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## 1 **CryoEM architecture of a native stretch-sensitive membrane microdomain**

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13

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15

### 16 **Abstract**

17 Biological membranes are partitioned into functional zones containing specific lipids and proteins,  
18 termed membrane microdomains. Their composition and organization remain controversial owing  
19 to a paucity of techniques that can visualize lipids *in situ* without disrupting their native behavior<sup>1,2</sup>.

20 The yeast eisosome, a membrane compartment scaffolded by the BAR-domain proteins Pil1 and  
21 Lsp1, senses and responds to mechanical stress by flattening and releasing sequestered factors<sup>3–</sup>  
22 <sup>7</sup>. Here, we isolated native eisosomes as helical filaments of Pil1/Lsp1 lattice bound to plasma

23 membrane lipids and solved their structures by helical reconstruction. We observe remarkable  
24 organization within the lipid bilayer density from which we could assign headgroups of PI(4,5)P<sub>2</sub>

25 and phosphatidylserine bound to Pil1/Lsp1 and a pattern of membrane voids, signatures of  
26 sterols, beneath an amphipathic helix. We verified these assignments using *in vitro* reconstitutions

27 and molecular dynamics simulations. 3D variability analysis of the native eisosomes revealed a  
28 dynamic stretching of the Pil1/Lsp1 lattice that affects functionally important lipid sequestration,

29 supporting a mechanism in which membrane stretching liberates lipids otherwise anchored by the  
30 Pil1/Lsp1 coat. Our results provide mechanistic insight into how eisosome BAR-domain proteins  
31 create a mechanosensitive membrane microdomain and, more globally, resolve long-standing

32 controversies about the architecture and nature of lipid microdomains.

33

## 34 Introduction

35 Over fifty years ago, it was proposed that the plasma membrane is compartmentalized into  
36 laterally heterogenous domains<sup>8,9</sup>. Although the biological evidence for membrane  
37 compartmentalization is overwhelming (e.g. localization of immune receptors to detergent-  
38 resistant or liquid-ordered membrane domains<sup>10-12</sup>, spatiotemporal organization of  
39 transmembrane proteins and plasma membrane lipids by the cortical actin cytoskeleton<sup>13-15</sup>) the  
40 determinants and the physical structure of the lipid organization within the membrane remain  
41 controversial. This is because almost all tools used to study membrane lipids also risk perturbing  
42 their behavior within the membrane context<sup>1,2,16</sup>.

43  
44 In *S. cerevisiae*, at least three membrane compartments have been identified: the membrane  
45 compartment containing Pma1 (MCP), the membrane compartment containing Can1 (MCC), and  
46 the highly dynamic membrane compartment containing TORC2 (MCT)<sup>3</sup>, in addition to the  
47 patchwork organization of many other integral membrane proteins<sup>17</sup>. The MCC microdomains,  
48 also known as eisosomes, are randomly distributed membrane furrows, about 300nm long and  
49 50 nm deep, scaffolded by the Bin/amphiphysin/Rvs (BAR) domain family protein Pil1 and its  
50 paralog Lsp1<sup>3,4,18</sup>. The eisosomes are unique among membrane compartments in their stability;  
51 under steady-state conditions, they do not change size or distribution within the membrane for  
52 the duration of the cell cycle and they are non-overlapping with sites of endocytosis, protecting  
53 their resident proteins from turnover<sup>19-21</sup>. The function of eisosomes remains mysterious, but they  
54 are clearly implicated in sensing and responding to plasma membrane stress: various stimuli  
55 including hypo-osmotic shock, heat shock, and mechanical pressure cause eisosomes to flatten  
56 and release the sequestered eisosome-resident proteins, enabling their signaling and transport  
57 functions<sup>3-5,18,22,23</sup>.

58  
59 Eisosomes are a plasma membrane feature that is unique to fungi and some algae<sup>24</sup>. However,  
60 they may have functional relatives in mammalian cells. They share many features with caveolae,  
61 including flattening in response to membrane stress<sup>25,26</sup>, their role in regulating plasma membrane  
62 homeostasis<sup>27,28</sup> and assembly dependent on negatively-charged lipids (phosphatidylserine and  
63 PI(4,5)P<sub>2</sub>)<sup>29-31</sup>. There are also several examples of mammalian membrane domains scaffolded  
64 by BAR-domain proteins including the t-tubules of cardiac and skeletal muscle tissue that are  
65 stabilized by BIN-1<sup>32-34</sup> and endosomal tubulation by SNX family proteins and pacsin-2<sup>35</sup>.

66

67 BAR domain proteins are a large and diverse family of proteins that play physiological roles in  
68 curvature sensing and/or induction at cellular membranes<sup>33,36</sup>. The majority have a characteristic  
69 banana-shape with a membrane-binding surface that displays dense positive charge, enabling  
70 the interaction with negatively-charged lipid headgroups<sup>33,35–37</sup>. They have been shown to induce  
71 curvature in membranes via the combination of a variety of mechanisms including 1) their own  
72 intrinsic curvature, 2) the insertion of amphipathic motifs into the membrane, and 3) their self-  
73 organization into large protein lattices<sup>33,35</sup>. While a wealth of data exists on the features of BAR  
74 domain proteins that mediate their curvature sensing/generation function, as well as their lipid  
75 binding affinities *in vitro*, what remains unknown is how BAR scaffolding impacts the organization  
76 of the complex mixture of lipids naturally found in cell membranes *in vivo*.

77  
78 Here we have isolated an intact membrane microdomain, scaffolded in helical tubules by the  
79 eisosome BAR-domain proteins Pil1/Lsp1. By solving high resolution structures using cryoEM  
80 and helical reconstruction, we have a unique opportunity to visualize the unperturbed eisosome  
81 membrane lipids which exhibit a surprising level of organization. A pattern of small membrane  
82 voids beneath the amphipathic helix of Pil1/Lsp1 forms a helical striation in the protein-bound  
83 cytoplasmic leaflet, but not the exoplasmic leaflet, and lipid headgroup density is observed within  
84 charged pockets of the Pil1/Lsp1. Using a combination of *in vitro* reconstitution and molecular  
85 dynamics (MD) simulations with various lipid combinations of known composition, we were able  
86 to identify which lipid species are responsible for the structural signatures we found in the native  
87 eisosome membrane microdomain, as well as observe a reduction in the dynamics of all the lipid  
88 species present at the Pil1 microdomains. The membrane voids are footprints of sterol molecules  
89 stabilized at the sites of bulky sidechains of the amphipathic helix and the bound lipid density is  
90 consistent with PI(4,5)P<sub>2</sub> and phosphatidylserine headgroups bound in each charged pocket.  
91 Remarkably, we also observe a dynamic stretching behavior of the Pil1/Lsp1 lattice in the native  
92 samples which alters the organization of these lipids in the cytoplasmic leaflet, revealing a  
93 possible mechanism of membrane tension sensing by the eisosomes.

94

### 95 **Isolation of native-like eisosome filaments**

96 Eisosome tubules were isolated from *S. cerevisiae* expressing a TAP-tag on the Target of  
97 Rapamycin Complex 2 (TORC2) subunit Bit61. Using a gentle purification procedure involving  
98 hand-grinding of flash frozen yeast under liquid nitrogen and an initial extraction buffer containing  
99 CHAPS detergent at less than 1/10x critical micelle concentration (0.5mM CHAPS in extraction

100 buffer versus CMC of ~6mM at 250mM NaCl)<sup>38</sup>, we were able to preserve a lattice of Pil1/Lsp1  
101 eisosome structural proteins in a near-native state, bound to the intact plasma membrane bilayer,  
102 observable as a two layer density within the protein tubule (Fig 1A). Pil1 is a common contaminant  
103 in *S. cerevisiae* pull-downs<sup>39</sup> and the amount of Pil1/Lsp1 protein isolated by our methods is below  
104 the detection limit of our protein gels, suggesting our eisosome filaments are a contamination of  
105 our intended target (Fig Ext Data 1A). Nevertheless, the large tubulated structures they form were  
106 salient features on the EM grid, enabling us to collect a sufficiently large dataset for structural  
107 determination via helical reconstruction (Fig Ext Data 1B).

108  
109 While eisosomes have been shown to have a furrow-like halfpipe structure *in vivo*<sup>29,40</sup>, our isolated  
110 eisosome tubules appear to be closed, continuous helices of Pil1/Lsp1 proteins (Fig 1A&B).  
111 Similar tubular structures have previously been shown in reconstituted Pil1 samples and  
112 eisosomes purified from yeast<sup>29</sup>. Presumably, once the eisosomes are freed from the plasma  
113 membrane by our gentle purification methods, the Pil1/Lsp1 lattice realigns to form a helical  
114 conformation around a lower-energy tubular state of the plasma membrane lipids to which it is  
115 bound. Alternatively, the eisosomes that we have isolated were already in a tubulated state *in*  
116 *vivo*, a behavior that has been observed in deletion strains of the eisosome-resident Sur7-family  
117 proteins, in palmitoylcarnitine-treated *S. cerevisiae*<sup>41</sup>, and upon Pil1 overexpression in *S.*  
118 *pombe*<sup>42,43</sup>.

119  
120 In our raw images and 2D classes, we noted a large variation in tubule diameters (Fig Ext Data  
121 1B&C). Using helical reconstruction, we were able to resolve 9 eisosome tubule structures with  
122 different helical symmetries and tubule diameters ranging from 308-372Å, but with a nearly  
123 identical lattice pattern of Pil1/Lsp1 dimers in all structures (Fig 1B, Ext Data 1D, Ext Data 2A,  
124 Supp Table 1&2). These tubule diameters are within the range of diameters of curvatures  
125 observed in eisosomes *in situ* (~300-600Å)<sup>44</sup>.

126  
127 **Structure of native Pil1/Lsp1 lattice**  
128 To improve resolution and enable 3D classification of the Pil1/Lsp1 dimers, we used a symmetry  
129 expansion and particle subtraction strategy, masking a central dimer and each of its contacting  
130 partners (a total of 7 dimers) combined with a small spherical mask to include the lipid bilayer  
131 beneath the central dimer (Fig Ext Data 2B). This allowed us to merge lattice pieces from the 9  
132 helical structures (resolution ranging from 3.7Å-7.2Å) into an expanded dataset (Supp Table 2).

133 Refinement of this dataset improved resolution to  $\sim 3.2\text{\AA}$ , enabling us to build and refine models  
134 of both Pil1 and Lsp1 proteins (Fig 1C, Ext Data 3A&B, Ext Data 4A).

135  
136 Because our samples most likely contain a mixture of both Pil1 and Lsp1 proteins and their  
137 sequence conservation is very high, we could not conclusively differentiate between these two  
138 proteins in our structures (Fig Ext Data 3C, Ext Data 5, 73% identity, *MUSCLE*). Although there  
139 is some evidence to suggest that Pil1 and Lsp1 form heterodimers in the cytosol prior to their  
140 assembly at the plasma membrane<sup>45</sup>, it is also known that they are capable of homodimerizing  
141 and tubulating membrane *in vitro*<sup>29</sup>. While deletion of Lsp1 does not cause profound alteration in  
142 eisosome structure at the plasma membrane, deletion of Pil1 prevents the formation of  
143 eisosomes<sup>40,46</sup>. Due to its essential role, we have chosen to base our interpretations on the model  
144 of a Pil1 homodimer.

145  
146 The overall “banana-shaped” BAR domain structure of the Pil1 dimer in our model corresponds  
147 well with the previous crystal structure of an Lsp1 dimer<sup>47</sup> (Fig 1C). Each monomer contributes  
148 three antiparallel alpha-helices that form kinked coiled-coil domains, while a fourth alpha-helix  
149 from each monomer sits atop the convex surface of the curved dimer. We were able to observe  
150 two additional structured regions of the protein in our maps: 1) a folded N-terminus (Nt) that forms  
151 lattice contact sites with the Nt of a neighboring dimer followed by 2) an Nt amphipathic helix (AH)  
152 buried within the lipid density of the “inner leaflet” of the bilayer that runs parallel to the BAR  
153 domain helices of each Pil1/Lsp1 monomer (Fig 1C, inset).

154  
155 Previous nanometer-resolution helical reconstructions of reconstituted Pil1 and Lsp1 proteins  
156 revealed a lattice pattern that could be fitted with the Lsp1 crystal structure, albeit with  
157 unaccounted density at the lattice contact sites<sup>29</sup>. Our structures clearly reveal three regions of  
158 contact between the central dimer and its neighbours (Fig Ext Data 6A). The first site of contact  
159 is a short stretch of interactions between the well-folded, domain-swapped Nt of monomer A  
160 (res1-8) in the central dimer and the equivalent Nt stretch (res1-8) of monomer B in neighboring  
161 dimer 2, including residue S6 which previously shown to be phosphorylated by Pkh1 and  
162 important for eisosome assembly in combination with other phosphorylated residues<sup>48,49</sup> (Fig Ext  
163 Data 6B). The remaining two contact sites are localized to the BAR domain tips, previously shown  
164 to be flexible in crystallographic studies<sup>47</sup>. A stretch of electrostatic interactions between residues  
165 171-186 on helix 3, as well as residue 145 on helix 2, of the BAR domain in monomer A of the

166 central dimer and the equivalent residues from monomer B of dimer 3 forms the second contact  
167 site (Fig Ext Data 6C). Finally, a hydrophobic interaction between Y155 at the tip of BAR domain  
168 helix 2 on monomer A of the central dimer with Y158 on monomer B of dimer 4, and vice versa,  
169 forms a third contact (Fig Ext Data 6D).

170

### 171 **Amphipathic helix associated with membrane void pattern**

172 As mentioned above, immediately following the Nt lattice contact site is a previously unrecognized  
173 AH (res 39-48), oriented parallel to the BAR domain (Fig 1C). N-terminal AHs are a common  
174 feature of the N-BAR family of BAR domain proteins (e.g. endophilin, amphiphysin) and are  
175 proposed to play crucial roles in curvature sensing/induction. Insertion of an AH into the  
176 membrane expands only one leaflet, thus inducing local curvature via a “wedging  
177 mechanism”<sup>35,50,51</sup>. Additionally, amphipathic helices can recognize membrane curvature through  
178 their preferred insertion at sites of lipid packing defects induced by high local curvature<sup>52</sup>.

179

180 By making one-pixel slices through the unsharpened maps parallel to the axis of the eisosome  
181 tubule, we could clearly see the well-defined protein density of the AH buried within the  
182 cytoplasmic leaflet of the membrane (visible as a uniform density of lower intensity) (Fig 1D, panel  
183 1, teal arrow). At slices  $\sim 5\text{\AA}$  deeper, an array of small voids in the membrane density begins to  
184 appear just below the AH. This pattern continues throughout the cytoplasmic leaflet, producing a  
185 fence-like striation in the membrane (Fig 1D, panels 2-5, violet arrows). However, this effect is  
186 asymmetric between the two leaflets: the pattern of voids is only present in the protein-bound  
187 leaflet, while the density of the exoplasmic leaflet is homogeneous throughout the lateral slices  
188 (Fig 1D, panels 6-10).

189

190 We wondered if these membrane voids could represent stably localized ergosterol molecules.  
191 Sterols, with their rigid ring structures, exhibit reduced Coulombic potential compared with tightly  
192 packed phospholipid tails<sup>53-55</sup>. Additionally, multiple examples of sterol/amphipathic helix  
193 interactions have been previously demonstrated. For example, transient interactions of sterols  
194 with the AH of Osh4 have been observed in MD simulations; cholesterol fills packing defects near  
195 aromatic side chains and wedges between acyl chains of poly-unsaturated PI(4)P<sup>56</sup>.

196

### 197 **Lipid binding pocket on Pil1/Lsp1 dimer**

198 Visualization of the electrostatic surface of the membrane-facing surface of the Pil1 dimer  
199 revealed two patches of intense positive charge adjacent to the amphipathic helices (Fig 1E).  
200 Remarkably, within this pocket, an unassigned density is coordinated by several charged residues  
201 that have been previously proposed to be involved in PI(4,5)P<sub>2</sub> binding<sup>29,47</sup> (Fig 1F). *In vitro*  
202 tubulation by Pil1 and Lsp1 is reportedly dependent on the presence of PI(4,5)P<sub>2</sub> in liposomes  
203 and defects in PI(4,5)P<sub>2</sub> regulation cause changes in eisosome morphology *in vivo*<sup>29,57,58</sup>.  
204 Additionally, Lsp1 has been shown to cluster PI(4,5)P<sub>2</sub> on giant unilamellar vesicles (GUVs) and  
205 prevent its lateral diffusion within the membrane<sup>59</sup>. Because of this well-documented structural  
206 and functional relationship between the eisosomes and PI(4,5)P<sub>2</sub>, we speculated that this  
207 unassigned density could represent one or more coordinated PI(4,5)P<sub>2</sub> headgroups.

208

### 209 **Eisosome reconstitution with known lipids**

210 In order to assign identities to the structural signatures we observe within the membrane, we  
211 chose to reconstitute eisosome filaments using lipid mixtures of known composition with  
212 recombinantly-expressed Pil1 protein. We tested several lipid mixtures with cholesterol and/or  
213 PI(4,5)P<sub>2</sub>, using as a constant component a mixture of 18:1 phosphatidylcholine (DOPC), 18:1  
214 phosphatidylethanolamine (DOPE), and 18:1 phosphatidylserine (DOPS).

215

216 Our final reconstructions were made with three lipid mixtures: 1) “minus PI(4,5)P<sub>2</sub>/plus sterol”,  
217 containing 30% cholesterol 2) “plus PI(4,5)P<sub>2</sub>/minus sterol”, containing 10% PI(4,5)P<sub>2</sub>, and 3)  
218 “plus PI(4,5)P<sub>2</sub>/plus sterol”, containing 10% PI(4,5)P<sub>2</sub> and 15% cholesterol (Supp Table 3&4). We  
219 used helical reconstruction to solve multiple structures of varying diameters, helical parameters,  
220 and resolutions. (Fig Ext Data 4B-D, 7A, Supp Table 1&2).

