A global *S. cerevisiae* small ubiquitin-related modifier (SUMO) system interactome

Tharan Srikumar, Megan Lewicki, Brian Raught

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**Review timeline:**

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**Editor: Thomas Lemberger**

**Transaction Report:**

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

1st Editorial Decision 13 December 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, several concerns on your work, which should be convincingly addressed in a revision.

The reviewers request several further analyses and validation to strengthen the conclusions. Reviewer #1 also asks for an estimation of the quality of the dataset in terms of sensitivity of the assays (estimated false negative rate).

We would also kindly ask you to deposit the interaction data in one of the databases of the Molecular interaction data should be deposited with a member of the International Molecular Exchange Consortium (IMEx, http://www.imexconsortium.org) and indicate the link to the data in a "Data availability" section within the Materials & Methods section.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

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REFEREE REPORTS:

Reviewer #1 (Remarks to the Author):

In this manuscript Srikumar and colleagues used affinity purification coupled to mass spectrometry to characterize the interaction network associated with series of components of the yeast SUMO system. The work is certainly interesting, full of potentially exciting results, but many of the observations are still a bit preliminary and not completely convincing to warrant publication in Molecular Systems Biology.

1) As the components of the SUMO system under investigation have very distinct subcellular locations (fig 2) the authors should show that the over-expressed versions (that have been used to generate the network) localize similarly (as the endogenous version). That is probably not be too difficult and would prove that over-expression of the baits did not affect their localization.

2) Generally the story is difficult to follow especially for the non-expert reader. A short introduction to explain the function of the series of baits used here (SUMO conjugating, ligases, proteases, deconjugating enzymes, etc) would help the reader get oriented with the discoveries. May be this could be done page 4 and 5 where the authors list the bait and the interactions found, i.e. it may be advisable to structure the paragraphs around the different protein functions (and not their names); similar comments for the networks, i.e. they could be organized according to protein/baits function.

Similarly the comments, p4-5 concerning the different subnetworks are difficult to understand:
- Ubc9: "the Ubc9 interactome were largely representative of the SUMO system interactors as a whole" is elusive what does that mean and was this expected? Does this tell us something concerning Ubc9 function? Unclear. How is this then possible that the overlap with Siz1 and Siz2 is only 20% (and only 40% with Ulp2 and 10% with Upl1). Again very unclear.
- Siz1 and Siz2: The interactions observed between Siz2 and components of the RNA polymerase are interesting results that may deserve some more validation: does that also happen with the endogenous versions, i.e. without over-expression? Do we know what the role of these interaction are? Are these components SUMOylated (may be one could look for motifs?). Figure 1F, the western blot is poorly convincing because of the contaminating band that co-elute almost at the same MW as Siz1. May be advisable to perform reverse IP, i.e. IP with anti-HA and WB with anti GFP, to control.

2) The various networks in supplemental figure 1 are confusing and poorly exemplify the point the authors try to make. For example the network in panel S1B does not show enrichment in proteins previously reported to be SUMO targets as claimed page 3 - it just show that a fraction of the node were previously known - enrichment requires the comparison to another set of irrelevant proteins, or network. Most important a p-value must be given. Similarly the panel S1C says physical interactions indicated in blue, genetic interaction in purple, novel interactions are highlighted in pink. This is wrong, the nodes and not the interactions (edges) are color coded.

3) Data quality. The authors should give an idea on the fractions of the know interactions that have been captured here (false negative or sensitivity).

4) Localization of the SUMO system. This part is poorly convincing, as apparently these localizations where already known from other studies. What are novelties in this paragraph? Not clear. If all is already known then this should be removed as this does not bring much: the AP-MS were done with over-expressed baits; the localization data here concern endogenously expressed GFP-fusions and thus do not help with the interpretation of the network. Siz2 localizes in additional nuclear bodies that do not overlap with Nop2; could this be PLMs?

5) Generally, the overall analysis is a bit disappointing and many more could have been done to prove the values of the dataset. For example, look for the presence of (or enrichment for)
SUMOylation motifs, or SUMO-interacting motifs, etc.

6) Figure 3E: it is completely unclear how the authors could derive such a model (including stoichiometries) from their data. Very generally there is a lack of clarity concerning what was previously known (from before) and what this work brings as novelities/discoveries. This requires much more clarity.

