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Lsm1 promotes genomic stability by controlling histone mRNA decay

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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 11 December 2010

Thank you for submitting your manuscript on Lsm1 regulation of histone levels and replication fork stability for consideration by The EMBO Journal. It has now been assessed by three referees with relevant expertise in mRNA degradation, histone regulation and replication fork stability, whose comments are copied below. As you will see, these referees consider your findings of interest in principle, but they do also raise a number of substantive concerns that would need to be addressed before publication in a broad general journal such as The EMBO Journal would be warranted. One recurrent main concern in this respect is that causal links between the different described events are not always decisively demonstrated - e.g. whether the lsm1 mutant effect is indeed specifically via histone mRNA regulation, whether altered histone mRNA levels directly lead to increased levels of free histone proteins, and whether there really is a direct effect on replication fork stability. Another issue is that several previous publications (some of which you cited while others have gone un-mentioned) have already reported certain findings pointing into similar directions. While we appreciate that scope and comprehensiveness of the current submission still set it apart from these earlier reports, it is nevertheless clear that in this light, discussion of your findings in a different context would be warranted (see esp. referee 2 for detailed suggestions), but also that stronger evidence for the proposed causalities would be even more important.

In this situation and with these reports at hand, I feel that we should be able to consider a revised version of this manuscript further but only if you should be able to address a the following main concerns to the referees' satisfaction: confirming altered mRNA half-lives/decay rates as asked for by referees 1 and 2; providing additional evidence for selectivity of the lsm1 mutation effect on histone mRNA (refs 1 and 2 - although I note that adding a microarray experiment as asked by referee 2 may exceed the scope of the current study); showing stronger evidence for altered histone levels being due to increased histone mRNA stabilization as asked for by referees 1 and 2; and trying to add more direct (mechanistic) evidence for effects on replication fork stability along the

lines of referee 3's comments. I realize that the latter may be technically quite challenging, but otherwise at least the claims in the title and throughout the text would clearly have to be modified to something more general such as 'DNA damage sensitivity' or 'genomic stability' rather than 'fork stability'. Finally, please heed the requests of all three reviewers for more careful and appropriate citation of background literature, and try to refocus the discussion to reflect the input provided by referee 2 point 1. In addition, please also try to address or respond to all the other specific points as completely and diligently as possible, keeping in mind that it is EMBO Journal policy to allow a single round of major revision only. 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). Finally, we will also require a brief section specifying the contributions of the individual authors on the paper. 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

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript Herrero and Moreno identify Lsm1 as a gene conferring rÈsistance to specific DNA damaging agents in yeast. Molecular and genetic assays demonstrate that this occurs through an effect on the stability of replication forks. Further analyses demonstrate that Lsm1 promotes the degradation of histone mRNAs thus preventing the accumulation of free histones, which itself provokes replication fork instability.
Overall, the manuscript is well written, the data are of very high quality and contain appropriate control. Thus, the manuscript is quite convincing. I believe that this manuscript is of interest because it provides a clear mechanistic explanation for a biological phenomenon, even if the effect of Lsm1 on replication fork stability and sensitivity to DNA damaging agents are somewhat indirect.
Nevertheless, some points need to be corrected:
- Page 9, line 10 from bottom and Figure 5C: The authors comment on the absence of increase of Cyc2 mRNA abundance in a delta-lsm1 mutant. The steady state mRNA level does not mean much as it is affected both by transcription and decay rates. In this specific case, the rate of decay is clearly affected by the delta-lsm1 mutation (Fig. 5C). The absence of a concomitant increase in the Cyc2 mRNA level thus indicates that transcription was also affected, most likely indirectly by the delta-lsm1 mutation (e.g., delta-lsm1 may stabilize an mRNA encoding a repressor of Cyc2 transcription). Overall, there is no evidence for specificity of the delta-lsm1 mutation that appears to affect the decay of all mRNAs tested, including histones and Cyc2. This is apparent from the decay rates. The authors should change their discussion and not comment on steady state levels but rather on decay rates. They may include plots of the data showing that mRNA half-lives are changed.
- Figure 6 and corresponding text: The Histone stability assay seems to have been performed in the absence of cycloheximide. It is thus impossible to conclude that histone protein degradation is impaired or that this degradation occurs normally but that newly synthesized proteins resulting from translation of stabilized histone mRNAs replenish the pool of histone. This experiment should be repeated with addition of cycloheximide (together with glucose or instead of glucose).
With these modifications, as well as the minor corrections listed below, the manuscript would certainly be of interest to the wide readership of the EMBO Journal.

