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Role of the import motor in insertion of transmembrane segments by the mitochondrial TIM23 complex

Dusan Popov-Celeketic, Karin Waegemann, Koyeli Mapa, Walter Neupert, Dejana Mokranjac

Corresponding author: Dejana Mokranjac, Ludwig Maximilians University

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Review timeline:

Transaction Report:

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

1st Editorial Decision   20 November 2010

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the three reports from the referees that were asked to assess it.

As the detailed reports are pasted below I would prefer not to repeat them here, but you will see that the reviewers, in principle, agree on the potential interest of the findings. However, all referees feel that additional work is needed to substantiate the conclusions drawn.

For example, referee 1 points out that in some instances, additional controls are required, including the analysis of un-arrested import. In addition, this referee also pinpoints several technical concerns that would need to be addressed. Referee 2 states that additional experiments aiming to address the role of the IMS domain of TIM14 in regulating insertion of lateral proteins should be conducted and suggests potential ways to achieve this. Please note that referee 3 also remarks on this aspect and while we would not insist on the elucidation of the full mechanistic details of how TIM14 controls release of laterally inserted proteins, we feel that the additional experiments suggested by referee 2 towards this end would strengthen the manuscript significantly. The most substantial concern of referee 3 -brought up with regard to several parts of the study- is that s/he feels that the use of non-specific cross-linking is not sufficient to substantiate the conclusions drawn and that those experiments would need to be complemented by alternative approaches.

From the analysis of these comments it becomes clear that significant revision is required before the manuscript becomes suitable for publication in EMBO reports. However, given the potential interest of your study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referee concerns must be addressed and their suggestions (as detailed above and in the referees' reports) taken on board, especially with regard to the concerns of referee 3 that alternative experimental approaches would be needed to substantiate the conclusions. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The mitochondrial TIM23 complex mediates both preprotein translocation into the matrix and lateral insertion of some preproteins into the inner membrane in an ATP-depend manner. Some precursors, however, are sorted laterally by the TIM23 complex into the inner membrane in an ATP-independent manner, raising question on the involvement of the import motor in this reaction. Here, the authors investigated this, and demonstrated that the import motor associates with the laterally sorted ATP-independent precursors even their insertion does not depend on ATPase activity of the motor. These results would provide important basis for the current discussion on the function model of the TIM23 complex in lateral sorting of preproteins into the inner membrane: the single-entity model and the modular model.

Comments,
1. The arrest and chase reactions in Fig.1B are not clearly demonstrated.
2. Fig. 1B & C, and Fig. 4C & D: why a significant amount of the import intermediate for 147DHFR and 127HDFR remained un-chased? Isn't this an artifact of DHFR-tag for the truncated precursors with shorter N-terminal segments?
3. Fig. 2: the authors should provide cross-linking data for un-arrested import as the control to show that they can be released completely from the TIM23 complex into the inner membrane.
4. Fig. 3A: 220DHRF-His efficiently provided mature form, whereas all other shorter versions had the intermediate forms (or even precursor form for 167DHFR-His). Are they completely released from the TIM23 complex under un-arrested condition? Cross-linking data under un-arrested condition should be shown for several precursors as the control.
5. It would be informative to know association of Tim21 and Pam17 with the import-arrested precursors.

6. Fig. 2F: Here, again, 147DHFR seemed to be released only inefficiently from the TIM23 complex, raising concern for artificial effect of the arrest/chase reaction. Detection of the cross-linking adducts during the course of in vitro import in the absence of NADPH/DHF would provide more convincing results than the arrest/chase reaction, although strict control of the import reaction seems to be difficult.

Referee #2 (Remarks to the Author):

The TIM23 complex in the mitochondrial inner membrane (IM) mediates both transocation across and lateral insertion into the IM in response to the destinations of the substrate mitochondrial precursor proteins. Currently there is a hot debate on the mechanism of the functional switch of the TIM23 complex between protein sorting to the matrix and that to the IM. One model (the modular model) suggests that the TIM23 complex exchanges between the complex lacking the entire import motor subunits (for IM sorted proteins) and that containing the motor components (for matrix-sorted proteins). The other model (the single-entity model) relies on the extensive conformational changes of the TIM23 complex that responds to demand or destinations of substrate proteins, sorting to the IM or matrix. The present work adopted the approach of chemical crosslinking to show that the arrested translocation intermediates for the IM are actually crosslinked to the motor components of the TIM23 complex. The results provide direct and strong evidence for the single-entity model of the TIM23 complex and appear to deserve urgent publication once the following points are clarified.

Fig. 1E - Is this observation with the ssc1-3 mutant also true for other fusion proteins?

Figs. 2 and 3 - Since those arrested fusion proteins consist of heterologous populations of m, i, and p forms, it should be important to clarify if the observed crosslinked products arose from the m (final) form or others

Fig. 4 - The negative role of the intermembrane space (IMS) domain of Tim14 is puzzling and could be a weakness of the manuscript. Does deletion of the IMS domain of Tim14 affect cell growth, BN-PAGE migration of the TIM23 complex, co-IP patterns between subunits of the TIM23 complex etc? Obviously, Fig. 4G and H are not sufficient to demonstrate that the IMS domain of Tim14 has a direct role in lateral IM insertion.

The lines 7-5 from the bottom in Page 6 require an appropriate reference.

Referee #3 (Remarks to the Author):

Popov- eleketi et al dissect the role of the mitochondrial ATPase complex in lateral release of imported proteins that carry hydrophobic transmembrane regions. The provide evidence that lateral release does not need the ATPase activity of the import motor but still components of the import motor are in the vicinity of the chain. One subunit has a domain that is exposed to the inter-membrane space and that when removed leads to enhanced lateral release.

