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O-GlcNAcylation of TAB1 modulates TAK1-mediated cytokine release

Shalini Pathak, Vladimir S. Borodkin, Osama Albarbarawi, David G. Campbell, Adel Ibrahim and Daan M. F. van Aalten

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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 09 December 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while all three referees consider the study as interesting in principle and while referee 2 is more positive it becomes clear from their reports that the depth of analysis not sufficient to support publication of the study here at this stage of analysis. I will not repeat all their individual points of criticism here, but the major issues put forward are that the physiological as well as the functional significance of TAB1 O-GlcNAcylation is not analysed to a sufficient level. Referees 1 and 3 make it very clear to the editor that they are not in favour of publication of the study here at this stage of analysis. Clearly, an extensive amount of further experimentation which includes crucial controls would be required to address these issues. Furthermore, the outcome of such experiments cannot be predicted at this point. In such a situation, I am sorry to say that we cannot consider (and thus commit to) a revision and I therefore see little choice but to come to the conclusion that we cannot offer to publish the manuscript at this point.

Still, given the interest expressed by the referees in principle we would not exclude the possibility to consider a new submission on the same topic should future studies allow you to strengthen the study considerably along the lines suggested by the reviewers and to analyse the functional and physiological significance of your findings in more depth. To be completely clear, however, I would like to stress that if you wish to send a new manuscript this will be treated as a new submission rather than a revision and will be evaluated again at the editorial level and reviewed afresh, also with

respect to the literature and the novelty of your findings at the time of resubmission. We will involve our original referees again if available at the time of resubmission. At this stage of analysis, though, I am sorry to have to disappoint you.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1

In this manuscript, the authors identified that TAK1 binding protein TAB1 is O-GlucNAcylated on Ser395, and that O-GlucNAcylation of TAB1 is required for full TAK1 activation. I think that the authors' analyses are basically interesting, but feel that several problems are not to be elucidated.

The major problem is that the authors only analyzed the effect of O-GlucNAcylation of TAB1 using mutant TAB1. Therefore, it is still unclear whether the effect of this mutant is caused by inhibition of O-GlucNAcylation, or by amino acid substitution-induced conformational change. To analyze this, I think that the authors should analyze the effect of O-GlucNAcylation itself in TAK1 activation. For example, the effect of IL-1 stimulation should be analyzed in low and high glucose containing medium. It is also required to analyze the same effect in OGT knockdown cells using RNAi.

My specific comments are as follows:

1) In Figure 2A, TAK1 activity was clearly suppressed in mutant TAB1 expressing cells. In Figure 2C, activating phosphorylation of TAK1 by NaCl was also suppressed. However, activating phosphorylation of TAK1 by IL-1 was not so suppressed (Figure 2B) compared with Figure 2C. The authors should explain this discrepancy.

2) In Figure 2A, I cannot distinguish legends of graph showing WT and S395A.

3) Page 13 line12, O-GlucNAcylation of TAB1 is "not" essential for NF-κB activation and IL-1α stimulated cytokine release.

Referee #2

In the manuscript entitled "O-GlcNAcylation of TAB1 modulates TAK1-mediated cytokine release" the authors demonstrated that Ser-395 of TAB-1 is a site for the O-GlcNAc modification. Using site-directed mutagenesis, the authors also provide data suggesting that O-GlcNAcylation of TAB1 at Ser-395 helps modulate TAK-1 mediated cytokine release upon stimulation. This work is interesting and important and is appropriate for publication in EMBO Journal, once a couple of concerns/questions are addressed.

Concerns.

1) The authors should provide at least one blot showing a competition with free GlcNAc to show that the O-GlcNAc signal is indeed specific to go along with figure 1. Figure 1C, the author should have a negative IP control with a non-specific antibody.

2) In figure 2A, could the authors include the negative control without transfection instead of stating in the text that it was in agreement with earlier studies (Mendoza et al, 2008)

3) Is TAK1 itself O-GlcNAcylated? Specifically, did the authors investigate TAK1 GlcNAcylation state in response to IL-1 stimulation and/or osmotic stress? Specific O-GlcNAcylation of TAK1 could be involved in TAK1 mediated cytokine release.