Minor points:
- Page 3, line 9: The exosome is described as an exonuclease while it was recently described to be both an exonuclease and endonuclease (Lebreton et al. Nature 2008).

- Page 3, the list of Dcp activator should also include Edc3 and Scd6 (e.g., Decourty et al. PNAS 2008). Similarly, interaction of Lsm1 with Pat1 was reported not only by Bonnerot et al., and Tharun et al. as quoted, but also by Bouveret et al. Finally, references for the Lsm2-8 complex are not the most appropriate (e.g., Tharun 2009 is a review on the Lsm1-7 complex!). Credits should probably be given to the original publications describing this Lsm2-8 complex, especially as they were published in The EMBO Journal (Mayes et al. EMBO J, 1999; Salgado-Garrido et al. EMBO J., 1999).
- Page 4, line 5: ´ S. cerevisiae lacks mRNA uridylation [a] should be rewritten to indicate that uridylation was not detected up to now but may occur and have gone undetected.
- Page 5, line 9: Petermann et al. lacks a date.
- Page 10, line 2 from bottom: The authors should probably indicate that other Lsm genes were identified by Libuda and Winston.
- Discussion is long and could be shortened.

Referee #2 (Remarks to the Author):

The study titled "Lsm1 promotes replication fork stability by controlling histone mRNA levels" by Herrero and Moreno [manuscript # EMBOJ-2010-76501] presents strong evidence for the significant contribution of posttranscriptional mechanisms in the regulation of histone mRNAs in yeast that were not thought to be important previously. Although the effect of lsm mutations on histone mRNA levels in yeast have been published previously (Mazzoni et al., 2005, Palermo et al., 2020, also see below), these studies were focused on the aging phenotypes of lsm mutants in yeast. Hence, the current study is the most comprehensive study of the effect of lsm1 mutants on histone mRNAs to date and its relationship to genomic stability and as such I recommend that this manuscript be published following some additional confirmatory experiments as suggested below to strengthen the authors' conclusions.

Major Concerns:

1.) Mammalian histone mRNAs are regulated both at the level of transcription and posttranscriptionally (Sittman et al., 1983), although posttranscriptional mechanisms have been the focus of most studies in the last two decades. Unlike their mammalian counterparts, yeast histone mRNAs are traditionally thought of being regulated primarily at the level of transcription due to their very short half-lives of these transcripts, particularly in response to replication arrest, while any contribution of posttranscriptional regulation of histone mRNAs was dependent upon the 3' ends of the histone mRNAs (Lycan and Osley, 1987). Two decades after this study, it was suggested that the poly-A polymerases Trf4/5 and the exosome may be playing a role in posttranscriptional regulation of histone mRNAs in yeast (Reis and Campbell, 2007). More recently, Lsm4 (Mazzoni et al., 2005) and Lsm1 have been implicated in the histone regulation in yeast (Palermo et al., 2010). These recent studies together with the current manuscript strongly suggest that the traditional view that yeast histone mRNAs are largely regulated at the level of transcription needs to be revised to accommodate the significant contribution of posttranscriptional mechanisms in their regulation. This would suggest that despite the significant differences in the structure of yeast and mammalian histone mRNAs, and in the mechanistic details of their regulation, the overall principles governing the regulation of yeast and mammalian histone mRNAs are essentially the same. This provides validation for the study of histone regulation and its impact on genomic stability in yeast as a good model for the study of histone regulation in mammals. However, the authors of the current manuscript fail to cite the relevant classic or recent studies listed above to place their findings in the context of published literature. Doing so will not only make their case stronger, but may also help reconcile the differences in some of the results obtained in these studies.

2.) Since the half lives of yeast histone mRNAs are believed to be very short, it would be informative to measure their half-lives in lsm1 mutants to quantitate the effect of Lsm1 on yeast histone mRNA stability.

3.) Lsm1 is likely to affect the levels of numerous mRNAs, one or more of which can potentially affect replication fork stability. Although it is plausible that the accumulation of excess histones in the lsm1 mutants may directly cause the replication fork instability, this is far from being a foregone conclusion. The authors have shown that lsm1 mutants accumulate excess histone transcripts, and

although they claim throughout the paper that this results in the accumulation of excess histone proteins, they have not shown this to be the case. Hence, to substantiate their claims further, they need to demonstrate that these excess histone mRNAs contribute to the actual accumulation of excess histone proteins in the lsm1 mutant.

