Conserved lipid transfer proteins of the Ups/PRELI family regulate lipid accumulation in mitochondria by shuttling phospholipids in a lipid-specific manner across the intermembrane space. Here, we combine structural analysis, unbiased genetic approaches in yeast and molecular dynamics simulations to unravel determinants of lipid specificity within the conserved Ups/PRELI family. We present structures of human PRELID1–TRIAP1 and PRELID3b–TRIAP1 complexes, which exert lipid transfer activity for phosphatidic acid and phosphatidylserine, respectively. Reverse yeast genetic screens identify critical amino acid exchanges that broaden and swap their lipid specificities. We find that amino acids involved in head group recognition and the hydrophobicity of flexible loops regulate lipid entry into the binding cavity. Molecular dynamics simulations reveal different membrane orientations of PRELID1 and PRELID3b during the stepwise release of lipids. Our experiments thus define the structural determinants of lipid specificity and the dynamics of lipid interactions by Ups/PRELI proteins.
Mitochondria are dynamic organelles involved in coordinating a plethora of cellular processes in both health and disease. Proper mitochondrial function requires a highly coordinated system of proteins and phospholipid supply. Phospholipids and their precursors must be delivered to mitochondria, shuttled between membrane leaflets and transported across the mitochondrial intermembrane space (IMS)\(^1\). In particular, the accumulation of the mitochondria-specific phospholipid cardiolipin (CL), as well as phosphatidylethanolamine (PE), which are synthesised within mitochondria, is necessary for normal cell function. Mitochondrial CL and PE deficiency results in abnormal mitochondrial morphology and is associated with embryonic lethality in mice\(^2\). The cellular consequences are typically altered respiratory pathways and protein import as well as oxidative stress leading to apoptotic cell death\(^3\)-\(^10\).

The first molecular details of phospholipid transport to and within mitochondria were recently uncovered with the identification of the Ups–Mdm35 family in yeast and the homologous PREL1–TRIAP1 system in humans\(^11\)-\(^14\). TRIAP1 (yeast Mdm35) and PRELI (yeast Ups) proteins reside within the IMS where they regulate phospholipid metabolism. Homologues of the Ups/PRELI protein family (PREL1D1, PRELID3a, PRELID1 in humans and Ups1, Ups2 and Ups3 in yeast) form complexes with TRIAP1/Mdm35 and together facilitate intra-mitochondrial transport in a lipid-specific manner. Specific phospholipids are extracted from the mitochondrial outer membrane (OM), carried across the IMS and inserted into the mitochondrial inner membrane (IM), towards the synthesis machineries of CL and PE\(^2\). It has been established that the Ups1–Mdm35 complex directly transfers phosphatidic acid (PA) between mitochondrial membranes allowing CL synthesis in the IM\(^13\). Similarly, the human homologue PREL1D1–TRIAP1 mediate PA transfer within the cardiolipin synthetic pathway\(^14\). More recently, Ups2–Mdm35 was shown to mediate the transfer of phosphatidylserine (PS) from donor to acceptor liposomes, in a similar manner to the Ups1–Mdm35 complexes (Supplementary Figure 1). Although these studies illuminated key features important for phospholipid transport to and within mitochondria, until recently, the Ups1–Mdm35 family in yeast and the homologous PRELI–TRIAP1 system in humans were the only ones to exchange phospholipids between mitochondria and the cytosol. The biological function of such a reverse transfer is not known and remains a matter of speculation. In order to unravel the structural determinants of the lipid specificity within Ups/PRELI-like proteins, we turned our attention to yeast orthologues of this family. Ups/PRELI proteins of yeast and human share the same structural fold and can functionally substitute for each other\(^6\),\(^15\).

We therefore performed an unbiased genetic screen in yeast to identify amino acids critical for the substrate specificity of Ups/PRELI proteins. The loss of the PRELID3b-homologue Ups2 is lethal in yeast cells lacking Phb1 or Phb2\(^21\), subunits of prohibitin membrane scaffolds in the IMS\(^25\). Cell growth can be restored by expression of Ups2, but only to a limited extent by the expression of the PREL1D1 homologue Ups1\(^2\). These differences are likely to be important for the lipid specificity of these lipid transfer proteins.
either flank the Ω loop, are located at the beginning of the C-terminal helix (α3) or in the β-sheet of the PRELI domain (Fig. 2b, Supplementary Figure 2A, Supplementary Table 1).

It is conceivable that a combination of mutations in Ups1 is required to alter its lipid specificity and to allow cell growth when expressed in ups2Δphb1Δ cells. Alternatively, Ups1 variants harbouring only individual mutations may be able to support growth of ups2Δphb1Δ cells. To distinguish between these possibilities, we mutated the most frequently amino acid residues of Ups1 by site-directed mutagenesis and expressed the obtained Ups1 variants in ups2Δphb1Δ cells (Fig. 2c). The growth of ups2Δphb1Δ cells was significantly improved upon expression of the mutant forms of Ups1 when compared to cells expressing wildtype Ups1, demonstrating that the replacement of single amino acids in Ups1 is sufficient to promote cell growth (Fig. 2c).

The ability of the Ups1 variants to substitute for the loss of Ups2 suggests that the mutations enabled Ups1 to transport PS, pointing to a critical role of specific residues flanking flexible loops and in the β-sheet of the PRELI domain of Ups1 for its lipid specificity.

To examine whether the mutation of these residues affects the ability of Ups1 for PA transfer, we expressed Ups1 variants in ups1Δ cells and assessed cell growth (Supplementary Figure 2B). Similar to Ups1, the Ups1 variants restored normal growth of ups1Δ cells, indicating that these mutants retain PA transfer activity. We therefore conclude that the amino acids K58, T76, T95, E108, F133 and M135 have a pivotal role in substrate selection. Mutations in these residues appear to allow PS transport by Ups1 without inhibiting its ability to transport PA.

