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Selective termination of lncRNA transcription promotes heterochromatin silencing and cell differentiation

Leila Touat-Todeschini, Yuichi Shichino, Mathieu Dangin, Nicolas Thierry-Mieg, Benoit Gilquin, Edwige Hiriart, Ravi Sachidanandam, Emeline Lambert, Janine Brettschneider, Michael Reuter, Jan Kadlec, Ramesh Pillai, Akira Yamashita, Masayuki Yamamoto & André Verdel

Corresponding author: André Verdel, UMR InsermU1209/CNRS5309/UGA

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1st Editorial Decision 16 February 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript although they also raise a number of concerns that you will have to address before they can support publication here.

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

-> Refs #1 and #2 both find that additional data is needed to unambiguously differentiate between transcription termination and RNA stability in Mmi1-dependent regulation of nam2 and the centromeric lncRNAs. I will therefore ask you for additional experimental data to address this.

-> Ref #3 is the more negative of the three and finds that more insight on the mechanism of termination would be required to make this study a strong candidate for publication here. While I realize that delineating the full mechanism will likely be outside the scope of a revision I would be happy to discuss what kind of data you would be able to include.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript

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will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind 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, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

The manuscript of Todeschini et al. describes the molecular function of lncRNA processing for promoting epigenetic silencing and cell differenciation in pombe.
Using RNA-IP coupled to RNA-sequencing, the authors isolated a series of RNA associated with Mmi1 protein, previously characterized as being involved in transcription termination. Interestingly, crystal structure analyses pinpointed a series of amino acids that are involved in the Mmi1 RNA recognition specificity. Mutations of those motifs allow the authors to directly connect mmi1 mutant phenotype to the RNA binding domain. The manuscript is then divided in 2 parts, the first one shows how Mmi1 binds to nam2 lncRNA. Cis and trans mutation shows misregulation of the downstream gene byr2 expression, indicating readthrough-mediated regulation. The second part describes how expression of the centromeric lncRNA is important into heterochromatin assembly independently of RNAi.
The manuscript is very well written and the results are of general interest as they present a clear step forward into the comprehension of the role of lncRNA transcription for gene silencing and epigenetic mark. This manuscript does warrant publication EMBO journal since it provides the first mechanistic insights into how pervasivness of non-coding transcription can affect cell differentiation and heterochromatin establishment in pombe. I recommend the authors to provide some clarifications both experimentally and in the text/figures prior publications.

Major critics:

1-The key experiment is the demonstration that mmi1 is indeed involved in transcription termination process of the Nam2 lncRNA. Figure 3B shows that RNAPIIser2 phosphorylation increases in the byr2 coding region when nam2 is mutated for mmi1 binding. This suggests that nam2 transcription elongates into the downstream byr2 region inhibiting its expression. This experiment is rather confusing to me since ser2P might also represent the downstream gene transcription and/or antisense transcription of byr2. More confusing is the reminiscence of high ser2P for probe 7 as if an alternative transcription process is occurring in this region that would be not controlled by nam2 readthrough.
Altogether, this experiment would benefit of 2 additional controls with RNAPII and ser5P occupancy allowing some indications on the quantity of readthrough polymerase and its directionality. I would recommend additional chip POLII and ser5P experiment to conclude more clearly. Technically, the readthrough could only be validated by measuring the nascent nam2 RNA, neither the presence of a long nam2 RNA form or increase of ser2P-POLII occupancy demonstrate a readthrough process since the longer form could appear because of RNA stabilisation (readthrough would be constant whatever the mmi context) and POLII occupancy would reflect an alternative transcription.

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in the attenuation of byr2. Indeed an antisense byr2 RNA has been annotated and interference with the elongated nam2 RNA might play a role in the regulation (double strand formation, titration of the antisense that might positively regulate byr2 etc..).

3-in the same lines, Figure 3b has some issues in the labelling (for Y and x axis.

