EMBO reports

Mitochondrial F_1F_O ATP synthase determines the local proton motive force at cristae rims

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DOI: 10.15252/embr.202152727

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Review Timeline:

Submission Date:	22nd Feb 21
Editorial Decision:	29th Mar 21
Revision Received:	16th Jul 21
Editorial Decision:	30th Aug 21
Revision Received:	31st Aug 21
Accepted:	10th Sep 21

Editor: Deniz Senyilmaz Tiebe/Martina Rembold

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. 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.)

1st Editorial Decision 29th Mar 2021

Dear Dr. Busch,

Thank you for the submission of your research manuscript to our journal, which was now seen by three referees, whose reports are copied below.

Referees express interest in the measurements of pH profiles of mitochondrial sub-compartments and the proposed roles of IF1 in regulation of local PMF. However, they also raise overlapping important concerns that need to be addressed to consider publication here.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Given these positive recommendations, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as 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 generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact the scientific editor handling your manuscript to discuss a revision plan should you need additional time, and also if you see a paper with related content published elsewhere.***

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 (where applicable).
2. Your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section should be separate. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by

eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Please note that for all articles published beginning 1 July 2020, the EMBO Reports reference style will change to the Harvard style for all article types. Details and examples are provided at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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).

3) 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. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/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.

5) 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 (<http://embor.embopress.org/authorguide>).

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.

- 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: <http://embor.embopress.org/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.

7) 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 <http://embor.embopress.org/authorguide#sourcedata>.

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <http://embor.embopress.org/authorguide#datacitation>.

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>).

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) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. 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.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

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 version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Mitochondrial energy conversion takes place in cristae membranes, invaginations of the inner membrane. There, the respiratory chain maintains a proton motive force - consisting of a membrane potential and a proton gradient - that powers ATP synthesis. Previous work in yeast has shown that no pronounced pH gradients exist along or over the inner membrane (PMID: 31964824), in line with a local kinetic coupling of the OXPHOS system. Likewise, individual cristae were shown in various human cells to maintain distinct membrane potentials, demonstrating that individual cristae operate as discrete units (PMID: 31609012 and 32067344). In this manuscript by Bettina Rieger et al, pH values have been determined in HeLa cells with the help of a pH sensitive GFP version. The data confirm a, the existence of only minor pH differences over or along the inner membrane also in human cells and b, that these values are no homogenous over the inner membrane. Moreover, the authors studied the pH differences at the IMS or matrix side on ATP synthase, which is a novel and elegant approach. By deleting IF1 from the cells, the authors provide new data on the physiological role of IF1 to modulate ATP synthase activity during glycolytic or OXPHOS conditions, which impacts the intra-mitochondrial proton concentrations.

Overall, the presented data are of very good quality and the manuscript provides new insights into the regulation of ATP synthase activity and intra-mitochondrial pH homeostasis. However, the conceptual advance at the current stage is rather limited and a few additional controls and textual edits are necessary to improve the manuscript.

Major points:
1. It is currently not clear, whether the GFP-tagged proteins assemble into the respective OXPHOS complexes. This information is important to validate central claims of this work. This is particularly important for the localisation of the probes and the question whether they report exclusively pH values in vicinity to the complexes. Hence, the authors should provide biochemical evidence for each GFP reporter and how efficiently it assembles, eg by western blot analyses of blue native page/size exclusion chromatography. Given that they used a strong viral promotor for expression, it is possible that a substantial fraction of the proteins is not assembled.

2. The new data on pH gradients in the absence of IF1 or upon expression of constitutively active IF2 are interesting, but currently lack additional experiments that are necessary to draw mechanistic conclusions. For example, analyses of membrane potential, steady state levels of mitochondrial proteins, activities of OXPHOS complexes or their coupling to produce ATP would be important controls. The data in Figure 2 A-D shows that the pH at SU e drops under OXPHOS conditions when oligomycin inhibits the ATP synthase, but not in the absence of IF1. This would suggest that either oligomycin cannot inhibit ATP synthase in this scenario or that the activity of the respiratory chain is decreased.

Minor points:
1. The title of the manuscript is misleading. There is currently no evidence that the authors measure pH values at the tips of the cristae membranes. Instead, micrographs presented in Figure 2 N show that the signal for ATP synthase is spread over the complete mitochondrial network. Possibly this observation can be explained by non-assembled reporter proteins (see point 1 above)?

2. Important recent work on the autonomous behaviour of cristae should be discussed (PMID: 31609012 and 32067344) in detail. Currently this work is not even cited. Moreover, the finding that pH gradients over the inner membrane are rather negligible is in line with previous findings, including the recent publication (PMID: 31964824). Therefore, the text needs to be adapted.

3. There are a few typos that should be corrected.

Referee #2:

The mitochondrial F1FO ATP-synthase (ATP synthase) utilizes a proton gradient to produce the bulk of cellular ATP under respiratory conditions. Upon growth at glycolytic conditions the ATP-synthase hydrolysis ATP to maintain a proton gradient across the inner membrane. According to the current understanding, the inhibitory factor 1 (IF1) blocks the ATPase activity to prevent futile ATP hydrolysis. Rieger and colleagues measured the impact of the ATP synthase and IF1 on the generation and maintenance of local proton gradients in the cell using GFP-based pH-sensitive probes. The author made unexpected findings on the function of the ATP synthase. First, they found that the ATP synthase determines local proton gradients in cristae tips. Second, the local proton gradient at the ATP synthase is small under respiratory growth conditions, but sufficient for ATP synthases. Finally, the study reveals that IF1 plays a central role for full ATP synthesis under respiratory conditions. Overall, this is an elegant study that reports exciting insights into the function of the ATP synthase in cells. The conclusions are well based on a set of highly sophisticated experiments. The reported findings are interesting for the broad readership of EMBO

Rep. Some points should be addressed to support their data.

1. The authors used a number of mitochondrial proteins fused to the reporter GFP to investigate the local pH values. Some of these proteins (subunit e, COXVIIIa) are much smaller than GFP. Therefore, the authors should investigate whether the used reporter proteins assemble into the mature complexes and localizes to the correct mitochondrial subcompartment.

2. Figs. 1D and 1F: The images should be larger.

3. Fig. S3C: The authors should show that IF1 associates with the ATP synthase. In the current version of Fig. S3C this is not clearly demonstrated. The molecular weight marker is missing. It is not clear whether the same gel was used. This question should be more carefully addressed.

4. The authors should speculate why there was no difference in local pH at subunit e in the the absence of IF1, regardless whether oligomycin was added or not (Fig. 2C).

Referee #3:

In this well-written and highly innovative manuscript, Rieger et al. show for the first time pH measurements in mitochondria sub-compartments supporting a role for ATP synthase and ATPIF1 in the regulation of the proton motive force under ATP synthesis (galactose) and hydrolysis (glucose) conditions. I enjoyed reading this report. The experimental work is well planned, and statistics/representation of results is beautifully done.

However, some of the conclusions are not clearly backed up by the results and some controls are missing.
1. In the definition of p in the introduction, authors note p= Δp = ΔΨm -2.3RT/F ΔpH (mV). For that reason, I interpreted that both mitochondrial membrane potential and pH should be considered for any conclusions on p (proton motive force). I understand the rationale of it, pointing to the conclusions made. However, I recommend concluding the results on pH solely, as no correlation was performed with membrane potential in this report.
2. Mitochondrial area shown in Figure S5 is surprisingly not changed when cells are kept in galactose (WT). At the same time, previous reports by the authors have shown increased mitochondrial mass under this condition. Because the conditions listed may also affect mitochondrial morphology, I suggest including pictures and quantification of additional mitochondrial morphology parameters, including length, circularity, and Aspect Ratio, and correlating these parameters with the pH changes observed.
3. The authors observed an increase in mitochondria connectivity. This raises the question if the changes in pH are contributed by mitochondrial architecture. In connection to this point, I wonder if the minor difference in SU e and SU pH under galactose correlates with hyperfused mitochondria and if the increase in mitochondrial mass observed in the IF1 KO also correlates with a more fused mitochondria phenotype? Are the H49K cells fragmented, and could this affect the measured pH?
4. The expression of ATPIF1 H49K was previously shown by the same group to change cristae structure (Weissert V, Rieger B, Morris S, Arroum T, Psathaki OE, Zobel T, Perkins G, Busch KB). Inhibition of the mitochondrial ATPase function by IF1 changes the spatiotemporal organization of ATP synthase. Biochim Biophys Acta Bioenerg. 2021 Jan 1;1862(1):148322). Did the authors

consider the possibility that such changes contribute to the pH alterations observed?
5. Concerning the constructs used, SU e and SU □□□can the authors confirm these subunits' proper assembly in CV? I suggest showing a native gel to demonstrate that the over-expressed peptides indeed assemble into the subunits or clarify if such verification was previously done elsewhere.
6. The authors described in the title and in the report that ATP synthase determines the local proton motive force in the cristae tips. Are the authors suggesting that the measurements performed in CV SU e and SU g can be extrapolated to the actual pH at the tip of the cristae? Is there an experiment showing this specific localization for the probe?
7. Previous work by the authors demonstrates the existence of different subpopulations of complex V, ATP synthase and ATP hydrolase, where ATP hydrolase has higher mobility than ATP synthase, and ATP synthase prevails at the cristae edges. (Salewskij K, et al. The spatio-temporal organization of mitochondrial F1FO ATP synthase in cristae depends on its activity mode. Biochim Biophys Acta Bioenerg. 2020) In that previous work, the authors used the glycolysis (2-DG) and ATP hydrolysis inhibitors (BMS-199264). Can the authors please provide evidence that galactose also promotes the same spatio-temporal organization of CV as did 2-DG?
8. As we need to take into consideration off-target effects of the drugs used as well as short-term effect versus long-term effect on biogenesis and cristae formation, I would like the authors to consider fact that localization of ATP synthase in the cristae tips was mainly observed for the dimers of the complex, which is not addressed in this report. Can the authors include BN for the conditions tested to provide evidence of accumulation of dimers?
9. I also suggest exploring ATP hydrolysis more carefully as the authors show only cells in glucose (synthesis and hydrolysis) versus galactose (synthesis only). Perhaps including an experiment where an ETC blocker is used to force hydrolysis.

Minor corrections:
1. Adding representative images for all panels is needed.
2. Table 1 from supplementary material is not cited in the report and legend is missing.
3. Supplementary Figure 5 should include images related to the quantifications, so the reader can appreciate morphological changes.
4. Supplementary Figure 3C shows no loading control. Also, an image should be shown to allow the reader to visualize the F1 particles.
5. Supplementary Figure 3A shows actin as a loading control and not VDAC as described in the figure legends.

detailed point-by-point responses

REFEREE #1:

Mitochondrial energy conversion takes place in cristae membranes, invaginations of the inner membrane. There, the respiratory chain maintains a proton motive force - consisting of a membrane potential and a proton gradient - that powers ATP synthesis. Previous work in yeast has shown that no pronounced pH gradients exist along or over the inner membrane (Todt-Ott 2020), in line with a local kinetic coupling of the OXPHOS system. Likewise, individual cristae were shown in various human cells to maintain distinct membrane potentials, demonstrating that individual cristae operate as discrete units (Wolf EMBO J 2020 and Kondadi EMBO Rep 2020). In this manuscript by Bettina Rieger et al, pH values have been determined in HeLa cells with the help of a pH sensitive GFP version. The data confirm a, the existence of only minor pH differences over or along the inner membrane also in human cells and b, that these values are no homogenous over the inner membrane. Moreover, the authors studied the pH differences at the IMS or matrix side on ATP synthase, which is a novel and elegant approach. By deleting IF1 from the cells, the authors provide new data on the physiological role of IF1 to modulate ATP synthase activity during glycolytic or OXPHOS conditions, which impacts the intra-mitochondrial proton concentrations.

Overall, the presented data are of very good quality and the manuscript provides new insights into the regulation of ATP synthase activity and intra-mitochondrial pH homeostasis. However, the conceptual advance at the current stage is rather limited and a few additional controls and textual edits are necessary to improve the manuscript.

Major points:
1. It is currently not clear, whether the GFP-tagged proteins assemble into the respective OXPHOS complexes. This information is important to validate central claims of this work. This is particularly important for the localisation of the probes and the question whether they report exclusively pH values in vicinity to the complexes. Hence, the authors should provide biochemical evidence **for each GFP reporter** and how efficiently it assembles, eg by western blot analyses of blue native page/size exclusion chromatography. Given that they used a strong viral promotor for expression, it is possible that a substantial fraction of the proteins is not assembled.

- ➔ We added two new Figures (**new Figure 2** and **Figure S1**) showing the assembly of tagged subunits and MPP-sEcGFP into the respective complexes. Complex V-SU gamma-sEcGFP and earlier SU-gamma-Dendra2 (CV-D) were detected in holo complex V (V–D) and in F1 subcomplexes (e.g. F1-c8 and subcomplexes containing subunits e, g and b according to (He et al., 2018)). Both subcomplexes are membrane-anchored (Song et al., 2018), but probably not assembled in dimeric and oligomeric ATP synthase assemblies. This also explains the higher mobility of tagged SU γ than tagged SU g (**new Figure 3**). Our single molecule studies with SU gamma in comparison to SU g/e show a higher mobility of ATP synthase F1-SU gamma and trajectories that follow the cristae compartment. Since also a portion of SU gamma-FP is assembled in the holo-complex, we suggest that SU gamma senses pH in fully assembled ATP synthase at the rims and along the entire cristae membrane either as F1-SU gamma, F1-c8-SU gamma and monomeric but probably not oligomeric forms of F1FO ATP synthase. CoxVIIIa-sEcGFP/GFP was assembled into the dimeric CIV complex and respiratory supercomplexes and SU e-sEcGFP into the monomeric and dimeric CV (Figure 1). Correct mitochondrial localization of our constructs CoxVIIIa-sEcGFP/GFP and MPP-sEcGFP were shown by Immuno-EM in Rosselin et al (Rosselin et al., 2017), of CV SU γ-EGFP in Wilkens et al (Wilkens et al., 2013) and of CV-SU e -GFP by STED in Kondadi et al. (Kondadi et al., 2020). Since subunit e lately assembles into the F1-FO complex with the peripherical stalk (He et al., 2018) and promotes oligomerization (He et al., 2018, Wagner et al., 2009, Pinke et al., 2020), the SU e-pH sensor is placed at the rims of the cristae, as the superresolution data also suggest (**new Figure 3**).

2. The new data on pH gradients in the absence of IF1 or upon expression of constitutively active IF2 are interesting, but currently lack additional experiments that are necessary to draw mechanistic conclusions. For example, analyses of membrane potential, steady state levels of mitochondrial proteins, activities of OXPHOS complexes or their coupling to produce ATP would be important controls.

- ➔ We added a new data (**new Figure 1**) which includes oxygen consumption rates, metabolic profiling, membrane potential measurements and quantification of mitochondrial morphology.

The data in Figure 2 A-D shows that the pH at SU e drops under OXPHOS conditions when oligomycin inhibits the ATP synthase, but not in the absence of IF1. This would suggest that either oligomycin cannot inhibit ATP synthase in this scenario or that the activity of the respiratory chain is decreased.

- ➔ Oligomycin as an inhibitor of ATP synthase indirectly suppresses the activity of the respiratory chain by a feedback mechanism. This is visible by decrease of oxygen consumption rates measured with an automatic flux analyzer (**Figure EV2**). We have added a related comment to the text. In this context, it should be mentioned that oligomycin increased the heterogeneity of $\Delta\Psi$m, as recent high-resolution imaging data suggest (Wolf et al., 2019).

Minor points:

1. The **title of the manuscript is misleading**. There is currently no evidence that the authors measure pH values at the tips of the cristae membranes. Instead, micrographs presented in Figure 2 N show that the signal for ATP synthase is spread over the complete mitochondrial network. Possibly this observation can be explained by non-assembled reporter proteins (see point 1 above)?

- ➔ The expression “tips” might be misleading, it is rather the “rims” of cristae which are disc like in HeLa mitochondria (Weissert et al., 2021). We have now added super-resolution data (**Figure 3**), where we show the localization and restrained movement of ATP synthase labeled at SU g. SU g is co-regulated and interacting with SU e (Carraro et al., 2014). See also #1.
- ➔ For SU y, our BN-PAGE data indeed suggest the existence of subcomplexes of F_1-SU gamma-sEcGFP. That explains the different distribution of SU y and SU g, which we now show in **Figure 3**.

Important recent work on the autonomous behavior of cristae should be discussed (Wolf EMBO J2020 and Kondadi EMBORep 2020) in detail. Currently this work is not even cited. Moreover, the finding that pH gradients over the inner membrane are rather negligible is in line with previous findings, including the recent publication (Todt PNAS2020). Therefore, the text needs to be adapted.

- ➔ We apologize for not including the recent seminal work on high-resolution membrane potential measurements showing cristae as single bioenergetic units (Toth et al, 2020 was cited in the first version, (Toth et al., 2020)). We have now included their results in the text as comments in the relevant passages.

