EMBO reports

Mitochondrial AAA-ATPase Msp1 detects mislocalized tail-anchored proteins through a dual-recognition mechanism

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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 10 October 2018

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, reading the reports it also becomes clear that some further work will be necessary to substantiate the importance of the hydrophobic patch for substrate recognition by Msp1.
It will also be important to provide further insight into the role of D12 in substrate binding, not only degradation, and further experiments are needed to rule out that the mutation changes stability or localization rather than activity.

Referee 2 raises further concerns regarding the reported interaction between Msp1 and Cis1. This referee points out that the hydrophobic region of Msp1 might also bind Cis1, providing an alternative explanation for the observed effects. I discussed this aspect further with the referees. Referee 1 and 3 indicated that the degradation of mistargeted TA proteins might be distinct from the Cis1-dependent degradation of mitochondrial precursor proteins and that studying the involvement of Cis-1 in TA protein degradation might be beyond the scope of the current manuscript. Therefore, if the analysis of Msp1 binding to Cis1 or the involvement of Cis1 in TA protein degradation is straightforward, it would certainly further strengthen the study if such an experiment was included, but it is not essential for the revision. The connection and possible involvement of Cis1 should however be discussed in the most appropriate manner.

Given the constructive referee comments and their support, we would thus like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will

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Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

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When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if

you have questions or comments regarding the revision.

REFEREE REPORTS

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Referee #1:

Msp1 facilitates the clearance of tail-anchor (TA) proteins that are mistargeted to the mitochondrial outer membrane (MOM). In this manuscript, Li et al. took a combined approach of the mutational analyses and site-specific photocrosslinking to identify Msp1 residues involved in substrate recognition. In parallel, the authors performed photocrosslinking of a model substrate Pex15Δ30 to reveal the region in the substrate TA proteins for recognition by Msp1. The results showed that substrate TA proteins exposed a hydrophobic patch in the cytosol, which was recognized by Msp1 through its conserved hydrophobic residues. Interestingly, the authors found that D12 on the intermembrane space (IMS) region of Msp1 also recognizes positively charged residues in substrate proteins, which are important for their mistargeting, as well. The authors further identified two new Msp1 substrate TA proteins by systematic screening with get3Δmsp1Δ strains. The experiments by heavy use of site-specific photocrosslinking are technically sound and provide important new insight into the mechanism of substrate recognition in Msp1-mediated quality control of mistargeted MOM proteins.

The manuscript is thus worth for urgent publication, but could become much stronger after consideration of the following points.

(1) In principle, site-specific photocrosslinking provides information on protein-protein interactions at the spatial resolution of amino-acid residues. Nevertheless, mapping of crosslinking-positive residues onto the X-ray structure of Msp1 (Fig. 2A and 2E) is not that precise. The authors state, "<I>this region (highlighted in green in Msp1 hexamer models) lines along the surface of Msp1 hexamer and extends to positions near pore loop 1. This spatial organization may facilitate substrate transfer from N-domain into the central pore</I>". However, the spatial proximity between the crosslinked hydrophobic residues and each pore loop are not readily understood from these panels. Crosslinking results should be mapped onto the X-ray structure of Msp1 in a semi-quantitative manner and at the amino-acid residue resolution. In relation to this, it is interesting to see if mutations of the hydrophobic residues near pore loop1 may not abolish substrate binding, but block efficient substrate transfer into the pore, leading to defective degradation.

(2) Although the transmembrane (TM) segments of Msp1 and Pex15Δ30 are crosslinked to each other, the authors did not discuss these physical contacts. Does this crosslinking reflect just physical proximity or something like more specific recognition? The residues upstream to the hydrophobic patch of Pex15Δ30 are crosslinked to Msp1, but the crosslinking residues were tracked down only up to residue 260 from the C-terminus. Perhaps, more N-terminal part can interact with Msp1? Even negative data of crosslinking involving residues N-terminal to residue 260 may be informative.

(3) Although BPA at residue 12 of Msp1 was crosslinked to Pex15Δ30 and replacement of D12 impaired degradation, the role of D12 is still elusive since the endogenous level of Msp1 with D12T mutation did not show defects in Msp1-mediated degradation. The authors discussed its role in the step other than rate-limiting step of degradation, but this was not experimentally demonstrated. To address this point, the authors can test the effect of D12T mutation in substrate binding, not only substrate degradation.

(4) In the case of ERAD, not only exposed hydrophobic regions but also some degrons play roles in efficient recognition for degradation. In the case of mistargeted TA proteins, it is not clear if the exposed hydrophobic residues represent the general element for Msp1 recognition. Perhaps, the hydrophobic patch of Pex15 play the role of degron, not a general feature of unfolded protein domains. This can be tested by introducing unrelated hydrophobic segments into Gem1, Tom7 or Fis1, or truncating the cytosolic domains of Gem1, Tom7 or Fis1 to expose their internal hydrophobic residues. It is also interesting to test the effects of positioning of the hydrophobic patch in substrate proteins; does it work only near the TM segment?

(5) Although the authors tested the roles of conserved hydrophobic residues in Msp1 in substrate recognition by replacement with moderately hydrophobic Ala. However, Ala replacement could affect the functional conformation of Msp1, and the authors only assessed this possibility by checking the oligomeric state of Msp1 by BN-PAGE analyses. The authors could test this point by analyzing the recombinant Msp1 variants (without the TM segment) with these mutations by CD or more primitive method like limited protease digestion. It is not necessary to test all the mutants.

Referee #2:

The conserved AAA-ATPase Msp1 removes mislocalized proteins from the mitochondrial outer membrane. It is largely unknown how Msp1 recognizes its substrates.
In this study, the authors employed the well-characterized, though artificial, Msp1 substrate GFP-Pex15Δ30 which accumulates on mitochondria under specific conditions. Following the degradation of this GFP-tagged model substrate upon cycloheximide-induced translation inhibition is THE assay of this study, which was carried out in a very large number of yeast strains harboring mutations in Msp1, in the model substrate or in targeting factors such as Pex19 or Get3. Based on the observation, that GFP-Pex15Δ30 degradation was slowed down in a number of these strains the authors propose that (i) a negative charge in the IMS-domain of Msp1 is critical for binding a positive stretch in the IMS-exposed C-tail of its substrates, (ii) a hydrophobic patch on the cytosolic site of Pex15 is critical for Msp1 recognition, (iii) hydrophobic residues in the N domain of Msp1 directly bind to substrate proteins, presumably thereby contacting the hydrophobic patch, and (iv) that even residues in the interior of the Msp1 complex contribute to substrate binding, potentially because they direct the substrates into the cavity of the hexamer. These data were complemented by a site-specific crosslinking screen presenting evidence for direct interactions of the N domain of Msp1 with its substrate. Moreover, a screen for endogenous Msp1 substrates suggested that Frt1 and Ysy6 are removed by Msp1 from the mitochondrial surface, in particular, when the GET pathway is perturbed.
This very data-rich study is certainly of interest for the Msp1 community. However, I am not convinced that the data shown here really help us to understand the molecular mechanisms by which Msp1 recognizes its substrates. A recent study published in Science (Weidberg and Amon, 2018) showed that Msp1 is targeted via Cis1 to Tom70, and that this recruitment is important for its binding to substrates. The defects described here in many of the point mutations might be caused by altered interactions of the Msp1 complex with other OMM proteins such as Cis1 and Tom70. The starting observation of this study was that 3 fold overexpression of a Tom70-Msp1 fusion made the N-domain of Msp1 obsolete. This experiment suggests that the N-domain is NOT essential for substrate recognition of Msp1. For most phenotypes reported in the various mutants in this study, it is not possible to tell whether they are caused by direct effects on a reduced substrate affinity or by indirect effects that compromise Msp1 activity rather non-specifically.

Specific points
1. The N domain of Msp1 is only critical if Msp1 levels are limiting. Doesn't this exclude an essential function of this region for substrate recognition?
2. Under stress conditions, yeast cells express Cis1 in order to recruit Msp1 to the TOM complex. Isn't it possible that the hydrophobic region in the N domain promotes Cis1 binding? This would explain many of the results of this study.
3. The point mutants analyzed here might lead to global structural perturbations and hence affect substrate interaction only indirectly. This is for example very likely for mutants E73A and L77A, both showing a strong aggregation phenotype in Fig. S2A. Basically all mutated residues are in contact to the AAA domain which might explain why they are conserved. How can the authors exclude that these residues predominantly stabilize the Msp1 complex structure rather than function in contacting substrates specifically. This also is in line with the observation that many of these positions show no strong substrate contacts in the crosslinking experiments.
4. The crosslinking experiment shown in Fig. 2 is elegant. However, the strongest crosslink is seen with residue 59 which is clearly outside of the conserved region and present in a helix that is N-terminal to the AAA domain. It is not clear to me why the authors assume that residues that mediate substrate recognition are particularly conserved. Isn't high conservation more an indication for catalytic residues (here unlikely) or stabilizing residues (here likely). Despite the elegance of the site-specific crosslinking approach used here, I am not convinced that it really helps to elucidate

how Msp1 binds its substrates.
5. From Fig. 3C, the authors claim that the D12T mutant fails to degrade Pex15 and Tom6, but still facilitates proteolysis of Tom5, Gem1 and Fis1. However, the data clearly show that also these three proteins are strongly stabilized in comparison to WT. This experiment rather suggests that the D12T mutant is not fully functional, for example because its positioning in the OMM is altered or because its structure is changed. Again, evidence for a specific role of the D12 residue for substrate binding is not convincingly shown.
6. Along the same lines: does the D12 mutant still bind Tom5/Gem1 or Fis1? The experiment shown in Fig. 3D could be performed with a D12T variant to test substrate binding of this mutant.
7. In Fig. 4, the authors study the relevance of a cytosol-exposed hydrophobic patch for Pex15 degradation. Insertion of charged residues into this patch causes mislocalization to the cytosol and abrogates Pex15 association with mitochondria. The authors therefore used alanine mutants which are associated with mitochondria but not degraded anymore by Msp1 (but by the proteasome). The authors conclude that the patch is critical for (direct) Msp1 recognition. However, isn't it likely that this patch just downstream of the TMD forms a membrane-associated helix? Removing this stretch might impact Msp1-mediated degradation even if this region is not directly recognized by Msp1.
8. For the interpretation of the data it is essential that all the protein versions used here are correctly localized in the outer membrane. The microscopic pictures are of convincing quality, however, they do not allow to assess whether the GFP tagged proteins are really correctly inserted into the OMM. From the data shown it cannot be excluded that some of the proteins used here are for example associated with the mitochondrial surface or mis-targeted to other mitochondrial compartments. In the past, methods had been developed by the Rapaport lab to validate whether tail anchored OMM proteins are correctly inserted, for example by cysteine-accessibility assays.

Minor points
9. Fig. S1B: The labeling of the samples used for figure S1B is odd. How can WT be also Δmsp1? What is the difference between lines 9 to 12 and 13 to 16? In order to assess the expression levels of Msp1 in the different strains (WT, knockin and epichromosome) it will be essential to show Western blots with Msp1-specific antibodies.
10. Page 3: „OMM contains essential TA proteins, including the fission receptor..." None of the factors described here is essential with the exception of Tom22. Thus "essential" should be replaced by "important" or something similar.
11. Page 4: "dimmers" should read "dimers"

Referee #3:

The AAA ATPase Msp1 extracts mistargeted tail-anchored proteins from the mitochondrial outer membrane for degradation. How Msp1 recognizes mislocalized proteins remained unknown. Using an impressive set of experiments Li and colleagues report that Msp1 binds to two regions of their substrates: a hydrophobic patch in the cytosolic exposed domain and positive charged residues exposed to the intermembrane space. Performing localization studies of a number of tail-anchored proteins in the a get3msp1 double knock out , the authors identified two additional Msp1 substrates. The findings shed new light how Msp1 recognize mislocalized proteins. Overall, the major conclusions are well-based on an elegant set of genetic and biochemical assays. The presented data are of high quality and the findings are very interesting. There are few points that should be addressed before publication.

The authors identified a hydrophobic patch in GFP-Pex15Δ30, which is the main recognition site for Msp1. How conserved is this sequence among tail-anchored proteins? Is it also present in the two newly identified Msp1 substrates Frt1 and Ysy6? Do mitochondrial tail-anchored proteins like shown for Gem1 in general lack this patch and therefore escape degradation by Msp1?

The authors should speculate why only a few tail-anchored proteins mislocalized to mitochondria, while the majority is not. Do the other tail-anchored proteins contain the hydrophobic patch?

All experiments were performed with tagged Msp1 variants. Previously, the authors showed that tagged Msp1 is rapidly degraded (Wu et al., J. Cell Biol. 2016). To exclude indirect effect due to altered stability of Msp1 variants, the authors have to analyze the stability of the Msp1 variants. Furthermore, the authors should confirm that selected untagged Msp1 variants similarly affect the

stability of the Pex15Δ30 substrate like the tagged Msp1 variants.

In Figure 6A, Far10, Sps2 and Vps64 should be removed since no GFP-signal is detected. In Figure S5C, a wild-type control has to be added to show that the proteins are mislocalized to mitochondria in the mutant strains.

In Figures 1, 6 and S1, a Msp1L122,123D mutant is depicted, which is not described in the text. The authors should mention in the mnuascipt why this Msp1 mutant was selected for their experiments.

Additional information has to be added to the figure legends. For instance, how much load and elution fraction is shown for the pulldown assays.

The manuscript (e.g. text and table S1) contains a few typos that need to be corrected.

1st Revision - authors' response 2 January 2019

Point-by-Point Response to the comments of the Referees
(The revised figures and texts are highlighted in red)

Referee #1:

Msp1 facilitates the clearance of tail-anchor (TA) proteins that are mistargeted to the mitochondrial outer membrane (MOM). In this manuscript, Li et al. took a combined approach of the mutational analyses and site-specific photocrosslinking to identify Msp1 residues involved in substrate recognition. In parallel, the authors performed photocrosslinking of a model substrate Pex15Δ30 to reveal the region in the substrate TA proteins for recognition by Msp1. The results showed that substrate TA proteins exposed a hydrophobic patch in the cytosol, which was recognized by Msp1 through its conserved hydrophobic residues. Interestingly, the authors found that D12 on the intermembrane space (IMS) region of Msp1 also recognizes positively charged residues in substrate proteins, which are important for their mistargeting, as well. The authors further identified two new Msp1 substrate TA proteins by systematic screening with get3Δmsp1Δ strains. The experiments by heavy use of site-specific photocrosslinking are technically sound and provide important new insight into the mechanism of substrate recognition in Msp1-mediated quality control of mistargeted MOM proteins.

The manuscript is thus worth for urgent publication, but could become much stronger after consideration of the following points.
(1) In principle, site-specific photocrosslinking provides information on protein-protein interactions at the spatial resolution of amino-acid residues. Nevertheless, mapping of crosslinking-positive residues onto the X-ray structure of Msp1 (Fig. 2A and 2E) is not that precise. The authors state, "*this region (highlighted in green in Msp1 hexamer models) lines along the surface of Msp1 hexamer and extends to positions near pore loop 1. This spatial organization may facilitate substrate transfer from N-domain into the central pore*". However, the spatial proximity between the crosslinked hydrophobic residues and each pore loop are not readily understood from these panels. Crosslinking results should be mapped onto the X-ray structure of Msp1 in a semi-quantitative manner and at the amino-acid residue resolution.

Thanks for suggesting summarizing the crosslinking data onto X-ray structures. We have revised **Figs 1E, 2A and 2E**. In **Fig 1E**, we mapped the critical residues to the hexameric structure to better appreciate their spatial relationship to pore loop 1. In **Fig 2A**, we mapped the poor loop residues positive for crosslinking to the structure. In **Fig 2E**, we mapped N-domain residues positive for crosslinking to the structure.

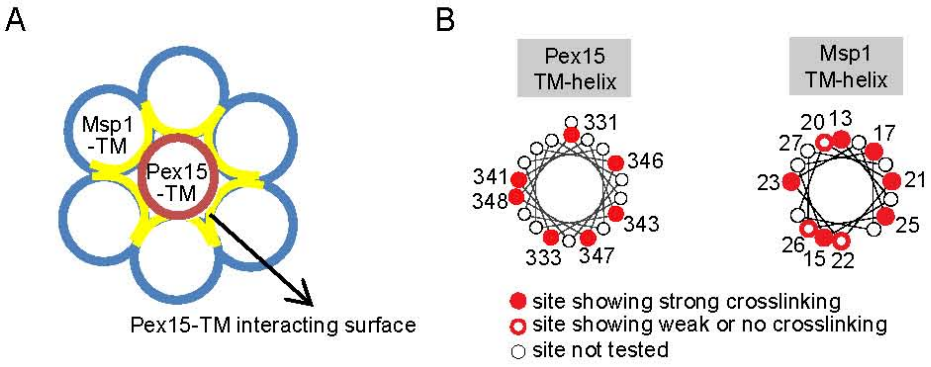
In relation to this, it is interesting to see if mutations of the hydrophobic residues near pore loop1 may not abolish substrate binding, but block efficient substrate transfer into the pore, leading to defective degradation.

From **Figs 1E and 2E**, we can see critical hydrophobic residues L69 and Y72 are proximal to pore loop 1. Y72 is in contact with pore loop 1. There are no additional hydrophobic residues to facilitate substrate transfer.

(2) Although the transmembrane (TM) segments of Msp1 and Pex15Δ30 are crosslinked to each other, the authors did not discuss these physical contacts. Does this crosslinking reflect just physical proximity or something like more specific recognition?

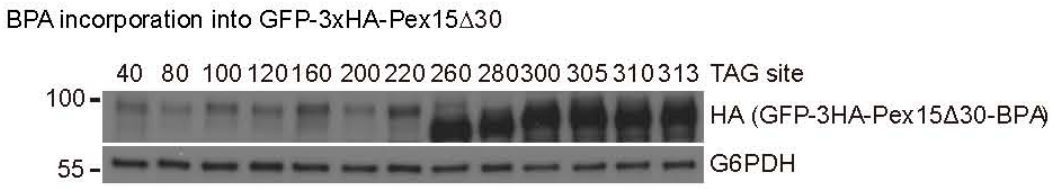
The TM segments of Msp1 and Pex15D30 have direct interactions, indicating Msp1 TM segment might play a role in substrate removal. We did not further characterize Msp1 TM segment for two reasons：

1. Msp1 TM segment is highly variable during evolution (Fig 1A) and we could not identify critical residues by performing alanine scan of the whole TM segment (summarized in Fig 1A and data in Fig EV2B).
2. We explored the possibility that Msp1 TM segments may assemble into a channel-like hexamer to help substrate dislocation across membrane. If this happens, we would expect Msp1 can only crosslink with Pex15 at one side of its TM segment (labeled as yellow in A). As shown in B, we plotted the crosslinking results in the TM-helixes and found Msp1 TM segment crosslinks with Pex15 in all the directions. Thus, a stable channel is unlikely formed by Msp1 TM segment.



The residues upstream to the hydrophobic patch of Pex15Δ30 are crosslinked to Msp1, but the crosslinking residues were tracked down only up to residue 260 from the C-terminus. Perhaps, more N-terminal part can interact with Msp1? Even negative data of crosslinking involving residues N-terminal to residue 260 may be informative.

We did try to map more N-terminal residues before 260. However, as shown below, when we incorporated BPA into more N-terminal positions, the expression level of Pex15Δ30 is greatly decreased (TAG sites 40-220). A recent paper reported the crystal structure of Pex15, showing its N-domain (residues 43-253) forms a folded domain (Gardner et al., Nature Communications 2018). Thus, incorporating BPA into this domain may disrupt folding and cause protein instability. In addition, we have shown in Fig 3 E and F that the C-terminal domain of Pex15Δ30 (residue 299-353) is sufficient for recognition and removal by Msp1. For these two reasons, we did not map into more N-terminal sites of Pex15.



(3) Although BPA at residue 12 of Msp1 was crosslinked to Pex15Δ30 and replacement of D12 impaired degradation, the role of D12 is still elusive since the endogenous level of Msp1 with D12T mutation did not show defects in Msp1-mediated degradation.

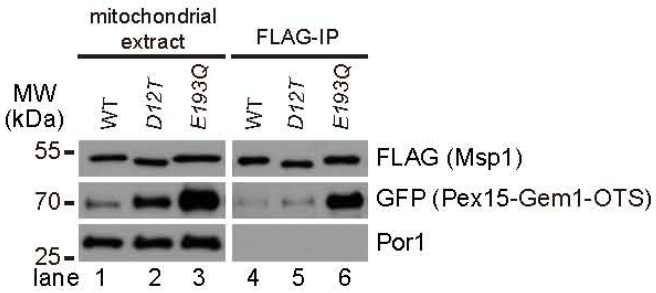
We apologize that we did not make some points very clear in the manuscript. In the crosslinking experiment as shown in Fig 2C, we have excluded most critical residues of Msp1 N-domain from BPA incorporation (except sites 86 and 95, which showed no or weak crosslinking) because incorporating BPA into these residue positions, which is similar to alanine mutation, may disrupt substrate interaction and cause false-negative crosslinking results. We thus did not examine crosslinking of BPA at residue 12 of Msp1. We have added a description to manuscript text (**page 7, lines 1-4 of the 3rd paragraph**).

Endogenous level of Msp1^{D12T} impaired the degradation of Pex15D30 (Fig 1B) and Frt1 (Fig 6 D and E) and could not rescue the growth defect of *get3Dmsp1D* cells as WT Msp1 did (Fig 6F). Thus, endogenous level of Msp1^{D12T} exhibited defects in Msp1-mediated degradation.

The authors discussed its role in the step other than rate-limiting step of degradation, but this was not experimentally demonstrated. To address this point, the authors can test the effect of D12T mutation in substrate binding, not only substrate degradation.

For the effect of D12T mutation in substrate binding, we have shown in Fig 1D lane 5 that the D12T mutation decreases the binding of Msp1^{E193Q} (substrate-trap mutant) to Pex15D30.

As shown bellow, we also tested the interaction of Msp1^{D12T} with substrate Pex15-Gem1-OTS. D12T mutation caused substrate accumulation (lane 2 vs. 1), but it only had marginal interaction with substrate as WT Msp1 did (lane 5 vs. 4). In contrast, the substrate-trap mutant E193Q caused substrate accumulation (lane 3 vs. 1) and had strong interaction with substrate (lane 6 vs. 4). Thus, Msp1^{D12T} is defective in substrate binding.

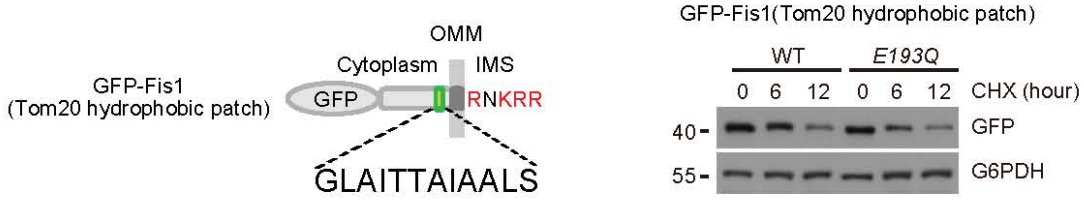


(4) In the case of ERAD, not only exposed hydrophobic regions but also some degrons play roles in efficient recognition for degradation. In the case of mistargeted TA proteins, it is not clear if the exposed hydrophobic residues represent the general element for Msp1 recognition. Perhaps, the hydrophobic patch of Pex15 play the role of degron, not a general feature of unfolded protein domains.

Thanks for raising these very interesting points. The hydrophobic patch unlikely serves as a degron because such a hydrophobic patch was not found in Msp1 substrates Frt1 and Ysy6. We have revised **Fig 6B** to show the hydrophobicity plot of Frt1 and Ysy6. In the small and heavily-charged cytoplasmic domain of Ysy6, only several distributed hydrophobic residues were found. Frt1 and Ysy6 may expose hydrophobic surfaces consisting of distributed residues, because hydrophobic residues in Msp1 N-domain, such as Y72, V81, and I93, remain essential for clearing these two proteins from mitochondria (Fig 6E).

This can be tested by introducing unrelated hydrophobic segments into Gem1, Tom7 or Fis1, or truncating the cytosolic domains of Gem1, Tom7 or Fis1 to expose their internal hydrophobic residues.

As shown bellow, we inserted a hydrophobic segment of 13aa from the transmembrane segment of Tom20 into Fis1. The chimeric protein was unstable but not degraded by Msp1. This is the same case as what we met when we mutated the hydrophobic patch of Pex15 (Fig 4B). Considering the existence of alternative pathways that can degrade OMM proteins with exposed hydrophobic regions (such as the Doa-Cdc48 pathway we reported previously), unfortunately the result only indicates the chimeric protein is a substrate more suitable for other pathways than Msp1 but cannot be used to draw conclusions about the substrate recognition mechanisms of Msp1.



It is also interesting to test the effects of positioning of the hydrophobic patch in substrate proteins; does it work only near the TM segment?

This is a very interesting question. Because Msp1 is anchored in the membrane and its N-domain is proximal to membrane, we speculate there could be spatial constraints for substrate recognition.

Indeed, when we placed the patch at the N-terminus of GFP-Fis1, Msp1 cannot degrade the chimeric protein. We have incorporated the result into **Fig 4H** (patch-GFP-Fis1 in revised **Fig 4H**).

(5) Although the authors tested the roles of conserved hydrophobic residues in Msp1 in substrate recognition by replacement with moderately hydrophobic Ala. However, Ala replacement could affect the functional conformation of Msp1, and the authors only assessed this possibility by checking the oligomeric state of Msp1 by BN-PAGE analyses. The authors could test this point by analyzing the recombinant Msp1 variants (without the TM segment) with these mutations by CD or more primitive method like limited protease digestion. It is not necessary to test all the mutants.

We agree that it is important to examine if alanine mutants affect general conformation of Msp1. We addressed this question from two aspects:

1. Protein thermo-stability reflects the folding status/structural stability. In **Appendix Figure S2 A-D**, we have prepared whole-cell protein extracts and examined the thermo-stability of Msp1 and Msp1^{E193Q}-FLAG and their V81A and I93A mutants. V81A and I93A mutants did not affect thermo-stability.
2. In **Appendix Figure S2E**, we generated recombinant Msp1 cytoplasmic domain (aa 33-345 that has been crystallized by Wohlever et al., Mol Cell 2017) and its V81A, I86A, I93A, and G95A variants and performed limited trypsin digestion. The variants had similar sensitivity to trypsin digestion as WT Msp1.

Referee #2:

The conserved AAA-ATPase Msp1 removes mislocalized proteins from the mitochondrial outer membrane. It is largely unknown how Msp1 recognizes its substrates.
In this study, the authors employed the well-characterized, though artificial, Msp1 substrate GFP-Pex15Δ30 which accumulates on mitochondria under specific conditions. Following the degradation of this GFP-tagged model substrate upon cycloheximide-induced translation inhibition is THE assay of this study, which was carried out in a very large number of yeast strains harboring mutations in Msp1, in the model substrate or in targeting factors such as Pex19 or Get3. Based on the observation, that GFP-Pex15Δ30 degradation was slowed down in a number of these strains the authors propose that (i) a negative charge in the IMS-domain of Msp1 is critical for binding a positive stretch in the IMS-exposed C-tail of its substrates, (ii) a hydrophobic patch on the cytosolic site of Pex15 is critical for Msp1 recognition, (iii) hydrophobic residues in the N domain of Msp1 directly bind to substrate proteins, presumably thereby contacting the hydrophobic patch, and (iv) that even residues in the interior of the Msp1 complex contribute to substrate binding, potentially because they direct the substrates into the cavity of the hexamer. These data were complemented by a site-specific crosslinking screen presenting evidence for direct interactions of the N domain of Msp1 with its substrate. Moreover, a screen for endogenous Msp1 substrates suggested that Frt1 and Ysy6 are removed by Msp1 from the mitochondrial surface, in particular, when the GET pathway is perturbed.

We sincerely thank this great summary of our results. There is one summary: (iv) that even residues in the interior of the Msp1 complex contribute to substrate binding, potentially because they direct the substrates into the cavity of the hexamer that we wish to explain a little bit. The result refers to Fig 2B that when Msp1^{E193Q} stably interacts with substrate Pex15Δ30, the interior pore loop residues of Msp1 can crosslink with Pex15Δ30. Msp1^{E193Q} is a Walker B mutation defective in ATP hydrolysis. It cannot continuously dislocate substrate through its central pore. But according to previous study of AAA-ATPase from Robert Sauer lab, the initial passing of substrate into the pore will not be prevented by Walker B mutation (Martin et al., Molecular Cell 2008). We thus did this crosslinking experiment with Msp1 pore loop residues to see if substrate can also enter the central pore of Msp1^{E193Q} hexamer. This is to confirm the ATPase domain of Msp1 has a similar working mode as other ATPases. This result has NO relationship with substrate recognition.

This very data-rich study is certainly of interest for the Msp1 community. However, I am not convinced that the data shown here really help us to understand the molecular mechanisms by which Msp1 recognizes its substrates. A recent study published in Science (Weidberg and Amon, 2018) showed that Msp1 is targeted via Cis1 to Tom70, and that this recruitment is important for its binding to substrates. The defects described here in many of the point mutations might be caused by altered interactions of the Msp1 complex with other OMM proteins such as Cis1 and Tom70. The starting observation of this study was that 3 fold overexpression of a Tom70-Msp1 fusion made the

N-domain of Msp1 obsolete. This experiment suggests that the N-domain is NOT essential for substrate recognition of Msp1. For most phenotypes reported in the various mutants in this study, it is not possible to tell whether they are caused by direct effects on a reduced substrate affinity or by indirect effects that compromise Msp1 activity rather non-specifically.

Specific points

1. The N domain of Msp1 is only critical if Msp1 levels are limiting. Doesn't this exclude an essential function of this region for substrate recognition?

We started with the result that Msp1$^{Tom70\text{-}N}$, in which we replaced the first 32 residues of Msp1 with that of Tom70, is functionally defective at endogenous expression level but can be compensated by overexpression (Fig EV1). This mutant is a PARTIAL but not a full N-domain mutant. This chimeric Msp1 only loses part of the N-domain (IMS residues and the TM segment) but contains an intact cytoplasmic N-domain containing all the critical hydrophobic residues (L69-L96). Thus, our result does not exclude an essential function of N-domain.

2. Under stress conditions, yeast cells express Cis1 in order to recruit Msp1 to the TOM complex. Isn't it possible that the hydrophobic region in the N domain promotes Cis1 binding? This would explain many of the results of this study.

We sincerely thank this great suggestion reminding us to test Cis1. We have added a citation of Cis1 in **page 4 lines 17-20** in the introduction section. Cis1 knockout has no effect on the degradation of GFP-Pex15D30 (**Appendix Figure S1**), suggesting Cis1 is not essential for removing mistargeted TA proteins.

3. The point mutants analyzed here might lead to global structural perturbations and hence affect substrate interaction only indirectly. This is for example very likely for mutants E73A and L77A, both showing a strong aggregation phenotype in Fig. S2A.

Msp1 is a dually localized protein to mitochondria and peroxisomes (Chen et al., EMBO J 2014; Okreglak & Walter, PNAS 2014). In Fig. S2A (now Fig EV2A), the "dots" of E73A and L77A mutants are not aggregates but their signals on peroxisomes. The dots are not limited to E73A and L77A but can also be seen in WT, LL2D and other Msp1 variants.

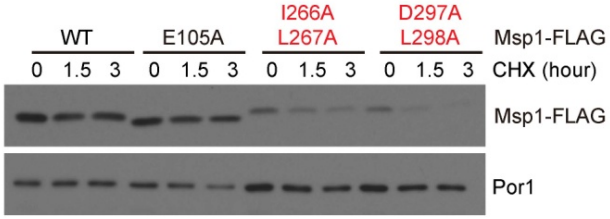
This also is in line with the observation that many of these positions show no strong substrate contacts in the crosslinking experiments.

We apologize that we did not make our experiment design very clear to avoid confusion. In the crosslinking experiment, we excluded most critical residues of Msp1 N-domain from BPA incorporation (except sites 86 and 95, which showed no (86) or weak (95) crosslinking) because incorporating BPA into these residue positions, which is similar to alanine mutation, may disrupt substrate interaction and cause false-negative results. Thus, we cannot conclude from the crosslinking experiment about the interaction between Msp1critical residues and substrate. We have added a description to the manuscript text (**page 7, lines 1-4 of the 3rd paragraph**).

Basically all mutated residues are in contact to the AAA domain which might explain why they are conserved. How can the authors exclude that these residues predominantly stabilize the Msp1 complex structure rather than function in contacting substrates specifically.

We agree that it is important to examine if N-domain mutants affect general conformation of Msp1. We discuss and address this question from the following aspects:

1. In our study, all the mutants shown were as stable as WT Msp1 (see the anti-FLAG blot in Figs 1B, EV2B, and 3A). We had 17 mutants affecting Msp1 stability, such as the two double mutants (highlighted in red) shown bellow. These mutants were excluded from analysis in the first place.



2. The N-domain mutants can form Msp1 hexamer normally (Fig 1C). Because hexamer assembly involves significant structural reorganization of Msp1 monomers (Wohlever et al., Mol Cell 2017), our results strongly support Msp1 structure is not significantly altered by the mutations.

3. We further examined if alanine mutants affect general conformation of Msp1. The results include:

a). Protein thermo-stability reflects the folding status/structural stability. In **Appendix Figure S2 A-D**, we prepared whole-cell protein extracts and examined the thermo-stability of Msp1 and Msp1^{E193Q}-FLAG and their V81A and I93A mutants. V81A and I93A mutants did not affect thermo-stability.

b). In **Appendix Figure S2E**, we generated recombinant Msp1 cytoplasmic domain (aa 33-345 that has been crystallized by Wohlever et al., Mol Cell 2017) and its V81A, I86A, I93A, and G95A variants and performed limited trypsin digestion. The variants had similar sensitivity to trypsin digestion as WT Msp1.

These new results support our N-domain mutants do not cause Msp1 misfolding and instability.

4. The crosslinking experiment shown in Fig. 2 is elegant. However, the strongest crosslink is seen with residue 59 which is clearly outside of the conserved region and present in a helix that is N-terminal to the AAA domain. It is not clear to me why the authors assume that residues that mediate substrate recognition are particularly conserved. Isn't high conservation more an indication for catalytic residues (here unlikely) or stabilizing residues (here likely). Despite the elegance of the site-specific crosslinking approach used here, I am not convinced that it really helps to elucidate how Msp1 binds its substrates.

Thanks for appreciating the elegance of our experimental approach. Msp1 critical residues for substrate binding locate to both sides of membrane (Fig 1), indicating direct interaction of Pex15 and Msp1 at both sides of membrane. Such interaction is quite unusual. We thus performed site-specific photocrosslinking experiments to confirm this point. Our crosslinking results (Fig. 2) served this purpose well.

Concerning the interpretation of the crosslinking results, the experiment has its limitation and we wish to make some points clear here. Substrate engagement by meiotic clade of AAA-ATPases is probably the most complicated among AAA-ATPases, because a stable ATPase hexamer is not present initially. Substrate engagement involves multiple steps from initial substrate detection to hexamer assembly and to full substrate engagement. The early and transient interactions are very difficult (almost impossible) to be captured in vivo. Our crosslinking experiment with Msp1^{E193Q} and substrate examines protein interaction of the last step (full substrate engagement). Interactions critical for initial substrate detection may or may not be present in the conformation. With this in mind, we can see strong crosslinking at a particular residue position (residue 59 as the reviewer pointed out) only indicates this residue is proximal to substrate in the conformation we examined but not necessarily mean this position is functionally important (it may or may not). For the same reason, the strong crosslinking of a non-essential residue (residue 59) does not necessarily mean other non-crosslinked positions are not functionally important. For these reasons, we avoided drawing too many conclusions than what we have summarized in the manuscript. There is no inconsistency in our crosslinking data.

Concerning the comment of conserved residues, conservation of residues often indicates functional importance. The function could be catalysis and structure-stabilization as the reviewer indicated and also be substrate recognition. We did not find particular reasons to restrict conserved residues to the former two functions but not to the last one.

5. From Fig. 3C, the authors claim that the D12T mutant fails to degrade Pex15 and Tom6, but still facilitates proteolysis of Tom5, Gem1 and Fis1. However, the data clearly show that also these three proteins are strongly stabilized in comparison to WT.

It seems we have some misunderstanding of Fig. 3C here. In Fig. 3C, D12T mutation impairs the degradation of substrates with outer membrane targeting sequences (OTSs) from Pex15, Tom5, Gem1 and Fis1, which all contain positively-charged IMS residues. Substrate without positively-charged IMS residues (Tom6-OTS) was not degraded by WT or D12T Msp1.

This experiment rather suggests that the D12T mutant is not fully functional, for example because its positioning in the OMM is altered or because its structure is changed.

D12 is in an IMS stretch of 12 residues, which is not a folded region and is in the other side of membrane from the folded cytoplasmic domain of Msp1. Its impact on Msp1 folding should be very limited. Importantly, Msp1^{D12T} is only defective in the interaction and degradation of substrates with positively-charged IMS tail. It is capable of removing substrate Ysy6 which has no IMS positive charges (Fig 6 D and E). This is strong evidence that Msp1^{D12T} localization at OMM is normal and its cytoplasmic domain is functional. Supporting this, D12T did not affect the assembly of Msp1 hexamer (Fig 1C lane 5).

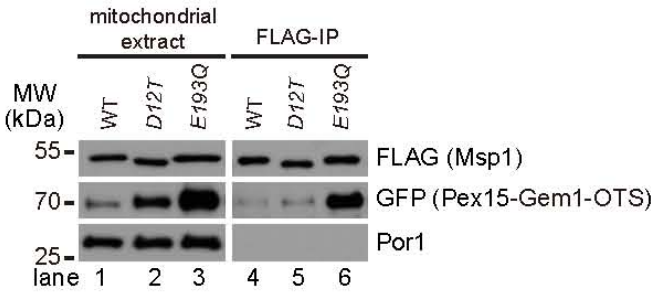
In addition, our hypothesis that D12 may facilitate substrate engagement through ionic interactions is based on multiple consistent results: 1. The negative charge of D12 is critical for

Msp1 to remove substrate Pex15D30 (Fig. 3A). 2. The positively-charged IMS tail of Pex15D30 correlates with its degradation by Msp1 (Fig 3 B and C). 3. D12T specifically impairs the degradation of multiple substrates with positively-charged IMS tails (Pex15D30, Frt1 and substrates in Fig. 3C) but does not affect the degradation of Ysy6 without IMS charges (Fig 6 D and E). Again, evidence for a specific role of the D12 residue for substrate binding is not convincingly shown.

We have shown in Fig. 1D lane 5 that D12T mutation decreases the binding of Msp1^{E193Q} (substrate-trap mutant) to Pex15D30. We also show in the reply to question 6 that D12T mutation causes substrate accumulation in cells but the mutant only has background interaction with substrate as WT Msp1 does, further supporting a defect of D12T mutant in substrate interaction.

6. Along the same lines: does the D12 mutant still bind Tom5/Gem1 or Fis1? The experiment shown in Fig. 3D could be performed with a D12T variant to test substrate binding of this mutant.

As shown below, the D12T mutation strongly stabilizes substrate Pex15-Gem1-OTS (lane 1 vs. 2), but did not proportionally enhance Msp1 interaction with substrate (lane 4 vs. 5). Thus, D12T mutation does not enhance substrate binding, and if we take enhanced substrate level into account, D12T mutation actually decreases substrate interaction, which is consistent with its proposed function. Substrate trap mutant E193Q is shown as a positive control for substrate interaction (lane 6).



7. In Fig. 4, the authors study the relevance of a cytosol-exposed hydrophobic patch for Pex15 degradation. Insertion of charged residues into this patch causes mislocalization to the cytosol and abrogates Pex15 association with mitochondria. The authors therefore used alanine mutants which are associated with mitochondria but not degraded anymore by Msp1 (but by the proteasome). The authors conclude that the patch is critical for (direct) Msp1 recognition. However, isn't it likely that this patch just downstream of the TMD forms a membrane-associated helix? Removing this stretch might impact Msp1-mediated degradation even if this region is not directly recognized by Msp1.

We understand and appreciate the referee's concern that mutating or deleting the hydrophobic patch of Pex15D30 may cause indirect effects, such as disturbing the true sequence recognized by Msp1. Our hypothesis that Msp1 may recognize this hydrophobic patch is based on a series of consistent results: 1. Hydrophobic residues in Msp1 N-domain is critical for substrate recognition (Fig 1). 2. Deleting the hydrophobic patch or reducing the hydrophobicity of the patch abolishes recognition and degradation of Pex15D30 by Msp1 (Fig. 4 A-E). 3. Most importantly, inserting the hydrophobic patch into mitochondrial TA proteins can turn them into Msp1 substrates and the conversion strictly requires patch hydrophobicity (Fig. 4 F-H). In the last experiment, we inserted the patch into two mitochondrial TA proteins with totally different sequences. The chance is very low that the patch happened to indirectly create Msp1 recognition motifs in unrelated sequences. Thus, the concern can be reduced by these consistent results.

8. For the interpretation of the data it is essential that all the protein versions used here are correctly localized in the outer membrane. The microscopic pictures are of convincing quality, however, they do not allow to assess whether the GFP tagged proteins are really correctly inserted into the OMM. From the data shown it cannot be excluded that some of the proteins used here are for example associated with the mitochondrial surface or mis-targeted to other mitochondrial compartments. In the past, methods had been developed by the Rapaport lab to validate whether tail anchored OMM proteins are correctly inserted, for example by cysteine-accessibility assays.

We have generated more than 100 Msp1 and substrate mutants and are not sure which protein is of particular concern about mistargeting. TA protein targeting is mediated by the transmembrane

segment and the flanking sequences (Borgese et al., JCB 2003; Rapaport, EMBO Rep 2003). The targeting sequences of TA proteins in Fig EV 3 B-F and Fig EV5 are intact.

Minor points

9. Fig. S1B: The labeling of the samples used for figure S1B is odd. How can WT be also msp1? What is the difference between lines 9 to 12 and 13 to 16?

We have labeled the genotype as : *msp1D* + Msp1-GFP rescue, "WT" means *msp1D* is rescued with wildtype Msp1-GFP. Lanes 9-12 and 13-16 are identical. We have deleted lanes 13-16 to simplify the figure.

In order to assess the expression levels of Msp1 in the different strains (WT, knockin and epichromosome) it will be essential to show Western blots with Msp1-specific antibodies.

Thanks for the suggestion. We have revised **Fig EV1D** as suggested.

10. Page 3: „OMM contains essential TA proteins, including the fission receptor..." None of the factors described here is essential with the exception of Tom22. Thus "essential" should be replaced by "important" or something similar.

Thanks for the suggestion. We have revised the manuscript as suggested.

11. Page 4: "dimmers" should read "dimers"

Thanks for the suggestion. We have revised the manuscript as suggested.

Referee #3:

The AAA ATPase Msp1 extracts mistargeted tail-anchored proteins from the mitochondrial outer membrane for degradation. How Msp1 recognizes mislocalized proteins remained unknown. Using an impressive set of experiments Li and colleagues report that Msp1 binds to two regions of their substrates: a hydrophobic patch in the cytosolic exposed domain and positive charged residues exposed to the intermembrane space. Performing localization studies of a number of tail-anchored proteins in the a get3msp1 double knock out , the authors identified two additional Msp1 substrates. The findings shed new light how Msp1 recognize mislocalized proteins. Overall, the major conclusions are well-based on an elegant set of genetic and biochemical assays. The presented data are of high quality and the findings are very interesting. There are few points that should be addressed before publication.

The authors identified a hydrophobic patch in GFP-Pex15Δ30, which is the main recognition site for Msp1. How conserved is this sequence among tail-anchored proteins? Is it also present in the two newly identified Msp1 substrates Frt1 and Ysy6? Do mitochondrial tail-anchored proteins like shown for Gem1 in general lack this patch and therefore escape degradation by Msp1?

Thanks for asking these great questions. The hydrophobic patch of Pex15 is not conserved among other TA proteins, including mitochondrial TA proteins and Msp1 substrates Frt1 and Ysy6. Frt1 and Ysy6 do not have hydrophobic patch near the TM segment. Ysy6 contains a small and highly-hydrophilic cytoplasmic domain with distributed hydrophobic residues. We have generated a new **Fig 6B** to illustrate sequence hydrophobicity of Frt1 and Ysy6 and have added the information to the manuscript.

Because the hydrophobic residues in Msp1 N-domain, such as Y72, V81, and I93, are generally required for the degradation of all the substrates, the hydrophobic surfaces exposed by substrates may consist of localized (Pex15) or distributed (Frt1 and Ysy6) hydrophobic residues. Pex15 is a special substrate that has a short hydrophobic patch to be easily identified and functionally confirmed.

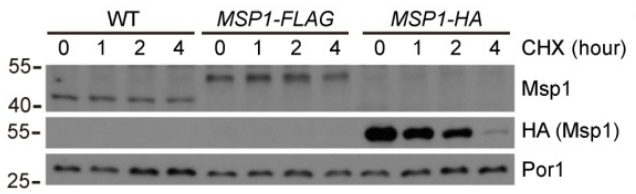
The authors should speculate why only a few tail-anchored proteins mislocalized to mitochondria, while the majority is not. Do the other tail-anchored proteins contain the hydrophobic patch?

Most TA proteins did not mislocalize to mitochondria in *get3D* cells probably because there are additional complexes to target TA proteins to the ER, such as the SND (Aviram et al., Nature 2016) and the EMC (Guna et al., Science 2018) complexes. We have added this to the manuscript (**page 12, the last 4 lines of the 2nd paragraph**).

All experiments were performed with tagged Msp1 variants. Previously, the authors showed that tagged Msp1 is rapidly degraded (Wu et al., J. Cell Biol. 2016). To exclude indirect effect due to altered stability of Msp1 variants, the authors have to analyze the stability of the Msp1 variants.

Yes, in Wu et al., J. Cell Biol. 2016, Msp1-HA had fast turnover rate and was a Doa1-Cdc48 substrate. At that time, Msp1-HA was not further characterized as other substrates. After Msp1 antibody was developed in the lab last year, we found Msp1 is stable and the HA tag destabilized the protein.

As shown below, FLAG and HA tagged Msp1 variants were probed with anti-Msp1 antibody, Msp1-FLAG was as stable as untagged Msp1. Msp1-HA was unstable (shown by the anti-HA blot) and had reduced protein level that can hardly be detected by our anti-Msp1 antibody. Thus, Msp1 stability is not affected by the FLAG tag. In addition, in Figs 1B and 3A, we have examined the stability of FLAG tagged Msp1 variants upon CHX treatment. All the FLAG tagged Msp1 variants are as stable as Msp1-FLAG.



Furthermore, the authors should confirm that selected untagged Msp1 variants similarly affect the stability of the Pex15Δ30 substrate like the tagged Msp1 variants.

We examined the degradation of GFP-Pex15D30 in WT, *msp1*^{V81A}, and *msp1*^{I93A} cells, with *msp1*^{E193Q}*-FLAG* cells as control. The turnover of GFP-Pex15D30 was impaired in cells with untagged mutant Msp1. We have supplemented the result as **Fig EV2C**.

In Figure 6A, Far10, Sps2 and Vps64 should be removed since no GFP-signal is detected.

Thanks for the suggestion. We have deleted Far10, Sps2 and Vps64 from Fig. 6A as suggested.

In Figure S5C, a wild-type control has to be added to show that the proteins are mislocalized to mitochondria in the mutant strains.

In Fig S5 (now Fig EV5), we overexpressed non-mitochondrial TA proteins in *get3Dmsp1D* cells to select candidates that mislocalize to mitochondria and obtained eight such proteins as shown in Fig S5C. These eight TA proteins were subsequently examined more rigorously at physiological expression level in Fig 6A. Because we have done the complete set of control in Fig 6A (WT, *get3D*, and *msp1D* cells), which is more informative than the suggested control for Fig S5C, we wish to keep Fig S5C in line with Fig S5 A and B.

In Figures 1, 6 and S1, a Msp1L122,123D mutant is depicted, which is not described in the text. The authors should mention in the mnuascipt why this Msp1 mutant was selected for their experiments.

Thanks for the suggestion. We have added a description in **page 5, lines 4-5 of the 2nd paragraph**.

Additional information has to be added to the figure legends. For instance, how much load and elution fraction is shown for the pulldown assays.

Thanks for the suggestion. We have revised the manuscript as suggested.

The manuscript (e.g. text and table S1) contains a few typos that need to be corrected.

Thanks for the suggestion. We have corrected the typos in the text and Tables.

2nd Editorial Decision 24 January 2019

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and support publication in EMBO reports without further revision.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can

proceed with the official acceptance of your study.

- Appendix: Please add page numbers to the table of content.

- Appendix figure S2 B, D: please indicate the number of experiments the quantification is based on and the meaning of the error bars. As far as I can see the graphs contain error bars?

- Please also upload the running title in our manuscript tracking system.

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- Please note that all corresponding authors are required to provide an ORCID ID for their name. This information is currently missing for Dr. Xi Wu. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

- You mention "data not shown" on page 7 and page 12. Please note that our editorial policies do not allow this. Therefore, please provide the data supporting the respective statements (or remove them from the article).

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- Our data editors from Wiley have already inspected the Figure legends for completeness and accuracy. Please see their suggested changes in the attached Word file. I have also taken the liberty to make some changes to the Abstract. Could you please review it and amend as you see best fit?

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS
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Referee #1:

This is a revised version of the manuscript that was previously submitted to EMBO Reports. The authors addressed most of my concerns raised before by performing several new experiments and the manuscript has been improved substantially. Some of the suggested experiments did not work, but due to understandable reasons, which was clearly explained. This is an important work that needs to be published urgently.

Referee #2:

The authors satisfyingly addressed the crucial points that I raised on the initial submission as far as this was possible. This study will help to better understand the molecular details by which the AAA protein Msp1 recognizes mistargeted TA proteins on the mitochondrial surface. I recommend publication of this study in its present form.

Referee #3:

In the revised version the authors addressed my comments satisfactorily. Importantly, they showed

that non-tagged versions of Msp1 similarly affect the stability of the Pex15delta30 substrate. Furthermore, the Flag-tagged Msp1 is not destabilized compared to endogenous Msp1, excluding indirect effects on its functionality due to Flag-tagging. The authors now clarify that Msp1 recognizes hydrophobic residues of its substrates since the hydrophobic patch of Pex15delta30 is not conserved among other tail-anchored proteins. Overall, the presented findings provide important insights how Msp1 recognizes mislocalized proteins in the outer mitochondrial membrane.

2nd Revision - authors' response 28 January 2019

The authors performed all minor editorial changes.

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Corresponding Author Name: Hui Jiang
Journal Submitted to: EMBO Reports
Manuscript Number:EMBOR-2018-46989

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	To quantify the percentage of cells with mitochondrial GFP-Pex15 in Fig 5D, we repeated the experiment 3 times. In each experiment, we quantified more than 100 cells for each genotype. Similarly in Fig 6E, experiments were repeated 3 times with more than 50 cells counted each time. Results were shown as means ± SEM. The error bars show the deviation between experiments is very small and experiments are of good reproducibility at the chosen sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	We calculated Stand Error of Mean (SEM) of the statisitical results.
Is the variance similar between the groups that are being statistically compared?	Yes. The SEM error bars in Fig 5D and 6E show the deviation between experiments is small and experiments are of good reproducibility.

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http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies: G6PDH (A9521, Sigma-Aldrich), HA-peroxidase (H6533, Sigma-Aldrich), and FLAG M2 (F1804, Sigma-Aldrich) Por1 (459500, Life Technologies), GFP (11814460001, Roche)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA