EMBO reports

Histone acetyltransferase 1 is a succinyltransferase for histones and non-histones and promotes tumorigenesis

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 1st Jul 2020

Dear Dr. Zhang

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

As you will see, while the referees agree that the study is potentially interesting, they also all point out that it requires significant revision before it can be considered for publication here. The major concerns regard the low fold-change in succinylation upon HAT1 KO, the reclassification of HAT1 as acyltransferase and potential indirect effects of reduced growth of HAT1 KO cells on metabolism to name a few. Referee 3 emphasized again in his/her further feedback that it will be important to address his/her point 1, i.e. to test whether HAT1 can also mediate other types of protein acylation since the succinyl-transferase activity of HAT1 could be part of its overall ability to mediate lysine acylation (in addition to its acetylation activity).

From the referee comments it is clear that a major revision will be required to address all concerns raised by the referees and to strengthen your data. Given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be October 1st in your case. Yet, given the current COVID-19 related lockdowns of laboratories, we have extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

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

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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

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

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

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

Data availability

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

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

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

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

10) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

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

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

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

Yours sincerely

Martina Rembold, PhD

Editor
EMBO reports

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Referee #1:

In this paper, the authors knocked out HAT1 in HepG2 cells, and conducted a quantitative succinylomic profiling to identify potential succinylated substrates regulated by HAT1. They validated histone H3K122 is a major succinylation sites regulated by HAT1, and such a succinylation plays a potential role in regulating gene expression. They further identified a non-histone, PGAM1, to be succinylated at K99, which links glycolysis to tumorigenesis. The authors did a lot of experiments, but their selling points are diverse and not very sharp. The story was loosely organized. However, this study deserves to be published in EMBO Reports, due to the importance of succinylation regulation. A minor but essential revision should be conducted.

1. The authors are weak in succinylomic data analysis. From Fig. 1a, actually I do not find a significant difference between HAT1-KO or WT cells. A 1.2-fold decrease is a very loose and arbitrary threshold to determine differentially down-regulated succinylated substrates and sites. I assume that there are a number of histone proteins identified from their succinylomic profiling, because many histone proteins are annotated in the KEGG pathway of systematic lupus erythematosus. So in their Fig. 1E, a GO-based enrichment analysis using GSEA will be more proper to identified differentially regulated biological processes.

2. In this paper, the authors told two stories on succinylation, including the regulation of histone and non-histone proteins. Frankly, if the authors can focus only one road, the study will be much better. When I read the manuscript, my first question is: which one is more important, regulation of gene regulation by modifying histones or regulation of glycolysis by modifying PGAM1?

3. Since the authors also conducted a ChIP-seq analysis, they should exploit that whether PGAM1 and the glycolysis pathway are also transcriptionally regulated by succinylation.

4. In the abstract, "...explore the succinylation activity of HAT1 in tumorigenesis" should be changed to "...explore the succinyltransferas activity of HAT1 in tumorigenesis".

Referee #2:

Yang and colleagues identified HAT1 as a lysine succinyltransferase. By in vitro enzymatic assay, they demonstrated that HAT1 prefer to catalyze lysine succinylation compared to acetylation.

The authors successfully figured out that T188 of HAT1 is important for the succinyltransferase activity but not for acetyltransferase function. Using this mutation, the authors validated and demonstrated that histone H3K122 and glycolytic enzyme PGAM1 K99 are bona fide substrates of HAT1. H3K122 succinylation positively affect expression of several genes such as CREBBP and RPTOR. PGAM1 K99 succinylation is important for regulating its glycolytic activity. In vitro and in vivo experiments demonstrated that HAT1 and its succinyltransferase activity promote tumor growth in liver and pancreatic cancers, and H3K122 and PGAM1 K99 succinylation may be the key substrates. This is an interesting paper which first time identified succinyltransferase as a new

enzymatic activity of HAT1 and its importance in tumorigenesis. The experiments are well-designed, and data are solid. Some control experiments are suggested to further strengthen the paper:

Major points:

1. Figure. 1. It is surprising that only one band respond to HAT1 knockout by Western blot (Fig. 1a) while more than 200 proteins were regulated by HAT1 in the proteomics study (Fig. 1b). Does that indicate most of the Ksucc substrates were modestly regulated by HAT1, or/and the stoichiometry of succinylation on these proteins are very low?

2. Figure. 1. Are the changes in Ksucc directly regulated by knocking out of HAT1, or indirectly by changed metabolism such as glycolysis or TCA cycle? Is succinyl-CoA levels changed in HAT1 KO cells? Comparing the Ksucc levels in HAT KO cells by over-expressing HAT1 or enzymatic dead mutant and quantification may be relevant experiments to do.

3. Figure 3. What is the rationale for choosing H3K122 site to study? Is this site most dynamically regulated by HAT1? Is histone H3K122ac affected by HAT1 knockout? H3K122ac ChIP should be carried out as a control (Fig. 3e).

4. Figure 4. What is the stoichiometry of succinylation on PGAM1 and other glycolytic enzymes?

5. Figure 4, 5. Are the activities of other glycolytic enzymes such as ENO1 and PKM regulated by HAT1 mediated Ksucc? The data seems to show that Ksucc of PGAM1 is responsible for the regulation of glycolysis by HAT1 (comparing Fig. 5c, 5f, and 4d).

6. Figure 6. Does the decrease in Ksucc of PGAM1 or H3K122 explain the compromised tumorigenic phenotype by HAT1 deletion? E.g. Can K99R of PGAM1 phenocopy HAT1 T188A in tumor growth?

Minor issue:

1. Fig. 2a. The claim that HAT1 deletion only reduces succinylation in H3 but not H4 is not convincing since the H4 signal is too weak to compare.

3. Fig. S4. Is H3K122 the only succinylation site regulated by HAT1? The ratios for 45 succinylation sites from quantitative proteomics should be shown.

4. Fig. 3. Representative tracks from H3K122 succ ChIP-seq should be shown in the manuscript.

Referee #3:

This manuscript reports the identification of HAT1 as a site-specific histone/protein succinyl-transferase. Using a large variety of methods and approaches, the authors aimed at demonstrating the functional significance of HAT1 succinyl-transferase activity, which could be distinguished from its role as a histone acetyl-transferase.

This manuscript deserves publication and should be of interest to a large audience.
However, there are important points that are in need of consideration before publication as

discussed below.

1 - There are two conceptually important points that need to be addressed at the beginning of the manuscript before specifically focussing the attention of histone/protein succinylation and its functional consequences.

First, throughout the manuscript the claim is that in addition to acetylation, HAT1 can mediate site-specific succinylation, but it is not clear whether HAT1 is also able to mediate other acylations, i. e. propionylation, butyrulation, crotonylation, etc…
Without any demonstration of the exclusive or preferential specificity of HAT1 to use succinyl-CoA in addition to acetyl-CoA, succinylation should be presented and discussed as an example of HAT1-mediated histone/protein acylations.
Therefore, at the beginning of the manuscript, the authors should test in vivo and in vitro the ability of HAT1 to also mediate other types of protein acylations.
In case HAT1 is found to mediate a broad range of protein acylations, the authors could use their subsequent work on succinylation as an example and discuss it as such.

Second, from a published parallel study of histone acetylation and butyrylation (pmid: 27105113), it appears clearly that the important point for in vivo gene activation is a combination of histone acetylation and butyrylation, which are dynamically present at given sites and that the presence of only one mark at the gene transcriptional start sites (TSSs) is rather associated with poor gene activity.
Therefore, the authors cannot exclude the possibility of dynamic alternative histone/protein modifications by acetylation and succinylation. Additionally, taking into account the paper mentioned above, these dynamic alternative modifications of a site by acetylation-succinylation could actually be more important for the measured functional consequences than the presence of one of these marks.

With respect to this latter point, the authors are invited to test whether the succinylated sites found on non-histone proteins, especially the one that is functionally considered, PGAM1 K99, have been also identified as acetylated sites. For this, they could check publicly available acetylomes.

2 - Although the authors showed that the HAT1 T188A mutant keeps it acetyl-transferase activity on H3K122 but loses its succinyl-transferase activity on this site, similar data are not shown for non-histone proteins.
More particularly, with respect to the presented functional studies on PGAM1, Fig. 5D suggests that HAT1 does not acetylate this protein.
However, the authors should know that anti-pan-Kac antibodies cannot detect all K-acetylated sites. Therefore, a negative result with Anti-pan-Kac antibodies is not a proof of the absence of acetylation. Famous examples are acetylated tubulin or HSP90, which are abundant proteins whose acetylation is detected only by some of the anti-pan-Kac antibodies.
The inability of the anti-pan-Kac/succ to detect all modified proteins can also be seen from the authors' own blots, for examples in Fig. 2A. Indeed, we can see on these blots that these antibodies detect H3 and H4, while H2A/H2B are barely detectable, although we know that they could be highly acetylated-acylated.
Here again, the authors could check the publicly available acetylome to see if the acetylation of PGAM1 has already been seen. Additionally, they are invited to moderate their conclusion on the specific role of succinylation but rather discuss a role for a combination of modifications.

3 - HAT1 depletion is shown in Fig.6B-F to severely affect HapG2 cell growth and survival. Taking

into account these dramatic effects, it is not clear how the authors could establish and keep in culture stable HAT1 KO cells shown in Fig. 1, 2, 3, 4 and 5?
Is there a possibility that the observed effects could be indirect and due to severe growth impairment and high rate of apoptosis?
The impaired cell growth could particularly be responsible for the observed metabolic defects reported in Fig. 4D.
The author should therefore find a way to conciliate the data shown in Fig. 6 with data shown in the preceding Figures.

4 - KAT2a and its target site, H3K79, were used here as a known succinyl-transferase and its corresponding target histone succinylated site.
This control is used to show that KAT2a knock-down does not affect H3K122 succinylation and therefore to conclude that HAT1 specifically succinylates this site. However, the authors do not show that, in their hands, KAT2a knock-down affects H3K79succ as expected. Without this control, their conclusion on the differential function of HAT1 and KAT2a could not be proposed.

Point by point response to referee comments

Referee #1:

In this paper, the authors knocked out HAT1 in HepG2 cells, and conducted a quantitative succinylomic profiling to identify potential succinylated substrates regulated by HAT1. They validated histone H3K122 is a major succinylation sites regulated by HAT1, and such a succinylation plays a potential role in regulating gene expression. They further identified a non-histone, PGAM1, to be succinylated at K99, which links glycolysis to tumorigenesis. The authors did a lot of experiments, but their selling points are diverse and not very sharp. The story was loosely organized. However, this study deserves to be published in EMBO Reports, due to the importance of succinylation regulation. A minor but essential revision should be conducted.

Question 1: The authors are weak in succinylomic data analysis. From Fig. 1a, actually I do not find a significant difference between HAT1-KO or WT cells. A 1.2-fold decrease is a very loose and arbitrary threshold to determine differentially down-regulated succinylated substrates and sites. I assume that there are a number of histone proteins identified from their succinylomic profiling, because many histone proteins are annotated in the KEGG pathway of systematic lupus erythematosus. So in their Fig. 1E, a GO-based enrichment analysis using GSEA will be more proper to identified differentially regulated biological processes.

Answer: Sincerely thanks for your kind comments and constructive advice. According to your comments, we re-performed the Western blot analysis in Fig 1A at a more suitable condition and improved the data (Fig. 1A), in which the pan-anti-Ksucc antibody showed the comprehensive and mixed succinylation of various proteins. Meanwhile, as a crucial PTM, succinylation can give targeted residues 2 negative charges (+1 to -1, higher than acetylation (+1 to 0) and monomethylation (no change)) and a larger structural group. Accordingly, the slight change of succinylation levels of targeted proteins may lead to significant function regulation (PMID: 27436229; PMID: 21151122).

The reviewer raised professional advice that the 1.2-fold threshold was relatively low and 1.5-fold and 2-fold threshold may better. We analyzed the succinylation proteomic data using the 1.2-fold, 1.5-fold and 2-fold threshold, and observed that the succinylation of 324 sites of 204 proteins for 1.2-fold, 200 sites of 147 proteins for 1.5-fold, and 46 sites of 41 proteins for 2-fold were down-regulated by HAT1 knockout, respectively. To more broadly explore the HAT1-mediated succinylation of targeted sites and proteins, we selected the 1.2-fold threshold ($P < 0.01$) for further analysis in this study, according to the reports (https://www.ncbi.nlm.nih.gov/pubmed/29324989, Brain 2018); https://www.ncbi.nlm.nih.gov/pubmed/28978618, Mol Cell Proteomics 2017; https://www.ncbi.nlm.nih.gov/pubmed/26911362, Scientific Reports 2016; https://www.ncbi.nlm.nih.gov/pubmed/27378549, Translational Psychiatry 2016; https://www.ncbi.nlm.nih.gov/pubmed/26486419, Molecular & Cellular Proteomics 2016; https://www.ncbi.nlm.nih.gov/pubmed/29044224, Scientific Reports 2017; https://www.ncbi.nlm.nih.gov/pubmed/30485681, Proteomics-Clinical Applications 2019; https://www.ncbi.nlm.nih.gov/pubmed/27436229, Nature Communications 2016; https://www.ncbi.nlm.nih.gov/pubmed/31746436, Oncology Reports 2020; https://www.ncbi.nlm.nih.gov/pubmed/29464899, Proteomics 2018). In the subsequent investigation, we validated that HAT1 significantly modulated the succinylation of histone H3 and PGAM1, and directly succinylated histone H3 on K122 and PGAM1 on K99, which contributed to the epigenetic regulation and glycolysis.

Besides, according to the advice, we completed the GO-based enrichment analysis using GSEA and showed in Fig 1E.

Question 2: In this paper, the authors told two stories on succinylation, including the regulation of histone and non-histone proteins. Frankly, if the authors can focus only one road, the study will be much better. When I read the manuscript, my first question is: which one is more important, regulation of gene regulation by modifying histones or regulation of glycolysis by modifying PGAM1?

Answer: Thanks for your kind comments. You raised a crucial point and aspect. In this study, we aimed to comprehensively explore the succinyltransferase activity of HAT1, including histones and non-histones. It has been reported that KAT2A is a histone H3K79 succinyltransferase in cancer progression (https://www.ncbi.nlm.nih.gov/pubmed/ 29211711, Nature 2017) and SIRT7 is a histone H3K122 desuccinylase (https://www.ncbi.nlm.nih.gov/pubmed/27436229, Nature Communications 2016), but the succinyltransferase of histone H3K122 remain unclear. In addition, it has been reported that SIRT5-mediated desuccinylation impacts diverse metabolic pathways (https://www.ncbi.nlm.nih.gov/pubmed/23806337, Molecular Cell 2013; https://www.ncbi.nlm.nih.gov/pubmed/24315375, Cell Metabolism 2013), but the succinyltransferase that can regulate metabolic pathway, including glycolysis, remains unclear. We found that HAT1, as a new histone succinyltransferase, catalyzed the succinylation of histone H3K122, modulating the epigenetics and gene regulation. HAT1, as a succinyltransferase, was able to catalyze the succinylation of PGAM1 (K99), contributing to glycolysis. Consequently, we identified the novel succinyltransferase activity of HAT1 for the different proteins but did not compare the importance. But our functional investigation showed that HAT1 and HTA1-mediated succinylation contributed to the tumor growth *in vitro* and *in vivo*, in which the succinylation of histone H3K122 and the levels of PGAM1-related metabolites were regulated in the *in vivo* system. Meanwhile, we also found that the succinylation of PGAM1 (K99) was crucial for tumor growth *in vivo* and *in vitro*. Hence, we showed the succinylation for both histone and non-histone to validate the succinyltransferase activity of HAT1 in this study.

