EMBO reports

Cristae undergo continuous cycles of membrane remodelling in a MICOS-dependent manner

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Transaction Report:

(This manuscript was transferred to *EMBO reports* following review at *The EMBO Journal*. Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18th Dec 2019

Thank you for the transfer of your manuscript from our sister journal The EMBO Journal to EMBO reports. Your manuscript had been evaluated by two mitochondria experts (referee 1 and 3) and by one imaging expert (referee 2) for potential publication in EMBO Journal. We note that after the revision, the referees appreciated that you provide evidence that mitochondrial cristae are dynamic and undergo continuous membrane remodeling in a MICOS-dependent manner. The referees however remained concerned that the current dataset failed to provide convincing evidence for bona fide membrane fusion and fission.

You have provided a revised version of your manuscript in which you toned down conclusions on fusion and fission alongside a point-by-point response. I have contacted former referee 1 and an additional advisor with expertise in mitochondrial dynamics.
Referee 1 concluded that even though s/he "[...] might not agree with every point that is discussed in the letter, I think that the manuscript contains a lot of interesting data. [...] I do think that by changing the title and part of the introduction and discussion the manuscript is much more accurate now [...]."
Also the advisor contacted has meanwhile replied and indicated that in his/her opinion the study '[...] will advance the field and provide new insights into the dynamic remodeling of the cristae membranes." The advisor agreed with the referees regarding the data on fusion and fission but considered the revised version sufficient to address these concerns.

Given the support from referee 1 and the advisor, we would thus like to invite you to submit a final revision of your study for publication in EMBO reports.

Please upload the revised text and address the following editorial concerns:

- Please provide up to five keywords.

- Figure 6A is never called-out in the text.

- Please remove all Appendix and movie legends from the manuscript file.

- Movies: our editorial assistants have already created the movies in the correct format, i.e., they have extracted the movie legends and zipped them together with the movie files. These .zip files have been uploaded to your manuscript.

- Please merge the Appendix legends with their figures and create a single pdf file called Appendix that includes a table of content including page numbers.

- I have checked the figure legends regarding completeness and accuracy. Please see my suggested changes in the attached document.

- 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 550x200-400 pixels large (width x height). 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.

Authors' response to referee reports:

Referee #1:

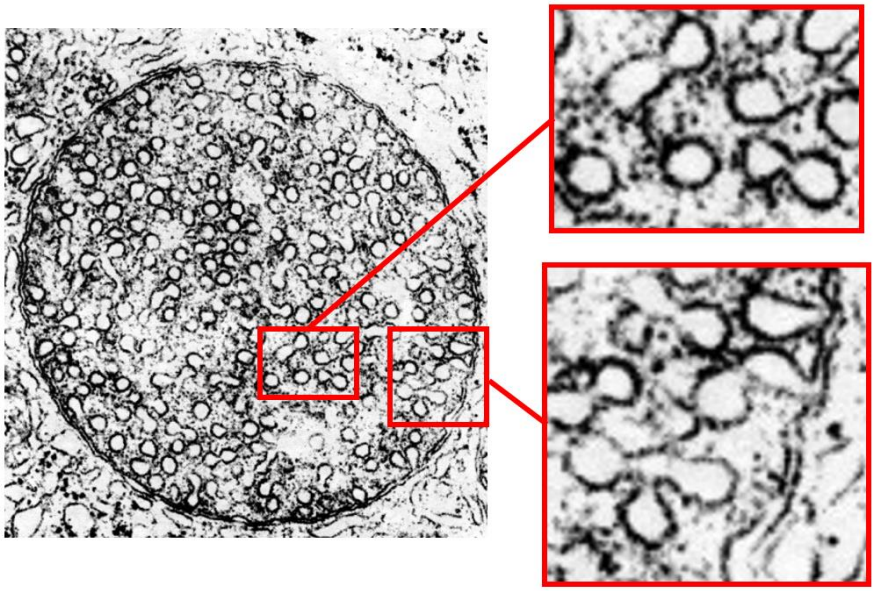
"The authors have addressed many of the critical points and substantiated the manuscript. I still think it is an extensive study with interesting data. "

Thank you for pointing this out.