Reviewer #2 (Remarks to the Author):

The authors used mass spectrometry and immunoaffinity purification of HA-ProtA tagged protein baits to identify interacting partners of the yeast Ubc9, E3 ligases Siz1, Siz2, and SUMO-specific proteases Ulp1, Ulp2. These analyses identified 452 high confidence interactions from 321 proteins and GO analyses revealed enrichment for proteins involved in several biological functions including ribosome biogenesis, chromatin remodelling and transcriptional regulation. Comparison of overlapping interactions identified subsets of proteins involved in specific functions (e.g. Siz2 but not Siz1 regrouped interactors such as RNA Pol I, II and II core transcription complexes). The authors also complement their interaction network with immunofluorescence data to confirm the nucleolar subcellular localization of Siz2 in the interphase. They also conducted IP pulldown experiments and in vitro binding assays to confirm specific interactions. Overall, these studies are well executed and provide valuable information on the protein-protein interactions associated with SUMO modifying enzymes in budding yeast. I recommend publication pending minor revisions outlined below:

1. The authors should indicate how they validated that the HA-ProtA tagged do not interfere with the functions of the protein baits.

2. Can the authors comment on the observation of sumoylation sites on their protein substrates? Presumably their MS analyses would be able to identify modified peptides.

3. On p. 4, last para. and Fig 1E, the authors indicated that the interactions are specific for Siz1-HA because the bands detected in the Siz2-HA lanes are judged to be non-specific (bands labelled with asterisks in Figure 1F). Is there any evidence suggesting that the asterisk-labelled band is not a modified form of Siz2? In Figure 1F (bottom panel), they show immunoblots of whole-cell lysates derived from cells expressing either Siz1-HA or Siz2-HA. The Siz2-HA lanes show multiple bands including some above and it is unclear how minor bands could not be related to Siz2-HA. Could it be that Siz2 is itself SUMOylated? The evidence for Siz1-HA interacting with Spt16-GFP (lane 13) is unclear. The asterisk-labelled non-specific band is very close to what is claimed to be Siz1-HA band.

4. On Fig. 3B, can the authors clarify if the nature of the tag (GFP or His-Flag) affects the SUMOylation pattern observed? For example CDC14 & Tof2 show SUMO bands in GFP Smt3 compared to His-Flag Smt3 whereas the opposite is observed for Net1.

5. On p. 8, the comment on "These data for the first time highlight specific roles for Ulp2 and Siz2 in the maintenance of rDNA repeats" should be moderated given prior reports (PLoS Genet, 4, e1000215, 2008, see Fig. 2H) indicating that levels of SUMO E3 activity (associated with Siz2) affect rDNA stability and copy number.

Reviewer #3 (Remarks to the Author):

The manuscript from Tharan et al using systematic proteomics approach to identify the interaction partners of the components in SUMO system. Previous reports mainly focused on identifying novel SUMO conjugates in budding yeast, plants and mammals. The manuscript sheds light on understanding how the SUMO machinery enzymes responsible for the conjugation and deconjugation steps through the angle of interaction proteins instead of substrates. The result showed only part of the newly identified interacting proteins overlapped with previously found
SUMO substrates. The rest identified proteins may be potential SUMO substrates due to their binding ability to the enzymes in SUMO system or mediate the recruitment of SUMO substrates as a bridge. Although the components of SUMO system in budding yeast are simpler than those in mammals, this manuscript still can be used as a good resource for studying SUMOylation in budding yeast. Furthermore, the orthologs of those identified proteins may provide the hint of novel SUMO function in other organisms.

Major criticisms:
1. According to the newly identified proteins which are interacted with the components of SUMO system, Tof2 and the RENT complex can bind to Ubc9 and be SUMOylated. The SUMOylated Tof2 and the RENT complex can serve as a bridge for binding to Lrs4, a component of the cohibin complex. The interaction among unmodified or SUMOylated proteins regulates the connection between rDNA and the inner nuclear membrane. One of the key points is the SUMO binding function of LRS4 to those SUMOylated proteins. Does Lrs4 contain SIM domain? This SUMO binding function should be responsible for the interaction between Lrs4 and the SUMOylated Tof2 and the RENT complex. The authors need to show that Lrs4 interacts with Tof2 and the RENT complex and this interaction is mediated by SIM domain.

2. The interaction between Ulp2 and Csm1 should be moved to the main text instead of supplementary table. In addition, although the interaction was already shown in previous yeast two-hybrid result, the authors need to provide biochemical binding assay to confirm this interaction.

3. The cohibin complex (Lrs4 and Csm1) bind to Ulp2, not Ube9. It is reasonable to assume that this complex should not be SUMOylated because of higher affinity to the Ulp2. On the other hand, Tof2 and the RENT complex should be SUMOylated due to their affinity to Ubc9. However, the SUMO conjugation patterns in Fig 3B are not very clear. Does SUMOylated Csm1 appear in longer exposure (because the faint signal showed in higher molecular weight)? Some bands with lower mobility were detected in cells expressing Cdc14, Tof2, and Net1 proteins even in the control lane without His-Flag-Smt3 or GFP-Smt3 expression. Are those signals from endogenous Smt3 conjugates or non-specific signal? Please use other antibodies (ex. anti-GFP, anti-His antibody) to confirm that these proteins are SUMOylated.

Minor points:
1. In Fig S1A, S1B, and S1C, the circles should be modified to fill with bright colors. The circle fill with dark color is not easy to read the black word inside the circle.

2. In Fig 2A, the author should put the image of Ulp1(150-621) localization in WT strain into the figure as a control for the normal localization of Ulp1(150-621) protein.

3. The purity of proteins in Fig 3 is not good. Please indicate which bands are those purified full length proteins or improve the protein purity in Fig 3A because there are too many degradation products.
12 March, 2013

Thank you for your email of Dec 13, 2012 inviting us to submit a revised version of our manuscript, “Physical and Functional organization of the small ubiquitin-related modifier (SUMO) system in S. cerevisiae” by Tharan Srikumar et al.

We appreciate the constructive comments from the referees, and were happy to read that they found our comprehensive analysis of SUMO system interactors to be a valuable resource, in principle.

In response to reviewer suggestions, we have re-written several sections of the manuscript, included additional experiments, and conducted a more in-depth analysis of the interaction data.

Importantly, our manuscript represents the first SUMO system interactome in any organism. Our AP-MS data identify >450 high confidence protein-protein interactions for the S. cerevisiae SUMO E2 Ubc9, the Siz-type E3s Siz1 and Siz2, and the SUMO-specific proteases Ulp1 and Ulp2.

We have also conducted live cell confocal microscopy to better characterize the intracellular localization of the SUMO system components. For the first time we report that Siz1 is specifically excluded from the nucleolus, while Siz2, Ubc9 and Ulp2 are not.

Finally, mining our new interactome, we provide a number of novel biological insights; (i) we validate several novel Siz1- and Siz2-specific interactors involved in transcriptional regulation. These data for the first time suggest that both Siz1 and Siz2 are likely to play important roles in transcription, but that they target different subsets of transcription proteins; (ii) we identify a new nucleoporin (Nup2) required for proper Ulp1 localization, and; (iii) we reveal a number of new details regarding the interactions between SUMO, the SUMO system components, and the RENT and cohibin complexes. In particular, we show that the cohibin component Csm1 interacts with Ulp2, and map this interaction to its N-terminus. We demonstrate that Lrs4 can bind directly to SUMO. We confirm that Ubc9 can bind directly to several members of the RENT complex, and that these proteins are sumoylated in vivo. Lastly, we demonstrate that disruption of Siz2 or Ulp2 results in a loss of proper rDNA repeat maintenance. Together these data significantly improve our understanding of how the SUMO system is likely to regulate nucleolar organization.
Our dataset also represents an important resource for the SUMO field. Since the SUMO system is highly evolutionarily conserved, many of the discoveries made here are likely to be applicable to all eukaryotes.

Please see our detailed responses to the reviewers below (reviewer comments in italics).

Reviewer #1 (Remarks to the Author):
In this manuscript Srikumar and colleagues used affinity purification coupled to mass spectrometry to characterize the interaction network associated with components of the yeast SUMO system. The work is certainly interesting, and full of potentially exciting results...

Thank you for the positive comments.

...but many of the observations are still a bit preliminary and not completely convincing to warrant publication in Molecular Systems Biology.

1.0 As described above, we have included some new findings in the revised manuscript; e.g. we provide a more thorough examination of the sumoylation of three different transcriptional regulatory proteins by specific SUMO E3 ligases, we include additional data on the SUMO-mediated regulation of rDNA repeats, and we have included additional analyses of the interactome data.

According to the journal website, “Reports are short publications focusing on a provocative and novel aspect of a study”. We would argue that our study is both novel and provocative, and that we have packed an impressive amount of data into this manuscript, as listed above.

1) As the components of the SUMO system under investigation have very distinct subcellular locations (fig 2) the authors should show that the over-expressed versions (that have been used to generate the network) localize similarly (as the endogenous version). That is probably not be too difficult and would prove that over-expression of the baits did not affect their localization.

1.1 The ProtA-HA proteins do indeed appear to be functional and localized properly, as: (i) The GFP/RFP-tagged proteins (which are expressed to similar levels and which have an epitope tag at the same location and of a similar size) used in this study match with previously reported immunofluorescence studies of endogenous SUMO system proteins, and with our own analysis of endogenous proteins (see Fig S2), and; (ii) we identified a number of previously identified SUMO system interactors. Also, the assumption that these baits are over-expressed is not necessarily warranted; we actually used very low levels of galactose in the culture media (0.05%) to induce relatively low protein expression levels.

2) Generally the story is difficult to follow especially for the non-expert reader. A short introduction to explain the function of the series of baits used here (SUMO conjugating, ligases, proteases, deconjugating enzymes, etc) would help the reader get oriented with the discoveries. Maybe this could be done on pages 4 and 5 where the authors list the bait and the interactions found, i.e. it may be advisable to structure the paragraphs around the different protein functions (and not their names); similar comments for the networks, i.e. they could be organized according to protein/baits
function. Similarly the comments, p4-5 concerning the different subnetworks are difficult to understand:
- Ubc9: "the Ubc9 interactome were largely representative of the SUMO system interactors as a whole" is elusive what does that mean and was this expected? Does this tell us something concerning Ubc9 function? Unclear. How is this then possible that the overlap with Siz1 and Siz2 is only 20% (and only 40% with Ulp2 and 10% with Upl1). Again very unclear.

1.2.1 We agree with the reviewer here, and have substantially re-written the manuscript in an attempt to make it clearer. While we are dealing with a very tight word limit, we hope that it is now easier to understand. Any specific suggestions to further improve clarity are welcome.

- Siz1 and Siz2: The interactions observed between Siz2 and components of the RNA polymerase are interesting results that may deserve some more validation: does that also happen with the endogenous versions, i.e. without over-expression? Do we know what the role of these interaction are? Are these components SUMOylated (may be one could look for motifs?).

1.2.2 Thank you for the positive comments. Of course, we fully agree that these results (along with many others in this large dataset!) are extremely interesting, and worthy of follow-up study. In the revised manuscript, we have included some additional data regarding the Siz2-polymerase connection, demonstrating that as predicted by our AP-MS data, Siz2 is in fact required for the sumoylation of Rpo21 (Fig 1). However, among the many choices for more in-depth follow-up, here we have chosen to focus on the role of the SUMO system in rDNA regulation (in particular because we also report for the first time that Siz1 is excluded from the nucleolus, while Siz2, Ubc9 and Ulp2 are not). While we plan on pursuing the connection between Siz2 and polymerases more in the future, a study of each and every new interesting set of interactions or new biological connections identified in our interactome is simply beyond the scope of the current manuscript.

3) Figure 1F, the western blot is poorly convincing because of the contaminating band that co-elute almost at the same MW as Siz1. May be advisable to perform reverse IP, i.e. IP with anti-HA and WB with anti GFP, to control.

1.3 As we point out in the figure, this antibody detects a non-specific band. However, the Siz1 protein clearly runs above it, and Siz2 runs below it. Since the non-specific band is present in every lane, it is actually not difficult to determine when Siz1 or Siz2 are present in the pulldowns; i.e. the presence of this contaminating band does not interfere with the interpretation of the data at all. Is there a specific lane where the presence/absence of Siz1/2 is not clear?

4) The various networks in supplemental figure 1 are confusing and poorly exemplify the point the authors try to make. For example the network in panel S1B does not show enrichment in proteins previously reported to be SUMO targets as claimed page 3 - it just show that a fraction of the node were previously known - enrichment requires the comparison to another set of irrelevant proteins, or network. Most important a p-value must be given. Similarly the panel S1C says physical interactions indicated in blue, genetic interaction in purple, novel interactions are highlighted in pink. This is wrong, the nodes and not the interactions (edges) are color coded.
1.4 We have now conducted a statistical analysis on our dataset (as described in Materials and methods), and provide a p-value for enrichment of SUMO targets. As expected, they are highly enriched (p<0.0001). The proportion of known SUMO targets in our dataset (77/321 ~24%) is much higher than the proportion of all known SUMO targets in the yeast proteome (613/5045 ~12%).

We have also changed the text to read “Proteins previously reported to have physical or genetic interactions with SUMO system components are highlighted...”

5) Data quality. The authors should give an idea on the fractions of the know interactions that have been captured here (false negative or sensitivity).

1.5 We have now included an additional set of spreadsheets (in Supplementary Table 1) listing all previously reported physical and genetic interactions for each SUMO system component.

Due to the dearth of published interaction data on the SUMO system it is difficult to assess a false negative rate for our study, but we can certainly compare our work to previous publications. Before our study, surprisingly few SUMO system interactors had been reported. For example, according to BioGRID and the Saccharomyces genome database (SGD), nine Siz1 physical interactions have been identified by AP-MS and AP-Western analyses. Our analysis found 7 of the same interactions, and added an additional ~70 putative Siz1 interactors. Another seven Siz1 interactions were detected using the yeast two hybrid (Y2H) system. Our analysis identified three of the same interactions. In the manuscript, we validate several of our new Siz1 interactors using co-IP Western (Fig 1), and all of the interactors that we tested proved to be bona fide Siz1 binding proteins as judged by this technique. We would thus predict our false positive rate to be very low.

Similarly, twenty AP-MS interactors were previously reported for Ulp1. We identified 12 of the same polypeptides, and identified an additional ~50 binding partners.

Only two physical interactions were previously reported for Ulp2 (both by Y2H). We detected one of these proteins in our AP-MS analysis, and identified an additional ~150 putative Ulp2 interacting partners.

Our data thus show excellent overlap with previous mass spectrometric-based studies and IP-Western data (with the addition of hundreds of new interactors) and a good, but somewhat lower level of overlap with yeast two hybrid data. This level of overlap (or even lower) has been observed in a number of other studies (see e.g. Breitkreutz et al. Science 328:1043). There are a number of likely reasons for the differences between AP-MS and Y2H data. For example, Y2H probably identifies some interactions that our method can miss, such as proteins that associate with the bait very weakly or transiently (and therefore do not make it through our purification protocol), or proteins that are normally expressed at low levels in log-phase yeast cultures (but which are artificially expressed at higher levels in the Y2H technique).

6) Localization of the SUMO system. This part is poorly convincing, as apparently these localizations were already known from other studies. What are novelties in this paragraph? Not clear. If all is already known then this should be removed as this does not bring much: the AP-MS were done with
over-expressed baits; the localization data here concern endogenously expressed GFP-fusions and thus do not help with the interpretation of the network. Siz2 localizes in additional nuclear bodies that do not overlap with Nop2; could this be PLMs?

1.6 We have re-written this section in an attempt to make it clearer. In fact, ours is the first study to use live cell imaging combined with confocal microscopy to study the entire SUMO system, and to use nucleolar markers to specifically look for localization in this organelle.

Also, as explained further in the manuscript, we used two sets of GFP/RFP-tagged proteins here; expressed from a plasmid AND endogenously tagged. Both sets of proteins shared identical localization. These data suggest that the gal-inducible HA-tagged proteins are in fact localized properly.

Budding yeast do not express a PML orthologue, and do not contain PML bodies.

7) Generally, the overall analysis is a bit disappointing and many more could have been done to prove the values of the dataset. For example, look for the presence of (or enrichment for) SUMOylation motifs, or SUMO-interacting motifs, etc.

1.7 As requested, we have added these data to Supplemental Table 1, listing all SUMO consensus sites and putative SIMs in our SUMO system interactome dataset.

Unfortunately, the presence of putative SUMO consensus sites or SUMO interacting motifs is just not very informative. As we previously reported, the standard “SUMO consensus site” sequence (hydrophobic-K-X-D/E) is associated with only about 40% of the identified SUMO conjugation sites in yeast proteins (Jeram et al. 2009 Proteomics, 9:922). In other words, the majority of SUMO conjugation sites do not conform to this sequence, and no additional strong consensus sequence has been identified to date.

Similarly, the SIM consensus sequence (usually written as I/L/V-I/L/V-X-I/L/V, or the reverse) is quite degenerate, and therefore also not very predictive. In fact, in another of our previous publications (Makhnevych et al. Mol Cell), we reported that the average yeast protein contains 2 or more putative SIMs.

Since these consensus sequences are not very useful or predictive, the field still generally relies on the use of mass spectrometry and mutational analysis to identify and confirm SUMO conjugation sites and SIMs.

8) Figure 3E: it is completely unclear how the authors could derive such a model (including stoichiometries) from their data. Very generally there is a lack of clarity concerning what was previously known (from before) and what this works brings as novelties/discoveries. This requires much more clarity.

1.8 We have attempted to make this section more clear in the revised manuscript. Previous publications have beautifully described how rDNA repeats are anchored to the inner nuclear membrane, via interactions between Fob1, RENT, cohinb and the INM proteins Heh1 and Nur1 (see e.g. work from the Mekhail, Moazed and Amon labs).
Our work demonstrates that: (i) the SUMO E2 protein Ubc9 can interact directly with the RENT components Tof2, Cdc14 and Net1 \textit{in vitro}, and that these proteins are sumoylated \textit{in vivo}; (ii) the SUMO-specific protease Ulp2 interacts with the cohibin complex, via binding to Csm1. We confirmed these data via IP-Western, and further demonstrate that this binding is mediated by the Csm1 N-terminus; (iii) we also demonstrate that the other cohibin subunit - Lrs4 - can bind directly to SUMO \textit{in vitro}. Taken together, these data allowed us to propose the working model in Fig 3.

We are not attempting to suggest any knowledge regarding the stoichiometry of the SUMO system proteins in this complex, but simply wished to convey our new findings in the form of a cartoon model. To avoid confusion, we have included a new statement in the Figure Legend (“Note: the stoichiometry of this complex, SUMO conjugation sites and SUMO binding sites remain to be identified.”).

Reviewer #2 (Remarks to the Author):

\textit{The authors used mass spectrometry and immunoaffinity purification of HA-ProtA tagged protein baits to identify interacting partners of the yeast Ubc9, E3 ligases Siz1, Siz2, and SUMO-specific proteases Ulp1, Ulp2. These analyses identified 452 high confidence interactions from 321 proteins and GO analyses revealed enrichment for proteins involved in several biological functions including ribosome biogenesis, chromatin remodelling and transcriptional regulation. Comparison of overlapping interactions identified subsets of proteins involved in specific functions (e.g. Siz2 but not Siz1 regrouped interactors such as RNA Pol I, II and II core transcription complexes). The authors also complement their interaction network with immunofluorescence data to confirm the nucleolar subcellular localization of Siz2 in the interphase. They also conducted IP pulldown experiments and in vitro binding assays to confirm specific interactions. Overall, these studies are well executed and provide valuable information on the protein-protein interactions associated with SUMO modifying enzymes in budding yeast. I recommend publication pending minor revisions outlined below:}

Thank you very much for the positive comments.

1. \textit{The authors should indicate how they validated that the HA-ProtA tagged do not interfere with the functions of the protein baits.}

2.1 It is entirely true that in any experiment in which an epitope tagged protein is used, we must consider the caveat that some function or activity could be affected by the additional amino acid sequence. However, we do have several lines of evidence to suggest that the epitope tags used in our study do not obviously disrupt the function of the proteins: (i) GFP-tagged SUMO system proteins (which are approximately the same size, and located at the C-terminus like the HA-ProtA tag) display the same intracellular localization as the endogenous proteins; (ii) Deletion of many of the SUMO pathway proteins is lethal, but haploid GFP strains, in which the tagged ORF is the only source of that protein, are viable (Huh \textit{et al.} Nature \textbf{425}:686); (iii) if the proteins were not folded properly, we would expect to see chaperones, heat shock proteins and other ER proteins as interactors – we don’t see them in our AP-MS data; (iv) finally, with the HA-ProtA epitope-tagged proteins we identified many previously reported SUMO system interactors.
Together these data strongly suggest that the epitope tags do not significantly affect the function or localization of the bait proteins.

2. Can the authors comment on the observation of sumoylation sites on their protein substrates? Presumably their MS analyses would be able to identify modified peptides.

2.2 Please see point 1.7 above.

The SUMO-specific proteases are extremely active in yeast. In our AP-MS approach, we lyse the cells using a gentle buffer system, in order to preserve as many protein-protein interactions as possible. Unfortunately, even in the presence of general protease inhibitors such as NEM, because the SUMO-specific proteases are so active, we routinely lose most sumoylation under these conditions.

When we are looking for SUMO conjugation sites, we actually use boiling SDS sample buffer to effect cell lysis, and thereby rapidly inactivate SUMO protease activity. Of course, this type of approach also efficiently denatures proteins and obliterates protein complexes. Unfortunately, until SUMO-specific protease chemical inhibitors are developed, we will have to live with this trade-off.

3. On p. 4, last para. and Fig 1E, the authors indicate that the interactions are specific for Siz1-HA because the bands detected in the Siz2-HA lanes are judged to be non-specific (bands labelled with asterisks in Figure 1F). Is there any evidence suggesting that the asterisk-labelled band is not a modified form of Siz2?

2.3.1 The antibody used here is directed against the HA epitope. The non-specific * band picked up by this antibody is present even in untransfected cells and cells expressing only Siz1-HA, so it cannot be a modified form of Siz2. It is possible that a modified form of Siz2-HA co-migrates with this band, however.

In Figure 1F (bottom panel), they show immunoblots of whole-cell lysates derived from cells expressing either Siz1-HA or Siz2-HA. The Siz2-HA lanes show multiple bands including some above and it is unclear how minor bands could not be related to Siz2-HA. Could it be that Siz2 is itself SUMOylated?

2.3.2 Siz2 is clearly modified in this sample. While these shifted bands most likely represent sumoylated Siz2, they could also represent a form of the protein modified with ubiquitin or another ubiquitin-like protein. The purpose of this experiment was simply to validate the Siz2-Spt16 interaction; we thus did not attempt to identify or characterize the modification on Siz2. (This is certainly an interesting observation, but beyond the scope of the current manuscript.)

The evidence for Siz1-HA interacting with Spt16-GFP (lane 13) is unclear. The asterisk-labelled non-specific band is very close to what is claimed to be Siz1-HA band.

2.3.3 Unfortunately, it is true that the non-specific band migrates close to Siz1-HA. However, as compared to Rpb3 and Top2, it is quite clear that there is an additional band in the Spt16 + Siz1 lane. We have also included a longer exposure of this blot in the supplementary material,
showing that even in a darker exposure, no additional signal is observed above the non-specific band (whereas other bands do definitely get darker).

4. On Fig. 3B, can the authors clarify if the nature of the tag (GFP or His-Flag) affects the SUMOylation pattern observed? For example CDC14 & Tof2 show SUMO bands in GFP Smt3 compared to His-Flag Smt3 whereas the opposite is observed for Net1.

2.4 It is possible, but not probable, that the epitope tags could affect SUMO conjugation efficiency in this experiment.

The His-tagged SUMO was over-expressed, while GFP-tagged SUMO expression was driven by a weaker promoter (and is therefore present at levels more closely corresponding to the endogenous SUMO, as detected by blotting with anti-SUMO). In the His-SUMO expressing cells, a clear shift in migration can be observed for the dark band just above the unmodified protein – this is due to the epitope tag. *(i.e. in this case, the His-SUMO conjugate accounts for the majority of the signal because it is expressed at much higher levels than the endogenous SUMO protein.)* In GFP-SUMO expressing cells, both GFP-SUMO and endogenous SUMO conjugates can be observed (note the same dark band is not shifted in this case). The GFP-tagged SUMO causes a more dramatic shift in migration, as is nicely observed in the Cdc14 and Tof2 blots. However, even in the Net1 blot, a lighter, more slowly migrating band can be observed. Unfortunately, this is the darkest exposure that we were able to obtain for this experiment.

Nevertheless, the goal here was to demonstrate that these proteins are SUMO conjugates, and we would suggest that we have done so, regardless of any effect that the tags might have on the SUMO pattern.

5. On p. 8, the comment on "These data for the first time highlight specific roles for Ulp2 and Siz2 in the maintenance of rDNA repeats" should be moderated given prior reports *(PLoS Genet, 4, e1000215, 2008, see Fig. 2H)* indicating that levels of SUMO E3 activity (associated with Siz2) affect rDNA stability and copy number.

2.5 Thank you for pointing this out. We have cited this reference, and changed the text accordingly.

Reviewer #3 (Remarks to the Author):

*The manuscript from Tharan et al using systematic proteomics approach to identify the interaction partners of the components in SUMO system. Previous reports mainly focused on identifying novel SUMO conjugates in budding yeast, plants and mammals. The manuscript sheds light on understanding how the SUMO machinery enzymes responsible for the conjugation and deconjugation steps through the angle of interaction proteins instead of substrates. The result showed only part of the newly identified interacting proteins overlapped with previously found SUMO substrates. The rest identified proteins may be potential SUMO substrates due to their binding ability to the enzymes in SUMO system or mediate the recruitment of SUMO substrates as a bridge. Although the components of SUMO system in budding yeast are simpler than those in mammals, this manuscript still can be used as a good resource for studying SUMOylation in budding yeast. Furthermore, the orthologs of those identified proteins may provide the hint of novel SUMO*
function in other organisms.

3.0 Thank you for the positive comments.

Major criticisms:
1. According to the newly identified proteins which are interacted with the components of SUMO system, Tof2 and the RENT complex can bind to Ubc9 and be SUMOylated. The SUMOylated Tof2 and the RENT complex can serve as a bridge for binding to Lrs4, a component of the cohibin complex. The interaction among unmodified or SUMOylated proteins regulates the connection between rDNA and the inner nuclear membrane. One of the key points is the SUMO binding function of LRS4 to those SUMOylated proteins. Does Lrs4 contain SIM domain? This SUMO binding function should be responsible for the interaction between Lrs4 and the SUMOylated Tof2 and the RENT complex. The authors need to show that Lrs4 interacts with Tof2 and the RENT complex and this interaction is mediated by SIM domain.

3.1 We have mutated the two predicted SIMs in Lrs4, but observed no effect on SUMO binding (data not shown). The Lrs4 SUMO binding site(s) thus appears to be a non-consensus type of SIM; this means that to map it would require a global alanine scanning (or similar) approach.

It must be pointed out here that we have already provided >450 new protein-protein interactions representing the first SUMO interactome map in any organism, conducted extensive live cell microscopy which identified new details regarding nucleolar localization of the SUMO system components, and then followed up on a number interesting observations. While characterizing the fine detail of the mechanism for how the SUMO system regulates rDNA is definitely of interest, we feel that this is beyond the scope of the current manuscript (especially since we have submitted this as a short Report...).

2. The interaction between Ulp2 and Csm1 should be moved to the main text instead of supplementary table. In addition, although the interaction was already shown in previous yeast two-hybrid result, the authors need to provide biochemical binding assay to confirm this interaction.

3.2 As suggested, we have moved the AP-MS data for this experiment to Figure 3. We have also provided additional in vivo mapping data, demonstrating that the Ulp2 interaction is mediated via the C-terminus of Csm1. Unfortunately, after numerous attempts we have been unable to express the Ulp2 protein in E. coli, so are unable to provide in vitro binding data. To address this point, we have added a caveat in the text, indicating that it is possible that the Csm1-Ulp2 interaction may not be direct.

3. The cohibin complex (Lrs4 and Csm1) bind to Ulp2, not Ubc9. It is reasonable to assume that this complex should not be SUMOylated because of higher affinity to the Ulp2. On the other hand, Tof2 and the RENT complex should be SUMOylated due to their affinity to Ubc9. However, the SUMO conjugation patterns in Fig 3B are not very clear. Does SUMOylated Csm1 appear in longer exposure (because the faint signal showed in higher molecular weight)?

3.3.1 We have not detected any sumoylated Csm1, even in longer exposures.
Some bands with lower mobility were detected in cells expressing Cdc14, Tof2, and Net1 proteins even in the control lane without His-Flag-Smt3 or GFP-Smt3 expression. Are those signals from endogenous Smt3 conjugates or non-specific signal? Please use other antibodies (ex. anti-GFP, anti-His antibody) to confirm that these proteins are SUMOylated.

3.3.2 Please see comment 2.4 above. These bands definitely appear to be conjugated to endogenous SUMO, as they are also present in the GFP-SUMO lanes, but not in the His-SUMO lanes. This is a very commonly used assay in the SUMO field, and we feel that the current data are actually quite convincing. Also, as pointed out in the manuscript, all three of these proteins were previously reported as putative SUMO conjugates – we have confirmed these findings here.

Minor points:
1. In Fig S1A, S1B, and S1C, the circles should be modified to fill with bright colors. The circle fill with dark color is not easy to read the black word inside the circle.

3.4 Thank you for this suggestion. We agree, and have changed the figures accordingly.

2. In Fig 2A, the author should put the image of Ulp1(150-621) localization in WT strain into the figure as a control for the normal localization of Ulp1(150-621) protein.

3.5 Agreed. We have now included the wt control in Fig 2A.

3. The purity of proteins in Fig 3 is not good. Please indicate which bands are those purified full length proteins or improve the protein purity in Fig 3A because there are too many degradation products.

3.6 The purity of these proteins is actually quite good, but there are indeed degradation products in the preparations. We have inserted an asterisk next to each full-length protein.

We again thank the editor and reviewers for the constructive comments, and trust that our manuscript is now acceptable for publication as a Report in Molecular Systems Biology.