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The Mitochondrial Contact Site complex, a determinant of mitochondrial architecture

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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.)

1st Editorial Decision 26 July 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see all three referees attest considerable interest in this dataset. However, both referees 1 and 2 raise largely overlapping experimental requests. We would like to invite you to submit a revision incorporating the experimental and minor textural issues raised.

Summary of major points that should be addressed in revision:

ref 1: describe SILAC better; ref 2: add Mass spec. source data
ref 3: quantify Immuno-gold EM data.
ref 1: PK digestion data for GFP-Tim23
ref 2: confirm IM/OM holocomplex.
ref 1 & 2: resolve inconsistencies in the co-IP data, in particular regarding the Fcj1-Mcs interaction.
ref 1 & 2: add more comprehensive Mcs interaction with Fcj1, Tob55 and Ugo1, as well as between Fcj1 and Tob55.
ref 1 & 2: does deletion of Mcs1 affect assembly of beta-barrel proteins in OM and Ugo1 function (mitochondrial fusion/fission)?
ref 1: confirm effect of Ugo1 & Tob55 deletion of CS formation.
ref 1: independent evidence for two distinct MICOS complexes.

Given the referees' positive recommendations, we are confident that we can proceed with a suitably

revised manuscript without delay.

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

Thank you for the opportunity to consider this exciting dataset for publication. In our view these revisions will further enhance the impact and scope of the work and we look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

Mitochondria are comprised of two membranes, the outer membrane (OM) and inner membrane (IM). The IM consists of the inner boundary membrane (IBM), which forms an envelope with the OM, and cristae, which protrude from the IBM into the interior. The cristae and IMB are connected by the crista junction (CJ) and the OM and IBM are close to each other at contact sites (CS). Although the CS structure has been observed by electron microscopy (EM) for a long time and is likely involved in protein import, protein components responsible for the CS formation has not been identified. In the present study, Hanner et al. utilized SILAC to search for yeast mitochondrial proteins enriched specifically in the CS fraction, which is also enriched with GFP-Tim23 spanning both OM and IM. They thus identified five new Mcs (mitochondrial contact site) proteins, whose initial characterization is reported here. Mcs proteins form the MICOS (mitochondrial contact site) complex in the inner membrane together with Fcj1 in the IM and Ugo1 and the TOB complex in the OM. Successful identification of long sought-after CS forming proteins should give significant impact on the field of mitochondrial biogenesis and mitochondrial structure-function relationship.

(1) In general, more description of the SILAC approach taken in this study should be included for the readers to follow the story easily. For example in Fig. 1C, how were SILAC ratios obtained? Cells with different expression of Tim23 and GFP-Tim23 were grown in media with different stable isotopes?

(2) The topology of GFP-Tim23 in mitochondria should be assessed by PK digestion and generation of the Tim23 fragment be shown (this was not shown previously).

(2) In Fig. 2A, IP of Msc10-6xHis pulled down Mcs29 and Mcs12, but IP of Mcs29-6xHis or Mcs12-6xHis did not pull down Msc10. Why? IP of any Mcs-6xHis pulled down Fcj1, but IP of Fcj1-6xHis did not pull down Mcs proteins, which is not consistent with the idea of Fcj1 interacting with or constituting the MICOS complex. Why?

(3) In Fig. 2C, why does Mcs10 so easily dissociate from the MICOS complex while it appears to interact with the other Mcs proteins stably as seen by co-IP after similar solubilization procedures? Majority of Mcs10 is found in fractions of about 200 kDa (?), not 100kDa as described in the text (page 10). Are MICOS I and II distinct complexes? The Mcs proteins may be just distributed

broadly, but not distributed in separate sets of fractions. Independent evidence (BN-PAGE etc.) is required to claim the presence of two distinct MICOS complexes. In relation to this, the description of "In any case, the molecular sizing profile nicely corresponds to the ability of Mcs10 to pull down virtually all of Mcs27 and part of Mcs12, but the reverse does not hold true (c. f. Fig. 2A, right panel). Mcs29 was recovered only to a lower extent in MICOS I and II, however more was present in the low molecular mass range." (pages 10-11) is confusing. Clearly more assessment is required to reconcile the mutually inconsistent results of co-IP experiments and gel-filtration analyses.

(4) Fig. 3 requires quantitative analyses. In Fig. 3B, since the number of cristae decreases by shifting from lactose to glycerol, expression levels of the Mcs proteins may change upon shifting among lactose, glycerol and glucose?

(5) In Fig. 4A, fraction numbers should be indicated (Fig. 1B, too). Distributions of Ugo1 and Fzo1 should be included in the analyses in Fig. 1B or Fig. 4A.

