The Yeast *RPL9B* Gene is Regulated by Modulation Between Two Modes of Transcription Termination

Rajani Kanth Gudipati, Helen Neil, Frank Feuerbach, Christophe Malabat and Alain Jacquier

*Corresponding author: Alain Jacquier, Institut Pasteur*

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**Transaction Report:**

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

1st Editorial Decision 13 October 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I have now received the final report from the three referees who evaluated your study and I enclose their comments below. As you will see they are in general positive regarding the study and require that a number of issues be addressed in a revised manuscript. These include further support for the regulation of *RPL9B* via two independent termination mechanisms. After discussing the study with the referees it seem that both referee #1 and #3 raise some concerns with the feasibility and chances of obtaining a clear outcome from the experiments suggested by deleting the Nrd1-Nab3 binding sites in *RPL9* by referee #2, it would be obviously a good experiment to do but I would appreciate your opinion if you believe this is doable, perhaps it is possible to show that Nrd1-Nab3 actually bind at these site. Given the support form the referees I would like to invite you to submit a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

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Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1

This manuscript describes a new pathway for autoregulation of the ribosomal protein gene RPL9B. The proposed mechanism is that when Rpl9 is over-expressed it binds (directly or indirectly) to a potential stem-loop structure present within the 3' UTR of RPL9B mRNA. This restricts transcription termination at downstream polyA sites. Instead Pol II is terminated at downstream Nrd1-dependent sites that act to recruit Rrp6 to degrade the RPL9B transcript. This work is elegantly performed and the results described support the presented model. Overall I am enthusiastic about this paper, even though there remain some minor issues, which need to be clarified prior to its publication.

1) The authors should apply the same method to analyze nrd1/nab3 and CPF-CF mutants as is presented in Fig1B and S1. Resolution on these figures is very clear whereas some bands on Fig1C and Fig1D are less so. E.g. in Fig1C, the authors claim that the lowest band represents a combination of species 1 and 2. It doesn't seem that bands 1 and 2 from 1B together should give such a strong signal. Therefore RNaseH digestion followed by Northern blotting on polyacrylamide gels should be used for the analysis shown in Fig1C and D. This would make these figures more convincing.

2) More evidence showing that expression of RPL9B is regulated by two independent termination mechanisms should be provided. Accumulation of species 3 should be visible in rrp6/CPF-CF mutants. Also deletion of RPL9A should alleviate the nrd1/nab3 phenotype. Both results would provide additional strong support for the proposed mechanism.

Referee #2

In this paper Gudipati et al. provide evidence for autoregulation of the Saccharomyces cerevisiae ribosomal protein gene RPL9. They identify regulatory sequences in the 3' UTR and go on to show that this regulatory element confers sensitivity to RPL9 gene copy number. While the details of this regulatory pathway are not completely worked out the authors propose a plausible model involving a balance between two opposing pathways for 3' end formation. In the presence of abundant Rpl9 protein a hairpin in the 3' UTR blocks access to a polyadenylation signal leading to read-through transcription and downstream termination through the Nrd1-Nab3 pathway. The paper would obviously be stronger if the authors could identify a protein bound to the regulatory element in the 3' UTR. Without this, the paper is still of some interest as the element seems to be conserved in different species of yeast.

Another major problem with the paper is that the evidence supporting a role for Nrd1 and Nab3 is very thin. The Northern blot in Figure 1C shows that depletion of Nrd1 and Nab3 seems to reduce the amount of band #3 which, because it is stabilized in an rrp6 null strain, the authors conclude is the Nrd1-Nab3 terminated transcript. This is possible, but given the long time taken to deplete Nrd1 and Nab3 this effect could be indirect. To eliminate this possibility the authors should show that deletion/mutation of Nrd1-Nab3 binding sites produces the same effect. In Figure 1 the authors show the positions of Nrd1 and Nab3 consensus binding motifs. Since several dataset have now been published (Wlotzka et al., EMBO, Jamonnak et al., RNA) showing actual in vivo binding sites for Nrd1 and Nab3 the authors should indicate which of the sites in RPL9 are bound in vivo and
knock out these sites. In addition, the authors should comment on why there is so much less total RPL9B RNA in the Nrd1-Nab3 depleted strain. How does depletion of the downstream-acting factors effect the accumulation of the upstream transcripts #1 and #2 terminated through the poly(A) pathway? Why isn't there more accumulation of band#4?

Minor Points:

On Page three the authors suggest a mechanism of termination by Sen1 that has not been shown by anyone. This sentence should start with "Although the actual mechanism of termination by Nrd1 complex has not been shown, one idea is that ..."

Page 4 end of second paragraph: "Thus, the possible involvement of the Nrd1 pathway in transcription termination of normal mRNAs remains a subject of debate..." This statement is untrue. As the authors state, CTH2 mRNA is terminated in this way, so while the mechanism may be rare, it is not debatable. In addition, several other instances of regulation of mRNA regulation by Nrd1 have recently been published (AHP1, URA1, SNA3; Jamonnak et al., RNA) and should be referenced by the authors.

Even among mutations that cause termination within the hairpin, which thus must be unfolded, there is variation in the abundance of transcripts terminated at each site and in the overall abundance of transcripts in each mutant, leading the authors to conclude that some of these mutants both destabilize the hairpin and block binding of the trans-acting factor. Although this is possible, it seems that if the trans-acting factor recognizes the folded hairpin, then it should not be bound in these mutants, or if it does bind it should not affect efficiency of termination within the hairpin itself. Further discussion is needed here.

Referee #3

Starting from unpublished deep sequencing data, the authors report the use of alternative pathways for transcription termination on RPL9B and propose autoregulation via switching to Nrd1-dependent termination and coupled degradation. A conserved stem-loop structure is strongly implicated in the regulation of termination.

A number of autoregulatory circuits involving RNA termination and/or 3' processing have been reported in yeast. Most involve components of the processing/surveillance system, making the present example more novel. The weaknesses of the MS are the lack of mechanistic information and the failure to find any support for direct binding of Rpl9, for which the authors compensate with extensive speculative discussion. The phenomenon reported will, however, be of considerable general interest and I would support publication after modest revision.

Specific points:

1) Do the rna14 and rna15 mutations give read-through to the same positions as depletion of Nrd1/Nab3?

2) Presentation of the northern data in Figs. 1, 3 and S2 in graphical form might have made the results clearer to a general audience. Moreover, some of the data, especially Fig. 3D, are not very pretty - making me wonder how often the gels have been run. I do not really doubt the conclusions, but some quantitation would not be amiss.

Minor points:

1) Fig. 2/S2: The northern data in Fig. S2 are more compelling than the growth tests in Fig. 2. The authors might consider swapping or combining them.

2) Results, line 6: missing bracket

3) Results, line 15: distant from -> separated by
Specific answer to Editor’s comment

“As you will see they are in general positive regarding the study and require that a number of issues be addressed in a revised manuscript. These include further support for the regulation of RPL9B via two independent termination mechanisms. After discussing the study with the referees it seem that both referee #1 and #3 raise some concerns with the feasibility and chances of obtaining a clear outcome from the experiments suggested by deleting the Nrd1-Nab3 binding sites in RPL9 by referee #2, it would be obviously a good experiment to do but I would appreciate your opinion if you believe this is doable”

Indeed, the difficulty in the experiment is not only experimental, but also conceptual as the actual recognition consensus sequences for these factors remains ill-defined. As you will see, we nevertheless attempted the experiment by introducing point mutations in all the guaa/g and ucuu sequences in the RPL9B 3'UTR. This indeed induced readthrough of site #3, although only partially (and we thus present this experiment only as a Supplementary Figure S2). But we also performed additional experiments (Figure 3) to strengthen the hypothesis.

“perhaps it is possible to show that Nrd1-Nab3 actually bind at these site.”

We now incorporate in Figure 1A the cross-link data for Nrd1 and Nab3 that were published (Jamonnak et al. 2011) during the course of the revision of our manuscript and that nicely corroborate our hypothesis since they mapped just upstream the termination sites of transcript #3.

point-by-point response to the referees’ comments

Referee #1

1) The authors should apply the same method to analyze nrd1/nab3 and CPF-CF mutants as is presented in Fig1B and S1. Resolution on these figures is very clear whereas some bands on Fig1C and Fig1D are less so. E.g. in Fig1C, the authors claim that the lowest band represents a combination of species 1 and 2. It doesn’t seem that bands 1 and 2 from 1B together should give such a strong signal. Therefore RNaseH digestion followed by Northern blotting on polyacrylamide gels should be used for the analysis shown in Fig1C and D. This would make these figures more convincing.

Yes, we agree with these comments. These RNase H digestions were repeated and analysed on acrylamide gels, as suggested. Due to the change in the structure of the manuscript, Fig1C has been replaced by Fig3A and Fig1D by Fig3B.

2) More evidence showing that expression of RPL9B is regulated by two independent termination mechanisms should be provided. Accumulation of species 3 should be visible in rrp6/CPF-CF mutants. Also deletion of RPL9A should alleviate the nrd1/nab3 phenotype. Both results would provide additional strong support for the proposed mechanism.

We have now performed three additional experiments to address this important point (provide more evidence for the two independent termination mechanism). First, we show that depletion of Nab3p induces readthrough at site #3 (Figure 3A). Second, mutation within potential Nab3 and Nrd1 binding sites in the 3'-UTR also induces some readthrough at site #3 (although not as strong as the depletion of Nab3p, likely reflecting the fact that these precise sites remain somewhat ill-defined; Supplementary Figure S2B). Third, we show that compromising the CPF/CF mode of termination by using a rna14-3 ts mutant results in the increase of transcript #3 at the expense of transcripts #1 and #2 (Figure 3B).
We have not performed specifically the experiment corresponding to the last suggestion (Also deletion of RPL9A should alleviate the nrd1/nab3 phenotype. Both results would provide additional strong support for the proposed mechanism) because the strains are too sick. Yet, one can see on Supplementary Figure S2B that the readthrough transcripts #4 accumulating when Nrd1-Nab3 binding sites are mutated decreases upon deletion of RPL9A, as is the case of transcript #3 (compare lanes 14 and 16 in Supplementary Figure S2B).

Referee #2:

the evidence supporting a role for Nrd1 and Nab3 is very thin. The Northern blot in Figure 1C shows that depletion of Nrd1 and Nab3 seems to reduce the amount of band #3, which, because it is stabilized in an rrp6 null strain, the authors conclude is the Nrd1-Nab3 terminated transcript. This is possible, but given the long time taken to deplete Nrd1 and Nab3 this effect could be indirect. To eliminate this possibility the authors should show that deletion/mutation of Nrd1-Nab3 binding sites produces the same effect.

This weakness was also pointed out by referee number 1 and, as described above, we have now performed three additional experiments to address this important point, experiments that are now illustrated in Figure 3A & 3B and Supplementary Figure S2B. Note that we also reduced the time of depletion of Nab3 from 12 to 9 hours. The elimination of the potential binding sites are shown in Supplementary Figure S2B (see comments to referee 1).

In Figure 1 the authors show the positions of Nrd1 and Nab3 consensus binding motifs. Since several dataset have now been published (Wlotzka et al., EMBO, Jamonnak et al., RNA) showing actual in vivo binding sites for Nrd1 and Nab3 the authors should indicate which of the sites in RPL9 are bound in vivo and knock out these sites.

The Nrd1 and Nab3 cross-link sites from Jamonnak et al are now included in Figure 1A (and nicely support the hypothesis).

In addition, the authors should comment on why there is so much less total RPL9B RNA in the Nrd1-Nab3 depleted strain.

With now 9 hours of depletion rather than 12 hours, this problem has been solved (see Figure 3A)

How does depletion of the downstream-acting factors effect the accumulation of the upstream transcripts #1 and #2 terminated through the poly(A) pathway? Why isn't there more accumulation of band#4?

If we understand well the point of the referee, he is wondering if there is a competition between the Nrd1 and the CPF/CF dependent termination pathways at sites #1 and #2. On Figure 3A & 3B, one can see that, indeed, the steady state level of transcript #2 increases in the Δrrp6 strain, whenever one of the two termination pathways is compromised, which might indicate such a phenomenon. However, we think that, at this stage, the data supporting this hypothesis are too thin and we thus preferred not to discuss it in the main text.

Concerning the question “Why isn't there more accumulation of band#4?“ a simple explanation, which we have not tested, would be that the very long 3'-UTR containing transcripts generated would be good substrates for degradation in the cytoplasm by the NMD pathway.

Minor Points:
On Page three the authors suggest a mechanism of termination by Sen1 that has not been shown by anyone. This sentence should start with "Although the actual mechanism of termination by Nrd1 complex has not been shown, one idea is that …"

Yes, this as been done as suggested.

Page 4 end of second paragraph: "Thus, the possible involvement of the Nrd1 pathway in transcription termination of normal mRNAs remains a subject of debate..." This statement is untrue. As the authors state, CTH2 mRNA is terminated in this way, so while the mechanism may be rare, it
is not debatable. In addition, several other instances of regulation of mRNA regulation by Nrd1 have recently been published (AHP1, URA1, SNA3; Jamonnak et al., RNA) and should be referenced by the authors.

Yes (the Jamonnak manuscript was published after we submitted our first version). The sentence now reads “However, it has recently been reported that, in a few cases, the Nrd1 termination pathway could be involved in termination of some mRNAs. Indeed, the recent genome-wide mapping of Nrd1-Nab3 cross-link sites has revealed the presence of these factors 3’ to several genes (such as AHP1, URA1, SNA3), suggesting a role for the Nrd1 pathway in termination of their transcription (Jamonnak et al, 2011).”

Even among mutations that cause termination within the hairpin, which thus must be unfolded, there is variation in the abundance of transcripts terminated at each site and in the overall abundance of transcripts in each mutant, leading the authors to conclude that some of these mutants both destabilize the hairpin and block binding of the trans-acting factor. Although this is possible, it seems that if the trans-acting factor recognizes the folded hairpin, then it should not be bound in these mutants, or if it does bind it should not affect efficiency of termination within the hairpin itself. Further discussion is needed here.

Our hypothesis implied that the mutation does not totally destabilize the hairpin, which thus would be in equilibrium between a folded and an unfolded state. In the folded state, the mutation might impair the binding of the trans-acting factors, while in the unfolded state, it unmask the cryptic poly(A) site. This precision is now added in the discussion of this point at the end of page 11 and the beginning of page 12.

Referee #3

1) Do the rna14 and rna15 mutations give read-through to the same positions as depletion of Nrd1/Nab3?

Our new data presented in Figure 3 and Supplementary Figure S2 indicate that, upon depletion of Nab3, termination reads through site #3 (as revealed by the appearance of band #4), whereas, inactivation of CPF/CF termination induces readthrough of sites #1 and #2 (Figure 3B) and results in termination at site #3 as evidenced by the absence of band #4 upon RNase H digestion with oligonucleotide RG055 (discussed and mentioned as “data not shown” at the end of page 9 and beginning of page 10: “After a 30 minutes shift to the semi-restrictive temperature (35°C), both transcripts #1 and #2 are decreased (compare lanes 5 and 6) and part of the termination probably occurs further downstream in a Nrd1-Nab3 dependent fashion, as evidenced by the increase of transcript #3 in the Δrrp6 background (compare lanes 7 and 8) and by the fact that no band corresponding to longer transcripts (such as band #4 in Figure 3A) was revealed after RNase H digestion in the presence of oligonucleotide RG055 (not shown).”

2) Presentation of the northern data in Figs. 1, 3 and S2 in graphical form might have made the results clearer to a general audience.

We think that the new data are clearer and, in our opinion, the gels show more information, giving qualitative in addition to quantitative information and are less prone to the biased interpretation by the authors and we thus greatly favour this representation.

Moreover, some of the data, especially Fig. 3D, are not very pretty - making me wonder how often the gels have been run. I do not really doubt the conclusions, but some quantitation would not be amiss.

We are not sure of what the referee meant here. In case the concern would be to known whether the experiment has been repeated, we provide a replicate of it below, although, admittedly, the gel is not “prettier” than the one included in the manuscript.
Minor points:

1) Fig. 2/S2: The northern data in Fig. S2 are more compelling than the growth tests in Fig. 2. The authors might consider swapping or combining them.

We followed the suggestion and the new Figure 2 combines data of Fig. 2 and S2 from the previous version.

2) Results, line 6: missing bracket
3) Results, line 15: distant from -> separated by

Thank you, this has been corrected.

2nd Editorial Decision 06 March 2012

Thank you for sending us your revised manuscript. In the meantime, my colleague Stan Gorski has left The EMBO Journal, and I have taken over the responsibility for this manuscript.

Referee 1 has now seen the manuscript again. In general, he/she is now positive about publication of your paper. Still, he/she feels that there are a few minor issues that need to be addressed (see below) before we can ultimately accept your manuscript, and I would like to ask you to deal with these issues in an amended version of the manuscript.

Furthermore, there are a number of editorial issues that need further attention:

* Please combine all supplementary files into one supplementary material file.

* Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication and explanation in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) or explanation in the figure legend to ask for the original scans. In the case of the present submission there are a number of panels that do not fully meet these requirements: Figure 1B, 2C, 4D, S2B. The molecular weight marker lanes should all come from the same gel. Please confirm/clarify this in the relevant figure legends and amend figure 1B according to the guideline mentioned above. According to our editorial policies we also need to see the original scans for the four panels in question.
Thank you for your kind cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEERE COMMENTS

Referee #1

This revised ms is much improved and appears to address all of the major issues raised in the 1st review round. We therefore recommend publication, but would suggest some minor adjustment/qualification. In particular the title is too general and should specify that these two modes of termination apply to the RPL9B gene specifically. Also the model in Fig 6 should indicate that direct binding of Rpl9p to the hairpin has not been demonstrated, indicating an indirect effect presumably through other factors yet to be identified.

2nd Revision - authors’ response 09 March 2012

Specific answer to Editor’s comment

“* Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication and explanation in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) or explanation in the figure legend to ask for the original scans. In the case of the present submission there are a number of panels that do not fully meet these requirements: Figure 1B, 2C, 4D, S2B. The molecular weight marker lanes should all come from the same gel. Please confirm/clarify this in the relevant figure legends and amend figure 1B according to the guideline mentioned above. According to our editorial policies we also need to see the original scans for the four panels in question.”

Indeed, all the different figures comply to the recommendations presented in Rossner and Yamada. In particular, all cropped and pasted parts of a given figure panel all provide from the same gel in every case (see annotated source data gels). Figure 1B (and 2B) have been amended according to the guidelines (white space added to clearly indicate the cropped and pasted parts, which all come from the same gel).

Note that, for Figure 3A and S2A, the different panels had not been rearranged in fact, but the white bars were only meant to delineate the different mutants/conditions used. To avoid confusions, the figures have been amended to remove those white bars.

Moreover, all figure legends have been amended, when appropriate, so as to make this point clear (that all parts are pasted from an unique original gel).
point-by-point response to the referees' comments

Referee #1

1) This revised ms is much improved and appears to address all of the major issues raised in the 1st review round. We therefore recommend publication, but would suggest some minor adjustment/qualification. In particular the title is too general and should specify that these two modes of termination apply to the RPL9B gene specifically.

The title has been changed to conform the referee’s request. It now reads “The Yeast RPL9B Gene is Regulated by Modulation Between Two Modes of Transcription Termination”

2) Also the model in Fig 6 should indicate that direct binding of Rpl9p to the hairpin has not been demonstrated, indicating an indirect effect presumably through other factors yet to be identified.

We have now added a question mark next to the arrows pointing Rpl9Bp to the RNA stem-loop and added in the figure legend “The bold, red question mark points to the fact that we were unable to show the direct interaction between Rpl9p and the RNA stem-loop structure, suggesting that this interaction might be indirect”.