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# PARP-1 Transcriptional Activity is Regulated by Sumoylation Upon Heat Shock

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**Review timeline:**

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|---|---|
| Submission date: | 13 March 2009 |
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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 17 April 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by two referees whose comments to the authors are shown below. We are still waiting for the report of referee 3 who is experiencing difficulties in getting back to us with his/her report as quickly as initially expected. As the other two reports are in fair agreement I am taking a preliminary decision on your manuscript now, based on the two enclosed reports, in order to save you from unnecessary loss of time. This decision is still subject to change should the third referee offer strong and convincing reasons for doing so.

As you will see the two referees consider the study as being a very interesting one in principle, but still feel that part of the data need to be strengthened considerably before they can support publication of the study. They both put forward a number of constructive suggestions how to improve the study. We will therefore be able to consider a revised version of this manuscript if you can address the referees' concerns in an adequate manner and to their satisfaction, and I would suggest at this point to start revising the paper along the lines suggested by the reviewers, and to also include satisfactory answers to any criticisms that might be raised in the third report, which we will forward to you as soon as we receive it.

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 as well as on the final assessment by the referees.

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

Yours sincerely,

Editor
The EMBO Journal

 

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Martin and co-workers report that poly(ADP-ribose) polymerase PARP-1 undergoes stress-inducible SUMO modification and that this modification is required for efficient transcriptional induction of hsp70.1. They also show that the stress-inducible PARP-1 sumoylation is facilitated by the SUMO E3-ligase PIASy, which is poly(ADP-ribosyl)ated and interacts with PARP-1. In fact, the PIASy, SUMO and Ubc9 (the E2 SUMO-conjugating enzyme) are all detected at the hsp70.1 promoter upon heat shock, whereas PARP-1 occupies the promoter constitutively and is released from it within 20-30 min of heat shock. Interestingly, RNF4, that has previously been shown to act as a poly-SUMO-targeted ubiquitin ligase, appears to be involved in the inducible hsp70.1 transcription through targeting PARP-1 for degradation by the ubiquitin-proteasome system. The authors conclude that the SUMO-specific ubiquitin E3-ligase RNF4 regulates both the abundance of sumoylated PARP-1 and the activation of hsp70.1 promoter in response to heat stress. The topic and obtained results are of great interest, highlighting the importance of complex post-translational regulation of the key proteins involved in the transcriptional control of gene expression under various cellular conditions. The Dejean laboratory has strong methodological background in analyzing different components of the SUMO machinery and they have made several fundamental discoveries in SUMO biology. In this study, they provide evidence for a novel mechanism by which PIASy-mediated SUMO modification of PARP-1 leads to its destruction by the RNF4-facilitated ubiquitin-proteasome system, thereby regulating the transcriptional activity of a stress-inducible gene, hsp70.1. A few severe technical shortcomings need to be addressed before the suggested conclusions are convincing (see below). Furthermore, understanding of the complex nature of the regulation through the two hierarchical post-translational modifications would be greatly improved in case a schematic model, based on the obtained results and previous studies, would be presented in the Discussion section of the manuscript.

Major comments:

1. A major finding of the manuscript, i.e. PARP-1-dependent effect on stress-inducible transcription of hsp70.1, is presented in Fig. 5A. Quantitative RT-PCR measurements of hsp70.1 expression were performed in untreated and heat-shocked PARP-1-/- MEFs expressing ectopically either wild type (WT) or K202R/K485R mutant (2KR) PARP-1. GAPDH was used as a control gene. The way the authors have chosen to present the data is strange, proper controls are missing and, therefore, the results are not convincing. It is not be necessary to interrupt the y-axis in panel A when they have not done so in panels B and C. Due to different levels of hsp70.1 expression in C (PARP-1-/- MEFs), WT and 2KR cells that are not exposed to heat shock, it seems that the differences in heat shock-induced effects are only minor. It would be important to see the original data obtained for GAPDH expression. In addition, the hsp70.1 expression analysis, using GAPDH as a control, should be available from PARP-1+/+ MEFs. The results shown in panels B and C, where PIASy was downregulated by siRNA in HeLa cells, are perhaps more obvious, but the differences are not necessarily PARP-1-specific as they can be due to other effects of PIASy. The authors should take serious measures to improve this part of the study and to provide stronger evidence for PARP-1 specificity.

2. Another major finding of the manuscript, i.e. stress-inducible RNF4-mediated ubiquitination of sumoylated PARP-1 is shown in Fig. 6. Here, it is absolutely necessary to show that SUMO-chains are formed also in vivo and, consequently, that RNF4 binding occurs in vivo. In these experiments, the 2KR mutant of PARP-1 should be included. In addition, it would be important to see the ChIP results of PARP-1 promoter binding (Fig. 5E) in the presence of RNF4 siRNA. The effects of siRNA-mediated RNF4 downregulation on hsp70.1 expression in heat-shocked cells are again minor given that the control (non-heat shock) levels are also lowered in the presence of RNF4 siRNA (Fig. 6E, F). Results on the ubiquitination of PARP-1 upon heat shock should be shown.

Minor comments:

 

1. The methodological procedures are not explained in detail. For example, the experiments using recombinant or in vitro translated proteins should be better described, since the patterns of sumoylated PARP-1 differ considerably between different figures.

2. In Fig. 3, analysis of all PARP-1 mutants should be shown in the same panel.

3. In Fig. 4C, the PARP-1 IP should be blotted with anti-SUMO to show that the modification is really due to sumoylation.

4. In the Discussion, if not already in the Intoduction, the authors should mention that heat shock-inducible SUMO-2 conjugation to PARP-1 was shown already earlier (Blomster et al., Mol. Cell. Proteomics, in press). The authors should also discuss their results in the light of another recent study by Petesch & Lis (Cell 134: 74-84, 2008), who elegantly showed that depletion of PARP-1 or its activity abolishes the loss of nucleosomes upon hsp70 activation. This is a very important paper to be referred to when the transcriptional induction of hsp70 is discussed. As indicated above, a schematic model of the previous work and the present results would be most helpful.

5. In general, one should indicate clearly what has been transfected to the cells, and the MW markers should be indicated in all figures.

Referee #2 (Remarks to the Author):

In this paper, the authors investigate the role of sumoylation in controlling PARP-1 activity in response to heat shock. This is an interesting topic and this paper provides important links between sumoylation and potential outputs in terms of parylation and transcriptional changes. In general, the data are of high quality and support the conclusions made. However, there are a few key areas where further experimentation is required to support the authors' claims. In particular, it would be useful to provide further data demonstrating the mutual functional consequences of sumoylation and parylation on PIASy and PARP-1 as this is the most novel aspect of this study.

Specific issues:
(1) Throughout the early part of the paper, PIASy is identified as an E3 ligase for PARP-1. However, the specificity of this activity should be determined. Ie other E3 ligases need testing to demonstrate that this is a specific effect. This included binding (Fig. 1), sumoylation (Fig. 2) and response to heat shock (Fig.3).

(2) In Fig. 1D, is it the loss of structural integrity of the RING domain or the loss of ligase activity that is crucial here- this should be discussed.

(3) In supplementary Fig. 1, there is an important issue. This figure does not prove that unmodified PARP-1 interacts and modification disrupts this, just that H2O2 controls the interaction by some means (maybe indirectly). To prove this, the experiment should be repeated with a mutant form of PARP1 that cannot be Parylated. An in vitro binding assay would also help.

(4) In Fig. 2A, why is the pattern of sumoylation the same if SUMO-2 is in chains and SUMO-1 is not in chains, as claimed in the discussion of Fig. 6?

(5) In Fig. 2F, molecular weight markers are needed. Why is there Parylated PARP-1 in lane 3 (and why is PIASy parylated)? No PARP-1 is added, so is it co-precipitated? If so, this implies that it was unParylated when immunoprecipitated as PIASy is not supposed to bind to Parylated PARP-1.

(6) What effect does Parylation have on PIASy activity as an E3 ligase? Also, does heat shock effect PIASy parylation?

(7) The data for the other mutants used in Fig. 3 should be added to supplementary data (ie rather than "not shown").

(8) In Fig. 4, a western blot is needed to prove the high molecular weight species are indeed SUMO conjugates. Also, is the parylation activity of PARP-1 affected by mutating the SUMO sites? This

 

should be tested in vitro for auto-parylation and transparylation activities.

(9) The key experiments in Fig. 5 need repeating with an "E-A" mutant, to rule out the differences occurring through alternative lysine modifications.

(10) The data in parts E-G should be repeated using real-time PCR. In particular, it is impossible to conclude anything about 5 min increases in PARP-1 binding from the single experiment depicted (an average of multiple experiments would need to be shown). Also, SUMO-2 should be analysed in ChIP in the same experiments.

(11) In Fig. 6D, it should be shown that heat shock increases ubiquitination in the absence of added RNF4 and that this is lost upon RNF4 depletion.

(12) Is sumoylated PARP-1 less stable than "bulk" PARP-1 as would be predicted by these results?

(13) Part F should be repeated in PARP-1 KO cells reconstituted with SUMO mutant forms of PARP-1. The prediction is that there should be no effect by RNF4 if the two are directly linked (at present, these might not be linked but be correlative).

(14) What is the role of HSF in these processes? Does it recruit PIASy or PARP-1?

Minor issues:
(1) The description of Fig. 1E is incorrect- the DNA binding domain alone is not tested.

(2) The top line in Fig. 6D was not readable.

Additional correspondence 27 April 2009

Thank you for your reply and for inviting us to revise our manuscript according to the reviewers' suggestions. We are happy that you and the reviewers consider our study interesting for possible publication in EMBO Journal.

Given the numerous and interesting points raised by the referees (more than 25 new experiments sugested in total, without referee #3), some of which, however, we consider well beyond the scope of the present work, we will do our best to address the principal issues raised. These are:

1. The demonstration of sumoylation leading to poly-chain formation on PARP1 in vivo;
2. Demonstration of the specific (or not) role of PIASy in mediating PARP1 sumoylation vis-à-vis the other PIAS E3 ligases;
3. Demonstration that a major part of the contribution of PIASy to transcriptional activation of hsp70 is mediated via PARP1;
4. Demonstration that RNF4 depletion impacts hsp70 promoter occupancy of PARP1;
5. A clearer demonstration of PARP1 ubiquitination upon heat shock;
6. Demonstration of the impact of sumoylation on PARP1 stability; and finally,
7. Demonstration of the role of PARP1 PARylation in regulating the interaction with PIASy.

We are conscious of the fact that some of these issues may be complicated by our finding that sumoylation renders PARP1 insoluble and thus may preclude some immunoprecipitation experiments, particularly upon heat shock. We are nevertheless confident in being able to satisfactorily improve the data of our manuscript, although we reckon that this will involve a number of time-consuming experiments.

We thank you again for your consideration.

 

Editor's response 08 May 2009

Thank you for your message and your suggestions for the revision. Let me first of all apologise for the delay in getting back to you with a reply. I was out of office the last two weeks. In the meantime I have now had a chance to look into the matter in depth.

First and further to my original decision letter I would like to mention that we have still not heard back from referee 3. We will not wait any longer for referee 3's input and the preliminary decision to consider a revised manuscript based on the two reports we received is therefore the formal initial decision on this manuscript.

Second, regarding your suggestions I would first of all like to point out that generally, we consider it important to address the main issues raised by referees rather than to perform every single experiment exactly as listed. Obviously, there are normally a number of different possibilities to address a given issue/concern experimentally in an adequate manner, which should all be fine. The seven suggestions you put forward sound fine to me. Let me also point out that from our side apart from strengthening the causality and specificity of the activity of PIASy in PARP-1 SUMOylation and on the downstream effects on hsp70 expression (along the lines you suggest) it would be particularly important to strengthen the RNF4/ubiquitination link and its functional consequences as suggested by referee 1 and as included in your list. Referee 2 also feels that the mutual effects of the PIASy/PARP-1 interaction, i.e. not only the functional consequences of PARP-1 SUMOylation, but also effect of parylation on PIASy activity should be analysed. While I appreciate that such a line of further experimentation may well go beyond the scope of this study it would still be good if this issue could be discussed in some depth unless you have some data in this direction already.

I also would like to point to the possibility to extend the deadline for submitting your revision to a maximum of 6 months in total upon request. After this time we would still be able to consider the revision, but we would need to take into account what has been published in the meantime.

I hope that these thoughts help you planning your revisions. Please do not hesitate to get back to us at any time should you have further questions or thoughts you would like to share with us or in case you would like to extend the deadline.

I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - authors' response 08 August 2009

Reply to: Referee #1 (Remarks to the Author):

*Martin and co-workers report that poly(ADP-ribose) polymerase PARP-1 undergoes stress-inducible SUMO modification and that this modification is required for efficient transcriptional induction of hsp70.1. They also show that the stress-inducible PARP-1 sumoylation is facilitated by the SUMO E3-ligase PIASy, which is poly(ADP-ribosyl)ated and interacts with PARP-1. In fact, the PIASy, SUMO and Ubc9 (the E2 SUMOconjugating enzyme) are all detected at the hsp70.1 promoter upon heat shock, whereas PARP-1 occupies the promoter constitutively and is released from it within 20-30 min of heat shock. Interestingly, RNF4, that has previously been shown to act as a poly-SUMOtargeted ubiquitin ligase, appears to be involved in the inducible hsp70.1 transcription through targeting PARP-1 for degradation by the ubiquitin-proteasome system. The authors conclude that the SUMO-specific ubiquitin E3-ligase RNF4 regulates both the abundance of sumoylated PARP-1 and the activation of hsp70.1 promoter in response to*

 

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*Major comments:*

*1. A major finding of the manuscript, i.e. PARP-1-dependent effect on stress-inducible transcription of hsp70.1, is presented in Fig. 5A. Quantitative RT-PCR measurements of hsp70.1 expression were performed in untreated and heat-shocked PARP-1-/- MEFs expressing ectopically either wild type (WT) or K202R/K485R mutant (2KR) PARP-1. GAPDH was used as a control gene. The way the authors have chosen to present the data is strange, proper controls are missing and, therefore, the results are not convincing. It is not be necessary to interrupt the y-axis in panel A when they have not done so in panels B and C. Due to different levels of hsp70.1 expression in C (PARP-1-/- MEFs), WT and 2KR cells that are not exposed to heat shock, it seems that the differences in heat shock-induced effects are only minor. It would be important to see the original data obtained for GAPDH expression. In addition, the hsp70.1 expression analysis, using GAPDH as a control, should be available from PARP-1+/+ MEFs. The results shown in panels B and C, where PIASy was downregulated by siRNA in HeLa cells, are perhaps more obvious, but the differences are not necessarily PARP-1-specific as they can be due to other effects of PIASy. The authors should take serious measures to improve this part of the study and to provide stronger evidence for PARP-1 specificity.*

As requested, we have added in Fig. 5A the data for the PARP-1+/+ MEFs in this experiment and presented the Y-axis without break. This shows that PARP plays an important role in the activation of HSP70 gene expression (as already published by Lis & Petesch, 2008, Cell, and Ouararhni et al., 2007, Genes Dev) and that restoration of PARP-1 in the -/- MEFs leads to (re-)activation, as expected (compare data obtained in control PARP-1+/+ with -/- MEFs), even though, due tothe lower expression of exogenous

| Cells | PARP-1 MEFs | +/+ | -/- | | |
|---|---|---|---|---|---|
| | pBABE- | C | C | WT | 2KR |
| Ct (GAPDH) | – heat shock | 17.692 | 17.135 | 17.472 | 17.146 |
| | + heat shock | 17.402 | 17.193 | 17.455 | 17.406 |

PARP-1, the rescue of PARP-1-/- MEFs with WT PARP-1 is incomplete). As requested, the GAPDH control Ct values are presented for the reviewer in the enclosed table: they do not explain the differences in HSP70.1 expression. Furthermore, we do consider that the PARP-1 dependent portion of the activation is significantly reduced by rescuing with the 2KR mutant instead of the WT PARP-1. We do agree that the difference in WT and 2KR activation is not heat shock-specific, but that the transcriptional effect, and consequently the effect of sumoylation, is strongly exacerbated by the stress. We thus consider this difference to be primarily SUMO-, rather than heat shock-specific, knowing that it is heat shock that leads to massive sumoylation. To further clarify this important point, we have added the following to the Discussion section (page 14):

> Yet, even in the absence of PARP-1, heat shock promotes significant promoter activation, thus suggesting the existence of PARP-1-independent mechanisms.

> Nevertheless, PIASy, sumoylation and RNF4 appear critically involved, as reducing their activity also reduces PARP-1-dependent promoter activation. That this occurs also in the absence of heat shock may suggest that the sumoylation of PARP-1 plays a similar role under basal conditions, but that heat shock, by strongly stimulating sumoylation, significantly enhances this effect.

PARP-1 specificity: we do not wish to claim that the PIASy effect on HSP70.1 promoter activation is wholly PARP-1-dependent. The reviewer, having raised this important issue, prompted us to include additional data (presented as Supplementary Fig. S5) showing that other factors present on the HSP70.1 promoter (besides the amply documented and cited HSFs), such as MEN1, Ku70/Ku80, and PRMT5, are all PIASy binding partners and some are known, or shown here, to be sumoylated. Clearly, therefore, the role of PIASy and sumoylation in regulating HSP70.1 promoter activation goes well beyond that of regulating PARP-1 function, i.e. the principal topic of the work presented here.

*2. Another major finding of the manuscript, i.e. stress-inducible RNF4-mediated ubiquitination of sumoylated PARP-1 is shown in Fig. 6. Here, it is absolutely necessary to show that SUMO-chains are formed also in vivo and, consequently, that RNF4 binding occurs in vivo. In these experiments, the 2KR mutant of PARP-1 should be included. In addition, it would be important to see the ChIP results of PARP-1 promoter binding (Fig. 5E) in the presence of RNF4 siRNA. The effects of siRNA-mediated RNF4 downregulation on hsp70.1 expression in heat-shocked cells are again minor given that the control (non-heat shock) levels are also lowered in the presence of RNF4 siRNA (Fig. 6E, F). Results on the ubiquitination of PARP-1 upon heat shock should be shown.*

- SUMO chains *in vivo*: We agree that the observation of 'laddering', as shown in the original Figure 4C (new Fig. 4E), does not exclude the possibility of multi-, or promiscuous modification by SUMO-2 (or by SUMO-1, for that matter). To demonstrate that these ladders most likely correspond to SUMO-2 chains, we have added a new figure (Fig. 4B) showing that overexpression of the poly-SUMO-2-specific de-sumoylating enzyme Senp6 (together with PARP-1, SUMO-2 and Ubc9) leads to the disappearance of such heat shock-induced laddering. Senp6 does not entirely abrogate PARP-1 sumoylation, as would be expected with Senp1 for example (effect shown in Figure 2B), because it does not cleave the SUMO-substrate bond as Senp1. Thus, the SUMO-modified PARP-1 species that remain in the presence of Senp6 correspond to multi-sumoylation of PARP-1, i.e. mono-sumoylation at several sites. Moreover, we have added a further panel to Figure 4 (panel F) showing that heat shock induces a robust laddering of endogenous PARP-1 by immunoprecipitation with anti-PARP-1 antibody and detection with anti-SUMO-2 antibody. To our mind this constitutes sufficient evidence that heat-shock induces poly-SUMO-2 modification of PARP-1. As requested by the reviewer we have included the 2KR SUMO-deficient mutant of PARP-1 in these experiments and we now show in the new Figure 4C that the abundance of highly sumoylated PARP-1 species induced by heat shock is significantly reduced when the PARP-1 2KR mutant is expressed instead of WT. In this context, we would also like to note that it may not be absolutely necessary to demonstrate poly-SUMO-2 chains. As Helle Ulrich has discussed in a recent review (Cell, 2008), one could also consider the possibility that RNF4 binds multiply-sumoylated substrates, and hence may even target (non-chain-forming-) SUMO-1 substrates. Our finding that PARP-1 is multiply modified also by SUMO-1 *in vitro* (see Figure 2A and new Figure 3B), makes PARP-1 an attractive candidate for such a mechanism and further, may explain why RNF4 knock-down affects non-heat shocked HSP70.1 promoter activity to a similar relative extent as that upon heat shock, as noted by the reviewer. Again, our point is that sumoylation's effects on PARP-1 function are not exclusive to heat shock, but are exacerbated by it.
- PARP-1/RNF4 interaction: in the revised version we have added a panel showing by coimmunoprecipitation of overexpressed RNF4 with PARP-1 in HeLa cell extracts (IP PARP-1, WB FLAG-RNF4; new Fig. 6A), that RNF4 interacts with PARP-1 *in vivo* and that, as reported for PML, this interaction does not require the integrity of RNF4 RING domain (use of the RNF4 C136/139/177/180S RING domain mutant deprived of ubiquitin E3 ligase activity published by H‰kli et al., 2005). We attribute the weakness of the obtained RNF4 signal in this experiment to the insolubility of sumoylated PARP-1 (see also Fig. 4D for this point) and the

necessity of carrying out extraction and immunoprecipitation under native, not denaturing conditions, as was possible for the immunoprecipitation of PARP-1 species modified by SUMO-2 (new Fig. 4F) or ubiquitin (new Fig. 6H).

Our findings that RNF4 interacts with PARP-1 (new Fig. 6A) and induces PARP-1 ubiquitination (old Fig. 6D, new Fig. 6G) show that RNF4 acts a ubiquitin E3 ligase for PARP-1. Importantly, we further show in this new Figure 6A and in the following new Figures 6B and 6C, that RNF4 induces PARP-1 degradation. We demonstrate that this degradation is dependent on RNF4 ubiquitin E3 ligase activity (new Fig. 6A and 6B), is mediated by the proteasome (use of the proteasome inhibitor MG132; new Fig. 6C), but is not due to PARP cleavage (new Fig. 6B). While PARP-1 K48-linked ubiquitination has been described previously (Wang et al., 2008), our work (strengthened by the new data) thus identifies for the first time a ubiquitin E3 ligase for PARP-1 and a caspase-independent mode of degradation of this protein.

- ChIP upon RNF4 siRNA: given the amount of material required, this experiment was not possible by transient RNF4 siRNA. Our attempts at using a stable RNF4 shRNA cell line (HeLa CL-6 used by Tatham et al., 2008) also failed because the level of RNF4 knockdown afforded by this cell line proved to be largely insufficient for conducting a meaningful experiment.

- Ubiquitination and heat shock: As requested by the referee, we have added a new panel to the revised version demonstrating that heat shock induces PARP-1 ubiquitination at the endogenous protein level (new Fig. 6H).

*Minor comments:*

*1. The methodological procedures are not explained in detail. For example, the experiments using recombinant or in vitro translated proteins should be better described, since the patterns of sumoylated PARP-1 differ considerably between different figures*.

More details on the *in vitro* methods have now been provided in the revised version in the figure legends. We do not fully agree that the band pattern varies considerably. Although the number and height of high-MW bands is variable in its extent (in function of *in vitro* reaction time), the patterns both *in vitro* (see Fig. 2A, Fig. 2D, new Fig. 3B and Fig. 6D) and *in vivo* (see e.g. Figs. 3C, 6F or S6) consistently show a major band at around 150 Kda (a di-modification) and another lesser, lower band between it and the unmodified PARP-1, with the latter being abolished by the K203R mutation. In this context, in the just published papers by the Sistonen (Blomster et al., 2009s; see their Fig. 2C) and Hottiger labs (Messner et al., 2009, FASEB J; their Fig. 2B), an additional band migrating similarly, as well as a doublet above the major band can also be seen. That the pattern complicates upon heat shock and/or at the endogenous protein level, we attribute to the concomitant ubiquitination and PARylation of PARP-1 as well as to promiscuous sumoylation at non-consensus sites.

*2. In Fig. 3, analysis of all PARP-1 mutants should be shown in the same panel.*

For this, we have re-done this *in vitro* experiment (new Fig. 3B). While not discussed (space non-permitting), this shows clearly that SUMO-1 and -2 show similar patterns at low modification levels, but that only the chain-forming SUMO-2 leads to very high-MW species at the long, one hour incubation time employed here.

*3. In Fig. 4C, the PARP-1 IP should be blotted with anti-SUMO to show that the modification is really due to sumoylation.*

To address the issue in this panel (now Fig. 4E), we have added a subsequent new panel (Fig.

4F; IP anti-PARP-1, WB anti-SUMO-2) showing clearly that PARP-1 is extensively modified by SUMO-2 upon heat shock *at the endogenous protein level.*

*4. In the Discussion, if not already in the Introduction, the authors should mention that heat shock-inducible SUMO-2 conjugation to PARP-1 was shown already earlier (Blomster et al., Mol. Cell. Proteomics, in press). The authors should also discuss their results in the light of another recent study by Petesch & Lis (Cell 134: 74-84, 2008), who elegantly showed that depletion of PARP-1 or its activity abolishes the loss of nucleosomes upon hsp70 activation. This is a very important paper to be referred to when the transcriptional induction of hsp70 is discussed. As indicated above, a schematic model of the previous work and the present results would be most helpful.*

During the preparation of the original, then the revised version of this manuscript, three papers on PARP-1 sumoylation have appeared in press (Blomster et al., Mol. Cell. Proteomics; Golebiowski et al., Science Signaling; Messner et al., FASEB J). The first two are methods papers identifying by a proteomic approach targets for heat shock-induced SUMO modification (among which PARP-1). The latter paper reports on PARP-1 sumoylation of a single site, K486, and raises in the discussion the precise issue we are addressing in our work: 'Understanding the balance between monosumoylation and polysumoylation of PARP1, as well as their functional differences will remain an exciting issue' . These three papers have now been cited in the Introduction, Results and Discussion section, where appropriate. The Petesch & Lis (2008) paper is now cited in the Introduction and mentioned again, together with the model (see below) in the Discussion.

Furthermore, as requested, we have included a new Figure (Fig. 7) depicting a model consistent with the results obtained in this manuscript. For clarity, this graphic does not include details on, for example, PARP-1 nucleosome/DNA/PAR interactions described in the literature, but these are discussed in the text.

*5. In general, one should indicate clearly what has been transfected to the cells, and the MW markers should be indicated in all figures.*

This has been done throughout, where appropriate.

Reply to : Referee #2 (Remarks to the Author):

*In this paper, the authors investigate the role of sumoylation in controlling PARP-1 activity in response to heat shock. This is an interesting topic and this paper provides important links between sumoylation and potential outputs in terms of parylation and transcriptional changes. In general, the data are of high quality and support the conclusions made. However, there are a few key areas where further experimentation is required to support the authors' claims. In particular, it would be useful to provide further data demonstrating the mutual functional consequences of sumoylation and parylation on PIASy and PARP-1 as this is the most novel aspect of this study.*

*Specific issues:*
*(1) Throughout the early part of the paper, PIASy is identified as an E3 ligase for PARP-1. However, the specificity of this activity should be determined. Ie other E3 ligases need testing to demonstrate that this is a specific effect. This included binding (Fig. 1), sumoylation (Fig. 2) and response to heat shock (Fig.3).*

As suggested by the reviewer, we have tested other SUMO E3s for their SUMO E3 ligase activity, both *in vitro* and *in vivo*.

We focused on the PIAS family members and, while PIAS-3 displayed no significant activity, PIAS1, PIASxa and -xß showed stimulatory activity on PARP-1 sumoylation *in vitro* (new Suppl. Fig. 2A). Further, we could observe that, like PIASy, PIASxa and -xß enhance heatshock-

induced PARP-1 sumoylation *in vivo* (new Suppl. Fig. 2B). Moreover, we demonstrate by co-immunoprecipitation that PIASxa also interacts with PARP-1 *in vivo* (new Suppl. Fig. 2C). Thus, other PIAS family members can function as SUMO E3 ligases for PARP-1. These data are discussed in the Results as follows (page 6):

> PIAS1, PIASxa and PIASxß, but not PIAS3, also displayed a stimulating effect on PARP-1 sumoylation *in vitro*. Moreover, PIASxa also interacts with PARP-1 in vivo (Suppl. Fig. 2), suggesting that other PIAS family members may function as SUMO E3 ligases for PARP-1 under these experimental conditions.

And page 7:

> Consistent with previous *in vitro* results, overexpression of PIASy (Figure 4G), PIASxa or PIASxß (Suppl. Fig. S2B, lanes 10 and 11) stimulated heat shockinduced PARP-1 sumoylation in co-transfection conditions.

It is possible that other SUMO E3 ligases not tested here, such as Pc2 or RanBP2, also enhance PARP-1 sumoylation. However, we show in the Fig. 4H (old Fig. 4E) that PIASy occupies a privileged position for this in the context of our study, i.e. heat shock, as knockdown of PIASy is sufficient for abrogating the major portion of heat shock-induced PARP-1 sumoylation.

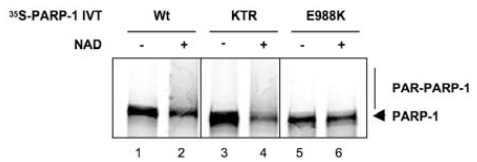
*(2) In Fig. 1D, is it the loss of structural integrity of the RING domain or the loss of ligase activity that is crucial here- this should be discussed.*

Our interest here was to map the domain(s) of PIASy interacting with PARP-1. Moreover, at present we are not aware of a RING finger mutation that would uncouple ligase function from structural integrity to address this issue in a meaningful way. Thus, the use of the word 'integrity' represents our cautious way of saying that we do not know whether ligase function, or just structure, is required for the interaction. Nonetheless, the C342F mutation is, in fact, ligase-dead (Bischof et al., 2006). To clarify this, we have amended this passage (page 5):

> To determine if the PIASy SP-RING finger domain is involved in the association, we expressed FLAG-HA-tagged PIASy wild-type (WT) or a derivative mutated in the SP-RING finger motif (Cys342Phe, abolishing E3 ligase activity; Bischof et al, 2006) in HeLa cells for immunoprecipitation experiments. Endogenous PARP-1 was detectable only in anti-HA immunoprecipitates from cells overexpressing wildtype PIASy (Fig. 1E, compare lanes 5 and 6), suggesting that the interaction requires the integrity and/or ligase function of the PIASy RING finger.

*(3) In supplementary Fig. 1, there is an important issue. This figure does not prove that unmodified PARP-1 interacts and modification disrupts this, just that H2O2 controls the interaction by some means (maybe indirectly). To prove this, the experiment should be repeated with a mutant form of PARP1 that cannot be Parylated. An in vitro binding assay would also help.*

We agree that H2O2, a global modulator of PARylation (and not just by PARP-1), may produce indirect effects. Accordingly, we performed the two suggested experiments.




Auto-PARylation of *in vitro* translated WT, K498/521/524R (KTR) and catalytically inactive E988K PARP-1.

- As shown in the figure added here for the reviewer, we generated the 'less-PARylatable' KTR (K498/521/524R) triple mutant that was described by Hottiger's lab (Altmeyer et al., 2009, Nucl

Acids Res.) and found it to undergo *in vitro* PARylation similar to the WT under our experimental conditions, and was hence unusable.

- An *in vitro* binding assay was performed and is now shown in new Fig. 1C. This shows that PIASy binds PARylated and non-PARylated PARP-1 equally well. To account for the observed differences between *in vivo* (Fig. S1) and *in vitro*, we thus modified the corresponding Results section as follows (page 5):

> To assess the impact of poly(ADP-ribosyl)ation on PIASy-PARP-1 interaction, we carried out a similar *in vitro* binding assay using auto-poly(ADP-ribosyl)ated PARP-1. Both poly(ADP-ribosyl)ated and non-poly(ADP-ribosyl)ated PARP-1 bound immobilized PIASy without apparent discrimination (Fig. 1C). In contrast, PIASy-PARP-1 co-immunoprecipitation *in vivo* was drastically reduced upon induction of poly(ADP-ribosyl)ation by treatment of cells with hydrogen peroxide, an effect that could be reversed by treatment of the cells with the poly(ADPribosyl) ation inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1-(2H)-isoquinolinone (DPQ) (Suppl. Fig. S1). Given that poly(ADP-ribosyl)ation does not affect PIASy-PARP-1 binding *in vitro*, these results suggest that stress-induced poly(ADP-ribosyl)ation can modulate the PIASy-PARP-1 interaction *in vivo*. Whether hydrogen peroxide-induced poly(ADP-ribosyl)ation of other substrates, or sequestration of poly(ADP-ribosyl)ated PARP-1 into PIASy inaccessible subcellular sites accounts for the reduced interaction, remains to be determined.

*(4) In Fig. 2A, why is the pattern of sumoylation the same if SUMO-2 is in chains and SUMO-1 is not in chains, as claimed in the discussion of Fig. 6?*

The similar pattern of modification of PARP-1 by SUMO-1 and SUMO-2 in this *in vitro* modification assay (Fig. 2A) is obtained after a short reaction time (15 min., now indicated in the corresponding figure legend). The revised Fig. 3B, another example of an *in vitro* reaction, shows clearly that at longer incubation times (60 min), SUMO-2 indeed forms more higher-MW conjugates than SUMO-1, which was also the case for Fig. 6D (old Fig. 6A). To demonstrate that the ladder of high MW conjugates of PARP-1 observed with SUMO-2 is due to the formation of SUMO-2 chains on PARP-1, we used Senp6, a de-sumoylase which specifically hydrolyzes SUMO chains but does not cleave the SUMO-substrate bond. Overexpression of Senp6 (together with PARP-1, SUMO-2 and Ubc9) leads to the disapperance of this heat shock-induced laddering, indicating that it is due to poly-SUMO-2 modification of PARP-1 (new Fig. 4B). The residual sumoylated PARP-1 species thus correspond to multi- (rather than poly-) modified PARP-1, i.e. PARP-1 mono-sumoylated at several sites (at least three, among which the identified lysines 203 and 486). Consequently, the similar pattern of PARP-1 modification observed in Fig. 2A after a short reaction time with SUMO-1 and with SUMO-2 is likely the consequence of promiscuous multi-sumoylation of the substrate, whereas, in Fig. 3B, the differential pattern, obtained after a longer reaction time, reflects the capacity of SUMO-2 to form chains (lanes 5-10, compare even with odd lanes).

*(5) In Fig. 2F, molecular weight markers are needed.*

-done

*Why is there Parylated PARP-1 in lane 3 (and why is PIASy parylated)? No PARP-1 is added, so is it co-precipitated? If so, this implies that it was unParylated when immunoprecipitated as PIASy is not supposed to bind to Parylated PARP-1.*

This is precisely the message of the figure: PIASy precipitates endogenous PARP-1 *in vivo* (which may be unPARylated at this point), such that addition of the PARylation mix (NAD+ etc.) in the subsequent *in vitro* reaction with the precipitates leads to PARylation of both PIASy and of the co-precipitated PARP-1 (Fig. 2F, lane 3).

*(6) What effect does Parylation have on PIASy activity as an E3 ligase? Also, does heat shock effect PIASy parylation?*

We have not explored the significance of PIASy PARylation further, but take this result to indicate that a functionally relevant, specific interaction between the two proteins occurs in vivo. We agree that the functional consequences and the regulation of PIASy PARylation are interesting questions to address in the future studies, but we feel that they are beyond the scope of the the present study. Nevertheless, we raise this interesting point in the revised Discussion (page 12):

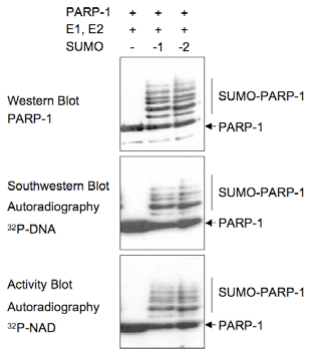
> Our demonstration here that PIASy may be poly(ADP-ribosyl)ated, besides confirming the physical interaction with PARP-1, could furthermore suggest that the activity of PIASy, like that of PARP-1 itself, is regulated by poly(ADPribosyl) ation. This could, for example, affect the DNA or chromatin binding of PIASy, as has been shown for p53 (Mendoza-Alvarez & Alvarez-Gonzalez, 2001), or its SUMO E3 ligase actvity. Conversely, recent *in vitro* results suggest that sumoylation does not affect poly(ADP-ribosyl)ation of PARP-1 (Messner et al, 2009). Nonetheless, given our finding that poly(ADP-ribosyl)ated PARP-1 exhibits reduced binding to PIAS *in vivo*, but not *in vitro*, it will be interesting to further explore the possible interplay between poly(ADP-ribosyl)ation and sumoylation.

*(7) The data for the other mutants used in Fig. 3 should be added to supplementary data (ie rather than "not shown").*

These results with the mutants K249R, K512R and K798R have now been shown in the new Supplementary Figure S3.