**Lipid transfer activities of Ups1 variants.** To analyse directly phospholipid transport by the Ups1 variants, we expressed them together with Mdm35 in a cell-free protein expression system in vitro and purified soluble heterodimeric complexes to near homogeneity (Supplementary Figure 3A). Lipid transport was quantitatively assessed in vitro monitoring the dequenching of NBD-labelled lipids upon transfer to acceptor liposomes lacking fluorescent lipids. Consistent with previous reports, Ups1–Mdm35 complexes transferred PS only inefficiently when compared to PA (~7.6% of PA transfer activity; Fig. 3a, b, Supplementary Figure 3D). The mutations K58V or E108D in Ups1 increased PS transport by ~200% (Fig. 3a, c). Although to a lesser extent, other Ups1 variants also showed increased PS transfer activity when compared to wildtype Ups1–Mdm35 complexes (Fig. 3a, c, Supplementary Figure 3D). The mutations K58V or E108D in Ups1 restored wildtype PS transfer activity, thus demonstrating that the replacement of single amino acids K58 and E108 of Ups1 and, to some extent, mutations of T76, T95 and M135 of Ups1 enable PS transfer, the mutations decreased the ability of Ups1 to transfer PA, with K58V and E108D showing the most pronounced effects (Fig. 3b, c, Supplementary Figure 3D). Thus, the relative PS/PA transfer activity of the Ups1 variants identified in the reverse genetic screen is significantly increased (Fig. 3d).

Although frequently identified in our reverse genetic screen, mutations in the amino acids T76, T95 and M135 had only a moderate effect on the PS transfer activity of Ups1. To gain further insight into the roles of these residues, we introduced the corresponding mutations and E108D in the Ups1 variant harbouring K58V. The mutations substantially increased PS transfer by Ups1–K58V, while the ability for PA transport remained largely unaffected, indicating that these residues limit PS transfer by Ups1–K58V (Fig. 3c, d, Supplementary Figure 3B, 3C, 3D). We also monitored PA/PS transfer by Ups1, Ups1–K58V and Ups1–K58VE108D using donor liposomes containing both non-fluorescent PS and PA (Supplementary Figure 3E, 3F). Whereas Ups1 transfers PA in a highly specific manner, Ups1–K58V and Ups1–K58VE108D transfer PS and PA, confirming their increased ability to transfer PS.

Together, these results establish the identified amino acid residues, in particular K58, as critical determinants for the lipid specificity of Ups1. Notably, the elevated PS transfer activity of various Ups1 variants enables them to substitute for Ups2 in vivo. Expression of the Ups1 variants K58V, E108D or K58VE108D in phb1Δups2Δ cells significantly increased mitochondrial PE levels (Supplementary Figure 2C, D).

K58 is a conserved determinant of PS specificity. Sequence alignments of the Ups/PRELI-family of lipid transfer proteins revealed that K58 is highly conserved among members of the...
Ups1/PRELID1 subfamily (see Supplementary Figure 10C). E108 shows a similar degree of conservation, whereas other amino acids identified in our genetic screen are less well conserved. To examine the role of K58 in lipid specificity of the human PRELID1, we replaced this amino acid by valine or threonine and monitored the lipid transfer activity of purified mutant PRELID1–TRIAP1 complexes in vitro (Fig. 4a, b, Supplementary Figure 4A, B). While efficiently mediating the transfer of PA, PRELID1 showed a low PS transfer activity, similar to Ups114. In contrast, substitution of K58 by valine or threonine substantially increased the ability of PRELID1 to transfer PS, but only moderately affected its PA transfer activity (Fig. 4a, b, Supplementary Figure 4B). We therefore conclude that the role of K58 as a critical determinant of PS transfer activity is conserved from yeast to humans.

To substantiate these findings, we attempted to obtain ligand-bound crystal structures of human PRELI-like proteins. Although structures of phospholipid-free form of yeast Ups1 and human PRELID3a19–21 have been determined, our initial electron density maps of the PRELID1 and PRELID3b structures showed that...
additional density existed within the cavity (Supplementary Figure 4C). Mass spectrometry (MS) identified that a fatty acid, likely an endogenous *E. coli* lipid molecule, was bound to PRELID1 and the abundant *E. coli* phospholipid phosphatidylglycerol (PG) was present in PRELID3b (Supplementary Figure 4D). We reasoned that the mutation of the critical K58 residue in PRELID1, which promoted PS transfer, would allow phospholipid exchange and subsequent crystallisation of a PS-bound ligand. We therefore incubated PRELID1–K58V with 1:2 DOPS-DDM micelles as described by Watanabe et al. for Ups1 and PA, and following nickel purification, and SEC were able to confirm by MS that DOPS was able to displace the *E. coli* derived fatty acid, albeit incompletely (Supplementary Figure 4D). PRELID1–K58V incorporating DOPS formed readily crystals and diffracted to 2.98 Å. Although occupancy of the bound PS molecule was not expected at 100% due to partial exchange in our sample (Supplementary Figure 4D), a difference map corresponding to a larger ligand molecule appeared during initial refinement stages. A molecule of PS was modelled into the omit map and this was further refined with ligand occupancy set to 0.3 in accordance with lipidomics analysis for the PS molecule (Fig. 4c). The PS molecule docked in the observed electron density was found enclosed within the cavity of PRELID1–K58V with the serine group positioned at the bottom of the pocket and the acyl chain pointing towards the entrance of the pocket (Fig. 4c, d). The overall structure of the PRELI domain is largely unchanged in PRELID1–K58V mutant, showing a rmsd of 0.9 Å over the equivalent backbone atoms. The R25 side-chain, which is located near the PA phosphate of the Ups1-PA structure, is shifted towards the serine moiety in PRELID1–K58V (Fig. 4d). The smaller V58 side-chain of the mutant now allows the phosphodiester linkage to pass comfortably through and extend deeper into the cavity and the acyl chains of PS occupy the same channels as for PA. Interestingly, in the PA-bound Ups1 structure, additional space was observed beyond the phosphate deeper into the cavity and in our PS-bound PRELID1 mutant this is now occupied by the serine group from PS.

**Fig. 3** Lipid transfer specificity of Ups1 mutants. a PS transfer of wildtype (WT) and mutant Ups1–Mdm35 complexes in vitro. Recombinant protein complexes (50 nM) were incubated with donor liposomes (12.5 µM) containing NBD-PS and Rhodamine-PE (PC/PE/CL/Lac-PE/NBD-PS/Rhodamine-PE = 50/35/5/5/5 mol%) and acceptor liposomes (50 µM; PC/PE/CL/Lac-PE/PS = 50/35/5/5/5 mol%) at 20 °C and NBD fluorescence was monitored. b PA transfer of wildtype (WT) and mutant Ups1–Mdm35 complexes in vitro. Recombinant protein complex (12.5 µM) were incubated with donor liposomes containing NBD-PA and Rhodamine-PE (12.5 µM; PC/PE/CL/Lac-PE/NBD-PA/Rhodamine-PE = 50/33/5/5/5/5 mol%) and acceptor liposomes (50 µM; PC/PE/CL/Lac-PE/PA = 50/35/5/5/5 mol%) and NBD fluorescence was monitored. c Relative PS (red) and PA (white) transfer activities of Ups1 variants relative to their PA transfer activities. See also Supplementary Fig. 3.
We therefore searched for residues that limit PA transfer by Ups2 in a reverse genetic screen for Ups2 mutants that can substitute for Ups1 in vivo. Similar to our previous screen, we exploited the requirement of Ups1 for the growth of \( \text{phb1}^{-}\Delta \) cells (Fig. 5a). \( \text{UPS2} \) was subjected to PCR mutagenesis and expressed in \( \text{ups1}^{\Delta \text{phb1}} \Delta \) cells harbouring plasmid-borne \( \text{PHB1} \). We screened for Ups2 variants that support cell growth upon loss of the \( \text{PHB1} \) encoding plasmid (Fig. 5a). We sequenced 112 mutant \( \text{UPS2} \) genes and identified in total 612 mutations (5.46 mutations per clone, Supplementary Data 1). The mutations

![Image](https://example.com/image1)

**Fig. 4** Characterisation of PS-bound PRELID3b mutants. **a** PS transfer by PRELID1, PRELID1-K58V (red triangles) or PRELID1-K58T (cyan diamonds). Assays are performed as in Fig. 3a, except that 120 nM protein was used. **b** PA and PS transfer activity of wildtype PRELID1 and mutant variants. Error bars represent mean ± SEM, \( n = 3 \). See also Supplementary Figure 4. **c** Left; PS headgroup orientation 1, with serine group protruding towards \( \beta \)-sheet of PRELID1-K58V (cyan). Right; PS headgroup orientation 2, with serine group protruding towards loop 2 of PRELID1. Electron density map for PS. The \( F_o - F_c \) difference Fourier map was calculated by omitting PS, and is shown in cyan sticks surrounded by blue meshes countered at 3.0\( \sigma \). **d** Comparison of PA-binding site in Ups1 and the PS-binding site of PRELID1-K58V-PS. Zoom in view of superposition of PRELID1-K58V-PS (cyan) and Ups1-PA (beige). All residues are numbered according to the protein sequence.
Fig. 5 Identification of Ups2 variants transferring PA. a Reverse genetic screen for Ups2 variants substituting for Ups1. ups1Δphb1Δ cells harbouring plasmid-borne PHB1 and URA3 were transformed with a linearised yeast plasmid and with PCR fragments of the UPS2 gene that were amplified by error-prone PCR. The plasmid encoding URA3 was excluded from cells upon growth of the transformants in the presence of 5′FOA. Cells were grown in the absence of inositol to further suppress the growth of the host strain. Plasmids encoding Ups2 mutants were isolated from growing cells and sequenced.

b The frequency of mutations in Ups2 variants. Amino acids that were found mutated in >10 Ups2 variants are highlighted. Secondary structure elements in Ups2 are shown.

c Growth of ups1Δphb1Δ cells expressing myc-tagged Ups1, Ups2 or the indicated Ups2 variants on selective, inositol-free medium containing glucose and 5′-FOA.

d Structural model of Ups2 (based on the PRELID3b) with amino acids highlighted that were found to be most frequently mutated.
affected a broad range of amino acid residues within Ups2, but those more frequently affected included I66, S76, M93, C101, E108 and I117 (Fig. 5b, Supplementary Table 2). Moreover, mutations accumulated in amino acid segments K64-L70 and R151-G159, which are part of the Ω loop and the C-terminal helix in Ups2, respectively (Fig. 5b, Supplementary Figure 5A).

Finally, truncations of the poorly conserved and unstructured C-terminal region of Ups2 were frequently found (Fig. 5b, Supplementary Figure 5A).

To assess the importance of individual Ups2 mutations for the growth of ups1Δphen1Δ cells, we expressed Ups2 variants harbouring the most frequent mutations or lacking the C-terminal 53 amino acid residues of Ups2 in ups2Δphen1Δ cells and monitored cell growth (Fig. 5c, Supplementary Figure 5B, 5C). With the exception of the Ups2 variant harbouring E108D, expression of all other Ups2 mutant forms allowed growth of ups2Δphen1Δ cells indicating that they retained PS transfer activity (Supplementary Figure 5B). In contrast to Ups2 or the C-terminal-truncated variant thereof, expression of most Ups2 variants also significantly improved growth of ups1Δphen1Δ cells (Fig. 5c), indicating that they can at least substitute for the loss of PA transfer by Ups1. We observed a moderately increased growth of ups1Δphen1Δ cells upon expression of Ups2 variants harbouring mutations in N21, M93 and E108 (Fig. 5c). Strikingly, Ups2 completely restored the growth of ups1Δphen1Δ cells, when I66 within the Ω loop was replaced with a less hydrophobic amino acid residue, suggesting a critical role of the Ω loop for substrate selection by Ups2. Consistently, many residues of the Ω loop of Ups2 were found to be mutated in our genetic screen for Ups2 variants that are able to substitute for Ups1 (Fig. 5b). We therefore replaced the complete Ω loop of Ups2 (amino acids 60–71) with the corresponding region of Ups1 (amino acids 60–71; see Fig. 6a) and examined the ability of the Ups2 loop-swap variant to restore growth of ups1Δphen1Δ cells. Cells harbouring the loop-swap variant grew similar to cells expressing Ups1 (Fig. 5c), indicating that the Ω loop of Ups1 confers PA transfer activity to Ups2.

Next, we monitored the PA/PS transfer activity of the loop-swap mutant of Ups2 in vitro. Ups2 showed an increased tendency to aggregate upon expression in a cell-free system or in E. coli, hampering the functional analysis of Ups2 variants. However, the solubility of Ups2 in vitro was significantly improved if the unstructured C-terminal region after W174 of Ups2 was removed (Supplementary Figure 6A), while preserving the functionality of Ups2 in vivo (Fig. 5c, Supplementary Figure 5B) and in vitro (Supplementary Figure 6B). We therefore introduced mutations in Ups2Δ174 and assessed the lipid transport activities of the variants in vitro. Ups2–Mdm35 complexes transferred PA only inefficiently when compared to PS (Fig. 6b, c, Supplementary Figure 6D). In contrast, we observed efficient PA transport by the loop-swap variant of Ups2 (~250% of Ups2), whereas its ability to transfer PS was significantly reduced (~30% of Ups2, Fig. 6b–d, Supplementary Figure 6D). Thus, replacement of the Ω loop of Ups2 by that of Ups1 converts the lipid transfer specificity of Ups2 from PS to PA (Fig. 6e, Supplementary Figure 6D). This indication is further substantiated by monitoring lipid transfer of non-fluorescent PA and PS (Supplementary Figure 6E, F). Accordingly, expression of the loop-swap variant of Ups2 significantly restored the CL levels in the cell lacking Ups1 (Fig. 6f), ensuring PA transfer activity of the variant in vivo. These experiments unravel the importance of the Ω loop for substrate specificity: while the Ω loop in Ups1 allows PA transfer, the corresponding region limits PA transfer activity in Ups2.

A sequence alignment of the Ω loops in Ups1/PRELID1 or Ups2/PRELID3b subfamilies did not reveal a sequence motif that is specifically conserved within subfamilies (Fig. 6g). However, we noted that Ω loops in Ups2/PRELID3b are substantially more hydrophobic than those in Ups1/PRELID1 which contain (an) additional charged residue(s) in the region (Supplementary Table 3, Fig. 6g). Moreover, Ups2 variants that were identified in the genetic screen and able to substitute for Ups1 often showed reduced hydrophobicity in the Ω loop by introducing a positively charged residue (Fig. 6g). We therefore propose that the hydrophobicity and charge distribution of the Ω loop are critical determinants for the substrate specificity of Ups/PRF lipid transfer proteins.

Molecular simulations define the roles of the Ω loop. To explore the mode of interaction between of PRELID3b–TRIAP1 and PRELID1–TRIAP1 complexes with a phospholipid bilayer, we performed coarse-grained molecular simulations with PS- and PA-containing membranes (Supplementary Figure 7, Supplementary Table 4, Supplementary Movies 1 and 2). The complexes were placed ~10 nm away from the bilayer and allowed to freely diffuse in solution. The simulations were performed with a single lipid-bound in the cavity (POPS for PRELID3b and POPA for PRELID1) (Supplementary Table 4, Supplementary Figure 7A, 7B). In all cases, the lipids remained bound in the cavity whilst the complex was free in solution (Fig. 7c, d, Supplementary Figure 7C, 7D).

The complexes contact the membrane several times before converging upon the stable binding mode, in which large aromatic residues are buried in the membrane (Fig. 7a, b, Supplementary Figure 7). The initial attachment to the membrane involves burial of one or more of these ‘anchoring’ residues before the insertion of the Ω loop and part of the C-terminal α3 helix in the bilayer (Fig. 7b, Supplementary Figure 8A). The next stage of the binding series involves an opening motion of the Ω loop, which allows the bound lipid to move down from the primary binding pocket, into a secondary site, before detaching fully. The pathway of the lipid during this process may be mapped in terms of close contact points between the lipid headgroup and the PRELI domain (Fig. 7c, d). These plots highlight several residues that were identified in the genetic screens as forming key contacts with the lipid substrate within the primary site. Residues in contact with the lipid during the exit pathway may also have important roles in substrate uptake and selectivity. K58 was notable in the PA release pathway as it maintained contact with the PA headgroup in the secondary site.

The binding orientation of PRELID1 and PRELID3b on the membrane is such that the lipid is primed in the preferred orientation to be readily released from the binding channel into the membrane (Fig. 7a, Supplementary Figure 7 and 8A, see Supplementary Movies 1 and 2). The hydrophobic lipid tails are able to partition into the bilayer, while the headgroup is released in the final step from the complex at the approximate height of the rest of the membrane lipid headgroups.

Interestingly, we found that PA was released from the complex with only partial burial of the protein, limited to the Ω loop and one end of the adjacent C-terminal helix, which are deeply buried into the hydrophobic region of the membrane (Fig. 7b, Supplementary Figure 8A). PS release, on the other hand, involved a more extensive overall interface of the protein surface on the membrane at the point of lipid release, consistent with the increased hydrophobicity of the Ω loop in the PS-specific Ups2/PRELID3b proteins. This could be quantified by measuring the number of contacts between each residue and the hydrophobic region of the membrane (Supplementary Figure 8A).

To further characterise this difference in behaviour, we performed simulations of the PRELID1–K58V complex with
Fig. 6 Lipid transfer specificity of Ω loop mutants of Ups1 and Ups2. a Amino acid sequence of the region surrounding the Ω loop in Ups1 and Ups2 and of the Ups2 loop-swap mutant. b PS transfer by Ups2 and loop-swap variant. The Ω loop was exchanged in a truncated version of Ups2 (Δ174) that is functionally active but shows reduced tendency to aggregate in vitro (Supplementary Figure 6A). Recombinant protein complexes (50 nM) were incubated with donor liposomes (12.5 µM) containing NBD-PS and Rhodamine-PE (PC/PE/CL/Lac-PE/NBD-PS/Rhodamine-PE = 50/23/15/5/5/2 mol%) and acceptor liposomes (50 µM; PC/PE/CL/Lac-PE/PS = 50/25/15/5/5 mol%) at 25 °C and NBD fluorescence was monitored. c PA transfer by Ups2 and the Ups2 loop-swap variant. Recombinant protein complexes (50 nM) were incubated with donor liposomes (12.5 µM) containing NBD-PA and Rhodamine-PE (PC/PE/CL/Lac-PE/NBD-PA/Rhodamine-PE = 50/23/15/5/5/2 mol%) and acceptor liposomes (50 µM; PC/PE/CL/Lac-PE/PA = 50/25/15/5/5 mol%) at 25 °C and NBD fluorescence was monitored. d PS and PA transfer activities of the Ups2 loop-swap variant normalised to control. Error bars represent mean ± SEM, n = 4. e PS/PA transfer activity ratio of Ups2 and the loop-swap variant. f Total CL levels in ups1Δphb1Δ cells expressing Phb1 and Ups1, Ups2 or Ups2 loop-swap. N = 3. Error bars represent mean ± SEM. *p < 0.05. g Sequence alignment of Ω loop regions of members of the Ups1/PRELID1 and Ups2/PRELID3b subfamilies in the indicated eukaryotic organisms. Mutations in Ω loop of Ups2 that were identified in the genetic screen are indicated below. Asterisks refer to the residues that were not found mutated.
**Fig. 7** Coarse-grained simulations of membrane association. 

