EMBO reports

Loss of colonic primary cilia promotes inflammation and carcinogenesis

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

Dear Dr. Hahne,

Thank you for transferring your manuscript to EMBO reports. I now went through your manuscript and the referee reports from The EMBO Journal (attached below). The referees acknowledge that the findings are of interest, but they have raised a number of remaining concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn.

EMBO reports emphasizes novel functional over detailed mechanistic insight, but asks for strong in vivo relevance of the findings, and clear experimental support of the major conclusions. Thus, we will not require addressing points regarding more mechanistic details experimentally. However, it will be necessary that in a revised manuscript you address all points questioning the main conclusions of the study, and all technical concerns, or points regarding the experimental designs, model systems used, or data presentation.

In this specific case, I ask you to address the remaining points of the referees in a detailed p-b-p-response (in particular those of referee #1) and by text changes, clarifications and data re-analysis or different data presentation (points of referee #2) in a further revised manuscript

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

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When submitting your revised manuscript, we will require:

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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 file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

I would suggest having 7-8 main figures (maybe Figs. 7 and 8 can be fused?) and 5 EV figures. The remaining data should then go to an Appendix file as described above, including the supplementary tables.

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Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) 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 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])

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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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11) 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.

12) Please order the manuscript sections like this using these names:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Data availability section (DAS) - Acknowledgements - Author contributions - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

Please also make sure that all the funding information is also entered into the online submission system and is complete and similar to the one in the manuscript text file (acknowledgements).

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (around 35 words).
- three to four short bullet points highlighting the key findings of your study
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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.

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Kind regards,

Achim

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The revised manuscript, now titled "Loss of colonic primary cilia promotes inflammation", did not sufficiently address my initial comments. No data has been provided on how PC loss in colonic fibroblasts results in the described phenotypes, and the increased inflammatory signaling has not been causally linked. Neither, I judge the argument plausible, that the current study represents a "conceptual advance over this previous paper". In their response the authors downplay that their previous study in EMBO Journal (Rocha et al, 2014) showed that „glycylation is responsible for the observed phenotypes, but not necessarily due to the observed loss of cilia, which at the time was a correlative, but not causative finding." However, I would like to kindly remind the authors of the title of their previous work which was: "Tubulin glycylases are required for primary cilia, control of cell proliferation and tumor development in colon" which clearly suggests such a causative link (although the responsible cell compartment now has to be re-interpreted). In my opinion, the authors have missed as chance to re-interprete and discuss the phenotype of full body KO of TTLL3 which is likely due to the role of PC in fibroblasts as shown here.

The newly added data does not provide a mechanistic link to distinguish the direct consequences of PC loss in fibroblasts that was also requested by reviewer 2. In this regard, the transcriptomic analysis in cultured fibroblast (Fig 6CD) is not conclusive because it only shows active inflammatory signaling by signals from inflamed colon, which is trivial in my opinion. Unfortunately, no comparison of isolated fibroblasts between PC proficient and deficient mice was performed which could have established a causative link. The observation that inflammatory signals act via IL6 as an upstream signal to reduce PC number (Fig 6 AD) is interesting but does not explain to the primary cause of the phenotypes.

Together, I therefore keep my initial opinion that this study is conceptionally not suitable for publication in EMBO Journal and should be published in a more organ specialized journal.

Referee #2:

This is now a well-executed and robust paper that should be of interest to EMBO J's readership. Some concerns remain in approximate order of importance:

One fundamental question that remains is: If cilia are downregulated in response to inflammatory cues, why does genetic ablation further exacerbate the consequences of this? If cilia are protective they would be expected to increase in number following insults. I understand that this is not the case and the mechanism has been delineated in detail in the revised manuscript, but the additive effects are not immediately intuitive.

Figure 5; I may misinterpret the analysis, but this shows that IL6 upregulation is specific to the floxed genotype. This would imply that IL6 is a *consequence* of ciliary deficiency under DSS treatment rather than its *cause* (as is shown in Figure 6). Is this the feed-forward loop the authors imply in Figure 8? This needs a really good explanation to alleviate any concerns on causality.

Why is the description of Figures 3E-I not together with the other AOM/DSS experiments? Along the same lines, the structure of

Figures 3 and 4 together is a bit odd; why are cilia quants again introduced in 4I after the genetic work has already been explained in the preceding panels? Wouldn't the KMs (and other similar data) for Ift88 and Kif3a be best represented side by side? I would prefer a structure in which 1) similar experiments in different genotypes are presented in parallel, and 2) baseline observations do not re-appear after the genetic functional experiments have already been shown.

The data in Figure 1 would benefit from quantification (how many CD140+ cells are ciliated versus Vim+ cells, etc).

Figure 2C; why are only Vim+ cells considered here whereas Figure 1 shows that this may not fully capture all ciliated fibroblasts. Same question applies to Figure 7B-C.

It would help if the authors explain what ABX464 targets when it is first mentioned.

Does the ABX compound prevent dysplasia in AOM/DSS treated animals?

Page 12, check sentence "Indeed, displayed..."

Point by point reply

Referee #1:

The revised manuscript, now titled "Loss of colonic primary cilia promotes inflammation", did not sufficiently address my initial comments. No data has been provided on how PC loss in colonic fibroblasts results in the described phenotypes, and the increased inflammatory signaling has not been causally linked.

Our reply:

The revised manuscript comprises two new figures, i.e. Figures 5 and 6 addressing this point. This has been appreciated by referee #2 stating "…the mechanism has been delineated in detail in the revised manuscript, …".

Neither, I judge the argument plausible, that the current study represents a "conceptual advance over this previous paper". In their response the authors downplay that their previous study in EMBO Journal (Rocha et al, 2014) showed that „glycylation is responsible for the observed phenotypes, but not necessarily due to the observed loss of cilia, which at the time was a correlative, but not causative finding." However, I would like to kindly remind the authors of the title of their previous work which was: "Tubulin glycylases are required for primary cilia, control of cell proliferation and tumor development in colon" which clearly suggests such a causative link (although the responsible cell compartment now has to be re-interpreted). In my opinion, the authors have missed as chance to re-interprete and discuss the phenotype of full body KO of TTLL3 which is likely due to the role of PC in fibroblasts as shown here.

Our reply:

Indeed, we observed that mice deficient for the tubulin glycylase Ttll3 display decreased number of primary cilia in the colon and increased susceptibility to chemically induced colon carcinogenesis (Rocha et al, 2014). Thus, loss of cilia could be the cause, because glycylation has been considered as cilia-specific. However, it cannot be excluded that this posttranslational modification has non-ciliary functions, especially because other substrates than tubulin can be glycylated. For instance, we observed glycylation of additional proteins in glycylase overexpressing cells (Fig. 1D in (Gadadhar et al, 2017)). The role of glycylation of these proteins is not yet explored and a contribution during colon carcinogenesis can therefore not be excluded. Our manuscript provides now evidence that indeed loss of primary cilia promotes colon carcinogenesis. We added this notion of page 5 of the manuscript.

The newly added data does not provide a mechanistic link to distinguish the direct consequences of PC loss in fibroblasts that was also requested by reviewer 2. In this regard, the transcriptomic analysis in cultured fibroblast (Fig 6CD) is not conclusive because it only shows active inflammatory signaling by signals from inflamed colon, which is trivial in my opinion. Unfortunately, no comparison of isolated fibroblasts between PC proficient and deficient mice was performed which could have established a causative link. The observation that inflammatory signals act via IL6 as an upstream signal to reduce PC number (Fig 6 AD) is interesting but does not explain to the primary cause of the phenotypes.

Our reply:

The crosstalk between primary cilia proficient and deficient fibroblasts will be part of a subsequent study. The activation loop between primary cilia and IL-6 production is further outlined in the response to referee #2.

Referee #2:

This is now a well-executed and robust paper that should be of interest to EMBO J's readership. Some concerns remain in approximate order of importance: One fundamental question that remains is: If cilia are downregulated in response to inflammatory cues, why does genetic ablation further exacerbate the consequences of this? If cilia are protective they would be expected to increase in number following insults. I understand that this is not the case and the mechanism has been delineated in detail in the revised manuscript, but the additive effects are not immediately intuitive.

Our reply:

We appreciate the positive comment of the referee on the revised version of the manuscript. DSS-induced inflammation is an established mouse model for acute and not chronic colitis. Thus, intestinal inflammation is followed by tissue repair and re-establishment of the colonic structure, which also includes primary cilia presence on colonic fibroblasts. As illustrated in Figure 8, primary cilia regulate through its presence or absence IL-6 signaling and thus the inflammatory response. Once fibroblasts loose primary cilia they contribute in the production of IL-6. The insult of inflammation is initiated by the loss of integrity of the mucosal barrier and promoted by an amplification loop between PC loss and IL-6 production. This circuit is accelerated in mice deficient for primary cilia. We modified the respective figure legend and extended the discussion on page 12 to clarify this.

Figure 5; I may misinterpret the analysis, but this shows that IL6 upregulation is specific to the floxed genotype. This would imply that IL6 is a *consequence* of ciliary deficiency under DSS treatment rather than its *cause* (as is shown in Figure 6). Is this the feed-forward loop the authors imply in Figure 8? This needs a really good explanation to alleviate any concerns on causality.

Our reply:

Indeed, the inflammatory response is present in both control and primary cilia-deficient mice, initiated by the insult of the mucosal barrier. But the inflammatory response, including IL6-signaling is augmented in primary cilia-deficient mice – as pointed out above. These mice display no signs of inflammation in steady state, when the mucosal barrier is intact.

Why is the description of Figures 3E-I not together with the other AOM/DSS experiments? Along the same lines, the structure of Figures 3 and 4 together is a bit odd; why are cilia quants again introduced in 4I after the genetic work has already been explained in the preceding panels? Wouldn't the KMs (and other similar data) for Ift88 and Kif3a be best represented side by side? I would prefer a structure in which 1) similar experiments in different genotypes are presented in parallel, and 2) baseline observations do not re-appear after the genetic functional experiments have already been shown.

Our reply:

Kif3a and Ift88 are essential for maintenance of primary cilia, but additional functions for both have been described. Validation of primary cilia deficiency is therefore required for both mouse models. The first set of experiments was performed using ColVIcre-Kif3a$^{flx/flx}$ and Kif3a$^{flx/flx}$ mice and findings were then validated using the respective Ift88$^{flx/flx}$ strains, which is not trivial. We followed the suggestion of the referee and regrouped specific figures according to the pathology they describe: Figure 3 for "Decreased number of primary cilia in colonic fibroblasts promotes CAC" and Figure 4 for "PC-deficient mice are more susceptible to DSS-induced colitis".

The data in Figure 1 would benefit from quantification (how many CD140+ cells are ciliated versus Vim+ cells, etc).

Figure 2C; why are only Vim+ cells considered here whereas Figure 1 shows that this may not fully capture all ciliated fibroblasts. Same question applies to Figure 7B-C.

Our reply:

The manuscript already includes Figures 3C and D showing quantification for primary cilia presence on vimentin and CD140a positive fibroblasts of ColVIcre-Kif3a$^{flx/flx}$ and Kif3a$^{flx/flx}$ mice, as well as Figures 3K and Expended View 2B of ColVIcre-Ift88$^{flx/flx}$ and Ift88$^{flx/flx}$ mice. Accordingly, about 30 and 35% of vimentin-positive and CD140a-positive cells, respectively, display primary cilia. Vimentin is a pan fibroblast marker, whereas CD140a and aIphaSMA describe subsets of fibroblasts. We added this information on page 6 on the manuscript. To obtain a global view on primary cilia presence on colonic fibroblasts, we have chosen the pan-fibroblast marker vimentin in Figures 2C and 7B-C.

It would help if the authors explain what ABX464 targets when it is first mentioned.

Does the ABX compound prevent dysplasia in AOM/DSS treated animals?

Our reply:

We actually wished to perform the suggested experiment, i.e. testing the effect of ABX464 on AOM/DSS induced carcinogenesis. This was, however, not approved by ABIVAX, who owns the compound ABX464. We discussed this issue with the editor and will remove the ABX464 experiment from the manuscript.

Page 12, check sentence "Indeed, displayed..."

Our reply:

We have modified the respective paragraph in the discussion.

Dear Dr. Hahne,

Thank you for the submission of your revised manuscript to our editorial offices. I now went through the manuscript and your final point-by-point-response, and I consider the remaining referee concerns as adequately addressed.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised version:

- Please provide the abstract written in present tense throughout.

- Please upload individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

- Please remove the bullet points, the synopsis blurb and the synopsis image from the main manuscript file. Please upload the synopsis image as separate file.

- Please provide the schematic summary (synopsis) figure in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels. Please make sure that the text in the figure in that format is not too small.

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Best,

Achim Breiling
Senior Editor
EMBO Reports

The authors have addressed all minor editorial requests.'

Dr. Michael Hahne
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CNRS UMR5535
1919 route de Mende
Montpellier 34293
France

Dear Dr. Hahne,

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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Achim Breiling
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- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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Each figure caption should contain the following information, for each panel where they are relevant:
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Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Merthods: Fibroblasts: Male C57BL/6 mice
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods: : Mouse strains: Kif3aflx/flx: L.S. Goldstein, University of California; IFT88flx/flx: B. K. Yoder, University of Alabama; Col6-Cre: G. Kollias BSRC Fleming Greece
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods: Mouse experiments were performed in strict accordance with the guidelines of the European Community (directive n°2010/63/EU) and the French National Committee (Project number APAFIS#18685) for care and use of laboratory animals.

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Materials and Merthods:

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Materials and Merthods:

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Merthods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and Merthods
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Merthods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Merthods; Figure Legends

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

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Merthods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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	
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	

Data Availability

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