The ER protein Ema19 facilitates the degradation of non-imported mitochondrial precursor proteins

Janina Laborenz, Yury Bykov, Katharina Knöringer, Markus Räschle, Sabine Filker, Cristina Prescianotto-Baschong, Anne Spang, Takashi Tatsuta, Thomas Langer, Zuzana Storchová, Maya Schuldiner, and Johannes Herrmann

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RE: Manuscript #E20-11-0748
TITLE: The ER protein Ema19 facilitates the degradation of non-imported mitochondrial precursor proteins

Dear Dr. Herrmann,

Two expert reviewers have evaluated your manuscript, and I am happy to report that both of them are basically enthusiastic. But as usual, they have suggestions for improvement. Reviewer #1 in particular has a number of concerns about the interpretation and the data presentation.

I will ask you to consider these comments carefully and make changes as appropriate to strengthen the manuscript. If you choose not to follow a particular recommendation, your reasoning should be explained in the cover letter for the revision.

I look forward to seeing a revised manuscript.

Best regards,
Ben Glick

Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Herrmann,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Protein targeting to precise locations is essential for cellular normal functions. In this study, Laborenz found a novel role of the ER integral membrane protein Ema19 in the clearance of mistargeted mitochondrial proteins. Ema19 was initially obtained by screening for protein depletion of which retarded import of mitochondrial proteins in vivo by using Ura3-fused mitochondrial precursor proteins expressed in ura3Δ cells. Here they found that in the absence of Ema19, several mitochondrial precursor proteins were stabilized, indicating the possibility that Ema19 promotes degradation of mitochondrial precursor proteins, likely their non-productive or mistargeted species. In particular, they found that newly synthesized ΔN-Oxa1, a non-mitochondrially targeted variant of Oxa1, was more rapidly degraded in the presence of Ema19 and that Ema19 was mainly localized in the ER. Thus, the authors speculated that Ema19 somehow interacts in the ER with mitochondrial proteins mistargeted to the ER, and facilitates their degradation. This is an interesting observation and would contribute to understanding the mechanism of how mistargeted mitochondrial precursor proteins are cleared in the cell. However, I am afraid that there is much room to be improved in this manuscript since mechanisms of how Ema19 works for degradation of presumably mistargeted mitochondrial precursor proteins are not clear. Perhaps the role of Ema19 is just indirect. In particular, I would like to see the following points more clearly shown in the manuscript.

(1) The authors' interpretation strongly relies on the exclusive localization of Ema19 on the ER. In other words, it is important to rule out such a possibility that a minor fraction of Ema19 is localized to mitochondria, while a major fraction of Ema19 can be found in the ER, and that the observed interactions of Ema19 with mitochondrial precursor proteins merely reflect those on mitochondria. In this sense, the microscopic images to show the localization of Ema19-EGFP (Figs. 2C and S4B) were too small. The authors should also eliminate such a mere possibility that attachment of the fluorescent protein to Ema19 could affect its localization to the ER.
(2) It would be nice to see if overexpression of Ema19 in wild-type cells would promote degradation of ΔN-Oxa1 and Erv1. This will complement the present observation by using ema19Δ cells. In relation to this, does overexpression of Ema19 in djp1Δ cells promote degradation of the Oxa1 precursor form? Does overexpression of Ema19 on the ura3Δ background with expressed Oxa1-Ura3 cause defects in the cell growth?
(3) Are IMS proteins other than Erv1 also affected by Ema19 depletion?
(4) It is not clear if the role of Ema19 in aberrant protein degradation is specific to mistargeted mitochondrial proteins. Does Ema19 depletion affect ERAD of aberrant ER proteins like Ste6* and CPY*?
(5) The quality or resolution of the EM images in Figs. 3B (no explanation for the arrows in the legend) and S4A is poor so that it is difficult to examine the strange internal structure of ema19Δ cells grown on glycerol. The shapes of organelles should be observed by fluorescence microscopy, too.

Other points.
(6) Fig. 1B, C - Microscopic images to show that Oxa1-split GFP is indeed visible on the ER are required.
(7) Figs. 1E and S1C - Comparison of ema19Δ cells with wild-type cells is required. The shapes of the cells should be indicated.
(8) Fig. 3 - Description of the methods for this figure (EM and lipidomics analysis) is too simple. More explanation should be included.
(9) Fig. 6B - The presence of non-degraded Erv1 on the ER should be shown.
(10) The numbers of experiments to check reproducibility are not clearly indicated.

Reviewer #2 (Remarks to the Author):

The manuscript by J. Laborenz et al. follows-up on the previous work by the same consortium (Hansen et al., Science 361, 1118-1122; 2018), which discovered a novel pathway for membrane targeting of mitochondrial precursor proteins (termed ER-SURF) and associated the co-chaperone Djp1 plus three so-called Ema proteins with this pathway. In the present comprehensive manuscript, Ema19 is the focus of interest.

Based on a series of well planned and executed biochemical, cell-biological, and genetic experiments the authors conclude that Ema19 is indeed involved in the ER-SURF pathway and plays a role in the degradation of on-imported mitochondrial precursor proteins on the ER surface.

Major points:
While the first conclusion is based on solid data, the second one leaves room for improvement. The latter conclusion heavily relies on Figure 6A and B, for which it remains open how many times the experiments were repeated. In fact, the two panels would benefit from an additional graphical representation with error bars. Furthermore, Hansen et al. suggested that Cdc48 and the proteasome are involved in the degradation of non-imported mitochondrial precursor proteins on the ER surface. Therefore, it would be exciting to know whether Ema19 is tied into the same or an alternative degradative pathway. At least, that should be re-visited in the Discussion.

Minor points:
On page 3, last section, first line, `by´ should be replaced by `be´.
On page 5, second paragraph, last line, `ER/stability´ should read `ER or stability´.
Table S5 is missing from the manuscript.

The ER protein Ema19 facilitates the degradation of non-imported mitochondrial precursor proteins

Janina Laborenz et al.

Point-by-point response to the comments raised on the initial submission:

Reviewer #1

Protein targeting to precise locations is essential for cellular normal functions. In this study, Laborenz found a novel role of the ER integral membrane protein Ema19 in the clearance of mistargeted mitochondrial proteins. Ema19 was initially obtained by screening for protein depletion of which retarded import of mitochondrial proteins in vivo by using Ura3-fused mitochondrial precursor proteins expressed in ura3Δ cells. Here they found that in the absence of Ema19, several mitochondrial precursor proteins were stabilized, indicating the possibility that Ema19 promotes degradation of mitochondrial precursor proteins, likely their non-productive or mistargeted species. In particular, they found that newly synthesized ΔN-Oxa1, a non-mitochondrially targeted variant of Oxa1, was more rapidly degraded in the presence of Ema19 and that Ema19 was mainly localized in the ER. Thus, the authors speculated that Ema19 somehow interacts in the ER with mitochondrial proteins mistargeted to the ER, and facilitates their degradation. This is an interesting observation and would contribute to understanding the mechanism of how mistargeted mitochondrial precursor proteins are cleared in the cell. However, I am afraid that there is much room to be improved in this manuscript since mechanisms of how Ema19 works for degradation of presumably mistargeted mitochondrial precursor proteins are not clear. Perhaps the role of Ema19 is just indirect. In particular, I would like to see the following points more clearly shown in the manuscript.

We thank the referee for her/his positive comments. We agree with the referee that the mechanisms by which Ema19 promotes precursor degradation are still to a large part elusive. We addressed her/his specific points as described in the following:

(1) The authors' interpretation strongly relies on the exclusive localization of Ema19 on the ER. In other words, it is important to rule out such a possibility that a minor fraction of Ema19 is localized to mitochondria, while a major fraction of Ema19 can be found in the ER, and that the observed interactions of Ema19 with mitochondrial precursor proteins merely reflect those on mitochondria. In this sense, the microscopic images to show the localization of Ema19-EGFP (Figs. 2C and S4B) were too small. The authors should also eliminate such a mere possibility that attachment of the fluorescent protein to Ema19 could affect its localization to the ER.

It is formally impossible to exclude that very minor amounts of Ema19 are present in mitochondria. However, there is no indication for the presence of mitochondrial Ema19 for several reasons: (1) Yeast cells expressing N-terminally tagged GFP-Ema19 only showed an ER staining but no staining in mitochondria (as shown in our study). (2) Yeast cells expressing C-terminally tagged Ema19-GFP likewise only showed fluorescence in the ER but not in mitochondria (as shown in our study). (3) In highly purified mitochondria, Ema19 was not identified by mass spectrometry (Morgenstern et al. 2017 Cell Reports 19, 2836-2852). This study contains the most comprehensive and thoroughly characterized proteome of mitochondria. (4) In human cells, Ema19 contains a C-terminal ER retrieval signal

characteristic for resident ER proteins. (5) Ema19 does not have a mitochondrial targeting sequence according to prediction programs such as TargetP. (6) Our observation, that Ema19 plays a role in the removal of non-imported mitochondrial precursors is also consistent with a localization of Ema19 outside of mitochondria.

In order to follow the suggestion of the referee, we now increased the size of the graphs which show the localization of Ema19 (Fig. 2C, Fig. S5).

(2) It would be nice to see if overexpression of Ema19 in wild-type cells would promote degradation of ΔN-Oxa1 and Erv1. This will complement the present observation by using ema19Δ cells. In relation to this, does overexpression of Ema19 in djp1Δ cells promote degradation of the Oxa1 precursor form? Does overexpression of Ema19 on the ura3Δ background with expressed Oxa1-Ura3 cause defects in the cell growth?

We agree that there will additional experiments necessary to elucidate the molecular function of Ema19 for quality control of mitochondrial precursor proteins. However, to our experience, the overexpression of ER proteins is often problematic as this might affect protein localization and function. We therefore felt, that it would be difficult to draw clear-cut solutions of such an experiment. We therefore decided not to overexpress Ema19. As now shown in Fig. 5A, we found that Ema19 interacts with the ER protein Spf1 which recently was identified as an extractor of mitochondrial precursor proteins in case these proteins are mislocalized to the ER surface. We will follow this interesting link up in the future but felt that it would go beyond this initial study.

However, we performed another experiment to better define the client spectrum of Ema19. To this end, we treated cells for 1 h with the uncoupler CCCP to dissipate the mitochondrial membrane potential before we identified Ema19 interactors by mass spectrometry. Under these conditions, we observed a considerable increase in the number of mitochondrial proteins that were associated with Ema19. This helps to define the client spectrum of Ema19 and supports our model according to which Ema19 interacts with extra-mitochondrial precursor proteins. These novel data are now shown as novel Figs. 5C and S6, and as additional data in Table S5.

(3) Are IMS proteins other than Erv1 also affected by Ema19 depletion?

Yes, also other the levels of other IMS proteins are diminished. We added the results for the IMS proteins Atp23 and Cmc1 to Fig. 4A, which shows that their levels are also reduced in *Δema19* cells.

(4) It is not clear if the role of Ema19 in aberrant protein degradation is specific to mistargeted mitochondrial proteins. Does Ema19 depletion affect ERAD of aberrant ER proteins like Ste6* and CPY*?

Several screens had been performed in the past to screen for components that are involved in ER-associated protein degradation. Ema19 was not picked up in these screens. However, *EMA19* and the ERAD gene *UBC6* were reported to genetically interact in a screen for epistatic interactions of ER components (Schuldiner et al. 2005. Cell 123, 507-519).

(5) The quality or resolution of the EM images in Figs. 3B (no explanation for the arrows in the legend) and S4A is poor so that it is difficult to examine the strange internal structure of ema19Δ cells grown on glycerol. The shapes of organelles should be observed by fluorescence microscopy, too.

We now made additional EM images from *Δema19* cells and show more images in Fig. S4. These images also include blow-ups to make the peculiar structures better visible to our readers. Since these structures are not visible in light microscopy and since the inclusions are not stained with BODIPY 493/503, we were unable to show these structures by fluorescence microscopy. We now also refer to the arrows in the legend of Fig. 3B.

Other points.
(6) Fig. 1B, C - Microscopic images to show that Oxa1-split GFP is indeed visible on the ER are required.

These images are seen as Figure 1E and Fig. S1C.

(7) Figs. 1E and S1C - Comparison of ema19Δ cells with wild-type cells is required. The shapes of the cells should be indicated.

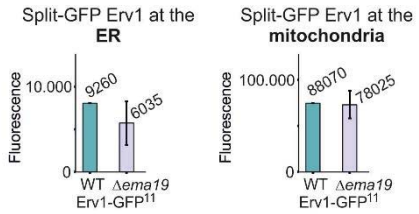
Images of both cell types are already shown as well as quantifications (as Fig. 1C, D).

(8) Fig. 3 - Description of the methods for this figure (EM and lipidomics analysis) is too simple. More explanation should be included.

We added further information to the legend of Figure 3 as suggested.

(9) Fig. 6B - The presence of non-degraded Erv1 on the ER should be shown.

We used the split-GFP approach to detect Erv1 on the ER. As shown in the Figure for inspection by the referee, this experiment suggests that Erv1 is also in proximity to the ER surface. The signal on the ER is about one tenth of that in the IMS of mitochondria (Mia40-GFP$^{1-10}$). This confirms the conclusion of this study that a considerable fraction of Erv1 comes into contact with the ER.



(10) The numbers of experiments to check reproducibility are not clearly indicated.
We now included this information

Reviewer #2

The manuscript by J. Laborenz et al. follows-up on the previous work by the same consortium (Hansen et al., Science 361, 1118-1122; 2018), which discovered a novel pathway for membrane targeting of mitochondrial precursor proteins (termed ER-SURF) and associated the co-chaperone Djp1 plus three so-called Ema proteins with this pathway. In the present comprehensive manuscript, Ema19 is the focus of interest.
Based on a series of well planned and executed biochemical, cell-biological, and genetic experiments the authors conclude that Ema19 is indeed involved in the ER-SURF pathway and plays a role in the degradation of on-imported mitochondrial precursor proteins on the ER surface.

We thank the referee for her/his very positive comments.

Major points:
(1) While the first conclusion is based on solid data, the second one leaves room for improvement. The latter conclusion heavily relies on Figure 6A and B, for which it remains open how many times the experiments were repeated. In fact, the two panels would benefit from an additional graphical representation with error bars. Furthermore, Hansen et al. suggested that Cdc48 and the proteasome are involved in the degradation of non-imported mitochondrial precursor proteins on the ER surface. Therefore, it would be exciting to know whether Ema19 is tied into the same or an alternative degradative pathway. At least, that should be re-visited in the Discussion.

We included the graphs with mean values and error bars as suggested. We also discussed a potential role of ERAD/Cdc48, of autophagy as well as of Spf1 as suggested.

In order to present further evidence for the function of Ema19, we added an additional proteomics experiment. To this end, we measured interactors of Ema19-GFP after treatment of cells for h our with CCCP. Uncoupling of the mitochondrial membrane potential increased the number of mitochondrial proteins that were co-isolated with Ema19. This nicely supports the proposed role of Ema19 as a factor that takes care of mitochondrial proteins that are stranded on the ER surface.

Minor points:
(2) On page 3, last section, first line, `by´ should be replaced by `be´.

We corrected this typo.
(3) On page 5, second paragraph, last line, `ER/stability´ should read `ER or stability´.

We corrected this typo.
(4) Table S5 is missing from the manuscript.

Table S5 is an additional Excel data with the mass spectrometry data. This table is provided as extra document and therefore missing in the pdf. However, it was uploaded together with the manuscript.

RE: Manuscript #E20-11-0748R
TITLE: "The ER protein Ema19 facilitates the degradation of non-imported mitochondrial precursor proteins"

Dear Dr. Herrmann,

As you will see, Reviewer #1 still has a few comments about issues that were not fully addressed in the revision. Please address these comments with changes to the text and figures, and with new experiments at your discretion, and then summarize the changes in a cover letter. I will evaluate the re-revised manuscript and render a decision.

Thanks for your patience with this process.

Best regards,
Ben Glick

Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Herrmann,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The authors responded to most of my concerns and questions. However, there are still points that need further responses from the authors.

(2) My comment: It would be nice to see if overexpression of Ema19 in wild-type cells would promote degradation of ΔN-Oxa1 and Erv1. This will complement the present observation by using ema19Δ cells. In relation to this, does overexpression of Ema19 in djp1Δ cells promote degradation of the Oxa1 precursor form? Does overexpression of Ema19 on the ura3Δ background with expressed Oxa1-Ura3 cause defects in the cell growth?
Response: These novel data are now shown as novel Figs. 5C and S6, and as additional data in Table S5.
My second comment: Fig. S6 should read Fig. S7.

(3) My comment: Are IMS proteins other than Erv1 also affected by Ema19 depletion?
Response: We added the results for the IMS proteins Atp23 and Cmc1 to Fig. 4A
My second comment: I could not find the results in Fig. 4A.

(6) My comment: Fig. 1B, C - Microscopic images to show that Oxa1-split GFP is indeed visible on the ER are required.
Response: These images are seen as Figure 1E and Fig. S1C.
My second comment: I meant as follows. Although the authors stated, "Using the precursor form of Oxa1 we only detected very low fluorescent levels in the WT but these were slightly increased in Δema19 cells (Fig. 1C, S1C). Consistent with previous reports, our data indicate that under normal conditions Oxa1 precursors associate with the ER surface only very transiently ", but it is difficult to judge that Oxa1-split GFP was localized in the ER in Fig. S1C.

(7) My comment: Figs. 1E and S1C - Comparison of ema19Δ cells with wild-type cells is required. The shapes of the cells should be indicated.
Response: Images of both cell types are already shown as well as quantifications (as Fig. 1C, D).
My second comment: I meant that the shapes of the cells had better be outlined by dotted lines or something.

(9) Comment: Fig. 6B - The presence of non-degraded Erv1 on the ER should be shown.
Response: We used the split-GFP approach to detect Erv1 on the ER. As shown in the Figure for inspection by the referee, this experiment suggests that Erv1 is also in proximity to the ER surface. The signal on the ER is about one tenth of that in the IMS of mitochondria (Mia40- GFP1-10). This confirms the conclusion of this study that a considerable fraction of Erv1 comes into contact with the ER.
My second comment: Interpretation of the split-GFP approach is not simple. I suggest the authors detect non-degraded Erv1 in the ER with antibodies against Erv1 or a tag attached to Erv1 by organelle fractionation or indirect fluorescence microscopy.

Specific points

Referee #1

The authors responded to most of my concerns and questions. However, there are still points that need further responses from the authors.

(2) My comment: It would be nice to see if overexpression of Ema19 in wild-type cells would promote degradation of ΔN-Oxa1 and Erv1. This will complement the present observation by using ema19Δ cells. In relation to this, does overexpression of Ema19 in djp1Δ cells promote degradation of the Oxa1 precursor form? Does overexpression of Ema19 on the ura3Δ background with expressed Oxa1-Ura3 cause defects in the cell growth?
Response: These novel data are now shown as novel Figs. 5C and S6, and as additional data in Table S5.
My second comment: Fig. S6 should read Fig. S7.

The referee is correct, this novel figure mentioned in the letter is S7. The text in the manuscript, though, was correct and did not need any correction.

(3) My comment: Are IMS proteins other than Erv1 also affected by Ema19 depletion?
Response: We added the results for the IMS proteins Atp23 and Cmc1 to Fig. 4A
My second comment: I could not find the results in Fig. 4A.

The novel figures were attached as TIF files and showed the signals for Atp23 and Cmc1 correctly. The figure embedded into the word file had not been replaced, though. We changed this now, too.

(6) My comment: Fig. 1B, C - Microscopic images to show that Oxa1-split GFP is indeed visible on the ER are required.
Response: These images are seen as Figure 1E and Fig. S1C.
My second comment: I meant as follows. Although the authors stated, "Using the precursor form of Oxa1 we only detected very low fluorescent levels in the WT but these were slightly increased in Δema19 cells (Fig. 1C, S1C). Consistent with previous reports, our data indicate that under normal conditions Oxa1 precursors associate with the ER surface only very transiently ", but it is difficult to judge that Oxa1-split GFP was localized in the ER in Fig. S1C.

These graphs show labeling experiments with split GFP fusions in which one part of GFP was on the cytosolic part of the ER protein Sec63, and the second part was fused to an Oxa1 version which was expressed without presequence. Since Sec63 is a resident and well-established ER protein, fluorescence is only seen here when the second part of the split GFP reporter (that on ΔN-Oxa1) is in proximity to the ER surface. Thus, signal intensity directly correlates with the amount of ΔN-Oxa1 on the ER. Moreover, the distribution of the GFP signal nicely shows the characteristic staining of ER in yeast cells (perinuclear and underneath the plasma membrane).

(7) My comment: Figs. 1E and S1C - Comparison of ema19Δ cells with wild-type cells is required. The shapes of the cells should be indicated.
Response: Images of both cell types are already shown as well as quantifications (as Fig. 1C, D).
My second comment: I meant that the shapes of the cells had better be outlined by dotted lines or something.

We added dotted lines into the figures as requested by the referee.

(9) Comment: Fig. 6B - The presence of non-degraded Erv1 on the ER should be shown.
Response: We used the split-GFP approach to detect Erv1 on the ER. As shown in the Figure for inspection by the referee, this experiment suggests that Erv1 is also in proximity to the ER surface. The signal on the ER is about one tenth of that in the IMS of mitochondria (Mia40- GFP1-10). This confirms the conclusion of this study that a considerable fraction of Erv1 comes into contact with the ER.
My second comment: Interpretation of the split-GFP approach is not simple. I suggest the authors detect non-degraded Erv1 in the ER with antibodies against Erv1 or a tag attached to Erv1 by organelle fractionation or indirect fluorescence microscopy.

We now performed this experiment as suggested by the referee and added it as novel Figure S6A.

RE: Manuscript #E20-11-0748RR
TITLE: "The ER protein Ema19 facilitates the degradation of non-imported mitochondrial precursor proteins"

Dear Dr. Herrmann,

I appreciate your attention to those final issues, and I am pleased to accept the revised manuscript for publication.

Thanks for sending this nice work to MBoC.

Sincerely,
Benjamin Glick
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Herrmann:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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