THE EMBO JOURNAL

Manuscript EMBO-2011-77167

Control of Ubp3 ubiquitin protease activity by the Hog1 SAPK modulates transcription upon osmostress

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Review timeline:

Submission date:	10 February 2011
Editorial Decision:	16 March 2011
Revision received:	19 May 2011
Editorial Decision:	10 June 2011
Accepted:	10 June 2011

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 16 March 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see in general the referees find the study to be potentially interesting but they require some further experimental analysis to make the study suitable for The EMBO Journal.

Among the issues raised by the referees it is important to show if the phosphorylation of Ubp3 is required for its recruitment to osmo-responsive genes or regulating its activity once there and to provide some insight into the targets of Ubp3 (especially mentioned by referee #3). Also the biological significance of the role of Ubp3 should be tested by looking into the sensitivity of yeast cells lacking Ubp3 to osmotic stress. Given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

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: http://www.nature.com/emboj/about/process.html

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:

In the article 'Control of Ubp3 ubiquitin protease activity by the Hog1 SAPK modulates transcription upon osmostress' the authors (Sole et al) show, very nicely, that Ubp3 is a novel target of Hog1. Ubp3 is phosphorylated by Hog1 at serine 695 and this (as well as the catalytic activity of Ubp3p) is necessary in order to activate osmoresponsive genes. Among the 10 de-ubiquitinases they tested only Ubp3p had an effect on induction of osmoresponsive stress genes and notably, deletion of BRE5, the cofactor of Ubp3, showed the same low induction of these genes as a ubp3 mutant. By immuno-precipitation experiments they show that Hog1p and Ubp3p interacts in a stress-dependent (0.4M NaCl) manner. Furthermore, Ubp3 is recruited to osmo-stress genes, as shown by chromatin immuno-precipitation (ChIP), and modulates both transcriptional initiation and elongation. Also, if ubp3 is deleted the density of RNAPII is significantly reduced at SLT1 and CTT1 promoters and in the open reading frames especially after osmo-stress, compared to isogenic wild-type strains. Importantly, if ubp3 is mutated at serine 695 (S695A) there is a reduction of RNAPII occupancy at these genes during stress conditions (0.4M NaCl). In addition, again by ChIP they show that recruitment of the transcription factors Msn2 and Tbp1 to the promoter of CTT1 is diminished in cells lacking Ubp3p. Strikingly and interestingly, the authors find that Ubp3p is phosphorylated at all times but is 'hyper-phosphorylated' after osmo-stress by Hog1p. Obviously, it would be very interesting to know on what amino-acid(s) the other phosphorylation(s) is/are and if this is regulated as well, but I believe that is beyond the scoop of this manuscript... Finally, the authors tested if a Ubp3-S695A mutant protein was as able to de-ubiquitnate RNAPII as an wild-type version of Ubp3p. I'm not sure if I entirely share the interpretations made by the authors in this experiment. Even though there are more ubiquitins cut off after stress in the wild type experiment than in the mutant experiment it seems like the efficiency of the mutant Ubp3 is very good already before stress. Also, it seems like there is less Ubp3 used in the mutant experiment than in the wild-type experiment. This is slightly puzzling to me. Maybe there is a simple explanation to this? Otherwise, I think this needs to be looked into. Finally, it would be appropriate to show a size ladder next to the lanes in Fig. 7B. A last thought is, can the authors provide proof on that Rsp5 is not over-ubiquitinated and thus serve as a substrate in this experiment (Fig.7B). All in all I think this is a very nice work and have only a few points which I think should be addressed.

Firstly, it would be interesting to know if the mutant allele ubp3-S695A is recruited to osmoresponsive genes during osmo stress.

Secondly, the issues raised on Fig 7B (see above) may be clarified.

Referee #2:

This is a fine study that links SAP kinase stress signaling to control of transcription through ubiquitylation. The authors make a compelling case for activation of the Ubp3 ubiquitin protease by the Hog1 SAPK in response to osmostress. Ubp3 has been implicated previously in both transcription initiation and elongation through its action on Tbp1 and RNA Pol II, respectively. In the present study, the authors demonstrate that Hog1 interacts with Ubp3 in a stress-dependent manner and that phosphorylation of Ubp3 by Hog1 on Ser695 enhances RNA Pol II occupancy on Hog1-driven genes. Ubp3 is also recruited to Hog1-driven genes in a Hog1-dependent manner. Ubp3 enhances both transcription initiation and elongation. The authors also show that Ubp3

isolated from osmotically stressed cells de-ubiquitinates RNA Pol II (Rpb1) more efficiently than that from unstressed cells. This enhanced activity is dependent on the Hog1 phosphorylation site.

The study is well conducted and clearly presented. I have two suggestions for improvement. First, there is a question regarding whether Hog1 phosphorylation of Ubp3 affects its recruitment to the DNA or its activity once it's there. The Ubp3 ChIP experiment in Figure 3A, which shows complete dependence on Hog1 for Ubp3 DNA occupancy is not useful to answer this question, because in a hog1Δ mutant there is no transcription. Moreover, the co-IP of Ubp3 with Pol II is unaffected by Hog1 or osmotic stress (Supp. Figure S5), suggesting that it may be recruited normally. However, the Pol II ChIP experiment in Figure 4B shows that the phosphorylation site mutant of Ubp3 is only partially impaired for induced RNA Pol II occupancy of the STL1 and CTT1 genes. It would be interesting to know if unphosphorylated Ubp3 is still recruited to these genes but is non-functional, or if it fails to be recruited. Because there is some induced Pol II occupancy remaining in the mutant, the experiment should give a meaningful result.

Second, it would have been nice if the question of the specific target of Ubp3 had been addressed in the study. The authors suggest that elongating RNA Pol II is a likely target, but considering the demonstrated specific effect on elongation of the STL1 gene (as compared to ADH1) when separated from its promoter (compare Fig. 6D to Supp. Fig. S4), it is difficult to see how such specificity might be achieved through targeting RNA Pol II. This is minimally worth comment in the discussion.

Referee #3:

The ubiquitin protease Ubp3 has previously been implicated in several distinct processes, including protection of transcription initiation factors and RNA Pol II, turnover of ribosomes during starvation, turnover of DNA damage repair factors, and regulation of vesicular sorting pathways. Here, Solé et al. extend the role of Ubp3 in regulating transcription by showing that it is required for full induction of several genes whose level of transcription increases in response to osmotic stress. The authors clearly demonstrate that the activity of Ubp3 is needed to deubiquitylate some factor associated with stress response gene activation, and that this activity is important for accumulating normal levels of transcription factors and Pol II at the promoters of some of these genes. Further, they show that this activity of Ubp3 is regulated by the SAPK Hog1, and that this regulation depends on a single serine residue, S695, presumably because this is the target of Hog1 phosphorylation. The data presented are of very high quality, and while some issues of interpretation need to be addressed, the overall conclusions drawn are convincingly supported and presented very clearly. The manuscript should therefore definitely be published based on the quality of the work, but it is less clear whether or not the identification of Ubp3 as a factor involved in the transcriptional stress response is sufficiently novel for publication in this journal. In favor of acceptance, the observation that the activity of a deubiquitylating enzyme can be regulated in response to an environmental cue is quite novel and important, as most of the attention focused on understanding regulation of ubiquitin-mediated proteolysis has thus far fallen on the various E3 ligases. However, Ubp3 has previously been shown to be involved in protecting Spt15 (Tbp1) and Pol II from degradation so a role in modulating transcription is already known. Further, the work presented here does not establish the target or targets of the regulated deubiquitylation by Ubp3, which might provide new mechanistic insight. Overall, the novelty and importance do seem to be sufficient for this journal, but this is not a clear case.

Issues to be addressed include:

1) The authors show that loss of Ubp3 leads to a decreased response to osmotic stress. Does this result in sensitivity of the yeast cells to stress at the level of growth or viability?

2) The northern blot data that is presented in several figures should be quantitated so that the reader does not have to estimate the ratio of signal to the loading control by eye. This is especially important in figures 4 and 6. A number is often presented below each lane, so this does not significantly affect the style of the presentation used here.

3) The authors should be more careful not to imply that Hog1 and Ubp3 have been shown to interact directly here, as the IP experiments presented used whole cell extracts so the interaction could be indirect.

4) The recruitment of Pol II to the promoters of genes assayed by ChIP seems puzzling; are the start sites for transcription for these genes known? The authors make several statements regarding roles of Ubp3 in initiation as distinct from elongation of transcription, but it is not clear that the ChIP probes actually discriminate between sites of initiation and elongation. What criteria are used to assign these labels? Are the authors trying to imply that Ubp3 promotes a transition from the initiation state to the elongation state, that Ubp3 is traveling with the elongating polymerase, or that Ubp3 promotes the rate or processivity of elongation? The usage of these terms should be clarified so that the reader can determine whether or not the data justify the conclusions. Related to this, the interpretation of the experiment shown in figure 6D seems simpler than is warranted. The data show that transcript level is affected, but this could be due to initiation, elongation, or stability of the message. The authors should comment on their interpretation of this experiment further, as well as their interpretation of the high level of STL1 transcript in unstressed cells that contain normal Ubp3.

5) The conclusions drawn from experiments with the C469A allele of UBP3 require the stability of this protein to be established. Has this been reported previously? If not, it needs to be shown.

Minor points

6) All of the ubiquitin proteases in yeast are tested for roles in the stress response except Doa4 and Ubp5. This is curious as Doa4 is the only one implicated in this response previously due to its known sensitivity to osmotic stress. Has this mutation been tested for the transcriptional response elsewhere or is there some other reason to omit these two proteases from the test?

7) The standard name for Tbp1 is Spt15, so the latter name should at least be mentioned. Also, it is labeled incorrectly as "Tpb1" on page 10.

8) The legend to figure 3 is confusing and should be clarified: "...Ubp3 binds to stress-dependent genes through the Hog1 SAPK allowing the recruitment of RNA Pol II." The data show that Ubp3 recruitment depends on Hog1 but not that Hog1 mediates the recruitment; the localization could be due to Hog1 kinase activity and not due to a direct interaction.

1st Revision - authors' response 19 May 2011

Referee #1

This referee found the work very nice and made some suggestions to improve the manuscript. We thank the referee for his/her positive overall view of our work and, we have followed the reviewer suggestions to improve the manuscript.

1) The reviewer pointed out that "*Strikingly and interestingly, the authors find that Ubp3p is phosphorylated at all times but is 'hyper-phosphorylated' after osmostress by Hog1p. Obviously, it would be very interesting to know on what amino-acid(s) the other phosphorylation(s) is/are and if this is regulated as well, but I believe that is beyond the scoop of this manuscript*".
Indeed, this is an interesting observation that suggests that Ubp3 can be regulated by alternative kinases in different growth conditions. Actually, we performed mass spectrometry analysis of purified Ubp3 from stressed and not stressed yeast cells to identify phosphopeptide variants and found several Ubp3 phosphorylation sites that were not osmo-regulated. Specifically, this analysis

showed that 11 sites on Ubp3 were phosphorylated under basal conditions (Ser20, Ser238, Ser243, Ser247, Ser257, Ser335, Ser339, Ser341, S343, Ser360 and Ser400). It is worth to mention that two of these serines (Ser339 and Ser400) were already reported (Soufi *et al.* Mol Biosyst. 2009 **5**:1337-46). We have mentioned this observation in the discussion.

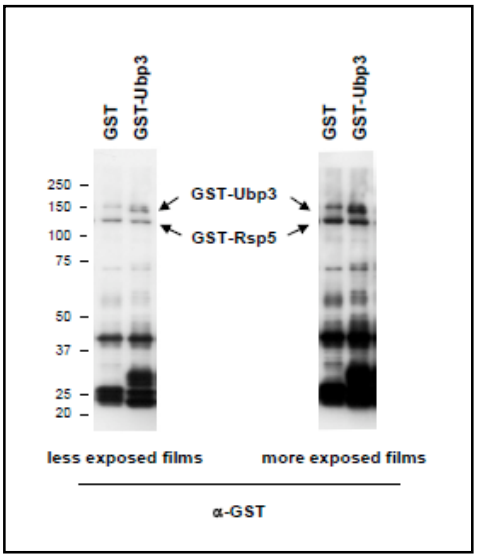
2) The reviewer asked whether the non-phosphorylated mutant allele Ubp3^{S695A} is recruited to osmoresponsive genes in response to stress. This was also requested by referee #2. We performed ChIP assays to assess HA-tagged Ubp3^{S695A} binding at S*TL1* and *CTT1* genes. Ubp3^{S695A} associated with osmo-dependent genes in response to stress, although it was bound in a slightly lesser extent than wild type at ORFs, possibly due to the reduced amount of RNA Pol II. This suggests that Hog1 phosphorylates Ubp3 to regulate its activity but not to recruit the de-ubiquitylase at chromatin. These results are now introduced in the main text of the manuscript and as Supplementary Figure 3.

3) The reviewer asked several questions on the RNA Pol II de-ubiquitylation assay presented as Figure 7B.

a) He/She pointed out that it seemed like the efficiency of the mutant Ubp3^{S695A} in deubiquitylating RNA Pol II was very good already before stress compared to wild type. The experiments with wild type and Ubp3^{S695A} can not be compared between them since they were performed in different days. Thus, the efficiency of deubiquitylation by Ubp3 (wild type or mutant allele) can only be assessed plus/minus stress. The α-GST blots were presented to compare +/- stress in each experiment to be able to calculate fold induction of wild type and Ubp3^{S695A} upon stress. Actually, the activity of wild type and Ubp3^{S695A} was very similar in basal conditions being the mutant slightly less active. This is a very valid point raised by the reviewer and we have now included the information in the revised version of the manuscript.

b) The reviewer asked to provide a proof on that Rsp5 was not over-ubiquitylated. We used the same amounts of ubiquitylation factors as previously reported (Somesh *et al.* 2005 Cell **121**:913-23) and thus we did not expect the over-ubiquitylation of Rsp5. Indeed, we probed using α-GST the presence of Rsp5 by Western blot on the ubiquitylation *in vitro* assays, and we did not observe a mobility shift in Rsp5 protein (see attached figure). Thus, it is unlikely that Rsp5 was over-ubiquitylated in the assays.

c) The reviewer suggested to include a size ladder next to the lanes in Figure 7B. We have done as suggested and include it in the revised manuscript.



Referee #2

The reviewer found this study a compelling case for activation of the Ubp3 de-ubiquitylase by the Hog1 SAPK in response to osmostress. He/She made two suggestions for improvement that we have followed.

1) He/She asked whether Hog1 phosphorylation of Ubp3 affected its recruitment to DNA or its activity once it's there. This question was also pointed out by reviewer #1. As described before, we performed ChIP experiments to assess the presence of HA-tagged Ubp3^{S695A} at the stress-responsive genes *STL1* and *CTT1* and found that binding of Ubp3^{S695A} was similar at promoters and slightly reduced at ORFs, possibly as a consequence of reduced RNA Pol II. We have now included this result in the manuscript as Supplementary Figure 3 and in the main text.

2) The second issue posed by the reviewer was regarding the specific target of Ubp3 during transcriptional elongation upon stress. "*The authors suggest that elongating RNA Pol II is a likely target, but considering the demonstrated specific effect on elongation of the STL1 gene (as compared to ADH1) when separated from its promoter (compare Fig. 6D to Supp. Fig. S4), it is difficult to see how such specificity might be achieved through targeting RNA Pol II. This is minimally worth comment in the discussion.*" The Hog1 SAPK interacts and travels with elongating RNA Pol II through the coding regions of osmoresponsive genes. Because Hog1 associates with ORF of osmoresponsive genes but is not recruited to constitutively expressed genes, we speculate that the SAPK itself dictates the specificity of targeting RNA Pol II (and presumably also Ubp3) only on those osmo-dependent ORFs. Actually, RNA Pol II association through the coding region of

the LexA-*STL1* gene increases in response to stress in a Hog1 dependent manner (Proft *et al.* Mol Cell. 2006 **23**:241-50). Thus, we may assume that the specificity of Ubp3 targeting RNA Pol II only in those osmo-dependent genes is achieved through the Hog1 SAPK. We have now included this discussion on the revised manuscript.

Referee #3

The referee pointed out that data was of very high quality, convincingly supported and clearly presented. He/She suggested clarifying several points that have been addressed as described below:

1) The reviewer asked whether loss of Ubp3 resulted in sensitivity of the yeast cells to stress at the level of growth or viability. *UBP3* or *BRE5* mutations did not display an osmo-sensitive phenotype in agar plate assay. Moreover, it is already published that Ubp3 is not necessary for optimal growth under osmostress conditions (Giaever *et al.* Nature 2002 **418**:387-91). It might exist different reasons for this lack of osmosensitivity. First, in *UBP3* deficient cells the transcription of osmoresponsive genes is clearly impaired but not totally abolished. Thus, the remaining transcription might be sufficient to allow cell survival upon stress. Second, there are several examples where transcriptional defects of osmo-stress genes do not correlate with cellular growth defect in stress conditions. For instance, a yeast strain lacking several transcription factors that are involved in the general transcriptional response to stress (i.e., *msn2 msn4*) is not osmosensitive (Estruch,F. FEMS Microbiol. Rev 2000 24:469-486). This is because such transcription factors can act in combination with others to elaborate a transcriptional network and deleting one of them is not enough to render osmosensitive cells. Third, it could be that the genes that have its transcription affected in the *UBP3* mutant are not the essential ones for cell survival, although are important for proper adaptation. This is a very valid point raised by the reviewer and we have now included the data as Supplementary Figure 1B and included the information in the revised manuscript.

2) The reviewer pointed out that the Northern blots that are presented in figures 4 and 6 should be quantified. We agree with the referee that presenting northern quantifications helps the reader to interpret the results. Therefore, we quantified the activation of the stress-dependent genes using as 100% reference the time of maximum gene expression of the wild type strain (Figure 4) or referenced to time 0 for each strain (Figure 6). These quantifications have been introduced now in the revised manuscript (see Figure 4 and 6).

3) The referee pointed out that "*The authors should be more careful not to imply that Hog1 and Ubp3 have been shown to interact directly here, as the IP experiments presented used whole cell extracts so the interaction could be indirect*". We completely agree with the referee that this is an important point and from the experiments presented we cannot conclude that both proteins are

interacting in a direct manner. Thus, to address this question, we have performed *in vitro* experiments using purified Ubp3 and Hog1 proteins from *E. coli* and showed that Ubp3 binds directly with the MAPK. This new result has been included in the manuscript as Supplementary Figure 7A and the manuscript has been modified accordingly.

4) The reviewer was concerned about how we discriminated transcriptional initiation and elongation processes.

a) He/She asked whether the transcription start sites for these genes are known. We know the transcription start site (TSS) of those genes thanks to a tiling array performed in our laboratory in response to stress. For *STL1* TSS is approximately 60 bp upstream of the SGD annotated start codon, while TSS of *CTT1* matches SGD annotation.

b) The reviewer asked which criteria is used to discriminate between initiation and elongation. As shown in previous studies (Proft *et al.* Mol Cell. 2006 **23**:241-50) we are able in our ChIPs to selectively assess binding to promoters and ORFs. The oligonucleotides were specially designed to discriminate between both regions. ChIP experiments were performed by shredding chromatin at an average fragment from 200 to 400 bp. PCR products of primers used range between 100 to 150 bp, and distance between promoter and coding region probes is 1Kb for *CTT1* and 2Kb approximately for *STL1*. Thus, promoter and coding regions are clearly distinguished.

c) The reviewer asked to clarify whether we implied that Ubp3 promotes a transition from the initiation state to the elongation state or that Ubp3 is travelling with the elongating polymerase or that Ubp3 promotes the rate or processivity of elongation. We have done as suggested. We believe that Ubp3 is playing roles at both initiation and elongation.

d) He/She asked about the interpretation of the experiment shown in figure 6D. The data presented in the Figure 6D shows that the elongation process is modulated by Ubp3. Here, we expressed *STL1* under an heterologous promoter (LexA) driven by LexA-VP16 activator as previously reported. The LexA system allows uncoupling of the processes of transcription initiation from elongation (Proft M. *et al*. Mol Cell. 2006 **23**:241-50). Thus, Figure 6D shows transcript levels in response to stress that can be attributed exclusively to an effect on elongation and not to changes in initiation. The use of a constitutive activator such as VP16 explains the observed basal transcription in this system. We have modified the text to clarify this point to readers. In addition, we have assessed RNA Pol II association through the ORF of the *LexA-STL1* gene in *ubp3* cells carrying a plasmid containing the wild type Ubp3 or the Ubp3^{S695A} allele. Cells carrying the Ubp3^{S695A} mutation showed reduced RNA Pol II at *STL1* coding region upon osmostress, compared to wild type. Thus, these experiments support our initial data and suggest that the phosphorylation of Ubp3 by Hog1 is crucial for RNA

Pol II binding during transcriptional elongation. We added this new result as Supplementary Figure 6 and clarified these experiments in the manuscript.

5) The reviewer pointed out that the conclusions drawn from experiments with the C469A allele of *UBP3* required to asses the stability of this protein. It was described that expression of wild type Ubp3 and the inactive form Ubp3^{C469A} are very similar (Cohen *et al.* Nat Cell Biol. 2003 **5**:661-7). We have now monitored the presence of HA tagged Ubp3^{C469A} and compared it with wild type Ubp3 by Western blot. The protein levels of both Ubp3 versions were similar in yeast cells. We have included this information as part of the Supplementary Figure S4.

As minor points the reviewer highlighted the following issues:

6) The reviewer asked to test transcription upon stress of *ubp4* (*doa4*) and *ubp5* mutant cells. Although it is true that Ubp4 (or Doa4) was suggested to be involve in osmotic stress (Fiorani *et al.* JBC 2004 **279**:21271-81), only a *doa4-10* mutant containing a nonsense mutation at Gln-388 was salt-sensitive but not the deletion of *DOA4*. Following the reviewer request we assessed expression of *STL1* and *CTT1* in *ubp4* and *ubp5* mutants by Northern blot and found that they did not have any particular effect on gene expression upon stress. These Northern blot experiments are now included as Supplementary Figure 1A and described in the result section of the revised manuscript.

7) The referee suggested to include the standard name for Tbp1 (Spt15) and indicated that it is labelled incorrectly as "Tpb1" on page 10. We have included the reviewer suggestions in the new version of the manuscript.

8) He/She suggested modifying the legend of Figure 3 to be clearer. We have done as suggested.

2nd Editorial Decision 10 June 2011

Your revised manuscript has been seen once more by two of the original referees who find that you have satisfactorily addressed their concerns. I am happy to accept the paper for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The authors of the revised manuscript has now adequately addressed my comments. Therefore I am satisfied and suggest that the work should be published.

Referee #3

The authors have adequately addressed the key points raised previously. While some issues remain regarding the target(s) of Ubp3 and the mechanism of its effects on transcription, these points have now been raised in the text of the manuscript as appropriate. The manuscript therefore appears to be suitable for publication.

