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The transcription factor Krox20 is an E3 ligase that sumoylates its Nab coregulators

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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 March 2011

Thank you for the submission of your research manuscript to EMBO reports. It was sent to three referees, and we have received reports from two of them, which you will find at the end of this email. As both are in fair agreement, I have made a decision now to avoid unnecessary loss of time.

Both referees consider the study preliminary for publication here at this stage and require a more conclusive demonstration of the SUMO E3 ligase activity of Krox20 before publication in EMBO reports can be considered. However, as both referees provide clear suggestions on how to strengthen the study, I would like to give you the opportunity to revise your manuscript. It will be crucial to provide in vitro sumoylation experiments and show sumoylation of endogenous Nab, which should be affected by Krox20 knockdown. If these concerns are adequately addressed, we would offer publication of your manuscript. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, the acceptance of your study will depend on the how well you address the referee concerns in the next, final version of your manuscript.

Do not hesitate to get in touch with me if I can be of any assistance during the revision process. Should you feel you will not be able to deal with these requests and choose to submit your paper elsewhere, I would welcome a message to that effect.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

While this is a potentially interesting observation there is no direct evidence presented to indicate that Krox20 functions as a SUMO E3 ligase. The transfection experiments show increased SUMO modification, but could be for reasons other than Krox20 being an E3 ligase (eg inhibiting a SUMO specific protease). What is required to unambiguously demonstrate that Krox20 is a SUMO E3 ligase is an in vitro SUMO modification reaction containing all of the components (ie SUMO activating enzyme, Ubc9, SUMO, Krox20 and substrate) as recombinant proteins purified from bacteria and show that Krox20 acts catalytically to increase SUMO modification of the substrate.

Referee #3:

This work concentrates on SUMO modification of Nab, which acts as a co-regulator on the transcription factor Krox20. The authors initially identified the SUMO conjugating enzyme Ubc9 as a binding partner of Krox20 and observed that co-expression of Krox20 with Nab leads to an increase in Nab sumoylation. From these data the authors conclude that Krox20 acts as a SUMO E3 ligase that may bridge Ubc9 to Nab. Based on further experiments they suggest that sumoylation of Nab affects transcriptional activity of Krox20.

A fundamental and major weakness of the work is that the main conclusion of Krox20 being a SUMO ligase is solely based on in vivo studies upon overexpression of Krox20. Convincing demonstration of E3 SUMO ligase activity requires in vitro sumoylation experiments with purified components. Moreover, to strengthen the in vivo data sumoylation of endogenous Nab must be demonstrated and the direct involvement of endogenous Krox20 in this process has to be confirmed by knock-down experiments of Krox20. Without these data the manuscript is clearly not a candidate for EMBO reports.

Correspondence

2 March 2011

We have now received the report of the third referee that was asked to assess your study. As his/her comments are in line with those of the other two, I have decided to forward them to you.

You will see that this referee raises also additional points, which would be informative to address. However, the crucial experiments on which acceptance of the manuscript would depend are those stated in my previous letter. Namely, the demonstration that Krox20 is a SUMO E3 ligase with in vitro experiments, that endogenous Nab is sumoylated and that this is affected by Krox20 knockdown. Please note that if the Krox20/Nab complex turns out to be the target for sumoylation, as referee 2 suggests, we feel this finding would not be sufficiently significant for publication in EMBO reports.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORT:

Referee #2:

The authors find that Krox20 interacts with the SUMO E2 conjugating enzyme Ubc9 in yeast two hybrid and upon Gst-pulldown. Interestingly, this interaction does not lead to Krox20 sumoylation, but instead to sumoylation of the Krox20 binding partner Nab (Nab1 and Nab2) in overexpression experiments. From these findings the authors conclude that Krox20 is a SUMO E3 ligase.

While the finding that Krox overexpression leads to Nab sumoylation upon overexpression of Nab1 and SUMO is convincing, the major conclusion that Krox20 is an E3 ligase is certainly not.

More likely is the interpretation that the Krox20/Nab complex is a target for sumoylation. While Krox20 may provide the Ubc9 binding site, Nab seems to contain the acceptor lysines. This in itself would be interesting, because it would be a nice example that recruitment and attachment can be split between complex subunits.

An E3 ligase is an enzyme. Enzymes function as catalysts and not as stoichiometric cofactors - one Krox20 would have to drive sumoylation of many Nab proteins. This needs to be demonstrated with purified enzymes *in vitro*.

It has not been tested whether overexpression or knock-down of known E3 ligases such as PIAS proteins would enhance or abolish sumoylation of Nab proteins in the presence of Krox.

Other SUMO and ubiquitin E3 ligases are characterized by massive autosumoylation - this does not seem to be the case for Krox20? While this is of course not an absolute requirement, it raises further doubts about the proposed function of Krox20 as an E3 ligase.

Does Nab1 or Nab2 itself bind to Ubc9 in pull-down assays? Other E3 ligase dependent targets usually do...

Evidence for endogenous Nab sumoylation is missing, sumoylation is only detected upon SUMO and Nab overexpression.

Minor points:

Gst-pulldown Ubc9/Krox20: input and pulled-down fraction need to be shown on same blot and information has to be provided as to how much input is shown.

RanGAP construct - what was used is the RanGAP1-C-term domain, not full length RanGAP1. This is no problem, but should be mentioned to avoid confusion (full length RanGAP1 is a 70 kD protein). What is a bit puzzling is that RanGAP1 sumoylation is only observed when SUMO is also transfected. RanGAP1 sumoylation usually can be observed with endogenous SUMO.

POINT BY POINT ANSWERS TO THE REFEREES' COMMENTS

Referee #1:

What is required to unambiguously demonstrate that Krox20 is a SUMO E3 ligase is an in vitro SUMO modification reaction containing all of the components (ie SUMO activating enzyme, Ubc9, SUMO, Krox20 and substrate) as recombinant proteins purified from bacteria and show that Krox20 acts catalytically to increase SUMO modification of the substrate.

As the referee requested, we have purified all the components for the in vitro sumoylation reaction (E1, Ubc9 and mature sumo, see Figure for referees 1, below; Krox20 and Nab, see Figure for referees 2) as purified recombinant proteins produced in *E. coli*. We have then conducted in vitro sumoylation assays that are presented in Fig. 2J. The data establish that Nab can be sumoylated in vitro with these sole components and that the modifications require Krox20. Furthermore the quantification of the amount of sumoylated Nab in respect to input Krox20 (ratio of approximately 9-fold) suggests that Krox20 acts as catalytic rather than stoichiometric factor, as expected for an E3 ligase (page 7).

Referee #2:

1. While the finding that Krox overexpression leads to Nab sumoylation upon overexpression of Nab1 and SUMO is convincing, the major conclusion that Krox20 is an E3 ligase is certainly not. More likely is the interpretation that the Krox20/Nab complex is a target for sumoylation. While Krox20 may provide the Ubc9 binding site, Nab seems to contain the acceptor lysines. This in itself would be interesting, because it would be a nice example that recruitment and attachment can be split between complex subunits. An E3 ligase is an enzyme. Enzymes function as catalysts and not as stoichiometric cofactors - one Krox20 would have to drive sumoylation of many Nab proteins. This needs to be demonstrated with purified enzymes in vitro.

See answer to referee #1.

2. It has not been tested whether overexpression or knock-down of known E3 ligases such as PIAS proteins would enhance or abolish sumoylation of Nab proteins in the presence of Krox.

We have tested the effect of transfecting expression constructs for the different mouse PIAS proteins in the co-transfection assay and have not observed any significant variations in the degree of Nab sumoylation (see Figure for referees 3). We have included a sentence in the manuscript indicating this observation (page 6).

3. Other SUMO and ubiquitin E3 ligases are characterized by massive autosumoylation - this does not seem to be the case for Krox20? While this is of course not an absolute requirement, it

raises further doubts about the proposed function of Krox20 as an E3 ligase.

In all experiments performed, both in vivo and in vitro, we have not observed any evidence of Krox20 sumoylation. Although this is usually the case for other E3 ligase, as the referee indicates, this is not an absolute rule. Therefore we do not think that this should be taken as an element against our interpretation of Krox20 ligase function, which, as indicated above, has been reinforced by the additional data provided in this new version of the manuscript.

4. Does Nab1 or Nab2 itself bind to Ubc9 in pull-down assays? Other E3 ligase dependent targets usually do...

Indeed the targets usually bind to Ubc9. However, these interactions are not always stable. To investigate this issue in the case of Nab, we have looked for interactions using the two-hybrid system. The data are presented in the Figure for referees 4 and did not reveal any specific interaction. This piece of information has now been included in the manuscript on page 5.

5. Evidence for endogenous Nab sumoylation is missing, sumoylation is only detected upon SUMO and Nab overexpression.

We now provide evidence for endogenous Nab sumoylation. This was obtained in P19 cells, either upon transfection of a Krox20 expression vector or upon endogenous Krox20 induction following serum stimulation after starvation. In the latter case, we could show that Nab sumoylation was indeed Krox20-dependent by knock-down of the latter using siRNAs. The new data are presented in Fig. 2 G-I.

Minor points:

6. Gst-pulldown Ubc9/Krox20: input and pulled-down fraction need to be shown on same blot and information has to be provided as to how much input is shown.

In Fig. 1B, inputs and pulled-down fractions were derived from the same blot, but are presented under each other for simplicity. The input corresponds to an amount of material equivalent to that used for pull-down. To make this clearer, we have added 100% at the level of the input in the revised Fig. 1.

7. RanGAP construct - what was used is the RanGAP1-C-term domain, not full length RanGAP1. This is no problem, but should be mentioned to avoid confusion (full length RanGAP1 is a 70 kD protein). What is a bit puzzling is that RanGAP1 sumoylation is only observed when SUMO is also transfected. RanGAP1 sumoylation usually can be observed with endogenous SUMO.

The fact that we used the C-terminal domain of RanGAP1 is now indicated in the text (page 6). It is true that RanGAP1 sumoylation can be observed with endogenous sumo. However, under our assay conditions, in which we overexpress the C-terminal domain of RanGAP1, we need to transfect low amounts of a sumo expression construct in order to observe efficient sumoylation of the protein.

Referee #3:

1. A fundamental and major weakness of the work is that the main conclusion of Krox20 being a SUMO ligase is solely based on in vivo studies upon overexpression of Krox20. Convincing demonstration of E3 SUMO ligase activity requires in vitro sumoylation experiments with purified components.

See answer to referee #1.

2. Moreover, to strengthen the in vivo data sumoylation of endogenous Nab must be demonstrated and the direct involvement of endogenous Krox20 in this process has to be confirmed by knock-down experiments of Krox20. Without these data the manuscript is clearly not a candidate for EMBO reports.

See answer to point 5 of referee #2.

FIGURES for referees

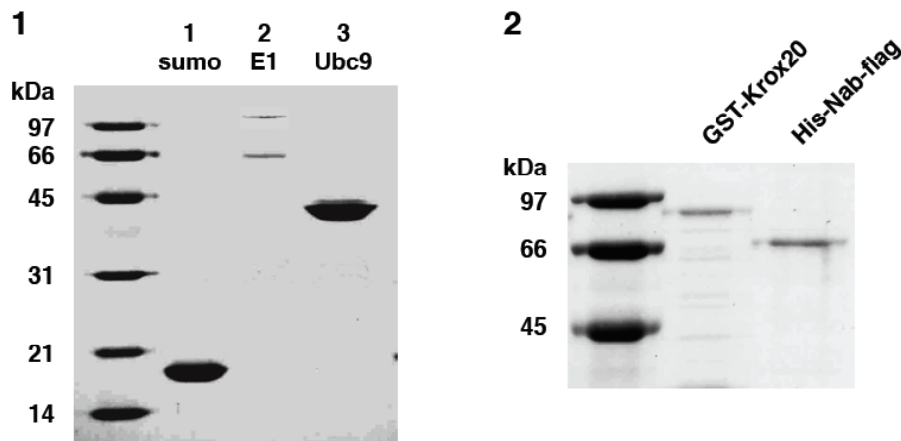


Figure for referees 1. Purification of the components of the in vitro sumoylation assay. Coomassie blue staining of a protein gel loaded with the different components purified from *E. coli*. 1: 2 µg mature sumo1; 2: 200 ng GST-Aos1/GST-Uba2; 3: 2 µg GST-Ubc9.

Figure for referees 2. Purification of Krox20 and Nab. Coomassie blue staining of a protein gel loaded with about ng of GST-Krox20 and His-Nab2-flag proteins purified from *E. coli*.

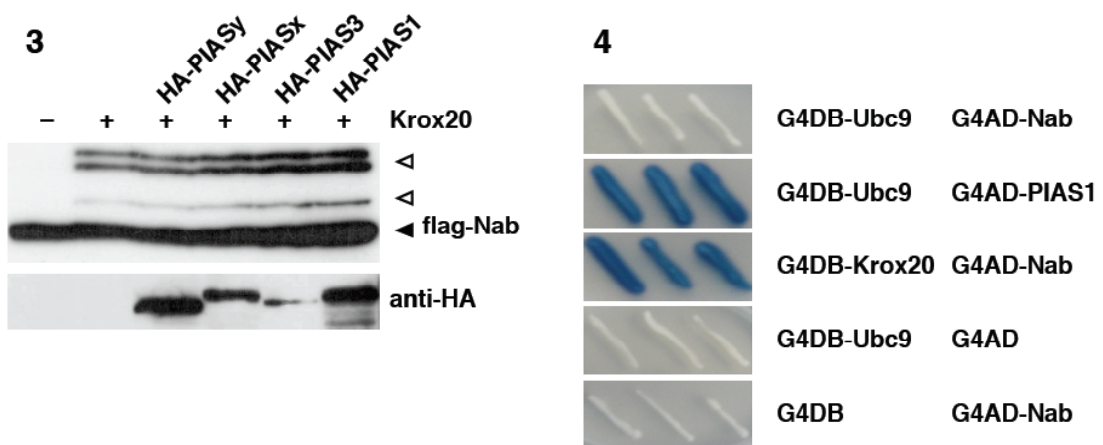


Figure for referees 3. Overexpression of PIAS does not affect Nab sumoylation in the co-transfection assay. 293T cells were transfected with flag-Nab and other expression vectors, including those for different PIAS proteins, as indicated and as described in Fig. 2A. The black arrowhead indicates non-modified Nab and white arrowheads modified Nab.

Figure for referees 4. Nab does not interact with Ubc9 in a two-hybrid system. In situ staining of β-gal activity in yeast to provide evidence for interaction (blue) or absence of interaction (white) between the indicated proteins, based on a Gal4 two-hybrid system (Gal4 DNA binding domain (G4DB), Gal4 activation domain (G4AD)).

2nd Editorial Decision

28 June 2011

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from referees 1 and 3 of the previous version. As you will see, they both consider the manuscript greatly improved although in need of some minor revision, as suggested by referee #3, and are in principle supportive of publication. Regarding referee 3's comments, I consider it would be outside of the scope of this manuscript to characterize the Krox20 domains that are essential for E3 ligase activity. However, it would be beneficial to the community to at least discuss the Krox20 domains that are present and which could be the active ones; does it have any homologous regions to other SUMO E3 ligases? Is there anything you can comment on in this respect? Regarding his/her point 2, please do note in the text the caveat that SUMO had to be overexpressed to detect endogenous NAb SUMOylation.

An important concern, however, is that regarding Figure 1 for referees, as it does indeed seem as if the higher band in lane 2 had been pasted in. Could you please send us the original blot(s) with which this figure was composed? It would be necessary to also send a correct version of this figure, to be included in the review process file that will accompany your manuscript and to be forwarded to the referee for his/her information. Please refer to the 'Electronic manipulation of images' subheading of our instructions to authors for more information.

I look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,
Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

I asked that the authors demonstrate that Krox20 had SUMO E3 ligase activity in vitro with bacterial expressed recombinant proteins. They have provided this data in fig 2J and while this single point experiment with GST tagged proteins (may have issues through GST dimerisation) is not ideal I believe they have fulfilled my requirement.

Referee #3:

In their revised version Garcia-Gutierrez and co-workers have significantly improved the manuscript. In particular they now provide evidence that Krox20 can act as a SUMO E3 ligase in an in vitro system (but see my comment below). Although this is an important finding per se, it would be more convincing to dissect the domains that are essential for this activity. Does Krox20 share any similarity to known ligases, such as RanBP2, PIAS family members or Topors.

What is still missing in the manuscript is the demonstration that endogenous Nap can be modified by endogenous SUMO. Experiments on sumoylation of endogenous Nap are still done in the presence of ectopically expressed SUMO. I acknowledge that due to the transient nature of sumoylation the demonstration of modification by endogenous SUMO is not a trivial task. However, since this is a critical point, the authors should at least more explicitly mention the fact that this still remains to be demonstrated.

Finally, I was somewhat puzzled by Figure 1 for referees. Here, the authors wish to demonstrate that components used for the in vitro sumoylation were purified to homogeneity. I was wondering why the upper band in lane 2 has a much brighter background. Was this band artificially inserted? I realize that this figure was provided for referees only, nevertheless the authors should clarify this issue.

2nd Revision - authors' response

01 July 2011

POINT-BY-POINT RESPONSE TO REFEREES' REMARKS

Referee #1 (Remarks to the Author):

I asked that the authors demonstrate that Krox20 had SUMO E3 ligase activity in vitro with bacterial expressed recombinant proteins. They have provided this data in fig 2J and while this single point experiment with GST tagged proteins (may have issues through GST dimerisation) is not ideal I believe they have fulfilled my requirement.

Although in figures for referees we have presented some of the purified proteins as GST fusions, for in vitro sumoylation assays the GST moiety was excised previously, except for Aosl and Uba2. This has been now clarified in the Supplementary Information.

Referee #3 (Remarks to the Author):

In their revised version Garcia-Gutierrez and co-workers have significantly improved the manuscript. In particular they now provide evidence that Krox20 can act as a SUMO E3 ligase in an in vitro system (but see my comment below). Although this is an important finding per se, it would be more convincing to dissect the domains that are essential for this activity. Does Krox20 share any similarity to known ligases, such as RanBP2, PIAS family members or Topors.

We have compared Krox20 with PIAS, RanBP2, Pc2 and Topors and no significant similarities have been found. This has been now included in the Discussion. We discussed previously the relevance of the zinc finger domain of Krox20 in Ubc9 binding and that other zinc-based structures of previously described ligases seem also to account for Ubc9 binding and ligase function. We have modified the text to emphasize this point.

What is still missing in the manuscript is the demonstration that endogenous Nap can be modified by endogenous SUMO. Experiments on sumoylation of endogenous Nap are still done in the presence of ectopically expressed SUMO. I acknowledge that due to the transient nature of sumoylation the demonstration of modification by endogenous SUMO is not a trivial task. However, since this is a critical point, the authors should at least more explicitly mention the fact that this still remains to be demonstrated.

Although we assume that faint sumoylation observed in lane 2 of Fig. 2A upon Nab and Krox20 overexpression is due to endogenous sumo, we agree with referee that sumoylation of endogenous Nab with endogenous sumo has not been demonstrated. We now mention in the Results section concerning Nab sumoylation this aspect.

Finally, I was somewhat puzzled by Figure 1 for referees. Here, the authors wish to demonstrate that components used for the in vitro sumoylation were purified to homogeneity. I was wondering why the upper band in lane 2 has a much brighter background. Was this band artificially inserted? I realize that this figure was provided for referees only, nevertheless the authors should clarify this issue.

We cannot explain this brighter background in the upper band of lane 2, as we manipulated general brightness/contrast to highlight the proteins of interest over slight contaminants in the preparations. These contaminants do not compromise our results, and we provide now (see below) a more realistic version of the figure for referees 1. We also provide the original scanning of the Coomassie blue staining of the electrophoresis gel, which demonstrate that the upper band in lane 2 was not artificially inserted.

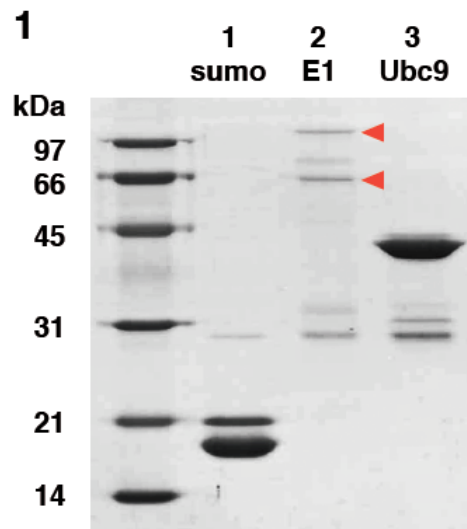
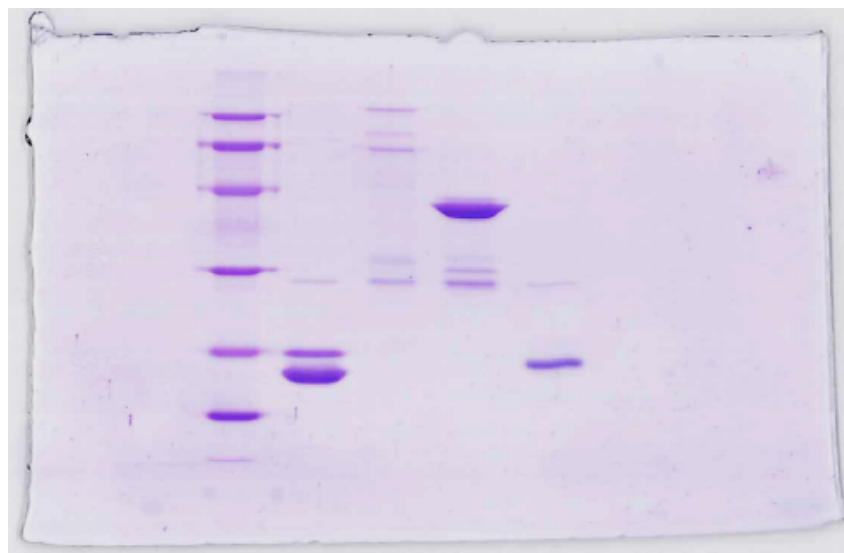


Figure for referees 1. Purification of the components of the in vitro sumoylation assay. Coomassie blue staining of a protein gel loaded with the different components purified from *E. coli*. 1: 2 μ g mature sumo1; 2: 200 ng GST-Aos1/GST-Uba2 (red arrowheads); 3: 2 μ g GST-Ubc9.



Original scanning of the Coomassie blue staining of the electrophoresis gel with components of the in vitro sumoylation assay.

3rd Editorial Decision

01 July 2011

Thank you for the submission of your revised manuscript, which I think satisfactorily addresses the referees' remaining concerns. Thus, I am very pleased to accept it 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,

Editor
EMBO Reports