EMBO reports

SIRT5 inhibits peroxisomal ACOX1 to prevent oxidative damage and is downregulated in liver cancer

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Transaction Report:

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

1st Editorial Decision 6 October 2017

Thank you for the submission of your manuscript to EMBO reports. We have now received the referee comments that are pasted below.

As you will see, all referees acknowledge that the findings are potentially interesting. However, both referees 3 and 4 point out that the peroxisomal localization of SIRT5 is not sufficiently convincing, and that it would need to be demonstrated that the observed effects on H2O2 homeostasis are really due to a role of SIRT5 in peroxisomes, as opposed to side effects or SIRT5 function in other cellular compartments. Addressing these points will entail a major round of significant revisions, but given the potentially interesting findings, I would like to invite you to fully address the referee concerns. The only exception is the comment by referee 2 regarding a role for SIRT5 in peroxisomal beta-oxidation and the ACOX1 succinylation sites, which would not need to be addressed experimentally.

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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

Regarding data quantification, please specify the number "n" for how many experiments were

performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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

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

REFEREE REPORTS

Referee #1:

This manuscript by Chen and colleagues examines the role of the sirtuin SIRT5 in peroxisomes. SIRT5 is a poorly understood sirtuin with unusual catalytic activities, functioning to remove succinyl, malonyl, and glutaryl groups from lysines on its target proteins, rather than acetyl groups. SIRT5 is primarily known as a mitochondrial protein, though it is present in other cellular compartments such as the cytosol and in the nucleus. Chen et al. show that a fraction of SIRT5 is peroxisomal, where it interacts with and inhibits the ACOX1 protein to suppress cellular ROS levels. The authors also provide evidence that suppression of SIRT5 levels may contribute to the pathogenesis of hepatocellular carcinoma.

Overall, this is a very important manuscript that makes a significant contribution to the understanding of SIRT5 biology. To my knowledge, no sirtuin has been shown to regulate targets in peroxisomes. Moreover, roles for SIRT5 in human cancer are an active area of investigation in many laboratories currently, an area where this manuscript contributes. I support publication in EMBO Reports, provided that the authors can address the following relatively minor concerns:

1. It would be of interest to know the spectrum of peroxisomal proteins potentially targeted by SIRT5. Can the authors cull such a list of candidates from the published SIRT5 proteomics studies and present such a table, perhaps in the supplemental data?
2. While the authors' immunofluorescence data are convincing, I would like to see the SIRT5 KD cells included as a negative control in Fig. 1F. In addition, cellular fractionation to elucidate localization of endogenous SIRT5 would be extremely helpful, particularly if mitochondria can be separated from mitochondria. The question is how much SIRT5 resides in one compartment versus the other?
3. The authors provide evidence that SIRT5 regulates the dimerization state of ACOX1. Can the authors use Pymol or the like to map the modification sites on ACOX1, for example to see whether

any lie at the dimerization interface? It would be extremely interesting to provide some structural insight into the basis for this mode of regulation. It would be nice to mutate the critical residues to show that this exerts the predicted effects on ACOX1 multimerization and activity, although I don't absolutely insist on this experiment.
4. A lot of the paper relies on ACOX1 activity assays. I would be more comfortable with this if the authors show someplace that this assay is specific, particularly in the setting of tissue lysates, for example by the use of a known specific ACOX1 inhibitor, or the use of ACOX1 KO tissue.
5. The authors should show some representative images of SIRT5 staining in HCC and normal in Fig. 6.

Other points
1. The authors' literature citations are not always exactly correct. For example, the Park 2013 Mol. Cell paper focused mostly on MEFs and liver (p.6). This paper should also be cited on p. 7.
2. Although the manuscript is generally very clear and well-written, there are some typos ("dimmer" rather than dimer on p. 13; "Hochest" rather than Hoescht in EV1).

Referee #2:

Dr Chen and colleagues have undertaken a well designed study where they have substantial biochemical and functional data to identify that acyl-CoA oxidase 1 (ACOX1) is a functional substrate that is desuccinylated and inactivated by SIRT5. The authors use multiple molecular manipulations in a full-factorial design to show the SIRT5 associates with the peroxisome and interacts with and desuccinylates ACOX1. The consequence of this action include the disruption of the homodimeric structure of ACOX1, and the reduction in ACOX1 generation of H2O2 as a byproduct of peroxisomal beta-oxidation. The authors also show that the genetic and pharmacologic increase in succinyl-CoA levels succinylates and activates ACOX1. As excess H2O2 can have detrimental effects on the cell the reserach team also show evidence of increase oxidative stress and propensity to ex-vivo tumor growth with the disruption of SIRT5 and show a correlation with reduced SIRT5 levels and increased ACOX1 activity in hepatocellur lines and suggest a correlation of poor outcome in human HCC carcinoma with reduced SIRT5 levels.

The only functional assays not performed is to evaluate whether SIRT5 levels modulate peroxisomal beta-oxidation and the sites of the succinylation were not confirmed.

I have some minor comments:
1. Top of page 12 - SDHA knockdown was said in increase global lysine succinlylation which was stated to be shown in Figure 3E. It shoudl be pointed out that this is shown in the input, as the inclination woudl be that this would be a separate immunblot rather than the loading control for an IP study.
2.Page 8, line 6. add ahRNA-2 was 'more' potent. the word more is missing.
3. Fig 1C and Fig 5C. The text nor the legend describe how many times these experiments were repeated. This should be added and it may be useful to quantitate these findings.
4. Fig 2A. HA-SIRT3 failed to express, and one cannot state it does not interact with ACOX1 if it is not present.

Referee #3:

In the manuscript by Chen et al., the authors describe a role for SIRT5 in the regulation of peroxisomal Acyl-CoA Oxidase 1 (ACOX1) function. SIRT5 is a member of the Sirtuin family of NAD+-dependent deacylases, involved in metabolism, stress response and genome stability. SIRT5 is one of the three mitochondrial members of the family, and its enzymatic activity targets a range of acyl groups including acetyl, malonyl and succinyl. The desuccinylase, demalonylase and deacetylase activities of SIRT5 have been characterized and linked to several metabolic pathways such as amino acid catabolism, urea cycle, TCA, cellular respiration as well as glucose and fatty acid metabolism. In this work, the authors show that SIRT5 desuccinylates ACOX1, the first enzyme of the β-oxidation pathway of very-long-chain-fatty acids (VLCFAs), a reaction that is

associated to H2O2 production. ACOX1 desuccinylation by SIRT5 inhibits its enzymatic activity by decreasing ACOX1 dimer formation, which results in decrease in H2O2 production and DNA damage signaling. SIRT5 downregulation in established cell lines (shRNA) or lack of SIRT5 in SIRT5KO mouse livers produce higher levels of ACOX1 succinylation, increased ACOX1 activity, increased H2O2 production and DNA damage signaling as well as anchorage-independent growth. The relevance of the negative regulation of ACOX1 by SIRT5 is reflected by the rescue of these SIRT5-deficient phenotypes upon shRNA-driven downnregulation of ACOX1. Consistent with the direct role of peroxisomal metabolism in development of liver diseases such as Hepatocellular carcinomas (HCC), the authors observe increased levels of succinyl-ACOX1, increased ACOX1 activity and decreased SIRT5 levels in a set of HCC tumors. Consistently, analysis of the outcome of these patients, associate high level of SIRT5 in these tumors with increased cell survival and decrease rate of recurrence.
This is a very interesting set of evidences, and link for the first time SIRT5 with peroxisomal metabolism. The authors have done in general a good work characterizing the ACOX1 desuccinylation activity of SIRT5, and have clearly established the direct link between succinylation of ACOX1 and ACOX1 activity. Moreover, the studies with HCC samples suggest that this functional link is very relevant in cancer. However, I have several concerns with the work as some claims are at this stage overstated and the manuscript has a number of relevant technical issues that should be addressed:
1) The main issue is that I believe the authors have not shown any convincing data demonstrating that SIRT5 really localizes to peroxisomes. This is a relevant issue as is a major claim of the manuscript. These claims of co-localization studies are based very poor IF studies that do not meet a minimal quality and resolution level (Figure 1E, 1F, S2, EV3). In the case of SIRT5 this is very obvious as in these IF experiments SIRT5 distribution is very diffuse and present in most of the cytoplasm. In fact, looking at the methods section is not even clear whether the images were made from a single focal plane (with a confocal microscope) or basically includes the whole cell. The authors only mention that it was done with an optical fluorescence microscope. This issue deserves more work including using other ways of demonstrating this claim such as FRET or similar assays. Even If the authors insist in develop further these colocalization studies, they should be performed in a single plane, with enough resolution and including a quantative statistical analysis used in these cases (e.g. Pearson correlation coefficient) to be convincing.

2) In fact, and linked to this previous issue, how do the authors explain that SIRT5 induces a similar level of H2O2 in peroxisomes, nucleus and cytoplasm (Figure 1A) and that all of them are neutralized by ACOX1 downregulation by shRNA (Figure 5A)? Even If this H2O2 can move from peroxisomes to the nucleus or cytoplasm, one would expect that, If produced in the peroxisome, peroxisomal H2O2 would be significantly higher. In fact, Figure EV3 shows that ACOX1 distribution is almost, but not completely, identical to the distribution of peroxisome marker PMP70. This is also in agreement with the clear effect of the mitochondrial (and nuclear) protein SDHA on succ-ACOX1 levels. Can the authors exclude that the link between SIRT5 and ACOX1 takes place in other non-peroxisomal compartments?
3) Does the catalytic-inactive SIRT5 mutant H158Y used in Figures 2E, 3A, 4A-B, bind to ACOX1? This is in fact a missing control of Figure 2E. If, in contrast to WT SIRT5, the H158Y mutant does not bind to ACOX1, the effect of active WT SIRT5 on ACOX dimerization may also be produced by a steric effect due to SIRT5 binding and not by SIRT5 enzymatic activity. This issue should be addressed.
4) In Figure 2A, the SIRT5 specificity claimed by the authors is not clear. No SIRT3 is shown even in the inputs and the levels of SIRT1 or SIRT7 are much lower than SIRT4 and 5. If the authors want to make this claim this should be repeated convincingly.
5) Figure 2D. I have several concerns. The first one is that the Acox1 levels in the IP are saturated and therefore the succ-ACOX1 differences between WT and KO are difficult to evaluate. In fact, looking carefully, KOs seem to show higher levels of ACOX1. The second one is that in fact, ACOX1 levels in the input are missing.
6) Figure 5D. The quality of the image should be improved maybe by increasing contrast. The images in S6 are much clearer. In its current form, is difficult to distinguish all the colonies quantified in Fig5E.
7) I have several concerns regarding figure 6A. One problem is that SIRT5 does not seem to be clearly decreased between peritumoral and tumor samples except for #1. The second one is that patient 4 is the one that has lower levels of SIRT5, but no change in succ-ACOX1 is observed. The authors should address these discrepancies.

Referee #4:

Protein succinylation is a posttranslational modification that can regulate multiple metabolic processes and the sirtuin 5 (SIRT5) catalyzes desuccinylation. Recent proteomic studies identified multiple succinylation sites in peroxisomal acyl-CoA oxidase 1 (ACOX1) that functions as a major H</sub>2</sub>O</sub>2</sub>-producer in peroxisomes. In this manuscript, Chen et al. report that SIRT5 is partially localized in peroxisomes and desuccinylates ACOX1, which leads to decrease the enzyme activity of ACOX1 by lowering its active dimer formation. The authors also show that the phenotypes upon deletion of SIRT5 including accumulation of H</sub>2</sub>O</sub>2</sub> and oxidative DNA damage were cancelled by knockdown of ACOX1. Together with a notion in regard to the correlation between downregulation of SIRT5 and higher succinylation and activity of ACOX1 in hepatocellular carcinoma (HCC), the authors conclude that SIRT5 regulates the homeostasis of peroxisomal H</sub>2</sub>O</sub>2</sub> and cellular ROS via controlling the succinylation of ACOX1.

The findings in this manuscript are interesting and extend our knowledge of the succinylation in regulation of protein functions. However, morphological data are ambiguous thus not sufficient to establish the exact localization of SIRT5 in or on peroxisomes. Moreover, there is no convincing evidence for assessing to what extent SIRT5 localized in peroxisomes is specifically involved in the various phenotypes induced by deletion of SIRT5 responsible for desuccinylation of ACOX1.

Major concerns:

1) Peroxisomal localization of SIRT5 (Figs. 1E, 1F, and S2). It is very difficult to see the peroxisomal localization of SIRT5 and HA-SIRT5. In Figure 1E, it looks like that the signal obtained by a mitochondrial localized HA-SIRT5 seems to be merged with that of PMP70. Photos with better quality should be provided to show peroxisomal localization of SIRT5. Selective permeabilization of plasma membrane using Streptolysin O or digitonin followed by washing out of the cytosol may improve immunostaining of SIRT5. Subcellular fractionation and immunoblot analysis would be better to identify peroxisomal localization of SIRT5. In addition, HA-SIRT5LQIV ^{del} seems to be localized in cytosol, however the selected boxed region is not suitable because the signal derived from HA-SIRT5LQIV ^{del} is weaker than that in other cellular regions. Does the LQIV ^{del} mutation affect the localization to mitochondria?

2) Based on the immunoprecipitation assay (Fig. 1D) and morphological analysis (Fig. 1E), the authors suggested that SIRT5 is imported by PEX7 as a PTS2-type peroxisomal matrix protein. However, there is no data demonstrating whether SIRT5 is localized inside peroxisomes. Is the SIRT5 localized on peroxisomal membrane or in the matrix of peroxisome?

3) The authors analyzed the succinylation (Figs. 2 and 3) and dimer formation (Fig. 4) of Flag-ACOX1 and endogenous ACOX1 by modulating the expression of SIRT5. However, there is no information concerning whether the bands indicated as "ACOX1" show the unprocessed A-chain of ACOX1 or the processed B-chain and C-chain. Does succinylation occur in both forms of ACOX1 equally and affect the processing of ACOX1? All of the band indicating ACOX1 and its succinylated form should be shown with molecular mass markers. This is a critical point to evidently demonstrate which part of ACOX1 is desuccinylated by SIRT5 in peroxisomes.

4) The author found that levels of H</sub>2</sub>O</sub>2</sub> were increased in peroxisomes, cytosol, and nuclei of HepG2 cells by stable SIRT5 knockdown and that the elevated level of H</sub>2</sub>O</sub>2</sub> was reduced by the knockdown of ACOX1 (Fig. 5A). However, it is generally accepted that catalase is most abundant protein in peroxisomes and its enzyme activity is very efficiently active. Why and how does less than 2-fold increase of ACOX1 activity by SIRT5 knockdown (Fig. 3C) give rise to significant elevation of H</sub>2</sub>O</sub>2</sub> in peroxisomes and even in cytosol and nucleus. The authors should clarify this point. The data concerning stoichiometry between ACOX1-mediated generation of H</sub>2</sub>O</sub>2</sub> and activity of catalase in HepG2 cells are required. In

addition, the data concerning to the levels of H</sub>2</sub>O</sub>2</sub> in other cell lines established from liver should be shown by the knockdown of SIRT5.

5) Various phenotypes induced by deletion of SIRT5 including accumulation of H</sub>2</sub>O</sub>2</sub> and oxidative DNA damage were cancelled by knockdown of ACOX1 (Fig. 5). Depletion of ACOX1 removes major H</sub>2</sub>O</sub>2</sub> source in peroxisomes, but concomitantly abrogates peroxisomal &[beta]-oxidation system, which leads to accumulation of very long chain fatty acids and various secondary effects. So, these data should not be interpreted to mean that SIRT5-regulated succiniylation and activation of ACOX1 affect cellular H</sub>2</sub>O</sub>2</sub> homeostasis. To determine actual contribution of ACOX1 in H</sub>2</sub>O</sub>2</sub> level upon SIRT5 knockdown, the authors should analyze H</sub>2</sub>O</sub>2</sub> production in HepG2 cell line with double knockdown of SIRT5 and ACOX1 upon re-introduction of a primarily peroxisome-localized SIRT5 variant (e.g. SIRT5-PTS1), which can restore desuccinylation reaction only in peroxisomes. SIRT5LQIV ^{del} could be used as a negative control.

Minor concerns:

1) Figure EV1. Cytosolic localization of Hyper-cyto is not clear. Hyper-cyto appears to be present in small cellular structures.
2) Fig. 1D. To support the interaction of SIRT5 with PEX7 via its PTS2-like sequence, HA-SIRT5 LQIV ^{del} should be added in the immunoprecipitation assay as a control.
3) On page 9, lines 13-14. Original paper should be cited to assure "Peroxisomes account for up to 35% of total H</sub>2</sub>O</sub>2</sub> generation."
4) On page 10, lines 2-4. What does it mean that the association of ACOX1 with PEX5 is much stronger than PEX7. PEX7 never binds directly ACOX1.
5) Fig. 2A. Both SIRT4 and SIRT5 were co-immunoprecipitated with Flag-ACOX1. Does SIRT4 localize to peroxisomes? Why did the authors focus on the function of SIRT5 in the post-translational modification of ACOX1?
6) On page 19, in the last paragraph. Does anyone previously mention the function of SIRT5 in peroxisomes? If so, the reference is required.
7) Fig. 3A. The data concerning the succinylation state of Flag-ACOX1 upon incubating either HA-tagged wild-type or SIRT5^{H158Y} should be provided.
8) Fig. 3B. Flag-ACOX1 used as a substrate in in vitro assay is not succinylated, which is not consistent with Fig. 2E.
9) Fig. 3E. The band showing the succinylation state of ACOX1 is not clear due to the heavy background.
10) Fig. 3C, D, and F. The succinylation state of Flag-ACOX1 in each condition should be shown to evaluate the correlation between succinylation level and enzyme activity of ACOX1.
11) Fig. 5A and B, and EV5A and B. It is not clear how the authors compared the data to analyze the statistical significance.

1st Revision - authors' response 3 January 2018

Referee #1:

This manuscript by Chen and colleagues examines the role of the sirtuin SIRT5 in peroxisomes. SIRT5 is a poorly understood sirtuin with unusual catalytic activities, functioning to remove succinyl, malonyl, and glutaryl groups from lysines on its target proteins, rather than acetyl groups. SIRT5 is primarily known as a mitochondrial protein, though it is present in other cellular compartments such as the cytosol and in the nucleus. Chen et al. show that a fraction of SIRT5 is peroxisomal, where it interacts with and inhibits the ACOX1 protein to suppress cellular ROS levels. The authors also provide evidence that suppression of SIRT5 levels may contribute to the pathogenesis of hepatocellular carcinoma.

Overall, this is a very important manuscript that makes a significant contribution to the understanding of SIRT5 biology. To my knowledge, no sirtuin has been shown to regulate targets in peroxisomes. Moreover, roles for SIRT5 in human cancer are an active area of investigation in many laboratories currently, an area where this manuscript contributes. I support publication in EMBO Reports, provided that the authors can address the following relatively minor concerns:

Response: We appreciate reviewer's efforts and praise to our study. Below, we address point-by-point the issues raised by the reviewer.

1. It would be of interest to know the spectrum of peroxisomal proteins potentially targeted by SIRT5. Can the authors cull such a list of candidates from the published SIRT5 proteomics studies and present such a table, perhaps in the supplemental data?

Response: Following reviewer's suggestion, we searched previous SIRT5 proteomics studies for proteins whose lysine succinylation was significantly increased by >2-fold ($P<0.01$) in Sirt5 knockout mouse livers (Rardin MJ, et al. *Cell Metab*. 2013. PMID:24315375). According to the PEROXISOME DB database (http://www.peroxisomedb.org/home.jsp), we listed potential peroxisomal substrates of SIRT5-regulated succinylation in new Appendix Table S1.

[redacted]

Protein	Description
ABCD3	ATP-binding cassette sub-family D member 3
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1
ACOX2	Peroxisomal acyl-coenzyme A oxidase 2
ACSL1	Long-chain-fatty-acid--CoA ligase 1
AMACR	Alpha-methylacyl-CoA racemase
CATA	Catalase
DECR2	Peroxisomal 2,4-dienoyl-CoA reductase
DHB4	Peroxisomal multifunctional enzyme type 2
DHRS4	Dehydrogenase/reductase SDR family member 4
ECHP	Peroxisomal bifunctional enzyme
GSTK1	Glutathione S-transferase kappa 1
HAOX1	Hydroxyacid oxidase 1

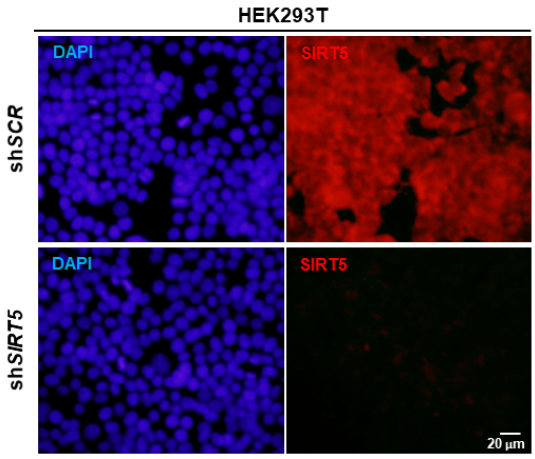
HYES	Bifunctional epoxide hydrolase 2
IDHC	Isocitrate dehydrogenase [NADP] cytoplasmic
NUDT7	Peroxisomal coenzyme A diphosphatase NUDT7
PAHX	Phytanoyl-CoA dioxygenase, peroxisomal
PECR	Peroxisomal trans-2-enoyl-CoA reductase
PRDX5	Peroxiredoxin-5, mitochondrial
SOX	Peroxisomal sarcosine oxidase
THIKA	3-ketoacyl-CoA thiolase A, peroxisomal
THIKB	3-ketoacyl-CoA thiolase B, peroxisomal
URIC	Uricase

2. While the authors' immunofluorescence data are convincing, I would like to see the SIRT5 KD cells included as a negative control in Fig. 1F. In addition, cellular fractionation to elucidate localization of endogenous SIRT5 would be extremely helpful, particularly if mitochondria (peroxisomal?) can be separated from mitochondria. The question is how much SIRT5 resides in one compartment versus the other?

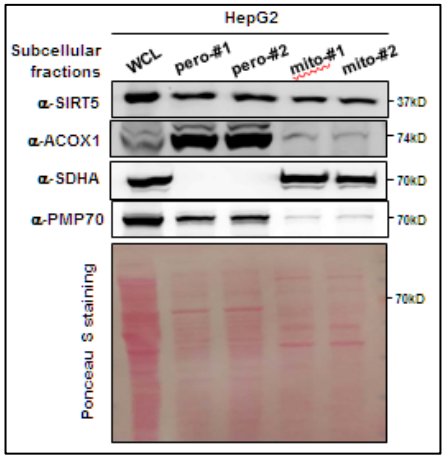
Response: According to the reviewer's suggestion, we have performed immunofluorescence staining in *SIRT5* knockdown HEK293T cells (new Appendix Figure S3).

Cellular fractionation was conducted in HepG2 cells to isolate peroxisomes by using the Peroxisome Isolation Kit (Sigma, Product Code PEROX1). As shown in new Figure 1F, as expected, ACOX1 and PMP70 were detected predominantly in the peroxisomal fraction, while SDHA was detected primarily in the mitochondrial fraction. Importantly, endogenous SIRT5 was detected in both the peroxisomal and mitochondrial fractions, clearly supporting the localization of SIRT5 in both subcellular compartments.

New Appendix Figure S3



New Figure



3. The authors provide evidence that SIRT5 regulates the dimerization state of ACOX1. Can the authors use Pymol or the like to map the modification sites on ACOX1, for example to see whether any lie at the dimerization interface? It would be extremely interesting to provide some structural insight into the basis for this mode of regulation. It would be nice to mutate the critical residues to show that this exerts the predicted effects on ACOX1 multimerization and activity, although I don't absolutely insist on this experiment.

Response: So far, only *Arabidopsis thaliana* acyl-CoA oxidase 1 dimer structure (RCSB PBD, 1W07) has been solved. Most of the lysine residues at the dimerization interface (e.g. K59/63/128/328) are not conserved in *Homo sapiens*.

Data for reviewer

K59 K63

```
Homo sapiens        5   LRRERDSASFNPELLTHILDGSPEKTRRRREIENMILNDPDFQMEDLNFLTRSQRVIEVAV  64
                        L  ER+ A F+ E +  +  GS         I  ++ +DP F+  +   L+R + ++  +
Arabidopsis thaliana 7   LADERNKAEFDVEDMKIVWAGSRHAFEVSDRIARLVASDPVFEKSNRARLSRKELFKSTL  66

Homo sapiens        65  RKSAIMVKKMREFGIADPDEIMWFKKLHLVNFVEPVGLNYSMFIPTLLNQGTTAQKEKWL  124
                        RK A   K++ E  + + +     +  H ++    V L++ MF+P +  QGT  Q++KWL
Arabidopsis thaliana 67  RKCAHAFKRIIELRLNEEEA——GRLRHFIDQPAYVDLHWGMFVPAIKGQGTEEQQKKWL  123

K128
Homo sapiens        125 LSSKGLQIIGTYAQTEMGHGTHLRGLETTATYDPETQEFILNSPTVTSIKWWPGGLGKTS  184
                          +  +QIIG YAQTE+GHG+++GLETTAT+DP+T EF++++PT T+ KWWPGGLGK S
Arabidopsis thaliana 124 SLANKMQIIGCYAQTELGHGSNVQGLETTATFDPKTDEFVIHTPTQTASKWWPGGLGKVS  183

Homo sapiens        185 NHAIVLAQLITKGKCYGLHAFIVPIREIGTHKPLPGITVGDIGPKFG——YDEIDNGYLK  241
                         HA+V A+LIT GK YG+H FIV +R +  H PLP ITVGDIG K G   Y+ +DNG+L
Arabidopsis thaliana 184 THAVVYARLITNGKDYGIHGFIVQLRSLEDHSPLPNITVGDIGTKMGNGAYNSMDNGFLM  243

Homo sapiens        242 MDNHRIPRENMLMKYAQVKPDGTYV-KPLSNKLTYGTMVFVRSFLVGEAARALSKACTIA  300
                         D+ RIPR+ MLM+ ++V  +G YV   +  +L YGTMV+VR  +V +A+ ALS+A  IA
Arabidopsis thaliana 244 FDHVRIPRDQMLMRLSKVTREGEYVPSDVPKQLVYGTMVYVRQTIVADASNALSRAVCIA  303

K328
Homo sapiens        301 IRYSAVRHQSEIKPGEPEPQILDFQTQQYKLFPLLATAYAFQFVGAYMKETYHRINEGIG  360
                         RYSAVR Q     G  E Q++D+ TQQ +LFPLLA+AYAF+FVG ++K  Y   + E +
Arabidopsis thaliana 304 TRYSAVRRQFGAHNGGIETQVIDYKTQQNRLFPLLASAYAFRFVGEWLKWLYTDVTERLA  363

Homo sapiens        361 QGDLSELPELHALTAGLKAFTSWTANTGIEACRMACGGHGYSHCSGLPNIYVNFTPSCTF  420
                          D + LPE HA TAGLK+ T+     GIE CR  CGGHGY  CSGLP ++   + P+CT+
Arabidopsis thaliana 364 ASDFATLPEAHACTAGLKSLTTTATADGIEECRKLCGGHGYLWCSGLPELFAVYVPACTY  423

Homo sapiens        421 EGENTVMMLQTARFLMKSYDQVHSGKLVCGMVSYLN——DLPSQRIQPQQVAVWPTMVDI  477
                        EG+N V+ LQ ARFLMK+  Q+ SGK+  G  +V+     L   R   Q+   W
Arabidopsis thaliana 424 EGDNVVLQLQVARFLMKTVAQLGSGKVPVGTTAYMGRAAHLLQCRSGVQKAEDWL———  478

Homo sapiens        478 NSPESLTEAYKLRAARLVEIAAKNLQKEVIHRKSKEVAWNLTSVDLVRASEAHCHYVVVK  537
                         +P+ + EA++ RA R+    AKNL K     +++E  +     DLV A+ AHC  +VV
Arabidopsis thaliana 479 -NPDVVLEAFEARALRMAVTCAKNLSK——FENQEQGFQELLADLVEAAIAHCQLIVVS  533

Homo sapiens        538 LFSEKLLK-IQDKAIQAVLRSLCLLYSLYGISQNAGDFLQGSIMTEPQITQVNQRVKELL  596
                         F  KL + I  K ++  L +LC +Y+LY  + ++ GDFL  + +T  Q +  N +++ L
Arabidopsis thaliana 534 KFIAKLEQDIGGKGVKKQLNNLCYIYALYLLHKHLGDFLSTNCITPKQASLANDQLRSLY  593

Homo sapiens        597 TLIRSDAVALVDAFDFQDVTLGSVLGRYDGNVYENLFEWAKNSPLNKAEVHESYK  651
                        T +R +AVALVDAF++ D  L SVLGRYDGNVY  LFE A   PLN + V + Y+
Arabidopsis thaliana 594 TQVRPNAVALVDAFNYTDHYLNSVLGRYDGNVYPKLFEEALKDPLNDSVVPDGYQ  648
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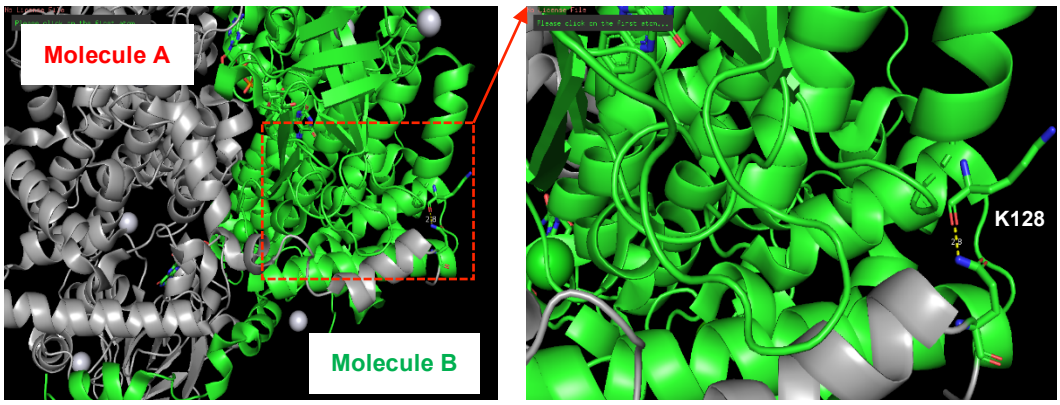
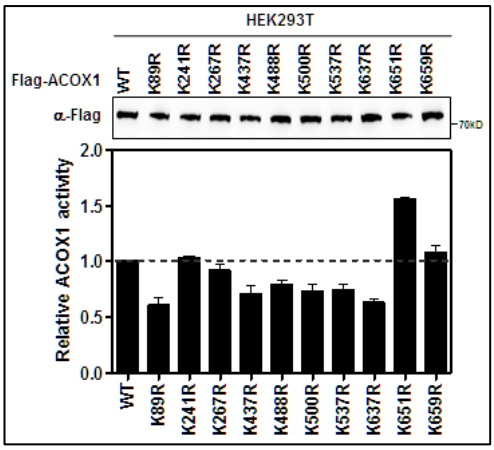


So far, only *Arabidopsis thaliana* acyl-CoA oxidase 1 dimer structure has been solved. However, most of the lysine residues at the dimerization interface (e.g. K59/63/128/328) are not conserved between *Arabidopsis thaliana* and *Homo sapiens*. Moreover, we selected 10 succinylated lysine residues in ACOX1 (Rardin MJ, et al. *Cell Metab*. 2013. PMID: 24315375; Weinert BT, et al. *Cell Rep.* 2013. PMID: 23954790.), and individually mutated K to R, which mimics the desuccinylation state. We found that ACOX1 enzyme activity may be regulated by succinylation of multiple residues rather than single lysine (new Appendix Figure S10). These new results are consistent with our model that enzyme-independent succinylation targets multiple residues rather than single lysine and, as a result, SIRT5-mediated desuccinylation and regulation of ACOX1 dimerization may involve multiple rather than single lysine residues.

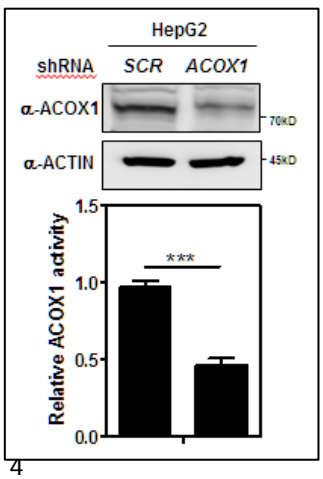
New Appendix Figure



4. A lot of the paper relies on ACOX1 activity assays. I would be more comfortable with this if the authors show someplace that this assay is specific, particularly in the setting of tissue lysates, for example by the use of a known specific ACOX1 inhibitor, or the use of ACOX1 KO tissue.

Response: The ACOX1 activity assay has been well developed, based on the H_2O_2-dependent oxidation of leuco-dichlorofluorescein catalysed by exogenous peroxidas (Small GM et al. *Biochem J*. 1985. PMD: 3994682). To address this reviewer's concern, we have measured ACOX1 activity in HepG2 stable cells with *ACOX1* knockdown (new Appendix Figure S8), providing additional control for the specificity of the assay.

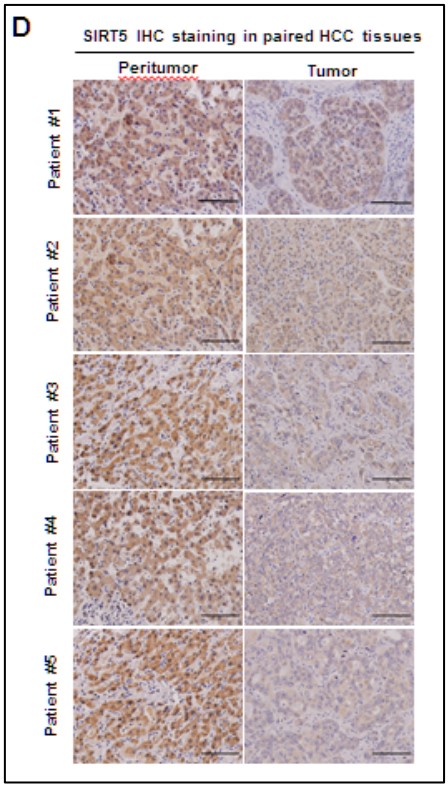
New Appendix Figure



5. The authors should show some representative images of SIRT5 staining in HCC and normal in Fig. 6.

Response: Representative images of SIRT5 staining in HCC and normal tissue have been included in new Figure 6D.

New Figure



Other points

1. The authors' literature citations are not always exactly correct. For example, the Park 2013 Mol. Cell paper focused mostly on MEFs and liver (p.6). This paper should also be cited on p. 7.

Response: Corrected. Thanks for pointing this out.

2. Although the manuscript is generally very clear and well-written, there are some typos ("dimmer" rather than dimer on p. 13; "Hochest" rather than Hoescht in EV1).
Response: Corrected. Thanks for pointing this out.

Referee #2:

Dr Chen and colleagues have undertaken a well-designed study where they have substantial biochemical and functional data to identify that acyl-CoA oxidase 1 (ACOX1) is a functional substrate that is desuccinylated and inactivated by SIRT5. The authors use multiple molecular manipulations in a full-factorial design to show the SIRT5 associates with the peroxisome and interacts with and desuccinylates ACOX1. The consequence of this action include the disruption of the homodimeric structure of ACOX1, and the reduction in ACOX1 generation of H2O2 as a byproduct of peroxisomal beta-oxidation. The authors also show that the genetic and pharmacologic increase in succinyl-CoA levels succinylates and activates ACOX1. As excess H2O2 can have detrimental effects on the cell the research team also show evidence of increase oxidative stress and propensity to ex-vivo tumor growth with the disruption of SIRT5 and show a correlation with reduced SIRT5 levels and increased ACOX1 activity in hepatocellur lines and suggest a correlation of poor outcome in human HCC carcinoma with reduced SIRT5 levels.

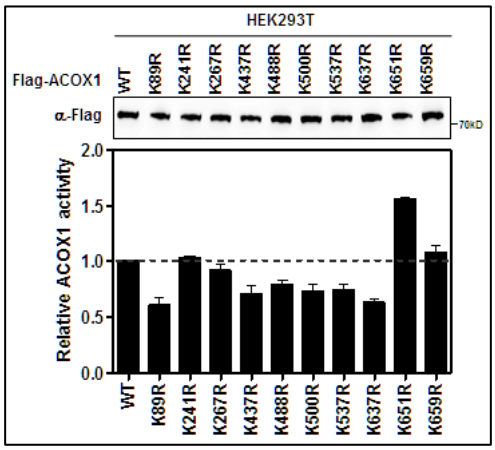
Response: We appreciate reviewer's efforts in reviewing our study. Below, we address point-by-point the issues raised by the reviewer.

The only functional assays not performed is to evaluate whether SIRT5 levels modulate peroxisomal beta-oxidation and the sites of the succinylation were not confirmed.

Response: As shown in new Appendix Table S1, there are quite some potential peroxisomal substrates of SIRT5-regulated succinylation. Assessment of the effect of SIRT5 on regulating peroxisomal beta-oxidation is of interest, but needs extensive efforts. The focus of our current study is to determine the role of SIRT5 in the regulation of ACOX1. We believe that we have provided sufficient evidence to show that SIRT5-dependent lysine succinylation increases the enzyme activity of ACOX1, the first rate-limiting enzyme in peroxisomal beta-oxidation, and thereby controls H_2O_2 production in the peroxisome.

To identify 'the sites of the succinylation', we generated 10 K-to-R mutants in ACOX1, and found that ACOX1 enzyme activity may be regulated by succinylation of multiple residues rather than single lysine (new Appendix Figure S10). This is consistent with the finding that succinylation is an enzyme-independent modification and targets multiple instead of individual lysine residues.

New Appendix Figure



New Appendix Table S1

Protein	Description
ABCD3	ATP-binding cassette sub-family D member 3
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1
ACOX2	Peroxisomal acyl-coenzyme A oxidase 2
ACSL1	Long-chain-fatty-acid--CoA ligase 1
AMACR	Alpha-methylacyl-CoA racemase
CATA	Catalase
DECR2	Peroxisomal 2,4-dienoyl-CoA reductase
DHB4	Peroxisomal multifunctional enzyme type 2
DHRS4	Dehydrogenase/reductase SDR family member 4
ECHP	Peroxisomal bifunctional enzyme
GSTK1	Glutathione S-transferase kappa 1
HAOX1	Hydroxyacid oxidase 1
HYES	Bifunctional epoxide hydrolase 2
IDHC	Isocitrate dehydrogenase [NADP] cytoplasmic
NUDT7	Peroxisomal coenzyme A diphosphatase NUDT7
PAHX	Phytanoyl-CoA dioxygenase, peroxisomal
PECR	Peroxisomal trans-2-enoyl-CoA reductase
PRDX5	Peroxiredoxin-5, mitochondrial
SOX	Peroxisomal sarcosine oxidase
THIKA	3-ketoacyl-CoA thiolase A, peroxisomal
THIKB	3-ketoacyl-CoA thiolase B, peroxisomal
URIC	Uricase

I have some minor comments:

1. Top of page 12 - SDHA knockdown was said in increase global lysine succinlylation which was stated to be shown in Figure 3E. It should be pointed out that this is shown in the input, as the inclination would be that this would be a separate immunblot rather than the loading control for an IP study.

Response: The elevated level of succinate by *SDHA* knockdown resulted in a remarkable increase of global lysine succinylation also in the input (Figure 3E). Thank the reviewer for pointing this out, and we have clarified this in the text.

2.Page 8, line 6. add shRNA-2 was 'more' potent. the word more is missing.

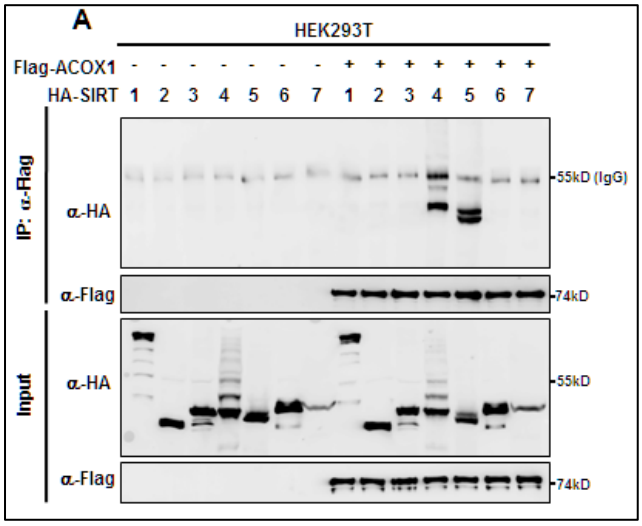
Response: Added. Thanks for pointing this out.

3. Fig 1C and Fig 5C. The text nor the legend describe how many times these experiments were repeated. This should be added and it may be useful to quantitate these findings.
Response: Thanks for point it out. We have added ‘n’ for every experiment which had been completed in biological triplicate.

4. Fig 2A. HA-SIRT3 failed to express, and one cannot state it does not interact with ACOX1 if it is not present.

Response: To address this reviewer's concern, we have solved the problem with SIRT3 ectopic expression and repeated the experiment in Figure 2A. As shown, Flag-ACOX1 could interact with HA-tagged SIRT4 and SIRT5, but not SIRT3 (new Figure 2A).

New Figure



Referee #3:

In the manuscript by Chen et al., the authors describe a role for SIRT5 in the regulation of peroxisomal Acyl-CoA Oxidase 1 (ACOX1) function. SIRT5 is a member of the Sirtuin family of NAD+-dependent deacylases, involved in metabolism, stress response and genome stability. SIRT5 is one of the three mitochondrial members of the family, and its enzymatic activity targets a range of acyl groups including acetyl, malonyl and succinyl. The desuccinylase, demalonylase and deacetylase activities of SIRT5 have been characterized and linked to several metabolic pathways such as amino acid catabolism, urea cycle, TCA, cellular respiration as well as glucose and fatty acid metabolism. In this work, the authors show that SIRT5 desuccinylates ACOX1, the first enzyme of the β-oxidation pathway of very-long-chain-fatty acids (VLCFAs), a reaction that is associated to H2O2 production. ACOX1 desuccinylation by SIRT5 inhibits its enzymatic activity by decreasing ACOX1 dimer formation, which results in decrease in H2O2 production and DNA damage signaling. SIRT5 downregulation in established cell lines (shRNA) or lack of SIRT5 in SIRT5KO mouse livers produce higher levels of ACOX1 succinylation, increased ACOX1 activity, increased H2O2 production and DNA damage signaling as well as anchorage-independent growth. The relevance of the negative regulation of ACOX1 by SIRT5 is reflected by the rescue of these SIRT5-deficient phenotypes upon shRNA-driven downnregulation of ACOX1. Consistent with the direct role of peroxisomal metabolism in development of liver diseases such as Hepatocellular carcinomas (HCC), the authors observe increased levels of succinyl-ACOX1, increased ACOX1 activity and decreased SIRT5 levels in a set of HCC tumors. Consistently, analysis of the outcome of these patients, associate high level of SIRT5 in these tumors with increased cell survival and decrease rate of recurrence.

This is a very interesting set of evidences, and link for the first time SIRT5 with peroxisomal metabolism. The authors have done in general a good work characterizing the ACOX1 desuccinylation activity of SIRT5, and have clearly established the direct link between succinylation of ACOX1 and ACOX1 activity. Moreover, the studies with HCC samples suggest that this functional link is very relevant in cancer. However, I have several concerns with the work as some claims are at this stage overstated and the manuscript has a number of relevant technical issues that should be addressed:

Response: We appreciate reviewer's efforts in reviewing and praise to our study. Below, we address point-by-point the issues raised by the reviewer.

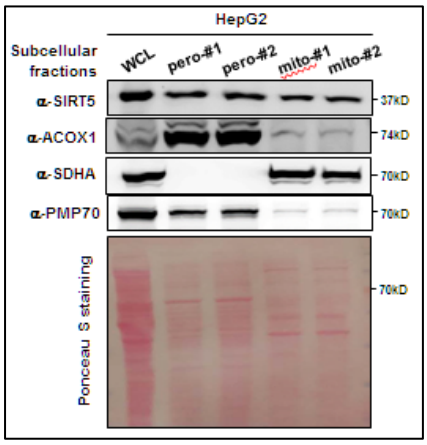
1) The main issue is that I believe the authors have not shown any convincing data demonstrating that SIRT5 really localizes to peroxisomes. This is a relevant issue as is a major claim of the manuscript. These claims of co-localization studies are based very poor IF studies that do not meet a minimal quality and resolution level (Figure 1E, 1F, S2, EV3). In the case of SIRT5 this is very obvious as in these IF experiments SIRT5 distribution is very diffuse and present in most of the cytoplasm. In fact, looking at the methods section is not even clear whether the images were made from a single focal plane (with a confocal microscope) or basically includes the whole cell. The authors only mention that it was done with an optical fluorescence microscope. This issue deserves more work including using other

ways of demonstrating this claim such as FRET or similar assays. Even If the authors insist in develop further these colocalization studies, they should be performed in a single plane, with enough resolution and including a quantative statistical analysis used in these cases (e.g. Pearson correlation coefficient) to be convincing.

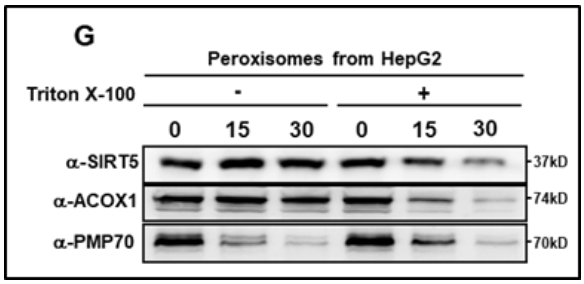
Response: All the IF images were obtained from a single focal plane with a confocal microscope and the highest resolution (630x). For clarification, we have included the detailed information in the figure legends of Figure 1E, 1F, EV3.

To provide convincing data demonstrating that SIRT5 localizes to peroxisomes, we conducted cellular fractionation in HepG2 cells and isolated peroxisomes. Endogenous SIRT5 can be detected in both the peroxisomal and mitochondrial fractions, supporting the localization of SIRT5 in both subcellular compartments (new Figure 1F). Furthermore, we performed peroxisome permeabilization assay and demonstrates that, like ACOX1, SIRT5 is a matrix protein rather than a membrane protein in peroxisomes (new Figure 1G).

New Figure 1F



New Figure 1G



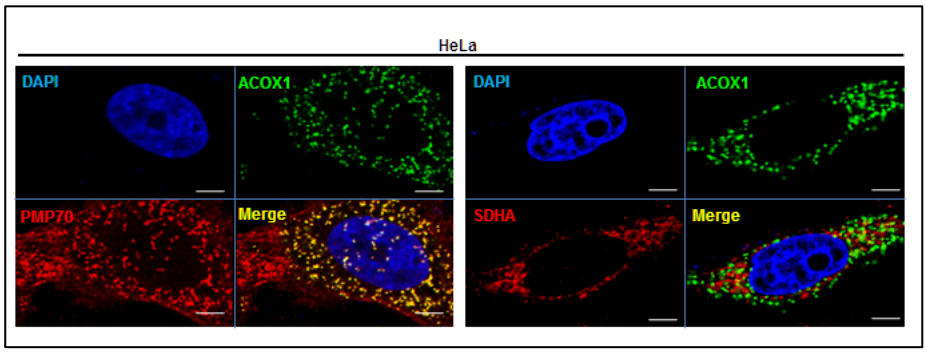
2) In fact, and linked to this previous issue, how do the authors explain that SIRT5 induces a similar level of H2O2 in peroxisomes, nucleus and cytoplasm (Figure 1A) and that all of them are neutralized by ACOX1 downregulation by shRNA (Figure 5A)? Even If this H2O2 can move from peroxisomes to the nucleus or cytoplasm, one would expect that, If produced in the peroxisome, peroxisomal H2O2 would be significantly higher. In fact, Figure EV3 shows that ACOX1 distribution is almost, but not completely, identical to the distribution of peroxisome marker PMP70. This is also in agreement with the clear effect of the mitochondrial (and nuclear) protein SDHA on succ-ACOX1 levels. Can the authors exclude that the link between SIRT5 and ACOX1 takes place in other non-peroxisomal compartments?

Response: Previous studies have shown that ACOX1 is predominantly located in the peroxisome (Poirier Y, et al. *Biochim Biophys Acta.* 2006. PMID: 17028011; Schlüter A, et al. *Nucleic Acids Res*. 2007. PMID:17135190. Wanders RJ, et al. *Biochim Biophys Acta*. 2006. PMID: 17055078). In agreement, we show that ACOX1 is clearly co-localized with PMP70 (a well-known peroxisomal marker), but not SDHA (a mitochondrial marker) (new Figure EV3D),

suggesting that regulation of ACOX1 should happen within the peroxisomal compartment. Furthermore, we have separated subcellular fractions of HepG2 cells, and discovered the presence of endogenous SIRT5 in both the peroxisomal and mitochondrial fractions (new Figure 1F), providing a strong evidence for the peroxisomal localization of SIRT5. Most likely, SIRT5 co-localizes with ACOX1 in peroxisomes to regulate its lysine succinylation and enzyme activity.

Sirt5 knockdown cells reflect a long-term H_2O_2 steady state rather than an instantaneous result (Figures 1A and 1B). H_2O_2 can move quickly from peroxisomes to the nucleus or cytoplasm (Costa A, et all. *Plant J.* 2010. PMID: 20230493). Besides ACOX1, we speculate *SIRT5* knockdown may influence other peroxisomal proteins or pathways related to H_2O_2 metabolism. Catalase is most abundant protein in peroxisomes and its enzyme activity is very efficiently active. This may explain why peroxisomal H_2O_2 is NOT significantly higher than the other two compartments.

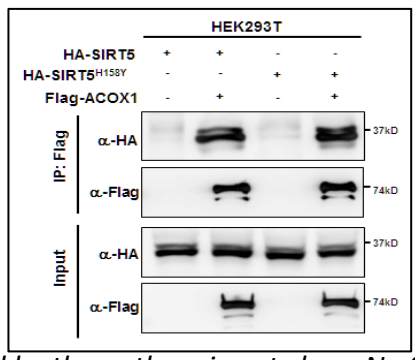
New Figure



3) Does the catalytic-inactive SIRT5 mutant H158Y used in Figures 2E, 3A, 4A-B, bind to ACOX1? This is in fact a missing control of Figure 2E. If, in contrast to WT SIRT5, the H158Y mutant does not bind to ACOX1, the effect of active WT SIRT5 on ACOX dimerization may also be produced by a steric effect due to SIRT5 binding and not by SIRT5 enzymatic activity. This issue should be addressed.

Response: To address this reviewer's question, we have examined and found that Flag-ACOX1 could interact with HA-tagged wild-type or mutant SIRT5 (new Appendix Figure S7).

New Appendix Figure S7

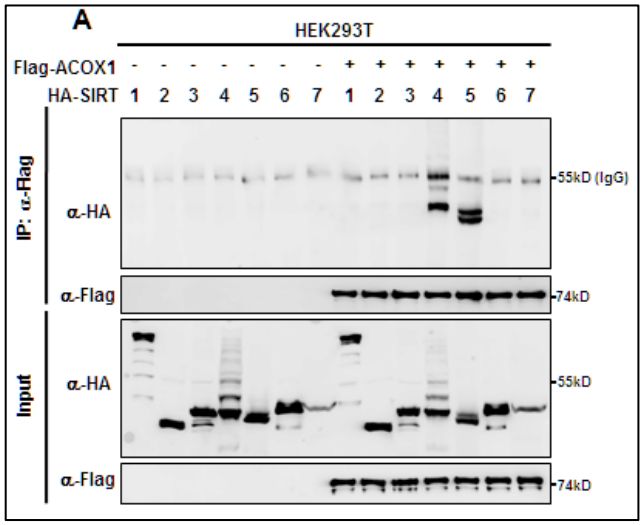


4) In Figure 2A, the SIRT5 specificity claimed by the authors is not clear. No SIRT3 is shown

even in the inputs and the levels of SIRT1 or SIRT7 are much lower than SIRT4 and 5. If the authors want to make this claim this should be repeated convincingly.

Response: To address this reviewer's concern, we ectopically expressed SIRT3 and repeated the experiment in Figure 2A. As shown, Flag-ACOX1 could interact with HA-tagged SIRT4 and SIRT5, but not SIRT3 (new Figure 2A).

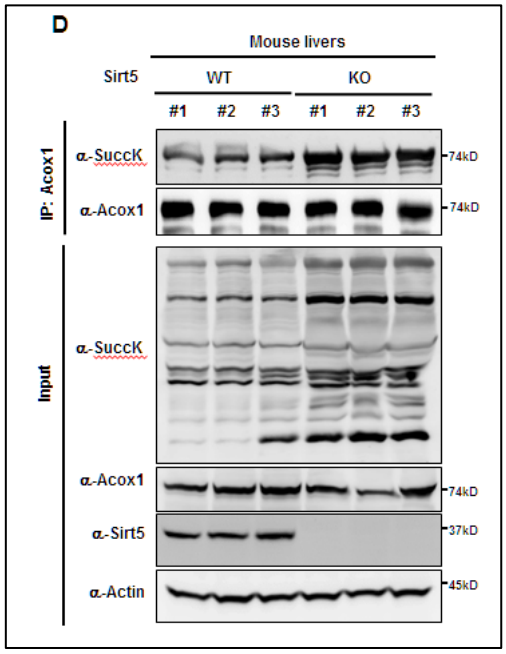
New Figure



5) Figure 2D. I have several concerns. The first one is that the Acox1 levels in the IP are saturated and therefore the succ-ACOX1 differences between WT and KO are difficult to evaluate. In fact, looking carefully, KOs seem to show higher levels of ACOX1. The second one is that in fact, ACOX1 levels in the input are missing.

Response: To address this reviewer's concern, we have repeated the experiment using less amount of IP samples for Acox1 protein and included the input for Acox1 (new Figure 2D).

New Figure 2D

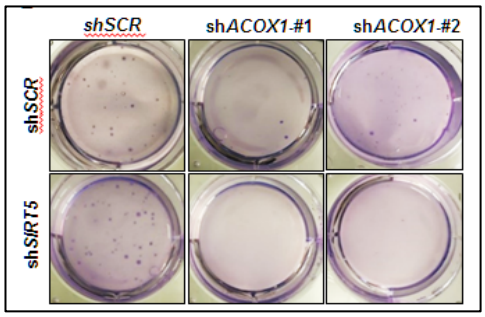


6) Figure 5D. The quality of the image should be improved maybe by increasing contrast. The images in S6 are much clearer. In its current form, is difficult to distinguish all the colonies

quantified in Fig5E.

Response: Upon request, we have increased the contrast to improve the image quality (new Figure 5D).

New Figure 5D



7) I have several concerns regarding figure 6A. One problem is that SIRT5 does not seem to be clearly decreased between peritumoral and tumor samples except for #1. The second one is that patient 4 is the one that has lower levels of SIRT5, but no change in succ-ACOX1 is observed. The authors should address these discrepancies.

Response: Among the examined 10 pairs of primary HCC tumors and their adjacent normal tissues, SIRT5 downregulation and increased ACOX1 succinylation were observed in 6 HCC samples, including #1, #2, #5, #7, #9, and #10 (Figure 6A). IHC staining in liver tissues in a larger study cohort consisting of 78 HCC patients confirmed that SIRT5 is generally down-regulated in liver tumor samples compared to peritumoral tissues (Figures 6C and 6D). These data support the notion that SIRT5 expression is commonly downregulated in HCC tumor samples.

It has to be noted that SIRT5 downregulation may not the sole reason causing the alteration in ACOX1 succinylation. Other factors may also change the lysine succinylation level of ACOX1 by different mechanisms. For instance, NAD^+ concentration which is normally lower in cancer cells than non-transformed cells (Djouder N, et al. *Mol Cell Oncol*. 2015. PMID: 27308492; Mederacke & Schwabe, et al. *Cancer Cell*. 2014. PMID: 25490440), may suppress SIRT5 catalytic activity. Second, SDH expression and/or activity will also change the level of succinate and subsequently global lysine succinylation. These may explain the discrepancies in Figure 6A as pointed by the reviewer. For clarification, we have added the related explanation in the discussion of our revision paper.

Referee #4:

Protein succinylation is a posttranslational modification that can regulate multiple metabolic processes and the sirtuin 5 (SIRT5) catalyzes desuccinylation. Recent proteomic studies identified multiple succinylation sites in peroxisomal acyl-CoA oxidase 1 (ACOX1) that functions as a major H2O2-producer in peroxisomes. In this manuscript, Chen et al. report that SIRT5 is partially localized in peroxisomes and desuccinylates ACOX1, which leads to decrease the enzyme activity of ACOX1 by lowering its active dimer formation. The authors also show that the phenotypes upon deletion of SIRT5 including accumulation of H2O2 and oxidative DNA damage were cancelled by knockdown of ACOX1. Together with a notion in regard to the correlation between downregulation of SIRT5 and higher succinylation and activity of ACOX1 in hepatocellular carcinoma (HCC), the authors conclude that SIRT5 regulates the homeostasis of peroxisomal H2O2 and cellular ROS via controlling the succinylation of ACOX1.

The findings in this manuscript are interesting and extend our knowledge of the succinylation in regulation of protein functions. However, morphological data are ambiguous thus not sufficient to establish the exact localization of SIRT5 in or on peroxisomes. Moreover, there is no convincing evidence for assessing to what extent SIRT5 localized in peroxisomes is specifically involved in the various phenotypes induced by deletion of SIRT5 responsible for desuccinylation of ACOX1.

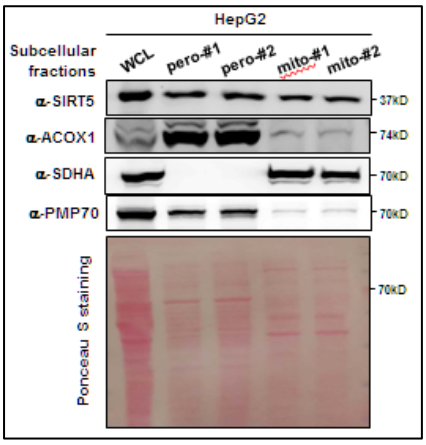
Response: We appreciate reviewer's efforts in reviewing and praise to our study. Below, we address point-by-point the issues raised by the reviewer.

Major concerns:

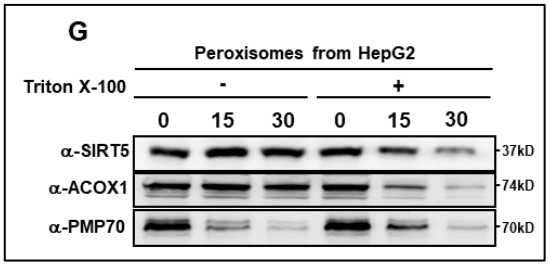
1) Peroxisomal localization of SIRT5 (Figs. 1E, 1F, and S2). It is very difficult to see the peroxisomal localization of SIRT5 and HA-SIRT5. In Figure 1E, it looks like that the signal obtained by a mitochondrial localized HA-SIRT5 seems to be merged with that of PMP70. Photos with better quality should be provided to show peroxisomal localization of SIRT5. Selective permeabilization of plasma membrane using Streptolysin O or digitonin followed by washing out of the cytosol may improve immunostaining of SIRT5. Subcellular fractionation and immunoblot analysis would be better to identify peroxisomal localization of SIRT5. In addition, HA-SIRT5 LQIV ^{del} seems to be localized in cytosol, however the selected boxed region is not suitable because the signal derived from HA-SIRT5LQIV ^{del} is weaker than that in other cellular regions. Does the LQIV ^{del} mutation affect the localization to mitochondria?

Response: To provide convincing data demonstrating that SIRT5 localizes to peroxisomes, we conducted cellular fractionation in HepG2 cells and isolated peroxisomes. Endogenous SIRT5 can be detected in both the peroxisomal and mitochondrial fractions, supporting the localization of SIRT5 in both subcellular compartments (new Figure 1F). Furthermore, we have performed peroxisome permeabilization assay and demonstrated that, like ACOX1, SIRT5 is a matrix protein rather than a membrane protein in peroxisomes (new Figure 1G).

New Figure

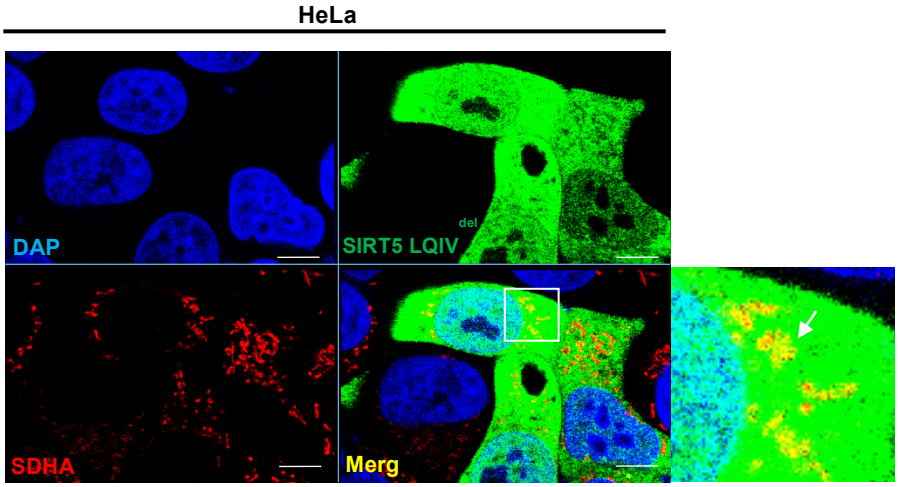


New Figure 1G



We also performed IF staining in cells ectopically expressing SIRT5 LQIV^{del} mutant. As shown below, SIRT5 LQIV^{del} mutant is diffusely distributed in the cell, and part of this mutant protein can co-localize with the mitochondrial marker SDHA.

Data for the reviewer



2) Based on the immunoprecipitation assay (Fig. 1D) and morphological analysis (Fig. 1E), the authors suggested that SIRT5 is imported by PEX7 as a PTS2-type peroxisomal matrix protein. However, there is no data demonstrating whether SIRT5 is localized inside peroxisomes. Is the SIRT5 localized on peroxisomal membrane or in the matrix of peroxisome?

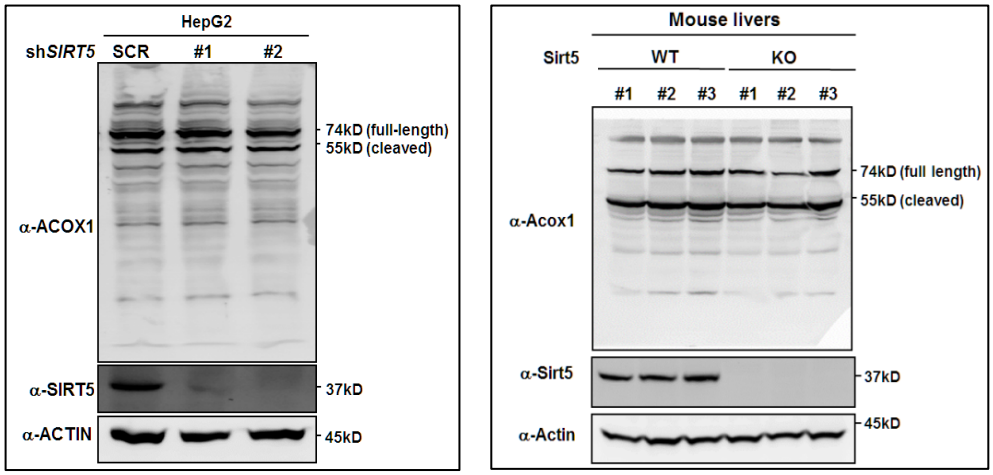
Response: To further address this question, we have permeabilized the membrane of purified peroxisomes with Triton X-100, followed by proteinase K treatment and western blotting analyses. This experiment showed that proteinase K could rapidly (within 15 min) degrade the peroxisome membrane protein PMP70, but not peroxisome matrix protein ACOX1 unless the peroxisome was permeabilized by Triton (new Figure 1G, above). Like ACOX1, SIRT5 was not degraded by Proteinase K treatment alone, but was degraded by Proteinase K after pretreatment with Triton. These results suggest that SIRT5 is a matrix

protein rather than a membrane protein in peroxisomes.

3) The authors analyzed the succinylation (Figs. 2 and 3) and dimer formation (Fig. 4) of Flag-ACOX1 and endogenous ACOX1 by modulating the expression of SIRT5. However, there is no information concerning whether the bands indicated as "ACOX1" show the unprocessed A-chain of ACOX1 or the processed B-chain and C-chain. Does succinylation occur in both forms of ACOX1 equally and affect the processing of ACOX1? All of the band indicating ACOX1 and its succinylated form should be shown with molecular mass markers. This is a critical point to evidently demonstrate which part of ACOX1 is desuccinylated by SIRT5 in peroxisomes.

Response: Full-length ACOX1 (72 kD) enters into peroxisomes where it is cleaved into B-chain (51 kD) and C-chain (21kD), or not cleaved as A-chain (72 kD). B- and C-chain of ACOX1 can form dimers with A-chain (Chu R, et al. *J Biol Chem.* 1995. PMID: 7876265). In this study, we have used an antibody which recognizes both A-chain and B-chain of ACOX1 (Proteintech, Product code: 10957-1-AP). Our data demonstrated that *SIRT5* knockdown or knockout did not affect the processing of ACOX1 both in cultured cells and mouse livers (please refer to 'Data to the reviewer'). Upon this reviewer's request, the molecular mass markers of all the bands indicating ACOX1 and its succinylated form have been marked in our revised paper.

Data for the reviewer

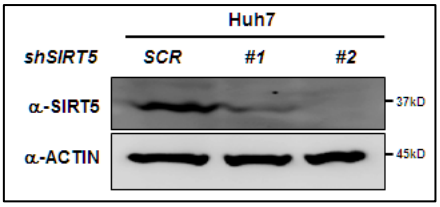


4) The author found that levels of H2O2 were increased in peroxisomes, cytosol, and nuclei of HepG2 cells by stable SIRT5 knockdown and that the elevated level of H2O2 was reduced by the knockdown of ACOX1 (Fig. 5A). However, it is generally accepted that catalase is most abundant protein in peroxisomes and its enzyme activity is very efficiently active. Why and how does less than 2-fold increase of ACOX1 activity by SIRT5 knockdown (Fig. 3C) give rise to significant elevation of H2O2 in peroxisomes and even in cytosol and nucleus. The authors should clarify this point. The data concerning stoichiometry between ACOX1-mediated generation of H2O2 and activity of catalase in HepG2 cells are required. In addition, the data concerning to the levels of H2O2 in other cell lines established from liver should be shown by the knockdown of SIRT5.

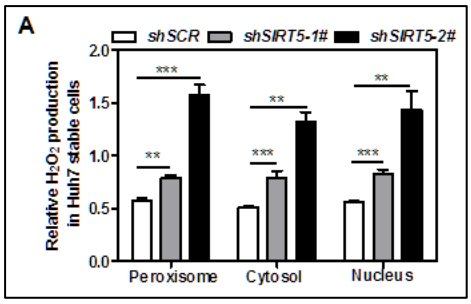
Response: The catalase activity is <u>*NOT*</u> affected by *SIRT5* knockdown (new Appendix Figure S4). So, the observed increase in peroxisomal H_2O_2 production in *SIRT5* knockdown HepG2 cells is most likely caused by activation of ACOX1 activity. Regarding the assay of 'the stoichiometry between ACOX1-mediated generation of H_2O_2 and activity of catalase' <u>*in vivo*</u>, it is technically very challenging and, to the best of our knowledge, has not been done and may not produce reliable result. If one purifies these enzymes and measure their activity *in vitro*, it would not reflect their activities *in vivo* (e.g. enzyme levels, modifications, the concentration of co-factors such as FAD+, and membrane/matrix association, etc). To assay their activity *in vivo*, one needs to use a specific inhibitor of ACOX1 which would perturb the relative contribution to the generation of H_2O_2.

Upon this reviewer's request, we have established *SIRT5* knockdown cells in Huh7 (new Appendix Figure S1), and detected the level of H_2O_2 in peroxisomes, cytosol, and nuclei (new Figure 1A). In agreement with our observations in HepG2 cells, SIRT5 depletion increased H_2O_2 production in Huh7 liver cancer cells.

New Appendix Figure



New Figure



5) Various phenotypes induced by deletion of SIRT5 including accumulation of H2O2 and oxidative DNA damage were cancelled by knockdown of ACOX1 (Fig. 5). Depletion of ACOX1 removes major H2O2 source in peroxisomes, but concomitantly abrogates peroxisomal &[beta]-oxidation system, which leads to accumulation of very long chain fatty acids and various secondary effects. So, these data should not be interpreted to mean that SIRT5-regulated succiniylation and activation of ACOX1 affect cellular H2O2 homeostasis. To determine actual contribution of ACOX1 in H2O2 level upon SIRT5 knockdown, the authors should analyze H2O2 production in HepG2 cell line with double knockdown of SIRT5 and ACOX1 upon re-introduction of a primarily peroxisome-localized SIRT5 variant (e.g. SIRT5-PTS1), which can restore desuccinylation reaction only in peroxisomes. SIRT5LQIV ^{del} could be used as a negative control.

Response: We appreciate this reviewer's constructive suggestion, and have carefully designed the experiments. To address this reviewer's question, at least 4 rounds of transfection and/or viral infection would be needed: (1) generation of SIRT5 KO cell pools; (2) put-back of wild-type or LQIV^{del} mutant SIRT5; (3) knockdown of *ACOX1* in putting-back cells; (4) transfection of Hyper-plasmids for H_2O_2 detection in living cells. Based on our previous experience, deletion of ACOX1 will greatly suppress cell growth in HepG2 cells. In addition to

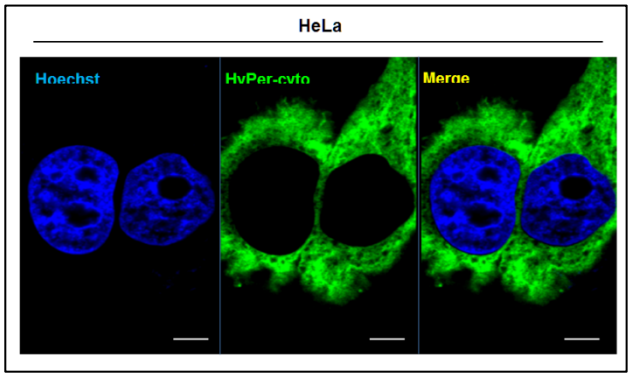
technical challenge, such extensive manipulation would unavoidably cause cellular stress and/or non-physiological protein expression level. We thus did not perform this experiment. Hopefully, this reviewer can understand our concern.

Minor concerns:

1) Figure EV1. Cytosolic localization of Hyper-cyto is not clear. Hyper-cyto appears to be present in small cellular structures.

Response: To address this reviewer's concern, we have repeated this experiment (new Figure EV1).

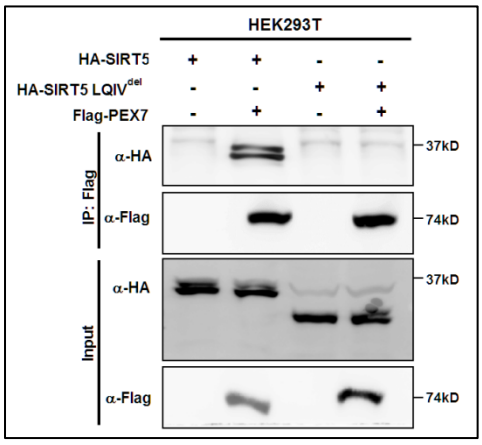
New Figure



2) Fig. 1D. To support the interaction of SIRT5 with PEX7 via its PTS2-like sequence, HA-SIRT5 LQIV ^{del} should be added in the immunoprecipitation assay as a control.

Response: Upon request, we have examined the interaction between PEX7 and wild-type or LQIV^{del} mutant SIRT5, and found that HA-SIRT5 LQIV ^{del} could not interact with Flag-PEX7 (new Figure 1D).

New Figure



3) On page 9, lines 13-14. Original paper should be cited to assure "Peroxisomes account for up to 35% of total H2O2 generation."

Response: This citation has been added (Boveris et al. *Biochem J*, 1972. PMID: 4404507). Thanks for pointing this out.

4) On page 10, lines 2-4. What does it mean that the association of ACOX1 with PEX5 is much stronger than PEX7. PEX7 never binds directly ACOX1.

Response: Corrected.

5) Fig. 2A. Both SIRT4 and SIRT5 were co-immunoprecipitated with Flag-ACOX1. Does SIRT4 localize to peroxisomes? Why did the authors focus on the function of SIRT5 in the post-translational modification of ACOX1?

Response: Our study was initiated from the observation that *SIRT5* knockdown cells exhibited higher H_2O_2 production in the peroxisome (Figures 1A-B). SIRT4 is a mitochondrial protein, and is not co-localized with the peroxisomal marker pDs-RED-Peroxi (Haigis MC, et al. *Cell*. 2006. PMID: 16959573). We thus did not further explore the protein association between ACOX1 and SIRT4.

6) On page 19, in the last paragraph. Does anyone previously mention the function of SIRT5 in peroxisomes? If so, the reference is required.

Response: To the best of our knowledge, no one has reported the function of SIRT5 in peroxisomes.

7) Fig. 3A. The data concerning the succinylation state of Flag-ACOX1 upon incubating either HA-tagged wild-type or SIRT5^{H158Y} should be provided.

Response: The data regarding the succinylation state of Flag-ACOX1 upon incubating either HA-tagged wild-type or SIRT5^{H158Y} has already been shown in Figure 2E.

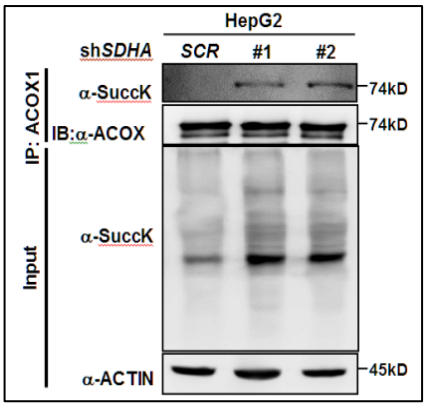
8) Fig. 3B. Flag-ACOX1 used as a substrate in in vitro assay is not succinylated, which is not consistent with Fig. 2E.

Response: Incubation with Succiny-CoA *in vitro* would dramatically increase the succinylation level of Flag-ACOX1, which may conceal the basal succinylation level.

9) Fig. 3E. The band showing the succinylation state of ACOX1 is not clear due to the heavy background.

Response: We have re-adjusted the image contrast to solve this problem (new Figure 3E).

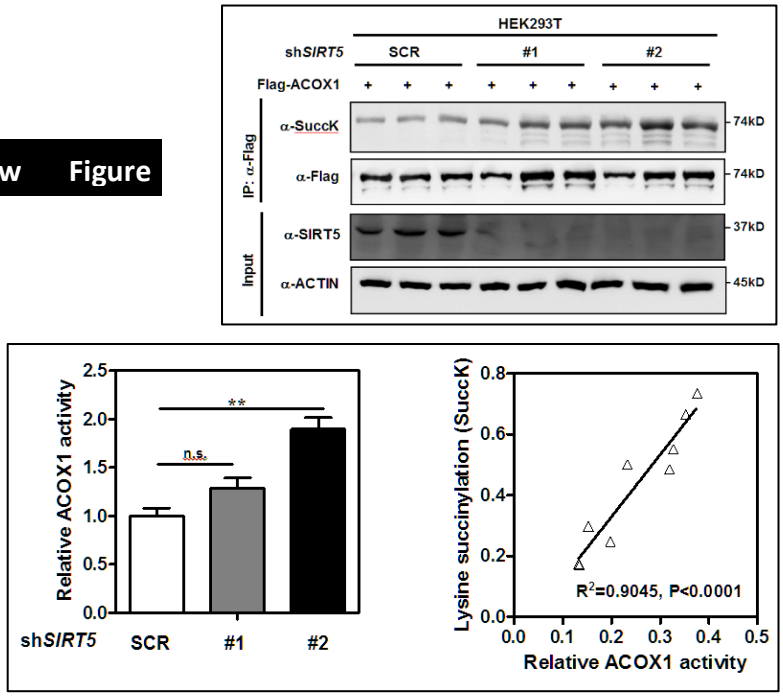
New Figure 3E



10) Fig. 3C, D, and F. The succinylation state of Flag-ACOX1 in each condition should be shown to evaluate the correlation between succinylation level and enzyme activity of ACOX1.

Response: Upon request, we have repeated the experiments, and the correlation between the succinylation level and enzyme activity of ACOX1 is now shown in new Figure 3C.

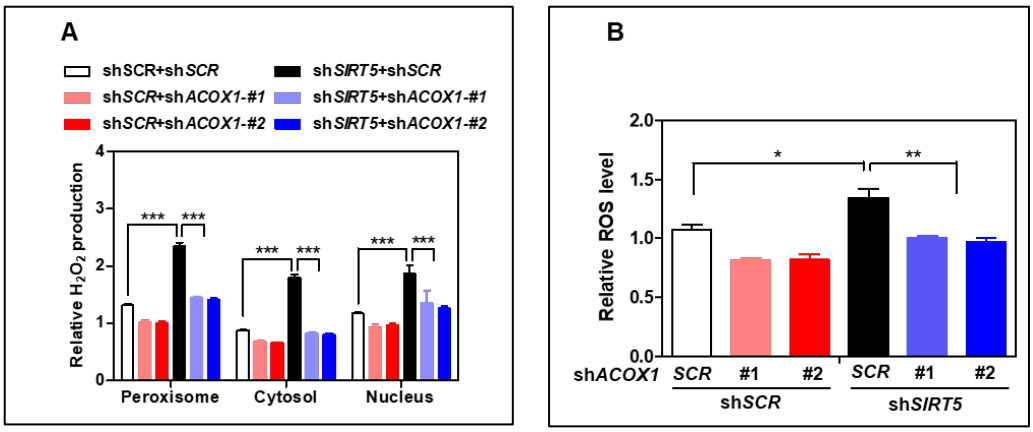
New Figure

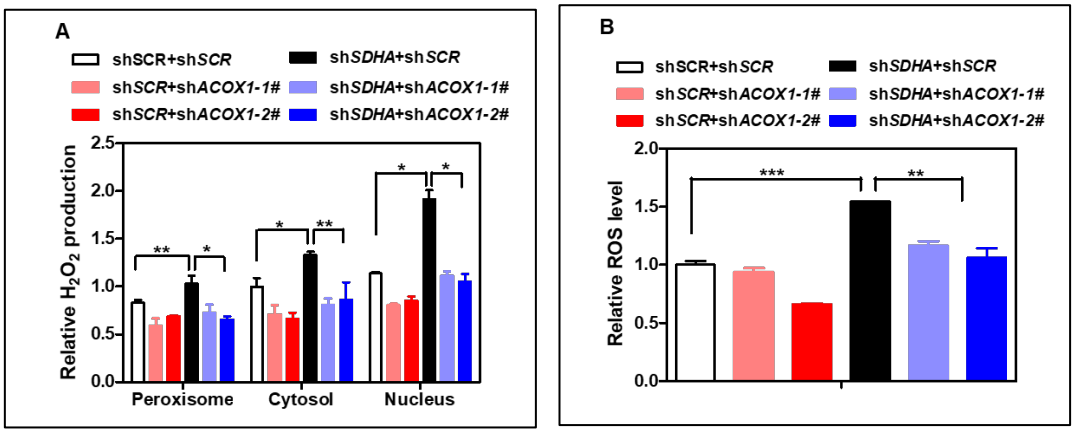


11) Fig. 5A and B, and EV5A and B. It is not clear how the authors compared the data to analyze the statistical significance.

Response: Upon clarification, we have re-marked the comparisons for the statistical significance in Fig. 5A and B, and EV5A and B.

[redacted]




A
shSCR+shSCR
shSCR+shACOX1-1#
shSCR+shACOX1-2#
shSDHA+shSCR
shSDHA+shACOX1-1#
shSDHA+shACOX1-2#
Relative H2O2 production
2.5
2.0
1.5
1.0
0.5
0.0
**
*
*
**
*
*
Peroxisome
Cytosol
Nucleus
B
shSCR+shSCR
shSCR+shACOX1-1#
shSCR+shACOX1-2#
shSDHA+shSCR
shSDHA+shACOX1-1#
shSDHA+shACOX1-2#
Relative ROS level
2.0
1.5
1.0
0.5
0.0
***
**

2nd Editorial Decision 25 January 2018

Thank you for the submission of your revised manuscript. We have now received the enclosed referee reports on it. As you will see, the referees overall support the publication of your study now, however, they still have a few concerns that need to be addressed and incorporated in the manuscript text before I can proceed with the official acceptance of your study.

I few other changes are also needed:

The statistical tests used to calculate p-values must be specified in the legends of Figs 1A-B, 3, 5, 6, EV4, EV5m, S2B, S8, S14. Our data editors have edited the figure legend text in the attached word file. Can you please have a very careful look if these suggested changes are all correct. If possible, please also provide table 1 in an editable word or excel file. Thank you.

In the source data for Fig 1F the alpha-SDHA and alpha-PMP70 have been mixed-up compared with the figure.

It would be better to call "raw data" source data in your source data files.

Please delete the list of abbreviations and instead spell out the full names in the manuscript text when they are used for the first time.

Please change the references to the numbered EMBO reports style that is part of EndNote.

I would like to suggest a few changes to the abstract. Please let me know whether you agree with these:

Peroxisomes account for ~35% of total H2O2 generation in mammalian tissues. Peroxisomal ACOX1 (acyl-CoA oxidase 1) is the first and rate-limiting enzyme in fatty acid β-oxidation and a major producer of H2O2. ACOX1 dysfunction is linked to peroxisomal disorders and hepatocarcinogenesis. Here we show that the deacetylase sirtuin 5 (SIRT5) is present in peroxisomes and that ACOX1 is a physiological substrate of SIRT5. Mechanistically, SIRT5-mediated desuccinylation inhibits ACOX1 activity by suppressing its active dimer formation in both cultured cells and mouse livers. Deletion of SIRT5 increases H2O2 production and oxidative DNA damage, which can be alleviated by ACOX1 knockdown. We show that SIRT5 downregulation is associated with increased succinylation and activity of ACOX1 and oxidative DNA damage response in hepatocellular carcinoma (HCC). Our study reveals a novel role of SIRT5 in inhibiting peroxisome-induced oxidative stress, in liver protection and in suppressing HCC development. (OK?)

I look forward to seeing a final version of your manuscript as soon as possible.
When you upload the new version of your manuscript you can bring forward all the old files and then replace only the ones that need to be replaced.
Let me know please if you have any questions or comments.

REFEREE REPORTS

Referee #1:

The changes made to the manuscript by Chen et al. have greatly strengthened it, and addressed my concerns. This submission reveals important new aspects of SIRT5 biology in the context of peroxisomes, and a function for this protein in ROS regulation via ACOX1. There are a few remaining minor issues that can be addressed by a bit of rewriting:
1. The statement that "acetylation, succinylation, malonylation, and glutarylation are structurally similar" is inaccurate. Acetylation is a small uncharged modification that masks the charge on a lysine at physiologic pH, whereas the other three modifications are bulkier, negatively charged modifications that reverse the charge on a lysine residue.
2. Close examination of Fig. S6 suggests that perhaps SIRT5 depletion does modestly increase

malonylation on ACOX1. The authors should probably say that the major effect of SIRT5 KD is on lysine succinylation. It is difficult to rule out an effect on other modifications without careful quantitative mass spec analysis in any case.
3. The SIRT5 signal in the tissue immunoblots in Fig. 6A is grossly blown out. It is impossible to make firm quantitative conclusions about relative SIRT5 levels in this context.
4. The authors indicate in the discussion section that no succinyl-transferase has been identified. Wang et al. very recently showed in Nature that aKGDH and GCN5 togetther succinylate nuclear histones.

Referee #2:

The authors have addressed my concerns. Thank you.

Referee #3:

In the new version of the manuscript, the authors have done a big effort to improve the manuscript. The new evidence included in this revised version has strengthened considerably the authors' claims and have addressed almost all my concerns satisfactorily. However, the SIRT5 localization to peroxisomes still needs some more evidence. The authors have clarified info regarding these IFs and have included a new IF of SIRT5 with the mitochondrial marker SDHA. Although indicative, these IF do not demonstrate unequivocally SIRT5 localization to Peroxisomes. The new cellular fraction included in 1F and 1G are more convincing and further support the authors' claim. In these fractions the authors tested a mitochondrial and a peroxisome marker but surprisingly did not test any non-mitochondrial/non-peroxisomal cytoplasmic or nuclear markers. Although the authors can discard that the co-localization between SIRT5 and ACOX1 does not take place in the mitochondria, they cannot exclude that the peroxisomal fractions also contain other cytoplasmic or even nuclear fractions. I am aware that the authors used a commercial kit to perform the fraction, but this does not demonstrate anything. These controls should be included in the experiment in figure 1F. In my opinion no significant levels of nuclear or cytoplamic markers would, together with the IFs, demonstrate definitively this co-localization.

Referee #4:

EMBO Report Ms. EMBOR- 2017-45124V2
Title: SIRT5 inhibits peroxisomal ACOX1 to prevent oxidative damage and is downregulated in liver cancer
Authors: Chen, X.-F. <I>et al.</I>

In the revised manuscript, the authors addressed most of the comments made by this reviewer. In revision, the authors improved the manuscript with respect to the intracellular localization of SIRT5 in peroxisomes by cell subcellular fractionation assay. In the revised manuscript, they also showed the data dealing with western blotting together with molecular-mass markers. However, this reviewer still has a couple of concerns in the revised manuscript and the authors' replies.

Major point:
1) Response to the major concern 3:
The authors explained that SIRT5-dependent desuccinylation regulates the activity of ACOX1 by showing the relationship between the activity of ACOX1 and the succinylated level of the unprocessed A-chain of ACOX1. However, the succinylated form of B-chain of ACOX1 was not provided, which is required for general readers to understand the physiological significance of the regulation of ACOX1 activity in a manner dependent on its desuccinylation. The authors showed in the data for the reviewer where the B-chain of ACOX1 is predominant in mouse liver, suggesting that the elevated activity of ACOX1 in the liver of SIRT5-knockout mouse is not explained solely by the desuccinylation of ACOX1-A-chain. Similarly, the correlation between ACOX1

succinylation level and its enzyme activity in HEK293 (Fig. 3C) might not be explained either by the data of the succinylation of the A-chain. Therefore, rather the succinylation of both A and B-chains of Flag-ACOX1 should be included if Flag-ACOX1 were processed in HEK293 cells as in HepG2 cells. Otherwise, the authors should clearly explain the reason why the authors focused on the modification of only the A-chain of ACOX1.

Minor points:
1) Molecular mass markers need to be indicated in the blot data detected with anti-succinyl-lysine antibody in Figs. 2D and 3E.
2) In newly added Fig. 2D, molecular mass markers of anti-Flag blots appear to be incorrect because PEX7 is a ~40-kD protein.

2nd Revision - authors' response 5 February 2018

Referee #1:

The changes made to the manuscript by Chen et al. have greatly strengthened it, and addressed my concerns. This submission reveals important new aspects of SIRT5 biology in the context of peroxisomes, and a function for this protein in ROS regulation via ACOX1. There are a few remaining minor issues that can be addressed by a bit of rewriting:

1. The statement that "acetylation, succinylation, malonylation, and glutarylation are structurally similar" is inaccurate. Acetylation is a small uncharged modification that masks the charge on a lysine at physiologic pH, whereas the other three modifications are bulkier, negatively charged modifications that reverse the charge on a lysine residue.

Response: Agree. We have corrected the statement in the introduction.

2. Close examination of Fig. S6 suggests that perhaps SIRT5 depletion does modestly increase malonylation on ACOX1. The authors should probably say that the major effect of SIRT5 KD is on lysine succinylation. It is difficult to rule out an effect on other modifications without careful quantitative mass spec analysis in any case.

Response: Agree. We have re-adjusted the original statement to '*SIRT5* knockdown did not obviously affect lysine malonylation, glutarylation, and acetylation of Flag-ACOX1 in HEK293T cells, implying that the major effect of SIRT5 depletion on ACOX1 posttranslational modification is lysine succinylation'.

3. The SIRT5 signal in the tissue immunoblots in Fig. 6A is grossly blown out. It is impossible to make firm quantitative conclusions about relative SIRT5 levels in this context.

Response: We agree that the immunoblot data in Figure 6A cannot bring a firm quantitative conclusion about SIRT5 protein expression in HCC patient samples. In fact, the quantitative conclusion that SIRT5 protein is commonly downregulated in HCC is based on IHC data using a specific antibody against SIRT5 in two separate study cohorts consisting of 78 and 118 HCC patient samples (Figures 6C and 6E).

4. The authors indicate in the discussion section that no succinyl-transferase has been identified. Wang et al. very recently showed in Nature that aKGDH and GCN5 togetther succinylate nuclear histones.

Response: We have included the recent finding about αKGDH and GCN5 in the discussion of our re-revised manuscript. Thank you for pointing this out.

Referee #2:

The authors have addressed my concerns. Thank you.

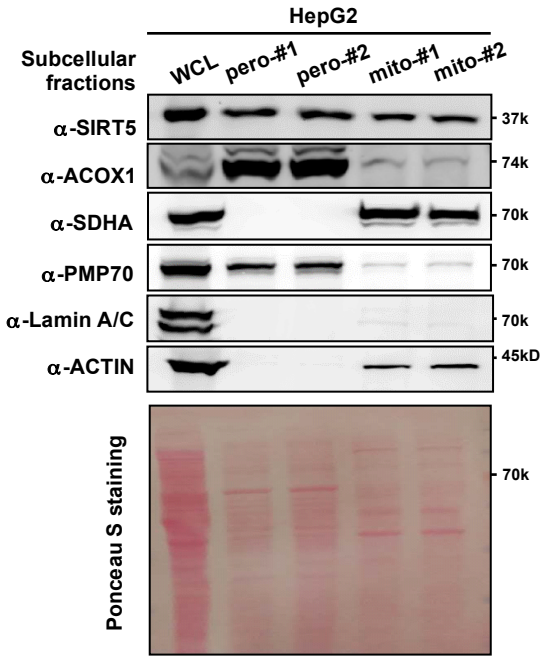
Response: Thank you very much.

Referee #3:

In the new version of the manuscript, the authors have done a big effort to improve the manuscript. The new evidence included in this revised version has strengthened considerably the authors' claims and have addressed almost all my concerns satisfactorily. However, the SIRT5 localization to peroxisomes still needs some more evidence. The authors have clarified info regarding these IFs and have included a new IF of SIRT5 with the mitochondrial marker SDHA. Although indicative, these IF do not demonstrate unequivocally SIRT5 localization to Peroxisomes. The new cellular fraction included in 1F and 1G are more convincing and further support the authors' claim. In these fractions the authors tested a mitochondrial and a peroxisome marker but surprisingly did not test any non-mitochondrial/non-peroxisomal cytoplasmic or nuclear markers. Although the authors can discard that the co-localization between SIRT5 and ACOX1 does not take place in the mitochondria, they cannot exclude that the peroxisomal fractions also contain other cytoplasmic or even nuclear fractions. I am aware that the authors used a commercial kit to perform the fraction, but this does not demonstrate anything. These controls should be included in the experiment in figure 1F. In my opinion no significant levels of nuclear or cytoplamic markers would, together with the IFs, demonstrate definitively this co-localization.

Response: Upon this reviewer's request, we have added β-ACTIN and Lamin A/C as the cytoplasmic and nuclear markers, respectively (new Figure 1F). Moreover, we have added the information about the commercial kit (Sigma, Product Code PEROX1) to perform the fraction into the 'Material and Method' of the re-revised paper.

New Figure 1F



Referee #4:

In the revised manuscript, the authors addressed most of the comments made by this reviewer. In revision, the authors improved the manuscript with respect to the intracellular localization of SIRT5 in peroxisomes by cell subcellular fractionation assay. In the revised manuscript, they also showed the data dealing with western blotting together with molecular-mass markers. However, this reviewer still has a couple of concerns in the revised manuscript and the authors' replies.

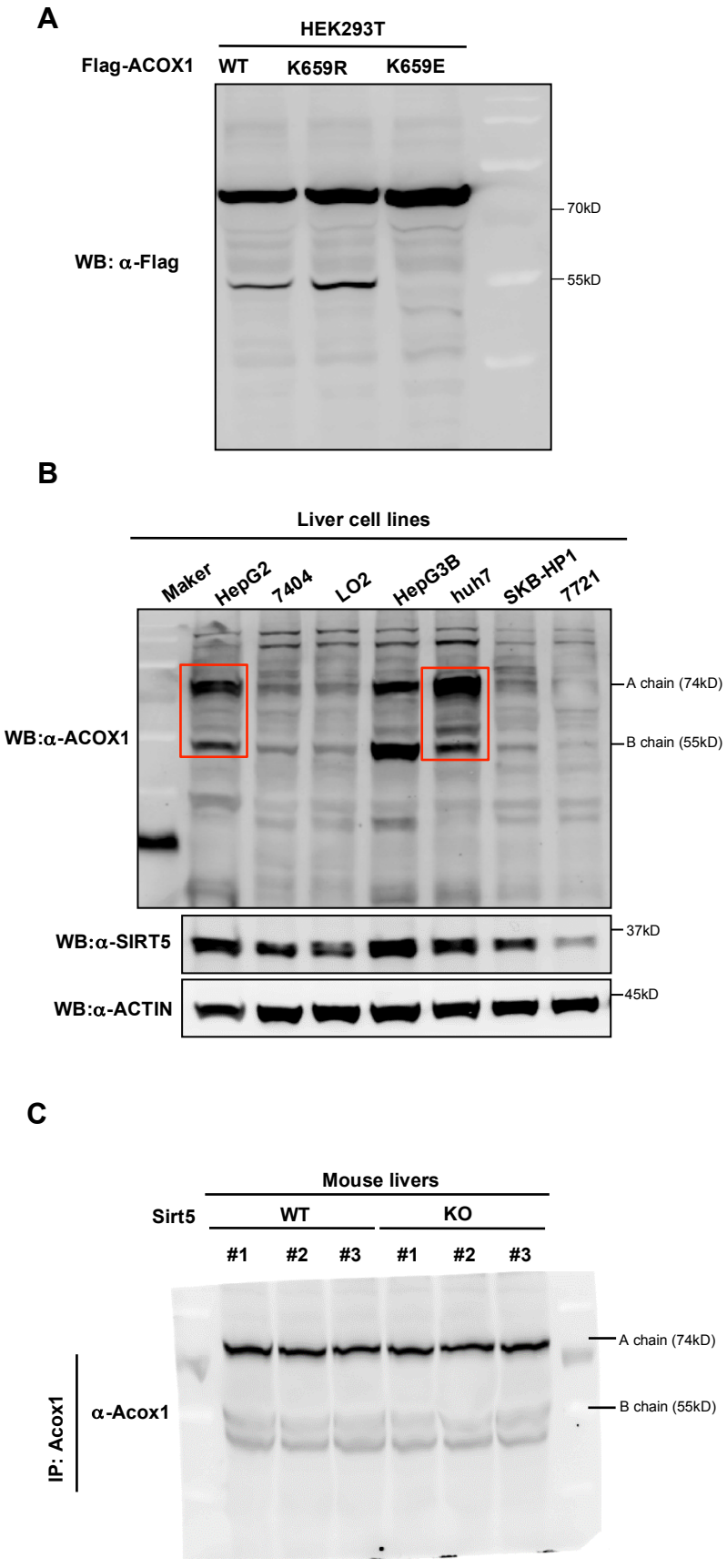
1) Response to the major concern 3:

The authors explained that SIRT5-dependent desuccinylation regulates the activity of ACOX1 by showing the relationship between the activity of ACOX1 and the succinylated level of the unprocessed A-chain of ACOX1. However, the succinylated form of B-chain of ACOX1 was not provided, which is required for general readers to understand the physiological significance of the regulation of ACOX1 activity in a manner dependent on its desuccinylation. The authors showed in the data for the reviewer where the B-chain of ACOX1 is predominant in mouse liver, suggesting that the elevated activity of ACOX1 in the liver of SIRT5-knockout mouse is not explained solely by the desuccinylation of ACOX1-A-chain. Similarly, the correlation between ACOX1 succinylation level and its enzyme activity in HEK293 (Fig. 3C) might not be explained either by the data of the succinylation of the A-chain. Therefore, rather the succinylation of both A and B-chains of Flag-ACOX1 should be included if Flag-ACOX1 were processed in HEK293 cells as in HepG2 cells. Otherwise, the authors should clearly explain the reason why the authors focused on the modification of only the A-chain of ACOX1.

Response: We appreciate the reviewer's constructive advice.

As shown below in 'Figures to the reviewer', the unprocessed A-chain (74 kDa), but not processed B-chain (55kDa) of Flag-ACOX1 is mainly expressed in transfected HEK293T (Figure A), also reflected by its endogenous expression in HepG2 and Huh7 cells (Figure B). Besides, the ACOX1 antibody(Santa) is good for western-blotting, and can well recognize the A and B chains of endogenous ACOX1 in liver cell lines, but this antibody did not work when used in co-immunoprecipitation (Figure C). So, we use another ACOX1 antibody (Proteintech), only the A-chain of endogenous Acox1 in mouse livers could be immunoprecipitated and detected by western-blotting by using this ACOX1 antibody (Figure C). Thus, we have mainly focused on the modification of the A-chain of ACOX1 in the continuation, although we believe that the A and B chains of ACOX1 has no preference on SIRT5-medieated acylation. This statement has been added into the re-revision.

New figure to this reviewer:



Minor points:
1) Molecular mass markers need to be indicated in the blot data detected with anti-succinyl-lysine antibody in Figs. 2D and 3E.
Response: Added.

2) In newly added Fig. 2D, molecular mass markers of anti-Flag blots appear to be incorrect because PEX7 is a ~40-kD protein.
Response: Corrected.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dan Ye
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45124-T

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Each experiment has independent triplicated repeats.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	At least three littermates were used for each experiment.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Yes, the criteria is pre-established when we need inclusion/exclusion
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	We have avoided any subjective bias.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Each experiment is random and has independent triplicated repeats
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Of course yes.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Shown in 'Materials and Methods ' on page 21
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines has been stated in 'Materials and Methods 'on page 21

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All the information has been stated in 'Materials and Methods 'on page 22
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animals studies is in complicance with ethical regulation.Animal experiments were performed at Fudan Animal Center in accordance with the animal ware fare guidelines
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO