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DeSUMOylating Isopeptidase: a second class of SUMO protease

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

1st Editorial Decision 01 September 2011

Please accept my apologies for the time it has taken me to contact you with a decision on your manuscript. We have now received the enclosed reports from two referees that were asked to assess your study. As you will see, although both the referees find the topic of interest and in principle suitable for us, they consider the study too preliminary for publication. Both of them request a number of technical improvements of the data and additional experiments to sufficiently characterize DeSI-1 as a SUMO specific isopeptidase.

Given that both referees provide constructive suggestions on how to strengthen the work, I would like to give you the opportunity to revise your manuscript. If the referee concerns are adequately addressed, we would be happy to accept your manuscript for publication. In this case, it would be important to address all referee concerns, including the control shRNA knockdown experiments by complementation with an shRNA-resistant DeSI-1 allele, the quantification of enzyme activity and use of PML and p63 as substrates in vitro, provide the total cellular pattern of modification after DeSI-1 overexpression and knockdown, and use shRNA to show the physiological significance of DeSI-1 to BZEL function. This last point is certainly worth trying, as it would increase the significance of the findings. However, publication would not depend on the successful outcome of this experiment, if all other issues are adequately addressed.

Please note that it is EMBO reports policy to undergo one round of revision only and thus,

acceptance of your study will depend on the outcome of the next, final round of peer-review. Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions.

The length of revised manuscripts must be a maximum of 29,500 characters (including spaces, figure legends and references), and thus you will need to shorten the main text. Shortening may be made easier by combining the Results and Discussion into a single section, which we require, and which eliminates the redundancy that is inevitable when discussing the same experiments twice. In addition, although basic Materials and Methods required for understanding the experiments performed must remain in the main text, additional detailed information may be included as Supplementary Material. We can publish only a maximum of 5 figures in the main text and you currently have 7. In this case, it would make sense to combine figures 3,4 and 5 into a single one, as they are very much related. Additional experiments requested by the referees would have to be incorporated to the existing figures, or some of the current (or new) figures can be included as supplementary information, if they are controls or not directly relevant to the main message of the study. Please also include the nature of the error bars in the legend to figure 7 (and in any new figure you may generate, if applicable).

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

Requires major revision but should ultimately be suitable for publication in ER.

All previous SUMO proteases that have been described belong to the ULP/SENP family of proteases. Thus, it is interesting to have learned from this work that another class of SUMO proteases appears to exist. [It should be pointed out that all previously verified SUMO proteases, which are in the ULP/SENP family, were identified by virtue of their homology to yeast Ulp1, the original SUMO protease identified (Li and Hochstrasser (1999) Nature 398:246). There is also a hint that another class of enzymes, the WLM-containing proteins, may be SUMO proteases (Mullen et al. (2010) MCB 30:3737). These studies should be cited and discussed.] The physiological significance of DeSI-1 to BZEL function remains unclear.

The activity of the enzyme against SUMO-BZEL is a little hard to judge. Ideally, one would like to see a quantitative in vitro comparison with a known SUMO protease such as SENP1, which from in vivo transfections seems to be significantly more active against SUMO1-BZEL than is DeSI-1.

I noticed that a crystal structure for DeSI had been deposited some time ago in the public databases by an overlapping set of authors. Is there another paper on this topic that is being submitted? It would important for the editors to know to what extent this would cover the same ground as this paper.

Overall, I am inclined toward recommending this for publication. However, several things still need to be done to make the paper acceptable: 1) perform a quantitative comparison of enzyme activity in vitro comparing to a known ULP/SENP enzyme and include all controls; 2) do controls for the RNA interference experiments; and 3) use their shRNA against DeSI-1 to evaluate effects on BZEL transcriptional repressor activity. All these are discussed in more detail below.

Specific comments:

Fig. 2C. It is stated in the text that this figure "confirms the desumoylating activity of DeSI-1 on BZEL," but these results do not show this. For example, DeSI-1 could be a site-specific protease that activates a true SUMO protease in the cell or removes an inhibitor from either the substrate or the protease. The writing should be more cautious here as well as in the descriptions of the data in the rest of this figure.

Fig. 2F. There is no control for off-target effects due to shRNA. I am not sure what the EMBO Reports policy is regarding RNAi: Are multiple different shRNAs needed? A rigorous complementation experiment with an shRNA-resistant DeSI-1 allele?

Fig. 3. The activity against SUMO-BZEL in 3a and the reactivity with SUMO1-VS in 3b are both quite low. It leaves the worry that SUMO-BZEL and SUMO conjugates generally are not the physiological targets of DeSI-1 but perhaps some other Ubl-modified proteins. What is the molar concentration of SUMO-BZEL relative to that of DeSI-1 in these experiments? Are all the components of the sumoylation kit from Active Motif purified from E. coli? Also, missing from the vinyl sulfone experiment in 3b is the C108S control to show that the modification is occurring where the authors think it is. This in vitro analysis is where a quantitative enzyme activity comparison to SENP1 is needed.

Fig. 6A. Could there be a weak activity against of DeSI-1 against PML-SUMO?

Fig. 7. An shRNA experiment would be more telling here. While the data hint that SUMO-BZEL might be less active as a repressor than the nonsumoylated form, such an overexpression approach does not allow the authors to argue that DeSI-1 is the SUMO protease responsible for activating BZEL. Since the authors have what appears to be a strong shRNA effect on DeSI-1, a blimp-1-luc transcriptional effect should be easy to test. Frankly, it is a little hard to understand how manipulating the SUMO status of probably 1% or less of BZEL can have much of an effect on overall BZEL-dependent transcriptional repression. Finally, BZEL and SUMO IP/IBs should be shown here.

In the Discussion, the authors say the dimeric nature of deSI-1 suggests its substrates will be dimers. Why would this be the case? I'm also curious: are the active sites in the deSI-1 dimer physically proximal to one another in the crystal structure? Generally speaking, the Discussion here adds very little to the paper.

Referee #2:

Shin and co-workers identify a potential novel desumoylating enzyme, which they designate DeSI-1. DeSI-1, which is also known as PPDE2, has not been functionally characterized previously, but bioinformatics has classified it as a deubiquitinating enzyme. Here the authors show that DeSI-1 does exhibits SUMO isopeptidase activity and can specifically remove SUMO paralogs from modified BZEL, which the authors have found to be a novel SUMO substrate. Desumoylation activity is shown both in vivo and in a reconstituted in vitro system demonstrating that DeSI-1 acts directly on SUMO conjugates. The authors further show that - in contrast to members of the SENP family of SUMO isopeptidase, DeSI-1 cannot catalyze the processing of SUMO precursors. Moreover, DeSI-1 is unable to desumoylate PML or p63 indicating that it exhibits substrate specificity. In summary, all experiments are well controlled and convincing. The identification of a novel class of SUMO isopetidase in mammalian cells is a highly significant and novel finding, which is of broad general interest. As outlined below, I only have some minor specific points that should be addressed.
Specific points:
1. So far the authors have only demonstrated desumoylating activity of DeSI-1 on the single substrate BZEL. To get a more general idea how DeSI-1 affects cellular sumoylation one would like to see the total cellular pattern of endogenous SUMO1 and SUMO2/3 modification following ectopic expression or knock-down of DeSI-1. It would also be helpful to see whether DeSI-1 demodifies PML or p63 in vitro. The experiment presented in Figure 6 has been done in cells upon transfection of PML/SUMO or p63/SUMO. The in vitro experiment would tell more about DeSI-1 substrate specificity. If DeSI-1 acts on PML/p63 in vitro, but not in vivo it might argue for spatial control of DeSI-1 activity.

Moreover, it would be interesting to study whether DeSI-1 can cleave polymeric SUMO2/3 chains or acts specifically on monomodified conjugates.

2. In the introduction the authors state "SENP6 and SENP7 are nuclear proteases with unknown function". However, several more recent papers have addressed the function of SENP6, in particular its role in trimming of SUMO2/3 chains. This statement should therefore be changed and the recent literature should be cited.

1st Revision - authors' response 05 December 2011

Response to the referees

We are grateful to reviewers, thoughtful and balanced suggestions for our manuscript. In response to reviewer's comments, we have made the following changes and submit the revised version.

Response to Reviewer 1.

1. All previous SUMO proteases that have been described belong to the ULP/SENP family of proteases. Thus, it is interesting to have learned from this work that another class of SUMO proteases appears to exist. [It should be pointed out that all previously verified SUMO proteases, which are in the ULP/SENP family, were identified by virtue of their homology to yeast Ulp1, the original SUMO protease identified (Li and Hochstrasser (1999) Nature 398:246). There is also a hint that another class of enzymes, the WLM-containing proteins, may be SUMO proteases (Mullen et al. (2010) MCB 30:3737).

➢ As suggested by the reviewer, we included the statement and reference explaining that SENPs were identified by virtue of homology to yeast Ulp1 (page 3, line 14-15). We also included the statement and reference that Wss1, a member of WLM-family proteins, was reported as a SUMO protease in yeast (page 8, line 7-10), and thank the reviewer for these points.

2. I noticed that a crystal structure for DeSI had been deposited some time ago in the public databases by an overlapping set of authors. Is there another paper on this topic that is being submitted? It would important for the editors to know to what extent this would cover the same ground as this paper.

➢ The crystal structure of DeSI-1 was determined by a group collaborating with us and the manuscript is in preparation. However, the cocrystal structure of DeSI-1 and SUMO-BZEL has not been determined yet and therefore the crystal structure information has not been very much helpful at this stage in explaining how DeSI-1 recognizes its substrate.

3. Fig. 2C. It is stated in the text that this figure "confirms the desumoylating activity of DeSI-1 on BZEL," but these results do not show this. For example, DeSI-1 could be a site-specific protease that activates a true SUMO protease in the cell or removes an inhibitor from either the substrate or the protease. The writing should be more cautious here as well as in the descriptions of the data in the rest of this figure.

➢ We agree with the reviewer on this point and as suggested, rephrased the conclusion of Fig. 2 as "Taken together, these results suggest the possibility that DeSI-1 may function as a desumoylase but not certainly deubiquitinase of BZEL."(page 5, line 15-16).

4. Fig. 2F. There is no control for off-target effects due to shRNA. I am not sure what the EMBO Reports policy is regarding RNAi: Are multiple different shRNAs needed? A rigorous complementation experiment with an shRNA-resistant DeSI-1 allele?

➢ As suggested by the reviewer, we employed two different shRNAs, shDeSI-1-1 and shDeSI-1-2, corresponding to nts 28-46 and 133-151 of the ORF of DeSI-1. Expression of either shRNAs resulted in an enhanced level of SUMO-modified BZEL, indicating that DeSI-1 acts as a

desumoylase of endogenouse BZEL. The new experimental data are presented as Fig. 5C and statement explaining the data is included in the result section (page 7, line 14-20) and in the legend to Fig. 5C.of this revised manuscript. In addition, these two shRNAs were used in experiments described in Fig. 5B and Supplementary Figure 3 of the revised version.

5. Fig. 3. The activity against SUMO-BZEL in 3a and the reactivity with SUMO1-VS in 3b are both quite low. It leaves the worry that SUMO-BZEL and SUMO conjugates generally are not the physiological targets of DeSI-1 but perhaps some other Ubl-modified proteins. What is the molar concentration of SUMO-BZEL relative to that of DeSI-1 in these experiments? Are all the components of the sumoylation kit from Active Motif purified from E. coli? Also, missing from the vinyl sulfone experiment in 3b is the C108S control to show that the modification is occurring where the authors think it is. This in vitro analysis is where a quantitative enzyme activity comparison to SENP1 is needed.

- For Fig. 3A experiment, 10 μM of SUMO-BZEL was used whereas 0, 0.1, and 0.4. μM of DeSI-1 were used. The concentrations of the proteins are included in the legend to Fig. 3A.

- All components of the sumoylation kit from Active Motif are purified from *E. coli*.

- As suggested by the reviewer, we performed the experiments using DeSI-1$_{C108S}$ mutant and as expected the new data show that DeSI-1$_{C108S}$ mutant is not able to form an adduct with SUMO-1-VS. The experimental data show that the desumoylase activity is mediated specifically by the isopeptidase activity of DeSI-1. The data are presented in Fig. 3B of the revised version and the statement relevant to the data was included in page 6, line 4.

- In response to reviewer's suggestion, we compared the desumoylase activity of DeSI-1 and SENP1 in vitro. 10 μM concentration of SUMO-BZEL was incubated with 0, 0.1, and 0.4. μM concentration of DeSI-1 or SENP1C. Under the condition, the desumoylase activity of DeSI-1 was found similar to that of SENP1C. The new experimental data is presented as Supplementary Fig.1 and the statement explaining the data is included in the results and discussion (page 5, line 34-36)

6. Fig. 6A. Could there be a weak activity against of DeSI-1 against PML-SUMO?

- To clarify the reviewer's concern, we repeated the experiments. The new data clearly show that DeSI-1 does not desumoylate SUMO-PML. The new data is presented as Fig. 4A of revised manuscript.

7. Fig. 7. An shRNA experiment would be more telling here. While the data hint that SUMO-BZEL might be less active as a repressor than the nonsumoylated form, such an overexpression approach does not allow the authors to argue that DeSI-1 is the SUMO protease responsible for activating BZEL. Since the authors have what appears to be a strong shRNA effect on DeSI-1, a blimp-1-luc transcriptional effect should be easy to test. Frankly, it is a little hard to understand how manipulating the SUMO status of probably 1% or less of BZEL can have much of an effect on overall BZEL-dependent transcriptional repression. Finally, BZEL and SUMO IP/IBs should be shown here.

- As suggested by the reviewer, we examined the effects of the depletion of endogenous DeSI-1 by introducing two different DeSI-1 specific shRNA, shDeSI-1-1 and shDeSI-1-2, on the repressor activity of BZEL. In this experiment, to maximize the effect of shRNAs, smaller amounts of BZEL expressing vector were used compared to those in Fig 5A. Upon introduction of BZEL expression vector, Blimp-1 promoter activity was slightly repressed and the additional introduction shRNA expression vector resulted in the enhanced suppression of the Blimp-1 promoter. These results as well as those in Fig. 5A support that DeSI-1- mediated desumoylation may regulate the transcription of endogenous blimp-1 gene. The new experimental data are presented as Fig. 5B and 5C and a statement explaining the new experimental data was included in the result section (page 7, line 14-20) and in the legend to Fig. 5B and C .

8. In the Discussion, the authors say the dimeric nature of deSI-1 suggests its substrates will be dimers. Why would this be the case? I'm also curious: are the active sites in the deSI-1 dimer physically proximal to one another in the crystal structure? Generally speaking, the Discussion here

adds very little to the paper.

➢ Upon rereading of the discussion section, we agree with the reviewer that this part adds very little to the paper. We omit this part of the discussion in the revised version. In answer to the reviewer's specific question in the crystal structure of the DeSI-1 dimer, active sites are physically proximal to one another and form a pocket. However, we feel that this information is beyond the scope of this paper and therefore choose not to include this part in this manuscript.

Response to Reviewer 2.

We appreciate the reviewer for helpful suggestions.
1. So far the authors have only demonstrated desumoylating activity of DeSI-1 on the single substrate BZEL. To get a more general idea how DeSI-1 affects cellular sumoylation one would like to see the total cellular pattern of endogenous SUMO1 and SUMO2/3 modification following ectopic expression or knock-down of DeSI-1.

➢ As suggested by reviewer, we examined the total cellular patterns of endogenous SUMO-1- and SUMO-2/3- modified proteins following the introduction of DeSI-1 shRNAs. However, the total cellular patterns of SUMO-1- and SUMO2/3- modified proteins were not altered significantly. These results suggest that DeSI-1acts on specific substrate proteins wereas SENP has more numerous substrates. The statement explaining the new experimental data was included in the results and discussion section (page 7, line 20-23).

2. It would also be helpful to see whether DeSI-1 demodifies PML or p63 in vitro. The experiment presented in Figure 6 has been done in cells upon transfection of PML/SUMO or p63/SUMO. The in vitro experiment would tell more about DeSI-1 substrate specificity. If DeSI-1 acts on PML/p63 in vitro, but not in vivo it might argue for spatial control of DeSI-1 activity.

➢ As suggested by reviewer, we performed an *in vitro* experiment to show that PML and ΔNp63, known substrates of SENPs, are not desumoylated by DeSI-1. Consistent with the *in vivo* data, PML and ΔNp63 were desumoylated by SENP1C and SuPr-1, respectively, but not by DeSI-*1 in vitro*. The experimental data was presented in the supplementary Fig. 2 of this revised manuscript and a statement explaining the experimental data was included in the results and discussion section (page 7, line 3-5).

Moreover, it would be interesting to study whether DeSI-1 can cleave polymeric SUMO2/3 chains or acts specifically on monomodified conjugates.

➢ As suggested by reviewer, using the poly SUMO-2/3 chain, we examined whether DeSI-1 has poly-SUMO 2/3 editing activity. Interestingly, DeSI-1, as well as SENP 6C, showed poly-sumo 2/3 deconjugation activity. The new experimental data was presented as Fig. 3D and the statement explaining the experimental data was included in the results and discussion section (page 6, line 17-24) and the legend to Fig. 3D.

3. In the introduction the authors state "SENP6 and SENP7 are nuclear proteases with unknown function". However, several more recent papers have addressed the function of SENP6, in particular its role in trimming of SUMO2/3 chains. This statement should therefore be changed and the recent literature should be cited.

➢ As suggested by reviewer, we changed the statement and cited the relevant literature (page 3, line 27-28).

In addition, as suggested by the editor, we changed the overall structure of the manuscript. Specifically;
1. The number of Figures was reduced from 7 to 5.
-Fig. 4 was moved to Fig. 2F and 2G of the revised version.
-Fig. 5 was moved Fig. 3C of the revised version.
2. Material and Methods section was moved to the Supplementary materials.
3. Three new Supplementary Figures were included.

2nd Editorial Decision 21 December 2011

Thank you for your patience while we have peer-reviewed your revised manuscript. As you will see from the reports below, the referees are now both positive about its publication in EMBO reports. Nevertheless, referee 1 asks for additional discussion of the merits of your study in comparison to the yeast Wss1 study and to provide statistical analysis of figure 5B. Please address both of these requests, as they will strengthen the novelty and significance of the study.

As the revisions required are minor, I am making an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once you have satisfactorily addressed these two issues, as well as a few other minor issues/corrections detailed below.

- As I mentioned in my decision letter of Sept 1, 2011, basic Materials and Methods required for understanding the experiments performed must remain in the main text, additional detailed information may be included as Supplementary Material. Thus, I must ask you to include a Methods section in the main manuscript file.

If all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The modified manuscript largely addresses my original concerns. I am at this point convinced that DeSI-1 is a deSUMOylating enzyme of a novel class, the first new class of such proteases to be identified. I think the data here are actually much better than for Wss1 in yeast, even though I brought up these latter data in my original review. It might be best to make clear that there were no direct biochemical data for Wss1 having deSUMOylating activity in the Mullen et al. paper (just some type of SUMO-dependent isopeptidase activity, although the data were not strong on this count either).

The DeSI-1 shRNA data on blimp1-luc activity in Fig. 5B are not overwhelming, but the trends are as predicted and it is likely to be statistically significant (this should be determined).

In summary, I support publication of the manuscript in EMBO Reports.

Referee #2:

The authors have adequately addressed all points raised in my initial comments. I therefore recommend publication of this manuscript in EMBO reports.

2nd Revision - authors' response 30 December 2011

We appreciate your efforts in the processing of our manuscripts. In response to your additional requests, we have made the following changes and submit the revised version.

1. As requested by the reviewer, we rephrased the statement regarding the possible desumoylase activity of Wss1 as “Recently, Wss1, a member of WLM family of metalloproteases was suggested to have a SUMO-dependent isopeptidase activity in yeast, although direct biochemical data supporting the desumoylating activity were not provided “(page 7, line 35-37).

2. Regarding the blimp-1-luc activity in Fig. 5B, we statistically analyzed the data using Student’s t test and the result is shown in Fig. 5B and the legend to Fig. 5B.

3. We included the material and methods for testing the desumoylase activity, such as in vitro desumoylation assays, labeling reactions and SUMO-2/3 chain editing assays, in the main text. Other material and methods for general experimental procedures, such as yeast two-hybrid screening, plasmids, isolation of murine tissue proteins, construction of shRNA-expressing plasmids, immunofluorescent microscopy, immunoprecipitation and immunoblot analysis, antibodies and expression and purification of recombinant proteins from E.coli, are included in the Supplementary Methods.

I appreciate your consideration.

3rd Editorial Decision 09 January 2012

We are very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

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Editorial office

