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# Structure and mechanism of a eukaryotic ceramide synthase complex

Xin Gong, Tian Xie, Qi Fang, Zike Zhang, Yanfei Wang, and Feitong Dong
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*Corresponding author(s): Xin Gong (gongx@sustech.edu.cn)*

## Review Timeline:


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| Submission Date: | 29th Jun 23 |
| Editorial Decision: | 17th Aug 23 |
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*Editor: William Teale*


## 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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Gong,

Thank you again for the submission of your manuscript entitled "Structure and mechanism of a eukaryotic ceramide synthase complex" (EMBOJ-2023-114889) and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, while all referees are generally supportive of publication in The EMBO Journal, all highlight some points for clarification and discussion that will require your attention before your manuscript can be published in The EMBO Journal.

Based on the overall interest expressed in the reports, I would like to invite you to address the comments of all referees in a revised version of the manuscript. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

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

Best regards,

William

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William Teale, Ph.D.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see

https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

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9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

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Further instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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IMPORTANT: When you send the revision we will require
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- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (15th Nov 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #1:

The manuscript by Tian Xie et al reports the cryo-EM structures of yeast Ceramide synthase (CerS) in complex with substrate C26-CoA. This work reveals yeast CerS holoenzyme as a dimer of Lac1-Lip1. It also importantly shows the detailed binding mode of the substrate C26-CoA as well as a potential lateral entrance for the sphingoid base. These together with biochemical analysis provide key insights into the ceramide formation reaction catalyzed by eukaryotic CerS. This work is a very nice study presenting important progress in the field. I would only suggest a few points that may need to be addressed.

Major:
1. Line 211-216: Lip1 F40 appears also involved with the interaction of Lac1 (Fig. 4b). The explanation of the effects of Lip1 F40 mutations should be careful as the effects may be indirect.
2. The authors solved the structure of Lac1-Lip1S74F and interestingly observed the collapse of the C26 acyl chain binding tunnel. From Fig. 4g, several residues lining the binding tunnel yet distant form S74F (e.g. W371, K293) show apparent conformational changes. The reason is worth investigation and discussion.
3. Line 350-353: about the catalytic model proposed, can the authors tell from the structure which is the more likely general base (D283 or D286) and which is the coordinating residue (H255 or H256)?
4. Fig 5b-d, the lipid-like density was shown multiple times, which appear redundant. Moreover, modeling a lipid there may help visualization and discussion.
5. Line 286-326, the section "Implications for mammalian CerS" should be shortened and moved to "Discussion" part since the analysis here is only based on predicted human CerS structures. For the same reason, I would suggest moving Fig 6 to supplementary figures.

Minor points:
1. Page 5 line 88-89, the authors should cite the literature here about the assay method they refer to. "develop" appears not accurate and the authors should tone it down.
2. Purified Lac1 or Lag1 shows two bands on SDS-PAGE analysis (Extended Data Fig. 2 a). What is reason for that?
3. Line 147-152: from gel filtration result (Extended Fig. 5e), it appears that the R78A/Y81A/Y125A/Y148A mutant doesn't apparently change the oligomer state so the conclusion on line 151 seems not that solid.

Referee #2:

Ceramides play a critical role in lipid biosynthesis and serve as important signaling molecules. Ceramide synthases, which catalyze ceramide formation, are potential drug targets for treating cancers and metabolic disorders. In this manuscript, the

