nature portfolio

Peer Review File

Oligomer-based functions of mitochondrial porin

Corresponding Author: Professor Toshiya Endo

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The work of Takeda et al. “Oligomer-based function of mitochondrial porin” provides novel insights into the oligomeric structure of the mitochondrial porin (Por1 in yeast and VDAC1 in human). The authors determined by cryo-EM the hexameric structure of yeast Por1, an oligomeric conformation never observed before, confirmed also by in vivo crosslinking experiments. This represents a more accurate description of the physiological conformation of such protein, since it has been obtained with protein purified from isolated yeast mitochondria, rather than with proteins reconstituted into lipid or detergent micelles. This novel hexameric structure provides crucial details which allow to better understand the function of a protein crucial in regulating crosstalk between cytosol and mitochondria. This will have a broader impact to the mitochondria community shedding light on the interconnection between different protein complexes in mitochondria and their involvement in multiple activities.
Based on the structural detail the authors reveal three novel roles of the porin oligomeric complex, independent from transport: regulation of mitochondria protein import, mitochondrial lipid composition, and retention of mitochondrial DNA. These are very intriguing findings that open novel avenues in the understanding of the regulation of mitochondria and revealing the key role of Porin in coordinating with other mitochondria complexes. Nevertheless, it remains unclear how those very divers roles of the oligomeric Por1 are coordinated and regulated, and how the hexameric structure is important for such functions.
1. The interaction between Porin and the unassembled Tom22 controls the balance between dimeric and trimeric TOM complex and this in turn influences the preference on the import of matrix or intermembrane space (IMS) proteins. Additionally, mutations of Por1 that interfere with hexamer formation, but do not affect its transport activity, impair import of IMS but not matrix proteins, suggesting a shift towards the dimeric TOM complex (known to mediate import preferentially of IMS protein based on previous study form the same group). The authors conclude that Tom22 prefers binding to the hexameric form of Por1 rather than the monomer, however it is possible that the mutation directly affects the interaction with Tom22. Moreover, the shift towards the dimeric TOM complex is inferred from the import efficiency of Tom22 but never directly proved.
Comments:
- Can the authors show direct interaction between Tom22 and the hexameric form of Por1(WT)?
- Can the authors directly show reduction of dimeric TOM complex in por1D? Why the deletion of Tom6 does not rescue the assembly of TOM dimer in the Por1(KE) mutant?
Minor points:
- The quantifications of protein import experiments require error bars.
- ED fig. 4e (right panel) is missing the legend for the graph
- Fig. 2B: The growth defects on SCLac at 30C and 37C perfectly recapitulates Por1 protein levels (fig. 2a). However, on SCD at 37C the single mutation D228K has a stronger phenotype compared to the R252E or double mutant. Do the authors have an explanation for this?
2. Por1 hexamer is involved in regulation of mitochondria lipid composition by mediating phospholipid scrambling between the protomers BC and B’C’. This conclusion is supported by identification of densities between protomers in the cryo-EM structure, identified as phospholipids, by molecular simulations, and by mitochondrial lipid composition analysis in Por1 mutant.
Comments:
- If the hexameric structure of Por1 is essential for lipid scrambling activity, any mutation disrupting the oligomer assembly, but not the NAD+ transport (for example the D228K/R252E (KE) mutation), should also affect lipid composition. It would be important to test other mutants in addition to the TDQ to confirm the role of the hexameric Por1 per se rather than the specific residues in lipid scrambling.

- Based on the structure of Por1, can the authors speculate on why does the Q73E mutation enhances scrambling activity? Is the negative charge of E interfering with the negative charge of the phospholipid headgroup?
Minor points:
- ED fig 5b: In the figure legend it is not mentioned what the insets in the graph are. Supposably they are a scale up of some parts of the main graph, but it should be clarified in the figure legend. Are the differences between WT and mutants statistically significant?
- Fig 3c: statistical analysis is required, mostly for PA.
3. Por1 is involved in mitochondrial DNA retention. Although supported by solid data, in my opinion this is the least convincing part of the manuscript. It is based on a single mutant of Por1 which assembles in higher-molecular weight complexes (or aggregates) and leads to mtDNA loss. However, there is no discussion on how this mutation affects structure of hexameric Por1, and why only this mutant, and not other mutation, or Por1 deletion for example, causes loss of mtDNA. The authors moved on and focused more on identifying nucleases required for the loss of mtDNA in Por1(QA), identifying 7 proteins, named mtDNA-losing (MDL) proteins. These proteins seem to be localised in mitochondria and their loss seems to lead to accumulation of mtDNA in the periphery of the organelle.
Comments:
- Provide structural insights on the Po1(QA) mutant and the effect of only this mutation on mtDNA retention. Provide some speculation on the potential physiological role of Por1(WT) in mtDNA retention.
Minor points:
- Fig ED 7e: the localisation of Abf2 is not only in the mitochondrial periphery but it seems to be still retained in the mitochondria. I would suggest rephrasing it in the text.
- Do the MDL deletions have an effect on mtDNA localisation with Por1(WT) (ED Fig 9)?

Overall, the conclusions are well supported by the data and are confirmed by complementary methodological approaches. The experimental approach is solid and described in detail. The results are presented with clarity and with sufficient context and referring to the adequate literature.

(Remarks on code availability)
N/A

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
This reviewer provides a focused technical report primarily assessing the HS-AFM part of the manuscript:

Sample preparation, HS-AFM operational parameters and conditions used for simulating AFM images are sufficiently described. The interpretation of the Por1 hexamer orientation (i.e. cytosolic side up) on the APTES-mica surface is sound, however the difference of the total height between the experimental and simulated topography (ED Fig. 1g vs h; ~7 nm vs 5 nm) should be discussed.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
Takeda, Shinoda et al determined the structure of the mitochondrial porin, a VDAC consisting of hexameric Por1 by cryo-EM at 3.2A resolution.

This is an excellent study with thorough experiments combining biochemical and biophysical techniques including coarse-grained molecular dynamics. The paper is well and clearly written. I found it easy to follow and a pleasure to read. The figures are clear and useful. The insights about the three major functions – Por1 binding to Tom22, maintenance of lipid composition, and mtDNA regulation – are properly argued and backed up by well-designed experiments. The conclusions are justified. In particular, I found the MD-assisted data interpretation about lipid scrambling, and the mutagenesis to explain the role of mtDNA integrity resourceful and convincing. This study advances the knowledge of VDACs and their role in mitochondrial metabolism and will therefore appeal to a wide audience.

Congratulations. This reviewer has no major nor minor critique and supports publication of the manuscript without further delays.

(Remarks on code availability)
I don't see any flaws in the methods, although I am not an expert in MD and cannot comment about the technical details of the course-grained MD experiment. The cryo-EM image processing follows standard procedures and is flawless.

Reviewer #5

(Remarks to the Author)
This paper present interesting new data and represents a significant advance.
I have some minor comments specifically relating to the section of lipid scrambling.

ref 24 is Jahn et al. (it is currently written as Ahn et al. – please fix the typo)

Lines 169-170: this statement is not correct. Jahn et al. showed that the import of PS across the OM in isolated mitochondria was reduced by an order of magnitude in mitochondria lacking Por1

Lines 181-184: this is an interesting result; the beta 1,2,18,19 interface was seen in previous structural work (and also in the current paper) but may be more available to lipids in human VDAC1 assemblies than it seems to be in the Por1 hexamer. It would be worth saying more on this point.

Phospholipid scrambling is principally analyzed by CGMD in this paper, with outcomes validated indirectly through lipid compositional changes in Figure 3. The depression of CL and increase of PA levels in Por1(TDQ)-expressing mitochondria is certainly suggestive, but also surprising as not changes are seen in the level of PE, a lipid that is generated from imported PS. This point deserves discussion, especially in light of the result of Jahn et al. reporting PS transport deficiency in mitochondria lacking porin. Are redundant scramblases somehow compensating?

(Remarks on code availability)
not applicable

Reviewer #6

(Remarks to the Author)
In this article, Endo and colleagues convincingly show that Por1 can form functional hexamers in yeast mitochondria. First, they determine the cryo-electron microscopy structure of Por1 in its hexameric form at 3.2 Å resolution from direct purification of Por1 from yeast mitochondria. Next, they assess the potential role of Por1 hexameric structure in three different cellular processes in which Por1 had been previously implicated: assembly of the TOM complex, lipid scrambling and regulation of mitochondrial DNA. In all cases, through careful mutational studies, they show that the correct assembly of the Por1 oligomer is required for its proper function. Overall, the manuscript is convincing, carefully written, and well thought-out. The results presented are important for scientists studying transport processes in mitochondria and represent a wonderful functional application of structural observations.I thoroughly enjoyed reading it and I recommend publication with only a few minor comments outlined below.

Minor

- Extended Data Fig. 4e lacks labels (colors) to interpret the curves
- The sentence in the Methods section "Previous all-atom molecular dynamics (MD) simulations of mouse VDAC1 showed that single point mutations can either enhance or suppress lipid scrambling activity 26" is incorrect, as ref. 26 does not discuss lipid scrambling by VDAC1.
- I found that several supplementary figures contain important data to follow the overall flow of the manuscript, and I suggest the authors, if space allows, to move some of these data to the main figures, eventually adding a few of them.

Stefano Vanni

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors addressed all points raised and the revised manuscript is acceptable for publication

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
The authors have sufficiently addressed my suggestions. No further comments from my side.

Reviewer #5

(Remarks to the Author)
I have a minor point regarding the response to one of points.

The authors write:
Since we analyzed lipid compositions of total cells, the PE levels analyzed here could be affected by not only the PE biosynthesis pathway by Psd1 within mitochondria but also the one by Psd2 outside mitochondria (Kennedy pathway). The latter pathway may mask a subtle change in the PE level as compared with the CL level, which is synthesized only inside mitochondria.

This point should be included in the manuscript as it is rather important.

Reviewer #6

(Remarks to the Author)
The authors have successfully addressed all my (minor) comments. I congratulate them again for this beautiful piece of work.

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The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

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NCOMMS-24-86792-T

Point-by-point responses to the reviewers' comments

Reviewer #1:

The work of Takeda et al. "Oligomer-based function of mitochondrial porin" provides novel insights into the oligomeric structure of the mitochondrial porin (Por1 in yeast and VDAC1 in human). The authors determined by cryo-EM the hexameric structure of yeast Por1, an oligomeric conformation never observed before, confirmed also by in vivo crosslinking experiments. This represents a more accurate description of the physiological conformation of such protein, since it has been obtained with protein purified from isolated yeast mitochondria, rather than with proteins reconstituted into lipid or detergent micelles. This novel hexameric structure provides crucial details which allow to better understand the function of a protein crucial in regulating crosstalk between cytosol and mitochondria. This will have a broader impact to the mitochondria community shedding light on the interconnection between different protein complexes in mitochondria and their involvement in multiple activities.
Based on the structural detail the authors reveal three novel roles of the porin oligomeric complex, independent from transport: regulation of mitochondria protein import, mitochondrial lipid composition, and retention of mitochondrial DNA. These are very intriguing findings that open novel avenues in the understanding of the regulation of mitochondria and revealing the key role of Porin in coordinating with other mitochondria complexes. Nevertheless, it remains unclear how those very divers roles of the oligomeric Por1 are coordinated and regulated, and how the hexameric structure is important for such functions.

We were glad to hear these positive remarks.

1. The interaction between Porin and the unassembled Tom22 controls the balance between dimeric and trimeric TOM complex and this in turn influences the preference on the import of matrix or intermembrane space (IMS) proteins. Additionally, mutations of Por1 that interfere with hexamer formation, but do not affect its transport activity, impair import of IMS but not matrix proteins, suggesting a shift towards the dimeric TOM complex (known to mediate import preferentially of IMS protein based on previous study form the same group). The authors conclude that Tom22 prefers binding to the hexameric form of Por1 rather than the monomer, however it is possible that the mutation directly affects the interaction with Tom22. Moreover, the shift towards the dimeric TOM complex is inferred from the import efficiency of Tom22 but never directly proved.

The Por1 KE mutation impairs the Por1-Tom22 interaction, but this does not mean that other hexamer-destabilizing Por1 mutations show similar defective interactions with Tom22. We thus agree with the referee that the KE mutation likely and directly affects the interaction with Tom22, so that Tom22 released from the TOM complex would not be stabilized by Por1(KE) and the TOM complex would shift from the Tom22-lacking dimer to the Tom22-containing trimer.

The dimer-to-trimer shift (not the trimer-to-dimer shift) of the TOM complex due to a failure in the Por1 interaction with free Tom22 was previously shown (Sakaue et al., *Mol Cell* 2019). In this manuscript, we also showed that, while *tom6*Δ promoted the shift from the TOM trimer to the dimer, *tom6*Δ*por1(KE)* suppressed this trimer-to-dimer shift (Supplementary Fig. 4c in the revised manuscript), indicating that the KE mutation suppresses the trimer-to-dimer shift of the TOM complex. The 400K and 400K* bands correspond to the TOM trimer, and the 100K band represents the TOM dimer.

We have thus altered the text, taking into account of these points, in the revised manuscript.

Can the authors show direct interaction between Tom22 and the hexameric form of Por1(WT)?

It should be interesting to show the suggested interaction between free Tom22 and hexameric, not dissociated Por1 directly by cyro-EM structure determination or MD simulation. However, at the moment, we are uncertain how long it will take for these approaches. In addition, although the KI mutation destabilizes the Por1 hexamer, this may not mean that Tom22 binds to only hexamer, not dissociated form of Por1 as explained above. We have changed the text in the revised manuscript accordingly.

Can the authors directly show reduction of dimeric TOM complex in por1D? Why the deletion of Tom6 does not rescue the assembly of TOM dimer in the Por1(KE) mutant?

Since in wild-type cells, the TOM trimer is a major form, the minor fraction of the TOM dimer is not visible in BN-PAGE. Therefore, it is difficult to see the reduction of the minor fraction of the Por1 dimer in *por1*Δ cells. The reduction by Por1 deletion (and likely Por1(KE) mutant) of the TOM dimer can be seen only in the background of *tom6*Δ, which destabilizes the TOM trimer. This was shown in Fig. 2E in our previous paper (Sakaue et al., *Mol Cell* 2019). We also found that the *tom6*Δ did not rescue the assembly of TOM dimer in *por1*Δ cells (likely Por1(KE) cells, as well) and discussed its possible reason (Sakaue et al., Mol Cell 2019); "Since the lack of Por1 together with Tom6 or Tom22 impaired the import via the trimer or dimer of the TOM complex, respectively, Por1 may play an active role in generating the functional dimer and trimer of the TOM complex" (quoted from the above reference).

The quantifications of protein import experiments require error bars.

We repeated the import experiments and added error bars (Fig. 2f and g in the revised manuscript). We also confirmed that protein levels of the MIA pathway substrates were reduced in Por1(KE) as well as *por1*Δ mitochondria, as compared with those in WT mitochondria. These results were added as a new Fig. 2e to the revised manuscript.

ED fig. 4e (right panel) is missing the legend for the graph.

We are sorry for the lack of explanation of the color cord. We have added the explanation to Supplementary fig. 4e (right) in the revised manuscript.

Fig. 2B: The growth defects on SCLac at 30C and 37C perfectly recapitulates Por1 protein levels (fig. 2a). However, on SCD at 37C the single mutation D228K has a stronger phenotype compared to the R252E or double mutant. Do the authors have an explanation for this?

The reason for this observation is not clear, but the D228K mutation may enhance the interaction between Por1 and Tom22 through R252, which could lead to increased trapping of Tom22 by Por1, thereby slowing down the assembly of newly imported Tom22 into the TOM complex (Supplementary Fig. 4e, right in the revised manuscript) and hindering proper trimer formation of the TOM complex. In contrast, the Por1(R252E) or Por1(D228K/R252E) mutant lacks R252, preventing Por1 from effectively trapping free Tom22 or significantly delaying the assembly of newly imported Tom22 into the TOM complex (Supplementary fig. 4e, right in the revised manuscript), as observed with Por1(D228K).

Por1 hexamer is involved in regulation of mitochondria lipid composition by mediating phospholipid scrambling between the protomers BC and B'C'. This conclusion is supported by identification of densities between protomers in the cryo-EM structure, identified as phospholipids, by molecular simulations, and by mitochondrial lipid composition analysis in Por1 mutant.

If the hexameric structure of Por1 is essential for lipid scrambling activity, any mutation disrupting the oligomer assembly, but not the NAD+ transport (for example the D228K/R252E (KE) mutation), should also affect lipid composition. It would be important to test other mutants in addition to the TDQ to confirm the role of the hexameric Por1 per se rather than the specific residues in lipid scrambling.

We performed a lipid composition assay for the Por1(KE) mutant according to the reviewer's suggestion. However, the Por1(KE) mutant did not show any significant alteration in CL or PA levels as compared with the Por1(WT) strain (Fig. 1 for Reviewers, below). Although both the TDQ and KE mutants exhibit defects in Por1 oligomerization, as assessed by BN-PAGE (Supplementary Figs. 4c and 6d in the revised manuscript), it is to be noted that the BN-PAGE is a relatively harsh method for detecting weakly assembled forms. Therefore, it remains possible that the KE mutant retains partial and weak hexamer formation sufficient to maintain scramblase activity at levels that preserve the steady-state lipid composition. In contrast, the TDQ mutant may be more severely impaired in hexamer formation than the KE mutant, leading to reduced scramblase activity and, consequently, a modest decrease in CL levels. However, decrease remains relatively limited, suggesting that Por1 alone may not fully account for lipid scramblase activity at the OM, consistent with previous findings (Li et al., PNAS 2023). It is also important to note that our phospholipid analysis was based on metabolic labeling to assess steady-state lipid composition, which does not directly measure the kinetics of lipid scrambling. Thus, even if the KE mutant exhibits reduced scramblase activity, such a defect might not be detectable under the steady-state conditions used here. Considering these results, we altered the related description in the revised manuscript as follows; these findings support the idea that the hexameric structure of Por1 is important for its lipid scramblase activity, although its precise relationship between hexamer stability and scramblase function remains to be elucidated.

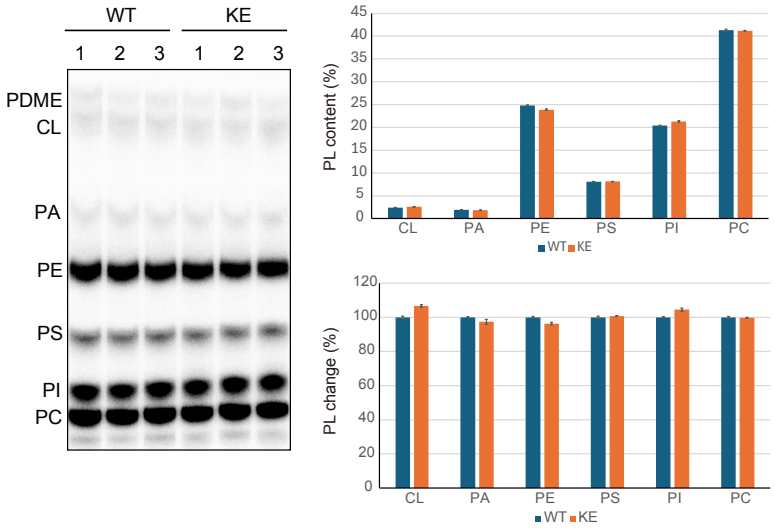

Fig. 1 for Reviewers

- Based on the structure of Por1, can the authors speculate on why does the Q73E mutation enhances scrambling activity? Is the negative charge of E interfering with the negative charge of the phospholipid headgroup?

A previous MD simulation study showed that a charge on E73 in VDAC accounts for the elevation of N-terminal protein dynamics as well as a thinning of the nearby membrane, which causes a transient approach of the phospholipid head groups towards E73 (Villinger

et al., PNAS 107, 22545-22551 (2010)). This fits our observation that the Q73E mutation in Por1 enhances the phospholipid scrambling activity.

Minor points:
- ED fig 5b: In the figure legend it is not mentioned what the insets in the graph are. Supposably they are a scale up of some parts of the main graph, but it should be clarified in the figure legend. Are the differences between WT and mutants statistically significant?

We have added an explanation to the legend of Supplementary Fig. 5b in the revised manuscript accordingly. We also added the results of statistical analyses to Supplementary Fig. 5b in the revised manuscript.

- Fig 3c: statistical analysis is required, mostly for PA.

We performed statistical analysis, the results of which are shown in Fig. 3c in the revised manuscript. The analysis revealed that the difference in CL levels between Por1(WT) and Por1(QA) cells is statistically significant, whereas the difference in PA levels is not. Since PA can be utilized in the synthesis of lipids other than CL, any potential accumulation of PA may not be detected. Accordingly, we have revised the description of lipid composition differences between Por1(WT) and Por1(QA) cells in the main text.

Por1 is involved in mitochondrial DNA retention. Although supported by solid data, in my opinion this is the least convincing part of the manuscript. It is based on a single mutant of Por1 which assembles in higher-molecular weight complexes (or aggregates) and leads to mtDNA loss. However, there is no discussion on how this mutation affects structure of hexameric Por1, and why only this mutant, and not other mutation, or Por1 deletion for example, causes loss of mtDNA. The authors moved on and focused more on identifying nucleases required for the loss of mtDNA in Por1(QA), identifying 7 proteins, named mtDNA-losing (MDL) proteins. These proteins seem to be localised in mitochondria and their loss seems to lead to accumulation of mtDNA in the periphery of the organelle.
Comments:
- Provide structural insights on the Po1(QA) mutant and the effect of only this mutation on mtDNA retention. Provide some speculation on the potential physiological role of Por1(WT) in mtDNA retention.

The structural reason for the rapid DNA loss with Por1(QA) mitochondria is unclear. We tried to determine the cryo-EM structure of the Por1(QA) mutant; however, gel-filtration profile) indicated that the purified Por1(QA) exists as a mixture of different oligomeric forms, which hampered further structural analysis. Therefore, it is not clear if Por1(QA) forms a large channel that facilitates the release of fragmented DNA to the cytosol, as previously suggested (Kim et al., Science 2019). To reflect this interpretation, we have added structural insights to the last part of the "Por1 plays a key role in maintaining mtDNA intergrity" section, as follows.

In the original manuscript — In the Por1(WT) strain, the MDL nucleases appear to be inactive or enzymatically latent within mitochondria, but they may become active or accessible in the Por1(QA) mutant

In the revised manuscript — In the Por1(WT) strain, the MDL nucleases appear to be inactive or enzymatically latent within mitochondria, but they may become active or accessible in the Por1(QA) mutant through an unknown signal transduction from Por1(QA) in the OM to the mitochondrial interior. The contribution of alteration of the Por1 oligomeric assembly by the QA mutation to such a signaling mechanism remains unclear.

Minor points:
- Fig ED 7e: the localisation of Abf2 is not only in the mitochondrial periphery but it seems to be still retained in the mitochondria. I would suggest rephrasing it in the text.

We have changed the sentence for ED fig. 7e in the manuscript accordingly.

- Do the MDL deletions have an effect on mtDNA localisation with Por1(WT) (ED Fig 9)?

We have analyzed the localization of mtDNA in the MDL deletions in Por1(WT) cells. The new results showed mtDNA is localized in both WT and MDL deletion mutant cells (Supplementary Fig. 9 in the revised manuscript)

Reviewer #2:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for his/her efforts in reviewing our manuscript.

Reviewer #3:

This reviewer provides a focused technical report primarily assessing the HS-AFM part of the manuscript:

Sample preparation, HS-AFM operational parameters and conditions used for simulating AFM images are sufficiently described. The interpretation of the Por1 hexamer orientation (i.e. cytosolic side up) on the APTES-mica surface is sound, however the difference of the total height between the experimental and simulated topography (ED Fig. 1g vs h; ~7 nm vs 5 nm) should be discussed.

We reasoned that the height difference between the experimental and simulated topography (ED Fig. 1g vs h in the original manuscript) is likely due to the presence of GDN micelles in the experimental data. This explanation has been incorporated into the main text of the revised manuscript. Since the orientation of the Por1 hexamer with GDN micelles may not be definitively clear, we have deleted the simulated topography viewed from the IMS side (ED Fig. 1i in the original manuscript).

Reviewer #4:

Takeda, Shinoda et al determined the structure of the mitochondrial porin, a VDAC consisting of hexameric Por1 by cryo-EM at 3.2A resolution.

This is an excellent study with thorough experiments combining biochemical and biophysical techniques including coarse-grained molecular dynamics. The paper is well and clearly written. I found it easy to follow and a pleasure to read. The figures are clear and useful. The insights about the three major functions – Por1 binding to Tom22, maintenance of lipid composition, and mtDNA regulation – are properly argued and backed up by well-designed experiments. The conclusions are justified. In particular, I found the MD-assisted data interpretation about lipid scrambling, and the mutagenesis to explain the role of mtDNA integrity resourceful and convincing. This study advances the knowledge of VDACs and their role in mitochondrial metabolism and will therefore appeal to a wide audience.

We were glad to hear this positive evaluation of our manuscript.

Reviewer #5:

This paper present interesting new data and represents a significant advance.
I have some minor comments specifically relating to the section of lipid scrambling.

ref 24 is Jahn et al. (it is currently written as Ahn et al. – please fix the typo)

We have corrected this typo.

Lines 169-170: this statement is not correct. Jahn et al. showed that the import of PS across the OM in isolated mitochondria was reduced by an order of magnitude in mitochondria lacking Por1

Thank you for pointing out this fact. We have corrected the description in the text accordingly.

Lines 181-184: this is an interesting result; the beta 1,2,18,19 interface was seen in previous structural work (and also in the current paper) but may be more available to lipids in human VDAC1 assemblies than it seems to be in the Por1 hexamer. It would be worth saying more on this point.

We have incorporated additional discussion on the role of the interfaces in lipid scrambling, which was suggested for human mitochondria (Jahn et al., 2023).

Phospholipid scrambling is principally analyzed by CGMD in this paper, with outcomes validated indirectly through lipid compositional changes in Figure 3. The depression of CL and increase of PA levels in Por1(TDQ)-expressing mitochondria is certainly suggestive, but also surprising as not changes are seen in the level of PE, a lipid that is generated from imported PS. This point deserves discussion, especially in light of the result of Jahn et al. reporting PS transport deficiency in mitochondria lacking porin. Are redundant scramblases somehow compensating?

Since we analyzed lipid compositions of total cells, the PE levels analyzed here could be affected by not only the PE biosynthesis pathway by Psd1 within mitochondria but also the one by Psd2 outside mitochondria (Kennedy pathway). The latter pathway may mask a subtle change in the PE level as compared with the CL level, which is synthesized only inside mitochondria.

Reviewer #6:

In this article, Endo and colleagues convincingly show that Por1 can form functional hexamers in yeast mitochondria. First, they determine the cryo-electron microscopy structure of Por1 in its hexameric form at 3.2 Å resolution from direct purification of Por1 from yeast mitochondria. Next, they assess the potential role of Por1 hexameric structure in three different cellular processes in which Por1 had been previously implicated: assembly of the TOM complex, lipid scrambling and regulation of mitochondrial DNA. In all cases, through careful mutational studies, they show that the correct assembly of the Por1 oligomer is required for its proper function. Overall, the manuscript is convincing, carefully written, and well thought-out. The results presented are important for scientists studying transport processes in mitochondria and represent a wonderful functional application of structural observations.I thoroughly enjoyed reading it and I recommend publication with only a few minor comments outlined below.

We were glad to hear these positive remarks.

Minor
- Extended Data Fig. 4e lacks labels (colors) to interpret the curves

We are sorry for this fault. We have added an explanation for the color code to the legend.

- The sentence in the Methods section "Previous all-atom molecular dynamics (MD) simulations of mouse VDAC1 showed that single point mutations can either enhance or suppress lipid scrambling activity 26" is incorrect, as ref. 26 does not discuss lipid scrambling by VDAC1.

We have changed the reference number.

- I found that several supplementary figures contain important data to follow the overall flow of the manuscript, and I suggest the authors, if space allows, to move some of these data to the main figures, eventually adding a few of them.

We would like to thank you for your suggestion. We have transferred ED Fig. 9 in the original manuscript to the main figure as new Fig. 6 in the revised manuscript.

Other changes

Since David Komander's group reported the human TOM-PINK1 structure including VDAC2 (Por1 homolog) dimer (Callegari et al., Science 2025) after submission of the original manuscript, we compared their dimeric VDAC2 with the hexameric Por1 determined in this work and found that the protomer interface is different between VDAC2 and Por1. The results are shown in Supplementary Fig. 2 in the revised manuscript.

NCOMMS-24-86792-T

Point-by-point responses to the reviewers' comments

Reviewer #1:

The authors addressed all points raised and the revised manuscript is acceptable for publication

We are glad to hear this comment that supports acceptance.

Reviewer #2:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for his/her efforts in reviewing our manuscript.

Reviewer #3:

The authors have sufficiently addressed my suggestions. No further comments from my side.

We are glad to hear this comment that supports acceptance.

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Reviewer #5:

I have a minor point regarding the response to one of points.

The authors write:
Since we analyzed lipid compositions of total cells, the PE levels analyzed here could be affected by not only the PE biosynthesis pathway by Psd1 within mitochondria but also the one by Psd2 outside mitochondria (Kennedy pathway). The latter pathway may mask a subtle change in the PE level as compared with the CL level, which is synthesized only inside mitochondria.

This point should be included in the manuscript as it is rather important.

Thank you for this comment. We have now included the description of the non-mitochondrial PE biosynthesis pathway in the revised manuscript.

Reviewer #6:

The authors have successfully addressed all my (minor) comments. I congratulate them again for this beautiful piece of work.

We are glad to hear this comment that supports acceptance.