Phosphorylation-dependent mitotic SUMOylation drives nuclear envelope-chromatin interactions

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Re: JCB manuscript #202103036

Dr. Richard W Wozniak
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Canada

Dear Dr. Wozniak,

Thank you for submitting your manuscript entitled "Phosphorylation-dependent mitotic SUMOylation drives nuclear envelope-chromatin interactions" and thank you for your patience with the peer review process. The manuscript was assessed by three expert reviewers, whose comments are appended to this letter. The points raised by the reviewers indicate the need for significant revision that involves additional experimental analysis. We have summarized our discussion of the reviews below to guide your revision efforts. We invite you to submit a revision if you are able to address these key concerns.

You will see that the reviewers shared interest in the proposal that Scs2 acts like a receptor that brings Siz2 activity to the INM in mitosis to support Sir4-subtelomeric chromatin association & Sir4 SUMOylation. However, they shared important concerns about the quality of the data and the strength of this model. In their view, the conclusions are weakened by the lack of quantification of the data and the lack of direct assessment of SUMOylation of substrates, instead relying on SUMO conjugate profiles. Rev#1 was additionally concerned about cell cycle effects (4th bulleted point, see similar point raised by Rev#2 #4) and asked for direct evidence for the association between Siz2 and Scs2 via a SUMO-SIM interaction (see also the same suggestion from Rev#3, part 2 of the bulleted points, #2).

For further consideration of this work at JCB, it will be essential to address the referees' core concerns with the lack of quantitation and robustness of the data. We also suggest adding a NE marker in the imaging studies, if possible. While the fractionation experiments to validate the localization as per Rev#3 (second part of the bulleted points, #1) will not be required, we feel that the reviewers' other points will need to be addressed convincingly, including the IPs for Siz2-Scz2 and clarifying whether phenotypes may be due to cell cycle effects in the mutants as per Rev#1. Looking at Opi1 localization (suggested by Rev #3) would help round out the proposed model and we encourage pursuing this analysis; mutational analysis of the FFAT motif suggested by the same reviewer would also be informative but is not essential to pursue during revision. Lastly, we agree with Rev#1 on the need to substantiate key (not all) conclusions with substrate blots.

Please let us know if you have any questions or anticipate any issues addressing these points. We would be happy to discuss the revisions further if needed.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Arshad Desai, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In their manuscript, Ptak et al. investigate the recruitment of SUMO E3 ligase Siz2 to the inner nuclear membrane (INM) during cell division in yeast. The author's model is based on the idea that initial SUMO modification events can trigger a 'sumoylation wave'. They claim to have "identified temporally and spatially controlled phosphorylation-dependent SUMOylation events", uncovering a mechanism by which phosphorylation of Siz2 mediates binding to Scs2 in a manner stabilised by SUMO-SIM interactions. The resulting sumoylation events at the nuclear envelope (NE) are then said to be involved in mediating interactions between the NE with both telomeres and INO1 respectively.

This is a very interesting topic and a suitable model system to study it. In spite of this, we believe the study is too preliminary and the data presented does not support the authors' main claims, with too many alternative models/hypotheses not discarded. We therefore cannot recommend this manuscript for publication. We highlight below some of the key aspects that preclude us from recommending publication:

• Without proper quantification (for NE localisation, phosphorylation, sumoylation), readers are left with single panel immunofluorescence images and blots along with the authors' qualitative assessment of the different outcomes. Not only quantifications are missing but also the fact that all the mutants are shown as separate blots/gels/panels makes it very complicated to assess critically and quantitatively.

• While we can understand how the authors initially refer to the four bands in the SUMO blots, using these blots as a proxy for sumoylation of specific proteins is not appropriate. For example, the authors do show Scs2 sumoylation (Figure 1E). However, this is done in ulp1 mutant background (without even comparing to wild type) and surprisingly, the shift that the SUMO-modified form experience differs by a big stretch depending on whether Scs2 is tagged in the N- or C-terminus. Additionally, they could have used the K180R mutant to confirm their observations. From this point onwards, we can't see why the authors keep using total SUMO blots rather than the substrate-specific blots.

• Had the authors used substrate-specific blots along the manuscript, we would be able to assess the feasibility of their model, which requires that a substantial amount of Scs2 is sumoylated in order to trigger the proposed downstream SUMO wave.

• Many results could be the consequence of different mutant backgrounds affecting cell cycle dynamics and consequently (and indirectly) affecting SUMO modification and/or NE localisation. Indeed, the authors use cyclin Clb2 levels, which peak during metaphase, as a reference. However, Clb2 levels peak at different times after α-factor release in different mutants (e.g. peak at 60 min in 1B but 50 mins in the Scs2K180R and ulp1K352E/V583H-V53 mutants in 1C and 1D respectively, while HA3-Scs2 increases the time till Clb2 peak to 70 min in ulp1K352E/V583H-V53 mutants in 1E). Therefore, changing cell cycle stage could easily explain different sumoylation levels in different figures.

• It appears to us that the authors do not have a clear criterium for what represents a 'significant' change and what doesn't. The analysis of Siz2 phosphorylation is a perfect example of this. While the authors claim that one specific Siz2 phospho-site mutant has impaired phosphorylation, it appears to us that all of the mutants have impaired phosphorylation. Same is true for the NE localisation.

