Supplementary Information

Structural and functional dissection of a higher-order oligomerization interface in yeast ceramide synthase

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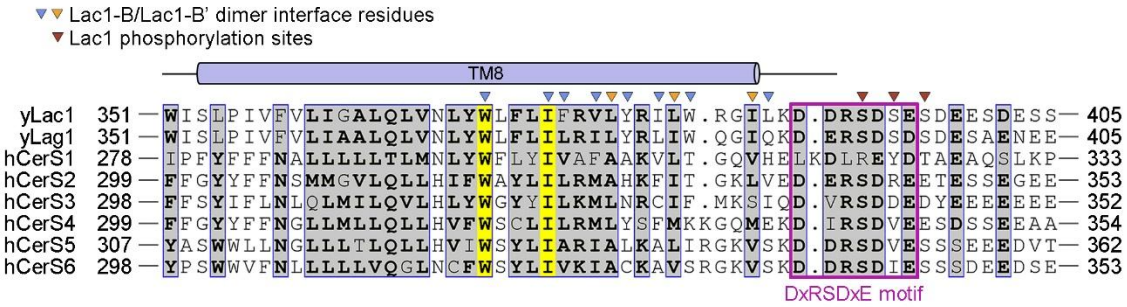
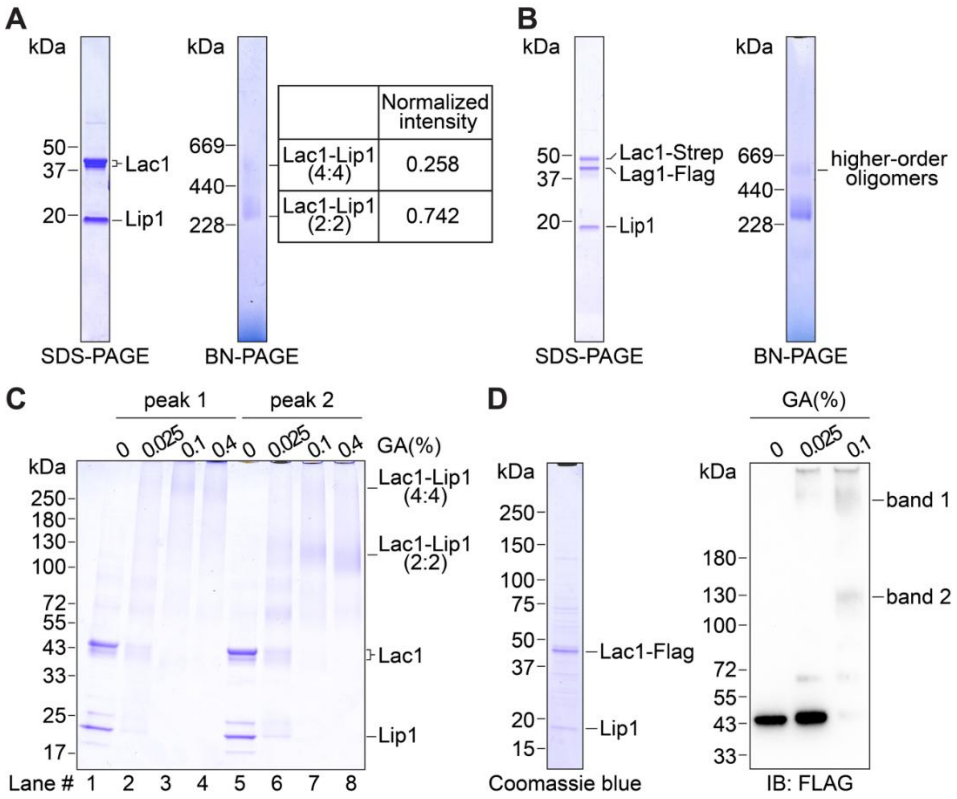
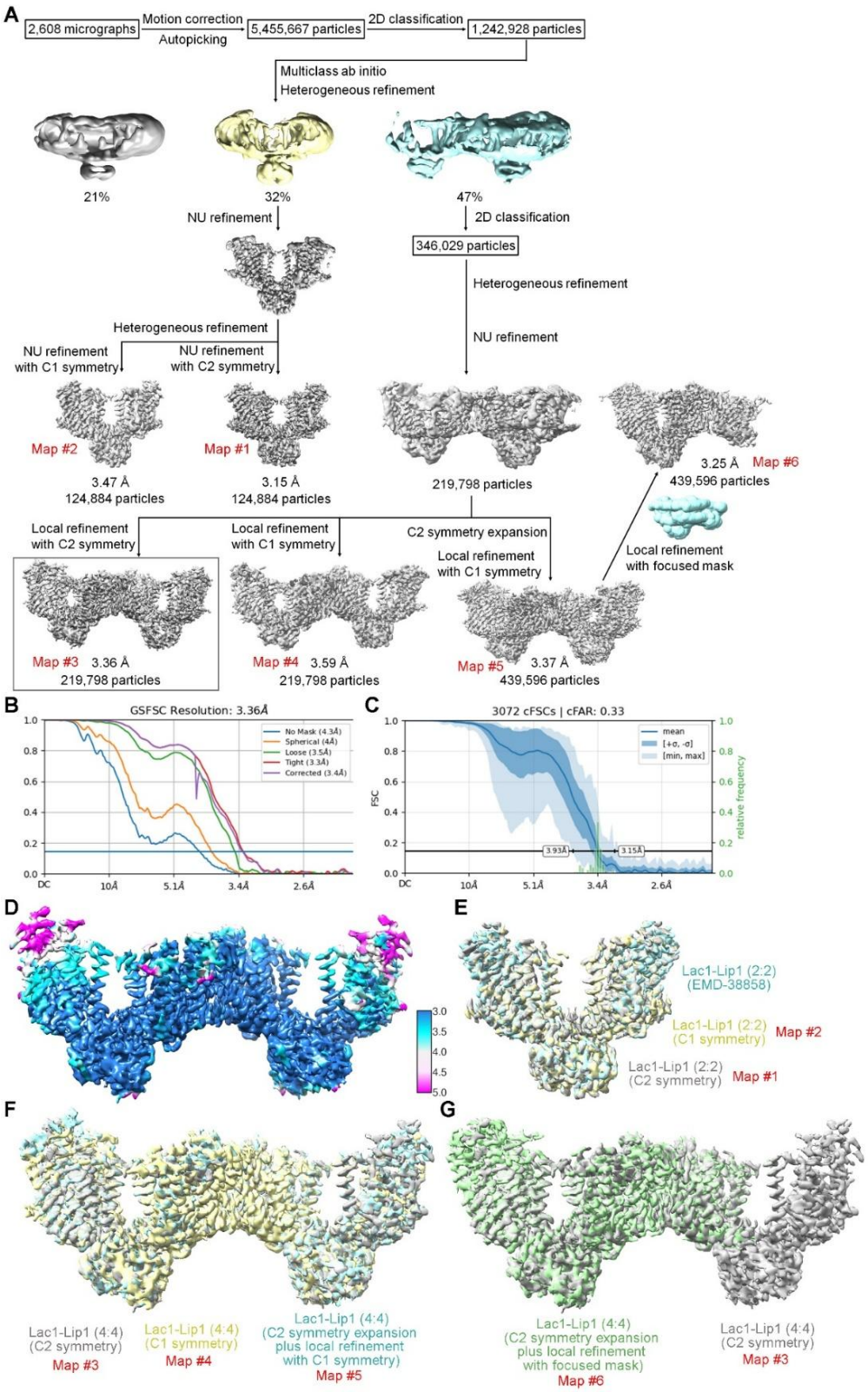


Supplementary Fig. 1 | Yeast Lac1 and Lag1 possess a conserved DxRSDxE motif. Sequence alignment of the C-terminal regions of yeast Lac1/Lag1 and human CerS proteins. The DxRSDxE motif is boxed in purple; the dimer-interface residues in TM8 of Lac1-B/Lac1-B' and the C-terminal phosphorylation sites are indicated.

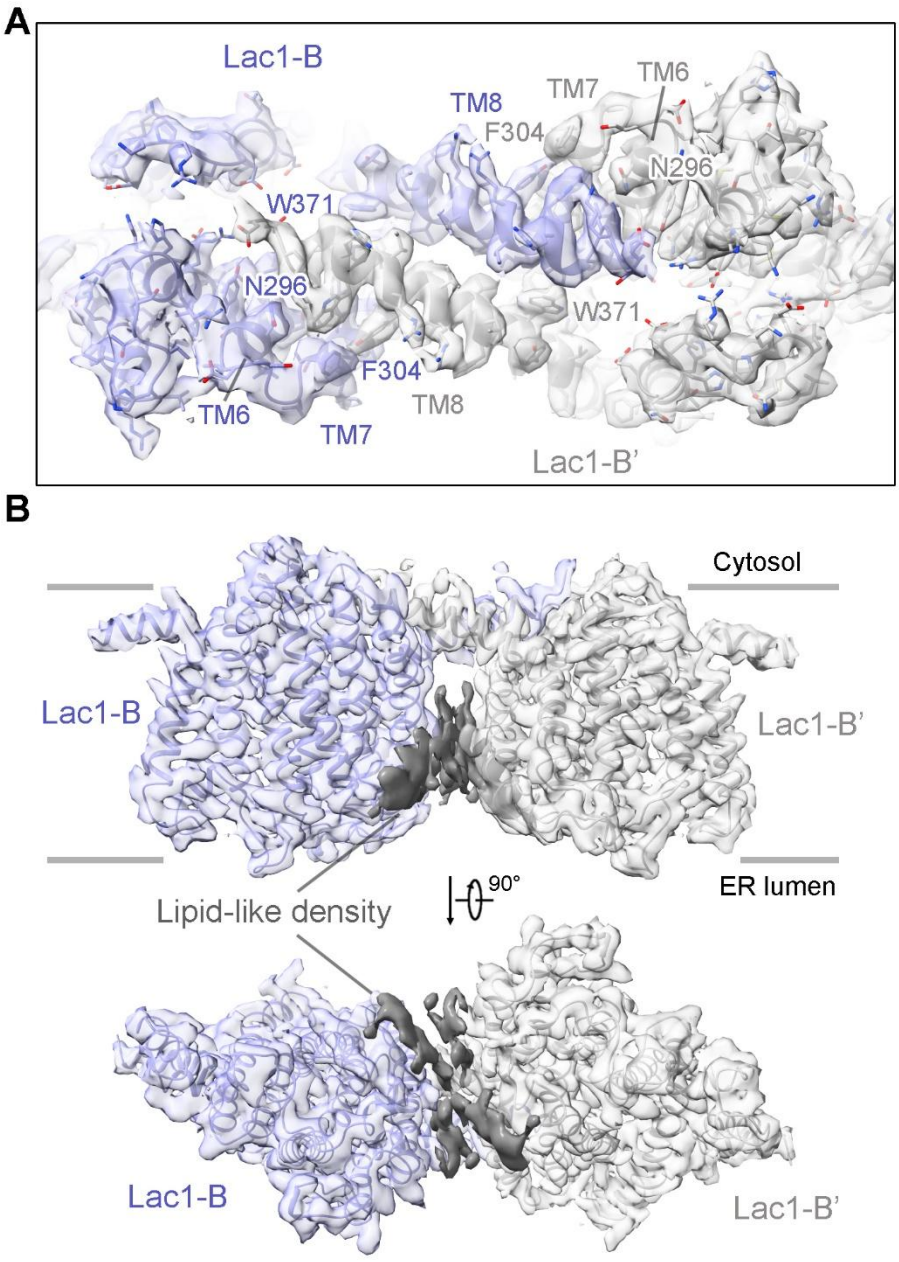


Supplementary Fig. 2 | Biochemical analysis of Lac1-Lip1 oligomeric states. **A,** Relative abundance of Lac1-Lip1 oligomeric species. A pool of all fractions from Fig. 1A was analyzed by SDS-PAGE (left) and BN-PAGE (right). Semi-quantitative densitometry of the representative BN-PAGE gel showed that 4:4 oligomers represent ~25% of the purified protein. **B,** Lac1-Lip1-Lag1 heterotetramers also assemble into higher-order oligomers. The heterocomplex was purified by tandem affinity purification and examined by SDS-PAGE (left) and BN-PAGE (right). Experiments were performed with three independent replicates. **C,** Glutaraldehyde (GA) cross-linking of purified Lac1-Lip1 complexes from SEC peaks 1 and 2, as indicated in Fig. 1A. Experiments were performed with three independent replicates. **D,** *In vivo* oligomeric states of the Lac1-Lip1 complex. The endogenously expressed complex was enriched via anti-Flag immunoprecipitation from ΔLag1 cells expressing Flag-tagged Lac1 at its native genomic locus, followed by cross-linking and Western blot analysis, which revealed both dimeric (band 2) and tetrameric (band 1) endogenous oligomers. Experiments were performed with three independent replicates. Source data are provided as a Source Data file.

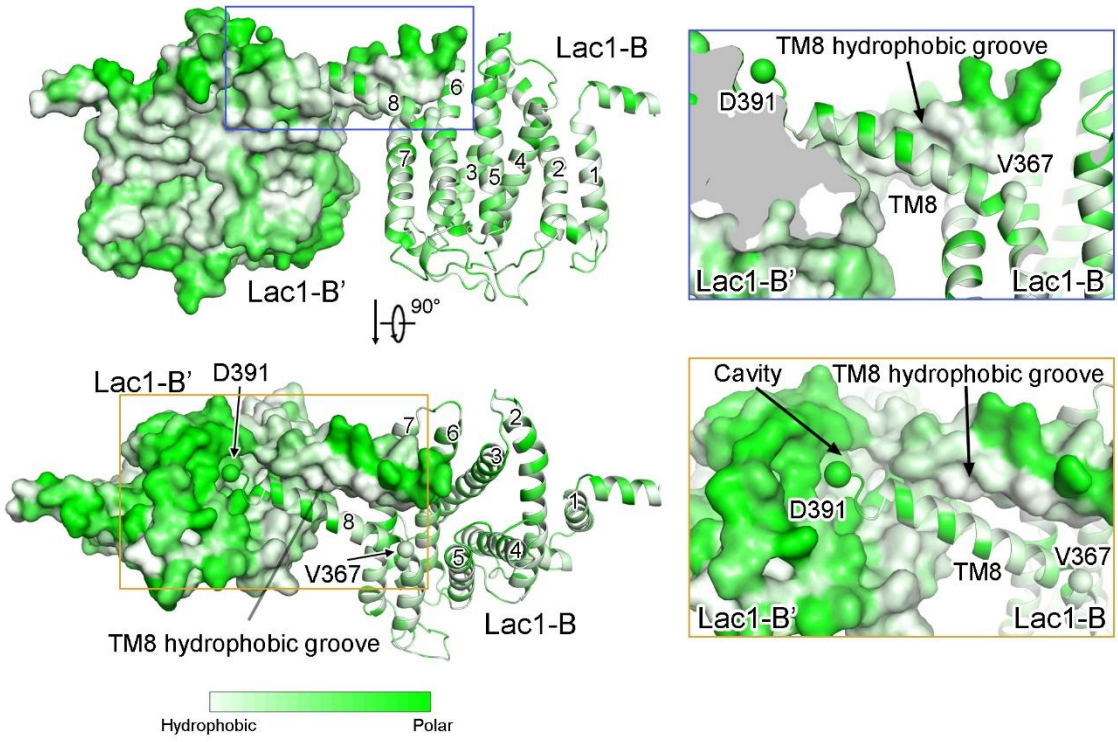


Supplementary Fig. 3 | Cryo-EM analysis of the Lac1-Lip1 complex. **A,** Cryo-EM data processing flowchart. Following careful evaluation, the C2-symmetry 4:4 Lac1-Lip1 map (Map #3) was selected

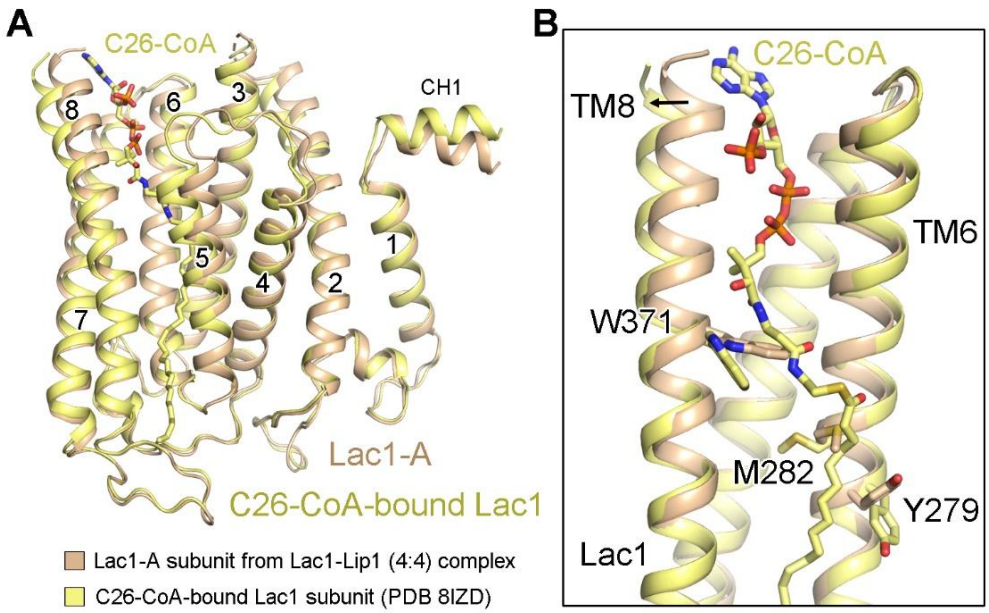
for further analysis in this study. **B,** Gold-standard FSC curve for the C2-symmetry 4:4 Lac1-Lip1 map (Map #3). **C,** cFAR plot of the C2-symmetry 4:4 Lac1-Lip1 map (Map #3). **D,** Local resolution map of the C2-symmetry 4:4 Lac1-Lip1 complex (Map #3). **E,** Comparison of the 2:2 Lac1-Lip1 maps obtained in this study (Map #1 and Map #2) with the previously published 2:2 map (EMD-38858). All three maps align closely. **F,** Comparison of the 4:4 Lac1-Lip1 maps resolved in this study (Map #3, Map #4, and Map #5). The maps show excellent superposition. **G,** Comparison of the 4:4 Lac1-Lip1 map (Map #3) with the locally refined map of a single protomer containing the Lac1-Lac1' interface (Map #6). The locally refined map aligns well with the full 4:4 map.



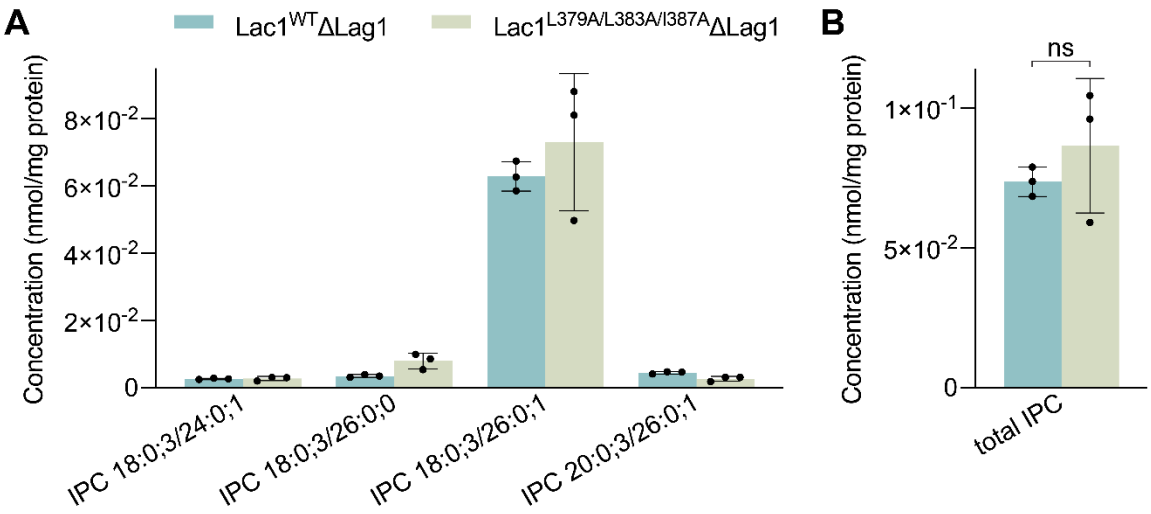
Supplementary Fig. 4 | Density map at the Lac1 dimer interfaces. **A,** Close-up view of the local map showing contacts among TMs 6, 7 and 8 that mediate Lac1 dimerization. **B,** Lipid-like densities resolved near the dimer interface within the luminal membrane leaflet.



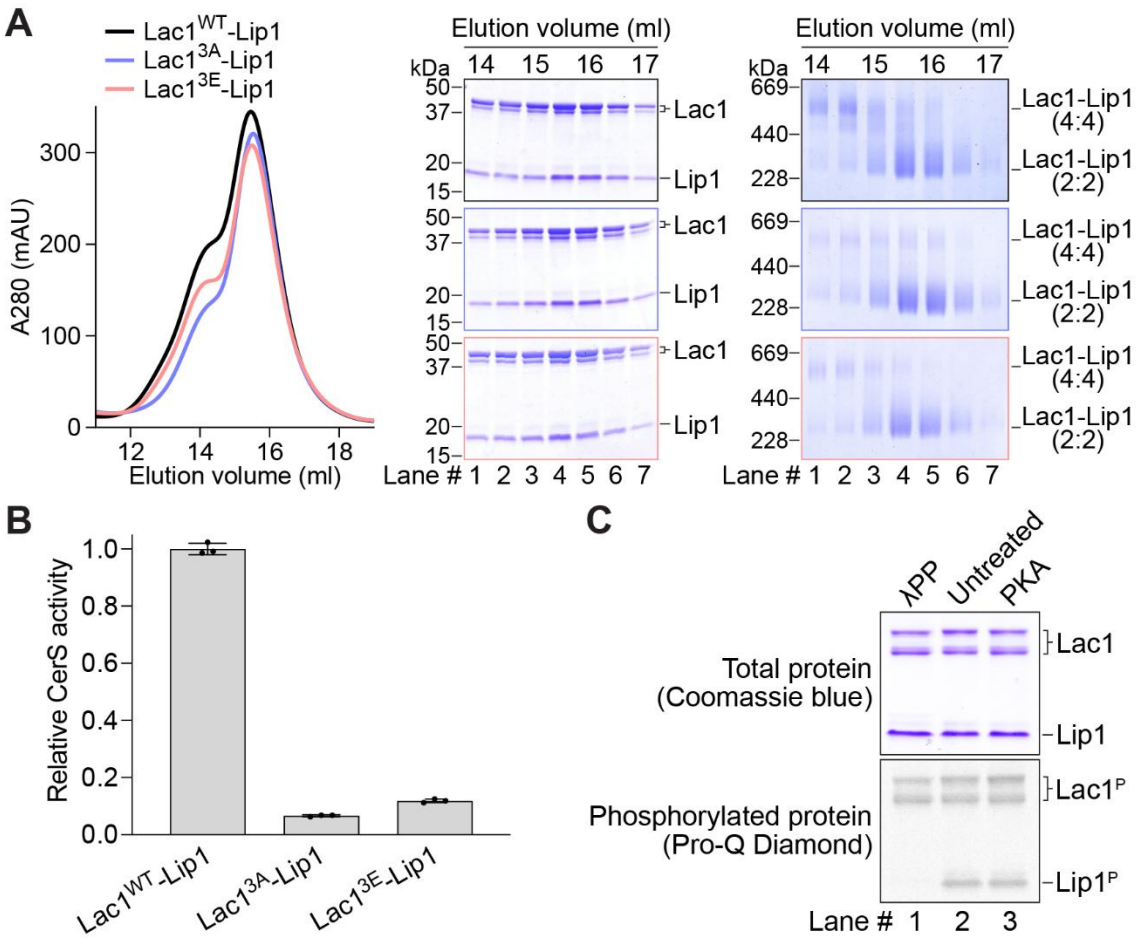
Supplementary Fig. 5 | The twisted TM8 of one Lac1 subunit docks into a predominantly hydrophobic groove on the adjacent Lac1 protomer at the dimer interface. Residues of both Lac1 subunits are colored according to the Eisenberg hydrophobicity scale (hydrophobic, gray; polar, green).



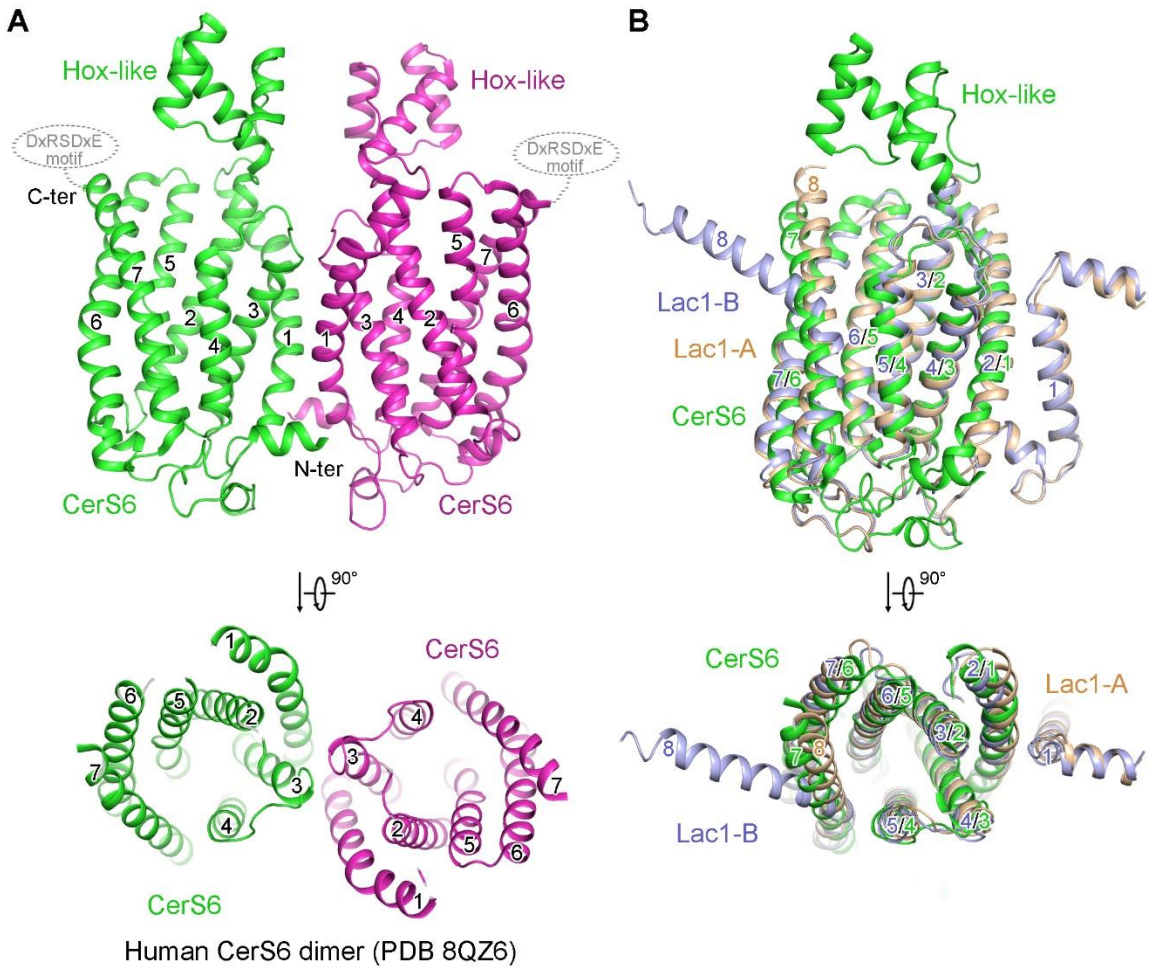
Supplementary Fig. 6 | The Lac1-A protomer in the 4:4 Lac1-Lip1 complex adopts an active conformation. **A,** Superimposition of Lac1-A from the 4:4 Lac1-Lip1 complex with C26-CoA-bound Lac1 (PDB 8IZD). The C26-CoA substrate is shown as sticks. **B,** Close-up view of the C26-CoA binding site highlighting residues that undergo notable conformational changes, depicted as sticks.



Supplementary Fig. 7 | LC-MS quantification of major IPC species (A) and total IPC levels (B) in Lac1^{WT}ΔLag1 and Lac1$^{L379A/L383A/I387A}$ΔLag1 cells after AbA treatment. Results are presented as average ± SD from three independent experiments (n = 3). Statistical significance was determined by two-tailed Student's t-test: 'ns' indicates no significance (P = 0.4170). Source data are provided as a Source Data file.



Supplementary Fig. 8 | Lac1-mediated dimerization of yeast CerS is independent of phosphorylation. **A,** SEC and BN-PAGE analyses reveal that both Lac1^{3A}-Lip1 and Lac1^{3E}-Lip1 mutants assemble into 4:4 Lac1-Lip1 complex, mirroring the WT complex. Experiments were performed with three independent replicates. **B,** *In vitro* CerS activity of the dephosphomimetic (Lac1^{3A}) and phosphomimetic (Lac1^{3E}) mutants compared to WT. Each data point represents the average ± SD of three independent experiments. **C,** Pro-Q Diamond staining following SDS-PAGE indicates that Lac1 in the purified WT complex is heavily phosphorylated. Protein kinase PKA or phosphatase λPP was utilized to phosphorylate or dephosphorylate the Lac1-Lip1 complex. Experiments were performed with three independent replicates. Source data are provided as a Source Data file.



Supplementary Fig. 9 | Human CerS6 dimerizes independently of the DxRSDxE motif. **A,** Overall structure of the dimeric human CerS6 (PDB 8QZ6). The C-terminal DxRSDxE motif is disordered. **B,** Superposition of Lac1-A and Lac1-B protomers from the 4:4 Lac1-Lip1 complex onto human CerS6 structure.

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

	4:4 Lac1-Lip1 complex (EMD-65613, PDB 9W3Z)
Data collection and processing	
Magnification	81,000
Voltage (kV)	300
Electron exposure (e^{-}/Å^2)	50
Defocus range (μm)	-2.0 to -1.0
Pixel size (Å)	1.072
Symmetry imposed	C2
Initial particle images (no.)	5,455,667
Final particle images (no.)	219,798
Map resolution (Å)	3.36 Å
FSC threshold	0.143
Map resolution range (Å)	3.0-5.0 Å
Refinement	
Initial model used (PDB code)	8Y2N
Model resolution (Å)	3.4 Å
FSC threshold	0.143
Model resolution range (Å)	
Map sharpening *B* factor (Å^2)	-96.6
Model composition	
Nonhydrogen atoms	14,854
Protein residues	1,800
Ligands	0
B factors (Å^2)	
Protein	43.91
R.m.s. deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.590
Validation	
MolProbity score	1.94
Clashscore	10
Poor rotamers (%)	0.00
Ramachandran plot	
Favored (%)	92.87
Allowed (%)	7.13
Disallowed (%)	0.00
Q-score	0.452

Supplementary Table 2 | Sphingolipid profile of Lac1^{WT}ΔLag1 and Lac1$^{L379A/L383A/I387A}$ΔLag1 yeast cells.

Species	nmol/mg protein						P value*
	Lac1^{WT}ΔLag1			Lac1$^{L379A/L383A/I387A}$ΔLag1			
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3	
dhCer 18:0;2/18:0;0	0.0020	0.0033	0.0017	0.0033	0.0012	0.0010	0.620
dhCer 18:0;2/24:0;0	0.0018	0.0025	0.0038	0.0020	0.0018	0.0022	0.301
dhCer 18:0;2/26:0;0	0.0448	0.0423	0.0363	0.0094	0.0107	0.0078	0.000
phCer 18:0;3/18:0;0	0.0005	0.0005	0.0008	0.0005	0.0007	0.0006	0.994
phCer 18:0;3/24:0;0	0.0263	0.0535	0.0224	0.0279	0.0195	0.0251	0.381
phCer 18:0;3/24:0;1	1.1241	0.8696	0.7399	0.6547	0.7898	0.8607	0.327
phCer 18:0;3/24:0;2	0.1283	0.1603	0.1351	0.1348	0.1375	0.1144	0.369
phCer 18:0;3/26:0;0	0.1926	0.1770	0.1331	0.0976	0.0794	0.0907	0.014
phCer 18:0;3/26:0;1	10.5886	9.2557	9.0175	4.5622	6.2256	5.3297	0.003
phCer 18:0;3/26:0;2	0.1842	0.1692	0.1578	0.0819	0.1071	0.1022	0.003
phCer 20:0;3/26:0;0	0.0262	0.0227	0.0179	0.0056	0.0082	0.0100	0.006
phCer 20:0;3/26:0;1	0.2663	0.1940	0.3264	0.1353	0.1599	0.0808	0.038
IPC 18:0;3/24:0;1	0.0025	0.0029	0.0026	0.0032	0.0031	0.0020	0.828
IPC 18:0;3/26:0;0	0.0032	0.0035	0.0040	0.0100	0.0086	0.0054	0.030
IPC 18:0;3/26:0;1	0.0586	0.0627	0.0674	0.0881	0.0811	0.0498	0.449
IPC 20:0;3/26:0;1	0.0041	0.0047	0.0048	0.0031	0.0031	0.0019	0.018
MIPC 18:0;3/26:0;0	0.0005	0.0004	0.0005	0.0004	0.0005	0.0003	0.571

*Statistical significance was determined using a two-tailed Student's t-test.

Supplementary Table 3 | List of primers used in this study.

Primer	Sequence (5'-3')
pCAG-Lac1-F	CGGGGTACCATGAGCACCATTAAGCCTAG
pCAG-Lac1-R	CCGCTCGAGGATGTCCTTCTTTGTGGGTGT
pCAG-Lac1-L379A/L383A/I387A-F	CTGATCTTTAGGGTGGCGTACAGGATTGCGTGGCGGGGCGCTCTGAAGGATGACCGG
pCAG-Lac1-L379A/L383A/I387A-R	CCGGTCATCCTTCAGAGCGCCCCGCCACGCAATCCTGTACGCCACCCTAAAGATCAG
pCAG-Lac1-Mut7A-F	GCTGCCGCAGCCGCAGCTGCAAGCGATGAGGAGTCTGATGAGAGCAG
pCAG-Lac1-Mut7A-R	TGCAGCTGCGGCTGCGGCAGCCTTCAGAATGCCCCGCCACAGAATC
pCAG-Lac1-3A-F	CTGAAGGATGACCGGGCCGACGCCGAGGCCGATGAGGAGTCTGAT
pCAG-Lac1-3A-R	ATCAGACTCCTCATCGGCCTCGGCGTCGGCCCGGTCATCCTTCAG
pCAG-Lac1-3E-F	CTGAAGGATGACCGGGAGGACGAGGAGGAGGATGAGGAGTCTGAT
pCAG-Lac1-3E-R	ATCAGACTCCTCATCCTCCTCCTCGTCCTCCCGGTCATCCTTCAG
pCAG-Lag1-F	CGGGGTACCATGACTAGCGCCACTGATAAGT
pCAG-Lag1-R	CCGCTCGAGCTCGCACTTCTCCTTGCT
pCAG-Lip1-F	CGGGGTACCATGAGCCAGCCTACACCTATT
pCAG-Lip1-R	CCGCTCGAGTCATCACATGTGGTAGATTGTGCTAT
XhoI-scLac1-F	GAAAATTCAATATAACATATGCTCGAGATGTCGACAATAAAGCCAAGCCCTTC
Flag-scLac1-R	GTAATCCTCGACAGAGCCAGCATCAACCTCATCAGAGCCAGAGCCAATATCCTTTTTCGTTGGAGT
SalI-Flag-R	GTAAAGACATAAGAGATCCGCGTCGACTTACTTGTCATCGTCGTCCTTGTAATCCTCGACAGAGCCAGC
scLac1-L379A/L383A/I387A-F	CTTATTTTTAGAGTTGCTTACAGGATTGCTTGGAGAGGTGCTCTCAAGGATGACAGA
scLac1-L379A/L383A/I387A-R	TCTGTCATCCTTGAGAGCACCTCTCCAAGCAATCCTGTAAGCAACTCTAAAAATAAG
5UTR-scLac1-KanR-F	ATACCTCCGGTAAACATTTAGATAGACACAGTATCAATAAACAAGAGCTGAAGGTACTTCAGGGTTGAA
3UTR-scLac1-KanR-R	TAAAGAATTAATGTGTAATGGTTATACTACTTAAAAACACCGTTTTCCTCCGATTCATTAATGCAGCTG
5UTR-scLac1-Flag-URA3-F	ATACCTCCGGTAAACATTTAGATAGACACAGTATCAATAAACAAGAGCTATGTCGACAATAAAGCCAAG
3UTR-scLac1-Flag-URA3-R	TAAAGAATTAATGTGTAATGGTTATACTACTTAAAAACACCGTTTTCCTTCAGTTTTGCTGGCCGCATC
5UTR-scLag1-KanR-F	TGTTGAGAGTGAACTCCAAGATACAGAGAAACTGAAGAAATAACGACAACGAAGGTACTTCAGGGTTGA
3UTR-scLag1-KanR-R	TCATACAGGGGGGAAATCATATGATGATACGTATTCTCCTTAAGATACGTCCGATTCATTAATGCAGCT