**a** Stages of membrane binding and PS release by the PRELID3b–TRIAP1 complex. Bound PS molecule coloured yellow except for phosphate (magenta) and serine (orange) particles. Final step shows a new PE lipid (magenta, transparent) bound in Site II. PRELID3b (cyan) and TRIAP (green) are shown as a backbone trace, with the Ω loop shown in dark blue. Membrane lipid phosphate groups are shown as grey spheres.

**b** Orientation of PRELID3b (cyan) and PRELID1 (yellow) on the membrane while lipid is in Site II. Anchoring aromatic residues are shown as purple spheres.

**c** Key residues of PRELID3b in contact with PS phosphate group are labelled for each binding site. Heatmap on the right shows contacts over time for the first simulation. Residues are coloured according to proportion of time in contact with lipid phosphate group over simulation (red minimum, blue maximum).

**d** As c but for PRELID1 and PA. PA is coloured grey with magenta phosphate group.
POPS (Supplementary Table 4). PRELID1−K58V was found to maintain the same upright orientation on the membrane indicating that the flattened orientation observed for PRELID3b during PS release is a property of the protein rather than a necessary requirement of the lipid substrate (Supplementary Figure 8B). Furthermore, key contacts formed by PRELID1 with the PA headgroup were lost for PRELID1−K58V with PS, which were not compensated for by the serine moiety (Supplementary Figure 8B). We also tested the PRELID3b−PRELID1-loop-swap mutant with bound PA (Supplementary Figure 9, Supplementary Table 4). This exhibited PA release in either an upright or flat conformation, which highlights the influence of the Ω loop in membrane-binding orientation. As a final comparison, we also simulated the behaviour of the Ups1−Mdm35 PA-bound complex and found the same upright orientation on the membrane (Supplementary Figure 9). Following lipid release, the open secondary binding site could either be occupied by new lipids or the Ω loop closes again. The final snapshots of each simulation are shown in Supplementary Figure 9, highlighting the stochastic nature of the process.

Discussion

The Ups/PRELI lipid transfer proteins are widely conserved in eukaryotes and can be classified into two subfamilies by sequence similarity (Ups1/PRELID1 and Ups2/PRELID3; see Supplementary Figure 10C). The crystal structures of PRELID11−173 and PRELID3b, together with previously reported PRELID3a, means that structural information is available for all human homologues of this lipid transfer protein family, which provides the framework to deepen our understanding of the structural determinants for lipid specificity. A structural comparison of the complete Ups/PRELI-like family highlights a conformationally flexible entrance to the phospholipid binding cavity. The crystal structures display a range of conformations for the Ω loop and the adjacent C-terminal helix α3 which cap the cavity (Fig. 1c, Supplementary Figure 10A). These two features are observed to progressively shift apart, akin to an index finger and thumb in a pinching motion, suggesting that conformational changes within the Ω loop and the C-terminal helix are important role for efficient substrate capture and delivery. The interface with TRIAP1 or Mdm35 remains unaltered despite these conformational differences within the PRELI domain. PRELID11−173 exhibits the most closed of all the structures while the structure of PRELID3b displays the most open entrance to the lipid binding cavity, and this likely contributes to its ability to readily accept the larger headgroup of PS.

The strong conservation throughout the PRELI family hampered the identification of critical residues involved in substrate selection from structural data alone. Insight came from reverse genetic screens in yeast for Ups1 and Ups2 variants with broadened or swapped lipid specificity. The gain-of-function Ups1 mutants from this approach can be categorised into two different functional regions: (a) residues facing to the inner substrate-binding cavity that tailor the cavity for selective accommodation of PA (K58, T95, E108) and (b) residues proximal to the Ω loop (K58, T76) and C-terminal α3 helix that likely have a role together in lipid extraction and capping an occupied binding cavity (F133, M135).

The critical K58 residue is conserved within Ups1/PRELID1 subfamily proteins but not in Ups2/PRELID3b, which harbour small polar or hydrophobic residues at this position. This is consistent with the role of the lysine residue being the key determinant for PA-specific transfer. K58 points into the lipid binding cavity of the PRELI domain and together with R25 make a specific electrostatic interaction with the negatively charged phosphomonoester of PA (Supplementary Figure 10B). Hydrogen bond interactions from basic side chains stabilise the −2 charge state of PA and this reinforces the electrostatic interaction and provides specificity for PA. In PS, the phosphate forms a phosphodiester linkage with a terminal serine moiety and therefore has a maximal charge state of −1 with a distinct charge density. Accommodation of this larger headgroup would not only be hindered by the bulkiness of the lysine side-chain, as observed in our structural observations (Fig. 4c, d), but the positive charge of K58 may capture the single-charged PS headgroup inappropriately and prevent it from being fully coordinated deeper within the cavity. Exchange of this residue for a small polar or hydrophobic side-chain would remove these limiting factors and promote accommodation of the PS headgroup. The side chains of T95 and E108 also protrude into the substrate-binding cavity near the PA headgroup and thus the changes to smaller residues at these positions (T95A or E108D) would also ease accommodation of the larger PS. The hydrophobic residues F133 and M135 are located at the beginning of the C-terminal α3. The role of these residues could be to either promote the interaction of the PRELI domain with the membrane prior to lipid loading/release or stabilise a key intermediate that allows access of the lipid molecule to the PRELI cavity. Consistent with this assumption, corresponding amino acid residues in PRELID1 are buried most deeply into the lipid bilayer while PA release in MD simulations. T76 flanks the Ω loop and makes direct contacts with one of the acyl chains of PA and therefore its role in substrate selection likely reflects an altered positioning of the acyl chains in a PS-bound PRELI domain. Molecular dynamics simulations with phospholipid-loaded PRELIDs provide further evidence for this, as a significant conformational change is observed within the Ω loop upon engagement with the bilayer, bringing T76 into direct contact with the PA headgroup during the final step of phospholipid release.

Our genetic screen for Ups2 mutants that are able to substitute for Ups1 revealed the primary importance of loop regions (the Ω loop, β5–β6, β7–C3) and the C-terminal helix in PS-specific transfer. The Ups2 variant carrying Ω loop from Ups1 is able to transfer PA, highlighting a pivotal role for the Ω loop in substrate selection. Notably, K58 is located in close proximity to the Ω loop raising the possibility that conformational changes in the loop region are communicated to residues involved in headgroup recognition of the lipid. Molecular simulations confirm that K58 maintains contact with the lipid headgroup throughout, from the bound conformation identified in the crystal structure via its route through the entire exit pathway. The Ω loop and the short alpha helix (α2) in Ups/PRELI proteins contain several hydrophobic residues that ensure lipid binding. However, the overall hydrophobicity in this region is significantly higher in Ups2/PRELID3b than in Ups1/PRELID1, which harbours additional charged residues in this region (Fig. 6g and Supplementary Table 3). In MD simulations, a more extensive interface with the membrane is generated with PRELID3b over PRELID1, reflecting the higher hydrophobicity (Fig. 7b, Supplementary Figure 8A). Consistent with a prominent role for substrate selection, the majority of mutations found in the Ups2 screen reduce the hydrophobicity of this region. Moreover, the comparison of the PRELI structures shows significant conformational plasticity in this region and suggests that a large conformational change is important for specific lipid transfer. We propose that these regions, especially the Ω loop, are involved in extraction of lipid molecules from membrane and loading of the lipid into inner cavity for specific capture. The small headgroup of PA give a cone-shaped structure, which prevents tight packing within a bilayer and presents a lower barrier to membrane insertion by PA-binding proteins. In the case of PS, which is more cylindrical,
headgroup packing is more efficient, and the bilayer leaflet less readily penetrated. It is therefore conceivable that higher hydrophobicity of the \( \Omega \) loop and neighbouring regions in PS-specific PRELI domains is required to facilitate the necessary penetration into the acyl region of PS-enriched membranes and the correct positioning of the headgroup in the binding cavity. This is consistent with the previous observations that mutations within the \( \Omega \) loop retard extraction of PA from membrane by Ups1–Mdm3519,20. Furthermore, the increased hydrophobicity of the PS-specific \( \Omega \) loop region likely inhibits release of the PRELI domain from a PA-enriched membrane, thereby retarding PA transport. Indeed, the Ups1 variant carrying the hydrophobic \( \Omega \) loop of Ups2 was not able to transfer PA efficiently (Supplementary Figure 6G, 6H). Moreover, the Ups1 loop-swap variant did not transfer PS as well, indicating that efficient PS transfer requires both the hydrophobic \( \Omega \) loop and the substrate-binding cavity that is tailored for PS.

Coarse-grain simulations have allowed us to explore molecular details of lipid unloading by PRELI domains beginning from free complexes in solution, requiring long timescales that would not be feasible for atomistic simulations. Thus, the PRELID11–TRIAP1 complexes were concentrated to 26 mg/ml using an Amicon 30 kDa MWCO ultrafiltration unit (Millipore). For crystallisation, the concentrated protein sample was mixed 1:1 with crystallisation liquor (100 mM CHES/NaOH pH 9.5, 200 mM NaCl, 40% (v/v) PEG 300) and dispensed into 1 \( \mu \)l sitting drops in the wells of MRC-MAXI 24 well plates, alongside a reservoir of 200 \( \mu \)l crystallisation liquor. Plates were left to equilibrate for 9 days at 20°C. Crystals were harvested and flash frozen in liquid nitrogen for data collection.

For expression of PRELID3b–TRIAP1 complex, TRIAP1 was cloned into vector pMALX(IE) including a N-terminal Maltron binding protein, and PRELID3b was cloned into vector pRSET-F. These were co-transformed in SHuffle E. coli cells, then co-expressed, harvested and Ni-NTA purified as described for PRELID1–TRIAP1. PRELID3b–MbpTRIAP1 complex was pulled down via N-terminally his-tagged PRELID3b. Following nickel affinity purification the sample was gel filtered using a Superdex 200 Hi Load preparative column (GE Healthcare). Fractions of the first peak corresponding to dimeric PRELID3b–MbpTRIAP1 complex were collected together and exchanged into crystallisation buffer (50 mM Tris-HCl pH 8.0, 5 mM NaCl, 5 mM Malto). PRELID3b–MbpTRIAP1 complexes were concentrated to 26 mg/ml using an Amicon 30 kDa MWCO ultrafiltration unit (Millipore). For crystallisation, the concentrated protein sample was mixed 1:1 with crystallisation liquor (100 mM CHES/NaOH pH 7) and dispersed into 1 \( \mu \)l sitting drops in the wells of MRC-MAXI 24 well plates, alongside a reservoir of 200 \( \mu \)l crystallisation liquor. Plates were left to equilibrate for 4 days at 20°C. Crystals were harvested and flash frozen with 25% glycerol in liquid nitrogen for data collection.

**Methods**

**E. coli strains and growth conditions.** *E. coli* strains used for expression of recombinant proteins are Shuffle T7 (NEB) and Rosetta-gami2 (DE3) (Merck). For preparation of DNA or cloning of genes XLI1 Blue strain was used. In general, these *E. coli* strains were cultivated in LB medium containing antibiotics (100 \( \mu \)g/ml ampicillin) for selection of plasmid at 37°C otherwise indicated in method details.

**S. cerevisiae strains and growth conditions.** Yeast strains used are based on S288c and listed in Supplementary Table S. Strain BY474175 were generated by tetrad dissection of a haploid carrying *HYN*, *NAT* and *URA3* after crossing PD49 (ups1A) and CG409 (pHBl + pCM189-PHBI). Cells harbouring UPS1 or UPS2 and PHBI were cultivated in synthetic complete medium lacking uracil and leucine supplemented with 2% glucose as a carbon source. For the experiments checking the growth phenotype of cells with impaired Ups1 function, myo-inositol was omitted from medium. See method details for precise growth conditions used in the genetic collection.

