

## Supplementary Information

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

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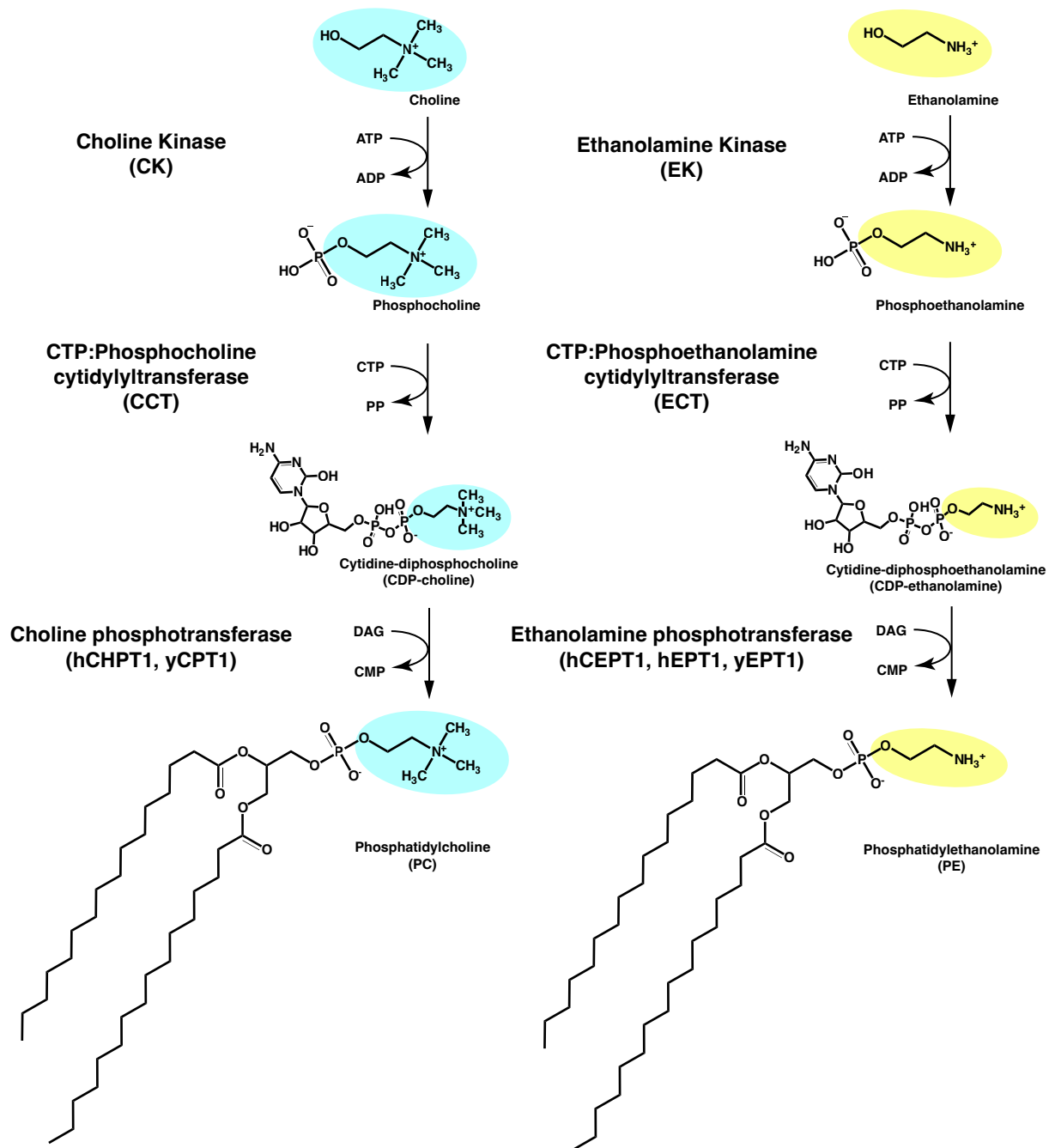
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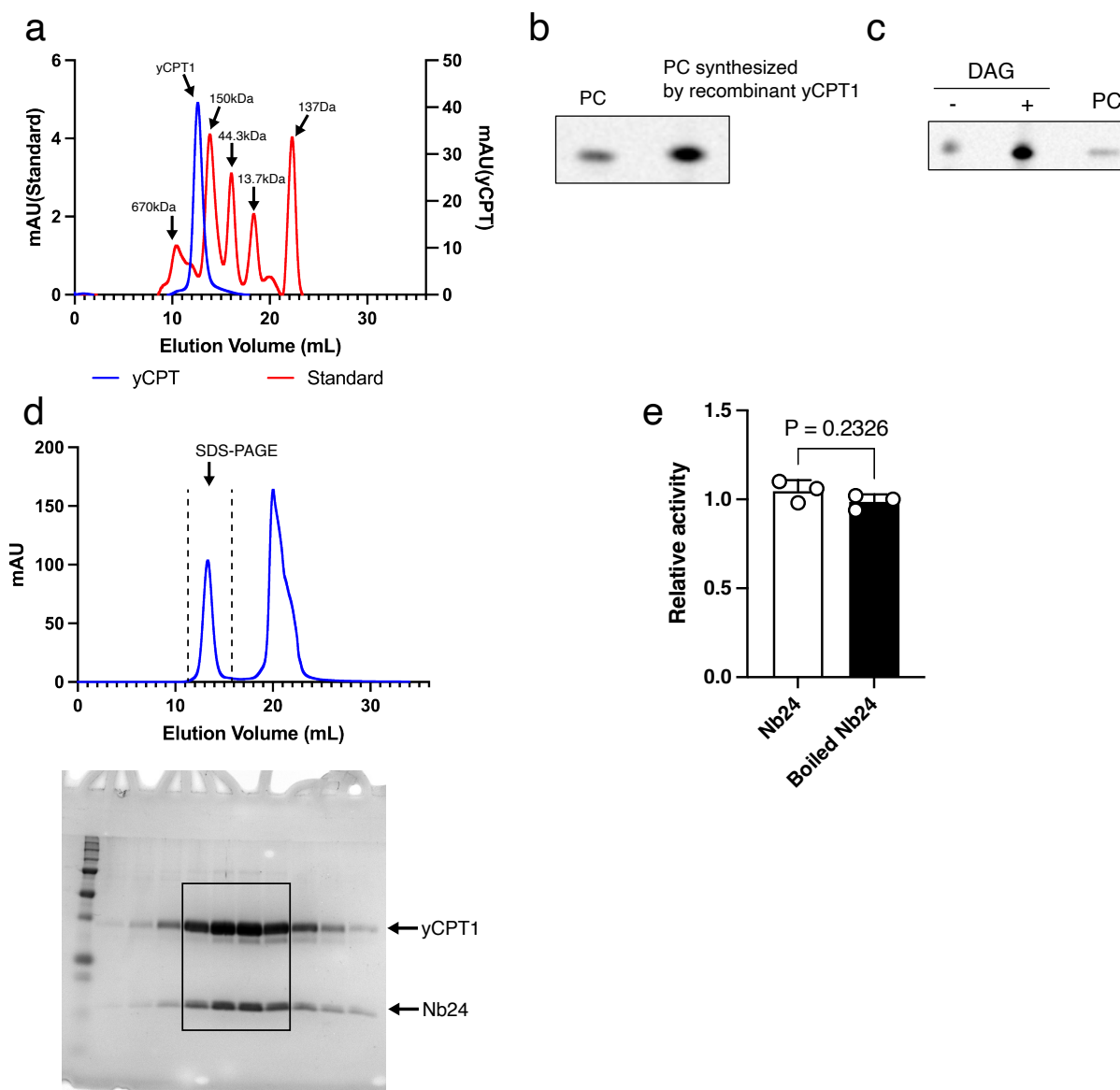
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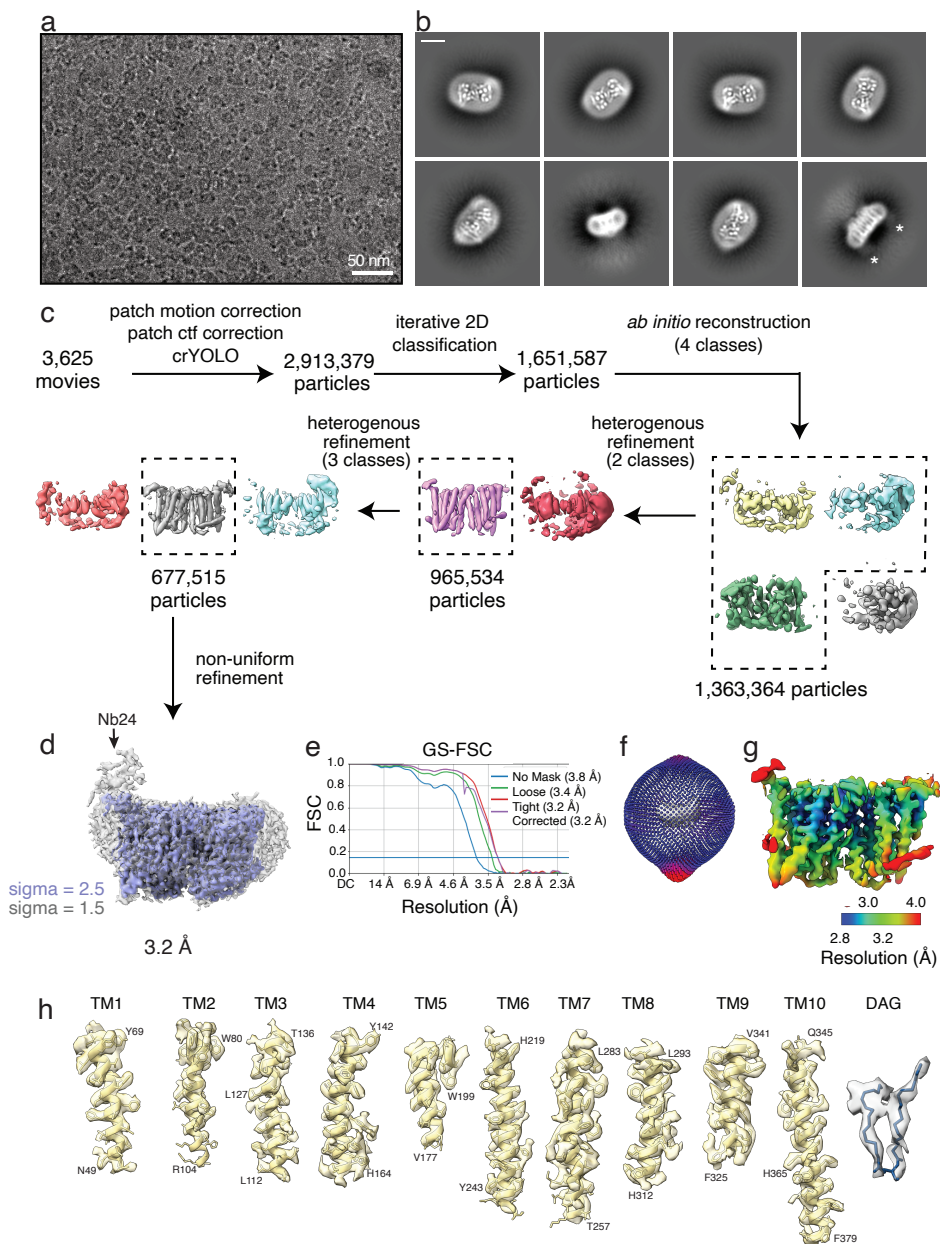
**Supplementary Figure 1. Depiction of Kennedy pathway for *de novo* phosphatidylcholine (PC) and phosphatidylethanolamine (PE) synthesis.** Choline or Ethanolamine is taken up by the cell and converted to phosphocholine or phosphoethanolamine by choline kinase or ethanolamine kinase respectively. These molecules are further converted to CDP-choline or CDP-ethanolamine by CTP:phosphocholine cytidyltransferase or CTP:phosphoethanolamine cytidyltransferase respectively. Choline phosphotransferase or ethanolamine phosphotransferase mediates the final step to conjugate these molecules with DAG to produce PC or PE.





**Supplementary Figure 2. Biochemical characterization of yCPT1.** **a.** Analytical size-exclusion chromatography profile of yCPT1 purified from Sf9 cells overlaid with trace from molecular weight standards. **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  $^{14}\text{C}$  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  $^{14}\text{C}$  labeled PC 16:0-20:4 from American Radiolabeled Chemicals **d.** Preparative size-exclusion chromatography of the yCPT1-Nb24 complex. The main peak fractions around 12ml, shown by dashed lines, were analyzed by SDS-PAGE to confirm co-migration of Nb24. The fractions indicated by the rectangle were used to prepare the cryo-EM sample. **e.** Binding of Nb24 has no impact on the enzymatic activity of purified recombinant yCPT1. yCPT1 was preincubated with either intact Nb24 (white) or boiled Nb24 (black) at  $100^\circ\text{C}$  for 10 min prior to the reaction. Data are

presented as the mean  $\pm$  SD from three independent measurements. Statistical significance was determined by unpaired two-sided t-test.

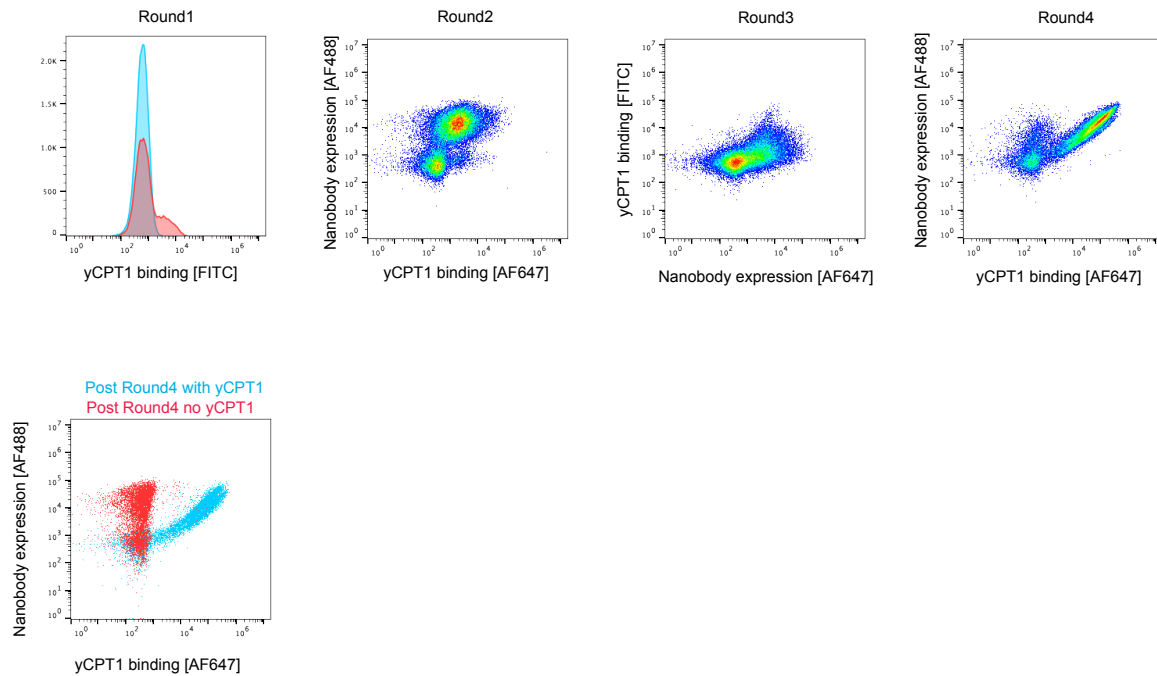


**Supplementary Figure 3. Cryo-EM processing of yCPT1-Nb24 in complex with DAG. a.**

Representative sub-area of a motion corrected micrograph of vitrified yCPT1-Nb24 particles. Scale bar 50 Å. **b.** Representative 2D classes. Position of Nb24 marked by \*. Box size 276 Å. **c.**

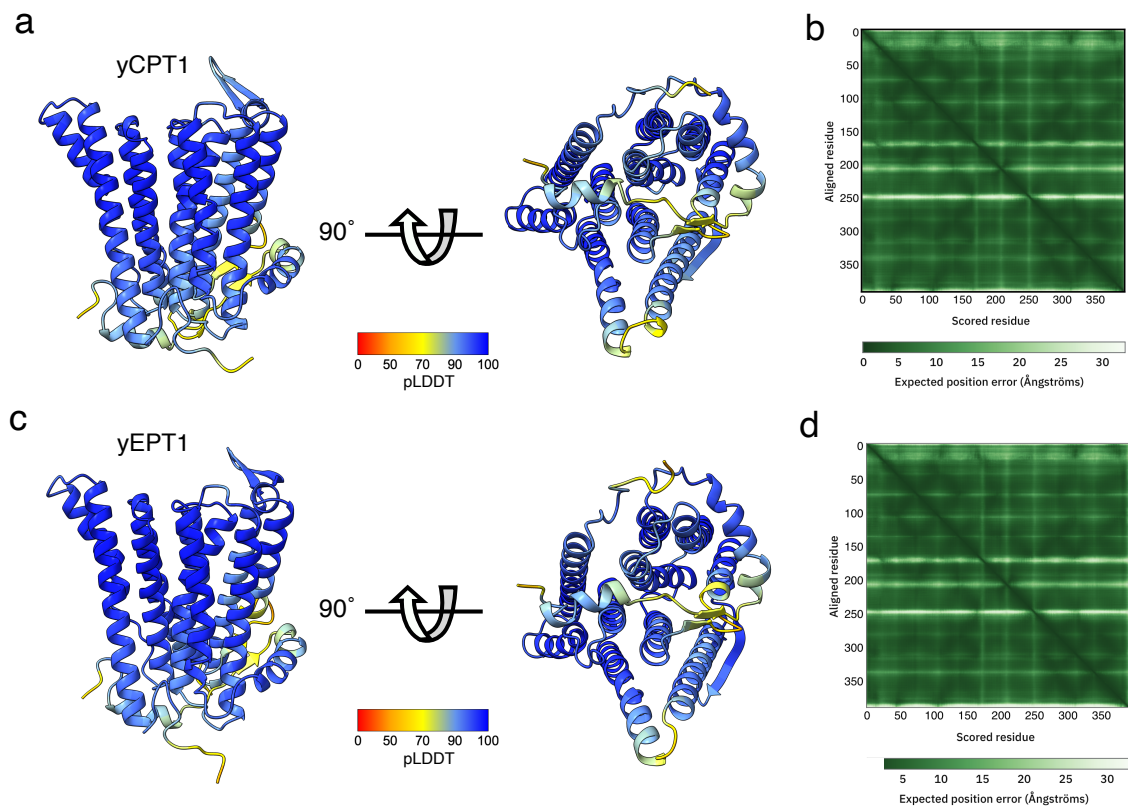
Cryo-EM processing workflow. 2,913,379 particles were selected from 3,625 micrographs using crYOLO. **d.** After iterative 2D classification and heterogenous refinement 677,515 good particles were subjected to a non-uniform refinement, reaching 3.2 Å resolution **e.** GS-FSC plot. **f.**

Particle orientation distribution plot. **g.** Local resolution estimation. **h.** Density of each TM helix and DAG are shown contoured at 3 sigma.

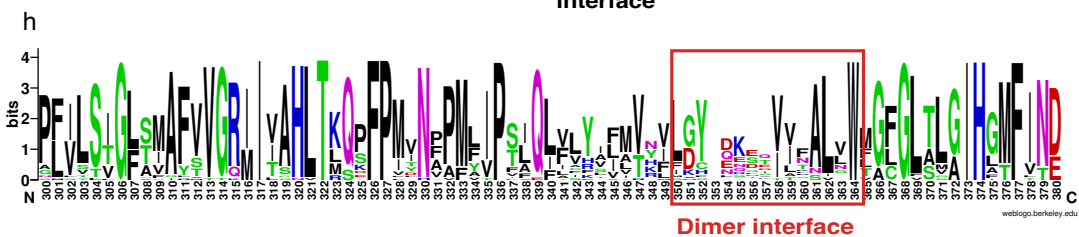
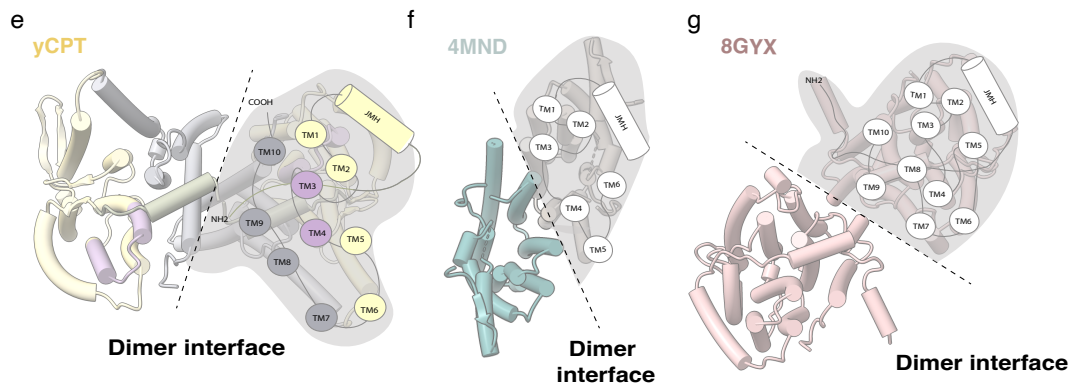
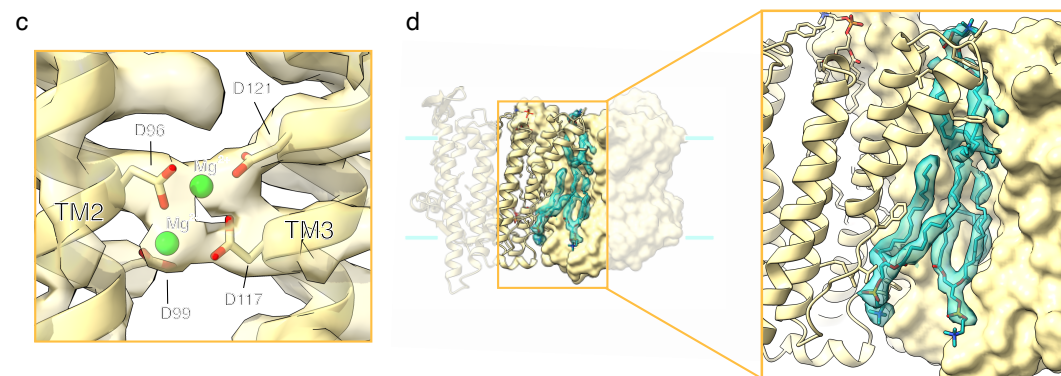
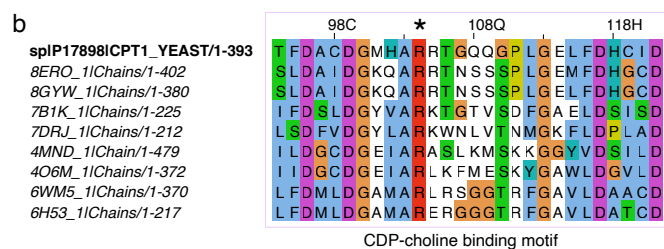
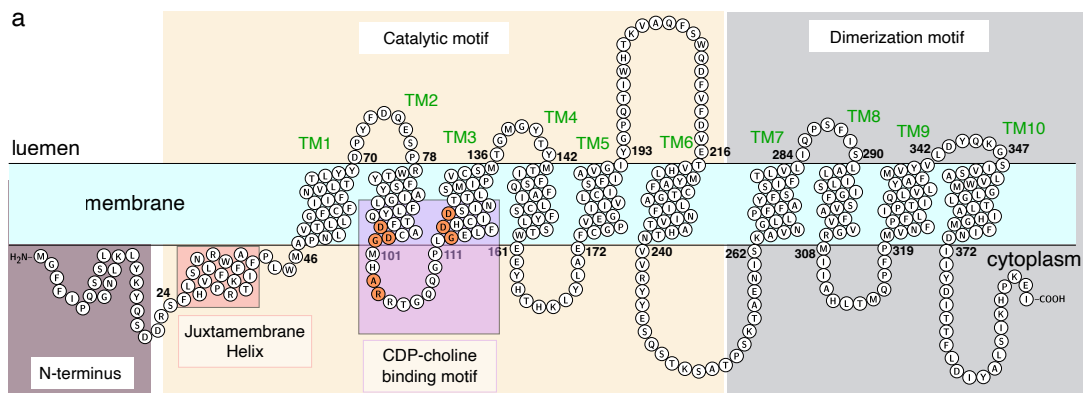


**Supplementary Figure 4. Yeast display selection and enrichment for yCPT1 nanobodies.**

(upper panels) Flow cytometry of enrichment of yeast clones binding to yCPT1. For the round1, pre-MACS(blue) and post-MACS(red) profiles are superposed. Note that FITC and AF647 are used alternately between rounds to monitor the yCPT1-bound yeast population. For the rounds 3 and 4, the top 0.1-0.2% population was gated for the subsequent round. (bottom) After round 4, the enriched clones show robust binding to yCPT1.

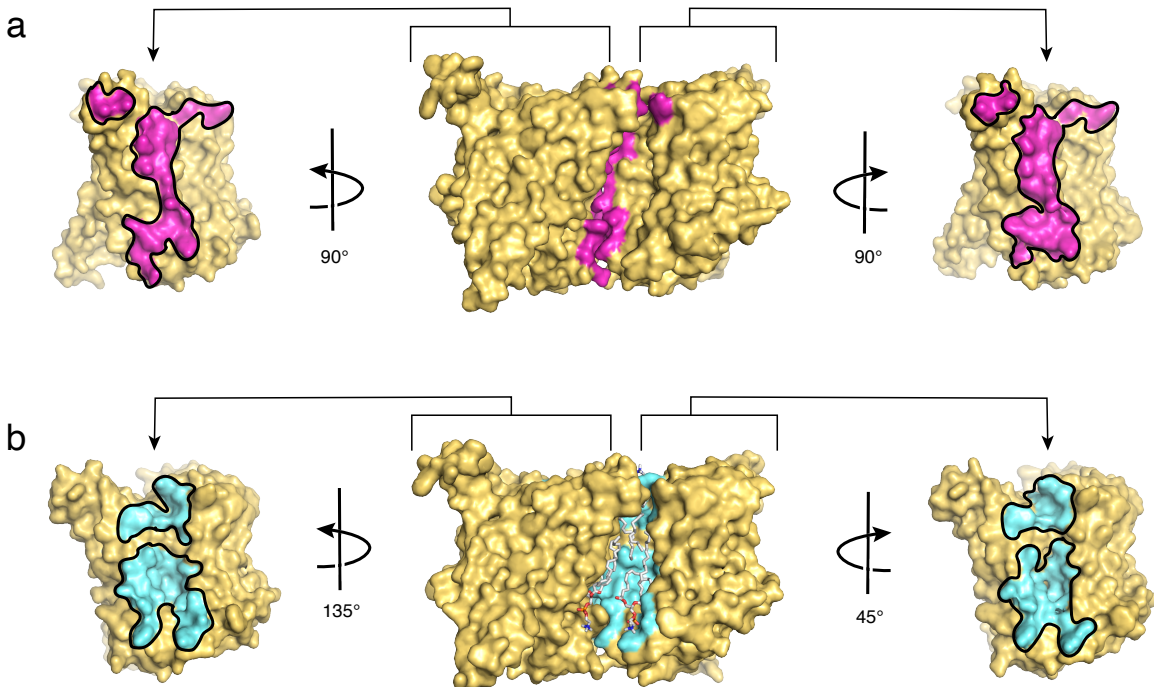


**Supplementary Figure 5. AlphaFold 2 models of yCPT1 and yEPT1.** **a.** AlphaFold 2 model of yCPT1 (UniProt P17898, AF-P17898-F1-v4) colored by pLDDT. **b.** Predicted aligned error (PAE) plot of yCPT1 model. **c.** AlphaFold 2 model of yEPT1 (UniProt P22140, AF-P22140-F1-v4) colored by pLDDT. **d.** Predicted aligned error (PAE) plot of yEPT1 model.



