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## Mechanisms of regulation of RNA polymerase III-dependent transcription by TORC1

Yuehua Wei, Chi Kwan Tsang

*Corresponding author: X.F. Steven Zheng, UMDNJ*

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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

27 February 2009

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Thank you for submitting your research manuscript for consideration to The EMBO Journal editorial office. As you will see from the enclosed reports, all three referees appreciate your findings that focus on functional interaction between TORC1 kinase and Maf1 in the regulation of PolIII transcription. Apart from this, all three referees request a significant amount of additional experimental work beside necessary improvement of data presentation and style to make to work accessible to a more general audience. Careful reading of specifically ref#2's report also reveals that the paper remains currently very descriptive and correlative, lacking thorough validation of causality. As this is very precisely detailed in the comments of this currently very critical referee there is no reason for me to repeat the shortcomings in very much detail. However, and appreciating the potential (!) interest in your study as also expressed from the other two referees, we still decided to offer you the chance to address all the criticisms raised during one round of major amendments. I have to remind you that the final decision on acceptance or rejection solely depends on the content of the final version of your manuscript that will certainly involve a re-assessment from the currently more reserved ref#2.

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):

Using ChIP assay, Wei et al., show in this manuscript the presence of TORC1 on the genes encoding 5S rRNA. This occupancy is reduced by starvation or in presence of rapamycin. Importantly, the authors report that the repression of transcription of Pol I- and Pol III-transcribed genes usually observed in presence of rapamycin is impaired if Tor1 is artificially present in the nucleus on the DNA (using several Tor1 mutants). Then, the authors focus their work on the Tor1-control of Maf1, the main regulator of Pol III transcription. The authors check the level of phosphorylation and the cellular localization of Maf1 in two different genetic backgrounds using several Tor1 mutants. In the two strains tested and in presence of rapamycin, the dephosphorylation of Maf1 is impaired (more or less, see my comments) if Tor1 is artificially present in the nucleus on the DNA. It has been reported that, in presence of rapamycin, Maf1 is translocated from the cytoplasm to the nucleus. The authors report that in a W303 context, Maf1 is always present in the nucleus and exclude from the nucleolus. Rapamycin induces a rapid accumulation of Maf1 in the nucleolus. The artificial presence of Tor1 to the DNA impaired the normal accumulation of Maf1 to Pol III-transcribed genes in presence of rapamycin. At last, Wei et al. deduce of co-immunoprecipitation and in vitro kination assays that TOR1C interacts with Maf1 and is able to phosphorylate this protein.

Overall, these studies reveal important aspects of the molecular functions of TOR1C and Maf1 that are clearly of great significance to the control of Pol I- and Pol III-transcribed genes in yeast and other organisms. However, experiments and manuscript need to be improved before publication.

1. Because the shapes of radiolabelled bands of fig. 7B are different from GST-Maf1 bands stained with Coomassie blue, it is essential to perform all these kination assays with GST alone (as controls) or GST fused to a control protein.
2. The figures 3A and 4A should be fused to allow a better comparison between western blots in the two genetic backgrounds. The main difference with the TOR1-RR mutant (absence or presence of Maf1 dephosphorylation caused by rapamycin in FM391 or W303 context respectively) has to be noted in the result part. The sentence page 11 line 7: "In the presence of TOR1-RR or TOR1-RR/NESdelta, Maf1 remained phosphorylated despite of rapamycin treatment" is only suitable to FM391/S288C strain and not for W303 strain. It is curious that Maf1 is mostly dephosphorylated in presence of rapamycin in TOR1-RR mutant in W303 context (fig. 4A) and that Pol III transcription is not inhibited in this condition in the same genetic background (see fig. 2A & B). Authors should discuss this phenomenon.
3. A control with a strain expressing Maf1-HA6 alone is indispensable to correctly interpret the fig. 7A upper panel.
4. The abstract does not carefully reflect the data shown in the manuscript. It gives the feeling that a big part of the work is dedicated to the TOR1C control of Pol I- and Pol III-transcribed genes and ribosome biogenesis but this is not the case. The abstract must be more focused on the results really presented in the manuscript (TOR1C control of Pol III transcription mediated by Maf1).
5. The authors report the occupancy of 5S rDNA by TORC1 as a novelty (abstract, page 7 and page 15). The Tor1 binding to the 35S rDNA promoter and the 5S rDNA in a rapamycin-sensitive manner was already reported by the Zheng lab in an article published in Nature journal (Li et al., 2006, see text and fig. 3e, f of this article). The novelties reported in the Wei et al. herein submitted manuscript are (1) the Tor1 binding to the 35S rDNA promoter and to the 5S rDNA in a NUTRIENT-sensitive manner and (2) the characterization of TORC1 binding to 5S rDNA.
6. All ChIP experiments have been performed with PCR. Real-time PCR (more sensitive and more accurate for quantification) should be used to confirm some results and to check whether Tor1 is present on tRNA genes. The last experiment is very important to test if the TOR1C control of Pol III transcription of tRNA genes is direct or indirect.

7. Throughout their manuscript, the authors refer to "Pol I or III genes" where they actually mean Pol I- or III-transcribed genes. Just as a globin gene encodes globin, a Pol III gene is a gene encoding a subunit of Pol III, which is not what is meant here. To avoid confusion, it is important that the term "Pol I or III genes" be replaced throughout with "Pol I or III-transcribed genes", or "class I or III genes".

8. Minor comments:

Page 9, line 11 and page 11, line 9: "Tor1-RR/NLS2mt" should be "Tor1-RR/NLSmt"

Page 12, line 5: "nucleus" should be "cytoplasm"

Page 14, line 2: "upper" should be "low"

Page 14, line 4: "low" should be "upper"

Page 15, line 7: "trascription" should be "transcription"

Page 17, line 19: CARA (for Constitutive Association of Rrn3 and A43) is a strain and not a Rrn3-A43 fusion protein as written (the strain synthesizes a Rrn3-A43 fusion protein)

Referee #2 (Remarks to the Author):

Wei et al. regulation of Pol III transcription by TOR1C at the chromatin.

The transcriptional synthesis of rRNA (Pol I) is thought to be co-regulated with the transcriptional synthesis of 5S rRNA and tRNAs (Pol III). This poorly understood aspect of growth control was approached by using yeast as a biological model. The data are interesting but unequally convincing, and lead to a rather vaguely formulated model, where Wei et al. propose that this co-regulation involves some direct functional interaction between the TORC1 kinase and Maf1, a negative regulator of Pol III. In its present form, this paper is hard to read, and I shall therefore begin by reformulating the main experimental claim, as I understand them, before analysing the data in more detail.

Experimental claims.

1. Using ChIP assays, Zheng et al. previously found that TORC1 is associated with the 35S rDNA promoter. This is lost when the Pol I dependent transcription of rDNA is blocked by nutrient depletion or Rapamycin. Not surprisingly, a Tor1 mutant lacking the Nuclear Export Signal (NES) leads to a constitutive association to the 35S rDNA, whereas a mutant excluding TORC1 from the nucleus has no detectable ChIP signal. Moreover, the 'HTH' domain of Tor1 is needed for its association to rDNA. Using the same experimental set up, Figure 1 shows a similar association of TORC1 to the 5S rDNA, transcribed by Pol III, but no CHIP signal was found for tDNA genes.
2. Preventing the association of TORC1 to the 5S and 35S rDNA genes impairs the synthesis of the corresponding rRNA, and also reduces the synthesis of tRNA, although the quantification of Figure 2 suggests a rather modest effect. Hence, it appears that TORC1 regulates the two main forms of Pol III transcription (tRNA and 5S rRNA) although it only binds 5S rDNA chromatin, not the tDNAs.
3. Preventing the association of TORC1 to the 5S and 35S rDNA genes leads to the accumulation of non-phosphorylated Maf1, which blocks Pol III in vivo. Towpik et al. (2008) recently showed that Maf1 phosphorylation and Pol III activity still responds to Rapamycin, even when Maf1 permanently resides in the nucleus. The present report extends Towpik's data by showing a major strain-dependent difference in the nucleo-cytoplasmic traffic of Maf1. Thus, Rapamycin does not (or not entirely) act by regulating the nucleo-cytoplasmic exchange of Maf1. Based on immunofluorescence data, Wei et al. suggest that TORC1 controls the nucleolar localisation of Maf1.
4. CoIP data are presented to suggest that Kog1 (a component of TorC1) and Maf1 may be part of the same multi-protein complex. Figure 7B shows that a bacterially expressed Maf1 can be phosphorylated in vitro by Tor1.
5. A model sketched out in Fig.7 posits that TORC1 turns down Pol III-dependent transcription by binding the 5S rDNA. This would allow TORC1 to directly phosphorylate Maf1, which would in turn 'prevent Maf1 from accumulating in the nucleolus and binding to Pol III-dependent genes' (legend of Figure 7), assuming, of course, that tRNA genes are themselves nucleolar. The model is vaguely formulated that I might have missed some essential aspect of it. However, it still doesn't understand if and how Wei et al. suggest that TORC1 regulates Pol III (including at the level of tRNA and other genes) by binding to the 5S rDNA chromatin, as implied by their title.

Specific comments.

Figure 1 documents the interesting finding that TORC1 associates with 5S rDNA

1. ChIP data do not show that a protein binds DNA, but provide evidence for an association close enough to warrant cross-linking. The HTH mutant supports a direct DNA binding effect, but there is no direct evidence that it is defective in DNA binding (as opposed to some association to chromatin). If Wei et al. want to argue that TORC1 is a DNA-binding protein (at the 35S and 5S rDNA), they should provide better evidence, such as a DNA shift assay, or strongly qualify their conclusion.

2. Tor1-delta controls, presented for IP made with Tor1 Ab (Figure 1B), are a useful but almost trivial control that the antibodies are indeed Tor-specific. It would be more pertinent to show this for the Kog1-Myc9 assay, to show that the signal observed is indeed Tor1-dependent. Showing the 35S rDNA (positive) controls along the 5S rDNA data would also help!

3. The negative ChIP results with tDNA are puzzling. It is not at all clear why the failure to detect TORC1 at tDNA genes could be explained by 'a low sensitivity of the ChIP assay' as suggested by the authors, especially since they detect Maf1 CHIP signals (Figure 6). Gene copy number is unlikely to be the explanation since the assay is based on signal enrichment. Negative results are interesting, and the authors should show them, perhaps with an appropriate control such as Pol I/Pol III epitope-tagged at one of the two common subunits (Rpc19 and Rpc40).

Figure 2 provides evidence that the synthesis of rRNA and tRNA correlates with the association of TORC1 to 5S and 35S rDNA, supporting the idea that TORC1 might regulate rDNA transcription by phosphorylating Pol I and Pol III or some associated factors such as Maf1.

1. The data are fine for 5S rRNA (Figure 2A), but not so for tRNA(Leu), where the quantification of Figure 2B reveals a rather modest effect (a 0.49 ratio seen in the absence of TOR1-RR, rising to 0.78 seen with the NES-delta form). If the authors argue that the presence of TORC1 at the 5S rDNA affects the Maf1-dependent control of tRNA genes (which, I think, is their conclusion), this quantification should be better documented.

2. The supplementary Figure might be presented in the main text. The experimental set-up is not obvious to me, since Tor1 can be inactivated without notably impairing growth (right?). I surely miss an important point, but please clarify the reasoning.

Figures 3, 4 and 6 present complex but interesting data, adding some evidence to the idea that Maf1 control on Pol III (in response to Rapamycin or to nutrient depletion) responds to TORC1 residence at rDNA. Figure 4 could possibly be left to the Supplement section. Some of these data make sense at the light of a paper by Towpik et al. (JBC 2008) based on Msn5 mutants. Referring to this paper at the beginning of this experimental section would probably simplify its presentation.

1. Maf1 nuclear localisation (in response to Rapamycin) and its association to 5S rDNA strongly depends on the strain background (S288C versus W303). It would be nice to know if these interesting differences are monogenic (the two 'wild' strains differ by their SSD1 gene, which interferes with Pol II and Pol III transcription- Stettler et al. MGG 1983). This could be checked by tetrad analysis, and by complementation by Msn5 or Ssd1.

2. Figure 6A documents the association of Maf1 to 5S rDNA (in response to Rapamycin) but the 35S rDNA control shown is 25S (internal). This implies, importantly, that Maf1 does not bind to 35S rDNA, but in that case it would be critical to use promoter 35S rDNA ChIP.

3. Figure 6C suggests that TORC1 must reside at rDNA (5S or 35S) to affect the Maf1-dependent control in response to Rapamycin. This is mostly based on the HTH Tor1 mutant (see my comment above on this mutant). It is essential to show the effect of the Tor1 mutants (NES, mt,HTH) on the Maf1 ChIP signal at the tRNA genes. Does they affect the tRNA signal as well?

Figure 5 shows that Rapamycin extends Maf1 to the nucleolus. This is consistent with the idea that Maf1 'binds' 5S rDNA in response to Rapamycin, and might be better presented with Figure 6C.

1. Nop1 was used as nucleolar marker. Nop1 occupies the whole nucleolus, and is thus not restricted to rDNA. Hmo1, a strong rDNA binding marker with a much more restricted distribution (Gadal et al., EMBO J. 2004) should be a much better indicator of rDNA.

2. It is suggested that tRNA genes are nucleolar and that the nucleolar localisation of Maf1 is needed for their activation. This is an interesting but controversial point, so far largely based on data by Engelke et al. If this is indeed the model proposed by Wei et al., they should extend their immunofluorescence data to Pol III, and see if it has a nucleolar localisation, depending on the rapamycin treatment.

Figure 7 presents data suggesting that Maf1 a substrate of Tor1. Since Maf1 and Tor1 are able to co-reside at the 5S rDNA, Co-IP would only support their hypothetical functional interaction if we had

an idea of the stringency of the conditions used. It is also shown that Tor1 is able to phosphorylate recombinant Maf1, but how can we tell that any Tyr or Ser containing substrate would not be phosphorylated under these conditions. These are important stringency controls. In their absence these potentially interesting data are rather preliminary and should be left out, or better substantiated.