**Protein expression and purification for structural studies.** A C-terminal truncation of PRELID1 spanning residues 1–173 (PRELID1-173) with an N-terminal histidine tag was cloned into the pET-duet-1 vector, and Cys112 and Cys115 were substituted for Ser by Q5 site-directed mutagenesis (NEB). TRIAP1 was cloned into the higher copy vector pRSET-Fb with an N-terminal histidine tag. The constructs were co-transformed into SHuffle E. coli cells, and co-expressed after induction overnight at 16°C with 0.1 mM IPTG at a cell density of OD600 0.6, in 1 L LB-preinoculated cultures. Cell pellets were resuspended in 20 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl) supplemented with 1x EDTA-free protease inhibitor cocktail (Sigma) and then disrupted by sonication. Lysates were centrifuged at 15,000 rpm for 30 min at 4°C and the clarified lysate was loaded on a NiNTA agarose column pre-equilibrated with 20 column volumes (CV) of wash buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 20 mM imidazole). This was then washed with 20 CV of wash buffer and eluted with 5 CV of elution buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 300 mM imidazole). Following nickel affinity purification the sample was further purified using a Superdex 75 Hi Load preparative gel filtration column (GE Healthcare) in gel filtration buffer (50 mM Tris, pH 8, 200 mM NaCl), to separate PRELID1-173–TRIAP1 complexes from the three free TRIAP1 in the sample. The PRELID1-173–TRIAP1 complexes were concentrated to 26 mg/ml using an Amicon 30 kDa MWCO ultrafiltration unit (Millipore). For crystallisation, the concentrated protein sample was mixed 1:1 with crystallisation liquor (100 mM CHES/NaOH pH 9.5, 200 mM NaCl, 40% (v/v) PEG 300) and dispensed into 1 \( \mu \)l sitting drops in the wells of MRC-MAXI 24 well plates, alongside a reservoir of 200 \( \mu \)l crystallisation liquor. Plates were left to equilibrate for 9 days at 20°C. Crystals were harvested and flash frozen in liquid nitrogen for data collection.
Cloning of genes in yeast expression vectors. C-terminal myc-tagged variants of yeast UPS2 had been cloned in YCplac111 vector under ADH1 promoter11. N-terminally his-tagged UPS2, a PCR fragment encoding the whole UPS2 gene was amplified from the plasmid YCplac111,UPS2/myc by error-prone PCR (in the presence of 0.25 mM MnCl2 and 0.05 U/µl Taq polymerase) with the primers TL1172 and TL 11722. GC410 (up2phphlb1a pCM189-PHB1) cells grown in SCD containing 1 µg/ml doxycycline and 15 µg/ml chloramphenicol were incubated for 48 h at 72 h at 30 °C. Appeared colonies on the plates (in total 133 colonies) were picked and streaked on SCD-leucine plus 60 µg/ml uracil, 1 µg/ml doxycycline and 0.1% 5′-FOA plates to purify as well as on SCD-uracil to check the loss of PHB1 plasmid. Colonies appeared on the plate were collected, and subjected to plasmid extraction using EZ-N-A-Prep Plasmid mini kit (Omega Bio-tek). To amplify the partial plasmids, they were individually transformed in E. coli XL1-blue and the transformants were subjected to plasmid extraction by the kit. UPS2 genes on the plasmids were sequenced to identify mutations (in total 119 clones).

To isolate UPS2 mutants suppressing the severe growth phenotype of the cell lacking PHB1, UPS2, a PCR fragment encoding the whole UPS2 gene was amplified from the plasmid YCplac111,UPS2/myc by error-prone PCR (in the presence of 0.25 mM MnCl2 and 0.05 U/µl Taq polymerase) with the primers TL12090 and TL 12091. TL51 (ups1A2phphlb1a pCM189-PHB1) cells grown in SCD containing 1 µg/ml doxycycline were isolated and transformed with 1 µg of the PCR fragment and 200 ng of a 7.5 kb fragment resulting from restriction digest of YCplac111,UPS2/myc by Sma1 and HindIII. Transformation mix was spread on ten SCD-leucine plates containing 60 µg/ml uracil and 1 µg/ml doxycycline. After incubation for 24 h at 30 °C, transformants were replica plated on SCD-leucine plus 60 µg/ml uracil, 1 µg/ml doxycycline and 0.1% 5′-FOA plates. The replica plates were incubated for 48 h at 72 h at 30 °C. Appeared colonies on the plates (in total 133 colonies) were picked and streaked on SCD-leucine plus 60 µg/ml uracil, 1 µg/ml doxycycline and 0.1% 5′-FOA plates to purify as well as on SCD-uracil to check the loss of PHB1 plasmid. Colonies appeared on the plate were collected, and subjected to plasmid extraction using EZ-N-A-Prep Plasmid mini kit (Omega Bio-tek). To amplify the partial plasmids, they were individually transformed in E. coli XL1-blue and the transformants were subjected to plasmid extraction by the kit. UPS2 genes on the plasmids were sequenced to identify mutations (in total 119 clones).

Cloning of genes for biochemical studies. N-terminally His-tagged variants of yeast UPS1 or UPS2 (ΔCysc all four cysteine codons have been replaced by serine codons) were cloned in pETDuet-1 vector (Merck) together with Mdm3511,15. N-terminally His-tagged variants of human PRELI1 or PRELI3d3 were cloned in pETDuet-1 vector together with TRIAP11,15. Point mutations were introduced by site-directed mutagenesis using Pfu Turbo DNA polymerase (Agilent) and the primers listed in Supplementary Table 7.

Expression in E. coli and purification of Ups2–Mdm35. Ups2–Mdm35 and its variants were expressed in E. coli Rosetta-gami2 (DE3) (Promega). After incubation at 37 °C for 3.5 h (OD600 ~ 0.5), the culture was shifted to 18 °C for 1 h and then Ups2 and Mdm35 were expressed by adding IPTG (0.2 mg/ml) for 14 h. E. coli cells were lysed in buffer C (50 mM Tris–HCl, pH 8, 500 mM NaCl, 1x Halt protease inhibitor mix, 1 mM PMSF, 20 mM imidazole, 100 U/ml Dnase I, 1 mM MgCl2, 25 µM wet cell weight) by Emulsiflex-C5 (Avanti Tech). The lysate was centrifuged at 10,000 x g for 20 min and the supernatant was recovered to a tube. Ni-sepharose high performance beads (GE healthcare) were added to the lysate (20 µl/ml) and his-tagged proteins were captured to beads by incubation for 1 h at 4 °C. After incubation, beads were recovered by centrifugation (1000g, 2 min), washed four times with 50 mM Tris–HCl, 500 mM NaCl, and eluted in buffer C (20 mM Tris–HCl pH 8.0, 500 mM NaCl, 300 mM imidazole, 20% glycerol). The elution was subjected to size exclusion chromatography using Superdex 200 increase 10/300 column (GE healthcare) in buffer B (10 mM Tris–HCl pH 8.0, 500 mM NaCl) and proteins were recovered from growing colonies.

Protein expression was achieved by shaking at 30 °C for 16 h in a protein expression mix based on S30 lysate of E. coli with T7 polymerase (Cuba Biotech, 35% (v/v) in reaction mix) in the presence of 15 µg/ml plasmid DNA but devoid of DTT. Protein purification of His–Ups1–Mdm35 complex was performed as described15. The purified protein sample was subjected to dialysis in storage buffer (10 mM Tris–HCl pH 7.4, 100 mM NaCl, 1 mM EDTA) for 4 h. Protein concentration was determined by absorption at 280 nm and extinction coefficient of the protein complex.
removed and 500 µl of pure water was added gently to the tube without mixing. The tube was centrifuged (15,000 x g, 10 min, TLA-55/50,000 rpm) and the supernatant was removed carefully without disturbing the pellet. The pellet was resuspended in 100 µl of pure water and the protein concentration was determined by Bradford assay. Aliquots of 100 µg protein were kept at −80 °C and were subjected to lipid analysis by qMS.

Quantitative mass spectrometry of phospholipids. Mass-spectrometric analysis of phospholipids was performed essentially as described. Briefly, lipids were extracted from samples in the presence of internal standards of major phospholipids (9 PC, 10 PE, 11 PS, 12 PE, 30 PE, 14 PI, 17 PI, 17-14, PI 17-14-1, PI 17-0-14-1 all from Avanti Polar Lipids) and CL (CL mix I, Avanti Polar Lipids LM-6003). Extraction was performed according to Bligh and Dyer with modifications. Final lipid samples were dissolved in 10 mM ammonium acetate in methanol and were splayed into a QTRAP 6500 triple quadrupole mass spectrometer (SCIEX) by nano-infusion spray device (TriVersa NanoMate with ESI-Chip type A, Advion). The quadrupoles Q1 and Q3 were operated at unit resolution. Phospholipid analysis was carried out in positive ion mode. PC analysis was carried out by scanning for precursors of m/z 184 at a collision energy of 37 eV. PE, PS, PI, PG, and PA measurements were performed by scanning for neutral losses of 141, 277, 183, 189 and 115 Da at CEi of 30, 30, 30, 25 and 25 eV, respectively. CL species were identified by scanning for precursors of the masses (m/z 465.4, 464.7, 491.4, 493.4, 495.4, 505.5, 519.5, 521.5, 523.5, 535.5, 547.5, 549.5, 551.5, 573.5, 575.5, 577.5, 579.5, 601.5, 603.5, 605.5, 607.5, 631.5, 715.3 and 715.3 Da) corresponding DAG-H2O fragments as singly charged ions at CEi of 45 eV. Mass spectra were analysed by the Analyst Software Version 1.2 (SCIEX) for identification, correction of isotopic overlap and quantification of lipids. Correction of isotopic overlap in CL species was performed using a spreadsheet calculating and subtracting theoretical amounts of [M-2H]+ and [M-4H]+ isotopes of each CL species that are isotopic to other CL species. Lipid amounts (pmol) were corrected for response differences between spectrometer (Thermo Scientific) and internal standards and endogenous lipids.

Identification of bound lipids to purified proteins. Overall, 20 µg of protein were added with 1 µg of trypsin and left incubating overnight at 37 °C. The mixture was then dried in a SpeedVac concentrator and re-dissolved in 100 µl acetonitrile (ACN) by sonication for 10 min. The lipid samples were separated on a C18 column (Acclaim PepMap 100, C18, 75 µm × 15 cm; Thermo Scientific) by Dionex UltiMate 3000 RSLC nano System, and then analysed by a hybrid LTQ-Orbitrap mass spectrometer (Thermo Scientific) via a dynamic nanospray source. The buffers and and gradient used for separation of lipids are adapted from ref. The quadrupoles Q1 and Q3 were operated at unit resolution. Phospholipid analysis was carried out in positive ion mode. PC analysis was carried out by scanning for precursors of m/z 184 at a collision energy of 37 eV. PE, PS, PI, PG, and PA measurements were performed by scanning for neutral losses of 141, 277, 183, 189 and 115 Da at CEi of 30, 30, 30, 25 and 25 eV, respectively. CL species were identified by scanning for precursors of the masses (m/z 465.4, 464.7, 491.4, 493.4, 495.4, 505.5, 519.5, 521.5, 523.5, 535.5, 547.5, 549.5, 551.5, 573.5, 575.5, 577.5, 579.5, 601.5, 603.5, 605.5, 607.5, 631.5, 715.3 and 715.3 Da) corresponding DAG-H2O fragments as singly charged ions at CEi of 45 eV. Mass spectra were analysed by the Analyst Software Version 1.2 (SCIEX) for identification, correction of isotopic overlap and quantification of lipids. Correction of isotopic overlap in CL species was performed using a spreadsheet calculating and subtracting theoretical amounts of [M-2H]+ and [M-4H]+ isotopes of each CL species that are isotopic to other CL species. Lipid amounts (pmol) were corrected for response differences between internal standards and endogenous lipids.

Formaldehyde precipitated lipids were subjected to lipid analysis by qMS. Lipid amounts (pmol) were corrected for response differences between spectrometer (Thermo Scientific) and internal standards and endogenous lipids.

Quantification and statistical analysis. Quantitative data are presented as arithmetic means ± standard error (SEM). The statistical significance in related figures was assessed using two-tailed Student’s t-test. P values and n in column plots from Student’s t-test were specified in corresponding figure legend.

Code availability. The script used to analyse protein–lipid interactions has been previously reported and is available from the corresponding authors upon request and with the permission of Heidi Koldsoe (Schroedinger Llc). Lipid contacts were calculated between each residue of the protein and the stated particle type of a given lipid using a distance cutoff of 0.6 nm to define a contact.

Data availability

The structure data for TRIAP1–PRELID3b, TRIAP1–PRELID1 and TRIAP1–PRELID1 (K57V) complexes have been deposited in the Protein Data Bank (with PDB ID codes: 614Y, 613V, 613Y). Other data are available from the corresponding authors upon reasonable request. The source data underlying Figs. 2b and 3b are provided as Supplementary Data 1. Uncropped gel images used to generate panels in Figure 2C, 3A, 4A, 5C, 6A, 6C and 6D are provided in Supplementary Figure 11. The source data of Figs. 3c, 4b, 6b, 6d and Supplementary Figure 2D, 3D, 3E, 4B, 6B, 6D, 6E and 6H are provided as a Source Data file.

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