4) The authors should look at the dynamics of TAB1 GlcNAcylation in response to IL-1 stimulation and osmotic stress during the time points they use. If the authors can show that GlcNAcylation of TAB1 changes during IL-1 stimulation or osmotic stress, this would further solidify their results in that GlcNAcylation of TAB1 is involved during TAK1 mediated cytokine release.

5) In figure 4, the authors show that GlcNAcstatin pS438 TAB1 phosphorylation and suggest that is due to the O-GlcNAc at S395. However, that experiment does not address that. Elevated O-GlcNAc might be altering the kinase or phosphatase activity at S438. The authors should show that the S395A mutant does the same thing as the GlcNAcstatin. This experiment has to be done.

Also of interest, the authors do not directly address how O-GlcNAcylation of TAB1 alters the phosphorylation on TAK1. Does the mutant bind differently to TAK1, does it alter it's cytolocalization? These questions should be addressed.

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Minor Comments

5) In figure 2A, the authors seems to have incorrectly labeled the figure. Instead of using dashed lines and symbols, the authors show consider using only 4 different symbols (circles, squares, diamonds, triangles).

6) Under the "O-GlcNAcylation of TAB1 modulates regulatory phosphorylation" section the first line states that TAB1 can be phosphorylated at T432. I believe the authors really mean T431 as indicated 2 lines later.

7) The graph in Fig 2A is hard to read. The authors might want to change the symbols to make them clearer. Also, Fig. 2F the X-axis is labeled 15 min 10 min 15 min. Is that correct?

Referee #3

In this manuscript, the authors demonstrate that TAK1-binding protein 1 (TAB1) is O-GlcNAcylated at Ser-395 and that this modification may be important for TAK1 activation. The results are potentially interesting. However, there are a number of concerns and weaknesses. Major concerns are; 1) there is no data showing that TAB1 is O-GlcNAcylated under physiological settings; 2) significance of TAB1 O-GlcNAcylation is not convincing due to inappropriate experimental designs and lack of controls; 3) the mechanism by which O-GlcNAcylation of TAB1 regulates TAK1 activity is not clear. Overall, this study is too preliminary.

Specific comments:

1) Why was IkBa not degraded upon IL-1 stimulation in Fig. 2E. As the authors describe in the text, IkB is degraded upon IKK-mediated phosphorylation. Therefore, analyzing the ratio of phosphrylated IkB vs. total IkB is not appropriate to assess the level of IKK activation.

2) In all experiments using TAB1-/- MEFs, TAB1+/+ and TAB1-/- MEFs transfected with empty vector should be included. Are the exogenously expressed TAB1 levels similar to the endogenous TAB1 levels?

3) It is important to determine the exact activity of TAB1 wild type and S395A for TAK1 activation. Dose-response curves should be determined.

4) Is TAB1 constitutively O-GlcNAcylated or in an IL-1 and osmotic stress-dependent manner?

5) Immunoblot analysis of TAB1 O-GlcNAcylation should be performed in Fig. 2B, 2C and Fig. 4.

6) The authors should test whether the S395A mutation affects the ability to associate with TAK1.

7) It is not clear whether increased phosphorylation of TAB1 at S438 is causally associated with reduced TAK1 activity. The additional experiments are needed.

8) JNK activation should also be examined.

Resubmission 21 August 2011

RESPONSE TO REFEREES

Referee #1

" To analyze this, I think that the authors should analyze the effect of O-GlucNAcylation itself in TAK1 activation. For example, the effect of IL-1 stimulation should be analyzed in low and high glucose containing medium. It is also required to analyze the same effect in OGT knockdown cells using RNAi."

The revised manuscript now contains a new section of R&D under the heading "*O-GlcNAcylation of TAB1 is inducible*". Here we show that we have generated an *O*-GlcNAc site-specific antibody, and use that to show that TAB1 *O*-GlcNAcylation can be induced by known activators of the TAK1 pathway (IL1/NaCl) and also by high glucose levels, or with a specific *O*-GlcNAcase inhibitor.

"1) In Figure 2A, TAK1 activity was clearly suppressed in mutant TAB1 expressing cells. In Figure 2C, activating phosphorylation of TAK1 by NaCl was also suppressed. However, activating phosphorylation of TAK1 by IL-1 was not so suppressed (Figure 2B) compared with Figure 2C. The authors should explain this discrepancy."