4.) Excess histone accumulation has been shown to affect the transcription of numerous genes (Singh et al., 2010). If lsm1 mutants indeed lead to the accumulation of excess histone proteins, it is possible that changes in the expression of some of these genes that are sensitive to histone levels may contribute to replication fork instability. Hence, it would be worthwhile to determine the mRNAs that are stabilized in the lsm1 mutant using microarray technology that is ubiquitously available. This would help confirm if the effect of Lsm1 on replication fork stability is indeed primarily via the regulation of histone mRNAs.

Minor Comments:

1.) On page 9, the authors claim that the "degradation of histone mRNAs takes place mainly by the 5' to 3' degradation pathway". How does this finding fit in with those of Lycan and Osley, 1987 and Reis and Campbell, 2007?

2.) On page 11, the authors mention that the "As previously shown, deletion of HHT2-HHF2 in the wild type strain had no effect in the resistance of the strain to MMS or HU". However, this reviewer was unable to find this data in the cited paper.

3.) On page 13, the authors state that "In animal cells, however, most of the regulation of histone synthesis occurs post-transcriptionally." As pointed out above, this appears to be the case simply because of the heavy focus on the study of posttranscriptional regulation of histone mRNAs in mammalian cells over the past two decades and several studies (Sittman et al., 1983; Ye et al., 2003; Su et al., 2003) have clearly shown the importance of transcriptional regulation in maintaining histone transcript levels.

4.) In figure 4a, is the HA tag present on the N- or C-terminus of histone H3? The positioning of the HA tag can presumably change the 5' or 3' end of the H3 transcript and could possibly interfere with the normal Lsm1 mediated regulation.

5.) In Figure 4c, can a longer exposure obtained after cutting out the top part of the Southern Blot be shown to demonstrate the different levels of ERCs in the three strains more clearly?

Referee #3 (Remarks to the Author):

In this manuscript, Herrero and Moreno reports the characterization of lsm1 , a yeast mutant isolated in a genome-wide screen for mutants that are hypersensitive to drugs interfering with DNA replication. Lsm1 is part of a complex involved in the degradation of mRNAs. Here, the authors show that lsm1 mutants accumulate Rad52 foci and H2A-P in response to genotoxic stress and fail to complete DNA replication, even though they are proficient to activate S-phase checkpoints and to repair double-strand DNA breaks. They also show that Lsm1 is important for the regulation of histone levels through the regulation of histone mRNA decay. From these data, the authors conclude that Lsm1 plays an important role in the maintenance of replication forks by regulating histone supply.

Overall, the manuscript is well written and the data are of high quality. However, the experiments performed by the authors are not sufficient to support the view that excess of free histones induces replication fork collapse in lsm1 cells exposed to HU or MMS. Indeed, none of the experimental approaches used here directly address the status of replication forks. Another plausible interpretation is that excess histone caused by replication inhibition is toxic to these cells, independently of replication fork collapse. In my opinion, since a link between Lsm1 and histone mRNA decay has already been established in yeast (Palermo et al., Cell Cycle 2010) and in human cells (Mullen and Marzluff, Gene Dev 2008), direct evidence of replication defects are required to warrant publication in the EMBO Journal.

Specific issues:

1) Page 6 (bottom): The statement that replication fork speed is reduced in lsm1 cells treated with MMS is too strong. The slower S-phase progression could also be due to reduced origin usage.

2) The ability of lsm1 mutants to activate Rad53 should be monitored in a time-course experiment and not for a single time point (Fig. 1B). Along the same line, the experiment performed in the presence of HU (Fig. 4C) is not convincing. The bulk of Rad53 should be hyperphosphorylated in HU-treated cells, which is not the case here.

3) Page 7: Increased phosphorylation of H2A in MMS-treated lsm1 cells does not necessarily reflect increased DSBs. This sentence should be toned down or the presence of DSBs actually demonstrated.

4) Fig. 4C: The difference between wt and fob1 cells is difficult to appreciate. A positive control for increased ERC level would have been useful. Again, the statement that Lsm1 is required for the stabilization of forks arrested at natural pause sites because of increased ERC levels and genetic interactions with mus81 should be toned down. Lsm1 could affect rDNA recombination in many ways and lsm1 mutants display negative genetic interactions with dozens of recombination and repair genes besides mus81.

1st Revision - authors' response 28 February 2011

We have now modified the manuscript to address the useful suggestions of the reviewers. We have included the following new data:

1. Fig. 1B. Time course of Rad53 phosphorylation in wt and *lsm1* mutant after MMS, phleomycin and HU treatment.
2. Fig. 4C. Southern blot to show the formation of DNA extra chromosomal circles (ERCs) in the *lsm1* mutant. In addition, we have used the strains *rtt101D* and *fob1D* as positive and negative controls, respectively.
3. Fig. 5B. Calculation of the half-lives of histone mRNAs in wt, *lsm1D*, *ski2D* and *lsm1D ski2D* double mutant.
4. Fig. 5C. Stability of H3 mRNA in wt and *lsm1D* cells following HU treatment.
5. Fig. 6A. Stability of H3 protein in wt and *lsm1D* cells in the absence and presence of cycloheximide.
6. Table S3. Gene expression profile of wt and *lsm1D* cells by microarray analysis.

We have also introduced the new references suggested by the reviewers 1 and 2. In our opinion, these changes have improved substantially the quality of the work and we trust the manuscript is now appropriate for publication in the *EMBO Journal.*

Response to editor´s comments:

In this situation and with these reports at hand, I feel that we should be able to consider a revised version of this manuscript further but only if you should be able to address a the following main concerns to the referees' satisfaction:

1. *Confirming altered mRNA half-lives/decay rates as asked for by referees 1 and 2; providing additional evidence for selectivity of the lsm1 mutation effect on histone mRNA (refs 1 and 2 - although I note that adding a microarray experiment as asked by referee 2 may exceed the scope of the current study).*

   We have measured the half-lives of histone mRNAs in the wild type, *ski2D*, *lsm1D* and *ski2 lsm1* double mutant. Histone mRNAs are stabilised approximately 2-fold in the *ski2D*, 4 to 6-

fold in *lsm1D* and 6 to10-fold in the double mutant. Therefore, although both, the 5´to 3´and the 3´to 5, pathways have an effect, the *lsm1* pathway (5´ to 3´) seems to have a higher impact.

We have confirmed by microarray analysis that histones are among the mRNAs upregulated in the *lsm1* mutant compared to the wild type. As predicted for a protein involved in mRNA decay many genes were upregulated in the *lsm1* mutant.

2. *Showing stronger evidence for altered histone levels being due to increased histone mRNA stabilization as asked for by referees 1 and 2.*

   We have performed the experiment suggested by reviewer #1 by adding cycloheximide instead of glucose to prevent *de novo* protein synthesis. In Figure 6A, we show that Histone H3 protein was unstable in the presence of cycloheximide in the wild-type and in the *lsm1* mutant, indicating that histone protein degradation is not impaired in the *lsm1* mutant and that the high levels of the histone H3 protein in this mutant is due to stabilization of the histone H3 mRNA.

3. *and trying to add more direct (mechanistic) evidence for effects on replication fork stability along the lines of referee 3's comments. I realize that the latter may be technically quite challenging, but otherwise at least the claims in the title and throughout the text would clearly have to be modified to something more general such as 'DNA damage sensitivity' or 'genomic stability' rather than 'fork stability'.*

   In order to have direct evidence of replication fork defects in the *lsm1* mutant we tried to use density transfer to analyse the progression of DNA replication forks (Tercero (2009). Methods Mol. Biol. 521, 203-213). For this purpose, we contacted Dr. Tercero at the CBMSO in Madrid who suggested to look at replication forks in *lsm1D* cells treated with HU. As shown in Figure 4A, in cells synchronised in G1 and treated with HU Rad53 remained phosphorylated after HU removal, pointing to a problem in DNA replication fork recovery that could be detected by density transfer. Dr. Tercero very kindly accepted to collaborate with us and constructed the *lsm1* mutant in the yeast W303 background lacking ARS608 and ARS609 DNA replication origins, that he normally uses for this experiment. However, this strain grew very slowly, the mother cells have a very big size and abnormal morphology and could not be properly synchronized in G1 with alpha factor, a requirement of the density transfer experiment. For this reason, we have been unable to obtain direct evidence of replication fork defects. Following your suggestion we have changed the title and the manuscript and use “genomic stability” rather than “fork stability”.

4. *Finally, please heed the requests of all three reviewers for more careful and appropriate citation of background literature, and try to refocus the discussion to reflect the input provided by referee 2 point 1.*

   We have included all the references suggested by the reviewers and have added a paragraph in the discussion reflecting the suggestion provided by reviewer #2 point 1.

