EMBO reports

Exosome-like nanoparticles from Mulberry bark prevent DSS-induced colitis via the AhR/COPS8 pathway

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Dear Prof. Zhang,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here. Please take particular care of the points of referee #1 regarding the WB data and reproducibility.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your 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 of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

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 & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets 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. ***

Moreover, I have these editorial requests:

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

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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Finally, please note that all corresponding and co-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 the ORCID ID to the account in our manuscript tracking system in our Author guidelines: http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

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,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The authors perform an extensive series of experiments to study the effect of exosomes derived from the bark of mulberry tree (MBELN) on intestinal epithelial cells and the gut microbiome in mice. They show that MBELN improves gut health and prevents (to some extent) DSS-induced colitis. MBELN is shown to induce a complicated pathway, part of which appears to involve activation of AhR by MBELN-derived HSPA8, culminating in transcriptional induction of CSN8. This, in turn, would cause cullin deneddylation and induction of antimicrobial peptides that improve the gut microbiome. There seem to be additional effects of MBELN directly on gut bacteria.

Many experiments are performed in this study - too many - but none go into the depth required to arrive at firm conclusions. Rather the study touches tangentially at many different issues performing what appear to be one-off experiments without much confirmation (e.g. proteomics, microbiome profiling). Also, in general, the quality of all Western blots shown is too low to allow reliable measurement of the small differences the authors claim to be induced by MBELN (AhR and CSN8 induction, ZO-1...). Size markers and specificity controls are missing throughout. It's not clear which of the bands is actually CSN8.

One would need to know a lot more about the reproducibility of the effects claimed. This should begin with different preparations of MBELN, showing that the activities ascribed to the isolate are consistent across different preparations. Dose responses would also be helpful. Validating HSPA8 as the active component would seem to be a whole paper on its own. The data shown in the manuscript is not convincing in this regard. It is not obvious how binding of HSPA8 (if it gets into cells) would activate AhR. Considering that AhR responds to small molecule ligands and MBELN is expected to contain many such compounds, HSPA8 seems an unlikely candidate. Besides, MBELN contains many different proteins and many appear to bind AhR (Suppl. Table 3), it is not clear why HSPA8 is the one and only mediator of the cellular effects.

The AhR-CSN8 interplay also needs much more extensive analysis (possibly another paper). How does AhR induce CSN8. What consequences does CSN8 induction have for the CSN holo-complex? Is more holo-complex formed? How does this lead to the induction of antimicrobial peptides?

MBELN effect on bacteria also needs much more detail (possibly a third paper). Is that also mediated by HSPA8? Why is MBELN specific to a subset of bacteria? What is the significance of the proteomics experiment summarized in Suppl. Table 2.

Referee #2:

In the paper "Exosomes-like nanoparticles from Mulberry bark prevent DSS-induced colitis by AhR/COPS8 signalling pathway", the author explored the possibility that mulberry bark derived exosomes-like nanoparticles (MBELN) may confer protection against mouse colitis via MBELN heat shock protein family A (Hsp70) member 8 (HSPA8) mediated activation of AhR signalling pathway. Moreover, they investigated the activation of intestinal epithelial cell AhR pathway as potential signalling leading to the induction of COPS8 that was found required for the protective effect of MBELN on mouse colitis.
The manuscript is very interesting and combines molecular data with microbiome analyses. However few points need to be addressed to strengthen the manuscript and make it easier to read and understand.

Major points:
• The authors tested the possible protective effects of MBELNs in mice, treating C57BL/6J mice with MBELNs for 15 days (line 121). However, in line 124 they assessed that the mice were sacrificed on day 30, did they give MBELNs to mice for 15 days, and then they sacrificed the mice after other 15 days? Please explain this point better. Moreover, the authors assessed that MBELNs treatment did not affect the health of mice, but in Fig 2SA the higher dose of MBLENs reduced the body weight of mice. How could the authors explain that? Please discuss this data.
• A lower magnification of the EM figure in S1-d will provide a larger view of isolated vesicles.
• Authors should discuss how come they find only 8 mRNAs inside the MBELN as showed in S1-I
• Authors should discuss how particles of about 150 nm are visible in figure S2-g as defined round red spots. Is possible that they represent membrane aggregations or collection of vesicles ?
• Please show protein modulation corresponding to mRNA modulation in samples of figure S2-i
• In the figures S3e-f the authors claim to have identified a unique protein profile of virulent L.monocytogenes-EGD that interacted with biotin labelled MBELNs when compared with non-virulent L.monocytogenes and they suggest that the interacting proteins may contribute to the efficiency in uptake of virulent versus non-virulent strains. The authors should explain how come they have a list of more than 200 proteins from Mass Spectrometry table II list and how come most of them are cytoplasmic and nuclear proteins. I believe that they have a lot of contamination and that critical controls are missing.
• The figure legend of figure 2C describe a western blot for COPS 8 in mice n=5 and MC38 cell line while the figure shows only the western blot from colon samples please add the figure.
• The results shown in figure 2e and 2e concerning the treatment with TCDD do not correspond ! What is the explanation of authors ? Moreover, to consider the effects of MG132 on COPS induction please analyse if the difference in COPS levels between cells treated with MBLEN and MBLEN+MG132 is significant.

• The authors identified COPS8 and Cullin1 from AhR pull down (figure 2f) but they did not investigate a direct interaction among them, to this aim a CO-IP should be done.
• To confirm AhR KO in MC38 cells is required a western blot for AhR, this could be shown as supplementary.
• The effects of COPS8 deficiency in Paneth cells is very interesting, in particular in the reduction of secretory granules, this aspect should be discussed.
• From the data in figure 6 it would appear that COPS8 ko expresses lower levels of ZO1 regardless of treatment. The same for dextran data thus supposing that COPS depletion affects NfKb and in general the response to pro-inflammatory stimulation including DSS treatment. The authors should investigate this aspect by analysing PBS and DSS treatment in mice with and without COPS8.
• In figure 6, please add the protein expression data concerning the results of NFkB fold change showed in figure 6-i
• Authors state that MBELN protein fraction that binds to recombinant hAhR on-chip was analysed using MS to characterize and identify the proteins present in the MBELN that are responsible of the effects on AhR signalling. The table III shows more than 200 proteins and from this long list they claim that they found HSPA8 as the contributor (Figure 7c), authors should explain how they arrived to this conclusion just excluding those proteins outside the range of 50-100 Kd.
• Figure 7g (line 444) is not shown in the panel
• The authors investigated whether HSPA8 plays a role in MBELN mediated deneddylation of CUL1 via the AhR pathway. To do this they treated MC38 +/- AhR and investigated the effects on the neddylation of Cullin 1, to confirm the role of COPS8 in the molecular pathway the authors should perform the same experiment with MC38 with COPS8 depleted.
• In the discussion, the authors state "Our finding that orally taking MBELNs leads to induction of an array of AMPs through the AhR/COP9/COPS8 pathways opens a range of clinical applications that could protect against a broad array of infection-causing agents, such as bacteria, fungi, parasites, viruses, and tumor cells ". Please rephrase this sentence excluding tumor cells.

Minor points:
• Check the sentence in line 210: MBEL treatment improved....The severity score showed lower levels
• Add molecular weight to the different western blots showed in the results.
• Please describe the histological data evaluated for the definition of the severity score
• Show the inputs for the IP.
• Indicate the time of treatment in cell lines
• In figure S2b, lung word is misplaced and it seems to indicate the colon
• In figure S3e the authors should pay attention to the legend of lines 1 and 2, because they wrote in both the same name, even if in figure legend they reported that one is virulent and the other is non-virulent.
• There is no correspondence between the graph and the number of replicates indicated in the manuscript. A) in figures 1j-l n =5 while in the legend and in the manuscript is indicated n=7. In the graph of figure 2C the replicates seem 3 instead of 5 indicated in the legend
• Line 217, change "was" with "were".
• Lines 245-246, about CUL1, please add a citation.

Referee #3:

In this report, Sriwastva et al. investigated whether and how exosome-like nanoparticles from Mulberry bark (MBELN) regulated intestinal homeostasis. They showed nicely that MBELN protected the intestines from DSS-induced inflammation mediated by HSPA8/AhR/COPS8. This is a well-designed and well-performed study. The approaches are comprehensive, and solid data support the conclusions. I only have a few minor concerns to be addressed to improve the quality of the manuscript further:
1) Fig1k, the histology was hard to read. In addition to histology, some cytokine data need to be shown the inflammation.
2) Fig 4j, it is interesting that although intestinal inflammation was more severe in CSN8 KO mice, Tregs were increased. It will be interesting to show if these Tregs express IFNg or IL17 to become proinflammatory?
3) In most Figs, please provide the information of how many times the experiments have been done?

Thank you for your initial evaluation of our manuscript entitled "Exosome-like nanoparticles from Mulberry bark prevent DSS-induced colitis via the AhR/COPS8 signaling pathway" (EMBOR-2021-53365V1). We appreciate your dedication to the peer-reviewing process and the time expended to provide us with your comments and feedback on our manuscript.
We have carefully followed all of the suggestions and constructive comments, and we made revisions and modifications accordingly. Below I am providing a point-by-point response to the reviewers' comments.

Referee #1:

- **The authors perform an extensive series of experiments to study the effect of exosomes derived from the bark of mulberry tree (MBELN) on intestinal epithelial cells and the gut microbiome in mice. They show that MBELN improves gut health and prevents (to some extent) DSS-induced colitis. MBELN is shown to induce a complicated pathway, part of which appears to involve activation of AhR by MBELN-derived HSPA8, culminating in transcriptional induction of CSN8. This, in turn, would cause cullin deneddylation and induction of antimicrobial peptides that improve the gut microbiome. There seem to be additional effects of MBELN directly on gut bacteria.**
  Response: Our primary focus for this manuscript was to address whether we could utilize a plant defensive strategy to prevent mouse colitis. Based on the findings we described in this study, we provide a foundation for us and other investigators to further investigate cellular and molecular mechanisms underlying each phenotype we described in this manuscript, including the direct effect of composition and function of gut bacteria we observed. As this reviewer notes, a number of additional findings not directly associated with the focus of this work should lead to further research and publications. Currently, several projects including whether the direct effect of MBELN on gut bacteria contributes to DSS induced colitis are underway to address these questions in my lab.

- **Many experiments are performed in this study - too many - but none go into the depth required to arrive at firm conclusions. Rather the study touches tangentially at many different issues performing what appear to be one-off experiments without much confirmation (e.g., proteomics, microbiome profiling). Also, in general, the quality of all Western blots shown is too low to allow reliable measurement of the small differences the authors claim to be induced by MBELN (AhR and CSN8 induction, ZO-1...). Size markers and specificity controls are missing throughout. It's not clear which of the bands is actually CSN8.**
  Response: Our primary focus for this manuscript was to test whether we could utilize a plant defensive strategy to broadly enhance prevention of mouse colitis. Therefore, many experiments were designed to provide a broad overview as to whether MBELN treatment would alter responses of recipient mice or cells to insults. These broad overarching experiments provided ample data, so we began to focus on the specific pathway (AhR in this case) and molecules (COPS8) in the AhR pathway. Finally, we addressed how MBELN plays a role in preventing DSS induced colitis and the pathway involved in this mechanism. In the revised manuscript, these key findings have been confirmed with more than one approach including PCR, western blots or confocal microscopy where appropriate. We believe the inclusion of these additional methods appropriately addresses the concerns of the

reviewer. We also performed new western blots for those that the reviewer indicated were unsatisfactory (Fig 2B, 2C, 2E, 2H, 6G, 7A for COPS8, 7B for COPS8 Appendix figure S2J) in previous version of the manuscript.

- **One would need to know a lot more about the reproducibility of the effects claimed. This should begin with different preparations of MBELN, showing that the activities ascribed to the isolate are consistent across different preparations. Dose responses would also be helpful.**
  Response: We have tested the reproducibility of the MBELN preparations. We used MBELN isolated from different lots of mulberry bark at different time points (spring, summer, fall and winter). We did not find a seasonal effect of the MBELN preparation on AhR activation. These data are presented in Fig EV1A and the results section, page 7, paragraph 1, line 200-205.

- **Validating HSPA8 as the active component would seem to be a whole paper on its own. The data shown in the manuscript is not convincing in this regard. It is not obvious how binding of HSPA8 (if it gets into cells) would activate AhR. Considering that AhR responds to small molecule ligands and MBELN is expected to contain many such compounds, HSPA8 seems an unlikely candidate. Besides, MBELN contains many different proteins and many appear to bind AhR (Suppl. Table 3), it is not clear why HSPA8 is the one and only mediator of the cellular effects?**
  Response: The reviewer is correct in that exosome-like nanoparticles are complex consisting of proteins, lipids, and nuclear acids. It is certainly a possibility that other factor besides HSPA8 also contribute to the phenotypes we observed. However, in this manuscript, among the proteins presented in Source Data for Appendix figure S3D, we selected HSPA8 because heat shock proteins play a critical role in AhR mediated activation(Kekatpure *et al*, 2009; Kudo *et al*, 2018). Our data show that HSPA8 has the higher affinity to bind AhR than the parental ligand. We further proved the concept of HSPA8 binding with AhR. We expressed HSPA8 protein (tagged with His and FLAG) in a bacterial system (*E. coli*). The purified MBELN-HSPA8 proteins (TEV protease cleaved and purified to eliminate His and MBP tagging) showed a strong binding capability with His-AhR immobilized on a NTA chip analyzed by Surface Plasmon Resonance (SPR). MC38 cells were treated with FLAG tagged MBELN-HSPA8 for 3 hr and confocal microscopy was performed and demonstrated that MBELN-HSPA8 and AhR were colocalized (Fig EV5G).

- **The AhR-CSN8 interplay also needs much more extensive analysis (possibly another paper). How does AhR induce CSN8? What consequences does CSN8 induction have for the CSN holo-complex? Is more holo-complex formed? How does this lead to the induction of antimicrobial peptides?**
  Response: These are all excellent questions raised by the reviewer. One of important findings described in this manuscript is that the expression of COPS8 is regulated by the AhR mediated pathway. COPS8 is one of the subunits in COP9 complex, and down-regulation of COPS8 alters COP9 complex formation(Qin *et al*, 2020; Su *et al*, 2009). It is conceivable that MBELN treatment will affect the composition and biological functions of the COP9 holocomplex. Therefore, our initial findings will open up a new avenue for further studying mechanism underling how induced COPS8 or COP9 holocomplex has an effect on

the expression/release of antimicrobial peptides. These points have been discussed in the revised manuscript, page 11-12, line 496-509.

- **MBELN effect on bacteria also needs much more detail (possibly a third paper). Is that also mediated by HSPA8? Why is MBELN specific to a subset of bacteria? What is the significance of the proteomics experiment summarized in Suppl. Table 2.**
  Response: Although we do not have evident supporting HSPA8 directly participates in inhibiting/promoting gut bacterial growth, antimicrobial peptides are well known in regulating the composition and homeostasis of gut bacteria. Our data show that HSPA8 plays a role in the induction of antimicrobial peptides via the AhR/COPS8 pathway. Our published data show that depending on the source of the edible plant exosome-like nanoparticles (ELNs), ELNs are selectively taken up by certain species of gut bacteria via different mechanisms.(Sundaram *et al*, 2019; Teng *et al*, 2018)
  The proteomics experiment we conducted was an attempt to show whether MBELN interacts in a different manner with protein from pathogenic Listeria monocytogenes when compared to non-pathogenic L. monocytogenes. This set data provides a foundation to further determine which protein(s) derived from pathogenic *Listeria monocytogenes* is/are targeted by MBELN for inhibiting *L. monocytogenes* growth. All these critical concerns as this reviewer pointed out have been discussed in the revised manuscript, page 13, line 568-572.

Referee #2:

In the paper "Exosomes-like nanoparticles from Mulberry bark prevent DSS-induced colitis by AhR/COPS8 signalling pathway", the author explored the possibility that mulberry bark derived exosomes-like nanoparticles (MBELN) may confer protection against mouse colitis via MBELN heat shock protein family A (Hsp70) member 8 (HSPA8) mediated activation of AhR signalling pathway. Moreover, they investigated the activation of intestinal epithelial cell AhR pathway as potential signalling leading to the induction of COPS8 that was found required for the protective effect of MBELN on mouse colitis.
The manuscript is very interesting and combines molecular data with microbiome analyses.
However few points need to be addressed to strengthen the manuscript and make it easier to read and understand.

Major points:

- **The authors tested the possible protective effects of MBELNs in mice, treating C57BL/6J mice with MBELNs for 15 days (line 121). However, in line 124 they assessed that the mice were sacrificed on day 30, did they give MBELNs to mice for 15 days, and then they sacrificed the mice after other 15 days? Please explain this point better.**
  Response: Yes, for purpose of toxicity or adverse effect assessment, mice were fed MBELN at two different doses (2×10^9 and 1×10^{10} MBELNs/100 μl/dose/mouse/day) for 15 days and the treatment stopped. We determined whether a 15-day treatment had any adverse effects such as skin rash, body weight changes, etc., during the next 15 days. Mice were sacrificed on day 30. This information has been modified in the revised manuscript page 4, line 121-124.

- **Moreover, the authors assessed that MBELNs treatment did not affect the health of mice, but in Fig 2SA the higher dose of MBLENs reduced the body weight of mice. How could the authors explain that? Please discuss this data.**
  Response: The reviewer is correct, there was a slight difference in changes in body weight, but it was not statistically significant, and moreover there was not any other adverse effects such as difficulty in mobility, diarrhea, skin rash or any health issues observed. We discussed this issue in the revised manuscript, the results section, page 4, line 124-131.

- **A lower magnification of the EM figure in S1-d will provide a larger view of isolated vesicles.**
  Response: A lower magnification of EM figure has been added in the Appendix figure S1D.

- **Authors should discuss how come they find only 8 mRNAs inside the MBELN as showed in S1-I.**
  Response: We apologize for the confusion. In previous version of manuscript (Appendix figure S1I), we presented the top 8 mRNAs. In the revised manuscript, in the Appendix figure S1I we represented the top 12 mRNAs that had the highest read count in MBELN. We also added a total mRNA profile in separate table (Source data for Appendix figure S3D)

- **Authors should discuss how particles of about 150 nm are visible in figure S2-g as defined round red spots. Is possible that they represent membrane aggregations or collection of vesicles?**
  Response: Individual MBELNs cannot be seen using a fluorescent microscope. It is likely these signals come from collection of MBELNs. We have discussed this issue in the results section, page 4, line 140-144.

- **Please show protein modulation corresponding to mRNA modulation in samples of figure S2-i.**
  Response: A western blot for CYP1a1 and IDO-1 has been added in Additional fig 2J and accordingly discussed in the results section page 5, line 171-174.

- **In the figures S3e-f the authors claim to have identified a unique protein profile of virulent *L. monocytogenes*-EGD that interacted with biotin labelled MBELNs when compared with non-virulent *L. monocytogenes* and they suggest that the interacting proteins may contribute to the efficiency in uptake of virulent versus non-virulent strains. The authors should explain how come they have a list of more than 200 proteins from Mass Spectrometry table II list and how come most of them are cytoplasmic and nuclear proteins. I believe that they have a lot of contamination and that critical controls are missing.**
  Response: The proteomics experiment we conducted attempted to show whether MBELN interacts with protein from pathogenic versus non-pathogenic *Listeria monocytogenes*. This set of MS data provides a foundation to further determine which protein(s) derived from pathogenic *Listeria monocytogenes* is/are targeted by MBELN for inhibiting *L. monocytogenes* growth. Like the proteomic data generated from many publications, the mass spectrometry data we have will need to be confirmed by western blot analysis of protein(s) we are interested in. As our primary focus in this manuscript was to study role of MBELN in

attenuation of DSS induce colitis. We did not further investigate the mechanism underlying the inhibition of bacterial growth.

- **The figure legend of figure 2C describe a western blot for COPS 8 in mice n=5 and MC38 cell line while the figure shows only the western blot from colon samples, please add the figure.**
  Response: Our apologies for this oversight. The western blot for COPS8 in MC38 cells has been added in Fig EV1C.

- **The results shown in figure 2e and 2e concerning the treatment with TCDD do not correspond! What is the explanation of authors? Moreover, to consider the effects of MG132 on COPS induction please analyze if the difference in COPS levels between cells treated with MBLEN and MBLEN+MG132 is significant.**
  Response: In the revised manuscript, the MG132 treatment led to a significant reduction of MBELN induced COPS8 expression. We performed analysis for COPS8 expression for the MBELN and MBELN+MG132 treatment, which suggested that MG132 treatment leads to a significant reduction in COPS8 expression (Fig 2E).

- **The authors identified COPS8 and Cullin1 from AhR pull down (figure 2f) but they did not investigate a direct interaction among them, to this aim a CO-IP should be done.**
  Response: We appreciate this suggestion. First, MS analyses data show that COPS8 and Cullin1 are detected in the AhR pull down complex. This result was further confirmed by a COPS8 and cullin1 western blot analysis of the AhR pull down complex, suggesting that they interact (Fig EV1E, discussed in the results section page 7, line 247-249). We have not conducted experiments to show whether this interaction is direct or indirect. We do not believe that without demonstrating a direct interaction on each other negatively affects our conclusion that "MBELN treatment leads to inducing the expression of COPS8 via the AhR signaling pathway".

- **To confirm AhR KO in MC38 cells requires a western blot for AhR, this could be shown as supplementary material.**
  Response: To confirm AhR KO in MC38 cells, a western blot for AhR from wild and AhR-KO MC38 cells has been added in the Fig EV1F and mentioned in result section page 7, line 256-258.

- **The effects of COPS8 deficiency in Paneth cells is very interesting, in particular in the reduction of secretory granules, this aspect should be discussed.**
  Response: In the revised manuscript, this issue has been discussed, in the discussion section, page 12, line 518-533.

- **From the data in figure 6 it would appear that COPS8 ko expresses lower levels of ZO1 regardless of treatment. The same for dextran data thus supposing that COPS depletion affects Nf-κB and in general the response to pro-inflammatory stimulation including DSS treatment. The authors should investigate this aspect by analyzing PBS and DSS treatment in mice with and without COPS8.**

Response: The analysis of ZO-1 protein expression and the serum FITC-dextran assay suggested that without DSS treatment, *COPS8*$^{\Delta IEC}$ mice had slightly decreased ZO-1 expression and higher serum FITC-dextran compared to *COPS8*$^{fl/fl}$ mice while it was not statistically significant in PBS treated groups of mice (Fig 6G-H). In contrast, *COPS8*$^{\Delta IEC}$ mice treated with DSS had significantly lower ZO-1 expressed and higher serum FITC-dextran than *COPS8*$^{fl/fl}$ mice (Figure 6G-H), suggesting *COPS8* plays a role in preventing increasing gut permeability at least by preventing reduction of ZO-1. In our experiment setting, COPS8 depletion does not affect the expression of *NF-kB* in the PBS group or DSS group, however COPS8 KO in *COPS8*$^{\Delta IEC}$ mice attenuates the MBELN induced inhibition of NF-κB expression (Fig EV4F) also mentioned in the results section page 10, line 400-413.

- **In figure 6, please add the protein expression data concerning the results of NFkB fold change showed in figure 6-i.**
  Response: Western blot for NF-kB have been added in Fig EV4F.

- **Authors state that the MBELN protein fraction that binds to recombinant hAhR on-chip was analysed using MS to characterize and identify the proteins present in the MBELN that are responsible for the effects on AhR signaling. Table III shows more than 200 proteins and from this long list they claim that they found HSPA8 as the contributor (Figure 7c), authors should explain how they arrived at this conclusion just excluding those proteins outside the range of 50-100 Kd.**
  Response: MS analysis shown several probable proteins binding with AhR. We first selected proteins in the range of 50-100 kDa, because in vitro studies suggest proteins in this range have an effect on AhR activation; this gives us 123 proteins. Heat shock proteins have been known to interact with AHR. Out of 123 proteins, HSPA8 showed the highest similarity of 71.2% amino acid sequence similarity (protein alignment analysis figure added in Fig EV5D) with homologous human HSP90aa1 protein, an AhR binding protein that provides stability to AhR. Subsequently, we performed docking analysis for AhR vs HSPA8 and AhR vs HSP90 which demonstrated, HSPA8 has higher binding affinity then homologous protein HSP90, with docking score of -336.21 vs HSP90 (docking score -282.25) (Added in Fig 7D). Collectively these analyses demonstrated HSPA8 could be an AhR interacting protein. Based on the computational lead, we cloned MBELN_HSPA8 into a bacterial system (*E. coli*) to get a purified active form of protein. SPR analysis and treatment of MC38 cells show similar results as the parent MBELN. The results have been section modified accordingly, line 427-442.

- **Figure 7g (line 444) is not shown in the panel.**
  Response: This was a typographical error and has been corrected. The data is represented in Fig 7E and Fig EV5H.

- **The authors investigated whether HSPA8 plays a role in MBELN mediated deneddylation of CUL1 via the AhR pathway. To do this they treated MC38 +/- AhR and investigated the effects on the neddylation of Cullin 1, to confirm the role of COPS8**

in the molecular pathway the authors should perform the same experiment with MC38 cells with COPS8 depleted.
Response: COPS8 play an essential role in cullin deneddylation and ubiqitinnation mediated protein degradation and OPS8 knock out results in impaired COPS8 holocomplex formation and cullin deneddylation.(Su *et al*, 2011) Intestinal specific COPS8 KO we present in the revised manuscript, shows that knock out of COPS8 leads to increasing the neddylation of Cullin 1 and attenuated MBELN mediated prevention of DSS induced mouse colitis (Fig EV2C and mentioned in the results section page 7, line 276-285). We believe these in vivo data presented in the revised manuscript are sufficient to support our conclusion.

- **In the discussion, the authors state "Our finding that orally taking MBELNs leads to induction of an array of AMPs through the AhR/COP9/COPS8 pathways opens a range of clinical applications that could protect against a broad array of infection-causing agents, such as bacteria, fungi, parasites, viruses, and tumor cells ". Please rephrase this sentence excluding tumor cells.**
  Response: Agree, corrected (Line 567).

Minor points:

- Check the sentence in line 210: MBEL treatment improved....The severity score showed lower levels
  Response: Corrected and modified accordingly (Line 214-218).

- **Add molecular weight to the different western blots showed in the results.**
  Response: Added.

- **Please describe the histological data evaluated for the definition of the severity score.**
  Response: Added to the methods section (Line 740-747).

- **Show the inputs for the IP.**
  Response: Added.