General comments: Wei et al. should be encouraged to write a more concise introduction, immediately presenting the known roles of Tor and of Maf1 in controlling and possibly coordinating Pol I and Pol III. Their discussion could also be improved to stress more clearly what is new in their data. As a minor point, and in keeping with recent recommendations by EMBO J., they should highlight the original papers on Tor (M. Hall et al.) and Paf1 (Pluta et al.), along with recent review papers.

Referee #3 (Remarks to the Author):

This manuscript provides new insight on the regulation of RNA polymerase III (Pol III) transcription, specifically on the role of TOR kinase in preventing repression of transcription. The study uses previously characterized rapamycin-resistant (RR) wildtype and mutant alleles of TOR1 to show that the nuclear, chromatin-binding form of Tor1 associates with 5S DNA in the context of the multisubunit complex, TORC1. The TOR1RR NES mutant blocks transcriptional repression, accumulates phosphorylated Maf1 and blocks Maf1 binding to tRNA and 5S RNA genes. Maf1 is shown to localize at the nucleolus under repressing conditions. Together, these data suggest that TORC1 phosphorylates Maf1 and prevents Maf1 function, specifically its association with nucleolar 5S PolIII genes. The demonstration that Kog1, a subunit of TORC1, can associate with Maf1 in vivo and that immunoprecipitated TOR1 can phosphorylate Maf1 in vitro suggests that TORC1 can phosphorylate Maf1 directly.

Several observations in the paper are especially significant: First, that TORC1 binds to the 5S DNA genes; Second, that Maf1 associates with the nucleolus under repressing conditions and Third, that purified TOR1 phosphorylates Maf1. Together, these data support a direct role for TOR1 in regulating pol III transcription and describe a mechanism by which 5S and tRNA transcription may be coordinated.

General comments:

The manuscript suffers from the numerous grammatical and typographical errors. At least one reference citation has been omitted (three groups showed that Maf1 is a phosphoprotein present in the cytoplasm under nutrient-rich conditions, Oficjalska-Pham et al, 2006; Roberts et al, 2006 and Moir et al, 2006, not two).

The manuscript contains inaccurate and confusing misstatements that detract from its readability (several of which are outlined below). These render the otherwise significant work unsuitable for publication as written.

Figure 2A and legend: The panel annotation is not clear. The dash, between vector and HTH could be understood to refer to "no plasmid" rather than TOR1-RR.

Page 10: The statements that "We metabolically labeled yeast cells with <sup>3</sup>H-Uracil to measure the rate of rRNA synthesis" and "To confirm this result, we performed Northern blot analysis of a pre-tRNA<sup>LEU3</sup>, which is commonly used to monitor the transcriptional rate of tDNAs" are not accurate. In this protocol, pulse labeling does not measure a rate of rRNA synthesis but provides a relative measure of rRNA synthesis. Similarly, the Northern does not monitor the transcriptional rate of tDNAs but detects a labile precursor tRNA species and is an indirect readout for tRNA synthesis.

Page 10: LEU3 refers to a protein coding gene not a tRNA gene. The tRNA gene is referred to as tRNA<sup>Leu3</sup>.

Page 10: "Indeed, the Pre-Leu showed the same expression pattern in cells carrying different TOR1-RR variants (Figure 2B)." is incorrect given the data shown in Figures 2A and 2B.

This section is would be more accurately and clearly written as follows: "To confirm this result, we performed Northern blot to detect a labile precursor tRNA species (pre-tRNA<sup>Leu3</sup>), which is commonly used to report tRNA synthesis (Oficjalska-Pham et al, 2006; Upadhya et al, 2002). Indeed, tRNA synthesis showed the same response to the TOR1-RR variants as 5S and 5.8S RNAs transcription: a requirement for functional NLS and HTH domains in TORC1 in order to block repression (Figure 2B).

Page10: "The parallel in the regulation of Pol I and Pol III by TORC1 further suggests that simultaneous binding of TORC1 to rDNA serves as a mechanism to coordinate ribosomal RNA synthesis." As there is no data reported for simultaneous binding to both loci, the sentence should probably read: " The parallel regulation of Pol I and Pol III transcription by TORC1 further suggests that binding of TORC1 to both the 25S rDNA promoter and 5S gene in the rDNA may serve as a mechanism to coordinate ribosomal RNA synthesis."

Page 12: "To our surprise, however, very little Maf1 was detected in the nucleus of W303a cells (Figure 4B). Instead, Maf1 was mainly localized in the nucleus." Figure 4B shows the first sentence to be in error and "nucleus" should be substituted with "cytoplasm".

Discussion, page 15: "In contrast, the cytoplasmic form of TORC1 is unable to promote Pol III-dependent gene expression." "promote" should be substituted with "regulate".

1st Revision - authors' response

14 April 2009

#### Responses to Referees' Comments

We are grateful to the reviewers for their constructive comments and insightful suggestions. We have performed additional experiments and revised the manuscript to address their concerns. Our new results lend further support to our central conclusions and improved the overall presentation. Below is our point-by-point response.

#### Referee #1

1. Because the shapes of radiolabelled bands of fig. 7B are different from GST-Maf1 bands stained with Coomassie blue, it is essential to perform all these kination assays with GST alone (as controls) or GST fused to a control protein.

Response: In Figure 6B, we included GST alone and GST-Fzo1 as controls to show that Tor1 specifically phosphorylated Maf1 protein in a rapamycin-sensitive manner. Phospho-Maf1 is known to have a slower electrophoretic mobility, which is likely to explain why the radioactively labeled bands are different from Coomassie blue-stained bands.

2. The figures 3A and 4A should be fused to allow a better comparison between western blots in the two genetic backgrounds. The main difference with the TOR1-RR mutant (absence or presence of Maf1 dephosphorylation caused by rapamycin in FM391 or W303 context respectively) has to be noted in the result part. The sentence page 11 line 7: "In the presence of TOR1-RR or TOR1 RR/NESdelta, Maf1 remained phosphorylated despite of rapamycin treatment" is only suitable to FM391/S288C strain and not for W303 strain. It is curious that Maf1 is mostly dephosphorylated in presence of rapamycin in TOR1-RR mutant in W303 context (fig. 4A) and that Pol III transcription is not inhibited in this condition in the same genetic background (see fig. 2A & B). Authors should discuss this phenomenon.

Response: We have moved the original Figure 4A to Figure 3B for a better comparison. As suggested by reviewer #2, we moved the original Figure 4B to Supplementary Figure 1. The slight difference in Maf1 phosphorylation in Tor1-RR strain upon rapamycin treatment is due to gel variation. We now show another set of data in Figure 3B. Our independent experiments indicate that Maf1 phosphorylation is resistant to rapamycin in W303a strain expressing Tor1-RR.

3. A control with a strain expressing Maf1-HA6 alone is indispensable to correctly interpret the fig. 7A upper panel.

Response: We repeated the experiment with Maf1-HA6 as a control. Our data show that Kog1-Myc9 was present in the IP only in the presence of Maf1-HA6 but not in its absence. A reverse IP shows essentially the same result (Figure 6A).

