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SENP3 is responsible for HIF-1 transactivation under mild oxidative stress via p300 de-SUMOylation

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(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 18 February 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports of all three reviewers, with the comments directly to the authors attached below. As you will see, all of these referees appreciate - in principle - your observation of ROS-mediated SENP3 stabilization and its potential role in HIF-1a regulation through p300 desumoylation. However they also raise a number of substantive issues that would have to be satisfactorily addressed before publication in The EMBO Journal may be warranted. While some of these points are aimed at improving and complementing the analysis to provide a more complete and comprehensive picture, there are also more serious concerns with the conclusiveness and interpretation of the experimental evidence for SENP3 regulation of HIF-1a and p300 (see in particular referee 2's comments).

Given the overall interest of the topic and the fact that the reviewers offer a number of rather specific suggestions for improvement, I feel inclined to give you the opportunity to respond to their criticisms in the form of a revised version of the manuscript. Thus, should you feel confident that you might be able to satisfactorily address the various issues raised, we should be happy to consider a revised manuscript further. I have to point out, however, that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you diligently answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In the manuscript entitled "SENP3 is responsible for HIF-1 transactivation under mild oxidative stress via p300 de-SUMOylation", Huang et al. describe the oxidation-induced stabilization of the SENP3 SUMO-specific protease. Once stabilized, SENP3 de-sumoylates the p300 coactivator, allowing efficient binding to HIF-1 , and transcription of HIF target genes. The manuscript contributes to the understanding of how cellular oxidative state contributes to HIF-mediated transcription.
The manuscript could be strengthened with the following experiments:

1. SENP3 clearly induces HIF activity by allowing binding of HIF to p300, as the numerous luciferase assays in the paper indicate. However, as SENP3 increases HIF-mediated transcription by modifying p300, a transcriptional regulator recruited by many transcription factors. Thus, a demonstration of HIF-dependence for SENP-induced transcriptional activity (Figure 4G), and promotion of tumor growth (Figure 7) should be provided.

2. Although Figure 7 provides evidence that SENP3 plays a role in HIF activation in a hypoxic tumor environment, there is no direct experiment provided to indicate that SENP3 is induced by hypoxia, and is involved in hypoxic activation of HIF-mediated transcription. Experiments that demonstrate hypoxia-mediated induction of SENP3 would thus strengthen the paper. Also, Does SENP3 induction by hypoxia involve ROS?

Referee #2 (Remarks to the Author):

In this manuscript Huang and co-workers investigate the involvement of the SUMO specific isopeptidase SENP3 in HIF-1alpha regulation. SENP3 has so far been described as a nucleolar SUMO isopeptidase involved in the control of rRNA processing. Here, the authors show that SENP3 is stabilized by mild oxidative stress leading to its accumulation and partial release from the nucleolus. Based on this finding the authors investigated a potential role of SENP3 in the regulation of HIF-1alpha activity. In reporter gene assays expression of SENP3 indeed enhances the transactivating potential of HIF-1alpha. However, this appears to be independent from SENP3-mediated desumoylation of HIF-1. Rather, subsequent experiment led them to propose that SENP3 controls HIF-1alpha through desumoylation of the HIF-1 co-activator p300, a known target of SUMO.
In summary, the part of the manuscript describing regulation of SENP3 by H2O2 is interesting and reveals a novel aspect in the control of SENP3 activity. However, the experiments aimed to link SENP3 function to HIF-1alpha regulation and in particular to p300 are not convincing in the current stage. One major flaw is that most data are based on overexpression of the respective proteins questioning the physiological relevance of the findings. In particular, several lines of evidence indicate that ectopic expression of SENPs affects SUMO conjugation in a rather general and unspecific way. Another weakness of the MS are the experiments, which wish to show that the sumoylation state of p300 affects its interaction with p300. Here, the technical quality of some key experiments is low and the data are far from convincing (see my detailed comments below).

Major points:

1. Figure 3C: Deconjugation of SUMO2 from HIF-1alpha by SENP3 is only shown under

conditions where all components are overexpressed. A key experiment would be to demonstrate enhanced SUMO conjugation of HIF-1alpha, preferentially at its endogenous level of expression, upon depletion of SENP3.

2. Figure 5G: Again, expression of HIF-1alpha target genes, such as VEGF or GLUT1 is only investigated in an experiment where SENP3 is overexpressed. It would be more important to see whether depletion of endogenous SENP3 affects the expression of these genes.

3. Figure 6B: These data question the specificity of ectopically expressed SUMO isopeptidases, since previous work by the same authors and others has demonstrated that SUMO modification of p300 can be reverted by ectopic expression of SENP1 or SENP2 (also known as SSP3) (Girdwood et al., Mol Cell 2003; Cheng et al, JBC, 2005). Now the authors show that expression of SENP3 also causes loss of p300-SUMO conjugates (Figure 6B). To support their conclusion that SENP3 is a physiologically relevant regulator of p300 the authors perform an RNAi experiment. However, the data aimed to demonstrate an increase in sumoylation of p300 upon RNAi-mediated depletion of SENP3 (Figure 6C, right) are not convincing. Under basal conditions there is virtually no difference in p300-SUMO3 conjugates in the presence or absence of SENP3. Additionally, one would also like to see p300-SUMO1 conjugates, a proper loading control and data on sumoylation of p300 upon depletion of SENP1 and SENP2. Finally, showing data on endogenous p300 modified by endogenous SUMO upon depletion of SENP3 would make a more convincing case.
4. Based on data in Figure 6D, E the authors propose a model in which sumoylation of p300 prevents binding to HIF-1alpha. However, none of the experiments clearly demonstrates the preferential binding of non-modified vs. modified binding p300 to HIF-1alpha. The experiments shown in Figure 6D do only indirectly address this point. Moreover, in the lower part of Figure 6D it is completely unclear, why the unmodified p300 - which even after depletion of SENP3 is still rather abundant and thus should not be affected in binding to HIF-1alpha - does not bind to it. Similarly, in the experiment shown in Figure 6E, meant to directly show a differential binding of non-modified and modified p300 to HIF-1alpha, even the unmodified p300 from control cells does not bind HIF-1alpha.

5. Figure 7: I do acknowledge that the xenograft experiment with SENP3-expressing cells generates a strong phenotype. While this correlates with a reduction of p300 sumoylation, the question again arises to what extent overexpression of SENP3 generally affects cellular sumoylation.

Additional points:

1. Figure 1F,G: One would also like to see ubiquitination of endogenous SENP3.

2. Figure 2: The effect of H2O2 should also be tested on the localization of endogenous SENP3.

3. Figure 3B is of very limited information. It just shows that - like HIF-1alpha - a subfraction of ectopiclly expressed SENP3 is found in the nucleoplasm.

4. The authors mention that "p300 is reported to have a SUMO1 modification (Girdwood et al, 2003), but whether it has SUMO2 or SUMO3 modification is not clear." However, Girdwood et al. did already show modification of p300 by all three SUMO paralogs.

Referee #3 (Remarks to the Author):

In this paper, the authors investigate the role of SENP3 in mediating the effects of mild oxidative stress. They focus their study on the key transcription factor HIF1 which has been shown to a key mediator of cellular oxidative stress responses. They make several interesting and significant discoveries. First they show that oxidative stress causes the release of SENP3 into the nucleoplasm, thereby opening up a number of interesting and novel regulatory activities for this enzyme. They go on to show that this enzyme affects HIF1 activity, not through a direct means but by causing the desumoylation of p300. This in itself is significant as it demonstrates a role for p300 sumoylation which has previously been rather enigmatic. Overall, this paper therefore provides an important advance that should be of wide general interest.
In general, the experiments are well controlled and the data support the conclusions. There are

however a few areas that should be addressed to make the paper more complete.
(1) Can the authors rule out that the effects they see are not due directly or indirectly to a change in SUMO-2/3 processing rather than SUMO conjugation? Similarly, can the authors be sure that there are no effects on the SUMO conjugation pathway itself following oxidative stress?
(2) In Fig. 1A, SENP3 stabilisation is not seen at 5 mins and plateaus at 30 mins as stated in the text. It should be 30 and 60 mins respectively.
(3) In Fig. 2B, it cannot be concluded that SENP3 and HIF1 colocalise as HIF1 is not in foci. High resolution confocal microscopy might help but otherwise the conclusions should be altered. If the authors want to claim that the two proteins associate, FRET and/or co-IPs would be appropriate alternatives.
(4) In Fig. 5A, E1A is not a specific p300 inhibitor, as this has pleiotropic effects. A better approach would be to use siRNA against p300. In part C, a western should be added to indicate equal expression of the p300 derivatives.
(5) In Fig. 6A, is the interaction between p300 and SENP3 dependent on oxidative stress as it should be according to the results presented? This should be shown. In part C, the sumoylation is reduced and not eliminated as the authors state (wording should be changed).
(6) In Fig. 6E, the top panel of the p300 IP should be increased in size to reflect the same portion of the gel as the lower panel so that it is clear that the band seen is just the top of the p300 bands.
(7) A key addition to Fig. 6 is the demonstration that recruitment of p300 to HIF-dependent target genes is affected by depletion of SENP3. Ideally HDAC6 occupancy would also be probed.
(8) Is the activity of p300 towards other TFs also affected? The generality of these effects should be investigated.
(9) The data in Fig.7 are interesting but would be more convincing if further links to the mechanistic details could be provided. For example, demonstrating changes in HIF target genes would be useful and correlating these with changes in p300 occupancy (to provide these correlations, more targets than VEGF would need testing). An additional approach would be to compare the effects of p300 and p300deltaCRD in promoting angiogenesis. The prediction is that the latter should be much more potent.

1st Revision - authors' response 30 May 2009

We appreciate the constructive comments by the reviewers. A point-by-point response to the reviewers' comments is listed below.

Referee #1:

1. SENP3 clearly induces HIF activity by allowing binding of HIF to p300, as the numerous luciferase assays in the paper indicate. However, as SENP3 increases HIF-mediated transcription by modifying p300, a transcriptional regulator recruited by many transcription factors. Thus, a demonstration of HIF-dependence for SENP-induced transcriptional activity (Figure 4G), and promotion of tumor growth (Figure 7) should be provided.

Response:
In order to address the question of HIF-dependence of SENP3-induced transcriptional activity, we knocked down HIF1 in HeLa cells and showed by real-time PCR that SENP3-induced increase in VEGF, Glut-1, and CA-9 transcription were dependent in on HIF1 expression (data were added to Figure 4G, right panel, in the revised version). Furthermore, we also knocked down HIF1 in HeLa cells over-expressing SENP3 and inoculated these cells in our xenograft model. We showed that the HIF1 -knock down cells could not develop tumors and were defective in VEGF production and CD31 expression (data were added to Figure 7A and B in the revised version), indicating that SENP3-induced tumor growth and angiogenesis was also dependent on HIF1 .

2. Although Figure 7 provides evidence that SENP3 plays a role in HIF activation in a hypoxic

tumor environment, there is no direct experiment provided to indicate that SENP3 is induced by hypoxia, and is involved in hypoxic activation of HIF-mediated transcription. Experiments that demonstrate hypoxia-mediated induction of SENP3 would thus strengthen the paper Also, Does SENP3 induction by hypoxia involve ROS?

Response:
We provided two new experiments to show that SENP3 can be induced by hypoxia and SENP3 was involved in hypoxic activation of HIF-1-mediated transcription in a ROS-dependent manner. We showed that either hypoxia or cobalt chloride can induce SENP3 accumulation, which was reversed by NAC. We also showed that hypoxia-induced HIF-1 transcativation was significantly repressed in SENP3-knocked down cells (Figure 1 in "figures for reviewer 1").

Referee #2:

In summary, the part of the manuscript describing regulation of SENP3 by H2O2 is interesting and reveals a novel aspect in the control of SENP3 activity. However, the experiments aimed to link SENP3 function to HIF-1alpha regulation and in particular to p300 are not convincing in the current stage. One major flaw is that most data are based on overexpression of the respective proteins questioning the physiological relevance of the findings. In particular, several lines of evidence indicate that ectopic expression of SENPs affects SUMO conjugation in a rather general and unspecific way. Another weakness of the MS are the experiments, which wish to show that the sumoylation state of p300 affects its interaction with p300. Here, the technical quality of some key experiments is low and the data are far from convincing (see my detailed comments below).
Major points:
1. Figure 3C: Deconjugation of SUMO2 from HIF-1alpha by SENP3 is only shown under conditions where all components are overexpressed. A key experiment would be to demonstrate enhanced SUMO conjugation of HIF-1alpha, preferentially at its endogenous level of expression, upon depletion of SENP3.

Response:
We performed the suggested experiment and showed that endogenous HIF1 could be conjugated by SUMO2/3, and depletion of SENP3 did not change the pattern of SUMO2/3 conjugation (Figure 1 in "figures for reviewer 2"). The discrepancy between the endogenous system and the overexpression one (Figure 3C in the revised version) might be explained by the quantity and the role of SENP3 under basal condition. These results were not conflicting with our conclusions, because SENP3's ability to enhance the transcriptional activity of HIF1 was not dependent on the SUMOylation status of HIF1 (Figure 4I), instead, SENP3 deconjugates SUMOylated p300 to enhance HIF1 transcription (Figure 5A & B).

2. Figure 5G: Again, expression of HIF-1alpha target genes, such as VEGF or GLUT1 is only investigated in an experiment where SENP3 is overexpressed. It would be more important to see whether depletion of endogenous SENP3 affects the expression of these genes.

Response:
We thought that the reviewer meant Figure 4G, not 5G. In order to address the question of SENP3-dependence of HIF-1 target gene expression, we knocked down endogenous SENP3 in HeLa cells and showed that H2O2-induced HIF-1 transactivation of VEGF, Glut-1, or CA-9 was dependent on SENP3 expression (data were added to Figure 4H, right panel, in the revised version).

3. Figure 6B: These data question the specificity of ectopically expressed SUMO isopeptidases, since previous work by the same authors and others has demonstrated that SUMO modification of p300 can be reverted by ectopic expression of SENP1 or SENP2 (also known as SSP3) (Girdwood et al., Mol Cell 2003; Cheng et al, JBC, 2005). Now the authors show that expression of SENP3 also causes loss of p300-SUMO conjugates (Figure 6B). To support their conclusion that SENP3 is a physiologically relevant regulator of p300 the authors perform an RNAi experiment. However, the data aimed to demonstrate an increase in sumoylation of p300 upon RNAi-mediated depletion of SENP3 (Figure 6C, right) are not convincing. Under basal conditions there is virtually no difference in p300-SUMO3 conjugates in the presence or absence of SENP3. Additionally, one

would also like to see p300-SUMO1 conjugates, a proper loading control and data on sumoylation of p300 upon depletion of SENP1 and SENP2. Finally, showing data on endogenous p300 modified by endogenous SUMO upon depletion of SENP3 would make a more convincing case.

Response:
It is true that previous work by Girdwood et al. demonstrated SUMO1, SUMO 2, and SUMO 3 conjugations of p300, and that SUMO1 modification of p300 could be reverted by over-expression of SENP2 (SSP3) (Mol Cell, 2003). We also demonstrated that SUMO1 modification of p300 could be removed by overexpression of SENP1 (Cheng et al, JBC, 2005). The present work addressed de-conjugation of SUMO2/3 from p300 by SENP3. In order to clarify the specificity of SENPs by non-overexpression approach, and to confirm that SENP3 was a physiologically relevant regulator of p300, we did new experiments. We compared global SUMO2/3 conjugation with p300-specific SUMO2/3 conjugation in SENP1-/-, SENP2-/- MEF cells and SENP3 knocked-down HEK293 cells respectively. Results (S. Figure2 in supplementary data and Figure 6C in the revised version) showed that SENP1 and SENP2 could affect global SUMO2/3 conjugation pattern (see the lysates), but SENP1 was not responsible for de-conjugation of SUMO2/3 from p300, however, SENP2 may de-conjugate to some extent under basal condition (see SUMO2/3 conjugation on p300 by IP). Moreover, in SENP1-/- and SENP2-/- MEF cells H2O2 could induce SENP3 expression and simultaneous de-conjugation of SUMO2/3 from p300 (S. Figure 2), but in SENP3 knocked-down HEK293 cells, H2O2 can no longer de-conjugate SUMO2/3 from p300 (Figure 6C in the revised version). These data confirmed the specificity of SENP3 in the removal of SUMO2/3 from SUMOylated p300 upon mild oxidative stress.
The reviewer thought that the results shown in Figure 6C (right panel) were not convincing, because under basal conditions there was virtually no difference in p300-SUMO2/3 conjugates in the presence or absence of SENP3. In the revised version we repeated the experiment. Instead of overexpressing p300 and SUMO2/3, we used siRNA to knock down SENP3 to see endogenous p300 and its SUMO2/3 conjugation. SUMO2/3 conjugates of p300 appeared to be slightly increased in SENP3 knocked-down cells under basal condition. This is similar to the previous situation in overexpression experiments (old Figure 6C). We believe that the small difference under basal condition is true and reasonable. Under basal condition, SENP3 protein level is very low, which is supported by SENP3 expression (Figure 1A) and global SUMO conjugation results (Figure 3A). Thus, SENP3's activity to de-conjugate SUMO2/3 from p300 is modest under basal conditions and becomes obvious only upon H2O2 treatment, supporting our hypothesis that de-conjugation of SUMO2/3 from p300 occurs predominantly under oxidative stress. In addition, other SENPs might modulate p300 SUMOylation under basal condition, as SENP2 appears to be able to remove SUMO2/3 from p300 to some extent (S. Figure 2, right, second lane).
The reviewer suggested that showing data on endogenous p300 modified by endogenous SUMO upon depletion of SENP3 would make a more convincing case. Thus, we performed experiments to demonstrate that SENP3 removes SUMO2/3 from endogenous SUMOylated p300. These new data replaced the previous figure (Figure 6C).

4. Based on data in Figure 6D, E the authors propose a model in which sumoylation of p300 prevents binding to HIF-1alpha. However, none of the experiments clearly demonstrates the preferential binding of non-modified vs modified binding p300 to HIF-1alpha. The experiments shown in Figure 6D do only indirectly address this point. Moreover, in the lower part of Figure 6D it is completely unclear, why the unmodified p300 - which even after depletion of SENP3 is still rather abundant and thus should not be affected in binding to HIF-1alpha - does not bind to it. Similarly, in the experiment shown in Figure 6E, meant to directly show a differential binding of non-modified and modified p300 to HIF-1alpha, even the unmodified p300 from control cells does not bind HIF-1alpha.

Response:
SUMOylated p300 constitutes less than 5% of total p300, a situation common to many SUMOylated substrates. Furthermore, binding of p300 to HIF1 is not stoichiometric due to the multiple binding partners of p300. Thus, it is difficult to demonstrate non-binding of endogenous SUMOylated p300 to endogenous HIF1 . We agree with the reviewer that the original Figure 6D lower panel is unclear. In the revised manuscript, we aimed to demonstrate that endogenous p300 bound to endogenous HIF1 is, at least predominantly, non-SUMOylated. We used anti-HIF1 to co-precipitate endogenous HIF1 and endogenous p300 that was bound to it. We showed that only the non-

SUMO2/3 modified endogenous p300 can be co-precipitated with endogenous HIF1 . The supernatant that was cleared of HIF1 -bound p300 was then immunoblotted with anti-p300 or anti-SUMO2/3. We showed that SUMO2/3 modified p300 can be readily detected in the supernatant, although there was still a great quantity of non-SUMO2/3 modified p300 in the supernatant due to the overabundance of p300 Figure 6E in the revised version . These new experiments provide better proof for our hypothesis that HIF1 -bound p300 was non-SUMO2/3 modified p300.
In addition, this new experiment strongly indicated that p300 bound to HIF-1 is only very small portion of unmodified p300, which is evidenced by that loading of the supernatant is 20 times less than the precipitates in this figure. Data with half loading of the supernatant is provided in Figure 2 for reviewer 2.
In response to the comment about old Figure 6E as to why the unmodified portion of p300 derived from SUMO2/3-intact samples is not binding with HIF-1 , we found that not all of the unmodified p300 would bind to HIF-1 . In this figure the control was SUMO2/3-intact, thus with p300 having more SUMO2/3 conjugation. In general, this sample should have reduced binding, but should not be no-binding to HIF-1. The absence of the p300 blot in the bottom panel was due to insufficient exposure. To improve the quality of these data, we repeated the experiments and showed a larger gel that included SUMO bands (Figure 6F in the revised version). An increased in vitro p300/HIF-1 binding in SUMO2/3-depleted sample provided direct evidence for "the preferential binding of non-modified vs modified p300 to HIF-1 ".

5. Figure 7: I do acknowledge that the xenograft experiment with SENP3-expressing cells generates a strong phenotype. While this correlates with a reduction of p300 sumoylation, the question again arises to what extent overexpression of SENP3 generally affects cellular sumoylation.

Response:
As suggested, we added an IB figure showing the SENP3 expression and global SUMO2/3 modification in cells prior to inoculation to make xenografts (Figure 7A left, in the revised version). We also added another IB figure showing the SENP3 expression and global SUMO2/3 modification in two groups of xenografts (Figure 7C left and middle, in the revised version).

Additional points:
1. Figure 1F,G: One would also like to see ubiquitination of endogenous SENP3.

Response:
Because the available anti-SENP3 antibody is not suitable for IP, we have to use a tagged SENP3 to demonstrate ubiquitination of tagged SENP3 by endogenous ubiquitin (new Figure 1G). As shown, ubiquitinated SENP3 accumulated after MG132 treatment and was marked reduced when H2O2 was added.

2. Figure 2: The effect of H2O2 should also be tested on the localization of endogenous SENP3.

Response:
We did the immunofluorescence experiment using anti-SENP3 antibody as suggested (see S. Figure 1).

3. Figure 3B is of very limited information. It just shows that - like HIF-1alpha - a subfraction of ectopiclly expressed SENP3 is found in the nucleoplasm.

Response:
This figure aimed to show that SENP3 interaction with HIF-1 in the nucleoplasm is enhanced under oxidative stress. This enhanced interaction is also demonstrated by a new co-IP data ("Figure 1 for reviewer 3").

4. The authors mention that "p300 is reported to have a SUMO1 modification (Girdwood et al, 2003), but whether it has SUMO2 or SUMO3 modification is not clear." However, Girdwood et al. did already show modification of p300 by all three SUMO paralogs.

Response:
We corrected this oversight in the new text.

Referee #3 (Remarks to the Author):

1. Can the authors rule out that the effects they see are not due directly or indirectly to a change in SUMO-2/3 processing rather than SUMO conjugation? Similarly, can the authors be sure that there are no effects on the SUMO conjugation pathway itself following oxidative stress?

Response:
We did experiment showing SUMO2/3 modification pattern in cells with depleted SENP3 by siRNA. SUMO2/3 monomers were decreased in SENP3 knockdown cells, indicating an increased conjugation. But un-processed SUMO2/3 monomers (usually on the top of the monomer band, labeled with , in SENP3 knockdown cells) were not observed (figure 1 in "Figures for reviewer 3"). As SENP3 has not been reported to function in SUMO procession (Miko lajczyk J. et al, JBC, 2007, 282: 26217), our result can be due to either that SENP3 has no function in SUMO2/3 procession, or that SENP3 is not completely depleted by siRNA in our experiment.

2. In Figure 1A, SENP3 stabilisation is not seen at 5 mins and plateaus at 30 mins as stated in the text. It should be 30 and 60 mins respectively.

Response:
Changed as suggested.

3. In Figure 2B, it cannot be concluded that SENP3 and HIF1 colocalise as HIF1 is not in foci. High resolution confocal microscopy might help but otherwise the conclusions should be altered. If the authors want to claim that the two proteins associate, FRET and/or co-IPs would be appropriate alternatives.

Response:
We agree with the reviewer and have changed the colocalization designation in the text. We also provided a co-IP data according to the reviewer's suggestion ("Figure 2 for reviewer 3"). It showed that overexpressed HIF-1 and SENP3 could interact, and this interaction is enhanced upon H2O2 exposure.

4. In Figure 5A, E1A is not a specific p300 inhibitor, as this has pleiotropic effects. A better approach would be to use siRNA against p300. In part C, a western should be added to indicate equal expression of the p300 derivatives.

Response:
We agree with the reviewer that E1a may have a pleiotropic effect. In a new experiment, we used siRNA against p300 in SENP3 overexpressing cells to show that expression of HIF1 target genes were greatly repressed (data are added to Figure 5B in the revised version). We also added an IB figure to show equal expression of p300 derivatives in Figure 5C as suggested.

5. In Figure 6A, is the interaction between p300 and SENP3 dependent on oxidative stress as it should be according to the results presented? This should be shown. In part C, the sumoylation is reduced and not eliminated as the authors state (wording should be changed).

Response:
It is true that the interaction between p300 and SENP3 can be detected by IP in the absence of H2O2 exposure. This is probably due to the fact that overexpression of SENP3 may mimic the effect of H2O2 on the accumulation and redistribution of SENP3. We agree with the comments about Figure 6C and change the text accordingly.

6. In Figure 6E, the top panel of the p300 IP should be increased in size to reflect the same portion

of the gel as the lower panel so that it is clear that the band seen is just the top of the p300 bands.

Response:
Figure 6E became new Figure 6F and was modified as suggested.

7. A key addition to Figure 6 is the demonstration that recruitment of p300 to HIF-dependent target genes is affected by depletion of SENP3. Ideally HDAC6 occupancy would also be probed.

Response:
We added a ChIP data showing that binding of p300 to the HRE DNA is increased by H2O2 exposure and decreased by SENP3 depletion (data is shown as new Figure 6G). We were not able to probe HDAC6 in these ChIP samples due to unavailability of HDAC6 antibody. However, our previous data demonstrated that HDAC inhibitor TSA could inhibit HIF-1 transactivation, which is consistent with other's findings that HDAC6 could promote HIF-1alpha stabilization and transcriptional activity (Nature Medicine, 2001,7:437; Cancer Res 2006; 66:1814). Moreover, SENP3 could still boost HIF-1 transactivation in cells treated with TSA, indicating that SENP3'effect is independent of HDAC (Figure 3 in "Figures for reviewer 3"). In contrast, as Girdwood D et al. reported, SUMOylated CRD recruits HDAC6 to ensure the transcriptional repression activity (Molecular Cell, 2003, 11: 1043). Given that these clues lead to contradictive conclusions towards HIF-1-p300 relation, the role of HDAC in SENP3-enhanced HIF-1 transactivation remains to be clarified in the future work.

8. Is the activity of p300 towards other TFs also affected? The generality of these effects should be investigated.

Response:
Based on the literature (for example, Freedman SJ et al, PNAS, 2002), we chose several TFs using available luciferase reporters to investigate the effects of SENP3 towards the non-HIF1 TFs. Results were shown in "Figure 4 for reviewer 3". P53, NF- B, and Stat3, but not AP-1, were affected by SENP3.

9. The data in Figure7 are interesting but would be more convincing if further links to the mechanistic details could be provided. For example, demonstrating changes in HIF target genes would be useful and correlating these with changes in p300 occupancy (to provide these correlations, more targets than VEGF would need testing). An additional approach would be to compare the effects of p300 and p300deltaCRD in promoting angiogenesis. The prediction is that the latter should be much more potent.

Response: We did a real time-PCR experiment showing expression of HIF target genes, such as VEGF, Glut-1 and CA-9 in different xenograft tissues (S. Figure 3 in supplemental data). These results provided further mechanistic detail to our hypothesis. However, we did not carry out overexpression of p300 or p300deltaCRD in the xenograft model because endogenous p300 is abundant and ectopically expressed p300 derivatives may not be able to cause phenotype change. Thus, silencing the endogenous p300 may be required. However, silencing of p300 may reduce cell survival. To address the issue of p300 in angiogenesis, we carried out experiments in cultured cells. First, endogenous p300 was knocked down by siRNA, and then siRNA-resistant full-length p300 or p300deltaCRD p300 were transfected in HeLa cells. Then, expression of three target genes was determined by real-time PCR. We were able to show results that support the reviewer's prediction that p300deltaCRD would promote HIF-1 target gene expression more strongly (data are shown as "Figure 5 for reviewer 3").

2nd Editorial Decision 22 June 2009

Thank you for submitting your revised manuscript. It has now been seen once more by the original referees 2 and 3, and I am happy to inform you that both of them consider the manuscript

significantly improved and most of their original concerns satisfactorily addressed. We should therefore be able to accept it for publication after a few remaining points have been addressed (see referee 3's comments below). In this respect, although referee 3 indicates a few outstanding experiments, s/he would not insist on their additional incorporation. However, s/he also requests a number of editorial changes, regarding some of the stated conclusions as well as the request to include some of the "referee only" supplementary data for the readers' information also as "real" supplemental figures.

I would thus like to ask you to make the requested editorial changes to the manuscript in a last round of revision, and to return the paper to us as soon as possible. When resubmitting the re-revised version, please also make sure to upload individual files for all of the main figures, to incorporate some of the "referee only" data into the supplement as requested and to remove the remaining "referee only" files. Once we will have received this final version, we should then be able to proceed with the acceptance of your paper.

I am looking forward to receiving your final version.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

In their revised manuscript Huang et al. have addressed the major points I had raised on the initial version of their manuscript. My major concern was that many experiments were performed by ectopic overexpression of SENP3, which may affect SUMO modification in a rather general and unspecific way. The authors address this point by adding several experiments, where they now additionally target SENP3 by RNAi. These data strengthen the manuscript considerably (for example new Figures 4H or 6C). Moreover, the authors have modified the part of the manuscript, which reports the impact of p300 sumoylation on HIF1 binding. Here they have repeated several experiments and now provide more convincing data to support their model.

Referee #3 (Remarks to the Author):

This is a much improved paper. However, there are a few areas that have not been fully addressed and other places where the text still needs changing. Also, some of the figures that were provided "for referees" really should be incorporated into the manuscript and discussed so that they are available to the readers.

Outstanding issues:
(1) Original point 3. The title of the section and the title of the legend still say SENP3 and HIF1 interact. This text needs altering as there is no evidence provided in the paper to substantiate this claim. Data is provided "for the referee" which does show that they can interact by co-IP (with overexpressed proteins) and this would need incorporating to begin to make these sorts of statements. However, H2O2 cannot be said to affect the interaction because there is more SENP3 in the input in lane 3. Thus any conclusions about inducible binding would need tempering.

(2) Original point 5. The authors have not shown that oxidative stress enhances interactions between p300 and SENP3 as requested. They argue that SENP3 overexpression mimics this due to mislocalisation. However, surely looking at endogenous SENP3 would resolve this problem, even if over-expressed p300 is needed for sensitivity purposes. SENP3 would then be relocalised following oxidative stress and interactions enhanced.

(3) Original point 8. I previously requested that the effect of SENP3 on other TFs controlled by p300 was shown. The authors have done this but the effects are not the same on all TFs. ie some go up, some go down and some are unaffected. This data should be included in the current manuscript (rather than as "data for referee") and the discrepancies discussed ie why the same thing does not happen as predicted.

(4) Original point 9. The data shown as "Fig.5 for reviewer" should be included and discussed in the context iof the current manuscript as this provides important corroborative information.

2nd Revision - authors' response 25 June 2009

We have revised the manuscript according to reviewer 3's suggestions as detailed below.

(1) Original point 3. The title of the section and the title of the legend still say SENP3 and HIF1 interact. This text needs altering as there is no evidence provided in the paper to substantiate this claim. Data is provided "for the referee" which does show that they can interact by co-IP (with overexpressed proteins) and this would need incorporating to begin to make these sorts of statements. However, H2O2 cannot be said to affect the interaction because there is more SENP3 in the input in lane 3. Thus any conclusions about inducible binding would need tempering.

Revision: We included the co-IP data as figure S2, and also added a sentence in the text to describe the result and changed the section title to "SENP3 participates in H2O2-induced global changes in SUMO2/3 modification and can regulate the SUMOylation status of HIF-1".

(2) Original point 5. The authors have not shown that oxidative stress enhances interactions between p300 and SENP3 as requested. They argue that SENP3 overexpression mimics this due to mislocalisation. However, surely looking at endogenous SENP3 would resolve this problem, even if over-expressed p300 is needed for sensitivity purposes. SENP3 would then be relocalised following oxidative stress and interactions enhanced.

Revision: We added a sentence to the discussion that "oxidative stress causes re-distribution of SENP3 from the nucleolus to the nucleoplasm, where SENP3 can regulate the SUMOylation status of p300."

(3) Original point 8. I previously requested that the effect of SENP3 on other TFs controlled by p300 was shown. The authors have done this but the effects are not the same on all TFs. ie some go up, some go down and some are unaffected. This data should be included in the current manuscript (rather than as "data for referee") and the discrepancies discussed ie why the same thing does not happen as predicted.

Revision: The effects of SENP3 on selected TFs controlled by p300 were shown in figure S5. We added the following paragraph to the results.
"As p300 serves as co-activator of multiple transcriptional factors (TFs), based on the literature (Freedman SJ et al, PNAS, 2002), we chose several TFs and used available luciferase reporters to investigate whether SENP3 affected their transcriptional activity. p53, NF- B, and Stat3, but not AP-1, were affected by SENP3, but the effect varies (Figure S4). This indicates that de-SUMOylation of p300 by SENP3 is not necessarily beneficial for all TFs. In addition, some of these TFs themselves may be direct substrates of SENP3, which makes the regulation more complex."

(4) Original point 9. The data shown as "Fig.5 for reviewer" should be included and discussed in the context iof the current manuscript as this provides important corroborative information.

Revision: We include these results as Figure 6E and modified the text accordingly.