Question 3: Since the authors also conducted a ChIP-seq analysis, they should exploit that whether PGAM1 and the glycolysis pathway are also transcriptionally regulated by succinylation.

Answer: Thank you for your professional comments and constructive advice. We checked our ChIP-seq data and failed to find the PGAM1 and other glycolytic enzymes. Meanwhile, ChIP assays showed that H3K122 succinylation and H3K122 acetylation were undetectable on the promoter of PGAM1 in liver cancer cells, in which HAT1 knockout and re-expression

of the HAT1 (T188A) mutant failed to affect this phenotype as well (Appendix Fig S8F). Consistently, HAT1 knockout and re-expression of the HAT1 (T188A) mutant failed to modulate the mRNA expression of PGAM1 in liver cancer cells (Appendix Fig S8G). Besides, we checked the ChIP-seq or ChIP analysis in available databases and literatures, and also did not found that PGAM1 was able to transcriptionally regulate by succinylation or acetylation. Thus, we conclude that HAT1 modulates the succinylation of PGAM1 at the post-translational level, but not at the transcriptional level mediated by histone H3K122 succinylation.

Question 4: In the abstract, "...Explore the succinylation activity of HAT1 in tumorigenesis" should be changed to "...explore the succinyltransferas activity of HAT1 in tumorigenesis".

Answer: Thanks for your kind comments. We revised it in the manuscript.

Referee #2:

Yang and colleagues identified HAT1 as a lysine succinyltransferase. By in vitro enzymatic assay, they demonstrated that HAT1 prefer to catalyze lysine succinylation compared to acetylation. The authors successfully figured out that T188 of HAT1 is important for the succinyltransferase activity but not for acetyltransferase function. Using this mutation, the authors validated and demonstrated that histone H3K122 and glycolytic enzyme PGAM1 K99 are bona fide substrates of HAT1. H3K122 succinylation positively affect expression of several genes such as CREBBP and RPTOR. PGAM1 K99 succinylation is important for regulating its glycolytic activity. In vitro and in vivo experiments demonstrated that HAT1 and its succinyltransferase activity promote tumor growth in liver and pancreatic cancers, and H3K122 and PGAM1 K99 succinylation may be the key substrates. This is an interesting paper which first time identified succinyltransferase as a new enzymatic activity of HAT1 and its importance in tumorigenesis. The experiments are well-designed, and data are solid. Some control experiments are suggested to further strengthen the paper:

Major points:

Question 1: Figure. 1. It is surprising that only one band respond to HAT1 knockout by Western blot (Fig 1A) while more than 200 proteins were regulated by HAT1 in the proteomics study (Fig 1B). Does that indicate most of the Ksucc substrates were modestly regulated by HAT1, or/and the stoichiometry of succinylation on these proteins are very low?

Answer: Sincerely thanks for your kind comments and professional advice. According to your advice, we re-performed the Western blot analysis in Fig 1A in a more suitable condition and improved the data (Fig 1A). Western blot analysis in Fig 1A using the pan-anti-Ksucc antibody showed the succinylation levels of multiple proteins and our data suggest that the HAT1 potentially regulates the succinylation of various proteins. Then we performed a succinylation quantitative proteomic, which could more specially and precisely understand the landscape of HAT1-mediated succinylation. The sensitivity of these two methods may be different.

Question 2: Figure. 1. Are the changes in Ksucc directly regulated by knocking out of HAT1, or indirectly by changed metabolism such as glycolysis or TCA cycle? Is succinyl-CoA levels changed in HAT1 KO cells? Comparing the Ksucc levels in HAT KO cells by over-expressing HAT1 or enzymatic dead mutant and quantification may be relevant experiments to do.

Answer: Thanks for your kind comments. The changes of succinylation may be directly regulated by HAT1 knockout. We validated it by using Western blot analysis and the succinylation quantitative proteomic. In addition, the levels of succinyl-CoA were not regulated by HAT1 knockout (Appendix Fig S2B). Meanwhile, we analyzed the HAT1-mediated succinylation on histone H3 and PGAM1 by *in vitro* succinylation assays and found that HAT1 was able to directly catalyze the succinylation of histone H3 and PGAM1 (Fig 2C and Fig 5D). Moreover, we have compared the succinylation levels of H3 and PGAM1 in HAT1 KO cells re-expressed HAT1 or related enzymatic dead mutant and found that the re-expression of the HAT1 (T188A) mutant in the HAT1 KO cells failed to

rescue the succinylation of H3 and PGAM1 compared with reconstituted expression of wild-type HAT1 (Fig 2G, Appendix Fig S4K and L, Fig 5A, and Appendix Fig S8A).

Question 3: Figure 3. What is the rationale for choosing H3K122 site to study? Is this site most dynamically regulated by HAT1? Is histone H3K122ac affected by HAT1 knockout? H3K122ac ChIP should be carried out as a control (Fig. 3e).

Answer: Thanks for your kind comments. We choose H3K122 succinylation to study for several reasons. Firstly, histone H3K122 is one of the most significant sites targeted by HAT1 depletion in the succinylation quantitative proteomic. Second, histone H3K122 acetylation play an important role in epigenetic regulation and gene expression modification and the succinylation is similar to the acetylation in some characters and functions (https://www.ncbi.nlm.nih.gov/pubmed/23415232, Cell 2013; https://www.ncbi.nlm.nih.gov/pubmed/29548294, Epigenetics Chromatin 2018; https://www.ncbi.nlm.nih.gov/pubmed/21151122, Nat Chem Biol. 2011; https://www.ncbi.nlm.nih.gov/pubmed/23954790, Cell Rep. 2013). Third, it has been reported that SIRT7, serving as an eraser, is able to catalyze the desuccinylase of histone H3 at the site of K122 (https://www.ncbi.nlm.nih.gov/pubmed/27436229, Nature Communications 2016). However, the writers for the succinylation of histone H3K122 have not been identified. Hence, we supposed that HAT1 might serve as the writer of histone H3K122 succinylation and validated that HAT1 could directly catalyze the succinylation of histone H3K122. The histone H3K122ac was not affected by HAT1 knockout (Fig 3A and Appendix Fig S5C). H3K122ac ChIP was carried out as a control (Fig. 3D, and Appendix Fig S5J and K).

Question 4: Figure 4. What is the stoichiometry of succinylation on PGAM1 and other glycolytic enzymes?

Answer: Thanks for your kind comments. The succinylation-site stoichiometry analysis was performed, and the results were listed in Appendix Table S5.

Question 5: Figure 4, 5. Are the activities of other glycolytic enzymes such as ENO1 and PKM regulated by HAT1 mediated Ksucc? The data seems to show that Ksucc of PGAM1 is responsible for the regulation of glycolysis by HAT1 (comparing Fig. 5c, 5f, and 4d).

Answer: Thanks for your kind comments. Our data of succinylation quantitative proteomic showed that HAT1 specifically mediated the succinylation of proteins involving glycolysis, including 7 of 10 key enzymes of glycolysis, such as GPI, TPI, GAPDH, PGK, PGAM, ENO1 and PKM (Fig 4A and Appendix Table S5). Then, we validated that HAT1 was able to modulate the succinylation of glycolytic enzymes, including PGAM1, ENO1 and PKM (Fig 5A, and Appendix Fig S8A-C). Importantly, we found that the HAT1-mediated succinylation significantly affected the enzyme activity of PGAM1 but slightly affected the enzyme activity of ENO1 and PKM in HepG2 cells (Appendix Fig S8D). Hence, we selected PGAM1 for further investigation and our data suggest that HAT1 contributes to glycolysis through the succinylation of PGAM1 in tumor cells.

Question 6: Figure 6. Does the decrease in Ksucc of PGAM1 or H3K122 explain the compromised tumorigenic phenotype by HAT1 deletion? E.g. Can K99R of PGAM1 phenocopy HAT1 T188A in tumor growth?

Answer: Thanks for your kind comments. In the nude mice tumorigenicity experiments, our data showed that the depletion of HAT1 significantly inhibited the tumor growth of HepG2 and PANC1 cells, in which the expression of Ki67 and succinylation of H3K122, and the levels of glycolytic markers related to PGAM1 were decreased as well in the tumor tissues (Fig 6D-H, and Appendix Fig S9A-E). Importantly, reconstituted expression of the HAT1 (T188A) mutant HAT1 failed to rescue this inhibition compared with the reconstituted expression of wild-type HAT1 in the HAT1 KO cells (Fig 6D-H, and Appendix Fig S9A-E).

Importantly, the nude mice tumorigenicity experiments showed that the reconstituted expression of PGAM1 (K99R) mutant failed to rescue the growth of PANC1 cells compared with the reconstituted expression of wild-type PGAM1 in the cells depleted endogenous

PGAM1 (Appendix Fig S9F-J). It suggests that K99R of PGAM1 can phenocopy HAT1 T188A in the tumor growth.

Minor issue:

Question 7: Fig. 2a. The claim that HAT1 deletion only reduces succinylation in H3 but not H4 is not convincing since the H4 signal is too weak to compare.