Referee #2:

The authors report the identification of novel lncRNA targets for Mmi1 and Rrp6 starting with a nice combination of RIP and bioinformatics. They back this up with a good structure-based mutational analysis that identifies mutations in the YTH domain with greatly reduced RNA binding. The analysis of the nam1 lncRNA makes a convincing case that transcription readthrough from the lncRNA can reduce expression of a downstream gene with a role in the initiation of sporulation. However, the major conclusion of the MS that the key function of Mmi1 is to terminate transcription on these lncRNA and on lncRNA from the centromeric regions could be more robustly demonstrated. Especially for very unstable lncRNAs, distinguishing between altered synthesis and rapid degradation can be challenging. There are some relatively straight forward analyses that would support the conclusions.

Specific points:
1) The Rrp6 result shown in Figure 3E raises some questions. The rrp6Δ mutation increases nam1-L detection to same extent as mmi1Δ - but the effects of rrp6Δ seem likely to reflect defects in degradation rather than increased transcription read-through. The nam1-1 construct clearly does result in transcription read-through and attenuation of Byr2 synthesis but carries multiple mutations. It would strengthen the MS to show that readthrough is also increased for mmi1Δ (and possibly rrp6Δ). The proposed role of Mmi1 in termination is a major conclusion of the MS and should be shown robustly.

2) Related to this is the suggestion that Mmi1 and Rrp6 also participate in termination on the nam5/6/7 lncRNAs. The extended transcripts are elevated in the mmi1-ts3 clr4Δ strain at 36{degree sign}C, but the obvious interpretation would be that this represents stabilization of these RNAs due to loss of exosome activity. It would be useful to demonstrate that loss of Rrp6 or functional Mmi1 indeed results in transcription read-through.

3) Fig. 3C: This is an important experiment and it would be useful to demonstrate that combining nam1-1 with Ttef increases byr2 mRNA or protein expression. The observation that the terminator reduces total RNA levels over the probe 1 region does not demonstrate increased mRNA expression.

4) P13: the authors write: "Thus, Rrp6, but not the deposition of the H3K9me mark, contributes to Mmi1/nam1-mediated control of sexual differentiation." Is there direct evidence that Rrp6 functions in the same pathway as nam1? Loss of Rrp6 apparently stabilizes the nam1-L transcript, but the data in Fig. 3C indicates that the lncRNA itself is not important.

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Mmi1 is a member of a conserved family of YTH RNA binding proteins. From several previous studies it is known that Mmi1 is involved in regulating a number of target RNAs in fission yeast including both meiotic mRNAs (that need to be silenced in vegetative growth) and some ncRNAs (that in turn have been shown to regulate protein-coding genes). Mmi1 has been found to be important for directing elimination of its target RNAs by the exosome, and both the exosome and Mmi1 have also been shown to be required for proper termination of target RNAs. However, the potential role of transcription termination in Mmi1-mediated gene regulation has remained unclear.

In this manuscript the authors provide evidence that Mmi1-dependent transcription termination can play a significant role in gene regulation. First they present a comprehensive analysis of Mmi1

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target RNAs identified through two approaches: pull-downs and bioinformatics. Both approaches identified a fair number of known targets (indicating the validity of the methods) as well as some novel targets, including five new ncRNA targets identified via both approaches. One of these ncRNAs (nam1) is shown to have a key role in regulating sexual differentiation: cells lacking Mmi1 have a defect in mating, and this defect is largely explained by loss of Mmi1-binding to nam1, which in turn regulates the expression of the byr2 gene, which lies just downstream of nam1 and is known to be essential for entry into sexual differentiation. The authors go on to demonstrate that it is Mmi1-dependent termination of nam1 transcripts that promotes expression of byr2, by suppressing read-through transcription. Consistent with previous reports, the exosome is also implicated in the control of termination, although the mechanism remains unclear. Furthermore, Mmi1 was also implicated in control of accumulation and termination of certain ncRNAs derived from the heterochromatic pericentromere regions, suggesting that Mmi1 may somehow work in concert with RNAi to regulate silencing in these regions.

The study is generally well done, and the demonstration that gene expression can be regulated by controlling termination of transcription of a ncRNA will be of broad interest. However I have two main reservations. Firstly, given what was already known, this main finding is somewhat incremental in nature - it is disappointing that no further light has been shed on the mechanism by which Mmi1 and the exosome bring about transcription termination. Secondly, the latter section of the paper on the pericentromeric ncRNAs feels rather under-developed, and if/how Mmi1 function might contribute to heterochromatic silencing at centromeres remains unclear. Without further mechanistic insight into at least one of these areas I am unconvinced that the manuscript represents a sufficient advance to warrant publication in EMBO Journal.