221

222 For all three lipid mixtures, the overall architecture of the Pil1 lattice is similar to the native  
223 samples. However, compared with the native samples, in “-PI(4,5)P<sub>2</sub>/+sterol” preparations, the  
224 amphipathic helices of the Pil1 dimers are not resolved (Fig 2A&B, Ext Data 3D, 7B). This could  
225 be because the AH is more mobile in this lipid mixture or because it is not inserted to the  
226 membrane. The AH (H<sub>0</sub>) of endophilin, for example, requires PI(4,5)P<sub>2</sub> for membrane  
227 penetration<sup>60</sup>. In the “+ PI(4,5)P<sub>2</sub>/-sterol” samples the AH density is improved, while the resolution  
228 AH density is highest and most resembles that of the native eisosomes in the “+PI(4,5)P<sub>2</sub>/+sterol”  
229 samples, suggesting that PI(4,5)P<sub>2</sub> is critical for AH stabilization (Fig 2C-D, Fig Ext Data 3E-F,  
230 7C&D). To probe the PI(4,5)P<sub>2</sub>-dependency of the AH insertion into the membrane, coarse



231 grained (CG) MD simulations were performed using a model tubule of Pil1 dimers and the exact  
232 lipid compositions of the reconstituted mixtures (“-PIP2/+sterol”, “+PIP2/-sterol”, and  
233 “+PIP2/+sterol”). The distances between the centre of mass (COM) of the AH and the head groups  
234 of the lipids were computed and, indeed, the averaged distance distributions show that in absence  
235 of PI(4,5)P<sub>2</sub> the amphipathic helices partially detach from the membrane (Figure 2E, Fig Ext Data  
236 8A-B).

237

### 238 **Membrane voids require PI(4,5)P<sub>2</sub> and sterol**

239 One feature that was notably absent from the “+PI(4,5)P<sub>2</sub>/-sterol” helices was the pattern of  
240 membrane voids in the cytoplasmic leaflet which we observe in the native eisosome filaments  
241 (Fig 2F-G). However, in the “+PI(4,5)P<sub>2</sub>/+sterol” helices, we can again observe the void pattern,  
242 supporting the notion that this pattern results from stable association of sterol molecules with the  
243 AH (Fig 2H). CG-MD simulations were performed with tubules of an identical lipid composition to  
244 the “+PI(4,5)P<sub>2</sub>/+sterol” mixture and the number of contacts between lipid headgroups and  
245 residues of Pil1 were measured for each lipid in terms of percentage of occupancy, i.e. the  
246 percentage of frames in which any lipid-protein contact is formed (Fig Ext Data 8C). Remarkably,  
247 sites of increased cholesterol occupancy were similar to the locations of the pattern of holes in  
248 the lipid density, with peaks at four clusters of residues, each containing aromatic sidechains: 1)  
249 residues 32F/33F, 2) residues 37G/38L/39A/40Y, 3) residues 42F/43R, and 4) residue 50F (Fig  
250 2I-J). To better understand the relationship between PI(4,5)P<sub>2</sub> binding and sterol dynamics, we  
251 reconstituted Pil1 scaffolds on pre-formed membrane lipid nanotubes to perform Fluorescence  
252 Recovery After Photobleaching (FRAP) assays using TopFluor-cholesterol in lipid mixtures with  
253 or without 1% PI(4,5)P<sub>2</sub> (Supp Video 1). For nanotubes with Pil1 but without PI(4,5)P<sub>2</sub> (-  
254 PI(4,5)P<sub>2</sub>/+sterol), there is a slight reduction in the mobile fraction of sterols, but this effect was  
255 pronounced in presence of Pil1 and 1% PI(4,5)P<sub>2</sub> (+1% PI(4,5)P<sub>2</sub>/+sterol) (Fig 2K, Supp Table 5).  
256 This indicates that, in the presence of PI(4,5)P<sub>2</sub>, the immobilized fraction of cholesterol interacts  
257 strongly with either Pil1 and/or other lipids bound to the protein. Notably, the Pil1 lattice remained  
258 highly stable on the nanotubes without PI(4,5)P<sub>2</sub> (Supp Video 2) indicating that the difference in  
259 sterol mobility is not due to unstable protein assembly.

260

### 261 **Lipid binding site in reconstituted eisosomes**

262 In the “+PI(4,5)P<sub>2</sub>/-sterol” structures, a clear triangular density, which we fitted with an inositol-  
263 1,4,5-phosphate (IP<sub>3</sub>) ligand to represent a PI(4,5)P<sub>2</sub> headgroup, was interacting with basic

264 residues R126, K130, and R133 on each protomer in the binding pocket we previously identified  
265 in the native structures (Fig 3A, Ext Data 3E). Interestingly, these conserved residues were  
266 previously shown to be important for eisosome assembly *in vivo* and membrane binding *in*  
267 *vitro*<sup>29,47</sup>. Comparing this additional density with the putative lipid density observed in the native  
268 eisosome filament, there is a clear overlap (Fig 3A). This extra density, not present in the “-  
269 PI(4,5)P<sub>2</sub>/+sterol” Pil1 filaments (Fig Ext Data 3D), likely reflects a PI(4,5)P<sub>2</sub> binding site in the  
270 native eisosomes. To confirm direct PI(4,5)P<sub>2</sub> and Pil1 interaction, we again utilized pre-formed  
271 membrane lipid nanotubes, in this case to measure lipid sorting coefficients of 1% TopFluor-  
272 PI(4,5)P<sub>2</sub> using a fluorescence ratiometric comparison with a reference lipid (Atto 647 N DOPE).  
273 This revealed a relative accumulation of PI(4,5)P<sub>2</sub> in regions with Pil1 scaffolds (Fig 3B, Fig Ext  
274 Data 9A-D). We also checked TopFluor-PI(4,5)P<sub>2</sub> diffusion in lipid nanotubes using FRAP assays  
275 and observed much slower recovery of PI(4,5)P<sub>2</sub> when Pil1 is bound, suggesting a strong  
276 interaction (Fig 3C, Supp Table 5). A similar observation has been previously reported with Lsp1<sup>59</sup>.

277  
278 In the “+PI(4,5)P<sub>2</sub>/+sterol” samples, a similar triangular density bound to R126, K130, and R133  
279 was observed and fitted with an IP<sub>3</sub> ligand, comparable to the PI(4,5)P<sub>2</sub> headgroup in “+PI(4,5)P<sub>2</sub>/  
280 sterol” samples; however, parallel slices through the AH region reveal a slight decrease in  
281 intensity of this PI(4,5)P<sub>2</sub> density in the “+PI(4,5)P<sub>2</sub>/+sterol” samples (Fig 3A and Ext Data 9E). In  
282 line with this observation, lipid sorting values for PI(4,5)P<sub>2</sub> in the “+1% PI(4,5)P<sub>2</sub>/+sterol” samples  
283 revealed PI(4,5)P<sub>2</sub> accumulation under Pil1 scaffolds, albeit to a lesser extent compared with  
284 “+1% PI(4,5)P<sub>2</sub>/-sterol” sample (Fig 3B). Additionally, in the CG-MD simulations for both the  
285 “+PI(4,5)P<sub>2</sub>/-sterol” and “+PI(4,5)P<sub>2</sub>/+sterol” systems, PI(4,5)P<sub>2</sub> occupancy was increased at  
286 charged residues in the lipid binding pocket, especially residues R43, Q60, R126, K130 and R133,  
287 while addition of cholesterol reduced occupancy for all these residues, complementing the lipid  
288 sorting observations (Fig 3D, Fig Ext Data 8D). Curiously, using FRAP, we actually observed a  
289 reduction in PI(4,5)P<sub>2</sub> mobility under the Pil1 scaffold in samples with cholesterol, suggesting that  
290 for the fraction of PI(4,5)P<sub>2</sub> that is immobilized (e.g. protein-bound), sterols might play a role in  
291 enhancing the Pil1-PI(4,5)P<sub>2</sub> interaction and perhaps stabilizing the AH (Fig 3C, Supp Table 5).

292  
293 In the “+PI(4,5)P<sub>2</sub>/+sterol” samples, we were surprised to observe an additional small density  
294 stabilized between the AH and the PI(4,5)P<sub>2</sub> headgroup, potentially coordinated by residues R43,  
295 K66, and/or R70 (Fig 3A). We speculated that this could be the signature of a lipid headgroup  
296 from one of the other lipids in our mixture. Phosphatidylserine (PS), in particular, is reported to

297 strongly partition with sterols both *in vitro* and *in vivo*<sup>61–63</sup>. Using lipid nanotubes with TopFluor-  
298 labeled PS, we observed PS sorting in both the “+1% PI(4,5)P<sub>2</sub>/-sterol” lipid mixture and the “+1%  
299 PI(4,5)P<sub>2</sub>/+sterol” mixture, with a significantly higher sorting coefficient in the presence of sterol,  
300 supporting the notion that our extra density is a PS headgroup (Fig 3B). CG MD simulations also  
301 suggest that DOPS exhibits a similar affinity to the charged lipid-binding pocket of Pil1 as  
302 PI(4,5)P<sub>2</sub>, but with alteration (either enhancement or reduction) of several interactions with  
303 charged residues in the lipid binding pocket upon the inclusion of sterols in the lipid mixture (Fig  
304 Ext Data 8E-F). Consequently, we fitted this density with a phospho-serine ligand to represent a  
305 PS headgroup (Fig 3A). Collectively, these data suggest that the inclusion of sterol in the lipid  
306 mixture increases the specificity of PI(4,5)P<sub>2</sub> and DOPS interaction with particular charged  
307 residues within the lipid binding pocket.

308  
309 Remarkably, an overall reduction in lipid dynamics was also observed by FRAP for TopFluor-PC,  
310 -PE, and -PS with 1% PI(4,5)P<sub>2</sub> and 30% cholesterol included in the membrane (Fig 3E-G). A  
311 significant portion of each of these lipids were immobilized in the presence of Pil1 indicating that  
312 the membrane is less diffusive under the Pil1 scaffold. While the fraction of these lipids that is  
313 immobilized by Pil1 is similar with or without PI(4,5)P<sub>2</sub>, the dynamics of the mobile lipid fraction  
314 for each of these lipids were decreased in the presence of PI(4,5)P<sub>2</sub>, again highlighting that  
315 PI(4,5)P<sub>2</sub> is crucial for formation of stable lipid microdomains under the Pil1 scaffold (Fig 3E-G,  
316 Supp Table 5). This implies that the binding of PI(4,5)P<sub>2</sub> and/or the stabilization of the AH of Pil1  
317 create a sieve-like lattice within the cytoplasmic leaflet that slows lipid dynamics in the membrane  
318 microdomain, enabling the sterol molecules to form semi-stable interactions with bulky side chains  
319 of the AH.

320

### 321 **Physiological implications of Pil1 lipid binding**

322 To understand the *in vivo* relevance of the lipid binding we observe, we produced mutant yeast  
323 strains that were predicted to affect the binding of Pil1 to different lipid species based on our  
324 structures and MD simulations (Fig 4A, Supp Table 6). Specifically, we tagged endogenous *PIL1*  
325 with *GFPenvy* in an *LSP1* deletion background and additionally introduced mutations to disrupt  
326 PI(4,5)P<sub>2</sub> binding (*pil1*<sup>R126A</sup> and *pil1*<sup>K130A/R133A</sup>), PS binding (*pil1*<sup>R43A</sup> and *pil1*<sup>K66A/R70A</sup>), and both  
327 PI(4,5)P<sub>2</sub> and PS binding (*pil1*<sup>K66A/R70A/K130A/R133A</sup>). We also produced a strain with mutations in the  
328 codons for the aromatic residues from each of the four sterol occupancy peaks observed in our  
329 CG-MD simulations (*pil1*<sup>F33A/Y40A/F42A/F50A</sup>) to disturb sterol localization.

330

331 Eisosome morphology and function was altered in all these mutants (Fig. 4B). Strains expressing  
332 *pil1*<sup>K66A/R70A</sup>, *pil1*<sup>K130A/R133A</sup>, and to a lesser degree *pil1*<sup>R43A</sup>, exhibited fewer eisosomes, but with an  
333 elongated morphology, while the *pil1*<sup>R126A</sup> and *pil1*<sup>K66A/R70A/K130A/R133A</sup> mutants displayed misshapen  
334 eisosomes and partial cytosolic mislocalization of the Pil1 protein. The *pil1*<sup>F33A/Y40A/F42A/F50A</sup> sterol  
335 binding-impaired mutants display unusual rod-like eisosomes that ingress towards the centre of  
336 the cell (Fig 4B, Fig Ext Data 9F). To further probe the role of these lipid binding sites, we chose  
337 to add an endogenous mScarlet-I tag to the eisosome resident protein Nce102. Nce102 re-  
338 localizes from the eisosomes into the bulk membrane (or vice versa) upon exposure to various  
339 membrane stressors including osmotic shocks, sphingolipid synthesis inhibition by the drug  
340 myriocin, and sphingoid base treatment<sup>5,64,65</sup>. Remarkably, all of the lipid binding-impaired  
341 mutants tested show a mislocalization of Nce102 already under steady state conditions, with  
342 nearly no co-localization in the *pil1*<sup>K66A/R70A/K130A/R133A</sup> (PI(4,5)P<sub>2</sub> and PS binding-impaired) and  
343 *pil1*<sup>F33A/Y40A/F42A/F50A</sup> (sterol binding-impaired) mutants (Fig 4C).

344

345 To determine if there are physiological consequences triggered by loss of lipid binding, we  
346 assessed growth of our mutant strains under a variety of stress conditions. All of our Pil1 lipid  
347 binding-impaired mutants exhibited myriocin-resistance (Fig 4D, Fig Ext Data 9G). This is in line  
348 with the previously described role of eisosomes in sphingolipid homeostasis<sup>64,66</sup>, and similar to  
349 what has been previously reported for a mutant lacking Pil1 and all eisosomal tetraspan proteins  
350 of the Nce102 and Sur7 families<sup>67</sup>. Interestingly, we observed that *pil1*<sup>K130A/R133A</sup>, and to a lesser  
351 degree *pil1*<sup>R43A</sup> and *pil1*<sup>K66A/R70A</sup>, grew better at low temperature relative to control. As cold  
352 resistance has been previously described for cells lacking the eisosome-localized PI(4,5)P<sub>2</sub>  
353 phosphatase Inp51, or Inp52<sup>68</sup>, it is possible that this cold resistance could be caused by either  
354 PI(4,5)P<sub>2</sub> dysregulation or Inp51/52 mislocalization.

355

356 We also checked growth with nystatin, an antimycotic that interacts with plasma membrane free  
357 ergosterol to induce cell lysis<sup>69,70</sup>. Strikingly, the sterol binding-impaired *pil1*<sup>F33A/Y40A/F42A/F50A</sup> mutant  
358 and the *pil1*<sup>R126A</sup> and *pil1*<sup>K66A/R70A/K130A/R133A</sup> mutants which exhibited cytosolic mislocalization were  
359 sensitive to nystatin, indicating a higher availability of free ergosterol at the plasma membrane  
360 (Fig 4D, Fig Ext Data 9G). However, none of the mutants showed resistance to the sterol  
361 synthesis inhibitor atorvastatin, indicating the nystatin sensitivity is not due to enhanced ergosterol  
362 synthesis<sup>71</sup>. It is tempting to speculate that that these mutations affect the lipid microdomain

363 generated by Pil1, rendering a normally sequestered population of ergosterol available for nystatin  
364 binding, though it is also possible that a more general disorganization of ergosterol localization  
365 occurs due to eisosome-dependent signaling dysregulation.

366

### 367 **3D variability analysis of native eisosome lattice**

368 With a clear understanding of the identities of the lipids that produce the signatures we see within  
369 the membrane and their functional importance, we returned to the native eisosome dataset to  
370 check for variability in the lipid organization. Using the symmetry expanded/density subtracted  
371 lattice particles, we performed 3D variability analysis. One component of our 3D variability  
372 analysis was particularly striking; we observed conformational flexibility within the Pil1 lattice that  
373 resembled a spring-like stretching and compression at the Nt contact sites (Supp Video 3).  
374 Because the AH is directly connected to this Nt region, we considered whether this stretching  
375 could be transmitted to the lipid bilayer. Remarkably, we could see that changes in the shape and  
376 size of the bound lipid density were synchronized with the stretching of the Nt contact sites.

377

378 To understand the relationship between the Nt stretching and the changes in the lipid density, we  
379 extracted 10 sets of non-overlapping particles along this variability dimension for further  
380 refinement (Fig Ext Data 4E-F, 10A). For these 10 classes, we also analyzed the tubule of origin  
381 for each particle to determine the contribution of tubule diameter to this stretching component.  
382 Particles from every tubule diameter are distributed across all 10 classes; however, particles from  
383 small diameter tubules are over-represented in the more compact classes, while those from large  
384 diameter tubules are over-represented in more stretched classes (Fig Ext Data 10B-C).

385

386 In the deepEMhancer sharpened maps, the putative lipid binding pocket of the most compressed  
387 class contained two clear densities, one triangular density that we could identify as a PI(4,5)P<sub>2</sub>  
388 headgroup with interactions at residues R126, K130, and R133, and the other smaller elongated  
389 density interacting with residue K66, which we had previously assigned as a PS headgroup in the  
390 "+PI(4,5)P<sub>2</sub>+sterol" reconstituted samples (Fig 5A, Fig Ext Data 3G). In contrast, the lipid binding  
391 pocket in the most stretched class was unoccupied in the sharpened maps, suggesting PI(4,5)P<sub>2</sub>  
392 and PS binding are disrupted upon stretching (Fig 5B, Fig Ext Data 3H).

393

394 To further investigate the differences between these classes, we analyzed slices through the  
395 membrane of the unsharpened maps. Strikingly, while the most compressed class retained the

396 pattern of membrane voids beneath the AH, indicating sterol binding, this pattern was blurred in  
397 the most stretched class, despite its slightly higher resolution (Fig 5C-D, Ext Data 10D-G, Supp  
398 Table 1). However, no overall changes in membrane thickness or overall intensity were observed  
399 in radial angle profile plots of these two classes (Fig Ext Data 10H). This suggests that sterols are  
400 mobilized rather than redistributed in the stretched class.

401

## 402 **Discussion**

403 Taken together, our observations provide clear evidence that the eisosome scaffold proteins  
404 Pil1/Lsp1 form a plasma membrane microdomain via their direct interactions with specific lipids.  
405 We believe this work provides an unprecedented level of detail into the organization and dynamics  
406 of plasma membrane lipids within this microdomain. Furthermore, our data allow us to propose a  
407 speculative model for how these eisosome microdomains could sense and respond to mechanical  
408 stress.

