EMBO reports

KLF10 promotes nonalcoholic steatohepatitis progression through transcriptional activation of zDHHC7

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1st Editorial Decision **29th Nov 2021**

Dear Dr. Lu,

Thank you for the submission of your research manuscript to our journal, which was now seen by three referees, whose reports are copied below.

We concur with the referees that the proposed role of KLF10 in nonalcoholic steatohepatitis progression through transcriptional activation of DHHC7 is in principle very interesting. However, referees raise significant and largely overlapping concerns that need to be addressed to consider publication here.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Should you be able to address all referee concerns satisfactorily, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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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:
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2. Your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section should be separate. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

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https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

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. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

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: <http://embor.embopress.org/authorguide#expandedview>.

- 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) 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 <http://embor.embopress.org/authorguide#sourcedata>.

8) 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 <http://embor.embopress.org/authorguide#datacitation>.

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>).

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

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. 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. ***

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.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

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.

Kind regards,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

This is extensive and very detailed work on the role of KLF10 in lipid-induced liver inflammation. Overall, the conclusions are supported by the data.

Minor comments:
1. Please include body weights for all mouse studies as body weight is an important denominator of liver weight, NAFLD, and NASH.
2. Please include all uncropped western blots with detailed legends in the supplement.

Referee #2:

Yang et al present evidence that the palmitoyl transferase zDHHC7 is a key player in nonalcoholic steatohepatitis. Upregulation of the transcription factor KLF10 transcriptionally activates zDHHC7, which is found to palmitoylate the fatty acid transporter CD36. CD36 palmitoylation promotes its cell surface expression and increases hepatic lipid uptake, leading to hepatic inflammation.

In general the conclusions of the manuscript are well-supported by the data presented. The authors have used appropriate model systems and the genetic evidence for the pathways identified is strong. Having identified upregulated KLF10 expression (Fig 1) they go on to overexpress KLF10 and demonstrate exacerbation of NASH induced by WD/CCl4 and exacerbation of NAFL promoted by a high fat diet (Fig 2). KLF20 overexpression is found to upregulate the Golgi-localised palmitoylating enzyme zDHHC7 through a KLF10 binding site in the zDHHC7 promoter (Fig 3). zDHHC7 is found to be required for cell surface expression of CD36 in cellular models (Fig S3) and KLF overexpression is found to increase palmitoylation and surface localisation of CD36 in the liver (Fig 4). KLF10-induced increases in CD36 palmitoylation and cell surface expression are found to be zDHHC7 dependent (Fig 5). CD36 knockout animals are found to be resistant to NASH induced by diet and KLF10 overexpression (Fig 6). Rexpression of wild type but not non-palmitoylated CD36 in vivo 'restores' diet and KLF10-induced NASH (Fig 7). KLF10 knockout in the liver is found to reduce zDHHC7 expression, CD36 palmitoylation,

The reliance on the pharmacological tool 2-bromopalmitate to support some conclusions is a weakness, as the specificity of this reagent is very poor. Genetic evidence supporting the proposed role for zDHHC7 in vivo (e.g zDHHC7 knockout) is not provided, but in general the evidence provided in cellular models that ZLF10 upregulates zDHHC7 to increase CD36 palmitoylation is strong.

Major
1. Fig 2B - KLF10 is almost undetectable in the control group. How is it possible to quantify this accurately to present a fold-change in expression?
2. The authors use a biotinylation assay to evaluate surface expression of proteins of interest - e.g. CD36 (Fig S3E, S3I, 5C, 7I, 8L and maybe others). Actin is presented as a loading control throughout. I have serious reservations about the validity of these assays, since actin is an intracellular protein and should not be labelled and purified in a cell surface fraction. All of these experiments need to be repeated and reanalysed with a suitable cell surface housekeeper (e.g. Na/K ATPase).
3. Fig 4 - no information is provided about the relative expression of CD36 in these models. The immunofluorescence presented in Fig 4A suggests expression is increased. Is this the case?
4. Fig 4A, 4F - CD36 is an integral membrane protein, yet the appearance of the CD36 immunofluorescent staining presented is not consistent with this. Particularly in the control group, this looks like a cytosolic localisation. If the CD36 was intracellular it would be punctate representing a vesicle / ER / Golgi location. I question the specificity of the antibody staining in this panel.
5. Fig 5D,E,F,G - It is a significant weakness of the experiments presented that they rely almost exclusively on the use of a very poor tool compound in 2-BP (see ACS Chem. Biol. 2013, 8, 9, 1912-1917). This is a largely discredited approach to manipulating palmitoylation in cells, and it detracts from the quality of the manuscript to rely on this tool. Replace these experiments with shRNA. The same for Figure S4.
6. Fig 6,7 - justify the choice of experimental model. Why are these experiments conducted in the presence and absence of KLF10 overexpression? If CD36 is an important end effector for development of NASH, the CD36 knockouts should be resistant to onset of NASH induced by HFD/CCl4 (without overexpression of KLF10). This important mechanistic insight is missing from the manuscript.
7. Fig 7 - is there a significant difference in NASH outcomes between the groups CD36-OE and mCD36-OE in the absence of KLF10 overexpression? According to the paradigm presented, if palmitoylation of CD36 is an important step in the pathway to NASH, these groups should be different from each other.
8. Discussion - the paper that identified zDHHCs 4 & 5 as palmitoylating CD36 specifically ruled out palmitoylation of CD36 by zDHHC7 (see figure 1 of Cell Reports Volume 26, Issue 1, Pages 209-221.e5). This should be acknowledged and discussed.

Minor
1. Lay summary - language is too technical throughout.
2. Throughout - the family of palmitoylating enzymes was renamed from 'DHHC' to 'zDHHC' several years ago when they were renumbered. Correct this throughout text, figures and legends.
3. Introduction 'Interestingly, recent studies suggest that CD36 protein can be palmitoylated, at cysteines residues (cys) 3, 7, 464, and 466' - the paper cited is 25 years old. Not recent!
4. Fig 1A - clarify n number. Legend indicates 3 per group, but the heat map appears to say 5 per group?
5. Fig 1C - is KLF10 expression normalised to the housekeeper in the bar chart? The same for other western blots (e.g. Fig 3B).
6. Fig 1D - if KLF20 is a transcription factor why is all the staining outside the nucleus? What does the bar chart show? KLF20 positive cells per field of view?
7. Fig 3A - what is the negative control used in these experiments?

Referee #3:

The manuscript by Yang et al. provides new insights in the role of KLF10 in the development of NASH. The authors found the KLF10 in liver is upregulated in a mouse model of NASH and in human NASH patients. Ectopic expression in mouse liver enhanced NASH progression. Mechanistically, the authors show that KLF10 induced expression of the palmitoyl transferase Dhhc7, which in turn triggered CD36 palmitoylation. Both, Dhcc7 induction and CD36 palmitoylation, were required for the KLF10-driven NASH progression. Liver-specific deletion of KLF10 reduced Dhcc7 expression and CD36 palmitoylation, rendering these mice resistant to the induction of NASH.
Regarding the data shown, the reviewer has very little criticism and it all appears to nicely support the drawn conclusions. However, a variety of interpretational and methodological issues and omissions should be corrected in order to evaluate whether this manuscript would be suitable for publication in EMBO reports.
1. Please provide detailed genetic information and background on the genetic mouse models used (CD36KO mice, KLF10flox/flox , and Alb-cre), even though purchased from a company, these details may be relevant
2. Since so central to the presented data, please provide detailed information (cloning, generation, titering etc.) on the used AAVs, which appear to be completely missing from the methods section. Were AAV's indeed used to infect primary mouse hepatocytes? At which time point after infection were hepatocytes in Fig. S6 analyzed?
3. Quantification of western blots do not appear plausible, such as in 2B and 2I, 3F, and S1J - just to name a few- differences appear rather big when looking at the blots but end up as 1.5-2 fold different in the densitometric analysis.
4. Individual n-numbers in each figure should be checked since for instance in Fig. 1A a heatmap with n=5 is presented but described in the figure legend with n=3, the same in Fig. S2A.
5. Please provide at least a list of genes regulated by KLF overexpression in HepG2 genes. Ideally you may deposited all RNAseq raw data to something like GEO.

