THE EMBO JOURNAL

Manuscript EMBO-2012-81286

Threonine-4 of mammalian RNA polymerase II CTD is targeted by Polo-like kinase-3 and required for transcriptional elongation

Corinna Hintermair, Martin Heidemann, Frederic Koch, Nicolas Descostes, Marta Gut, Ivo Gut, Romain Fenouil, Pierre Ferrier, Andrew Flatley, Elisabeth Kremmer, Rob D. Chapman, Jean-Christophe Andrau and Dirk Eick

Corresponding author: Dirk Eick, Helmholtz Center for Environmental Health

Review timeline:

Submission date:	05 March 2012
Editorial Decision:	14 March 2012
Revision received:	10 April 2012
Editorial Decision:	11 April 2012

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 14 March 2012

We have now received feedback from two expert reviewers that have evaluated your study on Thr4 phosphorylation in the Pol II CTD. I am pleased to inform you that both of them are generally very positive about this work, and we shall therefore be happy to consider the manuscript further for publication following revision of a few specific issues raised by the reviewers. In this respect, I feel that especially the two experiments suggested in referee 1's point 1 and referee 2's point 2 should at least be attempted in order to strengthen the understanding of Thr4 phosphorylation dependencies; whereas some other suggestions (e.g. ref 2 points 1 and 4) should at least be discussed in your response letter and revised manuscript. Related to that, please consider both referees' other presentational issues, including those on down-toning some interpretations and streamlining the introduction. Finally, for production purposes I would also ask you to amend the manuscript with brief 'Author Contribution' and 'Conflict of Interest' statements, and to submit the revised main article as a word document with separate image files for each of the main figures (whereas the supplement should remain in one single combined file, as is already the case).

I am therefore returning the manuscript to you for these revisions, hoping you will be able to return a revised manuscript as early as possible in order to facilitate timely publication. When preparing your letter of response, please be reminded that it will be necessary to diligently and comprehensively answer to all the points raised, and also bear in mind that this letter will form part of the Peer Review Process File available online to our readers 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).

Should you have any further questions regarding your revision, please do not hesitate to get back to

me directly. I am looking forward to receiving your revised manuscript!

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The paper by Hintermair et al describes the existence, importance, and effect on transcription of Threonine 4 CTD phosphorylation. It also identifies Polo-like kinase 3 as the likely responsible kinase. I was very impressed by this study, which is thorough, well conceived, and nicely carried out. The data are of very high quality, and the conclusions supported by the evidence. The story is also well presented. In my opinion, the paper can be published more or less as it is, but I have a few minor suggestions that should at least be considered by the authors.

1. The authors raise the possibility that Thr-4 phosphorylation is somehow coupled to Ser-2 phosphorylation (all Thr-4 phosphorylated CTDs are also Ser2-phosphorylated, for example). If so, it would nicely explain the apparent disagreement with the previous chicken study. It might be worth testing whether Thr-4 phosphorylation can be detected when Ser2 is mutated to alanine. If not, it would strongly support the idea that Ser2 phosphorylation is a requirement for Thr-4 phosphorylation (a peptide containing Ala2 and Thr4-p would have to be produced to make sure one of the Thr4-p specific antibodies can actually recognize Thr-4 phosphorylation if Ser2 has been mutated to alanine, of course).

2. I think the introduction is unnecessarily long (presently runs to 4 full pages). The reader does not need to know the finer details of phosphorylation at other CTD residues. It can be made much shorter and snappier to simply introduce the importance of understanding CTD modification, and in particular that Thr-4 phosphorylation is poorly investigated.

3. Does Flavopiridol inhibit Thr4 phosphorylation by Plk3 in vitro?

4. P. 12 line 4: "....either of both..". I suppose you mean "either OR both"?

5. Same page, line 18: replace "must not imply" with "need not imply"

6. Same page, last line: replace "tamper" with "affect".

7. The discussion can also be shortened significantly without affecting the message of the paper.

Referee #2

This manuscript provides evidence that phosphorylation of the RNA Pol II CTD at the Thr4 position is essential in mammalian cells, supports normal transcript elongation and depends, in part, on Polo-like kinase 3 (Plk3). Using an elegant system in which endogenous Pol II is inhibited by α-amanitin (and subsequently degraded), the authors show that an amanitin-resistant Rpb1 variant containing Ala substituted for Thr4 in all heptad repeats is incapable of supporting viability. They raise an antibody to CTD phosphorylated at Thr4 and show that this modification is highly conserved in eukaryotes, closely associated (and perhaps mechanistically coupled) with Ser2 phosphorylation, and concentrated towards the 3' ends of protein-coding genes. The T4A mutation disrupts normal Pol II distribution on a genome-wide basis (measured by ChIP-seq), in a manner they interpret as being consistent with a block to transcript elongation. Finally, they identify Plk3 as a Thr4 kinase in vitro, and show evidence that depletion of Plk3 diminishes Thr4 phosphorylation in vivo, whereas oxidative stress-known to induce Plk3 activity-causes increased Thr4 phosphorylation.

This is not the first report that Thr4 phosphorylation occurs-and appears to be essential-in vertebrate cells. A previous paper by Manley and co-workers suggested a specific role for this modification in 3'-end formation of histone mRNAs (Hsin et al., 2011). That paper implicated a different kinase, Cdk9, in Thr4 phosphorylation in vivo, but the evidence was circumstantial and did not include a direct demonstration that Thr4 was a Cdk9 target in vitro. The present study provides a more comprehensive analysis of Thr4 phosphorylation in vivo, and a comparison of Cdk9 and Plk3 activity towards Thr4 of the CTD in vitro. Additional experiments could be performed to resolve some of the discrepancies with Hsin et al., but overall this is a well-executed and reasonably interpreted study, which makes an important contribution to the understanding of transcriptional regulation by CTD phosphorylation, and will be of significant interest to scientists both in and out of the field.

My specific comments and concerns are as follows:

1. The initial description on page 8 of epitope-masking/ blocking effects detected in Fig. 2, is confusing. From this wording, I understood that the anti-phosphoThr4 antibody had trouble recognizing its epitope when both flanking sites, Ser2 and Ser5, were also phosphorylated. (Consulting Fig. 2 itself makes it clearer that either Ser2 or Ser5 modification alone can interfere with Thr4-P recognition.) When this issue is revisited in the Discussion on p. 15, they describe the data more accurately. The distinction is important, because it affects interpretation of the results of Cdk9 phosphorylating the CTD in vitro (Fig. 4A). Cdk9 can phosphorylate both Ser2 and Ser5 and might also phosphorylate Thr4 (consistent with flavopiridol-sensitivity reported by Hsin et al.), but the antibody might not be able to detect that modification. If Plk3 is more restricted in its specificity, it might only "look" like the better Thr4 kinase in vitro. The authors should at least discuss this possibility (and clarify the "and"/"or" confusion cited above.)

2. The effect of Plk3 knockdown on the Thr4-P signal is modest at best (Fig. 4B). (To my eye it does not look significantly greater than the apparent reduction in total Pol II.) Possible explanations mentioned in the text include incomplete knockdown or additional kinases contributing to the signal in vivo. To bolster the connection between Plk3 and Thr4-P they show that both increase upon oxidative stress (Fig. 4C). It seems to me this would provide an excellent system in which to test the dependence of Thr4-P on Plk3 more rigorously, i.e., by asking whether the increase caused by H2O2 is dampened by Plk3 knockdown.

3. The mapping of Thr4-P marks and analysis of the effects of the T4A mutation on Pol II dynamics on chromatin, by ChIP-seq, provide important information. I would avoid, however, some of the more dynamic language used to describe these, essentially static measurements. I would be hesitant, for example, to conclude that lack of Thr4-P "blocks elongation" or that presence of Thr4-P might "prevent initiation" at some genes. On the two genes used to suggest that Thr4-P might be playing such a repressive role, in Fig. 6E, the Pol II distributions look very different to me: the ATF3 data do suggest transcriptional induction, perhaps at the level of initiation; whereas DUSP1 looks to me more like a case of a paused Pol II that leaks into the body of the gene in the mutant. The authors are very conscientious, in the Discussion, in describing the pitfalls of over-reliance on phospho-specific antibodies, and on substitution mutants that might affect protein structure and function independent of post-translational modification. They should be similarly cautious about their genome-wide protein localization data.

4. The authors make the important point that substrate recognition by PLKs is often dependent on a phosphorylated Thr followed by a Pro (p. 19). Another feature of PLK function is that the most frequent "priming" kinases are CDKs, raising the obvious question of whether any of the CDKs known to phosphorylate the CTD (7, 8, 9, 12, 13) might stimulate subsequent phosphorylation by Plk3. (Priming by one CDK for another has been described within the context of the CTD.) In addition to the mechanistic insight this might provide, it could also explain why Thr4-P is flavopiridol-sensitive in vivo, even though Cdk9 and other flavopiridol-sensitive kinases do not appear to phosphorylate Thr4 in vitro.

List of responses to reviewer concerns
EMBO Journal Manuscript number 2012-81286
Hintermair et al., Threonine-4 of Mammalian RNA Polymerase II CTD is Targeted by Polo-like Kinase-3 and Required for Transcriptional Elongation

Responses are in italics

Editor
We have now received feedback from two expert reviewers that have evaluated your study on Thr4 phosphorylation in the Pol II CTD. I am pleased to inform you that both of them are generally very positive about this work, and we shall therefore be happy to consider the manuscript further for publication following revision of a few specific issues raised by the reviewers. In this respect, I feel that especially the two experiments suggested in referee 1's point 1 and referee 2's point 2 should at least be attempted in order to strengthen the understanding of Thr4 phosphorylation dependencies

Referee 1's point 1 we could experimentally address and the results will further strengthen the manuscript.

Referee 2's point 2 could be addressed partially. But we can now explain (including new data) the discrepancy of Cdk9 requirement for Thr4 phosphorylation observed by our and Jim Manley's lab.

'Contribution' and 'Conflict of Interest' statements

Is included in the revised version

Submit the revised main article as a word document with separate image files for each of the main figures (whereas the supplement should remain in one single combined file, as is already the case).

Revised version is submitted as a word document with separate image files, supplement as single PDF).

The word file of the manuscript text is 51,439 characters with spaces (excluding references, tables and supplementary material).

The transferral of copyright and page charge authorization and offprint form is added as signed PDF.

Referee #1

The paper by Hintermair et al describes the existence, importance, and effect on transcription of Threonine 4 CTD phosphorylation. It also identifies Polo-like kinase 3 as the likely responsible kinase. I was very impressed by this study, which is thorough, well conceived, and nicely carried out. The data are of very high quality, and the conclusions supported by the evidence. The story is also well presented. In my opinion, the paper can be published more or less as it is, but I have a few minor suggestions that should at least be considered by the authors.

We thank the reviewer for the kind words and the very constructive suggestions.

1.The authors raise the possibility that Thr-4 phosphorylation is somehow coupled to Ser-2 phosphorylation (all Thr-4 phosphorylated CTDs are also Ser2-phosphorylated, for example). If so, it would nicely explain the apparent disagreement with the previous chicken study. It might be worth testing whether Thr-4 phosphorylation can be detected when Ser2 is mutated to alanine. If not, it would strongly support the idea that Ser2 phosphorylation is a requirement for Thr-4 phosphorylation (a peptide containing Ala2 and Thr4-p would have to be produced to make sure one

of the Thr4-p specific antibodies can actually recognize Thr-4 phosphorylation if Ser2 has been mutated to alanine, of course).

We produced the recommended peptide (Ser2 mutated to alanine, Thr4-P). The Thr4-P specific mAb 6D7 recognize this peptide with similar specificity as the Thr4-P only peptide (see Figure S9), indicating that Ser2 is not required for peptide recognition by 6D7. The lack of recognition of mutant CTD ser2/ala by mAb 6D7 is therefore based on the missing Thr4 phosphorylation. This suggests that Ser2 phosphorylation (albeit not in the same repeat) is a prerequisite for Thr4 phosphorylation. This results fits with signal profiles for Ser2-P and Thr4-P in the 3' region of genes, but would also explain the sensitivity of Thr4-P to flavopiridol, an inhibitor of Ser2 phophorylation in vivo. The results also support the priming mechanism of Ser2-P for Thr4 phosphorylation suggested by Referee #2, point 4.

2. I think the introduction is unnecessarily long (presently runs to 4 full pages). The reader does not need to know the finer details of phosphorylation at other CTD residues. It can be made much shorter and snappier to simply introduce the importance of understanding CTD modification, and in particular that Thr-4 phosphorylation is poorly investigated.

We shortened the introduction and abridged the text from 4 to roughly 3.3 pages.

3. Does Flavopiridol inhibit Thr4 phosphorylation by Plk3 in vitro?

Flavopiridol efficiently inhibits Cdk9 at 1 μM in vitro, but has no inhibitory effect on Plk3. This new information is given in Figure S4,B.

4. P. 12 line 4: "....either of both..". I suppose you mean "either OR both"?

Corrected

5. Same page, line 18: replace "must not imply" with "need not imply"

Corrected

6. Same page, last line: replace "tamper" with "affect".

Corrected

7. The discussion can also be shortened significantly without affecting the message of the paper.

We have also shortened the discussion and omitted the first paragraph (half page) without loosing information.

Referee #2

This manuscript provides evidence that phosphorylation of the RNA Pol II CTD at the Thr4 position is essential in mammalian cells, supports normal transcript elongation and depends, in part, on Polo-like kinase 3 (Plk3). Using an elegant system in which endogenous Pol II is inhibited by alpha-amanitin (and subsequently degraded), the authors show that an amanitin-resistant Rpb1 variant containing Ala substituted for Thr4 in all heptad repeats is incapable of supporting viability. They raise an antibody to CTD phosphorylated at Thr4 and show that this modification is highly conserved in eukaryotes, closely associated (and perhaps mechanistically coupled) with Ser2 phosphorylation, and concentrated towards the 3' ends of protein-coding genes. The T4A mutation disrupts normal Pol II distribution on a genome-wide basis (measured by ChIP-seq), in a manner they interpret as being consistent with a block to transcript elongation. Finally, they identify Plk3 as a Thr4 kinase in vitro, and
show evidence that depletion of Plk3 diminishes Thr4 phosphorylation in vivo, whereas oxidative

stress-known to induce Plk3 activity-causes increased Thr4 phosphorylation.

This is not the first report that Thr4 phosphorylation occurs-and appears to be essential-in vertebrate cells. A previous paper by Manley and co-workers suggested a specific role for this modification in 3'-end formation of histone mRNAs (Hsin et al., 2011). That paper implicated a different kinase, Cdk9, in Thr4 phosphorylation in vivo, but the evidence was circumstantial and did not include a direct demonstration that Thr4 was a Cdk9 target in vitro. The present study provides a more comprehensive analysis of Thr4 phosphorylation in vivo, and a comparison of Cdk9 and Plk3 activity towards Thr4 of the CTD in vitro. Additional experiments could be performed to resolve some of the discrepancies with Hsin et al., but overall this is a well-executed and reasonably interpreted study, which makes an important contribution to the understanding of transcriptional regulation by CTD phosphorylation, and will be of significant interest to scientists both in and out of the field.

We thank the reviewer for the kind words and the very constructive suggestions.

My specific comments and concerns are as follows:

1. The initial description on page 8 of epitope-masking/ blocking effects detected in Fig. 2, is confusing. From this wording, I understood that the anti-phosphoThr4 antibody had trouble recognizing its epitope when both flanking sites, Ser2 and Ser5, were also phosphorylated. (Consulting Fig. 2 itself makes it clearer that either Ser2 or Ser5 modification alone can interfere with Thr4-P recognition.) When this issue is revisited in the Discussion on p. 15, they describe the data more accurately. The distinction is important, because it affects interpretation of the results of Cdk9 phosphorylating the CTD in vitro (Fig. 4A). Cdk9 can phosphorylate both Ser2 and Ser5 and might also phosphorylate Thr4 (consistent with flavopiridol-sensitivity reported by Hsin et al.), but the antibody might not be able to detect that modification. If Plk3 is more restricted in its specificity, it might only "look" like the better Thr4 kinase in vitro. The authors should at least discuss this possibility (and clarify the "and"/"or" confusion cited above.)

We apologize for the confusion and replaced "ser2 and Ser5" by "Ser2 or Ser5".

We agree with Referee #2 that the antibody cannot detect phosphorylation of Thr4 by Cdk9, if this kinase phosphorylates also Ser2 or Ser5 in the same repeat. We discuss this possibility in the Result section.
For the sensitivity of Thr4 phosphorylation to flavopiridol see also next point and the response to point 1 and 3 of Referee #1.

2. The effect of Plk3 knockdown on the Thr4-P signal is modest at best (Fig. 4B). (To my eye it does not look significantly greater than the apparent reduction in total Pol II.) Possible explanations mentioned in the text include incomplete knockdown or additional kinases contributing to the signal in vivo. To bolster the connection between Plk3 and Thr4-P they show that both increase upon oxidative stress (Fig. 4C). It seems to me this would provide an excellent system in which to test the dependence of Thr4-P on Plk3 more rigorously, i.e., by asking whether the increase caused by H2O2 is dampened by Plk3 knockdown.

We agree with Referee #2 that knockdown and induction experiments for Plk3 do not result in a clear picture whether Plk3 is the major and only kinase of Thr4. The proposed experiment of Referee #2 (treatment of cells with H_2O_2 after knockdown of Plk3) would probably also not lead to clear results, because the knockdown of Plk3 is always incomplete in our hands and it is unclear, whether the remaining Plk3 activity can substantially contribute to Thr4 phosphorylation. In addition, it is unclear to which extent the knockdown of Plk3 would interfere with induction of Plk3 by H_2O_2.
So we tried to get Plk3 knockout cells published several years before, but this was not successful yet. Knockout cells will answer the question, to which extent Plk3 contributes to Thr4 phosphorylation, and could also help to clarify the contribution of other cellular kinases. Since it is not in our hands, whether we can perform the experiments with Plk3 knockout cells in the very next future, I hope you agree that these experiments are beyond the experiments of the current manuscript.

Nevertheless, as asked by Referee #2 above: "Additional experiments could be performed to resolve some of the discrepancies with Hsin et al., …" *we were able to answer some of the concerns of*

Referee #2 regarding the sensitivity of Plk3 to flavopiridol and the function of Cdks of priming Thr4 phosphorylation (see Referee #1, points 1 and 3) (and new Figures S4B and S9).

3. The mapping of Thr4-P marks and analysis of the effects of the T4A mutation on Pol II dynamics on chromatin, by ChIP-seq, provide important information. I would avoid, however, some of the more dynamic language used to describe these, essentially static measurements. I would be hesitant, for example, to conclude that lack of Thr4-P "blocks elongation" or that presence of Thr4-P might "prevent initiation" at some genes.

We agree with Referee #2 to avoid a to dynamic language for the description of ChIP results. We rephrased "blocks elongation" *and* "prevent initation" *or used the subjunctive.*

On the two genes used to suggest that Thr4-P might be playing such a repressive role, in Fig. 6E, the Pol II distributions look very different to me: the ATF3 data do suggest transcriptional induction, perhaps at the level of initiation; whereas DUSP1 looks to me more like a case of a paused Pol II that leaks into the body of the gene in the mutant. The authors are very conscientious, in the Discussion, in describing the pitfalls of over-reliance on phospho-specific antibodies, and on substitution mutants that might affect protein structure and function independent of post-translational modification. They should be similarly cautious about their genome-wide protein localization data.

We agree with Referee #2 that the signal pattern of Pol II particular on the DUSP1 can be interpreted in different directions (increased initiation versus pausing). Therefore we omitted DUSP1 in Figure 6.

4. The authors make the important point that substrate recognition by PLKs is often dependent on a phosphorylated Thr followed by a Pro (p. 19). Another feature of PLK function is that the most frequent "priming" kinases are CDKs, raising the obvious question of whether any of the CDKs known to phosphorylate the CTD (7, 8, 9, 12, 13) might stimulate subsequent phosphorylation by Plk3. (Priming by one CDK for another has been described within the context of the CTD.) In addition to the mechanistic insight this might provide, it could also explain why Thr4-P is flavopiridol-sensitive in vivo, even though Cdk9 and other flavopiridol-sensitive kinases do not appear to phosphorylate Thr4 in vitro.

We really thank Referee #2 (and also Referee #1) for these suggestions. The new data added in Suppl. Figures S4B and S9 in fact indicate that phosphorylation of Ser2 appears to be a prerequisite for Thr4 phosphorylation. This observation also explains the flavopiridol sensitivity of Thr4 phosphorylation published by Hsin et al.
So, we follow Referee #2 and discuss Ser2-P as a priming mark for Thr4 phosphorylation.

We once gain want to thank both referees for the extremely rapid review process and for their very constructive comments.

2nd Editorial Decision 11 April 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees, 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, there I only need to ask you for one minor thing: we still require a brief but explicit 'author contribution' statement, to be included at the end of the manuscript text (after the acknowledgements). To expedite this, you may simply send this to us in the body of an email, from which we can easily copy it into the manuscript text file; alternatively

you may send as a new text document including this statement.

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

Yours sincerely,

Editor
The EMBO Journal

