Structure and mechanism of an intramembrane liponucleotide synthetase central for phospholipid biosynthesis

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Supplementary Figures 1-10 and Table 1.



Supplementary Figure 1 | The CDP-DAG mediated phospholipid biosynthesis and recycling pathways in prokaryotic and eukaryotic organisms. (a) Prokaryotic phospholipid biosynthesis pathways mediated by CDP-DAG. ACP, acyl carrier protein; PlsY/PlsB, Glycerol-3-phosphate acyltransferase; PlsC, 1-acyl-sn-glycerol-3-phosphate acyltransferase; Cds: CDP-DAG synthetase, encoded *CdsA* gene in prokaryotes (or by *CDS* genes in eukaryotes). PssA, bacterial phosphatidylserine synthase; Psd, phosphatidylserine decarboxylase; PgsA, phosphatidylglycerol phosphate (PGP) synthase; PgsA*, The *pgsA* gene in *M*. *smegmatis* encodes the phosphatidylinositol synthase enzyme. PgpA/B/C, PGP phosphatases; Cls, cardiolipin synthase. (b) Eukaryotic phospholipid biosynthesis and

recycling pathways mediated by CDP-DAG. GPAT, Glycerol-3-phosphate acyltransferase; AGPAT,

1-acylglycerol-3-phosphate-O-acyltransferase; PI kinase, phosphatidylinositol kinase; PIP kinase, phosphatidylinositol phosphate kinase; PLC, phospholipase C; DAGK, diacylglycerol kinase; PGS, PGP synthase; PSS, phosphatidylserine synthase found in *Saccharomyces cerevisiae* and some plants (such as wheat) with function similar to that of bacterial PssA; PSD, phosphatidylserine decarboxylase.



Supplementary Figure 2 | Role of methylmercury in stabilizing the crystal packing interfaces between adjacent TmCdsA molecules. (a) Overview of the crystal packing of TmCdsA. (b and c) Zoom-in views of the red (b) and blue-box (c) shaded interfacial regions shown in a, respectively. Mercury atoms are shown as purple spheres and the cysteine residues involved in binding methylmercury groups are presented as stick models.



Supplementary Figure 3 | **The experimental electron density maps of TmCdsA.** (**a**, **b**) The initial electron density map at 3.8 Å resolution output by the Phenix Autosol program with Autobuild model superposed on the map. The map is viewed along the membrane plane (a) or along the membrane normal from periplasmic side (b). (**c**, **d**) Anomalous difference Fourier maps of various mercury derivatives used as site-specific labels for the verification of sequence registration during structural model building and refinement.



Supplementary Figure 4 | Superposition of the overall structures of S200C/S223C (inactive) and S200C/S258C (active) mutants of TmCdsA. (a) Side view along the membrane plane. Color codes: blue, S200C/S223C; orange, S200C/S258C. Stereo images are presented. (b) Top view along the membrane normal. (c) A zoom-in view of the engineered C223 and C258 sites in the two structures. The two cysteine residues are highlighted as sphere models. The red arrows show the slight differences between the two structures.



Supplementary Figure 5 | The oligomeric states of TmCdsA and mutants in solution. (a, b) Crosslinker concentration and time-dependent crosslinking kinetics of wild type TmCdsA, S200C/S258C and S200C/S223C mutants. GA, glutaraldehyde. (c, d, e) Size-exclusion chromatography with multi-angle light-scattering (SEC-MALS) measurements on the protein masses of wild type TmCdsA (c), S200C/S258C (d) and S200C/S223C (e) mutants.

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DrCdsA	MPCGEA	AVETLSTRVL	TSVIGFLIVS	ALVW-IGWWA	MLPALIAVSF	45	FGLS <mark>E</mark> YFRML	DRNDLDVRRL	SLGVFGTALI	VASLPMFSEY	APWPGG	91
EcCdsA		MLKYRLI	SAFVLIPVVI	AALFLLPPVG	FAIVTLVVCM	37	LAAW <mark>E</mark> WGQLS	GFTTRSQRVW	LAVLCGLLLA	LMLFLLPEYH	RNIHQPLV	85
HiCdsA		<mark>MLKQR</mark> VL	SAIVLIAAVL	CALFL FTPFY	FALALGAVAI	37	LGIW <mark>E</mark> WTQFA	RLKQPLIRFF	VTTFLGVFIF	LWLYTEGNYL	DAGRVFEQHL	87
VcCdsA		<mark>MKQR</mark> II	TALILAPLVI	LGILYLPFAW	FMLALA <mark>VV</mark> TL	36	LGFW <mark>E</mark> WTQFV	NQP SRMLAMI	PALLVGGISV	ALIDFQFPAI	SNMNTAH	83
PaCdsA		<mark>MLKQR</mark> II	TALVLLPIAL	GG <mark>FF</mark> LLEGAF	FALFIGAVVS	37	LGAW <mark>E</mark> WARLA	GYE QQF GRVA	YAATVAVLMV	ALYHLPQLAG		77
SaCdsA		MKVRTL	TAIIALIVFL	PILL-KGGLV	LMIFANILAL	35	IALK <mark>E</mark> LLNMN	MIKFVSVPGL	ISAVGLIIIM	LPQHAGPWVQ	v	76
BcCdsA		MKQRII	TGVVAAALFI	PIVI-YGGVP	FTVLVYALAS	35	IGLY <mark>E</mark> LIRMN	KLTLISVP TV	LAAVLLWIIL	IPSSASELFT	<u>N</u>	76
AaCdsA	MRITQGERES	SGEFLMSREF	YGVLIGVTTL	LVIF-LPKSL	FLLVILF <mark>L</mark> CF	49	AISR <mark>E</mark> VSVA-	LGEN	EVFYFSPLVL	LTYYFADPL-		81
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EcCdsA	EISLWASLGW	WIVALLLVLF	YPGSAAIWRN	SKTLRLIFGV	LTIVPFFWGM	135	LALRAWHYDE	NHYSGAIWLL	YVMILVWGAD	SGAYMFGKLF	GKHKLAPKVS	185
HiCdsA	QLLLINAVSW	WGLALLUIS	YPKSAKFWSK	NPLLQLLFAF	STLIPFVAGV	137	LRLEHYTH	DPYHGLFLLL	YVFIL VWAAD	SGAYFSGRAF	GKRKLAPKVS	187
VcCdsA	FIVLGIGSLW	WLVSSGLAIT	YPRSRPLWEH	SSTVRHLFGL	FTLLPFFWSV	133	LFLRADTYLS	DPLYGAKLVL	FVCFLVWAAD	SGAYFVGKSL	GKHKMAPAVS	183
PaCdsA	-AVLLLALVW	WTLATVLVLT	YPESVGYW-G	GRWRRLGMGL	LILLPAWQGL	125	VLLKQWPL	ANGLII	AVMVLVWGAD	IGAYFSGKAF	GKRKLAPRVS	169
SaCdsA	-IQLKSLI	AMSFIVLSYT	VLSK-N	RFSFMD	AAFCLMSVAY	114	VG <mark>I</mark> GFMFFYE	TRSEGLHYIL	YAFLIVWLTD	TGAYLFGKMM	GKHKLWPVIS	164
BcCdsA	- IGLGKLEIT	FVIVLLLSY	TVLSKN	TFTFDN	ASFLLMATTY	117	VAMGFLYLNE	TRILGIKYVF	CALFVIWATD	SGAYF <mark>VG</mark> KAL	GKRKLWPEIS	167
AaCdsA	VFPLIGLL	SLYFAYKRWE		LNS	FFKSTFLLFY	112	PALFLVYLIK	IKEISTYYLL	IFIFGIWIND	VFAYYIGKNF	GKTPLFPKIS	162
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DrCdsA	PGKTVEGAIG	GLAFGFVVVL	VVSQLAGI	WTPLQA	FLYSVLVASA	229	SQLGDLSESL	IKRALRTKDS	GNSLPGHGGF	LDRLDSLMFA	VPATYFFLNI	SAFTN 28
EcCdsA	PGKTWQGFIG	GLATAAVISW	GYGMWANLDV	APVTL	LICSIVAALA	230	SVLGDLTESM	FKREAGIKDS	GHLIPGHGGI	LDRIDSLTAA	VPVFACLLLL	VFRTL 28
HiCdsA	PGKSWEGVIG	GLITALVLAF	IFIHFSNNTL	VG-DRNITGF	IILSVATVAI	236	SVLGDLTESM	FKRESGVKDS	SQLIPGHGGV	LDRIDSLTAA	VPFFSYFYFF	VL 28
VcCdsA	PNKTIEGLVG	GIVTAMLVGY	WVAECFGIQF	SSMPVM	LLIILLTVVI	229	SVLGDLVESM	FKRVSGIKDS	SNIIPGHGGI	LDRIDSLTAA	FPVFALLYFL	F 28
PaCdsA	PGKSWEGVYG	GLAASLAITL	AVGLYRGWSL	GALLLA	LLGAALVVFV	215	SIVGDLTESM	FKRQSGIKDS	SNLLPGHGGV	LDRIDSLTAA	IPVFAALLWA	AGWGAP - 27
SaCdsA	PNKTIEGFIG	GLFCSLIVPL	AMLYFVDFN-	MNVWIL	LGVTLILSLF	209	GQLGDLVE SG	FKRHFGVKDS	GRILPGHGGI	LDRFDSFMFV	LPLLNILLIQ	s 26
BcCdsA	PNKTIEGSLG	GIVCGIIVAL	VYNMFFPVE-	SNVVIL	IVLTIIISIF	212	GQIGDLVQSA	FKRHYGVKDS	GTILPGHGGI	LDRTDSWLFV	LPILYFLLQY	N 26
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DaCds2	35 SDSETKPE	VPVSADDTPE	VINKALSGIS	SRWKNWWVRG	TUTLAMISEE	FETTVLCDMV	LMMTVLCVOL	KCEOFTITIC	VEVVUEVDID	WEBTISWYEI	120
HsCds1	53 RTDSDIPE	TPPSSDRTPE	TLKKALSGLS	SRWKNWWTRG	TLTLTMISLE	FLITYMCSEM	LMLLVLGTOV	KCEHETTTC	VRVVHSVDLP	WERTLSWYEL	152
DmCds1	50 EVDELAKN	LPOGTONTPE	TLDSALKDLP	DRWKNWVTRG	TETWIMICGE	ALTIXCOPLA	LMITTLLVOV	KCFOFILSIC	VOUVETHELP	WERSLOWYEL	147
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Dacdsz HeCdel	LCVNIFFIGE	TVIDIFFILV	OPEROLOFIT	RINKFISFAL	YIACECMEVI	SLVKKHIKLQ	FIMFGWIHVI	L L T T V T Q S H L	VIONIFECMI	WEIVPISCVI	252
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TmCdsA ScCds1 DaCds2 HsCds1 DmCds1 Consistency TmCdsA ScCds1 DaCds2	150 VFDSFAYFTS VNDIFAYLCG CNDIMAYMFG CNDIMAYMFG CNDVMAYVFG V56*64**54* 200 VISFRTFLPF KPIYFHALNL YPFQIHSIAL	160 LKFGRTRISP ITFGKTKLI- FFFGRTPLI- FFFGRTPLI- 64**8*5970 210 ATTASLFAPF SSFASIMOPF	170 RYSPRKSLEG EISPKKTLEG KLSPKKTWEG KLSPKKTWEG 76**8*85** 220 CDIFESALKR GFFASGLKR GFFASGLKR	180 VIGGFLGVVI FLGAWFFTAL FIGGFFSTVV FIGGFFSTVV FIGGFATVL 79*7474777 230 HYGVKDSGKT TFKVKDFGHS AFKIKDFANT	190 YTFLYRLVVN ASIILTRILS FGILLSYVMA FGFIAAYVLS FGILFSYVLC 5568454974 240 LPGHGGMLDR IPGHGGITDR IPGHGGIMDR	DL PYTYLTCPVE GYSYFVCPVE KYQYFVCPVE NYQYFICPIQ 3736446655 250 IDGLLFVAPV VDCQFIMGSF FDCRYLMATF	DLHTNFFSNL FNNDSNRFTV YRSDVNSFVT YSEEQGRMTM 212322223 260 SYIVFKILE- ANLYYETFIS VNVYIASFI-	TCELNPVFLP DCQPSELFQL ECEPSELFQL SCVPSYLFTP 2633424622 	QVYRLPPIFF QDYSLPSVLQ QTYSLPPFLK QEYSLKLFGI 6264632311 270 VR STILMNLNDK IQQLLALRPD	LSVN DKVQINSITV SITGWTTVKL AVLRQERVSL GKTLNL 1110024535	197 325 331 349 342 425 425
TmCdsA ScCds1 DaCds2 HsCds1 DmCds1 Consistency TmCdsA ScCds1 DaCds2 HsCds1	150 VFDSFAYFTS VNDIFAYLCG CNDIMAYMFG CNDIMAYMFG CNDVMAYVFG VDS6464**54 200 VISFRTFLPF KPIYFHALNL YPFQIHSIAL	160 LKFGRTRIS ITFGKTKLI- FFFGRTPLI- FFFGRTPLI- 64**8*5970 210 AATVAIMDTF ATFASLFAPF SSFASIMGPF STFASLIGPF	170 RYSPRKSLEG EISPKKTLEG KLSPKKTWEG KLSPKKTWEG KLSPKKTWEG 76**8*85** 220 CDIFESALKR GFFASGLKR GFFASGFKR GFFASGFKR	180 VIGGFLGVVI FLGAWFFTAL FIGGFFSTVV FIGGFATVL 79*7474777 230 HYGVKDSGKT TFKVKDFGHS AFKIKDFANT AFKIKDFANT	190 YTFLYRLVVN ASIILTRIS FGILSYVMA FGFIAAYVIS FGILFSYVIC 5568454974 240 PGHGGMLDR IPGHGGIDR IPGHGGIMDR	DL PYTYLTCPVE GYSYFVCPVE KYQYFVCPVE NYQYFICPIQ 3736446655 250 IDGLLFVAPV VDCQFIMGSF FDCRYLMATF FDCQYLMATF	DLHTNFFSNL FNNDSNRFTV YRSDVNSFVT YSEEQGRMTM 212322223 260 SYIVFKILE – ANLYYETFIS VNVYIASFI- VHVYITSFI-	TCELNPVFLP DCQPSELFQL ECEPSELFQL SCVPSYLFTP 2633424622 	QVYRLPPIFF QDYSLPSVLQ QTYSLPPFLK QEYSLKLFGI 6264632311 270 VR	LSVN DKVQINSITV SITGWTTVKL AVLRQERVSL GKTLNL 1110024535	197 325 331 349 342 425 428 446
TmCdsA ScCds1 DaCds2 HsCds1 DmCds1 Consistency TmCdsA ScCds1 DaCds2 HsCds1 DmCds1	150 VFDSFAYFTS VNDIFAYLCS CNDIMAYMFG CNDIMAYMFG CNDVMAYVFG CNDVMAYVFG 200 VISFRTFLPF KPIYFHALNL YFFQIHSIAL YFFQIHSIAL YFFIWHSISL	160 LKFGRTRIS TFFGRTRII - FFFGRTPLI - FFFGRTPLI - 64 * * 8 * 5970 210 AATVAIMDTF ATFASLFAPF SSFASIMGPF SSFASIGPF	170 RYSPRKSLEG EISPKKTLEG KLSPKKTWEG KLSPKKTWEG 76**8*85** 220 CDIFESALKR GFFASGFKR GFFASGFKR GFFASGFKR	180 VIGGFLGVVI FLGAWFFTAL FIGGFFSTVV FIGGFATVL 79*7474777 230 HYGVKDSGKT TFKVKDFGHS AFKIKDFANT AFKIKDFANT AFKIKDFGDM	190 YTFLYRLVVN ASIILTRILS FGILLSYVMA FGFIAAYVLS FGILFSYVLC 5568454974 240 PGHGGMLDR IPGHGGIMDR IPGHGGIMDR IPGHGGIMDR	DL PYTYLTCPVE GYSYFVCPVE KYQYFVCPVE NYQYFICPIQ 3736446655 250 IDGLLFVAPV VDCQFIMGSF FDCRYLMATF FDCQYLMATF	DLHTNFFSNL FNNDSNRFTV YRSDVNSFVT YSEEQGRMTM 2123222223 260 SYIVFKILE – ANLYYETFIS VNVYIASFI- VHVYITSFI-	TCELNPVFLP DCQPSELFQL ECEPSELFQL SCVPSYLFTP 2633424622 	QVYRLPPIFF QDYSLPSVLQ QTYSLPPFLK QEYSLKLFGI 6264632311 270 VR	LSVN DKVQINSITV SITGWTTVKL AVLRQERVSL GKTLNL 1110024535 QIIELIDILI QQLHIFNSLK QQVQIYQSLK	197 325 331 349 342 425 428 446 439

Supplementary Figure 6 | **Alignment of the amino acid sequences of TmCdsA and other Cds homologs.** (a) TmCdsA aligned with various prokaryotic Cds homologs. The different background color codes indicate the varying degree of amino acid residue conservation among the Cds homologs, with the red color highlighting identical residues and blue color denoting unconserved residues. The regions covering transmembrane M1-M9 helices are labeled above the sequence as blue, green and red cylinders. The domain-specific color codes of the transmembrane helices

are consistent with those shown in Figure 3c, namely blue for NTD, green for MD and red for CTD. The solid red triangles mark the two highly conserved Asp residues (D219 and D249) that are directly involved in binding the Mg²⁺-K⁺ di-metal center and essential for the enzyme activity. Abbreviation of species names: Tm, *Thermotoga maritima*; Dr, *Deinococcus radiodurans*; Ec, *Escherichia coli*; Hi, *Haemophilus influenzae*; Vc, *Vibrio cholerae*; Pa, *Pseudomonas aeruginosa*; Sa, *Staphylococcus aureus*; Bc, *Bacillus cereus*; Aa, *Aquifex aeolicus*. The amino acid sequence of TmCdsA shares 27% identity or 49% similarity with that of EcCdsA which was originally purified and characterized in an early biochemical study¹. (b) TmCdsA aligned with various eukaryotic Cds homologs. Sc, *Saccharomyces cerevisiae*; Da, *Danio rerio*; Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*. The DmCds1 and DaCds2 are the two eukaryotic homologs reported to have essential roles in the recycling of phosphoinositide during signal transduction^{2,3}.



Supplementary Figure 7 | Probing the active site of TmCdsA with heavy surrogates of K⁺ and Mg²⁺ ions. (a) Anomalous difference Fourier peaks of Tl⁺ (red, +5.0 × σ level) and Ba²⁺ (green, +6.0 × σ level) ions bound to TmCdsA. (b) Isomorphous and anomalous difference Fourier peaks of Mn²⁺ and Cs⁺ ion bound to TmCdsA. The F_{Mn} - F_{Mg} map in blue is contoured at +5.5 × σ level, while the Mn-anomalous difference map in purple is at +4.0 × σ level. The F_{Cs} - F_K map in orange and the Cs-anomalous difference map in cyan are both contoured at +6.0 × σ level.



Supplementary Figure 8 | \mathbf{Rb}^+ and \mathbf{Mn}^{2+} ions simultaneously bind to the active site of TmCdsA. The F_{Rb+Mn} - F_{K+Mn} isomorphous difference map in blue is contoured at +3.0 × σ level. The anomalous difference map in purple using data collected at 1.54178 Å wavelength is contoured at +3.5 × σ level. Only \mathbf{Mn}^{2+} instead of \mathbf{Rb}^+ produces detectable anomalous signal under this wavelength. Both maps were computed to 4.5 Å resolution. The silver bullet models indicate the refined positions of \mathbf{Mg}^{2+} and \mathbf{K}^+ ions in the structure of S200C/S223C crystal. The peaks of \mathbf{Rb}^+ and \mathbf{Mn}^{2+} ions slightly deviate from the positions of \mathbf{K}^+ and \mathbf{Mg}^{2+} site due to minor changes of the unit cell dimensions in the derivatized crystal.



Supplementary Figure 9 | Analyses on the mono-dispersity of the wild-type, EDTA-treated TmCdsA proteins and the various mutants used for the activity assay. (a, b and c) Gel filtration (superdex 200 10/300 GL) profiles of the EDTA-treated TmCdsA and various mutants used for activity assay shown in Fig. 4e. For comparison, the elution profile of the wild-type protein is included in all three panels. (d) Dynamic light-scattering analyses on the main peak fractions (at around 12.8 ml) eluted from the gel filtration column for further verification of sample mono-dispersity. R_h (nm) represents the hydrodynamic radius of the particles in solution.



Supplementary Figure 10 | Substrate-dependent kinetic analyses of TmCdsA in the presence of 200 mM KCl and 2 mM MgCl₂. (a) CTP-dependent kinetic data measured in the presence of 2 mM DOPA. Each individual data points are plotted as mean value \pm standard error (SEM as indicated by the error bars. n=4 For the data points with [*CTP*] = 0.5, 1.0, 2.0 and 3.0 mM, while the other data points were measured with n=3 instead). For those data points with small errors, the error bars are buried within the symbols. (b) PA-dependent kinetic data measured in the presence of 4 mM CTP. For the data points with [*PA*] = 0.75, 1 and 1.5 mM, n=4, while the others are with n=3 instead. The kinetic parameters of the fitted curves are summarized in Supplementary Table 1.

Engrand		CTP Depender	nce	PA dependence			
Enzyme	V _{max}	$K_{ m half}/K_{ m m}^{\ \xi}$	Hill coefficient	V _{max}	$K_{ m half}/K_{ m m}^{\ \ \xi}$	Hill coefficient	
TmCdsA	1.52 ± 0.11	$K_{half} = 1.82$ ± 0.19	1.7 ± 0.2	2.55 ± 0.34	$K_{half} = 0.22$ ± 0.07	1.2 ± 0.4	
EcCdsA ^ξ	55.0	$K_m = 0.58 \pm 0.16$	N/A	55.0	$K_m = 0.28 \pm 0.05$	N/A	

Supplementary Table 1 | Kinetic parameters of TmCdsA and EcCdsA

The parameters are estimated by using the GraphPad Prism 6 software to fit the plots of initial velocity versus substrate (CTP or PA) concentration shown in Supplementary Figure 10. For CTP-dependent and PA-dependent kinetic data of TmCdsA, the allosteric sigmoidal model ($(Y = V_{max} * X^h / (K_{half}^h + X^h); h =$ Hill coefficient) is applied for data fitting. The error presented is \pm S. E. of the fit. Units for V_{max} are μ mol of CDP-DAG produced per min per mg enzyme. The unit for K_{half} or K_m is mM. [§] Data reported in the literature¹.

Supplementary References:

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