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Structural and Mechanistic Insights into MICU1 Regulation of Mitochondrial Calcium Uptake

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 11 September 2013

Thank you very much for submitting your structural study for consideration to The EMBO Journal editorial office.

Three referees have provided comments on your dataset and find the major message relevant and of potential interest for a general audience.

However, their detailed remarks also identify limitations that appear not that straightforward to overcome. In particular,

-ref#1 questions the fragment used for crystallization and requests at the minimum to clearly label and discuss potential drawbacks associated with this fact;
-ref#2 remains unconvinced that the proposed activation model is driven by physiological evidence, asking to remove it and discuss much carefully;
-#3 raises major issues that need to be addressed; the significant additional experimentation s/he requests should surface necessary and definitive molecular detail;

I realize that these are demanding and time-consuming experiments.

Given the indicated wider interest however, we would be prepared to re-assess a suitably revised version that would not necessarily depend on novel crystals from an extended fragment but should satisfy the crucial demands raised by refs#2 and #3 that would certainly become involved to reach a

final decision on a thoroughly revised version.

Please be reminded that The EMBO Journal considers only one round of timely limited major revisions.

Please do not hesitate to get in touch regarding potential timeline/feasibility of the requested experimental expansions if you deem necessary (preferably via E-mail).

REFEREE REPORTS

Referee #1:

The paper by Wang et al describes the crystal structure of MICU1. This is the first report of the structure of one of the components of the molecular machinery for mitochondrial Ca2+ uptake, which was only very recently identified at the molecular level. This is an achievement, and comes very timely with the excitement in this new area. The data are overall solid and well presented (but see the specific comments below), so the paper is in my view of interest to a broad audience.

Specific comments
- A major concern is the choice of the protein domain employed for the crystallization. While it is quite logical to remove the mitochondrial targeting sequence, it is a bit odd (unless a motivated answer is given) that by removing 97 aa the authors eliminated also the first part of the mature protein, also considering that this portion contains a conserved cysteine that could be involved in sulfur bridge formation, and thus oligomerization processes. Unless the entire mature protein is analyzed (and if this is not possible it should be clearly stated in the text), the possible problems in data interpretation should be at least mentioned.
- The cartoon in figure 5 speculates on the role of MICU1 in the regulation of mitochondrial Ca2+ entry and it is not supported by experimental data. Importantly, the authors imply (and discuss in detail in the Discussion) a putative role for LETM1 (and secondarily for other mechanisms, such as RyR1 and RAM). This is very misleading, also considering that the role of LETM1 in calcium homeostasis has been heavily questioned (see for example Hashimi et al, JBC 2013; Dimmer et al, Hum Mol Genet. 2008; Novikowsky et al, J. Gen. Physiol. 2013). The authors should focus on what their data tell them, thus on MICU1 and MCU, and not wander around a debated literature. Also in respect to the MCU complex, why do they show a monomer (apparently inactive in the cartoon), and a dimer (??), interacting with MICU? Do they have data supporting this view? If this is the case, it is mandatory to show them. If not, the authors should be more cautious in depicting the MCU structure. Overall, it seems to me that either the authors delete the figure or they make it more rigorous, adherent to real, widely accepted data.
- The buried surface after dimer formation is 1,122 A2 for the Ca-free and 434 A2 for the Ca-bound structure. Whilst the former value is consistent with the presence of a physiological dimer, the second value is very low, and it looks as if two independent monomers are present in the asymmetric unit of the crystal. This seems to be inconsistent with solution data and with the multiple hydrophobic interactions and hydrogen bonds described for the Ca2+-bound MICU1-xtal-deltaC dimer interface (Figure 3E).
- In the Discussion section, MICU1 equilibrium is reported as hexamer-high order oligomer. It is not clear what this high-order oligomerization looks like, since the hexamer-dimer equilibrium seems to be used to support the final speculation of the interaction with MCU (Fig. 5). In addition, the dimer in the crystal is obtained thank to the deletion of the C-terminal helix, and thus it should be at least mentioned that the biological relevance could be dubious.
- The text is a bit difficult to read in some parts, and some language editing would be beneficial.

Technical points:

- The crystallographic R factors for the Ca-free structure are quite high (0.252-0.305). In order to convince the reader and to give an idea of the quality if the structure, a stereo view of one or two portions of the electron density map, with coefficients (2Fobs-Fcalc), should be included in the supplementary materials.
- Some more data on the quality of the SAD map should be included in Table I, for example the value of the anomalous phasing power. Also the centric/acentric values of the figure of merits

should be reported.
- It is not clearly stated in the paper, but it seems that a hexamer is present in the asymmetric unit of the P21 crystal form, and a dimer in the I212121. This should be explicitly added in Table I.
- The IP experiments in figures 2H and supplementary Figure 7 lack of the necessary controls. Indeed, the authors should show the IPs also of cells transfected with MCU and MICU1 only to demonstrate that the binding of MICU1 to MCU is specific and not due to non specific binding of MCU to the beads.

Referee #2:

The authors report the X-ray crystallographic structures of human MICU1, the regulatory component of the mitochondrial calcium uniporter (MCU) in the apo and Ca2+ -bound states. The MICU1 structure consists of two canonical EF-hand Ca2+ -binding motifs, each paired with a structural (nonfunctional) EF-hand. It also contains a small N-terminal alpha/beta domain of unknown function and a C-terminal helix, which in the crystal, as well as in solution, contributes to its oligomerization, as the authors document via sedimentation velocity, light scattering and mutagenesis. The C-helix is also shown to be important for the regulatory activity of MICU1. Two molecules of MICU1 are arranged in a dimer, in which the EF-hand domains adopt different orientations depending on Ca2+. The dimers form a hexamer in the apo form and various oligomeric structures in the presence of Ca2+. The authors propose a model of the MICU1 function in which a hexameric apo form inhibits the uniporter, whereas its Ca2+ bound "higher-order oligomers" (as the authors call it) activate the uniporter.

The structures presented in this work provide a wealth of information about MICU1, and the work overall represents a major advance in the area of mitochondrial calcium signaling. The proposed model, however, is unconvincing because it hinges upon the extrapolation of the oligomerization properties of MICU1 observed in solution to the in vivo conditions. In the absence of any information about the oligomerization state of MICU1 in vivo the model (Fig. 5, Discussion, pages 14-15) remains highly speculative and should be deleted. Even without the model the work is of high importance and of great interest. More specific comments are listed below.

Major Comments:

(1) Page 11, top paragraph. The authors' conclusion about the oligomeric state of MICU1 in vivo is not warranted on the basis of the presented data. From the two observations that the C-helix contributes to the oligomerization in vitro and that it is necessary for the activity of MICU1, it does not follow that oligomerization is necessary for the activity. There may or may not be a cause-effect relation but this cannot be concluded on the basis of the presented data. This conclusion has to be modified. Unfortunately, this is the key conclusion in support of the model in Fig. 5. For that reason I suggest that Fig. 5 and the relevant discussion are deleted to reflect the tenuous, highly speculative nature of the proposed model.

(2) In several instances throughout the text the authors make unequivocal conclusions that are not well supported by the presented data. In particular, the statement that MICU1 forms "higher-order oligomers" in the presence of Ca2+ (pages 2, 5, 8, 13, 14, etc) is not supported by Fig. 2B, which shows that majority of the protein is distributed between two peaks, one corresponding to MW ~240 kDa (similar to that for the apo form), the other, a slower migrating species corresponding to MW ~90 kDa, likely a dimer. Other examples; on page 8, top paragraph the authors conclude that "the C-helix was necessary for both the hexamer .... and the high-order oligomerization". In fact, in Fig. 3G and Fig. S2C significant amounts of material are present with MW above 100 kDa and even above 200 kDa for both MICU-xtal-deltaC and its mutant F383A-H385A. While it is apparent from the presented data that the C-helix makes an important contribution to the oligomer formation, there are clearly other contributing factors. The authors should revise the manuscript with a special attention to these unnecessary misrepresentations of their results.

Minor comments:

(1) Fig. 2 A,B,E,F. Please change the labeling of the ordinate axis (UV) into something meaningful (e.g. Absorbance at 280 nm). Also please use the same scale for the abscissa in all four panels, for easier comparison of the distribution of various oligomers.

(2) Page 7, bottom paragraph. For most readers the term "coiled-coil" will imply the existence of a highly regular structural features, such as the 3-4 heptad repeat and a well defined supertwist. These features are apparently not present in the structure of MICU1, nor are they expected from the amino acid sequence of the C-helix. A more appropriate term here would be "helix bundle".

(3) Page 19. I am not aware of the application of sapphirine for optical windows. In particular, Beckman-Coulter uses sapphire or quartz. Please check if the information in the text is correct.

(4) Page 11 lines 6,7 from the bottom of page. The phrase: " which are not able to estimate the binding affinity of each site" is awkward. Please replace with: "could not be resolved"

Referee #3:

In this MS, Wang et al provide the first structural insights to the mitochondrial calcium uniporter. Specifically, they report crystal structures of Ca2+-free and Ca2+-bound human MICU1. The study is potentially highly relevant but the present draft needs to be improved in many regards.

Specific concerns:
-From the structural studies little can be learnt about the MICU1-MCU interaction that seems to control the uniporter function.
MCU has been predicted to oligomerize to form a channel. The stoichiometry among MICU1 hexamers and MCU is unclear.
The C-helix of MICU1 is proposed to interact with MCU. Therefore it would be important to evaluate the effect of calcium on MICU1 containing the C-helix.It is mentioned that the C-helix was removed to avoid protein aggregation but it is unclear whether aggregation did happen when MICU1-xtal was crystallized in the presence of calcium.
It is also unexplained why the first 96 amino acids of MICU1 were omitted in the crystallization study. Removal of <60 amino acids would have been sufficient to eliminate the mito targeting sequence and the hydrophobic domain.
How would the proposed model accommodate an N terminus that is membrane associated? Could this be discussed?

-The calcium measurements are not straightforward.
Calculation of percent stimulation from the aequorin luminescence values is not particularly meaningful. Traces would need to be calibrated in terms of uM calcium concentration. This would also be important since the effect of MICU1 overexpression seems to cause an unexpectedly large increase in the histamine-induced mitochondrial calcium signal.
It is unexplained what mechanism underlies the effect of MICU1 on mitochondrial calcium. Is it attributed to the role of MICU1 in gatekeeping described by both Mallilankaraman et al 2012 an Csordas et al 2013 or in the cooperative activation of the uniporter described by Csordas et al 2013?
It would be more straightforward to perform rescue experiments with the MICU1 mutants as described by Perocchi et al 2010 than pursuing overexpression studies.

-Calcium-induced conformational change
Where are EF1 and EF4 within the hexameric conformation - are they exposed to the solvent (from Fig1B, it looks as though EF1 is, but what about EF4)? Does Ca2+ need to bind both EF1 and EF4 for Ca2+ to induce a conformational change?
p8, bottom of page: "In sharp contrast, the deletion of the C-helix ...": since the EF hands have been proposed to be required for cooperativity (or for the transition from closed to high conductance states), it would be useful to discuss the findings in terms of the relevant literature which isn't done when 'cooperativity' is mentioned on P13 of the Discussion. The dimer formation in the presence of calcium also seems to favor large calcium uptake; this observation could also be discussed in terms of lit proposing a role for MICU1 in cooperative calcium uptake.

Minor:

-pseudo EF hands are called unexpected even though they are predicted in databases

-STIM analogy isn't really appropriate: STIM is transmembrane with calcium sensing on one side of the membrane and interaction with Orai on the other side (also, Orai is in the PM). Also, the oligomerization happens the opposite way.

-Including LETM1 and mRyR1 in a larger model of mito calcium uptake seems too speculative at this point, and takes the spotlight away from their structural findings and how these relate to the existing literature (which is insufficiently discussed), and also how to reconcile some of the proposed changes in the association of MICU1 monomers with the hydrophobic stretch in the N terminus.

-in the introduction the selection of many references seems to be random. For example for the role of calcium signal in mitochondrial apoptosis the first comprehensive reviews were:
Regulation of cell death: the calcium-apoptosis link.
Orrenius S, Zhivotovsky B, Nicotera P.
Nat Rev Mol Cell Biol. 2003 Jul;4(7):552-65.
Control of apoptosis by IP(3) and ryanodine receptor driven calcium signals.
Hajnoczky G, Csordas G, Madesh M, Pacher P.
Cell Calcium. 2000 Nov-Dec;28(5-6):349-63.

-Fig2A,B,E,F: expts are not well enough described. If this is not a standard technique, then it might be useful to better describe the expt. What are the red lines?
- p11: " The titration profile showed ... (Figure S5), which are not able to estimate the binding affinity of each site." The meaning of the underlined part is not clear.
- define 'resting state' (p12 - end of results, 1st para of Disc).

1st Revision - authors' response 04 December 2013

Referee #1:

The paper by Wang et al describes the crystal structure of MICU1. This is the first report of the structure of one of the components of the molecular machinery for mitochondrial Ca2+ uptake, which was only very recently identified at the molecular level. This is an achievement, and comes very timely with the excitement in this new area. The data are overall solid and well presented (but see the specific comments below), so the paper is in my view of interest to a broad audience.

Specific comments

1. A major concern is the choice of the protein domain employed for the crystallization. While it is quite logical to remove the mitochondrial targeting sequence, it is a bit odd (unless a motivated answer is given) that by removing 97 aa the authors eliminated also the first part of the mature protein, also considering that this portion contains a conserved cysteine that could be involved in sulfur bridge formation, and thus oligomerization processes. Unless the entire mature protein is analysed (and if this is not possible it should be clearly stated in the text), the possible problems in data interpretation should be at least mentioned.

Response:

We designed and evaluated four constructs (residues 53-476, residues 62-476, residues 80-476 and residues 97-476) based on a sequence alignment of MICU1 from different species (Figure S1). The construct (residues 97-476) described in our manuscript finally produced crystals that diffracted to 3.2 Å resolution. A protein secondary structure prediction (http://npsa-pbil.ibcp.fr/) suggests that residues 57-95 of MICU1 form a random coil. Additionally, Cys62 does not appear to be conserved from our analysis (Figure S1). We currently do not know the function of both this region and Cys62, which will be explored in our future studies.

The reason we have chosen this construct (residues 97-476) has been included into the revised manuscript, as follows: "To obtain Ca^{2+}-free MICU1 crystals, four constructs (residues 53-476, residues 62-476, residues 80-476 and residues 97-476) based on a sequence alignment of MICU1 from different species (Figure S1) were evaluated. One of the constructs (residues 97-476, referred to as MICU1-xtal hereafter; different constructs and names are shown in Figure S2A) successfully produced diffraction-quality crystals."

2. The cartoon in figure 5 speculates on the role of MICU1 in the regulation of mitochondrial Ca2+ entry and it is not supported by experimental data. Importantly, the authors imply (and discuss in detail in the Discussion) a putative role for LETM1 (and secondarily for other mechanisms, such as RyR1 and RAM). This is very misleading, also considering that the role of LETM1 in calcium homeostasis has been heavily questioned (see for example Hashimi et al, JBC 2013; Dimmer et al, Hum Mol Genet. 2008; Novikowsky et al, J. Gen. Physiol. 2013). The authors should focus on what their data tell them, thus on MICU1 and MCU, and not wander around a debated literature. Also in respect to the MCU complex, why do they show a monomer (apparently inactive in the cartoon), and a dimer (??), interacting with MICU? Do they have data supporting this view? If this is the case, it is mandatory to show them. If not, the authors should be more cautious in depicting the MCU structure. Overall, it seems to me that either the authors delete the figure or they make it more rigorous, adherent to real, widely accepted data.

Response:

We thank the reviewer for these kind suggestions. We also agree with the reviewer that the function of LETM1 currently remains under debate. To avoid potential confusion, Figure 5 has been deleted, and the Discussion section has been accordingly rewritten in the revised manuscript.

3. The buried surface after dimer formation is 1,122 A2 for the Ca-free and 434 A2 for the Ca-bound structure. Whilst the former value is consistent with the presence of a physiological dimer, the second value is very low, and it looks as if two independent monomers are present in the asymmetric unit of the crystal. This seems to be inconsistent with solution data and with the multiple hydrophobic interactions and hydrogen bonds described for the Ca2+-bound MICU1-xtal-deltaC dimer interface (Figure 3E).

Response:

Generally, there are four types of noncovalent interactions among proteins: hydrogen bonds, salt bridges, hydrophobic interactions and van der Waals interactions. The buried surface area of a dimer typically correlates with the total number of noncovalent interactions within the interface. Indeed, there are 92 and 67 noncovalent interactions (with a cut-off distance of 4.0 Å) for the Ca^{2+}-free and Ca^{2+}-bound MICU1 dimer, respectively. In Figure 3D and E, we only highlighted a few of the interactions that are potentially important for dimer formation.

4. In the Discussion section, MICU1 equilibrium is reported as hexamer-high order oligomer. It is not clear what this high-order oligomerization looks like, since the hexamer-dimer equilibrium seems to be used to support the final speculation of the interaction with MCU (Fig. 5). In addition, the dimer in the crystal is obtained thank to the deletion of the C-terminal helix, and thus it should be at least mentioned that the biological relevance could be dubious.

Response:

We agree with the reviewer that the Ca^{2+}-bound full-length MICU1 dimer is less likely to stably exist. To avoid potential confusion, Figure 5 and the corresponding description in the Discussion section have been deleted in the revised manuscript. We currently do not know how Ca^{2+}-bound MICU1 activates MCU. More studies are required to ascertain this crucial process in the near future.

5. The text is a bit difficult to read in some parts, and some language editing would be beneficial.

Response:

As suggested by the reviewer, our newly revised manuscript has been proofread by a professional English editing service.

Technical points:

6. The crystallographic R factors for the Ca-free structure are quite high (0.252-0.305). In order to convince the reader and to give an idea of the quality if the structure, a stereo view of one or two portions of the electron density map, with coefficients (2Fobs-Fcalc), should be included in the supplementary materials.

Response:

The Ca^{2+}-free MICU1 structure has been determined using SAD at a resolution of 3.2 Å. Additionally, there are six molecules (a total of 2280 residues) in the asymmetric unit. These factors may result in the slightly higher R factors for this structure.

As suggested by the reviewer, the $2\boldsymbol{F}_o$-$\boldsymbol{F}_c$ electron density maps for the Ca^{2+}-free and Ca^{2+}-bound MICU1 structures have been included in Figure S10.

7. Some more data on the quality of the SAD map should be included in Table I, for example the value of the anomalous phasing power. Also the centric/acentric values of the figure of merits should be reported.

Response:

As suggested by the reviewer, we have included the centric/acentric values of the figure of merits into the revised Table I. We used the program PHENIX to calculate the initial SAD phases to determine the Ca^{2+}-free MICU1 structure and performed the subsequent density modification. Unfortunately, this program did not generate phasing power statistics. The value of the mean figure of merit after density modification reaches to 0.638, and the resulting electron density map is sufficiently good to build a nearly complete model for the Ca^{2+}-free MICU1 molecule.

8. It is not clearly stated in the paper, but it seems that a hexamer is present in the asymmetric unit of the P21 crystal form, and a dimer in the I212121. This should be explicitly added in Table I.

Response:

A hexamer is present in the asymmetric unit of the Ca^{2+}-free MICU1 structure, and a dimer is present in the Ca^{2+}-bound MICU1 structure. This information has been included into the revised Table I.

9. The IP experiments in figures 2H and supplementary Figure 7 lack of the necessary controls. Indeed, the authors should show the IPs also of cells transfected with MCU and MICU1 only to demonstrate that the binding of MICU1 to MCU is specific and not due to non specific binding of MCU to the beads.

Response:

As suggested by the reviewer, these controls have been included in the revised Figure 2H and Figure S8.

Referee #2:

The authors report the X-ray crystallographic structures of human MICU1, the regulatory component of the mitochondrial calcium uniporter (MCU) in the apo and Ca2+ -bound states. The MICU1 structure consists of two canonical EF-hand Ca2+ -binding motifs, each paired with a structural (nonfunctional) EF-hand. It also contains a small N-terminal alpha/beta domain of unknown function and a C-terminal helix, which in the crystal, as well as in solution, contributes to its oligomerization, as the authors document via sedimentation velocity, light scattering and mutagenesis. The C-helix is also shown to be important for the regulatory activity of MICU1. Two molecules of MICU1 are arranged in a dimer, in which the EF-hand domains adopt different orientations depending on Ca2+. The dimers form a hexamer in the apo form and various oligomeric structures in the presence of Ca2+. The authors propose a model of the MICU1 function in which a hexameric apo form inhibits the uniporter, whereas its Ca2+ bound "higher-order oligomers" (as the authors call it) activate the uniporter.

The structures presented in this work provide a wealth of information about MICU1, and the work overall represents a major advance in the area of mitochondrial calcium signalling. The proposed model, however, is unconvincing because it hinges upon the extrapolation of the oligomerization properties of MICU1 observed in solution to the in vivo conditions. In the absence of any information about the oligomerization state of MICU1 in vivo the model (Fig. 5, Discussion, pages 14-15) remains highly speculative and should be deleted. Even without the model the work is of high importance and of great interest. More specific comments are listed below.

Major Comments:

(1) Page 11, top paragraph. The authors' conclusion about the oligomeric state of MICU1 in vivo is not warranted on the basis of the presented data. From the two observations that the C-helix contributes to the oligomerization in vitro and that it is necessary for the activity of MICU1, it does not follow that oligomerization is necessary for the activity. There may or may not be a cause-effect relation but this cannot be concluded on the basis of the presented data. This conclusion has to be modified. Unfortunately, this is the key conclusion in support of the model in Fig. 5. For that reason I suggest that Fig. 5 and the relevant discussion are deleted to reflect the tenuous, highly speculative nature of the proposed model.

Response:

As suggested by the reviewer, the model in Figure 5 and the relevant discussion are too speculative and have been deleted in the revised manuscript.

(2) In several instances throughout the text the authors make unequivocal conclusions that are not well supported by the presented data. In particular, the statement that MICU1 forms "higher-order oligomers" in the presence of Ca2+ (pages 2, 5, 8, 13, 14, etc.) is not supported by Fig. 2B, which shows that majority of the protein is distributed between two peaks, one corresponding to MW ~240 kDa (similar to that for the apo form), the other, a slower migrating species corresponding to MW ~90 kDa, likely a dimer. Other examples; on page 8, top paragraph the authors conclude that "the C-helix was necessary for both the hexamer .... and the high-order oligomerization". In fact, in Fig. 3G and Fig. S2C significant amounts of material are present with MW above 100 kDa and even above 200 kDa for both MICU-xtal-deltaC and its mutant F383A-H385A. While it is apparent from the presented data that the C-helix makes an important contribution to the oligomer formation, there are clearly other contributing factors. The authors should revise the manuscript with a special attention to these unnecessary misrepresentations of their results.

Response:

We thank the reviewer for these kind suggestions. The term “higher-order oligomer” is incorrect to describe our results and has been replaced by the more appropriate term “multiple oligomers”. We have accordingly revised the entire manuscript. Moreover, the results were carefully interpreted in the revised manuscript to avoid a misrepresentation of the results.

On page 8, the sentence "the C-helix was necessary for both the hexamer .... and the high-order oligomerization" has been rephrased to “the C-helix is necessary for the formation of the Ca^{2+}-free MICU1-xtal hexamer and substantially contributed to the formation of multiple oligomers of Ca^{2+}-bound MICU1-xtal.” in the revised manuscript.

Minor comments:

(3) Fig. 2 A,B,E,F. Please change the labelling of the ordinate axis (UV) into something meaningful (e.g. Absorbance at 280 nm). Also please use the same scale for the abscissa in all four panels, for easier comparison of the distribution of various oligomers.

Response:

As suggested by the reviewer, we have modified the Figure 2A, B, E, F accordingly in the revised manuscript.

(4) Page 7, bottom paragraph. For most readers the term "coiled-coil" will imply the existence of a highly regular structural features, such as the 3-4 heptad repeat and a well defined supertwist. These features are apparently not present in the structure of MICU1, nor are they expected from the amino acid sequence of the C-helix. A more appropriate term here would be "helix bundle".

Response:

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(5) Page 19. I am not aware of the application of sapphirine for optical windows. In particular, Beckman-Coulter uses sapphire or quartz. Please check if the information in the text is correct.

Response:

We thank the reviewer for pointing out this mistake. We have changed the word “sapphirine” into “sapphire” in newly revised manuscript.

(6) Page 11 lines 6,7 from the bottom of page. The phrase: " which are not able to estimate the binding affinity of each site" is awkward. Please replace with: "could not be resolved"

Response:

We thank the reviewer for nice suggestion. We have changed it accordingly in the revised manuscript.

Referee #3:

In this MS, Wang et al provide the first structural insights to the mitochondrial calcium uniporter. Specifically, they report crystal structures of Ca2+-free and Ca2+-bound human MICU1. The study is potentially highly relevant but the present draft needs to be improved in many regards.

Specific concerns:

1. From the structural studies little can be learnt about the MICU1-MCU interaction that seems to control the uniporter function. MCU has been predicted to oligomerize to form a channel. The

stoichiometry among MICU1 hexamers and MCU is unclear. The C-helix of MICU1 is proposed to interact with MCU. Therefore it would be important to evaluate the effect of calcium on MICU1 containing the C-helix. It is mentioned that the C-helix was removed to avoid protein aggregation but it is unclear whether aggregation did happen when MICU1-xtal was crystallized in the presence of calcium. It is also unexplained why the first 96 amino acids of MICU1 were omitted in the crystallization study. Removal of <60 amino acids would have been sufficient to eliminate the mito targeting sequence and the hydrophobic domain. How would the proposed model accommodate an N terminus that is membrane associated? Could this be discussed?

Response:

Our structural studies provide two important details regarding the function of the uniporter: (1) Ca^{2+}-free MICU1 formed a hexamer; and (2) Ca^{2+} binding to MICU1 resulted in large conformational changes that disrupt the hexamer. In combination with other techniques, we proposed a simple model that Ca^{2+}-free MICU1 formed a hexamer to bind and inhibit MCU through the C-helix. Ca^{2+} binding to MICU1 released the inhibition and resulted in the activation of the uniporter. Our model is consistent with a recent report by Csordas et al. 2013 (Cell metabolism, 17, 976-987).

The molecular weight of a MICU1 monomer is approximately 52 kDa and that of a MCU monomer is 40 kDa. Therefore, a hexamer of MICU1 and a tetramer of MCU (Raffaello et al., EMBO J, 2013) will correspond to approximately 470 kDa, which is consistent with blue native gel analysis (~480 kDa) of mouse liver mitochondria (Plovanich et al., PLoS ONE, 2013). Therefore, we speculate that the stoichiometry of the MICU1/MCU complex at low micromolar calcium concentration will be 6:4.

We attempted to crystallize the MICU1-xtal molecule in the presence of calcium but were not successful. As shown in Figure 2B and Figure S3B, Ca^{2+}-bound MICU1-xtal formed multiple oligomers. We are currently unsure whether Ca^{2+}-bound MICU1 will aggregate after binding MCU, which may require further studies to be clarified.

We have evaluated four different constructs for the crystallization of MICU1. The structure of the construct containing residues 97-476 was finally determined. We have included this information in the revised manuscript. The protein secondary structure prediction suggests that residues 28-56 and residues 57-95 of MICU1 form a transmembrane helix and a random coil, respectively. We speculate that residues 57-95 may form a loop to anchor the rest of MICU1. This model requires further biochemical verification in our future studies.

2. The calcium measurements are not straightforward. Calculation of percent stimulation from the aequorin luminescence values is not particularly meaningful. Traces would need to be calibrated in terms of uM calcium concentration. This would also be important since the effect of MICU1 overexpression seems to cause an unexpectedly large increase in the histamine-induced mitochondrial calcium signal. It is unexplained what mechanism underlies the effect of MICU1 on mitochondrial calcium. Is it attributed to the role of MICU1 in gatekeeping described by both Mallilankaraman et al 2012 an Csordas et al 2013 or in the cooperative activation of the uniporter described by Csordas et al 2013? It would be more straightforward to perform rescue experiments with the MICU1 mutants as described by Perocchi et al 2010 than pursuing overexpression studies.

Response:

As suggested by the reviewer, we have performed the rescue experiments for MICU1 wild type and mutants. We conducted the experiment as described in a recent study (Csordas et al., Cell Metabolism, 2013). Briefly, MICU1 was silenced by the transfection of siRNA into HeLa cells for 48 hours and was then rescued by MICU1 wild type and mutants. Measurements of mitochondrial Ca^{2+} were performed upon histamine treatment. The results have been updated in Figure 2I and Figure 3H of the revised manuscript.

3. Calcium-induced conformational change Where are EF1 and EF4 within the hexameric conformation - are they exposed to the solvent (from Fig1B, it looks as though EF1 is, but what

about EF4)? Does Ca2+ need to bind both EF1 and EF4 for Ca2+ to induce a conformational change?

Response:

We have included a supplemental figure (Figure S6A) to show the positions of the EF1 and EF4 sites. The EF1 sites are located in the periphery of the Ca^{2+}-free MICU1 hexamer and should be solvent exposed, whereas the EF4 sites lie closer to the centre and appear to be solvent inaccessible. Based on a structural comparison (Figure 1D and Figure 3C), we suggest that Ca^{2+} binding to the EF1 sites may induce a conformational change, whereas Ca^{2+} binding to the EF4 sites may play an important role in maintaining the protein stability.

4. p8, bottom of page: "In sharp contrast, the deletion of the C-helix ...": since the EF hands have been proposed to be required for cooperativity (or for the transition from closed to high conductance states), it would be useful to discuss the findings in terms of the relevant literature which isn't done when 'cooperativity' is mentioned on P13 of the Discussion. The dimer formation in the presence of calcium also seems to favour large calcium uptake; this observation could also be discussed in terms of lit proposing a role for MICU1 in cooperative calcium uptake.

Response:

We thank the reviewer for these kind suggestions. Our results suggest that both the EF hands and the C-helix are necessary for MCU activation. However, we currently do not know whether the EF hands and the C-helix cooperatively function during MCU activation. To be conservative, we have rephrased the sentence “In summary, our data indicated that calcium and the C-helix cooperatively induced the …...” as follows: “These results suggest that not only the EF hands but also the C-helix of MICU1 are necessary for the activation of MCU.” in the revised manuscript.

Our results suggest that Ca^{2+}-bound MICU1 must form a certain type of oligomer to regulate and activate the MCU channel. We are currently unsure whether dimeric Ca^{2+}-bound MICU1 is capable of activating the MCU channel. It is possible that the Ca^{2+}-bound MICU1 dimer is an intermediate state and acts as a building block to form other types of oligomers, as does the Ca^{2+}-free MICU1 dimer. More studies are required to clarify this speculation. Therefore, we summarized our speculation as follows: “MICU1 senses and binds Ca^{2+} and undergoes conformational changes, which result in the activation of the MCU channel that leads to massive calcium uptake. The details concerning the activation of MCU by Ca^{2+}-bound MICU1 await further elucidation.” in the revised manuscript.

Minor:

5. pseudo EF hands are called unexpected even though they are predicted in databases

Response:

As suggested by the reviewer, the word “Unexpectedly” has been replaced by the word “Furthermore” in the revised manuscript.

6. STIM analogy isn't really appropriate: STIM is transmembrane with calcium sensing on one side of the membrane and interaction with Orai on the other side (also, Orai is in the PM). Also, the oligomerization happens the opposite way.

Response:

We thank the reviewer for kind suggestion. STIM part in the discussion has been deleted in the revised manuscript.

7. Including LETM1 and mRyR1 in a larger model of mito calcium uptake seems too speculative at this point, and takes the spotlight away from their structural findings and how these relate to the existing literature (which is insufficiently discussed),and also how to reconcile some of the proposed changes in the association of MICU1 monomers with the hydrophobic stretch in the N terminus.

Response:

We agree with the reviewer that the proposed model is too speculative and has been deleted in the revised manuscript.

8. in the introduction the selection of many references seems to be random. For example for the role of calcium signal in mitochondrial apoptosis the first comprehensive reviews were:

Regulation of cell death: the calcium-apoptosis link. Orrenius S, Zhivotovsky B, Nicotera P. Nat Rev Mol Cell Biol. 2003 Jul;4(7):552-65. Control of apoptosis by IP(3) and ryanodine receptor driven calcium signals. Hajnoczky G, Csordas G, Madesh M, Pacher P. Cell Calcium. 2000 Nov-Dec;28(5-6):349-63.

Response:

We thank the review for good suggestion. We have replaced the reference accordingly in the revised manuscript.

9. Fig2A,B,E,F: expts are not well enough described. If this is not a standard technique, then it might be useful to better describe the expt. What are the red lines?

Response:

Fig. 2A, B, E and F were generated from multi-angle light scattering analysis. Multi-angle light scattering is a standard technique used to determine the absolute molar masses of macromolecules in solution and has been widely used. The red lines indicate the calculated MALS-determined molecular weights (i.e., the right vertical axis) over the prominent peaks (i.e., the horizontal axis). We have included this information in the revised figure legends.

10. p11: " The titration profile showed ... (Figure S5), which are not able to estimate the binding affinity of each site." The meaning of the underlined part is not clear.

Response:

We have rephrased the sentence as follows: “The titration profile indicated that multiple binding sites for Ca^{2+} exist in the MICU1-xtal or the MICU1-xtal-deltaC proteins (Figure S6B), and thus, the binding affinity of each site could not be resolved.” in the revised manuscript.

11. define 'resting state' (p12 - end of results, 1st para of Disc).

Response:

The phrase “resting state” at the end of the Results section has been rephrased to “our results indicated that MICU1 is less likely to bind Ca^{2+} in the resting cell.” in the revised manuscript.

The phrase “resting state” in the first paragraph of the Discussion section has been rephrased to “MICU1 constitutively suppressed mitochondrial calcium uptake in the cytosolic Ca^{2+} range of the resting cell” in the revised manuscript.

2nd Editorial Decision 29 December 2013

Thank you very much for your revisions.

These were assessed by one of the original referees, eventually supporting publication. However and as indicated in the attached remarks, this is conditioned on a few but crucial further clarifications/being explicit about current limitations and minor technical amendments. I strongly encourage you to consider and integrate these appropriately into an ultimate version of your study.

Before formal acceptance, please also notice that The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels/blots. As this entails presentation of un-cropped/unprocessed scans, I would be grateful for a PDF-file with this information.

Further, we would also be interested in an informative, 2-4 bullet point synopsis that summarises the major advance provided by your study. We would further be delighted for a graphical abstract in the format of 211x157 pixel for highlighting the study at our brand-new homepage (http://emboj.embopress.org).

Please allow me to congratulate you to your study. I look forward to receiving revised files, relevant source data, synopsis and graphical abstract soon.The editorial office will be in touch with necessary paperwork related to official acceptance upon their receipt though most likely NOT before the 7th of January 2014.

REFEREE REPORT:

The revision addressed many of the concerns but a few points remain to be clarified:

[Ca2+]m traces need to be calibrated in terms of uM calcium concentration.

Intro: "MICU1 contains only one membrane-spanning domain..." statement refers to a prediction made in the 2010 Perocchi paper. However, experimental follow-ups by the Mootha group found MICU1 to be a peripheral and not integral membrane protein (Csordas 2013, Sancak 2013).

The fact that only the 97-476 segment of MICU1 produced diffraction quality crystals is a limitation on the study and on its interpretation with regard to molecular topology. The sequence alignments show a conserved N-terminal segment (aa ~33-60), the role of which cannot be revealed in the structure of the full-length/native molecule. This should be pointed out somewhere; it will not take from the value of the presented data.

It is still not explained why the presence of the C-helix is not a problem when obtaining Ca2+-free MICU1 crystals and why in the presence of Ca2+ it causes protein aggregation; please, explain.

Pg. 8: "MICU1 was depleted by the transfection of HeLa cells with siRNA for 48 h and was then rescued by FL-MICU1or the mutants using a mitochondria-targeted aequorin (mt-AEQ)."
This doesn't seem to make sense; weren't these HeLa cells stably expressing mtAeq?

Pg 13: "Furthermore, in the presence of Ca2+, we observed that FL-MICU1, but not DeltaC-MICU1, associated with MCU (Figure S8)." The same was observed in the absence of Ca2+ as well... Interesting and should be discussed why the IP looked weaker in the presence of Ca2+ with FL-MICU1: is it only incidental or the association is weaker (or different stoichiometry?).

The Authors might want to mention in the text the recently described MICU2/3 and EMRE protein components of the uniporter.

2nd Revision - authors' response 31 December 2013

Referee comments:

The revision addressed many of the concerns but a few points remain to be clarified:

1. [Ca2+]m traces need to be calibrated in terms of uM calcium concentration.

Response:

To covert the luminescence value into actual calcium concentration, we have to repeat the siRNA knock out and rescue experiment, and meantime calibrate the standard curve of luminescence value versus calcium concentration. This will take quite a long time to finish this experiment.

In our current manuscript, the luminescence counts are enough to compare the functional difference of wild type and mutants. In our opinion, the additional calibration of uM calcium concentration is not absolutely required. Furthermore, for the purpose of studying the calcium variation in mitochondria, traces of the luminescence counts have been frequently used in many publications (Sancak et al., Science, 2013; Huang et al, Nature communications, 2013; Mallilankaraman et al., Nature Cell Biology, 2012; Perocchi et al., Nature, 2010).

2. Intro: "MICU1 contains only one membrane-spanning domain..."statement refers to a prediction made in the 2010 Perocchi paper. However, experimental follow-ups by the Mootha group found MICU1 to be a peripheral and not integral membrane protein (Csordas 2013, Sancak 2013).

The fact that only the 97-476 segment of MICU1 produced diffraction quality crystals is a limitation on the study and on its interpretation with regard to molecular topology. The sequence alignments show a conserved N-terminal segment (aa ~33-60), the role of which cannot be revealed in the structure of the full-length/native molecule. This should be pointed out somewhere; it will not take from the value of the presented data.

Response:

As suggested by the reviewer, we rephrased the sentence as follows: “MICU1 is a ~54 kDa protein with an amino-terminus mitochondrial targeting sequence, a predicted transmembrane helix (aa ~33-52) and a cytosolic C terminus (aa ~53-476) containing two classical EF-hand Ca^{2+}-binding domains (Aichberger et al, 2005; Linding et al, 2003; Perocchi et al, 2010). It localizes to the mitochondrial inner membrane and does not appear to participate in channel pore formation (Csordas et al, 2013; Sancak et al, 2013).” in the revised manuscript.

3. It is still not explained why the presence of the C-helix is not a problem when obtaining Ca2+-free MICU1 crystals and why in the presence of Ca2+ it causes protein aggregation; please, explain.

Response:

As shown in Figure S1, there are multiple hydrophobic residues in C-helix, indicating that the C-helix alone is easy to form oligomers in the solution. This hypothesis is supported by the fact that six C-helices are packed as a special helix bundle in our Ca^{2+}-free MICU1 structure. Upon Ca^{2+} loading, MICU1 molecule adopts large conformational changes, leading to the breakdown of the C-helix helix bundle because of steric hindrance. Therefore, C-helix mediated Ca^{2+}-bound MICU1 will form a mixture of oligomers (dimer, tetramer or other forms).

4. Pg. 8: "MICU1 was depleted by the transfection of HeLa cells with siRNA for 48 h and was then rescued by FL-MICU1or the mutants using a mitochondria-targeted aequorin (mt-AEQ)."This doesn't seem to make sense; weren't these HeLa cells stably expressing mtAeq?

Response:

No. We did not use HeLa cells stably expressing mtAeq. In our experiment, HeLa cells were transiently transfected with mtAeq. Detailed procedures are shown as follows: "For short-term silencing, HeLa cells were transfected with the siRNA version of an identical sh1 hairpin that was used in previous reports (Csordas et al, 2013; Perocchi et al, 2010) for 48 h using Oligofectamine (Invitrogen). The FL-MICU1 wild type and mutants were then co-transfected with a mitochondria-targeted aequorin for an additional 24 h using Fugene 6 Transfection Reagent (Promega)."

We rephrased the sentence to "MICU1 was depleted by the transfection of HeLa cells with siRNA for 48 h and was then rescued by the co-transfection of FL-MICU1 or the mutants with a mitochondria-targeted aequorin (mt-AEQ)." in the revised manuscript.

5. Pg 13: "Furthermore, in the presence of Ca2+, we observed that FL-MICU1, but not DeltaC-MICU1, associated with MCU (Figure S8)."The same was observed in the absence of Ca2+ as well... Interesting and should be discussed why the IP looked weaker in the presence of Ca2+ with FL-MICU1: is it only incidental or the association is weaker (or different stoichiometry?).

Response:

Our data showed that Ca^{2+}-bound MICU1 associated with MCU weaker than Ca^{2+}-free MICU1 did. This is consistent with recently published results (Figure 2E of Hoffman et al., Cell Reports, 2013). We currently do not have the explanation, which may require further studies to be addressed.

6. The Authors might want to mention in the text the recently described MICU2/3 and EMRE protein components of the uniporter.

Response:

We thank the reviewer for nice suggestion. We have included this information in the Introduction section of the revised manuscript.