Author's response to reviews

Title: NPC1L1 knockout protects against colitis-associated tumorigenesis in mice

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Author's response to reviews: see over
Dear Dr. Wagner, Dr. Polk and Dr. Eferl:

Thanks a lot for your comments and your comments are extremely beneficial to improve not only my manuscript, but also my future study. The answers to your comments are as below.

Thanks a lot for your consideration!

Best wishes,

Jianming He & Liqing Yu
Answers to Dr. D. Brent Polk:

Major Compulsory Revisions:

1. The main conclusion of the paper that NPC1L1−/− strongly protects against colitis-associated tumorigenesis should be tempered. The data suggest that this is a mild effect.

Answer: We removed “strongly” and revised the expression in the whole manuscript.

2. The conclusion that the tumors were smaller in the NPC1L1−/− mice is not supported by the data since there do not appear to be any significant differences in the size distribution of the tumors. Furthermore, there was no difference in the number of tumors in the 18 week group.

Answer: According to Fig 1C~F and table 1, tumors in NPC1L1−/− mice were really smaller than in WT mice. At the same time, we totally agree with Dr. Polk that there was not statistical significance in the tumor size between the two groups. In the revised manuscript, we emphasized that there was no statistical significance in the tumor size between the two groups.

3. It is unclear how the authors define malignant vs. non-malignant or how the malignant/tumor ratio was determined (Fig 2D), please clarify.

Answer: The malignant/tumor ratio was determined in this way. A) A pathologist who knew nothing about this experiment examined all slides
thoroughly. He counted the malignant tumors and the benign tumors in each slide and wrote them down. B) We calculated the malignant/tumor ratio of each mouse and presented the average malignant/tumor ratio of each group. We added a brief explanation in Methods-Histological Analysis.

4. Since inflammation plays a major role in promoting tumorigenesis in the AOM/DSS model, the role of NPC1L1 in regulating inflammatory severity following DSS alone should be specifically evaluated in more detail than that shown in Fig 3C. Also, what was the treatment of the mice included in this panel? The inflammatory markers in Fig 3D are non-specific and not helpful to evaluate inflammation.

Answer: Regretfully, the role of NPC1L1 in regulating inflammatory severity following DSS alone was not done. Fig 3C shows inflammation scores of 8 colorectums at 20W of WT and NPC1L1−/−. This was described in Methods-Histological Analysis. Fig 3D shows the inflammatory markers p-c-Jun1, 2, p-ERK3, 4 and Caspase-1 p205, 6. We agree that these three inflammatory markers are non-specific. But they are used as inflammatory markers in many papers1~6. They are also involved in carcinogenesis. These were discussed in Discussion.

References:


5. The conclusion that tumorigenesis is p53-independent is not strongly supported by the data, and previous studies with this model have already established that it is primarily beta catenin/Wnt driven. Please revise.

Answer: We agree that AOM-DSS induced tumorigenesis is primarily beta catenin/Wnt driven. We assayed p53 because it is a very important tumor suppressor. According to our results, there was not any detectable difference in p53 between WT and NPC1L1−/−. So, we think p53 was not involved in the mechanism of NPC1L1 knockout protecting against colitis-associated...
tumorigenesis. We also agree our data is not very consolidated to support it. Thus, we have revised the expression in the revised manuscript.

Minor Essential Revisions:

1. The data in Fig 1C is also included in Fig 1D-G; this is repetitive and unnecessary.

Answer: We removed it.

2. The axis labels in Fig 1D-G are confusing. Please label the x axis on each and clarify in the legend.

Answer: We revised the axis labels and its legend according to your suggestion.

3. Please comment on whether the WT and NPC1L1-/- mice were littermates, and also whether all experimental mice received AOM injections at the same time from the same lot. There can be substantial variability in AOM/DSS results between litters and also with different lots/ages of AOM.

Answer: We did AOM-DSS induced colitis-associated tumorigenesis as follows:

1. One WT mouse and one NPC1L1-/- mouse were mated in one cage to get NPC1L1+/ mice. 2. Then, NPC1L1+/ mice were mated with NPC1L1+/ mice to reproduce WT mice and NPC1L1+/ mice to be treated with AOM-DSS. (These
mean all AOM-DSS treated mice had the same grandfather and the same
grandmother. WT mice and NPC1L1\(^{-/-}\) mice were littermates.) 3. Six to seven
week old male mice were treated with AOM. Mice from one litter received AOM
injections at the same time. We revised Methods- Animals and diets to clarify
this.

4. The role of plasma lipid changes in tumorigenesis are unclear given
that there were no changes in plasma lipids in the 20 week group, which
was the group used to analyze histological tumor changes. Please
discuss.

Answer: Lipid metabolism is strongly associated with colorectal cancer and
cancer also caused aberrant lipid metabolism\(^1\sim4\). On week 20, mice had much
tumor burden for a long time and it also affected the plasma lipid. Thus, it is
hard to explain why there was not any significant difference in plasma lipid at
that time. We want to know what was changed by NPC1L1 knockout.
Therefore, we analyzed histological tumor changes of 20 week groups.

References:


2. Tornberg SA, Holm LE, Carstensen JM, Eklund GA. Risks of cancer of the colon and rectum in


5. The Western blot data for beta catenin in Fig 4 is unconvincing and difficult to interpret. Densitometry should be included and immunostaining of tumors would be helpful.

Answer: We agree. Densitometry of western blot in Fig 4 was added according to your suggestion.

Discretionary Revisions

The authors should comment on whether the NPC1L1 antagonist, ezetimibe would show similar results to those of the NPC1L1-/-.

Answer: We are going to do those experiments and do more in the mechanism if we get enough funds.
Answers to Dr. Robert Eferl:

Major Compulsory Revisions:

1. It seems that the authors have used separate mouse colonies (wt and NPCL1-/-) for their analysis. Although both were C57/BL6, this approach cannot be recommended because AOM/DSS-induced CRC formation is influenced by many modifier genes. Reasonable data can only be obtained with littermates, derived from heterozygous NPCL1+/- breedings that are kept in the same cage. This would account for a genetic drift of separate colonies and the influence of commensal bacteria on CRC formation. The tumor data including histopathological evaluation of low grade, high grade and invasive tumors have to be reproduced with littermate controls.

Answer: We agree that separate colonies should not be used in this experiment because other factors, such as bacteria, may influence at the outcome. We used littermates derived from NPC1L1+/- mice in this study and all mice in this study had the same grandfather and the same grandmother. (We got mice as follows: 1. One WT mouse and one NPC1L1+/- mouse were mated in one cage to get NPC1L1+/- mice. 2. Then, NPC1L1+/- mice were mated with NPC1L1+/- mice to reproduce WT mice and NPC1L1-/- mice to be treated with AOM-DSS. 3. Mice from one litter received AOM injections at the same time.) We revised Methods-Animals and diets to clarify this. Regretfully, we didn’t use heterozygous knockout mice in this study.
2. The protein expression data in Figure 4 are interesting but should be verified by IHC-stainings of tumors and adjacent tissue. Tumors from AOM/DSS-treated mice are difficult to isolate since they are not pedunculated like that of ApcMin mice. Therefore, contamination with adjacent non-tumor tissue cannot be excluded and IHC is recommended.

Answer: In our case, tumors in colon were big enough with clear boundary (It was indicated below.). Contamination should be minor. According to our protocol, contamination was not a major problem even in colorectal enterocytes. As shown in Fig 3A, that N-cadherin was not detected in membrane proteins from adjacent colorectal mucous membranes and tumors indicated that samples should not be contaminated with interstitial tissue.

Minor essential revisions

1. Proofreading by a native speaker is essential.

Answer: We revised it by a native speaker.

2. Page 8, 9 and discussion: replace ß-cantenin for ß-catenin.

Answer: We apologized for the spelling mistakes. We replaced them.
3. Supplementary Figure 1 A-E is not referred to in the manuscript text. It remains also unclear why the parameters in Figure 1A-E have been measured. Does the length of the small intestine influence CRC formation? A brief explanation would be helpful.

Answer: We added a brief explanation in the supplementary figure legend. We tried to add them in the main text at first but it broke the flow of ideas of the text. At last, we added them in the legend.

4. It seems that the livers of NPCL1-/- mice shown in Supplementary Figure 1F are consistently bigger than those of wt mice although measured weight values in the Figure legend and the bar diagram in Supplementary Figure 1D claim the opposite. How can this be explained?

Answer: We agree that Figure 1F makes reader think that the livers of NPCL1-/- mice were bigger than those of wt mice. But it is not true. Because most of the livers of WT mice were larger than cassettes for fixing, we folded them so that they could be put into cassettes. If you see the anterior side, it really seems that the livers of NPCL1-/- mice were consistently bigger than those of wt mice. However, if you look at the posterior side, you will see that because the livers of wt mice were folded. We have added the photograph of the posterior side to avoid misleading readers.