EMBO reports

Ronin governs the metabolic capacity of the embryonic lineage for post-implantation development

Kirill Salewskij, Theresa Gross-Thebing, Elizabeth Ing-Simmons, Binyamin Duethorn, Bettina Rieger, Rui Fan, Rui Chen, Niraimathi Govindasamy, Heike Brinkmann, Ludmila Kremer, Nannette Kuempel-Rink, Karina Mildner, Dagmar Zeuschner, Martin Stehling, Marion Dejosez, Thomas Zwaka, Hans Schöler, Karin Busch, Juan Vaquerizas, and Ivan Bedzhov
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1st Editorial Decision 10th May 2021

Dear Dr. Bedzhov,

Thank you for the submission of your manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, all referees acknowledge that the findings are interesting and novel. However, they also point out that the data do not always support the conclusions, and these issues must be addressed, ideally by providing stronger experimental evidence. I think all major points raised by referee 2 should be addressed experimentally, but please let me know in case you disagree and we can discuss this further. We can also video chat or talk on the phone about the revisions, if you prefer.

I would thus 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 major 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.

Regarding data quantification, please specify the number "n" for how many independent 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. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

IMPORTANT NOTE: 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:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
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.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

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.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) 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.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- 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.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records

from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) 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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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.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In the study by Salewskij et al. the authors propose that Ronin is key in driving the metabolic changes that occur during the transition from blastocyst to egg cylinder, by inducing the mitochondrial reorganisation necessary to meet the energy demands of rapid cell proliferation that occurs post-implantation. While the effect of Ronin on mitochondrial organisation has previously been demonstrated during other developmental processes, the study's contribution to the field remains novel and significant given the critical developmental transition it is shown to play a role in here.

The manuscript is well written and tells a coherent story, with the rationale and methodology behind each experiment made clear. The authors are measured in their use of language, taking care not to overstate their claims. It is suitable in scope for EMBO Reports, presenting a single key finding with clear supporting evidence.
The authors begin by demonstrating the efficacy of their inducible Ronin knockout ESC line and detailing its impact on cell proliferation. They then employ a rescue experiment, ruling out off target effects and demonstrating Ronin's ability to induce a proliferative state upon restoration.

The authors then offer insight into effects of Ronin knockouts using RNA-seq, detailing a broad range of transcriptional changes that occur. GO analysis was used to explore potential pathways and gene sets influenced by their knockout, revealing, among others, GO terms associated with ribosome complex assembly. While these data are interesting, given the OP Puro assay failed to demonstrate a robust functional effect on nascent protein production that could explain the difference in proliferation, the authors might consider shortening some of these sections and relegating part of the figures to the supplementary section in order to better align with EMBO Report's, concise and focused style.

The authors then turn their attention to other Ronin binding targets that were identified by analysing publicly available ChIP-seq data, exploring a possible effect on mitochondrial function. TEM show loss of cristae and Ronin KO results in general loss of mitochondrial ATP production and efficiency, offering an explanation for the reduced proliferation observed in their ESC culture model. In order to confirm whether these changes in mitochondrial structure also occur in vivo E4.5, E5.5, as well as diapause mouse embryos were examined by TEM, revealing similar differences to those found between fl/del and del/del cultured ESCs. A weakness of these experiments is that they are unable to provide direct evidence that Ronin is responsible for the mitochondrial changes observed in vivo, however, they do provide circumstantial evidence and offer credibility to the tissue culture model.

The role of Ronin in vivo is further explored through the generation of embryo / ESC chimeras, in order to elegantly sidestep the previous barrier of Ronin knockout embryos failing to progress beyond blastocyst stage. These experiments, further substantiate the results of the in vitro experiments, demonstrating that Ronin del/del cells are rapidly out-proliferated by their fl/del counterparts, and unable to enter a state of proliferation even in embryos transitioning from diapause to active development.

Minor comments:
In figures 5C and 6B it would be nice to see quantification of the differences in mitochondrial cristae along with an indication of the number of cells or mitochondria analysed.

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The study by Salewskij, Gros-Thebing and colleagues uncovers the role of the transcription factor Ronin in the regulation of metabolic output of mouse embryonic stem cells and early mouse development. The authors find that Ronin not only regulates the expression of ribosomal genes and thus metabolic remodelling at the onset of differentiation but also is required for the correct patterning and morphogenesis of the post implantation embryo.
This is an interesting and relevant study that sheds light on the link between metabolism and cell fate during early embryogenesis. However, there are some aspects that, if revised, would strengthen the study.

1. Cell growth and cycle analysis (lines 106-113; lines 166-167): The authors observe very clear differences in cell counts, but no significant difference in cell death levels or cell cycle profile when they compare deleted to control cells. In the micrographs shown in Figure 1 the main divergences in cell growth seem to occur between day 5 and 6 post Ronin deletion. If no differences in cell death or cell cycle analysis were found at days 4 and 6, it would be informative to look at these parameters at day 5 to pinpoint if changes in the cell death response or cell cycle profile account for the

differences observed. Additionally, the authors report here that Ronin depleted cells are quiescent and progress more slowly through the cell cycle, but this is not supported by the data presented. Looking at proliferation markers such as MCM2 or Ki67 could help to address this point. Alternatively, the authors may want to consider revising their statement.

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This study is novel and the conclusions are supported by strong evidence. It provides a clear answer to a long-standing question: the cause of acceleration of pluripotent cell proliferation rate in the post-implantation epiblast. The manuscript is overall excellent.
Major points:
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2/ Mouse EpiSC and conventional human PSCs are known to have low mitochondrial activity compared to naive ES cells, although their transcriptome corresponds to that of the post-implantation epiblast. What would be the effect of Ronin deletion if induced after the conversion from ES to EpiSC ?

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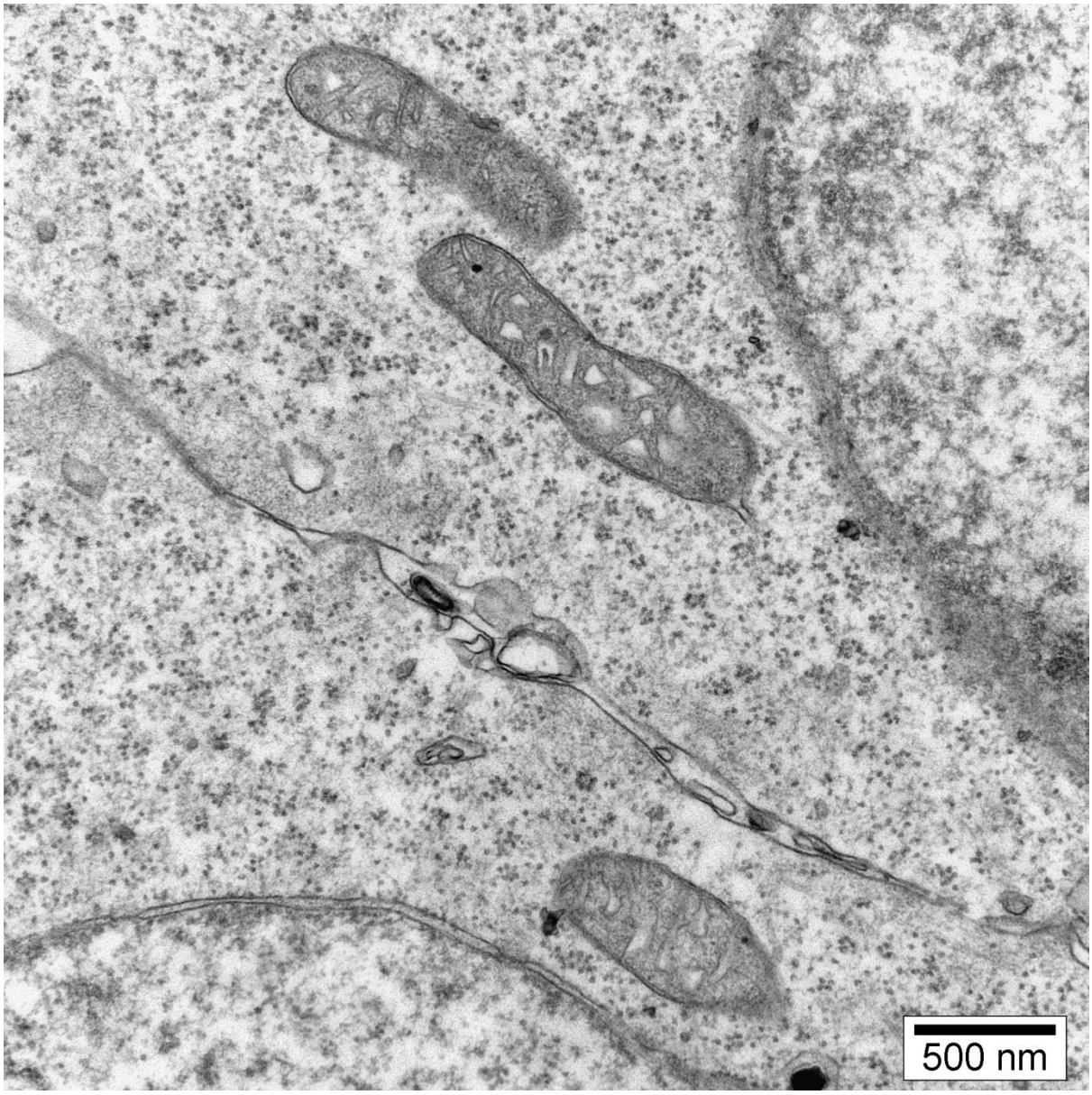
We completely agree with all remarks of Referee #1 and we are very grateful for his/her positive comments!

Minor comments:
In figures 5C and 6B it would be nice to see quantification of the differences in mitochondrial cristae along with an indication of the number of cells or mitochondria analysed.

We examined the structure of the mitochondria by transmission electron microscopy in the epiblast of E4.5 (n mitochondria = 55), diapause (n = 62) and E5.5 embryos (n = 65), as well as in Ronin fl/del, Day 4 del/del and Day 8 del/del ES cells (n mitochondria for each cell line > 150). We agree with Referee #1 that quantification of the number of the mitochondrial cristae will complement the results. However, as the transmission electron microscopy provides a single section through the mitochondria, it is practically impossible to get reliable quantifications even for controls. As you can see from the attached image, it is difficult to pinpoint the exact number of individual cristae. Proper quantitative analysis of these structures will require different experimental approach, such as 3D electron microscopy for acquiring the data and computational 3D reconstruction for the analysis. Therefore, we prefer to abstain providing specific numbers for the mitochondrial cristae, as in our view the results will not faithfully reflect the real numbers of these structures.


500 nm

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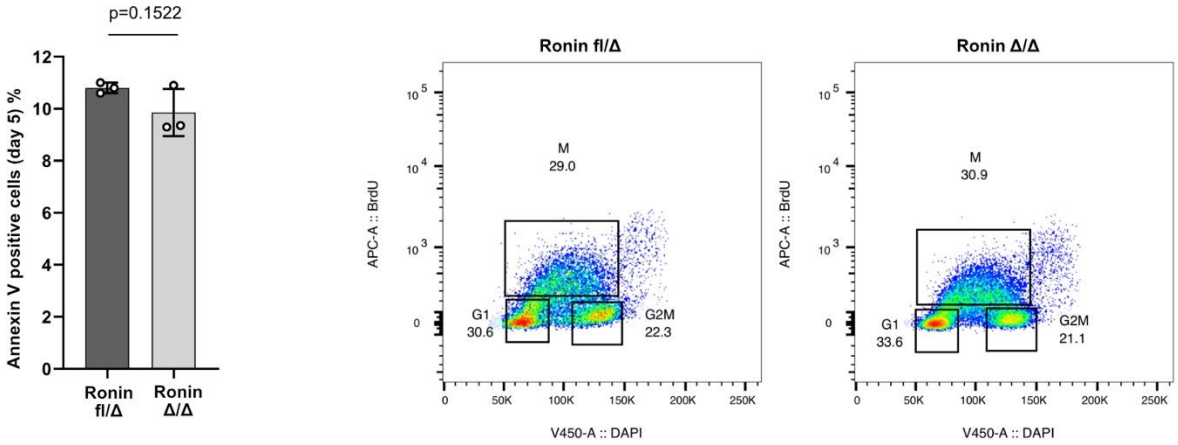
We are grateful to Referee #2 for his/her positive comments and constructive suggestions.

1. Cell growth and cycle analysis (lines 106-113; lines 166-167): The authors observe very clear differences in cell counts, but no significant difference in cell death levels or cell cycle profile when they compare deleted to control cells. In the micrographs shown in Figure 1 the main divergences in cell growth seem to occur between day 5 and 6 post Ronin deletion. If no differences in cell death or cell cycle analysis were found at days 4 and 6, it would be informative to look at these parameters at day 5 to pinpoint if changes in the cell death response or cell cycle profile account for the differences observed. Additionally, the authors report here that Ronin depleted cells are quiescent and progress more slowly through the cell cycle, but this is not supported by the data presented. Looking at proliferation markers such as MCM2 or Ki67 could help to address this point. Alternatively, the authors may want to consider revising their statement.

Referee #2 is correct; indeed, the decrease in the proliferation rate of Ronin KO cells becomes evident beyond day 4 of 4OHT treatment. This phenotype is manifested gradually, without a sharp increase in cell death or a particular block at specific phase of the cells cycle. Following the suggestion of Referee #2, we examined these parameters at day 5 of culture and we found no substantial difference between control and KO ES cells, in agreement with our conclusions in the manuscript. As the results of these analysis did not provide new information, we incorporated the data only into the Reviewers report (which will be also available to the readers).



As the cell cycle distribution (at all of the examined days of culture) was similar between control and ko cells, this suggested that that Ronin ko ESC progress more slowly through the cell cycle without being blocked in a particular phase. We agree with Referee #2 that the suggested slow progression requires additional experimental evidence that examines the dynamics of the cell cycle with single cell resolution (we consider that live imaging using fluorescent sensors is the most appropriate approach), therefore, we removed this statement from the manuscript.

2. Mitochondrial health and metabolic competency: The authors report severe impairment of cristae organization due to the downregulation of genes such as Opa1, Dnm1l and Micos10 in Ronin depleted cells. Although the mitochondrial membrane potential is found to be similar between control and Ronin depleted cells, loss the above mentioned genes have been associated with disrupted microdomains of mitochondrial membrane potential between cristae and inner boundary membranes (Wolf et al, EMBO J 2019). The distribution of TMRE appears not to be as homogeneous in treated vs control cells and this maybe why the variance of data is higher between treated vs control cells (Figure 5E). Doing mitochondria membrane potential analysis by flow cytometry could be

more informative as it gives single cell resolution.

We are very grateful to Referee #2 for the suggested experiment of measuring the membrane potential via flow cytometry! Indeed, this diminished the variability (3 independent experiments), but importantly the new analysis showed reduced TMRE intensity in Ronin KO ESC (updated Figures 5D and 5E). As the energy required for ATP production is derived from the $\Delta\Psi_m$, the new results suggest that Ronin ko cells exhibit a decreased energy availability for ATP synthesis. This fits well with the results of the Seahorse analysis, which also indicate general reduction of the mitochondrial ATP production rate and energy efficiency.

In lines 233-235, the authors refer that the structural changes are not pathological, but how is this observation supported by the data? There is no actual analysis of cytochrome c release and the authors actually associate the lower metabolic output to the disrupted cristae organization. Additionally, the authors cannot exclude that the levels of mitochondrial protein complexes are altered and contribute to this phenotype, as indicated by GO enrichment analysis shown in Figure 4E. The authors should consider revising this statement or adding additional supporting data.

We completely agree with Referee #2 and we removed this statement from the manuscript.

3. Quantifications: the authors should provide a quantification for the western blots shown in Figure 1A and the immunofluorescence images shown in the manuscript (Figure 1G, S1 and 2F).

The quantifications of Figure 1A, 1G and S1 (now EV1C) are presented in Figure EV1. As Ronin del/del cells exhibit strong proliferation defect, we are reluctant to make any strong statements about the result of the analysis displayed on Figure 2F. As stated in the text the lack of definitive endoderm markers may be an indirect effect of the slow growth rate; nonetheless, we cannot exclude that Ronin may play a yet unknown role in the specification or maintenance of the endoderm lineage. In addition, the expression pattern of Vimentin (intermediate filament) makes it impossible to accurately pinpoint which cells are positive. Therefore, quantifications based on the images of Figure 2F are very likely to be misleading. We consider that the investigation of potential Ronin function in the somatic lineages will require in-depth analysis in a separate study. Such analysis should also include the conditional deletion of Ronin in vivo, in the post-implantation embryo, in order to verify conclusions based on in vitro assays.

Minor comments:
- The number of observations/independent experiments should be reported for each figure. The error bars should also be specified.
- The authors should clarify what individual data points refer to - technical or biological replicates - and how quantifications were made (e.g. Figure 5E - Do individual points refer to cell colonies/ imaged microscopy fields?)
- Figure S4A refers to FPKM but no explanation to this parameter is given, which is helpful for non-experts in RNA-Seq data analysis.

The figure legends are updated according to the requirements of EMBO Reports. Figure 5E specifically is replaced by flow cytometry analysis (as suggested by Referee #2) and each data point represent an independent experiment. The legend of Figure S4A (now Figure EV4A) is also updated “normalized Ronin expression (fragments per kilobase of exonic sequence per million mapped reads; FPKM) in the epiblast of E4.5, diapause and E5.5 embryos, based on the RNA-seq dataset of Boroviak et al., 2015”.
To further clarify the usage of FPKM, we would like to mention here that the paired-end RNA-seq reads were aligned to the mouse genome, the number of reads overlapping exonic regions of each gene was counted, and normalized by library size and gene length to return expression values for each gene in FPKM (fragments per kilobase of exonic sequence per million mapped fragments

We thank again Referee #2 for his/her valuable suggestions that helped us to improve the manuscript.

Referee #3:

The manuscript of Salewskij et al. reports the results of a study showing that Ronin plays a key role in regulating the growth of mouse ES cells and that of post-implantation epiblast cells in mice. In ES cells, the authors show that Ronin regulates the formation of mitochondrial cristae. Ronin depletion results in a gradual loss of cristae, leading to a reduction of the mitochondrial ATP production rate. In vivo, they show that Ronin also governs post-implantation growth; Ronin-null ES cells fail to truly participate in post-implantation epiblast morphogenesis. This study is novel and the conclusions are supported by strong evidence. It provides a clear answer to a long-standing question: the cause of acceleration of pluripotent cell proliferation rate in the post-implantation epiblast. The manuscript is overall excellent.

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Major points:
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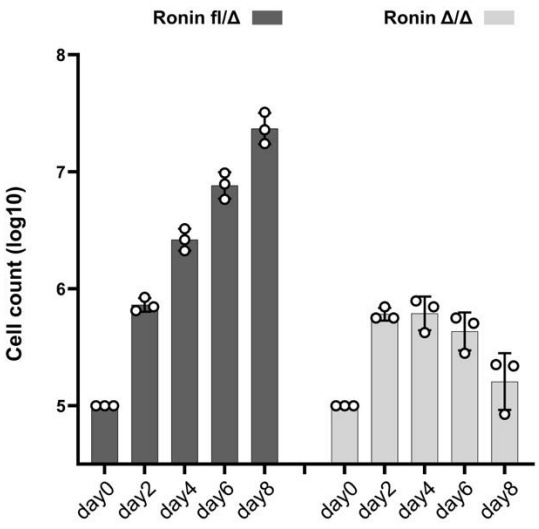
We referred to the publication of M. Snow (J Embryol Exp Morph, 1977) in the Discussion. The context of the specific paragraph in the Discussion was more appropriate for including M. Snow's work, rather than the Introduction. We thank Referee #3 for pointing us to fundamental study of M. Snow, which we overlooked in the original submission.

2/ Mouse EpiSC and conventional human PSCs are known to have low mitochondrial activity compared to naive ES cells, although their transcriptome corresponds to that of the post-implantation epiblast. What would be the effect of Ronin deletion if induced after the conversion from ES to EpiSC ?

