EMBO reports

Nutritional and metabolic control of germ cell fate through O-GlcNAc regulation

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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 Prof. Matsui,

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

As you will see, the referees acknowledge that you report interesting data on the effect of OGT and maternal ketogenic diet on PGC specification, but they also point out a number of important concerns.

One key concern relates to the use of OSMI-1 as sole approach to inhibit OGT. Referee 2 is concerned that the known cell toxicity of OSMI-1 might influence the results and their interpretation and both, referee 2 and referee 3 ask for orthogonal approaches to support the findings that are currently based on inhibitor treatment. This concerns must be addressed experimentally.

A second key concern relates to the mechanistic data presented and the proposed role of EZH2. You can either provide further data to substantiate the function of EZH2 in this context or alternatively focus your study on the role of OGT and maternal ketogenic diet. Referee 2 and 3 both supported such a focus on the in vivo data and OGT in further feedback to me and the publication of a shorter report is also be possible from our side.

Taken together, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) 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.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (June 17). Please discuss the revision progress ahead of this time with me if you require more time to complete the revisions.

I am also happy to discuss the revision further via e-mail or a video call, if you wish.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 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.

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We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

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2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
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- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
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Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
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12) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In their manuscript "Nutritional and metabolic control of germ cell fate through O-GlcNAc-mediated epigenetic regulation" Hayashi et al. describe how glucose metabolism can affect specification of primordial germ cells (PGCs) both in vitro and in vivo via an epigenetic mechanism. The authors first perform a screen with different perturbations of the glycolysis pathway and identify O-GlcNAc as potential regulator of PGC fate acquisition. The authors then go on to suggest that posttranscriptional regulation of EZH2 and its mediated H3K27me3 mark might be mechanistically responsible by modulating the expression of genes important for PGC specification. Finally they show that in vivo, OGT-conditional KO embryos have fewer PGCs and also that ketogenic diet of the mothers can have a detrimental effect on PGC numbers in the offspring.
This is a comprehensive and conceptually very interesting study, with potential long-term implications for reproductive health. While the study covers the topic with a large number of experiments addressing both functional and mechanistic aspects, it also has its weaknesses, specifically when it comes to the quality of some of the presented data and the interpretations deduced from them. While overall the experimental results point in the same direction, some controls and validations will need to be performed, in order to solidify the conclusions.

Major Points

1. Line 112-115 and Fig. EV1A/B: The authors list all the gene changes in the text but don't mention the unchanged expression of Stella when omitting Glucose and the increase in Stella expression when adding 2-deoxy-glucose. As Stella is a key early PGC marker, frequently used to define PGC fate, this should also mentioned in the text. Furthermore, it would be helpful to move figure panels EV1A/B to main Figure 1, so that the comparison of Glucose depletion to the different treatments in Figure 1 can be better made.

2. Fig. 1C: In order to know, if GlcNH2 supplementation can fully rescue the absence of Glucose, it would be necessary to also compare the data to a normal glucose control sample, both for Blimp1-mVenus as well as for gene expression.

3. Fig. 2D: For clarity, it would be good in the aggregate plot on top not only to show the DMSO (blue line), but as well show the aggregate plot for the OSM-1 treated peaks in a different colored line. That would clarify, if there is any shift in treatment vs. control.

4. Line 187-192: "Genes with increased H3K27me3 and decreased transcript levels following exposure to OSMI-1 were enriched in developmental genes such as Wnt- and Hox genes (Fig 2E and G; Table EV4). Genes with decreased H3K27me3 and increased transcript levels were enriched for pluripotency-related genes such as Sox2 and Klf4 (Fig 2F and H; Table EV4). These results showed that regulation of H3K27me3 by protein O-GlcNAcylation controls the expression of genes crucial for PGC specification."
The logic of this statement is not completely clear to me. It makes sense that a global reduction in H3K27me3 levels as shown in Fig. 2A-D after OSMI-1 treatment can cause de-repression of pluripotency genes as shown in Fig. 2F/H. However, it does not explain the gain in H3K27me3 and increased repression of developmental genes as shown in Fig. 2E/G, which is probably an

indirect secondary effect of the enhanced pluripotency gene expression. The authors should consider rephrasing the interpretation from these experiments related to the repression of developmental genes.

5. Fig. 3E: From the Sox2 quantification on the right it is apparent that the variation in Sox2 expression between replicates is too variable to be significantly different from the control. However the variation in expression of germ cell genes seems stable, which is counterintuitive, if they would be indeed targets of Sox2. The authors should therefore show if the Sox2 expression levels inversely correlate with the expression levels of the germ cell marker genes. If that anti-correlation is not observed, or more direct evidence such as a rescue of DZNEP or OSMI1 treatment by Sox2 knockdown is shown, the statement of Sox2 downregulation as the mechanism of action should be removed.

6. Fig. 4A-C: The IP and Western Blot experiments should be shown also in uncropped form in the supplementary Information. The currently shown bands are not clearly demarcated and some signal seems to be cut off. Also the error bars in the signal quantification in 4C seem unusually small. What has been compared here? Technical or biological replicates and how many?

7. Fig. 4E: The rescue of OSMI treatment by Ezh2 overexpression and OSMI treatment alone or with mCherry overexpression should be shown in comparison to the DMSO control (in 4D sample on the left). Only then it can be verified, if the Ezh2 overexpression can indeed rescue the OSMI treatment phenotype. As it stands right now, the experiment seems inconclusive.

8. Fig. 6: The result that ketogenic diet in mothers causes lower PGC specification efficiency in their offspring is very intriguing. Nevertheless, to rule out indirect effects such as reduced embryo growth or delays in development during the critical PGC specification period, the size of embryos of normal and ketogenic fed diet mothers should be also measured and compared as done for the later embryos (this can be done from the existing images).

Minor Points

1. Line 79-80: "In human PGCLCs, vitamin C supplementation enhances the activities of Jumonji (Jmj) C histone demethylases (Irie et al, 2015)..."
The reference given does not discuss vitamin C supplementation in the context of human PGCLCs. Please put the correct reference instead.

2. Fig. 1A: It should say Fructose-6-P instead of Fluctose-6-P.

3. Fig. 1E/F EV2B: Please make clear in the figure legends and explain in the main text + methods what Cyto+ and Cyto- means. People outside the PGCLC field would not understand otherwise that these are with and without cytokine controls. Please provide appropriate context by specifying, which cytokines are meant (all of them or only the BMPs) and that they are used to induce germ cell fate.

4. Line 166-167: "consistent with the results obtained under glucose deprivation (Fig EV3E and F)" should say instead (Fig. EV3D and E)

5. Fig. 3A, 5B, 6B: The enlarged images in the insets indicated by the yellow squares are missing. Please add them (better) or don't show the yellow squares (worse).

6. Fig. 3C: For consistency with the other panels Dppa3 should be named Stella. Otherwise it is hard to see the link between expression data and EZH2 CUT&RUN results. Also explain in the figure legend what p.c. and n.c. stand for (I assume positive and negative control) and correct alignment of the brackets and the misaligned significance star next to the Dppa3 EZH2 bar.

7. Figs. 4E and EV5C: Stella is misspelled.

8. Line 265: BIIMP1-positive should instead say BLIMP1-positive.

Referee #2:

Remarks to the Author,
In this manuscript titled "Nutritional and metabolic control of germ cell fate through O-GlcNAc-mediated epigenetic regulation", Hayashi et al. report that O-GlcNAcylation stabilizes the epigenetic regulator EZH2 and regulates the fate of primordial germ cells. And the authors use the heterozygous OGT knockout embryos from conditional tissue-specific OGT knockout mice to investigate the correlations between OGT and BLIMP1, and the roles of OGT on PGC specification. And they also use mice taking a ketogenic diet to investigate the effects of low carbohydrate nutrition status in fetal germ cells and find that a ketogenic diet decreases fetal germ cell development. The authors did a lot of work to link glucose metabolism and nutrition status with embryonic development through the regulatory roles of O-GlcNAcylation on EZH2 by OGT.
Comments:

1. The authors mainly use OSMI-1, an OGT inhibitor, as the major treatment to study the effects of O-GlcNAcylation. However, OSMI-1 shows strong cell toxicity that would decrease cell viability significantly. Therefore, in order to show the specificity, other treatments such as OGT knockdown, OGT overexpression, less toxic specific drugs, or OGA-related treatments should be included to confirm that OGT directly regulates the O-GlcNAcylation of EZH2 and affect the subsequent PGC formation or embryonic development.
2. In Fig1 F, why does OGT intensity increase while O-GlcNAc intensity decreases? The results seem to be controversial.
3. And for O-GlcNAcylation detection methods, other methods like Mass Spec or in vitro O-GlcNAcylation assays should also be included to confirm the direct O-GlcNAcylation modification of EZH2 by OGT.
4.The connections and interplay among OGT, BLIMP1, and EZH2 are a little bit confusing.
In the animal experiments, the authors report that the intensities of OGT and BLIMP1 show a positive correlation in the epiblast cells of embryos. And in Fig1D, OSMI-1 treatment decreases Blimp1 expression. Does OGT regulate BLIMP1 directly in the context of the manuscript? As previously reported, Blimp-1 interacts with OGT and is modified by O-GlcNAc, please refer to "Chen, Y.F., Shao, G.C., Li, J., Yang, A.Q., Li, J. and Ye, X.S., 2022. O-GlcNAcylation of blimp-1 in lymphocytes inhibits its transcriptional function and is associated with migration and invasion of breast cancer cells. Molecular Cancer Research, 20(4), pp.650-660."
And in Fig 3D, EZH2 inhibitor treatment decreases Blimp1 expression. What are the regulatory mechanisms between EZH2 and BLIMP1 in the manuscript context? Does OGT regulate BLIMP1 in a EZH2 dependent or independent manner?
In the text from line 194 to line 211, the authors report that "Immunostaining confirmed that inhibition of OGT by OSM1-1 caused a decrease of EZH2 protein in BLIMP1-negative cells, and of H3K27me3 on day 2 of PGCLC induction compared to cells grown in the absence of OSMI-1 (Fig 3A and B)." and "We, therefore, propose the following model in which O-GlcNAcylation contributes to the maintenance of EZH2 protein levels under normal culture conditions likely resulting in the upregulation of Blimp1 transcription, while inhibition of OGT by OSMI-1 results in decreased EZH2 expression likely resulting in impaired Blimp1 induction." Does OGT regulate EZH2 in a Blimp1-dependent or independent manner? What is the role of Blimp1 in the regulation of EZH2 by OGT.
The authors should clarify more clearly in terms of these parts and figure out the interplay among OGT, BLIMP1, and EZH2.
5. The quality of western blotting images should be improved, and the bands are relatively obscure.

Referee #3:

The paper by Hayashi et al., claims to establish a link between nutrient depravation, OGT activity, O-GlcNAc of EZH2 and Primordial Germ Cell induction both in vitro and in vivo. Most of the mechanistic insights in the paper rely on PGC-like cell induction in vitro and treatment with a number of metabolic and epigenetic inhibitors. On the other hand, in vivo analysis relies on the use of ketogenic diet for depleting glucose availability and an elegant Sox2Cre OGTflox mouse system for assessing the role of OGT during PGC specification. While the topic of this study is definitely very interesting I fear that the report suffers from significant flaws especially on the mechanistic side of the story. In my opinion the in vivo OGT-cKO as well as reduced number of PGCs when ketogenic diet is used on their own give a very interesting and strong phenotype that merits publication for example as a short report. However, there is little conclusive support for the proposed mechanistic model and the involvement of epigenetic regulation. Bellow I'm attaching my main points of criticism:
1. All mechanistic in vitro experiments rely exclusively on the use of small molecule inhibitors which can and often are non-specific. Mechanistic data in mouse stem cells can and shoulw be validated using orthogonal methods.
2. Conclusions from figure 1, 2 and 3 largely rely on immunofluorescent analysis of embryonic bodies with PGCLCs. I find these analysis very unconvincing. The DAPI signal is often blurry with weak nuclear staining. Surprisingly I can only visually see the effects of treatments on the cells very much in the middle of the EBs while PGCLCs are not present only in this compartment. Some of the treatments seem mainly to lead to death rather than specific effects e.g. Fig 3D. Fig 4D. Finally, EZH2 cKO mice show normal specification of PGCs in vivo (Lowe et al., 2022) how can this be in the light of your inhibition results?
3. Figure 4 A/B: The IP is not clean with no EZH2 detected in the input sample (A) and high background just bellow EZH2 in the IP sample of panel B. WB in C looks convincing and is important however neither A/B nor C seem to have biological replicates.
4. Figure 2: The Cut and Tag analysis does not show a convincing decrease of H3K27me3 especially since no spike-inns were used. I am hard pressed to see any difference in panel G. Moreover, in E there does not seem to be a significant overlap between upregulated/downregulated genes and loss/gain of H3K27me3 enrichment. No statistical test is performed here and the reverse analysis is not shown e.g. increased H3K27me3 and increased expression, this would be an necessary control analysis. Based on these data the claims are too strong.
5. Figure 3C: there doesn't seem to be a biological replicate of the EZH2 occupancy experiment.
6. While the effects of ketogenic diet on the number of PGCs seem robust and are very interesting I have doubts about BLIMP1, H3K27me3 and OGlcNAc quantifications. Figure 6 The effect size often seems minute (Blimp1 intensity) and the conclusions should be more nuanced. Looking at the images E-F it is clear that the DAPI signal is also lower in the ketogenic diet images. All Ifs should be normalized to the DAPI signal as is standard in such analysis. I suspect that this will remove the significance from this analysis.
7. Many of the conclusions from this report are not supported by the data:
a. Line 204-207: the proposed model has little link to real data, how could PRC2 mediate upregulation of Blimp1?
b. Line 318-319: There is no proof that the ketogenic diet effects are actually due to OGT activity.
c. Line 319-323: There is no data to show that e.g. 2DG results in EZH and H2K27me3 changes.

d. Line 323-324: Current data is not strong enough to conclude that PRC2 is important for inducing PGCs especially without genetic and in vivo evidence.
8. The authors have previously uncovered that 2DG treatment leads to a slight increase, not decrease in PGCLC number (Hayashi et al., 2017). How can this be explained when compared to this study?

All in all in my opinion this paper has reports on some intriguing effects of the ketogenic diet and the role of OGT on PGC specification. Beyond that the majority of mechanistic data is underwhelming this I do not support the publication of this manuscript.

Editor

One key concern relates to the use of OSMI-1 as sole approach to inhibit OGT. Referee 2 is concerned that the known cell toxicity of OSMI-1 might influence the results and their interpretation and both, referee 2 and referee 3 ask for orthogonal approaches to support the findings that are currently based on inhibitor treatment. This concerns must be addressed experimentally.

