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The mitochondrial calcium uniporter is a multimer that can include a dominant-negative pore-forming subunit

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Editor: Bernd Pulverer

1st Editorial Decision 11 February 2013

Thank you for submitting your manuscript 'The mitochondrial calcium uniporter is a multimer including a dominant-negative pore-forming subunit' for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below. I apologize for the slow turnaround in this case, due to the holiday period.

All three referees highlight the significant potential interest of this dataset. However, they all raise significant experimental and textural issues which preclude publication of the current manuscript. I list some of the key points below, but a number of additional controls and more minor issues would also have to be addressed.
ref1: > Check if the inner membrane potential is unaffected by changes in MCUb levels.
> Add MCUb with MCU in the planar lipid bilayers to show a fall in unitary conductance.
> Does Popen change or is the conduction pathway simply blocked?
> Biochemical data to address the stoichiometry of MCU: MCUb (see also ref 3)

ref 2: > The physiological relevance remains weak: only one cell type is studied and it is not stated what the relative expression level of MCU and MCUb are in HeLa cells.
> There is no experimental validation of the tetrameric structure; add titration of relative expression levels of the two isoforms and mitochondrial Ca2+ uptake rates in permeabilized cells.
> The FRET data does not prove MCUb oligomerization.
> Support or tone down suggestion that MCUb acts as a dominant negative
> Does MCUb affect permeation properties or gating properties?

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Ref 3:
> Document whether the observed mitochondrial calcium changes (Fig7, Supplementary Fig1) are not due to an altered deltapsi or changes in cytoplasmic calcium in the MCUb silenced and overexpressing cells.
> In Fig 6 clarify whether the presence of MCUb affected the channel activity induced by MCU addition
> Like ref 1: fuse into the bilayer MCU and MCUb premixed at different concentration ratios to test the idea that dependent on the stochiometry MCUb and MCU can form oligomers showing a range of different channel activities.

Ref 2 and 3 agree that the modelling should be toned down and supported by more developed biochemical data.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally suggest three months as standard revision time. Please contact us at this time if you require additional time for the revision. 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.

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

REFEREE COMMENTS

Referee #1

This is a very interesting and important contribution that provides fresh new insight into regulation of the ubiquitous mitochondrial uniporter channel. The authors show that a novel protein, called MCUb, oligomerises with the MCU pore-forming channel and, remarkably, acts as a dominant negative pore-forming subunit. Whilst MCUb has no channel activity on its own, it reduces mitochondrial calcium uptake. The experiments are well done and the findings will be of considerable interest.

I have the following comments.

1. Although the authors show interaction between MCU and MCUb, and that MCUb has no channel activity alone, it seems a bit of a jump to equate the fall in mitochondrial calcium uptake following overexpression of MCUb to direct inhibition of MCU channel activity. Have the authors checked that the inner membrane potential is unaffected by changes in MCUb levels? Also, is it possible to add MCUb with MCU in the planar lipid bilayers to indeed show a fall in unitary conductance? Does Popen change or is the conduction pathway simply blocked?

2. Molecular weights should be indicated on the gels. How do the levels of expression of MCU-GFP and MCUb-cherry compare? For the FRET studies, they should ideally be similar.

3. It is interesting that the ratio of MCU:MCUb varies between 3:1 (heart and lung) to > 40:1 (skeletal muscle). It would be important to know the stoichiometry of MCU: MCUb in each

tetramer. Although beyond the scope of this study, concatamers of MCU and MCUb could be informative.

Referee #2

In this manuscript, the co-discovers of the ion pore of the mitochondrial Ca2+ uniporter MCU explore its oligomeric status. They originally identified MCU as having two predicted transmembrane helices with a short linker between them with acidic residues that when mutated disrupted channel function. Here they have speculated, as they should, that MCU must be part of a complex to provide sufficient protein to constitute a permeation pathway. They approach this notion from several perspectives, some convincing and some not. First, they use co-immunoprecipitation and FRET. They conclude that >1 MCU can exist in a complex. They also describe an MCU homolog, MCUb, with ~50% identity. They show that it too can interact with MCU.

So far so good.

1. They demonstrate most convincingly in co-expression studies, using intact and permeabilized HeLa cells, that MCUb exerts an apparent dominant-negative effect. Furthermore, they demonstrate that mutation in MCU of a couple of residues near the so-called DIME motif to residues present in MCUb diminishes mitochondrial Ca2+ uptake. These data are convincing and interesting because they provide some information about a possible physiological function and the oligomerization of MCU. The authors show that the relative expressions of MCU and MCUb are different among mouse tissues, suggesting that the magnitude of MCU-mediated Ca2+ flux into mitochondria could be regulated in part in different tissues by distinct hetero-oligomeric subunit stoichiometries. This seems like a reasonable conclusion. One wishes therefore that this would have been explored further. I feel that a major weakness in the studies described here is that only one cell type is studied and that this physiological implication was not explored. Furthermore, it is not stated what the relative expression level of MCU and MCUb are in HeLa cells, which renders a full appreciation of the significance of the knockdown and over-expression studies less than optimal. For example, do HeLa cells even express MCUb? This is important because an implicit message from the title and abstract of the manuscript is that the uniporter always includes MCUb as a dominant negative subunit and that this is a physiological control mechanism in cells. Had this actually been demonstrated, the study would have had very high impact. It is disappointing that these issues were not pursued.

2. The authors also use molecular modeling to predict oligomerization of MCU, starting simply from the amino acid sequence. They conclude many things from modeling and molecular dynamics simulations, including the tetramerization of the MCU channel, the structure of the permeation pathway and the roles of specific side-chain interactions in Ca2+ permeation. Here, I think the authors are on quite shaky ground. Although they state in the Discussion that other stoichiometries are in principle thermodynamically possible, they claim that the tetramer "was by far the most likely quaternary structure", but they provide no evidence that other structures might not have been as good. Why not a hexamer? Has such predictive modeling ever been performed successfully for any ion channel (validated by cross-checking with the real crystal structure)? Importantly, there is no experimental validation of the predicted structure. Lacking therefore is any method for generating confidence that the modeling and simulations can provide real insights. Accordingly, this raises questions regarding the conclusions about Ca2+ permeation, pore structure and oligomerization. Regarding the oligomeric state. Success for other ion channels of unknown structures has been derived from good biochemistry, single molecule imaging and electrophysiology, but other than co-IP studies (which in fact do not provide information about direct protein interactions between MCU (MICU1 and MCU can co-IP each other but I don't imagine that the authors think MICU1 is part of the permeation pathway)), there is little biochemistry. The appearance of a high molecular weight band in SDS-PAGE is somewhat surprising...were these reducing or non-reducing conditions? But in any case, a band on a gel may contain other proteins that contribute to the apparent molecular weight. The electrophysiology is obviously challenging, but I wonder whether titration of relative expression levels of the two isoforms and the use of mitochondrial Ca2+ uptake rates in permeabilized cells could be used instead. Unfortunately, at the end of the day the evidence for tetramerization is not well supported.

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Minor

1. In presentation of the structure on p5, the authors refer to a selectivity filter, but one is not explicitly shown. The details of Ca2+ coordination are not shown. Despite assertions, a Ca2+ ion coordinated by the acidic residues is not shown. The figure legend indicates much smaller dimensions for the pore than is stated in the text (please specify whether the numbers used in the text refer to radius or diameter).
2. The authors see FRET between expressed MCUb and conclude that MCUb can self-oligomerize, but this result does not allow this conclusion. Because HeLa cells express MCU, it could be that MCUb oligomerizes with MCU, enabling FRET between MCUb. In order to reach the conclusion that MCUb can oligomerize, the authors would have to work in a system with MCU protein strongly reduced.
3. On p9 the authors conclude that lack of MCUb channel activity in bilayers indicates that it could be a dominant negative. I don't think any conclusion about how MCUb behaves in a hetero-oligomeric complex can be made from studies of homo-oligomeric MCUb. There are examples of silent ion channels that contribute to novel ion channel properties when hetero-oligomerized with another channel isoform subunit, i.e. do not act as dominant-negatives.
4. To ensure in the imaging experiments that the effect of MCUb to inhibit mitochondrial Ca2+ uptake was due to a dominant negative effect, the authors should perform western blotting to ensure that MCU protein levels were not reduced with MCUb expression.
5. The authors conclude in the Discussion that MCUb gets inserted into the MCU channel complex and alters the permeation properties, but this has not been shown. It could, for example, affect the gating properties and still account for the observed mitochondrial calcium phenotypes.
6. In Figure 2, it would be very useful in panels B, C, D to show where the lipid bilayer would be. In B, what are the solid balls? And what is the significance of the N-C terminus color gradient bar at the bottom? In A, it would be nice to have some residues labeled. The loops from the top view appear to be splayed outward, suggesting that the acidic residues could not be in close enough proximity to coordinate calcium. Some labeling would help to make things clearer to the reader. In C, it since the electrostatics of the outside of the channel are shown, it is equally informative to know about the electrostatics on the opposite, inner face of the pore. I wonder about this figure because it suggests that the external surface of the channel, which must face the lipid environment, is not hydrophobic...isn't this a concern? In D, what is the significance of showing the computed structure before and after insertion into membrane?

Referee #3

This MS describes the discovery of a dominant negative isoform (MCUb) of a recently described pore forming component of the mitochondrial calcium uniporter (MCU). The identification of MCUb is of great significance for understanding the molecular mechanisms of mitochondrial calcium uptake, which controls a range of cell functions. Most of the results show striking effects of MCUb and are clearly explained. Some specific concerns are listed below. The experimental results on MCUb are interrupted by description of an in silico model. This seems to break the flow and perhaps would be better positioned in the end of the manuscript.
Specific concerns:
-To support the specificity of the effects of MCUb targeting on the uniporter, it is necessary to document whether the observed mitochondrial calcium changes (Fig7, Supplementary Fig1) are not due to an altered deltapsi or changes in cytoplasmic calcium in the MCUb silenced and overexpressing cells.
-FRET studies demonstrate interaction between fluorescent protein tagged MCU molecules. Could you please provide evidence that the mCherry-or GFP-tagged MCU is properly folded and functional?
-No legend is provided for Fig3D.
-Pg 19: Electrophysiology methods: please, specify how much protein was added (to have a feeling what the ratio of first (MCUb) and second (MCU) channel introductions was). Fig6: Please clarify whether the presence of MCUb affected the channel activity induced by MCU addition?
-The bilayer studies support that MCUb does not form a channel. It would be nice to fuse into the bilayer MCU and MCUb premixed at different concentration ratios to test the idea that dependent on

the stochiometry MCUb and MCU can form oligomers showing a range of different channel activities. However, this experiment might be technically demanding.
-Does the modeling predict a stable closed conformation of the MCU channel? Recombinant MCU seems to produce opening events by itself when fused into lipid bilayer.
-Fig 1A: numbering the aa positions would make the figure easier to understand and to follow while reading the text.

1st Revision - authors' response 10 May 2013

(please see next page)

Response to referees

Referee #1 :

This is a very interesting and important contribution that provides fresh new insight into regulation of the ubiquitous mitochondrial uniporter channel. The authors show that a novel protein, called MCUb, oligomerises with the MCU pore-forming channel and, remarkably, acts as a dominant negative pore-forming subunit. Whilst MCUb has no channel activity on its own, it reduces mitochondrial calcium uptake. The experiments are well done and the findings will be of considerable interest.

I have the following comments.

1. Although the authors show interaction between MCU and MCUb, and that MCUb has no channel activity alone, it seems a bit of a jump to equate the fall in mitochondrial calcium uptake following overexpression of MCUb to direct inhibition of MCU channel activity. Have the authors checked that the inner membrane potential is unaffected by changes in MCUb levels?

The reviewer is right: mitochondrial membrane potential, representing the main driving force for organelle Ca^{2+} uptake, is a critical parameter. We thus checked whether regulation of MCUb levels affects $\Delta\psi_m$ with the potentiometric fluorescent probe TMRM: our results (new supplementary figure 9B) show that neither overexpression nor silencing of MCUb induce significant changes in mitochondrial membrane potential, thus strengthening the hypothesis that MCUb acts through an inhibition of MCU channel activity.

Also, is it possible to add MCUb with MCU in the planar lipid bilayers to indeed show a fall in unitary conductance? Does Popen change or is the conduction pathway simply blocked?

We followed the suggestion of the Reviewer and added MCUb to planar lipid bilayers containing active MCU, and observed no changes in either the conductance or the open probability and kinetic behaviour of the channel. These experiments are now shown in Figure 6A. Given that at least part

of the MCU homo-oligomer becomes assembled during *in vitro* expression (as indicated by the native PAGE of figure 4C), this result could reflect that subunit switch is very unlikely to occur in the bilayer once the channel is already assembled. Therefore, to obtain further evidence that indeed MCUb acts as dominant-negative subunit, we co-expressed MCU and MCUb *in vitro* using ratios of plasmid DNA yielding different protein expression levels (new supplementary figure S4A). In the 3:1 MCU:MCUb plasmid ratio, based on the protein expression levels (which are comparable for the two isoforms) we statistically expect mostly MCU/MCUb hetero-oligomers, and a minor fraction of MCU and MCUb homo-oligomers. We then incorporated MCU-only, MCUb-only or the co-expressed MCU and MCUb proteins into liposomes (new supplementary figure 4B) and studied their activities in electrophysiological experiments under the same ionic conditions, i.e. in 100 mM calcium gluconate solution. When the two proteins were co-expressed, the number of experiments in which we observed MCU activity in calcium gluconate (presumably due to the presence of "residual" homomeric MCU) became drastically reduced compared to MCU-only, new figure 6B and 6C). This fact and the observation that we could not reveal any difference with the few detected channel activities indicates that MCUb subunits, when forming heteromers with MCU, abolish the calcium permeation across the heteromeric channel. Thus, we conclude that MCUb indeed functions as dominant-negative subunit.

2. Molecular weights should be indicated on the gels. How do the levels of expression of MCU-GFP and MCUb-cherry compare? For the FRET studies, they should ideally be similar.

As suggested by the reviewer, we indicated the molecular weights on the gels.

We are aware that the FRET technique for studying protein-protein interactions can be tricky, and attention must be paid to experimental conditions, such as expression levels of the fluorophores. Unfortunately, MCUb chimeras express invariably at lower levels when compared to MCU chimeras: this is true with all the used tags (6xHis, GFP and mCherry) and can be appreciated by both Western blot (e.g. see whole lysate in figure 4A) and by fluorescence microscopy, where GFP- and mCherry-MCUb chimeras appear constitutively less "bright" than MCU chimeras. We don't have any validated explanation for this at the moment. However, we are confident that this difference in expression levels does not interfere with the results, and thus interpretation. Indeed,

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with the experimental settings we used, no positive correlation between expression levels and FRET efficiency was observed. In addition, MCU (higher expression) or MCUb (lower expression) can be equivalently used as donor or acceptor, leading to no differences in FRET efficiency (see figure 4B). Moreover, the compartmentalization of the protein in mitochondria enables the possibility of selectively bleaching and evaluating FRET in a specific cell region, thus having an internal control in each cell (i.e. the non-bleached region). We think that all these data, together with the confirmation through classical co-immunoprecipitation, consistently demonstrate the MCU/MCUb interaction.

3. It is interesting that the ratio of MCU:MCUb varies between 3:1 (heart and lung) to > 40:1 (skeletal muscle). It would be important to know the stoichiometry of MCU: MCUb in each tetramer. Although beyond the scope of this study, concatamers of MCU and MCUb could be informative.

Although the exact stoichiometry of the oligomer would be greatly informative, it is quite difficult to rigorously assess it. The *in situ* stoichiometry of the native channel is way complicated by the fact that the channel is part of a supercomplex containing channel-forming subunits and other regulatory proteins (MICU1, MICU2, MICU3, MCUR1). In this situation, the suggestion of making concatamers is particularly valuable, and we tried to pursue it. We generated MCU+MCU and MCU+MCUb constructs, although this approach is most likely complicated by the fact that MCU is post-translationally processed. The N-terminal mitochondrial targeting sequence is presumably proteolitically cleaved after mitochondrial import (this is consistent with the fact that MCU shows a specific band at 35KDa, while its predicted molecular weight is around 40KDa). Western blot analysis of MCU+MCU and MCU+MCUb revealed a number of specific bands, some corresponding to the monomer, others to the dimer. This migration pattern most likely reflects partial processing of the chimeras, thus complicating the approach and hampering conclusions we could obtain from it. We will dedicate further effort to the construction of effective (and uncleaved) concatamers, but for the time being we take the reviewer's advice to consider this extension beyond the scope of this study.

Referee #2 :

In this manuscript, the co-discovers of the ion pore of the mitochondrial Ca2+ uniporter MCU explore its oligomeric status. They originally identified MCU as having two predicted transmembrane helices with a short linker between them with acidic residues that when mutated disrupted channel function. Here they have speculated, as they should, that MCU must be part of a complex to provide sufficient protein to constitute a permeation pathway. They approach this notion from several perspectives, some convincing and some not. First, they use co-immunoprecipitation and FRET. They conclude that >1 MCU can exist in a complex. They also describe an MCU homolog, MCUb, with ~50% identity. They show that it too can interact with MCU.

So far so good.

1. They demonstrate most convincingly in co-expression studies, using intact and permeabilized HeLa cells, that MCUb exerts an apparent dominant-negative effect. Furthermore, they demonstrate that mutation in MCU of a couple of residues near the so-called DIME motif to residues present in MCUb diminishes mitochondrial Ca2+ uptake. These data are convincing and interesting because they provide some information about a possible physiological function and the oligomerization of MCU. The authors show that the relative expressions of MCU and MCUb are different among mouse tissues, suggesting that the magnitude of MCU-mediated Ca2+ flux into mitochondria could be regulated in part in different tissues by distinct hetero-oligomeric subunit stoichiometries. This seems like a reasonable conclusion. One wishes therefore that this would have been explored further. I feel that a major weakness in the studies described here is that only one cell type is studied and that this physiological implication was not explored. Furthermore, it is not stated what the relative expression level of MCU and MCUb are in HeLa cells, which renders a full appreciation of the significance of the knockdown and over-expression studies less than optimal. For example, do HeLa cells even express MCUb? This is important because an implicit message from the title and abstract of the manuscript is that the uniporter always includes MCUb as a dominant negative subunit and that this is a physiological control mechanism in cells. Had this

actually been demonstrated, the study would have had very high impact. It is disappointing that these issues were not pursued.

We agree that the discovery of this new MCU isoform opens a number of fundamental questions, in particular regarding the physiological significance of this complex in different tissues. We indeed proposed that MCU/MCUb expression ratio could be one of the determinants of the specific regulation of mitochondrial calcium uptake in different cell types. We are also aware that the situation is more complex than this, given the growing number of identified MCU regulators (MICU1, MICU2, MICU3 and MCUR1). Thus, the clarification of the physiological regulation of mitochondrial Ca^{2+} uptake will require a detailed analysis of the relative expression (and likely post-translational modification) of all regulatory elements. With this general caveat in mind, we addressed the point raised by the reviewer. We verified the relative MCU/MCUb expression in HeLa cells by real time PCR (figure 1D) and by Western blot (new supplementary figure 5). Indeed, we showed that MCUb is present (and of course this notion is confirmed by the fact that MCUb silencing has a functional consequence, i.e. it strongly increases mitochondrial calcium uptake) and shows an approximately 1:3 ratio compared to MCU, at least at the mRNA level. Moreover, in order to confirm the validity of our model, we extended mitochondrial Ca^{2+} measurements to both primary cells and cell lines, such as HEK 293 and neonatal mouse cardiac fibroblasts (new supplementary figure 7). The effect of the MCUb overexpression is essentially the same. In addition, we now mention in the Discussion section that recent recordings of the mitochondrial uniporter from mitoplasts isolated from different tissues by the Kirichok group (Fieni et al, 2012) highlighted an approximately 20-fold higher current density in mitochondria of skeletal muscle with respect to heart. Considering that we find an approx. 4-fold higher expression of MCU and a 3-fold lower expression of MCUb in skeletal muscle with respect to heart, the Kirichok data are compatible with our suggestion that MCU/MCUb ratios might indeed contribute to different tissue-dependent expression of active channels.

2. The authors also use molecular modeling to predict oligomerization of MCU, starting simply from the amino acid sequence. They conclude many things from modeling and molecular dynamics simulations, including the tetramerization of the MCU channel, the structure of the permeation

pathway and the roles of specific side-chain interactions in Ca2+ permeation. Here, I think the authors are on quite shaky ground. Although they state in the Discussion that other stoichiometries are in principle thermodynamically possible, they claim that the tetramer "was by far the most likely quaternary structure", but they provide no evidence that other structures might not have been as good. Why not a hexamer? Has such predictive modeling ever been performed successfully for any ion channel (validated by cross-checking with the real crystal structure)? Importantly, there is no experimental validation of the predicted structure. Lacking therefore is any method for generating confidence that the modeling and simulations can provide real insights. Accordingly, this raises questions regarding the conclusions about Ca2+ permeation, pore structure and oligomerization.

We are aware of the limitations of the computational approach to structure prediction, and fully taking over the reviewer's point we rewrote this section of the manuscript explaining the limits of the predictive model, remembering however that there are many successful and useful examples in the literature (see in this respect: "Kaihsu Tai, Philip Fowler, Younes Mokrab, Phillip Stansfeld, Mark S.P. Sansom, Chapter 12 Molecular Modeling and Simulation Studies of Ion Channel Structures, Dynamics and Mechanisms, In: Bhanu P. Jena, Editor(s), Methods in Cell Biology, Academic Press, 2008, Volume 90, Pages 233-265). We would like to underline that the built model rationalizes all mutation effects of MCU that have been reported in literature so far. As to the stoichiometry, although other numbers of monomers are in principle thermodynamically possible, the native PAGE we performed on the MCU expressed in vitro detected (with both MCU specific and anti-tag antibodies) both monomers and a higher molecular weight band compatible with a tetramer (new figure 4C). Indeed, we did not demonstrate the oligomerization of MCU by simple molecular modeling, but we first showed it through experimental approaches (i.e. co-IP and FRET). Altogether, we avoid drawing any conclusion based only on the computational model, but we rather used it to propose a mechanistic explanation of the MCUb dominant negative effect. Overall, as it guided the design of mutational analyses that were confirmed by direct experimental validation, we find the preliminary structural analysis useful. With all necessary cautions (that are underlined in the revised text), we believe that this model represents, so far, the best structural prediction that anticipates MCU crystal structure and can enable researchers to formulate theories and questions that are less obvious at the level of simple sequence alignment analysis.

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Regarding the oligomeric state. Success for other ion channels of unknown structures has been derived from good biochemistry, single molecule imaging and electrophysiology, but other than co-IP studies (which in fact do not provide information about direct protein interactions between MCU (MICU1 and MCU can co-IP each other but I don't imagine that the authors think MICU1 is part of the permeation pathway)), there is little biochemistry. The appearance of a high molecular weight band in SDS-PAGE is somewhat surprising...were these reducing or non-reducing conditions? But in any case, a band on a gel may contain other proteins that contribute to the apparent molecular weight. The electrophysiology is obviously challenging, but I wonder whether titration of relative expression levels of the two isoforms and the use of mitochondrial Ca2+ uptake rates in permeabilized cells could be used instead. Unfortunately, at the end of the day the evidence for tetramerization is not well supported.

Regarding the oligomeric state, we tried to be as prudent as possible in defining a precise stoichiometry: we define the functional channel as an "oligomer", most probably a tetramer based on the evidence summarized below. As already discussed in responding to reviewer #1, understanding the exact stoichiometry of the MCU complex is far from being trivial. The stoichiometry of the native channel is way complicated by the fact that the channel is part of a greater complex including pore subunits and other regulatory proteins (MICU1, MICU2, MICU3, MCUR1). Indeed, native gel electrophoreisis of purified liver mitochondria shows a MCU-reactive band at about 700 KDa (this has been shown by Dr. Mootha's group, and it is reproducible in our hands) that would correspond (quite unlikely) to a 20-mer. Rather, that band is reactive at least with MICU1, revealing that the native complex contains both channel forming and regulatory subunits. Considering this, it is pretty difficult to rigorously assess channel stoichiometry *in situ*. The most informative piece of evidence is in our view the analysis of the recombinant channel produced *in vitro*, where the contamination of other proteins is greatly reduced. We loaded this preparation on a native polyacrylamide gel without denaturing the samples and the calculated size of the detected band corresponds to the size of an MCU tetramer (new figure 4C). As to the electrophysiology, we followed the advice of the Reviewer and performed bilayer experiments whit protein preparations used obtained by *in vitro* co-expression of MCU and MCUb using ratios of plasmid DNA yielding different protein expression levels (new supplementary figure 4A). With a 3:1 ratio of MCU:MCUb, we expect the presence of mainly MCU/MCUb heterooligomers, and to smaller

extent MCU-only and MCUb-only homo-oligomers. We then incorporated MCU-only, MCUb-only or the co-expressed proteins into liposomes (new supplementary figure 4B) and studied their activities in electrophysiological experiments using the same ionic conditions, i.e. in 100mM calcium gluconate solution (new figure 6B). When the two proteins were co-expressed, the number of experiments in which we observed MCU activity in calcium (presumably due to the presence of "residual" homomeric MCU) became drastically reduced compared to MCU alone, (new figure 6C), but the conductance was unchanged. This, as for MCUb, also MCU/MCUb oligomers show no calcium permeability, or in principle such a dramatic (e.g. 20 fold) drop in the low (around 7 pS) conductance of MCU to bring it below the detectable limit (and physiological significance). We thus checked whether the lack of MCUb channel activity in calcium gluconate was due to misfolding of MCUb. To this end, we recorded the activity of the same protein preparation in a sodium-gluconate low divalent solution (10 pM calculated [Ca^{2+}]), given the known characteristic of calcium channels (Hess and Tsien, 1984, Lepple-Wienhues et al, 1996, Talavera and Nilius, 2006) and of MCU (Kirichok et al, 2004) to allow the passage of Na^{+} upon removal of Ca^{2+} (new supplementary figure 3). Indeed, a Na+ current was observed indicating that MCUb gives rise to a functional channel, albeit incapable of significant Ca^{2+} permeation. Altogether these new data indicate that MCUb subunits, by forming hetero-oligomers with MCU, abolish Ca^{2+}, but not Na^{+}, permeation across the channel. Thus, MCUb indeed functions as a dominant-negative channel subunit.

Minor

1. In presentation of the structure on p5, the authors refer to a selectivity filter, but one is not explicitly shown. The details of Ca2+ coordination are not shown. Despite assertions, a Ca2+ ion coordinated by the acidic residues is not shown. The figure legend indicates much smaller dimensions for the pore than is stated in the text (please specify whether the numbers used in the text refer to radius or diameter).

Figure 2A has been replaced with a clearer representation of the top view of predicted structure of the MCU tetramer in which it is possible to appreciate the four identical subunits delimiting the

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channel pore in which a calcium ion can be coordinated by acidic residues that also play a critical role in providing the electrostatic driving force to locate cations at the entrance of the channel pore. We apologize for the inconsistencies in pore dimension that has been now corrected.

2. The authors see FRET between expressed MCUb and conclude that MCUb can self-oligomerize, but this result does not allow this conclusion. Because HeLa cells express MCU, it could be that MCUb oligomerizes with MCU, enabling FRET between MCUb. In order to reach the conclusion that MCUb can oligomerize, the authors would have to work in a system with MCU protein strongly reduced.

We followed the very appropriate suggestion of the reviewer, also considering that endogenous MCU could at least in principal interfere with all FRET pairs we used. We have tested a siRNA targeting the 3'-UTR of the endogenous MCU mRNA, in order not to interfere with the overexpression of GFP- and mCherry-MCU chimeras. After validation, we repeated FRET experiments in control and MCU-downregulated cells. However, no significant changes in FRET efficiency values could be appreciated, even with the MCUb-MCUb pair (new supplementary figure 2). We think that, after CMV-driven overexpression, the vast majority of the newly synthetized channels are composed by the exogenous chimera, and thus the contribution of the endogenous protein is minimal.

3. On p9 the authors conclude that lack of MCUb channel activity in bilayers indicates that it could be a dominant negative. I don't think any conclusion about how MCUb behaves in a hetero-oligomeric complex can be made from studies of homo-oligomeric MCUb. There are examples of silent ion channels that contribute to novel ion channel properties when hetero-oligomerized with another channel isoform subunit, i.e. do not act as dominant-negatives.

To address this point, we set up the experiments with the co-expression system as described above. The use of co-expression with defined ratios of DNA/RNA e.g. in oocytes is a well-established method to assess the behaviour of a novel subunit. For example with AtKC1 (Jeanguenin et al, 2011, Plant Journal) a huge reduction of the current when co-expressing AtKC1-DN with inward

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shaker potassium channels is interpreted as hetero-tetramerization between the two types of subunits and a dominant negative action of AtKC1-DN. In our experiments we have a similar situation. The fact that the mutation of Glu residue in the pore alters calcium permeation in MCUb and MCU/MCUb hetero-oligomers is in agreement with findings describing single glutamate residue mutations in the L-type calcium channel leading to large reduction/loss of barium permeability (e.g. Yang et al, 1993; Tang et al, 1993).

4. To ensure in the imaging experiments that the effect of MCUb to inhibit mitochondrial Ca2+ uptake was due to a dominant negative effect, the authors should perform western blotting to ensure that MCU protein levels were not reduced with MCUb expression.

We performed Western blot analysis after modulation of MCUb expression, and we now show no difference in endogenous MCU levels (new supplementary figure 8).

5. The authors conclude in the Discussion that MCUb gets inserted into the MCU channel complex and alters the permeation properties, but this has not been shown. It could, for example, affect the gating properties and still account for the observed mitochondrial calcium phenotypes.

As discussed above, the new electrophysiological analyses of MCUb oligomers, and of MCU/MCUb mixed hetero-oligomers, has demonstrated the role of MCUb on MCU permeation properties, highlighting the most likely mechanism of the dominant-negative effect on ion channel activity. These new data are included and discussed in the revised manuscript. In addition, the mutagenesis experiment of Fig. 7D, that show the dominant-negative effect of mutation of residues proposed to be critical for cation permeation, also lends support to this hypothesis. Even with this new indication, however, the reviewer is in principle right, and thus now the possibility that MCUb could affect MCU gating properties is mentioned in the Discussion.

6. In Figure 2, it would be very useful in panels B, C, D to show where the lipid bilayer would be. In B, what are the solid balls? And what is the significance of the N-C terminus color gradient bar at the bottom? In A, it would be nice to have some residues labeled. The loops from the top view

appear to be splayed outward, suggesting that the acidic residues could not be in close enough proximity to coordinate calcium. Some labeling would help to make things clearer to the reader. In C, it since the electrostatics of the outside of the channel are shown, it is equally informative to know about the electrostatics on the opposite, inner face of the pore. I wonder about this figure because it suggests that the external surface of the channel, which must face the lipid environment, is not hydrophobic...isn't this a concern? In D, what is the significance of showing the computed structure before and after insertion into membrane?

We appreciate all the comments that helped us clarify the content of the manuscript. We updated figure 2B legend and shown lipid bilayer boundaries in panels B, C and D of figure 2. The color gradient bar located at the bottom of figure 2B has the purpose to help readers to locate N and C-terminal portion of the MCU monomers. Figure 2A has been replaced with a clearer representation of the predicted structure of the extracellular loop region of the MCU tetramer. Originally, figure 2C has been captured in order to better highlight the region of the protein complex (loop L1) that includes negatively charged amino acids that are known to play an essential role in MCU-mediated mitochondrial Ca2+ uptake. We now corrected the distorted perspective. Since we used Molecular Dynamics to refine the model structure and to investigate its stability in a lipid bilayer environment, we think that figure 2D gives readers useful insight on the behaviour of the MCU model in a lipid bilayer environment, with particular regards to the pore domain topology.

Referee #3 :

This MS describes the discovery of a dominant negative isoform (MCUb) of a recently described pore forming component of the mitochondrial calcium uniporter (MCU). The identification of MCUb is of great significance for understanding the molecular mechanisms of mitochondrial calcium uptake, which controls a range of cell functions. Most of the results show striking effects of MCUb and are clearly explained. Some specific concerns are listed below. The experimental results on MCUb are interrupted by description of an in silico model. This seems to break the flow and perhaps would be better positioned in the end of the manuscript.

Specific concerns:

-To support the specificity of the effects of MCUb targeting on the uniporter, it is necessary to document whether the observed mitochondrial calcium changes (Fig7, Supplementary Fig1) are not due to an altered deltapsi or changes in cytoplasmic calcium in the MCUb silenced and overexpressing cells.

We checked the mitochondrial membrane potential by using the potentiometric fluorescent probe TMRM. We couldn't observe any significant difference in fluorescence value neither when MCUb was overexpressed nor when it was silenced. This data are now shown in supplementary figure 9B. Moreover, we measured cytosolic Ca^{2+} transients in the same conditions: the data are now included in supplementary figure 9A and demonstrate that changes in mitochondrial Ca^{2+} uptake are not due to an altered cytosolic Ca^{2+} handling.

-FRET studies demonstrate interaction between fluorescent protein tagged MCU molecules. Could you please provide evidence that the mCherry-or GFP-tagged MCU is properly folded and functional?

Also this important control has been carried out. We measured mitochondrial Ca^{2+} transient and compared the effect of the different MCU and MCUb-chimeras. In all cases, mCherry- and GFP-MCU chimeras proved to be functional, exerting the same effect as wild-type protein (although a modest reduction in the effect was observed with GFP- or mCherry-MCU constructs, compared to the wild type protein or small tag (6xHis or Flag) chimeras (new supplementary figure 1A and 1B).

-No legend is provided for Fig3D.

We apologize for the missing legend, which is now in place.

-Pg 19: Electrophysiology methods: please, specify how much protein was added (to have a feeling what the ratio of first (MCUb) and second (MCU) channel introductions was). Fig6: Please clarify whether the presence of MCUb affected the channel activity induced by MCU addition?

Experimental details are now provided in the Materials and Methods section and in the figure legend.

-The bilayer studies support that MCUb does not form a channel. It would be nice to fuse into the bilayer MCU and MCUb premixed at different concentration ratios to test the idea that dependent on the stochiometry MCUb and MCU can form oligomers showing a range of different channel activities. However, this experiment might be technically demanding.

We followed the advice of the Reviewer and performed electrophysiological experiments where the preparations used obtained by *in vitro* co-expression of MCU and MCUb using ratios of plasmid DNA yielding different protein expression levels (new supplementary figure 4A). We chose this method and not pre-mixing of the already expressed proteins because the native gel suggests that at least a part of the protein is assembled already in the *in vitro* expression chamber. With a 3:1 plasmid ratio of MCU:MCUb, based on the protein expression levels, we statistically expect mostly MCU:MCUb hetero-oligomers, and a minor fraction of MCU and MCUb homo-oligomers. We then incorporated MCU-only, MCUb-only or the co-expressed proteins into liposomes (new figure 4B) and studied their activities in electrophysiological experiments using the same ionic conditions, i.e. in calcium gluconate solution. When the two proteins were co-expressed, the number of experiments in which we observed MCU activity in calcium gluconate (presumably due to the presence of “residual” homomeric MCU) became drastically reduced with respect to MCU alone, new figure 6C). This, as for MCUb, also MCU/MCUb oligomers show no calcium permeability, or a dramatic (e.g. 20 fold) drop in the low (around 7 pS) conductance of MCU that brings it below the detectable limit (and physiological significance). Altogether our new data indicate that MCUb subunits, when forming heteromers with MCU, abolish the Ca^{2+} permeation across the heteromeric channel. Thus, MCUb indeed functions as dominant-negative subunit.

-Does the modeling predict a stable closed conformation of the MCU channel? Recombinant MCU seems to produce opening events by itself when fused into lipid bilayer.

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Though Molecular Dynamics simulation clearly shows a tight packing of the C-terminal modeled portions of the membrane embedded MCU (figure 2D) we would like to underline that the built model is limited to the pore domain of this newly identified channel protein. The lack of structural information of other protein domains, that are part of the MCU channel protein, is a clear limitation to further explore the conformational landscape of such a complex machinery using computational techniques. We are planning to extend our predictive model and to use enhanced sampling techniques, such as accelerated MD, to give more insights on MCU biophysics.

-Fig 1A: numbering the aa positions would make the figure easier to understand and to follow while reading the text.

We now included the numbering of the indicated aminoacids

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2nd Editorial Decision 9 June 2013

I am very pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal pending the minor textural changes suggested by the referees. We particularly note the more accurate wording of the title suggested by referee 3 and the more thorough description of the tetrameric complex (ref 3).
Please review the abstract of the article carefully to ensure that it contains in concise form all the essential keywords that will allow maximal discoverability via PubMed text searches. Please change the sentence 'We have recently identified the channel responsible for Ruthenium Red-sensitive mitochondrial calcium uptake (MCU).' to 'The MCU channel is responsible for Ruthenium Red-sensitive mitochondrial calcium uptake') or similar - in addition, it would be useful to add the physiological function in this sentence to 'set the scene' for the general reader.

REFEREE COMMENTS

Referee #1

The authors have carried out new experiments that strengthen the conclusions drawn and have revised the paper appropriately. They have satisfactorily addressed my comments. This is a very nice study and should be published.

Referee #2

I am satisfied with the incorporation of new experimental data, revision of the manuscript, and responses to my previous comments. I feel that the manuscript is considerably improved snd should now be published.
I note a few small items that could be fixed:
The citation for MCUR1 and MICU2 is backwards in the text;
on p21, "infection" is used rather than transfection.
The methods refer to viruses that are not used, are they?

Referee #3

Most of my comments have been adequately addressed in the revision. The study is expected to make an important contribution to both the mitochondrial and calcium signaling research fields. However, a couple of minor points remain to be corrected.

-Right now the title suggests that MCU complex inevitably includes MCUb, but this is unlikely the case for example in skeletal muscle. Instead it could be said 'The MCU is a multimer that can include a dominant ..."

the [Ca2+]mt changes modulate avariety of key cellular processes, such as ... and the release of pro-apoptotic factors (Pinton et al., 2001; Scorrano et al., 2003).
This has been shown earlier by Szalai et al (EMBO J 1999, 18(22):6349-61)

"Overall, the biochemical and FRET data indicate that MCU monomers oligomerize both in vitro and in vivo in higher-order complexes, and thus support the tetrameric model of the computational analysis."
It is still not very well explained how the Authors arrived at a tetrameric structure, and how that's at the exclusion of other possibilities. One can't simply say that the 170kDa band on the native gel supports a tetrameric structure, because theoretically the band can be MCU + different proteins. It would be practical to better explain how the Authors confidently arrived at the model in Fig2 since the paper may be read by many non-experts in MD.

"Moreover, this complexity is currently growing: novel MCU interactors, MCUR1, (Plovanich et al., 2013) and MICU3, have been recently identified (Mallilankaraman et al., 2012a),..."

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Citation of the MICU2/3 and MCUR1 papers is inaccurate (the order of the citation in the text, and putting only MICU3 in the text).

Additional correspondence - author 13 June 2013

Thank you for your mail informing us that the paper by Raffaello et al. (ms. EMBOJ-2012-84304R) is accepted for publication in the EMBO Journal. Enclosed please find the final version of the manuscript that incorporates the modifications recommended by the reviewer, as detailed in the rebuttal letter. Thank you again for the constructive review process that allowed improving the paper.

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