• Direct evidence is needed for their proposed direct interaction between Siz2 and Scs2 and the potential SUMO-SIM involvement.
A few minor comments/questions:

• Fig 1A: how do authors know these are SUMO conjugates (no free SUMO) as stated in the text?
• Fig 3B: The pull-down should be presented in a single gel in order to compare phosphorylated vs non-phosphorylated forms. Showing separate blots makes them impossible to compare.
• Fig 6A - would want to see that total His8-SUMO (from Ni-NTA samples) and Sir4-V53 (from input) levels are similar in all the samples to know that band differences are not due to differences in starting protein levels.

Reviewer #2 (Comments to the Authors (Required)):

This manuscript from Ptak et al., identifies SUMOylation as an important mechanism influencing tethering of the genome to the nuclear envelope. The authors show yeast SUMO protein is concentrated at the nuclear envelope in mitosis and find that this timing coincides with the appearance of protein SUMOylation in the ~40-55kDa range. Elegantly, by analysis of mutants lacking proteins of this size that are known to be SUMOylated, they identify Scs2, vesicle-associated membrane protein as the major SUMOylated protein of this size in mitosis. Using a split GFP system, the authors show that Scs2 is in the inner nuclear membrane, in addition to the outer nuclear membrane. They show that phosphorylation pf the SUMO ligase Siz2 targets it to the inner nuclear membrane through association with Scs2. Siz2 then SUMOylates Scs2 and Sir4, and potentially other proteins too. The authors additionally show that one function of SUMOylation at the inner membrane is to recruit telomeres and the active INO4 gene. Interestingly, recruitment of telomeres to the nuclear envelope depends on SUMOylation of the telomere-associated protein Sir4. This is a very comprehensive study that uses a wide range of specific mutants to build a mechanistic model of how SUMOylation is enriched at the nuclear envelope and how this contributes to genome organisation. The paper is well written and the experiments support the conclusions. I have only a few suggestions for improvements.

1. It would be helpful to quantify the imaging data in some way. For all conclusions, the reader is presented with a single representative image. In particular, where a partial phenotype is observed or a rescue is concluded, quantification seems very important (i.e. Figures 4A and 4C).

2. It would be very nice to include a model in Figure 7 to summarise the findings in this paper.

3. Figure 3A. Pus1 and Hxk1 cell sizes seem different. Is the scale bar correct and does it apply to the whole panel?

4. Figure S5, SUMOylation seems to be maximal in S phase, but Figure S6E shows that Sir4 localization is lowest then. How can this be reconciled with the observation that Sir4 SUMOylation is functionally important in directing its localization?

5. Figures 3C and 3B are called out of order in the text.

Reviewer #3 (Comments to the Authors (Required)):

Genomes physically interact with the nuclear envelope and this can impact genome function. During the cell cycle, such interactions are temporarily lost and must be re-established. The authors describe cycle-regulated SUMOylation of nuclear envelope (NE) membrane proteins during mitosis in budding yeast. NE staining with anti-SUMO antibodies correlates with several novel bands on an anti-SUMO immunoblot. SUMOylation of the NE requires the SUMO E3 ligase Siz2 and is antagonized by the SUMO protease Ulp1. Furthermore, Siz2 relocalizes to the NE during mitosis. Based on published proteomics datasets, they test candidate targets and identify the VAP protein Scs2 as an ER/NE membrane protein that is SUMOylated during mitosis. Surprisingly, deletion of SCS2 or mutation of a SUMOylation site in Scs2 leads to loss of all NE SUMOylation, resulting in disappearance of the novel bands. This suggests that Scs2 plays a role in SUMOylation of several proteins on the NE.

The authors use clever yeast molecular genetics to establish that Scs2 localizes to the inner nuclear envelope. They also show that phosphorylaton of a single amino acid of Siz2 as well as a cryptic FFAT motif in Siz2 are required for Scs2 SUMOylation. Furthermore, Scs2 SUMOylation is required for NE recruitment of Siz2 though a SUMO interaction motif.

Finally, the authors explore the biological significance of mitotic SUMOylation of Scs2 and other NE proteins. They find that tethering of telomeres to the NE during anaphase and G1 and tethering of an active gene to the NPC during G1 and mitosis requires the Scs2-Siz2 complex. The tethering of telomeres correlates with SUMOylation of Sir4 and mutations that reduce Sir4 SUMOylation reduce Sir4 association with subtelomeric regions. This suggests that SUMOylaton of Scs2, and potentially other, downstream NE proteins, is important to re-establish NE tethering of chromatin following chromosome segregation.

The paper is technically excellent and the conclusions are sound. Although I see no major weaknesses, there are two important controls that should be performed prior to publication:
1. The microscopy images showing partitioning onto the NE should be quantified. This could be expressed as mean signal over the NE vs in the nucleus for a population of mitotic cells. This would make the quantitative defects seen in some experiments (e.g. Figure 4) more convincing.
2. The levels of Sir4 should be quantified in wild type and siz2 and scs2 mutants to confirm that they do not change.

Also, there are several questions that, if answered, would strengthen the paper:
1. To confirm that the additional SUMOylated species observed on the immunoblot are NE proteins, the authors could perform the immunoblot on fractionated lysates to examine the NE/ER microsomal fraction.
2. The interaction between Siz2 and Scs2 is based on microscopy. A stronger result would be co-immunoprecipitation, which has been performed with Scs2 and various binding partners in the past.
3. Given the known role of Scs2 in regulating INO1 transcription through interaction with Opi1, the authors should look at the localization of Opi1 during mitosis in the siz2 and scs2 mutants.
4. One of the most intriguing findings is the relationship between cell cycle-regulated phosphorylation of Siz2 and a FFAT + SUMO-dependent interaction between Siz2 and Scs2. This is unprecedented for FFAT-Scs2/VAP interactions and may reflect the relatively low affinity of the FFAT motif in Siz2 and Scs2. If so, substituting a high affinity FFAT motif might bypass the dependence on SUMOylation, making this interaction unregulated. Alternatively, it raises the possibility that other FFAT-Scs2 interactions are also modulated by SUMO. If so, then mutation of

the Scs2 SUMO site should disrupt those interactions as well.

Responses to Reviewers.

We thank all the reviewers for their time and helpful comments. Specific comments of the indicated Reviewer are quoted below and are followed by our response (*in italics*).

Reviewer #2 comments:

….. This is a very comprehensive study that uses a wide range of specific mutants to build a mechanistic model of how SUMOylation is enriched at the nuclear envelope and how this contributes to genome organisation. The paper is well written and the experiments support the conclusions. I have only a few suggestions for improvements.

1. It would be helpful to quantify the imaging data in some way. For all conclusions, the reader is presented with a single representative image. In particular, where a partial phenotype is observed or a rescue is concluded, quantification seems very important (i.e., Figures 4A and 4C).

We have now included line scans of mitotic nuclei that show the enrichment of SUMO and Siz2-GFP along the nuclear envelope (NE) in each of the relevant figures. For SUMO, scans were compared to DAPI to demarcate nuclear and NE-associated SUMO signals (previously Fig. 1 and 2, now Fig. 1-3). For the Siz2-GFP, siz2-GFP mutants, and Siz2-GFP in various mutants, including those in Fig. 3-5 (previously Figures 2-4), scans are compared to scans of NE localized Sur4-mCherry.

Also note that for Siz2-GFP/Sur4-mCherry (and indicated mutants) quantification all images were reacquired, and these new images have been incorporated into the figures.

2. It would be very nice to include a model in Figure 7 to summarise the findings in this paper.

This is now included as Figure 9.

3. Figure 3A. Pus1 and Hxk1 cell sizes seem different. Is the scale bar correct and does it apply to the whole panel?

Figure 3A is now Figure 4A. The sizes are the same. The confusion may be that the GFP_{11}-mCherry Hxk1 fusion is visible throughout the cell (mCherry). When this binds the GFP_{1-10}-Scs2 positioned on the cytoplasmic face of the NE/ER, the resulting complex labels the NE/ER, including the cortical ER adjacent to the plasma membrane, while the GFP_{11}-mCherry-Pus1 fusion (mCherry) is intranuclear and its binding to INM-associated GFP_{1-10}-Scs2 yields a uniform NE membrane signal. A comparison of the NE signals of the cells in question suggests they are of similar size.

Also note that scale bars throughout have been adjusted as they were originally presented as larger than their actual size.

4. Figure S5, SUMOylation seems to be maximal in S phase, but Figure S6E shows that Sir4 localization is lowest then. How can this be reconciled with the observation that Sir4 SUMOylation is functionally important in directing its localization?

The reviewer refers to Figure S6E that is not in the manuscript. We assume this is a typo and was meant to refer to Figure 6E (now Figure 7E).

We assume the reviewer is suggesting that Sir4 SUMOylation is maximal in S-phase based on data in Fig. S5. However, SUMOylation of Sir4 is not examined in this figure. This figure shows: 1) FACS data

indicating that each strain progressed similarly through mitosis, 2) cellular SUMOylation phenotypes for the indicated strains at various points in the cell cycle are consistent with the strain background, and 3) that the cellular levels of Sir4-V5 (total Sir4-V5 is shown, and this is predominantly unSUMOylated) do not appear altered during the cell cycle in the various strains examined.

The α-factor arrest release SUMOylation profiles shown are consistent with what we have observed in that, specific SUMOylated mitotic species, including Scs2-SUMO (see 60 min time point), are present in WT and sir4^{K1037R} cells, but absent in siz2^{S522A} cells. At 30-, and 90-min time points, consistent with S-phase, there are observable cell-cycle specific SUMOylated species (~ 50kDa), which may also be what the reviewer is referring to, but these are not dependent on Siz2 and are not mitotic. Thus, we observe specific, Siz2-dependent, SUMOylation events in mitosis that coincide with peak Sir4 subtelomere association. Furthermore, under conditions where mitotic, Siz2-dependent SUMOylation is prevented (siz2^{S522A} mutant), or where a specific Sir4 SUMOylation site is absent (sir4^{K1037R} mutant), Sir4 subtelomere association is compromised (now Fig. 7E).

5. Figures 3C and 3B are called out of order in the text.

This is now Figure 4, and the call order has been corrected.

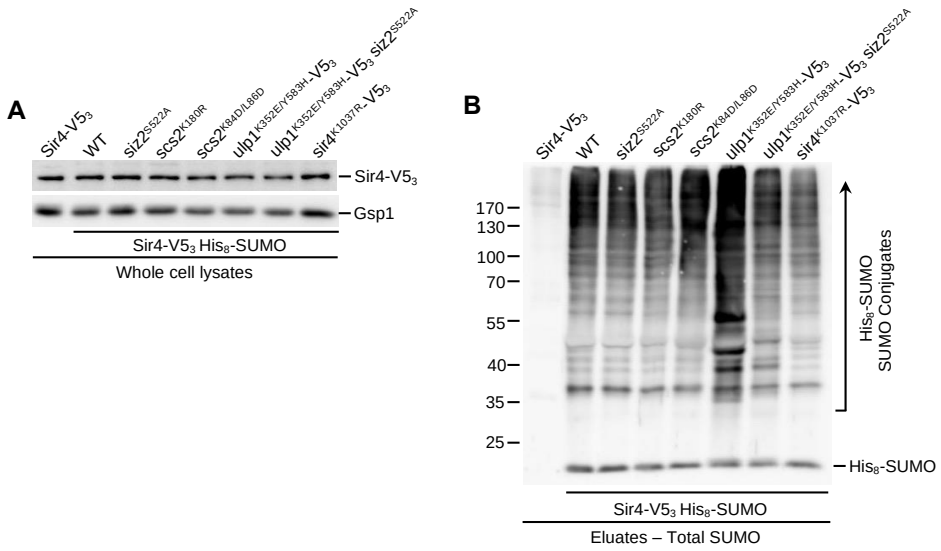
Reviewer #3 comments:

…The paper is technically excellent and the conclusions are sound. Although I see no major weaknesses, there are two important controls that should be performed prior to publication:
1. The microscopy images showing partitioning onto the NE should be quantified. This could be expressed as mean signal over the NE vs in the nucleus for a population of mitotic cells. This would make the quantitative defects seen in some experiments (e.g. Figure 4) more convincing.

As indicated in the response to reviewer 2, image quantifications are now included in each of the relevant figures.

2. The levels of Sir4 should be quantified in wild type and siz2 and scs2 mutants to confirm that they do not change.

As shown in Figure 7 (previously Figure 6), panels A-C, the levels of Sir4 are similar in the total cell lysates (the 'Load' fractions) of WT and the various mutant cells. To provide further evidence of this, we have included Reviewer Figure 1 below showing levels of Sir4-V$_5$ in whole cell extracts of WT and the various mutants used to assess Sir4 function in Figure 7, including siz2^{S522A}, scs2^{K180R}, scs2$^{K84D/L86D}$, ulp1$^{K352E/Y583H}$, siz2^{S522A} ulp1$^{K352E/Y583H}$, and sir4^{K1037R}. There appear to be no differences in the cellular levels of Sir4 among these mutants. Also shown are levels of SUMO-conjugates bound to NiNTA agarose beads for these same strains as requested by Reviewer 1 (see below).



Reviewer Figure 1. Panel A. Whole cell lysates from the indicated strains producing Sir4-V5$_3$ were examined by western blotting using anti-V5 antibody to detect Sir4-V5$_3$ and anti-Gsp1 (loading control). Similar levels of Sir4-V5$_3$ are detected in the various strains. Panel B. His$_8$-SUMO and His$_8$-SUMO conjugates were affinity-purified from the indicated strains containing Sir4-V5$_3$. Elutes from of NiNTA agarose beads were examined by western blotting using an anti-SUMO antibody. As shown, similar levels of His$_8$-SUMO and His$_8$-SUMO conjugates were bound for each strain except the ulp1$^{K352E/Y583H}$-V5$_3$. This is predicted as this strain contains higher cellular levels of SUMO conjugates (see Figure 2C).

Also, there are several questions that, if answered, would strengthen the paper:
1. To confirm that the additional SUMOylated species observed on the immunoblot are NE proteins, the authors could perform the immunoblot on fractionated lysates to examine the NE/ER microsomal fraction.

While we agree that such experiments would further support our conclusion that the Siz2-dependent mitotic SUMOylated species are associated with the NE, analysis by subcellular fractionation would require purification of yeast NEs separate from nucleoplasmic contaminants. We are aware of only one described procedure for this isolation (Rout et al. JCB 2000), and this is a complex, multi-step procedure. Considering this, and that SUMO modification is often labile, we feel that time invested in perfecting such a procedure, if done, would be confirmatory to our already comprehensive study and would be best invested in the future characterization of additional Siz2 targets.

2. The interaction between Siz2 and Scs2 is based on microscopy. A stronger result would be co-immunoprecipitation, which has been performed with Scs2 and various binding partners in the past.

The previous version of the manuscript (Fig. 3B) included immunoprecipitation (IP) analysis using strains producing TAP-tagged Scs2 and either Siz2-V5$_3$ or the siz2^{S522A}-V5$_3$ point mutant. Scs2-Tap binds to Siz2-V5, while the siz2^{S522A} point mutant, which fails to accumulate at the mitotic NE, showed reduced binding to Scs2-Tap and was detected at near background levels. These experiments were repeated, and additional controls were added. Figure 4C now presents data showing IP analysis of Scs2-TAP isolated from cells producing either Siz2-V5$_3$ or the siz2^{S522A}-V5$_3$ mutant, and scs2$^{K84D/L86D}$ -TAP (a mutation in the

FFAT binding domain of Scs2) producing Siz2-V5$_3$. Both mutations, as predicted, reduce Scs2-Siz2 interactions. We have also now included IP analysis examining the role of Scs2 SUMOylation and the SIM1 motif of Siz2 in the interaction of Scs2 with Siz2 (revised Fig. 5D). This is discussed further in response to point 4.

3. Given the known role of Scs2 in regulating INO1 transcription through interaction with Opi1, the authors should look at the localization of Opi1 during mitosis in the siz2 and scs2 mutants.

We agree with the reviewer that this represents an interesting question with regards to other functions of Scs2, however we do not see how this contributes to the main conclusions of the current study. Furthermore, studies by the Hochstrasser group (Felberbaum et al. 2012. MCB. 32:64-75) suggest that cells carrying the scs2^{K180R} mutation, which blocks Scs2 SUMOylation, do not exhibit an inositol auxotrophy. Thus, we believe that further analysis of the role of Siz2-mediated SUMOylation in the transcriptional regulatory functions of Scs2, specifically in binding the Opi1 repressor, is best addressed in a separate study.

4. One of the most intriguing findings is the relationship between cell cycle-regulated phosphorylation of Siz2 and a FFAT + SUMO-dependent interaction between Siz2 and Scs2. This is unprecedented for FFAT-Scs2/VAP interactions and may reflect the relatively low affinity of the FFAT motif in Siz2 and Scs2. If so, substituting a high affinity FFAT motif might bypass the dependence on SUMOylation, making this interaction unregulated. Alternatively, it raises the possibility that other FFAT-Scs2 interactions are also modulated by SUMO. If so, then mutation of the Scs2 SUMO site should disrupt those interactions as well.

We agree that a broader analysis of the contributions of SUMOylation to the interactions mediated by FFAT/MSP domains is likely to provide important new insights into the regulation of these interactions. However, engineering changes in the FFAT region of Siz2 or assessing the contributions of Scs2 SUMOylation in its association with other FFAT-containing proteins would require extensive further analysis that we believe is beyond the scope of this paper. Instead, to further evaluate the contribution of the SUMOylation to the interaction of Siz2 with Scs2 as suggested by the reviewer comments, we have used IP analysis to examine the interactions of Scs2-TAP with siz2$^{I472/473A}$-V5$_3$ (the SIM1 motif mutant) and scs2^{K180R}-TAP (the SUMO acceptor site mutation) with Siz2-V5$_3$ (Fig. 5D). We observed that mutations that disrupt the SIM motif of Siz2 or SUMOylation of Scs2 reduced cellular levels of the Scs2/Siz2 complex as compared to WT controls. Together with other data presented, these results provide a precedent for the contribution of SUMO-SIM interactions to interactions mediated by FFAT/MSP domain interactions.

Reviewer #1 comments:

…This is a very interesting topic and a suitable model system to study it. In spite of this, we believe the study is too preliminary and the data presented does not support the authors' main claims, with too many alternative models/hypotheses not discarded. We therefore cannot recommend this manuscript for publication. We highlight below some of the key aspects that preclude us from recommending publication:

• Without proper quantification (for NE localisation, phosphorylation, SUMOylation), readers are left with single panel immunofluorescence images and blots along with the authors' qualitative assessment

of the different outcomes. Not only quantifications are missing but also the fact that all the mutants are shown as separate blots/gels/panels makes it very complicated to assess critically and quantitatively.

As indicated in the response to Reviewer 2 above, we have now included line scans of nuclei to support our conclusions on the mitotic localization of Siz2-GFP and various siz2-GFP mutants. This was also done with the SUMO immunofluorescence analysis. Regarding western blots to examine SUMO conjugates or phosphorylation, in the absence of a direct indication of the figure(s) the reviewer is concerned with, we do not see where quantification of these data would provide further support for our conclusion. Where specific examples are raised below, we have addressed the reviewers concerns as requested.

• While we can understand how the authors initially refer to the four bands in the SUMO blots, using these blots as a proxy for SUMOylation of specific proteins is not appropriate. For example, the authors do show Scs2 SUMOylation (Figure 1E). However, this is done in ulp1 mutant background (without even comparing to wild type) and surprisingly, the shift that the SUMO-modified form experience differs by a big stretch depending on whether Scs2 is tagged in the N- or C-terminus. Additionally, they could have used the K180R mutant to confirm their observations. From this point onwards, we can't see why the authors keep using total SUMO blots rather than the substrate-specific blots.

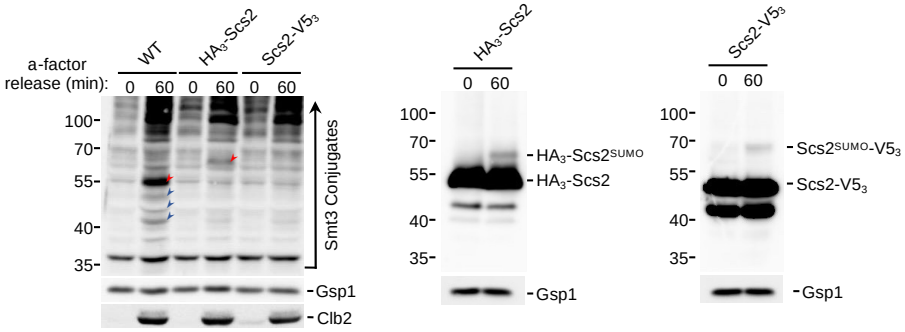
Our detection of the mitotic SUMOylation of a specific set of proteins, including four prominent SUMOylated species, provided a direct and multi-component read out to evaluate mechanistic steps involved in the targeting Siz2 to the NE and resulting SUMOylation events during mitosis (revised Fig. 1B, 2A, 2C, 2D, 3A, 3G, 4D, 5B, 5C, S1, S2, and S5). Thus, we have continued to use these data in the manuscript.

We agree with the reviewer's comment that it is important to examine specific mitotic SUMOylation targets, and, with this in mind, we focused specifically on Scs2 SUMOylation, as it is both a receptor for and a target of Siz2 during mitosis. As discussed below, we have included additional data that further supports these analyses. First, it is important to highlight previously published data from the Hochstrasser group that showed Scs2 SUMOylation and the visualization of Scs2-SUMO as an abundant SUMO species of ~55 kD in WT cells and, at significantly higher levels, in the ulp1^{K352E} mutant (Felberbaum et al. 2012. MCB. 32:64-75). We have reproduced these observations and further show that SUMOylation of Scs2 in WT and ulp1^{K352E} mutant cells occurs in mitosis and is dependent on Siz2 (revised Fig. 2-5).

This was confirmed by following Scs2 tagged with HA$_3$ at the N-terminus (HA$_3$-Scs2) or V5$_3$ at the C-terminus (Scs2-V5$_3$) in a ulp1$^{K352E/Y583}$-V5$_3$ background where mitotic SUMOylation of Scs2 is elevated. The difference in the mass of the SUMOylated forms of these two Scs2 fusions likely stems from multiple factors, including their different masses (V5 versus HA), the relative position of the tag (N- versus C-terminus) coupled with the SUMO modification, and their different amino acid residue composition. Notably, the increased mass of the SUMOylated, tagged Scs2 fusions detected using antibodies directed against their HA$_3$ or V5$_3$ tags directly correspond to the size of the major mitotic SUMOylated species detected with the SUMO-specific antibody (see revised Fig.2D). Furthermore, as the reviewer suggested, we show the formation of these SUMOylated species is dependent upon the K180 SUMO site of Scs2. This is now more clearly presented in Fig 2D. Importantly, in these Scs2-tagged strains, the 55 kD mitotic SUMOylation species detected in untagged strains is absent, consistent with it being Scs2. These observations, together with data showing that the 55 kD mitotic SUMOylation species is lost in the scs2Δ and scs2^{K180R} SUMO-site mutant, provide clear evidence that the 55 kD mitotic SUMOylation species we monitor during mitosis using anti-SUMO western blots is SUMOylated Scs2. We would emphasize that this approach of using SUMO blots to specifically monitor Scs2-SUMO was similarly used by the

Hochstrasser group (Felberbaum et al. 2012. MCB. 32:64-75). Furthermore, it is important to note that we choose to follow Scs2-SUMO and other mitotic SUMOylated proteins using the SUMO blot approach as tagged versions of Scs2, which would provide an alternative method for monitoring SUMO-Scs2, are functionally compromised. As shown in reviewer Figure 2 and Figure S1A of the manuscript, WT cells producing tagged Scs2, either at the N- or C-terminus, show reduced mitotic SUMOylation (compare anti-SUMO western blots of WT with Scs2-tagged strains). Only in the ulp1$^{K352E/Y583H}$-V5$_3$ mutant where Scs2 SUMOylation levels are elevated do we detect robust SUMOylation of the tagged Scs2 fusions (Fig. 2D).

Finally, we emphasize that we have also examined Siz2-mediated SUMOylation of Sir4 and its function in telomere anchoring to the NE during M- and G1-phase, and we show these processes are dependent on the mitotic NE recruitment of Siz2 and the SUMOylation of Scs2 (Fig. 7).



Reviewer Figure 2. Whole cell lysates from the indicated strains were produced after a-factor arrest (0 min) or 60 min post release from a-factor arrest. Lysates were probed by western blotting using anti-SUMO to observe SUMO conjugates, anti-Clb2 to assess cell cycle stage, anti-HA to probe for HA$_3$-Scs2, anti-V5 to probe for Scs2-V5$_3$, and anti-Gsp1 as a loading control. The red arrowheads identify the position of SUMOylated Scs2 and SUMOylated HA$_3$-Scs2. Blue arrowheads identify SUMOylated proteins whose modification are Siz2 and Scs2 dependent. Note that while SUMOylated HA$_3$-Scs2 and Scs2-V5$_3$ are observed when probing using an antibody that recognizes their respective tags, the level of HA$_3$-Scs2 is severely reduced relative to untagged Scs2 in the SUMO blots, while SUMOylated Scs2-V5$_3$ is not detected (left panel). These tag-dependent defects in Scs2 SUMOylation are accompanied by a severe reduction in the SUMOylation of the other Siz2 dependent mitotic targets. These effects also do not stem from a cell cycle defect as shown by similar Clb2 levels when comparing WT to the tagged Scs2 strains.

• Had the authors used substrate-specific blots along the manuscript, we would be able to assess the feasibility of their model, which requires that a substantial amount of Scs2 is SUMOylated in order to trigger the proposed downstream SUMO wave.

It is unclear to us how the reviewer concludes that the multiple NE SUMOylation events initiated at the NE by the binding of Siz2 to, and the SUMOylation of, Scs2 requires 'substantial' amounts of Scs2 to be SUMOylated (presumably they mean steady-state levels). We are uncertain what the reviewer's criteria are for 'substantial', however, it is important to consider that there is extensive literature showing that the functional impact of a SUMO modification(s) of a given protein is generally accomplished by low steady-state levels of this PTM (see Jentsch and Psakhye, Annu. Rev. Genet. 2013. 47:167–86; Flotho and Melchior. Annu. Rev. Biochem. 2013. 82:357–85; Chang and Yeh. Physiol Rev 2020. 100:1599–1619). In most instances, an explanation for this phenomenon remains outstanding. For Scs2, the levels of SUMOylation are also restricted to those Scs2 molecules present in the INM and

exposed to nuclear Siz2, while Scs2 present in the outer nuclear membrane, or the ER, is not predicted to be SUMOylated. The wave of SUMOylation we detect at the NE during mitosis is predicted to arise from the INM recruitment of Siz2 and SUMOylation of multiple proteins, including Scs2. This wave of INM SUMOylation events is conceptually similar to that reported to occur at single-stranded DNA repair sites, where the SUMOylation of multiple repair proteins by recruitment of a SUMO ligase recruitment to DNA contributes to the formation and stability of DNA repair complexes (Psakhye and Jentsch. 2012. Cell. 151:807–820.). In this case as well, only minor amounts of the repair proteins are detected with a SUMO modification at steady state.

• Many results could be the consequence of different mutant backgrounds affecting cell cycle dynamics and consequently (and indirectly) affecting SUMO modification and/or NE localisation. Indeed, the authors use cyclin Clb2 levels, which peak during metaphase, as a reference. However, Clb2 levels peak at different times after α-factor release in different mutants (e.g. peak at 60 min in 1B but 50 mins in the Scs2K180R and ulp1K352E/V583H-V53 mutants in 1C and 1D respectively, while HA3-Scs2 increases the time till Clb2 peak to 70 min in ulp1K352E/V583H-V53 mutants in 1E). Therefore, changing cell cycle stage could easily explain different SUMOylation levels in different figures.

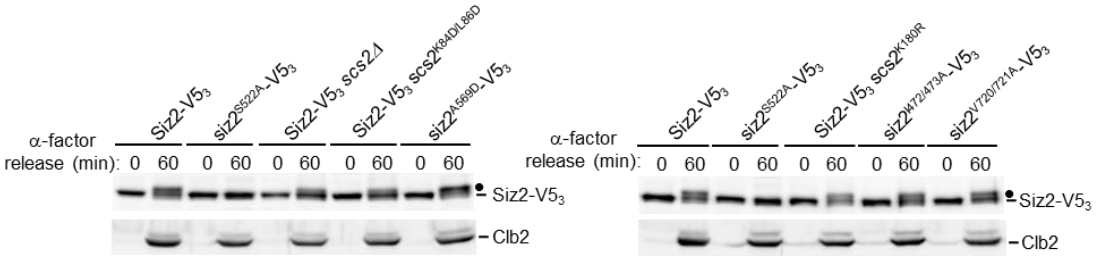
The 50 min Clb2 peak for the scs2^{K180R} blots was an error stemming from improper cropping of the western blot. This has been fixed (Fig. 2A; also see Fig. 2D, S2A). The weak 60 min Clb2 signal in the blot derived from ulp1$^{K352E/Y583}$-V5$_3$ cells stemmed from poor transfer. This arrest release experiment was repeated and shows peak Clb2 levels at 60 min. (Fig. 2C).

For all the relevant strains we interrogated in this manuscript, we examined of SUMO-conjugates at multiple time points throughout the cell cycle, beginning after release from G1-phase arrest through the following G1-phase of cell cycle. In each of the profiles, peak Clb2 levels occur between the 50 and 60 min time points. The exceptions include the HA$_3$-Scs2 ulp1$^{K352E/Y583}$-V5$_3$ and siz2^{S674A}-V5$_3$ strains that exhibit a mitotic delay (high Clb2 at 70 min) that is mirrored by a delay in peak mitotic SUMOylation. Beyond these two strains, cell cycle progression is reasonably consistent and implies that the various strains are progressing through the cell cycle at similar rates. Moreover, our time course captured samples at numerous points in the cell cycle, thus allowing the evaluation of SUMO-conjugate levels throughout the cell cycle. In the various strains where mitotic SUMOylation of Scs2 and other Siz2 target proteins was detected (e.g., WT and the ulp1^{K352E} mutants), this occurred at times corresponding with the trailing edge of peak Clb2 levels. This is discussed in the manuscript (see Fig. 1-5, S1, S2). By contrast, we did not detect increased levels of the Siz2 specific SUMOylation, including that of Scs2, at any point throughout the entire time course of arrest-release experiments, including mitosis (as defined by the rise and fall of Clb2 levels), in mutants where Siz2 is absent or fails to associate with the mitotic NE. Since we examined time points throughout the cell cycle in all mutants, we consider our interpretation reasonable.

• It appears to us that the authors do not have a clear criterium for what represents a 'significant' change and what doesn't. The analysis of Siz2 phosphorylation is a perfect example of this. While the authors claim that one specific Siz2 phospho-site mutant has impaired phosphorylation, it appears to us that all of the mutants have impaired phosphorylation. Same is true for the NE localisation.

First, we would emphasize to the reviewer that at no point in the manuscript do we use the term 'significant' as a descriptor for any observations in the absence of supporting statistical data. This was our criterion for the use of this term. Second, with regards to the specific example raised by this reviewer, we show that the gel mobility shift for Siz2-V5$_3$ seen during M-phase in WT cells (Fig. 3D, S2A) is not detected in a siz2 point mutation that removes a serine phosphorylation site (Fig. 3E, S2A;, siz2^{S522A}-V5$_3$). The requirement for Ser522 in the M-phase phosphorylation of Siz2 is quite clear (also see Reviewer Fig.

3). Our examinations of the phosphorylation states of various other non-phosphorylation site mutations were instead used as a qualitative assessment of whether these mutants failed to be phosphorylated at S522. If this were the case, such a mutant would be predicted to lack M-phase phosphorylation. In each of these various mutants, we observed M-phase specific changes in siz2 mobility similar to that seen with WT Siz2 (see Reviewer Fig. 3). While the levels of the slower migrating phosphorylated species that appear in individual samples of the time course experiments shown in the manuscript may vary somewhat between experiments and strains, our conclusion that these non-phosphorylation site mutant forms of siz2 are phosphorylated during mitosis seems reasonable. Regarding the NE localization of these mutants, as discussed above, we have now quantified the levels of NE-associated Siz2 and SUMO using line scans.



Reviewer Figure 3. Whole cell lysates from the indicated strains were produced after a-factor arrest (0 min) or 60 min post release from a-factor arrest. Lysates were probed by western blotting using anti-V5 to assess Siz2-V5$_3$ or mutant siz2-V5$_3$, anti-Clb2 to assess cell cycle stage, and anti-Gsp1 as a loading control. The dots indicate the position of mitotically phosphorylated Siz2-V5$_3$ or mutant siz2-V5$_3$.

• Direct evidence is needed for their proposed direct interaction between Siz2 and Scs2 and the potential SUMO-SIM involvement.

In Figure 4C, we show that Scs2-TAP affinity purified from cell lysates is associated with Siz2-V5$_3$. Moreover, the siz2^{S522A}, which fails to concentrate at the NE during mitosis, shows reduced binding to Scs2-TAP as compared to the WT Siz2. These observations, together with our analysis of scs2 point mutations and their effects on the mitotic recruitment of Siz2 to the NE, and data showing Siz2 function to mediates the SUMOylation of Scs2, provides strong support to our conclusion that Siz2 functionally interacts with Scs2. Regarding the involvement of the SUMO-SIM interaction, please see our response to point 4 of reviewer 3.

A few minor comments/questions:

• Fig 1A: how do authors know these are SUMO conjugates (no free SUMO) as stated in the text?

It has been previously established that cellular levels of free SUMO represent a small portion of the total SUMO. Moreover, free SUMO migrates at an apparent molecule mass of ~15 kD (which we can detect by western blots), while most of species detected with the anti-SUMO antibodies are of larger apparent mass. Moreover, the use of tagged SUMO or anti-SUMO antibodies (as used in this manuscript) to detect SUMO-conjugates by western blotting has long been established in the literature.

• Fig 3B: The pull-down should be presented in a single gel in order to compare phosphorylated vs non-phosphorylated forms. Showing separate blots makes them impossible to compare.

As indicated in the text, the results presented in Figure 4C (previously Fig. 3B) are intended to compare levels of Scs2 binding to Siz2 versus a Siz2 mutant (siz2^{S522A}) that fails to target to the NE during mitosis. As shown in Figure 4C, the results of these binding experiments are consistent with the mitotic NE association of WT Siz2 and the lack of NE binding of the siz2^{S522A} mutant. These experiments were not performed to examine the phosphorylation state of these Siz2 proteins, which are shown in Figure 3D-E, S2A.

• Fig 6A - would want to see that total His8-SUMO (from Ni-NTA samples) and Sir4-V53 (from input) levels are similar in all the samples to know that band differences are not due to differences in starting protein levels.

The requested data are shown in the reviewer Figure 1 and shown above in our response to reviewer 3, point 2.

September 2, 2021

RE: JCB Manuscript #202103036R

Dr. Richard W Wozniak
University of Alberta
5-14 Medical Sciences Building
Edmonton, Alberta T6G 2H7
Canada

Dear Dr. Wozniak:

Thank you for submitting your revised manuscript entitled "Phosphorylation-dependent mitotic SUMOylation drives nuclear envelope-chromatin interactions". The paper has now been seen again by the original reviewers and they all recommend acceptance and so we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

As you will see, reviewer #1 has one remaining concern regarding the added westerns of TAP showing the levels of Scs2 in the IPs. This reviewer feels that "...making conclusions from pull-downs conducted on separate blots is not appropriate and I would suggest a proper figure is presented so that the reader can compare the different mutants (on the same gel)." We agree with the reviewer that having the various mutants on the same blot would be the preferred method for presenting the data. Thus, if you have this data already in hand or can generate it quickly, we would encourage you to add it to the paper. However, since this assay is not essential for the main conclusions of the paper, we do not feel that publication should be unreasonably delayed and thus, if acquiring this data would be too time-consuming at this point, we will not require it for resubmission. However, in that case, you should adjust the text to reflect this caveat. Please be sure to include a comment in the final cover letter/rebuttal to indicate how this issue was addressed.

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2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis, including cropped blots. Thus, you will need to add molecular weight markers to the

blots in figures 3D, 3E, 4B, 5A, 7A-C, and the rightmost blots in supplementary figure 5.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
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g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section (that is, in addition to the supplementary figure legends).

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be

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Sincerely,

Arshad Desai, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This revised version by Ptak et al is sufficiently improved to warrant publication in JCB. While we still have our concerns with some of the figures and whether the data fully supports the authors' conclusions, we think they have addressed most of the concerns and the manuscript is improved as well as the new layout of the figures. Some of our impressions are highlighted below.

• Quantification has greatly improved the image analysis and allows more confidence in the claims made by the authors

• Figure 2D gives more confidence that the 55 kDa band is Scs2, with the single blot showing the differential shift in the tagged proteins that is removed with the K180R mutant and the individual blots of the tags showing the differential shift in the sumoylated bands is a good clarification of the initial query.

• Adding the WB of TAP to show the levels of Scs2 in the IP greatly increases confidence in the conclusions drawn. However, making conclusions from pull-downs conducted on separate blots is not appropriate and I would suggest a proper figure is presented so that the reader can compare the different mutants (on the same gel).

• The authors have sufficiently justified the use of Clb2 levels as a reference for cell cycle progression after correcting the errors in the previous draft's figures.

• We agree that, while the shift is subtle, it is reasonable to conclude that Siz2 is phosphorylated during mitosis in the mutant forms shown apart from S522A. The initial comment was observing that the other phosphorylation site mutants also have differences to the WT protein, and therefore also have impaired phosphorylation, albeit to a lesser extent than the S522A mutation. However, this is not essential for the conclusions of this paper and should not preclude publication.

Reviewer #2 (Comments to the Authors (Required)):

The authors have revised the manuscript to address all of the reviewers' comments.

Reviewer #3 (Comments to the Authors (Required)):

The authors have addressed my concerns and these changes make the paper more convincing.