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Peer Review File

Structural basis for catalysis and selectivity of phospholipid synthesis by eukaryotic choline-phosphotransferase

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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 manuscript titled “Structural basis for catalysis and selectivity of phospholipid synthesis by eukaryotic choline-phosphotransferase” reports structures of the yeast CDP-choline phosphotransferase and proposes hypotheses on mechanisms of substrate recognition and catalysis. The main results are three structures of yeast CPT1 in the presence of either DAG, CDP-choline and PC, or an inhibitor chelerythrine. cryoEM data collection, data analysis, model building and refinement are consistent with the current common practices and standards. The proposed mechanisms seem reasonable and I do not have any major concerns, except for a few minor complaints mostly on functional studies as listed below.

An enzymatic activity assay was used to evaluate activities of the wild type and mutant CPT1 to provide support for the structure-based hypotheses. The functional studies are quite rudimentary with only a single substrate concentration.

LC-MS/MS was applied to quantify lipid content in the purified protein, however, there is no mention of how detergent may affect the retention of the co-purified lipids. There is also no estimation on the number of bound lipids per protomer.

The proposed mechanism of CDP-choline recognition seems solid, so is the mobile histidine that facilitates extraction of the hydroxyl proton on DAG. However, the narrative on A or G at position 97 being part of the mechanism for achieving selectivity is not on a solid ground. Shouldn’t the exception on hEPT1 already have broken down the correlation?

It is intriguing to see that two acyl chains from a DAG could “stradle” TM6. How certain can the density be assigned to DAG? And could the authors comment on how a DAG could achieve this pose?

Line 32: change “those” to “that”?
The label “TM4” is on the wrong helix in Fig 2c.

Reviewer #2

(Remarks to the Author)
Phosphatidylcholine and the other phospholipids are the fundamental components of biological membranes. Choline-phosphotransferase (CPT) is a membrane-embedded enzyme catalyzing the final step of phosphatidylcholine biosynthesis. Through the catalytic function of CPT, cytidine-5'-diphosphate (CDP)-choline and 1,2-dacylglycerol (DAG) are converted into phosphatidylcholine and CMP. In the manuscript presented by Roberts, J. R. et al., the cryo-EM structures of a yeast (Saccharomyces cerevisiae) CPT1 in complex with substrates and an inhibitor were reported. The molecular bases underlying the DAG acyl-chain preference, choline/ethanolamine selectivity and inhibitor binding have been analyzed through structural analysis and biochemical assays. While new insights have been obtained through their work, there are several questions or suggestions for the authors to consider during revision.
1) In lines 153-154, it was stated that “the dimerization interface and contributions of lipids to support dimerization are not conserved between species.”. Have the authors tried to do sequence alignment among the CPT1 homologs from various fungal species to see if the dimerization motif found in yCPT1 is conserved among the homologs from fungi?
2) In the structure of yCPT1-DAG complex, the lipid density in the active-site cavity was assigned as DAG by taking account of the mass spectrometry analysis result of the purified yCPT1 sample (Supplmentary Fig. 6). As shown in the mass spec

data (Suppl. Fig. 6c), the content of DAG in the purified yCPT sample is fairly low at 0.02-0.15 pmol/nmol yCPT. Such an extremely low molar ratio of DAG:yCPT does not seem to support the assignment of the lipid density as DAG. Is it possible that the density may belong to PC as it is a dominating lipid species detected in the purified protein sample? To obtain the structure of yCPT1 in complex with DAG, it might be better to add sufficient exogenous DAG into the purified yCPT1 so that the internal lipid-binding site is loaded with DAG at high occupancy.
3) For the structure of yCPT1 in complex with CDP-choline, there is a lipid molecule bound to the active site nearby CDP-choline and it was assigned as the end-product of the reaction, namely PC. As the choline group of PC comes from CDP-choline, if the lipid belongs to PC, the CDP-choline should be converted into CMP after the choline-phosphate group is transferred to DAG. As a result, it should be CMP instead of CDP-choline bound to the active site along with PC. While the density of CDP-Choline fits well with the model, the density for the head group of PC is fairly weak and does not seem to fit well with the choline-phosphate group. Is it possible that the lipid density may belong to DAG or other lipids, or represent a mixture of DAG, PC and other phospholipid associated at the same site? Considering that there are no exogenous lipids added to the sample, it is hard to define the exact identity of the lipid molecule associated at the site nearby CDP-Choline if it is heterogenous. To overcome the problem and obtain the information relevant to the catalytic process, it might be better to load the two substrates (CDP-choline and DAG) simultaneously into the purified yCPT1 sample and solve the structure of yCPT1-CDP-choline-DAG complex. Alternatively, the two products, namely CMP and PC, could be loaded into yCPT1 for obtaining the structures of yCPT1 in complex with the products. Either the yCPT1-CDP-choline-DAG complex structure or the yCPT1-CMP-PC complex structure will be much more helpful in understanding the catalytic mechanism yCPT1 than the yCPT1-CDP-choline-PC complex.
4) In lines 220-221, “This ionic interaction is conserved among all reported structures of CDP-Aps and is likely critical for the catalytic reaction.”. To support the statement, evidences such as a sequence alignment figure or references of the previous works should to be cited at the end of the sentence.
5) In the structure of yCPT1 in complex with chelerythrine, the model of chelerythrine in one of the two monomers (monomer A) does not fit well with the density. The C4 atom of chelerythrine model is located outside the density. The model needs to be refined further to fit the density better. Moreover, there appears to be addition densities connected to the O26 atom of the chelerythrine molecule. It appears that the CHS model (https://www.rcsb.org/ligand/Y01) also fits the density well (maybe better than chelerythrine). Is it possible that that the binding site is a mixed binding site for chelerythrine and CHS (and/or lipids)? In that case, the author may need to generate a mask of chelerythrine and apply it in the skip-alignment 3D classification procedure to enrich the particles with chelerythrine and separate it from those associated with the other ligands. In case that the skip-alignment classification does not help to solve the problem, the sample may need to be prepared in a condition free of CHS or other chelerythrine-like additives and then supplied with chelerythrine for cryo-EM data collection.
6) For the three cryo-EM structures presented in the work, the maps were refined with no symmetry applied (C1). However, the two adjacent monomers within the homodimers apparently follow a C2 or pseudo-C2 symmetry. Have the authors tried to refine the maps with the C2 symmetry imposed, which may help to improve the resolution and map quality? In supplementary Table 1, the clashscore for the yCPT1 DAG structural model is a bit too high and may need to be reduced to a value around or below 4 through further refinement. For further refinement of the structural model against the cryo-EM map, application of non-crystallographic symmetry (NCS) restraints/constraints will help to improve the model quality (especially when the C2 symmetry is applied for generating the cryo-EM map).
7) In Fig. 1b, monomer B (in grey) is not distinguished well from the silver part of monomer A. It will be better to change it to a different color to visually separate it from the dimerization motif (and the other parts) of monomer A. In suppl. Fig. 2b an c, please explain in the legend what are the PC samples loaded as the references. Are they the standard radiolabeled PC sample? For suppl. Fig. 4e and f, the legends of these two panels are not provided. In line 43, “4NMD” is diffent from the one label in panel e (4MND). In the main text, there are also some other typos to be corrected. For instance, “contributing” in line 214 and “previously” in line 244.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have no further comments.

Reviewer #2

(Remarks to the Author)
While the authors have improved their work during the last revision, there might be a few minor issues to be settled for further revision.

1) In the structure of yCPT1 in complex with CDP-choline, the authors tried to explain and validate the assignment of the lipid molecule (trapped in the active-site cavity nearby CDP-chloine) as PC. Nevertheless, the fact is that the PC model does not fit very well with the density. In comparison, there is a PC molecule at the dimer interface, namely PCW1005 (chain A or B), which has a very good fitting with the density and can be used as a reference. Considering that no exogenous lipid molecules were added in the sample used for solving the complex structure, the lipid molecule trapped in the active site cavity should be from endogenous source and could be a mixture of different lipids instead of a single species. The weak density of the lipid head group also suggest that the occupancy may not be very high even if it is from PC. Therefore, the author should be open to the possibility of a mixed lipid species (such as PC and DAG, considering that the map is

reconstructed from numerous particles) or a partially occupied PC at this site. The point needs to be discussed in the revised manuscript, to explain the non-ideal fitting of PC model with the density.

2) The density of chelerythrine has been improved in the new map of yCPT1-chelerythrine complex. While the model of chelerythrine fits reasonably well with the corresponding density in chain B, the one in chain A does not fit very well, especially in the local region around the C4 atom of CTI1009. The problem can probably be solved by carrying out a rigid body refinement on the CTI1009 of Chain A, followed by the sphere refine on the ligand in the coot program to achieve an improved fitting of the model in the density.

3) The clashscore of yCPT1-DAG structure is still too high (10.24). Manual adjustment of the clashing residues/groups and further refinement need to be carried out to lower the clash score.

4) For the “Enzyme assay” in Materials and Methods session, the amount/concentration of enzyme used for the assays should be defined clearly in the text (lines 733, 739 and 742).

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed my previous questions/concerns in a constructive way. I don't have any further questions.

Reviewer #1 (Remarks to the Author):

The manuscript titled “Structural basis for catalysis and selectivity of phospholipid synthesis by eukaryotic choline-phosphotransferase” reports structures of the yeast CDP-choline phosphotransferase and proposes hypotheses on mechanisms of substrate recognition and catalysis. The main results are three structures of yeast CPT1 in the presence of either DAG, CDP-choline and PC, or an inhibitor chelerythrine. cryoEM data collection, data analysis, model building and refinement are consistent with the current common practices and standards. The proposed mechanisms seem reasonable, and I do not have any major concerns, except for a few minor complaints mostly on functional studies as listed below.

We appreciate the reviewer’s positive assessment of our experimental results.

1) An enzymatic activity assay was used to evaluate activities of the wild type and mutant CPT1 to provide support for the structure-based hypotheses. The functional studies are quite rudimentary with only a single substrate concentration.

To address this comment, we performed experiments titrating the substrate concentration in order to obtain a more comprehensive understanding of the mutant’s activity. Figure 2e-f now include a range of DAG concentrations, Figure 3h-i now include a range of CDP-choline or CDP-ethanolamine concentrations, and Figure 5b now includes a range of CDP-choline concentrations. We believe that these updated data support our findings about the altered enzymatic activity of the mutants.

2) LC-MS/MS was applied to quantify lipid content in the purified protein, however, there is no mention of how detergent may affect the retention of the co-purified lipids. There is also no estimation on the number of bound lipids per protomer.

It is not uncommon for membrane proteins to co-purify with lipid molecules even though these purifications are done using detergents. While it is hard to quantify how various detergents would affect the retention of co-purified lipids, in some cases, such as seen in our maps, density for co-purifying lipids is visible. In single particle cryo-EM analysis this can only happen when the lipids have high occupancy binding and/or play a structural role for the protein, otherwise the density for the lipid would be averaged out of the map. In our maps, we found densities for three phospholipids located on the outside of each protomer and one phospholipid (or DAG) bound inside each protomer. However, since we cannot exclude the possibility that there may be additional lipids loosely bound to the protein with low occupancy and/or not bound in a structured way (and therefore their position(s) averaged out of the maps), we would prefer not to include this estimation in the manuscript.

3)The proposed mechanism of CDP-choline recognition seems solid, so is the mobile histidine that facilitates extraction of the hydroxyl proton on DAG. However, the narrative on A or G at position 97 being part of the mechanism for achieving selectivity

is not on a solid ground. Shouldn't the exception on hEPT1 already have broken down the correlation?

We appreciate the feedback that the CDP-choline recognition seems reasonable, as does the mobile histidine likely being involved in the extraction of the hydroxyl proton of DAG. We agree with the reviewer that having an A or G at position 97 is a prediction rather than a strict rule for knowing the selectivity of an enzyme.

To better clarify this in the manuscript, we have edited the following statement (L. 270): "However, this prediction does not work for hEPT1, which with an Ala at this position would be predicted to be specific for larger CDP-choline, but instead is a promiscuous enzyme capable of using both CDP-choline and CDP-ethanolamine. This indicates that there are likely additional structural features that contribute to head-group selectivity."

To the following:

(now L. 277): "However, this is a prediction rather than a strict rule, since there are examples of promiscuous enzymes that can use both CDP-choline and CDP-ethanolamine as substrates. Nevertheless, even in the case of these promiscuous enzymes, having an A or G at position 97 correlates with which substrate is preferred. hCEPT1 and yEPT1 can catalyze both CDP-choline and CDP-ethanolamine although at different efficiencies (Hjelmstad and Bell, 1987; Henneberry and McMaster, 1999). hCEPT1 with an Ala at position 97 would be predicted to have higher specificity for CDP-choline over CDP-ethanolamine, which it does (Supplementary Fig. 12a) (Henneberry and McMaster, 1999). yEPT1 with a Gly at position 97 would be predicted to have higher specificity for CDP-ethanolamine versus CDP-choline, which it also does (Hjelmstad and Bell, 1987). Thus, the identity of residue 97 (A or G) clearly contributes to which substrate can be used or is preferred. However, for promiscuous enzymes there are likely additional structural features that also contribute to head-group selectivity."

We have also updated Supplementary Figure 12 to include a table describing the preference of yCPT1, yEPT1, hCHPT1, hCEPT1, and hEPT1 for: 1) PC or PE production, and 2) Whether there is an A or G in the position that would be predicted to correlate with substrate specificity.

4) It is intriguing to see that two acyl chains from a DAG could "straddle" TM6. How certain can the density be assigned to DAG? And could the authors comment on how a DAG could achieve this pose?

a. Both reviewers requested clarification about our certainty in assigning the density to DAG (see Reviewer 2-comment 2).

The reviewers are correct that this density does not have clear enough features for us to be completely certain that it represents DAG versus another phospholipid species. To address this concern, we have now done the following:

First, the experiments that we performed to address reviewer's 1 comment about the rudimentary nature of our functional studies (Reviewer 1-comment 1) now also give us confidence that our structural analysis of yCPT1 in detergent micelles was done in an environment of ~20-fold molar excess of DAG to yCPT1 (Figure 2f). A caveat of this calculation is that we have no way of quantifying how much of DAG was incorporated into the detergent micelles that surround yCPT1 and protein-free micelles; however, this large molar excess of DAG would increase the likelihood that DAG is present in the active site.

Second, to improve the structural features of this density in our map, we reconstituted yCPT1-Nb24 into a nanodisc assembled from a lipid composition of 8:2 DOPC:DAG. The rationale for this experiment was that the occupancy of DAG in the yCPT1 active site might improve by confining DAG to the same membrane environment as yCPT1 (versus relying on DAG being in the detergent micelles that surround yCTP1). Taking this approach, we determined a 3.7 Å resolution structure of the yCPT1 dimer embedded in the DAG-containing nanodisc (now Supplementary Figs. 7, 8). The structural organization of the yCPT1 in the nanodisc is globally the same as the 3.2 Å resolution structure we determined of yCPT1 in detergent micelles. Both structures of yCPT1 have a density in the same position that we had assigned to DAG in the detergent micelle yCPT1 structure. This density in yCPT1 nanodisc structure improved enough that we can now confidently fit the glycerol head group of DAG. While the head group region is better resolved, the densities for the two acyl chains are now less resolved; however, the angles of the chains coming from the glycerol head group still agree with the splayed density for the acyl chains that were present in the 3.2 Å resolution micelle map.