(6) Fig. 4E, how about the interactions of the other Mcs proteins (Mcs10, Mcs19 and Mcs29) with Fcj1 and Tob55? If the Mcs proteins binds to Tom55, does deletion of Mcs1 proteins affect the functions of the TOB complex i.e. assembly of beta-barrel proteins in the OM?

(7) Fig. 4G, how about the interactions of Ugo1 with the Mcs proteins? Does deletion of Mcs1 proteins affect the functions of the Ugo1 i.e. mitochondrial fusion?

(8) In Fig. 5, the authors analyzed the effects of deletion of the Mcs proteins on CJ formation. However, the authors should confirm that deletion of the Mcs proteins (and Tob55 and Ugo1) indeed leads to the loss of CS by EM analysis under hyperosmotic conditions as in Fig. 1A. Crista rims and crista branches should be defined and shown with arrows etc. in EM pictures. How about the roles of Tob55 and Ugo1 in the formation of CS or CJ?

(9) In the Discussion, do the authors want to claim that CS and CJ make a super complex or the same complex? At the moment, such a claim is not fully supported by experimental evidence.

Referee #2

Mitochondrial inner membranes are highly organized and display a unique morphology that contributes to the intrinsic functional compartmentation. Very little is known how this organization is achieved and which proteins or lipids contribute to the process of membrane shaping. In this manuscript Harner et al have taken an elegant biochemical approach to identify proteins that are involved in contact site formation. They purify mitochondrial contact sites biochemically and analyze their composition by mass spectrometry. These analyses lead to the identification of a protein complex termed MICOS of which the previously identified Fcj1 (Mitofilin in human) is a central constituent. Functional analyses indicate that MICOS is essential for christae morphology. Moreover, the authors show by pull-down analyses that subunits of MICOS are in physical contact with outer membrane proteins. While Fcj1 is found associated with Ugo1 other subunits such as Mcs19 and 27 bind to Tob55.

The manuscript defines for the first time a protein complex that is involved in christae organization. It is well written, reports on a novel and innovative approach, and the experiments are of high technical quality. Thus, the manuscript represents in principle an important contribution to molecular cell biology that will be of high interest to the broad readership of the EMBO Journal. However, I think that additional experiments are required to clarify some points. I am sure that the authors will be able to answer these points and that this important contribution to mitochondrial membrane biology will have significant impact on the field.

Specific points

1. The fractionation of contact sites on flotation gradients is the basis for this analysis. However the co-migration of proteins is not fully convincing as an argument for CS purification. The authors should address if a TOM-TIM23-spanning precursor is enriched in these fractions and can be

isolated. Other data to support this point, such as coisolation of IM-protein/OM-protein complexes from gradient fractions , would be appreciated.

2. The outer membrane spanning N-terminus of Tim23 is controversially discussed in the field. The authors should clarify why the GFP-Tim23 accumulates CS while Tim23, which also spans the outer membrane, does not to the same extend. Also, why are respiratory chain complexes enriched in the CS in the presence of GFP-Tim23. Why are Tim23 and Fcj1 not visible in the total of Figure 1 B? The Figure gives the impression that protein levels are very different in the two gradients (e.g Tom70, Tom40, Tom22...). The authors need to provide a steady state western blot analysis of mitochondrial proteins, especially those analyzed in the gradient, comparing wild type to GFP-Tim23 mitochondria to exclude that the Tim23 fusion has an effect on mitochondrial function and protein composition.

3. The authors need to provide the full data set of the mass spec analyses and statistics as supplemental material. This is essential to allow the reader to assess the quality of the analyses.

4. It is surprising that the Fcj1-6His is unable to co-isolate other Mcs protein (Figure 2A) but brings down Ugo1. Something seems to be wrong with the construct. If possible the authors should try using their Fcj antibody for CoIP.

5. The topology analysis in Figure 2 E is very important. The authors should provide molecular weight markers.

6. Figure 4E and G show that different Mcs proteins bring down different outer membrane proteins. The authors need to show if Fcj1 coisolates Tob55, as has been reported for Mitofilin previously (Xie et al., 2007), and if Mcs16 and 27 interact with Ugo1. It is not clear from the data that these components interact exclusively with other outer membrane proteins as shown in the model.

7. A major shortcoming of the manuscript is the missing functional analysis of mcs mutants. The authors need to address with import analyses if any of the import pathways is affected in mcs mutants. Does a lack of Tob55 binding affect beta barrel assembly or import? Is matrix transport affected by reorganization of christae? Is mitochondrial dynamics - fusion fission affected in mcs mutant mitochondria as suggested by the Ugo1 interaction.

8. The statistical analysis of EM micrographs suggests a reduction of CJ. However, the statement "complete loss" can not be based on sections of cells. I suggest to soften the statement or alternatively provide tomographies to support this statement.

Referee #3

The ultrastructure of the double-membraned mitochondrion is unique and how the inner membrane folds to incorporate regular shaped cristae is not well known. In this important study, Harner and colleagues identify and characterise a mitochondrial contact site complex in yeast important for the formation of crista junctions. The authors use an ingenious approach involving their Tim23 construct, which spans both outer and inner membranes, to enrich for proteins found at the contact sites between outer and inner membranes. SILAC studies identified candidate proteins and traditional biochemical techniques were employed to identify components of the complex and the fact that they interact with one another. One of the proteins is Fcj1, a protein previously implicated in cristae formation but other subunits are new. Further evidence supporting the role of these proteins at contact sites came from immunogold EM and a role in cristae junction formation supported following analysis of mitochondrial ultrastructure from knockout cell lines. The importance of the proteins in mitochondrial function was further demonstrated by phenotype studies that indicate impaired respiration and mtDNA inheritance following loss of MICOS components. The identification of these subunits serves as a great step to uncovering how these proteins might be involved in defining how a specific mitochondrial subcompartment is formed and it will also enable additional work to be performed in higher eukaryotes.

The manuscript is well written, the experimental approach is sound and the interpretation is well

balanced. I have a number of minor points that the authors could address:

1. The immunogold EM data shown in Fig. 3 looks quite nice although the membranes and cristae are not always clear (I do acknowledge that this is the case when performing this technique and the use of cryo-sectioning was performed so the membrane contrast is not great). For the sake of completeness, the authors could perform a quantification of spots to demonstrate an enrichment of the MICOS proteins at contact sites.

2. It would be useful to explain why fewer cristae might be seen in mitochondria from cells grown in glycerol compared to lactate or at least give a reference.

1st Revision - authors' response 16 September 2011

(Point-by-point response to Decision Letter):

We thank the reviewers for their careful and constructive evaluation of our manuscript. In the following we address point-by-point the various issues raised by them.

First of all we have dealt with the MAJOR POINTS to be addressed, as requested by the Chief Editor. We also reply to the comments of the individual reviewers.

(1) Ref 1: Describe SILAC better; ref 2: add Mass spec. source data
We included a more detailed description of the SILAC procedure in the text (Results section)in addition to the information provided in Material and methods section and added relevant mass spectrometry source data as DATASET Supplementary File F1(Excel file).

(2) Ref 3: Quantify immuno-gold EM data.

We have quantified the imuno-gold EM data and have added the requested information in Figure 3D and Supplementary Table S1.

(3) Ref 1: PK digestion data for GFP-Tim23

We have addressed this point in a different context in a publication which appeared in the meantime in EMBO J. For details please see: Harner, M., Neupert, W. and Deponte, M. (2011). Lateral release of proteins from the TOM complex into the outer membrane of mitochondria. EMBO J 30(16):3232-41. We have now included this reference in the text.

(4) Ref 2: Confirm IM/OM holocomplex.

We include additional experimental material confirming the existence of the IM/OM holocomplex (see Figure S4A). In this experiment, mitochondria from a strain deficient in Fcj1 were subfractionated and the vesicles separated by gradient centrifugation as described in Figure 1. The distribution of Mcs proteins and of Ugo1 are altered in comparison to wild type as to be expected when contacts between IBM and outer membrane are lost. Moreover, this experiment confirms the enrichment of contact sites in the sucrose gradient fractions of intermediate density.

(5) Ref 1 & 2: Resolve inconsistencies in the co-IP data, in particular regarding the Fcj1-Mcs interaction.

We have included new data in Figure 2A (right panel). Problem was that the presence of a C-terminal his-tag on Fcj1 leads to a reduced stability of the interactions of Fcj1 with the other Mcs proteins. We found out in the meantime that it is the C-terminal conserved domain of Fcj1 which is required for interaction of Fcj1 with itself and with other Mcs proteins. As shown now, all of the Mcs proteins interact with Fcj1 and Fcj1 with all Mcs proteins.

(6) Ref. 1 & 2: Add more comprehensive Mcs interaction with Fcj1, Tob55 and Ugo1, as well as between Fcj1 and Tob55.

We show in Fig. 4E interaction between Tob55 and Mcs19 as well as Mcs27, but observed no interaction of Tob55 with the other Mcs proteins. Further, we observed interaction of Ugo 1 with Fcj1 (Figure 4G), but not with one of the Mcs proteins.
In the meantime, in cooperation with Andreas Reichert, we have completed a detailed analysis of the various domains of Fcj1. We found that the full length Fcj1 and its isolated C-terminal domain, can bind Tob55 *in vitro*. Since this is a rather comprehensive and extensive study which is beyond the scope of this article and which could not be included due to length problems and since it is together with different coworkers, we have not included these data in the present manuscript.

(7) Ref 1 & 2: Does deletion of Mcs1 affect assembly of beta-barrel proteins in OM and Ugo1 function (mitochondrial fusion/fission)?

Deletion of Fcj1 which leads to severe reduction of steady state levels of the other Mcs proteins (Fig 2D) does not affect the assembly of beta-barrel proteins. These results are included in the detailed study of the effects of deletion of Fcj1 or domains of Fcj1 mentioned in connection with point 6. In none of the Mcs protein deletion strains the Ugo1 function is affected.

(8) Ref 1: Confirm effect of Ugo1 & Tob55 deletion of CS formation.

Deletion of Ugo1 leads to fragmentation of mitochondria, their lumen appears empty, but most interestingly very very rarely cristae-like structures are observed. This emphasizes the importance of Ugo1 in crista formation. We have added these results in Figure S4B. This raises the interesting question as to how an outer membrane protein affects formation of crista junctions and how this leads to loss of cristae, an intriguing question which remains to be addressed by future experiments. The effect of Tob55 deletion can unfortunately not be tested since Tob55 is an essential protein in yeast (responsible for the import of essential beta-barrel proteins). Down regulation of Tob55 would be possible, but we have great doubts that reliable conclusions would be possible on the basis of this procedure.

(9) Ref 1: Independent evidence for two distinct MICOS complexes.

We verified the presence of two distinct MICOS complexes by BN-PAGE. We added the requested information in Figure S2A.

(Point-by-point response to referees):

Referee #1

Mitochondria are comprised of two membranes, the outer membrane (OM) and inner membrane (IM). The IM consists of the inner boundary membrane (IBM), which forms an envelope with the OM, and cristae, which protrude from the IBM into the interior. The cristae and IMB are connected by the crista junction (CJ) and the OM and IBM are close to each other at contact sites (CS). Although the CS structure has been observed by electron microscopy (EM) for a long time and is likely involved in protein import, protein components responsible for the CS formation has not been identified. In the present study, Hanner et al. utilized SILAC to search for yeast mitochondrial proteins enriched specifically in the CS fraction, which is also enriched with GFP-Tim23 spanning both OM and IM. They thus identified five new Mcs (mitochondrial contact site) proteins, whose initial characterization is reported here. Mcs proteins form the MICOS (mitochondrial contact site) complex in the inner membr ane together with Fcj1 in the IM and Ugo1 and the TOB complex in the OM. Successful identification of long sought-after CS forming proteins should give significant impact on the field of mitochondrial biogenesis and mitochondrial structure-function relationship.

(1) In general, more description of the SILAC approach taken in this study should be included for the readers to follow the story easily.

For example in Fig. 1C, how were SILAC ratios obtained? Cells with different expression of Tim23 and GFP-Tim23 were grown in media with different stable isotopes?

Done. See point 1 of MAJOR POINTS.

(2) The topology of GFP-Tim23 in mitochondria should be assessed by PK digestion and generation of the Tim23 fragment be shown (this was not shown previously).

Done. See point 3 of MAJOR POINTS.

(2) In Fig. 2A, IP of Msc10-6xHis pulled down Mcs29 and Mcs12, but IP of Mcs29-6xHis or Mcs12-6xHis did not pull down Msc10. Why? IP of any Mcs-6xHis pulled down Fcj1, but IP of Fcj1-6xHis did not pull down Mcs proteins, which is not consistent with the idea of Fcj1 interacting with or constituting the MICOS complex. Why?

See point 5 of MAJOR POINTS. This is indeed a point which has puzzled us. There are several reasons why this is so. One might be that Mcs10 is expressed at a much higher level as compared to other Mcs proteins. Another one is that the presence of the C-terminal His-tag on Mcs29 and Mcs12 might destabilize the complex under the experimental conditions . It should, however, be noted that the functions of these proteins are not affected since the his-tagged variants are complementing the respective deletions.

(3) In Fig. 2C, why does Mcs10 so easily dissociate from the MICOS complex while it appears to interact with the other Mcs proteins stably as seen by co-IP after similar solubilization procedures?

Majority of Mcs10 is found in fractions of about 200 kDa (?), not 100kDa as described in the text (page 10). Are MICOS I and II distinct complexes? The Mcs proteins may be just distributed broadly, but not distributed in separate sets of fractions. Independent evidence (BN-PAGE etc.) is required to claim the presence of two distinct MICOS complexes. In relation to this, the description of "In any case, the molecular sizing profile nicely corresponds to the ability of Mcs10 to pull down virtually all of Mcs27 and part of Mcs12, but the reverse does not hold true (c. f. Fig. 2A, right panel). Mcs29 was recovered only to a lower extent in MICOS I and II, however more was present in the low molecular mass range." (pages10-11) is confusing. Clearly more assessment is required to reconcile the mutually inconsistent results of co-IP experiments and gel-filtration analyses.

See point 9 of MAJOR POINTS. Apparently the complex formed by the Mcs proteins is not a very stable entity, such as e.g. the cytochrome oxidase complex. This may lead to different results with the different techniques since the composition of the media used (salt concentrations, buffers etc.) is different in the gel filtration experiments as compared to co-isolation experiments , likewise the time periods in which the experiments are performed are different. We followed the suggestion of the referee to apply BN-PAGE which yielded very nice results and will be very useful for further analysis of the complex. Indeed the behavior of Mcs10 is highly intriguing, in particular since it has an exciting phenotype and is conserved throughout evolution.

(4) Fig. 3 requires quantitative analyses. In Fig. 3B, since the number of cristae decreases by shifting from lactose to glycerol, expression levels of the Mcs proteins may change upon shifting among lactose, glycerol and glucose?

See point 2 of MAJOR POINTS. The expression levels between growth on fermentable medium (glucose) and non-fermentable medium (lactate) are indeed different, e.g. the levels Fcj1 and Mcs19 are strongly reduced on fermentable carbon source.

(5) In Fig. 4A, fraction numbers should be indicated (Fig. 1B, too).
Distributions of Ugo1 and Fzo1 should be included in the analyses in Fig. 1B or Fig. 4A.

Done, with the exception of Fzo1 for which we do not have an antibody.

(6) Fig. 4E, how about the interactions of the other Mcs proteins (Mcs10, Mcs19 and Mcs29) with Fcj1 and Tob55? If the Mcs proteins binds to Tom55, does deletion of Mcs1 proteins affect the functions of the TOB complex i.e. assembly of beta-barrel proteins in the OM?

See points 6 & 7 of MAJOR POINTS.

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See point 7 of MAJOR POINTS. We followed the suggestion to include an improved explanation of the EM pictures. We greatly appreciate the suggestion of studying the morphology of the mutant strains by EM, we feel this is very important for obtaining further insights, but to do this properly will need many months of experimentation and quantitative and statistical evaluation of EM micrographs. Interestingly, as we show now in Figure 4SB Dugo1 cells lack not only crista junctions, like Dfcj1, but also internal membranes. It will be exciting to find out what is going on here biochemically.

(9) In the Discussion, do the authors want to claim that CS and CJ make a super complex or the same complex? At the moment, such a claim is not fully supported by experimental evidence.

Our results lead us to believe that the contact sites suggested by this study are part of a structure which serves to anchor the inner membrane to the outer membrane to allow the formation of crista junctions. Deletion of Fcj1 leads to loss of crista junctions and Fcj1 makes contact to the outer membrane via the TOB complex and Ugo1. As we discuss, there may exist further kind of contacts which may be structured in an entirely different manner and have different functions.

Referee #2

Mitochondrial inner membranes are highly organized and display a unique morphology that contributes to the intrinsic functional compartmentation. Very little is known how this organization is achieved and which proteins or lipids contribute to the process of membrane shaping. In this manuscript Harner et al have taken an elegant biochemical approach to identify proteins that are involved in contact site formation. They purify mitochondrial contact sites biochemically and analyze their composition by mass spectrometry.
These analyses lead to the identification of a protein complex termed MICOS of which the previously identified Fcj1 (Mitofilin in human) is a central constituent. Functional analyses indicate that MICOS is essential for christae morphology. Moreover, the authors show by pull-down analyses that subunits of MICOS are in physical contact with outer membrane proteins. While Fcj1 is found associated with Ugo1 other subunits such as Mcs19 and 27 bind to Tob55.

The manuscript defines for the first time a protein complex that is involved in christae organization. It is well written, reports on a novel and innovative approach, and the experiments are of high technical quality. Thus, the manuscript represents in principle an important contribution to molecular cell biology that will be of high interest to the broad readership of the EMBO Journal. However, I think that additional experiments are required to clarify some points. I am sure that the authors will be able to answer these points and that this important contribution to mitochondrial membrane biology will have significant impact on the field.

Specific points

1. The fractionation of contact sites on flotation gradients is the basis for this analysis. However the co-migration of proteins is not fully convincing as an argument for CS purification. The authors should address if a TOM-TIM23-spanning precursor is enriched in these fractions and can be isolated. Other data to support this point, such as coisolation of IM-protein/OM-protein complexes from gradient fractions , would be appreciated.

See point 4 of MAJOR POINTS. Unfortunately accumulation of a TOM-TIM spanning precursor as a marker is not practicable since the amounts of aminopterin needed for such an experiment would be extremely expensive. Question is also whether such structures would be stable during the long time period required for the analysis. This approach would also have the disadvantage that it would lead to the accumulation of a number of unrelated proteins, in particular TOM complex proteins and assiociated proteins into the contact sites. This is not the case with our approach of using GFP-Tim23.
The gradient resolves three fractions which contain outer membranes but not inner membranes and fractions which contain inner membranes but not outer membranes, and fractions which contain both types of membranes. Therefore, it did not seem useful to us to analyze these fractions for physical interaction of components derived from both membranes and to find interactions of outer and inner membranes since one could as well analyze whole mitochondria. The beneficial effect of analyzing the fraction which contains both types of membranes would be a certain increase in the yield of interacting proteins. Since the Mcs proteins are present virtually only in the intermediate fractions, one would not expect a significant increase of the ratio of Mcs to interacting outer membrane proteins.

2. The outer membrane spanning N-terminus of Tim23 is controversially discussed in the field. The authors should clarify why the GFP-Tim23 accumulates CS while Tim23, which also spans the outer membrane, does not to the same extend. Also, why are respiratory chain complexes enriched in the CS in the presence of GFP-Tim23. Why are Tim23 and Fcj1 not visible in the total of Figure 1 B? The Figure gives the impression that protein levels are very different in the two gradients (e.g Tom70, Tom40, Tom22...). The authors need to provide a steady state western blot analysis of mitochondrial proteins, especially those analyzed in the gradient, comparing wild type to GFP-Tim23 mitochondria to exclude that the Tim23 fusion has an effect on mitochondrial function and protein composition.

As to the topology of GFP-Tim23 we refer to the additional information which we have published in the meantime (EMBO J, 2011) which largely explains why others claim to have not been able to reproduce our results. The insertion of the N-terminus of Tim23 into the outer membrane is depending on the preprotein translocation load and is reversible, the spanning topology of GFP-Tim23 is permanent.

The fractions of intermediate density are not purified contact sites. There is always a certain and different amount of inner membranes adhering to contact sites, since these vesicles are generated by sonication.

Regarding protein levels on the gels, the bands are the result of immunoblotting and exposure on X-ray films. Only the relative distribution of one protein can be estimated. Since in the GFP-Tim23 mitochondria all Tim23 and thereby all Tim17 is shifted to contact sites, a larger amount of inner membrane proteins may be shifted to the middle of the gradient.

The question regarding possible alterations in the expression of inner membrane proteins due to the expression of GFP-Tim23 was studied in the above mentioned EMBO J paper. There was no obvious alteration, there was no effect on mitochondrial function and no effect on mitochondrial protein import. Interestingly, both wild type Tim23 and GFP-Tim23 when overexpressed, lead to the increased synthesis of Tim17, obviously a specific regulatory effect exerted by both types of proteins. Furthermore, GFP-Tim23 quantitatively forms a complex with Tim17, like wild type Tim23.

The referee is right in criticizing the controls for total in Fig. 1B two bottom panels. There are sometimes blotting artifacts. Of course in case of experiments which were used for quantitative evaluation we repeated these several times and discarded blots with blotting artifacts. It should be

realized that an experiment of this type including evaluation takes some two to three weeks of hard work.

3. The authors need to provide the full data set of the mass spec analyses and statistics as supplemental material. This is essential to allow the reader to assess the quality of the analyses.

See point 1 in MAJOR POINTS. In view of the size of the data set and the amount of results not included and not relevant in the present context, we are planning to publish these in a separate paper. The precision and reproducibility of this analysis is extremely high as for instance shown in Fig. 4 and Fig. S1. In addition, proteins shown previously to be located in a particular submitochondrial compartment by biochemical methods displayed identical abundance profiles in our experiment based on SILAC quantitation. This is true for components of the outer membrane, of the inner membrane and the matrix and for components of the endoplasmic reticulum. To demonstrate this, we have included now in Supplementary Table Y marker profile of a number of proteins in addition to what is shown in Supplementary figure S1 and by the data provided throughout the manuscript.

4. It is surprising that the Fcj1-6His is unable to co-isolate other Mcs protein (Figure 2A) but brings down Ugo1. Something seems to be wrong with the construct. If possible the authors should try using their Fcj antibody for CoIP.

We thank the referee for his advice. We have followed this and constructed an N-terminally his-tagged version of Fcj1. As now shown in Fig. 2A this has improved the analysis considerably. See also point 5 of MAJOR POINTS.

5. The topology analysis in Figure 2 E is very important. The authors should provide molecular weight markers.

We have now added the positions of the markers.

6. Figure 4E and G show that different Mcs proteins bring down different outer membrane proteins. The authors need to show if Fcj1 coisolates Tob55, as has been reported for Mitofilin previously (Xie et al., 2007), and if Mcs16 and 27 interact with Ugo1. It is not clear from the data that these components interact exclusively with other outer membrane proteins as shown in the model.

See point 6 of MAJOR POINTS. The referee is completely right in stating this and we do not suggest nor exclude that the other Mcs proteins interact with Tob55 or Ugo1 directly or indirectly.

7. A major shortcoming of the manuscript is the missing functional analysis of mcs mutants. The authors need to address with import analyses if any of the import pathways is affected in mcs mutants.
Does a lack of Tob55 binding affect beta barrel assembly or import? Is matrix transport affected by reorganization of christae? Is mitochondrial dynamics - fusion fission affected in mcs mutant mitochondria as suggested by the Ugo1 interaction.

In the first steps to functionally characterize the various Mcs proteins we have checked the effects on growth and mitochondrial ultrastructure. We entirely agree with the referee that a whole series of functional criteria must be analysed now which will include functions regarding bioenergetics (membrane potential, respiratory control, ATP production etc.), fusion and fission, transport of proteins and lipids, stability of mitochondria, formation of cristae, roles in apoptosis in metazoan cells, export of Fe-S- clusters etc. As far as we know so far, there is no effect of deletion of Mcs proteins on import of beta-barrel proteins and fusion and fission.

8. The statistical analysis of EM micrographs suggests a reduction of CJ. However, the statement "complete loss" can not be based on sections of cells. I suggest to soften the statement or alternatively provide tomographies to support this statement.

The referee is right of course. One should never say "complete". We simply were impressed that in delta Fcj1 and delta Mcs10 cells we could not find a single crista junction, like in the previous work by Rabl et al. So we write now: "virtually".

Referee #3

The ultrastructure of the double-membraned mitochondrion is unique and how the inner membrane folds to incorporate regular shaped cristae is not well known. In this important study, Harner and colleagues identify and characterise a mitochondrial contact site complex in yeast important for the formation of crista junctions. The authors use an ingenious approach involving their Tim23 construct, which spans both outer and inner membranes, to enrich for proteins found at the contact sites between outer and inner membranes. SILAC studies identified candidate proteins and traditional biochemical techniques were employed to identify components of the complex and the fact that they interact with one another. One of the proteins is Fcj1, a protein previously implicated in cristae formation but other subunits are new. Further evidence supporting the role of these proteins at contact sites came from immunogold EM and a role in cristae junction formation supported following analysis of mitochondrial ultrastructure from knockout cell lines. The importance of the proteins in mitochondrial function was further demonstrated by phenotype studies that indicate impaired respiration and mtDNA inheritance following loss of MICOS components. The identification of these subunits serves as a great step to uncovering how these proteins might be involved in defining how a specific mitochondrial subcompartment is formed and it will also enable additional work to be performed in higher eukaryotes.

The manuscript is well written, the experimental approach is sound and the interpretation is well balanced. I have a number of minor points that the authors could address:

1. The immunogold EM data shown in Fig. 3 looks quite nice although the membranes and cristae are not always clear (I do acknowledge that this is the case when performing this technique and the use of cryo-sectioning was performed so the membrane contrast is not great). For the sake of completeness, the authors could perform a quantification of spots to demonstrate an enrichment of the MICOS proteins at contact sites.

We appreciate this suggestion and have included a quantitation. See also point 2 of MAJOR POINTS.

2. It would be useful to explain why fewer cristae might be seen in mitochondria from cells grown in glycerol compared to lactate or at least give a reference.

We agree that this is a fascinating question. Why do mitochondria have crista junctions which have to be linked to the IBM? One reasonable answer is that mitochondria have to import a large number of hydrophobic membrane proteins into the cristae which apparently cannot pass through the matrix (in contrast to chloroplasts which can send membrane proteins through the stroma). Therefore, the formation of cristae might depend on intricate mechanisms and specificities to pass newly made membrane subunits of respiratory complexes from IBM through crista junctions into the crista, and also membrane lipids. Perhaps mutations in Mcs proteins lead to defects in these processes?

2nd Editorial Decision 26 September 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. As discussed by telephone today, it has now been seen again by referee1, whose comments are enclosed. As you will see, all the referee recommends publication after minor revision.

We hope to receive the final manuscript by tomorrow; you should be receiving a proof by about Thursday, assuming we can accept tomorrow. The paper should then be typeset rapidly and be published online no later than October 14th, all being well.

Please address all the minor issues raised by referee 1 in textural revision, including addition of the requested reference, in particular referring to the data in response to points 6 and 7 as 'data not shown', as also discussed by phone.

In fig 4 and possibly elsewhere panels are excised so narrowly that 'smiling' gels cause bands to run off the panel shown at the edges - we recommend showing more generous panels. Alternatively, please show uncropped images as 'source data'.

Please also submit all the relevant publication forms at this time (see below).

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

Please alert me separately upon arrival of the revision so that we can process the revision efficiently.

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

Yours sincerely,

Editor

The EMBO Journal

REFEREE COMMENTS

Referee #1

In principle, the revised manuscript has been improved significantly and, considering the given competitive circumstances, it can be published right now after very minor modifications I suggested below.

Here are my responses to the points the editor asked me for opinions:

(A) Request by Ref 2: Confirm IM/OM holocomplex.

'We include additional experimental material confirming the existence of the IM/OM holocomplex (see Figure S4A)'.

Actually, the authors could not show the two-membrane spanning intermediate enriched in the 'CS' fractions. Instead, the authors showed deletion of Fcj1, which caused loss of CS from EM analyses, resulted in different distribution of Ugo1 and Mcs proteins, which were overlapped in the presence of Fcj1.

However previously, a two membrane spanning intermediate (generated by ATP depletion) was already shown to be enriched in CS fraction by Pon et al (1989). Therefore I suggest the author to refer to this previous experiment.

(B) Request by Ref 1 & 2: Resolve inconsistencies in the co-IP data, in particular regarding the Fcj1-Mcs interaction.

'We have included new data in Figure 2A (right panel)'.

It was good that His-Fcj1 worked well. The authors had better use His-Fcj1 in Fig. 4G, but if they want to put such results in future manuscript, it could be OK. However, I still think that the authors had better at least mention the results supporting the interactions between Fcj1 and Ugo1/Tob55 referring to 'unpublished results'.

(C) Request by Ref 1: Confirm effect of Ugo1 & Tob55 deletion of CS formation.

'Deletion of Ugo1 leads to fragmentation of mitochondria, their lumen appears empty, but most interestingly very very rarely cristae-like structures are observed. This emphasizes the importance of Ugo1 in crista formation. We have added these results in Figure S4B'.

The new results showing that deletion of Ugo1 affects crista formation are highly interesting and have made the manuscript stronger.

(D) Request by Ref 1: Independent evidence for two distinct MICOS complexes.

'We verified the presence of two distinct MICOS complexes by BN-PAGE. We added the requested information in Figure S2A'.

These BN-PAGE analyses successfully showed the presence of two distinct MICOS complexes. However, asterisks and arrowheads should be explained in the legends.

(E) Request by Ref. 1 & 2: Add more comprehensive Mcs interaction with Fcj1, Tob55 and Ugo1, as well as between Fcj1 and Tob55 & (7) Does deletion of Mcs1 affect assembly of beta-barrel proteins in OM and Ugo1 function (mitochondrial fusion/fission)?.....the authors have responded that this data will be included in another study with different collaborators. If this data is essential, we can of course request that it be included here.

As I wrote above, the authors had better at least mention the results supporting the interactions between Fcj1 and Ugo1/Tob55 referring to 'unpublished results'.

2nd Revision - authors' response 29 September 2011

Comments to the points regarding the revised version.

Fulvio Reggiori has sent Figure 3, including Mcs29, in high resolution.

- We see possible running off of smiling bands only in case of ugo1 in Fig.4. We include a copy of the film showing that there is indeed a very minor signal in the bottom fraction which is not properly seen in Fig. 4.

Ad A. We tried to explain this in our previous response. In our view, such an experiment would only make sense if performed in vivo. This is not possible. The experiment by L. Pon et al. to which the referee is referring was performed with isolated mitochondria and since the conditions for generating mitochondrial vesicles were worked out for in vivo and since isolation of mitochondria may cause changes which interfere with reproducible fragmentation we have not used this approach. Bur it should be noted that the presence of the two membrane spanning Tim23 is an even more reliable as a marker. We have added a short description of the experiments by Pon et al.

The reference to the paper by Pon et al. was included in the manuscript anyway.
Ad B. We have added a sentence regarding the results demonstating interaction of Fcj1 with Tob55 (Körner, Reichert et al.)

Ad C. We appreciate the comment of the referees.

Ad. D. We apologize for have omitted this information.We added now the explanations of astrisks and arrowheads to the legend of Fig.S2:

Figure S2, related to Figure 2. Analysis of the MICOS complex. (A) Analysis by Blue native gel electrophoresis. Mitochondria isolated from wild type cells and cells lacking either Fcj1, Mcs27 or Mcs29 were lysed with digitonin, subjected to BN-PAGE followed by immunodecoration with antibodies against Fcj1, Mcs27 and Mcs29. In addition, mitochondria isolated from a strain containing a his-tagged version of Mcs10 were analyzed in the same way and then subjected to immunodecoration with antibodies against the his-tag. (B) Molecular sizing of the MICOS complex in mutants in which each one of the genes coding for the contact site proteins was deleted. The analysis was performed as described in Figure 2C. Arrow heads indicate positions of MICOS complexes I and II; Asterisks, unspecific cross reactions.
Ad E: Since there is no clear effect of Fcj1 deletion on the import of beta-barrel proteins we feel that this information is not essential. For Fcj1 interaction with Tob55 see point B.