authors determined the first structure of the yeast ceramide synthase Lac1-Lip1 complex with a C26-CoA substrate. The structure reveals the dimeric architecture of Lac1-Lip1, the catalytic reaction center, fatty acyl-CoA binding site, and a potential sphingoid base entry site. Based on the structure, and in combination with mutagenesis and functional studies, the authors proposed a plausible working mechanism for ceramide synthase. This work offers significant insights into the mechanism of ceramide synthase and lays the groundwork for the rational design of modulators. Overall, the data are of high quality, the findings are novel, and this work will produce a high impact in the field.
Several comments:
1. The activity reported in this study (fig.1b, c) seems considerably lower than those previously published (reference 28), exhibiting at least a hundred-fold difference. In addition, the sigmoidal curve shown in Fig 1b was not observed in the previous published work either. Just wondering whether the assay conditions could cause the difference?
2. It would be helpful to clarify the molecular basis of substrate preference.
In line 195, the authors wrote, "allowing the tunnel to perfectly accommodate a 26-carbon acyl chain." Based on Fig 3, it is unclear whether the acyl chain tunnel opens to the lumen. Considering that human homologues have varied substrate preferences, do they all share the same tunnel to coordinate different substrate lengths?
In fig. 3d, the authors present data up to C26, which displays the highest activity. Can the tunnel accommodate substrates longer than C26, and how would their activities compare? (In mammals, some ceramides have chains longer than C26)
3. The authors discovered an intriguing mutation, S74F, which causes the collapse of the fatty acyl-CoA C26 binding site and subsequently abolishes its function. Can the mutant protein accommodate a shorter substrate? It would be helpful to assess whether this mutant (or any other mutants in this region, such as F51, H52) affects the substrate selectivity as it interacts with the TM7/8 loop, which plays a critical role in substrate selectivity in human homologs.
Minor points
1. The authors attempted to disrupt the dimer interface of Lip1; however, the peak of the mutant protein remains at a similar position as the WT (Extended Data Fig.5b). Is there any evidence of the monomer formation for the mutant?
2. In ED figure 2, it might be useful to compare the peak positions of Lac1 and Lac1-Lip1.
3. In ED figure 6, please specify in the figure legend whether the peak fractions or a normalized, similar amount of protein was loaded on SDS-PAGE.

Referee #3:

Xie et al investigated the structure and catalytic mechanism of the yeast ceramide synthase enzyme, which is composed of two proteins, Lac1 and Lip1. Their study provides empirical structural information regarding the structural basis of the catalytic activities of yeast ceramide synthase. Cryo-Electron microscopy was used to elucidate the structure of Lac1-Lip1 at 3.09 Å, demonstrating that Lac1-Lip1 holoenzyme is a dimer of Lac1-Lip1 heterodimers in the membrane. A hydrophobic tunnel is apparent where the charged fatty acid is bound, exposing the acyl-CoA moiety toward the cytosolic leaflet. The resolved structure shows that the end of the hydrophobic cavity in Lac1 faces the Lip1 transmembrane domain (TM), and mutations in residues in the Lac1 cavity, or the Lip1 TM at the end of the cavity are also critical for catalytic activity. A lateral opening in Lac1 within the bilayer containing a lipid-like density is suggested to direct the sphingoid base substrate into the catalytic site. Finally, the authors compared their Lac1-Lip1 structures with the predicted structure (alpha-fold) of mammalian ceramide synthase 5, which is comprised by just one polypeptide. The comparison suggests that the yeast and mammalian enzymes are structurally similar, despite the yeast enzyme being composed of two polypeptides. Overall, we found this to be an excellent and interesting structural study of a eukaryotic ceramide synthase.
Comments:
The structural insights of membrane-embedded enzymes regarding the entry of substrates, exit of products, and the effects of heterogenous membrane environments are areas of intense interest. Predicted biochemical mechanisms of CerS were based largely on amino acid sequence motifs and this study provides empirical structural information on tertiary structures of Lac1-Lip1-Acyl-CoA and possible substrate entry mechanisms. We have two minor comments that the authors should consider addressing.
1. In Figure 5 c and d, the authors suggest that the lipid-like structures identify the sphingoid base substrate in the catalytic site. Can the authors provide further insight into how the chemical environment of the active site with the sphingoid base is established? For example, how are the hydrophilic amine and hydroxyl groups inserted deep into the hydrophobic membrane bilayer?
2. In Figure 6, the authors used the predicted structure of CerS5 (alpha-fold), which prefers a shorter chain length of acyl-CoA than C20. The importance of TM7/8 of Lac1 for interacting Lip1 may be more meaningful in other CerS enzymes such as mammalian CerS2 which prefers C24 acyl-CoA. Have the authors made this comparison (ie, with the predicted structure of CerS2 and are the results worth discussing?
3. Please provide evidence/reference to demonstrate that the interaction of TM of Lip1 and TM 2/4/5 of Lac1 is significant to stabilize the specific heterodimer.

