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p53 transcriptionally regulates SQLE to repress cholesterol synthesis and tumor growth

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1st Editorial Decision 15th Mar 2021

Dear Prof. Du,

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

I apologize for this unusual delay in getting back to you. It took longer than anticipated to receive the referee reports.

We concur with the referees that the proposed mechanism by which p53 suppresses cholesterol metabolism in principle very interesting. However, referees also raise significant concerns that need to be addressed to consider publication here. In particular,

1. stronger evidence supporting that p53 represses SQLE transcription independent of SREBP2 is required (with multiple SREBP2 RNAi's and rescue with SREBP2 restoration)
2. more mechanistic insight is required as to how p53 represses SQLE transcription (referee #1 point 2, referee #3 paragraph 3). Given the known role of p53 in regulation of cholesterol metabolism, this point is essential to address.
3. Currently, the luciferase assays do not sufficiently support the proposed inhibition of SQLE transcription by p53 (referee #1 point 2, referee #2, minor point 4, referee #3 paragraphs 3, 6, 7).

If you are unable to provide more insight into how p53 regulated cholesterol mechanism promotes liver cancer progression (referee #2, standfirst, and first major point, referee #3 paragraph 8), this will not preclude from publication.

Given these positive recommendations, we would like to invite you to submit a revised manuscript. 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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We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact the scientific editor handling your manuscript to discuss a revision plan should you need additional time, and also if you see a paper with related content published elsewhere.***

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 providing access to data deposited in public databases is missing

(where applicable).
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.

Please note that for all articles published beginning 1 July 2020, the EMBO Reports reference style will change to the Harvard style for all article types. Details and examples are provided at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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.

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 (). 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 (). Please find instructions on how to link your ORCID ID to

your account in our manuscript tracking system in our Author guidelines ().

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) 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 .

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 .

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).

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.

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

In the manuscript 'p53 transcriptionally regulates SQLE to modulate cholesterol and tumour growth' the authors show that p53 binds the promoter of SQLE, downregulate its expression and prevents cholesterol accumulation'. Previous studies have shown that p53 can control the cholesterol synthesis pathway by inhibiting SREBP mediated transcription. In this study the authors show that p53 directly regulates SQLE in an SREBP-independent manner in mice and in cell lines. The manuscript is well-written, has lots of validation experiments and is well-structured. The main novelty is the direct regulation of SQLE by p53. A worry is the contradiction of the data with the work by Jun et al showing that loss of SQLE promotes colon cancer. This needs to be more thoroughly discussed.

Major comments:
1. Figure 2G, the maturity status (P and M) of SREBP is not explained at that point in the text
2. There is considerable doubt in the field whether or not p53 can directly downregulate target genes (Fisher et al Oncogene 2017, Sullivan et al CDD, 2017). In this manuscript the authors show

that p53 directly binds to a p53 RE in SQLE. In luciferase assays the authors actually see an increased expression of SQLE and not a decrease in expression in the presence of p53. Many other p53 repressed genes have been evaluated and many turn out to not be direct repressed targets. The majority of these do have p53 REs in the promoter region, but are actually regulated by p130/107. Is downregulation of SQLE dependent on p130/107? In addition, does a high fat diet increase the amount of p53 bound to RE2?
3. Figure 6I is showing such a short amount of time and such small differences that a Kaplan Meyer is not suitable. With showing J, this figure is not necessary.
4. In Figure 7I it is not clear if authors only looked at macroscopic quantification or also included histological data to see if any of the terbinafine treated mice had tumours.
5. Contrary to promoting tumourigenesis, Jun et al discovered that reduced SQLE enhanced colorectal cancer progression. This paper is cited in the discussion once. The authors see that in the colorectal cancer cell line HCT116 cells, loss of p53 promotes SQLE expression and promotes tumour formation. In the Jun et al paper normal conditions, reveal that a reduced SQLE expression reduces p53 expression and that loss of SQLE promotes tumourigenesis of HCT116 cells. This needs to be further discussed and not just mentioned as one line in the discussion. Do the authors see an increase in SQLE in the colon in fig 2C?

Minor comments:
1. It would be useful to show the timeline of Supplemental figure 1 in the main figure
2. There are some typos in the text and figures. Page 13, second paragraph, examined should be examine, 6M Y-axis should read senescence
3. It is unclear why in figure 8B the liver weight/ body weight was determined, while in none of the other figures liver weight was corrected for body weight.

Referee #2:

In this Ms, Sun and colleagues describe a new mechanism by which p53 negatively regulates cholesterol metabolism. This is an interesting Ms that somehow supports the importance of the p53 pathway in cholesterol metabolism during cancer development, a notion that was previously identified by the Prives Laboratory and then confirmed by other laboratories (Freed-Pastor et al., Cell 2012; Turrell et al., Genes and Dev 2017; Moon et al., Cell 2019). Interestingly, the effect of WT-p53 on cholesterol metabolism observed by Sun and collaborators is different than the one previously reported since it does not involve SREBP2, a master regulator of cholesterol biosynthesis. Here, the authors show compelling evidence that p53 directly binds and represses SQLE transcription and that p53-mediated control of cholesterol metabolism is important for liver carcinogenesis. The molecular mechanism identified by Sun and collaborators is different from the one previously described, which involves p53-mediated activation of ABCA1, a retrograde cholesterol transporter which activity regulates SREBP2 maturation. However, because the links between p53 and the mevalonate pathway are not entirely new, it would have been nice to provide further information about the mechanisms by which cholesterol biosynthesis promotes liver cancer development in the context of p53-deficient liver tumors. Is this mechanism implicating the production of isoprenoids as previously shown for Mut-p53 or is it through other mechanisms? The authors suggest that this is somehow linked to p53's ability to control cellular senescence but the data are not strong enough to sustain this conclusion. Hence, this Ms is of general interest but still requires a few improvements before publication. Moreover, a particular effort should be done to

correct the (too) many English syntax errors.

Major comments :
- The authors should try to identify the mechanisms by which increased cholesterol metabolism promotes liver cancer development. Based on previous data linking Mut-p53 to the production of isoprenoids, the authors should evaluate whether these effects are not linked to increased geranylation of proteins (this could be done using pharmacological inhibitors of geranylgeranyl transferase such as GGTI-2133), an effect that was also previously linked to increased YAP-TAZ activity. Obviously, increased cholesterol metabolism and SQLE expression have an effect on cell proliferation but what is the underlying mechanism?
- Their preliminary data obtained in HepG2 cells suggest that SQLE depletion promotes SA-b-gal staining in p53 proficient, but not in p53 deficient cells, suggesting a potential effect on senescence. Although this increase appears relatively modest (15% increase), this is a potentially interesting concept. However, I believe this is an overstatement since SA-b-Gal staining is not sufficient to claim an effect on cellular senescence and the authors must use other senescence markers to sustain this conclusion. By the way, it is very surprising to see so many SA-bGal positive cells in their p53+/+ HepG2 population in basal conditions? Since SA-bGal staining is very sensitive to confluency, the authors should pay a particular attention to cell density when performing these analyses. Was that effect also observed in HCT116-p53+/+ siSQLE cells? Hence, it's a pity that the authors have not followed on that (potentially) interesting observation? Is that mechanism illustrating an interesting feed-back loop implicating the control of p53 activity and cholesterol metabolism in cellular senescence?

Minor comments :
- Fig 1H. It is very difficult to judge on filipin expression based on the IF images shown in this panel. Although the quantification of the staining looks pretty convincing, better images with better contrast should be shown.
- Fig 1I. I don't understand why the NES for cholesterol biosynthesis-related genes is negative despite they are up-regulated in p53 KO cells????
- Fig 2C. In this panel, the authors assessed SQLE expression in various tissues of p53 KO mice. The increased expression of SQLE in p53 KO liver is very significant but I was a bit puzzled by the increased SQLE protein levels observed in several tissues including lungs and kidneys. In these tissues, the mRNA levels of Sqle do not seem to be higher, and I was wondering therefore if the authors are suggesting that p53 controls SQLE expression both at the transcriptional and post-transcriptional levels? The authors should quantify the WB shown in this panel to make sure that these differences are not just reflecting experimental fluctuations in their immunoblotting experiments.
- Fig 3. Although most of their data support a model where WT-p53 represses SQLE expression through the binding to the R2 element located in intron 1, this element somehow activates transcription in a p53-dependent manner when it's located in front of the luciferase reporter both in HCT116 and HEK293 cells. The authors should try to provide an explanation to this observation in the discussion.
- Fig 5. In this figure, the authors show that SQLE expression is higher in HCC compared to adjacent non tumoral tissue. Although the immunoblots look relatively convincing, it would have been nice to confirm this result by IHC.
- Fig 6D. This colony formation assay suggests that SQLE depletion decreases the capacity of both p53+/+ and -/- cells to form colonies. However, it is difficult to appreciate this effect based on the pictures shown in the underneath panel. The pictures suggest that not only the number but also

the size of these colonies are very different in the 4 conditions (p53+/+ vs -/- with or wo the SQLE siRNA). The authors should show more representative pictures and/or quantify in a more appropriate manner the growth of these colonies.
- Fig 6 I. The authors measured tumor initiation and tumor volume in SQLE siRNA-treated HCT116. It is not clear what the authors exactly measured in Fig 6I? Is a detectable tumor based on palpation? This parameter of tumor growth is not extremely reliable and I believe the data showing tumor volume (Fig 6J) over time is sufficient. Nevertheless, I'm a bit skeptical that the siRNA is still efficiently depleting SQLE 28 days after transfection. I'm wondering why the authors have not considered using lentiviral delivery of shRNAs targeting SQLE which would have resulted in a more stable KD? Anyway, the authors should therefore show an immunoblot analysis of SQLE protein levels at the end of the experiment (day 28). If these data correspond to the results shown in panel 6L, then the authors should consider moving these data to panel 6J as validation results. They are less relevant to panel 6L. Actually, in this panel 6L, it is not indicated when exactly they measured intracellular cholesterol in these tumors? Were these analyses performed at the very end of the experiment (day 28)? The authors should clarify the legend of this figure...
- Fig 8E: in this figure, the authors characterized the effect of SQLE depletion on tumor development in vivo using p53 flox/flox; AlbCre mice. They performed Ki67 IHC on liver sections prepared from these animal models upon injection of AAV encoding shRNA SQLE. However, it is impossible to evaluate any effect on cell proliferation based on the pictures shown in this panel. The authors should show higher magnification microphotographs and should quantify these immunostainings. A quantification of the ORO staining would also clarify their conclusions on TG accumulation in these mice. p53 protein levels in the liver of these mice should also be assessed by immunoblotting as an important validation of the model.

English syntax errors must be corrected
They are too many to be listed but I've indicated here a few of them:
- In the main text "SQLE is the first oxygenation and rate-limited enzyme in cholesterol synthesis pathway". I guess the authors wanted to say that SQLE is an epoxidase?
- When the authors refer to HEPG2-p53 KO cells (generated by gene CrispR-Cas9 gene editing), they should not describe these cells as "p53-depleted cells" which is more appropriate for siRNA-mediated KD cells but less for KO cells.
- Fig 5A: "paracancerous tissue" should be replaced by non-cancerous adjacent tissue.

Bibliography
- More recent reviews describing the complex roles of p53 in metabolism have been published. The authors should consider adding these reviews or replacing the older ones.
- The metabolic functions of p53 in liver have also been reviewed recently (Krstic et al., 2018). I believe this review is highly relevant to this Ms and should be cited.

Referee #3:

The role of the tumor suppressor p53 in regulating cholesterol biosynthesis both as a wild-type protein and in tumor-associated mutants has been extensively studied. This has led to a key role for p53 regulation of the transcription factor SREBP2. In this manuscript, the authors propose an additional mechanism for this regulation that is independent of SREBP2 involving direct repression of the gene encoding the first enzyme in the cascade, SQLE.

Understanding mechanisms of action of p53 that are relevant to its tumor suppressor activity is an

important area of study. The ability of p53 to act as a direct transcriptional repressor has been controversial and the authors are providing new insights in this area. Thus, the manuscript attempts to provide new knowledge in a significant area. It is thus suitable for the readership of EMBO Reports. However, there are some substantial issues that need to be addressed before the study is suitable for publication. These are broadly related to tow main concerns.

First, whether p53 indeed represses SQLE expression and its underlying mechanism remains unclear.

Knockdown of SREBP2 or use of an SREBP2 inhibitor is used to show that SQLE basal expression is p53-dependent. It is unclear in these experiments (Figure 2H-I) what is the extent of the knockdown when comparing wild-type and p53-null cells. The immunoblots need to be quantitated, mRNA levels for SREBP2 need to be shown, and a restoration experiment with RNAI-resistant cDNA for SREBP2 need to be performed. Likewise, p53 levels appear to be changing with these treatments. p53 in the immunoblots needs to be quantitated as well.

Short term assays using inducible knockdown or increased expression of either p53 or SREBP2 would be most informative to reconcile some of these issues.

SREBP2 clearly regulates SQLE mRNA expression. It is difficult to interpret the findings in Figures 2H-K as basal level of SQLE is reduced in the various treatments and the conclusion is based on a fold-difference that is not quantitated or shown to be statistically significant.

Luciferase assays using this site of p53 occupancy show transcriptional activation rather than repression. The authors need to address this conundrum.

Second, the authors attempt to propose a model related to conditions of low versus normal sterol levels to explain findings. Yet, this is not directly experimentally addressed in these studies. There is a conceptual gap in that, it is suggested that p53-dependent down-regulation of SQLE occurs in normal sterol conditions. Yet, downregulation of SQLE leads to reduction in cholesterol levels. This should then now trigger the low-sterol response proposed by the Prives laboratory. There is a need for the authors to better justify how sterol conditions determine which mechanism is relevant and to integrate their findings better with the published literature.

Additional points:
The quality of the EMSA show in Figure 3E is poor and needs to be quantitated with statistics provided.

There are numerous spelling and grammatical errors. It is suggested that the authors have the manuscript edited by a native Englisher speaker.

The tumor data showing differences in levels of SQLE from normal tissue is intriguing. It is likely p53 status for these samples is available. This should be discussed. Otherwise this comparison does not shed new light on underlying mechanisms.

Response to Referee #1:

In the manuscript 'p53 transcriptionally regulates SQLE to modulate cholesterol and tumour growth' the authors show that p53 binds the promoter of SQLE, downregulate its expression and prevents cholesterol accumulation'. Previous studies have shown that p53 can control the cholesterol synthesis pathway by inhibiting SREBP mediated transcription. In this study the authors show that p53 directly regulates SQLE in an SREBP-independent manner in mice and in cell lines. The manuscript is well-written, has lots of validation experiments and is well-structured. The main novelty is the direct regulation of SQLE by p53. A worry is the contradiction of the data with the work by Jun et al showing that loss of SQLE promotes colon cancer. This needs to be more thoroughly discussed.

We very much appreciate the reviewer's positive comments on our work. We are also grateful for the reviewer's insightful and constructive critiques. As detailed below, we have performed a large number of experiments to address these comments, which we believe greatly improves the manuscript.

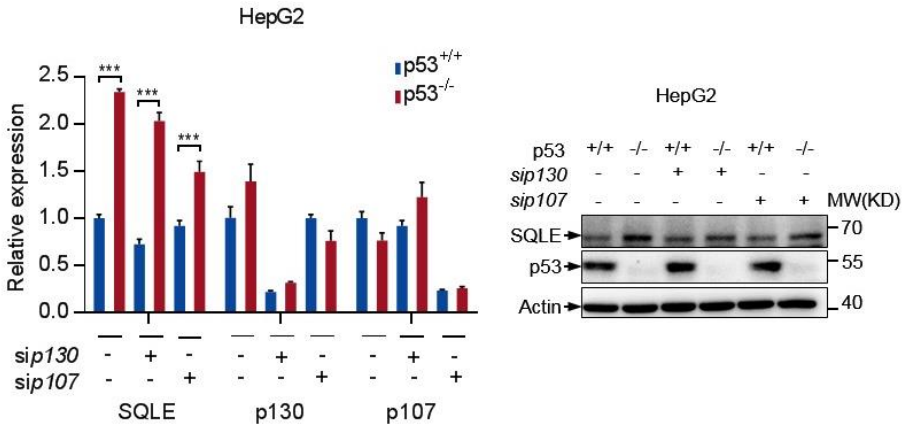
Major comments:
1. Figure 2G, the maturity status (P and M) of SREBP is not explained at that point in the text

We appreciate the referee for carefully reading the manuscript and for the constructive remarks. We have stated the status (P and M) of SREBP in the revised text.

2. There is considerable doubt in the field whether or not p53 can directly downregulate target genes (Fisher et al Oncogene 2017, Sullivan et al CDD, 2017). In this manuscript the authors show that p53 directly binds to a p53 RE in SQLE. In luciferase assays the authors actually see an increased expression of SQLE and not a decrease in expression in the presence of p53. Many other p53 repressed genes have been evaluated and many turn out to not be direct repressed targets. The majority of these do have p53 REs in the promoter region, but are actually regulated by p130/107. Is downregulation of SQLE dependent on p130/107?

