nature portfolio

Peer Review File

Molecular basis for the regulation of the SUMOylation activity of the Smc5/6 complex by Esc2

Corresponding Author: Professor David Reverter

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Summary
In this manuscript, Helena Borràs-Gas et al. investigate how Esc2 regulates Smc5/6 complex-mediated SUMOylation and propose that the SLD2 domain of Esc2 enhances Nse2(a subunit of the Smc5/6 complex)-dependent SUMO transfer by engaging both the Ubc9 backside and the C-terminal SIM2 region of Nse2. The study combines biochemical assays, structural modeling, and yeast genetics to support a model in which Esc2 acts as a SUMO-like cofactor for Nse2.
Overall, the manuscript contains potentially useful observations. In particular, it links Esc2 SLD2 functionally to the previously described Ubc9 backside/SIM-dependent mode of Nse2 activation and provides supportive in vivo data for the relevance of this interface under DNA damage conditions. However, in its current form, the manuscript overstates both its conceptual novelty and its mechanistic resolution. A central concern is that the proposed model substantially builds on a previously established Nse2 activation framework involving backside SUMO and SIM motifs (Doi: 10.1038/s41467-021-27301-9). In parallel, the SUMO-like domain of Esc2 has already been proposed to stimulate sumoylation by exploiting a noncovalent SUMO-binding site on the E2 enzyme Ubc9(Doi: 10.1101/gad.344739.120). Additionally, several of the strongest mechanistic claims, including direct Esc2–Nse2 interaction and simultaneous binding of Esc2 to Ubc9 and Nse2, are not directly demonstrated by the current data. As such, the manuscript would benefit from substantial revision to better align its claims, novelty, and interpretation with the evidence presented.

Major comments
1. The manuscript does not sufficiently distinguish the present model from prior mechanistic frameworks that already constrain its conceptual advance. Previous work has shown that Nse2 uses SIM motifs, including SIM2, to engage a second SUMO at the Ubc9 backside and thereby enhance E3-dependent discharge. In parallel, the SUMO-like domain of Esc2 has already been proposed to stimulate sumoylation by exploiting the noncovalent SUMO-binding site on the E2 enzyme Ubc9. Against this background, the current study is better viewed as an attempt to connect these two pre-existing lines of work by proposing that Esc2 SLD2 functionally couples to the established Nse2 SIM2/backside activation framework. This may represent a useful mechanistic refinement, but it is not equivalent to establishing a fundamentally new activation principle. The manuscript should therefore state more clearly that its main advance lies in proposing a more specific linkage between previously described mechanisms, rather than implying that the overall molecular logic of Nse2 activation is being uncovered here for the first time.
2. The claims of “molecular basis” and “simultaneous binding” are stronger than the current evidence supports. The Abstract and main text state that the study elucidates the molecular basis of Esc2-mediated regulation and that Esc2 simultaneously binds the Ubc9 backside and the SIM2 motif of Nse2. However, the supporting evidence is indirect, relying primarily on AlphaFold-based structural comparisons, mutational effect assays. No direct evidence for a ternary Esc2–Ubc9–Nse2 complex is presented. A crystal or cryo-EM structure of the relevant complex would substantially strengthen the proposed model, if feasible.
3. The specificity claim based on comparison with Siz1 is not yet convincing. The assay uses p53 rather than the canonical Siz1 substrate PCNA, limiting interpretation.

Minor comments
1. Because the current study is confined to the budding yeast system, the broader significance of the proposed mechanism remains uncertain. It would strengthen the manuscript if the authors could explore whether a similar regulatory logic may also apply to human homologs, or at least discuss this possibility more explicitly.

2. page 3, line 30: the notation “E2~SUMO” should be defined upon first use for clarity.
3. page 3, line 13: missing a closing parenthesis.
4. Please carefully distinguish throughout the manuscript between general references to SUMO and specific references to the yeast SUMO protein Smt3.
5. page 5, line 7: please write “rmsd” in uppercase and define it at first occurrence.
6. page 5, line 19: please clearly indicate which panels in Figure 1 are being referred to.
7. page 6, line 1-5: please clearly define the pairwise comparisons, state the observed differences for each condition, and explicitly connect these observations to the corresponding conclusions. As written, the logical progression from experiment to interpretation is not sufficiently clear.
8. page 6, line 4-5: “In contrast, both Esc2 constructs, including SLD1 and SLD2 domains, failed to increase the E2~SUMO thioester discharge” is not sufficiently accurate as written, because Esc2 constructs do enhance discharge in the presence of E3.
9. page 7, line 29 and 31: the notation for the SIM2 deletion mutant is inconsistent (“ΔSIM2Nse2” versus “Nse2ΔSIM2”). Please standardize this nomenclature throughout the manuscript and carefully check for similar inconsistencies elsewhere.
10. Figure 1b would benefit from clearer legend distinction. Readers should not have to rely primarily on the color across structural models to infer the intended correspondence. Please annotate the panel more explicitly, including the identities of the aligned domains.
11. Figure 2/3/4: please place the statistical symbols used for P values in an appropriate location.
12. No source data files were found.
13. Please ensure consistent nomenclature throughout the manuscript, not only for mutants. For example, the mutant labels in Figure 4c/e should be aligned with the naming conventions used elsewhere.

Reviewer #2

(Remarks to the Author)
In the manuscript “Molecular basis for the regulation of the SUMOylation activity of the Smc5/6 complex by Esc2”, the authors confirmed the regulation roles of Esc2 protein in Nse2-mediated SUMOylation. Using “Nse2/Arm-Smc5” as an E3, the in vitro data shown several truncated Esc2 proteins can stimulate the SUMO reaction, and when using siz1 as an E3, this stimulation was not observed, which demonstrated that Esc2 is a specifical Nse2 E3 stimulator. Based on a recent published structure (Varejao et al., 2021) and the AlphaFold3 prediction, the authors also demonstrate the stimulation is relied on a C-terminal SUMO-interacting motif (SIM2) of Nse2. Overall, the experiments are well performed and support their conclusions, but the findings are largely confirmatory.

Major points:
1, the statements like “SLD2 of Esc2 binds Nse2 through the SIM2 motif” in page 7 line 17, “these results highlight the specificity of the Esc2-Nse2 interaction” in page 8 line 27, need be very careful. There is no experimental evidence to support Esc2 can directly interact with Nse2 (or other components in Smc5/6 complex) in this manuscript or previous literature. Esc2 and Nse2 functional interaction is clear (as the data shown in the manuscript and in the literature), but these statements is a kind of mis-leading, let the readers think those two proteins can direct interact with each other.
2, the authors use “Nse2/Arm-Smc5” as an E3 SUMO ligase in their refined in vitro system, although it is known as a functional E3 ligase in vitro and well used in the field in the past. But this is not a physiological E3 in cells. In the past few years, several groups in the field have reported the Smc5/6 complex purification (Gutierrez-Escribano et al., 2020; Yu et al., 2021; Taschner et al., 2021; Hallett et al., 2022; Pradhan et al., 2023; Li et al., 2024). Using the Smc5/6 complex as a E3 may be a more physiological relative condition. Considering the Nse2 SIM2 motif is in the end of the protein, whether it is exposed for the interaction with backside SUMO or it’s hided in the complex is an interesting question? The authors have the refined in vitro system to test whether the stimulation still be maintained when using the Smc5/6 complex instead of “Nse2/Arm-Smc5” E3.
3, related to above point, the most important question is why the stimulation is Nse2 specifical. As the authors mentioned in the text, siz1 also contains a SIM motif can interact with backside SUMO, but Esc2 does not shown the stimulation. This suggests some unknow yet mechanism beside SLD2-backside SUMO interaction pe ser can lead the stimulation of Nse2-mediated SUMOylation. Other components in the Smc5/6 complex or the conformational change of Smc5/6 complex (by ATP binding/hydrolysis, DNA binding) may be related. The authors’ in vitro system put them in a good position to address this important question.

Minor points:
1, make sure the colors for the text and structures are consistent. Like in Fig1b, Fig4d,
2, it will be good to also show the experiment without the Esc2-SLD2 protein when test E3 mutants as in Fig 4C,
3, in the in vitro experiment, the authors used Esc2 SLD2 mutant are H339E, R401E, and K408E (e.g. in page 8, line 5), but for the in vivo mutants, the mutation are H399E/R401D/K408E (e.g. in page 9, line 9), is there a reason why they are slightly different?
4, in the Figure 5 legend, “Rmm3” should be “Rrm3”,
5, with Esc2-SLD2 mutations, it does not block the sumoylation of Sgs1-Top3-Rmi1 complex. The current drawing of the model shown in Fig6b need be modified.
6, the statistic labeling is off in most figures (Fig2, Fig3a, Fig4).

Reviewer #3

(Remarks to the Author)
Reverter and co-workers have addressed the molecular basis for the regulation of the sumoylation activity of the Smc5/6 complex by Esc,2 using AlphaFold modelling, mutagenesis, biochemistry and analysis of yeast mutant strains. They show that Esc2 does not contain intrinsic E3 ligase activity, but simultaneously binds to the backside of Ubc9 and the C-terminal SUMO interaction motif of Nse2 to coordinate the formation of a productive E3-E2-SUMO complex, promoting efficient SUMO transfer. This work is particularly important to help understand the role of SUMO to deal with replication stress. Overall, this is an interesting manuscript that I'm happy to support for publication after revision. It is not only relevant for the SUMO field, but also for the ubiquitin field as it enhances our understanding of E3 ligase stimulation.

Comments

1 - Consider producing full size Esc2 in insect cells since it is too large to produce in E. coli.

2 - Consider verifying sumoylation of other substrates in addition to Sgs1, especially Top3 and Rmi1, but also Pol2.

3 - p53 is not the most relevant substrate employed for in vitro experiments, since yeast doesn't have p53. It would be good to briefly explain at the start of the results section why p53 was chosen as model substrate instead of later in the results section.

4 - Please mention the number of replicates in the legend of Figure 5.

5 - Statistics – please indicate which two samples are being compared by adding lines in the relevant figure panels and add asterisks on top of these lines.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have reviewed the revised manuscript and have no further comments.

Reviewer #2

(Remarks to the Author)
Thank you for the authors' effects to address my comments. Two minor comments need be clarified before publication.
1) The NMR titration experiment's results have addressed my major concern about the Nse2-Esc2 interaction. For the broader readers, could the authors provide a little bit more explanation about the results? Like whether they can detect the Kd of Nse2-Esc2 two proteins' interaction? Why the Fig 1e and Fig 4f left looks different?
2) The hexameric Smc5/6 complex result is convincing; a gel picture of the purified complexes (WT and ΔSIM) will be useful in the Fig 5a.

Reviewer #3

(Remarks to the Author)
The authors have properly addressed my comments.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Summary
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Overall, the manuscript contains potentially useful observations. In particular, it links Esc2 SLD2 functionally to the previously described Ubc9 backside/SIM-dependent mode of Nse2 activation and provides supportive in vivo data for the relevance of this interface under DNA damage conditions. However, in its current form, the manuscript overstates both its conceptual novelty and its mechanistic resolution. A central concern is that the proposed model substantially builds on a previously established Nse2 activation framework involving backside SUMO and SIM motifs (Doi: 10.1038/s41467-021-27301-9). In parallel, the SUMO-like domain of Esc2 has already been proposed to stimulate sumoylation by exploiting a noncovalent SUMO-binding site on the E2 enzyme Ubc9 (Doi: 10.1101/gad.344739.120). Additionally, several of the strongest mechanistic claims, including direct Esc2–Nse2 interaction and simultaneous binding of Esc2 to Ubc9 and Nse2, are not directly demonstrated by the current data. As such, the manuscript would benefit from substantial revision to better align its claims, novelty, and interpretation with the evidence presented.

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R. We fully agree with the reviewer's standpoint. Our objective was to establish a mechanistic link between two well-characterized interactions: the non-covalent binding of Ubc9 to SUMO and the Nse2 SIM–SUMO backside interaction, in order to better understand how Esc2 stimulates the E3 SUMO ligase activity of Nse2. Previous studies have shown that Esc2 SLD2 binds to the Ubc9 backside in a manner analogous to SUMO (references 17, 22-24, cited in the main text). Our main contribution here is the identification of a direct interaction between Esc2 SLD2 and the Nse2 SIM2, which appears to functionally mimic the established SUMO backside-mediated stimulation of E3 activity through SIM binding.

We have revised the Abstract to clarify our contribution. *"Here, we uncover the molecular mechanism underlying Esc2-mediated stimulation of Nse2-dependent SUMOylation by identifying a direct interaction between the C-terminal SUMO-interacting motif (SIM2) of Nse2 and the Esc2 SLD2 domain. These coordinated interactions, facilitate the formation of a productive E3-E2~SUMO complex, thereby promoting efficient SUMO transfer."*

2. The claims of "molecular basis" and "simultaneous binding" are stronger than the current evidence supports. The Abstract and main text state that the study elucidates the molecular basis of Esc2-mediated regulation and that Esc2 simultaneously binds the Ubc9 backside and the SIM2 motif of Nse2. However, the supporting evidence is indirect, relying primarily on AlphaFold-based structural comparisons, mutational effect assays. No direct evidence for a ternary Esc2–Ubc9–Nse2 complex is presented. A crystal or cryo-EM structure of the relevant complex would substantially strengthen the proposed model, if feasible.

R. In the revised manuscript, we present direct evidence for SIM2 binding to the Esc2 SLD2 domain by performing NMR titration of a 13-mer SIM2 peptide with ^{15}N-labeled Esc2 SLD2, which reveals clear chemical shift perturbations upon peptide addition. Despite extensive efforts, we were unable to obtain crystals of either the binary 13-mer SIM2 peptide–Esc2SLD2 complex or the ternary Nse2/Arm-Smc5–Esc2SLD2–Ubc9-thioester complex. Nevertheless, the observed NMR chemical shift perturbations with the wild-type protein, in contrast to the Esc2-EDE triple mutant, provide strong evidence of a specific Nse2 SIM–Esc2 SLD2 interaction.

Please see the NMR experiments in the new Figure 1e and Figure 4f.

3. The specificity claim based on comparison with Siz1 is not yet convincing. The assay uses p53 rather than the canonical Siz1 substrate PCNA, limiting interpretation.

R. As suggested by the reviewer we have recapitulated similar assays with canonical Siz1 substrate PCNA, as presented in new *Supplementary Figure 5*. The results indicate a similar pattern in single turnover reactions to our standard non-specific substrate cp53.

We have modified the main text accordingly in page 9.

Minor comment

1. Because the current study is confined to the budding yeast system, the broader significance of the proposed mechanism remains uncertain. It would strengthen the manuscript if the authors could explore whether a similar regulatory logic may also apply to human homologs, or at least discuss this possibility more explicitly.

R. This is a good point that we did not discuss in the manuscript. We have added a sentence in the discussion about the comparison with the human homolog Nip45 and added a new reference.

We have modified the main text in page 13: *"Interestingly, since human Nse2 lacks the C-terminal SIM motif, an alternative mode of interaction has been proposed for Nip45 (Cho et al, Genes&Dev. 2024)".*

2. page 3, line 30: the notation "E2~SUMO" should be defined upon first use for clarity.

R. In the main text, we have added the word "thioester" after each "E2~SUMO" notation. Additionally, we have added the definition upon first use. Page 3: *(transient thioester bond between E2 and SUMO).*

3. page 3, line 13: missing a closing parenthesis.

R. Sorry for this typo.

4. Please carefully distinguish throughout the manuscript between general references to SUMO and specific references to the yeast SUMO protein Smt3.

R. Thanks for this advice. We have now checked throughout the manuscript this specific point.

5. page 5, line 7: please write "rmsd" in uppercase and define it at first occurrence.

R. Yes. We have written the abbreviation in uppercase and defined RMSD (root mean square deviation) upon its first use.

6. page 5, line 19: please clearly indicate which panels in Figure 1 are being referred to.

R. We have prepared a new Figure 1 with the NMR data and checked carefully the panel nomenclature.

7. page 6, line 1-5: please clearly define the pairwise comparisons, state the observed differences for each condition, and explicitly connect these observations to the corresponding conclusions. As written, the logical progression from experiment to interpretation is not sufficiently clear.

R. Thanks for the comment. To better comprehension, we have rephrased the following sentences in page 6: *"In contrast, both Esc2 SLD1–SLD2 tandem construct and the isolated SLD2 domain failed to increase the E2~SUMO thioester discharge in the absence of the E3 ligase"….*
*"Consistent with the results above, neither Esc2 construct promote enhanced discharge of the E2~SUMO thioester, whereas Nse2/Arm-Smc5 markedly accelerated discharge under the same conditions (**Figure 2b**)."*

8. page 6, line 4-5: "In contrast, both Esc2 constructs, including SLD1 and SLD2 domains, failed to increase the E2~SUMO thioester discharge" is not sufficiently accurate as written, because Esc2 constructs do enhance discharge in the presence of E3.

R. Sorry for this misunderstanding. As stated in the previous comment, we have rephrased the following sentences in page 6: *"In contrast, both Esc2 SLD1–SLD2 tandem construct and the isolated SLD2 domain failed to increase the E2~SUMO thioester discharge in the absence of the E3 ligase"….*

9. page 7, line 29 and 31: the notation for the SIM2 deletion mutant is inconsistent ("ΔSIM2Nse2" versus "Nse2ΔSIM2"). Please standardize this nomenclature throughout the manuscript and carefully check for similar inconsistencies elsewhere.

R. Sorry for this mistake. We have now standardized this nomenclature throughout the main text.

10. Figure 1b would benefit from clearer legend distinction. Readers should not have to rely primarily on the color across structural models to infer the intended correspondence. Please annotate the panel more explicitly, including the identities of the aligned domains.

R. Thanks for this advice. Figure 1b has been revised by directly labeling the aligned domains within the panel.

11. Figure 2/3/4: please place the statistical symbols used for P values in an appropriate location.

R. We apologize for the oversight. We have carefully revised the statistical significance symbols above the bar plots.

12. No source data files were found.

R. Sorry, but it was not requested in the initial submission. We have now included the source data in the new submission.

13. Please ensure consistent nomenclature throughout the manuscript, not only for mutants. For example, the mutant labels in Figure 4c/e should be aligned with the naming conventions used elsewhere.

R. Sorry for that. We have now standardized the mutant nomenclature everywhere.

Reviewer #2 (Remarks to the Author):

In the manuscript “Molecular basis for the regulation of the SUMOylation activity of the Smc5/6 complex by Esc2”, the authors confirmed the regulation roles of Esc2 protein in Nse2-mediated SUMOylation. Using “Nse2/Arm-Smc5” as an E3, the in vitro data shown several truncated Esc2 proteins can stimulate the SUMO reaction, and when using siz1 as an E3, this stimulation was not observed, which demonstrated that Esc2 is a specifical Nse2 E3 stimulator. Based on a recent published structure (Varejao et al., 2021) and the AlphaFold3 prediction, the authors also demonstrate the stimulation is relied on a C-terminal SUMO-interacting motif (SIM2) of Nse2. Overall, the experiments are well performed and support their conclusions, but the findings are largely confirmatory.

Major points:
1, the statements like “SLD2 of Esc2 binds Nse2 through the SIM2 motif” in page 7 line 17, “these results highlight the specificity of the Esc2-Nse2 interaction” in page 8 line 27, need be very careful. There is no experimental evidence to support Esc2 can directly interact with Nse2 (or other components in Smc5/6 complex) in this manuscript or previous literature. Esc2 and Nse2 functional interaction is clear (as the data shown in the manuscript and in the literature), but these statements is a kind of mis-leading, let the readers think those two proteins can direct interact with each other.

R. We agree with the reviewer’s point about the lack of direct interaction in the literature between Esc2 and Nse2. However, we now present direct evidence for SIM2 Nse2 binding to the Esc2 SLD2 domain using an NMR titration of a 13-mer SIM2 peptide with ^{15}N-labeled Esc2 SLD2, which reveals clear chemical shift perturbations upon peptide addition. The observed NMR chemical shift perturbations with the wild-type protein, in contrast to the Esc2-EDE triple mutant, provide strong evidence of a specific Nse2 SIM–Esc2 SLD2 interaction.

Please see the NMR experiments in the new Figure 1e and Figure 4f.

2, the authors use “Nse2/Arm-Smc5” as an E3 SUMO ligase in their refined in vitro system, although it is known as a functional E3 ligase in vitro and well used in the field in the past. But this is not a physiological E3 in cells. In the past few years, several groups in the field have reported the Smc5/6 complex purification (Gutierrez-Escribano et al., 2020; Yu et al., 2021; Taschner et al., 2021; Hallett et al., 2022; Pradhan et al., 2023; Li et al., 2024). Using the Smc5/6 complex as a E3 may be a more physiological relative condition. Considering the Nse2 SIM2 motif is in the end of the protein, whether it is exposed for the interaction with backside SUMO or it’s hided in the complex is an interesting question? The authors have the refined in vitro system to test whether the stimulation still be maintained when using the Smc5/6 complex instead of “Nse2/Arm-Smc5” E3.

R. Following the reviewer’s advice, we have recapitulated the single turnover reactions using the composite hexameric form of the Smc5/6 complex, in which Nse2 is fully integrated with all the other subunits (see new ***Figure 5a***). The E3 ligase activity of the hexameric Smc5/6 complex is also enhanced by the Esc2 SLD2 domain during the E3-dependent SUMOylation reaction.

3, related to above point, the most important question is why the stimulation is Nse2 specifical. As the authors mentioned in the text, siz1 also contains a SIM motif can interact with backside SUMO, but Esc2 does not shown the stimulation. This suggests some unknow yet mechanism beside SLD2-backside SUMO interaction pe ser can lead the stimulation of Nse2-mediated SUMOylation. Other components in the Smc5/6 complex or the conformational change of Smc5/6 complex (by ATP binding/hydrolysis, DNA binding) may be related. The authors’ in vitro system put them in a good position to address this important question.

R. We thank the reviewer for this insightful comment and agree that the selective stimulation of Nse2 by Esc2 may involve additional mechanisms beyond the SIM2–SLD2–Ubc9 backside interaction. Factors such as the architecture and conformational dynamics of the Smc5/6 complex, including ATP binding/hydrolysis and DNA binding, are currently under investigation in our laboratory and will be the focus of future work.

Minor points:

1, make sure the colors for the text and structures are consistent. Like in Fig1b, Fig4d,

R. Thanks for this advice. The colors in Figure 1a/1b and 4b are now consistent with the text.

2, it will be good to also show the experiment without the Esc2-SLD2 protein when test E3 mutants as in Fig 4C,

R. Thanks for this comment. This experiment has been reported previously in our earlier study (Varejao et al. Nature Communicaitons 2021).

3, in the in vitro experiment, the authors used Esc2 SLD2 mutant are H339E, R401E, and K408E (e.g. in page 8, line 5), but for the in vivo mutants, the mutation are H399E/R401D/K408E (e.g. in page 9, line 9), is there a reason why they are slightly different?

R. Sorry for that, it is a typo. The correct names for the mutants are H399E/R401D/K408E.

4, in the Figure 5 legend, “Rmm3” should be “Rrm3”,

R. Sorry for this typo. We have fixed it.

5, with Esc2-SLD2 mutations, it does not block the sumoylation of Sgs1-Top3-Rmi1 complex. The current drawing of the model shown in Fig6b need be modified.

R. We thank the reviewer for this valuable comment and agree that placing a cross over the arrow could be interpreted as complete inhibition of SUMOylation, whereas our data show a marked reduction, but not a complete loss, of SUMOylation in the esc2-EDE mutant. We have therefore modified the schematic accordingly (shown in new Figure 7).

6, the statistic labeling is off in most figures (Fig2, Fig3a, Fig4).

R. Sorry for this formatting error. We have fixed it.

Reviewer #3 (Remarks to the Author):

Reverter and co-workers have addressed the molecular basis for the regulation of the sumoylation activity of the Smc5/6 complex by Esc,2 using AlphaFold modelling, mutagenesis, biochemistry and analysis of yeast mutant strains. They show that Esc2 does not contain intrinsic E3 ligase activity, but simultaneously binds to the backside of Ubc9 and the C-terminal SUMO interaction motif of Nse2 to coordinate the formation of a productive E3-E2-SUMO complex, promoting efficient SUMO transfer. This work is particularly important to help understand the role of SUMO to deal with replication stress. Overall, this is an interesting manuscript that I’m happy to support for publication after revision. It is not only relevant for the SUMO field, but also for the ubiquitin field as it enhances our understanding of E3 ligase stimulation.

Comments

1 - Consider producing full size Esc2 in insect cells since it is too large to produce in E. coli.

R. We, and other groups, have tried unsuccessfully to produce the Esc2 full length for in vitro reactions, however it is quite unstable and cannot be detected.

2 - Consider verifying sumoylation of other substrates in addition to Sgs1, especially Top3 and Rmi1, but also Pol2.

R. Thanks for this advice. In the new Figure 6 (panel d), we now present in vivo SUMOylation results of Top3, which is another component of the STR complex, which displays z similar trend as Sgs1.

3 - p53 is not the most relevant substrate employed for in vitro experiments, since yeast doesn't have p53. It would be good to briefly explain at the start of the results section why p53 was chosen as model substrate instead of later in the results section.

R. Thanks for this advice. As used in our previous manuscripts, for convenience we are using the p53 C-terminal region as model substrate for SUMOylation in in vitro reactions. We have added at the beginning of the results an explanatory sentence*: "… within the C-terminal region of p53 …"*

4 - Please mention the number of replicates in the legend of Figure 5.

R. The legend of Figure 6 (previously Figure 5) has been updated to include the following statement: *"The experiments shown are representative of three independent biological replicates."*

5 - Statistics – please indicate which two samples are being compared by adding lines in the relevant figure panels and add asterisks on top of these lines.

R. We thank the reviewer for this suggestion. In our figures, statistical significance is indicated following the convention adopted throughout the manuscript, where asterisks placed above a bar denote a significant difference relative to the corresponding reference sample, which is left unmarked.

Editorial comment:

Regarding Fig. 1e and the left panel of Fig. 4f, the WT HSQC spectra appear to represent the same dataset displayed at different scales. Please clarify whether the WT data are identical and, if so, state explicitly in the legend that they are reproduced for comparison and present them at the same scale, or show the dataset only once.

R. The spectra in figures 1e and 4f are identical (WT Esc2 SLD2), but shown at different scale. Our goal in Fig 4f is to compare the spectra of the wild type Esc2 with the spectra of the triple mutant Esc2.

We have explicitly stated this point in the figure 4f legend: *"zoomed up spectra of Fig 1e, reproduced for comparison at the same scale*"

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I have reviewed the revised manuscript and have no further comments.

Reviewer #2 (Remarks to the Author):

Thank you for the authors' effects to address my comments. Two minor comments need be clarified before publication.

1) The NMR titration experiment's results have addressed my major concern about the Nse2-Esc2 interaction. For the broader readers, could the authors provide a little bit more explanation about the results? Like whether they can detect the Kd of Nse2-Esc2 two proteins' interaction? Why the Fig 1e and Fig 4f left looks different?

R. We have added a sentence in the NMR paragraph about the estimated K_D calculation (Page 5). *"The 20 residues with the largest CSP were fitted simultaneously giving an estimated K_D value of 900 μM which agrees with the magnitude of the observed CSPs."*

Figure 4f is a zoomed up spectra of Figure 1e (NMR spectra of WT Esc2 SLD2). We have now explicitly stated that in the figure 4f legend: *"zoomed up spectra of Fig 1e, reproduced for comparison at the same scale*"

2) The hexameric Smc5/6 complex result is convincing; a gel picture of the purified complexes (WT and ΔSIM) will be useful in the Fig 5a.

R. We have included the purification gel-filtration profile and a SDS-PAGE picture of WT and ΔSIM in the new Supplementary figure 5.

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R. The spectra in figures 1e and 4f are identical (WT Esc2 SLD2), but shown at different scale. Our goal in Fig 4f is to compare the spectra of the wild type Esc2 with the spectra of the triple mutant Esc2.

We have explicitly stated this point in the figure 4f legend: *"zoomed up spectra of Fig 1e, reproduced for comparison at the same scale*"

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Thank you for the authors' effects to address my comments. Two minor comments need be clarified before publication.

1) The NMR titration experiment's results have addressed my major concern about the Nse2-Esc2 interaction. For the broader readers, could the authors provide a little bit more explanation about the results? Like whether they can detect the Kd of Nse2-Esc2 two proteins' interaction? Why the Fig 1e and Fig 4f left looks different?

R. We have added a sentence in the NMR paragraph about the estimated K_D calculation (Page 5). *"The 20 residues with the largest CSP were fitted simultaneously giving an estimated K_D value of 900 μM which agrees with the magnitude of the observed CSPs."*

Figure 4f is a zoomed up spectra of Figure 1e (NMR spectra of WT Esc2 SLD2). We have now explicitly stated that in the figure 4f legend: *"zoomed up spectra of Fig 1e, reproduced for comparison at the same scale*"

2) The hexameric Smc5/6 complex result is convincing; a gel picture of the purified complexes (WT and ΔSIM) will be useful in the Fig 5a.

R. We have included the purification gel-filtration profile and a SDS-PAGE picture of WT and ΔSIM in the new Supplementary figure 5.

Reviewer #3 (Remarks to the Author):

The authors have properly addressed my comments