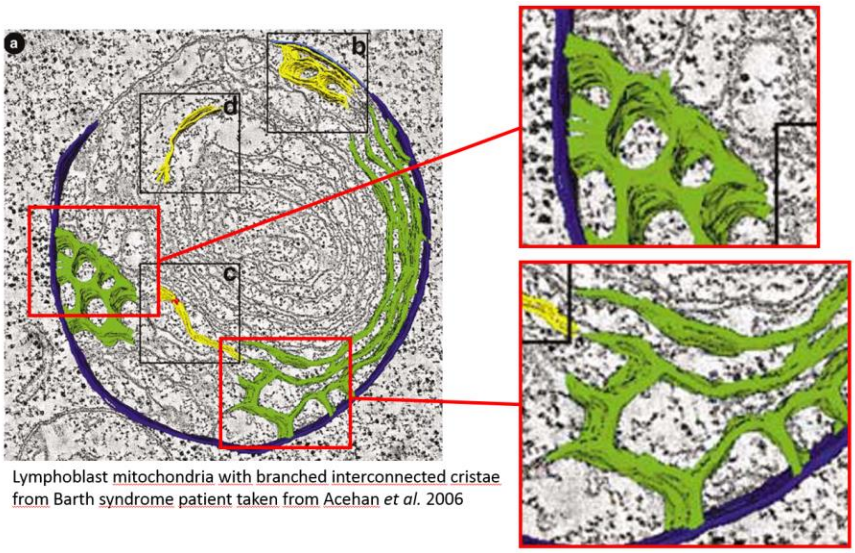
"Nonetheless, I'm also still not convinced that the data necessarily allow the conclusion of inner membrane fission and fusion. The authors made some effort to collect more data on that point. Additional marker proteins were included (ATP5I-PAGFP), more data on membrane potential fluctuations are shown and a potential PEG induced fusion was analyzed in WT and deltaMic13 cells (though the deltaMic13 fusion bar seems to be well within the margin of error of WT fusion, which makes this critical experiment difficult to interpret) but at the end off the day fission and fusion is not directly shown and doesn't become more real just by saying that the observed effects cannot be explained without fission and fusion. Why is it, for example, that in all EM investigations, the authors cite Mannella here, no signs of cristae membranes that are in the process of fission or fusion could be detected? In vesicle fusion as well as in membrane fission events in many membrane trafficking processes like synaptic vesicle fusion or clathrin-mediated endocytosis these states of membranes in the process of fusing or separating are readily observed. "

We see the points and have changed our conclusions accordingly. We still want to clarify that in the PEG fusion assay we have used a box plot diagram that rather shows the data range/percentiles (not error bars) and that statistical analysis clearly revealed that content mixing is significantly reduced (not blocked) in MIC13 KO cells compared to WT cells. The study from Carmen Manella (2001 IUBMB Life), we refer to, has observed that in isolated mitochondria the number of CJs and the morphology of cristae is dependent on the bioenergetic state (orthodox vs. condensed). Carmen Mannella nicely pointed out "...A serious drawback of electron microscopy is that changes in a particular specimen, for example, a specific mitochondrion, cannot be followed over time. Instead, inferences must be drawn from the behavior of populations of organelles..." (Mannella et al. 2001, IUBMB Life). In the same review he concluded that "...The membrane topologies observed in condensed (matrix contracted) and orthodox (matrix expanded) mitochondria cannot be interconverted by passive folding and unfolding. Instead, transitions between these morphological states likely involve membrane fusion and fission". Yet, we agree that this is also only a hint and

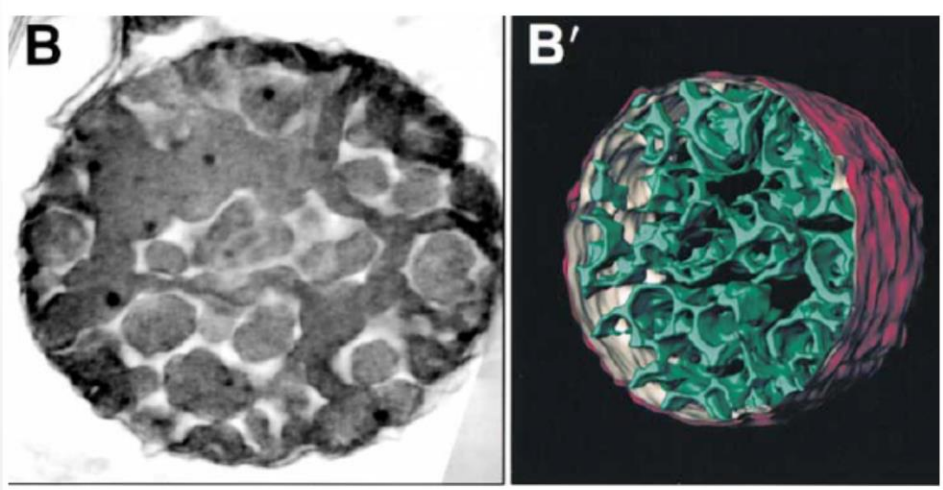
does not prove cristae fission/fusion formally also due to the limitation that it is not observed over time in living cells. Still, there are actually several other EM investigations that revealed striking membrane shapes of cristae in different mitochondria that could well represent fission/fusion intermediates. For your illustration I have put three examples (one under physiological condition, one from a patient, and one during apoptosis). :



Mitochondria with tubular cristae from adrenal gland taken from
Fawcett 1981



Lymphoblast mitochondria with branched interconnected cristae from Barth syndrome patient taken from Acehan *et al.* 2006



Apoptotic mitochondria with interconnected cristae taken from Scorrano et al. 2002

Albeit all these examples are not proving cristae fission and fission, we are convinced that with our novel observations studying cristae membrane remodelling events in living cells, it is time for formulating a working model for cristae fission and fusion that is unexpectedly linked to the dynamics of CJs and the MICOS complex. The experimental support is strong and explains many older observations reported by Mannella and others.

"To me it would make way more sense to publish the manuscript with a changed title and a toned down interpretation that is closer to the actual results. There is nothing wrong in implicating fission and fusion in the discussion but putting it as the major finding is to my understanding an overstatement. "

Thank you for this constructive suggestion. The revised version has been modified accordingly.

Referee #2:

"The authors have replied to my comments in every way possible. They provided raw imaging data to substantiate their claims, and they have analyzed their data in a blind fashion.
The blind analysis confirmed their observations, which is quite important. "

We are grateful for this positive feedback and the constructive advice.

"Unfortunately, from the raw data I am still unconvinced whether the authors have the required resolution and signal-to-noise ratios necessary to demonstrate cristae fusion or fission, as opposed to simpler spatial reorganization events. In this respect, it is interesting to note that the authors of the paper by Wang et al. (PNAS, 2019), whose labeling has a substantially higher signal-to-noise ratio, also advise caution in interpreting such images: "Time-lapse imaging may be able to capture a fusion event between distinct cristae protruding from opposing sides of the mitochondrion, although we cannot rule out the possibility that two opposing cristae happen to come close each other without fusion (Fig. 5A). " Overall, although I appreciate very much the effort of the authors, I am still unable to accept that the events they describe are always bona fide fusion and fission events. However, I would like to state that my opinion is that of an imaging specialist, but not a mitochondria expert. I suggest that mitochondria experts decide whether the manuscript is acceptable in its present form – as I do not see what the authors could do to improve it without efforts that would go beyond the purpose of one single paper. "

As pointed out in our response to reviewer 1 we agree and have adapted the overall conclusions accordingly. In the revised version we clearly state that due to technical limitations resulting from the achieved resolution of STED nanoscopy alone we only can conclude that two signals approach closer than 60 nm. We aimed to test content mixing by additional methods/dyes including novel data on TMRM (showing signal redistribution and fluctuation), photoactivation experiments (showing delayed distribution to distinct cristae consistent with the transient existence of isolated cristae) and the PEG fusion assay (showing MICOS-dependent mixing of two differently labelled cristae marker proteins). We now discuss these results more conservatively as support for our model (not as a proof). Thank you for acknowledging our immense efforts until this stage.

