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Structure and autoregulation of a P4-ATPase lipid flippase

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Summary

P4-ATPases are lipid flippases that drive active transport of phospholipids from exoplasmic or luminal leaflets to cytosolic leaflets of eukaryotic membranes. The molecular architecture of P4-ATPases and how they work in lipid recognition and transport has remained elusive. Using cryo-electron microscopy we have determined the structure of a P4-ATPase, specifically of the *Saccharomyces cerevisiae* Drs2p-Cdc50p, which is a phosphatidylserine and phosphatidylethanolamine specific lipid flippase. Drs2p-Cdc50p is autoinhibited by the Drs2p C-terminal tail and activated by phosphatidylinositol-4-phosphate (PI4P). We present three structures representing an autoinhibited, an intermediate, and a fully activated state. The analysis highlights specific features of P4-ATPases and reveals sites of autoinhibition and PI4P-dependent activation. We observe the opening of a putative flippase pathway engaging conserved residues Ile508 of transmembrane segment 4 and Lys1018 and polar residues of transmembrane segment 5 in the center of the lipid bilayer.

Introduction

Cells and organelles are defined by lipid bilayer membranes and membrane proteins. Lipid distributions between bilayer leaflets in eukaryotic membranes of the late secretory/endocytic pathways are asymmetric. These lipid gradients potentiate important biological processes such as membrane dynamics, endo- and exocytosis, and signalling¹⁻⁴. Due to membrane-fusion events and the downhill, bidirectional activity of lipid scramblases, lipid asymmetry must constantly be regulated and maintained. Members of two distinct membrane protein superfamilies drive the ATP-dependent unidirectional translocation of lipids against concentration gradients; namely ATP-binding cassette (ABC) transporters and P4-type ATPases that drive typically inward-to-outward (flop) and outward-to-inward (flip) translocation of lipids between bilayer leaflets, respectively¹⁻⁴. P-type ATPases couple transport to formation and breakdown of a phosphoenzyme in a functional cycle with so-called E1, E1P, E2P and E2 intermediate states (Extended Data Figure 1a), and P4-ATPases specifically to dephosphorylation of the E2P state^{5,6}, i.e. similar to the inward K⁺ transport of Na,K-ATPase, whereas E1P phosphorylation seems independent of transport (Extended Data Figure 1a and b)^{5,7}. While recent studies have shed light on the structure and function of lipid floppases^{8,9} and scramblases^{10,11}, P4-ATPases have so far been studied only by bioinformatics and functional assays. The transport mechanism remains debated with models implicating peripheral or centrally located lipid recognition sites and pathways¹²⁻¹⁴.

Most P4-ATPases are binary complexes, where a Cdc50-protein subunit is necessary for proper localization and function of the complex (Extended Data Figure 1c)^{15,16}. Mutant forms of mammalian lipid flippases have been implicated in disease, e.g. ATP8A1 and ATP8A2 in neurological disorders, ATP8B1 in progressive familial intrahepatic cholestasis type 1 (PFIC1), ATP10A in type 2 diabetes and insulin resistance, and ATP11A in cancer¹⁷. The trans-Golgi localized Drs2p-Cdc50p complex from the yeast *Saccharomyces cerevisiae* is well characterized. *In vivo*^{18,19} and *in vitro*^{20,21} studies show that Drs2p-Cdc50p primarily flips phosphatidylserine (PS) and to a lesser extent phosphatidylethanolamine (PE) from the luminal to the cytosolic leaflet, and indicate a role of this function in vesicle biogenesis at late secretory membranes²².

The C-terminus of Drs2p contains an autoinhibitory domain^{23,24}. Relief of autoinhibition requires the regulatory lipid phosphatidylinositol-4-phosphate (PI4P)^{6,23}. Binding of Gea2p (a guanine nucleotide exchange factor for the small GTPase Arf) to a basic segment of the C-terminus has been

reported to be necessary for activation *in vivo*²³, although not yet confirmed *in vitro*²⁴. Furthermore, interaction of Arl1p (a GTPase of the Arf family) with the extended N-terminus of Drs2p has been implicated in flippase activity *in vivo*²⁵. Whereas the first 104 amino acids of the N-terminus have little effect on *in vitro* activity, truncation of the C-terminus is activating, but the protein remains under regulation by PI4P²⁶ (Extended Data Figure 1d). While these studies highlight the components involved, a molecular mechanism of autoregulation for Drs2p-Cdc50p - and P-type ATPases in general - remains unknown.

Aiming for structural and mechanistic insights, we embarked on cryo-EM studies of beryllium fluoride (BeF₃⁻) stabilized Drs2p-Cdc50p complexes. Drs2p-Cdc50p was over-expressed in *S. cerevisiae* and purified in Lauryl Maltose Neopentyl Glycol (LMNG) detergent by affinity chromatography and gel filtration, resulting in a monodisperse sample containing both subunits (Extended Data Figure 2). Samples represent E2P phosphoenzyme-like states in the progressive steps from a fully autoinhibited P4-ATPase (E2P^{inhib}), to an intermediate activated state in the presence of PI4P (E2P^{inter}), and an outward-open and activated conformation represented by a C-terminally truncated enzyme, also in the presence of PI4P (E2P^{active}).

Overall structure and conformation

The structures of Drs2p-Cdc50p in the E2P^{inhib}, E2P^{inter}, and E2P^{active} conformations were determined at 2.8, 3.7 and 2.9 Å resolution, respectively, and reveal functional sites and the PI4P-dependent regulation of Drs2p-Cdc50p (Extended Data Table 1). Complete models, except for minor disordered regions at the termini, were obtained (Supplementary Video 1).

The structure of the Drs2p subunit is typical of P-type ATPases (see Supplementary Discussion) with 10 transmembrane (TM) helices and three cytosolic domains: the actuator (A) domain, the nucleotide-binding (N) domain and the phosphorylation (P) domain (Figure 1, Extended Data Figure 1c, e-g). The Cdc50p subunit has an ectodomain with two asymmetric lobes. The first is dominated by an antiparallel β-sandwich and the other contains little secondary structure apart from short helical segments (Extended Data Figure 3a). Two di-sulfide bonds identified earlier²⁷ are evident (Extended Data Figure 3b), and the four glycosylation sites have at least one *N*-acetylglucosamine unit revealed by the map (Extended Data Figure 3c-e). The fold of the ectodomain is similar to the lipid binding protein seipin, although loops of Cdc50p are considerably

longer (Extended Data Figure 3f). The two TM-helices of Cdc50p extend from the N- and C-terminus of the first lobe (Extended Data Figure 3a), interacting closely with each other and with TM10 of Drs2p (Extended Data Figure 3g).

Extensive quaternary interactions appear on the luminal face, where conserved regions of the Cdc50p ectodomain interact with all Drs2 loops (Figure 1b, Extended Data Figure 3h-k). The TM3-4 loop stretches into an intimate interaction site at the ectodomain of Cdc50p (Extended Data Figure 3h), and the N-terminus of Cdc50p extends along the cytosolic side of the TM-domain of Drs2p and makes contacts with a segment (residues 529-538) that connects TM4 and the phosphorylation site of the P-domain (Extended Data Figure 3a and i). The TM4-segment couples the phosphorylation site chemistry with conformational changes of the TM-domain in ion pumps²⁸, and it is 10 residues longer in P4-ATPases. For a detailed description of Drs2p-Cdc50p interactions, see Supplementary Information.

Autoinhibition and PI4P binding

Our samples map three distinct states leading from autoinhibition to activation. In E2P^{inhib}, the autoinhibitory C-terminus forms an extensive interface (residues 1252-1307) that spans the cytosolic domains (Figure 2a). A short helical segment of the C-terminal tail (H1^{C-tail}, residues 1252-1263) interacts with a unique helical insertion on the P-domain, while the rest of the tail extends to position a conserved GFAFS motif (residues 1274-1278) at the vertex between the cytosolic domains (Figure 2a) and overlap with the nucleotide binding site (Figure 2b). Autoinhibition is further stabilised by the clamping of a short loop region in the N-domain (698-704) over the GFAFS motif. Cleavage of the C-terminus at residue 1290 results in a 10-20 fold activation compared to wild-type Drs2p-Cdc50p²⁶ and it removes the bulk of the C-terminus that interacts with the A-domain (as well as unmodeled terminal residues). However, truncation at residue 1302 maintains autoinhibition²⁶ and preserves the interactions with the A-domain. This suggests an allosteric mechanism of autoinhibition, where the cytosolic domains are locked and prevented from undergoing conformational changes. The 16-residue linker between TM10 and the autoregulatory domain is not resolved sufficiently for modelling, but appears at a low-density threshold and in 2D class averages (Figure 2c).

Density is observed for PI4P bound between TM7, 8, and 10 of E2P^{inter} and E2P^{active}. Importantly, binding of PI4P is concurrent with the movement of the cytosolic ends of TM10 and the TM-helices of Cdc50p, and also the formation of an amphipathic helix just after TM10. The amphipathic helix propagates away from the autoinhibitory binding site and exerts a pull that dissipates the autoinhibitory interactions of H1^{C-tail} with the P-domain, thus releasing the P-domain. Additionally, the amphipathic helix provides a platform for interaction with the TM6-7 loop which moves in concert with the P-domain, explaining in part why C-terminal truncation at residue 1247 preserves PI4P dependence, cf. E2P^{active} (Extended Data Figure 1d), while truncation after residue 1232 leads to a reported PI4P-independent flippase²⁴. In E2P^{inter}, only the downstream interactions of the C-terminus with the N- and A-domain remain preserved (Figure 2d). Interestingly, H1^{C-tail} coincides with the previously reported Gea2p binding site²⁹, and its release thus mediated by PI4P exposes it for interaction. The PI4P binding site, however, is notably distinct from a previously proposed site at basic residues (1268-1273)²³.

The position of the PI4P glycerol backbone is stabilized by interactions with several positively charged residues located in the TM region of Drs2p, with selectivity for PI4P provided by the interaction of Tyr1235 and His1236 (displayed by the amphipathic helix) to the inositol-4-phosphate of PI4P (Figure 3a and b). Mutations of these residues strongly attenuate ATPase activity and the susceptibility of the C-tail to limited proteolysis, implying a loss of PI4P binding (Figure 3c, Extended Data Figure 4 and Supplementary information). Strikingly, the PI4P binding site of Drs2p is located in the same region as the C-terminal YY motif of the Na,K-ATPase α -subunit³⁰, which also affects transport function profoundly³¹ (Figure 3d).

In E2P^{inhib}, the amphipathic helix is not present, and the vacant PI4P site contains a lipid with a significantly smaller head group showing no specific interactions (Figure 3e). We modelled it as PS, which is the sole lipid added to the sample during purification, and presume that regular phospholipids are bound in a nonselective manner in the autoinhibited state.

A putative substrate entry site

E2P^{active} and E2P^{inter} are largely similar. In contrast, the lack of an autoinhibitory domain in E2P^{active} allows for further rearrangements of the N- and A-domains, translating into a more open conformation of the TM domain. Here, TM1 and in particular TM2 move away from the bulk TM

domain and expose TM4 to the luminal leaflet of the membrane. A conserved, unwound segment of TM4 (PISL) is exposed by this movement and has been implicated in lipid transport¹⁴ (Figure 4a and b). The luminal exposure of TM4 is lined by TM1-2-6, and we propose that the cleft marks the entry of a lipid transport pathway, only empty in the current E2P structures mimicked by BeF_3^- . The cleft partially overlaps with a previously proposed entry gate and residues important for lipid specificity¹². In particular Gln237, part of a conserved QQ-motif at the end of TM1, points into the open cleft, supporting its role in substrate specificity (Figure 4c). The cleft is also consistent with the proposed hydrophobic gate model⁷ with a central role for the conserved isoleucine of the PISL motif¹⁴, although the conformation of TM1-2 in $\text{E2P}^{\text{active}}$ (and the other E2P sub-states reported here) and earlier homology models are different.

Chimera constructs and mutants of the TM1-2 loop in P4-ATPases Dnf1p and Drs2p have indicated a role in lipid binding and specificity¹². A conserved arginine in the ecto-domain of Cdc50p (Arg151) reaches towards the proposed entry site where it could help orient the luminal loop between TM1 and 2 and guide lipid binding (Figure 4c). For comparison, mutations in the TM1-2 loop of Na,K-ATPase confer ouabain resistance³² and the cleft coincides with the ouabain binding site in the Na,K-ATPase (Figure 4d and f). The putative lipid entry pathway reaches the middle of the membrane, thus pointing to an alternating access mechanism. Extension of the cleft towards the cytosolic side overlaps with a lipid binding site and cyclopiazonic acid inhibitory site in SERCA³³ (Figure 4e and f). Such a proposed exit site at the cytoplasmic leaflet would be expected to emerge in a subsequent E2-E1 transition of the functional cycle and hints at possible evolutionary links of lipid flippases and ion pumps.

Unlike for the ion transporting P2-ATPases (Extended Data Figure 5b and c), negatively charged side chains are absent in the TM core of Drs2p, but the potentially positively charged Lys1018 of TM5 interacts with Asn1050 at a bulge of TM6 and could play a stabilizing, yet dynamic role (Figure 4g). Lys1018 has previously been implicated in transport in bovine ATP8A2⁷, and it projects a potentially positive charge at the middle of the transmembrane pathway (Figure 4a, b and g). The estimated pK_a ³⁴ of Lys1018 is around 6.8 for $\text{E2P}^{\text{inhib}}$ and 7.4 for $\text{E2P}^{\text{active}}$ indicating that it may switch between a neutral and a positively charged state as part of dynamic interactions with a negatively charged lipid head group.

Transport mechanism

The movements of the P and N domains in transition from a constrained $E2P^{\text{inhib}}$ to the $E2P^{\text{inter}}$ and $E2P^{\text{active}}$ states (Extended Data Figure 5d) are echoed in a concomitant movement of the adjacent TM6-7 loop towards the amphipathic helix formed upon PI4P binding (Extended Data Figure 5e). This suggests that binding of PI4P leads to the movement of the P-domain. Interestingly, the position of the dephosphorylation loop of the A-domain remains locally constant with respect to the P-domain (Extended Data Figure 1g). Removal of the C-terminus allows for increased mobility of the A-domain, which may explain why PI4P alone is not sufficient for full activation of the intact enzyme.

Taken together, we propose the following model for auto-regulation and exposure of the lipid entry site. $E2P^{\text{inhib}}$ has a closed TM domain with the cytosolic domains locked by the autoinhibitory C-terminus (Figure 5 and Supplementary Video 2). PI4P binding to the TM domain promotes the folding of an amphipathic helix on the C-terminal side of TM10. Formation of this helix has two effects: 1) it causes the remainder of the C-terminus to partially unfold, thus destabilizing its interaction with the P-domain through the displacement of H1^{C-tail} with the putative Gea2p binding site; and 2) it forms an interaction site for the TM6-7 loop, which then moves with the P-domain. Together these two movements shift the P and N domains towards the amphipathic helix. A progressive rotation of the A domain leads to a subtle movement of TM2 and to a lesser extent TM1 yielding the $E2P^{\text{inter}}$ state. Full displacement of the C-terminus leads to the $E2P^{\text{active}}$ state, where a further rotation of the N- and A-domains drives the opening of a putative substrate binding site through the movement of TM2 away from TM6. We anticipate that subsequent PS (or PE) substrate lipid binding will be associated with further conformational changes of the TM domain, as the enzyme dephosphorylates.

Conclusion

The structures of Drs2p-Cdc50p presented here provide insights into the architecture of the P4-ATPase lipid flippases with Cdc50p and lipid binding site. The structure of the autoinhibitory domain and conformational changes associated with PI4P binding reveal a mechanism of autoregulation of P4-ATPases and potential sites for intervention. Further structures capturing progressive states of substrate lipid binding, dephosphorylation, lipid translocation and cytoplasmic release may elucidate the lipid flippase mechanism.

Online material

Methods, along with any additional Extended Data display items, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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was performed by M.T. and J.A.L, with assistance from J.J.U. and J.L.K. Mutant forms were prepared and functionally characterized by T.D., C.M. and G.L. P.N. and J.A.L. supervised the project together with A.M. The manuscript was drafted by M.T., J.A.L. and P.N. All authors commented on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to P.N. pn@mbg.au.dk, A.M. arne.moeller@biophys.mpg.de, or G.L. guillaume.lenoir@i2bc.paris-saclay.fr

Figure Legends

Figure 1: Architecture of the Drs2p-Cdc50p complex

a, LocScale map³⁵ of E2P^{inhib} colored by domain. Drs2p: The A-, P- and N- domains are colored yellow, blue and red, respectively. The transmembrane domain is tan and the autoinhibitory C-terminus is green. Cdc50p is pink. Unmodeled map features corresponding to lipid or detergent are orange.

b, Cartoon representation of the refined E2P^{inhib} model, colors as in panel **a**.

Figure 2: Autoinhibition of Drs2p by its C-terminus

a, The autoinhibitory domain bound between the A-, P- and N- domains. Sequence motifs of the autoinhibitory domain are color-coded.

b, Alignment of the N-domains from E2P^{inhib} and AMPPCP-bound SERCA³⁶ (PDB 1T5S) highlighting the overlap of the autoinhibitory domain and the ATP site. Drs2p is colored as in **a**, SERCA is dark grey, and AMPPCP is yellow.

c, 2D class average of E2P^{inhib}; arrow indicating a fuzzy linker between TM10 and the autoinhibitory domain.

d, Partial release of autoinhibition by PI4P. Density for HC^{tail} is shown in light green to emphasize disassociation upon PI4P binding. The first and last residues modelled around the disordered linker are identified.

Figure 3: PI4P recognition and binding by Drs2p

a, The PI4P binding site of E2P^{active}. The EM density (purple) for the lipid is displayed at a lower threshold (0.75 r.m.s.d.) than for the protein (blue, 2.5 r.m.s.d.).

b, The PI4P binding sites in E2P^{inter} and E2P^{active} are consistent. Colors as in **a**. The lipid and protein EM densities are at similar thresholds (1.5 r.m.s.d.).

c, ATPase activity of wild-type and C-terminal mutants of Drs2p-Cdc50p (purified in DDM) plotted as the difference between the rate observed upon limited proteolysis with trypsin, in the presence of both PI4P and PS, and before adding the purified protein complexes to the assay medium (with PS but in the absence of PI4P; see Extended Data Figure 4b). Bars represent the mean $\pm$ SD of six replicates from two independent purification batches.

d, Superposition of Drs2p and the Na,K-ATPase. The C-terminus of the Na,K-ATPase³⁰ (PDB 3KDP) is shown in green and overlaps with PI4P.

e, Phospholipid binding at the PI4P site in E2P^{inhib}. Lys1224 is moved away and makes no direct contact. Arg1219 makes a nonselective contact with the glycerophosphate. Lipid density in grey (1.0 r.m.s.d.), and protein blue (2.0 r.m.s.d.).

Figure 4: Activation of Drs2p upon PI4P-binding and C-terminal truncation

a, A proposed lipid entry pathway is exposed upon full activation (PI4P binding, C-terminal truncation). TM4 is shown in yellow and the PISL-motif in orange. The asterisk marks the location of **c** and **g**.

b, Electrostatic surface of the entry pathway. Electrostatic surfaces from APBS^{37,38} are shown for all in Extended Data Figure 5a.

c, Interaction between Cdc50p Arg151 and the TM1-2 loop of Drs2p in E2P^{inhib}. Colors as in **a** with TM1-2 in tan.

d-f, Comparison of ouabain-bound E2P-Na,K-ATPase (**d**, PDB 4HYT), E2-SERCA with PE bound between TMs 2 and 4 (**e**, PDB 2AGV) and E2P^{active}-Drs2p-Cdc50p (**f**, this work). View and colors as in panel **a**. Na,K-ATPase: β - and γ -subunit pink, TM4 yellow, PEGL-motif orange, ouabain green. SERCA colors as **d** with PE depicted as green spheres. Ouabain site of Na,K-ATPase overlaps with the opening in E2P^{active}-Drs2p-Cdc50p. The bound PE in SERCA hints at a putative lipid exit site in Drs2p-Cdc50p.

g, The side chain of Lys1018 near the PISL-motif of TM4. The location is seen in **a**.

Figure 5: Proposed autoregulation mechanism

Schematic representation of Drs2p-Cdc50p, with functionally important domains and regions colored. Drs2p A-domain (yellow), P-domain (blue), N-domain red, TMs 1, 2 and 4 (yellow/orange, orange and wheat rods, respectively), TM6-7 loop (blue surface), TM10 amphipathic helix (green), C-terminus (dark green). Cdc50p (pink). Lipids as labelled. The grey surface denotes an auxiliary protein like Gea2p sequestering the C-terminus to prevent autoinhibition. Binding of PI4P triggers the formation of the C-terminal amphipathic helix, disrupts the binding of H1^{C-tail} to the P-domain, and results in a rigid body movement of the cytosolic domains and the TM6-7 loop. Full release of the autoinhibitory domain results in further rearrangements of the N- and A-domains, movements of TMs 1 and 2, and opening of a putative lipid entry site.

References

- 1 Hankins, H. M., Baldridge, R. D., Xu, P. & Graham, T. R. Role of flippases, scramblases and transfer proteins in phosphatidylserine subcellular distribution. *Traffic* **16**, 35-47, (2015).
- 2 Montigny, C., Lyons, J., Champeil, P., Nissen, P. & Lenoir, G. On the molecular mechanism of flippase- and scramblase-mediated phospholipid transport. *Biochim Biophys Acta* **1861**, 767-783, (2016).
- 3 Pomorski, T. G. & Menon, A. K. Lipid somersaults: Uncovering the mechanisms of protein-mediated lipid flipping. *Prog Lipid Res* **64**, 69-84, (2016).
- 4 Yang, Y., Lee, M. & Fairn, G. D. Phospholipid subcellular localization and dynamics. *J Biol Chem* **293**, 6230-6240, (2018).
- 5 Ding, J. *et al.* Identification and functional expression of four isoforms of ATPase II, the putative aminophospholipid translocase. Effect of isoform variation on the ATPase activity and phospholipid specificity. *J Biol Chem* **275**, 23378-23386, (2000).
- 6 Jacquot, A. *et al.* Phosphatidylserine stimulation of Drs2p.Cdc50p lipid translocase dephosphorylation is controlled by phosphatidylinositol-4-phosphate. *J Biol Chem* **287**, 13249-13261, (2012).
- 7 Coleman, J. A., Vestergaard, A. L., Molday, R. S., Vilsen, B. & Andersen, J. P. Critical role of a transmembrane lysine in aminophospholipid transport by mammalian photoreceptor P4-ATPase ATP8A2. *Proc Natl Acad Sci U S A* **109**, 1449-1454, (2012).
- 8 Lee, J. Y. *et al.* Crystal structure of the human sterol transporter ABCG5/ABCG8. *Nature* **533**, 561-564, (2016).
- 9 Perez, C. *et al.* Structure and mechanism of an active lipid-linked oligosaccharide flippase. *Nature* **524**, 433-438, (2015).
- 10 Brunner, J. D., Lim, N. K., Schenck, S., Duerst, A. & Dutzler, R. X-ray structure of a calcium-activated TMEM16 lipid scramblase. *Nature* **516**, 207-212, (2014).
- 11 Morra, G. *et al.* Mechanisms of Lipid Scrambling by the G Protein-Coupled Receptor Opsin. *Structure* **26**, 356-367 e353, (2018).
- 12 Baldridge, R. D. & Graham, T. R. Two-gate mechanism for phospholipid selection and transport by type IV P-type ATPases. *Proc Natl Acad Sci U S A* **110**, E358-367, (2013).
- 13 Jensen, M. S. *et al.* Phospholipid flipping involves a central cavity in P4 ATPases. *Sci Rep* **7**, 17621, (2017).
- 14 Vestergaard, A. L. *et al.* Critical roles of isoleucine-364 and adjacent residues in a hydrophobic gate control of phospholipid transport by the mammalian P4-ATPase ATP8A2. *Proc Natl Acad Sci U S A* **111**, E1334-1343, (2014).
- 15 Bryde, S. *et al.* CDC50 proteins are critical components of the human class-1 P4-ATPase transport machinery. *J Biol Chem* **285**, 40562-40572, (2010).
- 16 Coleman, J. A. & Molday, R. S. Critical role of the beta-subunit CDC50A in the stable expression, assembly, subcellular localization, and lipid transport activity of the P4-ATPase ATP8A2. *J Biol Chem* **286**, 17205-17216, (2011).
- 17 van der Mark, V. A., Elferink, R. P. & Paulusma, C. C. P4 ATPases: flippases in health and disease. *Int J Mol Sci* **14**, 7897-7922, (2013).
- 18 Alder-Baerens, N., Lisman, Q., Luong, L., Pomorski, T. & Holthuis, J. C. Loss of P4 ATPases Drs2p and Dnf3p disrupts aminophospholipid transport and asymmetry in yeast post-Golgi secretory vesicles. *Mol Biol Cell* **17**, 1632-1642, (2006).
- 19 Natarajan, P., Wang, J., Hua, Z. & Graham, T. R. Drs2p-coupled aminophospholipid translocase activity in yeast Golgi membranes and relationship to in vivo function. *Proc Natl Acad Sci U S A* **101**, 10614-10619, (2004).

- 20 Azouaoui, H. *et al.* A high-yield co-expression system for the purification of an intact Drs2p-Cdc50p lipid flippase complex, critically dependent on and stabilized by phosphatidylinositol-4-phosphate. *PLoS One* **9**, e112176, (2014).
- 21 Zhou, X. & Graham, T. R. Reconstitution of phospholipid translocase activity with purified Drs2p, a type-IV P-type ATPase from budding yeast. *Proc Natl Acad Sci U S A* **106**, 16586-16591, (2009).
- 22 Xu, P., Baldrige, R. D., Chi, R. J., Burd, C. G. & Graham, T. R. Phosphatidylserine flipping enhances membrane curvature and negative charge required for vesicular transport. *J Cell Biol* **202**, 875-886, (2013).
- 23 Natarajan, P. *et al.* Regulation of a Golgi flippase by phosphoinositides and an ArfGEF. *Nat Cell Biol* **11**, 1421-1426, (2009).
- 24 Zhou, X., Sebastian, T. T. & Graham, T. R. Auto-inhibition of Drs2p, a yeast phospholipid flippase, by its carboxyl-terminal tail. *J Biol Chem* **288**, 31807-31815, (2013).
- 25 Tsai, P. C., Hsu, J. W., Liu, Y. W., Chen, K. Y. & Lee, F. J. Arl1p regulates spatial membrane organization at the trans-Golgi network through interaction with Arf-GEF Gea2p and flippase Drs2p. *Proc Natl Acad Sci U S A* **110**, E668-677, (2013).
- 26 Azouaoui, H. *et al.* High phosphatidylinositol 4-phosphate (PI4P)-dependent ATPase activity for the Drs2p-Cdc50p flippase after removal of its N- and C-terminal extensions. *J Biol Chem* **292**, 7954-7970, (2017).
- 27 Puts, C. F. *et al.* Mapping functional interactions in a heterodimeric phospholipid pump. *J Biol Chem* **287**, 30529-30540, (2012).
- 28 Moller, J. V., Olesen, C., Winther, A. M. & Nissen, P. The sarcoplasmic Ca²⁺-ATPase: design of a perfect chemi-osmotic pump. *Q Rev Biophys* **43**, 501-566, (2010).
- 29 Chantalat, S. *et al.* The Arf activator Gea2p and the P-type ATPase Drs2p interact at the Golgi in *Saccharomyces cerevisiae*. *J Cell Sci* **117**, 711-722, (2004).
- 30 Morth, J. P. *et al.* Crystal structure of the sodium-potassium pump. *Nature* **450**, 1043-1049, (2007).
- 31 Poulsen, H. *et al.* Neurological disease mutations compromise a C-terminal ion pathway in the Na⁺/K⁺-ATPase. *Nature* **467**, 99-102, (2010).
- 32 Price, E. M. & Lingrel, J. B. Structure-function relationships in the Na,K-ATPase alpha subunit: site-directed mutagenesis of glutamine-111 to arginine and asparagine-122 to aspartic acid generates a ouabain-resistant enzyme. *Biochemistry* **27**, 8400-8408, (1988).
- 33 Obara, K. *et al.* Structural role of countertransport revealed in Ca(2+) pump crystal structure in the absence of Ca(2+). *Proc Natl Acad Sci U S A* **102**, 14489-14496, (2005).
- 34 Olsson, M. H., Sondergaard, C. R., Rostkowski, M. & Jensen, J. H. PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical pKa Predictions. *Journal of chemical theory and computation* **7**, 525-537, (2011).
- 35 Jakobi, A. J., Wilmanns, M. & Sachse, C. Model-based local density sharpening of cryo-EM maps. *Elife* **6**, (2017).
- 36 Sorensen, T. L., Moller, J. V. & Nissen, P. Phosphoryl transfer and calcium ion occlusion in the calcium pump. *Science* **304**, 1672-1675, (2004).
- 37 Dolinsky, T. J., Nielsen, J. E., McCammon, J. A. & Baker, N. A. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res* **32**, W665-667, (2004).
- 38 Jurrus, E. *et al.* Improvements to the APBS biomolecular solvation software suite. *Protein Sci* **27**, 112-128, (2018).

Methods

Biochemical studies of C-terminal Drs2p-Cdc50p mutants

Expression and streptavidin purification of wild-type Drs2p-Cdc50p and mutants for functional studies were carried out as described previously²⁰. Specifically, we used for this purpose a C-terminal Tobacco Etch Virus (TEV)-cleavable BAD tag and DDM was used throughout the purification procedure. Size-exclusion chromatography was performed on a Superdex 200 Increase 10/300GL column, with a mobile phase containing 0.5 mg/mL DDM and 0.025 mg/mL POPS in 50 mM MOPS-Tris buffer at pH 7.0, containing 100 mM KCl, 20% glycerol (w/v), and 5 mM MgCl₂ (SSR). ATP hydrolysis by PI4P-binding mutants was measured at 30 °C using an enzyme-coupled assay, by continuously monitoring the rate of NADH oxidation at 340 nm⁴³. The purified Drs2p-Cdc50p complexes were added at about 2 µg/mL, in a cuvette containing SSR buffer supplemented with 1 mM ATP, 1 mM phosphoenolpyruvate, 0.4 mg/mL pyruvate kinase, 0.1 mg/mL lactate dehydrogenase, 0.25 mM NADH, 1 mM NaN₃, 1 mg/mL DDM, and 0.1 mg/mL POPS. Trypsin and PI4P were subsequently added to concentrations of 0.05 mg/mL and 0.025 mg/mL, respectively. Conversion of NADH oxidation rates expressed in AU/s to ATPase activities in µmol/min/mg was based on the extinction coefficient of NADH (~6200 M⁻¹.cm⁻¹) and on Coomassie-Blue stained SDS-PAGE-based quantification of Drs2p using known amounts of SEC-purified Drs2p as standards.

For limited proteolysis experiments²⁶, wild-type Drs2p-Cdc50p and mutants were purified on streptavidin beads either in the presence or in the absence of POPS. Proteolysis with 40 U/mL thrombin took place for 1 hour at 20°C in the presence of 1 mg/mL DDM, and 0.05 mg/mL PS and/or 0.025 mg/mL PI4P when needed. Proteolysis was quenched by adding 1 mM PMSF.

Expression and purification of Drs2p-Cdc50p for structural studies

Protein expression in *S. cerevisiae*, membrane harvesting and solubilization were performed as previously described^{20,26,44} using a C-terminal thrombin cleavable BAD-tagged Drs2p construct yielding Drs2p ΔN104/Cdc50p, where Drs2p is missing the first 104 residues due to thrombin cleavage. A second construct with an additional thrombin site after residue 1247 in Drs2p resulted in the N- and C-terminally truncated Drs2p ΔNC/Cdc50p sample.

Affinity chromatography on streptavidin resin and detergent exchange: The BAD-tagged protein was batch bound to free streptavidin sepharose resin (typically 1 mL resin per 60 mL of

solubilized material) for 1 hour at 4°C. Detergent exchange into lauryl maltose neopentyl glycol (LMNG) was performed, by washing with 2 column volumes (CV) SSR with 1 mM DTT and 0.2 mg/mL LMNG, followed by washing with 10 CV SSR with 1 mM DTT and 0.1 mg/mL LMNG. The resin was resuspended in 1 CV SSR with 1 mM DTT, 0.1 mg/mL LMNG and 50 µg/mL Brain PS (Avanti Polar Lipids). 4 units/mL resin of bovine Thrombin (Calbiochem) was added to cleave the protein off the resin during an overnight incubation at 4°C. The protein was eluted from the resin in 10-20 CV of SSR with 1 mM DTT and 0.1mg/mL LMNG, and concentrated to 0.5-1 mL in a 100 kDa centrifugal concentrator (Vivaspin) with the sample typically reaching concentrations of 5-10 mg/mL.

Cleavage of double truncated construct: To produce the double-truncated Drs2p ΔNC/Cdc50p protein, after elution from the resin and concentration, 4U bovine thrombin per mL of resin used for the purification was added along with 0.025 mg/mL Brain PI4P (Avanti Polar Lipids), followed by incubation at room temperature for 1 hour, before quenching of the protease activity with 1 mM PMSF.

Size exclusion chromatography: For Drs2p ΔN104/Cdc50p, size exclusion chromatography (SEC) was run on a Superdex 200 Increase 10/300 column on an ÄKTA purifier system at 4°C in SSR with 0% glycerol, 1 mM DTT and 0.03 mg/mL LMNG. The peak fractions typically resulted in a Drs2p/Cdc50p concentration of 0.6 mg/mL, which was used directly for preparation of cryo-EM grids, or stored at -80°C for later use. For Drs2p ΔNC/Cdc50p, a first round of SEC had been run on a TSKg4000SW silica column on an ÄKTA purifier system at 4°C in SSR with 0% glycerol, 1 mM DTT and 0.03 mg/mL LMNG. The peak fractions were pooled and concentrated using a 50 kDa cut-off centrifugal concentrator to 8 mg/mL, and was stored at -80°C for later use. A second round of SEC was run on an analytical Superdex 200 Increase 3.2/300 column on an ÄKTA purifier system at 4°C in SSR with 0% glycerol, 1 mM DTT and 0.03 mg/mL LMNG, where 50 µL sample was injected, to remove the background detergent produced by concentrating the sample. Pooling of the peak fractions resulted in a protein concentration of 0.6 mg/mL, which was used directly for preparation of cryo-EM grids. Representative chromatograms and gels are shown in Extended Data Figure 2a-c.

Activity measurement on purified protein for structural studies

The activity of the purified Drs2p-Cdc50p used for structural studies was assayed using an arsenic-based Baginski Assay⁴⁵, a colorimetric assay for free inorganic phosphate. Drs2p-Cdc50p in LMNG to a final concentration of 10 µg/mL was added to a reaction buffer of SSR with 0% glycerol, 1 mM DTT, 0.02 mg/mL LMNG and 5 mM NaN₃ (final concentrations). PS (C8:0), Brain PI4P and BeF₃⁻ (BeSO₄ and KF in a 1:20 molar ratio) were added, when present, to final concentrations of 78 µg/mL, 20 µg/mL and 1 mM, respectively. After addition of protein to the reaction buffers, the samples were incubated on ice for 1 hour, before transfer to 30°C, and upon reaching this temperature, the reactions were initiated by addition of ATP to a concentration of 4 mM. At specific time points, 50 µL sample was transferred to a 96-well microplate, and mixed with 50 µL 1:5 solution of 30 mM ammonium heptamolybdate in H₂O, and 0.17 M ascorbic acid and 0.1% SDS in 0.5 M HCl. After 10 minutes at room temperature, 75 µL of arsenic solution (2% (w/v) anhydrous sodium metaarsenic, 2% (w/v) trisodium citrate dihydrate, 2% (v/v) glacial acid) was added to prevent further complexing of molybdate by phosphate. The plate was left at room temperature for 30 minutes, before measurement of the absorbance at 860 nm on a Wallac Victor 3 Multilabel plate reader (Perkin Elmer).

Negative stain electron microscopy

Copper G400-C3 grids were coated with 2% cellulidine, followed by evaporation of amorphous carbon using a Leica EM SCD500 high vacuum sputter coater. Before use, the grids were glow-discharged on a PELCO easiGlow Glow Discharge Cleaning System at 25 mA for 45 seconds. 3 µL of protein sample diluted to 20 µg/mL in detergent-free buffer was applied, followed by staining three times with 3 µL 2% uranyl formate solution, which had been stored at -80°C. Micrographs were collected on a Tecnai G2 Spirit (120kV) with a Tietz F416 CCD camera using Leginon⁴⁶. Imaging was performed at 67,000×magnification with a binned camera (pixel size 3.15 Å). Data processing including CTF-estimation, particle picking, extraction using a of 84-pixel box-size, and 2D classification was performed in cisTEM⁴⁷ (Extended Data Figure 2d-f).

Cryo-electron microscopy

Sample freezing: C-flat Holey Carbon grids, CF-1.2/1.3-4C (Protochips), were glow discharged on a PELCO easiGlow Glow Discharge Cleaning System at 15 mA for 45 seconds before addition of 3µL of 0.6 mg/mL Drs2p/Cdc50p in LMNG, which had been incubated on ice for at least 1 hour

with 1 mM beryllium fluoride (BeF_3^-), and 0.1 mg/mL Brain PI4P when indicated. The samples were vitrified on a Vitrobot IV (ThermoFisher) at 4°C and 100% humidity.

Data collection: The data was acquired on a Titan Krios with an X-FEG operated at 300 kV. Movies were acquired through a Gatan K2 camera with a Bioquantum energy filter operated at a slit width of 20 eV. Movies were collected in counting mode with a calibrated pixel size of 1.077 Å/pixel at a magnification of 130,000x (MPI for Biophysics, Frankfurt). 8 second exposures fractionated into 40 frames were collected through EPU at a dose rate of 1.4 or 1.5 $\text{e}^-/\text{Å}^2/\text{frame}$, corresponding to a total dose of 56 or 60 $\text{e}^-/\text{Å}^2$.

For Drs2p $\Delta\text{N104/Cdc50p}$ 765 movies were collected on samples with 0.1 mg/mL PI4P and 3069 movies were collected without PI4P. For Drs2p $\Delta\text{NC/Cdc50p}$ 2391 movies were collected on a grid with 0.1 mg/mL PI4P and 801 movies were on a grid without additional PI4P. However, upon initial processing the datasets resulted in identical reconstructions with the same density in the PI4P-binding site (likely because of the PI4P added during the purification of this sample), and they were treated as one dataset going forward.

Processing: For all three datasets, movie alignment with dose weighting using all frames and contrast transfer function (CTF) determination was performed in cisTEM through Unblur⁴⁸ and CTFFind4⁴⁹, respectively. After manual inspection of the micrographs 2050 were selected for Drs2p $\Delta\text{NC/Cdc50p}$, 2687 for Drs2p $\Delta\text{N104/Cdc50p}$ and 701 for Drs2p $\Delta\text{N104/Cdc50p}$ with PI4P. Using the cisTEM reference-free particle picker, a total of 1,047,615 particles were picked for Drs2p $\Delta\text{NC/Cdc50p}$ with PI4P and 2,156,578 for Drs2p $\Delta\text{N104/Cdc50p}$ and 578,440 for Drs2p $\Delta\text{N104/Cdc50p}$ with PI4P. The particles were extracted in cisTEM using a box-size of 256 pixels, and cisTEM was used for 2D classification although this was not used for selecting good particles.

Drs2p $\Delta\text{N104/Cdc50p}$ ($E2^{\text{inhib}}$): Three *ab initio* 3D references were generated in cryoSPARC⁵⁰ from all particles, resulting in one class corresponding to the protein particle, and two corresponding to junk. Three rounds of heterogeneous 3D classification in cryoSPARC were performed, where the first round had one protein class and four junk classes, while the next rounds only used two junk classes and one protein class. This resulted in 769,469 particles which were subjected to heterogeneous 3D refinement, resulting in an initial reconstruction at 3.2 Å from 752,881 particles.

540 These particles were re-extracted in Relion 3⁵¹ from movies aligned through the Relion
541 implementation of MotionCor2⁵² for per-particle CTF refinement with estimation of the beam tilt
542 and Bayesian Polishing⁵³ performed in Relion 3, before the final 3D refinement resulting in an
543 unmasked resolution of 3.0 Å and a masked resolution of 2.8 Å after postprocessing. Relion 3 was
544 used for estimation of the local resolution. The processing strategy is summarized in Extended Data
545 Figure 6.

546
547 *Drs2p ΔN104/Cdc50p with PI4P (E2P^{inter})*: Three *ab initio* 3D references were generated in
548 cryoSPARC⁵⁰ resulting in two junk and one protein-like class. These were used as reference in
549 three rounds of heterogeneous 3D classification that resulted in 291,944 protein particles. An initial
550 homogeneous 3D refinement of these, resulted in a reconstruction at 3.8 Å of 277,569 particles.
551 These particles were re-extracted in Relion 3⁵¹ from movies aligned through MotionCor2⁵²
552 implemented in Relion for per-particle CTF refinement with estimation of the beam tilt and
553 Bayesian Polishing performed in Relion 3⁵³, before the final 3D refinement resulting in an
554 unmasked resolution of 3.4 Å. However, this appeared to be a mixed state near TM10 of Drs2p, the
555 PI4P-binding site and in parts of the autoinhibitory domain. As simple 3D classification using the
556 refined map as reference failed to separate this heterogeneity, five new *ab initio* references were
557 generated in cryoSPARC⁵⁰ with similarity 1.0, to allow for very similar classes. These were then all
558 used as references for heterogeneous 3D refinement, resulting in one class corresponding to a PI4P-
559 bound structure, two classes identical to the determined autoinhibited structure in the absence of
560 PI4P, and two minor junk-classes. The PI4P-bound resulted in a 3.7 Å reconstruction from 78,981
561 particles from cryoSPARC, which was also refined in Relion 3 to an unmasked resolution of 3.9 Å
562 and a masked resolution of 3.7 Å after postprocessing. Relion 3 was used for estimation of the local
563 resolution. The processing strategy is summarized in Extended Data Figure 7.

564
565 *Drs2p ΔNC/Cdc50p with PI4P (E2^{active})*: Three *ab initio* 3D references were generated in
566 cryoSPARC⁵⁰ from all particles resulting in one class corresponding to the protein particle, and two
567 corresponding to junk. All particles were then subjected to heterogeneous 3D classification in
568 cryoSPARC, using each junk reference twice and the good class once, resulting in four junk classes
569 and one with particles corresponding to the protein. This class was then subjected to heterogeneous
570 3D refinement in cryoSPARC resulting in an initial reconstruction at 3.4 Å from 493,753 particles.
571 The 3D refinement was repeated in Relion 2.1⁵⁴ using a soft solvent mask, resulting in a

reconstruction of similar quality. To improve the map, particle sorting was performed in Relion 2.1 and particles with a z-score above 0.9 or a defocus higher than 2.0 μm were rejected, and the remaining 418,512 were re-extracted in Relion 3⁵¹ from movies aligned through Relion's own implementation of MotionCor2. Per-particle CTF refinement with estimation of the beam tilt and Bayesian Polishing performed in Relion 3⁵³, before the final 3D refinement resulting in an unmasked resolution of 3.1 Å and a masked resolution of 2.9 Å after postprocessing. Relion 3 was used for estimation of the local resolution. The processing strategy is summarized in Extended Data Figure 8.

Data collection and processing statistics are summarized in Extended Data Table 1.

Model building and refinement: The Drs2p $\Delta\text{NC}/\text{Cdc50p}$ with PI4P was built manually in COOT⁵⁵ guided by secondary structure predictions from RaptorX⁵⁶, and the structures of the Na^+/K^+ ATPase and SERCA in the E2P state (PDB 4HYT and 3B9B) for Drs2p, which was permitted by the shared topology, and similarity of the E2P-conformations. For Cdc50p, the positions of all glycosylations were visible as at least one sugar moiety, and were used in validation of the *de novo* traced structure along with the presence of the two di-sulfide bonds. The model was refined using Namdinator⁵⁷ and *phenix.real_space_refine*⁵⁸.

Drs2p $\Delta\text{N104}/\text{Cdc50p}$ was built by fitting the Drs2p $\Delta\text{NC}/\text{Cdc50p}$ model using Namdinator⁵⁷ followed by manual editing in COOT and manual tracing of the autoregulatory C-terminus. Coordinate refinement was carried out using *phenix.real_space_refine*⁵⁸. The Drs2p $\Delta\text{N104}/\text{Cdc50p}$ with PI4P model was built based on the other two structures with manual editing in COOT⁵⁵ followed by *phenix.real_space_refine*⁵⁸.

For Drs2p the 78 N-terminal residues of the construct, as well as the 46 C-terminal residues and 16 residue linker (20 in the structure of E2P^{inter}) between TM10 and the autoregulatory domain in Drs2p ΔN104 were too disordered for modeling or entirely missing from the density. For Cdc50p the 19 N-terminal (18 in the structure of E2^{active}) and the entire C-terminal tail of 33 residues (35 in the structure of E2^{inhib}) could not be modelled.

Model validation was performed using MolProbity⁵⁹ in Phenix⁶⁰.

Modelling and refinement statistics are summarized in Extended Data Table 1. Model-to-map FSC curves and representative densities from different areas of the maps are shown in Extended Data Figure 9.

References

- 39 Yan, R. *et al.* Human SEIPIN Binds Anionic Phospholipids. *Dev Cell* **47**, 248-256 e244, (2018).
- 40 Ashkenazy, H. *et al.* ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. *Nucleic Acids Res* **44**, W344-350, (2016).
- 41 Sievers, F. *et al.* Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* **7**, 539, (2011).
- 42 Afonine, P. V. *et al.* New tools for the analysis and validation of cryo-EM maps and atomic models. *Acta Crystallogr D Struct Biol* **74**, 814-840, (2018).
- 43 Moller, J. V., Lind, K. E. & Andersen, J. P. Enzyme kinetics and substrate stabilization of detergent-solubilized and membraneous (Ca²⁺ + Mg²⁺)-activated ATPase from sarcoplasmic reticulum. Effect of protein-protein interactions. *J Biol Chem* **255**, 1912-1920, (1980).
- 44 Azouaoui, H. *et al.* Coordinated Overexpression in Yeast of a P4-ATPase and Its Associated Cdc50 Subunit: The Case of the Drs2p/Cdc50p Lipid Flippase Complex. *Methods Mol Biol* **1377**, 37-55, (2016).
- 45 Baginski, E. S., Foa, P. P. & Zak, B. Determination of phosphate: Study of labile organic phosphate interference. *Clinica Chimica Acta* **15**, 155-158, (1967).
- 46 Suloway, C. *et al.* Automated molecular microscopy: the new Legimon system. *J Struct Biol* **151**, 41-60, (2005).
- 47 Grant, T., Rohou, A. & Grigorieff, N. cisTEM, user-friendly software for single-particle image processing. *Elife* **7**, (2018).
- 48 Grant, T. & Grigorieff, N. Automatic estimation and correction of anisotropic magnification distortion in electron microscopes. *J Struct Biol* **192**, 204-208, (2015).
- 49 Rohou, A. & Grigorieff, N. CTFFIND4: Fast and accurate defocus estimation from electron micrographs. *J Struct Biol* **192**, 216-221, (2015).
- 50 Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat Methods* **14**, 290-296, (2017).
- 51 Zivanov, J. *et al.* New tools for automated high-resolution cryo-EM structure determination in RELION-3. *Elife* **7**, (2018).
- 52 Zheng, S. Q. *et al.* MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat Methods* **14**, 331-332, (2017).
- 53 Zivanov, J., Nakane, T. & Scheres, S. H. W. A Bayesian approach to beam-induced motion correction in cryo-EM single-particle analysis. *IUCrJ* **6**, 5-17, (2019).
- 54 Fernandez-Leiro, R. & Scheres, S. H. W. A pipeline approach to single-particle processing in RELION. *Acta Crystallogr D Struct Biol* **73**, 496-502, (2017).
- 55 Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **66**, 486-501, (2010).
- 56 Wang, S., Li, W., Liu, S. & Xu, J. RaptorX-Property: a web server for protein structure property prediction. *Nucleic Acids Res* **44**, W430-435, (2016).

- 647 57 Kidmose, R. T. *et al.* Namdinator - Automatic Molecular Dynamics flexible fitting of
648 structural models into cryo-EM and crystallography experimental maps. *bioRxiv*, 501197,
649 (2018).
- 650 58 Afonine, P. V. *et al.* Real-space refinement in PHENIX for cryo-EM and crystallography.
651 *Acta Crystallogr D Struct Biol* **74**, 531-544, (2018).
- 652 59 Chen, V. B. *et al.* MolProbity: all-atom structure validation for macromolecular
653 crystallography. *Acta Crystallogr D Biol Crystallogr* **66**, 12-21, (2010).
- 654 60 Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular
655 structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-221, (2010).
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Data availability statement

Cryo-EM maps for the *S. cerevisiae* Drs2p-Cdc50p in the E2–BeF3 (E2inhib, EMD-4972), E2–BeF3-PI4P (E2Pinter, EMD-4973), and C-terminally truncated E2–BeF3-PI4P (E2Pactive, EMD-4974) forms are available on the EMDB server, and coordinates of the atomic structures have been deposited in the Protein Data Bank (PDB) under accession numbers 6ROH, 6ROI, and 6ROH.

Extended Data Figure legends

Extended Data Table 1: Cryo-EM data collection, refinement and validation statistics

* number in parenthesis corresponds to the final range/number.

The B-factor listed is the one used for refinement, while the one in the parentheses is the B-factor estimated by Relion PostProcessing.

Extended Data Figure 1: Activity of samples used for structural studies

a, Post-Albers cycle for the Na,K-ATPase. Sodium is transported during the E1 half cycle while potassium is transported during the E2 half cycle.

b, Post-Albers cycle for Drs2p-Cdc50p indicating the off-cycle regulation (grey). Lipid is transported during the E2 half cycle. Structures determined here are marked with red circles

c, Topology of Drs2p-Cdc50p with indication of the cleavages at the termini of Drs2p of the constructs used for structural studies (Δ N104: all constructs, Δ C1247: E2P^{active}). Cdc50p is pink, while for Drs2p the TM-domain is tan, the A-domain is yellow, the P-domain is blue, the N-domain is red and the autoinhibitory C-terminus is green.

d, Specific activity of Drs2p Δ NC/Cdc50p and Drs2p Δ N104/Cdc50p in LMNG measured by the Baginski Assay. Where present, PS C(8:0), Brain PI4P and BeF₃⁻ were added to final concentrations of 78 μ g/mL, 20 μ g/mL and 5mM, respectively.

e, Alignment of Drs2p-Cdc50p E2P^{active} and SERCA (PDB 3B9B). Drs2p-Cdc50p is green, SERCA is orange. (Superposition of C-alpha carbons excluding the N-domain)

f, The phosphorylation site of E2P^{active} with density for the BeF₃⁻ inhibitor, Mg²⁺ ion and coordinating residues. The characteristic E2P conformation of the dephosphorylation loop with the glutamate pointing away from the phosphorylation site is shown in stick representation.

g, The three Drs2p-Cdc50p structures aligned based on the P-domain, with Asp560-BeF₃⁻ and E342 shown to illustrate the similar conformations. Colors as in Figure 1a.

Extended Data Figure 2: Purification and negative stain electron microscopy of samples for structural studies

a, SEC chromatogram and gel of Drs2p Δ N104/Cdc50p. Red line on SDS-PAGE indicates the pooled fractions.

b, SEC of Drs2p Δ NC/Cdc50p with gels showing fractions. Red line on SDS-PAGE indicates the pooled fractions.

c, Analytical SEC of Δ NC purified from **b**, used for freezing grids.

d, A representative negative stained micrograph of autoinhibited Drs2p Δ N104/Cdc50p in LMNG.

e, Representative 2D class averages of the sample in **d** shows well defined and homogeneous particles with recognizable P-type ATPase features.

f, Enlarged 2D class average highlighting the recognizable domains of Drs2p-Cdc50p.

Extended Data Figure 3: Fold of Cdc50p

a, Cdc50p from E2P^{inhib} colored by rainbow. C α -carbons of asparagines carrying glycosylations are shown as black spheres. Drs2p is shown in grey.

b, Position of the disulfide sites in Cdc50p from E2P^{inhib}.

c-e, Glycosylation sites in Cdc50p from E2P^{inhib}. Map levels are 1.75 rmsd in panels c and d. Density for the glycosylation at Asn237 is only apparent at lower map levels (0.75 rmsd) and remained unmodeled.

f, Alignment of Cdc50p (purple cartoon) and a monomer of the lipid binding human seipin³⁹ (blue cartoon, PDB 6DS5), illustrating the similar folds, although loops of Cdc50p are more extensive. The sequence identity between the two proteins is only 4%. Transmembrane helices of seipin extending from similar positions as the helices of Cdc50p are not present in the structure, but may extend into the membrane in a similar way as observed for Cdc50p.

g, The TM-helices of the Drs2p-Cdc50p complex viewed from the luminal side. The TM helices of Drs2p are colored in rainbow, Cdc50p TM helices are pink.

h, Interaction between the Cdc50p ecto-domain (shown as surface) and the luminal TM3-4 loop of Drs2p (light green).

i, Interaction between the N-terminus of Cdc50p and the segment of Drs2p leading from TM4 to the phosphorylation site. Residues 529-538 are not present in P2 ATPases and are shown in dark blue.

j, Segments of Drs2p found to interact with Cdc50p mutants that disrupt complex formation are highlighted in green and the insert in Drs2p between TM4 and the phosphorylation site is blue. The structure shown in **c-e** is E2P^{inhib}, and Cdc50p is colored by conservation using ConSurf⁴⁰.

k, Part of a sequence alignment of Cdc50-proteins from *S. cerevisiae* and human CDC50A and CDC50B with residues important for complex-formation identified. Uniprot identifiers: Cdc50 – P25656, Lem3 – P42838, Crf1 – P53740, CDC50A – Q9NV96, CDC50B – Q3MIR4. The

alignment was performed using Clustal Omega⁴¹. For full sequence alignment see Supplementary Figure 1.

Extended Data Figure 4: Characterization of PI4P-binding mutants

a, Coomassie-blue stained SDS-PAGE of streptavidin-purified wild-type and mutant Drs2p-Cdc50p. Tobacco Etch Virus (TEV) protease is used to release the complex from streptavidin beads.

b, ATPase activity of PI4P-binding mutants, using an enzyme-coupled assay. The assay medium contained 1 mM ATP, 0.1 mg.mL⁻¹ POPS and 1 mg.mL⁻¹ DDM in SSR buffer. The rate of ATP hydrolysis was continuously recorded at 340 nm upon subsequent addition of 2 µg.mL⁻¹ of the purified complex, 5 µg.mL⁻¹ trypsin, and 25 µg.mL⁻¹ PI4P.

c, Size-exclusion chromatography on a Superdex 200 10/300GL column. The arrow indicates the dead volume (V₀).

d, Limited proteolysis of streptavidin-purified wild-type and mutant Drs2p-Cdc50p by thrombin. In the absence of PI4P WT and mutant forms of Drs2p are truncated to sizes corresponding to the loss of the first 104 residues from the N-terminus (ΔN, upper band in lanes 2, 4, 6 and 8) and to the loss of both the first 104 N-terminal and the last C-terminal 65 residues (ΔNC, upper band in lanes 2, 4, 6 and 8). Incubation with PI4P during limited proteolysis further promotes truncation in the C-terminus only for the WT Drs2p indicating an increased insensitivity of the Drs2p mutants for PI4P.

e, Control proteolysis experiment showing that PS does not alter cleavage of WT Drs2p-Cdc50p with thrombin, thereby demonstrating that C-terminal mutants Y1235A, Y1235F, and H1236A are specifically defective in PI4P binding.

Extended Data Figure 5: Proposed lipid translocation pathway and comparison between the three Drs2p-Cdc50p structures.

a, A proposed lipid translocation pathway is exposed upon activation by PI4P binding and C-terminal truncation. Electrostatic surfaces as calculated in APBS. Complement to Figure 4a.

b, Potassium binding in Na,K-ATPase³⁰ (PDB 3KDP), showing the coordination of ions in site I and site II by negatively charged and polar residues. TM4 is colored yellow with the PEGE-motif in orange, and the bound potassium as purple spheres.

c, Sites and residues of E2P^{active} corresponding to the ion binding sites of Na,K-ATPase in **b**. TM4 is yellow with the PISL-motif in orange, and stabilizing hydrogen bonds are shown.

d, Superposition of the three Drs2p-Cdc50p structures based on Cdc50p and TM7-10 of Drs2p. E2P^{inhib} is yellow, E2P^{inter} is blue, and E2P^{active} is green. The major conformational changes are in the cytosolic domains and TMs 1 and 2

e, Expanded view of **a** highlighting the change in the respective P-domains with the TM6-7 loop and the amphipathic helix (H1^{C-tail}) depicted in slightly darker colors and the autoinhibitory domain of E2P^{inhib} in dark green. Destabilisation or removal of the helical segment of the autoinhibitory domain (E2P^{inter} and E2P^{active}) leads to a rigid body movement of the P-domain.

Extended Data Figure 6: Processing pipeline for cryo-EM data of Drs2p-Cdc50p E2P^{inhib}

a, Representative motion-corrected and dose-weighted micrograph (defocus 1.6 μm) of autoinhibited Drs2p ΔN104 -Cdc50p in LMNG, frozen at a concentration of 0.6 mg/ml.

b, Fourier power spectrum of the micrograph shown in (A), with fit from CTFFIND 4.1 through cisTEM, which extends to 3 $\text{\AA}$.

c, Color code of processing software.

d, Data processing workflow with indication of the number of particles remaining after each step at which particles were discarded. The densities resulting from 3D refinement are shown in grey, while relevant masks are shown in light blue. The resolutions listed for 3D refinements are at FSC=0.143.

Extended Data Figure 7: Processing pipeline for cryo-EM data of Drs2p-Cdc50p E2P^{inter}

a, Representative motion-corrected and dose-weighted micrograph (defocus 1.5 μm) of Drs2p ΔN104 -Cdc50p with an intact C-terminus in LMNG, frozen at a concentration of 0.6 mg/ml in the presence of 75 $\mu\text{g/ml}$ Brain PI4P.

b, Fourier power spectrum of micrograph in (A), with fit from CTFFIND 4.1 through cisTEM, which extends to 5 $\text{\AA}$.

c, Color code of processing software.

d, Data processing workflow with indication of the number of particles remaining after each step at which particles were discarded. Densities resulting from 3D refinement are shown in grey, while relevant masks are shown in light blue. The resolutions listed for 3D refinements are at FSC=0.143. The 3.4 $\text{\AA}$ -refinement indicated a mixed state around TM10. To classify structural heterogeneity due to incomplete binding of PI4P, new ab initio references were generated in cryoSPARC allowing for high similarity, as the conformations were expected to be similar. Two different conformations

792 resulted: the autoinhibited one and a PI4P-bound. The autoinhibited was identical to E2P^{inhib} and
793 adding these particles to that dataset did not improve the reconstruction. The PI4P-bound
794 conformation was further refined in Relion.

795
796 **Extended Data Figure 8: Processing pipeline for cryo-EM data of Drs2p-Cdc50p E2P^{active}**

797 **a**, Representative motion corrected and dose weighted micrograph (defocus of 1.7 μ m) of C-
798 terminally truncated Drs2p Δ NC-Cdc50p in LMNG, frozen at a concentration of 0.6 mg/mL in the
799 presence of 75 μ g/mL Brain PI4P.

800 **b**, Fourier power spectrum of the micrograph shown in (A), as well as the fit from CTFFIND 4.1
801 through cisTEM, which extends to 3 $\text{\AA}$.

802 **c**, Color code of processing software.

803 **d**, Data processing workflow with indication of the number of particles remaining after each step at
804 which particles were discarded. Densities resulting from 3D refinement are shown in grey, while
805 relevant masks are shown in light blue. The resolutions listed for 3D refinements are at FSC=0.143.

806
807 **Extended Data Figure 9: Model validation and representative densities**

808 **a**, Cross-validation FSC curves for map-to-model fit produced by Mtriage⁴².

809 Curves representing model vs. full map are calculated based on the final model and the full, filtered
810 and sharpened map that it was refined against.

811 For the model vs. half-maps, the model (before the final refinement against) was refined against
812 half-map 1 filtered and sharpened as the full map, and FSC-curves were calculated using this
813 refined model against each half-map.

814 **b**, Representative densities from different areas of the three LocScale-maps. Each segment is
815 labelled with which residues are shown, and demonstrates the quality of the map in specific areas.
816 All are shown at a 1.5 r.m.s.d threshold.

a **b**

Extended data figure 1

Extended data figure 2

Extended data figure 3

Extended data figure 4

Extended data figure 5

Extended data figure 6

Extended data figure 7

d

Motion correction and CTF estimation of 765 movies

Manual selection of 701 good movies

Ab initio 3D references → 1 good classes, 2 junk

Reference-free autopicking of 578,440 particles

3 rounds of 3D classification with 1 good class and 2 junk classes (291,944 particles)

Homogeneous refinement of the good particles (277,569 particles)

Re-extraction in Relion 3.0 with motion correction through Relion's own MotionCorr2 CTF refinement and Bayesian Polishsing

3.8 Å

Ab initio 3D references → 4 good classes, 1 junk

3D refinement with solvent mask

3.4 Å

Heterogeneous refinement (Intermediate PI4P-bound: class 1, 94,070 particles – Autoinhibited: class 0+3, 154,903 particles)

3D refinement with solvent mask

3.9 Å

Local resolution estimation

Postprocessing using a tight mask: Final resolution: 3.7 Å

Extended data figure 8

d

Motion correction and CTF estimation of 3192 movies

Manual selection of 2687 good movies

Ab initio 3D references → 1 good classes, 2 junk

Reference-free autopicking of 1,047,615 particles

3D classification with 1 good class and 4 junk classes (558,130 particles)

Homogeneous refinement of the good class (493,753 particles)

3D refinement of particles from cryoSPARC using a solvent mask

3.4 Å

Particle sorting discarding particles with z-score above 0.9 or a defocus higher than 2.0 μm (418,512 particles from 2443 micrographs)

3D refinement with solvent mask

3.5 Å

Re-extraction in Relion 3.0 with motion correction through Relion's own MotionCorr2

3D refinement with solvent mask

3.1 Å

CTF refinement and Bayesian Polishing

Local resolution estimation

Postprocessing using a tight mask: Final resolution: 2.9 Å

Extended data figure 9

	Drs2p Δ N104/Cdc50p (autoinhibited) (EMD-4972) (PDB 6ROH)	Drs2p Δ N104/Cdc50p with PI4P (intermediate) (EMD-4973) (PDB 6ROI)	Drs2p Δ NC/Cdc50p with PI4P (activated) (EMDB-4974) (PDB 6ROJ)
Data collection and processing			
Magnification	130,000	130,000	130,000
Voltage (kV)	300	300	300
Microscope	Titan Krios	Titan Krios	Titan Krios
Electron exposure (e ⁻ /Å ²)	60	60	56
Defocus range (μm)	0.3-3.0 (0.4-2.5)*	0.4-2.4 (0.5-2.4)*	0.3-3.0 (0.4-2.0)*
Pixel size (Å)	1.077	1.077	1.077
Camera	Gatan K2	Gatan K2	Gatan K2
Number of movies	3069 (2050)*	765 (701)*	3192 (2443)*
Symmetry imposed	C1	C1	C1
Initial particle images (no.)	2,157,578	578,440	1,047,615
Final particle images (no.)	752,881	78,981	418,512
Map resolution (Å)	2.8	3.7	2.9
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	2.7-3.4	3.6-4.4	2.9-4.1
Refinement			
Model resolution (Å)	2.9	3.8	3.1
FSC threshold	0.5	0.5	0.5
Model resolution range (Å)			
Map sharpening <i>B</i> factor (Å ²) [#]	-40 (-76)	-60 (-115)	-35 (-69)
Model composition			
Non-hydrogen atoms	11758	11775	11465
Protein residues	1449	1447	1404
Ligands	2 H ₂ O, 1 Mg ²⁺ , 1 PS, 5 NAG, 2 BMA	2 H ₂ O, 1 Mg ²⁺ , 1 PI4P, 5 NAG, 2 BMA	2 H ₂ O, 1 Mg ²⁺ , 1 PI4P, 6 NAG, 4 BMA
<i>B</i> factors (Å ²)			
Protein	33.75/150.55/73.76	35.45/170.28/89.45	34.18/187.73/77.30
Ligand	55.17/92.52/75.64	68.87/120.20/89.63	51.25/111.21/76.81
Water	55.49/55.73/55.61	77.61/80.06/78.84	60.71/61.39/61.05
R.m.s. deviations			
Bond lengths (Å)	0.006	0.008	0.006
Bond angles (°)	0.867	0.992	0.902
Validation			
MolProbity score	1.40	1.61	1.36
Clashscore	4.00	5.05	3.58
Poor rotamers (%)	0.23	0.23	0.16
EMRinger	3.80	2.13	3.50
Ramachandran plot			
Favored (%)	96.60	94.99	96.64
Allowed (%)	3.40	5.01	3.36
Disallowed (%)	0.0	0.0	0.0

Supplementary Discussion

Comparison to other P-type ATPases

Drs2p-Cdc50p can be compared to Na,K-ATPase, which is also a P-type ATPase forming an $\alpha\beta$ binary complex. However, the fold of Cdc50p and its discrete interactions in the membrane and with the luminal face of Drs2p are fundamentally different from the β -subunit and regulatory FXYD subunit of Na,K-ATPase. The overall conformation of Drs2p is close to the ouabain-inhibited E2P-form of Na,K-ATPase⁶¹ (PDB 4HYT) and the outward-open E2-BeF₃⁻ form of SERCA⁶² (PDB 3B9B). E2P^{active} resembles 3B9B most closely, with an overall root mean square deviation (rmsd) of 4.4 Å (superposition of C α -atoms, excluding the N-domain, which is flexible) compared to the E2P^{inter} and E2P^{inhib} conformations with a rmsd of 4.8 Å and 5.2 Å, respectively (Extended Data Figure 1e). Other P-type ATPase conformations differ by a further margin of at least 0.8 Å in rmsd. The bulk of the A-domain is shifted by about 7 Å, but the (T/D)GE(S/T) loops of SERCA and Drs2p overlap, and density for the phosphate analogue BeF₃⁻ and bound Mg²⁺ is readily observable (Extended Data Figure 1f). The conformation of the dephosphorylation loops in the Drs2p structures are similar (Extended Data Figure 1g). Compared to the ion-transporting P-type ATPases, TM1 and 2 appear to be one turn shorter at the luminal side, suggesting they sit deeper in the membrane.

Interactions between Drs2p and Cdc50p

The three structures show an tight complex between Drs2p and Cdc50p with interactions on the cytosolic and luminal side and in the membrane. Contacts in the transmembrane regions of the two proteins appear to be fewer, as only TM10 of Drs2p interacts with the helices of Cdc50p (Extended Data Figure 3g). Interestingly, sequence alignments indicate that the TM4 segment as well as the TM3-4 loop are distinctly shorter in Neo1p and homologous lipid flippases that do not bind Cdc50p-subunits (Supplementary Data)⁶³. Furthermore, chimeras between Drs2p and Dnf1p of this loop result in an intact but non-functional flippase⁶⁴. These interactions suggest that Cdc50p is sensitive to the phosphorylation state of the P-domain on the cytosolic side, while on the luminal side it will sense the conformation of the transmembrane domain through the contact with the TM3-4 loop and other luminal loops, consistent with reported fluctuations in Cdc50p affinity for Drs2p during the transport cycle⁶⁵. However, structural analysis of other states are required to expand on these observations.

Mutations disrupting the interaction between Drs2p and Cdc50p have been identified in both the N-terminus of Cdc50p and in the interface with the TM3-4 loop of Drs2p (Extended Data Figure 3h-k).

In the *S. cerevisiae* phosphatidylcholine (PC) transporting Dnf1p-Lem3p, a Ser237Leu mutation in Lem3p (Ser196 in Cdc50p, located at the interface with the TM3-4 loop of Drs2p) disrupted the interaction between the two subunits, and Ala65Val (corresponding to Ala35 in Cdc50p near the TM8-9 loop of Drs2p), resulted in a mild reduction of interaction between the two subunits²⁷. Furthermore, combining Pro23His and Thr320Ala mutations in Cdc50p that are located at the Cdc50p N-terminus approaching the cytoplasmic end of Drs2p-TM4 and within the ecto-domain of Cdc50p (Figure 1d and e) rendered growth temperature-sensitive in a $\Delta cdc50\Delta lem3\Delta crf1$ background⁶⁶.

Mutations in the TM10 amphipathic helix disrupt PI4P activation

We further investigated the ability of Tyr1235Ala, Tyr1235Phe, and His1236Ala mutants to hydrolyse ATP. All three mutants were purified with a yield and stability similar to that of the wild-type complex (Extended Data Figure 4a and c). The PI4P induced ATPase activity of the Tyr1235Ala, Tyr1235Phe, and His1236Ala mutants however was strongly reduced, less than 10% of wild-type, i.e. consistent with a role in binding PI4P (Figure 3c and Extended Data Figure 4b). Owing to the requirement of both PI4P and PS (in addition to the removal of the C-terminal extension of Drs2p) for full activation of ATP hydrolysis by Drs2p-Cdc50p, we cannot exclude an effect on PS substrate lipid binding and/or transport. Binding of PI4P to Drs2p, however, specifically stimulates cleavage after Arg1290 in the regulatory C-terminal domain by limited proteolysis²⁶. Therefore, we subjected the wild-type Drs2p-Cdc50p and C-terminal mutants to limited proteolysis with thrombin. As expected, the wild-type lipid flippase displayed a typical proteolysis pattern, with PI4P-induced thrombin cleavage in the C-terminus of Drs2p (Extended Data Figure 4d) while PS had no effect on cleavage (Extended Data Figure 4e). Remarkably, this PI4P-induced cleavage was essentially abolished for all mutants, indicating that Tyr1235Ala, Tyr1235Phe, and His1236Ala mutations in the C-terminus of Drs2p specifically interfere with PI4P binding and cause a dramatic loss in PI4P-dependent ATPase activity.

a

b

Supplementary Figure 1: Sequence alignments for the P4-ATPase and Cdc50p subunits

a, Sequence alignment of the five *S. cerevisiae* P4-ATPases, the human ATP8A2, and *A. thaliana* ALA10, aligned by Clustal Omega^{41,67}, along with a structure-based sequence alignment between Drs2p from E2P^{active} and SERCA-BeF₃⁻ (PDB 3B9B) performed by the Dali Server's pairwise alignment⁶⁸ separately for the N-domain of Drs2p and for the rest of the protein, as the N-domain is very flexible and can distort the alignment. The structural alignment was adjusted to the full-length Drs2p sequence. For the rabbit SERCA it only includes the sequence of the structure that is shown, meaning that the C-terminal 7 residues are missing. The shading indicates conservation (blue 0% – red 100%). Above the sequences the domains and transmembrane helices of Drs2p are indicated in the same color scheme as Figure 1a. Uniprot identifiers: SERCA – P04191, Drs2p – P39524, Dnf1p – P32660, Dnf2p – Q12675, Dnf3p – Q12674, Neo1p – P40527, ATP8A2 – Q9NTI2, ALA10 – Q9LI83. **b**, Sequence alignment of Cdc50-proteins from *S. cerevisiae* and human CDC50A and CDC50B with residues important for complex-formation identified. Uniprot identifiers: Cdc50 – P25656, Lem3 – P42838, Crf1 – P53740, CDC50A – Q9NV96, CDC50B – Q3MIR4. The alignment was performed using Clustal Omega. The shading indicates conservation (blue 0% – red 100%).

Supplementary References

- 61 Laursen, M., Yatime, L., Nissen, P. & Fedosova, N. U. Crystal structure of the high-affinity Na⁺K⁺-ATPase-ouabain complex with Mg²⁺ bound in the cation binding site. *Proc Natl Acad Sci U S A* **110**, 10958-10963, (2013).
- 62 Olesen, C. *et al.* The structural basis of calcium transport by the calcium pump. *Nature* **450**, 1036-1042, (2007).
- 63 Palmgren, M., Osterberg, J. T., Nintemann, S. J., Poulsen, L. R. & Lopez-Marques, R. L. Evolution and a revised nomenclature of P4 ATPases, a eukaryotic family of lipid flippases. *Biochim Biophys Acta Biomembr*, (2019).
- 64 Baldridge, R. D. & Graham, T. R. Identification of residues defining phospholipid flippase substrate specificity of type IV P-type ATPases. *Proc Natl Acad Sci U S A* **109**, E290-298, (2012).
- 65 Lenoir, G., Williamson, P., Puts, C. F. & Holthuis, J. C. Cdc50p plays a vital role in the ATPase reaction cycle of the putative aminophospholipid transporter Drs2p. *J Biol Chem* **284**, 17956-17967, (2009).
- 66 Takahashi, Y., Fujimura-Kamada, K., Kondo, S. & Tanaka, K. Isolation and characterization of novel mutations in CDC50, the non-catalytic subunit of the Drs2p phospholipid flippase. *J Biochem* **149**, 423-432, (2011).
- 67 Apweiler, R. *et al.* UniProt: the Universal Protein knowledgebase. *Nucleic Acids Res* **32**, D115-119, (2004).
- 68 Holm, L. & Laakso, L. M. Dali server update. *Nucleic Acids Res* **44**, W351-355, (2016).