Following the suggestion of Referee #3 we examined the effects of Ronin deletion upon conversion of ESC to EpiSC. We "primed" the Ronin fl/del CreERT2 ESC by culture in EpiSC medium for 48h. After that we induced Ronin deletion via 4OHT treatment (day 0), while maintaining the EpiSC culture condition and analysed the proliferation rate up to day 8. We found that from day 4 the proliferation of Ronin del/del cells was suppressed and the cell number started to decline between day 6 and day 8 (please, see the figure bellow; the datapoints represent three independent experiments). As Referee #3 pointed out, the primed cells (mouse EpiSC and conventional human PSC) exhibit low mitochondrial activity. This raises several interesting questions: 1) does potential perturbation in the mitochondrial function in primed cells affects their viability and / or proliferation, despite of the low activity of the mitochondria; 2) does Ronin plays a yet unidentified role in primed cells; 3) is there changes in the Ronin target genes in naïve vs primed states of pluripotency and 4) what will be the effects of global Ronin depletion in the post-implantation epiblast. To address these questions, one has to carefully examine the cell behaviour, transcriptional and metabolic responses and also map the genome occupancy of Ronin binding in EpiSC. Such analyses have to be complemented with in vivo ablation of Ronin after implantation using Sox2-Cre mediated conditional deletion. Therefore, we prefer to include the preliminary results of Ronin deletion in primed cells only here (this document will be available to the readers as well), as the data in our view is insufficient to draw conclusions and the necessary workplan to do so is beyond the scope of the current manuscript. Nevertheless, we are interested to explore this research avenue in the future and we are grateful for Referee #3 for his/her suggestions!


Ronin fl/Δ
Ronin Δ/Δ
Cell count (log10)
8
7
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day6
day8
day0
day2
day4
day6
day8

1st Revision - Editorial Decision 20th Aug 2021

Dear Ivan,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees and I am happy to say that all support its publication now.

Only a few editorial requests still need to be addressed:

- Please add up to 5 keywords to your manuscript file.

- Hans Schöler's author contributions are missing, please add.

- The reference format is not exactly Harvard style. Not more than 10 authors may be listed before "et al", please correct.

- Please move the figure legends to after the Reference list.

- Fig EV4 only has one panel so doesn't really need the 'A' panel label.

- Please upload the source data as 1 file per figure and label all bands that are used in the figure panels.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript.

EMBO press 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 exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Please also check again the abstract that needs to be written in present tense. May be this sentence could be shortened to:
Here, we show that Ronin plays a critical role in the process by enabling active energy production, and the loss of Ronin causes a reversible quiescent state that promotes naïve pluripotency. (If this still correctly describes your data)

I look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

I thank the authors for responding to my comments. While a quantification of the number mitochondrial cristae would strengthen the results, I appreciate this is difficult ascertain from 2D sections alone.

I do not feel the lack of this quantification presents substantial grounds for rejection and therefore I would be happy for the manuscript to be accepted in its current form.

Referee #2:

The authors have addressed all the points raised in the original review. This is a very nice and interesting study.

Referee #3:

The authors have adequately responded to my comments

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 26th Aug 2021

Dr. Ivan Bedzhov
Max-Planck Institute for Moleculare Biomedicine
Münster
Germany

Dear Ivan,

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.

Please note that under the DEAL agreement of German scientific institutions with our publisher Wiley, you could be eligible for publication of your article in the open access format in a way that is free of charge for the authors. Please contact either the administration at your institution or our publishers at Wiley (emboreports@wiley.com) for further questions.

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Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Ivan Bedzhov
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2021-53048V1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple 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.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	cell proliferation, cell cycle, cell death, OP Puro, TMRE and Seahorse analysis were performed with three replicates ; RNA-seq analyisis were also performed with three replicates per condition
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Not applicable
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Not applicable
Is there an estimate of variation within each group of data?	Not applicable

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http://www.antibodypedia.com
http://1degreebio.org
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http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Not applicable

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies were validated for use by a supplyer and by other publications. Catalog numbers and dilitions are provided in the Matherials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Ronin flox/del mouse embryonic stem cells are generated by Dr. Marion Dejosez (Huffington Foundation Center for Cell-based Research in Parkinson's Disease, Department for Cell, Regenerative and Developmental Biology, Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA)

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	The mice used in this study were at the age from 6 weeks to 5 months. The animals were maintained under a 14-hour light/10-hour dark cycle with free access to food and water. Female mice were housed in groups of up to 4 per cage and male stud mice were housed individually. Embryos for experiments were obtained from wild-type B6C3F1 and CD1 strains.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments and husbandry were performed according to the German Animal Welfare guidelines and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (State Agency for Nature, Environment and Consumer Protection of North Rhine-Westphalia).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Data availablity section is provided
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	RNA-seq satasets were deposited to ArrayExpress
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

G- Dual use research of concern

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