We have redone these experiments and improved the figures as per suggestions of the other referees. A similar reduction in TAK1 autophosphorylation for the TAB1 *O*-GlcNAc deficient mutant is observed in response to IL1/NaCl.

"2) In Figure 2A, I cannot distinguish legends of graph showing WT and S395A."

The layout of this figure has been improved.

"3) Page 13 line12, O-GlucNAcylation of TAB1 is "not" essential for NF-κB activation and IL-1α stimulated cytokine release. "

This has been altered as per the referee's suggestion, and is also reflected in the title.

Referee #2

"1) The authors should provide at least one blot showing a competition with free GlcNAc to show that the O-GlcNAc signal is indeed specific to go along with figure 1. Figure 1C, the author should have a negative IP control with a non-specific antibody."

These controls have been performed and have been included in an expanded Fig. 2. The Ser395 *O*-GlcNAc site-specific antibody further confirms this result (included in the same figure).

"2) In figure 2A, could the authors include the negative control without transfection instead of stating in the text that it was in agreement with earlier studies (Mendoza et al, 2008)"

This control has been included as Fig. S1 in the Supplementary Material, showing that our observations are in agreement with the earlier studies cited by the referee.

"3) Is TAK1 itself O-GlcNAcylated? Specifically, did the authors investigate TAK1 GlcNAcylation state in response to IL-1 stimulation and/or osmotic stress? Specific O-GlcNAcylation of TAK1 could be involved in TAK1 mediated cytokine release."

We have investigated this by blotting for *O*-GlcNAc on IP-ed TAK1 and show the data in Fig. S2 in the Supplementary Material – TAK1 itself does not appear to be O-GlcNAcylated.

"4) The authors should look at the dynamics of TAB1 GlcNAcylation in response to IL-1 stimulation and osmotic stress during the time points they use. If the authors can show that GlcNAcylation of TAB1 changes during IL-1 stimulation or osmotic stress, this would further solidify their results in that GlcNAcylation of TAB1 is involved during TAK1 mediated cytokine release."

The revised manuscript now contains a new section of R&D under the heading "*O-GlcNAcylation of TAB1 is inducible*". Here we show that we have generated an *O*-GlcNAc site-specific antibody, and use that to show that TAB1 *O*-GlcNAcylation can be induced by known activators of the TAK1 pathway (IL1/NaCl) and also by high glucose levels, or with a specific *O*-GlcNAcase inhibitor.

"5) In figure 4, the authors show that GlcNAcstatin pS438 TAB1 phosphorylation and suggest that is due to the O-GlcNAc at S395. However, that experiment does not address that. Elevated O-GlcNAc might be altering the kinase or phosphatase activity at S438. The authors should show that the S395A mutant does the same thing as the GlcNAcstatin. This experiment has to be done."

These data are now included in a new Fig. 6 in the revised manuscript where we show the effects of presence/absence of *O*-GlcNAc on all known TAB1 phosphorylation sites.

"Also of interest, the authors do not directly address how O-GlcNAcylation of TAB1 alters the phosphorylation on TAK1. Does the mutant bind differently to TAK1, does it alter it's cytolocalization? These questions should be addressed."

We have addressed this issue by performing co-IP/IB studies (Fig. 4), showing that the amount of TAK1 associated with TAB1 does not change in an *O*-GlcNAc dependent manner. This is similar to TAB1 phosphorylation regulating TAK1 activity without alterations in the stoichiometry of the TAK1:TAB1 complex.

"5) In figure 2A, the authors seems to have incorrectly labeled the figure. Instead of using dashed lines and symbols, the authors show consider using only 4 different symbols (circles, squares, diamonds, triangles)."

This figure has been completely revamped, incorporating the referee's suggestions.

"6) Under the "O-GlcNAcylation of TAB1 modulates regulatory phosphorylation" section the first line states that TAB1 can be phosphorylated at T432. I believe the authors really mean T431 as indicated 2 lines later."

This has been corrected.

"7) The graph in Fig 2A is hard to read. The authors might want to change the symbols to make them clearer. Also, Fig. 2F the X-axis is labeled 15 min 10 min 15 min. Is that correct?"

This figure has been completely revamped, incorporating the referee's suggestions.

Referee #3

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In the new Fig. 4 in the revised manuscript, we no longer use total IkB as a reference as suggested by the referee.

"2) In all experiments using TAB1-/- MEFs, TAB1+/+ and TAB1-/- MEFs transfected with empty vector should be included. Are the exogenously expressed TAB1 levels similar to the endogenous TAB1 levels?"

This has been studied in detail, showing that we have matched expression levels precisely to that of exogenous TAB1. These important controls requested by the referee are discussed in the text, with all data shown in Fig. S1 of the Supplementary Material.

"3) It is important to determine the exact activity of TAB1 wild type and S395A for TAK1 activation. Dose-response curves should be determined."

These data are shown in Fig. S1 of the Supplementary Material.

"4) Is TAB1 constitutively O-GlcNAcylated or in an IL-1 and osmotic stress-dependent manner?"

The revised manuscript now contains a new section of R&D under the heading "*O-GlcNAcylation of TAB1 is inducible*". Here we show that we have generated an *O*-GlcNAc site-specific antibody, and use that to show that TAB1 *O*-GlcNAcylation can be induced by known activators of the TAK1 pathway (IL1/NaCl) and also by high glucose levels, or with a specific *O*-GlcNAcase inhibitor.

"5) Immunoblot analysis of TAB1 O-GlcNAcylation should be performed in Fig. 2B, 2C and Fig. 4."

These controls have been added.

"6) The authors should test whether the S395A mutation affects the ability to associate with TAK1."

We have addressed this issue by performing co-IP/IB studies (Fig. 4), showing that the amount of TAK1 associated with TAB1 does not change in an *O*-GlcNAc dependent manner. This is similar to TAB1 phosphorylation regulating TAK1 activity without alterations in the stoichiometry of the TAK1:TAB1 complex.

"7) It is not clear whether increased phosphorylation of TAB1 at S438 is causally associated with reduced TAK1 activity. The additional experiments are needed."

These data are now included in a new Fig. 6 in the revised manuscript where we show the effects of presence/absence of *O*-GlcNAc on all known TAB1 phosphorylation sites.

"8) JNK activation should also be examined."

Data showing effects on JNK activation have now been included as a new panel to Fig. 4.

2nd Editorial Decision 07 September 2011

Thank you for sending us your revised manuscript (previously EMBOJ-2009-73133) as a new

submission. Our original referees have now seen it again (referee 1 is the original referee 2 and vice versa). In general, the referees are now positive about publication of your paper. Still, referee 3 feels that there are a few remaining issues that need to be addressed, in part by additional experimentation (see below), before we can ultimately accept your manuscript. Referee 1 has minor suggestions. I would therefore like to ask you to deal with the issues raised in a revised manuscript.

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

1) Please reformat the manuscript, in particular the order of chapters, to EMBO Journal format (please see 'author instructions').

2) Please include an author contribution section and a conflict of interest statement into the main body of the manuscript text after the acknowledgements section.

3) Please upload one file per figure.

4) Prior to accepting manuscripts we always do a cross check to see if there is any similarity between the accepted manuscript text and previous published work. In your case, the cross check picked up such passages, mainly, but not exclusively in the materials & methods section (please see attachment). I therefore need to ask you to modify the highlighted passages.

5) We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer 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://www.nature.com/emboj/about/process.html

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.

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

Manuscript re-revision for EMBO J.

Title: O-GlcNAcylation of TAB1 modulates TAK1-mediated cytokine release

Authors: Shalini Pathak, Vladimir S. Borodkin, Osama Albarbarawi, David G. Campbell, Adel

Ibrahim and Daan M. F. van Aalten

General Comments:

The manuscript addresses how the increased O-GlcNAc modification of TAB1 at a single Serine (395) in response to IL-1 stimulation or osmotic stress is necessary for the full activation of TAK1. First the manuscript shows clearly by several methods that TAB1 is an O-GlcNAcylated protein under basal and stimulated conditions. This includes the mapping of a single site of O-GlcNAc on the protein. Then the authors establish how the increase in O-GlcNAc on TAB1 at this single site upon stimulation is required for the maximal kinase activity of TAK1 and therefore its downstream effects such as cytokine release and gene activation. Last, the manuscript shows how O-GlcNAcylation of TAB1 may regulate its phosphorylation at Serine 438, which represents an example of the interplay between these two post-translational modifications and opens questions for future investigations.