With the combination of more thorough biochemical assays and the new structure of yCPT1 in nanodiscs made from a mixture of DOPC:DAG we are confident that this density represents DAG rather than PC. The manuscript now includes the supplemental figures showing the structure of yCTP1 in nanodiscs (Supplement Figs. 7, 8) and we have added the following sentences into the results section:

(L. 166) – "To confirm our model of DAG binding to yCPT1, we prepared yCPT1 reconstituted in lipid nanodiscs enriched with C16-DAG and analyzed the structure by single-particle cryo-EM (Supplementary Figs. 7-8). In this structure, density for the glycerol head-group was clearly observed at the same pocket near the catalytic center that we assigned to DAG in the structure of yCPT1 in detergent micelles (Figs. 1-2 and Supplementary Fig. 7). While the acyl-chain densities were less visible in this yCPT1-nanodisc map due to the lower resolution, the angles of the chains coming from the glycerol head group agree with the splayed density for the acyl chains we observed in the 3.2 Å resolution map of yCPT1 in detergent micelles (Figs. 1-2, and Supplementary Fig. 7)."

b. We agree with the reviewer that the acyl chains of DAG adopt an interesting conformation. Indeed, this conformation is predicted to be energetically unfavorable for either phospholipids or diacylglycerols (Marsh, 2003). Still, this is the conformation we

see the DAG acyl chains adopt in the yCPT1 maps whether in detergent micelles or nanodiscs. We have now added a sentence in the manuscript highlighting that this is an unexpected conformation for the DAG.

The manuscript has been updated to include the following sentence:
(L. 176)- "In our model, the acyl chains of DAG adopt an unusual, splayed conformation, with the acyl chains "straddling" TM6."

Line 32: change "those" to "that"?
The label "TM4" is on the wrong helix in Fig 2c.

We thank the reviewer for noticing these errors and they have been corrected.

Reviewer #2 (Remarks to the Author):

Phosphatidylcholine and the other phospholipids are the fundamental components of biological membranes. Choline-phosphotransferase (CPT) is a membrane-embedded enzyme catalyzing the final step of phosphatidylcholine biosynthesis. Through the catalytic function of CPT, cytidine-5'-diphosphate (CDP)-choline and 1,2-dacylglycerol (DAG) are converted into phosphatidylcholine and CMP. In the manuscript presented by Roberts, J. R. et al., the cryo-EM structures of a yeast (Saccharomyces cerevisiae) CPT1 in complex with substrates and an inhibitor were reported. The molecular bases underlying the DAG acyl-chain preference, choline/ethanolamine selectivity and inhibitor binding have been analyzed through structural analysis and biochemical assays. While new insights have been obtained through their work, there are several questions or suggestions for the authors to consider during revision.

We thank the reviewer for their careful assessment of our work and their suggestions to strengthen the manuscript.

1) In lines 153-154, it was stated that "the dimerization interface and contributions of lipids to support dimerization are not conserved between species.". Have the authors tried to do sequence alignment among the CPT1 homologs from various fungal species to see if the dimerization motif found in yCPT1 is conserved among the homologs from fungi?

We appreciate the reviewer's suggestion to make a sequence alignment of CPT1 homologs from fungi. To do this, we curated the sequences of 169 CPT1 homologues from fungal species based on their sequence identity. We used sequences that ranged between 60%-90% identity to yCPT1. This showed that residues in the S. cerevisiae dimer interface are not conserved in other fungi, but are strongly conserved (88%) in the Saccharomyces genus, and, thus, we excluded the closest homologues in this genus. This alignment shows that residues involved in the S. cerevisiae CPT1 dimerization interface are not well conserved in other fungi. The data are now included as a logo plot in Supplemental Figure 4h. Thus, although existing as obligate dimers appears to be a

conserved structural feature of CDP-APs homologs (now Supplementary Fig. 4e-g), there does not seem to be a highly conserved dimerization motif.

To make this point in the manuscript we have updated the main text (L. 154) to read: "…the dimerization interface and contributions of lipids to support dimerization vary between species, including homologs in other fungi (Supplementary Fig. 4e-h)".

2) In the structure of yCPT1-DAG complex, the lipid density in the active-site cavity was assigned as DAG by taking account of the mass spectrometry analysis result of the purified yCPT1 sample (Supplementary Fig. 6). As shown in the mass spec data (Suppl. Fig. 6c), the content of DAG in the purified yCPT sample is fairly low at 0.02-0.15 pmol/nmol yCPT. Such an extremely low molar ratio of DAG:yCPT does not seem to support the assignment of the lipid density as DAG. Is it possible that the density may belong to PC as it is a dominating lipid species detected in the purified protein sample? To obtain the structure of yCPT1 in complex with DAG, it might be better to add sufficient exogenous DAG into the purified yCPT1 so that the internal lipid-binding site is loaded with DAG at high occupancy.

The concern about our confidence of assigning the density found in the yCPT1 active site was also shared by reviewer 1 and many of these questions have been addressed in comment 4 for Reviewer 1. We appreciated the reviewer's suggestion to make sure that there were sufficient amounts of exogenous DAG in the yCPT1 preparation used for structural analysis. We were able to test this when we expanded the activity assays as requested by Reviewer 1 (comment 1). In the new activity assays, we titrated the concentrations of DAG added to the purified yCPT1 samples (wild type and mutants) and then measured the release of CMP. Importantly, we found that even without adding additional substrate to 3 pmol of purified yCPT1, ~60 pmol of CMP was released. Thus, the purified yCPT1 we used for structural analysis already contained ~20-fold molar excess of DAG (Figure 2f). While a caveat of this calculation is that we are unable to quantify the amount of DAG accessible to yCPT1, the results of this analysis suggest that simply adding more DAG would not improve the occupancy of DAG in the active site because the protein sample already contains molar excess DAG.

To address this concern in a different way, we reconstituted yCPT1-Nb24 into nanodiscs using a lipid composition of 8:2 DOPC:DAG. The rationale for this experiment was that the occupancy of DAG in the yCPT1 active site would improve by having DAG in the same membrane environment as yCPT1. Taking this approach, we determined a 3.7 Å resolution structure of the yCPT1 dimer embedded in the DAG-containing nanodisc (Supplementary Figs. 7, 8). This analysis, as discussed in more detail in Reviewer 1-comment 4, allowed us to confidently fit the glycerol head group of DAG into this density in the active site.

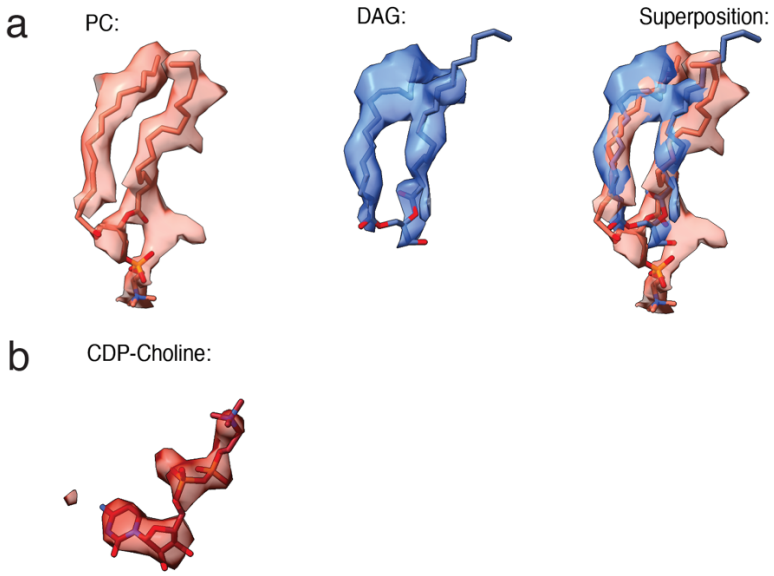
3) For the structure of yCPT1 in complex with CDP-choline, there is a lipid molecule bound to the active site nearby CDP-choline and it was assigned as the end-product of the reaction, namely PC. As the choline group of PC comes from CDP-choline, if the lipid belongs to PC, the CDP-choline should be converted into CMP after the choline-

phosphate group is transferred to DAG. As a result, it should be CMP instead of CDP-choline bound to the active site along with PC. While the density of CDP-Choline fits well with the model, the density for the head group of PC is fairly weak and does not seem to fit well with the choline-phosphate group. Is it possible that the lipid density may belong to DAG or other lipids, or represent a mixture of DAG, PC and other phospholipid associated at the same site? Considering that there are no exogenous lipids added to the sample, it is hard to define the exact identity of the lipid molecule associated at the site nearby CDP-Choline if it is heterogenous.

To overcome the problem and obtain the information relevant to the catalytic process, it might be better to load the two substrates (CDP-choline and DAG) simultaneously into the purified yCPT1 sample and solve the structure of yCPT1-CDP-choline-DAG complex. Alternatively, the two products, namely CMP and PC, could be loaded into yCPT1 for obtaining the structures of yCPT1 in complex with the products. Either the yCPT1-CDP-choline-DAG complex structure or the yCPT1-CMP-PC complex structure will be much more helpful in understanding the catalytic mechanism yCPT1 than the yCPT1-CDP-choline-PC complex.

The reviewer is wondering why we see CDP-choline and PC in the active sites of the yCPT1 structure (Figure 3), when it would potentially make more biochemical sense that either CMP and PC or CDP-choline and DAG would be present together. In other words, why is the end product, PC, still seen associated with yCPT1 when there is also CDP-choline present. While we appreciate the reviewer's suggestion to try to simultaneously load two substrates into yCPT1, the logistics of this experiment would be hard to properly control in order to achieve the desired structural result.

Our reasoning for assigning these densities to CDP-choline and PC is two-fold. First, it has been shown biochemically that CMP, the byproduct of the reaction, does not robustly inhibit yCPT1, having an apparent Ki > 2mM (Hjelmstad and Bell, 1987). In our experiment, we added 1mM CDP-choline to the sample prior to preparing cryo-EM grids. Even if all the CDP-choline was converted to CMP, it would not be expected to significantly inhibit yCPT1. This finding indicates that upon formation of PC and CMP during the yCPT1 catalytic cycle, CMP would not remain tightly associated with the enzyme (otherwise it would act as an inhibitor). However, PC has a significant interaction interface within the yCTP1 which would make its diffusion from the enzyme relatively slow (Figure 3c). Second, the density that we have assigned to PC, has features indicative of the PC headgroup, but not the DAG headgroup. This means that each density accommodates CDP-choline (rather than CMP) and PC (rather than DAG) (See Reviewer Figure 1 below). Thus, while we agree with the reviewer that it can be difficult to identify lipids in this resolution of map, we think that the combination of the reported biochemistry and the shapes of the density in the maps support our interpretation of the density.



Reviewer Figure 1: Comparison of densities attributed to PC and DAG in yCPT1. **a.** Density assigned to PC (left) in the CDP-choline and PC-bound yCPT1 map (Figure 3), density assigned to DAG (middle) in the DAG-bound map (Figure 2) and the superposition of both DAG and PC densities (right). **b.** Density assigned to CDP-choline in the CDP-choline bound yCPT1 map (Figure 3).

We have edited the main text (L. 207) to explain why we modeled PC rather than CMP into this density and more clearly state that this finding was unexpected:
"The resulting 2.8 Å resolution map has resolved densities that fit CDP-choline and PC. This finding was not anticipated since it would potentially make more biochemical sense that CMP and PC would be found together in the same structure. Our reasoning for assigning these densities to CDP-choline and PC is two-fold. First, it has been shown biochemically that CMP, the byproduct of the reaction, does not robustly inhibit yCPT1 (Hjelmstad and Bell, 1987). This finding indicates that upon formation of PC and CMP during the yCPT1 catalytic cycle, CMP does not remain tightly associated with the enzyme (otherwise it would inhibit the next reaction). PC, with a significant interaction interface within the yCPT1 (Figure 3c), likely diffuses from the enzyme slowly. Second, the density that we have assigned to PC, has features indicative of the PC headgroup (Fig 3b), but not density that would fit a DAG headgroup (Fig. 2b)."

4) In lines 220-221, "This ionic interaction is conserved among all reported structures of CDP-APs and is likely critical for the catalytic reaction.". To support the statement, evidence such as a sequence alignment figure or references of the previous works should to be cited at the end of the sentence.

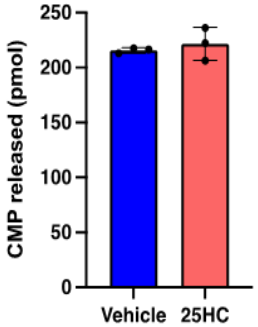
We thank the reviewer for these suggestions. We now cite manuscripts reporting the C DP-AP structures and include a sequence alignment to support our statement (now Supplementary Fig. 4b).

We have changed lines 220-221 to read (now L. 236):

“This ionic interaction is conserved among all reported structures of CDP-APs with CDP derivative bound and the perfect conservation of this residue among these proteins indicate that this interaction is likely critical for the catalytic reaction (Supplementary Fig. 4b) (Nogly *et al.*, 2014; Sciara *et al.*, 2014; Clarke *et al.*, 2015; Grāve, Bennett and Högbom, 2019; Belcher Dufrisne *et al.*, 2020; Centola *et al.*, 2021; Yang *et al.*, 2021; Wang and Zhou, 2023; Wang *et al.*, 2023).”

5) In the structure of yCPT1 in complex with chelerythrine, the model of chelerythrine in one of the two monomers (monomer A) does not fit well with the density. The C4 atom of chelerythrine model is located outside the density. The model needs to be refined further to fit the density better. Moreover, there appears to be addition densities connected to the O26 atom of the chelerythrine molecule. It appears that the CHS model (https://www.rcsb.org/ligand/Y01) also fits the density well (maybe better than chelerythrine). Is it possible that that the binding site is a mixed binding site for chelerythrine and CHS (and/or lipids)? In that case, the author may need to generate a mask of chelerythrine and apply it in the skip-alignment 3D classification procedure to enrich the particles with chelerythrine and separate it from those associated with the other ligands. In case that the skip-alignment classification does not help to solve the problem, the sample may need to be prepared in a condition free of CHS or other chelerythrine-like additives and then supplied with chelerythrine for cryo-EM data collection.

The reviewer is correct that there is likely some heterogeneity at O26. Since CHS was present in every protein purification used in this study and we did not observe a density like this in any other prep, we think that the additional density connected to the O26 atom of the chelerythrine molecule is most likely due to a heterogenous population of chelerythrine and DAG-bound yCPT1 particles. To better address this concern, we took two approaches. First, we biochemically tested whether CHS competes with DAG for the yCPT1 active site using an enzyme activity assay of yCPT1 in the presence of 25-hydroxy cholesterol, an analogue of CHS. We found that this compound has no impact on the activity of yCPT1. We include the result of this assay in a reviewer figure below (Reviewer Figure 2).



Reviewer Figure 2: yCPT activity in the presence of hydroxy cholesterol.* Vehicle=1% MeOH. The activity of yCPT1 is monitored by the release of byproduct CMP. The presence of 100uM 25-hydroxy-cholesterol (25HC) does not inhibit the reaction.

Secondly, as the reviewer helpfully suggested, we generated a mask of chelerythrine and did a 3D classification without additional alignment in Relion (v4.0.1). While this improved the density of the inhibitor (chelerythrine), we obtained the best resolved chelerythrine density when we took a similar approach in cryoSPARC (v4.5.3). In this case we used the maps of DAG-bound yCPT1 (described in Figure 2) and chelerythrine-bound yCPT1 (described Figure 4) as references for a reference-based 3D classification. This 3D classification strategy placed ~30% (130,293) of the particles into the DAG-bound yCPT1 3D class and the remaining ~70% (302,425) into the chelerythrine-bound yCPT1 3D class. We then took the particles in the chelerythrine-bound yCPT1 3D class and did a final ab initio reconstruction followed by a non-uniform refinement with C2 applied symmetry. The resulting yCPT1 map has a clearly defined density for chelerythrine. We have updated our maps (Figure 4b-d) and the processing figure (Supplementary Fig 14).

From these analyses we believe that the extra density near chelerythrine seen in our original maps was not derived from CHS, but came from a heterogenous mixture DAG-bound yCPT1 and chelerythrine-bound yCPT1 present in our data set that we were able to separate using 3D classification approaches.

6) For the three cryo-EM structures presented in the work, the maps were refined with no symmetry applied (C1). However, the two adjacent monomers within the homodimers apparently follow a C2 or pseudo-C2 symmetry. Have the authors tried to refine the maps with the C2 symmetry imposed, which may help to improve the resolution and map quality? In supplementary Table 1, the clashscore for the yCPT1 DAG structural model is a bit too high and may need to be reduced to a value around or below 4 through further refinement. For further refinement of the structural model against the cryo-EM map, application of non-crystallographic symmetry (NCS) restraints/constraints will help to improve the model quality (especially when the C2 symmetry is applied for generating the cryo-EM map).

a. Yes, the reviewer is correct that we had already attempted to refine the maps with C2 symmetry. While enforcing symmetry nominally improved the global resolution of the maps, it did not improve the actual quality of the map or our ability to build models into the density. For this reason, we continued with using C1 symmetry.

b. We were not able to significantly improve the clashscore for the yCPT1 DAG structure using the approaches suggested by the reviewer. The cause of the too-high clash score appears to be between the acyl chains of DAG and yCPT1 and not because of problems with the yCPT1 model.

7) In Fig. 1b, monomer B (in grey) is not distinguished well from the silver part of monomer A. It will be better to change it to a different color to visually separate it from the dimerization motif (and the other parts) of monomer A. In suppl. Fig. 2b an c, please explain in the legend what are the PC samples loaded as the references. Are they the standard radiolabeled PC sample? For suppl. Fig. 4e and f, the legends of these two panels are not provided. In line 43, “4NMD” is different from the one label in panel e

(4MND). In the main text, there are also some other typos to be corrected. For instance, “contributing” in line 214 and “previously” in line 244.

a. In Figure 1, we have changed the color of monomer B from gray to white to help differentiate this protomer from the dimerization motif.

b. In Supplementary Figure 2b and C, the reference lipid is 14C labeled PC 16:0-20:4 from American Radiolabeled Chemicals. We have amended the supplementary figure legend so that it reads:

> “**b.** The enzymatic activity of purified recombinant yCPT1 was measured using radiolabeled CDP-choline and DAG as the substrates. Radioactive phospholipids were analyzed by TLC. The reference PC sample is 14C labeled PC 16:0-20:4 from American Radiolabeled Chemicals **c.** Purified yCPT1 contain endogenous DAG from the Sf9 cells as the enzymatic reaction proceeds without adding extra DAG. The reference PC sample is 14C labeled PC 16:0-20:4 from American Radiolabeled Chemicals.”

c. We have added figure legends to panels e and f of Supplementary Figure 4 and corrected the error in the PDB code 4MND.

d. We thank the reviewer for bringing our attention to the listed typos and we they have been corrected.

References:

Belcher Dufrisne, M. *et al.* (2020) ‘Structural and Functional Characterization of Phosphatidylinositol-Phosphate Biosynthesis in Mycobacteria’, *Journal of Molecular Biology*, 432(18), pp. 5137–5151. Available at: https://doi.org/10.1016/j.jmb.2020.04.028.

Centola, M. *et al.* (2021) ‘Crystal structures of phosphatidyl serine synthase PSS reveal the catalytic mechanism of CDP-DAG alcohol O-phosphatidyl transferases’, *Nature Communications*, 12(1), p. 6982. Available at: https://doi.org/10.1038/s41467-021-27281-w.

Clarke, O.B. *et al.* (2015) ‘Structural basis for phosphatidylinositol-phosphate biosynthesis’, *Nature Communications*, 0, p. 8505. Available at: https://doi.org/10.1038/ncomms9505.

Grāve, K., Bennett, M.D. and Högbom, M. (2019) ‘Structure of Mycobacterium tuberculosis phosphatidylinositol phosphate synthase reveals mechanism of substrate binding and metal catalysis’, *Communications Biology*, 2, p. 175. Available at: https://doi.org/10.1038/s42003-019-0427-1.

Henneberry, A.L. and McMaster, C.R. (1999) ‘Cloning and expression of a human choline/ethanolaminephosphotransferase: synthesis of phosphatidylcholine and phosphatidylethanolamine.’, *Biochemical Journal*, 339(Pt 2), pp. 291–298.

Hjelmstad, R.H. and Bell, R.M. (1987) ‘Mutants of Saccharomyces cerevisiae defective in sn-1,2-diacylglycerol cholinephosphotransferase. Isolation, characterization, and cloning of the CPT1 gene.’, *Journal of Biological Chemistry*, 262(8), pp. 3909–3917. Available at: https://doi.org/10.1016/S0021-9258(18)61443-7.