Specific points:
1. Fig. EV2E - the effect of the point mutations on the RNA binding activity of Mmi1 is unconvincing; in particular, the signal for R381E looks at least as strong as WT for all but the highest concentration (where a bigger smear is seen). It would be helpful to quantify the signals in some way.

2. In Fig. EV2F - the localisation of Mmi1 is rather 'fuzzy' - while the localisation of the mutants is similarly 'fuzzy', it is not clear what can really be concluded from this.

3. Fig. 3A - the text states that mRNA expression levels of byr2 and nam1 are anti-correlated. This is true when comparing WT to mutant (in the mutant nam1 is increased and byr2 decreased), however it is not really apparent when comparing between time points in WT - in particular, at the 0.5hr time point nam1 and byr2 appear to increase together. If the model for regulation of byr2 by nam1 is correct, then at the onset of nitrogen starvation one would expect to see an increase in nam1 transcript and a decrease in byr2 transcript, but this is not seen in the data.

4. Fig. 4 and EV5 - the analyses of pericentromeric ncRNAs are done with a t.s. allele of mmi1 rather than the deletion mutant used throughout the rest of the study - is there a reason for this, and does the deletion mutant behave the same?

5. Fig. 4C is difficult to interpret, in part because of the large number of non-specific bands, and because the lanes are not labelled. Since PCR products 2 and 3 should both correspond to nam7-L, it is puzzling that cells lacking only clr4 give a signal for product 2 but not 3 - does this in fact represent detection not of nam7-L but a different dh-derived transcript in the clr4 mutant?

6. From the data in Fig. 4 and 5 the authors conclude that "Mmi1 contributes to heterochromatic gene silencing especially by promoting termination of transcription". However, while the data does indicate a role for Mmi1 in regulating expression and termination of the target nam5-7 ncRNAs, it remains unclear if or how this might contribute to heterochromatic silencing in the pericentromeric region more generally. Is there any consequence for the cell of the increased read-through transcription of nam7 in the absence of mmi1?

7. I think there is insufficient data to support the conclusion that Mmi1 "alternates" with RNAi to ensure continuous heterochromatic gene silencing during the cell cycle. From Fig. 5C it appears that nam5/6/7 levels are steady through the cell cycle in the absence of mmi1, i.e. there is no evidence of changes related to fluctuations in RNAi activity, while levels of dg transcripts, which are thought to

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change in line with RNAi activity, are unaffected by loss of mmi1. Thus it appears that the two machineries may just have different targets, rather than the implied alternating activity on common targets. Including an RNAi mutant in the time-course experiments for direct comparison with the mmi1 mutant might help to address this.

1st Revision - authors' response 16 May 2017

[Please see next page]

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Dear Dr. Nielsen,

We would like to thank you again for considering our manuscript for publication in the EMBO Journal. We also would like to thank all three referees for their overall positive and constructive comments.

We have now revised the manuscript following the referees' comments as well as your recommendations.

Please find below your editorial decision letter followed by a point-by-point response.

Sincerely,

André Verdel

P.S: please note that the text modified and added to the revised version of the MS appears in red.

Dear Andre,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

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website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Best regards,
Anne

Anne Nielsen PhD
Editor
The EMBO Journal

Referee #1:

The manuscript of Todeschini et al. describes the molecular function of lncRNA processing for promoting epigenetic silencing and cell differenciation in pombe. Using RNA-IP coupled to RNA-sequencing, the authors isolated a series of RNA associated with Mmi1 protein, previously characterized as being involved in transcription termination. Interestingly, crystal structure analyses pinpointed a series of amino acids that are involved in the Mmi1 RNA recognition specificity. Mutations of those motifs allow the authors to directly connect mmi1 mutant phenotype to the RNA binding domain. The manuscript is then divided in 2 parts, the first one shows how Mmi1 binds to nam2 lncRNA. Cis and trans mutation shows misregulation of the downstream gene byr2 expression, indicating readthrough-mediated regulation. The

second part describes how expression of the centromeric lncRNA is important into heterochromatin assembly independently of RNAi.
The manuscript is very well written and the results are of general interest as they present a clear step forward into the comprehension of the role of lncRNA transcription for gene silencing and epigenetic mark. This manuscript does warrant publication EMBO journal since it provides the first mechanistic insights into how pervasiveness of non-coding transcription can affect cell differentiation and heterochromatin establishment in pombe. I recommend the authors to provide some clarifications both experimentally and in the text/figures prior publications.

➔ We thank this referee for stating that this "*manuscript is very well written and the results are of general interest as they present a clear step forward into the comprehension of the role of lncRNA transcription for gene silencing and epigenetic mark*" and that "*this manuscript does warrant publication in the EMBO Journal*". Below, we provide the additional experimental data and clarifications asked by the referee.

Major critics:

1-The key experiment is the demonstration that mmi1 is indeed involved in transcription termination process of the Nam2 lncRNA. Figure 3B shows that RNAPIIser2 phosphorylation increases in the byr2 coding region when nam2 is mutated for mmi1 binding. This suggests that nam2 transcription elongates into the downstream byr2 region inhibiting its expression. This experiment is rather confusing to me since ser2P might also represent the downstream gene transcription and/or antisense transcription of byr2. More confusing is the reminiscence of high ser2P for probe 7 as if an alternative transcription process is occurring in this region that would be not controlled by nam2 readthrough.
Altogether, this experiment would benefit of 2 additional controls with RNAPII and ser5P occupancy allowing some indications on the quantity of readthrough polymerase and its directionality. I would recommend additional chip POLII and ser5P experiment to conclude more clearly. Technically, the readthrough could only be validated by measuring the nascent nam2 RNA, neither the presence of a long nam2 RNA form or increase of ser2P-POLII occupancy demonstrate a readthrough process since the longer form could appear because of RNA stabilisation (readthrough would be constant whatever the mmi context) and POLII occupancy would reflect an alternative transcription.

➔ We have conducted the two ChIP controls proposed by the referee. Importantly, the results of these experiments further support our initial conclusion that Mmi1 regulates the transcription termination of *nam1*. These experiments have been added to the MS (as Fig EV 4B and D) and the main text has been amended accordingly (page 13).

2-also the authors should discuss the possibility that the elongated RNAs per se is playing also a role in the attenuation of byr2. Indeed an antisense byr2 RNA has been annotated and interference with the elongated nam2 RNA might play a role in the regulation (double strand formation, titration of the antisense that might positively regulate byr2 etc..).

➔ We thank the referee for bringing up this point. Indeed, from our data we cannot exclude the possibility that *nam1* readthrough transcript can its self contribute to the silencing of *byr2*. To make this clear we have now added a sentence and additional references in the Discussion (page 20).

3-in the same lines, Figure 3b has some issues in the labelling (for Y and x axis.

➔ We have checked the labeling of Figure 3B Y and X axes. However, it looks fine to us both when looking at the digital and printed versions of the manuscript. If the issues are indeed with Figure 3B, please let us know more specifically what they are.

We did find that the labeling of the neighboring figure (Figure 3C) is rather confusing. This may be what the referee pointed to. We have now reorganized and reannotated Figure 3C to make it more readable.

Referee #2:

The authors report the identification of novel lncRNA targets for Mmi1 and Rrp6 starting with a nice combination of RIP and bioinformatics. They back this up with a good structure-based mutational analysis that identifies mutations in the YTH domain with greatly reduced RNA binding. The analysis of the nam1 lncRNA makes a convincing case that transcription readthrough from the lncRNA can reduce expression of a downstream gene with a role in the initiation of sporulation. However, the major conclusion of the MS that the key function of Mmi1 is to terminate transcription on these lncRNA and on lncRNA from the centromeric regions could be more robustly demonstrated. Especially for very unstable lncRNAs, distinguishing between altered synthesis and rapid degradation can be challenging. There are some relatively straight forward analyses that would support the conclusions.

➔ We would like to also thank this referee for his positive comments. Below, we provide additional data that further support our major conclusions, as asked by the referee.

Specific points:

1) The Rrp6 result shown in Figure 3E raises some questions. The rrp6Δ mutation increases nam1-L detection to same extent as mmi1Δ - but the effects of rrp6Δ seem likely to reflect defects in degradation rather than increased transcription read-through. The nam1-1 construct clearly does result in transcription read-through and attenuation of Byr2 synthesis but carries multiple mutations. It would strengthen the MS to show that readthrough is also increased for mmi1Δ (and possibly rrp6Δ). The proposed role of Mmi1 in termination is a major conclusion of the MS and should be shown robustly.

➔ We have now added a new series of ChIP experiments showing that the readthrough transcription at *nam1-byr2* locus observed in *nam1-1* cells also occurs in *mmi1Δ* cells. The data is presented in Figure EV 4C of the revised manuscript and a sentence describing these findings has been added to the Results section (page 13).

2) Related to this is the suggestion that Mmi1 and Rrp6 also participate in termination on the nam5/6/7 lncRNAs. The extended transcripts are elevated in the mmi1-ts3 clr4Δ strain at 36{degree sign}C, but the obvious interpretation would be that this represents stabilization of these RNAs due to loss of exosome activity. It would be useful to demonstrate that loss of Rrp6 or functional Mmi1 indeed results in transcription read-through.

➔ We now provide an additional series of ChIP experiments monitoring the level of elongating RNAPII at, and downstream of, pericentromeric *nam* regions. Importantly, these new experiments show that in *mmi1Δ clr4Δ* cells there is a clear Mmi1-dependent increase of the elongating RNAPII downstream the *nam* regions, when compared to wild-type cells and the *mmi1Δ* or *clr4Δ* mutants. These results further support the role of Mmi1 in promoting transcription termination at pericentromeric heterochromatin. The data is now presented in Figure EV 5G and the text in the Results section has been amended accordingly (page 16).

3) Fig. 3C: This is an important experiment and it would be useful to demonstrate that combining nam1-1 with Ttef increases byr2 mRNA or protein expression. The observation that the terminator reduces total RNA levels over the probe 1 region does not demonstrate increased mRNA expression.

➔ We now add a new series of data obtained by Northern blot, which show that the block of *nam1-1* readthrough transcription is accompanied by the accumulation of *byr2* mRNA. This data is now shown in Figure EV 4F and we have added in the Results section (page 14) a sentence describing this data.

4) P13: the authors write: "Thus, Rrp6, but not the deposition of the H3K9me mark,

contributes to Mmi1/nam1-mediated control of sexual differentiation." Is there direct evidence that Rrp6 functions in the same pathway as nam1? Loss of Rrp6 apparently stabilizes the nam1-L transcript, but the data in Fig. 3C indicates that the lncRNA itself is not important.

➔ We agree and thank the referee for bringing up this point. Indeed, our data demonstrate that Rrp6 is required for Mmi1-mediated control of *nam1* expression, and only suggest that Rrp6 acts in *nam1* pathway. We therefore rephrased the sentence (page 15), as follow: “Thus, Rrp6, but not the deposition of the H3K9me mark, contributes to Mmi1-mediated control of *nam1* expression.” Accordingly, we also changed the title of the paragraph (page 14).

Referee #3:

Mmi1 is a member of a conserved family of YTH RNA binding proteins. From several previous studies it is known that Mmi1 is involved in regulating a number of target RNAs in fission yeast including both meiotic mRNAs (that need to be silenced in vegetative growth) and some ncRNAs (that in turn have been shown to regulate protein-coding genes). Mmi1 has been found to be important for directing elimination of its target RNAs by the exosome, and both the exosome and Mmi1 have also been shown to be required for proper termination of target RNAs. However, the potential role of transcription termination in Mmi1-mediated gene regulation has remained unclear.

In this manuscript the authors provide evidence that Mmi1-dependent transcription termination can play a significant role in gene regulation. First they present a comprehensive analysis of Mmi1 target RNAs identified through two approaches: pull-downs and bioinformatics. Both approaches identified a fair number of known targets (indicating the validity of the methods) as well as some novel targets, including five new ncRNA targets identified via both approaches. One of these ncRNAs (nam1) is shown to have a key role in regulating sexual differentiation: cells lacking Mmi1 have a defect in mating, and this defect is largely explained by loss of Mmi1-binding to nam1, which in turn regulates the expression of the byr2 gene, which lies just downstream of nam1 and is known to be essential for entry into sexual differentiation. The authors go on to demonstrate that it is Mmi1-dependent termination of nam1 transcripts that promotes expression of byr2, by suppressing read-through transcription. Consistent with previous reports, the exosome is also implicated in the control of termination, although the mechanism remains unclear. Furthermore, Mmi1 was also implicated in control of accumulation and termination of certain ncRNAs derived from the heterochromatic pericentromere regions, suggesting that Mmi1 may somehow work in concert with RNAi to regulate silencing in these regions.

The study is generally well done, and the demonstration that gene expression can be

regulated by controlling termination of transcription of a ncRNA will be of broad interest. However I have two main reservations. Firstly, given what was already known, this main finding is somewhat incremental in nature - it is disappointing that no further light has been shed on the mechanism by which Mmi1 and the exosome bring about transcription termination. Secondly, the latter section of the paper on the pericentromeric ncRNAs feels rather under-developed, and if/how Mmi1 function might contribute to heterochromatic silencing at centromeres remains unclear. Without further mechanistic insight into at least one of these areas I am unconvinced that the manuscript represents a sufficient advance to warrant publication in EMBO Journal.

➔ We also thank this referee for stating that "*The study is generally well done, and the demonstration that gene expression can be regulated by controlling termination of transcription of a ncRNA will be of broad interest.*" Below, we now provide further experiments and clarifications asked by the referee.

Specific points:
1. Fig. EV2E - the effect of the point mutations on the RNA binding activity of Mmi1 is unconvincing; in particular, the signal for R381E looks at least as strong as WT for all but the highest concentration (where a bigger smear is seen). It would be helpful to quantify the signals in some way.

➔ We have quantified the binding of Mmi1 WT and mutants to RNA. The quantification is now presented in Figure EV 2F of the revised version of the manuscript and mentioned in the Results section (page 10).

2. In Fig. EV2F - the localisation of Mmi1 is rather 'fuzzy' - while the localisation of the mutants is similarly 'fuzzy', it is not clear what can really be concluded from this.

➔ We have redone the immunofluorescence experiments in order to improve the quality of the images. New images now replace the ones initially presented in Figure EV 2G. These images show no significant change in the subcellular localization of the Mmi1 point mutants, indicating that their loss of binding to RNA is not caused by a change in their cellular localization, as previously stated in the manuscript.

3. Fig. 3A - the text states that mRNA expression levels of byr2 and nam1 are anti-correlated. This is true when comparing WT to mutant (in the mutant nam1 is increased and byr2 decreased), however it is not really apparent when comparing between time points in WT - in particular, at the 0.5hr time point nam1 and byr2 appear to increase together. If the model for regulation of byr2 by nam1 is correct, then at the onset of nitrogen starvation one would expect to see an increase in nam1 transcript and a decrease in byr2 transcript, but this is not seen in the data.

➔ Indeed, the data presented in Figure 3A shows that, when comparing wild-type to mutant, the RNA levels of *byr2* and *nam1* are anti-correlated, but this anti-correlation is not observed when comparing in wild-type cells the level of the same RNAs at 0.5 hr after the induction of sexual differentiation. Why this is the case is subject to speculation. *Byr2* is among the "master" genes expressed at the onset of sexual differentiation in response to environmental changes. In this context, *nam1*-mediated gene silencing of *byr2* is most likely only part of the regulatory system that tightly controls *byr2* expression*.* Importantly, regardless of what are the details of *byr2* regulation, our findings demonstrate that *nam1*-dependent regulation is critical for the proper control *byr2* expression, especially when the cells enter sexual differentiation.

4. Fig. 4 and EV5 - the analyses of pericentromeric ncRNAs are done with a t.s. allele of mmi1 rather than the deletion mutant used throughout the rest of the study - is there a reason for this, and does the deletion mutant behave the same?

➔ We would like to point out first that we did not make such strain because the deletion of *mmi1* requires to also delete the gene *mei4* (which encodes a transcription factor under the control of Mmi1 and that activates meiotic genes) to avoid *mmi1Δ* cells from undergoing untimely meiosis. This genetic constraint first described by our collaborators (Harigaya et al, Nature, 2006) is also mentioned in the Material and Methods section of this manuscript. This is the reason why we initially decided to make *mmi1-ts3 clr4Δ* double mutant cells instead of the triple mutant *mmi1Δ mei4Δ clr4Δ*.

To respond to the referee's question, we have now made the *mmi1Δ mei4Δ clr4Δ* mutant strain and reanalyzed the requirement of Mmi1 in heterochromatin gene silencing at pericentromeres. Importantly, we observe in *mmi1Δ mei4Δ clr4Δ* cells, as in *mmi1-ts3 clr4Δ* cells, a clear increase of the levels of *nam7* readthrough transcripts, compared to WT, *mmi1Δ* and *clr4Δ* mutants. This data is mow presented in Fig EV 5F and mentioned of the Results section (page 16).

5. Fig. 4C is difficult to interpret, in part because of the large number of non-specific bands, and because the lanes are not labelled. Since PCR products 2 and 3 should both correspond to nam7-L, it is puzzling that cells lacking only clr4 give a signal for product 2 but not 3 - does this in fact represent detection not of nam7-L but a different dh-derived transcript in the clr4 mutant?

➔ We have reannotated Figure 4C to make it more easy to analyze. The new version of the figure now replaces the previous Figure 4C. In addition, we would like to confirm that in this experiment the setup used for the RT-PCR allows to detect only *nam7* readthrough transcripts and not any other *dh* transcripts transcribed downstream of *nam7.* We have now made this clear in the Material and Methods

section (page 25 and 26).

Regarding the statement that "*it is puzzling that cells lacking only clr4 give a signal for product 2 but not 3*", we would like to bring the following clarification. In *clr4Δ* cells, transcription of *nam7* strongly increases (because of the complete loss of heterochromatin) and this comes with an increase in the level of *nam7* transcripts as well as *nam7* readthrough transcripts. However, because in *clr4Δ* cells Mmi1 still binds to RNA targets and to pericentromeric chromatin (Fig 5A), we believe that Mmi1 continues to promote *nam7* transcription termination in these cells, although not efficiently enough to terminate all of *nam7* transcription as early as in wild-type cells. In agreement with this possibility, in *clr4Δ* cells product #2 accumulates but not product #3, which corresponds to longer *nam7* readthrough transcripts that accumulate only in the double mutant *mmi1Δ clr4Δ* cells.

6. From the data in Fig. 4 and 5 the authors conclude that "Mmi1 contributes to heterochromatic gene silencing especially by promoting termination of transcription". However, while the data does indicate a role for Mmi1 in regulating expression and termination of the target nam5-7 ncRNAs, it remains unclear if or how this might contribute to heterochromatic silencing in the pericentromeric region more generally. Is there any consequence for the cell of the increased read-through transcription of nam7 in the absence of mmi1?

➔ We investigated the consequence of *mmi1* deficiency on the function of the centromeres and found that, in absence of Mmi1, a fraction of the cells show centromeric defects. However, this defect is, at least partially, indirect and caused by the expression of the gene *rec8*, encoding a meiotic cohesin (which under vegetative growth is normally silenced by Mmi1-mediated degradation of *rec8* mRNAs). Similar findings have been reported recently (Folco, Nature, 2017). Several other meiotic genes upregulated in *mmi1Δ* cells are susceptible to contribute to the observed centromere defects (Hiriart, EMBOJ, 2012; Folco, Nature, 2017). Because of the misexpression of diverse meiotic chromatin regulators, it is in fact very challenging (if not impossible) to use *mmi1Δ* cells to discriminate between the defects caused by the desilencing of some pericentromeric repeats from the ones caused by the misexpression of meiotic chromatin regulators.

To bypass this difficulty, we then took the opposite strategy, which consists in overexpressing Mmi1 (using the strong promoter *nmt1*). Remarkably, overexpression of Mmi1 level leads to a reduction of H3K9me2 level at *nam5/6/7* repeats as well as at *dg* repeats, suggesting that although Mmi1 localizes at specific places it may indeed have a more general impact on pericentromeric heterochromatin. Furthermore, these data also suggest that Mmi1-mediated gene silencing may be competing with RNAi-mediated heterochromatin formation. A possibility that we had already evoked in the Discussion section of the manuscript and which is now further supported by these new findings. This new data is now

presented in Fig EV 5E, the Results (page 16) and the Discussion sections (pages 21 and 22).

7. I think there is insufficient data to support the conclusion that Mmi1 "alternates" with RNAi to ensure continuous heterochromatic gene silencing during the cell cycle. From Fig. 5C it appears that nam5/6/7 levels are steady through the cell cycle in the absence of mmi1, i.e. there is no evidence of changes related to fluctuations in RNAi activity, while levels of dg transcripts, which are thought to change in line with RNAi activity, are unaffected by loss of mmi1. Thus it appears that the two machineries may just have different targets, rather than the implied alternating activity on common targets. Including an RNAi mutant in the time-course experiments for direct comparison with the mmi1 mutant might help to address this.

➔ We agree. We have removed the term "alternate" from the Abstract (page 3), Results (page 17) and Discussion (page 18). Moreover, a sentence in both the Introduction and the Discussion sections was rephrased (respectively, page 8 and 21) and a sentence added in the Discussion to further clarify this last part of the study (page 22).

2nd Editorial Decision 08 June 2017

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by all three original referees and their comments are shown below. As you will see the referees find that all major criticisms have been sufficiently addressed and they recommend the manuscript for publication. However, before we can go on to officially accept your manuscript there are a few editorial issues concerning text and figures that I need you to address.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to receiving your final revision.

REFEREE REPORTS

Referee #1:

The revised manuscript of Touat-Todeschini et al. shows that the selective termination of lncRNA transcription promotes epigenetic silencing and cell differentiation in pombe.

The authors respond well to all the comments and issues raised by the referees, including mine, and I support now publication in EMBOj since it provides a novel mechanism on how lncRNA targeted by Mmi1 YTH factors can modulate epigenetic local environment to control cell differentiation. The dissection of such regulatory pathway provides the community an excellent base to identify similar mode of action in other eukaryotes and is the starting point for further characterization of other factors involved and the relationships with the lncRNA itself (and/or its transcription) and protein domain(s) involved in the target selective recognition.

Referee #2:

In the revised MS, each of the specific points raised in my initial review has been satisfactorily addressed. I am therefore happy to recommend publication.

Referee #3:

In the revised manuscript the authors have added several new pieces of data that strengthen the conclusion that Mmi1 contributes to transcription termination of target RNAs. All of the specific technical/interpretation issues raised in my original review have been addressed. I have only one minor additional comment on the revised data: in relation to point 1, Fig. EV 2F, it is unclear how the quantification has been done (i.e. which dose point(s) it represents) - this needs to be explained more fully in the methods.

My more general reservations have not really been addressed: it is disappointing that the authors have not been able add any functional insight into how Mmi1 contributes to termination, and/or the significance of its role at pericentromeres. Nevertheless, the illustration that gene expression can be regulated via control of neighbouring ncRNA transcription termination will be of interest to the readership of EMBO Journal.

2nd Revision - authors' response 14 June 2017

We are glad to see that all three referees recommend the manuscript for publication.

We have now addressed all the editorial issues that you pointed out in your letter (below). In addition, we added further details (in red in the text) on how the quantification was done in Fig. EV

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2F, as requested by referee#3. Finally, we also added the PDB accession number for the structure of Mmi1 YTH domain.

Once again, I would like to thank you for having considered our manuscript for publication in The EMBO Journal.

3rd Editorial Decision 19 June 2017

I am pleased to inform you that your manuscript has now been accepted for publication in The EMBO Journal.

If you have any questions, please feel free to contact me. Thank you for your contribution to The EMBO Journal and congratulations on this nicely executed work!

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Journal Submitted to: EMBOJ
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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The data shown in figures should satisfy the following conditions:

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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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