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A long Nuclear Retained Non-Coding RNA Regulates Synaptogenesis by Modulating Gene Expression

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1st Editorial Decision 12 March 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has been evaluated by three referees and I enclose their reports below. As you will see from their comments the referees find the study to be potentially interesting but require some further experimental analysis before it can be further considered for the EMBO Journal.

The referees provide mixed recommendations regarding publication and overall raise two main issues, one is the further analysis of the tissue specific functions of Malat1, while this a good suggestion and would potentially strengthen the manuscript, I find that it is beyond the scope of the current study and therefore not a specific requirement for publication in the journal. The second issue is that there is currently a lack of molecular insight into how Malat1 regulates genes expression. This more mechanistic aspect of the study should be experimentally addressed to provide more insight why Malat1 is recruited to sites of transcription and how the ncRNA affects gene expression, this should also include the splicing microarray dataset mentioned in the discussion. Should you be able to address these issues, we would be wiling to consider a revised 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:
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Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript by Bernard et al. addresses the role of the long nuclear-retained non-coding RNA (ncRNA) Malat1 in synapse formation. Malat1 was originally identified as a nuclear speckle-localized ncRNA overexpressed in different types of cancer. Speckles are sites of storage/modification and/or assembly from where pre-mRNA processing factors are recruited to active sites of transcription. In the present study, the authors provide evidence suggesting that Malat1 is highly abundant in brain tissue where it modulates the recruitment of a subset of RNA-processing factors (SF2/ASF and SC35) to nuclear speckles in order to control the expression of genes involved in regulating synapse formation and maintenance. Although such a model is attractive, a weakness of this study is that it lacks mechanistic information on how Malat1 and the synaptic proteins may be functionally linked. The data presented does not provide sufficient information to support a direct connection between Malat1 and the synaptic proteins. The manuscript could be improved and more informative if several changes are made. My specific comments are indicated below:

1) Quantification of the data from Figure 1a will strengthen the claim that Malat1 is highly abundant in brain tissue.

2) In Figure 2 the authors use the transcriptional inhibitors DRB and α-amanitin interchangeably. However, they do not provide an explanation for that in the text. Why using DRB in Figure 2C if mRNA levels were measured using α-amanitin?

3) Is data in Figure 2C normalized to an internal control? What does "relative value" mean?

4) In Figures 2D-S the authors claim that SF2/ASF is recruited to nuclear speckles after 15 minutes of recovery from transcription inhibition. However, it is not clear to me how they know that SF2/ASF resumes its localization to speckles if they did not use any marker protein for these structures. In order to make a claim supporting temporal regulation of the recruitment of SF2/ASF and Malat1 to nuclear speckles, they should perform the experiment using a speckle marker that is not affected by transcriptional inhibition.

5) In Figure 3 they measure LacI-mCherry fluorescence as a reporter for transgene locus decondensation (transcriptional activation). They show that in response to activation with doxycycline, the reporter locus is decondensed (There is more LacI-mCherry fluorescence). Are LacI-mCherry protein levels similar in all conditions? In supplementary Figure 4 they show evidence for reporter locus activation, but it's not clear why they applied doxycycline for two hours instead of 3 hours as in Figure 3. How many cells were analyzed to generate Figure 3Q?

6) The authors claim that Malat1 ncRNA is not involved in the recruitment of transcription factors to the active transcription site. However, they only tested the protein CDK9 in supplementary Figure 2. They should test a battery of proteins involved in Pol II transcription in order to strengthen their claim.

7) In Table 1 they show groups of genes downregulated in Malat1 knock-down Neuro2A cells. Are

endogenous expression levels of Malat1 in Neuro2A cells comparable to its levels in MEFs? Since results in Table 1 rely in the successful knock-down of Malat1 in Neuro2A cells, how effective is Malat1 knock-down in these cells?

8) The authors claim that either knock-down or overexpression of Malat1 in hippocampal neurons induce synapse-associated phenotypes. How effective is Malat1 knock-down in hippocampal neurons? Since Malat1 is an abundant RNA in neurons, how overexpressed is Malat1 in the experiment in Figure 4L? They use synapsin labeling to score for synaptic phenotypes. Are synapsin levels affected by Malat1 knock-down? What about other synaptic proteins? They should score for phenotypes using several markers.

9) Is data in Figure 4N normalized to an internal control? What is "Expression ratio"?

10) Does Malat1 knock-down or overexpression in hippocampal neurons impairs synaptic activity?

11) Data presented here is not sufficiently integrated in the context of the literature. In particular, one of the main conclusions of the paper is that Malat1facilitates recruitment of SF2/ASF ans SC35 to nuclear speckles. However, the role of the SR proteins in pre-mRNA processing is not described in the text. They should include a section (Introduction) describing the role of SR proteins in regulating gene expression.

Referee #2 (Remarks to the Author):

General comments:

This is an interesting paper that begins to explore the functional role of the noncoding RNA Malat1 in cell biology and particularly in synaptogenesis. The results show that Malat1 RNA is expressed in many tissues, especially in neurons, is localized in nuclear speckles in an RNA polymerase II-dependent transcription-dependent manner (which is in itself intriguing), is involved in the recruitment of SR type pre-mRNA processing factors to transcription sites as well as the expression of genes involved in nuclear processes and synapse function, and affects synaptic density.

These findings are well supported by the data presented. The paper is succinct, well written, and represents a major advance on the hitherto very limited knowledge of the functions of this interesting and widely expressed noncoding RNA, although it also raises additional unanswered questions, especially with regard to the general role of Malat1 in the modulation of transcript profiles in different types of cells.

Specific comments:

1. I suggest that the functional and visual differences between paraspeckles and nuclear speckles, and the relationship of the latter to interchromosomal granules* be explained in the Introduction. At present, the statement that "The long Malat1 transcript has been localized to nuclear speckles or interchromatin granule clusters (IGCs) ...." is ambiguous in respect of whether these are different compartments or different names for the same compartment.

2. The Northern data presented in Figure 1A, as well as previous studies, indicate that Malat1 is widely expressed in different tissues and presumably has a role that it is relatively generic in different contexts. Thus, although not commented on in the paper, it is a surprise that "non-neuronal cells in brain sections showed low or undetectable levels of Malat1 ncRNA" (Results p. 5), which shows that Malal1 is not ubiquitously expressed in the brain and begs the question of what subset(s) of cells in other tissues it may be expressed in. Moreover, since the results presented in this paper indicate that Malat1 "regulates synapse formation by modulating the expression of genes involved in synapse formation and/or maintenance" (Abstract, p. 2), the question arises as to what (presumably non-neuronal) function(s) Malat1 fulfils in other tissues where it is relatively highly) expressed, such as heart, kidney and lung (Fig. 1A). Given the reasonable hypothesis (Discussion, p. 11) that "Malat1 regulates gene expression by modulating the differential association or activity of SR-splicing factors" and the reasonable suggestion (Discussion, p. 12) that "it is likely to play broader regulatory roles by affecting different genes in a tissue specific manner", I suggest that the paper

could be considerably strengthened by undertaking similar RNA FISH analyses, developmental expression profiles, and Malat1 knockdowns / overexpression / microarray interrogations in (one or two) other tissues to try to gain more insight into what types of cells or cell states express Malat1, and what types of transcripts are affected by alterations of its expression. This would not be a trivial undertaking, perhaps beyond the scope of the present paper, but would go a long way to generalizing an understanding of Malat1 function.

3. In the Discussion (p. 12), reference is made to the results of alternative splicing microarrays (data not shown). Why are these data not shown?

4. As an aside, it was an interesting experience to read and review a paper presented in landscape format - a first for me. I though at first that this may be because the figures were presented in landscape format (which would therefore avoid assembling a composite PDF file), but no, 3 of the 4 figures are in portrait layout. Intriguing.

Referee #3 (Remarks to the Author):

In this study, the authors examined the role of a long nuclear non-coding RNA Malat-1 in splicing factor recruitment to nascent RNA and gene expression. On a cell line expressing an inducible locus, the authors observed reduced recruitment of SR proteins to nascent RNA in the absence of Malat-1 (degraded by an antisense oligo). However, gene expression from the locus was unaffected.

The authors went on to detect changes in gene expression by microarray in hippocampal neurons. GO analysis suggests that affected genes involved in synaptogenesis were selectively affected. Consistently, Malat-1 knockdown appears to reduce synaptic density. The authors concluded that Malat-1 is selectively involved in regulated gene expression critical for synapse function in neurons.

The major problem I have is the disconnection between Malat-1 mediated recruitment of SR protein factors and Malat-1 regulated gene expression. The authors made a lot of hand waving speculations to force the link without data to support their conclusions.

I am also confused by some specific interpretation of the data. For example, the authors suggested that SF2/ASF recovered ahead of Malat-1 on nuclear speckles 15 min after washing away the transcription inhibitor DRB (Fig. 2M). Does this mean that the recruitment of SF2/ASF (or SR proteins in general) to nascent RNA does not need Malat-1. This would suggest that SR protein recruitment has nothing to do with the function of Malat-1. This would further disconnect the front parts of the paper to later ones with regard to Malat-1 dependent gene expression.

The authors did not fully describe the gene expression profiling experiment. Did the microarray experiment only detect gene down-regulation in response to Malat-1 depletion? I would image that many genes were also up-regulated. Were those un-regulated genes indirectly induced? How do we know if any gene was directly affected by Malat-1?

To me, GO analysis does not mean much. It appears that Malat-1 is quite ubiquitously expressed in many cell types and thus may affect gene expression in a general way. As a result, its down-regulation may affect many genes critical for the cellular function of specific cell type under investigation, in this case, the hippocampal neuron. Thus, the conclusion drawn could be misleading.

1st Revision - authors' response 06 July 2010

We greatly appreciate the comments of the reviewers and editor. Below are our responses to each of the comments. The changes have made this a significantly better paper.

Editor's comments: *There is currently a lack of molecular insight into how Malat1 regulates genes expression. This more mechanistic aspect of the study should be experimentally addressed to provide more insight why Malat-1 is recruited to sites of transcription and how the ncRNA affects gene expression; this should also include the splicing microarray dataset mentioned in the discussion.*

Answer: In the first version of the manuscript, we showed that Malat1 co-localized with SR proteins in nuclear speckles and that its depletion affects the recruitment of SR-proteins to an actively transcribing reporter gene locus (NB: At present we do not have any data indicating that Malat1 is recruited to the sites of transcription, as stated in the editor's comment). We further showed that in neurons, Malat1 controls the expression of a subset of genes specifically involved in synaptic function. Importantly, while most studies on ncRNAs provide little to no functional data; our work provided significant functional insights into the role of this highly expressed ncRNA. We now provide further experimental data showing Malat1 specifically controls the recruitment of SR proteins to the reporter gene locus without affecting the recruitment of transcription factors and RNA polymerase II complex. As discussed in the text, it has already been demonstrated that changes in the recruitment of SR proteins to transcription sites or pre-mRNA can affect several aspects of pre-mRNA biogenesis and maturation including transcription, splicing, RNA transport and mRNA decay (Long and Caceres, 2009 The Biochemical journal 417, 15-27). As you will see in the accompanying manuscript (that is being reviewed right now by another journal), we have demonstrated that in cancerous cell lines, Malat1 interacts with SR proteins and depletion of Malat1 affects phosphorylation of SR proteins, alternative splicing of pre-mRNAs and finally transport of poly A+ RNAs. In the present manuscript, we have demonstrated that in neuronal cells, Malat1 controls the expression of synapse-specific genes, which is a more physiologically relevant system. Taken together, we hypothesize that Malat1 regulates the expression of synapse-specific genes by modulating SR protein activity. The direct demonstration of this hypothesis is a full project on its own that will be the next step of our work.

As requested by the editor, this new version of the manuscript provides a significant amount of new data:

-the demonstration that, as opposed to the SR proteins, the recruitment to the site of transcription of several proteins involved in RNA Pol-II transcription are not dependent on Malat1.
-Experimental evidence showing that over-expression of Malat1 in neurons can be detected by in situ hybridization.
-The complete analysis of the genes impacted by Malat1 knockdown, which further demonstrates that in neurons, the synaptic genes are preferred targets of Malat1-dependent regulation.

Referee #1

1) *Quantification of the data from Figure 1a will strengthen the claim that Malat1 is highly abundant in brain tissue.*

Answer: We have now quantified the data and added a histogram showing the quantification of the northern blot in supplementary figure 1.

2) *In Figure 2 the authors use the transcriptional inhibitors DRB and alpha-amanitin interchangeably. However, they do not provide an explanation for that in the text. Why using DRB in Figure 2C if mRNA levels were measured using alpha-amanitin?*

Answer: Both *alpha-amanitin* and DRB efficiently inhibits RNA pol II-mediated transcription. Alpha-amanitin directly inhibits RNA pol II by binding to the polymerase resulting in its degradation. DRB is a kinase inhibitor that preferentially inhibits pTEF-B kinase (CDK9/Cyclin-T that phosphorylates the serine-2 in the CTD of RNA pol II) activity, which is required for the RNA pol II-mediated transcriptional elongation. However, α-amanitin-mediated transcription inhibition is irreversible, where as transcription in the DRB-treated cells can be reactivated upon removal of the drug from the media. We used alpha-amanitin in Figs. 2B-C, and DRB in Fig. 2H-S. We used DRB in Fig 2H-S experiments, because we wanted to study the recovery kinetics of Malat1 to nuclear speckles during transcription reactivation.
We have now explained the rationale of using 2 different drugs in the results section.

3) *Is data in Figure 2C normalized to an internal control? What does "relative value" mean?*

Answer: In all the qPCR experiments, b-actin was used as internal control. In Fig. 2C, Malat1, mCAT2 and 7SK RNA levels were normalized to b-actin mRNA and were presented relative to RNA levels in untreated (control) cells.
We have now added this information in the legend.

4) *In Figures 2D-S the authors claim that SF2/ASF is recruited to nuclear speckles after 15 minutes of recovery from transcription inhibition. However, it is not clear to me how they know that SF2/ASF resumes its localization to speckles if they did not use any marker protein for these structures. In order to make a claim supporting temporal regulation of the recruitment of SF2/ASF and Malat1 to nuclear speckles, they should perform the experiment using a speckle marker that is not affected by transcriptional inhibition.*

Answer: We have now added a supplementary figure (Figure S3) using a speckle marker (SON) that is continued to localize to speckles during transcriptional inhibition (Sharma et al., 2010 Mol. Biol Cell 21(4) 650-63). This figure shows that SF2/ASF resumes its localization to speckle 15 minutes after the recovery of inhibition.

5) *In Figure 3 they measure LacI-mCherry fluorescence as a reporter for transgene locus decondensation (transcriptional activation). They show that in response to activation with doxycycline, the reporter locus is decondensed (There is more LacI-mCherry fluorescence). Are LacI-mCherry protein levels similar in all conditions?*

Answer: The LacI-mCherry fusion protein binds to the Lac-Operator DNA sequences, present in the reporter gene. By the help of LacI-mCherry signal, we visualize the reporter gene locus (Janicki et al., 2004 Cell **116**, 683-698). However, the LacI-mCherry is not transcribed by the reporter locus, but from a plasmid that was integrated in a different genomic region in the cells. Therefore, transcriptional activation of the reporter locus by DOX does not alter the cellular levels of LacI-mCherry protein. The increased size of the LacI-mCherry signal observed in the transcriptionally active reporter locus is due to decondensation of the reporter locus chromatin

5-2) *In supplementary Figure 4 they show evidence for reporter locus activation, but it's not clear why they applied doxycycline for two hours instead of 3 hours as in Figure 3.*

Answer: We have analyzed the transcriptional activation of the reporter locus at different time points (1 hrs to 4 hrs) both by RT-PCR or by microscopic analyses and the results were comparable between each time point.