409

410 While the Pil1/Lsp1 lattice is capable of self-organization on the membrane surface in the absence  
411 of PI(4,5)P<sub>2</sub>, we find that PI(4,5)P<sub>2</sub> is necessary for the stabilization of the AH within the cytosolic  
412 leaflet. When PI(4,5)P<sub>2</sub> is bound and the AH is inserted, semi-stable interactions of sterols, mostly  
413 with bulky side-chains of the AH, are able to form and the mobility of lipids (and likely the  
414 eisosome-resident proteins, like Nce102) within the membrane microdomain is reduced. Notably,  
415 the stabilized lipid interactions we observe are limited to the cytoplasmic leaflet, demonstrating  
416 clear membrane asymmetry in the intra-leaflet lipid dynamics.

417

418 Under mechanical stress, Pil1/Lsp1 lattice stretching could conceivably be communicated to the  
419 lipids in the cytoplasmic leaflet through the direct connection between the Nt lattice contact sites  
420 and the AH. In our native structures, we see that lattice stretching is correlated with a mobilization  
421 of PI(4,5)P<sub>2</sub> and a loss of the sterol patterning within the cytoplasmic leaflet (Fig 5E), which implies  
422 an increased mobility of all of the lipids within the eisosome. Given that all of our Pil1 lipid binding-  
423 impaired mutants, even those with mild morphology defects, exhibit mislocalization of the tension-  
424 responsive protein Nce102, we propose that this lipid mobilization represents a general  
425 mechanism to free sequestered factors to initiate their membrane stress-sensitive signaling  
426 functions. These sequestered factors need not be proteins; for example, our mutants predicted to  
427 be compromised in sterol coordination are hypersensitive to Nystatin, a macrolide  
428 antibiotic that binds sterols in the plasma membrane leading to cell leakage and death, suggesting

429 that sequestration of sterol at eisosomes can also regulate the amount of free sterol in the  
430 PM. Lastly, although we have presented eisosome lattice stretching as a possible trigger leading  
431 to release of sequestered factors, other membrane perturbations including changes in lipid  
432 dynamics triggered, for example, by thermal shock or amphiphilic toxins, could also be sensed by  
433 this system.

434

435 This remarkable function of AHs to organize the lipids within the membrane and connect  
436 mechanical stretching to the dynamics of those lipids may be a conserved feature of  
437 mechanosensitive proteins. In fact, AHs from many mechanosensitive ion channels, including  
438 Piezo1<sup>72-74</sup>, Piezo2<sup>75</sup>, TREK/TRAAK<sup>76,77</sup> and OSCA1.2<sup>78</sup> have bulky side chains inserted to the  
439 membrane and Piezos, in particular, have been proposed to modulate and be sensitive to the lipid  
440 composition of the membranes in which they function<sup>79-81</sup>.

441

442 Although the eisosomes are a fungi-specific membrane feature, many of the principles of lipid  
443 coordination by BAR domain proteins and other proteins that form lattices on membrane surfaces  
444 are likely to be conserved. For example, caveolae in mammalian cells, scaffolded by the  
445 interaction between caveolins (integral membrane proteins) and cavins (peripheral membrane  
446 proteins) are proposed to concentrate cholesterol, PS, and PI(4,5)P<sub>2</sub><sup>31,82,83</sup>, as well as flatten in  
447 response to sterol removal from the plasma membrane<sup>84</sup>, suggesting that they share many  
448 features with the eisosome membrane microdomain. In fact, the organization and alteration of the  
449 dynamics of membrane lipids through interactions between membrane scaffolding proteins,  
450 charged lipids, and sterols could extend across a wide variety of structurally and functionally  
451 diverse proteins that have been proposed to associate with membrane microdomains, from focal  
452 adhesion proteins<sup>85,86</sup> to ESCRTs<sup>87,88</sup> to myelin-specific proteins<sup>89,90</sup>. Our detailed characterization  
453 of the eisosome membrane microdomain provides a novel context for understanding how protein-  
454 lipid interactions participate in cell signaling functions.

455 *Methods:*

## 456 **Yeast strains**

457 Yeast strains used for endogenous protein purification were constructed using classical  
458 recombination methods. Yeast strains with point mutations were constructed using CRISPR-Cas9  
459 based methods. Strains used in this study are listed in Supplementary Table 6.

460

## 461 **Native eisosome purification**

462 Yeast expressing Bit61-TAP from the endogenous locus were grown to optical density (OD<sub>600</sub>) of  
463 6-8, harvested by centrifugation at 6000rpm for 10 mins, flash frozen in liquid nitrogen, and stored  
464 at -80°C. Cells were lysed by manual grinding with mortar and pestle under liquid nitrogen, then  
465 resuspended by slow rotation at 4°C in 1.5 volumes of extraction buffer (50mM PIPES pH 7,  
466 300mM NaCl, 0.5mM CHAPS, 0.5mM DTT plus 1mM PMSF and 1x Complete protease inhibitor  
467 cocktail (-EDTA) (Roche)). Lysates were cleared by centrifugation at 12000rpm (xg) for 10 mins,  
468 and supernatants were incubated with IgG-coupled Dynabeads M270 (ThermoFisher Scientific)  
469 for 2 hrs at 4°C. Beads were washed 5 times with wash buffer (50mM PIPES pH 7, 300mM NaCl,  
470 1mM CHAPS, 0.5mM DTT) at 4°C, then incubated with TEV protease (0.1 mg/ml) for 1hr at 18°C.  
471 Eluate was collected at 4°C then used immediately for cryoEM grids preparation.

472

## 473 **Plasmids and protein purification**

474 *PIL1* was cloned into pCoofy6 vector (a gift from Sabine Suppmann, Addgene plasmid # 43990)  
475 as described in Scholz et al.<sup>91</sup> (doi: 10.1186/1472-6750-13-12) with following primers: LP1-  
476 Sumo3-Pil1-fwd 5'-gtgtccagcagcagaccggtggaatgcacagaactactctttaag, LP2-ccdB-Pil1-rev 5'-  
477 cccagaacatcaggtaatggcgtaagctgtgtgttggggaag, LP2-ccdB-fwd 5'-cgccattaacctgatgttctggg,  
478 and LP1-Sumo3-rev 5'-tccaccggtctgctgctggaacac. After PCR-amplification using Q5 High-Fidelity  
479 2X Mastermix (#M0492, New England Biolabs Inc.) gel-purified DNA fragments were assembled  
480 using RecA recombinase (#M0249, New England Biolabs Inc.). A plasmid containing mCherry  
481 was a kind gift from Alphée Michelot (Aix-Marseille Université). The mCherry was cloned into the  
482 C-terminus of Pil1 with following primers: LP1-Pil1-linker-mCherry-fwd 5'-  
483 tctctcccaacaacaacagctgagctcgctgcagcaatggtgag, LP2-mCherry-rev 5'-  
484 tgggtgctcgagtgcggccgcaagcctagttccggactgtacagctc, LP1-Pil1-rev 5'-agctgtgtgttggggaagagac,  
485 and LP2-pCoofy-vector-fwd 5'-gcttgccgcccgcactcgagcaccac. The DNA fragments were amplified  
486 and assembled as above.

487



488 Recombinant Pil1 and Pil1-mCherry were expressed in BL21(DE3)pLysS (#200132, Agilent  
489 Technologies Inc.) in auto induction LB medium (#AIMLB0210, Formedium Ltd.) overnight at  
490 20°C. Cells were lysed in lysis buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 2 mM MgAc, 30 mM  
491 imidazole) supplemented with 1% Triton X-100, 1 mM PMSF and cOmplete protease inhibitor  
492 cocktail (#5056489001, Roche) by sonication on ice. Proteins were first purified with HisTrap Fast  
493 Flow column (#GE17-5255-01, Cytiva) in Äkta Pure system (Cytiva) using gradient of imidazole  
494 from 30 mM to 500 mM. Proteins were subsequently dialyzed overnight with 20 mM HEPES, pH  
495 7.4, 75 mM KCl, 2 mM MgAc buffer and further purified with HiTrap Q sepharose HP column  
496 (#17115401, Cytiva). A KCl gradient from 75 mM to 500 mM was used to elute the protein. To  
497 cleave off the Sumo3 tag, SenP2 protease in final concentration of 30  $\mu\text{g/ml}$  was then added and  
498 protein were dialyzed with 20 mM HEPES, pH 7.4, 150 mM KoAc, 2 mM MgAc buffer overnight.  
499 Finally, protein samples were cleaned with Superdex 200 Increase 10/300 GL column (#GE28-  
500 9909-44, Cytiva) equilibrated with 20 mM HEPES, pH 7.4, 150 mM KoAc, 2 mM MgAc buffer.  
501 Proteins were concentrated to 20-25  $\mu\text{M}$ , snap-frozen with liquid nitrogen and stored in -80°C.

502

### 503 **Reconstitution of Pil1 tubules**

504 Lipids used are listed in Supplemental Table 3. To reconstitute Pil1 tubules using large unilamellar  
505 vesicles (LUVs) for cryoEM, lipids were mixed in chloroform to final concentration of 3.8 mM with  
506 desired molar ratios. Chloroform was evaporated under argon gas flow and subsequently for three  
507 hours in 30°C vacuum oven. A lipid film was hydrated in reaction buffer (20 mM HEPES, pH 7.4,  
508 150 mM KoAc, 2 mM MgAc), subjected to 10 cycles of freeze-thaw and extruded through a 200  
509 nm pore-sized polycarbonate filter (Cytiva Inc.) using a mini-extruder (Avanti Polar Lipids Inc.).  
510 Lipid compositions used in cryoEM studies are listed in Supplemental Table 4. To produce  
511 samples for cryoEM studies, a mixture of 15-20 $\mu\text{M}$  recombinant Pil1 and  $\sim 2\text{mg/mL}$  LUVs were  
512 incubated at 30°C for 1hr before freezing.

513

514 To reconstitute Pil1 tubules on pre-formed membrane nanotubes for fluorescence microscopy  
515 experiments, supported lipid films over silica beads were formed from multilamellar vesicles by  
516 mixing lipids in chloroform to final concentration of 1 mg/ml and evaporating the solvent as  
517 described previously<sup>92</sup>. Briefly, lipid films were hydrated using 5 mM HEPES, pH 7.4 buffer.  
518 Multilamellar vesicles were then mixed with silicon dioxide microspheres (Corpuscular #140256-  
519 10 or Sigma-Aldrich #904384) and dried for 30 minutes in 30°C vacuum oven. Imaging chamber  
520 was prepared by attaching a sticky-Slide VI 0.4 (Ibidi GmbH, #80608) on a 24 x 60 mm

521 microscope cover glass. Sample chambers were passivated for 10 minutes with 2 g/l bovine  
522 serum albumin solution and subsequently washed several times with reaction buffer (20 mM  
523 HEPES, pH 7.4, 150 mM KoAc, 2 mM MgAc). Lipid-coated silica beads were then hydrated by  
524 adding a small amount of beads in the sample chamber and allowing beads to roll through the  
525 chamber. Several different lipid compositions were used in these experiments (See Supplemental  
526 Table 4). 0.01 mol% of Atto647N DOPE was added to each lipid mixture to visualize nanotubes  
527 and as the reference fluorescent lipid for lipid sorting coefficient measurements.

528

### 529 **CryoEM grid preparation and data collection**

530 5ul of fresh sample was applied to untreated Lacey Carbon film on copper mesh grids (Jena  
531 Bioscience #X-170-CU400), blotted for 3-4s, then re-applied, blotted for 2-4s (2<sup>nd</sup> blot), and finally  
532 plunge-frozen in a Leica GP2 plunge system at 18°C, 90% humidity.

533

534 Native eisosome filaments were imaged by targeted acquisition using SerialEM with a 300kV  
535 Titan Krios fitted with a Gatan K2 Quantum direct electron detector (Heidelberg). A total of 2827  
536 movies were collected, each with a total dose of 40 e-/Å<sup>2</sup>, a target defocus range of -0.8 to -1.8  
537 μm and a pixel size of 1.327Å (105kx magnification).

538

539 Reconstituted Pil1 filaments were imaged using EPU software with a 300kV Titan Krios and a  
540 Falcon 4 direct detector (DCI Lausanne). Three datasets were collected: 1) Pil1 + “minus  
541 PI(4,5)P2” liposomes (21,386 movies), 2. Pil1 + “minus cholesterol” liposomes (22,960 movies),  
542 and 3) Pil1 + “PI(4,5)P2/cholesterol” liposomes (22,408 movies). For each movie, a total dose of  
543 50 e-/Å<sup>2</sup>, a target defocus range of -0.6 to -1.8 μm and a pixel size of 0.83Å (96,000x  
544 magnification) was used.

545

### 546 **CryoEM data processing**

547 The pipeline for cryoEM data processing is outlined in Extended Data Figures 1,2, and 7.

548

549 For native eisosomes, movies were aligned in using MotionCor2<sup>93</sup> and CTF correction was  
550 completed using Gctf<sup>94</sup>. Filaments were handpicked using manual picking in RELION v2.1.0. 2D  
551 classification was run iteratively in RELION 2.1. For particles in each clean RELION 2D class,  
552 power spectra were summed by class, then manually sorted into identical “types”. Helixplorer-1<sup>95</sup>  
553 was used to estimate helical parameters, which were used with particles from each “type” for 3D

554 auto-refinement with helical parameters in RELION. All helix types were then corrected for  
555 handedness and aligned along the D symmetry axis (using C symmetry worsened resolution). A  
556 mask was generated in RELION covering the central third of the helix and a final round of helical  
557 refinement was completed either with the mask (to optimize resolution) or unmasked (to be used  
558 for particle subtraction). Resolution estimates for masked maps are based on gold standard FSC  
559 values with a 0.143 cutoff on post-processed maps with the one-third mask used for refinement,  
560 a manually chosen initial threshold, and auto-bfactor calculation.

561  
562 Symmetry expansion and density subtraction on native filaments was completed with unmasked  
563 maps from the final iteration of 3D auto-refinement. Helical parameters for each helix type were  
564 used for symmetry expansion but using C symmetry instead of D symmetry. A mask for density  
565 subtraction was generated in Chimera v1.5 by the addition of two zone maps: 1) an 8Å zone using  
566 models of a central Pil1/Lsp1 dimer and the six dimers with which it shares lattice contact sites  
567 and 2) a spherical zone of 60Å centered on the amphipathic helices of the Pil1/Lsp1 dimer. This  
568 initial mask was then extended with a soft edge, then used for density subtraction and reboxing  
569 of the particles in RELION v3.1.3. Subtracted particles were then used to reconstruct a volume  
570 and particles for all helix “types” and the reconstructed volume were imported into cryoSPARC  
571 v4.1.2 for further processing. Homogenous refinement was completed with all particles with the  
572 reconstructed volume as the initial model using C2 symmetry. This map was used for refinement  
573 of the Pil1 and Lsp1 native models (See “Model building’ for details). This map was then symmetry  
574 expanded in C2 for 3D variability analysis. 3D variability analysis was completed with 5  
575 components requested. Manual inspection revealed one component that exhibited lattice  
576 stretching. This component was used for 3D variability display in intermediate mode with 10 non-  
577 overlapping frames used to generate particle subsets. Each particle subset was then used for  
578 masked local refinement with a mask covering the central dimer (generated in Chimera using the  
579 Pil1/Lsp1 dimer with a 10Å zone and extended with a soft edge in RELION). These maps were  
580 used for refinement of the Pil1 compact and stretched (native) models (See “Model building’ for  
581 details). Sharpening with deepEMhancer<sup>96</sup> was used to improve the resolution of lipid headgroups  
582 in the lipid binding pocket. Resolution estimates are based on gold standard FSC values with a  
583 0.143 cutoff using an optimized mask automatically generated during refinement.

584  
585 For reconstituted Pil1 filaments, all data processing was completed in cryoSPARC v4.1.2. Movies  
586 were processed on-the-fly with CryoSPARC Live v3.2.2 , using patch motion correction and patch

587 CTF estimation. Filaments were picked using Filament Tracer, then cleaned and sorted using  
588 iterative rounds of 2D classification. Clean classes were used to calculate average power spectra,  
589 which were then manually sorted into identical “types”. Helixplorer-1 was used to estimate helical  
590 parameters, which were used with particles from each “type” for helical refinement. After initial  
591 refinement all helix types were corrected for handedness and aligned along the D symmetry axis.  
592 A mask on the central third of the helix was created in Relion v3.1.3 and used for a final round of  
593 helical refinement. These maps were used for refinement of the Pil1 lattice (-PIP2/+sterol  
594 reconstituted), Pil1 lattice (+PIP2/-sterol reconstituted), and Pil1 lattice (+PIP2/+sterol  
595 reconstituted) models (See “Model building” for details). Resolution estimates are based on gold  
596 standard FSC values with a 0.143 cutoff using an optimized mask automatically generated during  
597 refinement.

598  
599 Figures were made in Chimera v1.16 or Chimera-X v1.5. Parallel slice images were made in FIJI  
600 using the Reslice tool without interpolation. 3D intensity plots were made in FIJI using the 3D  
601 surface plot tool on the slice with maximum sterol void intensity using identical display conditions  
602 on both maps. Radial angle profile plots were made in FIJI using the Radial Profile Extended  
603 plugin with an angle of 40 degrees, corresponding to the size of the sphere used in density  
604 subtraction to include the lipid density under the central dimer.

605

### 606 **Model building**

607 Structure predictions for Pil1 and Lsp1 from the AlphaFold database (<https://alphafold.ebi.ac.uk/>)  
608 were used as starting models, with the C-terminal region removed, starting from residue 275, for  
609 which no density was observed. Iterative rounds of model building, performed in Coot v.0.8.9.2,  
610 and real-space refinement, performed with Phenix-1.20-4459, were completed until no  
611 improvement in the model was observed. The model quality and fit to density were performed  
612 using Molprobit and Phenix-1.20-4459.

613

614 Ligand constraints for inositol 2,4,5-triphosphate and the phospho-serine were produced using  
615 phenix.elbow. Refinements with ligands were performed with these ligand constraints. For lipid  
616 headgroup ligands in native eisosome compact dimer model, ligands refined in the Pil1  
617 “+PI(4,5)P2/+sterol” reconstituted map were placed in the in the deepEMhancer sharpened native  
618 eisosome compact map, then adjusted in Coot with rigid body fitting.

619

620 An electrostatic potential map of the model surface was calculated using the Coulombic potential  
621 function of ChimeraX v1.5.

622

### 623 **Lipid diffusion measurements with Fluorescence Recovery After Photobleaching (FRAP)**

624 Lipid nanotubes were prepared as described above and 200 nM of Pil1-mCherry was incubated  
625 with nanotubes for 30 minutes. FRAP experiments were performed with Olympus IX83 wide-field  
626 microscope equipped with an Olympus Uapo N 100x 1.49 oil objective and an ImageEM X2 EM-  
627 CCD camera (Hamamatsu). The system was controlled by the Visiview software (Visitron  
628 Systems GmbH). Five frames were captured before a small protein-coated region was bleached.  
629 Subsequently, the recovery of fluorescence intensity was measured by capturing images every  
630 500 ms for 1-2 minutes. The FRAP data was analyzed with Fiji/ImageJ by measuring intensity  
631 over time from the bleached region (region-of-interest), the region on nanotube outside the  
632 photobleached region (bleaching correction) and the region outside nanotube (background). After  
633 background subtraction and bleaching correction, the data for normalized to the intensity values  
634 before photobleaching. Graphs were generated with Origin Pro 2022 (OriginLab Corp.). To  
635 calculate recovery halftimes and mobile fractions, we used either one-phase exponential  
636 equation:

$$637 \quad y = A1 \times e^{t/\tau} + y0$$

638 or two-phase exponential equation:

$$639 \quad y = A1 \times e^{t/\tau1} + A2 \times e^{t/\tau2} + y0$$

640 where A is value of y in plateau, t is time,  $\tau$  is a time constant and y0 is the value of y when t=0.

641

642 Recovery halftimes were then calculated with equation:

$$643 \quad \text{halftime} = \tau \times \ln(2)$$

644

### 645 **Lipid sorting coefficient measurements.**

646 Lipid nanotubes were prepared as described earlier and incubated with 200-400 nM of Pil1-  
647 mCherry for 30 minutes until protein scaffolds were formed and visible by fluorescence  
648 microscopy. Imaging was performed using an inverted spinning disk microscope assembled by 3i  
649 (Intelligent Imaging Innovation) consisting of a Nikon Eclipse C1 base, a 100x 1.3 NA oil  
650 immersion objective. Sorting coefficients were calculated for each of the mentioned lipids using  
651 Atto 647N DOPE as the reference lipid with the following equation:

$$652 \quad \text{Sorting coefficient} = \frac{(F_{\text{Tested lipid}}/F_{\text{Atto 647N DOPE}}) \text{ under Pil1}}{(F_{\text{Tested lipid}}/F_{\text{Atto 647N DOPE}}) \text{ bare lipid nanotube}}$$

653

654 where  $F_{\text{Tested lipid}}$  and  $F_{\text{Atto 647N DOPE}}$  are the integrated fluorescence densities of the lipid problem  
655 and lipid reference integrated from the lipid nanotube plot profiles (See Fig Ext Data 9D) after  
656 background subtraction and neglecting the polarization factor<sup>97</sup>. Data and graphs were generated  
657 using Origin Pro 2022 (OriginLab Corp.).

658

### 659 **Molecular dynamics simulations**

660 In accordance with the experimental models, three systems with different lipid compositions were  
661 modelled and simulated. Coarse grained (CG) molecular dynamics (MD) simulations of Pil1 tubule  
662 interacting with the tubule membranes were performed, in duplicates.

663

664 The tubule membranes were built using the BUMPY Software<sup>98</sup>. The systems, without the protein,  
665 were solvated with water and minimized using the steepest descent algorithm<sup>99</sup>. Four equilibration  
666 steps were performed, as follows: i) A first equilibration with a timestep of 5fs was run for 10ns,  
667 imposing position restraints with a force constant (fc) of 300kj/mol/nm<sup>2</sup> on the lipid tails, to allow  
668 formation of membrane pores to equilibrate lipid and water content between the tubule lumen and  
669 the external region. 2) A second equilibration step of 5ns was performed using the previous  
670 settings but increasing the fc at 500kj/mol/nm<sup>2</sup> and the timestep to 10fs. 3) A third equilibration  
671 step was run for additional 10ns after removal of position restraints to allow the closure of the  
672 pores. The Berendsen barostat and the v-rescale thermostat<sup>100,101</sup> - with a temperature of 303K -  
673 were used.

674

675 From the CryoEM structure of Pil1 tubule (21 dimers), CG mapping was performed using  
676 Martinize2<sup>102</sup>, imposing an elastic network within the dimers. The CG protein model was then  
677 manually positioned around the tubule membrane using VMD v1.9<sup>103</sup>. The final system was  
678 solvated with water beads and neutralized adding Na<sup>+</sup> and Cl<sup>-</sup> ions. Each system was minimized  
679 and equilibrated in seven steps: 1) A first equilibration with a timestep of 5fs was run for 10ns,  
680 imposing position restraints with a force constant (fc) of 300kj/mol/nm<sup>2</sup> on the lipid tails, to allow  
681 waterpore formation. The Berendsen barostat<sup>100</sup> was applied to all the direction, with  $\tau_p=5$ , and  
682 the v-rescale thermostat was used, setting the temperature at 303K<sup>101</sup>. 2) A second equilibration  
683 step of 5ns was performed using the previous setting but increasing the fc at 500kj/mol/nm<sup>2</sup>, and

684 the timestep at 10fs. 3) A third equilibration step was run for other 10ns increasing the fc at  
685 1000kj/mol/nm<sup>2</sup>, to maintain the waterpores open and to allow for the solvent equilibration. 4)  
686 Starting from the 4th step, the fc on the tails was progressively reduced to slowly induce a slowly  
687 closure of the pores. Finally, a fc of 500kj/mol/nm<sup>2</sup> was applied, to run an equilibration of 5ns. 5)  
688 An equilibration decreasing the fc at 300kj/mol/nm<sup>2</sup> was carried out for other 5 ns. 6) An  
689 equilibration removing the fc on the lipid tails was performed to allow the complete closure of  
690 waterpores. 7) A final equilibration step of 10ns was run without restraints, increasing the  
691 timesteps to 20fs.

692  
693 For systems containing cholesterol the equilibration procedure was extended including an  
694 additional first equilibration step, with a reduced timestep of 2fs.

695  
696 For production, the Parrinello-Rahman barostat<sup>104</sup> was used with  $\tau_p=12$ . For each system, two  
697 replicates of approximately 10 $\mu$ s were performed. The simulations were carried out using the  
698 GROMACS Software v2021.5<sup>99</sup> and the Martini3 force field<sup>105,106</sup>.

699  
700 To compute the lipids' occupancy, the PyLipID python package was used<sup>107</sup>. The analysis was  
701 performed selecting the head groups of lipids. The values were averaged over time and over the  
702 dimers. To investigate differences in the insertion of the amphipathic helices based on the  
703 different lipid membrane composition, the distances between the centre of mass (COM) of the  
704 amphipathic helices and the head groups of the lipids were calculated using gmx mindist tool of  
705 GROMACS 2021.5 Software<sup>99</sup>.

## 706 707 **Spot assays**

708 Saturated overnight yeast cultures (30°C, SC medium) were diluted to OD<sub>600</sub> 0.1 in the morning  
709 and grown into mid log phase (OD<sub>600</sub> 0.5-0.8). Log phase cells were diluted to OD<sub>600</sub> 0.1, and a  
710 10-fold dilution series was spotted onto pre-dried SC media plates containing treatment  
711 substances, or vehicle. Plates were incubated at 30°C, except low (15°C) temperature plates, and  
712 imaged when differences were most apparent (typically after 24h for Nystatin, 48h for controls  
713 and Atorvastatin, 144h for myriocin, and 168h for 15°C). Substance stocks used in this study:  
714 Myriocin (Sigma M1177) 2.5mM in MeOH, Nystatin (Sigma 475914) 50mM in DMSO, Atorvastatin  
715 (Sigma PHR1422) 20mM in DMSO.

716

## 717 **Fluorescence microscopy and image evaluation**

718 Logarithmically growing overnight yeast cultures (30°C, SC medium) were diluted and grown to  
719 OD<sub>600</sub> 0.6. For Fluorescence live cell microscopy, cells were loaded into a Concanavalin coated  
720 flow chamber (ibidi  $\mu$ -Slides VI 0.4 ibi Treat). Microscopy was performed at room temperature  
721 with a ZEISS LSM 980 microscope with Airyscan 2, using a 63x 1.4 NA oil immersion Objective.  
722 Images were taken as z-series to generate 2D SUM projections. For determining colocalisation  
723 between Pil1-GFP and Nce102-mScarlet-I, cells were first segmented based on Nce102-  
724 mScarlet-I signal using Cellpose<sup>108</sup>. Segmented cells that were intersected by the image borders,  
725 and cells that featured Nce102-Scarlet signal below the fixed threshold (thresholded area = 0) for  
726 calculating Manders' colocalization coefficient M1 (fraction of Nce102-Scarlet overlapping with  
727 Pil1-GFP) were excluded. Manders' coefficients of single cells were obtained by analysis of 3D  
728 stacks in Fiji, using the BIOP version of the JACoP plugin<sup>109</sup> with fixed manual thresholds for Pil1-  
729 GFP and Nce102-mScarlet-I, and graphs were generated with Origin Pro 2022 (OriginLab Corp.).

730

## 731 **Statistics and reproducibility**

732 For MD simulations, the sample size was determined empirically, considering the time necessary  
733 for equilibration of the lipids. For our systems that is about 10 microseconds. Two replicas were  
734 performed for each lipid system. The FRAP data is combined from three individual experiments  
735 with individual measurements from these experiments pooled together for the analysis. The raw  
736 data is available upon reasonable request. For lipid sorting co-efficients, in all conditions N=2.  
737 Statistical significance was determined with Tukey's HSD test following one-way ANOVA  
738 assuming normal distribution. Box plots elements are defined as follows: Center line is the  
739 median, box limits are 25% to 75% lower and upper quartiles, small square box inside the box  
740 limits is the mean, whiskers are the range within 1.5 IQR, circular points are datapoints, and points  
741 outside the 1.5 IQR are outliers. Yeast growth assays and microscopy were repeated at least  
742 three times on different days, yielding similar results. For calculating Manders' overlap coefficient,  
743 microscopy data from several days was pooled to analyze at least 100 cells per mutant.

744

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766

#### 767 **Author contributions**

768 J.M.K. and R.L. designed the project. J.M.K. designed experiments, processed EM data,  
769 built/refined structural models, interpreted results, and prepared manuscript. M.H. prepared  
770 reconstituted samples and designed and interpreted FRAP experiments. L.X. optimized/prepared  
771 native samples, produced CRISPR mutants. J.A. designed and interpreted MD simulations.  
772 J.E.M. designed and interpreted lipid sorting experiments. M.G.T. performed and interpreted  
773 growth assays and fluorescence microscopy. L.F.E. coded tools to assist in sorting native  
774 eisosome data. R.L. supervised the project, S.V. supervised MD simulations, A.R. supervised *in*  
775 *vitro* reconstitution studies, A.D. supervised EM data processing. All authors discussed the results  
776 and commented on the manuscript.

777

#### 778 **Competing interests**

779 The authors declare no competing interests.

780

#### 781 **Data availability**

782 Sharpened maps used for refinement and all associated helical maps and deepEMhancer  
783 sharpened maps have been deposited in the Electron Microscopy Data Bank under the following  
784 accession codes: Eisosome native (EMD-18307), Pil1 -PIP2/+sterol reconstituted (EMD-18308),  
785 Pil1 +PIP2/-sterol reconstituted (EMD-18309), Pil1 +PIP2/+sterol reconstituted (EMD-18310),  
786 Eisosome native compact (EMD-18311), Eisosome native stretched (EMD-18312).

787

788 Models have been deposited in the Protein Data Bank: Pil1 lattice (native) (PDB 8QB7), Lsp1  
789 lattice (native) (PDB 8QB8), Pil1 lattice (-PIP2/+sterol reconstituted) (PDB 8QB9), Pil1 lattice  
790 (+PIP2/-sterol reconstituted) (PDB 8QBB), Pil1 lattice (+PIP2/+sterol reconstituted) (PDB 8QBD),  
791 Pil1 lattice compact (native) (PDB 8QBE), Pil1 dimer compact with lipid headgroups (native) (PDB  
792 8QBF), and Pil1 lattice stretched (native) (PDB 8QBG).

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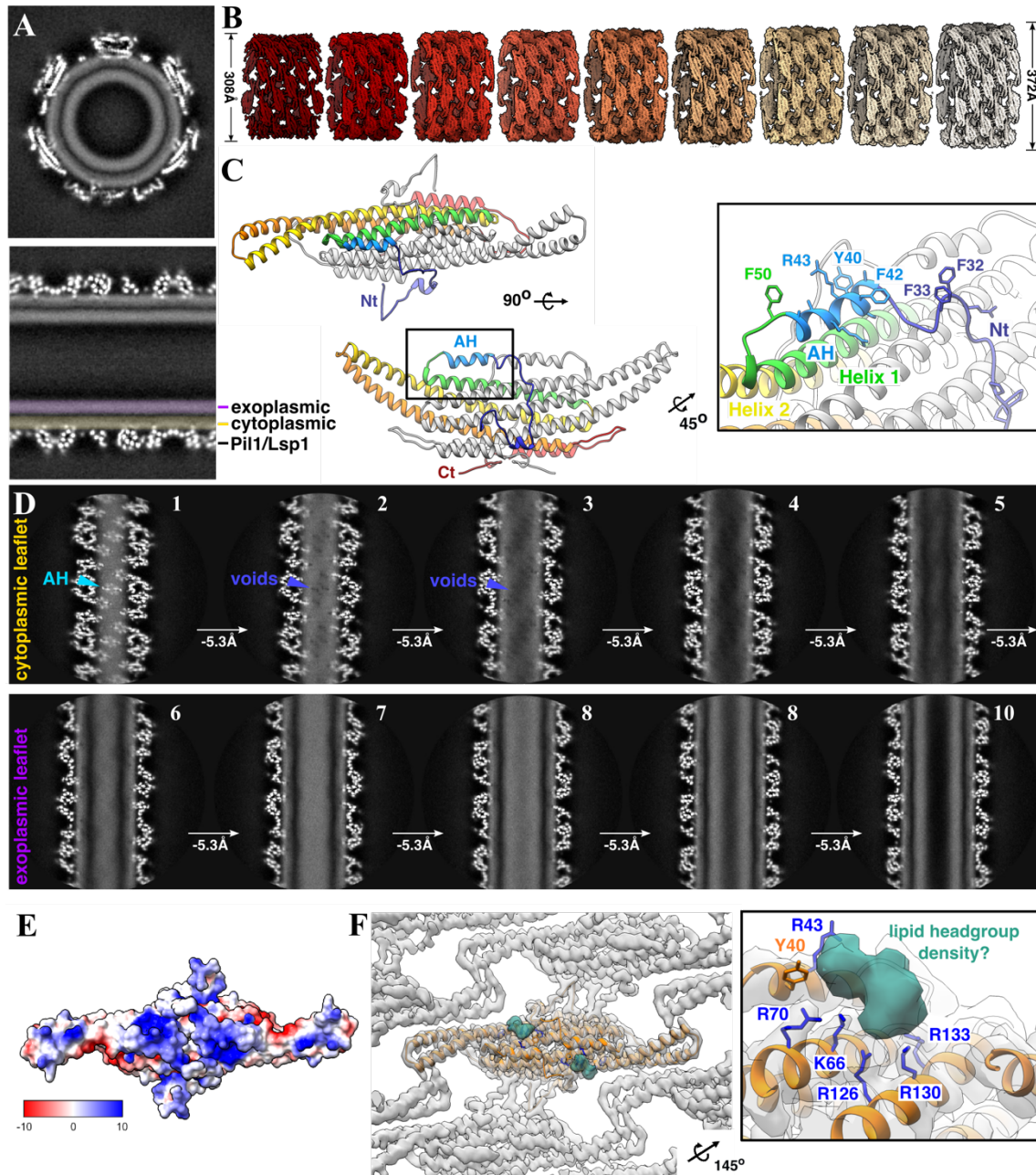
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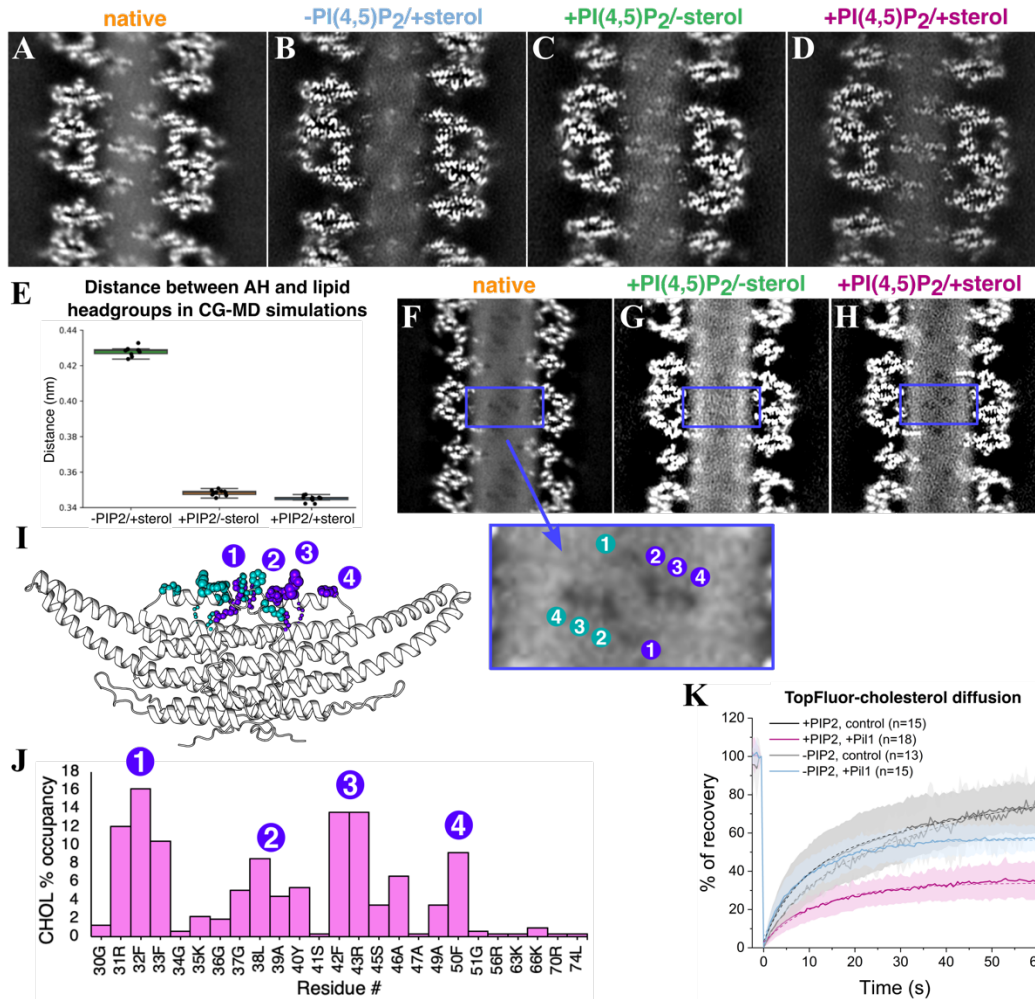
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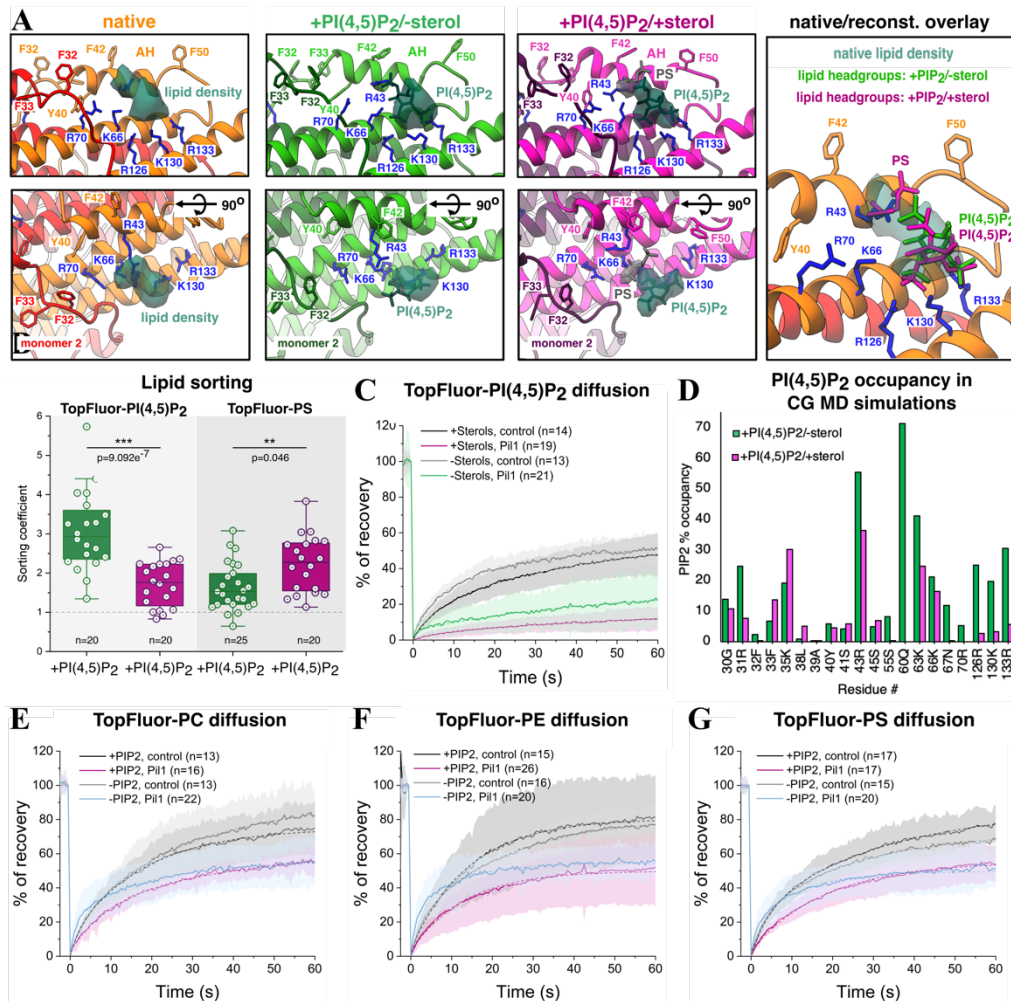


1050 **Figure 1. Native-like eisosomes retain unperturbed plasma membrane microdomain.** **A.** Central  
 1051 (“transverse” above and “sagittal” below) slices of helical reconstruction of a native eisosome with  
 1052 membrane bilayer visible. **B.** Sharpened maps of 9 helical structures of varying diameter. **C.** Model of Pil1  
 1053 dimer, rainbow coloring on chain A from Nt (blue) to Ct (red). [inset] Zoom on AH. **D.** Series of one-pixel  
 1054 slices through helical reconstruction of native eisosome, separated by  $\sim 5.3\text{\AA}$  depth. Cyan arrow indicates  
 1055 AH (panel 1), violet arrow indicates membrane voids (panels 2-3). Void pattern continues through  
 1056 cytoplasmic leaflet (top panels 1-5) but is absent in exoplasmic leaflet (bottom panels 6-10). **E.** Electrostatic  
 1057 surface prediction of Pil1 model. **F.** Unassigned putative lipid density (sea green) in deepEMhancer  
 1058 sharpened map localized to charged pocket. [inset] Charged residues coordinating unassigned density.

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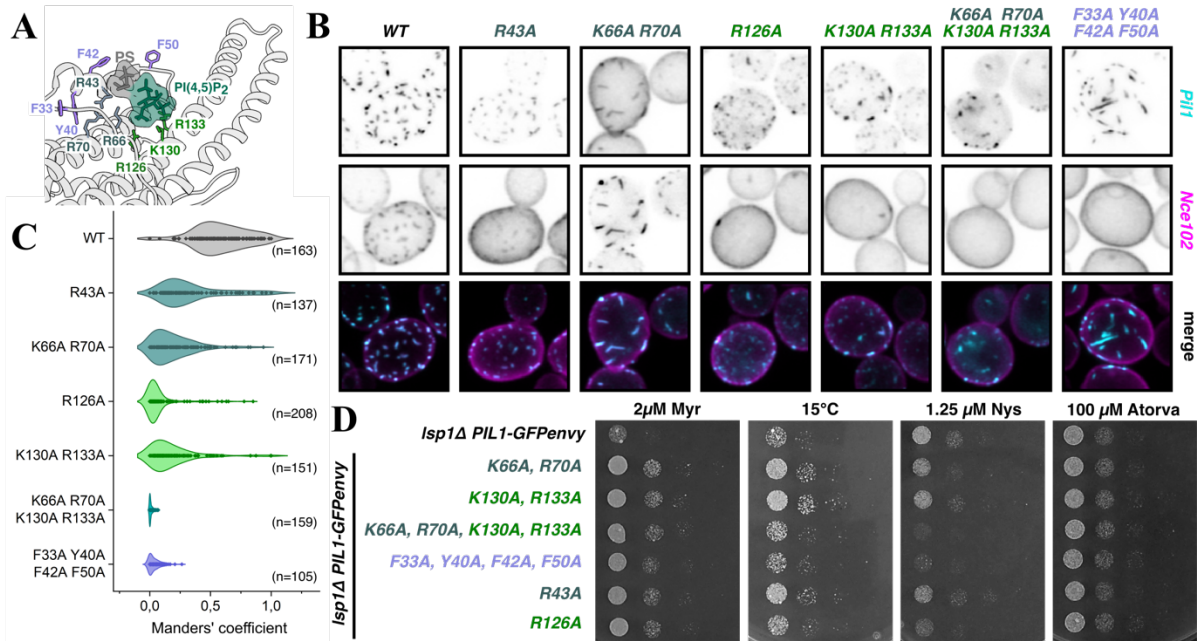


1060 **Figure 2. Sterols are stabilized by the Pil1/Lsp1 amphipathic helix within the eisosome membrane**  
 1061 **microdomain. A-D.** Parallel slice at maximum amphipathic helix density of unsharpened maps of native  
 1062 (A), “-PI(4,5)P<sub>2</sub>/+sterol” reconstituted (B), “+PI(4,5)P<sub>2</sub>/-sterol” reconstituted (C), and “+PI(4,5)P<sub>2</sub>/+sterol”  
 1063 reconstituted (D) eisosomes. **E.** Distance between the center of mass of the AH and lipids’ head groups in  
 1064 CG simulations of different lipid compositions. **F-H.** Membrane voids pattern within the cytoplasmic leaflet  
 1065 in native (F), “+PI(4,5)P<sub>2</sub>/-sterol” reconstituted (G), and “+PI(4,5)P<sub>2</sub>/+sterol” reconstituted eisosomes (H). **I.**  
 1066 CG-MD snapshot showing AH-cholesterol interactions. Inset image: membrane voids with numbers  
 1067 indicating individual sterol dwell sites **J.** Occupancy of cholesterol at amphipathic helix residues in  
 1068 +PI(4,5)P<sub>2</sub>/+sterol system in CG-MD simulations. **K.** FRAP of TopFluor-cholesterol in control samples  
 1069 without protein and with Pil1 in presence and absence of 1% PI(4,5)P<sub>2</sub>. Solid lines indicate a mean of n  
 1070 number of measured nanotubes with standard deviation shown. Dashed lines indicate the fitted data.



1071 **Figure 3. Reconstitution of purified Pil1 with lipids of known composition enables identification of**  
 1072 **structural signatures in native membrane.** **A.** Lipid headgroup densities (sea green for PI(4,5)P<sub>2</sub>, grey  
 1073 for PS) in lipid binding pockets of native (orange), “+PI(4,5)P<sub>2</sub>/-sterol” reconstituted (lime green), and  
 1074 “+PI(4,5)P<sub>2</sub>/+sterol” reconstituted (magenta) eisosome maps. Rightmost panel: overlay of native  
 1075 eisosome density with fitted model of IP<sub>3</sub> in “+PI(4,5)P<sub>2</sub>/-sterol” reconstituted structure (lipid headgroups  
 1076 indicated in lime green) and IP<sub>3</sub> plus phospho-serine of “+PI(4,5)P<sub>2</sub>/+sterol” structure (lipid headgroups  
 1077 indicated in magenta). **B.** Lipid sorting co-efficients of PI(4,5)P<sub>2</sub> and DOPS in “+1% PI(4,5)P<sub>2</sub>/-sterol” and  
 1078 “+1% PI(4,5)P<sub>2</sub>/+sterol” reconstituted Pil1 tubules. [Statistical significance: Tukey’s HSD test following  
 1079 one-way ANOVA assuming normal distribution, \*\*\**p* < 0.001, \*\**p* < 0.01, N=2]. **C.** FRAP of TopFluor-  
 1080 PI(4,5)P<sub>2</sub> in “+1% PI(4,5)P<sub>2</sub>/-sterol” and “+1% PI(4,5)P<sub>2</sub>/+sterol” reconstituted Pil1 tubules. **D.** PI(4,5)P<sub>2</sub>  
 1081 lipid occupancy for residues <5Å from PI(4,5)P<sub>2</sub> headgroup with >5% occupancy in CG-MD simulations.  
 1082 **E-G.** FRAP of TopFluor-PC (**E**), TopFluor-PE (**F**) and TopFluor-PS (**G**) in control samples without protein  
 1083 and with Pil1 in “+1% PI(4,5)P<sub>2</sub>/-sterol” and “+1% PI(4,5)P<sub>2</sub>/+sterol” lipid nanotubes. Solid lines indicate a  
 1084 mean of n number of measured nanotubes with standard deviation shown. Dashed lines indicate the fitted  
 1085 data.

1086



1087

1088 **Figure 4. Lipid binding-impaired mutants affect morphology and function of eisosomes *in vivo*. A.**

1089 A cartoon of the lipid binding pocket in the native sample. Sterol binding residues in violet, proposed PS

1090 binding residues in grey, PI(4,5)P<sub>2</sub> binding residues in sea green. **B.** Eisosome morphology in *Isp1Δ* yeast

1091 expressing, from their endogenous locus, Pil1-GFP with indicated lipid-binding mutations and Nce102-

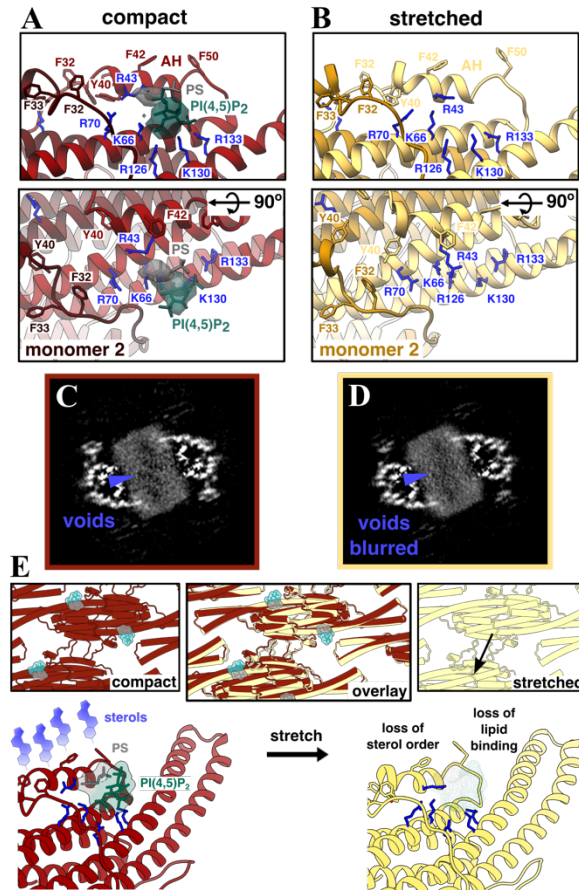
1092 mScarlet-I (summed stacks). Merged represents summed stacks of Pil1-GFP (cyan) and mScarlet-I

1093 (magenta) signals **C.** Manders' thresholded fraction of Nce102-mScarlet-I that colocalizes with Pil1-GFP

1094 lipid binding-impaired mutants in single cells (Manders' M1 colocalization coefficient). The shaded area

1095 represents the probability for datapoints of the population to take on this value. **D.** Growth assays of *Isp1Δ*

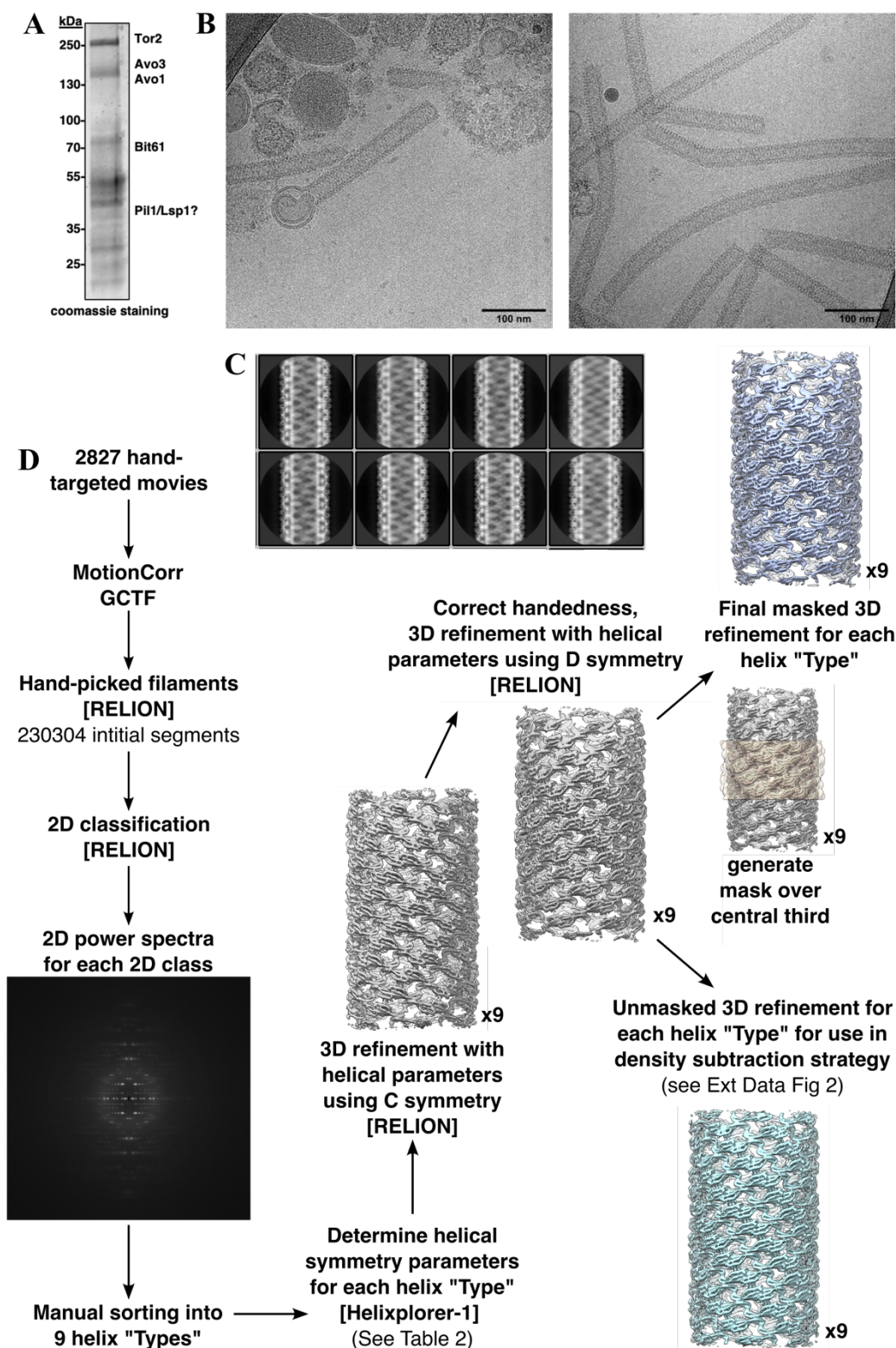
1096 yeast expressing Pil1-GFP lipid binding-impaired mutants. Myr: myriocin, Nys: nystatin, Atorv: atorvastatin.



1097

1098 **Figure 5. 3D variability analysis reveals Pii1/Lsp1 lattice stretching and its effects on membrane**  
 1099 **organization within native eisosomes.** **A.** Well-defined two-part density (sea green for PI(4,5)P<sub>2</sub>, grey  
 1100 for PS) occupying charged pocket in deepEMhancer sharpened map of most compact lattice conformation  
 1101 (model in dark red). **B.** No clear density observed in charged pocket in sharpened map of most stretched  
 1102 conformation (model in light yellow). **C-D.** Membrane void pattern, or lack thereof, in corresponding slices  
 1103 of unsharpened maps of most compact (**C**) and most stretched (**D**) conformation. **E.** Model illustrating  
 1104 membrane stretching destabilizes lipid headgroup and sterol binding (compact model in dark red, stretched  
 1105 model in light yellow, PI(4,5)P<sub>2</sub> headgroup in sea green, PS headgroup in grey, sterols in violet).

## Ext Data Fig 1

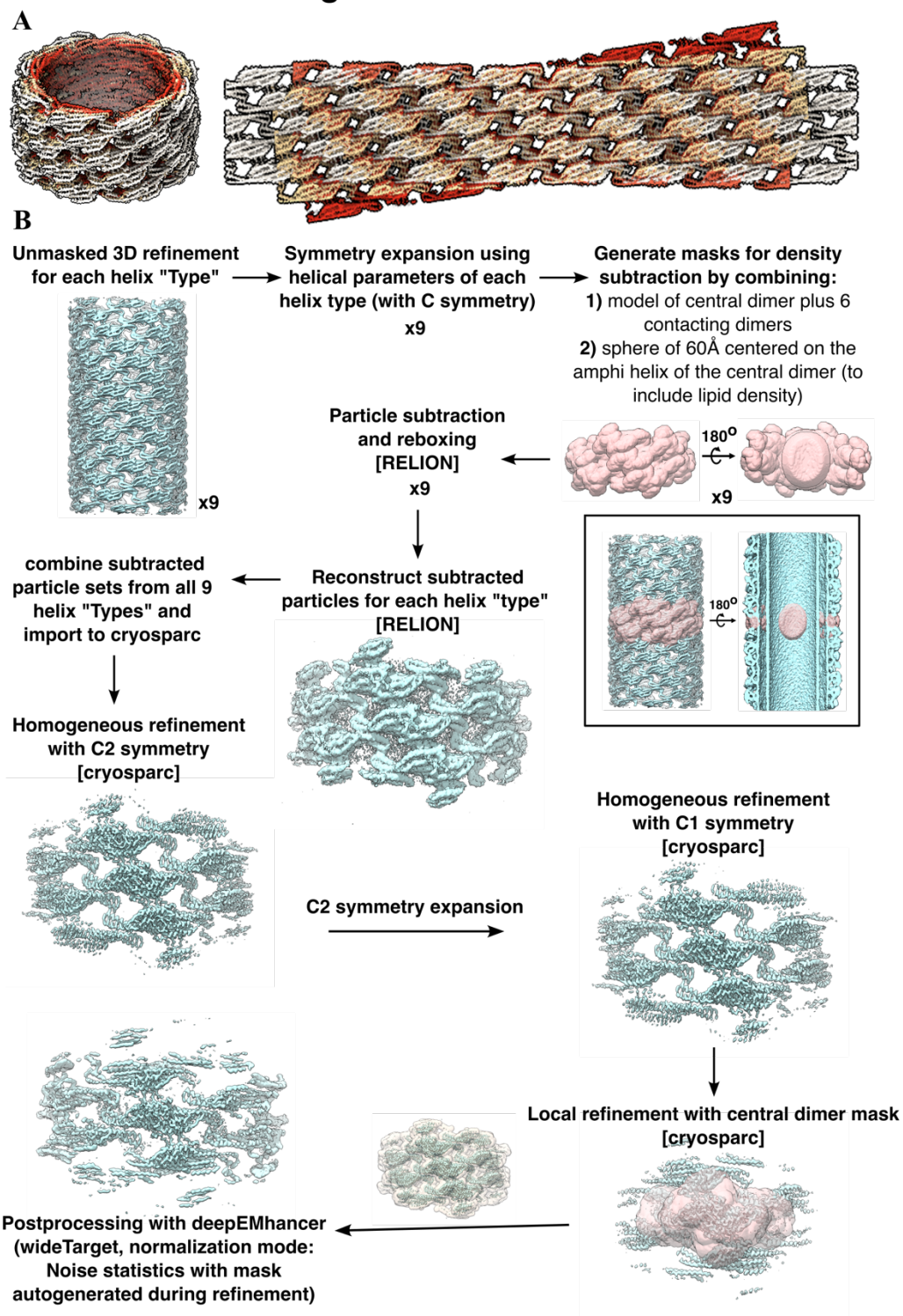


**Figure Extended Data 1. CryoEM data processing of native eisosome filaments. A.** Coomassie staining of protein gel of Bit61-TAP purification of eisosome filaments. **B.** Raw



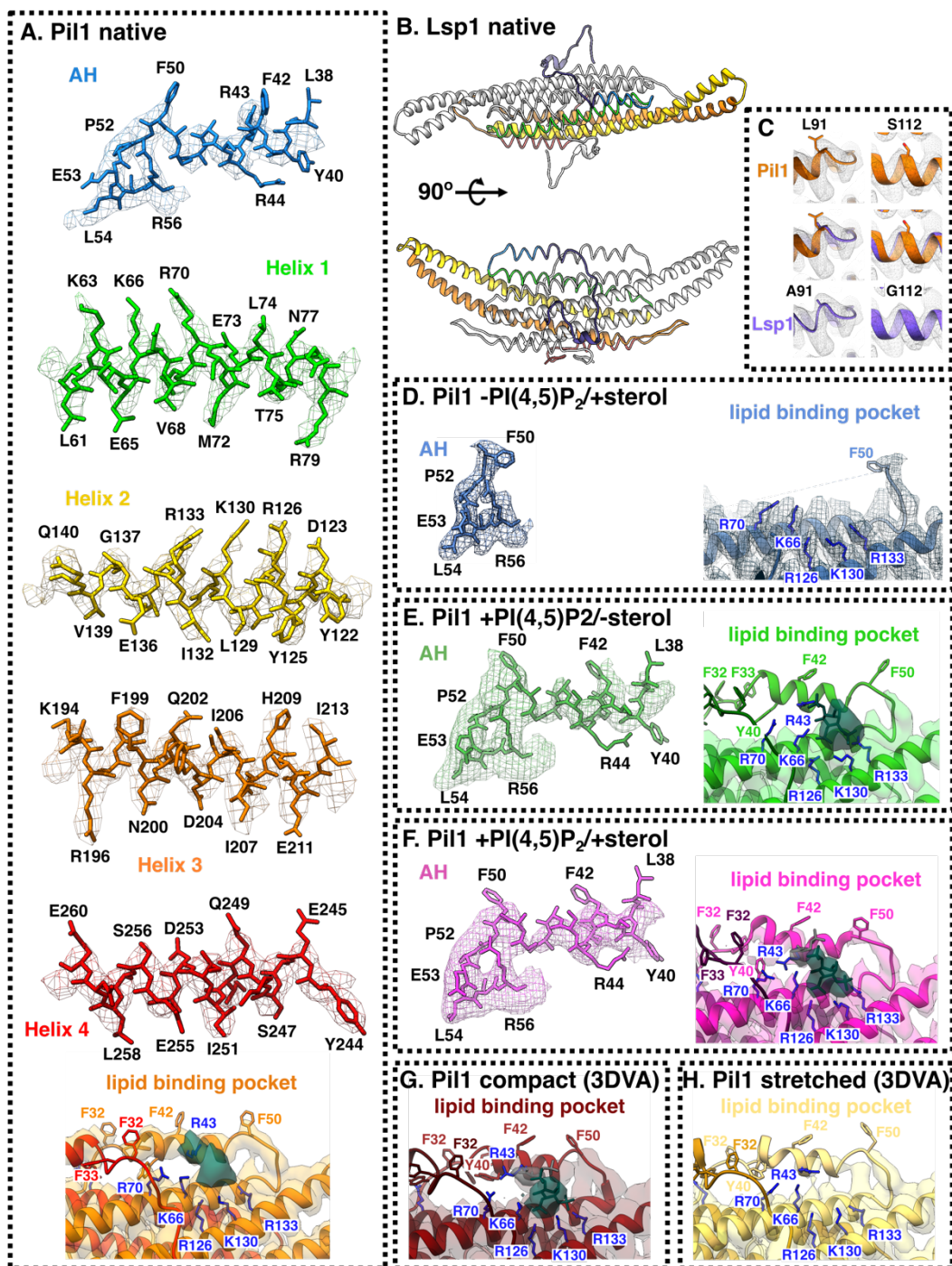
micrographs with native eisosome filaments and other putative contaminants visible. **C.** Example 2D classes with varying filament diameters. **D.** Helical reconstruction data processing strategy.

## Fig Ext Data 2



**Figure Extended Data 2. Symmetry expansion of native eisosome filaments. A.** Unrolled and aligned helical structures of native eisosome filaments of different diameters. **B.** Data processing strategy for symmetry expansion of helical reconstructions

### Fig Ext Data 3



**Figure Extended Data 3. Map-to-model fit.** **A.** Pil1 native eisosome map-to-model fit. **B.** Lsp1 model. **C.** Fit comparison of divergent residues in Pil1 and Lsp1. **D.** -PI(4,5)P<sub>2</sub>+sterol reconstituted map-to-model fit of unresolved amphipathic helix and unoccupied lipid binding pocket. **E.** +PI(4,5)P<sub>2</sub>-sterol reconstituted map-to-model fit of amphipathic helix and lipid binding

pocket bound to PI(4,5)P<sub>2</sub> headgroup. **F.** +PI(4,5)P<sub>2</sub>/+sterol reconstituted map-to-model fit of amphipathic helix and lipid binding pocket bound to PI(4,5)P<sub>2</sub> and PS headgroups. **G.** Most compact class of 3D variability analysis (3DVA) map-to-model fit of lipid binding pocket bound to PI(4,5)P<sub>2</sub> and PS headgroups. **H.** Most stretched class of 3DVA map-to-model fit of unoccupied lipid binding pocket.

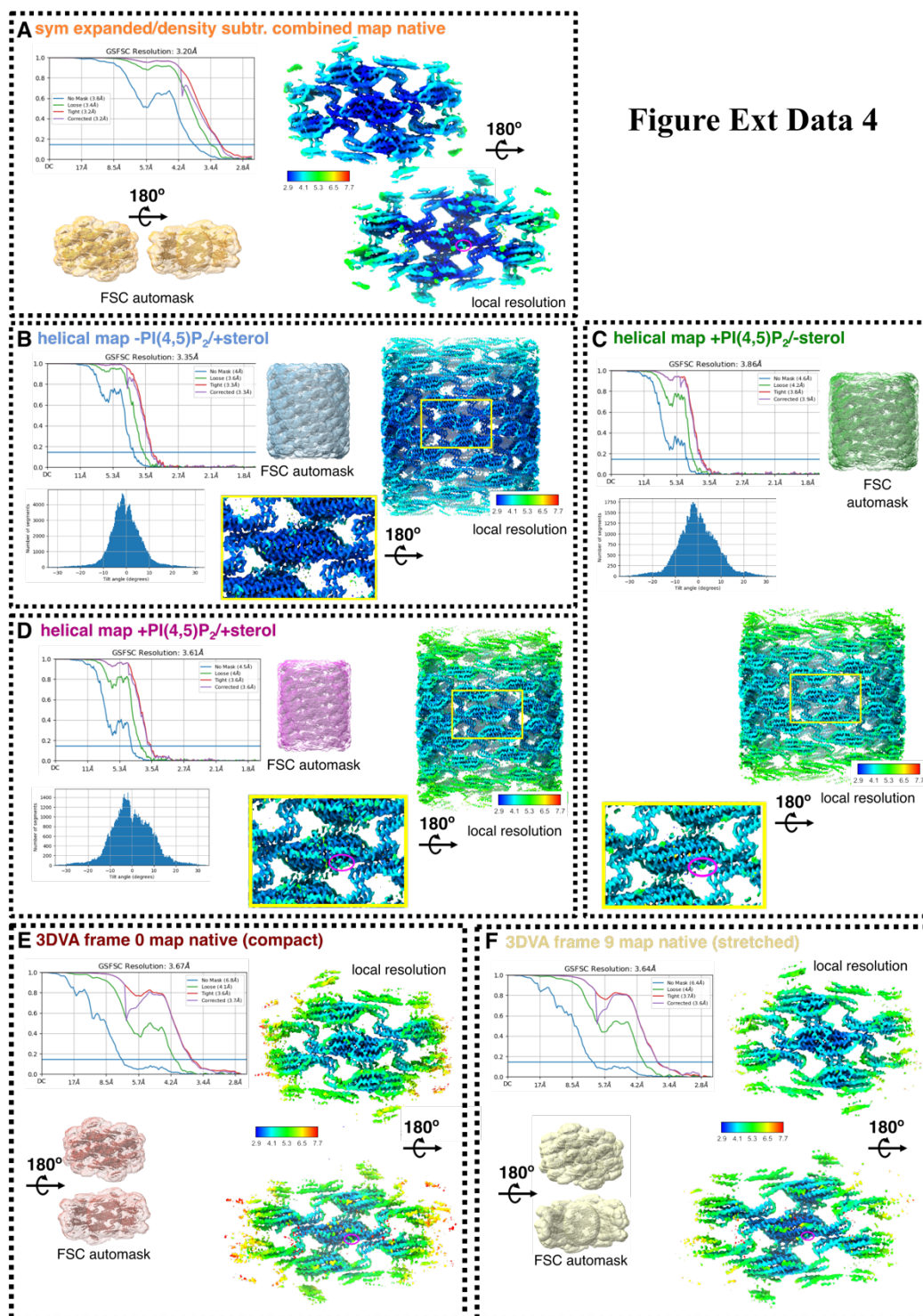
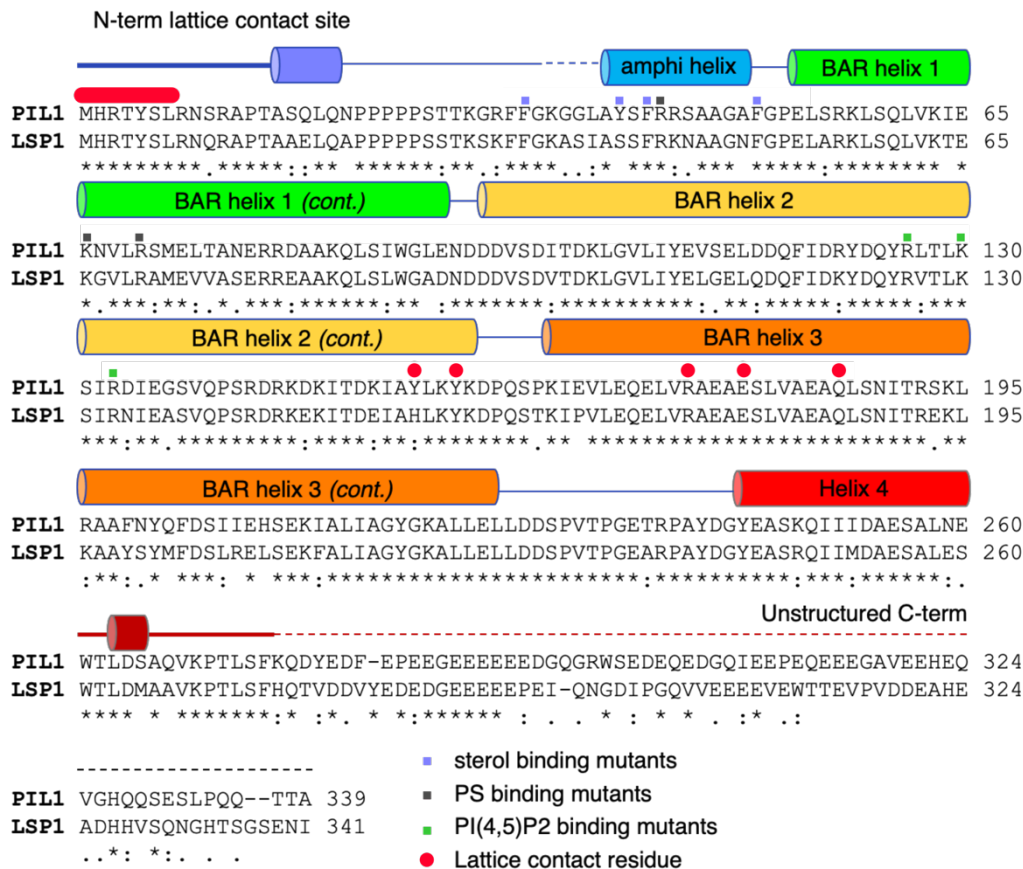


Figure Ext Data 4

**Figure Extended Data 4. Map quality and local resolution.** **A.** Gold standard Fourier shell correlation (GSFSC) plots, orientation distribution plot, auto-generated mask for average resolution determination at FSC 0.143, and local resolution map for symmetry expanded native map. Magenta circle highlights lipid binding pocket. **B-D.** GSFSC plots, helical symmetry error

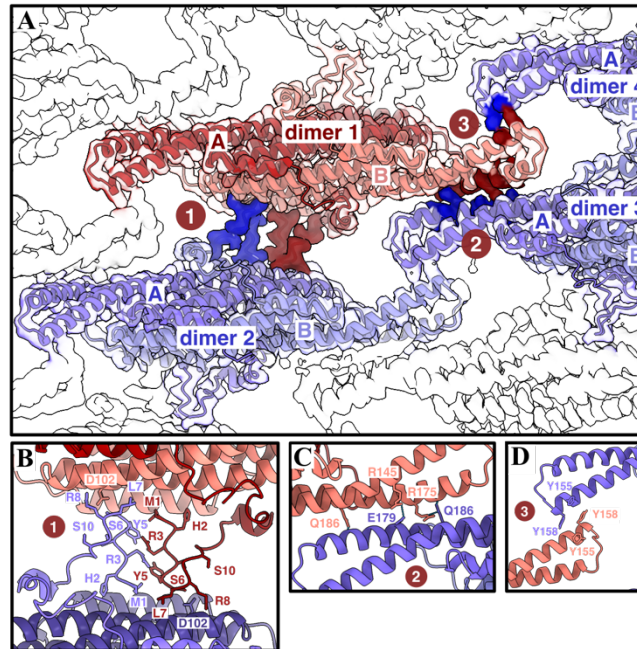
plot, auto-generated mask for average resolution determination at FSC 0.143, and local resolution map for helical maps of -PI(4,5)P2/+sterol (**B**), +PI(4,5)P2/-sterol (**C**), and +PI(4,5)P2/+sterol (**D**) reconstituted Pil1 tubules. Magenta circles highlight lipid binding pocket. **E-F**. GSFSC plots, orientation distribution plot, auto-generated mask for average resolution determination at FSC 0.143, and local resolution map for 3DVA frame 0 (most compact) (**E**) and 3DVA frame 9 (most stretched) (**F**) refined maps.

## Figure Ext Data 5



**Figure Extended Data 5. Sequence alignment of *S. cerevisiae* Pil1 and Lsp1.** Violet squares indicate sterol binding residues, grey squares indicate proposed PS binding residues, green squares indicate PI(4,5)P<sub>2</sub> binding residues, red circles indicate residues that form lattice contacts.

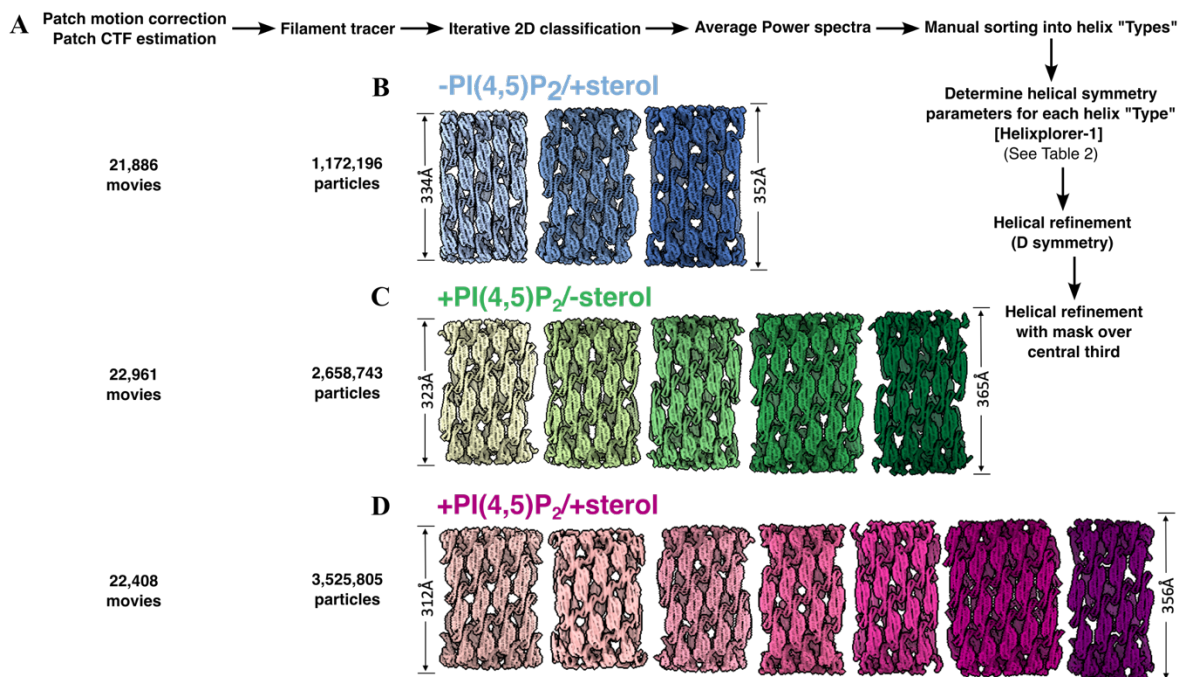
## Fig Ext Data 6



**Figure Extended Data 6. Lattice contact sites between Pil1 dimers.** **A.** Overview of contact sites. **B.** Site 1: Nt contact sites formed by res 1-8. **C.** Site 2: Electrostatic interactions between res171-186 at helix 3 of BAR domain. **D.** Site 3: Hydrophobic interactions between Y155 and Y158 at BAR domain tips.

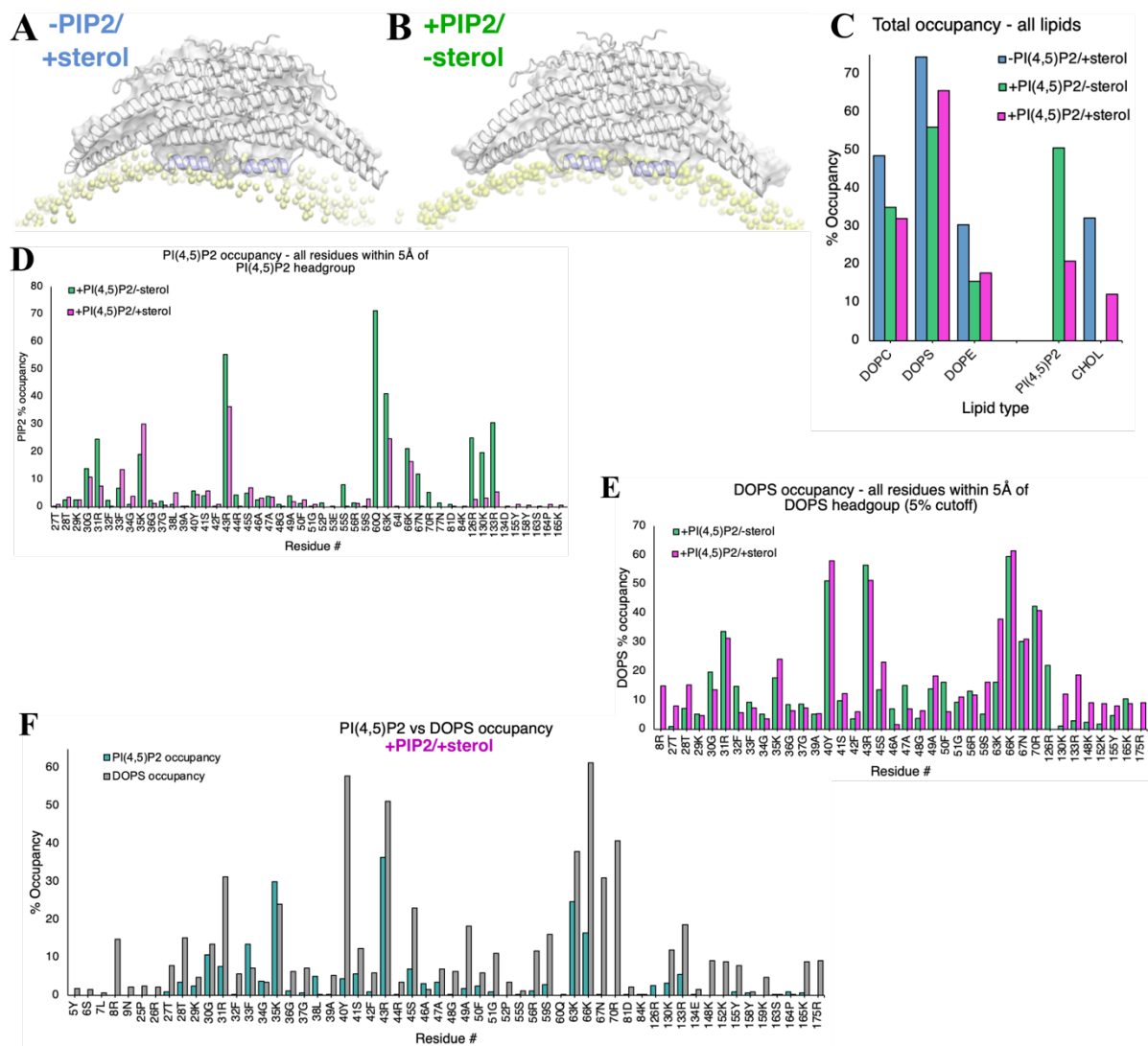


## Figure Ext Data 7



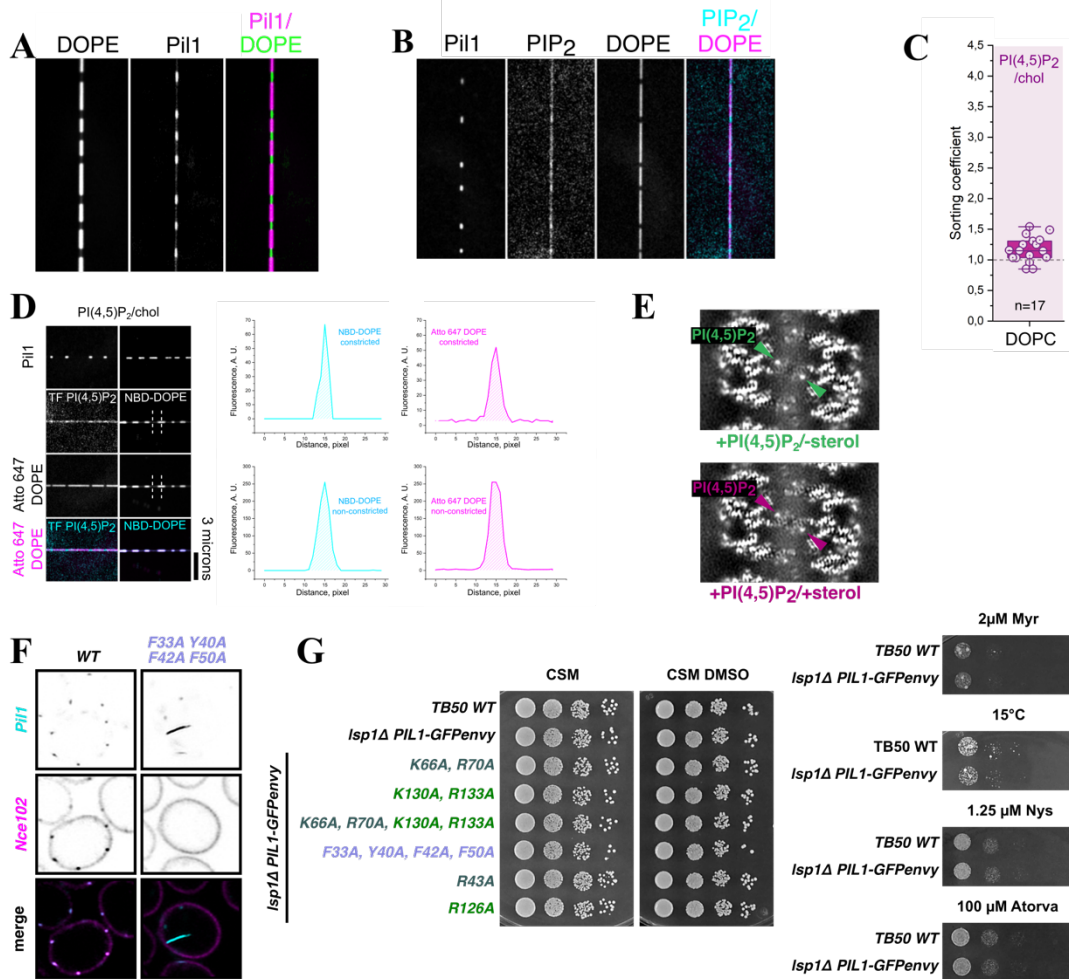
**Figure Extended Data 7. CryoEM data processing of reconstituted Pil1 filaments with lipid mixtures of known composition. A.** Helical reconstruction data processing strategy for reconstituted Pil1 tubules. **B-D.** All helical reconstructions from -PI(4,5)P<sub>2</sub>+sterol (**A**), +PI(4,5)P<sub>2</sub>-sterol (**B**), and +PI(4,5)P<sub>2</sub>+sterol (**C**) reconstituted Pil1 tubules.

## Fig Ext Data 8



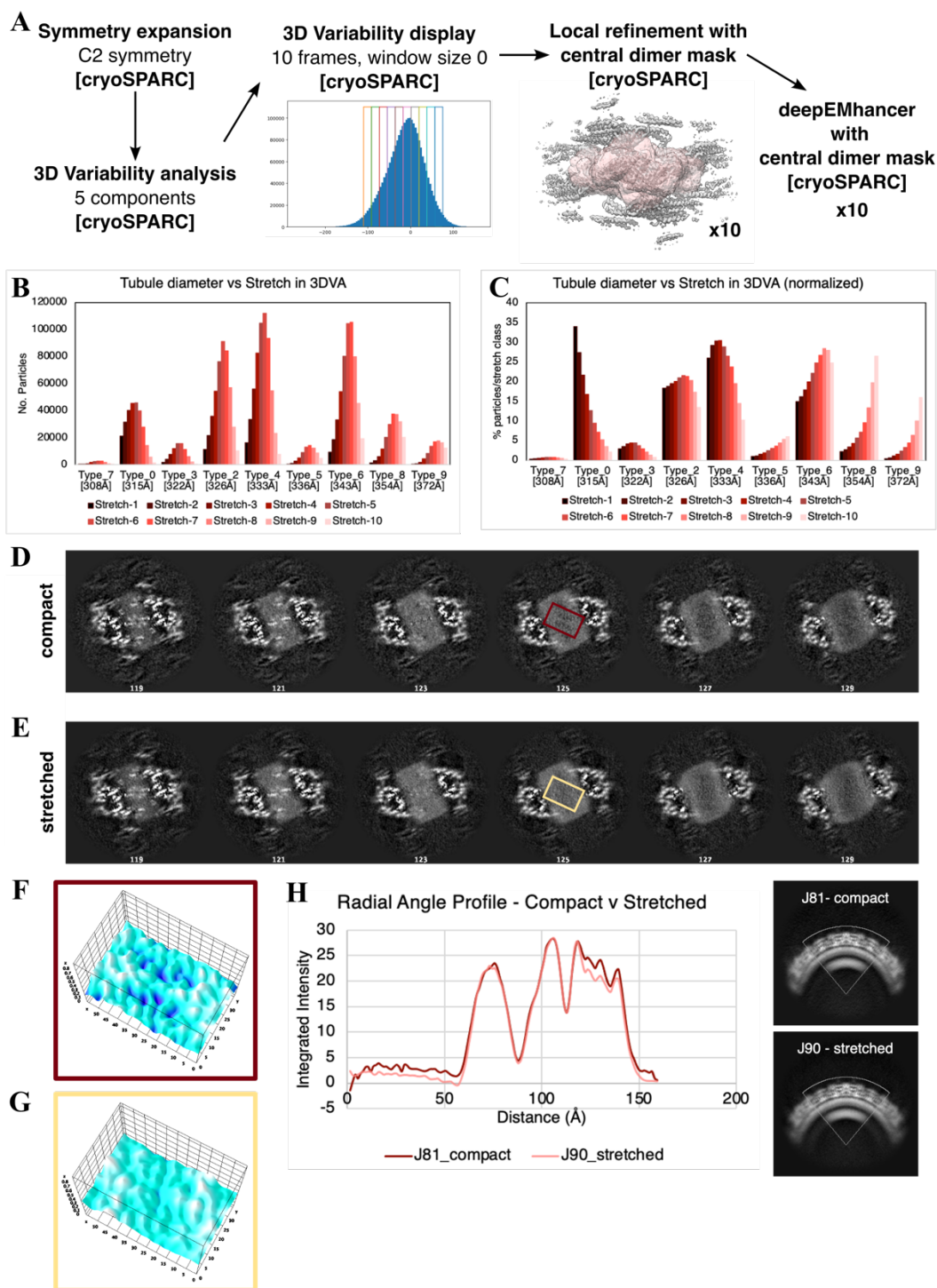
**Figure Extended Data 8. Coarse-grained molecular dynamics simulations.** **A-B.** Snapshots of the amphiphilic helices (blues) in membrane (yellow) in the “-PIP2/+sterol” system (**A**) and in the +PIP2/-sterol system (**B**). **C.** Total occupancy per lipid for all lipids in each CG-MD system. **D.** PI(4,5)P<sub>2</sub> occupancy reported for all residues <5Å from PI(4,5)P<sub>2</sub> headgroups. **E.** DOPS lipid occupancy for residues <5Å from DOPS headgroup with >5% occupancy in CG-MD simulations. **F.** Comparison of PI(4,5)P<sub>2</sub> occupancy and DOPS occupancy for residues <5Å from PI(4,5)P<sub>2</sub> and/or DOPS headgroup.

## Fig Ext Data 9



**Figure Extended Data 9. Lipid sorting co-efficients and lipid binding-impaired mutants. A.** Example lipid nanotube with DOPE-Atto647N and Pil1-mCherry bound **B.** Example lipid nanotube with Pil1-mCherry, fluorescent TopFluor-PI(4,5)P<sub>2</sub>, and fluorescent DOPE-Atto647N. **C.** Lipid sorting co-efficient of DOPC in “+1% PI(4,5)P<sub>2</sub>+sterol” reconstituted Pil1 tubules. [N=2]. **D.** Amphipathic helix slice through unsharpened maps of “+PI(4,5)P<sub>2</sub>-sterol” (left panel) and “+PI(4,5)P<sub>2</sub>+sterol” (right panel), with arrows indicating presumed PI(4,5)P<sub>2</sub> density in each reconstruction (green and lilac, respectively). **E.** Demonstration of the fluorescence plot profiles of the lipid of interest and reference lipid used to extract the integrated fluorescence densities (hatched areas) to measure lipid sorting coefficients. **F.** Central confocal slice of *Isp1Δ* yeast expressing Pil1-GFP variants and Nce102-Scarlet-I from their endogenous loci, highlighting cytosolic ingress of eisosomes in sterol binding-impaired mutant Pil1<sup>F33A/Y40A/F42A/F50A</sup>. **G.** Controls for growth assays of *Isp1Δ* yeast expressing Pil1-GFP lipid binding-impaired mutants.

## Figure Ext Data 10



**Figure Extended Data 10. 3D variability analysis. A.** 3DVA data processing strategy. **B.** Frequency of particles from each helical reconstruction type found in each 3DVA class (1=most compact, 10=most stretched). **C.** Percent of particles normalized to total particles in each helix type found in each 3DVA class (1=most compact, 10=most stretched). **D-E.** One pixel slices

separated by  $\sim 2.6\text{\AA}$  through unsharpened maps of most compact (**D**) and most stretched (**E**) 3DVA classes with region of maximum sterol void pattern highlighted (most compact: red box, most stretched: yellow box) **F**. 3D surface intensity plot of highlighted region (from panel D) of most compact class (red box) **G**. 3D surface intensity plot of highlighted region (from panel E) of most stretched class (yellow box). **H**. Radial angle profile plots of bilayer within most compact and most stretched classes.

**Supplementary Video 1. FRAP analysis example +PI(4,5)P<sub>2</sub>+sterol.** Example FRAP of nanotube produced with +PI(4,5)P<sub>2</sub>+sterol lipid mixture with 1% TopFluor-cholesterol (top panel, cyan) with Pil1-mCherry (middle panel, magenta) assembled on nanotube surface. Control lipid 0.1% DOPE-Atto647n shown in bottom panel (yellow).

**Supplementary Video 2. FRAP analysis example -PI(4,5)P<sub>2</sub>+sterol.** Example FRAP of nanotube produced with -PI(4,5)P<sub>2</sub>+sterol lipid mixture with 1% TopFluor-cholesterol (top panel, cyan) with Pil1-mCherry (middle panel, magenta) assembled on nanotube surface. Control lipid 0.1% DOPE-Atto647n shown in bottom panel (yellow).

**Supplementary Video 3. 3DVA native eisosome lattice.** 3D variability component from analysis using symmetry expanded/density subtracted particles from native eisosomes, demonstrating stretching at Nt lattice contact sites that expands the Pil1/Lsp1 lattice and is correlated with changes in the putative lipid-binding pocket.

**Table 1. CryoEM data collection, refinement, and validation statistics.**

**Table 2. Symmetry parameters and diameters of helical structures.** Helix type (numbered from smallest to largest diameter), alternative name, point group symmetry, helical rise and twist, helix diameter and resolution of helical map.

**Table 3. Lipids used in this study.** IUPAC names, abbreviations used in text, catalogue numbers and supplier.

**Table 4. Lipid mixtures used in this study.** Mixture name used in text, components of lipid mixtures, and molar ratio (mol %).

**Table 5. FRAP halftimes in seconds and percentages of mobile fractions.** Values are derived from one-phase exponential (one half-time shown) or two-phase exponential (two half-times shown) equations (see Materials and methods). Standard error of fitted values (s.e.) and the goodness of fits (R-squared) are shown.

**Table 6. Yeast strains used in this study.** Strain name, genotype, and source.

	#1 Pil1 lattice (native) (EMD-18307) (PDB 8QB7)	#2 Lsp1 lattice (native) (PDB 8QB8)	#3 Pil1 lattice (- PIP2/+sterol reconstituted) (EMD-18308) (PDB 8QB9)	#4 Pil1 lattice (+PIP2/- sterol reconstituted) (EMD-18309) (PDB 8QBB)
<b>Data collection and processing</b>				
Magnification	10500x	10500x	96000x	96000x
Voltage (kV)	300	300	300	300
Electron exposure (e <sup>-</sup> /Å <sup>2</sup> )	40.00	40.00	50	50
Defocus range (µm)	-0.8 to -1.8	-0.8 to -1.8	-0.6 to -1.8	-0.6 to -1.8
Pixel size (Å)	1.33	1.33	0.83	0.83
Symmetry imposed	Symmetry expanded, C1	Symmetry expanded, C1	Helical, D1	Helical, D3
Rise (Å)	-	-	5.044	14.547
Twist (deg.)	-	-	136.5	83.250
Initial particle images (no.)	1211972	1211972	1172196	2685743
Final particle images (no.)	1211972	1211972	176005	85456
Map resolution (Å)	3.20	3.20	3.35	3.86
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.972-42.269	2.972-42.269	1.762-29.519	1.747-34.472
<b>Refinement</b>				
Initial model used (PDB code)	Pil1 (P53252) AlphaFold2 database			
Model resolution (Å)	3.3	3.5	3.3	3.9
FSC threshold	0.143	0.143	0.143	0.143
Map sharpening B factor (Å <sup>2</sup> )	-106	-106	-101.9	-101.3
<b>Model composition</b>				
Non-hydrogen atoms	30058	29750	27874	30394
Protein residues	3794	3794	3500	3794
Ligands	0	0	0	I3P: 14
<b>B factors (Å<sup>2</sup>)</b>				
Protein	7.58/145.95/75.53	40.94/191.65/92.81	36.10/208.72/84.74	81.87/246.84/120.61
Ligand	-	-	-	168.85/168.85/168.85
<b>R.m.s. deviations</b>				
Bond lengths (Å)	0.002	0.003	0.002	0.003
Bond angles (°)	0.508	0.65	0.467	0.553
<b>Validation</b>				
MolProbity score	1.47	1.59	1.38	1.58
Clashscore	8.68	11.82	6.96	7.54
Poor rotamers (%)	0.43	0	0	0
<b>Ramachandran plot</b>				
Favored (%)	98.51	99.26	99.19	97.03
Allowed (%)	1.49	0.74	0.81	2.97
Disallowed (%)	0	0	0	0

	#5 Pil1 lattice (+PIP2/+sterol reconstituted) (EMD-18310) (PDB 8QBD)	#6 Pil1 lattice compact (native) (EMD-18311) (PDB 8QBE)	#7 Pil1 compact dimer with lipids headgroups (native) (PDB 8QBF)	#8 Pil1 lattice stretched (native) (EMD-18312) (PDB 8QBG)
<b>Data collection and processing</b>				
Magnification	96000x	105000x	105000x	105000x
Voltage (kV)	300	300	300	300
Electron exposure (e <sup>-</sup> /Å <sup>2</sup> )	50	40	40	40
Defocus range (µm)	-0.6 to -1.8	-0.8 to -1.8	-0.8 to -1.8	-0.8 to -1.8
Pixel size (Å)	0.83	1.327	1.327	1.327
Symmetry imposed	Helical, D1	C1, symmetry expanded	C1, symmetry expanded	C1, symmetry expanded
Rise (Å)	5.41	-	-	-
Twist (deg.)	133.60	-	-	-
Initial particle images (no.)	3525805	1211972	1211972	1211972
Final particle images (no.)	77414	63118	63118	77457
Map resolution (Å)	3.61	3.67	3.67	3.64
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	1.754-50.022	3.302-61.571	3.302-61.571	3.261-61.394
<b>Refinement</b>				
Initial model used (PDB code)	-	-	-	-
Model resolution (Å)	3.6	3.9	3.7	3.8
FSC threshold	0.143	0.143	0.143	0.143
Map sharpening B factor (Å <sup>2</sup> )	-91.1	-99.6	-99.6	-94.6
<b>Model composition</b>				
Non-hydrogen atoms	30548	30058	4364	30058
Protein residues	3794	3794	542	3794
Ligands	IP3:14 SEP:14	0	IP3:2 SEP:2	0
<b>B factors (Å<sup>2</sup>)</b>				
Protein	66.75/175.85/99.10	73.61/172.32/104.67	73.61/172.32/104.84	63.31/156.79/96.07
Ligand	123.82/123.82/123.82	-	109.94/109.94/109.94	-
<b>R.m.s. deviations</b>				
Bond lengths (Å)	0.003	0.003	0.006	0.004
Bond angles (°)	0.578	0.67	1.101	0.699
<b>Validation</b>				
MolProbity score	1.43	1.63	1.74	1.67
Clashscore	7.95	13.07	14.61	12.9
Poor rotamers (%)	0	0.85	1.07	0
<b>Ramachandran plot</b>				
Favored (%)	99.26	98.14	97.77	97.77
Allowed (%)	0.74	1.86	2.23	2.23
Disallowed (%)	0	0	0	0



	Name	Alt. Name	Point group symmetry	Helical rise	Helical twist	Helix Diameter	Reso
<b>Native</b>	Type 1	7	D2	11.215	54.344	307.9	7.16
<b>Native</b>	Type 2	0	D1	5.515	-53.522	314.9	3.84
<b>Native</b>	Type 3	3	D4	21.344	-50.942	322.0	4.28
<b>Native</b>	Type 4	2	D7	37.299	36.720	325.6	3.72
<b>Native</b>	Type 5	4	D1	5.173	-136.534	332.6	3.72
<b>Native</b>	Type 6	5	D1	5.135	49.337	336.2	4.37
<b>Native</b>	Type 7	6	D2	10.067	-48.121	343.2	3.72
<b>Native</b>	Type 8	8	D1	4.860	-46.583	353.9	4.02
<b>Native</b>	Type 9	9	D8	37.570	-57.772	371.6	4.28
<b>Reconstituted lipid mixture: -PIP2/+sterol</b>	Type 1	III	D7	36.538	-14.466	334.2	3.89
<b>Reconstituted lipid mixture: -PIP2/+sterol</b>	Type 2	II	D1	5.044	-136.500	340.9	3.35
<b>Reconstituted lipid mixture: -PIP2/+sterol</b>	Type 3	I	D2	9.762	131.906	351.9	3.57
<b>Reconstituted lipid mixture: +PIP2/-sterol</b>	Type 1	b	D1	5.420	133.600	323.1	3.91
<b>Reconstituted lipid mixture: +PIP2/-sterol</b>	Type 2	a	D4	21.004	39.094	332.0	3.88
<b>Reconstituted lipid mixture: +PIP2/-sterol</b>	Type 3	d	D1	5.078	-136.523	345.3	4.65
<b>Reconstituted lipid mixture: +PIP2/-sterol</b>	Type 4	c	D3	14.547	-83.250	351.9	3.86
<b>Reconstituted lipid mixture: +PIP2/-sterol</b>	Type 5	e	D1	4.714	-81.141	365.2	4.5
<b>Reconstituted lipid mixture: +PIP2/+sterol</b>	Type 1	D	D2	11.142	-137.653	312.1	3.76
<b>Reconstituted lipid mixture: +PIP2/+sterol</b>	Type 2	K	D1	5.740	152.300	318.7	6.81
<b>Reconstituted lipid mixture: +PIP2/+sterol</b>	Type 3	A&C	D1	5.408	133.595	325.4	3.61
<b>Reconstituted lipid mixture: +PIP2/+sterol</b>	Type 4	FGH	D4	20.968	-140.956	332.0	3.79
<b>Reconstituted lipid mixture: +PIP2/+sterol</b>	Type 5	J	D1	5.077	-136.507	343.1	4.52
<b>Reconstituted lipid mixture: +PIP2/+sterol</b>	Type 6	E	D1	4.980	-161.053	349.7	3.8
<b>Reconstituted lipid mixture: +PIP2/+sterol</b>	Type 7	B	D3	14.548	276.716	356.3	4.03

Table 1. Lipids used in this study. <https://doi.org/10.1101/2023.08.25.554800>; this version posted August 26, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

UP AC Name	Abbreviation	Catalogue #	Purchased from
1,2-dioleoyl-sn-glycero-3-phosphocholine	DOPC	#850375	Avanti Polar Lipids Inc.
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine	DOPE	#850725	Avanti Polar Lipids Inc.
1,2-dioleoyl-sn-glycero-3-phospho-L-serine	DOPS	#840035	Avanti Polar Lipids Inc.
brain L- $\alpha$ -phosphatidylinositol-4,5-bisphosphate	PI(4,5)P2	#840046	Avanti Polar Lipids Inc.
1-oleoyl-2-(6-[4-(dipyrrometheneboron difluoride)butanoyl]amino)hexanoyl-sn-glycero-3-phosphoinositol-4,5-bisphosphate	TopFluor-PI(4,5)P2	#810184	Avanti Polar Lipids Inc.
23-(dipyrrometheneboron difluoride)-24-norcholesterol	TopFluor-cholesterol	#810255	Avanti Polar Lipids Inc.
1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycerol-3-Phosphocholine	NBD-PC	#810133	Avanti Polar Lipids Inc.
1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphoethanolamine	NBD-PE	#810156	Avanti Polar Lipids Inc.
1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphocholine	TopFluor-PC	#810281	Avanti Polar Lipids Inc.
1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphoethanolamine	TopFluor-PE	#810282	Avanti Polar Lipids Inc.
1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phospho-L-serine (ammonium salt)	TopFluor-PS	#810283	Avanti Polar Lipids Inc.
ATTO 647N-labelled 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine	Atto 647N-DOPE		ATTO-TEC GmbH

**CryoEM studies**

Name	Lipid mixture	molar ratio
-PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:Cholesterol	30:20:20:30 mol%
+PI(4,5)P2/-sterol	DOPC:DOPE:DOPS:brain PI(4,5)P2	50:20:20:10 mol%
+PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:Cholesterol:brain PI(4,5)P2	35:20:20:15:10 mol%

**Lipid sorting coefficient and FRAP studies\***

Name	Lipid mixture	molar ratio
-PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:cholesterol:TopFluor-cholesterol	30:20:20:29:1 mol%
+1% PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:cholesterol:TopFluor-cholesterol:brain PI(4,5)P2	29:20:20:29:1:1 mol%
+1% PI(4,5)P2/-sterol	DOPC:DOPE:DOPS:TopFluor-PI(4,5)P2	59:20:20:1 mol%
+1% PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:cholesterol:TopFluor-PI(4,5)P2	29:20:20:30:1 mol%
+1% PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:cholesterol:brain P(4,5)P2:TopFluor-PC	28:20:20:30:1:1 mol%
+1% PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:cholesterol:brain P(4,5)P2:TopFluor-PE	29:19:20:30:1:1 mol%
+1% PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:cholesterol:brain P(4,5)P2:TopFluor-PS	29:20:19:30:1:1 mol%
+1% PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:cholesterol:brain P(4,5)P2:NBD-PE	29:19:20:30:1:1 mol%
+1% PI(4,5)P2/+sterol	DOPC:DOPE:DOPS:cholesterol:brain P(4,5)P2:NBD-PC	29:19:20:30:1:1 mol%

\*0.01 mol% of Atto647N DOPE was added to each lipid mixture for these experiments

Table 5. FRAP halftimes in seconds and percentages of mobile fractions.

TopFluor-cholesterol				
	halftime 1 (s.e.), s	halftime 2 (s.e.), s	mobile fraction (s.e.), %	R-squared
+PIP2, control	5.6 (0.4)	64.1 (8.6)	86.4 (0.5)	0.993
+PIP2, Pil1	8.7 (0.3)	-	34.0 (0.1)	0.953
-PIP2, control	5.0 (1.0)	38.5 (3.6)	92.5 (0.9)	0.987
-PIP2, Pil1	6.7 (0.2)	-	56.3 (0.1)	0.971

TopFluor-PIP2				
	halftime 1 (s.e.), s	halftime 2 (s.e.), s	mobile fraction (s.e.), %	R-squared
+sterol, control	4.8 (0.3)	35.4 (1.2)	60.8 (0.4)	0.998
+sterol, Pil1	4.6 (0.6)	61.9 (4.4)	20.7 (0.6)	0.994
-sterol, control	4.6 (0.3)	27.3 (2.0)	56.7 (0.4)	0.994
-sterol, Pil1	28.5 (1.0)	-	27.5 (0.3)	0.973

TopFluor-PC				
	halftime 1 (s.e.), s	halftime 2 (s.e.), s	mobile fraction (s.e.), %	R-squared
+PIP2, control	1.3 (0.4)	12.3 (0.4)	75.1 (0.3)	0.991
+PIP2, Pil1	14.4 (0.4)	-	58.7 (0.3)	0.996
-PIP2, control	2.7 (0.3)	18.8 (0.5)	90.7 (0.5)	0.998
-PIP2, Pil1	1.0 (0.1)	13.2 (0.4)	55.9 (0.2)	0.968

TopFluor-PE				
	halftime 1 (s.e.), s	halftime 2 (s.e.), s	mobile fraction (s.e.), %	R-squared
+PIP2, control	9.7 (0.1)	-	80.4 (0.2)	0.988
+PIP2, Pil1	9.0 (0.3)	-	49.9 (0.2)	0.926
-PIP2, control	1.9 (0.3)	15.6 (0.3)	81.6 (0.2)	0.995
-PIP2, Pil1	0.8 (0.1)	7.6 (0.5)	54.4 (0.1)	0.951

TopFluor-PS				
	halftime 1 (s.e.), s	halftime 2 (s.e.), s	mobile fraction (s.e.), %	R-squared
+PIP2, control	2.9 (0.2)	21.7 (0.5)	86.5 (0.2)	0.997
+PIP2, Pil1	12.7 (0.2)	-	55.4 (0.2)	0.982
-PIP2, control	2.6 (0.2)	17.7 (0.4)	73.0 (0.1)	0.997
-PIP2, Pil1	0.7 (0.2)	7.8 (0.4)	49.6 (0.1)	0.954

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Name	Genotype	Source
TB50a	MATa <i>leu2-3,112 ura3-52 rme1 trp1 his3</i>	Loewith lab
TB50α	MATa <i>leu2-3,112 ura3-52 rme1 trp1 his3</i>	PMID:26028537
CG1-37	TB50a <i>BIT61-TAP::kanMX4</i>	This study
JK001	TB50α <i>PIL1-GFPenvy::HIS3MX6 lsp1::HphMX4</i>	This study
AB001	TB50α <i>pil1-R43A-GFPenvy::HIS3MX6 lsp1::HphMX4</i>	This study
AB002	TB50α <i>pil1-R126A-GFPenvy::HIS3MX6 lsp1::HphMX4</i>	This study
AB003	TB50α <i>pil1-K130A/R133A-GFPenvy::HIS3MX6 lsp1::HphMX4</i>	This study
XZ001	TB50α <i>pil1-K66A/R70A-GFPenvy::HIS3MX6 lsp1::HphMX4</i>	This study
XZ002	TB50α <i>pil1-K66A/R70A/K130A/R133A-GFPenvy::HIS3MX6 lsp1::HphMX4</i>	This study
XZ003	TB50α <i>pil1-F33A/Y40A/F42A/F50A-GFPenvy::HIS3MX6 lsp1::HphMX4</i>	This study
MGTY064	TB50α <i>pil1-GFPenvy::HIS3MX6 lsp1::HphMX4 NCE102-mScarlet-I::Hph</i>	This study
JK003	TB50α <i>pil1-R43A-GFPenvy::HIS3MX6 lsp1::HphMX4 NCE102-mScarlet-I::Hph</i>	This study
JK004	TB50α <i>pil1-R126A-GFPenvy::HIS3MX6 lsp1::HphMX4 NCE102-mScarlet-I::Hph</i>	This study
JK005	TB50α <i>pil1-K130A/R133A-GFPenvy::HIS3MX6 lsp1::HphMX4 NCE102-mScarlet-I::Hph</i>	This study
MGTY056	TB50α <i>pil1-K66A/R70A-GFPenvy::HIS3MX6 lsp1::HphMX4 NCE102-mScarlet-I::Hph</i>	This study
MGTY062	TB50α <i>pil1-K66A/R70A/K130A/R133A-GFPenvy::HIS3MX6 lsp1::HphMX4 NCE102-mScarlet-I::Hph</i>	This study
MGTY061	TB50α <i>pil1-F33A/Y40A/F42A/F50A-GFPenvy::HIS3MX6 lsp1::HphMX4 NCE102-mScarlet-I::Hph</i>	This study