Answer: Thanks for your kind comments. We re-performed the Western blot analysis in a more suitable condition and improved the data (Fig 2A and Appendix Fig S4A), in which the histone H3 and H4 signals could be clearly observed. And we revised "HAT1 deletion only reduces succinylation in H3 but not H4" to "HAT1 knockout significantly reduced the levels of histone H3 succinylation in HepG2 cells, which was more obvious than the levels of histone H4 succinylation" in the revised manuscript.

Question 8: Fig. S4. Is H3K122 the only succinylation site regulated by HAT1? The ratios for 45 succinylation sites from quantitative proteomics should be shown.

Answer: Thank for your kind comments. H3K122 may not be the only succinylation site of histone regulated by HAT1. In this study, the succinylation quantitative proteomic identified 45 histone succinylation sites, in which 9 histone succinylation sites were targeted by HAT1 and the ratios were shown (Appendix Fig S5B).

Question 9: Fig. 3. Representative tracks from H3K122 succ ChIP-seq should be shown in the manuscript.

Answer: Thanks for your kind comments. The representative tracks from H3K122 succinylation ChIP-seq were shown (Appendix Fig S5I).

Referee #3:

This manuscript reports the identification of HAT1 as a site-specific histone/protein succinyl-transferase. Using a large variety of methods and approaches, the authors aimed at demonstrating the functional significance of HAT1 succinyl-transferase activity, which could be distinguished from its role as a histone acetyl-transferase.

This manuscript deserves publication and should be of interest to a large audience.

However, there are important points that are in need of consideration before publication as discussed below.

Question 1: There are two conceptually important points that need to be addressed at the beginning of the manuscript before specifically focusing the attention of histone/protein succinylation and its functional consequences. First, throughout the manuscript the claim is that in addition to acetylation, HAT1 can mediate site-specific succinylation, but it is not clear whether HAT1 is also able to mediate other acylations, i. e. propionylation, butyrulation, crotonylation, etc... Without any demonstration of the exclusive or preferential specificity of HAT1 to use succinyl-CoA in addition to acetyl-CoA, succinylation should be presented and discussed as an example of HAT1-mediated histone/protein acylations. Therefore, at the beginning of the manuscript, the authors should test in vivo and in vitro the ability of HAT1 to also mediate other types of protein acylations. In case HAT1 is found to mediate a broad range of protein acylations, the authors could use their subsequent work on succinylation as an example and discuss it as such.

Answer: Sincerely thanks for your kind comments and constructive advice. According to your advice, we assessed the ability of HAT1 to mediate other types of protein acylations *in vivo* and *in vitro*. Western blot analysis using pan-acylation antibodies showed that the acetylation levels were decreased upon the deletion of HAT1 as a positive control, but the HAT1 knockout failed to affect the levels of other acylations, including propionylation, butyrylation, and crotonylation in the HepG2 cells (Appendix Fig S2C). Meanwhile, HAT1 knockout significantly reduced the levels of histone H3 succinylation and acetylation, and reconstituted expression of the HAT1 (T188A) mutant, which failed to affect histone H3 acetylation, reduced histone H3 succinylation compared with reconstituted expression of wild-type HAT1 in the cells depleted endogenous HAT1 (Fig 2B and G, Appendix Fig S4B-E,

and Appendix Fig S4K and L). However, HAT1 knockout and HAT1 (T188A) mutant could not affect the levels of propionylation, butyrulation, and crotonylation of histone H3 in the cells (Appendix Fig S4M). Similarly, *in vitro* acylation assays revealed that HAT1 directly catalyzed the succinylation of histone H3, but failed to catalyze the propionylation, butyrulation, and crotonylation of histone H3 (Fig 2C and Appendix Fig S4F). For PGAM1, our data showed that the succinylation of PGAM1 was inhibited by depletion of HAT1 in the HepG2 cells, and re-expression of the HAT1 (T188A) mutant in the cells depleted endogenous HAT1 failed to rescue the succinylation of PGAM1 compared with reconstituted expression of wild-type HAT1 (Fig 5A and Appendix Fig S8A and B). Meanwhile, the acetylation of PGAM1 was observed, but not propionylation, butyrulation, and crotonylation, but the acetylation of PGAM1 was not able to be affected by HAT1 knockout and HAT1 (T188A) mutant in the system (Appendix Fig S8E). Similarly, *in vitro* acylation assays revealed that HAT1 directly catalyzes the succinylation of PGAM1, but failed to catalyze the acetylation, propionylation, butyrulation, and crotonylation of PGAM1 (Fig 5D and Appendix Fig S8J-L). Taken together, we conclude that HAT1 is able to modulate the succinylation, but not propionylation, butyrulation, and crotonylation, in the liver cancer cells. And HAT1 directly catalyzes the succinylation, but not acetylation, propionylation, butyrulation, and crotonylation, of histone H3 and PGAM1.

Question 2: Second, from a published parallel study of histone acetylation and butyrylation (pmid: 27105113), it appears clearly that the important point for in vivo gene activation is a combination of histone acetylation and butyrylation, which are dynamically present at given sites and that the presence of only one mark at the gene transcriptional start sites (TSSs) is rather associated with poor gene activity. Therefore, the authors cannot exclude the possibility of dynamic alternative histone/protein modifications by acetylation and succinylation.

Answer: Sincerely thanks for your kind comments and constructive advice. In this study, for histone H3, we found that HAT1 depletion and HAT1 (T188A) mutant significantly reduced H3K122 succinylation, but not H3K122 acetylation, on the promoter region of the representative gene, including CREBBP, BPTF and RPTOR, in the cells (Fig 3D and Appendix Fig S5J and K). The reconstituted expression of the HAT1 (T188A) mutant, which

failed to affect histone H3 acetylation, reduced histone H3 succinylation compared with reconstituted expression of wild-type HAT1 in the cells depleted endogenous HAT1 (Fig 2G and Appendix Fig S4K and L). For PGAM1, the acetylation of PGAM1, but not its propionylation, butyrulation and crotonylation, was observed, but the acetylation of PGAM1 could not be affected by HAT1 knockout and HAT1 (T188A) mutant (Appendix Fig S8E). The *in vitro* acylation assays revealed that HAT1 directly catalyzes the succinylation of PGAM1, but failed to catalyze the acetylation, propionylation, butyrulation, and crotonylation of PGAM1 (Fig 5D and Appendix Fig S8J-L). Thus, we conclude that HAT1 is able to catalyze the succinylation, excluding the possibility of acetylation, propionylation, butyrulation, and crotonylation of histone H3 and PGAM1.

Question 3: Additionally, taking into account the paper mentioned above, these dynamic alternative modifications of a site by acetylation-succinylation could actually be more important for the measured functional consequences than the presence of one of these marks. With respect to this later point, the authors are invited to test whether the succinylated sites found on non-histone proteins, especially the one that is functionally considered, PGAM1 K99, have been also identified as acetylated sites. For this, they could check publicly available acetylomes.

Answer: Sincerely thanks for your kind comments and constructive advice. Additionally, we identified that HAT1 could catalyze the succinylation of PGAM1 K99 in this study. The acetylation of PGAM1 was observed, but the acetylation of PGAM1 was not able to be affected by HAT1 knockout and HAT1 (T188A) mutant (Appendix Fig S8E). The *in vitro* acylation assays revealed that HAT1 directly catalyzed the succinylation of PGAM1, but failed to catalyze the acetylation, of PGAM1 (Fig 5D). Meanwhile, we checked publicly available acetylomes and literatures and found that PGAM1 could be acetylated at K251, K253 and K254, but not K99 (https://www.uniprot.org/uniprot/P18669#ptm_ processing; PMID: 22157007). Thus, we conclude that the PGAM1 K99 is succinylated, but not acetylated.

Question 4: Although the authors showed that the HAT1 T188A mutant keeps it acetyl-transferase activity on H3K122 but loses its succinyl-transferase activity on this site, similar data are not shown for non-histone proteins. More particularly, with respect to the presented functional studies on PGAM1, Fig. 5D suggests that HAT1 does not acetylate this protein. However, the authors should know that anti-pan-Kac antibodies cannot detect all K-acetylated sites. Therefore, a negative result with Anti-pan-Kac antibodies is not a proof of the absence of acetylation. Famous examples are acetylated tubulin or HSP90, which are abundant proteins whose acetylation is detected only by some of the anti-pan-Kac antibodies.

The inability of the anti-pan-Kac/succ to detect all modified proteins can also be seen from the authors' own blots, for examples in Fig. 2A. Indeed, we can see on these blots that these antibodies detect H3 and H4, while H2A/H2B are barely detectable, although we know that they could be highly acetylated-acylated.

Here again, the authors could check the publicly available acetylome to see if the acetylation of PGAM1 has already been seen. Additionally, they are invited to moderate their conclusion on the specific role of succinylation but rather discuss a role for a combination of modifications.

Answer: Thanks for your kind comments. You raised a crucial point and constructive advice. According to the comments, we performed additional experiments and checked the publicly available acetylome. Our data showed that the acetylation of PGAM1 was observed, but the acetylation of PGAM1 was not able to be affected by HAT1 knockout and HAT1 (T188A) mutant (Appendix Fig S8E). It suggests that the acetylation antibody is available to the detection of PGAM1. The *in vitro* acylation assays revealed that HAT1 directly catalyzes the succinylation of PGAM1, but failed to catalyze the acetylation, of PGAM1 (Fig 5D). Meanwhile, we checked publicly available acetylomes and literatures and found that PGAM1 could be acetylated at K251, K253 and K254, but not K99 (https://www.uniprot.org/uniprot/P18669#ptm_processing; PMID: 22157007). In addition, about the results of Fig 2B, the H2A/H2B are barely detectable may due to the antibody or the basic acylation levels, and similar results could be found in the previous studies

(https://www.ncbi.nlm.nih.gov/pubmed/29211711, Nature 2017). We revised the conclusion on the specific role of succinylation but rather discuss a role for a combination of modifications that "Taken together, we conclude that HAT1 contributes to the glycolysis through modulation of PGAM1 succinylation, but not acetylation, in tumor cells" in the part of result and discussion (Page 16, line 11; Page 20, line 19).

Question 5: HAT1 depletion is shown in Fig.6B-F to severely affect HapG2 cell growth and survival. Taking into account these dramatic effects, it is not clear how the authors could establish and keep in culture stable HAT1 KO cells shown in Fig. 1, 2, 3, 4 and 5? Is there a possibility that the observed effects could be indirect and due to severe growth impairment and high rate of apoptosis? The impaired cell growth could particularly be responsible for the observed metabolic defects reported in Fig. 4D. The author should therefore find a way to conciliate the data shown in Fig. 6 with data shown in the preceding Figures.

Answer: Thanks for your kind comments. You raised an essential point. On the one hand, to assess the effect of HAT1 and HAT1-mediated succinylation on the HapG2 cell proliferation, a total of 5×10^4 cells, including wild type HepG2 cells and HAT1 KO HepG2 cells, were plated into 96-well plates and the significant difference of HapG2 cell proliferation was observed after 4 days (Fig 6). Meanwhile, in the nude mice tumorigenicity analysis, a total of 5×10^6 cells, including wild type HepG2 cells and HAT1 KO HepG2 cells were subcutaneously injected into the nude mice and the effect of HAT1 and HAT1-mediated succinylation on the tumor growth of HepG2 cells *in vivo* was observed 30 days after injection (Fig 6). On the other hand, in the analysis of the preceding Figures, such as Fig. 1, 2, 3, 4, and 5, the experiment and analysis were performed by collecting and using the same number of the cells, including wild type HepG2 cells and HAT1 KO HepG2 cells, and thereby the cell growth failed to affect the results in the system. Meanwhile, as Western blot analysis, the same total proteins were presented by β-actin, suggesting that the cell growth fails to affect the results in the system. A similar design and results could be found in the previous studies (https://www.ncbi.nlm.nih.gov/pubmed/29211711, Nature 2017; https://www.ncbi.nlm.nih.gov/pubmed/29883613, Molecular Cell 2018;

https://www.ncbi.nlm.nih.gov/pubmed/27436229, Nature Communications 2016). For example, it has been reported that KAT2A depletion and KAT2A mutant modulate the succinylation and succinylation-mediated transcriptional gene expression, and can affect cancer cell growth as well (PMID: 29211711). It has been reported that SIRT7 knockout modulates the succinylation and succinylation-related gene chromatin compaction/genome stability, and also affects cancer cell survival (PMID: 29883613). In addition, it has been identified that EP300 knockout modulates lysine 2-hydroxyisobutyrylation and 2-hydroxyisobutyrylation-mediated ENO1 activity and glycolysis, and affects cancer cell proliferation as well (PMID: 27436229).

Question 6: KAT2a and its target site, H3K79, were used here as a known succinyl-transferase and its corresponding target histone succinylated site. This control is used to show that KAT2a knock-down does not affect H3K122 succinylation and therefore to conclude that HAT1 specifically succinylates this site. However, the authors do not show that, in their hands, KAT2a knock-down affects H3K79succ as expected. Without this control, their conclusion on the differential function of HAT1 and KAT2a could not be proposed.

Answer: Thanks for your kind comments. According to the comments, we added this control and re-performed the experiment. Our data showed that KAT2A knockdown could significantly reduce the succinylation levels of histone H3K79 (H3K79succ) and the acetylation levels of histone H3K9 (H3K9ac), but failed to affect succinylation levels of histone H3K122 (H3K122succ) in HepG2 cells (Appendix Fig S5N).

1st Revision - Editorial Decision 12th Nov 2020

Dear Dr. Zhang

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

As you can see, the referees find that the study has been significantly improved during revision and recommend publication without further revision.

Before I can accept the manuscript, I need you to address some minor points below:

- Author Contributions: please use abbreviations instead of the full author names.

- Please reformat the references so that the first 10 authors are listed followd by et al.

- Please add all information on funding to the relevant sections in our online submission system.

- As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find my suggestions in the attached document. I have also added some more comments throughout the manuscript and in the figure legends. Please review these. Moreover, in order to maximize the impact of your study, I would recommend to have it proofread by a native speaker or by an English editing service.

- Appendix Figure S8 currently runs over three pages. You might want to consider splitting it into three different figures.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

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

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In the revision, the authors considerably improved the manuscript by adding new results on the data analysis, interpreting the their logic on succinylation of histone and non-histone proteins potentially regulated by HAT1, and their ChIP-seq analysis. They addressed all my concerns in a neat manner. I think the current form is ready for publication.

Referee #2
[recommended publication without further revision]

Referee #3:

Most of the concerns raised previously have been taken into account and the manuscript has now been much improved. I can therefore recommend this manuscript for publication.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 1st Dec 2020

Dr. Xiaodong Zhang
Nankai University
Department of Cancer Research
Tianjin
China

Dear Dr. Zhang,

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Yours sincerely,

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The data shown in figures should satisfy the following conditions:

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2. Captions

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

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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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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.

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