4. The abstract does not carefully reflect the data shown in the manuscript. It gives the feeling that a big part of the work is dedicated to the TORC1 control of Pol I- and Pol III-transcribed genes and ribosome biogenesis but this is not the case. The abstract must be more focused on the results really presented in the manuscript (TOR1C control of Pol III transcription mediated by Maf1).

Response: We have revised the abstract accordingly.

5. The authors report the occupancy of 5S rDNA by TORC1 as a novelty (abstract, page 7 and page 15). The Tor1 binding to the 35S rDNA promoter and the 5S rDNA in a rapamycin-sensitive manner was already reported by the Zheng lab in an article published in Nature journal (Li et al., 2006, see text and fig. 3e, f of this article). The novelties reported in the Wei et al. herein submitted manuscript are (1) the Tor1 binding to the 35S rDNA promoter and to the 5S rDNA in a NUTRIENT-sensitive manner and (2) the characterization of TORC1 binding to 5S rDNA.

Response: We are more explicit about what is new and what is already published.

6. All ChIP experiments have been performed with PCR. Real-time PCR (more sensitive and more accurate for quantification) should be used to confirm some results and to check whether Tor1 is present on tRNA genes. The last experiment is very important to test if the TOR1C control of Pol III transcription of tRNA genes is direct or indirect.

Response: We have performed real-time PCR, confirming Tor1 association of 35S and 5S rDNA chromatin (Figure 1D). We have also performed real-time PCR to test the association of Tor1 to tRNA genes (Figure 1F) but couldn't identify any specific association.

7. Throughout their manuscript, the authors refer to "Pol I or III genes" where they actually mean Pol I- or III-transcribed genes. Just as a globin gene encodes globin, a Pol III gene is a gene encoding a subunit of Pol III, which is not what is meant here. To avoid confusion, it is important that the term "Pol I or III genes" be replaced throughout with "Pol I or III-transcribed genes", or "class I or III genes".

Response: We have replaced "Pol I or III genes" with "Pol I- or Pol III-transcribed genes" throughout the manuscript.

8. Minor comments:

Page 9, line 11 and page 11, line 9: "Tor1-RR/NLS2mt" should be "Tor1-RR/NLSmt"

Page 12, line 5: "nucleus" should be "cytoplasm"

Page 14, line 2: "upper" should be "low"

Page 14, line 4: "low" should be "upper"

Page 15, line 7: "transcription" should be "transcription"

Page 17, line 19: CARA (for Constitutive Association of Rrn3 and A43) is a strain and not a Rrn3-A43 fusion protein as written (the strain synthesizes a Rrn3-A43 fusion protein)

Response: We have corrected all these errors.

Referee #2:

Specific comments.

Figure 1 documents the interesting finding that TORC1 associates with 5S rDNA

1. ChIP data do not show that a protein binds DNA, but provide evidence for an association close

enough to warrant cross-linking. The HTH mutant supports a direct DNA binding effect, but there is no direct evidence that it is defective in DNA binding (as opposed to some association to chromatin). If Wei et al. want to argue that TORC1 is a DNA-binding protein (at the 35S and 5S rDNA), they should provide better evidence, such as a DNA shift assay, or strongly qualify their conclusion.

Answer: We agree that TORC1 could be associated with rDNA either directly or indirectly. To avoid any confusion, we have generally referred to TORC1 association with rDNA chromatin. We have also changed the title to reflect this point.

2. Tor1-delta controls, presented for IP made with Tor1 Ab (Figure 1B), are a useful but almost trivial control that the antibodies are indeed Tor-specific. It would be more pertinent to show this for the Kog1-Myc9 assay, to show that the signal observed is indeed Tor1-dependent. Showing the 35S rDNA (positive) controls along with the 5S rDNA data would also help!

Answer: Kog1 associated with either Tor1 or Tor2 to form TORC1. Therefore, Kog1-Myc9 is expected to still bind to 5S rDNA chromatin in tor1 strain in the form of TORC1 containing Tor2. As requested, we have added new data in Figure 1E to show Kog1-Myc9 association with 35S rDNA promoter along with the 5S rDNA.

3. The negative ChIP results with tDNA are puzzling. It is not at all clear why the failure to detect TORC1 at tDNA genes could be explained by 'a low sensitivity of the ChIP assay' as suggested by the authors, especially since they detect Maf1 ChIP signals (Figure 6). Gene copy number is unlikely to be the explanation since the assay is based on signal enrichment. Negative results are interesting, and the authors should show them, perhaps with an appropriate control such as Pol I/Pol III epitope-tagged at one of the two common subunits (Rpc19 and Rpc40).

Answer: Also refer to Reviewer #1 point 6. We have performed real-time PCR that is a more sensitive and quantitative method but are still unable to show any specific Tor1 association with tDNA (Figure 1F). In our hands, we can clearly detect proteins bound to tDNA as shown with Maf1 (Figure 5).

Figure 2 provides evidence that the synthesis of rRNA and tRNA correlates with the association of TORC1 to 5S and 35S rDNA, supporting the idea that TORC1 might regulate rDNA transcription by phosphorylating Pol I and Pol III or some associated factors such as Maf1.

1. The data are fine for 5S rRNA (Figure 2A), but not so for tRNA(Leu), where the quantification of Figure 2B reveals a rather modest effect (a 0.49 ratio seen in the absence of TOR1-RR, rising to 0.78 seen with the NES-delta form). If the authors argue that the presence of TORC1 at the 5S rDNA affects the Maf1-dependent control of tRNA genes (which, I think, is their conclusion), this quantification should be better documented.

Answer: The effect of TORC1 inhibition on tRNA synthesis is moderate but reproducible. Other investigators in the field have reported similar observations (Molecular cell 22, 623-32; PNAS 103, 15044-9). The differential effects of rapamycin on 5S rDNA and tDNAs could reflect distinct regulatory mechanisms: one requires TORC1 association with chromatin (5S rDNA), resulting in robust regulation; the other does not (tDNA), resulting in only moderate control. We have included a sentence in the Discussion section to reflect this point.

2. The supplementary Figure might be presented in the main text. The experimental set-up is not obvious to me, since Tor1 can be inactivated without notably impairing growth (right?). I surely miss an important point, but please clarify the reasoning.

Answer: We have moved the original Supplementary Figure 1 to Figure 2A. We have also described the experimental strategy in detail in the text. Both Tor1 and Tor2 can form TORC1. In a strain without Tor1, Tor2 can still form TORC1. Therefore, cell growth remains relatively normal. We then use rapamycin to transiently knock down this endogenous TORC1 (containing Tor2). This allows us to assay for the functions of TORC1 contains different Tor1-RR variants (they do not bind to FKBP12-rapamycin).



Figures 3, 4 and 6 present complex but interesting data, adding some evidence to the idea that Maf1 control on Pol III (in response to Rapamycin or to nutrient depletion) responds to TORC1 residence at rDNA. Figure 4 could possibly be left to the Supplement section. Some of these data make sense at the light of a paper by Towpik et al. (JBC 2008) based on Msn5 mutants. Referring to this paper at the beginning of this experimental section would probably simplify its presentation.

Answer: We have followed the suggestion by putting the original figure 4B to Supplementary Figure 1 and simplifying the presentation by referring Towpik et al. (JBC 2008) in the beginning. As suggested by referee #1 (point 2), we have moved the original Figure 4A to Figure 3B for a better comparison of Maf1 phosphorylation in two different genetic backgrounds.

1. Maf1 nuclear localisation (in response to Rapamycin) and its association to 5S rDNA strongly depends on the strain background (S288C versus W303). It would be nice to know if these interesting differences are monogenic (the two 'wild' strains differ by their SSD1 gene, which interferes with Pol II and Pol III transcription- Stettler et al. MGG 1983). This could be checked by tetrad analysis, and by complementation by Msn5 or Ssd1.

Answer: This is a great suggestion. We have introduced a centromere plasmid-borne SSD1 and confirmed that it can suppress the rapamycin hypersensitivity of the W303a tor1 strain (Supplementary Figure 2A). Interestingly, SSD1 partially restored cytoplasmic localization of Maf1 in W303a strain, suggesting that SSD1 is indeed involved in determining Maf1 localization (Supplementary Figure 2B).

2. Figure 6A documents the association of Maf1 to 5S rDNA (in response to Rapamycin) but the 35S rDNA control shown is 25S (internal). This implies, importantly, that Maf1 does not bind to 35S rDNA, but in that case it would be critical to use promoter 35S rDNA ChIP.

Answer: We have now included the control data on Maf1 association with 35S rDNA promoter (35S-P) in Figure 5A and 5B.

3. Figure 6C suggests that TORC1 must reside at rDNA (5S or 35S) to affect the Maf1-dependent control in response to Rapamycin. This is mostly based on the HTH Tor1 mutant (see my comment above on this mutant). It is essential to show the effect of the Tor1 mutants (NES, mt, HTH) on the Maf1 ChIP signal at the tRNA genes. Does they affect the tRNA signal as well?

Answer: Maf1 ChIP data in cells expressing RR/NES, RR/NLSmt and RR/HTH at 5SrDNA, and tDNAs are shown in Figure 4D and 5C, respectively.

Figure 5 shows that Rapamycin extends Maf1 to the nucleolus. This is consistent with the idea that Maf1 'binds' 5S rDNA in response to Rapamycin, and might be better presented with Figure 6C.

Answer: 5S rDNA association of Maf1 in Figure 6C of the original manuscript is now presented in Figure 4D in conjunction with other data on Maf1 nucleolar localization.

1. Nop1 was used as nucleolar marker. Nop1 occupies the whole nucleolus, and is thus not restricted to rDNA. Hmo1, a strong rDNA binding marker with a much more restricted distribution (Gadal et al., EMBO J. 2004) should be a much better indicator of rDNA.

Answer: Nucleolus becomes very compact after rapamycin treatment. We found Maf1 localization is essentially the same as Nop1 under such condition. Therefore, we do not expect to detect a different localization pattern with Hmo1 as a marker.

2. It is suggested that tRNA genes are nucleolar and that the nucleolar localisation of Maf1 is needed for their activation. This is an interesting but controversial point, so far largely based on data by Engelke et al. If this is indeed the model proposed by Wei et al., they should extend their immunofluorescence data to Pol III, and see if it has a nucleolar localisation, depending on the rapamycin treatment.

Answer: We have analyzed the localization of Pol III-specific subunit Rpc82 by IF (Supplementary Figure 3). We found that Rpc82 is present in the nucleolus, which is not affected by rapamycin

treatment. Since Rpc82 localization is not sensitive to rapamycin, it does not indicate where tRNA is transcribed. Because rapamycin similarly affects Maf1 nucleolar localization, and its association with rDNA and tDNA, the simplest model is that tDNAs are regulated inside the nucleolus by Maf1 availability.

Figure 7 presents data suggesting that Maf1 is a substrate of Tor1. Since Maf1 and Tor1 are able to co-reside at the 5S rDNA, Co-IP would only support their hypothetical functional interaction if we had an idea of the stringency of the conditions used.

Response: We used a commonly used, relatively mild condition for the co-IP, which is necessary to preserve an intact TORC1 complex.

It is also shown that Tor1 is able to phosphorylate recombinant Maf1, but how can we tell that any Tyr or Ser containing substrate would not be phosphorylated under these conditions. These are important stringency controls. In their absence these potentially interesting data are rather preliminary and should be left out, or better substantiated.

Response: We have included both GST and GST-Fzo1(AA1-343) as controls. In particular, GST-Fzo1 has a similar molecular weight as GST-Maf1. However, only Maf1 is phosphorylated by Tor1 and this phosphorylation is sensitive to rapamycin (Figure 6B). It has been well documented that Tor kinase is highly specific toward its substrates. Tor is also known for not being able to phosphorylate commonly used artificial substrates such as MBP and enolase.

General comments: Wei et al. should be encouraged to write a more concise introduction, immediately presenting the known roles of Tor and of Maf1 in controlling and possibly coordinating Pol I and Pol III. Their discussion could also be improved to stress more clearly what is new in their data. As a minor point, and in keeping with recent recommendations by EMBO J., they should highlight the original papers on Tor (M. Hall et al.) and Maf1 (Pluta et al.), along with recent review papers.

Response: We incorporated these suggestions into the manuscript, which has indeed improved the presentation.

Referee #3:

General comments:

The manuscript suffers from the numerous grammatical and typographical errors. At least one reference citation has been omitted (three groups showed that Maf1 is a phosphoprotein present in the cytoplasm under nutrient-rich conditions, Oficjalska-Pham et al, 2006; Roberts et al, 2006 and Moir et al, 2006, not two).

Response: We have done more careful proofreading and corrected the error in citing the original work on Maf1 phosphorylation.

The manuscript contains inaccurate and confusing misstatements that detract from its readability (several of which are outlined below). These render the otherwise significant work unsuitable for publication as written.

Figure 2A and legend: The panel annotation is not clear. The dash, between vector and HTH&#x0394; could be understood to refer to "no plasmid" rather than TOR1-RR.

Response: Annotation corrected.

Page10: The statements that "We metabolically labeled yeast cells with <sup>3</sup>H-Uracil to measure the rate of rRNA synthesis" and "To confirm this result, we performed Northern blot analysis of a pre-tRNA<sup>LEU3</sup>, which is commonly used to monitor the transcriptional rate of tDNAs" are not accurate. In this protocol, pulse labeling does not measure a rate of rRNA synthesis but provides a relative measure of rRNA synthesis. Similarly, the Northern does not monitor the transcriptional rate of tDNAs but detects a labile precursor tRNA species and is an

indirect readout for tRNA synthesis.

Response: Statements corrected

Page 10: LEU3 refers to a protein coding gene not a tRNA gene. The tRNA gene is referred to as tRNA<sup>Leu3</sup>.

Response: Corrected

Page 10: "Indeed, the Pre-Leu showed the same expression pattern in cells carrying different TOR1-RR variants (Figure 2B)." is incorrect given the data shown in Figures 2A and 2B.

Response: Corrected

This section is would be more accurately and clearly written as follows: "To confirm this result, we performed Northern blot to detect a labile precursor tRNA species (pre-tRNA<sup>Leu3</sup>), which is commonly used to report tRNA synthesis (Oficjalska-Pham et al, 2006; Upadhya et al, 2002). Indeed, tRNA synthesis showed the same response to the TOR1-RR variants as 5S and 5.8S RNAs transcription: a requirement for functional NLS and HTH domains in TORC1 in order to block repression (Figure 2B).

Response: Corrected

Page10: "The parallel in the regulation of Pol I and Pol III by TORC1 further suggests that simultaneous binding of TORC1 to rDNA serves as a mechanism to coordinate ribosomal RNA synthesis." As there is no data reported for simultaneous binding to both loci, the sentence should probably read: " The parallel regulation of Pol I and Pol III transcription by TORC1 further suggests that binding of TORC1 to both the 25S rDNA promoter and 5S gene in the rDNA may serve as a mechanism to coordinate ribosomal RNA synthesis."

Response: Corrected.

Page 12: "To our surprise, however, very little Maf1 was detected in the nucleus of W303a cells (Figure 4B). Instead, Maf1 was mainly localized in the nucleus." Figure 4B shows the first sentence to be in error and "nucleus" should be substituted with "cytoplasm".

Response: Corrected

Discussion, page 15: "In contrast, the cytoplasmic form of TORC1 is unable to promote Pol III-dependent gene expression." "promote" should be substituted with "regulate".

Response: Corrected.

2nd Editorial Decision

22 April 2009

Your revised manuscript has now been re-reviewed by one of the original referees whose comments you will find enclosed. As you will see, this scientist still appreciates the science and also notices at least some improvement of its presentation. Nevertheless, this very experienced person still raises very strong objections related to (a) title expressing what has actually been shown by the data, (b) structure and correctness of the information provided in the abstract (c) formulating a specific scientific question (d) precise description what had actually been shown by the data (e) clearly distinguishing current knowledge (including accurate referencing of published results) from the real novelty your manuscript provides. I have to emphasize that this referee does NOT really favour offering you a second chance to revise the paper, given the very explicit and strong input/guidance provided by all three referees already after initial peer-review. Further, the journal policy does usually permit only one round of major revisions, so the natural fate of your work would be a

rejection. However, and only based on the premise that your work does contain valuable scientific information (that the other two as well as this referee have outlined) I urge you to carefully attend again to the original critiques as well as the rather explicit comments provided here to improve accuracy and style of your scientific presentation.

Finally, I do have to mention that the assessment of your ultimate version will involve the very same scientist and we will have no hesitation to reject the paper if this referee remains unconvinced.

Yours sincerely,

Editor  
The EMBO Journal

## REFEREE REPORTS

Referee #2 (Remarks to the Author):

All three referees reacted to the first draft of this paper by a long catalogue of comments that probably reflected the fact that it was rather hard to read. I summarised my own feeling as follows: the paper contains interesting and challenging data sometimes presented in a very complex way... its final model is not clearly formulated.... Even the title of the paper is very ambiguous.

I'm happy to say that the new version is notably improved, with some interesting new data, such as the effect of SSD1 and the nucleo-nucleolar distribution of Pol III. The logic of the paper is more apparent, but I remain confused by the title and by the vague and sometimes ambiguous formulation of the Abstract. In short, the data presented are interesting and the overall approach original. Yet, I am afraid that the Ms does not yet qualify for a publication in EMBO Journal, unless the points below are substantially addressed.

1. The title now claims that TORC1 regulates Pol III dependent transcription (NB: the full word RNA polymerase III should appear in the title) due to its binding to chromatin. I suppose they mean rDNA chromatin, otherwise this title would be totally misleading, since the paper actually shows that TORC1 does NOT bind tRNA genes.

2. The second sentence of the abstract recalls that TORC1 is associated to 35S rDNA (Li et al., 2006) but the same paper also showed that it binds to 5S rDNA. The Abstract is formulated in such a way that the latter observation might appear to have been made in the present Ms.

3. Maf1 phosphorylation, nucleolar localisation and binding to Pol III transcribed genes (meaning, I believe, tRNA genes, which are not themselves bound by TORC1) require TORC1 association with rDNA. It is unclear if the authors mean binding to 35S rDNA or to 5S rDNA, but Figure 7, and several sentences in the text, suggests the latter. I don't think that this is justified by the data. This ambiguity must be clarified.

4. The tor1-HTH ( $\Delta$ ) mutation (please follow the standard yeast genetic nomenclature...) is crucial to the whole argument since it prevents TORC1 to bind rDNA, but supposedly does not impair its nuclear/nucleolar localisation. However, the latter point has not been established, unless I overlooked some crucial data. Moreover, tor1-HTH ( $\Delta$ ) also prevents TORC1 from binding the 35S rDNA promoter (Li et al., Nature 2006), which brings us back to the point above: its effects on tRNA expression could be mediated via the 35S rDNA.

5. Nucleolar localisation of tRNA transcription: Engelke et al. (2006) have argued that tRNA genes are nucleolar, but I know of no other evidence supporting this important but controversial conclusion. Wei et al. find Rpc82 (a Pol III subunit) to be uniformly distributed in the nucleoplasm and the nucleolus. The data are clean and deserve more than a Supplementary figure. My own interpretation is that Pol III must in part be nucleolar (5S rDNA) and in part nucleoplasmic (tRNA and other Pol III transcript). Wei et al. take the opposite view tRNA and 5S rDNA are both nucleolar but, in that case, why is Pol III so abundant in the nucleoplasm? Please clarify.

6. 'TORC1 is in close proximity to Maf1 in the Pol III regulatory pathway' (Abstract). Does this

pretty vague statement mean that Maf1 phosphorylation is due to TORC1, and only occurs when the latter is bound to the 5S rDNA (or 35S rDNA) region? Please clarify.

7. The revised introduction adequately reviews the TOR and Maf1 field, but fails to clearly and simply state what is the question asked (i.e., how does TORC1 control tDNA transcription, and why is it dependent on its binding to rDNA). The authors should better spell out the novelty of their data (which is real) relatively to their own previous work (Li et al. 2006) or to work by other labs on the regulation of Pol III in response to rapamycin, and on the role of Maf1.

2nd Revision - authors' response

30 April 2009

Response to reviewer 2's comments

Question 1: The title now claims that TORC1 regulates Pol III dependent transcription (NB: the full word RNA polymerase III should appear in the title) due to its binding to chromatin. I suppose they mean rDNA chromatin, otherwise this title would be totally misleading, since the paper actually shows that TORC1 does NOT bind tRNA genes.

Answer: We have changed the title to "Mechanisms of regulation of RNA polymerase III-dependent transcription by TORC1", which reflects more accurately our findings.

Question 2: The second sentence of the abstract recalls that TORC1 is associated to 35S rDNA (Li et al., 2006) but the same paper also showed that it binds to 5S rDNA. The Abstract is formulated in such a way that the latter observation might appear to have been made in the present Ms.

Answer: We have completely rewritten the abstract to accurately reflect the new findings.

