] Similarly, it would be useful to have lists of up-regulated and down-regulated genes in a spreadsheet.
[3] The comparison with meiotically induced genes (figure 2A) would be illustrated better using Venn diagrams. Also, a p-value showing the statistical significance of the overlap should be shown.
[4] A similar presentation and analysis should be applied to the different groups of co-regulated genes identified by microarray analysis (early, middle, late, -N). (The authors mention that 'most' of the up-regulated genes belonged to the 'early' group)

Minor points:
P10. 'exosom', should read 'exosome'
P14. 'weather' should say 'whether'

1st Revision - authors' response 24 December 2010

Point-By-Point Response
Referee #1

1 The authors state that disassembly of Red1 dots "apparently inactivates Red1 function" (p4), but the only evidence to support this claim is correlative, rather than indicating a clear cause and effect. The molecular function of Red1 is in any case not defined beyond its broad involvement in DSR-RNA turnover, so it seems a little premature to be discussing its inactivation.

We have modified the sentence accordingly to more accurately reflect our findings (page 5, line 1).

2 The authors note that about half of the mRNAs that decrease in abundance in the red1 deletion strain are encoded by sub-telomeric genes. This observation would seem to warrant more attention in the discussion - can the authors suggest a possible explanation?

It is intriguing that a fraction of telomeric genes were directly or indirectly under the control of Red1. However, expression analyses indicated that both increased (e.g. *SPBPB2B2.03c*) and decreased (e.g. *SPBPB10D8.02c*) genes were present near telomeres of chromosome 1 and 2, and some of the decreased genes in *red1*Δ cells were classified as upregulated genes during nitrogen starvation/meiosis. In addition, these downregulated genes at telomeric regions have not been studied so far. From the available yet limited information about these genes, we still have not found any explanation for the mechanism of Red1-mediated up- and down-regulated genes at telomeric domains. Thus, we prefer not to discuss the potential mechanisms in the present study. Instead, this observation is now under further investigation, and we are addressing this point in another study that we are currently performing.

3 Red1 is described as being "homologous to human CCDC131" (p6), but in the following sentence it is pointed out that the similarity between the two proteins is limited to the zinc finger. Unless there is further evidence of conservation of function, it seems very premature to suggest that the proteins are homologous.

We have modified this statement in the manuscript and have now suggested that the Red1 Zn-finger domain is conserved in various species and that CCDC131 is one of the proteins that has a ZnF domain homologous to the Red1 Zn-finger motif (page 6, line 15).

4 It is not clear from the Materials and Methods section whether the epitope-tag alleles used to generate the data of Figure 2D retain their authentic 3' UTRs (and hence their DSR elements). In

fact, if the method of Bahler et al was followed, as suggested, the modified alleles should lack their original UTRs. The authors should clarify this point and, if appropriate, suggest why the Red1 pathway operates on mRNAs that lack DSR elements.

We did not construct either the Mei4-HA or Mcp5-myc strains, because we obtained them from the National BioResourse Project of Japan. Mcp5-tag proteins were originally described in Saito et al. 2006, and the authentic *mcp5*$^+$ 3' UTR is described as being present at the 3' end of the myc tag (Also see the strain list in Supplementary Table SI).

As for Mei4, the DSR region of *mei4*$^+$ is present within the coding sequence, and a previous study has clearly shown that the *mei4*$^+$ 3' UTR is dispensable for Mmi1-dependent mRNA elimination. Therefore, *mei4*$^+$ mRNA is subjected to selective elimination even in the absence of the authentic 3' UTR. In addition, since Mei4-HA has not been described so far, we checked the *mei4*$^+$ gene of the mei4-HA strain, and found that the *mei4*$^+$ 3' UTR is present as in wild-type. We have now modified the Materials and methods section to clarify the point (page 20, line 7).

5 The sentence beginning "These results suggest the following interpretations ..." (p11) lists three interpretations, only the second of which is suggested by the data described in the section of which the sentence forms a part.

We have modified the sentence accordingly to suggest only the second interpretation (page 11, line 19).

6 It might be unreasonable to expect a full biochemical characterization of Red1, but it would at least be interesting to know whether its zinc finger does indeed possess RNA-binding activity, and whether any of the co-immunoprecipitation data described reflect interactions mediated via RNA (are they sensitive to incubation with RNAses?).

We examined whether Red1 interactions with Mmi1, Rrp6, and Pla1 were sensitive to RNase treatment. As shown in Supplementary Figure S4, these interactions were refractory to the enzymatic digestion of RNase A and RNase T1, suggesting that RNA molecules do not support these associations.

To investigate whether Red1 had RNA-binding activity, we expressed and purified the Red1 zinc finger domain from *E. coli*, and then performed an RNA gel shift assay. Unfortunately, however, we could not obtain any solid data showing the activity *in vitro*. We think that we need to change or modify our protocols (e.g. gel shift, expression vector, or expression system), but we have not yet optimized them. Once we do we would definitely like to include the *in vitro* experiments in another Red1 paper addressing the biochemical properties of Red1 and the Red1 complex. However, to address this issue in a different way, we carried out RNA-IP experiments to test whether Red1 co-precipitated with meiotic mRNAs. As shown in Figure 6F, we found that Red1 IP concentrated target mRNAs more efficiently than Red1 mutant IP. Although indirect, this implies that the Red1 zinc finger has RNA-binding activity or facilitates RNA-binding activity of Red1 immunoprecipitates (page 14, line 6).

7 The text (p10) indicates that Red1 co-localized with Pla1, but the data presented (Fig. 4A) suggest that the co-localization is at best partial, despite the co-immunoprecipitation of the two proteins. As both proteins are nuclear, some level of coincidence of signal would be expected, considering the small dimensions of the yeast nucleus. How have the authors quantified the extent of co-localization?

We compared Red1 dots with other nuclear bodies, centromeres, telomeres, and nuclear foci with unknown function, to assess whether Red1 foci coincided accidentally with these structures. We found that Red1 dots hardly co-localized or overlapped with these nuclear domains (nuclei containing a coincided/overlapped focus: with centromeres, 2.8 %; with telomeres, 4.4 %; and with unknown structures, 7.9 %). In stark contrast, all nuclei contained coincided/overlapped foci when we compared Red1 localization with Pla1. Therefore, we concluded that Red1 co-localizes with Pla1. The co-IP experiments also support this conclusion.

Referee #2

Major issues:
1 A mmi1 deletion is lethal, mainly due to the "overexpression" of Mei4 which triggers the expression of a large set of meiotic genes. That is why in a mei4 mutant background is possible to delete mmi1, as the authors point out in the manuscript (Fig 3D). Interestingly, when red1 is deleted there is more mei4 mRNA and Mei4 protein than in mmi1 mutant cells, but delta-red1 cells are viable. The authors should find an explanation for this observation.

The genes involved in DSR-mediated mRNA removal can be classified into three categories: essential genes (*mmi1*$^+$, *rna15*$^+$, *pla1*$^+$, and *dis3*$^+$), non-essential genes but required for normal growth (*red1*$^+$ and *rrp6*$^+$), and non-essential genes dispensable for normal growth (*pab2*$^+$). Thus, there is no correlation between meiotic mRNA elimination and viability/cell growth. In addition, we are not sure whether Mei4 mis-expression is responsible for the growth defect of *red1*Δ cells because *mei4*Δ did not rescue the growth retardation of the *red1*Δ cells (our unpublished result). Furthermore, the expression analyses of the recent report (St-Andre et al, J Biol Chem. 2010) and our study do not support that Mei4, which is expressed due to the inactivation of mRNA elimination by DSR, is fully functional since all the previously known targets of Mei4 (*mde1*$^+$*-3*$^+$, *mde5*$^+$*-9*$^+$, *spo6*$^+$, *mes1*$^+$, *and sec9*$^+$) except *mde10*$^+$ are not accumulated in *pab2*Δ and *red1*Δ cells. Considering this information, we hypothesize that, like Rna15 and Pla1, Mmi1 has at least two different roles, pre-mRNA processing and meiotic mRNA elimination. However, we do not have any data explaining the reason why *mei4* mutation rescues *mmi1* deficiency, and so we decided not to discuss this issue because our argument seems premature.

2 Figure 6 and the Red1 Zinc-finger domain: in panel E, the authors should show the control of delta-red1 cells, so it can be compared the accumulation of the meiotic mRNAs in delta-red1 cells with Red1-H637I cells (without that is impossible to deduce that the H637I mutant has a completely inactive Red1).

We have performed the experiment as suggested by referee #2, and we found that the zinc-finger mutation did not abolish Red1 activity completely, as shown in Figure 6E.

3 Also, in this section, the authors conclude that the Red1 zinc-finger domain has an RNA-binding activity, but they do not show any conclusive experiment in that direction; a simple RNA-IP comparing wt and H637I Red1 should be shown.

We carried out RNA-IP experiments to test whether Red1 coprecipitated with meiotic mRNAs. We found that Red1 IP concentrated target mRNAs more efficiently compared to Red1 mutant IP (Figure 6F). This suggests that Red1 zin finger has RNA-binding activity or facilitates RNA-binding activity of Red1 immunoprecipitates (page 14, line 6).

4 The authors argument that Red1 is not degraded during meiosis, based only on a snapshot western blot of meiotic cells (Figure 7B), but is impossible to observe any fluorescence of the Red1-tomato cells during conjugation or before meiosis I (Figure 7A). Although their hypothesis is probably correct, a western blot of a complete meiosis time-course should be shown; alternatively, they could show a western blot of a time-course of nitrogen starvation in the lys1::matPc cells (that they use in Figure 7D).

We have taken the pictures of fluorescent images using the same conditions both in mitosis and meiosis. As referee#2 pointed out, it is really difficult to observe Red1-tdTomato in meiosis under these conditions. However, we noticed that longer exposure enabled us to see the dispersed signal of Red1 in the nucleus. The reason we did not include the photo is that the images are not good enough to be included. Actually, we have identified a Red1-binding protein, which will be described in another manuscript, and found that the dispersion of the Red1 binding protein in meiosis is much more obvious than that of Red1 under a microscope. We therefore believe that Red1 is indeed dispersed during meiosis.

In addition, we performed western blotting of a time-course of nitrogen starvation in the *lys1*::*matPc* cells as suggested. These results are shown in Supplementary Figure S8 and indicate that the protein levels of Red1-GFP were not significantly changed during the pheromone activation, further supporting our idea that Red1 protein becomes dispersed during meiosis (page 15, line 19).

Minor issues:
1 There are several typos in the text that need to be corrected (i.e. exosom in page 10, exsosome in page 16).

The typos have now been corrected in the modified manuscript.

2 p.3, second paragraph. The reference Hariyaga & Yamamoto is not appropriate as a general revision, since it is a specific revision of pombe meiosis.

We have now cited two more relevant papers that describe general revision of meiosis as suggested (page 3, line 17).

3 Supplementary Fig S3D, second cluster (from NCRNA.70 to AC977.02): the drawing is reversed, with the complementary strand-encoded genes drawn on top.

The reason several genes with "c", which stands for complementary, are drawn on top is that the SPAC212 cosmid was reversed from its original orientation. To the best of our knowledge, gene structures and directions, but not systematic gene IDs, are corrected. This might be a little confusing, but the drawings in Supplementary Figure S3D reflect the latest information of the fission yeast genome database.

Referee #3

[1] It would be useful to have a spreadsheet with the microarray data as supplementary data accompanying the manuscript (indicating the expression value in each of the two experiments, the common name and the systematic name of each of the genes). I am aware that the authors have deposited the data in a public database, but this would make the data more accessible.

As suggested, we have the data set in the supplementary data set, which contains the results of the two independent expression analyses.

[2] Similarly, it would be useful to have lists of up-regulated and down-regulated genes in a spreadsheet.

We have prepared two lists containing the lists of up-regulated and down-regulated genes, and the lists are shown in Supplementary Tables SII and SIII.

[3] The comparison with meiotically induced genes (figure 2A) would be illustrated better using Venn diagrams. Also, a p-value showing the statistical significance of the overlap should be shown.

In figure 2A, we carried out the expression profiling of vegetative wild-type and vegetative *red1*Δ strains, picked up the genes accumulated in *red1*Δ compared to wild-type cells, and showed the result as a pie chart. Thus, we do not think that the suggested presentation and calculating p-value can be applied to illustrate the expression analyses.

[4] A similar presentation and analysis should be applied to the different groups of co-regulated genes identified by microarray analysis (early, middle, late, -N). (The authors mention that 'most' of the up-regulated genes belonged to the 'early' group)

In Supplementary Tables SIII and SIV, the categories of each gene classification (e.g. early, middle, transient, or unassigned) are now available and can be easily checked. In addition, we mentioned "most of the other highly upregulated mRNAs" in the manuscript, but this sentence seems a little confusing. So we modified the manuscript to clearly state "most of the highly upregulated" but not "most of the up-regulated" genes (page 7, line 20.

Minor points:
P10. 'exosom', should read 'exosome'

P14. 'weather' should say 'whether'

We have corrected these typos in the revised manuscript.

2nd Editorial Decision/Decision Letter 18 January 2011

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by one of the original reviewers (see comment below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

For production of the manuscript, we will require individual files for each of the main figures. In addition, we would appreciate if the two supplementary information files 'Supplementary Figures S1-S8' and 'Supplementary Tables SI-SIII' could be merged into one single PDF to minimize the number of files that our readers would have to download. In order to allow you to upload the requested files, I am therefore returning the manuscript to you once more with the 'revision upload' link given below, kindly asking you to resubmit the paper in final format at your earliest convenience. Once we will have received these files, we shall then be able to swiftly proceed with formal acceptance and publication of the manuscript!

REFEREE REPORT

Referee #1 (Remarks to the Author):

The authors have satisfactorily addressed each of the points raised in my review of the earlier version of the manuscript, by appropriate modification of the text and inclusion of additional data.

