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Redox regulation of SUMO enzymes is required for ATM activation and survival in oxidative stress

Nicolas Stankovic-Valentin, Katarzyna Drzewicka, Cornelia König, Elmar Schiebel and Frauke Melchior

Corresponding author: Frauke Melchior, University of Heidelberg

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1st Editorial Decision 01 December 2015

Thank you again for submitting your manuscript on the functional significance of SUMO E1/E2 redox regulation for our consideration. We have now heard back from three expert referees, whose comments are copied below for your information. I am pleased to inform you that all of them consider this work potentially interesting and would in principle support publication after revision in The EMBO Journal. Nevertheless, while the reviewers all appreciate the first part of the manuscript identifying and characterizing a Ubc9 mutant that is no longer redox-sensitive, they retain significant concerns regarding the conclusiveness of the second part related to the DNA damage response and ATM activation. Since these concerns are clearly explained in the three reports, I will not repeat them in detail here, but would like to invite you to address them by revising the manuscript in line with the referees' comments and suggestions.

Thank you again for the opportunity to consider this work for The EMBO Journal! I look forward to your revision.

REFEREE COMMENTS

Referee #1:

Summary
In this study, the authors assessed the physiological relevance of the redox regulation of SUMO enzymes, which they had previously observed: the formation of a disulfide bond between the SUMO

E1 and E2 enzymes, which results in the transient/partial inactivation of the sumoylation pathway upon oxidative stress. This modification is present in different cell lines and a primary event in the sumoylation process. To address its physiological relevance, authors performed an in vitro screening to identify mutant forms of Ubc9, the E2 SUMO enzyme, that are resistant to oxidation. Together with the help of the Ubc9 structure, they identified a mutant localized in a loop proximal to the catalytic cysteine, which decreases the stability of the intermolecular disulfide bond linking Ubc9 to Uba2 without affecting intrinsic SUMO ligase activity. Expression of this mutant in the U2OS cell line confirmed that the Ubc9 mutant oxidation is impaired upon H2O2 treatment, as featured by a swift reduction of the intermolecular disulfide bond once formed. This translates in the decrease of cell fitness of the Ubc9 mutant upon oxidative challenge and 20% oxygen exposure. To explain this phenotype, the authors turned to the DNA repair pathway, which is known to be regulated by sumoylation and to be required for cell fitness during mild oxidative challenge. They show that the expression of the Ubc9 mutant drastically alters the kinetic of activation of the ATM pathway, leading to defective activation of the Chk2-H2AX axis. As ATM can be independently activated by DNA-strand break and oxidative stimuli, the authors ask which of these pathways is affected by the Ubc9 oxidation mutant. As Ubc9 mutant toxicity is prevented by the antioxidant NAC, the authors infer that the oxidative-dependent activation of ATM is preferentially affected by the lack of SUMO enzymes redox regulation.

This study provides a novel and interesting approach to define the physiological scope of their previously described SUMO enzymes redox switch. It is well written and the overall approach is adequately and rigorously undertaken. The study convincingly shows that one of the consequences of a defective Ubc9 redox switch is an impaired activation of the ATM pathway. Some elements still remain to be clarified though, concerning this later aspect.

1. How much of the Ubc9 D100A phenotype rely on ATM deficient activation?
Is it possible to rescue the fitness defect of Ubc9 mutant by artificially promoting activation of the ATM pathway? Is there any effect on the ATR pathway?

2. The authors suggest that ATM oxidative activation is specifically affected in Ubc9 mutants. However, the experimental scheme doesn't fully allow this conclusion. Indeed, the authors assess the sensitivity of the Ubc9 mutant to different chemotherapeutic drugs that partly operate through H2O2 production, and then show that the observed effect is rescued by NAC. This experiment shows that the redox regulation of SUMO enzymes is important for the response to AraC, which suggests that this oxidation event could be a relevant therapeutic target for cancer therapy. However, the downstream effect on ATM activation can hardly be inferred from data. First, is ATM activation indeed impaired in the Ubc9 mutant upon AraC/VP16 treatment and rescued by NAC? Second is the formation of ATM redox dimers impaired in the Ubc9 mutant upon H2O2 or drug treatment? In another angle, if ATM oxidative activation is preferentially affected in the Ubc9 mutant, is it possible to bypass ATM through the MRN-dependent pathway? Conversely, does Ubc9 mutant affect ATM activation by bleomycin. Since H2O2 activate ATM through either oxidative stress or DSB-dependent pathways, would it help to use diamide as an oxidant to measure a 'pure' oxidative activation of ATM in the context of Ubc9 mutant.

Minor comments:
1. In the in vitro activity assay, 1mM of H2O2 is used to make the redox dimer. This is a high dose of oxidant. Is this amount required to produce the disulfide in vitro, which contrasts with the 250 uM used in cells? Any explanations ?
2. p9 fig 2D is 3D

Referee #2:

This is an interesting study that draws upon previous observations by this group that oxidative stress transiently inactivates SUM01 and SUM02 enzyme activity by inducing disulfide bond formation between the catalytic cysteines. Here they employed a random mutagenesis screen to identify a SUM02 variant (Ubc9D100A) resistant to oxidative inactivation. This and other mutants were very thoroughly characterized in vitro and in vivo for activity. This mutant represented a very useful resource to investigate the significance of SUMO oxidation in damage signalling and cell survival.

They provide some evidence that failure to form the SUMO E1-E2 disulfide in mutant expressing cells results in a defect in the initiation or maintenance of ATM activity after H2O2 exposure. Finally they demonstrate that the D100A mutant sensitizes cells to chemotherapeutics that generate ROS.

Specific comments
This is a carefully carried out study that identifies a SUMO-E2 variant that is resistant to redox-switch (SUMOE1-E2 disulfide) a mechanism designed to protect the cell. Overall the quality of the data is good and the experimental plan provides new mechanistic insight into the redox regulation of SUMOE1/E2 in the response to oxidative stress. While the data point to a role for SUMOE1-E2 disulphide formation in the activation or maintenance of ATM activation, the quality of these results is not convincing. This is particularly true for the data in Fig 6.

1. They claim that Ubc9Wt and Ubc9Da show "striking differences in the timing and maintenance of DNA damage response". Reference to Fig6A reveals that this focuses almost entirely on a single time point at 30 min where the pS1981ATM signal is extremely weak for the U6C9D4 sample. This blot needs to be more convincing.

2. Moving from ATM activation to downstream phosphorylation of H2AX in Fig 6B. Here signalling is shown only at one time point 30 min. Should include 60 and 120 min.

3. Not clear why there is a discrepancy between comet assay data, similar levels of DNA damage in UbC9WT and UbC9DA after 30 min, and 3 hr data for damage foci where UbC9DA cells with foci was significantly lower than Wt. The latter suggests less damage. The data in Fig6D for the two markers of foci (γH2AX and 53BP1) do not correspond. Wt do but the data for DA mutant are very variable. Why were the same time points not chosen? Should be addressed.

4. Have they used any other antioxidants to look for protection of Ubc9DA cells?

Referee #3:

Based on their previous findings that SUMO E1 and E2 enzymes (Uba2 and Ubc9) can be transiently inactivated by reactive oxygen species (ROS) via formation of a disulfide bond between their catalytic cysteines, the authors, in this manuscript, aimed to reveal molecular details of this redox switch and its biological effects on cell signaling in response to oxidative stress induced by varied conditions. They first defined that Uba2-Ubc9 oxidation may play a general role in ROS dependent signaling. Using a series of reasonable methods, they then identified a catalytically fully active SUMO E2 enzyme variant (Ubc9 D100A) that was not inactivated by ROS, and proved that Ubc9 D100A was much better suitable to study consequences of impaired redox regulation. They thus had elucidated the sensing mechanism of this thiol switch. They further studied a role for reversible Uba2- Ubc9 oxidation in ROS-dependent events, focusing on DNA damage repair (DDR). They demonstrated that oxidation of the Uba2 and Ubc9 is required to maintain ATM activity upon oxidative stress, and that Ubc9 D100A sensitizes cells to chemotherapeutics. Because ROS play an intriguing role in cells, it is important to understand how those redox sensitive proteins work and are regulated by ROS. The manuscript did provide interesting novel insight into this aspect. The first part of work in the sites and mechanism of redox sensing is delicately conducted, though not straightforward, and the data are convincing and fairly interesting.

Major concerns:
The second part regarding to how this Uba2-Ubc9 oxidation is in light to signaling is not directly supportive to the first part. A gap exists in how the cellular effects induced by the replacement of Ubc9 with the mutant D100A can be attributed to disregulation of SUMOylation, globally or specifically in DDR proteins. A global SUMOylation pattern in "Ubc9 DA" cells, compared with "Ubc9 wt" cells is helpful. Given that SUMO E1 and Ubc9 have both been observed at sites of DNA damage (Galanty et al., 2009), and SUMOylation-deSUMOylation is essential events in the assembly and disassembly of repair complexes (Psakhye & Jentsch, 2012, Wu et al., 2014), it is needed to have a test, or at least a preliminary exclusion, whether a few members of DDR complex or ATM itself have changed SUMOylation. These results will better bridge the gap and strengthen

the significance the authors' findings.

Minor points:
Although the authors concluded that the rate of disulfide formation was undistinguishable between Ubc9 wt and Ubc9 D100A (Fig. 4B), it is visible that the mutant showed stronger and speedier oxidation in this figure (the thicker band at 5 min and overall). Is there contradiction with the notion that the mutant is resistant to oxidation? How would the authors explain this?

In Discussion on page 17, the text showed twice "during SUMO thioester transfer or disulfide bond formation..." Does it mean these cysteines may involve in both thioester transfer and disulfide bond formation? This reviewer might misunderstand, but the readers might be confused either.

Also on page 17, line 6, you used "far removed from..." Could it be "far remote from"?

1st Revision - authors' response 26 March 2016

Step-by-step response to the reviewers
We would like to thank the reviewers for their insightful and constructive comments and suggestions, which helped to strengthen and expand previous conclusions. Most importantly, we can now conclude that SUMO E1-E2 oxidation acts to maintain / rather than induce ATM phosphorylation. After a summary of all changes including many new experiments, please find our step-by step response.

Overview - changes in figures:

Figures 1-3, 5:	unchanged Figure 4: panel B and C replaced by new panel B (new time course blots and quantification, oxidation/reduction)
Figure 6:	Panel A unchanged (blots moved closer to each other), Panel B replaced by new experiment (longer time course), Panel C unchanged (blots moved closer to each other), Panel D new experiments (Hydroxyurea versus H2O2 tested for pATM), Panel E new experiment (Hydroxyurea effect on ATR).
Figure 7:	Panel A: IFs from old Figure 6D, Quantification of new experiments (more replicates, different time points) Panel B: New experiments (Comet assays after 0.5 and 7 h)
Figure 8:	Panel A: new experiment (Diamide induces disulfide, but not pATM), Panel B: new experiments (ocadaic acid), Panel C: old Figure 7A
Figure 9:	revised Model from old Figure 7B
Suppl Fig 1-4:	unchanged
Suppl Fig 5:	new (SUMO blots)
Suppl Fig 6-8:	unchanged, old Suppl Figures 5-7
Suppl Fig 9:	new (no disulfide after Hudroxyurea)
Suppl Fig 10:	new (Inhibition of ATM reduces γH2Ax Foci after H2O2)
Suppl Fig 11:	new (Diamide induces DTT-sensitive ATM oligomers)
Suppl Fig 12:	unchanged, old suppl Figure 8
Suppl Fig 13:	new (induction of ATM upon Ara-C treatment, but not with NAC)

Referee #1:
Summary .....
This study provides a novel and interesting approach to define the physiological scope of their previously described SUMO enzymes redox switch. It is well written and the overall approach is adequately and rigorously undertaken. The study convincingly shows that one of the consequences of a defective Ubc9 redox switch is an impaired activation of the ATM pathway. Some elements still remain to be clarified though, concerning this later aspect.

1. How much of the Ubc9 D100A phenotype rely on ATM deficient activation?

While we can certainly not exclude the possibility that the long-term toxicity of Ubc9 D100A in chronic or acute oxidative stress has multiple reasons, we now included two experiments that strengthen our conclusion that the ATM pathway is one major target: a) We prevented ATM activation in wt cells using the ATM inhibitor KU-55933 and quantified the percentage of cells with γH2AX foci upon H2O2 treatment (supplemental Fig. 10). In the presence of the inhibitor, wt Ubc9 cells show the same phenotype as Ubc9 D100A cells without inhibitor: the number of cells with γH2AX foci is much lower compared to the cells without treatment. Thus, direct inactivation of ATM resembles the phenotype that we observed in the D100A cells.

b) Conversely, we inhibited de-phosphorylation of ATM upon treatment with H2O2 by adding okadaic acid (as described in Goodarzi et al. (2004), Embo J) to Ubc9 D100A cells. This led to a significant increase of cells with γH2AX foci, similar to the number observed in wt Ubc9 cells (Figure 8B). This experiment reveals that events immediately downstream of ATM phosphorylation are not defective in Ubc9 D100A cells.

Is it possible to rescue the fitness defect of Ubc9 mutant by artificially promoting activation of the ATM pathway?

Due to intrinsic and technical problems, we have not been able to rescue fitness in clonogenic survival assays. Long-term analyses with okadaic acid cannot be done as it rapidly induces apoptosis. We tried several other long-term experiments unsuccessfully, such as depletion of a specific phosphatase scaffold subunit (PPP2AR1) by RNAi and overexpression of phosphomimetic Chk2 ((Shang et al (2014), Oncogenesis 3(2), e85). Moreover, overexpression of activated ATM or Chk2 does by no means reflect the local and transient activation of ATM upon DNA damage.
Is there any effect on the ATR pathway?

The ATR pathway is not affected. H2O2 did not induce pATR to significant levels, and there was no difference between wt Ubc9 and Ubc9 DA cells (data not shown). We thus activated the ATR pathway using hydroxyurea and analysed phosphorylation of Chk1 as a readout (Figure 6E). phospho-Chk1 levels are induced to comparable levels in wt Ubc9 and Ubc9 DA cells. Of note, hydroxyurea treatment does not induce the Uba2-Ubc9 disulfide (Supplemental Fig. 9).

2. The authors suggest that ATM oxidative activation is specifically affected in Ubc9 mutants. However, the experimental scheme doesn't fully allow this conclusion. Indeed, the authors assess the sensitivity of the Ubc9 mutant to different chemotherapeutic drugs that partly operate through H2O2 production, and then show that the observed effect is rescued by NAC. This experiment shows that the redox regulation of SUMO enzymes is important for the response to AraC, which suggests that this oxidation event could be a relevant therapeutic target for cancer therapy. However, the downstream effect on ATM activation can hardly be inferred from data. First, is ATM activation indeed impaired in the Ubc9 mutant upon AraC/VP16 treatment and rescued by NAC?

We now investigated phosphorylation of ATM upon AraC treatment (Supplemental Figure 13). pATM was indeed induced in wt cells but to a much smaller extent in DA cells. We could indeed detect a moderate increase in wt but not in Ubc9 D100A cells after 3 h, which was abolished by NAC.

Second is the formation of ATM redox dimers impaired in the Ubc9 mutant upon H2O2 or drug treatment?

DTT-sensitive oligomers of ATM are formed to similar extent in wt and in Ubc9 DA cells upon H2O2 or diamide treatment (Supplemental Figure 11). Of note, in contrast to what has been observed in HEK293T cells (Guo et al. Science, 2010), diamide does not lead to ATM phosphorylation in our U2OS cells.

More importantly, as outlined below, we have no evidence that SUMO E1~E2 disulfide formation promotes redox-dependent ATM activation (it is not upstream of ATM). Instead it seems to protect phosphorylated ATM from dephosphorylation.

In another angle, if ATM oxidative activation is preferentially affected in the Ubc9 mutant, is it possible to bypass ATM through the MRN-dependent pathway?

Here may be a misunderstanding - we never wanted to imply that our pathway affects oxidative activation of ATM. We described the two known pathways of ATM activation (via the MRN pathway and via direct ATM oxidation) and left it open whether SUMO E1~E2 oxidation is upstream of either pathway, represents a novel branch of ATM activation, or acts downstream of ATM activation by protecting the activated form of ATM.

With the help of the reviewer's suggestions, we now provide strong evidence that it is the protection of the phosphorylated species and not the activation that is impaired in our Ubc9 D100A cell line.

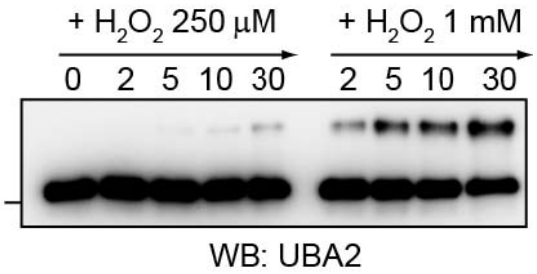
Conversely, does Ubc9 mutant affect ATM activation by bleomycin.
Bleomycin is known to induce ROS production locally (Mawatari et al. (2008), Aisa Pac J Clin Nutr, Gao et al. (2009), Free Radical Biology & Medicine) and is thus not suitable to distinguish a) DNA damage only effects from b) ROS only effects and from c) ROS + DNA damage effects.
To distinguish these scenarios, we followed the suggestion of this reviewer to test diamide (see below) and we tested Hydroxyurea. Hydroxyurea does not induce E1~E2 disulfides, but can activate ATM. As shown in the new Figure 6D, ATM is phosphorylated to comparable levels in Ubc9 wt and Ubc9 DA cells. We conclude from this and the diamide experiment described below that Ubc9 DA cells are affected only when ROS and DNA damage occur simultaneously.

Since H2O2 activate ATM through either oxidative stress or DSB-dependent pathways, would it help to use diamide as an oxidant to measure a 'pure' oxidative activation of ATM in the context of Ubc9 mutant.

We thank the reviewer for this suggestion, which helped significantly to better understand the role of the E1~E2 disulfide in ATM phosphorylation. Diamide induced a rapid and very efficient Uba2~Ubc9 disulfide, which was more rapidly formed - but also much less stable - in Ubc9 DA cells compared to wt cells. During the time window where the Uba2~Ubc9 disulfide was present we did not observe phosphorylation of ATM. From this, we conclude that E1-E2 disulfide formation is not required for phosphorylating ATM.

Minor comments:
1. In the in vitro activity assay, 1mM of H2O2 is used to make the redox dimer. This is a high dose of oxidant. Is this amount required to produce the disulfide in vitro, which contrasts with the 250 µM used in cells? Any explanations ?

SUMO E1~E2 disulfide formation is indeed much slower and less sensitive in vitro than in cells, especially when one works with endogenous enzyme concentrations (100 -300 nM). This suggests that the formation of the disulfide is an assisted process in cells. We envision two possibilities that we will explore in the future: either oxidation is facilitated by enzymes such as peroxiredoxins, or unknown scaffold proteins locally enrich SUMO E1 and E2 enzymes in an orientation that favors disulfide formation.



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thank you for pointing this out

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This is an interesting study that draws upon previous observations by this group that oxidative stress transiently inactivates SUM01 and SUM02 enzyme activity by inducing disulfide bond formation between the catalytic cysteines. .....

Specific comments: This is a carefully carried out study that identifies a SUMO-E2 variant that is resistant to redox-switch (SUMOE1-E2 disulfide) a mechanism designed to protect the cell. Overall the quality of the data is good and the experimental plan provides new mechanistic insight into the redox regulation of SUMOE1/E2 in the response to oxidative stress.

While the data point to a role for SUMOE1-E2 disulphide formation in the activation or maintenance of ATM activation, the quality of these results is not convincing. This is particularly true for the data in Fig 6.

1. They claim that Ubc9Wt and Ubc9Da show "striking differences in the timing and maintenance of DNA damage response". Reference to Fig6A reveals that this focuses almost entirely on a single time point at 30 min where the pS1981ATM signal is extremely weak for the U6C9D4 sample. This blot needs to be more convincing.

Our original design of figure 6A seems to have led to a misunderstanding. There were two panels with different time points of the same experiment (in the revised figure 6A, we moved them closer to each other). At 30 min, there is no significant difference between wt and D100A cells. But at 60 min, 120 min and 240 min, we can clearly see the presence of Phospho-ATM in the Ubc9 wt cells, but not in the Ubc9 D100A. This is perfectly in line with a new experiment that compares pATM levels upon H2O2 and HU. This experiment is now added as Figure 6D.

1. *Moving from ATM activation to downstream phosphorylation of H2AX in Fig 6B. Here signalling is shown only at one time point 30 min. Should include 60 and 120 min.*

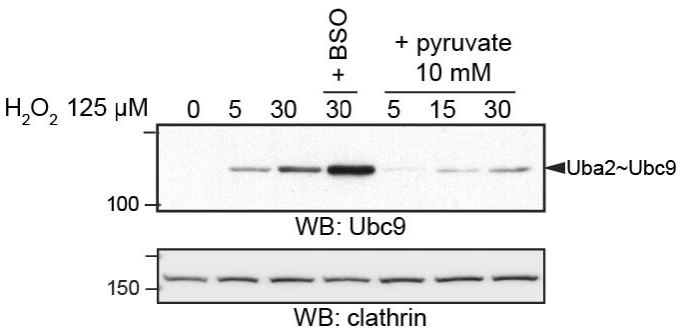
2. *Not clear why there is a discrepancy between comet assay data, similar levels of DNA damage in UbC9WT and UbC9DA after 30 min, and 3 hr data for damage foci where UbC9DA cells with foci was significantly lower than Wt. The latter suggests less damage.*

We agree and have repeated the experiment with time points up to 240 min (new Figure 6B).
This is one of the key points of the paper -even though Ubc9 D100A cells have as much damage as wt Ubc9 cells, they cannot maintain assembly of DNA damage repair foci (based on number of cells with 53BP1 and the gamma-H2AX foci).
To clarify this issue, we performed additional comet assays, in which we included a 7 h time point. Again, damage is very comparable. Nevertheless, the difference between wt and DA cells regarding 53BP1 and gamma-H2AX foci is highly significant.

The data in Fig6D for the two markers of foci (γH2AX and 53BP1) do not correspond. Wt do but the data for DA mutant are very variable. Why were the same time points not chosen? Should be addressed.

We repeated the γH2AX experiment with the same time points as the 53BP1 experiment and with more biological replicates. The conclusions remain the same - there are significantly less cells with foci in Ubc9 DA - compared to wt cells after H2O2 treatment.

4. Have they used any other antioxidants to look for protection of Ubc9DA cells?
No, we have not yet. N-acetyl cysteine increases the glutathione pool in cells and is a well-established tool in the redox field. We know from our published and current work that glutathione is the physiologically relevant antioxidant for the SUMO E1-E2 disulfide in cells, and thus feel that NAC is a very suitable tool. An alternative that we will explore in the future is pyruvate (which is present at 1 mM in standard cell culture media). As shown for the reviewer's attention in the figure below, pyruvate also counteracts disulfide formation by H2O2.



Referee #3:

.....

Because ROS play an intriguing role in cells, it is important to understand how those redox sensitive proteins work and are regulated by ROS. The manuscript did provide interesting novel insight into this aspect. The first part of work in the sites and mechanism of redox sensing is delicately conducted, though not straightforward, and the data are convincing and fairly interesting.
Major concerns: The second part regarding to how this Uba2-Ubc9 oxidation is in light to signaling is not directly supportive to the first part. A gap exists in how the cellular effects induced by the replacement of Ubc9 with the mutant D100A can be attributed to disregulation of SUMOylation, globally or specifically in DDR proteins. A global SUMOylation pattern in "Ubc9 DA" cells, compared with "Ubc9 wt" cells is helpful.

We thank the reviewer for the suggestion. We have now included immunoblots comparing SUMOylation patterns in wt Ubc9 and Ubc9 DA cells. As shown in supplemental Fig. 5, we did not observe any significant differences in the pattern of SUMO conjugates without or upon treatment with 250 µM H2O2. Because immunoblotting of total lysates detects only the most abundant SUMO targets, this finding does obviously not exclude that individual low-abundant SUMO targets differ between Ubc9 wt and D100A cells.

Given that SUMO E1 and Ubc9 have both been observed at sites of DNA damage (Galanty et al., 2009), and SUMOylation-deSUMOylation is essential events in the assembly and disassembly of repair complexes (Psakhye & Jentsch, 2012, Wu et al., 2014), it is needed to have a test, or at least a preliminary exclusion, whether a few members of DDR complex or ATM itself have changed SUMOylation. These results will better bridge the gap and strengthen the significance the authors' findings.

There is currently no evidence that ATM itself is the relevant SUMO target. SUMOylation of ATM has not been described so far, even in large unbiased SUMO proteomic screens, and we have not been able to detect SUMOylation of ATM after IPs.
Finding the relevant SUMO target(s) that are needed to be desumoylated for sustained ATM signalling will of course the next big step in our project. This will however be very challenging and is clearly beyond the scope of this manuscript:

a) many proteins change their SUMOylation status after treatment with H2O2. Some prominent examples have been published (e.g., PTEN, HIPK2), and from unpublished work that we carried out with Ron Hay, we have a list of 300 proteins that gain or loose SUMO within the first 15 min after H2O2 treatment in HeLa cells.
b) to identify those proteins that depend on SUMO E1~E2 oxidation, rather than on other H2O2 dependent mechanisms (inactivation of phosphatases, activation of kinases, inactivation or activation of SUMO proteases, activation of PIAS E3 ligases....), we need to compare the SUMO proteome of wt and Ubc9 D100A cells. However, to obtain sufficient cells expressing wt and mutant at endogenous levels for an unbiased SILAC approach is technically rather challenging. This is especially true for the mutant cells that are difficult to maintain, as up to 200 15 cm plates are needed for duplicate experiments with all controls to detect low abundant SUMO targets.
c) Once we find proteins that differ between wt and D100A cells, we need to identify those that are directly affected by SUMO E1~E2 disulfide formation. Again, this will not be

trivial, considering that many proteins are only modified upon recruitment to damaged sites. Lack of phosphorylated ATM may cause reduced SUMOylation of many DNA repair proteins, but these may not be the ones that explain why ATM phosphorylation is not maintained in mutant cells.

As described above, experiments done in response to the reviewers clarified one important aspect - we have now strong evidence that SUMO E1-E2 oxidation is needed to maintain phospho ATM. This also allowed us to discuss more clearly how ROS-induced deSUMOylation may contribute to DNA damage response, and we hope that this reviewer will be satisfied with this.

Minor points: Although the authors concluded that the rate of disulfide formation was undistinguishable between Ubc9 wt and Ubc9 D100A (Fig. 4B), it is visible that the mutant showed stronger and speedier oxidation in this figure (the thicker band at 5 min and overall). Is there contradiction with the notion that the mutant is resistant to oxidation? How would the authors explain this?

We thank the reviewer for pointing this out. We repeated the experiment with earlier time points. Indeed, the Ubc9 DA variant can be oxidized faster than the wt enzyme, both in vitro (new figure 4B) and in cells (Figure 5B, same as in first submission). One hypothesis for the dual effect (faster oxidation and faster reduction) is that the mutation of Ubc9 aspartate 100 to alanine, a much smaller and uncharged amino acid, improves access of hydrogen peroxide or diamide to the catalytic cysteines in the transiently interacting E1-E2 pair, which would result in faster oxidation. For the same reasons, this mutation may allow better access of the negatively charged glutathion to the oxidized E1~E2 disulfide.

In Discussion on page 17, the text showed twice "during SUMO thioester transfer or disulfide bond formation..." Does it mean these cysteines may involve in both thioester transfer and disulfide bond formation? This reviewer might misunderstand, but the readers might be confused either.

This is indeed the case - as we described in the introduction of our manuscript, the same two cysteines that are needed in E1 (Uba2/Aos1) and E2 (Ubc9) for SUMO thioester formation and transfer can form a disulfide with each other in oxidative stress. In consequence, the conformation in which both enzymes can be oxidized by H2O2 must resemble the conformation in which SUMO is transferred from the E1 cysteine to the E2 cysteine. E1 and E2 enzymes that are engaged in this disulfide are catalytically inactive as long as the disulfide persists.
We changed the discussion in some places for clarity and to incorporate new data.

Also on page 17, line 6, you used "far removed from..." Could it be "far remote from"?
We have corrected the manuscript.

2nd Editorial Decision 18 April 2016

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

REFEREE COMMENTS

Referee #1:

the authors have satisfactorily addressed the question raised in the initial evaluation

Referee #2:

Given that the authors state clearly that "SUMO E1-E2 oxidation acts to maintain rather than induce ATM phosphorylation, title might be modified to reflect this to "Redox-regulation of SUMO

enzymes is required for maintenance of ATM activity and survival in oxidative stress"

Referee #3:

The revised version of this manuscript clarified some critical points and made the findings more solid and conclusions more convincing. Although the links between the disulfide formation in Ubc9 and ATM signaling remain indirect, the reasons the authors argued for technical challenges are acceptable. The new figure 4B provided an important redox feature of the DA mutant, which is interesting and revealed more detailed mechanisms that will better illustrate redox regulation of SUMOylation.

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This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The experiments were performed at least in three independent replicates (except Suppl. Fig. 8A and Suppl. Fig. 10, n = 2)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	see p25
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes
Is there an estimate of variation within each group of data?	Yes, indicated on the figures (S.E.M or box plots (Fig. 5D, left panel))
Is the variance similar between the groups that are being statistically compared?	We used the Welch's t-test to take into account a possible unequal variance

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	see p21 and p22 for antibodies
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	see p23 for cell sources. Cells are regularly tested for mycoplasma contamination using the following kit: Venor GeM (Minerva Biolabs, 11-1050)

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA