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SUMO2 is essential while SUMO3 is dispensable for mouse embryonic development

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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.)

Editor: Esther Schnapp

1st Editorial Decision 13 February 2014

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees acknowledge that the findings are interesting. However, they also have several suggestions for how the study could be further improved. Referee 1 is not convinced that the data support the hypothesis that the total amount of all SUMO proteins is crucial for embryo survival, and also asks for a better analysis of the Sumo2+/-;Sumo3-/- mutant embryos. Referee 2 points out that it should be investigated whether SUMO1 can compensate for SUMO2/3 loss, which might support the hypothesis that total SUMO level and not specific paralogs are important for embryo development. This referee further suggests to examine SUMO paralog expression in adult tissues, and asks for some missing information that needs to be provided.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the

completeness of your responses included in the next, final version of the manuscript.

Please provide the missing quantifications, and please specify "n" (for the number of experiments, but also the number of cells) in each relevant figure legend. This information is currently missing and needs to be provided in the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have any further questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

It is well known that SUMO2 and SUMO3 are very close members of SUMO. In this manuscript, Wang et al generate SUMO2 and SUMO3 knockout mice, and evidently show that SUMO2 knockout mice die before E10 days and SUMO2 knockout mice looks normal. This is an interesting phenomenon to show that SUMO2 not SUMO3 is essential for embryonic development. Although the authors did some experiments to explain why SUMO2 is so critical in embryo, some major issues still need to be clarified.
1. Authors show that the SUMO2 is a predominant SUMO protein in early embryo, and then claim that the decrease of total amount of SUMO proteins, which is majority contributed by SUMO2 expression, in SUMO2 knockout mice might be a major reason for the death of SUMO2-/-. The supportive evidence is that no SUMO2+/-:SUMO3-/- mice are found at birth after crossing SUMO2+/-;SUMO3+/- with SUMO3-/-. However, authors show SUMO3 only accounts for 2-3% of total SUMO proteins at early embryo (Figure 4A). It looks quite hard to understand why so little amount of SUMO protein contributed by SUMO3 could make such critical phenotype in SUMO2+/-;SUMO3-/- but not in SUMO2+/-;SUMO3+/-?
2. As authors only mention in this manuscript that SUMO2+/-;SUMO3-/- mice are not found after birth, I am wondering whether the phenotypes shown in SUMO2+/-;SUMO3-/- mice are the similar as that in SUMO2-/- mice? And also die at E10?
3. In Figure 4B and 4C, the authors should save the un-conjugated SUMO2/3 band in the blot.

Referee #2:

Wang et al. introduce SUMO2 and SUMO3 KO mouse lines and provide a basic characterization of these KO lines. Key findings are (i) that SUMO2 is essential for normal mouse embryonic development while SUMO3 is not, and (ii) that SUMO2 is much more abundant than SUMO3 or SUMO1. Based on these data and additional experiments, the authors conclude that absolute combined SUMO1/2/3 levels are of critical importance for mouse embryonic development, and not necessarily the presence or absence of an individual paralogue. The mechanism of the embryonic lethality of SUMO2 KOs was not further studied.

Overall, I think the SUMO2 and SUMO3 KO mouse lines described here will be interesting to the SUMO research field, although a conditional KO approach would have been more helpful. Further the notion of strikingly different levels of the different SUMO paralogues is interesting, as is the likely conclusion that the combined SUMO1/2/3 levels and not the individual paralogues are of critical importance in embryonic development.

I have the following comments:

1. It seems to me based on the primer sequences given that there may be a confusion with the SUMO nomenclature in this paper. My understanding is that the SUMO2/3 nomenclature introduced initially was inverted in the current databases. Initially, mature SUMO2 and SUMO3 were defined as 92 amino acid and 93 amino acid proteins. This nomenclature is often used in the SUMO field and corresponds to the original characterization of the SUMO2 and SUMO3 genes as SMT3A and

SMT3B, respectively. I would like to ask the authors to check this and state explicitly which nomenclature they follow and what database gene names they refer to.

2. The gene targeting strategy used to mutate the SUMO genes involved the insertion of stop codons in exon 1 of the two SUMO genes. I think the exact mutated sequence has to be provided (i.e. the exact location of the stop codons in the sequences).

3. Lack of SUMO1 expression is compensated by SUMO2/3. It would be important to know if the reverse is true, i.e. if SUMO1 protein levels and SUMO1 conjugation increases in the absence of SUMO2/3. This seems to be easy to check and should be included in the present paper. Corresponding data might support the authors' conclusion about the role of absolute SUMO levels vs. individual SUMOs.

4. The Western blot data shown in Figure 4 B/C should be quantified. The blots show substantial variation between individuals of the same genotype, which leads to some skepticism regarding the actual differences between genotypes, which the authors use for their argumentation.

5. Does the notion of SUMO2 dominance also hold up for adult tissues? I think it would be interesting to check this in analogy to the experiment shown in Figure 4A. Figure S4 indicates that the relative abundance of SUMO3 may be much more profound in adult tissues than in the embryo.

Overall, I think this study will attract attention because it describes interesting new mouse KO tools for the SUMO community and information on the relative abundance of the different SUMO paralogues and its possible functional relevance. As I outlined above, I think the genetic description of the KO lines has to be improved, and the analysis of the relative abundance of the different SUMOs has to be extended somewhat. Of note, the present paper contains very little mechanistic information on the roles of SUMO2 and SUMO3. For example, it remains entirely unclear why the loss of SUMO2 has such profound effects on embryogenesis. Nevertheless, I am inclined to support publication of the study in EMBO Reports, but I would like to see the authors' response to the comments I made above.

Referee #3:

In this study, Wang and co-workers demonstrate that Sumo-2 that is the predominantly expressed Sumo isoform during mouse embryogenesis, but Sumo-3, is dispensable for mouse embryonic development. Furthermore, their data suggest that expression levels, not functional differences between Sumo-2 and Sumo-3, are critical for normal embryogenesis. This is an important and high-quality work.

Minor point:
The sentence "... The severe defects in zebrafish embryonic development induced by Ubc9 or SUMO1-3 deficiency could be rescued by human or zebrafish SUMO1, SUMO2, or SUMO3..." is unclear. I wonder whether the Ubc9 deficiency in zebrafish can be rescued by any SUMO. Please, explain, correct and clarify!

1st Revision - authors' response 03 April 2014

Response to the points of criticism of the referees

Referee #1:

It is well known that SUMO2 and SUMO3 are very close members of SUMO. In this manuscript, Wang et al generate SUMO2 and SUMO3 knockout mice, and evidently show that SUMO2 knockout mice die before E10 days and SUMO2 knockout mice looks normal. This is an interesting phenomenon to show that SUMO2 not SUMO3 is essential for embryonic development. Although

the authors did some experiments to explain why SUMO2 is so critical in embryo, some major issues still need to be clarified.
1. Authors show that the SUMO2 is a predominant SUMO protein in early embryo, and then claim that the decrease of total amount of SUMO proteins, which is majority contributed by SUMO2 expression, in SUMO2 knockout mice might be a major reason for the death of $SUMO2^{-/-}$. The supportive evidence is that no $SUMO2^{+/-}$;$SUMO3^{-/-}$ mice are found at birth after crossing $SUMO2^{+/-}$;$SUMO3^{+/-}$ with $SUMO3^{-/-}$. However, authors show SUMO3 only accounts for 2-3% of total SUMO proteins at early embryo (Figure 4A). It looks quite hard to understand why so little amount of SUMO protein contributed by SUMO3 could make such critical phenotype in $SUMO2^{+/-}$;$SUMO3^{-/-}$ but not in $SUMO2^{+/-}$;$SUMO3^{+/-}$?

This is a very important question put forward by Referee #1. Considering SUMO3 only accounting for 2-3% of total SUMOs in early embryo, we were also surprised about the critical phenotype in $Sumo2^{+/-}$;$Sumo3^{-/-}$ compared to $Sumo2^{+/-}$;$Sumo3^{+/-}$ embryos. To better understand these unexpected results, we massively activated breeding of $Sumo2^{+/-}$;$Sumo3^{+/-}$ with $Sumo3^{-/-}$ mice, after submission of the manuscript. First, we found that at E15.5 and E17.5 $Sumo2^{+/-}$;$Sumo3^{-/-}$ embryos did not show any obvious phenotype. This suggests that $Sumo2^{+/-}$;$Sumo3^{-/-}$ embryos developed the critical phenotype much later than $Sumo2^{-/-}$ embryos. However, at E19.5 $Sumo2^{+/-}$;$Sumo3^{-/-}$ embryos were smaller than littermates of other genotypes. Notably, we found two $Sumo2^{+/-}$;$Sumo3^{-/-}$ mice after birth in a total of 35 litters (progeny:145). These rare $Sumo2^{+/-}$;$Sumo3^{-/-}$ mice were much smaller than other littermates (Fig. S5). These new results suggest that there are critical threshold levels of SUMOylation for embryogenesis at different stages and further support our conclusion that sufficient levels of SUMO2/3 are indispensible for embryonic development and that this is particularly critical at late state of embryogenesis. We have added a few sentences to report and discuss these new findings (page 9, first paragraph).

2. As authors only mention in this manuscript that $SUMO2^{+/-}$;$SUMO3^{-/-}$ mice are not found after birth, I am wondering whether the phenotypes shown in $SUMO2^{+/-}$;$SUMO3^{-/-}$ mice are the similar as that in $SUMO2^{-/-}$ mice? And also die at E10?

This question was answered above.

3. In Figure 4B and 4C, the authors should save the un-conjugated SUMO2/3 band in the blot.

In the new Figures 4B,C, we have provided the entire blots including the molecular weight range covering unconjugated SUMO, as suggested. It demonstrates that at E8.5 most of SUMO2/3 is conjugated to target proteins. This situation is different in adult animals when most of SUMO2/3 is present as free SUMO (Fig. S4).

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Wang et al. introduce SUMO2 and SUMO3 KO mouse lines and provide a basic characterization of these KO lines. Key findings are (i) that SUMO2 is essential for normal mouse embryonic development while SUMO3 is not, and (ii) that SUMO2 is much more abundant than SUMO3 or SUMO1. Based on these data and additional experiments, the authors conclude that absolute combined SUMO1/2/3 levels are of critical importance for mouse embryonic development, and not necessarily the presence or absence of an individual paralogue. The mechanism of the embryonic lethality of SUMO2 KOs was not further studied.

Overall, I think the SUMO2 and SUMO3 KO mouse lines described here will be interesting to the SUMO research field, although a conditional KO approach would have been more helpful. Further the notion of strikingly different levels of the different SUMO paralogues is interesting, as is the likely conclusion that the combined SUMO1/2/3 levels and not the individual paralogues are of critical importance in embryonic development.

I have the following comments:

1. It seems to me based on the primer sequences given that there may be a confusion with the SUMO nomenclature in this paper. My understanding is that the SUMO2/3 nomenclature introduced initially was inverted in the current databases. Initially, mature SUMO2 and SUMO3 were defined

as 92 amino acid and 93 amino acid proteins. This nomenclature is often used in the SUMO field and corresponds to the original characterization of the SUMO2 and SUMO3 genes as SMT3A and SMT3B, respectively. I would like to ask the authors to check this and state explicitly which nomenclature they follow and what database gene names they refer to.

We have checked the nomenclature, as suggested. We have used the SUMO nomenclature in accordance with the NCBI database. We have added a sentence to clarify this issue (page 10, second paragraph).

2. The gene targeting strategy used to mutate the SUMO genes involved the insertion of stop codons in exon 1 of the two SUMO genes. I think the exact mutated sequence has to be provided (i.e. the exact location of the stop codons in the sequences).

This information has been added, as requested (page 10, second paragraph; Table S1; primers for gene targeting).

3. Lack of SUMO1 expression is compensated by SUMO2/3. It would be important to know if the reverse is true, i.e. if SUMO1 protein levels and SUMO1 conjugation increases in the absence of SUMO2/3. This seems to be easy to check and should be included in the present paper. Corresponding data might support the authors' conclusion about the role of absolute SUMO levels vs. individual SUMOs.

This is indeed an important question put forward by the referee. We have performed new Western blot analyses and added the requested information (Fig. 4B,C). When corrected for β-actin as loading control, we did not see a significant increase in SUMO1 conjugation in SUMO2 or SUMO3 mutant embryos. This suggests that SUMO1 cannot compensate for SUMO2 or SUMO3 loss. We have added a few sentences to the text to report these data and our conclusion (page 7, second paragraph).

4. The Western blot data shown in Figure 4 B/C should be quantified. The blots show substantial variation between individuals of the same genotype, which leads to some skepticism regarding the actual differences between genotypes, which the authors use for their argumentation.

We have run new Western blots with SUMO2 and SUMO3 mutant embryos collected from 2-3 litters and quantified data corrected for β-actin as loading control, as suggested (new Figures 4B,C).

5. Does the notion of SUMO2 dominance also hold up for adult tissues? I think it would be interesting to check this in analogy to the experiment shown in Figure 4A. Figure S4 indicates that the relative abundance of SUMO3 may be much more profound in adult tissues than in the embryo.

We have analyzed SUMO1-3 expression in brains, hearts, and kidneys of adult animals, as suggested (Fig. 4A, adult) and also at postnatal day 0 (Fig. 4, P0). As in embryos, SUMO2 is the predominantly expressed SUMO isoform in adult organs, accounting for about 65%-70% of total SUMOs. However, SUMO3 expression is considerably higher in the adult than in the embryonic state. At P0, relative levels of SUMO1-3 are similar to those found at E8.5.

Overall, I think this study will attract attention because it describes interesting new mouse KO tools for the SUMO community and information on the relative abundance of the different SUMO paralogues and its possible functional relevance. As I outlined above, I think the genetic description of the KO lines has to be improved, and the analysis of the relative abundance of the different SUMOs has to be extended somewhat. Of note, the present paper contains very little mechanistic information on the roles of SUMO2 and SUMO3. For example, it remains entirely unclear why the loss of SUMO2 has such profound effects on embryogenesis. Nevertheless, I am inclined to support publication of the study in EMBO Reports, but I would like to see the authors' response to the comments I made above.

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In the cited paper of Hao Yuan et al., the authors did indeed not investigate Ubc9 deficiency. We have, therefore, modified the respective sentence, as suggested. The modified sentence reads: The severe defects in zebrafish embryonic development induced by SUMO1-3 deficiency could be rescued by any human or zebrafish SUMO1, SUMO2, or SUMO3. We thank the referee for pointing to this mistake.

We appreciated the constructive criticisms of three referees that helped us to improve the quality of this manuscript considerably.

2nd Editorial Decision 22 April 2014

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the comments from both referees who were asked to assess it, and both support publication of the paper now. I am therefore 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.