Marsh, D. (2003) ‘Lipid–binding proteins: Structure of the phospholipid ligands’, *Protein Science*, 12(9), pp. 2109–2117. Available at: https://doi.org/10.1110/ps.0396803.

Nogly, P. *et al.* (2014) ‘X-ray structure of a CDP-alcohol phosphatidyltransferase membrane enzyme and insights into its catalytic mechanism’, *Nature Communications*, 5(1), p. 4169. Available at: https://doi.org/10.1038/ncomms5169.

Sciara, G. *et al.* (2014) ‘Structural basis for catalysis in a CDP-alcohol phosphotransferase’, *Nature Communications*, 5(1), p. 4068. Available at: https://doi.org/10.1038/ncomms5068.

Wang, L. and Zhou, M. (2023) ‘Structure of a eukaryotic cholinephosphotransferase-1 reveals mechanisms of substrate recognition and catalysis’, *Nature Communications*, 14(1), p. 2753. Available at: https://doi.org/10.1038/s41467-023-38003-9.

Wang, Z. *et al.* (2023) ‘Structural basis for catalysis of human choline/ethanolamine phosphotransferase 1’, *Nature Communications*, 14(1), p. 2529. Available at: https://doi.org/10.1038/s41467-023-38290-2.

Yang, B. *et al.* (2021) ‘The phosphatidylglycerol phosphate synthase PgsA utilizes a trifurcated amphipathic cavity for catalysis at the membrane-cytosol interface’, *Current Research in Structural Biology*, 3, pp. 312–323. Available at: https://doi.org/10.1016/j.crstbi.2021.11.005.

Reviewer #1 (Remarks to the Author):

I have no further comments.

We thank the reviewer for their original comments.

Reviewer #2 (Remarks to the Author):

While the authors have improved their work during the last revision, there might be a few minor issues to be settled for further revision.

1) In the structure of yCPT1 in complex with CDP-choline, the authors tried to explain and validate the assignment of the lipid molecule (trapped in the active-site cavity nearby CDP-chloine) as PC. Nevertheless, the fact is that the PC model does not fit very well with the density. In comparison, there is a PC molecule at the dimer interface, namely PCW1005 (chain A or B), which has a very good fitting with the density and can be used as a reference. Considering that no exogenous lipid molecules were added in the sample used for solving the complex structure, the lipid molecule trapped in the active site cavity should be from endogenous source and could be a mixture of different lipids instead of a single species. The weak density of the lipid head group also suggest that the occupancy may not be very high even if it is from PC. Therefore, the author should be open to the possibility of a mixed lipid species (such as PC and DAG, considering that the map is reconstructed from numerous particles) or a partially occupied PC at this site. The point needs to be discussed in the revised manuscript, to explain the non-ideal fitting of PC model with the density.

As requested, we have added the following points in the results and discussion sections:

(L207) The resulting 2.8 Å resolution map has resolved densities that fit CDP-choline and a lipid species that, although the density for this lipid is weak, we modeled as PC. (Fig. 3b, d). The weakness of this density suggests either low occupancy of PC or that there could be a heterogenous population of PC and DAG at this position.

(L221) Despite these indicators, the assignment of this density to PC will require further study to fully test this model.

(L354) Unexpectedly, we observed a PC-like lipid density at the DAG binding site in the CDP-choline bound structure of yCPT1. According to the reaction cycle, PC is the end product of the catalytic reaction of yCPT1 and would have been released from the enzyme. While the weak density at this position of the map suggests low occupancy or perhaps a mixed population of lipid species, it is intriguing that CDP-choline and a lipid molecule can stably occupy the binding site simultaneously. Further study will be needed to explore this observation.

2) The density of chelerythrine has been improved in the new map of yCPT1-chelerythrine complex. While the model of chelerythrine fits reasonably well with the corresponding density in chain B, the one in chain A does not fit very well, especially in the local region around the C4 atom of CTI1009. The problem can probably be solved by carrying out a rigid body refinement on the CTI1009 of Chain A, followed by the sphere refine on the ligand in the coot program to achieve an improved fitting of the model in the density.

This has been addressed. Fig. 4 and Fig. S14 have been updated and the updated yCPT1-chelerythrine model has been uploaded to the PDB.

3) The clashscore of yCPT1-DAG structure is still too high (10.24). Manual adjustment of the clashing residues/groups and further refinement need to be carried out to lower the clash score.

This has been addressed. The clashscore of yCPT1-DAT is now 6.75. This value has been updated in the cryo-EM table (Supplemental Table 1) and the updated model has been uploaded to the PDB.

4) For the “Enzyme assay” in Materials and Methods session, the amount/concentration of enzyme used for the assays should be defined clearly in the text (lines 733, 739 and 742).

These values have now been added to the Material and Methods section:

(now L762) ….and 0.2µM enzyme.

(now L768) …..and 2µM for A97G mutant and 0.3µM for other enzymes

(now L770) The content of CMP released from CDP-choline was quantified by LC-MS/MS as described below. For ethanolamine phosphotransferase assay, 0.3µM WT and 2µM A97G enzyme…..