5. *Finally, we will also require a brief section specifying the contributions of the individual authors on the paper.*

   A brief section with the author’s contributions has been added after the acknowledgements section.

Response to referees’ comments

Referee #1:

Overall, the manuscript is well written, the data are of very high quality and contain appropriate control. Thus, the manuscript is quite convincing. I believe that this manuscript is of interest because it provides a clear mechanistic explanation for a biological phenomenon, even if the effect of Lsm1 on replication fork stability and sensitivity to DNA damaging agents are somewhat indirect. Nevertheless, some points need to be corrected:

- *Page 9, line 10 from bottom and Figure 5C: The authors comment on the absence of increase of Cyc2 mRNA abundance in a delta-lsm1 mutant. The steady state mRNA level does not mean much as it is affected both by transcription and decay rates. In this specific case, the rate of decay is clearly affected by the delta-lsm1 mutation (Fig. 5C). The absence of a concomitant increase in the Cyc2 mRNA level thus indicates that transcription was also affected, most likely indirectly by the delta-lsm1 mutation (e.g., delta-lsm1 may stabilize an mRNA encoding a repressor of Cyc2 transcription). Overall, there is no evidence for specificity of the delta-lsm1 mutation that appears to affect the decay of all mRNAs tested, including histones and Cyc2. This is apparent from the decay rates. The authors should change their discussion and not comment on steady state levels but rather on decay rates. They may include plots of the data showing that mRNA half-lives are changed.*

We agree with the reviewer. Therefore, we have removed the data previously shown in Figure 5C and deleted all the information about this experiment from the results and discussion. We have measured the half-lives of the histone mRNAs in the wild type, lsm1 , ski2 and lsm1 ski2 double mutants (Figure 5B). The half-live of the histone mRNAs increased approximately 4 to 6-fold in the lsm1 mutant.

- *Figure 6 and corresponding text: The Histone stability assay seems to have been performed in the absence of cycloheximide. It is thus impossible to conclude that histone protein degradation is impaired or that this degradation occurs normally but that newly synthesized proteins resulting from translation of stabilized histone mRNAs replenish the pool of histone. This experiment should be repeated with addition of cycloheximide (together with glucose or instead of glucose).*

We have performed the experiment suggested by the reviewer with the addition of cycloheximide instead of glucose to prevent de novo protein synthesis. The result of this experiment is shown in Figure 6A. Histone H3 protein was unstable in the presence of cycloheximide both in the wild-type and in the lsm1 mutant, indicating that histone protein degradation is not impaired in the lsm1 mutant and that the high levels of the histone H3 protein in this mutant are caused by stabilization of the histone H3 mRNA.

Minor points:

- *Page 3, line 9: The exosome is described as an exonuclease while it was recently described to be both an exonuclease and endonuclease (Lebreton et al. Nature 2008).*

On page 3, line 11 we have added the sentence: "that has been recently shown to have also endonucleolytic activity (Lebreton et al., 2008).

- *Page 3, the list of Dcp activator should also include Edc3 and Scd6 (e.g., Decourty et al. PNAS 2008). Similarly, interaction of Lsm1 with Pat1 was reported not only by Bonnerot et al., and Tharun et al. as quoted, but also by Bouveret et al. Finally, references for the Lsm2-8 complex are not the most appropriate (e.g., Tharun 2009 is a review on the Lsm1-7 complex!). Credits should probably be given to the original publications describing this Lsm2-8 complex, especially as they were published in The EMBO Journal (Mayes et al. EMBO J, 1999; Salgado-Garrido et al. EMBO J., 1999).*

On page 3, line 19 we have added "Edc3 and Scd6 (Decourty et al. 2008)" and the references to Bouveret et al. 2000, Mayes et al. 1999 and Salgado-Garrido et al. 1999.

- *Page 4, line 5: S. cerevisiae lacks mRNA uridylation; should be rewritten to indicate that uridylation was not detected up to now but may occur and have gone undetected.*

On page 4, line 8 we have added the sentence: "In S. cerevisiae mRNA uridylation has not been detected".

- *Page 5, line 9: Petermann et al. lacks a date.*

Page 5, line 11 we have added Petermann et al., 2010.

- *Page 10, line 2 from bottom: The authors should probably indicate that other Lsm genes*

were identified by Libuda and Winston.

On page 11, line 15 after Libuda and Winston, 2010 we have changed the sentence: "Interestingly, LSM1 was found among the genes that suppressed this loss of viability" to "Interestingly, LSM1, LSM6 and LSM7 were found among the genes that increased viability".

- *Discussion is long and could be shortened.*

We have shortened the discussion.

Referee #2:

...Hence, the current study is the most comprehensive study of the effect of lsm1 mutants on histone mRNAs to date and its relationship to genomic stability and as such I recommend that this manuscript be published following some additional confirmatory experiments as suggested below to strengthen the authors' conclusions.

Major Concerns:

1) Mammalian histone mRNAs are regulated both at the level of transcription and posttranscriptionally (Sittman et al., 1983), although posttranscriptional mechanisms have been the focus of most studies in the last two decades. Unlike their mammalian counterparts, yeast histone mRNAs are traditionally thought of being regulated primarily at the level of transcription due to their very short half-lives of these transcripts, particularly in response to replication arrest, while any contribution of posttranscriptional regulation of histone mRNAs was dependent upon the 3' ends of the histone mRNAs (Lycan and Osley, 1987). Two decades after this study, it was suggested that the poly-A polymerases Trf4/5 and the exosome may be playing a role in posttranscriptional regulation of histone mRNAs in yeast (Reis and Campbell, 2007). More recently, Lsm4 (Mazzoni et al., 2005) and Lsm1 have been implicated in the histone regulation in yeast (Palermo et al., 2010). These recent studies together with the current manuscript strongly suggest that the traditional view that yeast histone mRNAs are largely regulated at the level of transcription needs to be revised to accommodate the significant contribution of posttranscriptional mechanisms in their regulation. This would suggest that despite the significant differences in the structure of yeast and mammalian histone mRNAs, and in the mechanistic details of their regulation, the overall principles governing the regulation of yeast and mammalian histone mRNAs are essentially the same. This provides validation for the study of histone regulation and its impact on genomic stability in yeast as a good model for the study of histone regulation in mammals. However, the authors of the current manuscript fail to cite the relevant classic or recent studies listed above to place their findings in the context of published literature. Doing so will not only make their case stronger, but may also help reconcile the differences in some of the results obtained in these studies.

We are very grateful to this reviewer for this very useful comment. We have included a paragraph in the discussion with this content.

2) Since the half lives of yeast histone mRNAs are believed to be very short, it would be informative to measure their half-lives in lsm1 mutants to quantitate the effect of Lsm1 on yeast histone mRNA stability.

We have measured the half lives of histone mRNAs in the wild type, ski2 , lsm1 and ski2 lsm1 double mutant. Histone mRNAs are stabilised approximately 2-fold in the ski2 , 4 to 6-fold in lsm1 cells and 6 to 10-fold in the double mutant. Therefore, although both, the 5¥to 3¥and the 3¥to 5, pathways have an effect the lsm1 pathway (5¥ to 3¥) seems to have a higher impact. This data has been added to Figure 5B.

3) Lsm1 is likely to affect the levels of numerous mRNAs, one or more of which can potentially affect replication fork stability. Although it is plausible that the accumulation of excess histones in the lsm1 mutants may directly cause the replication fork instability, this is far from being a foregone conclusion. The authors have shown that lsm1 mutants accumulate excess histone transcripts, and although they claim throughout the paper that this results in the accumulation of excess histone

proteins, they have not shown this to be the case. Hence, to substantiate their claims further, they need to demonstrate that these excess histone mRNAs contribute to the actual accumulation of excess histone proteins in the lsm1 mutant.

We have performed one new experiment suggested by referee #1 (Figure 6A), showing that histone H3 protein is degraded in the lsm1 mutant after the addition of cycloheximide. Therefore, accumulation of histones in the lsm1 mutant seems to be due to stabilization of histone mRNAs rather than to a defect in protein degradation.

4) Excess histone accumulation has been shown to affect the transcription of numerous genes (Singh et al., 2010). If lsm1 mutants indeed lead to the accumulation of excess histone proteins, it is possible that changes in the expression of some of these genes that are sensitive to histone levels may contribute to replication fork instability. Hence, it would be worthwhile to determine the mRNAs that are stabilized in the lsm1 mutant using microarray technology that is ubiquitously available. This would help confirm if the effect of Lsm1 on replication fork stability is indeed primarily via the regulation of histone mRNAs.