5-3*) How many cells were analyzed to generate Figure 3Q?*

Answer: ~25 cells/experiment were analyzed and the experiment was repeated three times independently. We have now added this information in the legend.

6) *The authors claim that Malat1 ncRNA is not involved in the recruitment of transcription factors to the active transcription site. However, they only tested the protein CDK9 in supplementary Figure 2. They should test a battery of proteins involved in Pol II transcription in order to strengthen their claim.*

Answer: We have now provided new data (Supplementary figure 4) showing that the recruitment of RNA Polymerase II and transcriptional activator (rtTa) to the transcriptionally active reporter locus remains unaffected in Malat1 depleted cells.

7-1) *In Table 1 they show groups of genes downregulated in Malat1 knock-down Neuro2A cells. Are endogenous expression levels of Malat1 in Neuro2A cells comparable to its levels in MEFs?*

Answer: In culture, Malat1 RNA is detected ≈2.5 PCR cycles after b-actin mRNA in Neuro2A and after ≈4 cycles in wtMEFs. We have now added a sentence in the legend to the supplementary figure 1.

7-2) *Since results in Table 1 rely in the successful knock-down of Malat1 in Neuro2A cells, how effective is Malat1 knock-down in these cells?*

Answer: Supplementary Table 2 shows that there is ~60% inhibition of Malat1 level upon transfection of Neuro2A with antisense vs. scramble oligonucleotides.

8-1) *The authors claim that either knock-down or overexpression of Malat1 in hippocampal neurons induce synapse-associated phenotypes. How effective is Malat1 knock-down in hippocampal neurons?*

Answer: Figure 4N shows a histogram of the level of Malat1 upon knock-down with two different oligos (a mean of 60% inhibition) and supplementary figure 5 shows ISH and qPCR analysis of Malat1 inhibition in hippocampal neurons.

8-2*) Since Malat1 is an abundant RNA in neurons, how overexpressed is Malat1 in the experiment in Figure 4L?*

Answer: As explained in the method sections, the over-expression of Malat1 was first examined by ISH, and the quantification of synapses was performed on the same neurons. We have now added an image of a neuron over-expressing Malat1 in supp figure 5 D, E.

8-3) *They use synapsin labeling to score for synaptic phenotypes. Are synapsin levels affected by Malat1 knock-down? What about other synaptic proteins? They should score for phenotypes using several markers.*

Answer: We have now added the values of the ratio of Synapsin I mRNA upon Malat1 knockdown in the text. This shows that the level of synapsin I mRNA is not changed upon Malat1 kock-down. We have only used Synapsin I because it is a general marker of synapse that does not discriminate between inhibitory and excitatory synapses. It is expressed from earliest stages of synapse formation (cf. Ahmary et al. Nat Neurosci 3: 445) and therefore identifies nascent and mature synapses (like in Graf et al. 2004). We have now added a sentence in the text.

9*) Is data in Figure 4N normalized to an internal control? What is "Expression ratio"?*

Answer: We have now added in the legend of figure 4N that the levels are expressed relative to the Actin mRNA level.

10) *Does Malat1 knock down or over-expression in hippocampal neurons impair synaptic activity?*

Answer: Malat1 knockdown in hippocampal neurons leads to a decrease of Neuroligin-1 mRNA level and to a decreased synaptic density. It has already been demonstrated that decrease of Neuroligin-1 mRNA level leads to a decrease of synaptic density and an impairment of synaptic activity (Graf et al. 2004; Chih et al. 2005). We think that understanding the impact of Malat1 knockdown on synaptic activity will be beyond the scope of the current study.

11) *Data presented here is not sufficiently integrated in the context of the literature. In particular, one of the main conclusions of the paper is that Malat1 facilitates recruitment of SF2/ASF and SC35 to nuclear speckles. However, the role of the SR proteins in pre-mRNA processing is not described in the text. They should include a section (Introduction) describing the role of SR proteins in regulating gene expression.*

Answer: We have included a description about the role of SR proteins in pre-mRNA processing in the discussion.

Referee #2

1. *I suggest that the functional and visual differences between paraspeckles and nuclear speckles, and the relationship of the latter to interchromosomal granules* be explained in the Introduction.*

Answer: As per the reviewer's suggestion, we have modified the text accordingly

2. *The Northern data presented in Figure 1A, as well as previous studies, indicate that Malat1 is widely expressed in different tissues and presumably has a role that it is relatively generic in different contexts. Thus, although not commented on in the paper, it is a surprise that "non-neuronal cells in brain sections showed low or undetectable levels of Malat1 ncRNA" (Results p. 5), which shows that Malal1 is not ubiquitously expressed in the brain and begs the question of what subset(s) of cells in other tissues it may be expressed in. Moreover, since the results presented in this paper indicate that Malat1 "regulates synapse formation by modulating the expression of genes involved in synapse formation and/or maintenance" (Abstract, p. 2), the question arises as to what (presumably non-neuronal) function(s) Malat1 fulfils in other tissues where it is relatively highly) expressed, such as heart, kidney and lung (Fig. 1A). Given the reasonable hypothesis (Discussion, p. 11) that "Malat1 regulates gene expression by modulating the differential association or activity of SR-splicing factors" and the reasonable suggestion (Discussion, p. 12) that "it is likely to play broader regulatory roles by affecting different genes in a tissue specific manner", I suggest that the paper could be considerably strengthened by undertaking similar RNA FISH analyses, developmental expression profiles, and Malat1 knockdowns / overexpression / microarray interrogations in (one or two) other tissues to try to gain more insight into what types of cells or cell states express Malat1, and what types of transcripts are affected by alterations of its expression. This would not be a trivial undertaking, perhaps beyond the scope of the present paper, but would go a long way to generalizing an understanding of Malat1 function.*

Answer: While we agree with the reviewer that the understanding of Malat1 function in other cell-types and tissues would strengthen the work, we find that it is beyond the scope of the current study.

3. *In the Discussion (p. 12), reference is made to the results of alternative splicing microarrays (data not shown). Why are these data not shown?*

Answer: The microarray data showing the Malat1-mediated changes in alternative splicing of endogenous gene pre-mRNAs in HeLa cells is part of another manuscript that is presently being reviewed by another Journal (please see the attached copy of the manuscript). In that manuscript, we performed a detailed characterization of the role of Malat1 in SR protein activity.

4. *As an aside, it was an interesting experience to read and review a paper presented in landscape format - a first for me. I though at first that this may be because the figures were presented in landscape format (which would therefore avoid assembling a composite PDF file), but no, 3 of the 4 figures are in portrait layout. Intriguing.*

Answer: We apologize for this formatting error. We have now made the corrections in the new version of the manuscript.

Referee #3

1. *The authors suggested that SF2/ASF recovered ahead of Malat-1 on nuclear speckles 15 min after washing away the transcription inhibitor DRB (Fig. 2M). Does this mean that the recruitment of SF2/ASF (or SR proteins in general) to nascent RNA does not need Malat-1. This would suggest that SR protein recruitment has nothing to do with the function of Malat-1. This would further disconnect the front parts of the paper to later ones with regard to Malat-1 dependent gene expression.*

Answer: Malat1 ncRNA is a component of nuclear speckles and co-localized with several of the speckle markers including SF2/ASF. Nuclear speckles are irregularly shaped and highly dynamic subnuclear domains that are enriched with pre-mRNA splicing/processing factors (Hall et al., 2006 Anat Rec A Discov Mol Cell Evol Biol *288*, 664-675; Lamond and Spector, 2003 Nat Rev Mol Cell Biol *4*, 605-612). Nuclear speckles do not represent major sites of transcription, but are thought to be predominantly involved in the assembly, modification and/or storage of the pre-mRNA splicing machinery. It has been proposed that speckles are sites from where splicing factors are recruited to proximal, active transcription sites located elsewhere in the nucleus (Lamond and Spector, 2003 Nat Rev Mol Cell Biol *4*, 605-612; Misteli 2000 Journal of cell science *113*, 1841-1849). In the present manuscript, results described in Fig 2D-S indicates that Malat1 may not directly be involved in the relocalization of SF2/ASF to nuclear speckles. However, the Malat1 knockdown analyses in the reporter cell line indicates that Malat1 is involved in the recruitment of SF2/ASF to the transcription site.

2.1. *The authors did not fully describe the gene expression profiling experiment. Did the microarray experiment only detect gene down-regulation in response to Malat-1 depletion? I would imagine that many genes were also up-regulated.*

Answer: We have now analyzed the genes that were up-regulated upon Malat1 knock down and added the Table 2 and supplementary table 2 that describe the GO groups significantly enriched in the genes that are up-regulated by Malat1 knock down.
Supplementary table 3 shows examples of genes that are up-regulated, down-regulated or un-regulated (not effected) upon Malat1 knock down.

2.2. *Were those un-regulated genes indirectly induced? How do we know if any gene was directly affected by Malat-1?*

Answer: The DNA array experiment does not allow us to know if a gene was directly affected by Malat-1. We have now added a sentence in the discussion: "Future analysis will determine which steps of mRNA biogenesis are altered in neuronal cells that are depleted of Malat1 ncRNA and if the effect on neuronal genes is direct"

3.1 *To me, GO analysis does not mean much.*

Answer: We agree with this reviewer that the GO analysis on it's own does not mean much, However, it is supported by our primary experimental data. The results of our GO analysis are actually supported by the data of gene regulation that we have obtained in MEFs and by experimental analysis of the effect of Malat1 knockdown on synapses in primary hippocampal neurons.

3.2 *It appears that Malat-1 is quite ubiquitously expressed in many cell types and thus may affect gene expression in a general way. As a result, its down-regulation may affect many genes critical*

for the cellular function of specific cell type under investigation, in this case, the hippocampal neuron. Thus, the conclusion drawn could be misleading.

Answer: Malat1 is ubiquitously expressed, and we also hypothesized that its down-regulation may affect gene expression. Actually, both our DNA microarray analysis and our other experiments confirmed that Malat1 is involved in the regulation of general steps of gene expression. Yet, the same DNA array analysis and our experimental data also demonstrate that down regulation of Malat1 does not affect gene expression in a very general way but rather impacts specific functions of the cells (Synapse formation in the neurons and nuclear function).
In the brain, there are several examples of ubiquitously expressed proteins involved in basal cellular function whose down-regulation has cell-specific phenotypes. We have now added the following text in the discussion:

"Several examples are known of genes ubiquitously expressed, but whose altered expression specifically affect neuron-specific functions eventually leading to neurological diseases. The Survival of MotoNeuron (SMN) protein is expressed in all cells and it is involved in pre-mRNA splicing, but mutations reducing its expression lead to the selective dysfunction of motoneurons and ultimately to Spinal Muscular Atrophies (Burghes & Beattie, 2009). The Fragile X Mental Retardation Protein (FMRP) is an RNA-binding protein expressed in many tissues and involved in mRNA trafficking. The absence of FMRP leads to synaptic dysfunction and severe cognitive deficiency (Bassell & Warren, 2008). Superoxide Dismutase 1 (SOD1) is an ubiquitously expressed metalloprotein, but its mutation specifically induces the death of motoneurons and amyotrophic lateral sclerosis (Pasinelli & Brown, 2006). Therefore, understanding how Malat1 controls the expression of neuron-specific genes will provide insight into the more general question of how the alteration of the expression of a ubiquitously expressed ncRNA functions in a cell-type specific manner.

2nd Editorial Decision 16 July 2010

I have now looked through your revised manuscript in detail and your responses to the referees' comments. I find that you have satisfactorily addressed the concerns raised and I am happy to accept the manuscript for publication in The EMBO Journal. It will make a great contribution. You will receive the official acceptance letter in the next day or so.

Best wishes,

Editor,
The EMBO Journal