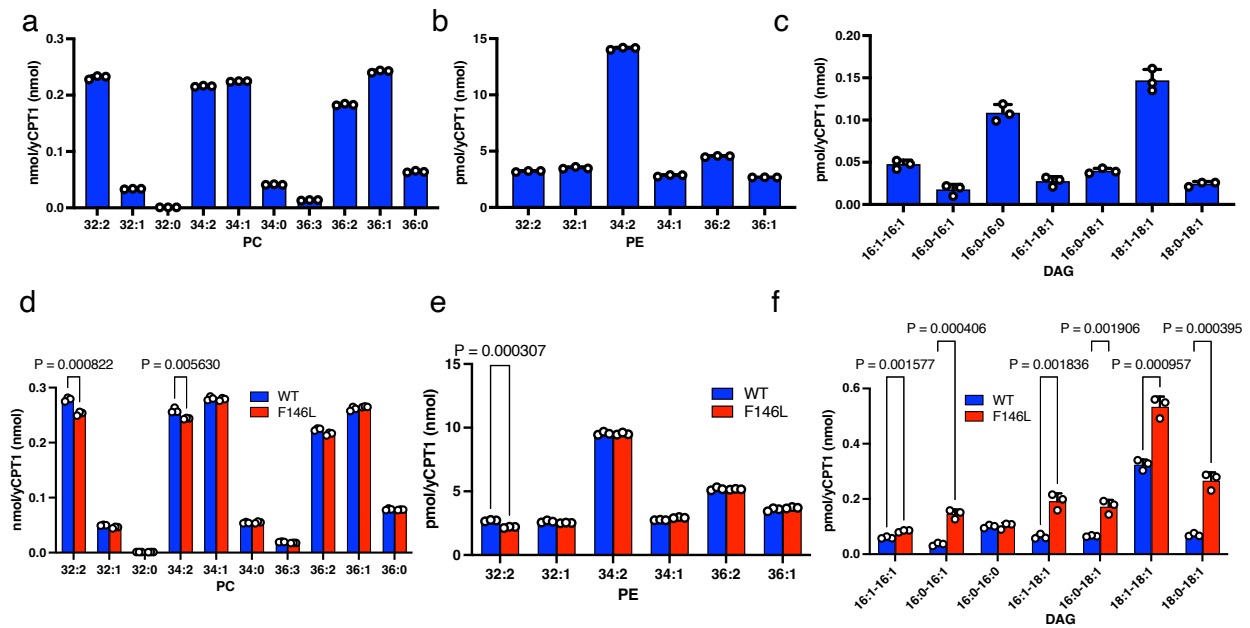
**Supplementary Figure 6. Overall architecture of yCPT1.** **a.** Snake plot of yCPT1 with each motif colored in the same way as in Fig.1: yellow, catalytic motif; purple, CDP binding motif; grey, dimerization motif; ruby red, N-terminal stretch. The juxtamembrane helix is represented in a red rectangle and CDP-AP signature motif residues are colored in orange. **b.** Sequence alignment of the CDP-choline binding motif of yCPT1 and other members of the CDP-alcohol phosphotransferase family currently available in the PDB. The conserved residue Arg104 is indicated with an asterisk. **c.** Magnesium coordination at the signature motif with the cryo-EM density map surrounding the magnesium ions. **d.** Phospholipid molecules embedded between monomers of yCPT1. Each protomer is represented as cartoon and surface, and the phospholipids are shown as sticks with the cryo-EM density. **e-g.** Distinct dimer interface of yCPT1(e) in comparison to prokaryotic or other eukaryotic CDP-APs. - *A. fulgidus* IPCD/DIPPS colored in teal (4MND)(g), Human CEPT1 colored in pink (8GYX)(g). yCPT1 colored same as map and models shown in panel A. **h.** Weblogo plot of sequence conservation among 169 fungal species with 60-90% identity to yCPT1. The dimer interface motif is indicated in red (residues 350-364).



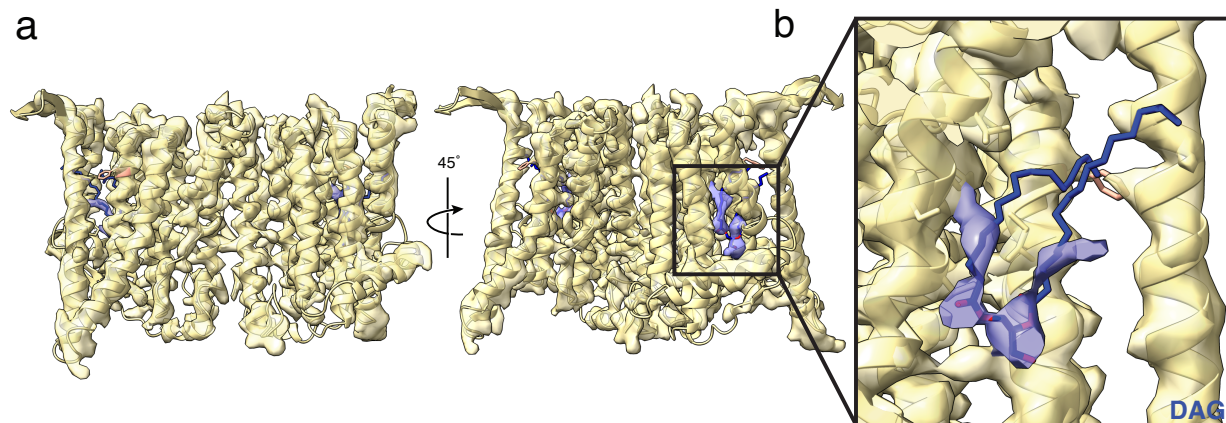


**Supplementary Figure 7. Dimerization interface of the yCPT1.** **a.** The dimer interface mediated through the protein interactions are shown in magenta. Interface residues are defined as the ones within 4 Å of each other. **b.** Dimer interface mediated through contact with phospholipid molecules are shown in cyan. For clarity, the lipid molecules are removed in the split subunit view. The interface residues are defined as the ones within 4 Å from the neighboring subunit or the lipid molecules.

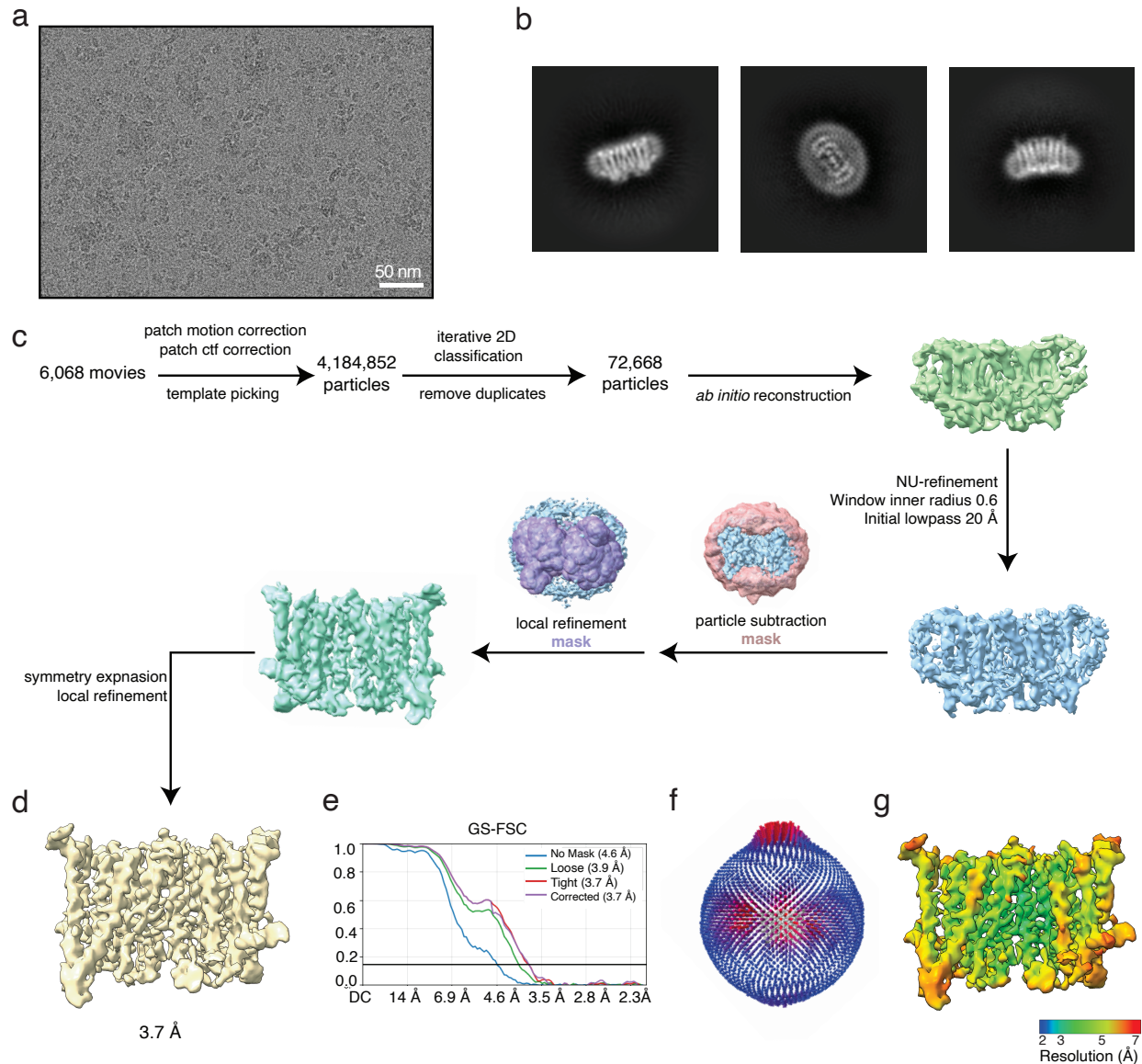




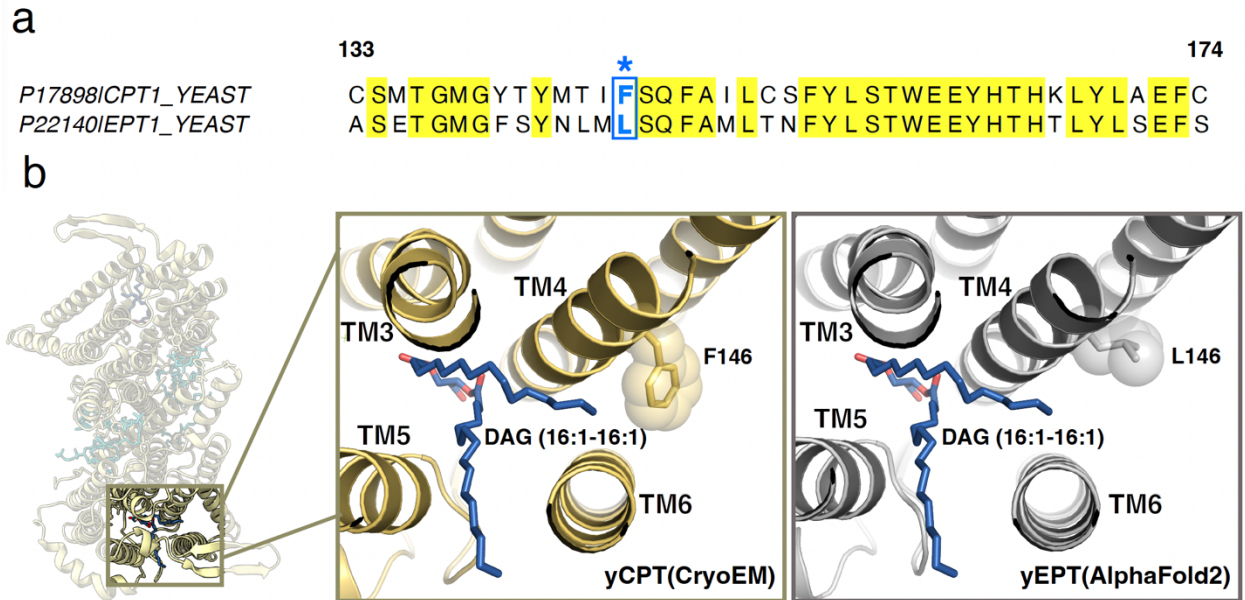
**Supplementary Figure 8. Quantification of PC, PE, and DAG from the purified proteins.** Lipids were extracted from 900 pmol of purified yCPT1 WT (blue) or yCPT1 F146L mutant (red). The amounts of PC (a, d), PE (b, e), and DAG (c, f) were determined by LC-MS/MS. Data are presented as the mean  $\pm$  SD from three independent measurements. Statistical significance was determined by unpaired two-sided t-test.



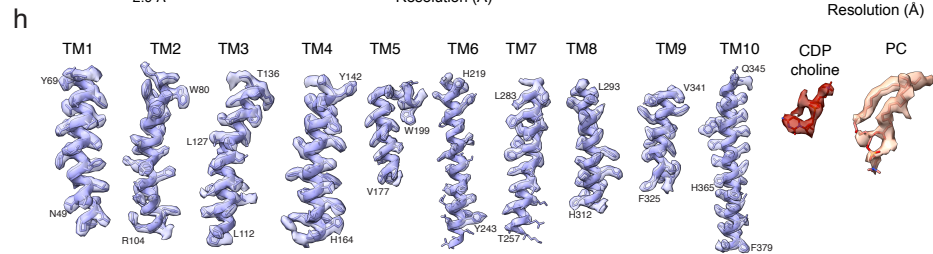
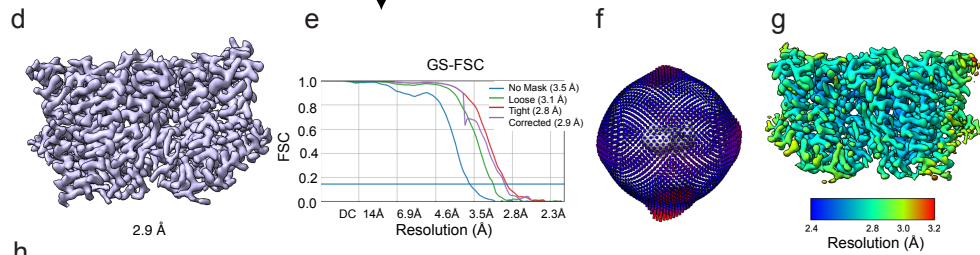
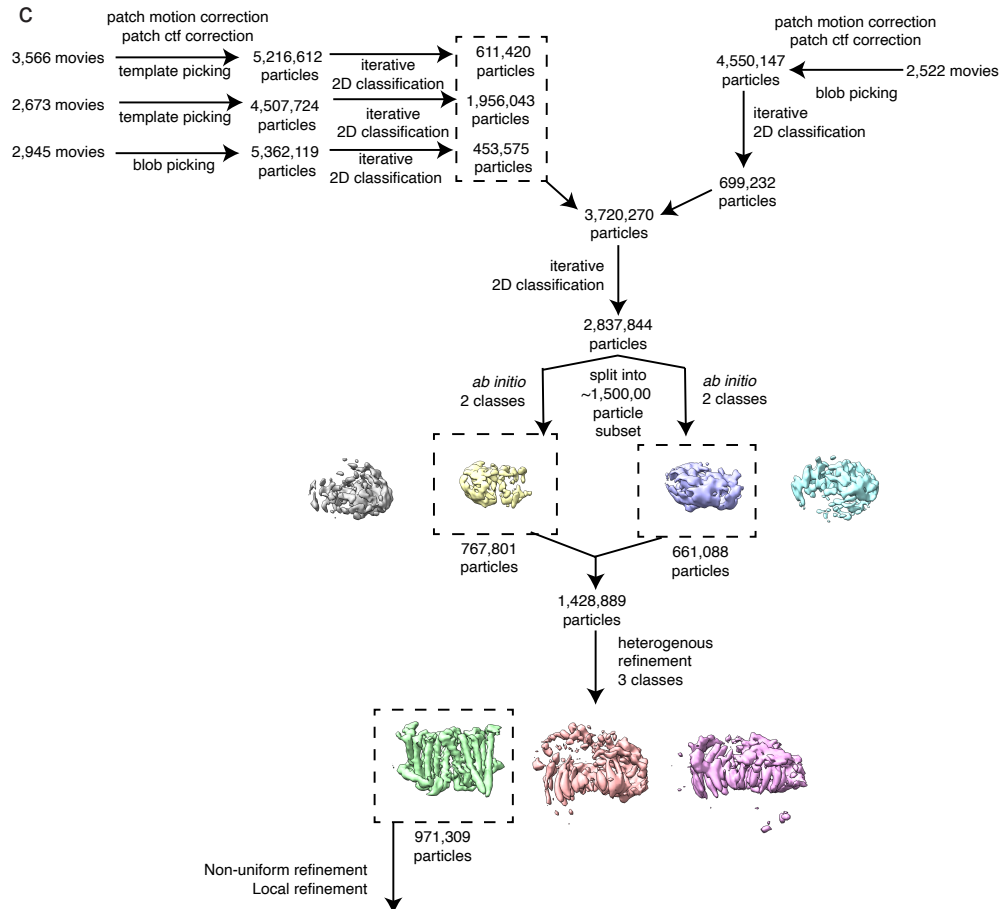
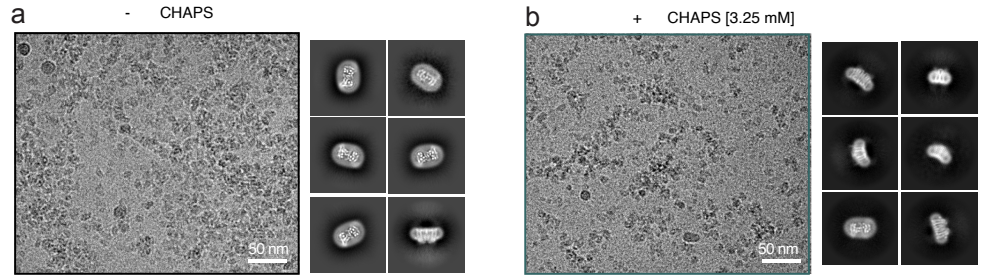
**Supplementary Figure 9. DAG binding mode of  $\gamma$ CPT1 in nanodiscs.** **a.** 3.7 Å resolution cryo-EM density map of dimeric  $\gamma$ CPT1 in a nanodisc assembled from a mixture of 8:2 DOPC:DAG and belted with MSP1E3D1 and atomic model of dimeric  $\gamma$ CPT1 (PDB: 8UL9). Structure is viewed from the membrane plane (left) and rotated 45° to better show the DAG binding site (right) which is expanded in panel b. **b.** The cryo-EM density of DAG is shown in blue with the model also shown in blue.



**Supplementary Figure 10. Cryo-EM processing of yCPT1 in nanodiscs.** **a.** Representative sub-area of a motion corrected micrograph of vitrified yCPT1-Nb24 in nanodiscs assembled from a mixture of 8:2 DOPC:DAG and belted with MSP1E3D1. Scale bar, 50 nm. **b.** Representative 2D classes. Nb24 is not visible in the averages. Box size 276 Å. **c.** Cryo-EM processing workflow. 4,184,852 particles were selected from 6,068 micrographs and underwent extensive 2D classification and particle subtraction to remove density from the nanodisc. **d.** 3.7 Å resolution 3D EM map determined using 72,668 particles (yCPT1 dimers) that were symmetry expanded for a total of 143,336 particles. They symmetry expanded particles were subjected to a local refinement. **e.** GS-FSC plot of map shown in panel d. **f.** Particle orientation distribution plot. **g.** Local resolution estimation.



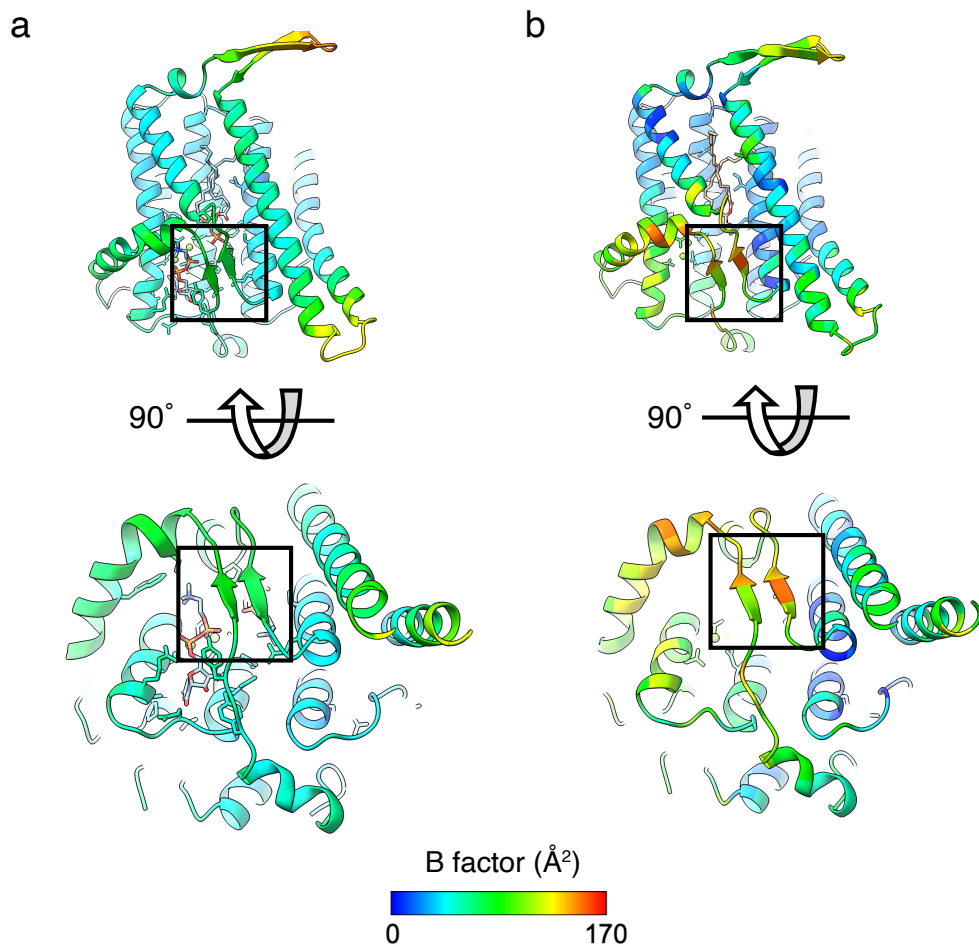
**Supplementary Figure 11. Structural comparison between the cryo-EM yCPT1 and AF2-predicted yEPT1 structures.** **a.** Sequence alignment of yCPT1 (UniProt ID: P17898) and yEPT1 (UniProt ID: P22140) around TM4 region. Identical residues between these proteins are highlighted yellow. yCPT1 F146 and yEPT1 L146 are marked by an asterisk and shown in blue. **b.** Comparison of the DAG-binding site of cryo-EM structure of yCPT1 and AF2-predicted structure of yEPT1.



**Supplementary Figure 12. Cryo-EM processing of  $\gamma$ CPT1 in complex with CDP-choline. a. b.**

Sub-area of motion corrected micrographs of vitrified  $\gamma$ CPT1 in the presence (a) and absence (b) of 3.25 mM CHAPS. Scale bars, 50 nm. Representative class averages, box size 276 Å. c. Cryo-EM processing workflow. Initial particles were picked by cryoSPARC template picking, or crYOLO model as specified. ~3.7 million total particles were extracted and pooled from the 4 datasets. d. and e. After iterative 2D classification and heterogenous refinement 971,309 good particles were subjected to a non-uniform refinement reaching 2.9 Å GS-FSC resolution. f. Orientation distribution plot. g. Local resolution estimation. h. Density of each TM helix and CDP choline are shown contoured at 6 sigma. Density of PC is shown contoured at 3 sigma.



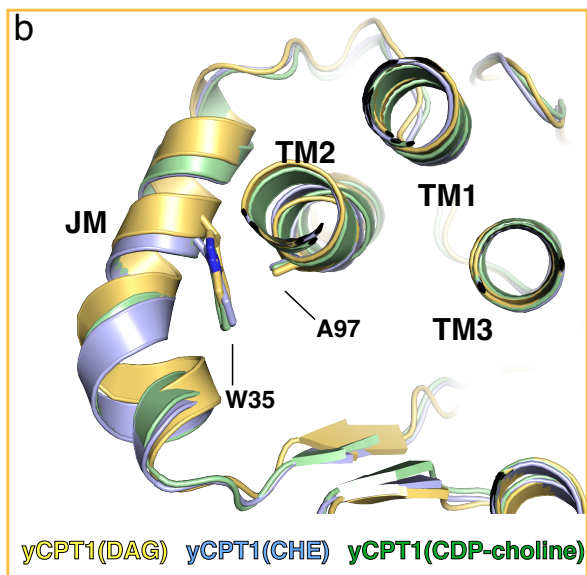


**Supplementary Figure 13. B-factor comparison between γCPT1 bound with CDP-choline/PC and DAG. a.** B-factor plot of γCPT1 bound with CDP-choline and PC. **b.** B-factor plot of γCPT1 bound with DAG alone and no CDP-choline. The enclosed area is rigidified upon binding of CDP-choline, while the same area is the most flexible region in the DAG-bound form.

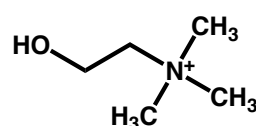
a

|        | PC  | PE  | A97 equivalent |
|--------|-----|-----|----------------|
| yCPT1  | +++ | -   | A              |
| yEPT1  | +   | +++ | G              |
| hCHPT1 | +++ | -   | A              |
| hCEPT1 | +++ | +   | A              |
| hEPT1  | -   | +++ | G              |

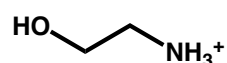
b



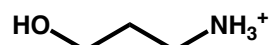
c



choline



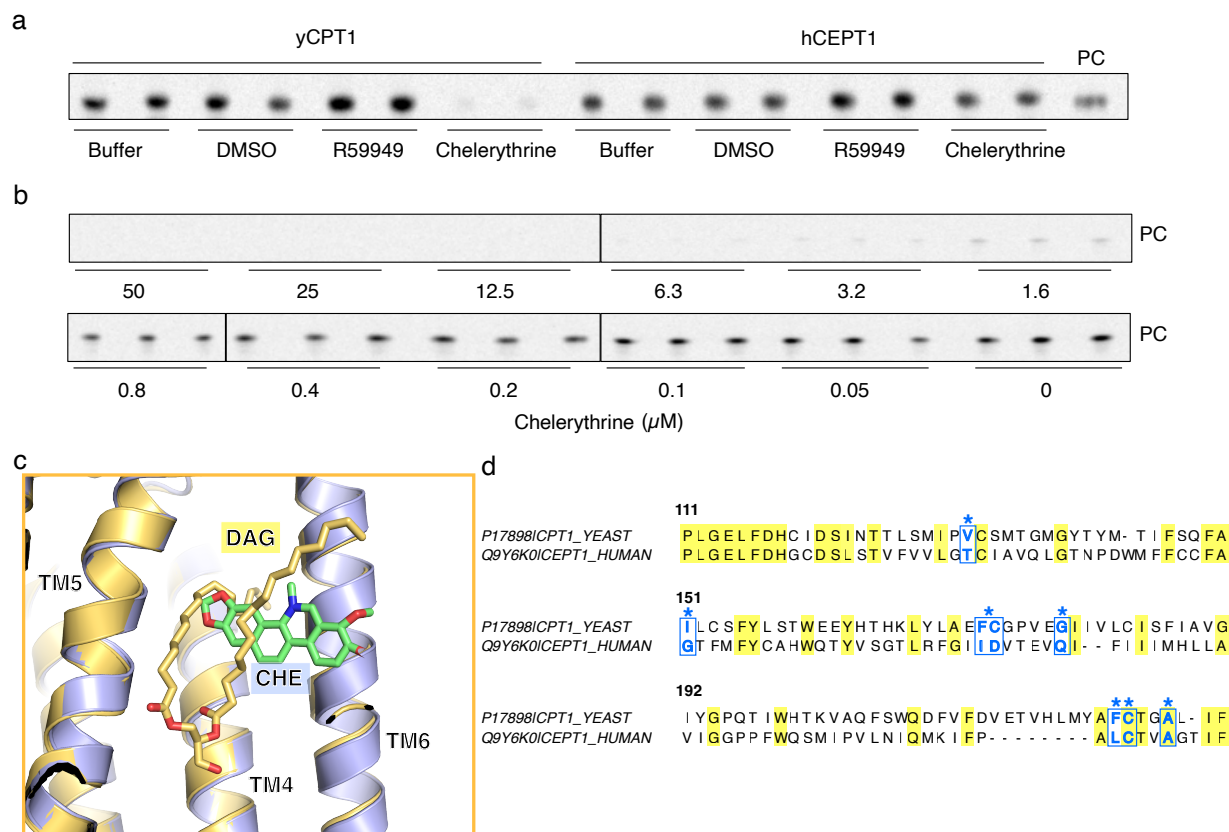
ethanolamine



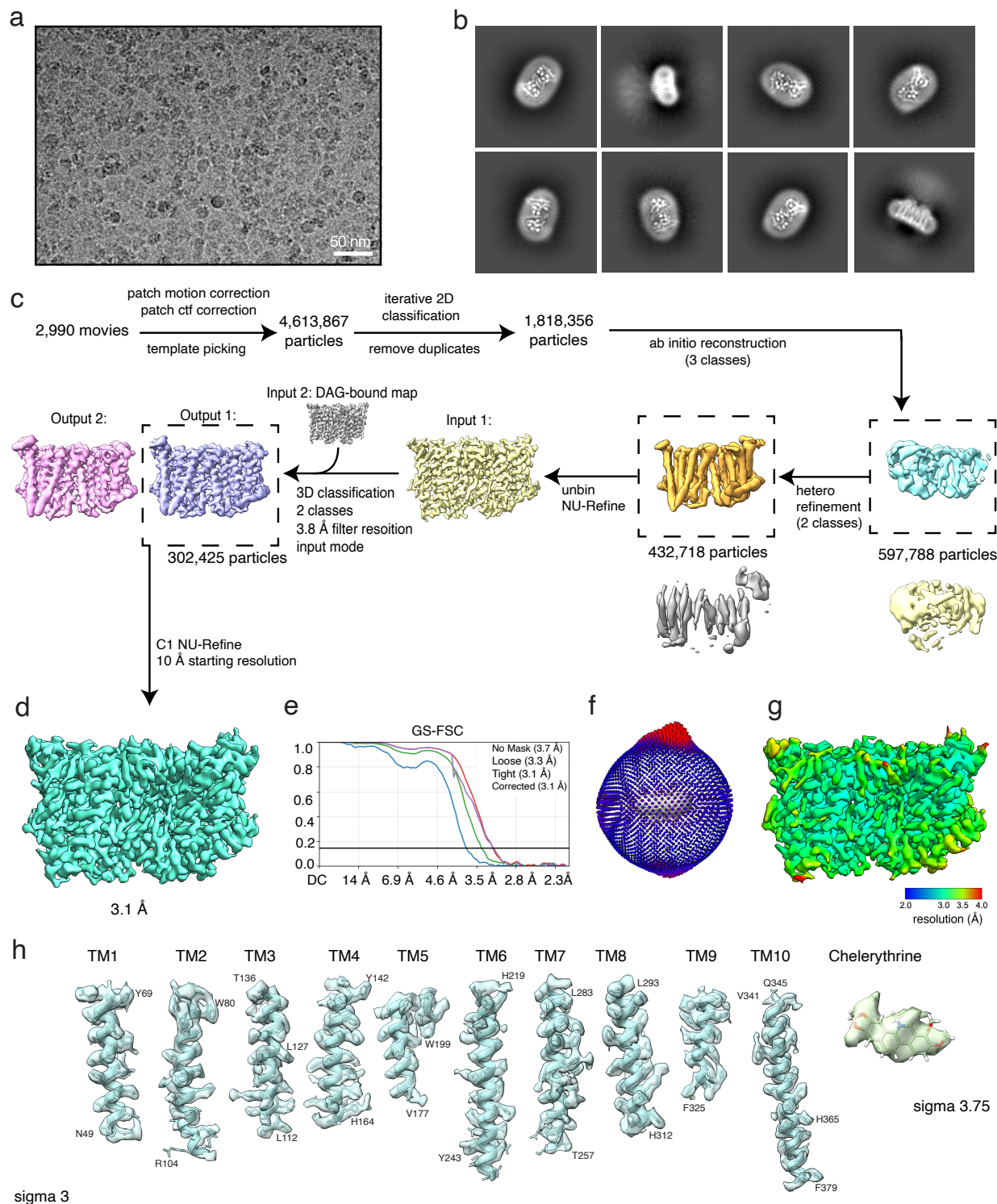
propanolamine

**Supplementary Figure 14. Specificity determinant for the lipid head group.** **a.** The selectivity matrix for the human and yeast enzymes. The preference for PC and PE production and the residue at the equivalent position to Ala97 in yCPT1 are noted. yCPT1 (UniProt ID:P17898), yEPT1 (UniProt ID: P22140), hCHPT1 (UniProt ID: Q8WUD6), hCEPT1 (UniProt ID: Q9Y6K0), and hEPT1 (UniProt ID: Q9C0D9). References for PC and PE activity: yCPT1 and yEPT1<sup>1</sup>, hCHPT1<sup>2</sup>, hCEPT1<sup>3</sup>, hEPT1<sup>4</sup>. - = no preference, += slight preference, +++ = strong preference. **b.** Structural comparison of yCPT1 in complex with various substrates. Note that the position of W35 is constant. Yellow, yCPT1 bound to DAG; blue, yCPT1 bound to chelerythrine (CHE); green, yCPT1 bound to CDP-choline. **c.** Chemical structures of choline, ethanolamine, and propanolamine.



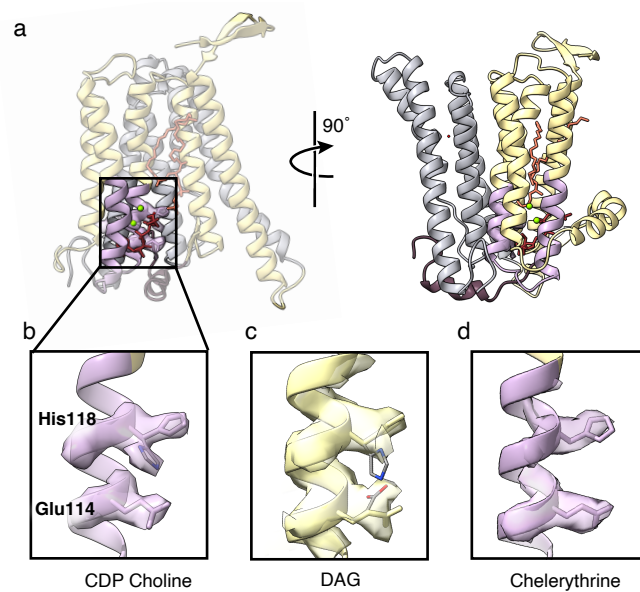


**Supplementary Figure 15. Specific inhibition of yCPT1 by chelerythrine.** **a.** TLC analysis of radiolabeled PC shows chelerythrine specifically inhibits yCPT1, while it doesn't suppress hCEPT1 enzymatic activity. R59949 doesn't inhibit either yCPT1 or hCEPT1. **b.** PC synthesis activity of the purified yCPT1 is suppressed by chelerythrine concentration dependently. Chelerythrine concentration is titrated from 0,05μM to 50μM. **c.** Superposition of yCPT1 structures bound to either DAG or chelerythrine. Chelerythrine occupies the DAG binding pocket and overlaps where DAG binds. **d.** Sequence alignment of yCPT1 (UniProt ID: P17898) and hCEPT1 (UniProt ID: Q9Y6K0) around TMs 4-6. Identical residues are highlighted yellow and chelerythrine-binding residues are marked by asterisk and shown in blue.



**Supplementary Figure 16. Cryo-EM processing of yCPT1 in complex with chelerythrine.** **a.** Sub-area of motion corrected micrograph of vitrified yCPT1 particles. Scale bar, 50 nm. **b.** Representative 2D classes. Box size 276 Å. Scale bar 50 Å. **c.** Cryo-EM processing workflow. ~4.6 million initial particles were picked from 2,990 micrographs using crYOLO. **d. and e.** After iterative 2D classification and heterogenous refinement 302,425 good particles were subjected

to a final *ab initio* reconstruction and then non-uniform refinement with C2 symmetry imposed, reaching 3.1 Å GS-FSC resolution. **f.** Orientation distribution plot. **g.** Local resolution estimation. **h.** Density of each TM helix and chelerythrine are shown contoured at 3 sigma and 3.75 sigma respectively.



**Supplementary Figure 17. Conserved Histidine residue can adopt different rotameric conformations depending on bound substrate. a.** Overall structure of  $\gamma$ CPT1 monomer in complex with CDP-choline. The catalytic site is highlighted in the left panel and enlarged in (b). **b, c, d.** Comparison of the cryoEM density maps and models of His118 and Glu114 of  $\gamma$ CPT1 bound with CDP-choline (b), DAG (c), and chelerythrine (d).

**Supplementary Table 1. Cryo-EM data collection, refinement and validation statistics**

|                                           | yCPT1 DAG<br>(EMDB-42357)<br>(PDB 8UL9) | yCPT1 DAG<br>in nanodisc<br>(EMD-46969) | yCPT1 CDP<br>choline/PC<br>(EMDB-42496)<br>(PDB 8URP) | yCPT1<br>Chelerythrine<br>(EMDB-42500)<br>(PDB 8URT) |
|-------------------------------------------|-----------------------------------------|-----------------------------------------|-------------------------------------------------------|------------------------------------------------------|
| <b>Data collection and processing</b>     |                                         |                                         |                                                       |                                                      |
| Magnification                             | 81,000                                  | 81,000                                  | 81,000                                                | 81,000                                               |
| Voltage (kV)                              | 300                                     | 300                                     | 300                                                   | 300                                                  |
| Electron exposure (e-/Å <sup>2</sup> )    | 60                                      | 60                                      | 60                                                    | 60                                                   |
| Defocus range (μm)                        | 1.5-2.4                                 | 1.5-2.4                                 | 1.5-2.4                                               | 1.5-2.4                                              |
| Pixel size (Å)                            | 1.08                                    | 1.08                                    | 1.08                                                  | 1.08                                                 |
| Symmetry imposed                          | C1                                      | C1                                      | C1                                                    | C2                                                   |
| Initial particle images (no.)             | 2,913,379                               | 4,184,852                               | 3,720,270                                             | 4,613,867                                            |
| Final particle images (no.)               | 677,515                                 | 72,668                                  | 971,309                                               | 302,425                                              |
| Map resolution (Å)                        | 3.2                                     | 3.7                                     | 2.9                                                   | 3.1                                                  |
| FSC threshold                             | 0.143                                   | 0.143                                   | 0.143                                                 | 0.143                                                |
| Map resolution range (Å)                  | 2.8-7.9                                 | 3.2-12                                  | 2.6-6.8                                               | 2.9-9.5                                              |
| <b>Refinement</b>                         |                                         |                                         |                                                       |                                                      |
| Initial model used                        | AlphaFold2(P17898)                      |                                         | PDB 8UL9                                              | PDB 8UL9                                             |
| Model resolution (Å)                      | 3.20                                    |                                         | 2.90                                                  | 3.10                                                 |
| FSC threshold                             | 0.143                                   |                                         | -                                                     | -                                                    |
| Model resolution range (Å)                | XXX                                     |                                         | XXX                                                   | XXX                                                  |
| Map sharpening B factor (Å <sup>2</sup> ) | 148.9                                   |                                         | 130.6                                                 | 144.4                                                |
| Model composition                         |                                         |                                         |                                                       |                                                      |
| Non-hydrogen atoms                        | 6674                                    |                                         | 6747                                                  | 6622                                                 |
| Protein residues                          | 774                                     |                                         | 774                                                   | 774                                                  |
| Ligands                                   | 14                                      |                                         | 16                                                    | 14                                                   |
| B factors (Å <sup>2</sup> )               |                                         |                                         |                                                       |                                                      |
| Protein                                   | 78.91                                   |                                         | 62.61                                                 | 68.23                                                |
| Ligand                                    | 82.18                                   |                                         | 72.25                                                 | 73.85                                                |
| R.m.s. deviations                         |                                         |                                         |                                                       |                                                      |
| Bond lengths (Å)                          | 0.004                                   |                                         | 0.002                                                 | 0.002                                                |
| Bond angles (°)                           | 0.504                                   |                                         | 0.455                                                 | 0.516                                                |
| Validation                                |                                         |                                         |                                                       |                                                      |
| MolProbity score                          |                                         |                                         |                                                       |                                                      |
| Clashscore                                | 6.75                                    |                                         | 5.50                                                  | 5.02                                                 |
| Poor rotamers (%)                         | 2.33                                    |                                         | 1.60                                                  | 1.90                                                 |
| Ramachandran plot                         |                                         |                                         |                                                       |                                                      |
| Favored (%)                               | 95.97                                   |                                         | 97.14                                                 | 97.27                                                |
| Allowed (%)                               | 4.03                                    |                                         | 2.86                                                  | 2.73                                                 |
| Disallowed (%)                            | 0                                       |                                         | 0                                                     | 0                                                    |

**Supplementary Table 2.** Primer Sequences, Custom Synthesis from IDT.

| Primer Name       | Oligonucleotide Sequence (5' to 3') |
|-------------------|-------------------------------------|
| yCPT-W35A-Q5-Fwd  | GTATTGAGGCCCTTTGCCAGAAAGTTTGCGACT   |
| yCPT-W35A-Q5-Rvs  | ATGGTTTGAAAGGAAAGAGCGGTCATCACTTTG   |
| yCPT-A97G-Q5-Fwd  | TACCAGACTTTTGATGGCTGCGATGGAATGCAT   |
| yCPT-A97G-Q5-Rvs  | TAAAAACAAACCTATAGCATAGGAGAAATAGGT   |
| yCPT-E114A-Q5-Fwd | CAGGGCCCTCTAGGGGCCTTGTTTGACCATTGT   |
| yCPT-E114A-Q5-Rvs | CTGGCCTGTACGACGTGCATGCATTCCATCGCA   |
| yCPT-H118A-Q5-Fwd | GGGGAATTGTTTGACGCCTGTATTGATTCAATC   |
| yCPT-H118A-Q5-Rvs | TAGAGGGCCCTGCTGGCCTGTACGACGTGCATG   |
| yCPT-F146L-Q5-Fwd | ACATATATGACCATTCTGTCACAATTTGCGATA   |
| yCPT-F146L-Q5-Rvs | ATTCCTGTCTGTTCCATGACAGGTATGGGTTAC   |

**Supplementary Table 3. MRM transitions for lipids, succinic acid, and CMP**

| <i>Compound</i>      | <i>MRM transition</i> | <i>Ion mode</i> | <i>DP (V)</i> | <i>CE (V)</i> |
|----------------------|-----------------------|-----------------|---------------|---------------|
| <i>PC 32:2</i>       | 732.5 > 184           | positive        | 60            | 40            |
| <i>PC 32:1</i>       | 734.5 > 184           | positive        | 60            | 40            |
| <i>PC 32:0</i>       | 736.5 > 184           | positive        | 60            | 40            |
| <i>PC 34:2</i>       | 758.5 > 184           | positive        | 60            | 40            |
| <i>PC 34:1</i>       | 760.5 > 184           | positive        | 60            | 40            |
| <i>PC 34:0</i>       | 762.5 > 184           | positive        | 60            | 40            |
| <i>PC 36:3</i>       | 784.5 > 184           | positive        | 60            | 40            |
| <i>PC 36:2</i>       | 786.5 > 184           | positive        | 60            | 40            |
| <i>PC 36:1</i>       | 788.5 > 184           | positive        | 60            | 40            |
| <i>PC 36:0</i>       | 790.5 > 184           | positive        | 60            | 40            |
| <i>PC 36:0-d70</i>   | 861.1 > 184           | positive        | 60            | 40            |
| <i>PE 32:2</i>       | 688.5 > 547.5         | positive        | 60            | 25            |
| <i>PE 32:1</i>       | 690.5 > 549.5         | positive        | 60            | 25            |
| <i>PE 34:2</i>       | 716.5 > 575.5         | positive        | 60            | 25            |
| <i>PE 34:1</i>       | 718.5 > 577.5         | positive        | 60            | 25            |
| <i>PE 36:2</i>       | 744.5 > 603.5         | positive        | 60            | 25            |
| <i>PE 36:1</i>       | 746.5 > 605.5         | positive        | 60            | 25            |
| <i>PE 33:1-d7</i>    | 711.5 > 570.5         | positive        | 60            | 25            |
| <i>DAG 16:1-16:1</i> | 582.5 > 311.2         | negative        | 60            | 35            |
| <i>DAG 16:0-16:1</i> | 584.5 > 311.2         | negative        | 60            | 35            |
| <i>DAG 16:0-16:0</i> | 586.5 > 313.2         | negative        | 60            | 35            |
| <i>DAG 16:1-18:1</i> | 612.5 > 311.2         | negative        | 60            | 35            |
| <i>DAG 16:0-18:1</i> | 612.5 > 339.2         | negative        | 60            | 35            |
| <i>DAG 18:1-18:1</i> | 638.5 > 339.2         | negative        | 60            | 35            |
| <i>DAG 18:0-18:1</i> | 640.5 > 339.2         | negative        | 60            | 35            |
| <i>succinic acid</i> | 117 > 73              | negative        | -70           | -24           |
| <i>CMP</i>           | 324 > 112             | positive        | 60            | 20            |



Supplementary References:

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