Response: We applied knockdown of OGT by siRNA and overexpression of OGA to confirm the importance of O-GlcNAcylation in PGC formation. We added the data in Fig.2.

A second key concern relates to the mechanistic data presented and the proposed role of EZH2. You can either provide further data to substantiate the function of EZH2 in this context or alternatively focus your study on the role of OGT and maternal ketogenic diet. Referee 2 and 3 both supported such a focus on the in vivo data and OGT in further feedback to me and the publication of a shorter report is also be possible from our side.

Response: As suggested by the editor, we focused on the OGT function in vitro and in vivo, and the effect of maternal ketogenic diet in vivo. All data concerning EZH2 were deleted in the revised manuscript.

*****IMPORTANT NOTE:

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

Response: We added data in the experiments in Fig.1F (1G in the revised version) and 6G (4G in the revised version), and deleted data in Fig.2B, 3B, 7E, 7H, EV4, and EV5B.

Referee #1:

Major Points

1. Line 112-115 and Fig. EV1A/B: The authors list all the gene changes in the text but don't mention the unchanged expression of Stella when omitting Glucose and the increase in Stella expression when adding 2-deoxy-glucose. As Stella is a key early PGC marker, frequently used to define PGC fate, this should also mentioned in the text. Furthermore,

it would be helpful to move figure panels EV1A/B to main Figure 1, so that the comparison of Glucose depletion to the different treatments in Figure 1 can be better made.

Response: We mentioned unchanged expression of Stella after glucose depletion (line 112-114), and moved Figure EV1A, B to main Fig.1.

2. Fig. 1C: In order to know, if GlcNH2 supplementation can fully rescue the absence of Glucose, it would be necessary to also compare the data to a normal glucose control sample, both for Blimp1-mVenus as well as for gene expression.

Response: We deleted data concerning GlcNH2 supplementation in Fig. 1C.

3. Fig. 2D: For clarity, it would be good in the aggregate plot on top not only to show the DMSO (blue line), but as well show the aggregate plot for the OSM-1 treated peaks in a different colored line. That would clarify, if there is any shift in treatment vs. control.

Response: We deleted all data in Fig.2, and focused on the OGT function in vitro and in vivo, and the effect of maternal ketogenic diet in vivo.

4. Line 187-192: "Genes with increased H3K27me3 and decreased transcript levels following exposure to OSMI-1 were enriched in developmental genes such as Wnt- and Hox genes (Fig 2E and G; Table EV4). Genes with decreased H3K27me3 and increased transcript levels were enriched for pluripotency-related genes such as Sox2 and Klf4 (Fig 2F and H; Table EV4). These results showed that regulation of H3K27me3 by protein O-GlcNAcylation controls the expression of genes crucial for PGC specification."
   The logic of this statement is not completely clear to me. It makes sense that a global reduction in H3K27me3 levels as shown in Fig. 2A-D after OSMI-1 treatment can cause de-repression of pluripotency genes as shown in Fig. 2F/H. However, it does not explain the gain in H3K27me3 and increased repression of developmental genes as shown in Fig. 2E/G, which is probably an indirect secondary effect of the enhanced pluripotency gene expression. The authors should consider rephrasing the interpretation from these

experiments related to the repression of developmental genes.

Response: We deleted all data in Fig.2 and related descriptions in the text as mentioned above.

5. Fig. 3E: From the Sox2 quantification on the right it is apparent that the variation in Sox2 expression between replicates is too variable to be significantly different from the control. However the variation in expression of germ cell genes seems stable, which is counterintuitive, if they would be indeed targets of Sox2. The authors should therefore show if the Sox2 expression levels inversely correlate with the expression levels of the germ cell marker genes. If that anti-correlation is not observed, or more direct evidence such as a rescue of DZNEP or OSMI1 treatment by Sox2 knockdown is shown, the statement of Sox2 downregulation as the mechanism of action should be removed.

6. Fig. 4A-C: The IP and Western Blot experiments should be shown also in uncropped form in the supplementary Information. The currently shown bands are not clearly demarcated and some signal seems to be cut off. Also the error bars in the signal quantification in 4C seem unusually small. What has been compared here? Technical or biological replicates and how many?

7. Fig. 4E: The rescue of OSMI treatment by Ezh2 overexpression and OSMI treatment alone or with mCherry overexpression should be shown in comparison to the DMSO control (in 4D sample on the left). Only then it can be verified, if the Ezh2 overexpression can indeed rescue the OSMI treatment phenotype. As it stands right now, the experiment seems inconclusive.

Response: We also deleted all data in Fig.3 and Fig.4 and related descriptions in the text as mentioned above.

8. Fig. 6: The result that ketogenic diet in mothers causes lower PGC specification efficiency in their offspring is very intriguing. Nevertheless, to rule out indirect effects such as reduced embryo growth or delays in development during the critical PGC specification period, the size of embryos of normal and ketogenic fed diet mothers should be also measured and compared as done for the later embryos (this can be done from the existing images).