- **Indicate the time of treatment in cell lines.**
  Response: Added in the results and the methods section (Line 165 & 715).

- **In figure S2b, lung word is misplaced and it seems to indicate the colon.**
  Response: Corrected in revised manuscript Appendix figure S2B.

- **In figure S3e the authors should pay attention to the legend of lines 1 and 2, because they wrote in both the same name, even if in figure legend they reported that one is virulent and the other is non-virulent.**
  Response: Agree, Appendix figure S3E is corrected.

- **There is no correspondence between the graph and the number of replicates indicated in the manuscript. A) in figures 1j-I n =5 while in the legend and in the manuscript is**

indicated n=7. In the graph of figure 2C the replicates seem 3 instead of 5 indicated in the legend.
Response: Agree, Figure 1j-l is updated and for Figure 2c, the legend is corrected as N=3.

- **Line 217, change "was" with "were".**
  Response: Correction made in the revised manuscript line 224.

- **Lines 245-246, about CUL1, please add a citation.**
  Response: Citation (Lyapina et al., 2001) added for Cullin 1 in line 253 of revised manuscript.

Referee #3:

In this report, Sriwastva et al. investigated whether and how exosome-like nanoparticles from Mulberry bark (MBELN) regulated intestinal homeostasis. They showed nicely that MBELN protected the intestines from DSS-induced inflammation mediated by HSPA8/AhR/COPS8. This is a well-designed and well-performed study. The approaches are comprehensive, and solid data support the conclusions. I only have a few minor concerns to be addressed to improve the quality of the manuscript further:

- **Fig1k, the histology was hard to read. In addition to histology, some cytokine data need to be shown the inflammation.**
  Response: A new image for HE staining has been included (Fig 1K) and the ELISA for IL-1β and IL-6 cytokines has added in revised manuscript (Fig 1M), the results section page 6, line 216-218.

- **Fig 4j, it is interesting that although intestinal inflammation was more severe in CSN8 KO mice, Tregs were increased. It will be interesting to show if these Tregs express IFNg or IL17 to become proinflammatory?**
  Response: Thank you for pointing out this, Fig 4J lower panel indicates that *COPS8*$^{\Delta IEC}$ mice had higher percentage of Th17 cells. (Fig 4J lower panel)

- **In most Figs, please provide the information of how many times the experiments have been done?**
  Response: Agree with your suggestions and we added info to each figure legends.

References:

Kekatpure VD, Dannenberg AJ, Subbaramaiah K (2009) HDAC6 modulates Hsp90 chaperone activity and regulates activation of aryl hydrocarbon receptor signaling. *J Biol Chem* 284: 7436-7445

Kudo I, Hosaka M, Haga A, Tsuji N, Nagata Y, Okada H, Fukuda K, Kakizaki Y, Okamoto T, Grave E *et al* (2018) The regulation mechanisms of AhR by molecular chaperone complex. *J Biochem* 163: 223-232

Qin N, Xu D, Li J, Deng XW (2020) COP9 signalosome: Discovery, conservation, activity, and function. *J Integr Plant Biol* 62: 90-103

Su H, Huang W, Wang X (2009) The COP9 signalosome negatively regulates proteasome proteolytic function and is essential to transcription. *Int J Biochem Cell Biol* 41: 615-624

Su H, Li J, Menon S, Liu J, Kumarapeli AR, Wei N, Wang X (2011) Perturbation of cullin deneddylation via conditional Csn8 ablation impairs the ubiquitin-proteasome system and causes cardiomyocyte necrosis and dilated cardiomyopathy in mice. *Circulation research* 108: 40-50 %U https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3017673/

Sundaram K, Miller DP, Kumar A, Teng Y, Sayed M, Mu J, Lei C, Sriwastva MK, Zhang L, Yan J *et al* (2019) Plant-Derived Exosomal Nanoparticles Inhibit Pathogenicity of Porphyromonas gingivalis. *iScience* 21: 308-327

Teng Y, Ren Y, Sayed M, Hu X, Lei C, Kumar A, Hutchins E, Mu J, Deng Z, Luo C *et al* (2018) Plant-Derived Exosomal MicroRNAs Shape the Gut Microbiota. *Cell Host Microbe* 24: 637-652 e638

Dear Prof. Zhang,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, referees #2 and #3 now fully support the publication of your study. Referee #1 has remaining concerns and suggestions to improve the manuscript, I ask you to address in a final revised version. Please also provide a point-by-point response regarding these remaining points.

Moreover, I have these editorial requests I also ask you to address:

- Please shorten the title to not more than 100 characters (including spaces).

- Please shorten the abstract to not more than 175 words.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the diagrams in the Appendix), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment' or 'independent replicate', but clearly state if these were biological or technical replicates. If statistical testing was done but there is no significant difference, please also mark this in the diagrams (n.s.). It seems presently some diagrams have only partial stats.

- Please call the 'Disclosure of Potential Conflicts of Interest' 'Conflict of interest statement'.

- Please remove the sentence 'Expanded View for this article is available online' from the manuscript main text.

- It seems author Michael Merchant is missing from the author contributions. Please check.

- Please add the paragraph on funding to the acknowledgements.

- Presently, the RCS (9543880) grant is not mentioned in the manuscript text. Moreover, the NIH grant P20GM103436 was not entered in the submission system. Please make sure that all funding information is entered into our system upon submission and is identical to the information in the manuscript text.

- It seems there is presently no callout for Fig. 2I. Please check.

- Several Western blots (e.g. those in Fig. EV1C) are too closely cropped. Please show more of the original blots in these panels.

- Please provide the source data for all the Western blots (shown in main, EV and Appendix figures). The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the revised manuscript. Please include size markers for scans of entire blots, label the scans with figure and panel number and send one PDF file per figure. The images for the Appendix should be combined in one file.

- Please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently some of the scale bars have text near them (see e.g. Fig. 3C). Please check.

- Please clearly separate the images in Fig. 1I that come from different photos (as in Fig. 6B).

- Upon quality control of the figures, we found a checkerboard pattern in the images shown in Fig. 1K (please see the example attached). Could you please explain the origin of these patterns?

- Appendix Tables S1, S2 and S3 are datasets. Please upload the original excel files of these tables as dataset files, using the nomenclature Dataset EVx. Please put their legend on the first TAB. Then remove these from the Appendix and update all callouts (again using Dataset EVx) and make sure that the datasets are called out. There seems to be no callout for Appendix Table S4 (which should then be Appendix Table S1), but one for 'Supplemental Table 4'. Please check.

- Moreover, there is a dataset file uploaded. Please also name this using the nomenclature Dataset EVx, and put a legend on the first TAB. Please make sure this is then correctly called out (using again Dataset EVx).

- Could the sequence alignments in Fig. EV5 (D and E) be moved to the Appendix?

- Please completely fill in the author checklist. Presently, there are no entries or incomplete entries for B5 and C6.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Best,

Achim Breiling
Editor
EMBO Reports

Referee #1:

I am sorry to say, the revised manuscript does little to increase my confidence that the story purported here is correct. Much more data would be need on so many fronts to convince me that there is a true basis to the pathway depicted here. In the rebuttal, the authors admit that their experimentation is "broad and merely provides a foundation". To me, this is a weakness not s strength. There are certainly bits of descriptive data that appear well documented, for example, the effects of MBELN on gut morphology in various mouse models. Still, there is insufficient proof of the overarching story that MBELN-derived HSPA8 activates AhR culminating in transcriptional induction of CSN8 and CRL regulation. Especially the claim that HSPA8 is the active component is so novel that it cannot be accepted without considerably more evidence supporting it. HSPA8 is a "professional" protein binder, interacting with many different proteins. It is thus not surprising it also interacts with AhR, if tested in vitro (especially if the AhR was denatured which it might well be on the SPR chip). And modeling is one of those things one can't really be certain about. The authors would need to test the modeling results with targeted point mutations etc...

Referee #2:

The authors have addressed all the criticisms raised and now the paper is ready for publication.

Referee #3:

All my previous concerns have been addressed appropriately.

Editor
Achim Breiling
EMBO Reports

RE: Manuscript ID: EMBOR-2021-53365V2

Title: Exosomes-like nanoparticles from Mulberry bark prevent DSS-induced colitis via AhR/COPS8 signalling pathway

Dear Editor, Achim Breiling;

We are grateful to you and the reviewers for the time and effort in reviewing our manuscript. Additionally, we sincerely appreciate the encouraging comments regarding our research and the valuable suggestions for clarification. We appreciate this opportunity to address the comments of the reviewer one.

Reviewer one:

Criticism:

I am sorry to say, the revised manuscript does little to increase my confidence that the story purported here is correct. Much more data would be needed on so many fronts to convince me that there is a true basis to the pathway depicted here. In the rebuttal, the authors admit that their experimentation is "broad and merely provides a foundation". To me, this is a weakness not s strength. There are certainly bits of descriptive data that appear well documented, for example, the effects of MBELN on gut morphology in various mouse models. Still, there is insufficient proof of the overarching story that MBELN-derived HSPA8 activates AhR culminating in transcriptional induction of CSN8 and CRL regulation. Especially the claim that HSPA8 is the active component is so novel that it cannot be accepted without considerably more evidence supporting it. HSPA8 is a "professional" protein binder, interacting with many different proteins. It is thus not surprising it also interacts with AhR, if tested in vitro (especially if the AhR was denatured which it might well be on the SPR chip). And modeling is one of those things one can't really be certain about. The authors would need to test the modeling results with targeted point mutations etc...

Response: First, this reviewer stated that “HSPA8 is a "professional" protein binder, interacting with many different proteins”. This statement must be based on proteins from mammalian hosts, since no protein interaction studies have been conducted with plant HSPA8. There is no scientific experimental evidence to deny or support the reviewer’s statement. The HSPA8 we identified in this study is a plant protein, not a mammalian protein and there is no scientific literature indicating that plant HSPA8 is a "professional" protein binder interacting with many different proteins from a mammalian host, must less a “professional” protein binder in a plant system.

Second, we appreciate the fact that we would need to “test the modeling results with targeted point mutations etc...”. In this study, the modeling results we presented have been confirmed with data generated from our surface plasmon resonance (SPR) experiment. This reviewer points to the fact that the AhR bound to the SPR chip might be denatured. There is no end to the speculations that could be generated regarding protein being denatured or how it was bound in an unusual fashion to the SPR chip. SPR experiments are an accepted methodology in scientific research. Collectively, our findings open up a new avenue to investigate which amino acid(s) in the HSPA8 domains are critical for binding to the AhR and activate its downstream genes such COPS8.

As this reviewer suggested in his/her comments based on our first version of this manuscript; “Validating HSPA8 as the active component would seem to be a whole paper on its own”. This reviewer’s statement alone indicates that much work needs to be done to more fully define the functions of plant HSPA8. What we hope to achieve with the current manuscript is the initiation of the process of exploring HSPA8 functions and activity.

In addition, the positive or negative outcome generated from these experiments, as this reviewer suggested, will not affect our central conclusion “Exosomes-like nanoparticles from Mulberry bark prevent DSS-induced colitis via the AhR/COPS8 signaling pathway”. Further, there are always more experiments that could be run to validate or further validate a hypothesis or a set of conclusions. However, given the comments of the other reviewers and the response provided above to Reviewer #1, we are of the opinion that the results and conclusions of our manuscript are substantial enough and valid to merit publication although we trust the reviewer one judgment on this matter.

Prof. Huang-Ge Zhang
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505 S. Hancock ST
Louisville, KY 40202
United States

Dear Prof. Zhang,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

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Corresponding Author Name: Huang-Ge Zhang
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2021-53365V1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ 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?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	On the basis of prevoiusly published artcicle with similar study design samples size for included mice were selected.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	yes
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Mice of 6-8 weeks 8 week old were selected for the experiment, without any sign of adverse sign or diseased state. We did not encounter any event which require exclusion of mice from the study.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Mice were assigned randomly to the respective groups and were kept in 12 hrs of light and dark cycle in a in a pathogen free facility.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were blindly and randomly assined to groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes
4.b. For animal studies, include a statement about blinding even if no blinding was done	The assigned groups and their name was coded for for respective group of treatment to minimize bias. The results were recorded in code and evaluated by blinded investigator to reduce any biasness.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, a t-test was used to compare the means of two groups and one-way Analysis of Variance test to compare multiple experimental groups. The significance is showed as P≤0.05*, P≤0.01** and P<0.001*** were considered to be statistically significant.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	5. No.Antibody- Application; Company(Catalog) 1.COPS8- WB, IF, IP; Abcam(ab77300) 2.COPS5- WB; BIOMOL(PW8365) 3.COPS6- WB; Santa Cruz(Sc-393023) 4.COPS7- WB; Santa Cruz(sc-398882) 5.Cullin 1- WB; EPITOMICS(2436-1) 6.Cullin 3- WB; EPITOMICS(2506-1) 7.CYP1a1- WB; Invitrogen(PA5-15213) 8.IDO-1- WB; Proteintech(66528-1-IG) 9.AhR- WB, IF, IP; Invitrogen(MA1-514) 10.Nf-kB- WB; BD Biosciences(610869) 11.β-actin- WB; Santa Cruz(sc-47778) 12.α-tubulin- WB; Santa Cruz(sc-5286) 13.Zonula occludens-1 (ZO-1)- WB; Invitrogen(33-9100) 14.IL-17a- ELISA; eBioscience™(14-7175-81) 15.IL-6- ELISA; eBioscience™(14-7069-81) 16.TNF-α- ELISA; eBioscience™(14-7325-81) 17.IL-10- ELISA; eBioscience™(14-7101-81) 18.IL-1b- ELISA; eBioscience™(14-7012-81)
	20.PE anti-mouse IL-17A- FC; BioLegend(506904) 21.APC anti-mouse IL-17A- FC; BioLegend(506916) 22.FITC anti-mouse IL-17A- FC; BioLegend(506908) 23.FITC anti-human IFN-γ- FC; BioLegend(502507) 24.APC anti-human IFN-γ- FC; BioLegend(506510) 25.PE anti-mouse FOXP3- FC; BioLegend(126404) 26.APC anti-CD11b- FC; BioLegend(101212) 27.FITC anti-CD11b- FC; BioLegend(101206) 28.PE anti-CD11b- FC; BioLegend(101208) 29.APC anti-CD4- FC; BioLegend(100412) 30.FITC anti-CD4- FC; BioLegend(100406) 31.PE anti-CD4- FC; BioLegend(100512) 32.PE/Cyanine7 anti-CD3- FC; BioLegend(100220) 33.APC/Cyanine7 anti-CD3- FC; BioLegend(100222) 34.PE/Cyanine7 anti-Gr-1- FC; BioLegend(108416) 35.FITC anti-Ly-6G- FC; BioLegend(127606) 36.PE anti-Ly-6G- FC; BioLegend(127608) Abbreviation: WB-western blot; IF-Immunofluorescence; IP-Immuno-precipitation; FC- Flow
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were purchased from ATCC

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	1. C57BL/6J- Female were obtained from Jackson Laboratories. 2. The COPS8-floxed mouse model (COPS8flox/flox) was originally created as described previously .(Menon et al., 2007). To delete COPS8 in IECs, Villin-Cre+/COPS8flox/flox mice (termed COPS8ΔIEC) were generated by crossing COPS8flox/flox mice with Villin-Cre transgenic mice. The Villin-Cre mice were in a C57BL/6 background. Littermate Villin-Cre- / COPS8flox/flox mice (termed COPS8fl/fl) were obtained and used as controls. (Menon S, Chi H, Zhang H, Deng XW, Flavell RA, Wei N (2007) COP9 signalosome subunit 8 is essential for peripheral T cell homeostasis and antigen receptor-induced entry into the cell cycle from quiescence. Nature Immunology 8: 1236-1245
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. All the mice were housed in a pathogen free facility on a 12 h light/dark cycle. (IACUC Protocol: 21918)
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Comply

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
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.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable

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	Gene microarray data were deposited at NCBI,Gene Expression Omnibus . As of yet not available online
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Comply
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

G- Dual use research of concern

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