Question 3: Maf1 phosphorylation, nucleolar localisation and binding to Pol III transcribed genes (meaning, I believe, tRNA genes, which are not themselves bound by TORC1) require TORC1 association with rDNA. It is unclear if the authors mean binding to 35S rDNA or to 5S rDNA, but Figure 7, and several sentences in the text, suggests the latter. I don't think that this is justified by the data. This ambiguity must be clarified.

Answer: Maf1 is found at 5S but not 35S rDNA chromatin, while TORC1 is found at both chromatin sites, suggesting that Maf1 is regulated by TORC1 at 5S rDNA chromatin. However, we cannot rule out the possibility that TORC1 at 35S rDNA transiently regulates Maf1. We have modified our model (Figure 7) and included a brief discussion to reflect this point (Top paragraph, Page 18).

Question 4: The tor1-HTH $\Delta$  mutation (please follow the standard yeast genetic nomenclature...) is crucial to the whole argument since it prevents TORC1 to bind rDNA, but supposedly does not impair its nuclear/nucleolar localisation. However, the latter point has not been established, unless I overlooked some crucial data. Moreover, tor1-HTH $\Delta$  also prevents TORC1 from binding the 35S rDNA promoter (Li et al., Nature 2006), which brings us back to the point above: its effects on tRNA expression could be mediated via the 35S rDNA.

Answer: We have previously shown that Tor1(HTH) mutant is still localized to the nucleus (Supplementary Figure 6, Li et al, Nature 2006), indicating that HTH mutation specifically affects Tor1 association with rDNA chromatin.

Question 5: Nucleolar localisation of tRNA transcription: Engelke et al. (2006) have argued that tRNA genes are nucleolar, but I know of no other evidence supporting this important but controversial conclusion. Wei et al. find Rpc82 (a Pol III subunit) to be uniformly distributed in the nucleoplasm and the nucleolus. The data are clean and deserve more than a Supplementary figure. My own interpretation is that Pol III must in part be nucleolar (5S rDNA) and in part nucleoplasmic (tRNA and other Pol III transcript). Wei et al. take the opposite view tRNA and 5S rDNA are both nucleolar but, in that case, why is Pol III so abundant in the nucleoplasm? Please clarify.

Answer: We have moved the Rpc82 data to Figure 4A. We understand the skepticism on nucleolar localization of tRNA genes (tDNA) in the absence of supporting data from other laboratories. Rpc82 localization throughout the nucleus is indeed suggestive of Pol III function outside the nucleolus. Accordingly, we have removed statements related to tDNA localization in the nucleolus and offered an alternative explanation on how TORC1 association with rDNA chromatin might affect tRNA genes (Top paragraph, Page 18). Our data is consistent with the model that TORC1 regulates Maf1 phosphorylation in an rDNA chromatin-dependent manner, and that Maf1 in turn controls tDNA transcription (Figure 7). This new model can satisfactorily explain how TORC1 regulates tDNA without tDNA localization in the nucleolus.

Question 6: 'TORC1 is in close proximity to Maf1 in the Pol III regulatory pathway' (Abstract). Does this pretty vague statement mean that Maf1 phosphorylation is due to TORC1, and only occurs when the latter is bound to the 5S rDNA (or 35S rDNA) region? Please clarify.

Answer: We have removed this vague statement from the Abstract and inserted a discussion of the role of TORC1 in Maf1 phosphorylation in Page 15.

Questions 7: The revised introduction adequately reviews the TOR and Maf1 field, but fails to clearly and simply state what is the question asked (i.e., how does TORC1 control tDNA transcription, and why is it dependent on its binding to rDNA). The authors should better spell out the novelty of their data (which is real) relatively to their own previous work (Li et al. 2006) or to work by other labs on the regulation of Pol III in response to rapamycin, and on the role of Maf1.

Answer: We have now clearly stated the questions asked with TORC1 and Maf1 in the Introduction, and spelled out the novelty of the new data in both Abstract and Introduction.

3rd Editorial Decision

20 May 2009

I do apologize for the delay in getting back to you with a final decision on your revised manuscript. The reason for this was that we carefully evaluated the still negative assessment of the critical referee at the editorial level. I have to stress that I discussed your work in detail with our Executive Editor. I am glad to inform you that in light of the initial requests from the referees and your responses provided during revision, together with the clear novelty (that ref#2 does not deny and indeed appreciates) we decided to eventually publish your paper in The EMBO Journal.

Irrespective of this decision, we would still encourage you to consider some of the points this referee raised, but refrain from re-publication of data that were already presented in your original 2006 paper. We would particularly encourage clarification of Maf1 localization (or lack thereof) in the nucleolus when it is supposedly being phosphorylated by 5S-associated TORC1. While you show that chromatin-association of Tor (or rather, presence of the HTH domain) is important for Maf regulation, this does not prove it necessarily happens at the 5S locus - it might be tempting to speculate that but it could occur elsewhere, on chromatin.

I kindly ask you to revise and modify the manuscript according to this, and the referee's requests as you find appropriate and provide us with the ultimate version of your work as soon as you can.

Yours sincerely,

Editor  
The EMBO Journal

## REFEREE REPORTS

Referee #2 (Remarks to the Author):

#### General comment

This work sheds new light on the Maf1-dependent regulation of Pol III in response to rapamycin, via the TOR cascade. The connection between Maf1 and TOR has already been established (2006) by three different labs, and recent data from Towpick et al. (2008) have suggested that this is not simply a matter of nuclear vs. cytoplasmic localisation of Maf1. Wei et al. now argues that Maf1 phosphorylation status depends on TORC1 being present at the 5S rDNA chromatin, by a direct phosphorylation by the Tor1 kinase (or, possibly, via another kinase?), which ultimately excludes Maf1 from the nucleolus and prevent its inhibition of Pol III. These are interesting and reasonably convincing data. However, the model presented has weaknesses, especially since unlike what they say in their rebuttal, Wei et al. have maintained its more speculative aspects (Tor1 at the 5S rDNA, key involvement of the nucleolar localisation of Maf1) that, in my opinion, are not or poorly supported by their data. This work is nevertheless interesting and deals with an important aspect of growth control, but I'm afraid that a publication in EMBO J. would be premature at this point.

#### Background

In their Nature paper (2006), Zheng and his co-workers found that, under normal growth conditions, TORC1 is associated to rDNA chromatin at the 5S and 35S promoter. They also found that this binding depends on a HTH motif present on the Tor1 kinase (concerning 5S rDNA, this is detailed in Figure 1 of the present paper). Rapamycin excludes TORC1 from the nucleus, which simply explains the lack of Pol I dependent transcription of 35S rDNA) and the Pol III-dependent transcription (5S rDNA), assuming them to be activated by TORC1. Other labs have reported that rapamycin also turns off Pol III and that this depends on its inhibition by Maf1. As a phosphoprotein, Maf1 resides in the cytoplasm but, in response to rapamycin, Maf1 is not phosphorylated and accumulates in the nucleus, accounting for a general inhibition of Pol III under these conditions. However, recent data (Towpick et al. 2008) have suggested that this is not simply a matter of nuclear vs. cytoplasmic localisation of Maf1.

#### New data presented

1. Chip data reveal that tRNA genes are not bound by TORC (unlike 5S rDNA).
2. Mutants lacking the Tor1 NLS or the HTH motif do not turn off tRNA transcription if exposed to rapamycin. Conversely, a mutant lacking the Tor1 NES (where TORC1 permanently resides in the nucleus) turns on this synthesis in the presence of rapamycin. This strongly suggests that Maf1 phosphorylation requires TORC1 to bind the rDNA chromatin.
3. Tor1 and Maf1 show some degree of co-immunoprecipitation and Maf1 is phosphorylated by Tor1 in vitro, which argues that TORC1 may directly phosphorylate Maf1 in vivo.
4. In W303 (but not in S288C), Maf1 is nuclear even under favourable conditions, which is due to a defective *ssd1-d* allele (NB: Sutton et al., 1991, the reference to the much later work of Kaeberlin & Guarente 2002 seems irrelevant), in keeping with previous work (Stettler et al., 1993; Reinke et al., 2004) linking Pol III transcription and TOR regulation to *Ssd1*. Thus, the inhibitory effect of Maf1 on Pol III is not simply due to its presence in the nucleus (see also Towpick et al. (2008)).
5. Rapamycin transiently recruits Maf1 to 5S rDNA and tRNA chromatin, which again, depends on TORC1 binding the rDNA chromatin. In W303, this correlates with a partial redistribution of Maf1 to the nucleolus. No data is shown for S288C, and this nucleolar localisation could arguably reflect the fact that Maf1 binds the 5S rDNA chromatin. It is (implicitly) assumed that this transient association to the nucleolus permanently switch off Pol III-dependent transcription, perhaps because Maf1 irreversibly dissociates/degrades Pol III from its template (NB: this may be tested by Pol III chipping).

#### Specific comments

As I understand it, the main (and important) point of this Ms is that, in growing cells, TORC1 must reside at the rDNA chromatin (presumably, but not necessarily, at the 5S rDNA) to phosphorylate Maf1 and, therefore, to exclude it from the nucleus (and/or the nucleolus). The model proposed (Figure 7) is based on four main points restated below, as quoted from page 19.

1. Under favourable growth conditions, TORC1 is localized in the nucleus and is associated with both 5S rDNA and 35S promoter chromatin to regulate (NB: activate?) the synthesis of ribosomal RNAs. Upon starvation (NB: in response to rapamycin?), TORC1 dissociates from rDNAs and exits from the nucleus, resulting in transcriptional repression (NB: also of Pol III at the 5S rDNA promoter?).

2. At 5S rDNA, TORC1 regulates Maf1 by phosphorylation, preventing Maf1 from accumulation in the nucleolus and association with Pol III-transcribed genes. Maf1 should also be absent from the nucleolus in S288C, which is only shown for W303 (NB: Figure 4C is an enlargement of 4B, not 4A, otherwise the data make no sense, there are still several errors in the text, please check carefully!). This is important given the difference in the SSD1 alleles of these two 'wild types'.

if TORC1 phosphorylates MAF1 at the 5S rDNA (in fast growing cells), why is MAF1 not detected at 5S in fast growing cells? How could MAF1 be a target of TORC1 at the rDNA (i.e. in the nucleolus) without being detected in nucleolus? There are some ways out of these difficulties, but they should be argued.

COMMENT: Three Tor1 mutations were used in this work. In the NES mutant (lacking a nuclear export signal), TORC1 is constitutively present in the nucleus and bound to rDNA. In the NLS mutant (lacking a nuclear localisation signal), TORC1 is unable to reach the nucleus and, consequently, the rDNA. An internal deletion of the HTH domain prevents Tor1 from being associated to either 5S rDNA or 35S rDNA. The NLS and HTH mutants are predicted to have the same physiological response (which is the case), but the stronger predictions are, of course, those made with the HTH mutation, which prevents TORC1 from being recruited to the rDNA chromatin. In their rebuttal (but not in the Ms itself), Wei et al. refer to the supplementary data of their Nature paper (2006) showing that the TORC1 mutant form is present in the nucleus in growing cells. Given the importance of these data in the context of this ms, I strongly recommend to show then, with appropriate controls, in the present paper.

Wei et al. should also consider the possibility that the mutant, in addition from preventing TORC1 to be recruited to rDNA, impairs the ability to phosphorylate MAF1. This possibility could have been tested using the in vitro assay, and should at the very least be mentioned as a caveat.

3. In certain yeast background, TORC1-regulated phosphorylation also promotes Maf1 nuclear export, which further reduces its accessibility to Pol III (NB : their genetic complementation data show this to depend on Ssd1. The role of Ssd1 could be highlighted, even in the abstract).

4. It has been reported that tDNAs are clustered in the nucleolus (Thompson et al, 2003). Phosphorylation of Maf1 at rDNA chromatins may be sufficient to control Maf1 nucleolar localization, which in turn controls the transcription of tRNA genes.

COMMENT: since the 5S rDNA and tRNAs represent about 90 % of the yeast Pol III transcripts, point 4 obviously predicts that the bulk of Pol III should be nucleolar. When testing this by Pol III immunofluorescence (at my request), Zheng et al. found Pol III to be (roughly) equally distributed between the nucleolus and the nucleoplasm, consistent with the canonical view that 5S rDNA is nucleolar and tRNA genes nucleoplasmic. The nucleolar localisation of tRNA genes still remains a possibility, in view of the seemingly persuasive data presented by Engelke's group (Thompson et al., 2003), but Zheng's data on Pol III actually argue against this possibility, and thus against the last element of their own model.

3rd Revision - authors' response

28 May 2009

Detailed changes:

1. The issue of Maf1 nucleolar localization and regulation of tRNA genes.

We are puzzled by reviewer 2's conflicting comments regarding the study by Thompson et al that tDNAs are localized to the nucleolus. Nonetheless, we do think Maf1 availability in the nucleolus is the most likely mechanism for TORC1 to control tDNA transcription inside the nucleolus. The model in Figure 7 has been updated accordingly.

2. Does TORC1 phosphorylate Maf1 at 5S rDNA chromatin?

Although HTH mutation prevents Tor1 from binding to both 5S and 35S rDNA, Maf1 is only detected at 5S rDNA. Therefore, it is most logical to propose that TORC1 complex phosphorylates Maf1 at 5S rDNA chromatin, which is reflected in the modified model in Figure 7. We are mindful of and discussed the limitation of our method that does not allow us to completely rule out the possibility that TORC1 complex also phosphorylates Maf1 at 35S rDNA promoter.

3. The SSD1 result should be highlighted in the abstract.

We have now included the SSD1 result in the abstract, and used the names of different SSD1 alleles.

4. The Maf1 nucleolar localization result is not shown in SC288C strain.

Maf1 is normally absent from the nucleus thus also the nucleolus in this strain (Figure 3). When



treated with rapamycin, Maf1 is clearly localized in the nucleolus as indicated by the ChIP result (Figure 5A). So the results are already in the manuscript.  
5. Figures 4A-C have been changed