Gut microbiota directs PPARg-driven reprogramming of the liver circadian clock by nutritional challenge

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Transaction Report:
(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 April 2016

Thank you for the submission of your manuscript to our journal. We have now received the referee reports that are copied below.

As you will see, all referees acknowledge that the findings are potentially interesting and novel. However, they also suggest at least one additional experiment to strengthen the study. Both referees 1 and 3 point out that PPARg inhibitors or liver-specific PPARg KO mice should be used to demonstrate that microbiota mediate their effects on the liver circadian clock through PPARg. This is the most important concern that should be addressed. A complete list of all gene expression changes should also be included in the manuscript, and/or the data deposited elsewhere, and all missing quantifications and statistical analyses must be provided.

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

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 25,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5
expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Please also change the reference style to the numbered EMBO reports style that can be found in EndNote.

Regarding data quantification, please remember to specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please also include scale bars in all microscopy images.

We now 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. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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.

REFEREE REPORTS

Referee #1:

Murakami and co-workers provide a piece of solid evidence suggesting that a possible signaling from the gut microbiota to the liver PPARg is in the mechanism(s) that mediates a previously described high fat diet (HFD)-induced reprogramming of the liver circadian clock. Notably, control chow-fed mice with microbial transfer from HFD-fed donors (HF-R) yield a similar metabolic phenotype to that of HFD-fed mice, and the further transcriptome analysis indicate a possible involvement of PPARg pathway in this effect. In support of the authors' hypothesis, PPARg activation occurs in the liver of HF-R mice at ZT12. Finally, the authors confirmed the relevance of gut microbiota in the observed liver reprogramming by using animals treated with antibiotics. I have a few comments that I'd like the authors to consider.

Major note:
To consolidate the proposed role of PPARg signaling in HF-R mice, a pharmacological blockade of this signaling would be required. Does a PPARg antagonist (GW9662) treatment reduce PPARg activity (ChIP) and its target expression (cidec and so on) in HF-R mice? The lab has demonstrated in the past that it has all the required tools and experience for the PPARg antagonist treatment (Eckel-Mahan et al. Cell 2013).

Minor notes:
1) It is not clearly discussed why the phenotype at ZT0 is not as obvious as that at ZT12 in HF-R liver.

2) Can the authors write in more detail on the method for microbiota transplantation? The methods only say that the animals were colonized with microbiota by gavaging freshly harvested donor feces suspended in PBS for 5 days, once a day. Concentrations of donor feces in PBS? How much mg per kg body weight per day?

Referee #2:

The manuscript by Murakami et al. elucidates an exciting finding that gut microbiota could
modulate the liver circadian rhythm. By using comparative analysis between (i) high-fat diet (HFD)-fed mice and (ii) mice transplanted with HFD-fed mice, the authors elegantly demonstrate that gut microbiota mediate their effects through the liver PPARγ and its downstream genes. The observation that microbiota ablation could normalize the observed circadian phenotype provide strong support for the study's conclusion. However, some of the aspects in the study require more analysis and discussion (see below).

Major comment:
1. The link between gut microbiota and modulation PPARγ appears to be disconnected. Did the author evaluate gut-derived metabolites (metabolomics) that could be activating PPARγ in the liver? For instance, it was proposed that bacterial-derived SCFA are potential activator of PPARγ (den Besten et al. 2015. Diabetes. 64(7):2398-408).

2. If gut microbiota can influence hepatic circadian rhythm, then what would be the implication to liver health? Authors need to discuss if microbiota-driven effects on the liver clock can be considered as detrimental (e.g. inducing dysregulation of liver circadian rhythm, thus promoting metabolic syndrome).

3. Figure 1E is not adequately discussed in the manuscript. It is unclear what "delta" is supposed to represent. No statistics was presented in the figure as well.

4. The genes associated with liver circadian rhythm are not given adequate discussion, introduction or citation/reference.