This work provides preliminary mechanistic insight into the function of the mitochondrial ATPase. The experiments are clear and the MS largely well written. The work is based almost exclusively on non-specific cross-linking tools. The cross-linking studies presented provide some interesting preliminary observations on a possible non-ATP requiring role for the ATPase. However, these experiments do not provide any real mechanistic insight into the phenomenon revealed and would therefore have to be considered as preliminary. Additional experiments with different biochemical approaches and careful quantification would be required to provide a real mechanistic insight into this role of the ATPase in general and that of the TIM14 subunit more specifically.

Major comments
Fig.1
There is no quantification of the import/chase experiments in these panels. There is a comparison with various proteins that are seen at various levels and undergo varying degrees of chase. Values of various proteins should be normalized and quantification data shown together with the qualitative data.

Fig2
Below each cross-linking panel indicate the antibody used
The authors attribute complexes to various cross-links but the reader has no information on how these are deduced. They are based on past work but the way they are presented they seems arbitrary.

Fig2
As with the previous data these cross-linking experiments are qualitative data with no quantification. In some experiments several cross-links are seen but the population of the cross-linked complex of interest is absolutely a minor sub-populations. It is difficult in this sense to draw conclusions about what complex is significant and the result of mechanistically non-relevant proximity. This becomes particularly important since the authors use a non-specific cross-linking approach.

Fig2
What are the cross-linking results when components of the ATPase complex have been removed by deletion or the ts hsp70 is used at non-permissive conditions?

Fig2F
The quality of this experiment is extremely low and should be repeated.

p9 top
....This demonstrates that the components of the import motor are a genuine part of the translocase during lateral insertion of transmembrane segments.....
No firm conclusions can be drawn from generalized cross-linking. The statement must be reworded to reflect this.

p9 and Fig3A
....However, their crosslinking patterns differed and also the intensities of their crosslinking adducts increased from the shortest to the longer precursors, yet disappeared with the longest one..... were very similar when the different precursors were accumulated in transit (Fig 3B-D).....
What are these cross-links shown? Why are there more than one cross-linked populations? How do the various identified cross-linked complexes compare in terms of size? i.e. are there complexes that contain Tim44 and Tim14 etc at the same time? It's very difficult to do this comparison by eye on gels that have different markers and have been run at different lengths.

p. 9
....Thus it appears that the translocase remains in the same conformation from the point when the stop-transfer....
One can not draw conclusions about conformation from a cross-linking experiment like this one! Rephrase.

p. 11
....One possibility is that the IMS domain of Tim14 affects the rate of lateral insertion by affecting the conformation of the entire TIM23 complex. We therefore compared the crosslinking pattern of Tim23 in wild type and Tim14 60 mitochondria. This assay was previously shown to be very sensitive to changes in the conformation of the translocase (Popov- eleketi et al, 2008). The crosslinking patterns of Tim23 in the two types of mitochondria were essentially indistinguishable. Thus the IMS domain of Tim23 appears to have a more direct role in the process of lateral insertion.....
Judging the conformational state of the translocase from cross-linking data is very qualitative. Moreover, even these data are not shown here and the authors reference another citation. If these assays can really follow conformation they should be shown here. I should think that some additional, proper conformational assay would be necessary to draw a conclusion.

p.11 and Fig. 4H
...These results indicate that it is the IMS domain of Tim14 that is crosslinked to the laterally sorted precursor....
Not really, since:
a. There is clearly no direct proof that lack of cross-linking in the IMS deletion mutant is directly linked to the absence of the IMS domain. One cannot rule out in an experiment like this that the

conformation of the protein is altered and therefore overall cross-linking is affected. The authors can only address this by site-specific cross-linking or if they invest some serious effort in mapping by mass spectrometry WHERE the cross-links in TIM14 are.
b. The cross-linking profile they get with the deltaIMS mitochondria is completely different to the one they get with the wild type mitochondria.

p.11
the main solid observation on TIM14 that the authors provide is that TIM14 slows down lateral release. Why? How do they rationalize this finding? What is the mechanistic implication?

p. 12 Conclusions
......We show here that the components of the import motor are found in close vicinity of the laterally sorted proteins even when the ATPase activity of the import motor is not required in the process. Furthermore, the IMS-exposed domain of Tim14 modulates the rate of lateral insertion of transmembrane segments. Thus the function of the import motor of the TIM23 complex extends beyond the ATP-dependent action during the translocation of proteins across the inner membrane into the matrix.....
The authors show vicinity to the translocating chain of the ATPase components using cross-linking. They deduce from this that vicinity equals direct mechanistic involvement. I do not find enough evidence here to justify this conclusion.

Minor comments
p.3
The underlYing molecular mechanisms of the switching between these different modes of transport have remained largely unclear.

p8
...Crosslinking was performed with isolated intact mitochondria followed by isolation of precursor proteins and their crosslinking adducts on NiNTA-Agarose beads....
Mention the cross-linking approach used, e.g. homobifunctional generalized etc

p.11 ...We expressed and arrested in vivo cytb2(1-107)DHFR in wild type and Tim14 60 cells.... (Fig 4G, upper panel)...... (Fig 4G, lower panel). These results indicate that it is the IMS domain of Tim14 that is crosslinked to the laterally sorted precursor....

There are no upper and lower panels in 4G. Is 4H meant here?

1st Revision - authors' response   18 February 2011

We thank the Referees and the Editorial Board of EMBO Reports for carefully evaluating our manuscript and for their thoughtful comments. These suggestions have definitely contributed to improvement of the manuscript and to clarify certain aspects.
Below we address point by point the various comments and suggestions raised by the Referees.

Referee #1 (Remarks to the Author):

The mitochondrial TIM23 complex mediates both preprotein translocation into the matrix and lateral insertion of some preproteins into the inner membrane in an ATP-depend manner. Some precursors, however, are sorted laterally by the TIM23 complex into the inner membrane in an ATP-independent manner, raising question on the involvement of the import motor in this reaction. Here, the authors investigated this, and demonstrated that the import motor associates with the laterally sorted ATP-independent precursors even their insertion does not depend on ATPase activity of the motor. These results would provide important basis for the current discussion on the function model of the TIM23 complex in lateral sorting of preproteins into the inner membrane: the single-entity model and the modular model.

Comments,
1. The arrest and chase reactions in Fig.1B are not clearly demonstrated.

When the PK-treated samples are analyzed it is seen that at the zero time point of the chase there is very little of PK-protected material. During the chase period, the signal of the mature form constantly increases. In contrast, the signal of the intermediate form initially increases and then decreases as it is converted to the mature form. We have changed the panel with one of longer exposure of the same experiment which demonstrates this more clearly. Furthermore, chase experiments performed in wt and *ssc1-3* mitochondria are now having more time points which, together with quantifications done after the suggestion of Referee 3, demonstrate this more clearly.

2. Fig. 1B & C, and Fig. 4C & D: why a significant amount of the import intermediate for 147DHFR and 127HDFR remained un-chased? Isn't this an artifact of DHFR-tag for the truncated precursors with shorter N-terminal segments?

Experiments shown in Figure 4 (now in Supplementary Figure 3) present normal import of these precursors. Like the –DHF/NADPH lanes in Figure 1, they demonstrate that these precursors are not completely matured by IMP even under "normal" import conditions. This has been observed before and is not an artifact of DHFR-tag but is due to the requirement for folded HBD for efficient processing by IMP (see for example Glick et al, Prot Sci (1993); Voos et al, JCB (1993); Stuart et al, Eur J Biochem (1994); Chacinska et al, Cell (2005); Tamura et al, JCB (2009)). Since shorter constructs have an incomplete HBD domain they are processed only inefficiently. Even longer constructs which have the complete HBD, such as b_2(1-220)DHFR, are not completely matured (references above but see also Figure 1). We have added a sentence in the text to clarify this point.

3. Fig. 2: the authors should provide cross-linking data for un-arrested import as the control to show that they can be released completely from the TIM23 complex into the inner membrane.

This is an excellent suggestion. We have expressed b_2(1-107)DHFRHis and b_2(1-147)DHFRHis in the presence and in the absence of aminopterin ie under arrest and non-arrest conditions. We observe crosslinks to Tim23 only if the precursors were expressed in the presence of aminopterin demonstrating that the precursor proteins have to be in the translocase for efficient crosslinking. This is now included as Supplementary Figure 2.

4. Fig. 3A: 220DHRF-His efficiently provided mature form, whereas all other shorter versions had the intermediate forms (or even precursor form for 167DHFR-His). Are they completely released from the TIM23 complex under un-arrested condition? Cross-linking data under un-arrested condition should be shown for several precursors as the control.

See our response to the comment 3.

5. It would be informative to know association of Tim21 and Pam17 with the import-arrested precursors.

We did attempt these experiments for the original submission of this manuscript. Unfortunately, our antibodies to Tim21 and Pam17 are of insufficient quality, even after affinity purification, to be used for crosslinking experiments shown here. We did, however, previously analyze the association of these two proteins with the TIM23 complex saturated with matrix and laterally sorted precursor proteins (Popov-Celeketic et al, EMBO J (2008)). This analysis revealed that Tim21 is present in the translocase under all conditions analyzed, whereas Pam17 is absent from the translocase involved in transport into the matrix.

6. Fig. 2F: Here, again, 147DHFR seemed to be released only inefficiently from the TIM23 complex, raising concern for artificial effect of the arrest/chase reaction. Detection of the cross-linking adducts during the course of in vitro import in the absence of NADPH/DHF would provide more convincing results than the arrest/chase reaction, although strict control of the import reaction seems to be difficult.

We agree with the Referee that such experiments would be very difficult to control due to the constant supply of new translocating chains from the TOM complex and we therefore did not attempt to perform them. We would, however, like to point out that the kinetics of import during the chase reaction after the DHF/NADPH arrest do not differ dramatically, if at all, from the kinetics of regular import ie without the initial arrest (see for example import kinetics of b_2(1-147)DHFR now presented in Supplementary Figure 3). Also, it is generally observed in the field that the overall efficiency of import and its kinetics are very dependent on the precursor protein analyzed.

Referee #2 (Remarks to the Author):

The TIM23 complex in the mitochondrial inner membrane (IM) mediates both transocation across and lateral insertion into the IM in response to the destinations of the substrate mitochondrial precursor proteins. Currently there is a hot debate on the mechanism of the functional switch of the TIM23 complex between protein sorting to the matrix and that to the IM. One model (the modular

model) suggests that the TIM23 complex exchanges between the complex lacking the entire import motor subunits (for IM sorted proteins) and that containing the motor components (for matrix-sorted proteins). The other model (the single-entity model) relies on the extensive conformational changes of the TIM23 complex that responds to demand or destinations of substrate proteins, sorting to the IM or matrix. The present work adopted the approach of chemical crosslinking to show that the arrested translocation intermediates for the IM are actually crosslinked to the motor components of the TIM23 complex.

The results provide direct and strong evidence for the single-entity model of the TIM23 complex and appear to deserve urgent publication once the following points are clarified.

Fig. 1E - Is this observation with the ssc1-3 mutant also true for other fusion proteins?
We have analyzed additional fusion proteins that are laterally sorted by the TIM23 complex in an ATP-independent manner and got essentially the same results as for b_2(1-147)DHFR. These results are presented in Supplementary Figure 1.

Figs. 2 and 3 - Since those arrested fusion proteins consist of heterologous populations of m, i, and p forms, it should be important to clarify if the observed crosslinked products arose from the m (final) form or others
One way to address this question is to inhibit cleavage by MPP and subsequently analyze the size of crosslinked adducts. Since MPP is essential for viability of yeast cells the experiment can not be done *in vivo*. It is however possible to inhibit MPP *in vitro* by incubating mitochondria in the presence of EDTA and o-Phe. This procedure removes metal ions that are essential for MPP activity. We managed to establish conditions for simultaneous NADPH/DHF arrest and MPP inhibition, Response Figure 1A. Furthermore we demonstrate that the precursor can be chased into mitochondria even if MPP remains inactive during the chase period, Response Figure 1A. However, when we performed crosslinking under conditions which inhibit MPP, crosslinking was inhibited as well, Response Figure 1B, lower panel. It is currently not clear why that would be the case.
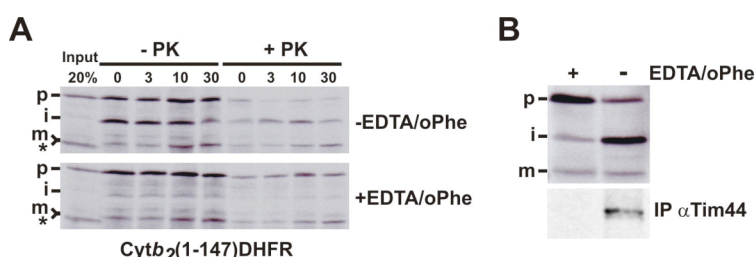
Response Figure 1. Inhibition of MPP during the arrest and chase of cytb_2(1-147)DHFR. (A) lower panel: ^{35}S-labelled precursor protein cytb$_2$(1-147)DHFR was preincubated with DHF and NADPH, in the import buffer lacking divalent ions and containing EDTA and o-Phe, for 10 min at 25°C. During the same time, mitochondria were preincubated in the same import buffer with EDTA alone for 5 min at 25°C before addition of o-Phe. The precursor protein was subsequently bound to energized mitochondria for 15 min. Mitochondria were reisolated, washed to remove nonspecifically bound material, resuspended in the import buffer preincubated with EDTA and o-Phe, and incubated further in the absence of DHF and NADPH. At indicated time points, samples were removed and treated with proteinase K (PK) where indicated. Samples were analyzed by SDS-PAGE followed by autoradiography. Upper panel: Samples were treated in the same way except that EDTA and o-Phe were omitted. (B) ^{35}S-labelled precursor protein cytb$_2$(1-147)DHFR was arrested in mitochondria as described above, upper panel. Samples were crosslinked with DSG, solubilized in SDS-containing buffer followed by immunoprecipitation with antibodies to Tim44, lower panel. Samples were analyzed by SDS-PAGE and autoradiography.

We also expressed and arrested *in vivo* cytb$_2$(1-167)D19DHFR, a matrix-targeted precursor protein whose precursor and mature forms correspond in size to the precursor and intermediate forms of cytb$_2$(1-147)DHFR, Response Figure 2. The crosslinking adducts of cytb$_2$(1-167)D19DHFR run at sizes indistinguishable from those of the adducts of cytb$_2$(1-147)DHFR, suggesting that it is either the precursor or the intermediate form of cytb$_2$(1-147)DHFR that is crosslinked to TIM23 subunits. In any case it seems clear that it is not the mature form that is crosslinked. We would leave the decision as to whether to include this Figure to the manuscript to the Referees and the Editor.
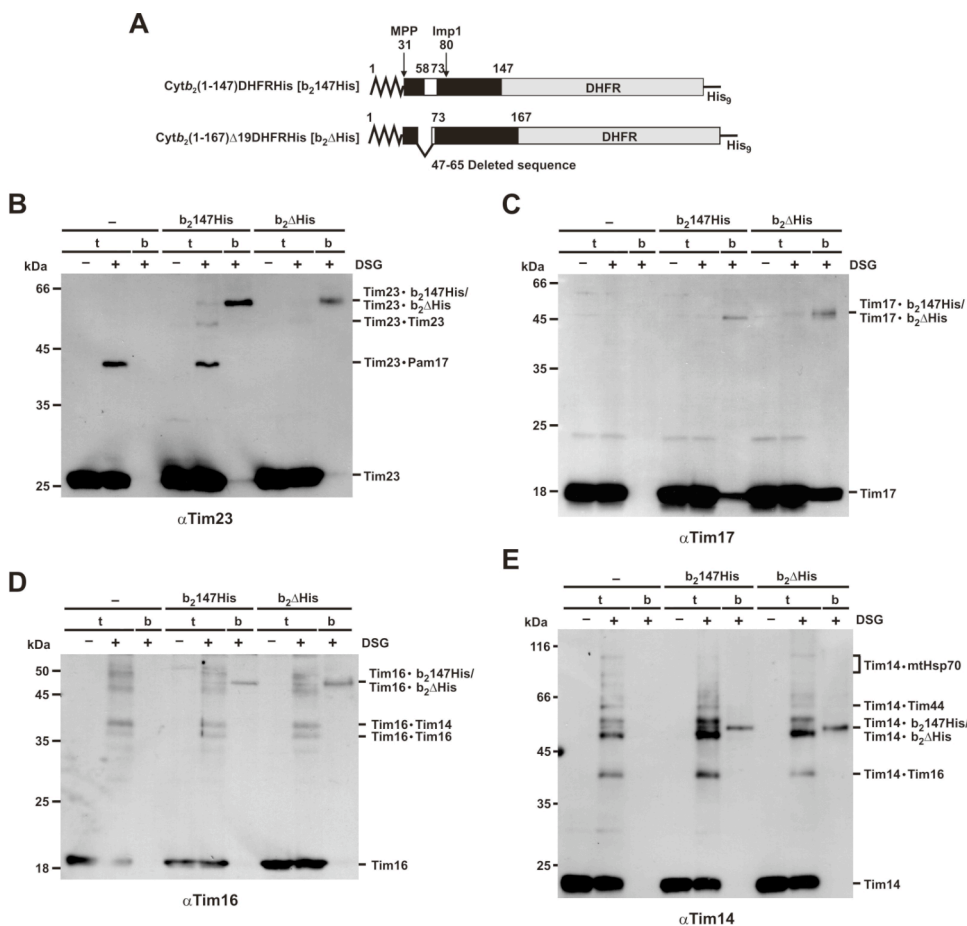
Response Figure 2. (A) Schematic representation of precursor proteins used. (B-E) Indicated precursor proteins were expressed in yeast cells in the presence of aminopterin and isolated mitochondria were subjected to crosslinking with DGS. Part of the samples was immediately prepared for SDS-PAGE, t - totals, and the rest was solubilized with SDS-containing buffer and incubated with NiNTA beads. Specifically bound material was eluted with Laemmli buffer containing 300 mM imidazole, b - bound. Samples were analyzed by SDS-PAGE and immunodecoration with antibodies to Tim23 (B), Tim17 (C), Tim16 (D) and Tim14 (E).

Fig. 4 - The negative role of the intermembrane space (IMS) domain of Tim14 is puzzling and could be a weakness of the manuscript. Does deletion of the IMS domain of Tim14 affect cell growth, BN-PAGE migration of the TIM23 complex, co-IP patterns between subunits of the TIM23 complex etc? Obviously, Fig. 4G and H are not sufficient to demonstrate that the IMS domain of Tim14 has a direct role in lateral IM insertion.

We agree with the Referee that "direct" is somewhat overstated here and have rephrased the respective sentences. We have also performed the suggested experiments with the following results. The assembly of the TIM23 complex is not affected by the deletion of the IMS domain of Tim14 as judged by coIPs from digitonin solubilized mitochondria, as previously shown (Mokranjac et al, JBC (2007)). We have now performed similar experiments with preincubation of isolated mitochondria at 37°C and still observed no effect of deletion of the IMS domain of Tim14 on the coIP patterns between TIM23 subunits. We have previously observed that the Tim14-Tim16 dimer is stable upon solubilization of mitochondria with Triton X-100 (Kozany et al, NSMB (2004) and tested now if these harsher solubilization conditions would show a destabilization of the Tim14-Tim16 dimer in mutant mitochondria, as for example seen for Tim16-Mdj2 complex (Mokranjac et al, JBC (2005)). However, even under these conditions the Tim14-Tim16 dimer remained stable. We also performed BNPAGE analysis as suggested. It should be noted here that the TIM23 complex falls largely apart under conditions of BNPAGE and only various subcomplexes are observed (Chacinska et al, Cell (2005); Tamura et al, JCB (2006)). Under these conditions, the Tim14-Tim16 subcomplex, probably representing a tetramer (Mokranjac et al, EMBO J (2006)), was absent. Also, yeast cells expressing Tim14 lacking its IMS domain show a temperature sensitive growth on lactate medium. These results, now included in Figure 4 and Supplementary Fig 3, further support the notion that the IMS domain of Tim14 has a more direct role in the process of lateral insertion.

The lines 7-5 from the bottom in Page 6 require an appropriate reference.
The reference has been added.

Referee #3 (Remarks to the Author):

Popov-Čeleketić et al dissect the role of the mitochondrial ATPase complex in lateral release of imported proteins that carry hydrophobic transmembrane regions. The provide evidence that lateral release does not need the ATPase activity of the import motor but still components of the import motor are in the vicinity of the chain. One subunit has a domain that is exposed to the inter-membrane space and that when removed leads to enhanced lateral release.

This work provides preliminary mechanistic insight into the function of the mitochondrial ATPase. The experiments are clear and the MS largely well written. The work is based almost exclusively on non-specific cross-linking tools. The cross-linking studies presented provide some interesting preliminary observations on a possible non-ATP requiring role for the ATPase. However, these experiments do not provide any real mechanistic insight into the phenomenon revealed and would therefore have to be considered as preliminary. Additional experiments with different biochemical approaches and careful quantification would be required to provide a real mechanistic insight into this role of the ATPase in general and that of the TIM14 subunit more specifically.

Major comments
Fig.1
There is no quantification of the import/chase experiments in these panels. There is a comparison with various proteins that are seen at various levels and undergo varying degrees of chase. Values of various proteins should be normalized and quantification data shown together with the qualitative data.
The quantifications of chase reactions are now shown together with the films. They demonstrate that the chase in ss1-3 mitochondria, under nonpermissive conditions, is indistinguishable from that in wt mitochondria for ATP-independent, laterally sorted precursor proteins. According to the suggestion of Referee 2, we analyzed chase reactions of additional ATP-independent, laterally sorted precursor proteins and obtained essentially the same results as for b_2(1-147)DHFR. These results are now included in Figure 1 and Supplementary Figure 1.
Fig2
Below each cross-linking panel indicate the antibody used
The authors attribute complexes to various cross-links but the reader has no information on how these are deduced. They are based on past work but the way they are presented they seems arbitrary.
We have indicated antibodies used for decorations below the corresponding panels in addition to the already existing labeling on the right side of the panel. All crosslinking adducts labeled on the right sides of the films were previously identified, by several groups including our own, and they are certainly not arbitrary. Due to the space restrictions we feel that is not appropriate to extensively elaborate on the previous literature, especially since this is not of immediate importance for this manuscript.
Fig2
As with the previous data these cross-linking experiments are qualitative data with no quantification. In some experiments several cross-links are seen but the population of the cross-linked complex of interest is absolutely a minor sub-populations. It is difficult in this sense to draw conclusions about what complex is significant and the result of mechanistically non-relevant proximity. This becomes particularly important since the authors use a non-specific cross-linking approach.
Crosslinking is one of the rare techniques which enables detection of protein-protein interactions in the intact cellular milieu. Though relatively nonspecific crosslinkers such as glutaraldehyde can be used, we prefer to use crosslinkers with well defined side chain specificity and a known spacer arm length. DSG, used in this study, is a crosslinker which is highly specific for amino groups and which has a short spacer arm (7.7 Å). Furthermore, we use low concentrations of crosslinker, short incubation times and incubation at low temperatures which all contribute to the high specificity of reactions. Thus, if the crosslinks are obtained, they are result of relevant interactions. The efficiency of crosslinking of course depends on the serendipitous positioning of Lys side chains but the fact that the crosslinking adducts can be detected speaks for a close vicinity of two proteins.
Fig2

What are the cross-linking results when components of the ATPase complex have been removed by deletion or the ts hsp70 is used at non-permissive conditions?

All components of the import motor of the TIM23 complex are essential for viability of yeast cells and it is thus not possible to use deletion strains. It is also questionable what the analysis of crosslinking in ssc1-3 under nonpermissive conditions would bring as expression of precursor proteins would have to be induced in the rapidly dying cells. We did however previously analyze the crosslinking patterns of Tim44 and Tim14 in isolated mitochondria from ssc1-3 cells and observed that, upon preincubation at 37°C, the ATP-dependence of crosslinking adducts between import motor components is lost as are the crosslinks to mtHs70 (Mokranjac et al. EMBO J (2003)). This further supports the functional significance of the observed crosslinks.

Fig2F

The quality of this experiment is extremely low and should be repeated.

For the initial submission, the experiment has been performed several times to confirm the results and a representative experiment was presented. We changed the respective panels with longer exposures when available. It should be stated that such experiments require enormous amounts of noncommercial, affinity purified antibodies which are not available in unlimited amounts. Furthermore, one regularly needs over one month exposures to obtain reasonable signals. We do not find that the quality of this experiment is lower than those regularly published in high impact journals such as EMBO Reports.

p9 top

....This demonstrates that the components of the import motor are a genuine part of the translocase during lateral insertion of transmembrane segments.....

No firm conclusions can be drawn from generalized cross-linking. The statement must be reworded to reflect this.

We reworded the sentence to "This further supports the notion that …", as suggested. We would however like to add that in the publication in which we originally proposed the single entity model (Popov-Celeketic et al, EMBO J (2008)) we showed, by coIPs, that the components of the import motor are present in the TIM23 complex saturated with the laterally sorted proteins. We now extend this study and show here the functional significance of this association.

p9 and Fig3A

....However, their crosslinking patterns differed and also the intensities of their crosslinking adducts increased from the shortest to the longer precursors, yet disappeared with the longest one..... were very similar when the different precursors were accumulated in transit (Fig 3B-D).....

What are these cross-links shown? Why are there more than one cross-linked populations? How do the various identified cross-linked complexes compare in terms of size? i.e. are there complexes that contain Tim44 and Tim14 etc at the same time? It's very difficult to do this comparison by eye on gels that have different markers and have been run at different lengths.

The cytb2 part of the chimeras contains several Lys residues which can be involved in crosslinking with the components of the TOM and TIM23 machineries. Since more than one Lys residue in each chimera is available for crosslinking it is not unexpected that several crosslinking adducts are observed for each chimera, Figure 3A. Furthermore, when the chimeras of different lengths are arrested in mitochondria for example Lys residue 83 would be in a different molecular environment if it is present in cytb_2(1-107)DHFR as compared to same residue present in cytb_2(1-147)DHFR, Response Figure 3. Therefore we stated that the "crosslinking patterns (of different chimeras) differed and also the intensities of their crosslinking adducts increased from the shortest to the longer precursors, yet disappeared with the longest one".
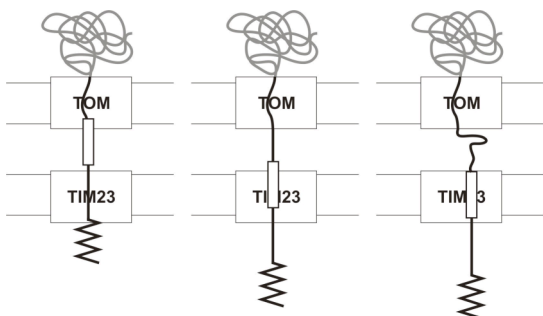
Response Figure 3. Schematic presentation of arrested chimeras of different lengths.

From all panels in Figure 3, it is obvious that under the crosslinking conditions used the vast majority of proteins remain not crosslinked (this further lowers the likelihood of nonspecific interactions). It is thus not very likely that a single polypeptide chain of the arrested chimera would be crosslinked to Tim14 via one Lys residue and to Tim44 via another Lys residue. Furthermore, the sizes of the NiNTA pulled down crosslinking adducts correspond in size to the size of analyzed Tim protein plus the size of the chimera. This also speaks against the simultaneous crosslinking of three proteins.

p. 9

....Thus it appears that the translocase remains in the same conformation from the point when the stop-transfer....

One can not draw conclusions about conformation from a cross-linking experiment like this one! Rephrase.

We have previously shown that the crosslinking pattern of the TIM23 complex, particularly when analyzed by antibodies to Tim23, depends on and is very specific for the activity state of the complex (Popov-Celeketic et al, EMBO J (2008)). We observed dimerization of Tim23 only when laterally sorted substrates are arrested in the translocase. Since we now see that this crosslinking pattern remains the same irrespective of the length of arrested chimera we are convinced that this is a reasonable conclusion. However, due to the space limitations (see comments from the Editor) we removed this sentence from the text since it does not bear immediate relevance for this manuscript.

p. 11

....One possibility is that the IMS domain of Tim14 affects the rate of lateral insertion by affecting the conformation of the entire TIM23 complex. We therefore compared the crosslinking pattern of Tim23 in wild type and Tim14Δ60 mitochondria. This assay was previously shown to be very sensitive to changes in the conformation of the translocase (Popov-Čeleketić et al, 2008). The crosslinking patterns of Tim23 in the two types of mitochondria were essentially indistinguishable. Thus the IMS domain of Tim23 appears to have a more direct role in the process of lateral insertion.....

Judging the conformational state of the translocase from cross-linking data is very qualitative. Moreover, even these data are not shown here and the authors reference another citation. If these assays can really follow conformation they should be shown here. I should think that some additional, proper conformational assay would be necessary to draw a conclusion.

As stated above we have previously shown that the crosslinking pattern of Tim23 is changing during changes of the activity state of the translocase. It does not seem appropriate to us that the same experiments are published twice. However, one can see in Figs 2A and 3B that Tim23 dimers are visible only in the presence of laterally sorted substrates. It would of course be excellent to have additional assays to follow conformational changes of the translocase *in organello* but development of such assays is far from trivial. For example *in vivo* FRET assays could not be used since tagging of the majority of the TIM23 components results in nonfunctional proteins. We have recently established *in vitro* FRET-based assays to follow the conformational changes of mtHsp70 during its ATP-hydrolysis dependent cycle (Mapa et al, Mol Cell (2010)) but this was only possible due the availability of the components involved in the recombinant form and in large amounts. For the membrane-integrated part of the complex this is clearly much more complicated and certainly beyond the scope of this manuscript.

p.11 and Fig. 4H

...These results indicate that it is the IMS domain of Tim14 that is crosslinked to the laterally sorted precursor....

Not really, since:

a. There is clearly no direct proof that lack of cross-linking in the IMS deletion mutant is directly linked to the absence of the IMS domain. One cannot rule out in an experiment like this that the conformation of the protein is altered and therefore overall cross-linking is affected. The authors can only address this by site-specific cross-linking or if they invest some serious effort in mapping by mass spectrometry WHERE the cross-links in TIM14 are.

b. The cross-linking profile they get with the deltaIMS mitochondria is completely different to the one they get with the wild type mitochondria.

We agree with the Referee that "indicate" is too strong of a word here and have rephrased the sentence. The crosslinking pattern in Tim14D60 is different from the wild type only by a shift of the crosslinks to lower molecular weights due to the smaller size of Tim14 protein.

p.11

the main solid observation on TIM14 that the authors provide is that TIM14 slows down lateral release. Why? How do they rationalize this finding? What is the mechanistic implication?

The TIM23 complex is possibly the only translocase in the cell which sorts proteins into two different compartments but, in addition, is able to differentiate between two types of transmembrane segments. Some transmembrane segments are laterally released into the inner membrane whereas others are allowed to pass through TIM23 complex to be subsequently inserted from the matrix side in a process known as conservative sorting. Excellent work from the Pfanner group has recently demonstrated that even within one precursor protein with multiple transmembrane segments some are laterally released whereas others are conservatively sorted (Bohnert et al. Current Biology (2010)). It is thus quite clear that recognition of transmembrane segments and the entire process of lateral insertion by the TIM23 complex have to be tightly regulated, likely on multiple levels. We are presenting experimental evidence here which, in our opinion, allows us to propose a role for Tim14 in this process. The data presented here are consistent with a model in which Tim14 is part of a "scanning machinery" which recognizes and subsequently laterally inserts some transmembrane segments. We have added a sentence in the text to explain this.

We would also like to add here that in this manuscript we are primarily discussing an even more basic question – whether the import motor is at all present in the translocase during the lateral insertion or not. Demonstrating and acknowledging its presence is only the first and obviously a difficult step towards clarifying the roles of the individual subunits in the process.

p. 12 Conclusions

......We show here that the components of the import motor are found in close vicinity of the laterally sorted proteins even when the ATPase activity of the import motor is not required in the process. Furthermore, the IMS-exposed domain of Tim14 modulates the rate of lateral insertion of transmembrane segments. Thus the function of the import motor of the TIM23 complex extends beyond the ATP-dependent action during the translocation of proteins across the inner membrane into the matrix.....

The authors show vicinity to the translocating chain of the ATPase components using cross-linking. They deduce from this that vicinity equals direct mechanistic involvement. I do not find enough evidence here to justify this conclusion.

From the data presented in the manuscript it is clear that vicinity to the translocating chain is only one of the evidences to support our conclusions. We also demonstrate that mutations of import motor can specifically affect the process of lateral insertion without influencing translocation into the matrix, the well established role of the import motor. Furthermore, we have previously shown by coIPs that the components of the import motor are present in the translocase during lateral insertion (Popov-Celeketic et al, EMBO J (2008)).

Minor comments

p.3

The underlYing molecular mechanisms of the switching between these different modes of transport have remained largely unclear.

The milestone publication from the Rapoport lab on the crystal structure of bacterial SecYEG protein translocase (van den Berg et al, Nature (2004)) has raised a hot debate as to the molecular identity of the lateral gate and the oligomeric state of the active translocase. Thus, even in this system which is understood in much more detail the molecular mechanisms remain unclear.

p8

...Crosslinking was performed with isolated intact mitochondria followed by isolation of precursor proteins and their crosslinking adducts on NiNTA-Agarose beads....

Mention the cross-linking approach used, e.g. homobifunctional generalized etc

A more precise description of DSG is now given in Methods section.

p.11 ...We expressed and arrested in vivo cytb2(1-107)DHFR in wild type and Tim14Δ60 cells.... (Fig 4G, upper panel)...... (Fig 4G, lower panel). These results indicate that it is the IMS domain of Tim14 that is crosslinked to the laterally sorted precursor....

There are no upper and lower panels in 4G. Is 4H meant here?

Yes, we apologize for this mistake.

Comment of the Editor

Also, the length of the revised manuscript may not exceed 27,500 characters (including spaces) and, including figures, the paper must ultimately fit onto maximally seven pages of the journal. Should

you find the length constraints to be a problem, you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

Due to space limitations, some experiments from the original submission and the majority of newly included ones are now presented in the Supplementary Material. Also, data referring to the matrix targeted precursor in Fig 1 was completely removed as the results on the ATP-dependent, laterally sorted precursor demonstrate the same point. The text has been shortened in parts of less immediate importance due to the given length limit.

2nd Editorial Decision 04 March 2011

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. I am happy to tell you that all referees now support publication of your manuscript in EMBO reports. Referee 2 only suggests to incorporate the response figure 2 into the supplementary information and I would kindly ask you to do this (and discuss the data in the main manuscript) before we proceed with the official acceptance of your manuscript.

Please submit the final version through our website again. On a more formal note, please also indicate in the manuscript (either in the figure legends or in the materials and methods section) how many independent times each experiment has been performed. It seems as if at the moment, this information is missing, but if I just overlooked it, please accept my apologies and ignore this point.

I look forward to seeing a new revised version of your manuscript as soon as possible.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The paper is well revised and answers satisfactorily the points that reviewers have raised. I believe this is an important paper to solve the problem about the sorting machinery of mitochondrial inner membrane proteins.

Referee #2 (Remarks to the Author):

This is a revised manuscript, which was improved significantly by adding new results in response to my previously raised concerns. Although the role of the intermembrane-space domain of Tim14 in lateral inner-membrane sorting is still vague, further efforts to probe its mechanism would take a substantially long time and delay possible publication. I suggest that Response Figure 2 be included in the supplemental data.

Referee #3 (Remarks to the Author):

The authors have addressed all the points raised previously diligently and provide an updated and more clear version of their MS. I think that their data make an important contribution to the

mechanistic understanding of ATPase independent lateral sorting of mitochondrial proteins during the import reaction.

2nd Revision - authors' response   16 March 2011

Please find enclosed the final version of our manuscript entitled "Role of the import motor in insertion of transmembrane segments by the mitochondrial TIM23 complex" by Duöan Popov-eleketi , Karin Waegemann, Koyeli Mapa, Walter Neupert and myself for publication in EMBO Reports.

According to the suggestion of Referee #2 and yourself we introduced Response Figure 2 into the manuscript. It is now shown as Supplementary Figure 3. You will notice that Supplementary Figure 3 is not identical to the Response Figure 2. This is because the Figure in the Response Letter had partly the panels which were already shown in the Figure 2 of the main text. As this is clearly unacceptable for publication, the figure in the Supplementary material now presents the results of a different experiment but of the same type. With regard to your suggestion to indicate how many times each experiment has been performed, we added a sentence in the Material and Methods section to address it. I would also like to add here that it is our policy in the lab that the critical experiments are not only repeated by one person but also by at least one additional colleague to a) insure their reliability and b) make sure that the protocols are understandable and reproducible. I am sorry if my (really unacceptable) laziness to invest time in scanning additional films and making extra figures for Response letter, which may have not been published at all, made you suspicious and apologize truly for this.

We do hope that you will now find the manuscript acceptable for publication in EMBO Reports and thank you for all your work with it.

3rd Editorial Decision   18 March 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. This also includes the two response figures that are currently in the point-by-point response. I am fully aware that one of them is now also present in the supplementary information, but we felt that this would be necessary, as not every reader will download the Review Process Files.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Finally, we provide a short summary of published papers on our website to emphasize the major findings in the paper and their implications/applications for the non-specialist reader. To help us prepare this short, non-specialist text, we would be grateful if you could provide a simple 1-2 sentence summary of your article in reply to this email.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports