EMBO reports

Human mitochondrial ADP/ATP carrier SLC25A4 operates with a ping-pong kinetic mechanism

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Dear Prof. Kunji,

Thank you for submitting your research manuscript for consideration by EMBO reports. It has now been seen by three experts in the field, and we have received the full set of their reports, which are included below.

As you will see, referees #1 and #2 are very positive about this study, they acknowledge the significance of the topic and the technical quality of the investigation, they find the manuscript well-written, and they recommend its publication after minor revision. Referee #3, on the other hand, identifies contradictions with the published literature and does not agree with your interpretation of the presented data.

Given the referee reports, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed in their reports) must be 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. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we usually recommend a revision within 3 months (July 2nd). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

Please note that you can publish your study either as a Report or as an Article. For Reports, the manuscript should not exceed 27,000 characters (including spaces but excluding Materials & Methods and References) and 5 main plus 5 Expanded View figures. The Results and Discussion sections must be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For an Article there are no length limitations, but it should have more than 5 main figures and the Results and Discussion sections must be separate. In both cases, the entire Materials and Methods must be included in the main manuscript file.

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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 unless you opt out of this (please see below for further information).

4) A complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF (please see below for more information).

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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

7) Please note that a "Data availability" section at the end of Materials and Methods is now mandatory. In case you have no data that require deposition in a public database, please state so instead of refereeing to the database: "Our study includes no data deposited in public repositories." under the heading "Data availability".
See also). Please note that the Data availability statement is restricted to new primary data that are part of this study.

8) We request authors to consider both actual and perceived competing interests. Please review the new policy () and update your competing interests statement if necessary. Please name this section 'Disclosure and competing interests statement' and place it after the Acknowledgements section.

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter plots showing the individual data points.

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Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

This manuscript by Cimadamore-Werthein, Baron et al is focused on kinetic analysis of the mitochondrion ADP/ATP carrier SLC25A4. Proposed transport mechanisms for this important protein have diverged significantly from earlier models invoking simultaneous binding of both substrates to a homodimeric transporter to more recent models based on a large body of structural and functional data, with seminal contributions from the Kunji group, revealing a monomeric transporter with a single binding site for ATP/ADP operating with an alternating access mechanism. While the latter is now widely accepted as being correct, experimental determination of transport kinetics has remained challenging. Here the authors use human SLC25A4 reconstituted in liposomes as the basis of a robotic transport assay that overcomes several challenges that have complicated accurate determination of reaction initial velocities and have led to error prone results and skewed interpretations of reaction kinetics. Assuming equivalent binding and transport steps for ATP and ADP, the assay measures uptake of radiolabeled ATP into proteoliposomes with different internal ATP concentrations. The authors are able to measure initial reaction rates based the linear time dependent accumulation of radiolabeled ATP inside liposomes. From these initial reaction rates at multiple external substrate concentrations, the authors are able to derive Michaelis Menten parameters for each internal ATP concentration. Plotting Km and Vmax values for each of these reveals parallel Lineweaver Burke plots, consistent with transport rates being independent of 'counter substrate' concentrations (internal ATP) and with a single binding site alternative access scheme. This represents an important and hitherto missing piece of functional evidence in further support of the so called 'ping-pong' kinetic model for transport that is likely applicable to other mitochondrial SLC25 family transporters. As such, the data presented are very much suitable for publication in EMBO reports as is. The manuscript is succinct and clearly written and was a pleasure to read. Parameterization of equations used for data fitting and their derivation are clearly explained. I only have minor points for clarification.

Minor Points:
1. Was there any reason only a single biological replicate is reported for the 2.5mM internal concentration of ATP?
2. Could the assay not be adapted for different concentrations of ADP inside by the addition of gramicidin or valinomycin allowing for Na+ or K+ influx into liposomes preloaded with Na+ inside and K+ outside or vice versa to mitigate the mentioned potential buildup with the ATP/ADP exchange?