We have compared the levels of mRNA in the wild type and the lsm1 mutant by microarray analysis and have found that histones are found among the 2888 genes upregulated in the lsm1 mutant. As predicted for a protein involved in mRNA decay many genes were upregulated in the lsm1 mutant.

Minor Comments:

1) On page 9, the authors claim that the "degradation of histone mRNAs takes place mainly by the 5' to 3' degradation pathway". How does this finding fit in with those of Lycan and Osley, 1987 and Reis and Campbell, 2007?

We have measured the half lives of histone mRNAs in the wild type, ski2 , lsm1 and ski2 lsm1 double mutant. Histone mRNAs are stabilised approximately 2-fold in the ski2 , 4 to 6-fold in lsm1 and 6 to10-fold in the double mutant. Therefore, although both, the 5¥to 3¥and the 3¥to 5, pathways have an effect, the lsm1 pathway (5¥ to 3¥) seems to have a bigger impact.

On page 9, line 22: we have changed the original sentence: "We found that deletion of LSM1 highly increases the abundance and stability of all histone mRNAs analyzed (Figure 5B). Deletion of SKI2 did not affect the steady-state levels of histone mRNAs and resulted in a slight effect on histone mRNA stability. These results indicated that degradation of histone mRNAs takes place mainly by the 5¥ to 3¥ degradation pathwayÖto "We found that deletion of LSM1 increased the stability of all histone mRNAs analyzed four to six-fold (Figure 5B), whereas deletion of SKI2 affected histone mRNA stability to a lesser extend (two-fold). Double mutant lsm1 ski2 exhibited a strong accumulation of histone mRNAs (six to ten-fold). These results indicated that in budding yeast degradation of histone mRNAs takes place mainly by the 5¥ to 3¥ degradation pathway and to a lower extent by the 3¥to 5¥degradation pathway".

2) On page 11, the authors mention that "As previously shown, deletion of HHT2-HHF2 in the wild type strain had no effect in the resistance of the strain to MMS or HU". However, this reviewer was unable to find this data in the cited paper.

The reviewer is correct. Gunjan and Verrault (2003) showed that overexpression of HHT2-HHF2 in the wild-type had no effect in the sensitivity/resistance to MMS or HU but they did not check behaviour of the HHT2-HHF2 deletion. Therefore, on page 11, line 19 we have deleted this reference and changed the original sentence: "As previously shown, deletion of HHT2-HHF2 in the wild-type strain had no effect in the resistance of the strain to MMS or HU (Gunjan and Verreault, 2003)" to " Deletion of HHT2-HHF2 in the wild-type strain had no effect in the sensitivity of the strain to MMS or HU".

3) On page 13, the authors state that "In animal cells, however, most of the regulation of histone synthesis occurs post-transcriptionally." As pointed out above, this appears to be the case simply because of the heavy focus on the study of posttranscriptional regulation of histone mRNAs in mammalian cells over the past two decades and several studies (Sittman et al., 1983; Ye et al., 2003; Su et al., 2003) have clearly shown the importance of transcriptional regulation in maintaining

histone transcript levels.

On page 13, line 16 we have corrected this sentence to "In animal cells, regulation of histone synthesis occurs both transcriptionally and post-transcriptionally (Sittman et al., 1983; Ye et al., 2003; Kaygun and Marzluff, 2005; Mullen and Marzluff, 2008). Posttranscriptional regulation consists of rapid degradation of histone mRNAs, both after inhibition of replication and at the end of a normal S-phase. Histone mRNAs are the only mRNAs in mammalian cells that are not polyadenylated. Instead, they end in a stem-loop structure that is recognized by the Stem Loop Binding Protein (SLBP). It has been shown that histone mRNA degradation in human cells requires the protein Upf1, which interacts with the SLBP at the 3¥end of histone mRNAs after treatment with HU (Kaygun et al. 2005).

4) In figure 4A, is the HA tag present on the N- or C-terminus of histone H3? The positioning of the HA tag can presumably change the 5' or 3' end of the H3 transcript and could possibly interfere with the normal Lsm1 mediated regulation.

The HA tag is present in the C-terminus of histone H3. We used the construction described by Gunjan and Verrault (2003).

5) In Figure 4C, can a longer exposure obtained after cutting out the top part of the Southern Blot be shown to demonstrate the different levels of ERCs in the three strains more clearly?

We have included a new exposure of the Southern blot in Figure 4C. In addition, we have used the strain rtt101 as a positive control. On page 8, line 16 we have added the following sentence: "As a positive control we used rtt101 that has been previously shown to accumulate ERCs (Luke et al., 2006)."

Referee #3:

Overall, the manuscript is well written and the data are of high quality. However, the experiments performed by the authors are not sufficient to support the view that excess of free histones induces replication fork collapse in lsm1 cells exposed to HU or MMS. Indeed, none of the experimental approaches used here directly address the status of replication forks. Another plausible interpretation is that excess histone caused by replication inhibition is toxic to these cells, independently of replication fork collapse. In my opinion, since a link between Lsm1 and histone mRNA decay has already been established in yeast (Palermo et al., Cell Cycle 2010) and in human cells (Mullen and Marzluff, Gene Dev 2008), direct evidence of replication defects are required to warrant publication in the EMBO Journal.

In order to have direct evidence of replication fork defects in the lsm1 mutant we tried using density transfer to analyse the progression of DNA replication forks (Tercero (2009). Methods Mol. Biol. 521, 203-213). For this purpose, we contacted Dr. Tercero at the CBMSO in Madrid who suggested to look at replication forks in cells treated with HU. As shown in Figure 4A, Rad53 remained phosphorylated after HU removal, pointing to a problem in DNA replication fork recovery that could be detected by density transfer. Dr. Tercero very kindly constructed the lsm1 mutant in the W303 background lacking ARS608 and ARS609 DNA replication origins. However, this strain grew very slowly, the mother cells have a very big size and abnormal morphology and could not be properly synchronized in G1 with alpha factor, a requirement of the density transfer experiment. Therefore, since we have been unable to perform this experiment we have turn down our claims in the revised manuscript.

Specific issues:

1) Page 6 (bottom): The statement that replication fork speed is reduced in lsm1 cells treated with MMS is too strong. The slower S-phase progression could also be due to reduced origin usage.

We have deleted the sentence "The reduction of fork speed in lsm1 cells treated with MMS suggested the existence of defects in replication fork stability."

2) The ability of lsm1 mutants to activate Rad53 should be monitored in a time-course experiment and not for a single time point (Fig. 1B). Along the same line, the experiment performed in the presence of HU (Fig. 4C) is not convincing. The bulk of Rad53 should be hyperphosphorylated in HU-treated cells, which is not the case here.

We have performed the time course experiment which is shown in Figure 1B. Phospho-Rad53 (Rad53-P) is more intense in the lsm1 mutant than in the wild-type after treatment with HU, as also shown in Figure 4A. We added the following sentence on page 5, line 19: "In fact, compared to the wild-type strain, in the lsm1 mutant we observed a further increase in the degree of Rad53 activation after treatment with HU, as revealed by the stronger intensity of the Rad53 phosphorylated form."

3) Page 7: Increased phosphorylation of H2A in MMS-treated lsm1 cells does not necessarily reflect increased DSBs. This sentence should be toned down or the presence of DSBs actually demonstrated.

On page 7, line 11 we have replaced "revealing" for "suggesting".

4) Fig. 4C: The difference between wt and fob1 cells is difficult to appreciate. A positive control for increased ERC level would have been useful.

We have included an improved exposure of the Southern blot in Figure 4C. In addition, we have used the strain rtt101 as a positive control. On page 8, line 16 we have added the following: "As a positive control we used rtt101 that has been previously shown to accumulate ERCs (Luke et al., 2006)."

Again, the statement that Lsm1 is required for the stabilization of forks arrested at natural pause sites because of increased ERC levels and genetic interactions with mus81 should be toned down. Lsm1 could affect rDNA recombination in many ways and lsm1 mutants display negative genetic interactions with dozens of recombination and repair genes besides mus81.

On page 8, line 18 we have changed the sentence "These results indicated that Lsm1 is required for the stabilization of replication forks that move through damaged and paused DNA sites. A further confirmation of this conclusion came from the analysis of synthetic genetic interactions of lsm1 with mms4 and mus81 ," to "These results suggested that Lsm1 is required for the stabilization of replication forks that move through damaged and paused DNA sites, which is supported by the analysis of synthetic genetic interactions of lsm1 with mms4 and mus81 ,"

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Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and it has now been seen once more by one of the original referees (see comments below). As a result, I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

Referee #1

(Remarks to the Author)
In this revised version of their manuscript, Herrero and Moreno have answered my comments in a suitable manner. If the other referee's are also convinced the author's answers to their criticisms, I believe that the manuscript should be published in the EMBO Journal.