We thank the referee for this insightful suggestion. To answer this question, we knocked down p130/107 using siRNA. As shown in the figure below (panel a and b), in p130 /107-depleted cells, p53 still led to reduction in SQLE both mRNA and protein levels. However, silencing p107 diminished the difference of SQLE levels between $p53^{+/+}$ cells and $p53^{-/-}$ cells, which suggest that downregulation of SQLE by p53 may be partially dependent on p107. Moreover, p130/107 knockdown decreased SQLE expression in $p53^{-/-}$ cells but not $p53^{+/+}$ cells, which means p53 may also be involved in p130/107-regulated SQLE. Overall, these data suggest that p53 coordinates with p130/107 to regulate SQLE.

Because of the large amount of data that are already in the revised manuscript, these data are not included. But we will present these data if the referee prefers.



$p53^{+/+}$ and $p53^{-/-}$ HepG2 cells were treated with control, p130 siRNA or p107 siRNA as indicated for 48 hr. mRNA levels were detected by qRT-PCR (panel a) and proteins expression (panel b) was analyzed by western blot.

In addition, does a high fat diet increase the amount of p53 bound to RE2?

To address this important issue, we performed ChIP assay using liver tissues from normal or high fat diet (HFD) p53 wildtype mice. As shown in revised Fig. EV3F, a high fat diet didn't increase the amount of p53 bound to RE2.

3. Figure 6I is showing such a short amount of time and such small differences that a Kaplan Meyer is not suitable. With showing J, this figure is not necessary.

As the referee suggested, we removed Figure 6I in revised manuscript.

4. In Figure 7I it is not clear if authors only looked at macroscopic quantification or also included histological data to see if any of the terbinafine treated mice had tumours.

We thank the referee for this comment. We apologized that we haven't stated this clearly in the previous version of the manuscript. In Figure 7I (revised Fig. 7L), we looked at macroscopic quantification to determine whether the mice had tumor or not, which was also confirmed by H&E staining.

5. Contrary to promoting tumourigenesis, Jun et al discovered that reduced SQLE enhanced colorectal cancer progression. This paper is cited in the discussion once. The authors see that in the colorectal cancer cell line HCT116 cells, loss of p53 promotes SQLE expression and promotes tumour formation. In the Jun et al paper normal conditions, reveal that a reduced SQLE expression reduces p53 expression and that loss of SQLE promotes tumourigenesis of HCT116 cells. This needs to be further discussed and not just mentioned as one line in the discussion. Do the authors

see an increase in SQLE in the colon in fig 2C?

We thank the refer for raising this point. In the Jun et al paper, they found that SQLE reduction promoted CRC (colorectal cancer) aggressiveness through a combination of senescence bypass, anoikis resistance, EMT (epithelial-mesenchymal transition) and the generation of MCSCs (migrating cancer stem cell)(Jun et al., 2020). However, they discovered that reduction SQLE decreased p53 level, promoted cell survival and invasiveness when HCT116 cells were cultured in ULA surface plates to mimic an anoikis condition. Here we found that p53 repressed SQLE expression to mediate tumor suppression, which is consistent with the study by Moon et al (Moon et al., 2019). These observations indicate that when cells are under non-apoptotic condition, upregulation of SQLE by p53 loss to maintain cell growth and cellular cholesterol level. However, when cells are under anoikis condition, cholesterol-dependent reduction of SQLE protects cell from death through reducing p53 level, subsequently leads to cell survival and invasiveness. The reciprocal regulation between SQLE and p53 is likely a key mechanism that modulates cell growth and invasion. We have now discussed this in the revised manuscript.

As suggested by the referee, we have examined SQLE expression in colon tissue. As shown in revised Fig. 2C, p53 deficiency led to an increase in SQLE both mRNA and protein levels in mice colon tissue.

Minor comments:

1. It would be useful to show the timeline of Supplemental figure 1 in the main figure

As suggested, we have now shown the timeline in the main figure (revised Fig. 1A).

2. There are some typos in the text and figures. Page 13, second paragraph, examined should be examine, 6M Y-axis should read senescence

We appreciate the referee for reading our manuscript carefully. We have now thoroughly checked the manuscript for typos and grammatical mistakes.

3. It is unclear why in figure 8B the liver weight/ body weight was determined, while in none of the other figures liver weight was corrected for body weight.

We thank the referee for this insightful comment. As suggested, we have now replaced figure 8B with the data of liver weight. Please see the revised Fig. 8B.

Response to Referee #2:

In this Ms, Sun and colleagues describe a new mechanism by which p53 negatively regulates cholesterol metabolism. This is an interesting Ms that somehow supports the importance of the p53 pathway in cholesterol metabolism during cancer development, a notion that was previously identified by the Prives Laboratory and then confirmed by other laboratories (Freed-Pastor et al., Cell 2012; Turrell et al., Genes and Dev 2017; Moon et al., Cell 2019). Interestingly, the effect of WT-p53 on cholesterol metabolism observed by Sun and collaborators is different than the one previously reported since it does not involve SREBP2, a master regulator of cholesterol biosynthesis. Here, the authors show compelling evidence that p53 directly binds and represses SQLE transcription and that p53-mediated control of cholesterol metabolism is important for liver carcinogenesis. The molecular mechanism identified by Sun and collaborators is different from the one previously described, which involves p53-mediated activation of ABCA1, a retrograde cholesterol transporter which activity regulates SREBP2 maturation. However, because the links between p53 and the mevalonate pathway are not entirely new, it would have been nice to provide further information about the mechanisms by which cholesterol biosynthesis promotes liver cancer development in the context of p53-deficient liver tumors. Is this mechanism implicating the production of isoprenoids as previously shown for Mut-p53 or is it through other mechanisms? The authors suggest that this is somehow linked to p53's ability to control cellular senescence but the data are not strong enough to sustain this conclusion. Hence, this Ms is of general interest but still requires a few improvements before publication. Moreover, a particular effort should be done to correct the (too) many English syntax errors.

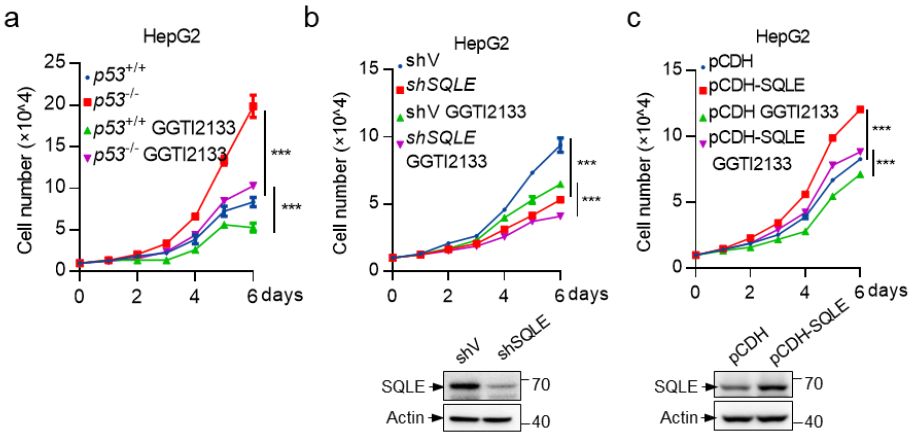
We thank the referee for considering the topic to be interesting and appreciate the insightful and constructive comments on our work.

Major comments :
- The authors should try to identify the mechanisms by which increased cholesterol metabolism promotes liver cancer development. Based on previous data linking Mut-p53 to the production of isoprenoids, the authors should evaluate whether these effects are not linked to increased geranylation of proteins (this could be done using pharmacological inhibitors of geranylgeranyl transferase such as GGTI-2133), an effect that was also previously linked to increased YAP-TAZ activity. Obviously, increased cholesterol metabolism and SQLE expression have an effect on cell proliferation but what is the underlying mechanism?

We thank the reviewer for the insightful question on how increased cholesterol metabolism promotes liver cancer development. Cholesterol is an important

component of cellular membranes and serves as a precursor for steroid hormones and bile acids. Cholesterol can also modulate signaling pathways involved in tumorigenesis and cancer progression. Additionally, cholesterol-derived metabolites play complex roles in supporting cancer progression and suppressing immune responses. Geranylgeranylation of proteins, a branch of the cholesterol synthesis pathway, is required for maintaining the stemness of breast cancer cells (Freed-Pastor et al., 2012). To evaluate whether the effect of SQLE and p53 on cell proliferation is linked to increased geranylation of proteins, we treated cells with geranylgeranyl transferase inhibitor GGTI-2133. As shown in the figure below, GGTI-2133 treatment decreased cell proliferation in both $p53^{+/+}$ and $p53^{-/-}$ cells and diminished the difference between these two cell lines (panel a). GGTI-2133 addition reduced cell growth and diminished the difference between control cells and SQLE-depleted cells (panel b). Similar results were obtained when we treated SQLE stably expression cells with GGTI-2133 (panel c). These data suggest that the effect of SQLE and p53 on cell growth is partially dependent on geranylation of proteins.

Because of the large amount of data that are already in the revised manuscript, this data is not included. But we will present these data if the referee prefers.



a. Proliferation of $p53^{+/+}$ and $p53^{-/-}$ HepG2 cells treated with DMSO or GGTI-2133 (1μM).
b. Growth of $p53^{+/+}$ HepG2 cells stably expressing control or SQLE shRNA in the presence or absence of GGTI-2133 (1μM). SQLE protein was examined by western blotting.
c. $p53^{+/+}$ HepG2 cells stably overexpressing SQLE or vector control were treated with or without GGTI-2133 (1μM). Cell proliferation is shown. SQLE protein is also shown.

- Their preliminary data obtained in HepG2 cells suggest that SQLE depletion promotes SA-b-gal staining in p53 proficient, but not in p53 deficient cells, suggesting a potential effect on senescence. Although this increase appears relatively modest (15% increase), this is a potentially interesting concept. However, I believe this is an overstatement since SA-b-Gal staining is not sufficient to claim an effect on cellular senescence and the authors must use other senescence markers to sustain this conclusion. By the way, it is very surprising to see so many SA-bGal positive cells in their p53+/+ HepG2 population in basal conditions? Since SA-bGal staining is very sensitive to confluency, the authors should pay a particular attention to cell density

when performing these analyses. Was that effect also observed in HCT116-p53+/+ siSQLE cells? Hence, it's a pity that the authors have not followed on that (potentially) interesting observation? Is that mechanism illustrating an interesting feed-back loop implicating the control of p53 activity and cholesterol metabolism in cellular senescence?

We appreciate the referee for this helpful suggestion. The induction of senescence in SQLE knockdown cells was also examined by the marked accumulation the promyelocytic leukaemia protein nuclear bodies (Ferbeyre et al., 2000, Pearson et al., 2000). As shown in revised Fig EV5I, knockdown of SQLE in HepG2 cells strongly enhances the formation of the PML-NBs in $p53^{+/+}$ cells. Similar to the SA-β-Gal staining data (revised Fig. 6L and Fig. EV5H), in p53-deficient cells, senescence decreased markedly and SQLE depletion lost its ability to induce this phenotype.

We thank the referee for raising this important issue. High population of SA-β-Gal positive cells in $p53^{+/+}$ HepG2 cells may be due to high cell density. We have reperformed this experiment in HepG2 cells with low cell density. Please see revised Fig. 6L.

Moreover, we investigated whether cholesterol is important for p53-induced cell senescence. When cells were cultured in LPDS medium, cholesterol addition indeed decreased p53-induced cell senescence, but not in p53-deficient cells (revised Fig. EV5J).

In sum, p53 is critical for the induction and maintenance of senescence (Campisi and d'Adda di Fagagna, 2007, Vousden and Prives, 2009, Ben-Porath and Weinberg, 2005). Our study found that p53 repressed SQLE expression, and downregulation of SQLE induced senescence through p53. Additionally, cholesterol could reduce p53-induced cell senescence under low-sterol conditions. As the referee mentioned, this may suggest there is an interesting feed-back loop implicating the control of p53 activity and cholesterol metabolism in cellular senescence.

Minor comments :

- Fig 1H. It is very difficult to judge on filipin expression based on the IF images shown in this panel. Although the quantification of the staining looks pretty convincing, better images with better contrast should be shown.

As suggested, we have now shown the images with better contrast in Fig. 1H (revised Fig. 1J).

- Fig 1I. I don't understand why the NES for cholesterol biosynthesis-related genes is negative despite they are up-regulated in p53 KO cells????

A positive value indicates correlation with the first phenotype and a negative value indicates correlation with the second phenotype. Therefore, the data in previous version of manuscript means cholesterol biosynthesis genes are correlated with $p53^{-/-}$

phenotype (second phenotype). For better visualization, we re-analyzed the data and replaced Fig. 1I with the figure with a positive value of NES (revised Fig. 1L). We apologize for this and thank the referee for raising this issue.

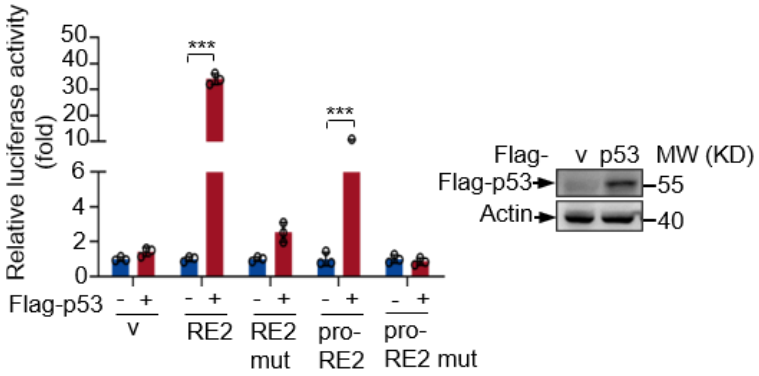
- Fig 2C. In this panel, the authors assessed SQLE expression in various tissues of p53 KO mice. The increased expression of SQLE in p53 KO liver is very significant but I was a bit puzzled by the increased SQLE protein levels observed in several tissues including lungs and kidneys. In these tissues, the mRNA levels of Sqle do not seem to be higher, and I was wondering therefore if the authors are suggesting that p53 controls SQLE expression both at the transcriptional and post-transcriptional levels? The authors should quantify the WB shown in this panel to make sure that these differences are not just reflecting experimental fluctuations in their immunoblotting experiments.

We thank the referee for raising this issue. We agree with the referee, the transcriptional regulation of SQLE by p53 may be tissue-type dependent. In lung and kidney tissues, p53 controls SQLE expression might be at post-transcriptional levels. We are currently investigating the mechanism by which p53 regulates the protein expression of SQLE in these two tissues. For this study, we have now removed the data from these two issues in revised Fig. 2C if the referee agrees.

As suggested, we have quantified the WB bands using Image J software (revised Fig. 2C).

- Fig 3. Although most of their data support a model where WT-p53 represses SQLE expression through the binding to the R2 element located in intron 1, this element somehow activates transcription in a p53-dependent manner when it's located in front of the luciferase reporter both in HCT116 and HEK293 cells. The authors should try to provide an explanation to this observation in the discussion.

We thank the referee for raising this point. In reporter assays, luciferase expression driven by genomic regions of p53-repressive target genes (EPCAM, CPS-1, OTC, ARG1, ME1 and PDK2) can be either promoted (for example, EPCAM response element (Sankpal et al., 2009) and CPS-1, OTC, ARG1 response element (Li et al., 2019), or suppressed (for example, ME1 (Jiang et al., 2013) and PDK2 response elements (Contractor and Harris, 2012) by p53. Like the EPCAM response element, SQLE response element (RE2) increased luciferase expression in response to p53 (revised Fig. 3H). This may be due to an unknown enhancer element is required for p53 to have a repressive effect in vivo. Thus, we examined whether SQLE promoter was required for the repressive effect of p53.We cloned SQLE promoter plus p53 response elements (RE1 or RE2) and performed luciferase reporter assays. SQLE promoter indeed decreased the RE2 luciferase activation derived by p53, despite the fact that luciferase expression driven by RE2 plus SQLE promoter was still promoted by p53 (Please see Figure below). This data indicates that the promoter of SQLE gene may functionally influence p53-mediated SOLE expression through RE2.



Luciferase reporter assay with indicated response elements constructs in human HEK293T cells co-transfected with or without Flag-p53. Renilla vector pRL-CMV was used as a transfection internal control. Relative luciferase activity (fold change) is shown. pro-RE2 /RE2 mut means SQLE promoter plus RE2/RE2 mut construct.

To further corroborate p53-drived SQLE-RE2 luciferase expression is dependent on p53 transcriptional function, we used an inhibitor of p53 transcriptional activity, pifithrin-α (PFTα) (Komarov et al., 1999) PFTα treatment impeded p53-induced luciferase expression from RE2 (revised Fig. 3J). This result provides an additional piece of evidence for p53-mediated repression of SQLE is dependent on transcriptional function of p53. Although we are highly interested, we feel that fully addressing it is a long-term project. We hope the referee agrees.

As suggested, we have discussed this observation in revised manuscript.

- Fig 5. In this figure, the authors show that SQLE expression is higher in HCC compared to adjacent non tumoral tissue. Although the immunoblots look relatively convincing, it would have been nice to confirm this result by IHC.

We thank the referee for this comment. We agree that it's better to confirm this result by IHC. However, we apologize that we unfortunately run out of the human samples. Alternately, we analyzed mRNA expression of SQLE in HCC using two public gene-expression databases (TCGA and Oncomine). SQLE expression increased in HCC compared with adjacent normal tissues (Fig. 5B and Fig. EV4A left panel). Additionally, we are trying to get new human samples but this may take a while. We could certainly perform the analysis once we get them if the referee strongly prefers.

- Fig 6D. This colony formation assay suggests that SQLE depletion decreases the capacity of both p53+/+ and -/- cells to form colonies. However, it is difficult to appreciate this effect based on the pictures shown in the underneath panel. The pictures suggest that not only the number but also the size of these colonies are very different in the 4 conditions (p53+/+ vs -/- with or wo the SQLE siRNA). The authors should show more representative pictures and/or quantify in a more appropriate

manner the growth of these colonies.

We thank the referee for this comment. To better address this comment, we repeated this experiment. Number of colonies with a diameter greater than 10μm were quantified. Please see the revised Fig. 6D. We have described this accordingly in revised Figure legends.

- Fig 6 I. The authors measured tumor initiation and tumor volume in SQLE siRNA-treated HCT116. It is not clear what the authors exactly measured in Fig 6I? Is a detectable tumor based on palpation? This parameter of tumor growth is not extremely reliable and I believe the data showing tumor volume (Fig 6J) over time is sufficient. Nevertheless, I'm a bit skeptical that the siRNA is still efficiently depleting SQLE 28 days after transfection. I'm wondering why the authors have not considered using lentiviral delivery of shRNAs targeting SQLE which would have resulted in a more stable KD? Anyway, the authors should therefore show an immunoblot analysis of SQLE protein levels at the end of the experiment (day 28). If these data correspond to the results shown in panel 6L, then the authors should consider moving these data to panel 6J as validation results. They are less relevant to panel 6L. Actually, in this panel 6L, it is not indicated when exactly they measured intracellular cholesterol in these tumors? Were these analyses performed at the very end of the experiment (day 28)? The authors should clarify the legend of this figure...

We thank the referee for these helpful comments. For the Fig. 6I in previous version of the manuscript, referee#1 has the same suggestion (major comment 3). We strongly agree with referee#1 and referee#2 that Fig. 6I is not necessary. As suggested, we have now removed it and kept the Fig 6J (revised Fig. 6I) showing tumor volume over time in revised manuscript.

In Fig. 6L (revised Fig. 6K), we measured the cholesterol level and protein expression at the end of the experiment (day 28). We have now moved the western blot data to Fig 6J (revised Fig. 6I). As described previously, siRNA works efficiently for xenograft tumor models (Du et al., 2013, Jiang et al., 2013). We apologize that we didn't state it clearly in our previous version of manuscript. We have now clarified this in revised Figure legends of Fig. 6I.

- Fig 8E: in this figure, the authors characterized the effect of SQLE depletion on tumor development in vivo using p53 flox/flox; AlbCre mice. They performed Ki67 IHC on liver sections prepared from these animal models upon injection of AAV encoding shRNA SQLE. However, it is impossible to evaluate any effect on cell proliferation based on the pictures shown in this panel. The authors should show higher magnification microphotographs and should quantify these immunostainings. A quantification of the ORO staining would also clarify their conclusions on TG accumulation in these mice. p53 protein levels in the liver of these mice should also be assessed by immunoblotting as an important validation of the model.

We thank the referee for these constructive suggestions. As suggested, we have now shown higher magnification microphotographs and quantified the Ki67 immunostainings (revised Fig. 8D). We have quantified the triglyceride (TG) level in these liver tissues using Triglyceride Quantification Colorimetric/Fluorometric Kit(Biovision, K622). Please see the revised Fig. 8F, knockdown SQLE decreased liver triglyceride accumulation, especially in p53 deficient mice. As suggested, we examined p53 protein expression in liver tissues (revised Fig. 8G).

English syntax errors must be corrected
They are too many to be listed but I've indicated here a few of them:
- In the main text "SQLE is the first oxygenation and rate-limited enzyme in cholesterol synthesis pathway". I guess the authors wanted to say that SQLE is an epoxidase?
- When the authors refer to HEPG2-p53 KO cells (generated by gene CrispR-Cas9 gene editing), they should not describe these cells as "p53-depleted cells" which is more appropriate for siRNA-mediated KD cells but less for KO cells.
- Fig 5A: "paracancerous tissue" should be replaced by non-cancerous adjacent tissue.

We very appreciate the referee for carefully reading our manuscript. We have now thoroughly checked the manuscript for typos and grammatical mistakes, and corrected the description as referee suggested. We would like to use an editing service if necessary.

Bibliography
- More recent reviews describing the complex roles of p53 in metabolism have been published. The authors should consider adding these reviews or replacing the older ones.

We thank the referee for this suggestion. We have now added some recent reviews about the role of p53 in metabolism in revised manuscript.

- The metabolic functions of p53 in liver have also been reviewed recently (Krstic et al., 2018). I believe this review is highly relevant to this Ms and should be cited.

We thank the referee for this suggestion. We have read this knowledgeable review carefully which is very relevant to our manuscript. We have now cited it in revised manuscript.

Response to Referee #3:

The role of the tumor suppressor p53 in regulating cholesterol biosynthesis both as a wild-type protein and in tumor-associated mutants has been extensively studied. This has led to a key role for p53 regulation of the transcription factor SREBP2. In this manuscript, the authors propose an additional mechanism for this regulation that is independent of SREBP2 involving direct repression of the gene encoding the first enzyme in the cascade, SQLE.

Understanding mechanisms of action of p53 that are relevant to its tumor suppressor activity is an important area of study. The ability of p53 to act as a direct transcriptional repressor has been controversial and the authors are providing new insights in this area. Thus, the manuscript attempts to provide new knowledge in a significant area. It is thus suitable for the readership of EMBO Reports. However, there are some substantial issues that need to be addressed before the study is suitable for publication. These are broadly related to tow main concerns.

We thank the referee for considering the topic to be important and our manuscript to be suitable for EMBO Reports. We also thank the referee for the constructive comments on our work. As detailed below, we have performed numerous experiments to address these comments, which we believe improve the manuscript greatly.

First, whether p53 indeed represses SQLE expression and its underlying mechanism remains unclear.

We thank the referee for this comment. We performed several experiments to further investigate whether p53 represses SQLE expression and its underlying mechanism.

To ascertain the inhibitory effect of p53 on SQLE, we compared the SQLE expression in isogenic $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells. The absence of p53 resulted in an increased level of both SQLE mRNA and protein (revised Fig. 1I, 2D). Furthermore, we generated p53 knockout human hepatocellular carcinoma cell line HepG2 using CRISPR/Cas9 system. p53 knockout elevated SQLE expression (revised Fig. 1H, 2E). Similar results were obtained in BEL-7402, SK-HEP-1, and MCF7 cells when p53 was depleted using siRNA or shRNA in these cells (revised Fig. EV2A-D). Moreover, we also examined SQLE expression in various tissues from $p53^{-/-}$ and $p53^{+/+}$ mice. The tissues from $p53^{-/-}$ mice-- including liver, brain, spleen, and colon had higher levels of SQLE, compared with those in the corresponding tissues from $p53^{+/+}$ mice (revised Fig. 2C). These results suggest that p53 indeed represses SQLE

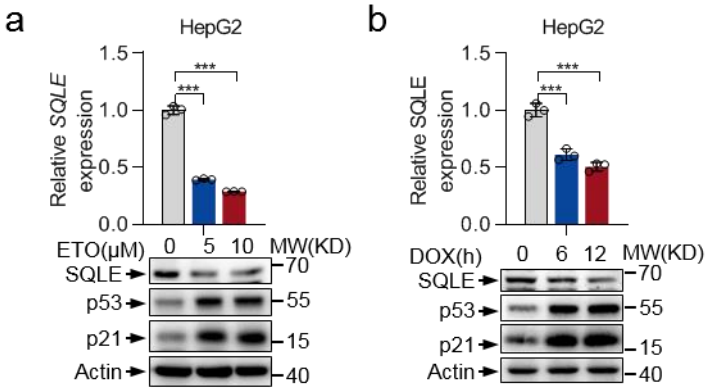
expression.

To investigate how p53 inhibits SQLE expression, we analyzed SQLE gene and identified two putative p53 response elements (RE1 and RE2) in the first intron of SQLE gene (revised Fig. 3A). We performed luciferase reporter assay, ChIP assay and EMSA experiments. As shown in revised Fig. 3, p53 functionally bound to SQLE-RE2, which is independent of SREBP2. Similar results were observed when we examined whether the effect of p53 on SQLE was conserved in mice (revised Fig. EV3D-H). These data suggest that SQLE is a p53 target gene.

To further corroborate p53-mediated inhibition of SQLE through p53 transcriptional function, we used an inhibitor of p53 transcriptional activity, pifithrin-α (PFTα) (Komarov et al., 1999). PFTα restored p53-inhibited SQLE expression. As a control, p53-induced expression of p21 was inhibited by PFTα (revised Fig. EV3A). In addition, PFTα treatment impeded p53-induced luciferase expression from RE2 (Fig. 3J). Moreover, ChIP assay showed that PFTα reduced the amount of p53 bound to SQLE-RE2 as well as p21-RE (revised Fig. EV3C). These results provide an additional piece of evidence for p53-mediated repression of SQLE is dependent on transcriptional function of p53.

In addition, we treated $p53^{+/+}$ HepG2 cell with genotoxic agents etoposide (ETO) and doxorubicin (DOX) which can stabilize the short-lived p53 protein. When cells were treated with ETO (Figure below, panel a) or DOX (Figure below, panel b), p53 was stabilized and SQLE expression was decreased. These results indicate p53 may also repress SQLE on DNA damage.

Taken together, these results demonstrate that p53 inhibits SQLE expression, which is dependent on p53 transcriptional function.



a. *$p53^{+/+}$* HepG2 cells were treated with increasing amount of ETO for 12 hours. mRNA and protein were examined respectively. **b**. *$p53^{+/+}$* HepG2 cells were treated with 1μg/ml DOX for indicated durations and were analyzed by qRT-PCR and western blotting.

Knockdown of SREBP2 or use of an SREBP2 inhibitor is used to show that SQLE basal expression is p53-dependent. It is unclear in these experiments (Figure 2H-I) what is the extent of the knockdown when comparing wild-type and p53-null cells. The immunoblots need to be quantitated, mRNA levels for SREBP2 need to be shown, and

a restoration experiment with RNAI-resistant cDNA for SREBP2 need to be performed. Likewise, p53 levels appear to be changing with these treatments. p53 in the immunoblots needs to be quantitated as well.

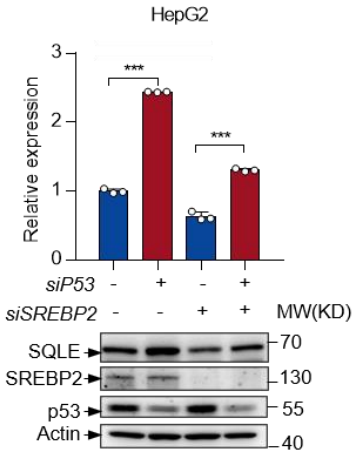
We thank the referee for these comments. We have quantitated SQLE and p53 in the immunoblots and shown mRNA levels of SREBP2. Please see revised Fig. 2H (Figure 2H), revised Fig. EV2E (Figure 2I).

As suggested, we performed a restoration experiment with RNAi-resistant cDNA for SREBP2. As shown in revised Fig. 2K, knockdown SREBP2 reduced SQLE expression in both p53 wt and knockout cells, enforced expression of SREBP2 restored SQLE expression. Moreover, p53-mediated SQLE downregulation still existed in both SREBP2-depleted cells and SREBP2-enforced expression cells (revised Fig. 2K).

Short term assays using inducible knockdown or increased expression of either p53 or SREBP2 would be most informative to reconcile some of these issues.

As recommended by the referee, we performed several experiments to further investigate whether p53 represses SQLE expression dependent on SREBP2. First, we generated a p53 Tet-on expression system in a p53-null lung cancer cell line H1299. As shown in revised Fig. EV2F, doxycycline-induced ectopic p53 expression still reduced SQLE levels even in the absence of SREBP2. Similarly, we used siRNA to knockdown both p53 and SREBP2 expression in HepG2 cells. Please see the Figure below, the downregulation of SQLE by p53 was also observed in SREBP2 siRNA transfected cells. Because of several data with similar conclusion that are already in the revised manuscript, this data is not included. But we will present this data if the referee prefers. Additionally, we used two more sets of siRNA oligonucleotides (siSREBP2#2, siSREBP#3) targeting different regions of SREBP2. p53 deficient still let to a noticeable increase in SQLE levels in SREBP2-depleted cells (revised Fig. 2I and 2J).

Taken together, these data indicate that p53 represses SQLE expression independent of SREBP2.

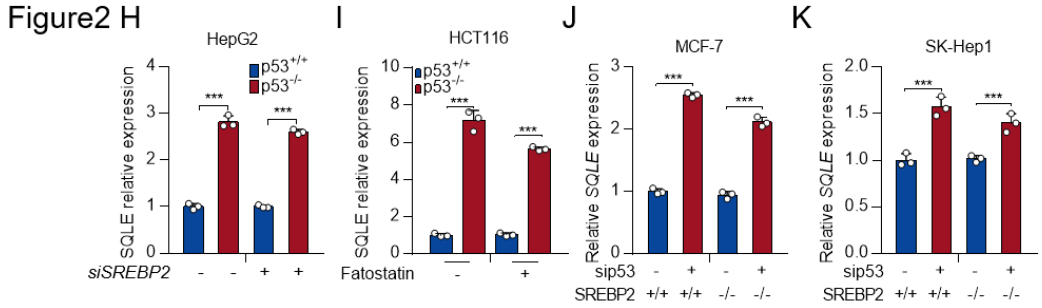


p53$^{+/+}$ and *p53*$^{-/-}$ HepG2 cells were treated with control siRNA, p53 siRNA or SREBP2 siRNA as

indicated. mRNA and protein expression were examined respectively.

SREBP2 clearly regulates SQLE mRNA expression. It is difficult to interpret the findings in Figures 2H-K as basal level of SQLE is reduced in the various treatments and the conclusion is based on a fold-difference that is not quantitated or shown to be statistically significant.

We apologize that it was difficult to interpret the results in Figures 2H-K of previous version of manuscript. For better visualization, we re-analyzed these data. Please see the figures below. The $p53^{+/+}$ condition (columns 1 and 3 in each figure) has been set to 1. We compared SQLE mRNA levels in $p53^{-/-}$ versus $p53^{+/+}$ background individually (column 2 vs column 1; column 4 vs column 3). In SREBP2-depleted (figure below, 2H), Fatostatin treatment (figure below, 2I), or SREBP2 knockout cells (figure below, 2J and 2K), p53 is still able to suppress SQLE expression.



H mRNA levels of *p53*$^{+/+}$ and *p53*$^{-/-}$ HepG2 cells treated with control siRNA or SREBP2 siRNA for 72 hours as indicated.

I *p53*$^{+/+}$ and *p53*$^{-/-}$ HCT116 cells were treated with Fatostatin (10 μM) for 24 h. mRNA expression were analyzed by qRT-PCR.

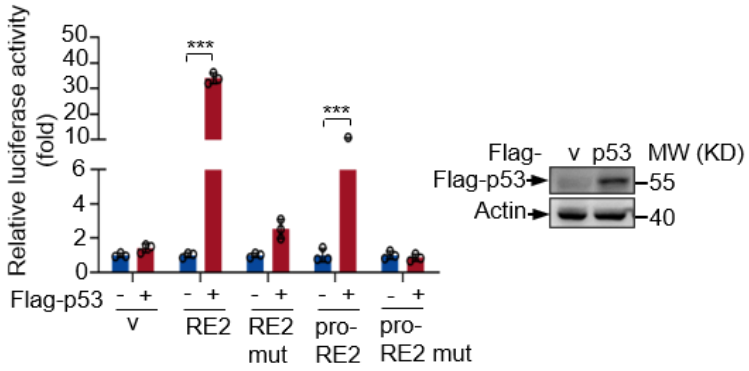
J and **K** Control and *SREBP2* knockout MCF-7 cells (**J**) or SK-HEP-1 cells (**K**) using sgRNA CRISPR/Cas9 were treated with control siRNA or p53 siRNA for 48 hours. mRNA expression were examined as indicated.

Luciferase assays using this site of p53 occupancy show transcriptional activation rather than repression. The authors need to address this conundrum.

We thank the referee for raising this point. To address this concern, we used an inhibitor of p53 transcriptional activity, pifithrin-α (PFTα) (Komarov et al., 1999). PFTα treatment impeded p53-induced luciferase expression from RE2 (revised Fig. 3J). In addition, the tumor-associated p53 mutant (p53R175H) which lost the transcriptional activity failed to active SQLE-RE2 luciferase expression (revised Fig. 3I). These results indicate that p53-induced SQLE-RE2 luciferase expression is dependent on p53 transcriptional activity.

In reporter assays, luciferase expression driven by genomic regions of p53-repressive target genes (EPCAM, CPS-1, OTC, ARG1, ME1 and PDK2) can be either promoted (for example, EPCAM response element (Sankpal et al., 2009) and

CPS-1, OTC, ARG1 response element (Li et al., 2019), or suppressed (for example, ME1 (Jiang et al., 2013) and PDK2 response elements (Contractor and Harris, 2012) by p53. Similar to the EPCAM response element, SQLE response element (RE2) increased luciferase expression in response to p53 (revised Fig 3H). This may be due to an unknown enhancer element is required for p53 to have a repressive effect in vivo. Thus, we examined whether SQLE promoter was required for the repressive effect of p53.We cloned *SQLE* promoter plus p53 response elements (RE1 or RE2) and performed luciferase reporter assays. *SQLE* promoter indeed decreased the RE2 luciferase activation derived by p53, despite the fact that luciferase expression driven by RE2 plus SQLE promoter was still promoted by p53 (Please see Figure below). This data indicates that the promoter of *SQLE* gene may functionally influence p53-mediated SOLE expression through RE2. Although we are highly interested, we feel that fully addressing it is a long-term project. We hope the referee agrees.

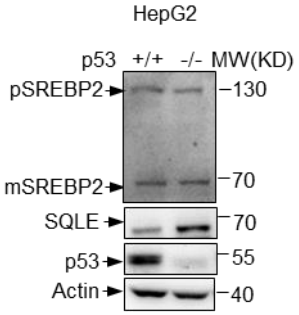


Luciferase reporter assay with indicated response elements constructs in human HEK293T cells co-transfected with or without Flag-p53. Renilla vector pRL-CMV was used as a transfection internal control. Relative luciferase activity (fold change) is shown. pro-RE2 /RE2 mut means SQLE promoter plus RE2/RE2 mut construct.

Second, the authors attempt to propose a model related to conditions of low versus normal sterol levels to explain findings. Yet, this is not directly experimentally addressed in these studies. There is a conceptual gap in that, it is suggested that p53-dependent down-regulation of SQLE occurs in normal sterol conditions. Yet, downregulation of SQLE leads to reduction in cholesterol levels. This should then now trigger the low-sterol response proposed by the Prives laboratory. There is a need for the authors to better justify how sterol conditions determine which mechanism is relevant and to integrate their findings better with the published literature.

We thank the referee for raising this important issue. We agree with the referee that p53-dependent downregulation of SQLE leads to reduction in cholesterol levels, which may affect cellular sterol conditions (revised Fig. 4C-F). To address this concern, we examined SREBP2 maturation in $p53^{+/+}$ and $p53^{-/-}$ cells under normal sterol conditions. As shown in Figure below, p53 had minimal effect on the maturation of SREBP2 under normal sterol conditions. This result suggests the

p53-mediated SQLE inhibition may not trigger the low-sterol response and induce SREBP2 maturation. Overall, the findings by Prives group and our work suggest that p53 tightly controls SQLE expression and cholesterol synthesis under both normal and low sterol conditions through different mechanisms.

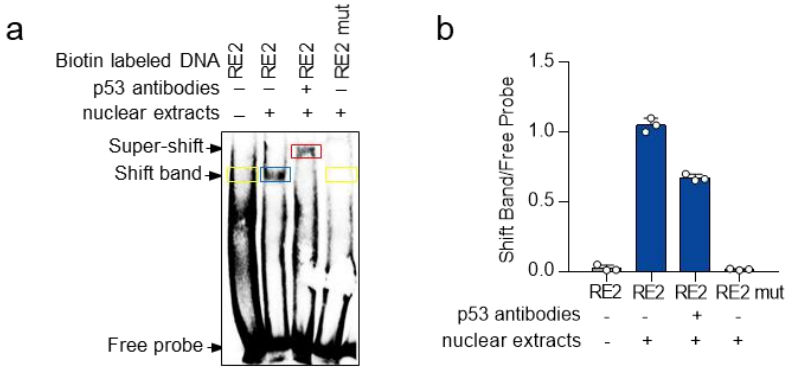


Protein expression of *p53*$^{+/+}$ and *p53*$^{-/-}$ HepG2 cells were analyzed by western blotting. pSREBP2: premature SREBP2; mSREBP2: mature SREBP2.

Additional points:

The quality of the EMSA show in Figure 3E is poor and needs to be quantitated with statistics provided.

We thank the referee for this comment. We apologize for the image with high background for EMSA in Figure 3E (panel a). Please see the figure below for quantitation (panel b).



For better visualization, we marked out the band in EMSA data. As shown in revised Fig. 3E, the band marked out with blue box (shift band) means the binding between nuclear extracts and RE2. The band marked out with red box (super-shift band with anti-p53 antibodies) means p53 as the protein presented in the EMSA band, which proves that p53 can bind to RE2. We wish the referee agree with us to do so.

There are numerous spelling and grammatical errors. It is suggested that the authors

have the manuscript edited by a native Englisher speaker.

We thank the refer for this comment. We have now thoroughly checked the manuscript for typos and grammatical mistakes. We would like to use an editing service if necessary.

The tumor data showing differences in levels of SQLE from normal tissue is intriguing. It is likely p53 status for these samples is available. This should be discussed. Otherwise this comparison does not shed new light on underlying mechanisms.

We thank the referee for this comment. In revised Fig. 5B, 5C, and Fig. EV4A, 4B, the human cohort data we analyzed showed that SQLE is upregulated in various tumors compared with their counterparts' adjacent normal tissues. We agree with the referee that these data only indicate the important role of SQLE in tumorigenesis. Unfortunatelythe p53 status for these cohort data is not available.

In revised Fig. EV2K and 2L, we have analyzed SQLE expression in human patients using a human HCC database and a human BRCA (The Cancer Genome Atlas, TCGA), in which p53 status is available. SQLE expression levels were higher in human carcinomas harboring p53 mutations (mut) than those with wildtype p53 (wt).

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1st Revision - Editorial Decision 13th Jul 2021

Dear Dr. Du,

Thank you for submitting your revised manuscript. It has now been seen by all 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.

• Please address the remaining minor concerns of referee #2. (please see the attached pdf)
• The section on the cover page entitled as "Disclosure" should be moved after the Author Contributions section and should be renamed as Conflict of Interest.
• As per our format requirements, in the reference list, citations should be listed in alphabetical order and then chronologically, with the authors' surnames and initials inverted; where there are more than 10 authors on a paper, 10 will be listed, followed by 'et al.'. The author names should not be written with capital letters. Please see https://www.embopress.org/page/journal/14693178/authorguide#referencesformat
• Please make the dataset GSE176112 publicly available and remove the reviewer password from the manuscript.
• We note that the figures are provided in .pptx format, which is not allowed. For publication, we require TIFF, PDF or EPS files in PC or Macintosh format, preferably from PhotoShop or Illustrator software. Please see https://www.embopress.org/page/journal/14693178/authorguide#figureformat
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• 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

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have addressed the concerns and provide more mechanistical insights as well as more controls. I do believe the data shown to the reviewers only should be taken up in the manuscript.

Referee #2:

The authors have significantly improved their initial Ms and have made a substantial effort to respond to most of the concerns raised by the reviewers. I believe they provide enough convincing data to support their main conclusions about the importance of p53-mediated repression of SQLE in hepatocytes and liver cancer. I believe the Ms is now ready for publication with a few minor improvements (including text editing). Below is a detailed answer to my previous main comments + a few constructive recommendations to further improve the quality of this nice work.

c Major comments :-The authors should try to identify the mechanisms by which increased cholesterol metabolism promotes liver cancer development. Based on previous data linking Mutp53 to the production of isoprenoids, the authors should evaluate whether these effects are not linked to increased geranylation of proteins(this could be done using pharmacological inhibitors of geranylgeranyl transferase such as GGTI-2133), an effect that was also previously linked to increased YAP-TAZ activity. Obviously, increased cholesterol metabolism and SQLE expression have an effect on cell proliferation but what is the underlying mechanism?

We thank the reviewer for the insightful question on howincreased cholesterol metabolism promotes liver cancer development. Cholesterol is an important component of cellular membranes and serves as a precursor for steroid hormones and bile acids. Cholesterol can also modulate signaling pathways involved in tumorigenesis and cancer progression.Additionally, cholesterolderived
metabolites play complex roles in supporting cancer progression and suppressing immune responses.Geranylgeranylation of proteins, a branch of the cholesterol synthesis pathway, is required for maintaining the stemness of breast cancer cells (Freed-Pastor et al., 2012). To evaluate whether the effect of SQLE and p53 on cell proliferationislinked to increased geranylation of proteins, we treated cells withgeranylgeranyl transferase inhibitorGGTI-2133.As shown in the figure below, GGTI-2133 treatment decreased cell proliferation in both p53+/+and p53-/-cells and diminished the difference between these two cell lines (panel a).GGTI-2133 addition reducedcell growth and diminished the difference between control cells and SQLEdepleted cells (panel b). Similar results were obtained when we treated SQLE stably expression cells with GGTI-2133 (panel c). These data suggest that the effect of SQLE and p53 on cell growthis partially dependent on geranylation of proteins.
Because of the large amount of data that are already in the revised manuscript, this dataisnot included. But we will present thesedata if the referee prefers.
a. Proliferation of p53+/+and p53-/-HepG2 cells treated with DMSO or GGTI-2133 (1μM).
b. Growth of p53+/+HepG2 cells stably expressing control orSQLE shRNA in the presence or absence
ofGGTI-2133 (1μM).SQLE protein was examined by western blotting.

c. p53+/+HepG2 cells stably overexpressing SQLE or vector control were treated with or without GGTI-2133
(1μM). Cell proliferation is shown. SQLE protein is also shown.

The data shown in panel A are interesting and support the notion that the mechanism by which deregulation of cholesterol metabolism upon p53 inactivation contributes to growth involves increased geranylation of proteins. I feel that these data are important and should be added in the main figure 5. To gain space in this figure if needed, I suggest to move the panels corresponding to data obtained in HCT116 to the supp figures since these data only confirm those obtained in HEPG2 cells, a cellular model more relevant for HCC. The authors should then comment on these results in the discussion section of their Ms. I have the feeling that data shown in panels B and C are less meaningful as they basically "only" demonstrate that the effect of the drug is not due to unspecific/off targets. Panels B and C could eventually be removed from the Ms or added as Supp. Figs to gain space.

Their preliminary data obtained in HepG2 cells suggest that SQLE depletion promotes SA-b-gal staining in p53 proficient, but not in p53 deficient cells, suggesting a potential effect on senescence. Although this increase appears relatively modest (15% increase),this is a potentially interesting concept. However, I believe this is an overstatement since SA-b-Gal staining is not sufficient to claim an effect on cellular senescence andthe authors must use other senescence markersto sustain this conclusion. By theway, it is very surprising to see so many SA-bGal positive cells in their p53+/+ HepG2 population in basal conditions?Since SA-bGal staining is very sensitive to confluency, the authors should pay a particular attention to cell density when performing these analyses. Was that effect also observed in HCT116-p53+/+ siSQLE cells? Hence, it's a pity that the authors have not followed on that (potentially) interesting observation? Is that mechanism illustrating an interesting feed-back loop implicating the con

We appreciate the referee for this helpful suggestion. The induction of senescence in SQLE knockdown cells was also examined by the marked accumulation the promyelocytic leukaemia protein nuclear bodies(Ferbeyre et al., 2000, Pearson et al., 2000). As shown in revised Fig EV5I,knockdown of SQLEin HepG2cells strongly enhances the formation of the PML-NBsin p53+/+cells. Similar to the SA-β-Gal staining data (revised Fig.6L and Fig.EV5H),in p53-deficient cells, senescence decreased markedly and SQLE depletion lost its ability to induce this phenotype.

We thank the referee for raising this important issue. High population of SA-β-Gal positive cells in p53+/+HepG2 cells may be due to high cell density. We have reperformed this experimentin HepG2 cells with low cell density. Please seerevised Fig. 6L.
Moreover, we investigated whether cholesterolis important for p53-induced cell senescence. When cells were cultured in LPDS medium, cholesterol addition indeed decreased p53-induced cell senescence, but not in p53-deficient cells(revised Fig. EV5J).
In sum, p53 is critical for the induction and maintenance of senescence (Campisi and d'Adda di Fagagna, 2007, Vousden and Prives, 2009, Ben-Porath and Weinberg, 2005). Our study found that p53 repressedSQLE expression,and downregulation of SQLE inducedsenescence through p53. Additionally, cholesterol could reduce p53-induced cell senescenceunder low-sterol conditions. As the referee mentioned, this may suggestthere is an interesting feed-back loop implicating the control of p53 activity and cholesterol metabolism in cellular senescence.

The data provided in response to this criticism, including the evaluation of PML-nuclear bodies, are convincing and provide further evidence that p53-mediated control of cholesterol metabolism plays a significant role in the control of cellular senescence. Their data also point at a potential

feed-back mechanism by which perturbations of cholesterol metabolism promote cellular senescence in a p53-dependent manner. A few comments about those links should be added in the
discussion.

Most of the minor points have been addressed, except
1- the discrepancy between the observed repression of SQLE transcription by p53 and the induction by ectopic p53 of the p53-RE-Luc construct. I don't think that their new data provide a better explanation for this discrepancy. The failure of this reporter construct to recapitulate the behavior of the endogenous SQLE promoter raises obvious questions about the regulation of this promoter, a notion that will likely be the topic of future research. Nevertheless, I believe that the other data provided by the authors in this improved version of the Ms significantly reinforce the notion that p53 is directly involved in the repression of SQLE.
2- Fig 6D: my comment on the difficulty to appreciate the size/number of the colonies has not been completely addressed. I believe the readers will have hard time appreciating the claimed differences based on the microphotographs shown in Fig6D. I think the best would be to show representative images at much higher magnification in the Supp Figures rather than showing the images currently included in Fig 6D. Alternatively, just showing the quantification of these assays is sufficient in my opinion. My remark also concerns the complementary images shown in Fig EV5.
3- I still have the feeling that the images corresponding to their histological / IHC analyses of their tumors (in particular those shown in fig 7 and fig 8 should be enlarged and shown at higher magnification. In these figures, some panels could be easily moved to the sup data section to gain space (panels 7B, 7J, 8A (the genotyping panel).

Referee #3:

The authors have addressed the concerns of the previous review. The manuscript is acceptable for publication.

Response to Referee #2:

The data shown in panel A are interesting and support the notion that the mechanism by which deregulation of cholesterol metabolism upon p53 inactivation contributes to growth involves increased geranylation of proteins. I feel that these data are important and should be added in the main figure 5. To gain space in this figure if needed, I suggest to move the panels corresponding to data obtained in HCT116 to the supp figures since these data only confirm those obtained in HEPG2 cells, a cellular model more relevant for HCC. The authors should then comment on these results in the discussion section of their Ms. I have the feeling that data shown in panels B and C are less meaningful as they basically "only" demonstrate that the effect of the drug is not due to unspecific/off targets. Panels B and C could eventually be removed from the Ms or added as Supp. Figs to gain space.

We thank the referee for this insightful suggestion. We have added this data in the main figure 6 (revised Fig. 6A), and commented on these results in the discussion section.

The data provided in response to this criticism, including the evaluation of PML-nuclear bodies, are convincing and provide further evidence that p53-mediated control of cholesterol metabolism plays a significant role in the control of cellular senescence. Their data also point at a potential feed-back mechanism by which perturbations of cholesterol metabolism promote cellular senescence in a p53-dependent manner. A few comments about those links should be added in the discussion.

We thank the referee for this suggestion. We added a few comments in the discussion.

Most of the minor points have been addressed, except
1- the discrepancy between the observed repression of SQLE transcription by p53 and the induction by ectopic p53 of the p53-RE-Luc construct. I don't think that their new data provide a better explanation for this discrepancy. The failure of this reporter construct to recapitulate the behavior of the endogenous SQLE promoter raises obvious questions about the regulation of this promoter, a notion that will likely be the topic of future research. Nevertheless, I believe that the other data provided by the authors in this improved version of the Ms significantly reinforce the notion that p53 is directly involved in the repression of SQLE.

We appreciate that the referee agrees that the question about the regulation of SQLE promoter would be the topic of future research. And we thank the referee for the positive comments on our data which provides the evidence that p53 is directly involved in the repression of SQLE.

2- Fig 6D: my comment on the difficulty to appreciate the size/number of the colonies has not been completely addressed. I believe the readers will have hard time appreciating the claimed differences based on the microphotographs shown in Fig6D. I think the best would be to show representative images at much higher magnification

in the Supp Figures rather than showing the images currently included in Fig 6D. Alternatively, just showing the quantification of these assays is sufficient in my opinion. My remark also concerns the complementary images shown in Fig EV5.

We thank the referee for this insightful suggestion. We deleted the microphotographs of colonies and only showed the quantification of these assays in revised Fig 6D and EV5D as suggested.

3- I still have the feeling that the images corresponding to their histological / IHC analyses of their tumors (in particular those shown in fig 7 and fig 8 should be enlarged and shown at higher magnification. In these figures, some panels could be easily moved to the sup data section to gain space (panels 7B, 7J, 8A (the genotyping panel), .

As the referee suggested, we have now shown these images at higher magnification. Please see the revised Figures.

2nd Revision - Editorial Decision 2nd Aug 2021

Dear Dr. Du,

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!

Before we can transfer your manuscript to our production team, we need to sort out one more thing. The labels of your synopsis image are difficult to read when resized to the final publication format (550px wide, 300-600 px high, please see attached). Please increase the font size of the labels. You can send the file to me per email. Thanks!

Kind regards,

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--
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1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ 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?
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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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5. For every figure, are statistical tests justified as appropriate?	Results are shown as mean ± s.d.. All statistical methods used were specified in the figure legends. All statistical analysis was performed and P values were obtained using GraphPad Prism software 7.0. When P value <0.05, differences were considered significant. Statistical significance is shown as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
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12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes we have reported them in materials and methods.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	GEO data are deposited and accession number is available in the "Data Availability" Section
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