5. Is the complete list of genes in Figure 2A available for review (or uploaded in databases)?

Minor comment:
6. Typographical error in page 11: (i) 'shacked for 15 seconds'. (ii) 'the addiction of isopropanol'. (iii) 'depc' have to be capitalized.

Referee #3:
In the manuscript "Gut microbiota directs PPARγ-driven reprogramming of the liver circadian clock by nutritional challenge" by Murakami et al. the relationship between diet-gut microbiota interaction and metabolic circadian rhythm in the liver is investigated. Recipient mice were transplanted with gut microbiota from donors fed high-fat diet or chow diet and metabolic regulation in the liver was studied over the day. In addition, the effect of antibiotics treatment on circadian rhythms of hepatic PPARγ and PPARγ targets was studied in mice fed chow or high-fat diet. The manuscript shows that high-fat diet and microbiota from mice fed high-fat diet have partly overlapping effect on liver metabolism, in particular on lipid metabolism, and that gut microbiota from donors fed high-fat diet enhances circadian fluctuation of PPARγ and PPARγ target genes compared to gut microbiota from donors fed chow diet. The circadian rhythm of PPARγ regulated genes in mice fed high-fat diet was abolished by antibiotics treatment.

The observations made are potentially interesting and important. However, I have some comments, suggestions and concerns that needs to be addressed prior publication.
1. Page 1: The title of the paper is confusing and should be simplified. In addition it makes mechanistic claims that are not fully supported by the data presented (see paragraph 11 below).
2. Page 4: The phenotype (at least BW) of the donor mice should be shown (maybe in methods section).
3. Page 4: The gut microbiota composition of the recipient mice should be shown and commented.
4. Page 4: Reference to Sayin et al is wrong. In this study no HFD was used. A subsequent paper from the same group investigates FXR in relation to HFD.
5. Page 4: The section about FXR does not connect to the rest of the manuscript. It if should be kept its relevance needs to be commented in the discussion.
6. Page 5: Section title is confusing
7. Page 5: What was the total number of regulated transcripts (up and down) in the different groups? What pathways were not shared but unique for either diet or FT? Could be displayed e.g. as a Venn diagram.
8. Page 6: Were the 12.8% PPARγ target genes a significant overrepresentation? What was the p value for overrepresentation?
9. Page 6: The increased levels of long chain fatty acids in the liver are not surprising since these mice had steatosis! I'm not sure that the metabolome analysis of the liver is relevant for the manuscript.

10. Page 8: Please show data on liver steatosis and fat depots for the antibiotics experiment. This is important since the circadian phenotype could be related to obesity and steatosis and not to total body weight.

11. Discussion/general: The authors claim that the metabolic shift observed is dependent on PPARg. To prove this mechanism they need to perform additional experiments including e.g. liver specific PPARg KO mice. In its current form the manuscript only shows correlations.

1st Revision - authors' response 03 June 2016

Thank you for sending the reviewers comments and your valuable input. We have now completed the revision of our manuscript “Gut microbiota directs PPARg-driven reprogramming of the liver circadian clock by nutritional challenge” by Murakami et al. In order to generate a revised version that would meet all the criteria for acceptance, we took the needed time to complete all the requested experiments and modified the text/figures in accordance. I am particularly pleased with the outcome. The paper is now much improved, where all the additional experiments confirm our initial conclusions and conceptual message.

Specifically, this new version includes additional experimental data obtained using the selective PPARg inhibitor GW9662 in the fecal transplanted animals. This final evidence further strengthens our study demonstrating that gut microbiota-driven reprogramming of the liver clock is mediated by the transcription factor PPARg. Moreover, as requested by the referees, we included the complete list of genes regulated by fecal transplantation or diet and the corresponding gene ontology analysis. Finally, we addressed all the reviewers' suggestions and concerns as detailed in the attached point-by-point rebuttals.

We thank the reviewers for the constructive critiques and you for the insight. We hope that you share our enthusiasm for the findings and that you feel that this revised paper is now suitable for publication in EMBO Reports.

ANSWERS TO THE REVIEWERS COMMENTS

Referee #1
We thank Reviewer #1 for the constructive comments and suggestions. We have been able to address all the points raised as reported below.

Major note: To consolidate the proposed role of PPARg signaling in HF-R mice, a pharmacological blockade of this signaling would be required. Does a PPARg antagonist (GW9662) treatment reduce PPARg activity (ChIP) and its target expression (cidec and so on) in HF-R mice? The lab has demonstrated in the past that it has all the required tools and experience for the PPARg antagonist treatment (Eckel-Mahan et al. Cell 2013)

The point is well taken. To satisfactorily address this question we have used the selective PPARg antagonist (GW9662) and treated fecal transplanted mice. GW9662 is demonstrated to selectively block the binding of co-activators to PPARg without affecting the capacity of PPARg to bind DNA (Leesnitzer et al. Biochemistry 2002). We had successfully used the same approach in our study by Eckel-Mahan et al. (Cell 2013). We have included a new set of data in Figure 4E, and we have discussed them in the Results section (Page 8). Our data demonstrate that the increase in Pparg and Cidec liver gene expression at ZT12 is fully prevented when HF-R mice are treated with GW9662. This result consolidates the notion that microbiota-driven reprogramming by a high fat feeding depends on PPARg signaling.

Minor notes: 1) It is not clearly discussed why the phenotype at ZT0 is not as obvious as that at ZT12 in HF-R liver.
As shown in the western analyses from nuclear extracts and in ChIP experiments both in HFD (Eckel-Mahan et al., 2013) and HF fecal transplant (Fig 4B&4D), PPARγ displays a rhythmic oscillation with a peak at ZT12. This profile explains the diurnal oscillation of PPARγ target genes that show a peak in their expression at ZT12 as well, following PPARγ nuclear accumulation and chromatin recruitment. The difference in PPARγ chromatin recruitment and protein levels is not significant at ZT0 and indeed expression changes are not significant at this specific ZT.

2) Can the authors write in more detail on the method for microbiota transplantation? The methods only say that the animals were colonized with microbiota by gavaging freshly harvested donor feces suspended in PBS for 5 days, once a day. Concentrations of donor feces in PBS? How much mg per kg body weight per day?

We appreciate the concern of the reviewer about this important methodology and we have addressed this issue by providing a more detailed description of the fecal transplant protocol in the Materials and Methods section (Page10-11). Specifically, the recipients were subjected to oral gavage with fresh harvested feces, approximately 800-1000mg per kg body weight per day (0.8-1 fecal pellet per animal per day).

Referee #2:
We would like to thank this reviewer for the constructive comments. By providing additional data and amending the text we have responded in full to all the queries.

Major comment:
1. The link between gut microbiota and modulation PPARγ appears to be disconnected. Did the author evaluate gut-derived metabolites (metabolomics) that could be activating PPARγ in the liver? For instance, it was proposed that bacterial-derived SCFA are potential activator of PPARγ (den Besten et al. 2015. Diabetes. 64(7):2398-408).

We thank the reviewer for pointing out this important issue. We added this point in the Discussion (Page 9-10). While the metabolome analysis in the feces or serum from portal vein could not be performed, we speculate that SCFA produced by microbial fermentation might be one of the key mediators that links gut microbiota and host liver PPARγ reprogramming. First, as the reviewer pointed out, SCFA are modulators of PPARγ in this model (den Besten et al. 2015. Diabetes). It is conceivable that PPARγ signaling is altered by different SCFA profiles induced by dietary changes. Second, SCFA transported via bloodstream is a substrate for lipogenesis in the liver and adipose tissues (den Besten et al. 2013 Am J Physiol Gastrointest Liver Physiol, Backhed et al. 2010 Biochim Biophys Acta, Singh et al. 2015 Cell Met). Our data suggest that SREBP1 pathway is enhanced in HF-R and that increased long chain fatty acids are activators of PPARγ. Thus, we speculate that bacterial-derived SCFA activates PPARγ in the host liver both in a direct manner, as PPARγ ligands, and in an indirect manner, via the activation of the SREBP1 pathway.

2. If gut microbiota can influence hepatic circadian rhythm, then what would be the implication to liver health? Authors need to discuss if microbiota-driven effects on the liver clock can be considered as detrimental (e.g. inducing dysregulation of liver circadian rhythm, thus promoting metabolic syndrome).

Following this reviewer’s advice, we have added a discussion about the possible detrimental effect of the HF microbiota on liver health (Page 10). Our results demonstrate that HFD-induced gut dysbiosis affected hepatic diurnal rhythmicity and induced hepatosteatosis and an increase in fat depots. Blocking the PPARγ signaling at its peak time point reverted the fat depot phenotype in HF-R mice (Fig 4F). Thus, the changes in circadian gene expression associated to HFD-driven microbiome seem to contribute to fatty liver development and possibly to further metabolic diseases.

3. Figure 1E is not adequately discussed in the manuscript. It is unclear what "delta" is supposed to represent. No statistics was presented in the figure as well.

We apologize for the confusion. We have clarified this point in the manuscript (Page 5) and in the legend of Figure 2 (now Fig 2B). The figure represents the difference between HF and CC qPCR values (delta (HF-CC)) in each condition (Diet and FT) at each time point. Because each value is derived from the subtraction of average value of CC from that of HF at each time point, no statistical analysis is appropriate.
4. The genes associated with liver circadian rhythm are not given adequate discussion, introduction or citation/reference.

As requested by this reviewer, we have added references and a discussion about this point in the manuscript (Pages 4-5; please see also our response to point 3). In the HF-R liver, we have observed a mild phase-shift in the expression of core clock genes. This mirrors the expression profiles of the same genes in HFD-fed mice. This indicates that the metabolic alteration triggered by HF-driven microbiota affects molecular circadian functions that in turn can affect circadian physiology governed by the clock.

5. Is the complete list of genes in Figure 2A available for review (or uploaded in databases)?

As requested, we have uploaded the complete list of genes of Fig 2A (Figure 3A in the revised version of the manuscript). The data are now available in Source data files for Fig3 and in the Gene expression Omnibus database GSE 52333 for HFD data and GSE 82250 for FT data.

Minor comment:

6. Typographical error in page 11: (i) 'shacked for 15 seconds'. (ii) 'the addiction of isopropanol'. (iii) 'depc' have to be capitalized.

We thank the reviewer for pointing these typos. We have now corrected all of these typographical errors.

Referee #3

We thank this referee for all the constructive suggestions and comments aimed at improving the quality of our manuscript. We have addressed all the points as reported here:

1. Page 1: The title of the paper is confusing and should be simplified. In addition it makes mechanistic claims that are not fully supported by the data presented (see paragraph 11 below).

While we appreciate the point raised by this reviewer, we have ultimately decided not to change the title of the manuscript. First, we didn’t feel it is confusing and have asked several colleagues who also felt not to be confusing. Second, we have tried other options and all would not be satisfactorily. However as suggested by this reviewer at point 11, we performed the fecal transplantation experiment and have treated the animals with a specific PPAR blocker. Please, see our response to point 11 for details. Therefore, we believe that our “mechanistic claims” are now justified.

2. Page 4: The phenotype (at least BW) of the donor mice should be shown (maybe in method section).

We added the body weight of the donor mice in the in the results section at Page3.

3. Page 4: The gut microbiota composition of the recipient mice should be shown and commented.

This point is well taken and we thank the reviewer for this request. We have included a cladogram showing the CC-R and HF-R mice microbiota composition in Fig. 1D and we commented the new data in the main manuscript at Page 4. This data also proves the efficiency of our fecal transplantation method.

4. Page 4: Reference to Sayin et al is wrong. In this study no HFD was used. A subsequent paper from the same group investigates FXR in relation to HFD.

We thank this reviewer to point out the mistake in the reference “Sayin et al.”. We have changed the sentence to ‘the gut microbiota elicit profound effect on bile acid metabolism and Farnesoid X receptor (FXR) signaling.’ We also added the most recent “Ava Parseus et al., 2016: Microbiota-induced obesity requires farnesoid X receptor” as shown at Page 4 and in the reference list.

5. Page 4: The section about FXR does not connect to the rest of the manuscript. If it should be kept its relevance needs to be commented in the discussion.

We appreciate the concern of this reviewer about the FXR results at Page 4. We believe that our gut FXR pathway data further supports the notion that the fecal transplantation partially reproduced the microbial environment of donors in the recipient mice. In addition, we think that this result is important in light of the finding that gut microbiota is able to promote an obesity-phenotype through FXR (Ava Parseus et al., 2016 Gut). As requested, we added a comment at Page 4.
6. Page 5: Section title is confusing
We changed the section title at page 5 with a new one: “HFD and HFD-induced Dysbiosis share a specific transcriptional program.”

7. Page 5: What was the total number of regulated transcripts (up and down) in the different groups? What pathways were not shared but unique for either diet or FT? Could be displayed e.g. as a Venn diagram.
As requested by the reviewer, we added the total number of transcripts upregulated and downregulated in HF-fed mice and HF-fecal transplanted mice in the text (Page 5) and the KEGG pathways exclusive for the 2 conditions (Fig EV2). In HF ‘up’ groups, Insulin signaling pathway and immune signaling pathways, such as chemokine and Toll like receptor pathways, were uniquely upregulated in Diet groups but no in FT groups, whereas lipid metabolism-related pathways are shared by both diet and FT. On the other hand, in CC ‘up’ group, the ‘circadian rhythm’ pathway is specifically upregulated in diet. This is consistent with the fact that dampening of clock genes amplitude was observed upon HF feeding (Eckel-Mahan K 2013 Cell, Kohsaka 2007 Cell Metabolism) but not in HF-R.

8. Page 6: Were the 12.8% PPARg target genes a significant overrepresentation? What was the p value for overrepresentation?
The 12.8% ppar targets is a highly significant overrepresentation with a p value of 0.006. We added the p value in the main text at Page 6.

9. Page 6: The increased levels of long chain fatty acids in the liver are not surprising since these mice had steatosis! I'm not sure that the metabolome analysis of the liver is relevant for the manuscript.
The liver metabolome analysis was performed (and reported in the paper) not simply to underlie the steatotic phenotype but – and most importantly - to point out the overlap between the effect of HFD and HF fecal transplant showing specific metabolites. Moreover, the list of overlapping metabolites at ZT12 was mainly characterized by long chain fatty acids and some of them have been demonstrated to be potential PPAR ligands. This is relevant as PPAR is a key player of the liver diurnal reprogramming in both HF feeding and HF fecal transplant.

10. Page 8: Please show data on liver steatosis and fat depots for the antibiotics experiment. This is important since the circadian phenotype could be related to obesity and steatosis and not to total body weight.
This point is well taken. We report on the significant differences between HFD control and HFD + antibiotics in serum glucose levels (Fig EV4). We are not able to show the fat depots of the antibiotics-treated mice because the adipose tissue was not collected during the experiment.

11. Discussion/general: The authors claim that the metabolic shift observed is dependent on PPARg. To prove this mechanism they need to perform additional experiments including e.g. liver specific PPARg KO mice. In its current form the manuscript only shows correlations.
We thank this referee for pointing out this important issue. The correlative nature of microbiome studies has been a trend of the past several years. We have improved our study by providing mechanistic experiments aimed at findings causality. Instead of using PPAR liver-specific KO mice which would have the confounding effect of being chronically deficient in PPAR , we used a pharmacological approach that is physiologically much more powerful. By performing fecal transplantation and then treating the recipients with the specific PPAR inhibitor GW9662 (as suggested by the referee #1) we have been able to prove that our initial conclusions were valid (please see Materials and Methods Page 11 for experimental details). Indeed, blocking PPAR significantly abolishes the HF-R phenotype in term of fat depot and completely prevented the increase in ppar and PPAR -target genes expression at ZT12 (Fig 4E&4F).
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, and congratulations for your first EMBO press paper!

REFEREE REPORTS

Referee #1

Authors replied adequately all the questions I raised. So I think that this paper is now adequate for publication.

Referee #3

The questions I raised in my review of the ms have been adequately addressed and I find the ms ready for publication.
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 NIH'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 > 2, 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 authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:
- A specification of the experimental system investigated (e.g. cell line, species name).
- The (co)author(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/changed/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 or unpaired), simple linear regression, and analysis of variance; whether variability is homogenous as determined by the Bartlett test, or not.
- Definition of error bars as s.d. or s.e.m.
- Exact statistical test results, e.g., P-value < x; are there adjustments for multiple comparisons?
- Are tests one-sided or two-sided?
- Are tests independent or paired?
- Definition of "center value" 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be found. Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

B - Statistics and general methods

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? (Do not worry if you cannot see all your text once you press return)

Before performing the experiment we ran a power analysis.

1.b. For animal studies, include a statement about sampling size or estimate even if no statistical methods were used.

We chose the number of animals in accordance with guidelines of the Institutional Animal Care and Use Committee at the University of California at Irvine (Material and Methods, Page 10).

Operations to minimize the effects of subjective bias when assigning samples to treatments (e.g. randomization procedure)? If yes, please describe.

The treatments were assigned randomly to each experimental group.

For animal studies, include a statement about randomization even if no randomization was used.

The treatments were assigned to each experimental unit with a known, often equal, probability of receiving a given treatment.

4. Were any steps taken to minimize the effects of subjective bias during group allocation and/or in assessing results (e.g. blinding of the investigator)? If yes, please describe.

One of the two first authors of the paper was blind to the treatment and/or the outcome of the experiment, particularly when there is any subjective element in assessing the results.

4.a. For animal studies, include a statement about blinding even if no blinding was done.

No blinding experiments were performed "blindly" with respect to the treatments when possible and particularly when there is any subjective element in assessing the results.

5. For every figure, are statistical tests justified as appropriate?

Yes, they are (Materials and Methods, Page 14).

6. Are the data from the assays of the tests (e.g., "normal distribution") described any methods used to assess this?

Yes, and if the data were not distributed normally we used a different test (Materials and Methods, Page 14).

If there is an estimate of variation within each group of data?

Yes, the program Sigma Stat (Systat) software used to run the statistics considers the variation within each group.

If the variance similar between the groups that are being statistically compared?

Yes, the statistic program Sigma Stat (Systat) software performs a variance test before running the statistics.

C - Reagents

For the data (bacterial, viral, and cancer cell lines), are these in vitro reagents either of high quality and standardized to meet national or international standards? (Do not worry if you cannot see all your text once you press return)

Is the data (bacterial, viral, and cancer cell lines) use from a high-quality, peer-reviewed source?

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D- Animal Models

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), Uniprot (see link list at top right).

7. Identify the source of all cells and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

8. 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.

9. Include a statement confirming that consent to publish was obtained.

10. Report any restrictions on the availability (e.g., to the use of human data or samples).

11. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

12. For phase 1 and II randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit this CONSORT checklist (see link list at top right) with your submission. See author guidelines, under Reporting Guidelines. Please confirm you have submitted this list.

13. 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.

14. Provide accession codes for deposited data. See author guidelines, under Data Accessibility.

E- Human Subjects

15. Identify the committee(s) approving the study protocol.

16. 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.

17. 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.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under Data Accessibility.

19. 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.

20. 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.

21. 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.

22. 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.

23. 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.

G- Dual use research of concern

24. Could your study fall under dual use research restrictions? Please check biosafety guidelines (see link list at top right) and list of select agents and toxins [IAW06/OCDE (see link list at top right)]. According to this biosafety guidelines, provide a statement only if it could.

No, it does not fall under dual use research restrictions.