EMBO reports

Fasting-sensitive SUMO-switch on Prox1 controls hepatic cholesterol metabolism

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Stephan,

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

As you will see, the referees acknowledge that the findings are potentially interesting, but they also raise a number of concerns and I list some of them below.

- Provide more evidence how SUMOylation affects Prox1 activity/localization
- Test other dietary regimes (atherogenic diet, chow diet)
- Why was only the SUMO siteK 556 chosen for further studies?

Upon further discussion with the referees, we agreed that the first point should be experimentally addressed. More information on how SUMOylation affects the molecular localization of Prox1 on chromatin and the molecular interaction with LHR-1, HNF4alpha and PXR should be provided. The data should be complemented with results from alternative diets, if possible, and the different diets used in Fig. 3A and Fig. 5 need to be justified and explained.
The importance of the modification on K353 does however not need to be experimentally tested (referee 2, point 1). A discussion why K556 was chosen for further studies is sufficient.

I am also happy to discuss the revisions further in a Zoom call, if you wish. Just send me an e-mail and we can arrange this.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. 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.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (January 10th, 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

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We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Specifically, we would kindly ask you to provide public access to the the following datasets:
- mass spec data
- RNAseq data

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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10) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
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Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
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- Please also include scale bars in all microscopy images.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

12) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

In the current study, the authors explored SUMOylation targets in the liver controlled by fasting/refeeding. They identified Prox1, a transcription factor that is SUMOylated at lysine 556 in a feeding-dependent manner. However, in high fat fed mice, Prox1 SUMOylation remains high at the fasted state. By generating hepatocyte- selective knock-in of a SUMOylation-deficient Prox1 mutant into mice fed a high-fat/high- fructose diet, the authors reveal that blocking Prox1 SUMOylation decreases circulating cholesterol levels that is associated with up-regulation of liver bile acid detoxifying pathways during fasting. Thus, using a combination of biochemical approaches and mouse genetic models, the authors have identified a novel function of Prox1 SUMOylation in liver cholesterol/bile acid metabolism.

In general, the study is well executed. What is less clear is how SUMOylation affects Prox1 activity. The molecular details of the proposed mechanism can be further characterized.

1. Cholesterol levels don't vary much with fasting or feeding, as shown in the control mice in Fig. 5B (high fat/high fructose diet) and Fig. S4A (chow fed). As such, it's unclear why the reduction in total cholesterol can only be observed at the fasted state under high fat/high fructose diet. In Fig. 3A, it was shown that high fat diet caused continuous Prox1 K556 SUMOylation. Why high fat/high fructose diet was used instead in Fig. 5?
2. Along the same line, wouldn't it be more informative to challenge the mice with an atherogenic diet (e.g., with 0.1% cholesterol) if the authors believe cholesterol metabolism is a primary pathway targeted by Prox1?
3. In Discussion, the authors suggest that de-SUMOylation likely leads to loss of the repression function of Prox1 on selective nuclear receptors, such as LRH1 and SXR/PXR. These molecular mechanisms can be readily tested. For example, does Prox1K556R affects its interaction with LRH1 and/or SXR/PXR activity in a report-based assay?

Referee #2:

Summary

The authors aimed to identify the dynamic role of SUMOylation and the role that it has over transcriptional control of liver metabolism under two metabolic states: fasting vs feeding. There, they performed a SUMO proteome analysis in the mouse liver under fasted vs refed conditions to determine proteins that were differentially SUMOylated. They identified the transcription factor Prospero Homeobox Protein 1 (Prox1) as the major SUMOylation protein that became SUMOylated under refed conditions. The authors then generated a conditional Knock In mouse of Prox1 with a site-specific mutation at lysine 556 abolishing that SUMOylation site. These mice failed to become SUMOylated under re-fed conditions and had lower levels of circulating cholesterol though no differences were seen in total cholesterol and cholesterol esters in the liver. An RNA-seq analysis revealed differential gene expression in bile acid detoxification and the authors postulated that this may be the reason why cholesterol levels were reduced in the Prox1 mutant. In summary this manuscript was very preliminary and underdeveloped. There are several critical concerns that will need to be addressed before it is suitable for publication. Due to these concerns, I do not recommend for publication in EMBO Reports.

Major Comments
1. The authors state that two SUMO sites were identified on Prox1 (353 vs 556) yet the authors only focused on site 556. What was the rationale? The authors then claimed that mutating the 556 site led to the ablation of SUMOylation and thus postulated that this was relevant site. This does not however disprove the importance of site 353. The authors should also generate mutant models in which 353 is targeted to truly gauge the regulation of this protein.

2. More detailed methods are needed. What chromosome was the construct targeted to? How were the AAV CRE injected? What concentration of AAV was used? What gender were the mice? Were these experiments done in both males and females?

3. Given that diet-induced-obesity caused no changes in Prox1 SUMOylation between fasted and refed states, it was unclear why the authors focused on this metabolic state instead of under chow fed conditions when Prox1 became SUMOylated under refed conditions.

4. Figures were very poorly organized, and it was difficult to follow the logic of the authors. For example, in Figure 3A, it was unclear that the western blot to the right was also a part of 3A. Then in Figure 3B, a densitometry was shown to reflect total Prox 1 from Fig.3A but then qPCR results were also apart of Fig3B? The grouping logic was very confusing. Also, relative log2FC is not very conventional to depict qPCR or western blot densitometry data.

Minor Comments
1. Many grammar errors throughout. Please proof your manuscript carefully before submission.
2. Figure 1B: The ponceau stain is very poor in the S2-IP blot and should be redone.

Referee #3:

EMBOR-2022-55981V1
Fasting-sensitive SUMO-switch on Prox1 controls hepatic cholesterol metabolism by Ana Jimena Alfaro Nunez, ..., Frauke Melchior, and Stephan Herzig

This well-executed and rationalized study led by experts in the fields of SUMOylation and regulation of metabolism/metabolic mouse models shows that specific SUMOylation of prospero homeobox protein 1 (Prox1) can indeed regulate hepatic fasting metabolism in vivo. Prox1 was firstly shown to be markedly SUMOylated at lysine 556 in the liver of ad libitum and re-fed mice, with the modification being abolished upon fasting. Even more interestingly, diet-induced obesity rendered the hepatic SUMOylation of Prox1 almost inert to fasting. Hepatocyte-selective knock-in of a SUMOylation-deficient Prox1 mutant mice in turn led on fed a high-fat/high-fructose diet to a reduction of systemic cholesterol levels that associated with an induction of liver bile acid detoxifying gene expression during fasting. This manuscript beautifully fulfils the attributes of physiological relevance, wide interest, notable conceptual advance and impact on the research community. This is a manuscript with an emphasis on functional insight over extensive mechanistic detail, as the actual role of SUMOylation on the Prox1's interactions with other proteins or chromatin was left unstudied. I do not have any major concerns regarding the story, but hope that the authors will consider the following points:
- Results, under the subtitle/Prox1 is modified by SUMO at lysine 556 in response to nutrient availability: "...These results suggested that under conditions of diet-induced obesity the SUMO-switch on Prox1 was mostly un-responsive to nutrient availability..." Instead of using the expression "mostly unresponsive", it would be perhaps more pertinent to clearly compare to the effect of re-feeding on the Prox1 SUMOylation of mice on chow and HFD, as based on Fig. 3A, there is still a significant increase in the Prox1 SUMOylation upon re-feeding of HFD-fed mice.
- Last Result page: " ..Because SUMO conjugation did not affect the stability, localization nor binding affinity to chromatin of Prox1 (Fig. S6A and B)...". I do not see how the stability was examined here in the Fig. S6. The localization probably refers to

intracellular localization, not localization on chromatin? By the way, why were the immunofluorescence studies carried out in HeLa cells instead of sticking to the HepG2 cells that were used as a proxy of hepatocytes? Where is the evidence for unaltered binding affinity to chromatin of the SUMOylated Prox1? I my opinion binding affinity cannot be simply judged by salt extraction of nuclei with a couple of different salt concentrations. Please, clarify the meaning and basis of the above sentence. It would be pertinent to mention e.g. in the discussion that the modification can alter the distribution of the Prox1 on chromatin as has been proven with ChIP-seq to be the case with several transcription factors. I assume that the label CE (in panel B) refers to cytoplasmic extracts (in the legend the label seems to C).
- P. 3 of discussion: it is stated that Cyp3a4 is up-regulated in the Prox1 mutant mouse model. However, the CYP3A4 is a human enzyme, i.e. there is no Cyp3A4 in mouse and no orthologous CYP3A pairs between mouse and human. Please, clarify the statement.
- The rodent homolog of human xenobiotic receptor SXR (steroid and xenobiotic receptor) was initially dubbed PXR (pregnane X receptor). It would be pertinent to add the abbreviation PXR at least in parenthesis when the receptor is dealt with for the first time in the manuscript. The findings of this work might at least in part mechanistically explain the results of a recent work reporting that nutritional status modifies the PXR-regulated transcriptome both qualitatively and quantitatively (PMID: 31723190). The authors might want to consider addition of this reference to the discussion.

We thank the editor and the reviewers very much for their critical but constructive comments. We have addressed all raised issues by additional experiments and/or changes in the original text version. Please find our point-by-point response below:

Response to Editor:

- Provide more evidence how SUMOylation affects Prox1 activity/localization

We appreciate this comment by the editor and have performed the following experiments accordingly:

1. We have performed an over-expression study of Prox1 wild-type (wt) or SUMO-deficient mutant (K556R) in HepG2 cells showing that both the un-modified and SUMOylated species localize within the nucleus (in manuscript: Fig. EV5B). These data suggest that the SUMO modification on Prox1 does not affect its nuclear localization and that Prox1 in general is involved in the transcriptional regulation of down-stream target genes.

2. In addition, by using a cycloheximide pulse and chase study with HEK293A cells over-expressing Prox1 wt, K556R or E558A, we demonstrated that the stability of Prox1 is not affected by SUMO conjugation (in manuscript: Fig. EV5A).

3. A protocol to immuno-precipitate HA-tagged Prox1 was established to investigate whether SUMOylation affects the interaction of Prox1 with Myc-tagged PXR, HNF4a or LRH-1 overexpressed in HEK293A cells. Interestingly, the interaction of Prox1 with LRH-1 was affected by the loss of SUMOylation (in manuscript: Fig. 6D), while the interaction with HNF4a or PXR was not affected (in manuscript: Fig. EV5D).

Overall, these in vitro data reveal a mechanism by which SUMOylation could affect the activity of Prox1. We suggest that the repressor activity of Prox1 on LRH-1 could be mediated by SUMOylation of Prox1 on lysine 556 by directly affecting the interaction strength between these two transcription factors.

- Test other dietary regimes (atherogenic diet, chow diet). The data should be complemented with results from alternative diets, if possible, and the different diets used in Fig. 3A and Fig. 5 need to be justified and explained.

We thank the editor for this helpful comment. We have performed a high cholesterol (2%) diet challenge with our SUMO-deficient Prox1 knock-in mouse model for 14 weeks. Both male and female mice expressing the SUMO-deficient Prox1 in the liver coped with the high cholesterol challenge to the same degree as the control animals (in manuscript: Appendix Fig. S3 and 4). In line with this, the liver transcriptome of control and mice expressing the mutant Prox1 was comparable (data not shown).

In addition, we have incorporated data from wild-type mice fed a high cholesterol diet (2%) (in manuscript: Fig. 3A and EV2A). The SUMO switch on Prox1 was fully responsive to fasting cues in the liver of mice fed a high cholesterol diet. This observation could explain why we did not identify any phenotype with our knock-in mouse model upon this dietary challenge.

We have also incorporated data from wild-type mice fed a high fat/high fructose diet as a second model of diet induced obesity (in manuscript: Fig. 3C and EV2C). In line with the original high fat diet model, the SUMO-switch on Prox1 was also blunted with this model of diet induced obesity. A high fat/high fructose study was chosen to challenge our SUMO-deficient Prox1 knock-in mouse model as this combinatorial diet better represents the "Western diet" and is commonly used in studies of lipoprotein metabolism (Kleinert *et al*, 2018; Sachs *et al*, 2021). Consequently, this model was subsequently chosen to conduct the metabolic phenotyping experiments.

- Why was only the SUMO site K556 chosen for further studies?

As requested, the rationale for choosing SUMO site K556 is now outlined in the manuscript. Lysine 556 is a better candidate for SUMO conjugation as it resides in an unstructured region whereas lysine 353 is part of an α-helix. Lysin 556 has also been identified as the main SUMOylation site of Prox1 using an E.Coli expression-modification system (Pan *et al*, 2009).

We feel that these new data have strengthened the manuscript and further support our key findings:

1. Hepatic Prox1 is a highly efficient SUMO target. Given the crucial role of Prox1 in the regulation of lipid metabolism, it is important to communicate that it exists as both an unmodified and a SUMOylated version in hepatocytes.

2. The SUMO conjugation of Prox1 is dramatically affected by fasting. This observation further emphasizes the participation of Prox1 in the regulation of liver metabolism in response to environmental cues.

3. Indeed, the transcriptional activity of Prox1 on genes involved in the bile acid detoxifying pathway is altered when the conjugation of Prox1 with SUMO is genetically blocked. In line with this, the interaction of Prox1 with a key nuclear receptor (LRH1) regulating cholesterol and bile acid metabolism is affected by SUMO conjugation.

Referee #1:

In the current study, the authors explored SUMOylation targets in the liver controlled by fasting/refeeding. They identified Prox1, a transcription factor that is SUMOylated at lysine 556 in a feeding-dependent manner. However, in high fat fed mice, Prox1 SUMOylation remains high at the fasted state. By generating hepatocyte- selective knock-in of a SUMOylation-deficient Prox1 mutant into mice fed a high-fat/high- fructose diet, the authors reveal that blocking Prox1 SUMOylation decreases circulating cholesterol levels that is associated with up-regulation of liver bile acid detoxifying pathways during fasting. Thus, using a combination of biochemical approaches and mouse genetic models, the authors have identified a novel function of Prox1 SUMOylation in liver cholesterol/bile acid metabolism.

In general, the study is well executed. What is less clear is how SUMOylation affects Prox1 activity. The molecular details of the proposed mechanism can be further characterized.

We thank referee #1 for the overall support and for confirming that our study was well executed. As outlined in our response to the editor above, we have attempted several experimental approaches to further delineate the mechanistic relevance of Prox1 SUMOylation in this context.

1. Cholesterol levels don't vary much with fasting or feeding, as shown in the control mice in Fig. 5B (high fat/high fructose diet) and Fig. S4A (chow fed). As such, it's unclear why the reduction in total cholesterol can only be observed at the fasted state under high fat/high fructose diet.

We suggest that the functionality of the SUMO-switch on Prox1 relies on its sensitivity to fasting cues to support the detoxification of bile acids that accumulate when food is not available. In the context of diet induced obesity, the SUMO-switch on Prox1 is less responsive to fasting cues. Our engineered de-SUMOylation of Prox1 promotes the metabolism of bile acids during fasting thus affecting the total levels of cholesterol. Note, that we identify no transcriptional changes in lean nor obese mice expressing the mutant Prox1 in the re-fed state further supporting that the functionality of the SUMO-switch on Prox1 relies on its responsiveness to fasting signals.

In Fig. 3A, it was shown that high fat diet caused continuous Prox1 K556 SUMOylation. Why high fat/high fructose diet was used instead in Fig. 5?

In the context of diet-induced obesity the response of the SUMO-switch on Prox1 fasting signals is impaired. We chose to continue with the high fat/high fructose diet to induce a metabolic burden at the level of both lipid intake and de novo synthesis mimicking a western diet. In general, the combination diet is easier to handle (pellets are more stable) and it is a common protocol in diabetes/lipoprotein research (Kleinert et al, 2018; Sachs et al, 2021).

2. Along the same line, wouldn't it be more informative to challenge the mice with an atherogenic diet (e.g., with 0.1% cholesterol) if the authors believe cholesterol metabolism is a primary pathway targeted by Prox1?

We thank the reviewer for this suggestion. We have now performed a high cholesterol (2%) diet challenge with our SUMO-deficient Prox1 knock-in mouse model for 14 weeks (in manuscript: Appendix Fig. S3 and 4). However, we identified no metabolic differences between the control and the mice expressing the SUMO-deficient Prox1. Note that the response of the SUMO-switch on Prox1 to fasting cues is fully active in the liver of mice challenged with a high-cholesterol diet (in manuscript Fig. 3 A).

3. In Discussion, the authors suggest that de-SUMOylation likely leads to loss of the repression function of Prox1 on selective nuclear receptors, such as LRH1 and SXR/PXR. These molecular mechanisms can be readily tested. For example, does Prox1K556R affects its interaction with LRH1 and/or SXR/PXR activity in a report-based assay?

We appreciate the referee's suggestion. To address this point, we performed a set of co-immunoprecipitation assays of HA-tagged Prox1 and Myc-tagged PXR, HNF4a or LRH-1 using HEK293A cells. The interaction of Prox1 with LRH-1 was weaker with the SUMO-deficient (KR) mutant than with wt Prox1 (in manuscript: Fig. 6D), while the interaction with HNF4a or PXR was comparable between Prox1 wt and the KR mutant (in manuscript: Fig. EV5D). These results suggested that Prox1 SUMOylation specifically affects the interaction with distinct transcription factors and may thereby determine specific subsets of downstream target genes.

Referee #2:

Summary

The authors aimed to identify the dynamic role of SUMOylation and the role that it has over transcriptional control of liver metabolism under two metabolic states: fasting vs feeding. There, they performed a SUMO proteome analysis in the mouse liver under fasted vs refed conditions to determine proteins that were differentially SUMOylated. They identified the transcription factor Prospero Homeobox Protein 1 (Prox1) as the major SUMOylation protein that became SUMOylated under refed conditions. The authors then generated a conditional Knock In mouse of Prox1 with a site-specific mutation at lysine 556 abolishing that SUMOylation site. These mice failed to become SUMOylated under re-fed conditions and had lower levels of circulating cholesterol though no differences were seen in total cholesterol and cholesterol esters in the liver. An RNA-seq analysis revealed differential gene expression in bile acid detoxification and the authors postulated that this may be the reason why cholesterol levels were reduced in the Prox1 mutant. In summary this manuscript was very preliminary and underdeveloped. There are several critical concerns that will need to be addressed before it is suitable for publication. Due to these concerns, I do not recommend for publication in EMBO Reports.

Major Comments

1. The authors state that two SUMO sites were identified on Prox1 (353 vs 556) yet the authors only focused on site 556. What was the rationale? The authors then claimed that mutating the 556 site led to the ablation of SUMOylation and thus postulated that this was relevant site. This does not however disprove the importance of site 353. The authors should also generate mutant models in which 353 is targeted to truly gauge the regulation of this protein.

We thank referee #2 for the critical but constructive comments. Although we cannot make conclusions regarding the potential post-translational modifications on Prox1 at lysine residue 353, we have confirmed that the main SUMOylation site on hepatic Prox1 is lysine 556. The major modified species of Prox1 is abolished in the liver of mice expressing the K556R mutant in hepatocytes (in manuscript Fig. 4B).

2. More detailed methods are needed. What chromosome was the construct targeted to? How were the AAV CRE injected? What concentration of AAV was used? What gender were the mice? Were these experiments done in both males and females?

We thank this referee for pointing out that relevant information was missing. It has been added to the manuscript.

3. Given that diet-induced-obesity caused no changes in Prox1 SUMOylation between fasted and refed states, it was unclear why the authors focused on this metabolic state instead of under chow fed conditions when Prox1 became SUMOylated under refed conditions.

We indeed started by characterizing a cohort of lean mice fed a standard chow diet expressing the SUMO-deficient Prox1 (K556R) mutant in hepatocytes. We analyzed the metabolic state in the fasted and re-fed states. However, we did not identify any metabolic differences, nor any differences in the transcriptome between control or mice expressing the SUMO-deficient Prox1 in the re-fed state. Given these results, we hypothesized that the functionality of the SUMO-switch on Prox1 rather relies on its sensitivity to fasting cues. Thus, the altered response of the SUMO switch on Prox1 we observed in obese animals offered the opportunity to explore the role of Prox1 SUMOylation with our SUMO-deficient Prox1 knock-in mouse model during fasting.

4. Figures were very poorly organized, and it was difficult to follow the logic of the authors. For example, in Figure 3A, it was unclear that the western blot to the right was also a part of 3A. Then in Figure 3B, a densitometry was shown to reflect total Prox 1 from Fig.3A but then qPCR results were also apart of Fig3B? The grouping logic was very confusing. Also, relative log2FC is not very conventional to depict qPCR or western blot densitometry data.

We thank again this referee for pointing out these key points, they have been corrected in the manuscript. We have re-arranged the figures and specified the type of data presented in each figure. We are now presenting our qPCR data as relative to time point zero or control.

Minor Comments

1. Many grammar errors throughout. Please proof your manuscript carefully before submission.

The manuscript has been reviewed thoroughly, we apologize for the amount of grammar errors in our first submission.

2. Figure 1B: The ponceau stain is very poor in the S2-IP blot and should be redone.

We agree, the ponceau staining is not ideal. However, we were not able to re-run the SUMO2 IP.

Referee #3:

EMBOR-2022-55981V1
Fasting-sensitive SUMO-switch on Prox1 controls hepatic cholesterol metabolism by Ana Jimena Alfaro Nunez, ..., Frauke Melchior, and Stephan Herzig

This well-executed and rationalized study led by experts in the fields of SUMOylation and regulation of metabolism/metabolic mouse models shows that specific SUMOylation of prospero homeobox protein 1 (Prox1) can indeed regulate hepatic fasting metabolism in vivo. Prox1 was firstly shown to be markedly SUMOylated at lysine 556 in the liver of ad libitum and re-fed mice, with the modification being abolished upon fasting. Even more interestingly, diet-induced obesity rendered the hepatic SUMOylation of Prox1 almost inert to fasting. Hepatocyte-selective knock-in of a SUMOylation-deficient Prox1 mutant mice in turn led on fed a high-fat/high-fructose diet to a reduction of systemic cholesterol levels that associated with an induction of liver bile acid detoxifying gene expression during fasting. This manuscript beautifully fulfils the attributes of physiological relevance, wide interest, notable conceptual advance and impact on the research community. This is a manuscript with an emphasis on functional insight over extensive mechanistic detail, as the actual role of SUMOylation on the Prox1's interactions with other proteins or chromatin was left unstudied.

We highly appreciated referee #3's positive comments on the execution and rationale of our study.

I do not have any major concerns regarding the story, but hope that the authors will consider the following points:

- Results, under the subtitle/Prox1 is modified by SUMO at lysine 556 in response to nutrient availability: "...These results suggested that under conditions of diet-induced obesity the SUMO-switch on Prox1 was mostly un-responsive to nutrient availability..." Instead of using the expression "mostly unresponsive", it would be perhaps more pertinent to clearly compare to the effect of re-feeding on the Prox1 SUMOylation of mice on chow and HFD, as based on Fig. 3A, there is still a significant increase in the Prox1 SUMOylation upon re-feeding of HFD-fed mice.

We thank the referee for pointing out this statement. Indeed, the levels of Prox1 SUMOylation are also promoted by feeding in obese mice. We have re-phrased this paragraph in the manuscript to clearly describe the data.

- Last Result page: " ..Because SUMO conjugation did not affect the stability, localization nor binding affinity to chromatin of Prox1 (Fig. S6A and B)...". I do not see how the stability was examined here in the Fig. S6. The localization probably refers to intracellular localization, not localization on chromatin? By the way, why were the immunofluorescence studies carried out in HeLa cells instead of sticking to the HepG2 cells that were used as a proxy of hepatocytes? Where is the evidence for unaltered binding affinity to chromatin of the SUMOylated Prox1? I my opinion binding affinity cannot be simply judged by salt extraction of nuclei with a couple of different salt concentrations. Please, clarify the meaning and basis

of the above sentence. It would be pertinent to mention e.g. in the discussion that the modification can alter the distribution of the Prox1 on chromatin as has been proven with ChIP-seq to be the case with several transcription factors. I assume that the label CE (in panel B) refers to cytoplasmic extracts (in the legend the label seems to C).

We agree with the referee, and we have clarified our statements in the manuscript. Using a cycloheximide pulse and chase study with HEK293A cells over-expressing Prox1 wt, K556R or E558A, we have demonstrated that the stability of Prox1 is not affected by SUMO conjugation (in manuscript: Fig. EV5A). We have also analyzed the localization of Prox1 wt and K556R mutant in HepG2 cells and show that the nuclear localization of Prox1 is not affected by SUMO conjugation (in manuscript: Fig EV5B).

We have also clarified our statement regarding the salt nuclear extractions. Indeed, SUMO-conjugation could alter the distribution of Prox1 on specific chromatic regions and this could not be assessed by salt nuclear extractions. Our experiment was meant as a first step to identify whether SUMO-conjugation could impair the binding affinity of Prox1 to either DNA or DNA-bound proteins. With our gradient salt extractions of endogenous nuclear proteins, we could show that both the un-modified and SUMOylated species of Prox1 have a similar binding strength to chromatin (in manuscript: Fig EV5C).

- P. 3 of discussion: it is stated that Cyp3a4 is up-regulated in the Prox1 mutant mouse model. However, the CYP3A4 is a human enzyme, i.e. there is no Cyp3A4 in mouse and no orthologous CYP3A pairs between mouse and human. Please, clarify the statement.

We thank the referee for pointing out this mistake. The manuscript has been corrected. We have identified an over-expression of Cyp3a11 as well as Cyp3a44.

- The rodent homolog of human xenobiotic receptor SXR (steroid and xenobiotic receptor) was initially dubbed PXR (pregnane X receptor). It would be pertinent to add the abbreviation PXR at least in parenthesis when the receptor is dealt with for the first time in the manuscript. The findings of this work might at least in part mechanistically explain the results of a recent work reporting that nutritional status modifies the PXR-regulated transcriptome both qualitatively and quantitatively (PMID: 31723190). The authors might want to consider addition of this reference to the discussion.

We thank the referee for pointing us to this publication. The authors studied the liver transcriptome of mice injected with the PXR synthetic agonist pregnenolone-16-α-carbonitrile (PCN) after 5 h fasting and 1 h after a glucose administration (2 g/kg oral gavage). This study shows that the PCN mediated activity of PXR differs between both conditions, having more pronounced effects after glucose treatment. As we could not demonstrate that the interaction of Prox1 with PXR is modulated by SUMOylation, we decided to rephrase our conclusions. Therefore, we did not mention this publication in our discussion.

References

Kleinert M, Clemmensen C, Hofmann SM, Moore MC, Renner S, Woods SC, Huypens P, Beckers J, de Angelis MH, Schürmann A *et al* (2018) Animal models of obesity and diabetes mellitus. *Nat Rev Endocrinol* 14: 140-162

Pan M-R, Chang T-M, Chang H-C, Su J-L, Wang H-W, Hung W-C (2009) Sumoylation of Prox1 controls its ability to induce VEGFR3 expression and lymphatic phenotypes in endothelial cells. *Journal of Cell Science* 122: 3358-3364

Sachs S, Niu L, Geyer P, Jall S, Kleinert M, Feuchtinger A, Stemmer K, Brielmeier M, Finan B, DiMarchi RD *et al* (2021) Plasma proteome profiles treatment efficacy of incretin dual agonism in diet-induced obese female and male mice. *Diabetes, Obesity and Metabolism* 23: 195-207

Dear Stephan,

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

As you will see, both referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

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Please also deposit the RNA-seq data in a public repository and add this information to the data availability section.

- Please rename the Conflict of Interest section to 'Disclosure Statement and Competing Interests'. . For more information see https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

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- Please note that per our editorial policies all statements need to be supported by data. In this context we note 3 statements that are based on 'data not shown' (pages 13-15). Please either remove the statement or provide the data.

- The information on funding should be part of the Acknowledgment section.

- You have callouts to Tables EV1 and EV2 but the tables are not provided.

- Appendix:
- Please provide it as a .pdf file.
- Moreover, all materials and methods need to be part of the main manuscript methods.
- Figure S1: please only define significance values that are shown in the figure panels, i.e., please remove *P < 0.05
- Figure S2: If the information given under D) 'Every dot represents on individual mouse...' is valid for A-D, then please move it to the end of the Figure legend and precede the statement with 'Data information:'
- S3A: *P < 0.05 is specified in the figure legend but not part of the figure panels. Please remove it.
- S4: Precede the information about the quantification for (B-C) with 'Data information: (B-C) etc'. Same for S5
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We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

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Martina Rembold, PhD
Senior Editor
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****

Referee #1:

the authors have addressed my main concerns.

Referee #2:

The authors addressed all my comments satisfactorily and the manuscript is now suitable for publication.

The authors have addressed all minor editorial requests.

Prof. Stephan Herzig
Helmholtz Munich
Institute for Diabetes and Cancer
Ingolstaedter Landstraße 1
Neuherberg 85764
Germany

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- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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Each figure caption should contain the following information, for each panel where they are relevant:
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