6. Please incorporate interpretation of data of others in regard to genomic binding of KLF10 in liver, which does exist (PMID: 34402428). Was KLF10 binding to the Dhcc7 promoter also observed by others?
7. ChIP results (3I) should not be visualized by semiquantitative PCR in 2021, please repeat by using quantitative PCR.
8. An interpretation of the described results in light of what is known about KLF10 in liver regarding its regulation by ChREBP (PMID: 21856285) and regarding its known role in circadian control and metabolism (PMIDs: 20385766, 34402428, 33396939) is missing.
9. Accordingly, the underlying reasons for conflicting findings with PMID: 34402428 should be at least discussed.
10. The idea that a single target gene of KLF10, Dhhc7, mediates a CD36-dependent mechanism that is responsible for most of this transcriptions factors effects on triglyceride accumulation and fibrosis should be discussed in light of the identified overall KLF10 responsive genes (Fig. 1A and Table S4)
Minor concerns:
A) Fig. 3G has a small and misplaced 2.0 in its right x-axis
B) Fig. 5 contains 'member' protein which probably refers to 'membrane...

Title: KLF10 promotes nonalcoholic steatohepatitis progression through transcriptional activation of zDHHC7

No. EMBOR-2021-54229-T

Dear Editors and Reviewers:

Thank you very much for giving us an opportunity to revise our manuscript. We appreciate the constructive comments and suggestions by the Editors and the Reviewers regarding our manuscript. We have read those comments carefully and addressed them at our best. Especially, we have performed additional experiments to address the concerns raised by Reviewer 2. We have also provided more experimental, interpretational and methodological information to address the concerns raised by Reviewers 1 and 3. In addition, the title, text and figures, have been revised according to the Editors' and Reviewers' suggestions. Those changes or corrections have been highlighted in RED in the revised manuscript. We hope our study in current form is of potential interest to your readership.

Thank you very much for your consideration.

Referee #1:

This is extensive and very detailed work on the role of KLF10 in lipid-induced liver inflammation. Overall, the conclusions are supported by the data.

Reply: We greatly appreciate the positive comments from Reviewer #1.

Minor comments:

1. Please include body weights for all mouse studies as body weight is an

important denominator of liver weight, NAFLD, and NASH.

Reply: We appreciated the comment. We apologize for the missing data regarding body weight. We have added the results and descriptions in the revised manuscript. Please see the data in the Figures 2E, 2M, 6E, 7C, 8C, EV1B and EV1F.

2. Please include all uncropped western blots with detailed legends in the supplement.

Reply: We appreciated the comment. All of all uncropped western blots were included in the Figure EV5.

Referee #2:

Yang et al present evidence that the palmitoyl transferase zDHHC7 is a key player in nonalcoholic steatohepatitis. Upregulation of the transcription factor KLF10 transcriptionally activates zDHHC7, which is found to palmitoylate the fatty acid transporter CD36. CD36 palmitoylation promotes its cell surface expression and increases hepatic lipid uptake, leading to hepatic inflammation.

In general the conclusions of the manuscript are well-supported by the data presented. The authors have used appropriate model systems and the genetic evidence for the pathways identified is strong. Having identified upregulated KLF10 expression (Fig 1) they go on to overexpress KLF10 and demonstrate exacerbation of NASH induced by WD/CCl4 and exacerbation of NAFL promoted by a high fat diet (Fig 2). KLF20 overexpression is found to upregulate the Golgi-localised palmitoylating

enzyme zDHHC7 through a KLF10 binding site in the zDHHC7 promoter (Fig 3). zDHHC7 is found to be required for cell surface expression of CD36 in cellular models (Fig S3) and KLF overexpression is found to increase palmitoylation and surface localisation of CD36 in the liver (Fig 4). KLF10-induced increases in CD36 palmitoylation and cell surface expression are found to be zDHHC7 dependent (Fig 5). CD36 knockout animals are found to be resistant to NASH induced by diet and KLF10 overexpression (Fig 6). Rexpression of wild type but not non-palmitoylated CD36 in vivo 'restores' diet and KLF10-induced NASH (Fig 7). KLF10 knockout in the liver is found to reduce zDHHC7 expression, CD36 palmitoylation,

The reliance on the pharmacological tool 2-bromopalmitate to support some conclusions is a weakness, as the specificity of this reagent is very poor. Genetic evidence supporting the proposed role for zDHHC7 in vivo (e.g zDHHC7 knockout) is not provided, but in general the evidence provided in cellular models that ZLF10 upregulates zDHHC7 to increase CD36 palmitoylation is strong.

Reply: We greatly appreciate the positive comments from Reviewer #2. We have performed additional experiments to address the concerns, including the use of zDHHC7 knockdown cellular models.

Major

1. Fig 2B - KLF10 is almost undetectable in the control group. How is it possible to quantify this accurately to present a fold-change in expression?

Reply: We appreciated the comment. We apologize for the unclear

western blots and have repeated the experiments by increasing the loading amount of proteins. The quantitation was re-analyzed accordingly. Please see the results in the Figure 2B and 2J in the revised manuscript.

2. The authors use a biotinylation assay to evaluate surface expression of proteins of interest - e.g. CD36 (Fig S3E, S3I, 5C, 7I, 8L and maybe others). Actin is presented as a loading control throughout. I have serious reservations about the validity of these assays, since actin is an intracellular protein and should not be labelled and purified in a cell surface fraction. All of these experiments need to be repeated and reanalysed with a suitable cell surface housekeeper (e.g. Na/K ATPase).

Reply: We appreciated the comment. We agree with the Reviewer that β-actin might not be suitable as a loading control for membrane proteins. Therefore, we repeated these experiments using Na/K ATPase (ATP1a1) as a loading control and re-analyzed the quantitation. Please see the results in the Fig 4C, 4H, 5C, 5F, 7I, 8L, EV2L, EV2P, and EV4E.

3. Fig 4 - no information is provided about the relative expression of CD36 in these models. The immunofluorescence presented in Fig 4A suggests expression is increased. Is this the case?

Reply: We appreciated the comment. We have examined the total protein expression of CD36 as suggested, which showed that KLF10 overexpression did not affect total CD36 expression in the liver. Please see the results in the Figure 4C and 4H.

In the immunofluorescence presented in Fig 4A, we aimed to detect the expression of CD36 on the plasma membrane, using β-catenin as a

marker for hepatocyte surface [Zhao L, et al. J Hepatol. 2018;69(3):705-717]. The staining and quantitation results indicated that KLF10 overexpression led to an increased localization of CD36 on the plasma membrane (Figure 4A and 4B).

4. Fig 4A, 4F - CD36 is an integral membrane protein, yet the appearance of the CD36 immunofluorescent staining presented is not consistent with this. Particularly in the control group, this looks like a cytosolic localisation. If the CD36 was intracellular it would be punctate representing a vesicle / ER / Golgi location. I question the specificity of the antibody staining in this panel.

Reply: We appreciated the comment. In the Figure 4A and 4F, the CD36 immunofluorescent staining looks like a cytosolic localization particularly in the control group. This could be due to the lower palmitoylation of CD36 in the control mice. As indicated by our results and previous study [Zhao L, et al. J Hepatol. 2018;69(3):705-717], lower palmitoylation decreased CD36 protein hydrophobicity and reduced its localization on the plasma membrane.

Furthermore, we performed CD36 immunostaining in HepG2 cells (Figure 5J), showing that CD36 is punctate representing a vesicle / ER / Golgi location, as described by the Reviewer. The CD36 antibody in our experiments (1: 100, Abcam, Cat#: ab23680) has also been used for immunofluorescence staining in previous studies [Nagao M, et al. Diabetes. 2020; 69(6):1193-1205; Hao JW, et al. Nat Commun. 2020; 11(1):4765; Wang J, et al. Cell Rep. 2019;26(1):209-221].

5. Fig 5D,E,F,G - It is a significant weakness of the experiments presented

that they rely almost exclusively on the use of a very poor tool compound in 2-BP (see ACS Chem. Biol. 2013, 8, 9, 1912-1917). This is a largely discredited approach to manipulating palmitoylation in cells, and it detracts from the quality of the manuscript to rely on this tool. Replace these experiments with shRNA. The same for Figure S4.

Reply: We appreciated the comment. To rule out the non-specific effects of 2-BP, we have performed additional experiments using zDHHC7 shRNA as suggested. In agreement, knockdown of zDHHC7 suppressed the roles of KLF10 overexpression on CD36 palmitoylation, plasma membrane localization, cellular lipid droplets accumulation and TG retention (Figure 5H-5L). Moreover, KLF10 overexpression-induced the interaction of CD36 with Fyn and Lyn, activation of JNK signaling, and suppression of AMPK signaling were also attenuated in HepG2 cells transfected with shRNA targeting zDHHC7 (Figure EV3F-3J). Taken together, our results demonstrated that zDHHC7 is required for KLF10-mediated CD36 palmitoylation and function.

6. Fig 6,7 - justify the choice of experimental model. Why are these experiments conducted in the presence and absence of KLF10 overexpression? If CD36 is an important end effector for development of NASH, the CD36 knockouts should be resistant to onset of NASH induced by HFD/CCl4 (without overexpression of KLF10). This important mechanistic insight is missing from the manuscript.

Reply: We appreciated the comment. In Figure 6 and 7, KLF10 overexpression or not was performed in CD36 wild-type, knockout and palmitoylation sites-mutant mice, respectively. In the Figure 6 of our original manuscript, our results showed that liver fibrosis, liver/body

weight ratio, hepatic TG contents, and serum AST and ALT levels were lower in CD36 knockout mice, compared with wild-type mice (Figure 6D-6G, column 1 versus column 3). These results demonstrated that CD36 knockout mice are resistant to the onset of NASH induced by WD/CCl_4 treatment. We apologize for the missing statistic comparison in the bar graph. We have added the comparisons and their descriptions in the revised Figure 6 and Figure 7.

7. Fig 7 - is there a significant difference in NASH outcomes between the groups CD36-OE and mCD36-OE in the absence of KLF10 overexpression? According to the paradigm presented, if palmitoylation of CD36 is an important step in the pathway to NASH, these groups should be different from each other.

Reply: We appreciated the comment. There is a significant difference in NASH outcomes between the groups CD36-OE and mCD36-OE in the absence of KLF10 overexpression. We have added the statistic comparisons in the bar chart of Figure 7A-7D (column 4 versus 2). Our results suggest that palmitoylation of CD36 is an important step in NASH progression, which is consistent with previous study [Zhao L, et al. J Hepatol. 2018; 69(3):705-717]. Thanks for the valuable suggestions.

8. Discussion - the paper that identified zDHHCs 4 & 5 as palmitoylating CD36 specifically ruled out palmitoylation of CD36 by zDHHC7 (see figure 1 of Cell Reports Volume 26, Issue 1, Pages 209-221.e5). This should be acknowledged and discussed.

Reply: We appreciated the comment. In the Figure 1F of Wang's study [Wang J, et al. Cell Rep. 2019; 26(1):209-221], the authors showed that

knockdown of zDHHC7 did not affect palmitoylation of CD36 in HEK293T cells. They further identified that DHHC4 and DHHC5 are required for CD36 palmitoylation in adipocytes. We speculate that the palmitoyl-acyltransferases for target proteins might be cell- or tissue-specific. For instance, zDHHC5 was shown to facilitate STAT3 palmitoylation in oligodendrocytes [Ma Y, et al. Glia. 2022; 70(2):379-392], while only overexpression of zDHHC7 and zDHHC3 could increase the palmitoylation level of STAT3 in HEK293T cells [Zhang M, et al. Nature. 2020; 586 (7829):434-439]. We have acknowledged and discussed this issue in the 'Discussion' section.

Minor

1. Lay summary - language is too technical throughout.

Reply: We appreciated the comment. We have revised the Lay summary as suggested.

2. Throughout - the family of palmitoylating enzymes was renamed from 'DHHC' to 'zDHHC' several years ago when they were renumbered. Correct this throughout text, figures and legends.

Reply: We appreciated the comment. We have corrected this throughout text, figure and legends as suggested.

3. Introduction 'Interestingly, recent studies suggest that CD36 protein can be palmitoylated, at cysteines residues (cys) 3, 7, 464, and 466' - the paper cited is 25 years old. Not recent!

Reply: We appreciated the comment. We apologize for the mistake and have revised the sentence as suggested.

4. Fig 1A - clarify n number. Legend indicates 3 per group, but the heat map appears to say 5 per group?

Reply: We appreciated the comment. We apologize for the mistake and have revised the Legend of Figure 1A as suggested.

5. Fig 1C - is KLF10 expression normalised to the housekeeper in the bar chart? The same for other western blots (e.g. Fig 3B).

Reply: We appreciated the comment. For the bar chart in Figure 1C and others, the expression of KLF10 and target proteins were quantified and normalized to the housekeeper as indicated. We have added the descriptions about normalization of western blots in each Figure Legend.

6. Fig 1D - if KLF10 is a transcription factor why is all the staining outside the nucleus? What does the bar chart show? KLF10 positive cells per field of view?

Reply: We appreciated the comment. We re-examined the KLF10 protein expression between the livers of NASH and NAFL mice using immunohistochemistry, which showed that KLF10 staining was mainly localized in the nucleus. Please see the new data in the Figure 1D, in which the bar chart showed the fold change of KLF10 positive cells per field (NASH mice vs. NAFL mice).

7. Fig 3A - what is the negative control used in these experiments?

Reply: We appreciated the comment. In the Figure 3A and 3B, mouse primary hepatocytes were transfected with lentiviruses expressing KLF10 or empty vector as a negative control. We have added the detailed

information in the ‘Methods’ and ‘Figure Legend’ sections in the revised manuscript.

Referee #3:

The manuscript by Yang et al. provides new insights in the role of KLF10 in the development of NASH. The authors found the KLF10 in liver is upregulated in a mouse model of NASH and in human NASH patients. Ectopic expression in mouse liver enhanced NASH progression. Mechanistically, the authors show that KLF10 induced expression of the palmitoyl transferase Dhhc7, which in turn triggered CD36 palmitoylation. Both, Dhcc7 induction and CD36 palmitoylation, were required for the KLF10-driven NASH progression. Liver-specific deletion of KLF10 reduced Dhcc7 expression and CD36 palmitoylation, rendering these mice resistant to the induction of NASH.

Regarding the data shown, the reviewer has very little criticism and it all appears to nicely support the drawn conclusions. However, a variety of interpretational and methodological issues and omissions should be corrected in order to evaluate whether this manuscript would be suitable for publication in EMBO reports.

Reply: We greatly appreciate the positive comments from Reviewer #3. We have added more information to address interpretational and methodological issues as suggested.

1. Please provide detailed genetic information and background on the

genetic mouse models used (CD36KO mice, KLF10flox/flox , and Alb-cre), even though purchased from a company, these details may be relevant

Reply: We appreciated the comment. CD36 KO mice (B6/JGpt-*Cd36*^{em11Cd1894}/Gpt, Strain No. T010474), KLF10 flox/flox (B6/JGpt-*Klf10*^{e}m1Cflox/Gpt, Strain No. T018201), and Albumin-Cre mice (B6/JGpt-H11$^{em1Cin(Alb-iCre)}$/Gpt, Strain No.T003814) based on C57BL/6J background. were obtained from Gempharmatech Co., Ltd. (Nanjing, Jiangsu, China). We have added this information in the 'Methods' section in the revised manuscript.

2. Since so central to the presented data, please provide detailed information (cloning, generation, titering etc.) on the used AAVs, which appear to be completely missing from the methods section. Were AAV's indeed used to infect primary mouse hepatocytes? At which time point after infection were hepatocytes in Fig. S6 analyzed?

Reply: We appreciated the comment. In our study, AAV was used to infect mouse livers, whereas lentivirus was used to infect cultured cells, including mouse primary hepatocytes and HepG2 cells. The detailed information of AAVs and lentivirus has been provided in the 'Methods' section in the revised manuscript.

3. Quantification of western blots do not appear plausible, such as in 2B and 2I, 3F, and S1J - just to name a few- differences appear rather big when looking at the blots but end up as 1.5-2 fold different in the densitometric analysis.

Reply: We appreciated the comment. We have re-quantified the western blots results as suggested.

4. Individual n-numbers in each figure should be checked since for instance in Fig. 1A a heatmap with n=5 is presented but described in the figure legend with n=3, the same in Fig. S2A.

Reply: We appreciated the comment. We apologize for the mistake and have checked the Figure Legends as suggested. The n-numbers have been added in each Figure Legend.

5. Please provide at least a list of genes regulated by KLF overexpression in HepG2 genes. Ideally you may deposited all RNAseq raw data to something like GEO.

Reply: We appreciated the comment. The top 10 upregulated genes and 10 downregulated genes by KLF10 overexpression have been shown in Figure 3A. Besides, our RNA-Seq raw data have been deposited in the NCBI's Gene Expression Omnibus database (GEO GSE197053 and GSE197054).

6. Please incorporate interpretation of data of others in regard to genomic binding of KLF10 in liver, which does exist (PMID: 34402428). Was KLF10 binding to the Dhcc7 promoter also observed by others?

Reply: We appreciated the comment. We have incorporated our results with recent studies regarding KLF10 and added some discussions about this issue in the 'Discussion' section as suggested. The regulation of zDHHC7 by KLF10 has not been reported before. However, as suggested by the Reviewer, we searched the ChIP-seq database (Cistrome, http://cistrome.org/db/#/) and found that the zDhhc7 promoter contains potential KLF10 binding site, which is consistent with our

experimental results. The ChIP-Seq analysis is shown as below.

Gene	Score	Coordinate	Visualize
ZDHHC7	1.647	chr16:84974174-85011534	WashU UCSC

7. ChIP results (3I) should not be visualized by semiquantitative PCR in 2021, please repeat by using quantitative PCR.

Reply: We appreciated the comment. The Figure 3I is the result of PCR gel, whereas Figure 3J is the result of quantitative real-time PCR. We have corrected the Figure Legend to make it clear.

8. An interpretation of the described results in light of what is known about KLF10 in liver regarding its regulation by ChREBP (PMID: 21856285) and regarding its known role in circadian control and metabolism (PMIDs: 20385766, 34402428, 33396939) is missing.

Reply: We appreciated the comment. We apologize for the missing information about studies on KLF10's metabolic roles. We have added the suggested literatures and discussed the Questions 8 and 9 in the 'Discussion' section.

9. Accordingly, the underlying reasons for conflicting findings with PMID: 34402428 should be at least discussed.

Reply: We appreciated the comment. In the findings of PMID: 34402428, the authors showed that KLF10 can mitigate glucose intolerance and hepatic steatosis in mice challenged with a sugar beverage. However, another study reported that hepatic KLF10 expression is elevated in the obese and diabetic mice [Yang X, et al. Diabetologia. 2017;

60(12):2443-2452]. Overexpression of KLF10 in the liver of C57BL/6J mice increased blood glucose levels and impaired glucose tolerance, while hepatic KLF10 knockdown in db/db and diet-induced-obese mice decreased blood glucose levels and improved glucose tolerance [Yang X, et al. Diabetologia. 2017; 60(12):2443-2452]. Although the reasons behind these inconsistent results remain unclear, the feeding and treatment conditions are different between these studies. High sugar consumption usually leads to simple steatosis, whereas western diet-feeding plus CCl4 treatment were used in our study to explore the potential function of KLF10 in the steatosis-to-NASH progression. Therefore, we speculate that the role of KLF10 in metabolic liver diseases might be diverse in the context of different physiological and pathological conditions.

10. The idea that a single target gene of KLF10, Dhhc7, mediates a CD36-dependent mechanism that is responsible for most of this transcriptions factors effects on triglyceride accumulation and fibrosis should be discussed in light of the identified overall KLF10 responsive genes (Fig. 1A and Table S4)

Reply: We appreciated the comment. We totally agree with the Reviewer that KLF10 may exert its pathogenic roles in NASH progression through additional targets. For instance, as described in the manuscript, we also found that Gadd45g was upregulated by KLF10 overexpression or in the livers of NASH mice (Table EV4). This protein has been shown to regulate cytokine expression in T cells [Schmitz I. et al. Adv Exp Med Biol. 2013; 793:51-68]. Whether KLF10 could promote hepatic inflammatory response through Gadd45g in the development of NASH remains to be

determined. Therefore, further studies are still needed to explore more transcriptional targets of KLF10 in the regulation of NASH progression. Our transcriptome screening may provide a valuable resource to investigate this issue. We have added some discussion about this issue in the 'Discussion' section as suggested.

Minor concerns:

A) Fig. 3G has a small and misplaced 2.0 in its right x-axis

Reply: We appreciated the comment. We have revised the Figure 3G as suggested.

B) Fig. 5 contains 'member' protein which probably refers to 'membrane...

Reply: We appreciated the comment. We have corrected the Figure 5C as suggested.

Dear Dr. Lu,

Thank you for submitting your revised manuscript. It has now been seen by one of the original referees.

As you can see, the referee finds that the study is significantly improved during revision and recommends publication. However, I need you to address the editorial points below before I can accept the manuscript.

• We note that there are currently 6 keywords and we can only accommodate 5. Please reduce the number of keywords to 5.
• We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Also, please rename the 'Conflict of Interests' section as 'Disclosure statement and competing interests'.
• We note that Table EV1 is too long to be an EV Table. Therefore, please upload it as Dataset EV1. Please upload the remaining 3 EV tables as individual files called Table EV1-Table EV3.
• Please change the title "Supplementary Figures and Tables" to "Expanded View Figure Legends".
• Please split the source data as one file per figure.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #2:

The authors have done an excellent job responding to reviewers' comments with additional experiments and analysis.

Title: KLF10 promotes nonalcoholic steatohepatitis progression through transcriptional activation of zDHHC7

No. EMBOR-2021-54229V2

Dear Editors:

We are glad to know that our manuscript would be accepted after some minor revisions. We appreciate the valuable comments and suggestions from the Editors and Reviewers during the revision process. We have revised the manuscript as suggested and provided a point-to-point response below. We hope our study in current form is of potential interest to your readership.

Thank you very much for your consideration.

The Editorial Points:

- We note that there are currently 6 keywords and we can only accommodate 5. Please reduce the number of keywords to 5.

Reply: We have reduced the number of keywords to 5. Thanks.

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Also, please rename the 'Conflict of Interests' section as 'Disclosure statement and competing interests'.

Reply: We have renamed the section as 'Disclosure statement and competing interests'. We declare that we have no conflict of interest.

- We note that Table EV1 is too long to be an EV Table. Therefore, please upload it as Dataset EV1. Please upload the remaining 3 EV tables as individual files called Table EV1-Table EV3.

Reply: The original Table EV1 have been uploaded as Dataset EV1. The remaining 3 EV tables have been revised as Table EV1-EV3. Thanks.

- Please change the title "Supplementary Figures and Tables" to "Expanded View Figure Legends".

Reply: The title has been changed to "Expanded View Figure Legends". Thanks.

- Please split the source data as one file per figure.

Reply: The source data have been split as one file per figure. Thanks.

- Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes

activated.

Reply: We have seen the points and clarified them in the figure legends using track changes activated.

2nd Revision - Editorial Decision **11th Apr 2022**

Dear Dr. Lu,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

--
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Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Table EV2-EV4

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods
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Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Materials and Methods
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods, Figure Legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Materials and Methods
Studies involving **human participants**: 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.	Yes	Materials and Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
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State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	Materials and Methods
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Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Figure Legends