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U2 snRNA is inducibly pseudouridylated at novel sites by Pus7p and snR81 RNP

Guowei Wu, Mo Xiao, Chunxing Yang and Yi-Tao Yu

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Review timeline:

Submission date:	22 September 2010
Editorial Decision:	24 September 2010
Additional Correspondence:	24 September 2010
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Additional Correspondence:	09 November 2010
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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 24 September 2010

Thank you for submitting your research manuscript (EMBOJ-2010-76058) to our editorial office. I have now had the opportunity to carefully read it and discuss it with the other members of our editorial team. Our conclusion is, I am afraid to say, that we cannot offer publication in The EMBO Journal.

Your study reports for the first time an example of RNA pseudouridylation that is not constitutive but inducible by environmental conditions. We appreciate that this is an interesting and potentially also important observation. However, we are unfortunately not convinced that this finding by itself is sufficient to justify publication in a broad general journal such as The EMBO Journal at this point. While we realize that you have elucidate some of molecular details of these inducible modifications (imperfect consensus sequences, responsible enzyme activities), major open questions such as how stresses lead to the broadening of the modification consensus remain open. The most important concern that in our view precludes consideration at this stage is however the fact that the functional significance of any of these inducible modifications remains to be established; in the absence of any sort of further insight into their importance I am afraid we must consider the study thus somewhat too preliminary for The EMBO Journal at this point, and I have therefore decided to return the manuscript to you with the message that we will not be able to consider it for publication.

Please note that we publish only a small percentage of the many manuscripts submitted to The EMBO Journal, and that as editors we can therefore subject only those to external review that are likely to obtain enthusiastic responses both from our reviewers and readers. I am sorry to disappoint you on this occasion and hope that this negative decision does not prevent you from considering our

journal for publication of other studies in the future.

Yours sincerely,

Editor
The EMBO Journal

Additional Correspondence 24 September 2010

Indeed, it's very disappointing news, but I truly believe that we have made remarkable discoveries that change current views on RNA modifications. I think the RNA modification/editing field will really appreciate our work. I understand that the functional aspect is also important, and we did actually find that the inducible pseudouridines are functionally relevant (They contribute to splicing). So if we include this piece of data, will it be OK to have it reviewed?

Additional Correspondence 24 September 2010

Thanks for your response. Adding data such as those you are mentioning might help but we would need to see them before being able to reassess whether or not full review would be warranted. But I would certainly be open to look at an amended resubmission. I wonder if you also tested some general fitness/growth effects e.g. for strains with abolished inducible pseudouridylation under/after stressed conditions?

Additional Correspondence 24 September 2010

We have looked at the effect of adding the two pseudouridines (through targeted pseudouridylation by an artificial box H/ACA RNP) on pre-mRNA splicing using a sensitive reporter system (Act1-Cup1). We found that the effect, although mild, is clear: a reduction in splicing of a mutant Act1-Cup1 was observed, even cells were in the log phase (unstressed conditions). A growth defect of the mutant on copper-medium was also observed. We did not observe the effect on wild-type pre-mRNA splicing. We reasoned that there are probably a lot more induced modifications in the spliceosomal snRNAs that are yet to be identified. A combination of all these modifications may result in a profound effect on splicing and growth. With regard to the experiment you mentioned, we did observe that deletion of the enzymes (snR81 and Pus7) resulted in mild but consistent effect on cell growth, consistent with the results and hypothesis described above.

Additional Correspondence 29 September 2010

Thank you for sending your revised/amended manuscript containing additional data as to the functional significance of induced pseudouridylation sites in yeast U2. I have now looked at this new information, and conclude that it would now be worth subjecting the study to external peer review at The EMBO Journal. As you certainly understand, we cannot predict the outcome of the reviewing process, which may turn out to be the same as our original editorial decision.

For now we will be happy to use the single complete file you have provided in your response email, and we have therefore uploaded it into our manuscript tracking system. I will be in touch again as soon as I hear back from the referees.