3. There are a few typos that should be corrected.

- ➔ In the current version we have used a professional proofreader, remaining typos are on our account alone

REFEREE #2:

The mitochondrial F1FO ATP-synthase (ATP synthase) utilizes a proton gradient to produce the bulk of cellular ATP under respiratory conditions. Upon growth at glycolytic conditions the ATP-synthase hydrolysis ATP to maintain a proton gradient across the inner membrane. According to the current understanding, the inhibitory factor 1 (IF1) blocks the ATPase activity to prevent futile ATP hydrolysis. Rieger and colleagues measured the impact of the ATP synthase and IF1 on the generation and maintenance of local proton gradients in the cell using GFP-based pH-sensitive probes. The author

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1. The authors used a number of mitochondrial proteins fused to the reporter GFP to investigate the local pH values. Some of these proteins (subunit e, COXVIIIa) are much smaller than GFP. Therefore, the authors should investigate whether the used reporter proteins assemble into the mature complexes and localizes to the correct mitochondrial sub-compartment.

- ➔ **Please see also response to referee #1. We added BN-PAGE analysis of the assembly of labeled SU into complex (new Figure)**
- ➔ Assembly into complexes and subcomplexes of CoxVIIIa-GFP, and CV SU gamma-GFP was shown by 2D-BN-PAGE in (Muster et al., 2010)
- ➔ Correct localization of CoxVIIIa-pHL/GFP and MPP-pHL were shown by Immuno-EM in Rosselin et al (Rosselin et al., 2017), of CV SU γ-EGFP in Wilkens et al (Wilkens et al., 2013) and of CV-SU e -GFP by STED in Kondadi et al. (Kondadi et al., 2020). These findings were cited in the manuscript now

2. Figs. 1D and 1F: The images should be larger.

→ the images were enlarged. Former Figure 1G-I were moved to **new Fig. 6**

3. Fig. S3C: The authors should show that IF1 associates with the ATP synthase. In the current version of Fig. S3C this is not clearly demonstrated. The molecular weight marker is missing. It is not clear whether the same gel was used. This question should be more carefully addressed.

- ➔ We added the image of the full gel showing that IF1 is associated with complex V (**Figure 2A**). since the data are so substantial, we moved Figure S3c to Figure2.

4. The authors should speculate why there was no difference in local pH at subunit e in the absence of IF1, regardless whether oligomycin was added or not (Fig. 2C).

- ➔ We suspect that the reviewer is referring to former Fig. 2B (**now Fig.7B**), since no oligomycin treatment was shown in Fig. 2C. Under OXPHOS conditions, pH at ATP synthase is determined by proton pumping from the ICS into the matrix and diffusion of protons from the CIV, CIII, and CI sources to ATP synthase. One would expect forward and reverse ATP synthase to be active in IF1KO under OXPHOS conditions because of the low delta pH. These activities apparently cancel each other out, since oligomycin, which blocks both activities, has no effect. The lower pH in IF1-H49K cells at SU e could be explained by a shorter diffusion range between proton source and sink (CV) due to the altered cristae ultrastructure (Weissert et al., 2021). However, in our previous work (Rieger et al., 2014), we also observed that the incubation time with oligomycin had a strong influence on the local pH. An exposure of more than 30 min strongly decreased the pH at SU e. Here, a new steady-state possibly plays in over time. Wolf et al. 2019 also noted that oligomycin markedly increased the heterogeneity of the mitochondrial membrane potential.

REFEREE #3:

In this well-written and highly innovative manuscript, Rieger et al. show for the first time pH measurements in mitochondria sub-compartments supporting a role for ATP synthase and ATPIF1 in the regulation of the proton motive force under ATP synthesis (galactose) and hydrolysis (glucose) conditions. I enjoyed reading this report. The experimental work is well planned, and

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- ➔ We added $\Delta\Psi m$ data, and extended the discussion

2. Mitochondrial area shown in Figure S5 is surprisingly not changed when cells are kept in galactose (WT). At the same time, previous reports by the authors have shown increased mitochondrial mass under this condition. Because the conditions listed may also affect mitochondrial morphology, I suggest including pictures and quantification of additional mitochondrial morphology parameters, including length, circularity, and Aspect Ratio, and correlating these parameters with the pH changes observed.

- ➔ Mitochondrial mass/footprint quantification and network analysis have now been added to Figure 1. We do not know exactly what work the reviewer has in mind. Neither in (Weissert et al., 2021), nor in (Rieger et al., 2019) we found an increase in mitochondrial footprint/mass under respiratory conditions. Moreover, here we compared a switch from high glucose (25 mM) to galactose (10 mM), in our previous work we had a different condition (Minimal Essential Medium Earle's Salt (5.6 mM glucose + L-glutamine) supplemented with 10% FCS (fetal calf serum), 1% HEPES and 1% NEAA (non-essential amino acids)). In the present study, we found a decrease in mitochondrial mass under respiratory conditions in WT cells, but higher cross-linking, which is consistent with what has been reported previously (Rossignol et al., 2004) (**new Figure 1)**

3. The authors observed an increase in mitochondria connectivity. This raises the question if the changes in pH are contributed by mitochondrial architecture. In connection to this point, I wonder if the minor difference in SU e and SU γ pH under galactose correlates with hyperfused mitochondria and if the increase in mitochondrial mass observed in the IF1 KO also correlates with a more fused mitochondria phenotype? Are the H49K cells fragmented, and could this affect the measured pH?

- ➔ Indeed, we found a more fused phenotype for Gal-WT cells than for HGlc-WT. No difference in branch length or network number was found between Gal-wt and Gal-IF1-KO, though. IFH49K-Gal cells showed no morphological difference to WT-Gal cells, but internal architecture likely is affected (Weissert et al., 2021). We discuss this in the current version. See also comment to 4.

4. The expression of ATPIF1 H49K was previously shown by the same group to change cristae structure (Weissert V, Rieger B, Morris S, Arroum T, Psathaki OE, Zobel T, Perkins G, Busch KB). Inhibition of the mitochondrial ATPase function by IF1 changes the spatiotemporal organization of ATP synthase. Biochim Biophys Acta Bioenerg. 2021 Jan 1;1862(1):148322). Did the authors consider the possibility that such changes contribute to the pH alterations observed?

- ➔ Thank you for mention this important point. We did not include ultrastructural constraints in our discussion, but have done so now. In our previous paper, we found a range of IF1 effects on ultrastructure: from almost no changes to reduction and restructuring of cristae (Weissert V, Rieger B, Morris S, Arroum T, Psathaki OE, Zobel T, Perkins G, Busch KB). This implies that the distance between primary (CIV) and secondary protons pumps (CV) is probably shortened and as a result also the diffusion distance which will influence the steady state pH.

5. Concerning the constructs used, SU e and SU γ, can the authors confirm these subunits' **proper assembly in CV**? I suggest showing a native gel to demonstrate that the over-expressed peptides indeed assemble into the subunits or clarify if such verification was previously done elsewhere.

- ➔ Please see answer to referee #1
- ➔ Assembly into complexes and subcomplexes of Co8a-GFP, and CV SU gamma-GFP was shown by 2D-BN-PAGE in (Muster et al., 2010)
- ➔ Correct localization of Co8a-pHL/GFP and MPP-pHL were shown by Immuno-EM in Rosselin et al (Rosselin et al., 2017), of CV SU γ-EGFP in Wilkens et al (Wilkens et al., 2013) and of CV-SU e -GFP by STED in Kondadi et al. (Kondadi et al., 2020)

6. The authors described in the title and in the report that ATP synthase determines the local proton motive force in the cristae tips. Are the authors suggesting that the measurements performed in CV SU e and SU g can be extrapolated to the actual pH at the tip of the cristae? Is there an experiment showing this specific localization for the probe?

- ➔ We include a new Figure that shows that the localization and mobility of CV labeled at Su g compared to CV-SU γ is confined. This indicates that SU g is mainly part of dimers and oligomers of CV which are localized at the rims/tips of cristae (Davies et al., 2012, Davies et al., 2011)

7. Previous work by the authors demonstrates the existence of different subpopulations of complex V, ATP synthase and ATP hydrolase, where ATP hydrolase has higher mobility than ATP synthase, and ATP synthase prevails at the cristae edges. (Salewskij K, et al. The spatio-temporal organization of mitochondrial F1FO ATP synthase in cristae depends on its activity mode. Biochim Biophys Acta Bioenerg. 2020) In that previous work, the authors used the glycolysis (2-DG) and ATP hydrolysis inhibitors (BMS-199264). Can the authors please provide evidence that galactose also promotes the same spatio-temporal organization of CV as did 2-DG?

- ➔ We now include a quantitative analysis of the spatiotemporal organization of complex V (SU-γ -HaloTag) in galactose and compare the mean diffusion coefficient with that of the same construct in glucose. CV displays slower movement under respiratory conditions in line with stimulated activity of the synthase. We have added a new Figure (**new Fig. 4**)and chapter addressing these points.

8. As we need to take into consideration off-target effects of the drugs used as well as short-term effect versus long-term effect on biogenesis and cristae formation, I would like the authors to consider fact that localization of ATP synthase in the cristae tips was mainly observed for the dimers of the complex, which is not addressed in this report. Can the authors include BN for the conditions tested to provide evidence of accumulation of dimers?

- ➔ We now include a semi-quantitative analysis of the BN-Gel showing an increase in oligomers in Gal vs. HGlc wt cells and in IF-OE cells vs. HGlc (**Fig.2**).

9. I also suggest exploring ATP hydrolysis more carefully as the authors show only cells in glucose (synthesis and hydrolysis) versus galactose (synthesis only). Perhaps including an experiment where an ETC blocker is used to force hydrolysis.

- ➔ As suggested, we inhibited the respiratory chain at complex III with Antimycin A. Cells were grown with low glucose (5.5 mM). The pH at SU e under low glucose was similar as what we have measured before (Rieger et al., 2014). Antimycin A treatment decreased the pH from pH 7.11 ± 0.03 (SE) to pH 6.99 ± 0.015 (SE). A similar response was reported by the Demaurex group (pH 7.25 ± 0.06 and pH 6.97 ± 0.06) (Rosselin et al., 2017). A drop of matrix pH after antimycin A application was observed before (Rosselin et al., 2017) (Santo-Domingo et al., 2013). KCN and oligomycin effects were described in our 2014 paper (Rieger et al., 2014)

Minor corrections:

1. Adding representative images for all panels is needed.
   ➔ Done

2. Table 1 from supplementary material is not cited in the report and legend is missing.
   ➔ Fixed

3. Supplementary Figure 5 should include images related to the quantifications, so the reader can appreciate morphological changes.
   ➔ We show quantification of mitochondrial morphology in **new Figure 1**

4. Supplementary Figure 3C shows no loading control. Also, an image should be shown to allow the reader to visualize the F1 particles.
   ➔ The Coomassie stained gel was added to show equal loading. Also, full gel images are displayed now.

5. Supplementary Figure 3A shows actin as a loading control and not VDAC as described in the figure legends.
   ➔ fixed

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1st Revision - Editorial Decision 30th Aug 2021

Dear Dr. Busch,

Thank you for submitting your revised manuscript. It has now been seen by two of the original referees.

I apologize for the delay in getting back to you. It took longer than anticipated to receive the referee reports.

As you can see, the referees find that the study is significantly improved during revision and recommends publication. However, I need you to address the editorial points below before I can accept the manuscript.

• Please address the remaining concerns of referee #1 by textual alterations as suggested by the referee by keeping 'track changes' on.
• Please provide 3-5 keywords for your study. These will be visible in the html version of the paper and on PubMed and will help increase the discoverability of your work.
• We note that the format of Data Availability section is currently incorrect. As per our guidelines, Data Availability section is reserved for the new primary dataset that is generated in this study and deposited in a public data repository. If no dataset is generated/deposited, please make a statement that no data were deposited in a public database.
• Please remove the 'Competing Financial Interest section.
• As per our format requirements, in the reference list, citations should be listed in alphabetical order and then chronologically, with the authors' surnames and initials inverted; where there are more than 10 authors on a paper, 10 will be listed, followed by 'et al.'. Please see https://www.embopress.org/page/journal/14693178/authorguide#referencesformat
• The movies need to be zipped with their legends.
• Please add a 'Table of Contents' to the Appendix file. Moreover, the table should be renamed to "Appendix Table S1" and the figure should be "Appendix Figure S1"
• We note the phrase 'data not shown' on page 5, which is not allowed by the journal policy. Please either remove the statement or show the data.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

In this revised version, the authors have addressed the main points raised on the initial version. Importantly, they have now provided evidence on the assembly of the GFP-tagged subunits and found that CoxVIIIa-sEcGFP/GFP was assembled mainly into the dimeric CIV complex (and also partly into respiratory supercomplexes) and SU e-sEcGFP into the monomeric and dimeric CV. Their other construct, SU gamma-sEcGFP, did hardly (a few percent at best) assemble into a fully mature ATP synthase, but rather into subassmblies. Hence this probe cannot be used to measure pH values precisely at the ATP synthase. The authors did a great job in describing this well, but did not change consistently the subsequent text according to these new findings. Specifically, an unassembled reporter construct impacts the interpretation of the data: a, the observed differences in diffusion between SUe/g and SUgamma could be explained by their assemblyb status, and b, the observed pH gradients over the inner membrane specifically at ATP synthase, which they cannot measure. Instead, it appears that their SUgamma reporter can detect pH values close on the cristae membrane.
Because these aspects are essential parts of this work, the text needs to be substantially revised. Examples of these uncorrected statements are: .."the pH probe at SU gamma senses the pH in fully assembled ATP synthase at the n-side of rims and along the entire cristae membrane either as F1-SU gamma or F1-c8-SU gamma", "To gain more insights on the local pH at ATP synthase, we also determined pH values atsubunit gamma of ATP synthase in WT, IF1-KO and IF1-H49K cells.", "The determined pH values at ATP synthase subunits SU e and SU gamma were then used to calculate the corresponding delta pH." etc.

Referee #2:

The revised manuscript by Rieger and colleagues strongly improves. Most importantly, the authors show now that the GFP-tagged proteins are assembled into the mature complexes. The authors satisfactorilly addressed my concers in the revised version. This an elegant study that report interesting findings. I recommend publication of the manuscript in EMBO Rep.

response to Referee #1

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Because these aspects are essential parts of this work, the text needs to be substantially revised. Examples of these uncorrected statements are: ..

"the pH probe at SU gamma senses the pH in fully assembled ATP synthase at the n-side of rims and along the entire cristae membrane either as F1-SU gamma or F1-c8-SU gamma",

- Changed to: the pH probe at SU gamma senses the pH mainly as F_1-SU gamma or F_1-c_8-SU gamma and partially in fully assembled ATP synthase at the *n*-side of cristae membranes.

"To gain more insights on the local pH at ATP synthase, we also determined pH values at subunit gamma of ATP synthase in WT, IF1-KO and IF1-H49K cells.",

- Changed to: The C-terminus of SU gamma fused to sEcGFP extruded from the F_1-head (F1-SU g or F1-c8-SU g, such that the sensor can detect pH values close on the cristae membrane.

"The determined pH values at ATP synthase subunits SU e and SU gamma were then used to calculate the corresponding delta pH." etc.

- Changed to: The determined pH values at ATP synthase subunits SU e and SU gamma were then used to calculate the corresponding deltapH across the crista membrane. It has to be kept in mind, though, that due to assembly of SU g into ATP synthase subcomplexes, this was not necessarily the DpH across single ATP synthase molecules.
- Further adaptations see marked text

Referee #2:

The revised manuscript by Rieger and colleagues strongly improves. Most importantly, the authors show now that the GFP-tagged proteins are assembled into the mature complexes. The authors satisfactorilly addressed my concers in the revised version. This an elegant study that report interesting findings. I recommend publication of the manuscript in EMBO Rep.

2nd Revision - Editorial Decision 10th Sep 2021

Karin Busch
Westfalian Wilhelms University of Munster, Germany
Germany

Dear Dr. Busch,

Since my colleague Deniz Senyilmaz Tiebe is currently not in the office, I have stepped in as secondary editor for your manuscript, which I am very pleased to accept for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Yours sincerely,

Martina Rembold, PhD
Senior Editor
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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:
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  - are tests one-sided or two-sided?
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  - definition of 'center values' as median or average;
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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?	repetitive experiments until a quasi normal distribution was achieved (exception: single molecule diffusion analysis)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	not applicable
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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.	evaluated by two persons independently
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Is there an estimate of variation within each group of data?	can be provided upon request

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Is the variance similar between the groups that are being statistically compared?	some conditions show higer variation, we assume that this is metabolic variance

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).	IF1 was detected with anti-ATPIF1 antibody (CD6P1Q) purchased from Cell Signaling (#13268), VDAC was detected with an anti-VDAC antibody (Cell signaling #4661), Subunit was detected with an anti-ATP5B antibody (Proteintech #17247-1-AP), subunit e with an anti-ATP5I antibody (Abcam #122241) and polyclonal anti SU (ATP5C1) was a gift from Ilka Wittig. GFP/sEcGFP was detected with polyclonal anti-GFP antibody (host: rabitt, #AB10145, purchased from Merck Millipore.) To detect CIV, anti-MTOC1 (a-Cox1, host mouse, mono clonal) from Thermo (#459600) was used. Monoclonal anti-actin was purchased from Sigma Aldrich (#A1978, host mouse). For the secondary antibody Peroxidase AffiniPure Goat Anti-Mouse IgG + IgM (H+L) (111-035-068) or Goat Anti-Rabbit IgG (H+L) (111-035-045) from Jackson Immuno Reasearch were used.
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 cell lines were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, for mycoplasma contamination is regularly tested by PCR (PromoceII, PK-CA91-1048

* 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.	not applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	not applicable
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.	not applicable

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
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
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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	no applicable
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).	we include movies in expanded view
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.	not applicable