Response: We measured the size of ES, MS, LS embryos in normal and ketogenic diet fed

mother, and confirmed their comparable size. We showed the data in Fig. EV4D in the revised version.

Minor Points

1. Line 79-80: "In human PGCLCs, vitamin C supplementation enhances the activities of Jumonji (Jmj) C histone demethylases (Irie et al, 2015)..." The reference given does not discuss vitamin C supplementation in the context of human PGCLCs. Please put the correct reference instead.

Response: We cited the reference by Irie et al. for hPGCLCs (line 75).

2. Fig. 1A: It should say Fructose-6-P instead of Fluctose-6-P.

Response: We corrected it.

3. Fig. 1E/F EV2B: Please make clear in the figure legends and explain in the main text + methods what Cyto+ and Cyto- means. People outside the PGCLC field would not understand otherwise that these are with and without cytokine controls. Please provide appropriate context by specifying, which cytokines are meant (all of them or only the BMPs) and that they are used to induce germ cell fate.

Response: We defined Cyto+ as the culture medium with BMP4, BMP8a, LIF, SCF, and EGF in method section (line 315-316) as well as the legends for Fig.1F.

4. Line 166-167: "consistent with the results obtained under glucose deprivation (Fig EV3E and F)" should say instead (Fig. EV3D and E)

Response: We corrected the figure citation (line 161-162).

5. Fig. 3A, 5B, 6B: The enlarged images in the insets indicated by the yellow squares are missing. Please add them (better) or don't show the yellow squares (worse).

Response: The figures included the inlets of the enlarged images, but we more clearly highlighted by thin squares with orange dotted lines.

6. Fig. 3C: For consistency with the other panels Dppa3 should be named Stella. Otherwise it is hard to see the link between expression data and EZH2 CUT&RUN results. Also explain in the figure legend what p.c. and n.c. stand for (I assume positive and negative control) and correct alignment of the brackets and the misaligned significance star next to the Dppa3 EZH2 bar.

Response: We delated Fig.3 as mentioned above.

7. Figs. 4E and EV5C: Stella is misspelled.

Response: We delated Fig.4 and EV5 as mentioned above

8. Line 265: BIIMP1-positive should instead say BLIMP1-positive.

Response: We corrected it (line 206).

Referee #2:

1. The authors mainly use OSMI-1, an OGT inhibitor, as the major treatment to study the effects of O-GlcNAcylation. However, OSMI-1 shows strong cell toxicity that would decrease cell viability significantly. Therefore, in order to show the specificity, other treatments such as OGT knockdown, OGT overexpression, less toxic specific drugs, or OGA-related treatments should be included to confirm that OGT directly regulates the O-GlcNAcylation of EZH2 and affect the subsequent PGC formation or embryonic development.

Response: We applied knockdown of OGT by siRNA and overexpression of OGA to confirm the importance of O-GlcNAcylation in PGC formation. We added the data in Fig.2 in the revised version. We delated data concerning EZH2 and focused on the OGT function in vitro and in vivo, and the effect of maternal ketogenic diet in vivo.

2. In Fig1 F, why does OGT intensity increase while O-GlcNAc intensity decreases? The results seem to be controversial.

Response: After we reanalyzed the data with three biological replicates and DAPI normalization, the increased OGT in 2DG or Cyto- conditions was not observed. We updated Fig.1G in the revised version.

3. And for O-GlcNAcylation detection methods, other methods like Mass Spec or in vitro O-GlcNAcylation assays should also be included to confirm the direct O-GlcNAcylation modification of EZH2 by OGT.

Response: We delated data concerning EZH2 as mentioned above.

5. The connections and interplay among OGT, BLIMP1, and EZH2 are a little bit confusing. In the animal experiments, the authors report that the intensities of OGT and BLIMP1 show a positive correlation in the epiblast cells of embryos. And in Fig1D, OSMI-1 treatment decreases Blimp1 expression. Does OGT regulate BLIMP1 directly in the context of the manuscript? As previously reported, Blimp-1 interacts with OGT and is modified by O-GlcNAc, please refer to "Chen, Y.F., Shao, G.C., Li, J., Yang, A.Q., Li, J. and Ye, X.S., 2022. O-GlcNAcylation of blimp-1 in lymphocytes inhibits its transcriptional function and is associated with migration and invasion of breast cancer cells. Molecular Cancer Research, 20(4), pp.650-660."

And in Fig 3D, EZH2 inhibitor treatment decreases Blimp1 expression. What are the regulatory mechanisms between EZH2 and BLIMP1 in the manuscript context? Does OGT regulate BLIMP1 in a EZH2 dependent or independent manner?

In the text from line 194 to line 211, the authors report that "Immunostaining confirmed that inhibition of OGT by OSM1-1 caused a decrease of EZH2 protein in BLIMP1-negative cells, and of H3K27me3 on day 2 of PGCLC induction compared to cells grown in the absence of OSMI-1 (Fig 3A and B)." and "We, therefore, propose the following model in which O-GlcNAcylation contributes to the maintenance of EZH2 protein levels under normal culture conditions likely resulting in the upregulation of Blimp1 transcription, while inhibition of OGT by OSMI-1 results in decreased EZH2 expression likely resulting in impaired Blimp1 induction." Does OGT regulate EZH2 in a Blimp1-dependent or independent manner? What is the role of Blimp1 in the regulation of EZH2 by OGT.

The authors should clarify more clearly in terms of these parts and figure out the interplay among OGT, BLIMP1, and EZH2.

Response: We delated data concerning EZH2 as mentioned above. OGT may directly or indirectly control Blimp1 expression. We discussed a possible indirect regulation of BLIMP1 expression by O-GlcNAcylation in line 259-262.

6. The quality of western blotting images should be improved, and the bands are relatively obscure.

Response: We deleted the western blot data in Fig. 4.

Referee #3:

While the topic of this study is definitely very interesting I fear that the report suffers from significant flaws especially on the mechanistic side of the story. In my opinion the in vivo OGT-cKO as well as reduced number of PGCs when ketogenic diet is used on their own give a very interesting and strong phenotype that merits publication for example as a short report. However, there is little conclusive support for the proposed mechanistic model and the involvement of epigenetic regulation. Bellow I'm attaching my main points of criticism:

Response: As suggested, we focused on the OGT function in vitro and in vivo, and the effect of maternal ketogenic diet in vivo. All data concerning EZH2 were deleted in the revised version.

1. All mechanistic in vitro experiments rely exclusively on the use of small molecule inhibitors which can and often are non-specific. Mechanistic data in mouse stem cells can and shoulw be validated using orthogonal methods.

Response: We applied knockdown of OGT by siRNA and overexpression of OGA to confirm the importance of O-GlcNAcylation in PGC formation, and showed data in Fig.2 in the revised version. Meanwhile, the roles of EZH2 and H3K27me in regulation of candidate downstream genes were omitted.

2. Conclusions from figure 1, 2 and 3 largely rely on immunofluorescent analysis of embryonic bodies with PGCLCs. I find these analysis very unconvincing. The DAPI signal is often blurry with weak nuclear staining. Surprisingly I can only visually see the effects of treatments on the cells very much in the middle of the EBs while PGCLCs are not present only in this compartment. Some of the treatments seem mainly to lead to death rather than specific effects e.g. Fig 3D. Fig 4D. Finally, EZH2 cKO mice show normal specification of PGCs in vivo (Lowe et al., 2022) how can this be in the light of your inhibition results?

Response:
By editing the manuscript according to the reviewers' comments, we deleted most of the immunostained PGCLC data including Fig. 3D and Fig.4D as well as data concerning the EZH2 inhibitor, and we normalized the remaining immunostained PGCLC data in Fig.1G, and all of the immunostained embryo data in Fig. 3D, Fig.4D, G, and Fig.EV4A by DAPI signals.

3. Figure 4 A/B: The IP is not clean with no EZH2 detected in the input sample (A) and high background just bellow EZH2 in the IP sample of panel B. WB in C looks convincing and is important however neither A/B nor C seem to have biological replicates.

Response: We deleted the western blot data in Fig. 4.

4. Figure 2: The Cut and Tag analysis does not show a convincing decrease of H3K27me3 especially since no spike-inns were used. I am hard pressed to see any difference in panel G. Moreover, in E there does not seem to be a significant overlap between upregulated/downregulated genes and loss/gain of H3K27me3 enrichment. No statistical test is performed here and the reverse analysis is not shown e.g. increased H3K27me3 and increased expression, this would be an necessary control analysis. Based on these data the claims are too strong.
5. Figure 3C: there doesn't seem to be a biological replicate of the EZH2 occupancy experiment.

Response: We deleted the data in Fig. 2 and Fig.3 related to EZH2.

6. While the effects of ketogenic diet on the number of PGCs seem robust and are very interesting I have doubts about BLIMP1, H3K27me3 and OGlcNAc quantifications. Figure 6 The effect size often seems minute (Blimp1 intensity) and the conclusions should be more nuanced. Looking at the images E-F it is clear that the DAPI signal is also lower in the ketogenic diet images. All Ifs should be normalized to the DAPI signal as is standard in such analysis. I suspect that this will remove the significance from this analysis.

Response: We reanalyzed the immunostaining data with normalization to the DAPI signal, in which the DAPI signals were comparable between ketogenic and control diet, and confirmed the significant decline of BLIMP1 and O-GlcNAc intensity by ketogenic diet. We presented the updated data in Fig. 1G, 3D, 4D and G, and Fig,EV4A in the revised version. We deleted data concerning H3K27me3.

7. Many of the conclusions from this report are not supported by the data:
a. Line 204-207: the proposed model has little link to real data, how could PRC2 mediate upregulation of Blimp1?

Response: We deleted the description related to EZH2.

b. Line 318-319: There is no proof that the ketogenic diet effects are actually due to OGT activity.

Response: We deleted the sentence.

c. Line 319-323: There is no data to show that e.g. 2DG results in EZH and H2K27me3 changes.
d. Line 323-324: Current data is not strong enough to conclude that PRC2 is important for inducing PGCs especially without genetic and in vivo evidence.

Response: We deleted the discussion related to EZH2.

7. The authors have previously uncovered that 2DG treatment leads to a slight increase, not decrease in PGCLC number (Hayashi et al., 2017). How can this be explained when compared to this study?

Response: In the previous study, we evaluated PGCLC formation at day4 after their induction, while we showed the effects of the inhibitors at day 2 in this study. On day4, the percentage of BLIMP1-positive cells in aggregates was indeed increasing, but the expression of PGC-related genes such as *Blimp1, Stella,* and *Nanos3* was generally decreased (Hayashi et al., 2017, Fig S9B and D). Therefore, we concluded that normal germ cell formation was not progressing in glucose-depleted or glycolysis-inhibiting condition.

Dear Prof. Matsui

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study but request some further textual and experimental (ref #3) changes, that should be addressed. Please also discuss the limitations pointed out by referee #1 in the text/discussion section.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study:

- Your manuscript will be published in our 'Reports' section. Therefore, please combine Results and Discussion.

- Please remove the 'Author Contributions' section from the manuscript file and make sure that the author contributions in our online submission system are correct and up-to-date. The information you specified in the system will be automatically retrieved and typeset into the article. You can enter additional information in the free text box provided, if you wish.

- References: please use the abbreviation 'et al' if there are more than 10 authors. You can download the respective EndNote file from our Guide to Authors
https://endnote.com/style_download/embo-reports/

- Please arrange figure panels in a way that they can be called out in an alphabetical order. In this respect we note that Fig 1C is called out before 1A.

- Approval of experiments with mice: Please provide the reference number for approval in the methods section and in the Author Checklist.

- Please note that the Data availability section may only refer to datasets produced in the current study. Please refer to the public databases used in another section, unless this is relevant for the generation of the new datasets? Please clarify.

- Tables EV1-EV3 need renaming to Dataset EV1-EV3 as they have multiple sheets and are larger datasets. Please upload these as file type 'Dataset'. The colour should be removed and the legend should be in a separate tab of the .xls file. Please do not forget to change the name in the .xls file itself and update all callouts in the text.

- Tables EV4 and EV5 need renaming to Tables EV1 and EV2.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

- Finally, EMBO Reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height) in PNG for JPG format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

Limitations:
The observed effect sizes of O-GlcNAC perturbation are relatively modest and based mostly on quantification of

immunostainings, which can be subjective. Also the new qPCR results in Figure 2 based on OGT siRNA and OGA overexpression show relatively small impact on PGCLC marker expression. It cannot be ruled out that the phenotypes are mostly due to general cell / embryo health and not necessarily a PGC-specification-specific effect.
PROS: Nevertheless, the authors have responded adequately to all reviewers' questions and rewritten the paper by removing the more problematic part on Ezh2 and rather focused on the effect of ketogenic diet on PGC specification in vivo. The findings overall are novel and of general interest and therefore warrant publication of in the current form.

Referee #2:

The manuscript is now suitable for publication in EMBO reports. I have no other concerns.

Referee #3:

The revised manuscript by Hayashi et al., addresses most of my concerns mainly by improving IF analysis and removing the mechanistic EZH2 data. The authors have also performed a set of siRNA/overexpression experiments. These are particularly important since they seek to confirm the phenotype observed upon OGT inhibition. The effect sizes are rather small, therefore it is important to include at least 3 technical replicates in these experiments and actually show that the O-GlcNac levels are reduced by IF. Whith this data in hand, the results indicate that OGT is one of (but probably not the only) mediator of glycolytic block phenotype on PGC specification. In my opinion once the missing control is included and conclusion is nuanced this manuscript merits publication in EMBO reports as the ketogenic diet results are of interest to the field .

Minor comments:
-The experiments on simple siRNA KD which fail to give a robust downregulation should go to the EV figures.
- Text on line 113-115 should be simplified, there is absolutely no data claiming that 2DG effect on Stella especially since the -Glucose also doesn't affect Stella expression. In fact this is a marker of pluripotent cells which is in line with the results from the RNAseq.

Responses to the comments

Editor:

-Your manuscript will be published in our 'Reports' section. Therefore, please combine Results and Discussion.

We combined the Results and Discussion sections.

-Please remove the 'Author Contributions' section from the manuscript file and make sure that the author contributions in our online submission system are correct and up-to-date. The information you specified in the system will be automatically retrieved and typeset into the article. You can enter additional information in the free text box provided, if you wish.

We deleted Author Contributions from the manuscript file.

-References: please use the abbreviation 'et al' if there are more than 10 authors. You can download the respective EndNote file from our Guide to Authors
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The style of the References was adapted to the Author Guidelines.

-Please arrange figure panels in a way that they can be called out in an alphabetical order. In this respect we note that Fig 1C is called out before 1A.

We moved the old Figure 1C to Figure 1A and rearranged panels in Figure 1.

-Approval of experiments with mice: Please provide the reference number for approval in the methods section and in the Author Checklist.

The approval numbers of the animal experiments were added to the methods section and to the Author Checklist.

-Please note that the Data availability section may only refer to datasets produced in the current study. Please refer to the public databases used in another section, unless this is relevant for the generation of the new datasets? Please clarify.

The description regarding the use of the existing data files was moved to the methods of RNA-seq.

-Tables EV1-EV3 need renaming to Dataset EV1-EV3 as they have multiple sheets and are larger datasets. Please upload these as file type 'Dataset'. The colour should be removed and the legend should be in a separate tab of the .xls file. Please do not forget to change the name in the .xls file itself and update all callouts in the text.

Tables EV1-EV3 was replaced to Datasets EV1-EV3, and the corresponding citations in the manuscript were edited.

-Tables EV4 and EV5 need renaming to Tables EV1 and EV2.

Tables EV4 and EV5 were renamed to Tables EV1 and EV2 and the corresponding citations in the manuscript were edited.

-I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

All comments were addressed and the detailed responses were provided.

- Finally, EMBO Reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height) in PNG for JPG format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

A) a short (1-2 sentences) summary of the findings and their significance

The hexosamine biosynthetic pathway and protein O-GlcNAcylation play a role in PGC specification, and the maternal ketogenic diet also controls fetal PGC development through this pathway, suggesting the possibility that nutritional status in early pregnancy regulates fetal germ cell formation and differentiation.

B) 2-3 bullet points highlighting key results

- Glucose regulates PGC formation via the hexosamine biosynthetic pathway and protein O-GlcNAcylation in vitro.

- Suppression of carbohydrate metabolism and O-GlcNAcylation *in vivo* by Ogt-cKO or ketogenic diets reduces PGC formation and germ cell development.

C) a synopsis image that is 550x300-600 pixels large (width x height) in PNG for JPG format.

We attached a synopsis image.

Referee #1:

-Limitations:
The observed effect sizes of O-GlcNAC perturbation are relatively modest and based mostly on quantification of immunostainings, which can be subjective. Also the new qPCR results in Figure 2 based on OGT siRNA and OGA overexpression show relatively small impact on PGCLC marker expression. It cannot be ruled out that the phenotypes are mostly due to general cell / embryo health and not necessarily a PGC-specification-specific effect.

We discussed the limitations of this study noted by the Referee #1 to the Results and Discussion section (lines 261 - 264) as suggested.

-PROS: Nevertheless, the authors have responded adequately to all reviewers' questions and rewritten the paper by removing the more problematic part on Ezh2 and rather focused on the effect of ketogenic diet on PGC specification in vivo. The findings overall are novel and of general interest and therefore warrant publication of in the current form.

We appreciate the positive comment to our manuscript by the Referee #1.

Referee #3:

-The revised manuscript by Hayashi et al., addresses most of my concerns mainly by improving IF analysis and removing the mechanistic EZH2 data. The authors have also performed a set of siRNA/overexpression experiments. These are particularly important since they seek to confirm the phenotype observed upon OGT inhibition.

We appreciate the positive comment to our manuscript by the Referee #3.

-The effect sizes are rather small, therefore it is important to include at least 3 technical replicates in these experiments and actually show that the O-GlcNac levels are reduced by IF. Whith this data in hand, the results indicate that OGT is one of (but probably not the only) mediator of glycolytic block phenotype on PGC specification. In my opinion once the missing control is included and conclusion is nuanced this manuscript merits publication in EMBO reports as the ketogenic diet results are of interest to the field .

According to the Referee #3's comments, we included three technical replicates in the qPCR experiments using the PGCLC aggregates with the siRNA treatment and/or lentivirus overexpression in Fig 2B and 2C and Fig EV3G and 3H. Also, immunostaining analysis was added to show a decrease in the OGT and O-GlcNAc levels in the PGCLC aggregates with the siOgt treatment and/or OGA overexpression (Fig 2D and 2E).

-Minor comments:
The experiments on simple siRNA KD which fail to give a robust downregulation should go to the EV figures.

The results related to PGCLC aggregates with simple siRNA KD were moved to Fig EV3.

-Text on line 113-115 should be simplified, there is absolutely no data claiming that 2DG effect on Stella especially since the -Glucose also doesn't affect Stella expression. In fact this is a marker of pluripotent cells which is in line with the results from the RNAseq.

We edited the related description in line 112 - 114 as suggested.

Prof. Yasuhisa Matsui
Institute of Development, Aging and Cancer, Tohoku University
Seiryo-machi 4-1
Sendai, Miyagi 980-8575
Japan

Dear Prof. Matsui,

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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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

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1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Data Availability Section

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Table EV5

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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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

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)
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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.	Not Applicable	

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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)
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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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	Figure legends, Materials and Methods
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?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
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.	Yes	Materials and Methods
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	Figures

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	Figure legends
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 approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods (approval number: 2019AcA-026-01)
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)
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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	

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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	
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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	