Best regards,

Editor
The EMBO Journal

2nd Editorial Decision 20 October 2010

Your amended manuscript has now been seen by three expert reviewers, whose comments are copied below. As you will see, all three referees feel that your discovery of inducible RNA pseudouridylation is potentially very interesting and important, and we shall in this light be happy to consider a manuscript revised along the reviewer comments further for publication. In this respect, while the majority of specific comments pertain to aspects of discussion and presentation, referee 2 also raises a number of more substantive issues (points 4-7). Given the interest of the primary observation of inducible RNA modification and the fact that decisively addressing these points might amount to a substantial extension of this work, I appreciate that this may beyond the scope of the present manuscript and would therefore not insist on these points when considering a revised version. Having said that, the manuscript may certainly be further strengthened by any additional information on these points that you may already have or be able to obtain relatively quickly, especially regarding point 7 (reversibility of inducible modifications) and possibly point 6 (other targets such as 25S rRNA, also asked by referee 1).

Please be reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer/discuss all the various points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In your revised manuscript, please also include sections containing Conflict of Interest and Author Contributions statements. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision or its timeline.

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 is a landmark study by Yu and colleagues. They demonstrate that under stress conditions U2 snRNA in yeast becomes hyper-pseudouridylated at minimally two extra sites. They document that these two sites, U56 and U93, loosely match consensus sites for pseudouridylation at position 35 (catalyzed by the protein-only pseudouridine synthase Pus7p) and U1051 of 25S rRNA (guided by snR81 and catalyzed by Cbf5p in a snoRNP complex), respectively. Using mutant enzymes and site-directed mutagenesis of U2 snRNA and/or snR81, the authors prove in an elegant series of experiments, that the reactions are indeed catalyzed by these enzymes/RNPs and that imperfect sequence matches are key to stress inducible modification at those sites. Importantly, pseudouridylation of U93 in U2 snRNA impacts splicing in vivo demonstrating the functional importance of inducible RNA modification.

These are paradigm-shifting discoveries, as RNA modification was generally assumed to be constitutive. All experiments are convincing and well controlled. In fact, the primer extensions of CMC modified U2 snRNA are simply gorgeous. The revelations are remarkable on several levels,

first, this is the first documentation of inducible RNA modification, second, the modification occurs through two completely independent mechanisms, third, yet both mechanisms depend on imperfect recognition, and fourth, this opens an entire new field of investigation and underlines the importance of RNA modification. Is RNA modification reversible? ...

I only have a couple of suggestions.

1. In Fig. 5D and E where the 3' pseudouridylation pocket of snR81 (which is responsible for the inducible modification of U93) is mutated, it would have been interesting to test if and how the modification of U1051 in 25S rRNA (whose modification is usually guided by that pocket) is affected. In particular, does that modification become also inducible with an imperfect match? Along the same line, what happens to that modification in heat shock, is it reduced, i.e., is it interdependent with that of U93 in U2 snRNA?

2. The study raises the obvious question of what happens in mammalian cells? Are the equivalent sites in U2 also modified under stress? At least, are there imperfectly matching snoRNAs for those sites?

3. Perhaps another point that could be added to the discussion in the context of mechanisms of induced pseudouridylation by Pus7p and snR81 RNP: Under nutrient deprivation, rRNA and tRNA synthesis are dramatically down regulated consequently freeing up some Pus7p and snR81 RNP which also function in modification of these RNAs (hence, addressing point 1 might be interesting).

Referee #2 (Remarks to the Author):

The manuscript titled; U2 snRNA is inducibly pseudouridylated at novel sites by Pus7p and snR81 RNP Guowei Wu, Mu Xiao, Chunxing Yang, and Yi-Tao Yu details biochemical and genetic evidence for the existence of conditionally induced pseudo-uridines at two conserved uridines in U2 snRNA. This is the first study to show evidence of such changes and suggests a novel and unexplored regulatory mechanism that is potentially very exciting and interesting. These findings establish the phenomenon and show that it can involve either protein or RNA based pseudouridylation events, however the paper is burdened by a great amount of detail without penetrating the mechanism or the function of this regulation. Admittedly it is early in the process so it may be that the description of the phenomenon is interesting enough.

Specific point:

1. The conditions for inducing the changes in the modifications are not adequately described. Generally cells per ml is better than OD and will allow researchers to duplicate the conditions..

2. Figures 3D and Figure 7 are cited in the text but are not in the merged document. There is a legend for 3D. On pg 7 there is a citation for Figure 7 that seems designed to explain the logic behind the experiments in Figure 4. The experiments in Fig 4 will be challenging to the general reader and will need a stronger introduction of the 5' and 3' pseudo-uridylation "pocket".

3. The mutations presented in Figure 5A are difficult to see, and will be more so if the figure is reduced.

4. The copper assay in Figure 6C shows a clear effect on splicing of a GAG 3'ss which is very interesting but many other mutant substrates are readily available in the ACT-CUP series and could be analyzed. In particular the lack of analysis of branchpoint mutants is confusing given the role of U2 in splicing.

5. Pertaining to this observation it is not clear why the modification would reduce recognition of the mutant 3'ss unless it somehow increases the fidelity of the second step. Second step fidelity can be tested in other ways to try to connect this modification regulation to a function.

6. An analysis of other potential sites in known RNA targets of pus 7 and snR81 is also not explored-ie. does 25S have imperfect sites that may also be modified by snR81 under altered growth

conditions? Might not modification of other targets affect splicing (or other processes).

7. Are the induced changes stable? ie. Do the heat-shock induced changes at U56 disappear (at a rate faster than simple dilution by growth? if cells are shifted back to lower temp?

Referee #3 (Remarks to the Author):

Manuscript Summary:
This work describes the inducible pseudouridylation of novel nucleotides in U2 snRNA. Novel sites U56 and U93 are pseudouridylated by Pus7p and snR81, respectively, when the yeast cell encounters stressful or limiting growth conditions. Both novel nucleotide modification sites exhibit imperfect consensus sequences for Pus7P and snR81 directed modification. Mutational analysis revealed the importance of these imperfect consensus sequences for inducible modification. Thus for the first time, it has been demonstrated that both protein-only and RNA-guided ribonucleotide modification can occur at novel nucleotides induced by stressed growth conditions.

Manuscript Comments:
These experiments are well done and convincing. The mutational analysis strongly supports the author's conclusions. Their observations are novel and have important implications for both protein-only and RNA-guided nucleotide modification. This submitted manuscript is both suitable for and worthy of publication in the EMBO Journal. Several comments are listed below.

1. There are two modifications at U93 and U94. The appearance of 94 in particular appears variable depending upon the experiment. The authors indicate in the Results that the significance of this modification will be explored in the Discussion but there really is no significant discussion of this point.

2. Is it possible to correlate the mutations made in the target site to create imperfect base pairing with the available crystal structure of the H/ACA snoRNP? Does the positioning of these mismatches in the guide snoRNA:substrate duplex suggest how these novel sites then become inducible in stress conditions?

3. The authors suggest that additional components are likely to be important for the induction process in addition to imperfect base pairing. Any suggestions as to what these may be?

1st Revision - authors' response 30 October 2010

As indicated in our response letter, we have revised our manuscript according to the editor's suggestions and reviewers' comments.

The major revisions are:

1. We have added a description of the result of pseudouridylation of 25S rRNA at position 1051. (last paragraph on Page 11).

2. We have discussed possibilities as to why Ψ93 is induced while Ψ42 and Ψ1051 are not (Page 15/16).

3. We have added a discussion about the possibility that Pus7p and snR81 RNP are freed up (due to down regulation of tRNA and rRNA, which are also natural substrates of Pus7p and snR81 RNP, respectively) under induced conditions, making them more readily available for U2 (Page 17).

We have also corrected the typos and made some minor changes suggested by the referees.

Our point-by-point response to the reviewers' comments are detailed below.

Referee #1 (Remarks to the Author):

This is a landmark study by Yu and colleagues. They demonstrate that under stress conditions U2 snRNA in yeast becomes hyper-pseudouridylated at minimally two extra sites. They document that these two sites, U56 and U93, loosely match consensus sites for pseudouridylation at position 35 (catalyzed by the protein-only pseudouridine synthase Pus7p) and U1051 of 25S rRNA (guided by snR81 and catalyzed by Cbf5p in a snoRNP complex), respectively. Using mutant enzymes and site-directed mutagenesis of U2 snRNA and/or snR81, the authors prove in an elegant series of experiments, that the reactions are indeed catalyzed by these enzymes/RNPs and that imperfect sequence matches are key to stress inducible modification at those sites. Importantly, pseudouridylation of U93 in U2 snRNA impacts splicing in vivo demonstrating the functional importance of inducible RNA modification.

These are paradigm-shifting discoveries, as RNA modification was generally assumed to be constitutive. All experiments are convincing and well controlled. In fact, the primer extensions of CMC modified U2 snRNA are simply gorgeous. The revelations are remarkable on several levels, first, this is the first documentation of inducible RNA modification, second, the modification occurs through two completely independent mechanisms, third, yet both mechanisms depend on imperfect recognition, and fourth, this opens an entire new field of investigation and underlines the importance of RNA modification. Is RNA modification reversible? ...

I only have a couple of suggestions.

1. In Fig. 5D and E where the 3' pseudouridylation pocket of snR81 (which is responsible for the inducible modification of U93) is mutated, it would have been interesting to test if and how the modification of U1051 in 25S rRNA (whose modification is usually guided by that pocket) is affected. In particular, does that modification become also inducible with an imperfect match? Along the same line, what happens to that modification in heat shock, is it reduced, i.e., is it interdependent with that of U93 in U2 snRNA?

Re: This is a great point. We have tested U1051 using the 3'-pocket-mutated snR81, but the results were negative—no pseudouridine was detected at U1051, even when cells were grown to saturation. Although it is not clear as to why this site was not pseudouridylated under induced conditions, we note two major defferences between U2 and 25S rRNA. First, while U2 is transcribed by Pol II, 25S rRNA is transcribed by Pol I, and second, U2 is localized to Cajal bodies and the nucleoplasm, whereas 25S rRNA resides in the nucleoli. Because of these differences, it is possible that U1051 and U93 respond to stresses differently. A detailed analysis is necessary to address this problem. The result and discussion have now been incorporated into the revision. However, because of the negative result of induced formation of Y1051, we did not show the data, but we are willing to show it if necessary.

Under saturation or heat-shock, pseudouridylation at U1051 does not appear to be reduced in wild-type strain (wild-type snR81). It appears to be independent of U93 of U2.

2. The study raises the obvious question of what happens in mammalian cells? Are the equivalent sites in U2 also modified under stress? At least, are there imperfectly matching snoRNAs for those sites?

Re: Great questions. We are planning on addressing these questions in the future.

3. Perhaps another point that could be added to the discussion in the context of mechanisms of induced pseudouridylation by Pus7p and snR81 RNP: Under nutrient deprivation, rRNA and tRNA synthesis are dramatically down regulated consequently freeing up some Pus7p and snR81 RNP which also function in modification of these RNAs (hence, addressing point 1 might be interesting).

Re: Great comment. We have taken the reviewer's advice, and have added this point to Discussion. Even though Y93 of U2 is independent of Y1051 of 25S rRNA, I think this comment is still valid. We have discussed this possibility in the revision.

Referee #2 (Remarks to the Author):

The manuscript titled; U2 snRNA is inducibly pseudouridylated at novel sites by Pus7p and snR81 RNP Guowei Wu, Mu Xiao, Chunxing Yang, and Yi-Tao Yu details biochemical and genetic evidence for the existence of conditionally induced pseudo-uridines at two conserved uridines in U2 snRNA. This is the first study to show evidence of such changes and suggests a novel and unexplored regulatory mechanism that is potentially very exciting and interesting. These findings establish the phenomenon and show that it can involve either protein or RNA based pseudouridylation events, however the paper is burdened by a great amount of detail without penetrating the mechanism or the function of this regulation. Admittedly it is early in the process so it may be that the description of the phenomenon is interesting enough.

Specific point:

1. The conditions for inducing the changes in the modifications are not adequately described. Generally cells per ml is better than OD and will allow researchers to duplicate the conditions..

Re: This is now fixed in the revision (Materials and methods).

2. Figures 3D and Figure 7 are cited in the text but are not in the merged document. There is a legend for 3D. On pg 7 there is a citation for Figure 7 that seems designed to explain the logic behind the experiments in Figure 4. The experiments in Fig 4 will be challenging to the general reader and will need a stronger introduction of the 5' and 3' pseudo-uridylation "pocket".

Re: Thanks. These have now been fixed.

3. The mutations presented in Figure 5A are difficult to see, and will be more so if the figure is reduced.

Re: We have changed the font size for these mutated nucleotides (Figs. 5A and 5D), and they should be fine now.

4. The copper assay in Figure 6C shows a clear effect on splicing of a GAG 3'ss which is very interesting but many other mutant substrates are readily available in the ACT-CUP series and could be analyzed. In particular the lack of analysis of branchpoint mutants is confusing given the role of U2 in splicing.

Re: We have tested several of these mutants, and only the gAG 3'SS mutant (the second-step pre-mRNA mutant) showed a clear phenotype as shown. At this point, we don't know the exact mechanism behind it. However, given that Y93 is located far away from the branch site recognition region (in U2), we think that it was not completely unexpected that we did not see an obvious phenotype for the branch site mutants (pre-mRNAs).

5. Pertaining to this observation it is not clear why the modification would reduce recognition of the mutant 3'ss unless it somehow increases the fidelity of the second step. Second step fidelity can be tested in other ways to try to connect this modification regulation to a function.

Re: Great point. We don't know the exact mechanism, but agree with the reviewer's comments about the possibilities. We plan to address this problem in the future.

6. An analysis of other potential sites in known RNA targets of pus 7 and snR81 is also not explored- ie. does 25S have imperfect sites that may also be modified by snR81 under altered growth conditions? Might not modification of other targets affect splicing (or other processes).

Re: We have tested U1051 using the 3'-pocket-mutated snR81, but did not observe induced modification. We have provided a couple of explanations (see our response to Point 1 of reviewer #1). It is likely that there are some naturally occurring inducible sites in 25S rRNA (and possibly other RNAs as well) similar to those of U56 and U93 in U2, and induced modification at these sites may be functionally relevant. Our future experiments will be directed towards addressing this issue.

7. Are the induced changes stable? ie. Do the heat-shock induced changes at U56 disappear (at a rate faster than simple dilution by growth? if cells are shifted back to lower temp?

Re: Great questions. We have done some preliminary experiments with the intent of addressing this important issue. Specifically, we grew cells to saturation, and then transferred cells to fresh medium containing thiolutin (to block the synthesis of new U2 RNA). From this point on, we examined the Y93 level. While our results suggested a moderate reduction of Y93 within 2 hours, the experiments did not allow us to check U2 at longer time points due to poor growth and low quality of RNA (RNA degrades at a substantial level after two hours). Although it's quite possible that Y93 is reversible, we don't feel comfortable to make any conclusion at this point. This issue will be carefully addressed in the future.

Referee #3 (Remarks to the Author):

Manuscript Summary:

This work describes the inducible pseudouridylation of novel nucleotides in U2 snRNA. Novel sites U56 and U93 are pseudouridylated by Pus7p and snR81, respectively, when the yeast cell encounters stressful or limiting growth conditions. Both novel nucleotide modification sites exhibit imperfect consensus sequences for Pus7P and snR81 directed modification. Mutational analysis revealed the importance of these imperfect consensus sequences for inducible modification. Thus for the first time, it has been demonstrated that both protein-only and RNA-guided ribonucleotide modification can occur at novel nucleotides induced by stressed growth conditions.

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1. There are two modifications at U93 and U94. The appearance of 94 in particular appears variable depending upon the experiment. The authors indicate in the Results that the significance of this modification will be explored in the Discussion but there really is no significant discussion of this point.

Re: Good point. Indeed, the appearance of Y94 varies from time to time. However, because there is no good explanation for it at this point, we do not discuss it further in Discussion. As such, we deleted "(see Discussion)" from the sentence on page 7.

2. Is it possible to correlate the mutations made in the target site to create imperfect base pairing with the available crystal structure of the H/ACA snoRNP? Does the positioning of these mismatches in the guide snoRNA:substrate duplex suggest how these novel sites then become inducible in stress conditions?

Re: We think it is possible. However, because we don't have much expertise in crystal structure and modeling, it may take a substantial amount of time for us to do this. We will, however, try to collaborate with a crystallographer in the future, focusing on the structural aspect of inducible modification.

3. The authors suggest that additional components are likely to be important for the induction process in addition to imperfect base pairing. Any suggestions as to what these may be?

Re: It could be some specific factor(s) (e.g., a chaperone) associated with the specific pseudouridylation pocket (e.g., the 3' pocket of snR81) or present in specific nuclear subcompartments (Cajal bodies, nucleoplasm, etc). The discussion is now expanded in the revision.

Additional Correspondence 09 November 2010

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by one of the original reviewers, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal. Before we will able to send you a formal letter of acceptance, I only I need to remind you that we require an explicit 'author contribution' statement, to be included at the end of the manuscript text. To simplify things, you could simply send this to us in the body of an email, we can easily copy such a passage into the manuscript text file; alternatively you can send as a new text document including this statement.

Furthermore, in case you haven't already done so, please also send us the signed License To Publish.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,

Editor
The EMBO Journal