*(8) In Fig. 4, a western blot is needed to prove the high molecular weight species are indeed SUMO conjugates. Also, is the parylation activity of PARP-1 affected by mutating the SUMO sites? This should be tested in vitro for auto-parylation and transparylation activities.*

High-MW species: In this panel (Fig. 4C, now Fig. 4E), an anti-SUMO western blot would not be informative from a whole cell extract, but to address the issue, we have added a subsequent new panel (Fig. 4F; IP anti-PARP-1, WB anti-SUMO-2) showing clearly that PARP-1 is extensively modified by SUMO-2 upon heat shock at the endogenous protein level. Similar findings, albeit under overexpression conditions (and cited in the revised version), have also been reported recently by the Sistonen lab (Blomster et al., 2009, Mol. Cell. Proteomics).
PARylation: To address this question, we have added a supplementary figure (Suppl. Fig. S4) showing that the PARP-1 2KR (K203/486R) mutant exhibits the same auto-PARylation activity as WT. For the reviewer, we are including here an additional figure showing that PARP-1 sumoylation has no effect on its DNA-binding and poly(ADP-ribosyl)ation activity *in vitro*.



For this, products of recombinant PARP-1 sumoylated *in vitro*, separated by SDS-PAGE and blotted to nitrocellulose membrane and renatured, were visualized by Western blotting with anti-PARP-1 antibody (top panel), analyzed for their DNA-binding activity (Southwestern blot with 32P-DNA probe, middle panel) and poly(ADP-ribosyl)ation activity (activity blot with 32P-NAD, lower panel). Finally, Hottiger's lab has also shown recently that sumoylation does not affect PARP-1 PARylation (Messner et al., 2009, FASEB J).

*(9) The key experiments in Fig. 5 need repeating with an "E-A" mutant, to rule out the differences occurring through alternative lysine modifications.*

We agree this to be a potential caveat in the presented results, but given the recent publication from the Hottiger lab (Messner et al., 2009, FASEB J) investigating PARP-1 acetylation in SUMO modification site proximity, we reckon this issue has been addressed. We feel that the study of other potential lysine modifications is beyond the scope of the present work.

*(10) The data in parts E-G should be repeated using real-time PCR. In particular, it is impossible to conclude anything about 5 min increases in PARP-1 binding from the single experiment depicted (an average of multiple experiments would need to be shown). Also, SUMO-2 should be analysed in ChIP in the same experiments.*

We are confident that the semi-quantitative PCR results presented in Fig. 5 E-G are an accurate reflection of HSP70.1 promoter occupancy since the results of panel E (PARP-1) confirm exactly the previously published data from the Hamiche lab obtained by qRT-PCR (Ouararhni et al., 2006, Genes Dev.). Given that PIASy and Ubc9 show similar promoter occupancy kinetics, we reckon that SUMO-2 will follow these, but could not re-do this timecourse for lack of sufficient quantity of ChIP-grade anti-SUMO-2 antibody.

*(11) In Fig. 6D, it should be shown that heat shock increases ubiquitination in the absence of added RNF4 and that this is lost upon RNF4 depletion.*

As requested by the referee, we have added a new panel to the revised version demonstrating that heat shock induces PARP-1 ubiquitination at the endogenous protein level (new Fig. 6H). To test RNF4 effect on endogenous PARP-1 ubiquitination upon heat shock as in Fig. 6H, we used a stable RNF4 shRNA cell line (HeLa CL-6 used by Tatham et al., 2008). Our attempts failed, however, because the level of RNF4 knockdown afforded by this cell line proved to be largely insufficient for conducting a meaningful experiment.

Our findings that RNF4 interacts with PARP-1 (new Fig. 6A) and induces PARP-1 ubiquitination (old Fig. 6D, new Fig. 6G) show that RNF4 acts a ubiquitin E3 ligase for PARP-1. Importantly, we further show in this new Figure 6A and in the following new Figures 6B and 6C, that RNF4 induces PARP-1 degradation. We demonstrate that this degradation is dependent on RNF4 ubiquitin E3 ligase activity (new Fig. 6A and 6B), is mediated by the proteasome (use of the proteasome inhibitor MG132; new Fig. 6C), but is not due to PARP cleavage (new Fig. 6B). Although PARP-1 K48-linked ubiquitination has already been described (Wang et al., 2008), our work (strengthened by the new data) thus identifies for the first time a ubiquitin E3 ligase for PARP-1 and a caspase-independent mode of degradation of this protein.

*(12) Is sumoylated PARP-1 less stable than "bulk" PARP-1 as would be predicted by these results?*

Yes, it is. In the new Supplementary Fig. S6, we show by monitoring PARP-1 stability upon cycloheximide treatment that under non-stressed conditions the highly sumoylated forms of PARP-1 are less stable than the unmodified form (upper panel). Of note, this treatment does not cause any global disappearance of proteins conjugated to SUMO in our experimental conditions (lower panel), suggesting that disappearance of the higher-MW PARP-1-SUMO conjugates is not the consequence of simple de-sumoylation.

*(13) Part F should be repeated in PARP-1 KO cells reconstituted with SUMO mutant forms of PARP-1. The prediction is that there should be no effect by RNF4 if the two are directly linked (at present, these might not be linked but be correlative).*

True, if one assumes that all (or most) of RNF4's effect on HSP70 promoter activity is due to PARP-1 only. But we do not anticipate the PARP-1 2KR mutant to restore the effect of RNF4 siRNA completely, because this mutant is only partially sumoylation-deficient (cf. Fig. 2B, C). Moreover, we now show that other factors than PARP-1, such as MEN1, Ku70/Ku80 and PRMT5, present on the HSP70.1 promoter, are PIASy binding partners, with some known, or shown here, to be sumoylated (new Suppl. Fig. S5). This indicates that the role of sumoylation in regulating HSP70.1 promoter activation likely goes well beyond that of just regulating PARP-1 function and therefore, that the SUMO-triggered RNF4/ubiquitination pathway may also target other transcriptional regulators operating on this promoter. Thus, we do not expect that the effect of RNF4 is explained exclusively by its activity on PARP-1. This point is raised in the Results (page 9):

> To further characterize other possible effects on HSP70.1 promoter activation, we also tested the interaction of PIASy with other factors known to be present on this promoter. These included the DNA repair factors Ku70 and Ku80, the argine methyl transferase PRMT5 (Ouararhni et al, 2006) and the tumor suppressor MEN1, a homologue of the Drosophila Menin protein, that is recruited to the HSP70 promoter upon heat shock (Papaconstantinou et al, 2005). Indeed, all four of these proteins interacted with PIASy *in vivo* (Supplementary Figure S5A, B and C), with Ku70, Ku80 (Gocke et al, 2005; Yurchenko et al, 2006), as well as PRMT5 (Suppl. Fig. S5D) also being SUMO substrates themselves. These results suggest that PIASy and, by extension, sumoylation targets several other factors, besides PARP-1, present on the HSP70.1 promoter.

And in the Discussion (page 14):

> Our finding that other factors associated with heat shock protein promoters are sumoylated or are PIASy binding partners (e.g. MEN1, Ku70/80, PRMT5) suggests that SUMO-triggered, RNF4-mediated ubiquitination may similarly play a wider role by regulating the activity of other proteins besides PARP-1.

*(14) What is the role of HSF in these processes? Does it recruit PIASy or PARP-1?*

We agree that investigating the role of other factors, not even just the HSFs, present on heat shock-inducible promoters with regard to sumoylation, PIAS or PARP-1 interaction will be a fruitful endeavour. At present, however, we have not extended such studies beyond the inclusion of an additional Supplementary figure S5 showing that other previously demonstrated HSP70.1 promoter factors are sumoylated and/or PIASy binding partners (c.f. response 13 above). This suggests strongly that HSP70.1 gene expression is controlled by a complex network of factors present on this promoter. Understanding the interplay between these factors (and their sumoylation) remains an exciting issue for future studies.

*Minor issues:*

*(1) The description of Fig. 1E is incorrect- the DNA binding domain alone is not tested.*

True, this has been corrected as follows (Results, page 5):

> As shown in Figure 1F, both the PARP-1 amino-terminus that encompasses the DNA-binding domain, as well as the auto-modification (BRCT) domain, bound $^{35}$S-labelled PIASy protein in GST pulldown assays, suggesting that these domains, either together or separately, are critical for PIASy interaction.

*(2) The top line in Fig. 6D was not readable.*

-apologies: a PDF conversion error.

2nd Editorial Decision 21 August 2009

Thank you for sending us your revised manuscript. Our original referees have now seen it again. In general, the referees are now positive about publication of your paper. Still, referee 2 feels that there are a few issues that still need to be addressed (see below) before we can ultimately accept your manuscript. Specifically, he/she feels that point 9-11 (original numbering) have not been addressed adequately. Referee 1 has one minor issue. I know that you have replied to points 9-11 of referee 2 already in your point-by-point response. Still, as he/she feels strongly about these issues I would like to ask you to address/respond to these points and the issue brought up by referee 1 in an amended manuscript.

Furthermore, there is one remaining editorial issue that needs further attention.

Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans (for our records).

In the case of the present submission there are a number of panels that do not appear to fully meet these requirements: figure 2B, figure 4B

I therefore like to kindly ask you to send us a new version of the manuscript that contains suitably amended versions of these figures. I feel that it would also be important to explain the assembly procedure for these figures in the figure legends (i.e. that all lanes come from the same gel). Please be reminded that according to our editorial policies we also need to see the original scans for the figures in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final print version of the paper.

Please let us have a suitably amended manuscript as soon as possible. Thank you very much for your cooperation.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The revised manuscript is significantly improved and I agree with most of the authors' responses. However, one important issue needs to be resolved. The results shown in Fig. 5A do not support the conclusions made that the effect on hsp70.1 transcription is enhanced by heat shock. The ratio between non-heat shock and heat shock samples seems the same in WT and 2KR. The authors should revise the text accordingly or they should provide more convincing data.

 

Referee #2 (Remarks to the Author):

This is an improved paper and a lot of my points have been addressed adequately. However, tehre are a few outstanding issues which should be taken care of. The points below refer to the original criticisms that I raised.

(6) The authors have not looked at the effect of Parylation on the activity of PIASy. It would have added further significance to this manuscript but is not critical as the emphasis of the manuscript is in the other direction. nevertheless, demionstrating reciprocal regulatory activities would have been a good addition, although I can accept why the authors feel that this would be excessive.

(9) The recent paper by the Hottinger group shows that sumoylation affects acetylation most likely through a site distant from the site of SUMO conjugation. However, what this does not do is rule out the possibility of other lysine modifications occuring through the sumoylated lysine residue. It is for this reason that it is essential to use the "E mutants" to rule out such possibilities. Only after testing the E mutant and showing identical properties ie loss of sumoylation and similar functional defects, can it be completely concluded that SUMO is the relevant modification. I would therefore urge the authors to include this experiment, otherwise the conclusions need tempering.

(10) The authors need to show reproducibility by some means and this has not been done. It is often difficult to see identical time course data, especially semi-quantitatively (hence my suggestion of using real time). Nevertheless it would be reassuring to demonstrate reproducibility, even by indicating that repeat experiments have been done (which should be a pre-requisite for inclusion of the data anyway). This is currently not even indicated.

(11) The authors have shown increased ubiquitination as requested but not formally proven the involvement of RNF4. They use a stable cell line and conclude that the knockdown was not efficient to see an effect. However, in Fig. 6F, siRNA duplexes against RNF4 efficiently deplete RNF4 so it is unclear why they have not used this approach to demonstrate involvement of RNF4. Although highly suggestive of a role for RNF4, the overexpression experiments would be greatly enhanced by the inclusion of this simple experiment. If it does not work under the conditions of Fig.6F, where RNF4 is efficiently depleted, then the involvement of RNF4 has to be questioned.

2nd Revision - authors' response 25 August 2009

We have responded below to the remaining issues raised by the referees and have amended the text accordingly.

Concerning the figures: as you requested, we have revised Figs. 2B and 4B and have included the original scans, indicating the lanes used. Non-contiguous lanes are now clearly separated by blank spaces. Both figures were assembled from single blots and in the corresponding legends we have added: ‘Composite figure from a multi-lane, single blot with enhanced contrast.’ (pp. 24, 25).

Finally, in the abstract we have replaced ‘RNF4…regulates the abundance of SUMOmodified PARP-1 species’ with ‘RNF4…regulates the stability of PARP-1’, to more forcefully summarize the results of the revised version.

We hope you agree with the changes made in this revision and find that we have now satisfactorily addressed the remaining concerns. We thank you again for your consideration.

 