Oxidative stress protein Oxr1 promotes V-ATPase holoenzyme disassembly in catalytic activity-independent manner

Md. Murad Khan, Seowon Lee, Sergio Couoh-Cardel, Rebecca A. Oot, Hyunmin Kim, Stephan Wilkens, Soung-Hun Roh

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Corresponding authors: Soung-Hun Roh (shroh@snu.ac.kr), Stephan Wilkens (wilkenss@upstate.edu)

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Editor: Daniel Klimmeck

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.)
Dear Dr Roh,

Thank you for the submission of your manuscript (EMBOJ-2021-109360) to The EMBO Journal for consideration. Your study has been sent to three experts, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and value of your results.

Given the referees' positive recommendations and detailed comments, I would like to invite you to submit a revised version of the manuscript, addressing the remaining minor issues raised.

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.

When submitting your revised manuscript, please carefully review the instructions below.

Please let me know at any time if you have additional questions related to the reviewers’ input or formatting changes required.

Thank you for the opportunity to consider your work for publication.
I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instruction for the preparation of your revised manuscript:

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 response 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.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). 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.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

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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 

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can 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 or a single pdf per main figure 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 

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as "Figure EV1, Figure EV2” etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

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10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen: http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

Further information is available in our Guide to Authors: https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 14th Dec 2021.

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Referee #1:

This is an extensive study that combines structural studies utilizing cryo-EM and biochemical and genetic studies using yeast S. cerevisiae. The studies are broken into 3 segments. The first segment deals with the assembly of the v-ATPase under in vitro conditions with studies using cryo-EM. The studies indicate that while V1 may be locked into a single rotamer conformation, that rotamer conformation is relaxed with assembly into the entire V1Vo. The second segment addresses the asymmetry mismatch of Vo c-subunits. This study incorporates the cryo-EM data with genetic studies. The results provide a better understanding on how asymmetry mismatch occurs in a seemingly symmetric system. The third segment identifies and defines a role of the Oxr1 protein in the assembly of V1Vo. This study used cryo-EM, biochemistry, and genetics. The results provide a role of the Oxr1 protein which up to this report, was undefined.

These studies, results, and conclusions presented here were all well done, unambiguous, and clear. All of the data is important and new providing new information on the V1Vo ATPase. The most intriguing results were derived from the last segment which gave good evidence for the role of Oxr1 in the cell. Oxr1 is a protein of unknown function required for oxidative damage
resistance. With the knowledge that it is involved in assembly of V1Vo, this places V1Vo as critical for resistance to oxidative damage. The results presented here will open up a number of avenues into the role of V1Vo in this process. These results make this especially interesting to a broad audience.

The results and conclusions from the first 2 segments are a bit more nuanced but nonetheless, new and important. Overall, the manuscript provides new and important results, much of which, will be of interest to a broad audience.

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Khan et al present a new mechanism for regulation of V-ATPase assembly and activity that involves Oxr1, a protein associated to oxidative stress protection. The findings are novel and of most relevance to the field of bioenergetics, V-ATPase are conserved molecular motors essential for numerous cellular process and human conditions including cancer, osteoporosis, renal tubular acidosis, and neurovegetative diseases.

Oxr1 is the first non-V-ATPase protein shown to inhibit V1 and drive disassembly of the V1Vo complex. Until know, and for more than 20 years, the only inhibitory protein known to silence V1 was the V1 subunit H (autoinhibition of the complex). The mechanism proposed by Khan and collaborators is novel and significantly distinct from the autoinhibitory mechanism. For example, Oxr1 binding causes dissociation of V1 subunit H and retention of V1 subunit C (opposite to the autoinhibitory mechanism). In addition, Oxr1 locks V1 in a different rotary conformational state (state 2). The authors used Cryo-EM and biochemicals students to support their conclusions. The data is robust and the experiments conducted thoroughly. Other important finding of the study is that it also provides structural data that explains how the V-ATPase exclusive subunit c' in the proteolipid ring of Vo helps alleviate the symmetry mismatch between the 3 rotary steps in V1 and 10 rotatory steps in Vo.

This reviewer's request (minor) is to address in the discussion whether and how Oxr1-mediated disassembly could be reversed?

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Khan et al., have used single particle cryoEM with mutational and functional analysis to investigate the mechanisms by which V-ATPases in yeast are regulated. The manuscript report three principle finding each of increasing interest and significance. 1) The first finding is that autoinhibited V1 in state 2 and autoinhibited Vo in nanodiscs in state 3 can reassemble and form a functional holoenzyme which exists in all three states but specifically state 1 indicating that assembly does not need ATP hydrolysis. 2) The asymmetry of the c-ring is likely to counter-act the symmetry mismatch between Vo and V1. They also showed through mutations that removing the asymmetry in glutamate distribution, the enzyme was inactive. 3) They observed and identified an unknown protein bound to V1 which is likely to be a new way to inhibit V1 or disassemble the V-type ATPase which is independent of function. The paper is well written, it contains lots of detail but in a way a reader not familiar with the field can follow easily. The experiments are all well executed and explained. The story presented is complete. I learnt a lot from reading the manuscript which I enjoyed very much. Congratulations.

Minor comments:
Page 2, last paragraph, 1st line: Why is V-ATPase from yeast "an excellent model system for the mammalian enzyme"? Why is it excellent? How is it similar? In what ways can you transfer the knowledge from this paper to the mammalian enzyme?
In the manuscript there was several mentions about mammalian enzyme and that the yeast was more understood. What I couldn't understand was why the topic of the mammalian enzyme kept coming up. What in your results would be applicable to the mammalian enzyme? How can you compare them?

Page 14, Last paragraph:
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Figure S4B: the left panel has 11 side chains shown, the others 10.

Fig S6C and Fig S7: What is conA? Why does it cause inactivity of V-type ATPases.
Authors’ response to reviewers’ comments

We would like to thank the reviewers for their constructive comments and suggestions on our manuscript, entitled “CryoEM Structures of Reconstituted V-ATPase and Oxr1-bound V1 Reveal a Novel Mechanism of Regulation”. We replied to the reviewers’ comments in a point-by-point manner in the following rebuttal. Also please note that we corrected a few minor errors and inconsistencies in the figures as listed following the responses to the reviewers’ comments.

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We thank the reviewer for the positive evaluation of our study.

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Authors' response:
We thank the reviewer for the positive evaluation of our study

Q: This reviewer's request (minor) is to address in the discussion whether and how Oxr1-mediated disassembly could be reversed?

Authors' response:
The reviewer brings up an important question. We expect that there is a mechanism in vivo with which Oxr1 can be released from V1. One reason for our assumption is that while wild type V1 does not associate with Vo in vitro (due to the high stability of the ADP inhibited conformation of V1), enzyme re-assembly happens within a few minutes in vivo in presence of glucose. However, the mechanism of reactivation and reassembly of wild type V1 in vivo is not yet known, and we therefore can not speculate as to the presence of a mechanism to reverse Oxr1 mediated disassembly, or even whether such a mechanism actually exists. Indeed it is possible that Oxr1-mediated disassembly is not reversed. As Oxr1-mediated disassembly differs from canonical reversible disassembly in that it occurs independent of ATP hydrolysis, we propose (on page.18) that the role of Oxr1 may be in quality control. Oxr1 may serve to remove non-functional V1-ATPase that is damaged by eg. oxidative stress. In this case, the V1-ATPase could be turned over permitting assembly of new, functional V1 with the Vo remaining on the membrane. The details of this mechanism are of great interest and will be the focus of future studies in our lab. We have added the following sentence to the Discussion to address the issue:

"However, whether mechanisms exist to release Oxr1 and repair and reactivate damaged V1 to allow reassembly of active holo V-ATPases is currently unknown."

Referee #3:

Khan et al., have used single particle cryoEM with mutational and functional analysis to investigate the mechanisms by which V-ATPases in yeast are regulated. The manuscript report three principle finding each of increasing interest and significance. 1) The first finding is that autoinhibited V1 in state 2 and autoinhibited Vo in nanodiscs in state 3 can reassemble and form a functional holoenzyme which exists in all three states but specifically state 1 indicating
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Minor comments:
Q: Page 2, last paragraph, 1st line: Why is V-ATPase from yeast "an excellent model system for the mammalian enzyme"? Why is it excellent? How is it similar? In what ways can you transfer the knowledge from this paper to the mammalian enzyme?

Authors' response:
Thank you for pointing this out. The structure, mechanism and (from what we know so far) regulation of the eukaryotic V-ATPase is highly conserved from yeast to mammals. Yeast is an excellent model system because (i) unlike for the mammalian V-ATPase, it is relatively straightforward to purify milligram amounts of the yeast complex (and sub-complexes) for biochemical studies, (ii) yeast is amenable to genetic manipulation, and (iii) yeast, unlike mammalian cells, can grow without the enzyme (conditional lethal vma- phenotype), thus allowing functional studies by genetic means and by biochemical studies of sub-complexes of the enzyme. In fact, functional complementation of subunit deletions in yeast has been used to confirm putative V-ATPase subunits from higher organisms, including human (Lu, X (1998) Immunity, Sun-Wada G-H (2003) Gene, for example). Moreover, in cases where disease-causing missense mutations do not lead to increased protein turnover, the yeast system has been used to analyze molecular mechanisms of such mutations (Zirngibl, RA (2019) JBC). We have expanded the text referred to by the reviewer as follows (page 2):

“Eukaryotic V-ATPases are highly conserved. The relative ease of yeast genetics and the ability to purify milligram amounts of yeast V-ATPase and its subcomplexes make Saccharomyces cerevisiae a powerful model system to study the structure, catalytic mechanism and regulation of the enzyme from higher organisms, including mammals.”

Q: In the manuscript there was several mentions about mammalian enzyme and that the yeast was more understood. What I couldn't understand was why the topic of the mammalian enzyme kept coming up. What in your results would be applicable to the mammalian enzyme? How can you compare them?

Authors' response:
As mentioned in the response to the previous comment, V-ATPase is highly conserved from yeast to mammals. Many if not all mechanistic and regulatory features that were initially described for the yeast enzyme were subsequently observed for the mammalian complex, such as reversible disassembly, presence of an assembly chaperone, enzyme targeting to different organelles via isoforms of the Vo-a subunit etc. We think that is therefore reasonable to assume that the structural and mechanistic findings described in our manuscript deserve to be put in context of what is known about the mammalian complex. For example, the here identified TLDc domain of Oxr1 is conserved from yeast to mammals, and we therefore think that it is reasonable to include the mammalian V-ATPase in the discussion of the possible physiological role(s) of the TLDc containing proteins characterized in mammalian cells so far. Up to this point, studies in higher organisms have shown TLDc domain containing protein interaction with V-ATPase and found that loss of function phenocopies V-ATPase mutations. Given our results, we have proposed a quality control model for the function of Oxr1 that could be applied to mammalian systems as well. Our study extends the information regarding this new class of V-ATPase interacting proteins by providing novel information that 1.) illuminates the (highly conserved) binding site for Oxr1 and 2.) defines the physical basis of the impacts of Oxr1 binding on V-ATPase function. We feel that this study provides important information that will add to the evolving pool of information on how TLDc proteins impact V-ATPase in physiological processes from yeast to humans. Moreover, the asymmetry of the c-ring glutamates is conserved as well, and we therefore utilized the recent structures of human, rat and bovine V-ATPase to draw parallels to the yeast enzyme here characterized by site directed mutagenesis - a study that would be difficult to conduct with the mammalian system. To illustrate the structural conservation of the TLDc domains of yeast Oxr1, Drosophila skywalker, zebrafish Oxr2 and human NCOA7 and the conservation of residues that constitute the binding site of Oxr1 on the V-ATPase, we have added a new EV figure associated with main text Figure 3 (Figure EV3 in the revised manuscript).

Q: Page 14, Last paragraph:
You hypothesise in Roh et al., 2020 that the asymmetry in positions of glutamates in the c-ring is the cause of autoinhibition of passive proton transport across free Vo. In your switch mutant you removed the asymmetry. Do you see passive proton transport across free Vo? If no, what impact does this have on your hypothesis from Roh et al.,2020?

Authors’ response:
The reviewer brings up an important question. However, to answer this question, a series of experiments would need to be conducted to test the reviewer’s hypothesis. As we point out in Roh et al., 2020, autoinhibition of passive transport has at least two origins: (i) the interaction of the rotor subunit ‘d’ with the stator subunit ‘a’ (via a’s N-terminal domain, aNT), and (ii) the unequal spacing of c-ring glutamates. To test the reviewer’s hypothesis, we would need to purify and reconstitute Vo and Vo-d from wild type and the deletion, addition, and switch-mutants and compare their passive proton conductance activities, a project that we feel goes beyond the scope of the current study.
Q: Figure S4B: the left panel has 11 side chains shown, the others 10.

Authors’ response:
Thank you for catching this error. The eleventh side chain, which belongs to Ser184, was displayed unintentionally. We have corrected the error in the revised figure (now Figure EV2).

Q: Fig S6C and Fig S7: What is ConA? Why does it cause inactivity of V-type ATPases.

Authors’ response:
As described on page 7 of the original manuscript, ConA stands for Concanamycin A, a macrolide isolated from Streptomyces that inhibits eukaryotic V-ATPases (yeast and mammalian) by binding to the c subunits of the Vo. It is thought that ConA inhibits the enzyme by blocking c-ring rotation. A highly ConA sensitive enzyme preparation therefore indicates tight coupling between Vo proton channel and V1-ATPase.

Changes made to title and figures:
We have changed the original title of the manuscript from “CryoEM Structure of Reconstituted V-ATPase and Oxr1-bound V1 Reveal Mechanism of Activity Regulation” to “CryoEM Structures of Reconstituted V-ATPase and Oxr1-bound V1 Reveal a Novel Mechanism of Regulation” in order to highlight the novelty of the Oxr1-mediated regulation and to comply with the 100 character limit for manuscript titles. We also fixed several minor errors and inconsistencies in the figures as follows: Figure 2A, black font of labels was changed to red font for consistency; Figure 2B, glutamic acid labels next to red dots were added for clarity, and c” was added before mutant residue names for consistency; Figure 2C, the rotation angles were corrected from 120-123-117º to 120-117-123º (the angles were correct in the original main text and supplement figure S4, which is now Figure EV2); Figure 4C, α6×His was changed to α5×His as the antibody was raised against a penta-His tag; Figure 4D, right panel, HWT was changed to Hwt for consistency; Figure 5, inhibitory ADP was drawn on the wrong A/B interface in panel A, and we changed the label on panel B from V1(C)Oxr1p to V1(C)Oxr1 for consistency; Appendix Figure 3B (formerly supplement figure S5C) had labels Oxr1_deg and Oxr1_nat reversed. We moved the following figures from the original supplement to EV figures: EV1 was Figure S2, EV2 was S4, EV3 is new, EV4 is a combination of former S6 and S7, and EV5 was S8. Finally, we added a description of the mass spectrometry experiment in the Methods section and we included the Excel file of the peptide identifications as Appendix Table S2.
Dear Dr Roh,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed. Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
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https://www.embopress.org/doi/full/10.15252/embj.2019103932

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.
Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

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

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

* 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.

9. For experiments involving live animals, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

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’. Please confirm you have followed these guidelines.

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

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 NRRMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Report any restrictions on the availability (and/or on the use) of human data or samples.

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

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.

E- Human Subjects

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., PNA-Sig data: Gene Expression Omnibus GSE33462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for ‘Data Deposition’.

19. Data deposited 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

20. Deposition is strongly recommended for any datasets that are central and integral to the study. Please consider the journal’s data policy. If restructured 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).

21. 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).

22. Computational models that are central and integral to the 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 MIMAR 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 BioModelsDB (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.

F- Data Accessibility

23. Provide a "Data Availability" section at the end of the Materials & Methods, listing the deposition codes for data included at the end of the Materials & Methods section.

G- Dual use research of concern

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