Referee #2:

The mitochondrial ADP/ATP carrier (SLC25A4) is the prototype of proteins in the mitochondrial carrier family that transport nucleotides and various metabolites across the inner membrane. It is long believed to function as a homodimer in sequential mode in which the two exchanged substrates bind from the opposite sides of the membrane and move across the membrane simultaneously. The Kunji lab previously provided biochemical, biophysical and structural data that suggest a monomeric operation mode. In this model, there is only one single substrate binding site. An alternating access mechanism was proposed in which a central substrate binding site is used for substrate binding from one side of the membrane. Following conformational changes and the release of the substrate at the other side of the membrane, the transport counterion binds to the carrier to

complete the exchange process. However, this so-called ping-pong transport mechanism does not fit with previously published transport kinetic data, likely due to a number of confounding factors that might have deviated the final interpretation. In this manuscript, the authors reanalyzed the kinetic properties of human ADP/ATP carrier by using recombinant expression in yeast and reconstitution in proteoliposomes of defined lipid composition and buffer conditions. They captured time frames during the transport process in which the transport rate is linear. By measuring the uptake of radioactively labelled ATP, they found that the Km/Vmax ratio is constant when different internal concentrations of ATP are applied. The kinetic data support a ping-pong kinetic mechanism in which substrate exchange across the membrane occurs consecutively rather than simultaneously.
This is a well written short article with high-quality data that provide kinetic support for a monomeric operation mode of ADP/ATP carrier. The study is highly significant and has broad implications for the understanding of other mitochondrial carriers. I recommend the publication of the manuscript in EMBO report. I have only a few minor comments.

1. It would be helpful if the authors can explain why they choose to use a truncated version of Ant1.
2. I suggest moving Fig. 2A to Figure 1.
3. I also suggest that the authors include what is expected with regards to the Lineweaver-Burk lines and Km/Vmax values if the substrates are exchanged by a sequential mechanism. This may help those not in the field of enzymatic kinetics to better understanding the data.

Referee #3:

In this manuscript, the authors Cimadamore-Werthein, et. al studied kinetic properties of the human mitochondrial ADP/ATP carrier by using proteoliposomes and transport robotics. Based on the Km/Vmax ratio in varied internal ATP concentrations, the authors conclude that the carrier operates in a ping-pong mechanism rather than the early proposed sequential kinetic mechanism. In fact, the sequential kinetic mechanism on homodimer has not been mentioned for many years. In this proposed model, the c-state conformation always couples with m-state, so the ratio of the two states should always be 50%:50%. Therefore, the simplest and strongest evidence against this sequential mechanism is that the ADP/ATP carrier predominantly adopts the c-state or ADP-waiting conformation in rest condition (~98% based on previous NMR experiment, Nat Struct Mol Biol 2015, 22:636). Moreover, as early as 2007 (PNAS 104:10830), the same group of this manuscript reported that the carrier functions as monomer, which itself also excludes the possibility of sequential kinetic mechanism of homodimer. Therefore, the aim of this work seems not reasonable, especially when it's put within the context of the big publications of the authors.
The reviewer also wants to point out that although forming monomer excludes the possibility of sequential kinetic mechanism, the ping-pong mechanism does not exclude the possibility of forming dimmer. The ADP/ATP carrier functioning as monomer does not exclude the possibility that they still can form homodimer, especially when a large number of evidence suggests its existence (Biochem. Biophys. Res. Commun.1982,109:471; Biochemistry 1985,24:3821; Biochemistry 2016, 55:6238; etc). Forming homodimer does not mean that the movements of the two protomers have to couple with each other. Their association could be less specific, but still could be favorable mainly through the entropy contribution. Therefore, the reviewer think whether the ADP/ATP carrier exits in monomer or homodimer is still an issue of intensive controversy, and the author should not present it as a matter of fact. Moreover, the authors stated that the carrier has one single binding site, which is not supported by early biochemical results (Eur J Biochem 1970,16:313) and more recent results (Structure 2015, 23:1394; Comput Struct Biotechnol J 2022,20:1829).
Due to the above concerns, the current version of the manuscript is not acceptable, and the reviewer recommends the authors to interpret their results from a more reasonable angle and present their opinions in a more scientific and open-minded way when they resubmit their manuscript.

Dear Dr. Papaioannou,

We thank you and the reviewers for taking time to consider our manuscript. We are pleased that reviewers 1 and 2 appreciate the quality and significance of our work, but are surprised by the comments of reviewer 3. None of the latter deal with any aspect of the methods, experimental data or conclusions of this paper, but rather with secondary issues unrelated to the core message, which deals with the transport kinetics of the mitochondrial ADP/ATP carrier.

We have addressed the individual points below.

Referee #1:

This manuscript by Cimadamore-Werthein, Baron et al is focused on kinetic analysis of the mitochondrion ADP/ATP carrier SLC25A4. Proposed transport mechanisms for this important protein have diverged significantly from earlier models invoking simultaneous binding of both substrates to a homodimeric transporter to more recent models based on a large body of structural and functional data, with seminal contributions from the Kunji group, revealing a monomeric transporter with a single binding site for ATP/ADP operating with an alternating access mechanism. While the latter is now widely accepted as being correct, experimental determination of transport kinetics has remained challenging. Here the authors use human SLC25A4 reconstituted in liposomes as the basis of a robotic transport assay that overcomes several challenges that have complicated accurate determination of reaction initial velocities and have led to error prone results and skewed interpretations of reaction kinetics. Assuming equivalent binding and transport steps for ATP and ADP, the assay measures uptake of radiolabeled ATP into proteoliposomes with different internal ATP concentrations. The authors are able to measure initial reaction rates based the linear time dependent accumulation of radiolabeled ATP inside liposomes. From these initial reaction rates at multiple external substrate concentrations, the authors are able to derive Michaelis Menten parameters for each internal ATP concentration. Plotting Km and Vmax values for each of these reveals parallel Lineweaver Burke plots, consistent with transport rates being independent of 'counter substrate' concentrations (internal ATP) and with a single binding site alternative access scheme. This represents an important and hitherto missing piece of functional evidence in further support of the so called 'ping-pong' kinetic model for transport that is likely applicable to other mitochondrial SLC25 family transporters. As such, the data presented are very much suitable for publication in EMBO reports as is. The manuscript is succinct and clearly written and was a pleasure to read. Parameterization of equations used for data fitting and their derivation are clearly explained. I only have minor points for clarification.

We thank the reviewer for their detailed analysis of our manuscript and especially for the comment that this work is an 'important and hitherto missing piece of functional evidence in further support of the so called 'ping-pong' kinetic model for transport that is likely applicable to other mitochondrial SLC25 family transporters'.

Minor Points:

1. Was there any reason only a single biological replicate is reported for the 2.5mM internal concentration of ATP?

Our original internal ATP concentration series (0.1, 0.25, 0.5 and 1.0 mM) provided enough coverage to determine the kinetic mechanism of the carrier. To ensure our interpretation was correct at higher concentrations, we decided to test an internal ATP concentration of 2.5 mM, which is ~1000 times the Km of transport. As this single data set was consistent with the other data, we have included it for completeness, but have clearly stated that it represents one biological repeat, consisting of three technical repeats (still representing 21 individual data points).

2. Could the assay not be adapted for different concentrations of ADP inside by the addition of gramicidin or valinomycin allowing for Na+ or K+ influx into liposomes preloaded with Na+ inside and K+ outside or vice versa to mitigate the mentioned potential build up with the ATP/ADP exchange?

The reviewer is correct that these ionophores would prevent the build-up of a membrane potential, but they would also lead to changes in sodium or potassium concentrations, especially on the inside of the proteoliposomes, which have a very small volume. The activity of mitochondrial carriers is known to be sensitive to changes in ionic strength, because of the multiple ionic interactions involved in the mechanism, such as salt bridge network formation and substrate binding. Thus, to keep the experiment as simple as possible and to avoid any potential technical artefacts, we decided to use ATP homo-exchange, where the transport rates are solely dependent on the applied substrate gradients.

Referee #2:

The mitochondrial ADP/ATP carrier (SLC25A4) is the prototype of proteins in the mitochondrial carrier family that transport nucleotides and various metabolites across the inner membrane. It is long believed to function as a homodimer in sequential mode in which the two exchanged substrates bind from the opposite sides of the membrane and move across the membrane simultaneously. The Kunji lab previously provided biochemical, biophysical and structural data that suggest a monomeric operation mode. In this model, there is only one single substrate binding site. An alternating access mechanism was proposed in which a central substrate binding site is used for substrate binding from one side of the membrane. Following conformational changes and the release of the substrate at the other side of the membrane, the transport counterion binds to the carrier to complete the exchange process. However, this so-called ping-pong transport mechanism does not fit with previously published transport kinetic data, likely due to a number of confounding factors that might have deviated the final interpretation. In this manuscript, the authors reanalyzed the kinetic properties of human ADP/ATP carrier by using recombinant expression in yeast and reconstitution in proteoliposomes of defined lipid composition and buffer conditions. They captured time frames during the transport process in which the transport rate is linear. By measuring the uptake of radioactively labelled ATP, they found that the Km/Vmax ratio is

constant when different internal concentrations of ATP are applied. The kinetic data support a ping-pong kinetic mechanism in which substrate exchange across the membrane occurs consecutively rather than simultaneously.

This is a well written short article with high-quality data that provide kinetic support for a monomeric operation mode of ADP/ATP carrier. The study is highly significant and has broad implications for the understanding of other mitochondrial carriers. I recommend the publication of the manuscript in EMBO report. I have only a few minor comments.

We thank the reviewer for their supportive comments on the manuscript, kindly stating that this work is 'highly significant and has broad implications for the understanding of other mitochondrial carriers'.

1. It would be helpful if the authors can explain why they choose to use a truncated version of Ant1.

The full-length mammalian protein does not express in mitochondria of *S. cerevisiae* (Hatanaka et al., 2001; Jaiquel Baron et al., 2021). However, the truncation of the human carrier leads to high levels of expression and to complementation, and allows the purification of folded and functional protein (Jaiquel Baron et al., 2021). We have added a sentence to the manuscript to this effect.

2. I suggest moving Fig. 2A to Figure 1.

We thank the reviewer for their suggestion, and agree that it makes more sense to present Fig. 2A in Fig. 1, which we have now done.

3. I also suggest that the authors include what is expected with regards to the Lineweaver-Burk lines and Km/Vmax values if the substrates are exchanged by a sequential mechanism. This may help those not in the field of enzymatic kinetics to better understanding the data.

We thank the reviewer for their suggestion. We have added an additional supplementary figure to the manuscript, as suggested.

Referee #3:

In this manuscript, the authors Cimadamore-Werthein, et. al studied kinetic properties of the human mitochondrial ADP/ATP carrier by using proteoliposomes and transport robotics. Based on the Km/Vmax ratio in varied internal ATP concentrations, the authors conclude that the carrier operates in a ping-pong mechanism rather than the early proposed sequential kinetic mechanism. In fact, the sequential kinetic mechanism on homodimer has not been mentioned for many years. In this proposed model, the c-state conformation always couples with m-state, so the ratio of the two states should always be 50%:50%. Therefore, the simplest and strongest evidence against this sequential mechanism is that the ADP/ATP carrier predominantly adopts the c-state or ADP-waiting conformation in rest condition (~98%

based on previous NMR experiment, Nat Struct Mol Biol 2015, 22:636). Moreover, as early as 2007 (PNAS 104:10830), the same group of this manuscript reported that the carrier functions as monomer, which itself also excludes the possibility of sequential kinetic mechanism of homodimer. Therefore, the aim of this work seems not reasonable, especially when it's put within the context of the big publications of the authors.

We thank the reviewer for taking the time to read this paper, but we fundamentally disagree with their comments. Their remarks do not address any aspect of the methods, experimental data or conclusions of the paper, related to the kinetics of the mitochondrial ADP/ATP carrier, but rather deal with secondary issues. It is true that the kinetic mechanism has not been addressed for the longest time, but it was entirely proper to do so, as our results clearly contradict the earlier published data.

The reviewer also wants to point out that although forming monomer excludes the possibility of sequential kinetic mechanism, the ping-pong mechanism does not exclude the possibility of forming dimmer. The ADP/ATP carrier functioning as monomer does not exclude the possibility that they still can form homodimer, especially when a large number of evidence suggests its existence (Biochem. Biophys. Res. Commun.1982,109:471; Biochemistry 1985,24:3821; Biochemistry 2016, 55:6238; etc). Forming homodimer does not mean that the movements of the two protomers have to couple with each other. Their association could be less specific, but still could be favorable mainly through the entropy contribution. Therefore, the reviewer think whether the ADP/ATP carrier exits in monomer or homodimer is still an issue of intensive controversy, and the author should not present it as a matter of fact. Moreover, the authors stated that the carrier has one single binding site, which is not supported by early biochemical results (Eur J Biochem 1970,16:313) and more recent results (Structure 2015, 23:1394; Comput Struct Biotechnol J 2022,20:1829).

This work does not address whether the carrier operates as a monomer or dimer nor does it aim to identify the binding site of the carrier. It provides, in the words of one of the other reviewers, an ‘important and hitherto missing piece of functional evidence in further support of the so called 'ping-pong' kinetic model for transport’.

Due to the above concerns, the current version of the manuscript is not acceptable, and the reviewer recommends the authors to interpret their results from a more reasonable angle and present their opinions in a more scientific and open-minded way when they resubmit their manuscript.

A constant Km/Vmax ratio, which results in parallel lines in a Lineweaver-Burk plot, can only mean that substrate exchange follows a ping-pong kinetic mechanism. It is not a question of being reasonable. We are also not obliged to explain or justify the data of others in the publications the reviewer has mentioned, as none of them address the kinetic mechanism. However, in good scientific practice, we are allowed to present the most consistent mechanistic model that agrees with our kinetic data. It is somewhat ironic that we are accused of not being scientific or open-minded, since our results here overthrow a ~40-year-old dogma in the field.

We prefer to comment on the cited papers of the referee’s report below.

Biochem. Biophys. Res. Commun.1982,109:471 This paper does not deal with the kinetic mechanism of the ADP/ATP carrier, but investigates the oligomeric state by neutron scattering, claiming it was a dimer. At the time, the protein was purified by negative chromatography, meaning that large amounts of lipids and detergent co-purified, which could not be fully masked by deuterium, leading to an overestimation of the size, as found later (Nury et al., 2008).

Eur J Biochem 1970, 16:313 This paper does not deal with the kinetic mechanism, but instead studies the competition between atractyloside and substrates, showing that they compete for the same site, which has been supported by the structures (Pebay-Peyroula et al., 2003; Ruprecht et al., 2014).

Nat Struct Mol Biol 2015, 22:636 In this NMR study the claim is made that AAC is "refolded" from inclusion bodies in Fos-choline-12, which is one of the harshest detergents. Under these conditions, the specific inhibitor carboxyatractyloside has a Kd of 20 uM, which is at least four orders of magnitude higher than that of the native protein, demonstrating that the protein is misfolded, Thus, none of the claims in this paper are valid, as all subsequent work has also shown. We and others have already addressed these and other issues with this work extensively (Chipot et al., 2018; Dehez et al., 2017; King et al., 2018; Kurauskas et al., 2018a; Kurauskas et al., 2018b).

PNAS 2007, 104:10830 This paper does not address the kinetic mechanism, but demonstrates that the mitochondrial ADP/ATP carrier functions as a monomer in mitochondrial membranes, which is consistent with our proposed structural mechanism (Figure 3).

Biochemistry 2016, 55:6238 This paper does not address the kinetic or molecular mechanism of the carrier. In this paper coarse grain dynamics are performed on a set of inactive carriers to show that they clump together to form dimers, trimers, tetramers, and hexamers, possibly because ionic strength and pH are difficult to model well in coarse grain simulations. Importantly, they show that there is no conserved dimerization interface, as carriers interact with all three domains randomly. Still, none of these conclusions are relevant, as in reality the carriers cycle continuously between a cytoplasmic and matrix conformation, each carrier being in a different dynamic state at any one time, precluding stable interactions. We have addressed these and other issues with this study previously (Kunji and Ruprecht, 2020).

Biochemistry 1985, 24:3821 This paper shows that the carrier from beef heart mitochondria has three tightly bound cardiolipins per protein, as revealed by ^{31}P NMR. This claim has indeed been confirmed by structural studies (Nury et al., 2005; Pebay-Peyroula et al., 2003; Ruprecht et al., 2014; Ruprecht et al., 2019), but it does not address the kinetic mechanism or oligomeric state of the protein.

Structure 2015, 23:1394 This is a study with a completely different carrier, the Mg-ATP/Pi carrier, and does not address any aspects of our paper. Again, this work used fos-choline-12 and thus has no relevant functional information, because the protein was misfolded (see comments and papers above).

Comput Struct Biotechnol J 2022, 20:1829 This is a molecular dynamics paper that does not address the kinetic mechanism. However, it does claim that the carrier in the cytoplasmic state has a second binding site, involving the positively charged residues of the cytoplasmic network. However, the cytoplasmic network is universal to all SLC25 carriers, thus providing no molecular basis for substrate specificity, but it does have a defined and experimentally proven role in the mechanism (King et al., 2016; Ruprecht et al., 2019). The suggested second binding site is located in the region that forms the cytoplasmic gate, meaning that substrate binding and closure of the carrier are mutually exclusive.

In fact, MD simulations have identified hundreds of potential binding sites and substrate poses, but none of these studies are carried out in a relevant time frame to assess their significance for the transport mechanism, which occurs on millisecond time scale rather than microsecond time scale. Moreover, we have not been able to confirm the involvement of these additional sites in substrate binding using thermostability shift assays, which only detect significant substrate-carrier interactions (Mavridou et al., 2022). Thus, the ADP/ATP carrier has only one substrate binding site in agreement with a ping-pong kinetic mechanism, demonstrated unequivocally by this work.

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Dear Edmund,

Thank you for submitting your revised manuscript for consideration by EMBO reports. It has now been seen by the three referees who were asked to re-evaluate your manuscript, and we have received the full set of their reports that I have already shared with you (included again below), as well as cross-referee comments from them. Referees #1 and #2 mention that their concerns have been addressed, and they now support publication of the paper in EMBO reports, while referee #3 suggests that a clarification of the data interpretation be added to the manuscript.

I would also like to thank you for your feedback on the outcome of the above process and your willingness to include a statement in the Discussion of your manuscript clarifying that the data presented in this paper do not directly address the question of the oligomeric state of the ADP/ATP carrier, in response to the criticism of referee #3. Please include your suggested text in a revised manuscript, and make sure that the changes are highlighted (or "tracked") to be clearly visible. Please also include in your re-submission a response/rebuttal note to the comments of referee #3.

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

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Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
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Referee #1:

The authors have adequately addressed the minor points I had, and I look forward to seeing this manuscript out in its final form.

Referee #2:

The reviewer has no further comments.

Referee #3:

The authors didn't address most of my concerns. As I stated what I really conerned are not the methods and results of this work but the proposed transport mechanism framework the authors used to understand these results. The authors proposed a symmetric transport mechanism, based on which they insisted that the carrier can not exist as dimeric form, and that's the reason why thet symmetrized the asymmetric m-state structure in their previous Cell paper. However, I couldn't find the basis for their proposal for a symmetric transport mechanism. In fact there are a variety of evidences suggesting an asymmetric transport mechanism. Therefore, the authors' proposal could quite possibly be wrong, or at least the authors should soften their statement as possibly as they can in their manuscirpt.

Referee #1

The authors have adequately addressed the minor points I had, and I look forward to seeing this manuscript out in its final form.

Thank you for your helpful comments to improve our manuscript.

Referee #2

The reviewer has no further comments.

Thank you for your helpful comments to improve our manuscript.

Referee #3

The authors didn't address most of my concerns. As I stated what I really conerned are not the methods and results of this work but the proposed transport mechanism framework the authors used to understand these results. The authors proposed a symmetric transport mechanism, based on which they insisted that the carrier can not exist as dimeric form, and that's the reason why thet symmetrized the asymmetric m-state structure in their previous Cell paper. However, I couldn't find the basis for their proposal for a symmetric transport mechanism. In fact there are a variety of evidences suggesting an asymmetric transport mechanism. Therefore, the authors' proposal could quite possibly be wrong, or at least the authors should soften their statement as possibly as they can in their manuscirpt.

In this paper, we do not address whether the mechanism is symmetric or asymmetric.

To comply with the reviewer’s request, we have added the following line to the discussion:

The data presented in this paper do not directly address the question of the oligomeric state of the ADP/ATP carrier. However, they provide further support for the notion that the carrier functions as a monomer with a single substrate binding site, consistent with a large number of structural and functional data gathered over recent years.

2nd Revision - Editorial Decision **16th May 2023**

Dear Edmund,

Thank you for the submission of your revised manuscript to our offices. Browsing through the manuscript, we have noticed that there are still two issues that need to be fixed before we can proceed with acceptance of your paper. In particular:

1. Please note that you can either publish the study as a Report or as an Article. For Reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding Materials & Methods and references) and 5 main plus 5 Expanded View figures. The Results and Discussion sections must be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For an Article there are no length limitations, but it should have more than 5 main figures and the Results and Discussion sections must be separate. In both cases, the entire Materials and Methods must be included in the main manuscript file.

2. The author contributions statement should be removed from the manuscript file. Instead, we use CRediT to specify the contributions of each author in the journal submission system. Please use the free text box to provide more detailed descriptions. See also our guide to authors:
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.

Please address both issues in a revised manuscript. I look forward to seeing the new version as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
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All editorial and formatting issues were resolved by the authors.

Prof. Edmund Kunji
University of Cambridge
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United Kingdom

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Best regards,

Ioannis

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Best,
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Hannah Sonntag, PhD
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