Referee #3:
"The main message of the re-revised manuscript is that the cristae of mitochondria undergo a continuous cycle of fusion and fission in a MICOS-dependent manner. Although I am a believer that cristae do have the ability to fuse and separate, I do not observe any solid evidence in this manuscript that this is what occurs. The evidence present is all circumstantial. "

It is encouraging the reviewer is a believer in this dynamic process (as we are), yet the prevailing dogma in the mitochondrial field is that cristae are rather stable and static entities under physiological conditions. We would like to emphasize that fission/fusion is not the main message here (although certainly an important one based on which we now formulated a working model) but that the dynamic nature of CJs and of cristae are the main focus of the paper. Here I am also referring to the first paragraph of our response letter. We think that cristae fission/fusion as a model is important to discuss and to propose. Yet, our study has other very important main findings that go beyond this aspect. As pointed out above we have adapted the overall conclusions accordingly and hope that the reviewer sees that the comprehensive novel data should be made more accessible to a broad audience.

"My major concern with this manuscript is that the data presented has been over interpreted to fit the authors preconceived ideas. I am happy to accept that the data presented shows that cristae are dynamic and in continuous motion which occurs in a MICOS-dependent manner. I am not happy with the notion that the data presented provides evidence of cristae fission and fusion. To accept the later interpretation, I would like to see evidence that fusion of two mitochondria populations containing MICOS complexes with different labelled tag can fuse to make one type of MICOS complex and that when the authors say two cristae have fused in an X or Y configuration, this is supported by EM analyses using correlative SR light/EM techniques. "

See also our responses to reviewers #1 and #2 above. We agree that correlative light/EM approaches are an interesting other approach that, however, would go beyond the scope here in our opinion.

"Other possibilities would be to provide images of the quality of figure 5A in the related PNAS paper provided but with additional panels which shows the two merged cristae move together after a merging event. Also, proper description of the realistic resolution of each technique used needs to be added to the text so non-specialist can accurately assess the reliability of the data and conclusions presented. "

Overall, these are interesting suggestions. We actually do see cristae moving together after apparent mergence but this would still not allow us to prove a fusion event. For instance, we see cristae moving towards each other and merging by forming apparent letters in the shape of 'X' and 'Y'. These complex cristae shapes sometimes move together for short times followed by spatial separation of cristae. Yet, these cristae could just form close contacts, appear as 'letters' and move together. Overall, we have provided 15 movies to observe CJ and cristae dynamics in WT and KO cells.

We have added info on the resolution of STED (60 nm) in the revised version pointing out this technical limitation.

"Thus, in it's the current form, I can not recommend the publication of this article (despite the impressive amount of work performed), unless the focus on cristae fusion and fission is removed. "

We have accordingly changed the interpretations and the focus of the manuscript (see also first paragraph of our response letter).

1st Revision - authors' response 19th Dec 2019

The authors performed the requested editorial changes.

2nd Editorial Decision 13th Jan 2020

Thank you for your patience while we have editorially reviewed your revised manuscript. I am now writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed.

2nd Revision - authors' response 13th Jan 2020

The authors performed the requested editorial changes.

3rd Editorial Decision 14th Jan 2020

Thank you for implementing the final revisions. I am now 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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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Andreas Reichert
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49776

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size for various experiments were chosen in the range typically used in the mitochondrial biology field involving biochemistry experiments, electron micrsocopy and super-resolution imaging
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No inclusion/exclusion criteria was used
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.	Quantification of cristae, CJs dynamics and PEG experiment was done blindly in order to avoid biased results.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	Quantification of cristae, CJs dynamics and PEG experiment was done blindly in order to avoid biased results.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes, appropriate statistics are included
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, statistics are included for each figure in the respective legends.

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Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	N/A

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).	The details of the antibodies are listed in the materials 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.	Hela and HEK293 cells were obtained fom ATCC. WT HAP1 cells and KOs in HAP1 cells were obtained from Horizon Discovery company. All the cell lines used in the manuscript were checked for mycoplasma contamination routinely.

* 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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	N/A
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).	N/A
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).	N/A
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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