**Response to reviewers' comments:**

**Referee #1:**

*The manuscript by Tian Xie et al reports the cryo-EM structures of yeast Ceramide synthase (CerS) in complex with substrate C26-CoA. This work reveals yeast CerS holoenzyme as a dimer of Lac1-Lip1. It also importantly shows the detailed binding mode of the substrate C26-CoA as well as a potential lateral entrance for the sphingoid base. These together with biochemical analysis provide key insights into the ceramide formation reaction catalyzed by eukaryotic CerS. This work is a very nice study presenting important progress in the field. I would only suggest a few points that may need to be addressed.*

We thank this reviewer for his/her appreciation of our work. We've revised the manuscript based on the suggestions raised by this reviewer to improve the quality of the study.

*Major:*
*1. Line 211-216: Lip1 F40 appears also involved with the interaction of Lac1 (Fig. 4b). The explanation of the effects of Lip1 F40 mutations should be careful as the effects may be indirect.*

We thank this reviewer for the insightful suggestion. The original statement has been revised as "*Data on these two Lip1 mutants suggest that Lip1 can enhance the catalytic activity of the complex probably by engaging in Lac1 interaction and acyl chain binding ...*" in the revised manuscript.

*2. The authors solved the structure of Lac1-Lip1S74F and interestingly observed the collapse of the C26 acyl chain binding tunnel. From Fig. 4g, several residues lining the binding tunnel yet distant form S74F (e.g. W371, K293) show apparent conformational changes. The reason is worth investigation and discussion.*

We thank this reviewer for the constructive comment. This might be explained as that the mutation of Lip1$^{S74F}$ initiated the direct conformation change of the Lac1 TM7/8 loop, thus leading to the subtle perturbation of the overall structure of Lac1 and causing the local conformational changes for the side chains of the residues lining the binding tunnel yet distant from Lip1$^{S74F}$ (e.g. W371, K293, etc.). The corresponding discussion has been added in the revised manuscript in lines 286-289.

*3. Line 350-353: about the catalytic model proposed, can the authors tell from the structure which is the more likely general base (D283 or D286) and which is the coordinating residue (H255 or H256)?*

We thank this reviewer for the insightful comment. Due to the absence of structures representing distinct catalytic states, such as those in complex with a sphingoid base substrate, it remains challenging to determine the specific aspartate residue that serves as the general base as well as the specific histidine residue that functions as the coordinating residue. It is even plausible that both aspartate residues and both histidine residues cooperate in the catalytic process. Further studies are needed to elucidate the precise catalytic mechanism of ceramide synthase.

*4. Fig 5b-d, the lipid-like density was shown multiple times, which appear redundant. Moreover, modeling a lipid there may help visualization and discussion.*

Point taken. The original Fig 5 was simplified and revised as suggested by this reviewer.

*5. Line 286-326, the section "Implications for mammalian CerS" should be shortened and moved to "Discussion" part since the analysis here is only based on predicted human CerS structures. For the same reason, I would suggest moving Fig 6 to supplementary figures.*

Point taken. The original Fig. 6 was moved to the supplementary materials. The section “Implications for mammalian CerS” was shortened and moved to the Discussion section.

*Minor points:*
*1. Page 5 line 88-89, the authors should cite the literature here about the assay method they refer to. "develop" appears not accurate and the authors should tone it down.*

Point taken. In the revised manuscript, we have cited the relevant literature about the assay method, and we have replaced the phrase "develop" with "employ".

*2. Purified Lac1 or Lag1 shows two bands on SDS-PAGE analysis (Extended Data Fig. 2 a). What is reason for that?*

We thank this reviewer for the insightful question. We have conducted an investigation using a Lac1 construct with a deletion of the N-terminal 40 residues, and found that this Lac1-ΔN40 construct migrated as a single band at a position similar to the lower band observed for the WT Lac1 (Figure shown below, panel A). This suggests that the lower band of purified WT Lac1 protein may be attributed to degradation originating from the N-terminal region of Lac1 protein. Consistent with this, the N-terminal 70 residues of Lac1 were not resolved in the EM map, indicating the high flexibility of this region. Additionally,

we observed that the Lac1$^{\Delta N40}$-Lip1 complex exhibited similar enzymatic activity compared to the WT Lac1-Lip1 complex (Figure shown below, panel B). Since the N-terminal degradation/truncation of Lac1 did not affect the conclusions of the original manuscript, we did not include this analysis in the manuscript.

We thank this reviewer for the constructive comment. And we are sorry for the overinterpretation of the results for the R78A/Y81A/Y125A/Y148A mutant in the original manuscript. The corresponding content was modified to as "*Although the mutated complex remained partially as dimer in SEC, this mutant displayed prominently reduced expression level, relatively poor solution behavior with broad SEC peak, and approximately 5% of the enzymatic activity of the WT complex (Fig EV3E, F). The data implies that the Lip1 homo-dimerization interface might also be important for the proper folding and enzymatic activity of the complex.*" in the revised manuscript to avoid overinterpretation.

We thank the reviewer for his/her time and constructive comments.

**Referee #2:**

*Ceramides play a critical role in lipid biosynthesis and serve as important signaling molecules. Ceramide synthases, which catalyze ceramide formation, are potential drug targets for treating cancers and metabolic disorders. In this manuscript, the authors determined the first structure of the yeast ceramide synthase Lac1-Lip1 complex with a C26-CoA substrate. The structure reveals the dimeric architecture of Lac1-Lip1, the catalytic reaction center, fatty acyl-CoA binding site, and a potential sphingoid base entry site. Based on the structure, and in combination with mutagenesis and functional studies, the authors proposed a plausible working mechanism for ceramide synthase. This work offers significant insights into the mechanism of ceramide synthase and lays the groundwork for the rational design of modulators. Overall, the data are of high quality, the findings are novel, and this work will produce a high impact in the field.*

*Several comments:*

*1. The activity reported in this study (fig.1b, c) seems considerably lower than those previously published (reference 28), exhibiting at least a hundred-fold difference. In addition, the sigmoidal curve shown in Fig 1b was not observed in the previous published work either. Just wondering whether the assay conditions could cause the difference?*

We appreciate the insightful comment from the reviewer. The Lac1-Lip1 complex protein from ref28 exhibited over a hundred-fold higher activity compared to our purified Lac1-Lip1 complex. Additionally, the Triton X-100 purified Lac1-Lip1 complex from ref28 displayed reasonable ceramide synthase activity despite no visible Lip1 on the protein gel. However, in our study, we observed that the Lac1 protein alone had no activity. Furthermore, the sigmoidal curve observed in Figure 1C of our study was not observed in ref28. We suspect that the differences in CerS activity between our study and ref28 may be attributed to several factors, including variations in the fusion of affinity tags to different regions of the Lac1 protein, differences in protein expression systems, usage of detergents during protein purification, variations in protein quantification methods, and differences in assay systems. Further investigation is necessary to thoroughly explore and clarify the discrepancies between our current study and ref28.

> *2. It would be helpful to clarify the molecular basis of substrate preference.*
> *In line 195, the authors wrote, "allowing the tunnel to perfectly accommodate a 26-carbon acyl chain." Based on Fig 3, it is unclear whether the acyl chain tunnel opens to the lumen. Considering that human homologues have varied substrate preferences, do they all share the same tunnel to coordinate different substrate lengths?*
> *In fig. 3d, the authors present data up to C26, which displays the highest activity. Can the tunnel accommodate substrates longer than C26, and how would their activities compare? (In mammals, some ceramides have chains longer than C26)*

We thank this reviewer for the critical comments. As suggested by this reviewer, the original Fig. 3A was modified to show that the acyl-chain binding tunnel opens to the ER lumen.

Since both the hydrophilic reaction chamber (Fig. 2C) and the upper portion of the acyl-chain binding tunnel (Fig. 3E) exhibit high conservation among yeast and human CerS homologs, it is likely that human CerS homologs utilize the same reaction chamber and tunnel as the Lac1-Lip1 complex to coordinate acyl-CoA substrates. However, the residues lining the lower portion of the acyl chain binding tunnel are not conserved in yeast and human CerS homologs (Fig. 3E). This observation leads us to suspect that this variable region of human CerS homologs might play a role in accommodating acyl-CoA substrates of different lengths. To fully understand the mechanism of substrate selectivity for human CerS homologs, further structural and biochemical studies are required.

The close proximity of the distal C26 atom of the C26 acyl chain to the side chains of surrounding hydrophobic residues (such as L341, F343, and I352 of Lac1, and F40 of Lip1) suggests that the tunnel may not have sufficient space to accommodate substrates longer than C26 (Fig. 3C). Furthermore, it is worth noting that *S. cerevisiae* predominantly produces C26 ceramides and does not naturally synthesize longer chain ($\geqslant$C28) ceramides. Therefore, we suspect that the CerS activity of the Lac1-Lip1 complex with longer acyl-CoA substrates, such as C28-CoA, might be significantly lower compared to its activity with C26-CoA substrate.

*3. The authors discovered an intriguing mutation, S74F, which causes the collapse of the fatty acyl-CoA C26 binding site and subsequently abolishes its function. Can the mutant protein accommodate a shorter substrate? It would be helpful to assess whether this mutant (or any other mutants in this region, such as F51, H52) affects the substrate selectivity as it interacts with the TM7/8 loop, which plays a critical role in substrate selectivity in human homologs.*

We appreciate the valuable comment from the reviewer. As recommended, we have examined the enzymatic activity of the F51A, F51R, H52A, and S74F mutants using C14- to C26-CoA substrates (as described in lines 267-271 of the revised manuscript). Our data showed that the F51A mutant displayed similar acyl-CoA substrate selectivity as the WT complex, and the other three mutants were essentially inactive with all the acyl-CoA substrates tested (see Appendix Fig S4C in the revised manuscript).

*Minor points*

*1. The authors attempted to disrupt the dimer interface of Lip1; however, the peak of the mutant protein remains at a similar position as the WT (Extended Data Fig.5b). Is there any evidence of the monomer formation for the mutant?*

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*2. In ED figure 2, it might be useful to compare the peak positions of Lac1 and Lac1-Lip1.*

Point taken. The figure has been revised in accordance with the suggestion made by this reviewer in the revised manuscript.

*3. In ED figure 6, please specify in the figure legend whether the peak fractions or a normalized, similar amount of protein was loaded on SDS-PAGE.*

Point taken. The figure legend was revised as "*Similar amounts of purified proteins were subjected to SDS-PAGE gels for analysis.*" in the revised manuscript.

We thank the reviewer for his/her time and constructive comments.

## Referee #3:

*Xie et al investigated the structure and catalytic mechanism of the yeast ceramide synthase enzyme, which is composed of two proteins, Lac1 and Lip1. Their study provides empirical structural information regarding the structural basis of the catalytic activities of yeast ceramide synthase. Cryo-Electron microscopy was used to elucidate the structure of Lac1-Lip1 at 3.09 Å, demonstrating that Lac1-Lip1 holoenzyme is a dimer of Lac1-Lip1 heterodimers in the membrane. A hydrophobic tunnel is apparent where the charged fatty acid is bound, exposing the acyl-CoA moiety toward the cytosolic leaflet. The resolved structure shows that the end of the hydrophobic cavity in Lac1 faces the Lip1 transmembrane domain (TM), and mutations in residues in the Lac1 cavity, or the Lip1 TM at the end of the cavity are also critical for catalytic activity. A lateral opening in Lac1 within the bilayer containing a lipid-like density is suggested to direct the sphingoid base substrate into the catalytic site. Finally, the authors compared their Lac1-Lip1 structures with the predicted structure (alpha-fold) of mammalian ceramide synthase 5, which is comprised by just one polypeptide. The comparison suggests that the yeast and mammalian enzymes are structurally similar, despite the yeast enzyme being composed of two polypeptides. Overall, we found this to be an excellent and interesting structural study of a eukaryotic ceramide synthase.*

*Comments:*
*The structural insights of membrane-embedded enzymes regarding the entry of substrates, exit of products, and the effects of heterogenous membrane environments are areas of intense interest. Predicted biochemical mechanisms of CerS were based largely on amino acid sequence motifs and this study provides empirical structural information on tertiary structures of Lac1-Lip1-Acyl-CoA and possible substrate entry mechanisms. We have two minor comments that the authors should consider addressing.*

*1. In Figure 5 c and d, the authors suggest that the lipid-like structures identify the sphingoid base substrate in the catalytic site. Can the authors provide further insight into how the chemical environment of the active site with the sphingoid base is established? For example, how are the hydrophilic amine and hydroxyl groups inserted deep into the hydrophobic membrane bilayer?*

We thank this reviewer for the constructive comment. The sphingoid base substrates are amphipathic molecules with hydrophilic amine and hydroxyl groups and hydrophobic aliphatic chains. The surface of transmembrane region of the Lac1-Lip1 complex embedded in membrane bilayer remain largely hydrophobic, ensuring the tethering of sphingoid base substrate to the Lac1-Lip1 complex. Meanwhile, the polar residues Ser186 and Gln227, located close to the entrance of the lateral opening, might provide a relative hydrophilic environment to help orientate and coordinate the hydrophilic amine and hydroxyl groups of sphingoid base. Altogether, the predominantly hydrophobic property of the transmembrane region and the hydrophilic feature near the lateral opening on Lac1 provide a suitable environment to coordinate a sphingoid base substrate.

> *2. In Figure 6, the authors used the predicted structure of CerS5 (alpha-fold), which prefers a shorter chain length of acyl-CoA than C20. The importance of TM7/8 of Lac1 for interacting Lip1 may be more meaningful in other CerS enzymes such as mammalian CerS2 which prefers C24 acyl-CoA. Have the authors made this comparison (ie, with the predicted structure of CerS2 and are the results worth discussing?*

We thank this reviewer for the insightful comment. As suggested by this reviewer, we've superimposed the predicted human CerS2 model ($hCerS2^{AF}$) with the Lac1-Lip1 structure (Figure shown below, panel A), and also the $hCerS2^{AF}$ with the $hCerS5^{AF}$ (Figure shown below, panel B). Although CerS2 prefers C24-CoA and CerS5 prefers C16-CoA, $hCerS2^{AF}$ and $hCerS5^{AF}$ share similar structural features in the TM6/7 loop and the N-terminal region. The precise mechanism of acyl-CoA selectivity in human CerS homologs would need to be explored in the future.

We thank this reviewer for the constructive comment. As suggested by this reviewer, we created two Lip1 mutants at the TM interaction interface, namely $Lip1^{V37F/K41F}$ and $Lip1^{V37Y/K41Y}$. Our results indicated that both mutants partially impaired the formation of the complex between Lac1 and Lip1, providing evidence for the importance of the TM interaction interface in the formation of the Lac1-Lip1 complex (see lines 248-252 and Appendix Fig S4A in the revised manuscript).

We thank the reviewer for his/her time and constructive comments.

Dear Xin,

Thank you submitting a revised version of your manuscript. It was sent to the same three reviewers that originally appraised your work; their comments are attached to the bottom of this email. As you will see, all three referees are satisfied with the changes you made. Before we can move forwards towards publication of your manuscript, though, there are some remaining editorial points which need to be addressed. In this regard, would you please:

- choose only five keywords,
- limit the listed authors to ten for each reference (+ et al. where necessary), and
- ensure datasets EMD-35862, EMD-35863 and 8IZD; 8IZF are not referred to in the data availability statement, and made publicly available.

I look forward to receiving these changes. EMBO Press is an editorially independent publishing platform for the development of EMBO scientific publications.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also figure legend guidelines: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (18th Jan 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #1:

All my questions have been adequately addressed. I would recommend publication.

Referee #2:

The authors have adequately addressed my comments.

Referee #3:

The authors have adequately addressed my concerns.

All editorial and formatting issues were resolved by the authors.

Dear Xin,

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Congratulations on a study that I am sure will be really well-appreciated!

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If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

Yours sincerely,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

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## EMBO Press Author Checklist

| |
|---|
| Corresponding Author Name: Xin Gong |
| Journal Submitted to: EMBO Journal |
| Manuscript Number: EMBOJ-2023-114889 |

**USEFUL LINKS FOR COMPLETING THIS FORM**
The EMBO Journal - Author Guidelines
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**Reporting Checklist for Life Science Articles (updated January**
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
**Please note that a copy of this checklist will be published alongside your article.**

**Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

**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 $\chi^2$ 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.

**Please complete ALL of the questions below.**
**Select "Not Applicable" only when the requested information is not relevant for your study.**

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| Newly Created Materials | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| New materials and reagents need to be available; do any restrictions apply? | Not Applicable | |

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| Include a statement about **sample size** estimate even if no statistical methods were used. | Not Applicable | |
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| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Not Applicable | |
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