The manuscript is well written and all the experiments clearly support all conclusions.

The authors have addressed properly nearly all the comments previously made by the reviewers on the first revision and therefore the manuscript is recommended for publication.

Some Minor Specific Comments:

1) In Figure 5, part C, assuming that a statistical analysis was performed in all time points, the authors may have forgotten to add an asterisk on top of the grey bar corresponding to the 24h TNFα secretion since this time clearly shows a difference similar to the one observed for 16h.

2) Authors should explain figure 6 more in the body of the text. Specifically as it pertains to the phosphorylation status (site-specific) of the O-GlcNAc mutant in response to stimulus. For example: "In the presence of NaCl phosphorylation of S438 was observed to increase in both WT and S395A TAB mutant when compared to control. However, in the presence of both NaCl and GlcNAstatin there is a further increase in phosphorylation of S438 in WT, but this further increase was not seen in the S395A mutant. Suggesting the O-GlcNAc at S395 may regulate TAB1 phosphorylation at S438." Some additional statement are needed in the text as none can be found in regards to the data shown in fig 6 as it relates to the S395A mutant.

3) In Fig. 6, the blot is labeled S431, should this be T431?

4). It is unclear to this reviewer how fig 6 and the corresponding text in the body of the manuscript sufficiently addresses reviewer #3 comment (#7).

Referee #2

I think that the authors' additional experiments and comments are adequate. Therefore, I think that this manuscript is suitable for publication in the EMBO Journal.

Referee #3

The manuscript is significantly improved and several major concerns have been appropriately addressed. However, there are still some concerns.

Specific comments:

1) The mechanism by which O-GlcNAcylation of TAB1 regulates TAK1 activity is still unclear. The results regarding with the relationship between O-GlcNAcylation and phosphorylation of TAB1 in Fig. 6 are very preliminary. If the phosphorylation of S423 is inhibitory to TAK1 activity, a double S395 O-GlcNAcylation and S423 phosphorylation mutant of TAB1 should not reduce IL-1 and NaCl-induced TAK1 activation. The analysis of such double mutants should greatly strengthen

the manuscript.

2) Fig. S1D is not clear. Is this activity of TAK1 without stimulation or with IL-1 stimulation?

3) The authors should show the absolute values of TAK1 activity in several figures. It is not clear whether Fig. S1D is consistent with Fig. 4A.

4) The method for TAK1 activity measurement is confusing. In some figures and Method section, glutathione-sepharose beads were used. Is GST-TAK1 or GST-TAB1 used for those experiments?

Additional correspondence (author) 12 September 2011

Thank you for sending us these referee reports. We're glad to see the referees find the revised version of our manuscript significantly improved, and recommend publication after a number of further issues have been resolved.

I writing to explain to you how we are planning to address these further issues, in particular regarding the two comments of the referees that could require further work.

Could you please let me know whether you would find the proposed course of action acceptable so we can proceed along these lines and hopefully return a final revision of the manuscript to you within the next few weeks.

See below for individual proposed responses to the referees/editorial comments.

1) Please reformat the manuscript, in particular the order of chapters, to EMBO Journal format (please see 'author instructions').

>Will be done

2) Please include an author contribution section and a conflict of interest statement into the main body of the manuscript text after the acknowledgements section.

>Will be done

3) Please upload one file per figure.

>Will be done

4) Prior to accepting manuscripts we always do a cross check to see if there is any similarity between the accepted manuscript text and previous published work. In your case, the cross check picked up such passages, mainly, but not exclusively in the materials & methods section (please see attachment). I therefore need to ask you to modify the highlighted passages.

I have carefully studied this document. Apart from the materials and methods, which contains some sentences detected in other publications (apart from a whole section in red - I will take this up with the postdoc involved), I am unable to find more than a combination of previously published words in our document, and not the examples of "passages" (implying whole sentences or paragraphs) that you refer you. All "matches" there are in the R&D section are in the "<1%" catergory. Would it be possible for you to very specifically define what you are referring to?

5) We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation

would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

>Yes we would be happy to do this.

Referee #1

[...]Some Minor Specific Comments:

1) In Figure 5, part C, assuming that a statistical analysis was performed in all time points, the authors may have forgotten to add an asterisk on top of the grey bar corresponding to the 24h TNFα secretion since this time clearly shows a difference similar to the one observed for 16h.

>we will look into this and if necessary do extra experiments (1-2 weeks of work)

2) Authors should explain figure 6 more in the body of the text.
Specifically as it pertains to the phosphorylation status (site-specific) of the O-GlcNAc mutant in response to stimulus. For example: "In the presence of NaCl phosphorylation of S438 was observed to increase in both WT and S395A TAB mutant when compared to control. However, in the presence of both NaCl and GlcNAstatin there is a further increase in phosphorylation of S438 in WT, but this further increase was not seen in the S395A mutant. Suggesting the O-GlcNAc at S395 may regulate TAB1 phosphorylation at S438." Some additional statement are needed in the text as none can be found in regards to the data shown in fig 6 as it relates to the S395A mutant.

>We will include the further sentence and discussion suggested by the referee

3) In Fig. 6, the blot is labeled S431, should this be T431?

>That is indeed a typo that will be fixed

4). It is unclear to this reviewer how fig 6 and the corresponding text in the body of the manuscript sufficiently addresses reviewer #3 comment
(#7).

>See below for a response to that comment.

Referee #3

[...]Specific comments:

1) The mechanism by which O-GlcNAcylation of TAB1 regulates TAK1 activity is still unclear. The results regarding with the relationship between O-GlcNAcylation and phosphorylation of TAB1 in Fig. 6 are very preliminary. If the phosphorylation of S423 is inhibitory to TAK1 activity, a double S395 O-GlcNAcylation and S423 phosphorylation mutant of TAB1 should not reduce IL-1 and NaCl-induced TAK1 activation. The analysis of such double mutants should greatly strengthen the manuscript.

>This work did not aim to uncover the precise details of how O-GlcNAcylation regulates phosphorylation of TAB1, nor to uncover the precise molecular (structural?) mechanism by which TAB1 O-GlcNAc regulates TAK1 activity. Although we appreciate it would strenghten the manuscript as the referee suggests, we would like to avoid doing these experiments. First of all, S423 phosphorylation is NOT affected by S395 O-GlcNAcylation, as shown by Fig. 6, so it is not clear why the referee asks us to make this double mutant. Furthermore, we feel this is an example of "mission creep" - where results of experiments suggested by referees in the first round, lead to further suggestions for experiments by referees in the second round. It would take us 4-8 weeks to carry out this extra experiment.

2) Fig. S1D is not clear. Is this activity of TAK1 without stimulation or with IL-1 stimulation?

>This is with IL1 stimulation (the legend refers to panel A where this is mentioned) and this will be made clearer in a revised figure legend.

3) The authors should show the absolute values of TAK1 activity in several figures. It is not clear whether Fig. S1D is consistent with Fig. 4A.

>This will be done.

4) The method for TAK1 activity measurement is confusing. In some figures and Method section, glutathione-sepharose beads were used. Is GST-TAK1 or GST-TAB1 used for those experiments?

>This will be fully explained in the revised manuscript.

Additional correspondence (editor) 15 September 2011

Thank you for your message outlining your suggestions for the revision. I have now had a chance to look into this in depth.

With respect to referee 3 point 1 I think that the referee may have referred to the wrong residue. I agree with you that S423 phosphorylation is not affected by S395 O-GlcNAcylation, but the referee may have intended to refer to S438. In any case, I have now considered your request to refrain from doing further experiments along these lines, and I have decided to follow your suggestion and not to insist on this.

With respect to the CrossCheck results, I agree with you that the key issue lies within the materials & methods section. So, I would suggest going through the text once more, and to this section in particular, to modify the text where needed and appropriate.

I agree with all the other points that you have listed.

We are looking forward to your amended manuscript in due course.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - authors' response 06 December 2011

RESPONSE TO REFEREES/EDITORIAL COMMENTS

1) Please reformat the manuscript, in particular the order of chapters, to EMBO Journal format (please see 'author instructions').

This has been done in the revised version

2) Please include an author contribution section and a conflict of interest statement into the main body of the manuscript text after the acknowledgements section.

This has been included in the revised version

3) Please upload one file per figure.

This has been done at the time of electronic resubmission

4) Prior to accepting manuscripts we always do a cross check to see if there is any similarity between the accepted manuscript text and previous published work. In your case, the cross check picked up such passages, mainly, but not exclusively in the materials & methods section (please see attachment). I therefore need to ask you to modify the highlighted passages.

As previously discussed, the section in materials and methods has been adjusted.

5) We now encourage the publication of source data, would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures?

Yes we would be happy to do this.

Referee #1

1) In Figure 5, part C, assuming that a statistical analysis was performed in all time points, the authors may have forgotten to add an asterisk on top of the grey bar corresponding to the 24h TNFα secretion since this time clearly shows a difference similar to the one observed for 16h.

These experiments have been repeated and the appropriate asterisk has been added.

2) Authors should explain figure 6 more in the body of the text. Specifically as it pertains to the phosphorylation status (site-specific) of the O-GlcNAc mutant in response to stimulus. For example: "In the presence of NaCl phosphorylation of S438 was observed to increase in both WT and S395A TAB mutant when compared to control. However, in the presence of both NaCl and GlcNAstatin there is a further increase in phosphorylation of S438 in WT, but this further increase was not seen in the S395A mutant. Suggesting the O-GlcNAc at S395 may regulate TAB1 phosphorylation at S438." Some additional statement are needed in the text as none can be found in regards to the data shown in fig 6 as it relates to the S395A mutant.

The sentence and discussion suggested by the referee has been included in the revised manuscript.

3) In Fig. 6, the blot is labeled S431, should this be T431?

This typo has been corrected.

4). It is unclear to this reviewer how fig 6 and the corresponding text in the body of the manuscript sufficiently addresses reviewer #3 comment (#7).

See our below for a response to the issue raised by referee #3.

Referee #2

No issues to address.

Referee #3

1) The mechanism by which O-GlcNAcylation of TAB1 regulates TAK1 activity is still unclear. The results regarding with the relationship between O-GlcNAcylation and phosphorylation of TAB1 in Fig. 6 are very preliminary. If the phosphorylation of S423 is inhibitory to TAK1 activity, a double S395 O-GlcNAcylation and S423 phosphorylation mutant of TAB1 should not reduce IL-1 and NaCl-induced TAK1 activation. The analysis of such double mutants should greatly strengthen the manuscript.

As discussed in our e-mail exchange previously, this work did not aim to uncover the precise details of how O-GlcNAcylation regulates phosphorylation of TAB1, nor to uncover the precise molecular

(structural?) mechanism by which TAB1 O-GlcNAc regulates TAK1 activity. Although we appreciate it would strenghten the manuscript as the referee suggests, we have not included these experiments as discussed in our e-mail exchange. First of all, S423 phosphorylation is NOT affected by S395 O-GlcNAcylation, as shown by Fig. 6, so it is not clear why the referee asks us to make this double mutant. Furthermore, we feel this is an example of "mission creep" - where results of experiments suggested by referees in the first round, lead to further suggestions for experiments by referees in the second round. It would take us 4-8 weeks to carry out this extra experiment (and it would undoubtedly lead to further interesting questions and experiments!).

2) Fig. S1D is not clear. Is this activity of TAK1 without stimulation or with IL-1 stimulation?

This is with IL1 stimulation (the legend refers to panel A where this is mentioned) and this has been made clearer in a revised figure legend.

3) The authors should show the absolute values of TAK1 activity in several figures. It is not clear whether Fig. S1D is consistent with Fig. 4A.

Absolute values of TAK1 activity are now shown on the vertical axis.

4) The method for TAK1 activity measurement is confusing. In some figures and Method sections, glutathione-sepharose beads were used. Is GST-TAK1 or GST-TAB1 used for those experiments?

This is now explicitly stated in the revised manuscript.

