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End-joining inhibition at telomeres requires the translocase and polySUMO-dependent ubiquitin ligase Uls1

Rachel Lescasse, Sabrina Pobiega, Isabelle Callebaut, Stéphane Marcand

Corresponding author: Stéphane Marcand, CEA

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 03 October 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the comments of three expert referees, which you will find copied below. As you will see, all three referees consider the study interesting and of potential importance, and would therefore support publication pending adequate revision. In this regard, while the reviewers agree that many of the arising further-reaching questions should be left for future work, they nevertheless point out a few aspects of the proposed model that should be more directly tested at this stage in order to strengthen the conclusions of the study. In particular, it would appear to be important to address the role of Uls1 SIMs (see referees 2 and 3), to add some insights into the NHEJ inhibition by Rap1 polysumoylation/Uls1 inactivation (referees 2 and 3), and to test the role of Rap1 poly-sumoylation in regulation of its levels at telomeres (referees 1, 2, 3).

Should you be able to provide some additional data to address these questions (as well as adequately respond to the various more specific queries of the reviewers), then we should be happy to consider a revised version of the manuscript further for publication. Since it is our policy to allow a single round of major revision only, please however make sure to diligently answer to all the points raised at this stage. We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you again for the opportunity to consider this work for publication, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this beautifully executed and clearly presented study, Lescasse and colleagues describe a novel aspect of telomere protection - the Uls1-dependent removal of poly-sumoylated Rap1 from chromosome ends. The experiments are well controlled and the main conclusions of the manuscript are well supported by the data. I strongly believe that this work will be received with considerable interest by researchers in several fields including telomere biology, DNA damage responses and protein modifications.

My only significant comment relates to the extent to which the model in figure 6 is supported by the data versus relying on inferences. I agree with the authors that a role for Uls1 in 'sweeping' polysumoylated Rap1 off the DNA is a likely possibility given earlier findings and the present manuscript. However, they have not shown that polysumoylated Rap1 accumulates on chromosomes and that Uls1 favors its removal from DNA. Neither Shah et al. 2010 nor Uzunova et al. 2007 provide evidence that the mutations at positions 975 and 1333 specifically reduce or abolish translocase and ligase activity. They may simply cause misfolding and destabilize the protein. Is there any separation of function data that would support that the mutations specifically abolish the respective function rather than making inactive protein? In the absence of such evidence it is speculative that Uls1 removes Rap1 from telomeres. This should be investigated in a follow-up study and it should be made clear in the current manuscript that this aspect of the model is speculative.

The identification and naming of a new domain in Uls1 as REPULS may be premature as no function has been assigned to this part of the protein and the deletion has no phenotype in the context of this study.

The ~100 fold difference in end fusions between RAP1 deletion and ULS1 deletion is further supported by the smear in RAP1delta (complex mixture of fusions) versus individual bands in the PCR from ULS1delta cells.

Referee #2 (Remarks to the Author):

In this study, Lescasse et al. show that the inactivation of the STUbl protein Uls1 impairs NHEJ inhibition at telomeres. Inhibition of NHEJ by Uls1 requires its translocase activity and it ubiquitin ligase activity. The requirement for Uls1 is bypassed by an Smt3 mutation preventing the formation of poly-SUMO chains. Because Rap1 has been reported by this group to be essential for telomere NHEJ inhibition and the existence of negative interactions between rap1-hypomorphic alleles and usl1 mutations, the authors explore the possibility that Rap1 is a target of Uls1. They show that Rap1 is polysumoylated (when SMT3 is overproduced) and that increased levels of polysumoylated Rap1 are obtained in the absence of Uls1. In agreement with these results, mutating K240 and K246 of Rap1 at the same time strongly decreases polysumoylation of Rap1, and bypasses the requirement of Uls1 in NHEJ inhibition. Otherwise, the rap1-2R mutant does not affect Rap1 core functions.

From these results they build a model in which Uls1 contributes to inhibition of NHEJ by preventing the accumulation of polysumoylated Rap1. The basic findings of this study are important advance and will be of general interest. They provide a (negative) function for the sumoylation of a telomeric protein, a field that is rapidly growing. However, there are several key questions left unanswered. Indeed, their model implies that:

1) polysumoylation of Rap1 affects Rap1 ability to inhibit NHEJ
2) Uls1 recognizes through its SIM motif polysumoylated Rap1
3) Uls1 through its translocase activity displaces polysumoylated Rap1 molecules from the telomeric DNA and target polysumoylated Rap1 to degradation

At this stage the MS is minimal and would be significantly strengthened if these points could be addressed. The proposed experiments are within the capabilities of this group.

Major questions:

1) What subpathway of NHEJ inhibition is affected by the inactivation of Uls1
2) What is the effect of mutating the SIM domain of Uls1
3) Is the polysumoylation of Rap1 or the absence of Uls1 associated to increased levels of Rap1 at telomeres. In the absence of this demonstration, the question can be also addressed by measuring the telomeric level of rap1-2R mutant.
4) Is Uls1 at telomeres?

Besides this question, one wonders what is the positive role of Rap1 sumoylation but this latter point would deserve a complete study. I will be satisfied if the authors can address the major points.

Other questions
1) In the His-SMT3 pulldowns, how much of the total sample is loaded for the input? In the pulldown lanes (in cells expressing HIS-SMT3), sumoylated Rap1 is clearly detected. Should it be detected in the input as well?
2) Growth defect is very mild in the rap1DAmP Uls1 double mutant. Could the authors measure Rap1 levels in the rap1-DAmP ? Could the authors better comment the phenotype of the rap1DAmP Uls1 in NHEJ inhibition (the double mutant shows less fusions than the uls1 alone).
3) Rap1 is essential, I could not figure out how the rap1 mutants were expressed in the mutant cells. Are the rap1 mutant integrated in the genome? Or carried by a plasmid that complements a rap1 strain? Expression of the mutant should be shown.

Overall, this is a very exciting paper

Referee #3 (Remarks to the Author):

This study examined the role of the STUbL and translocase Uls1 in the inhibition of NHEJ at telomeres. The findings that uls1 cells exhibit increased levels of telomere-telomere-fusions and that this defect is suppressed by lif1 nicely support a role of Uls1 in NHEJ inhibition at telomeres. Additional studies using point mutations in Uls1 suggest that both its Ubiquitin E3 ligase and the translocase activities are required for this role. The authors further suggest that such a role of Uls1 is mediated by removing poly-sumoylated forms of Rap1, a main inhibitor of NHEJ, from the telomeres. This notion is supported by two results. First, in the absence of poly-SUMO chains or in rap1-2KR cells the level of telomere-telomere fusions is decreased in uls1 cells. Second, depletion of Uls1 results in the accumulation of poly-sumoylated Rap1. The manuscript is clearly written and the findings are exciting and suitable for publication at EMBO J. A few points do need to be addressed by either re-writing and/or additional experiments. In particular, several aspects of the model can be tested, and the authors may have already done so.
1. The authors' data suggest that poly-SUMO chains are involved the observed NHEJ inhibition. To drive the point home, it will be informative to test if the four previously identified SIMs in Uls1 are required for its telomere functions.
2. Do Siz E3 ligases mutations suppress the telomere fusions in uls1 ? If yes, this would further strength the notion that removing sumoylated Rap1 is the reason for the suppression by rap1-KR.
3. While this reviewer does not think that this paper necessarily has to provide more mechanism results to be published. Important points need to be discussed (even better if there can be some data). For example, how does sumoylation of Rap1 impair its function in NHEJ inhibition? Have the authors tested the interaction with the factors that Rap1 works with, e.g. Rif2 and Sir4? Are they still recruited? In addition, as the proposed mechanism is that Uls1 acts through ubiquitination of sumoylated Rap1 and proteasomal degradation, whether the authors can detect a decrease of ubiquitination of Rap1 in uls1 cells.

Minor points.
1. The negative interaction between uls1 and rap1-DAmP allele in exponential growing cells and the suppression by the smt3-3R allele are interesting. However, this part of the manuscript seems a little off-topic and incomplete since it apparently is independent of the telomere fusion inhibition

and further investigations on this are lacking.
2. WB results showing a reduced sumoylation of Rap1-2K (Fig. 5) should already be mentioned in the context of Fig.4 since it is needed for the conclusion that the suppression of the uls1 by rap1 2-309, -1R, -2R is indeed due to abolishing Rap1 sumoylation.
3. The suggested role for removing sumoylated Rap1 in stationary cells would suggest that there is more modified Rap1 in these cells compared to exponentially growing cells even in the wild type. Have the authors tested this?

1st Revision - authors' response 21 December 2012

Thank you for giving us the opportunity to submit a revised version of our manuscript on Uls1 function at telomeres. We would also like to thank the referees for their assessment of this work and for their comments to improve it.

We added several new experiments. In short:
- The presence of Uls1 at telomeres was observed by ChIP (Figure 2E)
- Siz1 E3 ligase loss suppresses the telomere fusions caused by Uls1 loss (Figure 3C)
- Rap1 poly-SUMO conjugates are also detected in uls1- stationary cells (Figure 5)
- Sir4 but not Rif2 remains proficient to inhibit NHEJ at telomeres in the absence of Uls1. In uls1-sir4- cells, telomere fusions are frequent and their occurrence is still suppressed by a lack of poly-SUMO chains and by rap1 alleles (Figure 6A & B)
In addition, we determined the doubling time of the uls1- rap1-DAmP cells (Figure 3D) and observed that Rap1 level is not significantly reduced in rap1-DAmP cells (Figrue 3E). In supplemental data, we show that Uls1 loss does not significantly change telomere length in the presence or absence of Sir4 and Rif2 (Figure S2).

Following is our point-to-point response to the refereesí comments:

Referee #1
1) We agree and unfortunately there is no known Uls1 function that would requires only one of its two activities. We added a cautionary note in the second paragraph of the second section of the Results (page 6):
" Although an effect of these point mutations on protein folding cannot be ruled out, this result suggests that both enzymatic activities of Uls1 are required for efficient NHEJ inhibition at telomeres"
We deleted the ambiguous reference to Shah et al. (2010) but we maintained the reference to Uzunova et al. (2007) since they were the first to create the C1333S allele (they observed that in this mutant and in uls1- cells, accumulation of high molecular weight poly-SUMO conjugates is similar; page 34172)

2) It is not unusual to name a new domain before knowing its function. We choose this name thinking that it will be easy to memorize.

3) This correlation between the quantity and the quality of the PCR products is indeed very useful. We added a sentence in the first paragraph of the Results (page 5):
"(Figure 1B, second panel from top, 34 PCR cycles; rarer fusions are amplified as discrete bands)"

Referee #2
1) To address this point, we determined the genetic interaction between uls1, rif2 and sir4. In the absence of Uls1, NHEJ inhibition at telomeres still relies on Sir4 but the Rif2 pathway and the pathway independent of Rif2 and Sir4 are defective. The new data are presented in Figure 6 and in a new section at the end of the Results (pages 10-11). They are discussed in a new (the third)

paragraph in the Discussion (page 12).

2) Addressing Uls1 SIMs function was inconclusive. We created an allele where all 4 previously identified SIMs are mutated. But this mutant proved to be insufficient neither to cause telomere fusions nor to abolish Uls1 interaction with SUMO in a yeast two-hybrids assay. This suggests that Uls1 possesses additional, less canonical SIMs. Recent works by Sun and Hunter (JBC 2012 vol287 p42071) and by Vogt and Hofmann (MMB 2012 vol832 p249) indicate that an acid beta strand in non-globular context might be enough to form a SIM. The Uls1 N-terminal region from aa1 to aa460 could fit this context but the precise identification of new SIMs within this domain remains to be done. To point into this direction, we wrote in the first paragraph of the second section of the Results (page 5):
"At least four SUMO interacting motifs (SIM) are present in the amino-terminal region of the protein (Uzunova et al., 2007). An allele mutated within these SIMs still interacts with SUMO in a yeast 2-hybrid assay (data not shown), suggesting that Uls1 may possess additional, less canonical SIMs that remain to be located (Vogt and Hofmann, 2012; Sun and Hunter, 2012)."

3) In the absence of Uls1, poly-SUMOyated Rap1 molecules remain a small fraction of the total Rap1 molecules and should not influence the overall level of Rap1 at telomeres (as further suggested by the lack of telomere elongation in uls1- cells). An important point that was not explicit in the original manuscript is that telomere fusions are cumulative events in non-dividing cells. Thus even if at a given time a telomere has a low probability of being affected, over a long period of time the progressive accumulation of fusions can become significant. To address this, we added the following sentence at the end of the second paragraph of the Discussion (page 12):
"In addition, because telomere fusions occurring in nondividing cells are cumulative events, even low steady level of SUMOylated Rap1 and relatively infrequent and transient telomere exposure to NHEJ could result in a significant accumulation of fusions over time."
We also develop this point further at the end of the next paragraph, discussing the loss of Rif2 function in the absence of Uls1.

4) We addressed this by ChIP and observed that Uls1 is present at telomeres. The new data is shown in Figure 2E and is described in a new paragraph at the end of the second section of the Result (page 7).

i) The input sample is 1/2000th of the total extract subjected to the pull down. This information is added in the legend of Figure 5. Rap1 SUMO conjugates are too rare to be detected without enrichment. It is a common situation for many SUMOylated proteins.

ii) Relative Rap1 levels are not significantly reduced in the rap1-DAmP cells (the new data is in Figure 3E and is described in the first paragraph of the fourth section of the Results). Loss of the 3í UTR may alter translation quality (for instance the coupling between translation rate and protein folding) but this remains to be addressed.
Fusions are indeed slightly and reproducibly less frequent in uls1- rap1-DAmP cells than in uls1- cells. The cause of this different is unclear to us. Perhaps the growth defect of the double mutant influences stationary phase entry and indirectly the rate of fusions but other scenarios can be imagined.

iii) All rap1 alleles are integrated at the endogenous locus. We added this information in the Materials & Methods.

Referee #3
1) See point 2) for Referee #2

2) Siz1 loss suppresses the occurrence of telomere fusions in cells lacking Uls1. Siz2 loss causes a more limited suppression. The new data is shown in Figure 3C and is described in a new paragraph at the end of the third section of the Results (pages 7-8).

3) Among those directions, we addressed the genetic interactions with Rif2 and Sir4. See point 1) for Referee #2

i) We agree that the data with the rap1-DAmP allele are only hinting at a role for Uls1 and poly-SUMOylation in Rap1 essential transcription function but we think it is important to suggest such possibility.

ii) The genetic approach of Figure 4 allows first the identification of lysines 240 and 246 as putative SUMOylation sites. Figure 5 comes next to support this interpretation.

iii) Protein extraction from stationary cells proved to be relatively inefficient but still we can detect the accumulation of poly-SUMOylated Rap1 molecules in uls1- cells in this context. The new data is in Figure 5 (lower panels). The added protocol step for this condition is indicated in the Materials & Methods (page 14).

Finally, I would like to thank you again for allowing us to submit this revised version and I am looking forward to hear from you about the status of our manuscript.

Acceptance letter 21 January 2013

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of 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.

Thank you again for this contribution to The EMBO Journal, and congratulations on a successful publication! Please consider us again in the future for your most interesting work.

Referee #2

(Remarks to the Author)
In the revised version, Lescasse et al. have responded to the criticisms that were raised.
Briefly, they have determined the genetic interaction between uls1, rif2 and sir4.
They deduced that Rap1 poly-SUMOylation antagonizes NHEJ inhibition by Rif2 and by the Rif2-Sir4 independent pathway but not by the Sir4 pathway.
They have mutated the 4 SIM domains but this Uls1 mutant did not abolish Uls1 interaction with SUMO.
They now precise that poly-SUMOyated Rap1 molecules remain a small fraction of the total Rap1 molecules, a question that was opened in the original version. The question remains how a very small fraction of sumoylated Rap1 can decrease the efficiency of NHEJ inhibition but this point is convincingly discussed in the discussion.
They show that Uls1 is clearly present at telomeres.
My minor comments were adressed.

