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1 **Self-assembly of the general membrane-remodeling** 2 **protein PVAP into 7-fold virus-associated pyramids**

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28 Key words: archaea, virus, viral egress

29

30 **Abstract**

31 Viruses have developed a wide range of strategies to escape from host cells in which they
32 replicate. Some archaeal viruses employ for egress a pyramidal structure with sevenfold
33 rotational symmetry. Virus-associated pyramids (VAPs) assemble in the host cell membrane
34 from the virus-encoded protein PVAP, and open at the end of the infection cycle. We characterize
35 this unusual supramolecular assembly using a combination of genetic, biochemical and electron
36 microscopic techniques. By whole-cell electron cryo-tomography, we monitor morphological
37 changes in virus-infected host cells. Subtomogram averaging reveals the VAP structure. By
38 heterologous expression of *PVAP* in cells from all three domains of life we demonstrate that the
39 protein integrates indiscriminately into virtually any biological membrane, where it forms
40 sevenfold pyramids. We identify the protein domains essential for VAP formation in PVAP
41 truncation mutants by their ability to remodel the cell membrane. Self-assembly of PVAP into
42 pyramids requires at least two different, in-plane and out-of-plane, protein interactions. Our
43 findings allow us to propose a model of how PVAP arranges to form sevenfold pyramids and
44 suggest how this small, robust protein may be used as a general membrane remodeling system.

45

46 **Significance statement**

47 The archaeovirus SIRV2 has developed unique mechanisms to penetrate the plasma membrane
48 and S-layer of its host *Sulfolobus islandicus* to leave the cell after replication. SIRV2 encodes
49 10kDa protein PVAP, which assembles into 7-fold symmetric virus-associated pyramids (VAPs)
50 in the host cell plasma membrane. Towards the end of the viral replication cycle, these VAPs
51 open to form pores through the plasma membrane and S-layer, allowing viral egress. Here we
52 show that PVAP inserts spontaneously and forms VAPs in any kind of biological membranes. By
53 electron cryo-tomography we have obtained a 3D map of the VAP and present a model
54 describing the assembly of PVAP into VAPs. Our findings open new avenues for a large variety of
55 biotechnological applications.

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62 **Introduction**

63 Release of virus particles from infected cells is the last essential step of the viral replication
64 cycle. In the course of this process, virions face the challenging task of crossing the cell envelope.
65 Viruses have developed an arsenal of diverse strategies to overcome this problem. Most
66 bacterial viruses are lytic and induce lysis of the infected cell with help of the holin-endolysin
67 system (1), while others disrupt the host cell envelope via inhibition of the murein biosynthesis
68 pathway (2). The morphological and genomic properties of archaeal viruses (3) suggested that
69 their egress from host cells may have unusual traits that are different from those of bacterial
70 viruses. Indeed, while most archaeal viruses exit cells without lysis, some, in particular the rod-
71 shaped or icosahedral viruses that respectively infect *Sulfolobus islandicus* (SIRV2) and
72 *Sulfolobus* turreted icosahedral virus 1 (STIV1), are lytic and exploit a special mechanism of
73 virion egress (4-8). During the infection cycle of these viruses, pyramidal protrusions with
74 sevenfold rotational symmetry form in the host cell membrane. As the final step of the infection
75 cycle the VAPs open outwards along the seams of their seven facets, creating ~100 nm apertures
76 through which the newly formed virions escape from the host cell (4, 7). VAPs consist of
77 multiple copies of a ~10 kDa virus-encoded protein, which we term PVAP (SIRV2_P98) (7-9).
78 Surprisingly, PVAP assembles into membrane pyramids even when expressed heterologously in
79 archaeal and bacterial expression systems, demonstrating that no other viral proteins are
80 required for VAP formation (7). The mechanism by which VAPs self-assembles in the membrane
81 remains unknown.

82 In the present study we used electron cryo-tomography to investigate morphological features of
83 SIRV2 replication and the formation of VAPs at different time points after infection. By
84 subtomogram averaging, we determined a first 3D map of the VAP. This map, in combination
85 with secondary structure predictions of PVAP and expression of *wild type PVAP* or a variety of
86 truncation mutants in archaeal, bacterial and eukaryotic cells allows us to propose a model of
87 how PVAP arranges to form the sevenfold pyramids. These insights are fundamental for
88 understanding how this mechanism can be exploited as a universal tool to engineer the
89 formation and controlled opening of large pores in biological or artificial lipid bilayers.

90

91 **Results**

92 **SIRV2 induces morphological changes of the host cell**

93 We analyzed morphological changes in *S. islandicus* during SIRV2 infection and the time points
94 of VAP formation and opening by whole cell electron cryo-tomography at 0.5, 3, 6 and 12 hours
95 post infection (h.p.i.). This allowed us to monitor morphological changes at high resolution and
96 to compare these with previous results obtained by thin-sectioning of chemically fixed cells
97 during the final stages of SIRV2 infection (4). Up to 3 h.p.i. infected cells were indistinguishable
98 from uninfected control cells and no virions were visible in the cytoplasm (Figs. 1a). This
99 suggests that SIRV2 does not enter the cell as an intact virus particle, in accordance with a
100 previous study, in which we have directly observed infection of *S. islandicus* by SIRV2 in the
101 electron microscope (10). Electron-dense ~100 nm globules were the only conspicuous features
102 in the cytoplasm of controls and infected cells (Fig.1a), similar to those reported for *S.*
103 *solfataricus* cells (11). However, each cell, infected or not, contained one or a few of these
104 globules, which are therefore unrelated to virus replication. Their size and density above the
105 cytoplasmic background indicates that they may be storage granules (12).

106

107 At 3-6 h.p.i., about half way through the infection cycle, newly assembled virions became visible
108 in the cytoplasm as described earlier (4). They were organized in up to three bundles per cell,
109 each consisting of roughly 50 rod-shaped particles (Fig.1b). Starting from 3 h.p.i., VAPs of
110 various sizes formed in the plasma membrane of the host cells, most of which had penetrated
111 the S-layer (Fig. 1b, 2 a-d). Each cell contained on average about 10 VAPs. The height of the VAPs
112 (measured from the membrane to the tip of the pyramid) ranged from ~20nm to 150nm. At an
113 early stage of formation, the VAPs in the plasma membrane had not yet punctured the S-layer of
114 the host cell (Supplementary Fig. 1), but they already had the distinct features of hollow
115 heptagonal pyramids, corroborating the earlier assumption that VAPs grow from the base by
116 gradual expansion of their triangular facets (7). Very occasionally, VAPs contained a spherical
117 storage granule (Fig. 2c). These granules likely correspond to the previously described intra-
118 pyramidal bodies (IPB) in STIV-induced VAPs (11). Analysis of cells at distinct time points post
119 infection showed that at 6 h.p.i. a number of VAPs had opened. The fraction of open VAPs
120 increased, until at 12 h.p.i. all VAPs had unfolded, like the petals of a flower (Fig. 1c, 2e-h). The
121 VAPs appeared to open as the pyramidal structures broke along the seams of the triangular
122 pyramid faces (Fig. 2f). The VAP facets curved outwards with counter-clockwise handedness
123 when viewed from the cell exterior (Fig. 2f), as suggested by electron micrographs of isolated
124 VAPs (7). As the VAPs opened, the virion bundles disintegrated and individual virions diffused
125 into the medium through the open VAPs (Fig. 3).

126

127

128

129 **VAP structure**

130 The shape of isolated VAPs has previously been investigated by negative staining and electron
131 microscopy (7). To gain insight in the structure of VAPs *in situ*, closed or open VAPs were imaged
132 by whole-cell electron cryo-tomography (Fig. 4a-c). The triangular faces of all VAPs appeared to
133 consist of two distinct layers, irrespective of their conformation or stage of assembly. The
134 $\sim 4.5 \pm 1$ nm outer layer was continuous with, and indistinguishable from, the cell membrane. The
135 inner layer had a thickness of $\sim 4.0 \pm 1$ nm (Fig. 4). The centre-to-centre distance between the two
136 layers was 10.0 ± 1 nm, leaving a 5.8 ± 1 nm gap of lower density. At the base of each VAP, the
137 inner layer extended up to 15 nm beyond the outer layer into the cytoplasm. This suggests that
138 the inner layer consists of a protein sheet that is attached and runs parallel to the cytoplasmic
139 membrane surface (Fig. 4).

140

141 In order to verify that both layers of the VAP consist only of PVAP protomers, we analysed
142 archaeal (*S. acidocaldarius*) or bacterial (*E. coli*) PVAP expression mutants constructed in a
143 previous study (7). Whole-cell electron cryo-tomography of transformed cells revealed the
144 distinctive two layers in all VAPs (Fig. 4c, S3), indicating that both consist solely of PVAP
145 (Supplementary Fig. 3).

146

147 **Subtomogram averaging of VAPs**

148 A 3D map of the VAP was obtained by averaging 57 tomographic subvolumes of closed VAPs in
149 the membrane of PVAP-expressing *E. coli* cells. Sections through the averaged volume parallel to
150 the membrane showed clear sevenfold symmetry (Supplementary Fig. 4), which was applied to
151 the final average to further improve the signal-to-noise ratio (Fig. 4d-k, Supplementary movie 1).
152 The local resolution estimated by the program ResMap (13) was between 36 and 58 Å, with a
153 mean resolution of ~ 43 Å (Supplementary Fig. 5). Since VAPs come in different sizes, only the
154 upper parts of the pyramids, to a height of ~ 62 nm and an outer diameter of ~ 96 nm, were used
155 for averaging. At the tip, the inner opening angle of the pyramid was $\sim 80^\circ$ (Fig. 4d). As observed
156 in the raw tomograms, the structure consisted of two layers (Fig. 4d, e, i-k). The outer layer
157 formed a continuous envelope, consisting of seven triangular facets (Fig. 4f-k). As seen from the
158 outside, the facets had a perceptible counter-clockwise handedness (Fig. 4f) and each facet was
159 slightly convex towards the inside (Fig. 4f, g, j, k). The angle at the tip of the triangular facets was
160 35° , in accordance with previous measurements on isolated VAP fragments (7). Overall, the
161 entire structure had the appearance of a tent or teepee (Fig. 4g). The inner, cytosolic layer of the
162 pyramid consisted of seven triangular sheets parallel to the membrane on the outside (Fig. 4i-k).
163 Cross-sections through the sevenfold averaged volume revealed narrow connecting densities
164 between the two layers of the pyramid, suggesting that they are physically linked (Fig. 4k).

165

166 **Membrane remodelling by PVAP**

167 *In silico* secondary structure predictions (14) and hydrophobicity analysis (15) of PVAP suggest
168 that the protein consists of an N-terminal transmembrane helix (residues 5-34), followed by
169 three hydrophilic α -helices of two to three turns each, separated by short linker regions
170 (Supplementary Fig. 2). The PSORT-server (16) indicated that PVAP does not contain a pre-
171 sequence. This is consistent with a previous study of the N-terminal amino acid sequence of
172 purified PVAP of SIRV2 with Edman degradation, which did not indicate a cleavable signal
173 sequence (9). Thus, we assumed that the hydrophobicity of its predicted N-terminal
174 transmembrane segment drives the spontaneous insertion of PVAP into the lipid bilayer. To test
175 this hypothesis, *PVAP* was expressed in the *Saccharomyces cerevisiae*, an eukaryotic organism.
176 Cells were harvested 16 hours after induction of *PVAP* expression, high-pressure frozen and
177 freeze-substituted. In addition, sections prepared by the Tokuyasu method (17) were
178 immunolabeled with antibodies raised against PVAP. All samples were analysed by transmission
179 electron microscopy. Surprisingly, VAPs were found in most, if not all, cellular membranes. In
180 terms of size and appearance, the pyramids were indistinguishable from those that assembled in
181 *E.coli* after PVAP expression or in *S. islandicus* after SIRV2 infection. PVAP-specific antibodies
182 labeled VAPs in the nuclear envelope, the endoplasmic reticulum, Golgi apparatus, intracellular
183 vesicles and mitochondria (Fig. 5).

184

185 **Role of PVAP domains in VAP assembly**

186 To identify which parts of the PVAP are required for VAP assembly, truncated mutants lacking
187 the last 10, 20, 30, 40 or 70 C-terminal residues ($\Delta C10$, $\Delta C20$, $\Delta C30$, $\Delta C40$ or $\Delta C70$) were
188 constructed (Fig. 6). EM analysis of *E.coli* cells transfected with these constructs revealed VAPs
189 only in case of the $\Delta C10$ mutant (Fig. 6).

190

191 By contrast, VAPs did not form after truncation of 20 to 70 C-terminal residues (*PVAP* $\Delta C20$,
192 $\Delta C30$, $\Delta C40$ or $\Delta C70$, Fig. 6), corresponding to one to three C-terminal α -helical segments.
193 Instead, expression of these constructs resulted mostly in protein aggregates. In addition,
194 constructs lacking 20-40 C-terminal residues caused the inner membrane of *E.coli* to form large
195 invaginations, suggesting that these variants still interact with the membrane (Fig 6*b*). The effect
196 was most pronounced for *PVAP* $\Delta C20$. In contrast, *PVAP* $\Delta C70$ did not produce any membrane
197 invaginations (Fig 6*b*).

198

199 Expression of a *PVAP* construct lacking the predicted N-terminal transmembrane helix
200 (*PVAP* $\Delta N30$) likewise did not result in VAP formation. There was no sign of any interaction with

201 the membrane (Fig. 6b), indicating that the N-terminal transmembrane domain is indeed
202 required for membrane insertion of PVAP protomers.

203

204 We asked if the PVAP transmembrane domain is essential for VAP formation or could be
205 replaced by any other transmembrane domain. To characterize the role of the PVAP
206 transmembrane domain in VAP formation we constructed a chimera by fusing the *E. coli* flagellar
207 regulator *Flk*, a gene encoding a single trans-membrane helix inner membrane protein (18) to
208 *PVAPΔN30*, replacing the N-terminal transmembrane helix (residues 1-30) of PVAP(18). After
209 expression, this fusion construct (PVAPtmFlk) was indeed inserted into the membrane, as
210 judged by Western blot analysis of cell fractions with SIRV2-PVAP antibody (Supplementary Fig.
211 6). However, there was no evidence of VAPs in these cells (Fig 6b).

212 Taken together these findings indicate that the N-terminal domain is essential for membrane
213 insertion of PVAP and for the interaction between PVAP protomers, which results in the
214 assembly of a protein sheet on the inner membrane surface. The C-terminal domain of PVAP
215 (except the last 10 residues, which are predicted to be disordered) is required for VAP
216 formation. Without this domain, the protein aggregates instead of forming VAPs.

217

218 **PVAP oligomers**

219 To characterize the oligomerization of PVAP *in vitro*, we fused a His-tag to the C-terminus and
220 expressed the protein heterologously in *E. coli*. Isolated membranes were solubilized with the
221 detergent N-laurylsarcosine. PVAP was purified by nickel affinity chromatography and size
222 exclusion chromatography. The single peak in the gel filtration profile corresponds to a
223 molecular mass of ~70 kDa (Supplementary Fig. 7). SDS-PAGE analysis of peak fractions show
224 discrete PVAP bands at ~10, 20, 30 and 70 kDa (Supplementary Fig. 7), indicating that in
225 detergent solution PVAP forms different oligomers, the largest of which is most likely a
226 heptamer. A PVAP heptamer is also suggested by gel filtration chromatography.

227

228 **Discussion**

229 The VAP, an archeoviral egress structure that takes the shape of a large sevenfold pyramid in the
230 host membrane, is without parallel in biology. It consists of multiple copies of PVAP, a 10 kDa
231 membrane protein, which forms VAPs in the membrane, evidently without the need for any
232 other cellular component.

233

234 How does the 10 kDa PVAP assemble to form sevenfold pyramids in the membrane? To address
235 this question, we investigated the VAP structure itself by electron cryo-tomography, and have

236 studied the membrane insertion and biochemical properties of PVAP. We have demonstrated
237 that PVAP forms VAPs in archaeal, bacterial and eukaryotic membranes, into which it inserts
238 indiscriminately, and that, with the exception of the last 10 C-terminal residues, the entire length
239 of the protein is required for VAP assembly. Finally, we have shown that PVAP forms oligomers,
240 most likely heptamers, in detergent solution.

241

242 **PVAP is a universal membrane remodeling system**

243 Sequence analysis of PVAP suggested that the protein does not contain a signal sequence and
244 thus most likely integrates spontaneously into the archaeal membrane. A similar mechanism of
245 membrane insertion has been found for tail-anchored (TA) proteins (19) and for bacterial pore-
246 forming toxins (bPTFs) (20). TA proteins are indigenous proteins, which contain a single C-
247 terminal transmembrane segment. They are inserted into their target membrane in a Sec-
248 independent, but organelle-specific manner, occasionally aided by cytoplasmic chaperones (19).
249 Similar to PVAP, bPTFs are expressed as monomers and insert into the target membrane, where
250 they assemble into pore-forming oligomers, either to kill other bacteria or, in case of pathogens,
251 to lyse the host membrane and thus to aid bacterial proliferation (20).

252

253 Overexpression of *PVAP* in the archaeon *S. acidocaldarius*, the bacterium *E. coli* and the
254 eukaryote *S. cerevisiae* resulted in the formation of VAPs in the plasma membranes of all hosts.
255 Even more remarkably, VAPs were observed in virtually all cellular membranes of the eukaryote
256 *S. cerevisiae*, including the nuclear envelope, the ER, the mitochondrial outer membrane and the
257 plasma membrane. This demonstrates that, in contrast to other known type of protein
258 spontaneously integrating into membranes, PVAP is able to insert into practically any biological
259 lipid bilayer, solely by virtue of its N-terminal transmembrane segment. Once inserted into the
260 bilayer, it forms sevenfold pyramids, irrespective of fundamental differences in lipid or protein
261 composition of the target membrane. These characteristics render PVAP a unique, universal
262 membrane remodeling tool.

263

264 **Supramolecular organisation of VAPs**

265 Whole-cell electron cryo-tomography and subtomogram averaging revealed that the VAPs
266 consist of the same two layers of roughly equal thickness in all endogenous and heterologous
267 expression hosts. The outer layer was continuous with the plasma membrane, whereas the inner
268 layer formed a discontinuous sheet at the cytoplasmic membrane surface. As PVAP must insert
269 with its N-terminal TM segment into the plasma membrane, the outer layer most likely consists
270 of multiple copies of this part of PVAP plus interspersed membrane lipid. In turn, the
271 cytoplasmic protein sheet must consist of the tightly associated C-terminal domains of the

272 protein. The low-density region between the two layers would then account for the linker region
273 between the cytoplasmic domains and the N-terminal trans-membrane segments (Fig. 7a).

274

275 Recently it was suggested that the opening of STIV-induced VAPs depends on polymerization of
276 ESCRT-III homologs, resulting in the 'stripping' of VAPs from their cytoplasmic membrane (21).
277 Our observation that the outer layer of the double-layered VAP structure is continuous with the
278 plasma membrane (Fig 4) does not support this model. Moreover, global analysis of host gene
279 expression during the SIRV2 infection cycle documented that ESCRT-III-like proteins were
280 down-regulated in infected cells (22). Thus, it is possible, although not very likely, that
281 mechanisms of VAP opening differ in SIRV2-infected and STIV-infected cells.

282

283 Our tomograms of closed and open VAPs throw some light on the mechanism by which the
284 pyramids open to facilitate viral egress. The seven edges of the closed pyramid are slightly
285 curved in a right-handed fashion (Fig. 4 f). In the open state, the edges of the seven individual
286 facets are curved in the same way (Fig. 4 f). In the open VAP, each facet curls outwards (Fig. 4 f).
287 This suggests that in the closed state, the VAP structure is under mechanical tension. This
288 tension is likely to provide the energy required for VAP opening. As the tip of the VAP displays
289 the point of strongest membrane curvature in the whole assembly, it most likely serves as
290 predetermined breaking point, at which the pyramid would begin to unfold along its seams.

291

292 **VAP assembly and opening**

293 We propose a model for VAP assembly (Fig. 7). Upon synthesis in the cytosol, PVAP integrates
294 spontaneously into the cell membrane, depending solely on the hydrophobicity of its N-terminal
295 transmembrane helix segment. In the membrane, tight interactions between PVAP protomers
296 result in the formation of protein sheets, which consist of the observed two layers. We propose
297 that this interaction involves both the N-terminal transmembrane helices and the C-terminal
298 hydrophilic PVAP domains, as indicated by the expression of truncation constructs.

299

300 In order to assemble into a pyramid instead of a flat sheet, at least two different kinds of
301 interactions between PVAPs are necessary, one in-plane interaction within the triangular facets,
302 and one out-of-plane interaction, at the edges of the pyramid. The interaction at the edges is
303 most likely weaker than the in-plane interaction, so that the pyramids open along these lines.
304 Since all VAPs observed in *S. islandicus* or in heterologous systems grow to roughly the same size,
305 the expansion of VAP must be constrained in some way.

306

307 At present it is unknown if the pyramids are built in a one-by-one self-assembly process from
308 individual PVAP protomers or if, upon membrane insertion, the protomers assemble into
309 heptamers, which then combine into pyramids in a second stage of assembly. The prior
310 formation of heptamers in the membrane is suggested by the gel filtration experiments, which
311 show one homogenous peak of ~70 kDa in detergent solution. Given that detergent mimics the
312 hydrophobic membrane environment, it is not unlikely that the same interactions that give rise
313 to the heptamer in a detergent micelle would also promote the formation of heptamers in the
314 membrane, which may thus be the building blocks of the pyramids.

315

316 VAP opening presumably involves a host or virus-specific factor, since the pyramids only open in
317 virus-infected *Sulfolobus* cells but remain closed in PVAP-expressing bacteria and yeast. Once the
318 mechanism that triggers VAP opening is elucidated, this system could be utilised to introduce
319 ~100 nm apertures in any lipid bilayer. VAPs might then be used for targeted drug delivery,
320 releasing compounds from liposomes upon a specific signal. In addition, the PVAP
321 transmembrane domain has the ability to insert into all types of biological membranes and may
322 therefore be fused to proteins that otherwise cannot be reconstituted into lipid bilayers. This
323 system thus has interesting potential applications in basic research, biotechnology and therapy.

324

325

326 **Materials and Methods**

327

328 **Virus and host strains** *S. islandicus* LAL 14/1 cells were grown, synchronized and infected with
329 SIRV2 as described previously (7) and in the Supplementary Information (SI).

330

331 **Plasmid constructs and transformation of *S. acidocaldarius*** SIRV2_ORF98 (NCBI RefSeq ID:
332 NP_666583) was amplified from SIRV2 genomic DNA and cloned into the pSVA1450 plasmid
333 behind an araS promoter, which yielded pTQ26. pTQ26 was transformed to *S. acidocaldarius*
334 M31 as described in the SI.

335

336 **Plasmid constructs and transformation of *E.coli*** SIRV2_ORF98 was amplified from SIRV2
337 genomic DNA with different primers resulting in 3' truncated PCR products of 267, 237, 207, 177
338 and 87 bp. A 5' truncation of ORF98 was created by amplification of a 216 bp product starting at
339 position 81. The same sequence was fused with the 75 bp transmembrane segment of the *E.coli*
340 *Flk* gene as described in the SI. All PVAP gene mutants were cloned into the T7 promoter-driven
341 expression vector pSA4. Expression was induced with isopropyl β -d-1-thiogalactopyranoside
342 (IPTG). Analysis of PVAP expressing cultures by high pressure freezing, freeze substitution and
343 western blotting was performed as described in ref (9) and (7).

344

345 **Plasmid constructs and transformation of *S. cerevisiae*** SIRV2_ORF98 was amplified from
346 SIRV2 genomic DNA and cloned in the expression vector pCM190. *S. cerevisiae* was transformed
347 with this plasmid. A pre-culture was grown in selective medium as described in the SI. After one
348 day, cells were diluted 1/1000 in medium without doxycyclin.

349

350 **Immuno-electron microscopy** Yeast cells were fixed, washed, pelleted in gelatin and the
351 gelatine pellet was solidified on ice and cut into small blocks as described in the SI. These were
352 infiltrated 2.3M sucrose, mounted on aluminium pins and frozen in liquid nitrogen. Thin sections
353 were cut and picked up in a 1:1 mixture of 2.3M sucrose and 2% methylcellulose. Labelling for
354 PVAP was done as described previously (7).

355

356 **High-pressure freezing and freeze-substitution** *E.coli* cells were taken up in cellulose
357 capillary tubes and *S. cerevisiae* cultures were concentrated by filtration. Samples were high-
358 pressure frozen and freeze-substitution was performed in anhydrous acetone containing 2%
359 osmium tetroxide. Afterwards the samples were washed with dry acetone and embedded
360 stepwise in EPON. After heat polymerization thin sections were cut, collected on 200 mesh

361 Formvar-coated copper grids and post-stained as described in the SI. Images were recorded with
362 a JEOL 1010 electron microscope equipped with an Olympus Keen View camera.

363

364 **Whole cell cryo-tomography** *S. islandicus* cells were harvested, concentrated by low-speed
365 centrifugation and plunge-frozen. *E. coli* cells overexpressing *PVAP* were harvested at the same
366 conditions, washed once in 50mM Tris, 300mM NaCl, pH7 and plunge-frozen in the same buffer.
367 Before freezing, suspensions were mixed with an equal volume of 10 nm colloidal protein-A gold
368 suspension. Tomograms were recorded with a Polara G2 Tecnai field emission transmission
369 electron microscope equipped with a Gatan Tridiem energy filter and 2x2 k CCD camera.
370 Tomographic tilt series of zero-loss filtered images were recorded and tomograms
371 reconstructed as described in the SI.

372

373 **Subtomogram averaging** For subtomogram averaging of VAPs, 57 pyramid volumes were cut
374 out from a single tomogram of a PVAP overexpressing *E. coli* cell, aligned and averaged applying
375 7-fold rotational symmetry using the PEET software as described in the SI. The resolution of the
376 map was estimated using the ResMap software (13).

377

378 **PVAP purification** A codon-optimized SIRV_ORF98 gene was synthesized and inserted in the
379 plasmid pET26b. *E. coli* BL21DE3/Rosetta/pLysS cells were transformed with this plasmid.
380 Protein expression was induced with 1mM IPTG. After 2 hours, cells were pelleted, resuspended
381 in lysis buffer and disrupted with a Microfluidizer. The membrane fraction was pelleted by
382 centrifugation, and diluted in 50 mM Tris pH 7.0, 300 mM NaCl to a protein concentration of 5
383 mg/ml. 1.5% N-laurylsarcosine was added. After high-speed centrifugation the supernatant was
384 loaded onto a Ni-NTA column followed by several washing steps. The protein was eluted in
385 buffer containing 500 mM imidazole and concentrated using Amicon spin columns with a 30 kDa
386 cutoff prior to loading onto a gel filtration column, as described in more detail in the SI.

387

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392 Data deposition: The map and fitted model reported in this paper have been deposited
393 in the Electron Microscopy Data Bank (accession no. 5844).

394

395

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446

447 **Figure Legends**

448

449 **Fig. 1. Morphological changes of *S. islandicus* during infection with SIRV2.** Tomographic
450 slices of typical archaeal cells at 0.5 (a), 3-6 (b) and 9 (c) hours post infection with SIRV2. Black
451 arrowheads, closed VAPs; white arrowheads, open VAPs. Scale bars, 500 nm.

452

453 **Fig. 2. VAPs in closed and open conformation. (a, c, e, g)** Tomographic slice and segmented,
454 surface-rendered volumes (b, d, f, h) of VAPs in the membrane of SIRV2-infected *S. islandicus*
455 cells. VAPs are either closed (a-d) or open (e-h). The S-layer is purple, the cell membrane blue
456 and the VAP is yellow. Scale bars, 200nm

457

458 **Fig. 3. SIRV2 virion egress.** Rendered tomographic volume of a SIRV2-infected *S. islandicus* cell,
459 12 h post infection. SIRV2 virions (orange, brown, purple) are released through open VAPs
460 (yellow) that create ~ 100 nm apertures in the plasma membrane and S-layer (green). Orange,
461 virions inside the cell; purple, virions escaping from the cell; brown, virion outside the cell.
462 Transparent blue, viral or host DNA. Scale bar: 500 nm.

463

464 **Fig. 4. VAP structure.** Tomographic slices through closed (a) and open (b) SIRV2-induced VAPs
465 of *S. islandicus* and VAPs formed after PVAP expression in *E. coli* (c), indicating two layers, one of
466 which is continuous with the cell membrane (black arrowheads), while the other (white
467 arrowheads) forms a sheet at the cytoplasmic surface of the membrane. VAPs in *S. islandicus*
468 protrude through the S-layer (white arrows). (d-k) 3D map of VAP obtained by subtomogram
469 averaging, with sevenfold symmetry applied. Tomographic slice perpendicular to the pyramid
470 base (d) and successive tomographic slices parallel to the base (e) show the two layers in the
471 walls of the pyramid. (f) Top-view of the 3D map in solid representation shows that the edges of
472 the of the seven pyramidal facets are slightly curved counter-clockwise (dotted lines). (g-k)
473 Different orientations of the 3D map in surface representation. Transparent mesh and golden
474 surface show different threshold levels. Black and white arrowheads indicate outer and inner
475 layer, respectively. Open arrowheads indicate connections between inner and outer layers of the
476 VAP. Scale bars, 200 nm (a-c), 50 nm (d-k).

477

478 **Fig. 5. VAP formation in *S. cerevisiae*.** PVAP expression in *S. cerevisiae* causes VAP formation in
479 various cellular membranes. (a,b) Immuno-labelling of unfrozen cryo-sections with anti-PVAP
480 antibodies. (a) VAP in the endoplasmic reticulum. (b) VAPs in mitochondrial membranes (c)

481 Freeze-substituted cell with VAP in the nuclear envelope. The inset shows an enlarged VAP. P,
482 plasma membrane; CW, cell wall; M, mitochondrion; N, nucleus. Arrows indicate VAPs. Scale
483 bars, 200 nm.

484

485 **Fig. 6. Expression of PVAP variants in *E.coli*.** Several PVAP truncation mutants were
486 constructed. (a) Schematic representation of PVAP constructs expressed in *E.coli*. (PVAP) wild
487 type PVAP. (PVAP Δ C10) PVAP lacking 10, (PVAP Δ C20) 20, (PVAP Δ C30) 30, (PVAP Δ C40) 40, or
488 (PVAP Δ C70) 70 C-terminal residues. (PVAP tm Flk) PVAP construct in which the transmembrane
489 segment is replaced by that of the *E.coli* membrane protein Flk. (PVAP Δ N30) PVAP lacking the N-
490 terminal transmembrane segment. (b) Electron micrographs of thin sections through *E.coli* cells
491 expressing PVAP constructs as in A. VAPs (open arrows) and invagination of the membrane
492 (black arrow) are shown; Bars, 200 nm; bar in inset, 100 nm.

493

494 **Fig. 7. Model of VAP assembly.** (a) Predicted secondary structure of PVAP, with N-terminal
495 trans-membrane helix and three short C-terminal α -helices. (b) Schematic top view of the VAP,
496 with its seven facets in different colours. Strong interactions between individual PVAP
497 protomers stabilise the integrity of each facet, whereas weak interactions at the seams (white
498 dashed lines) between two neighbouring facets form predetermined break points. The black
499 dashed indicates the cross section through the VAP in in c. (c) PVAP protomers (green) insert
500 spontaneously into the plasma membrane (blue), with the short C-terminal helices exposed to
501 the cytoplasm. Close contacts between PVAP molecules in each facet (aqua and orange outline)
502 exclude S-layer proteins (grey), and combine into a protein sheet below the plasma membrane.
503 Addition of PVAP units at the base pushes the pyramid outwards.

504

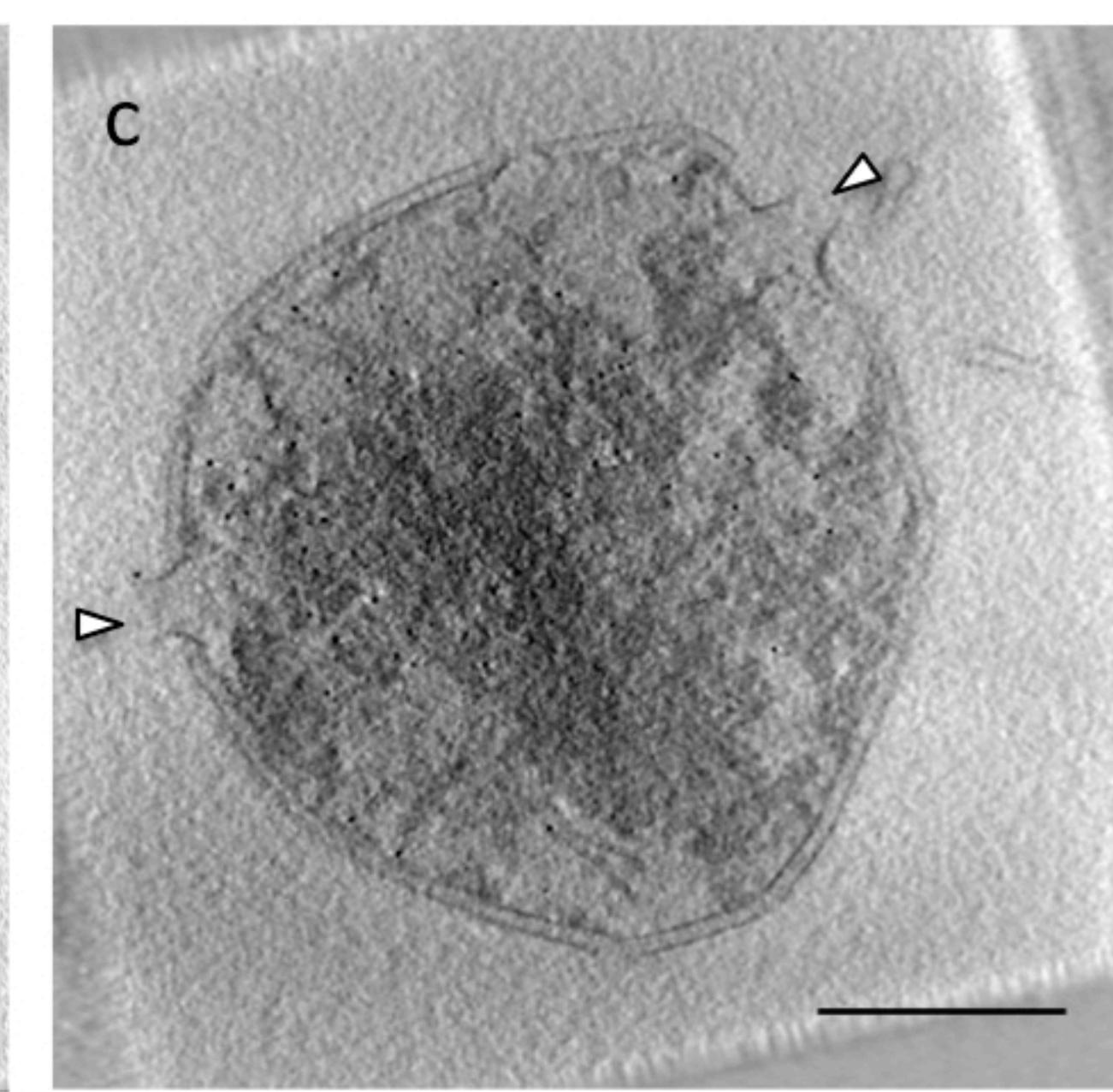
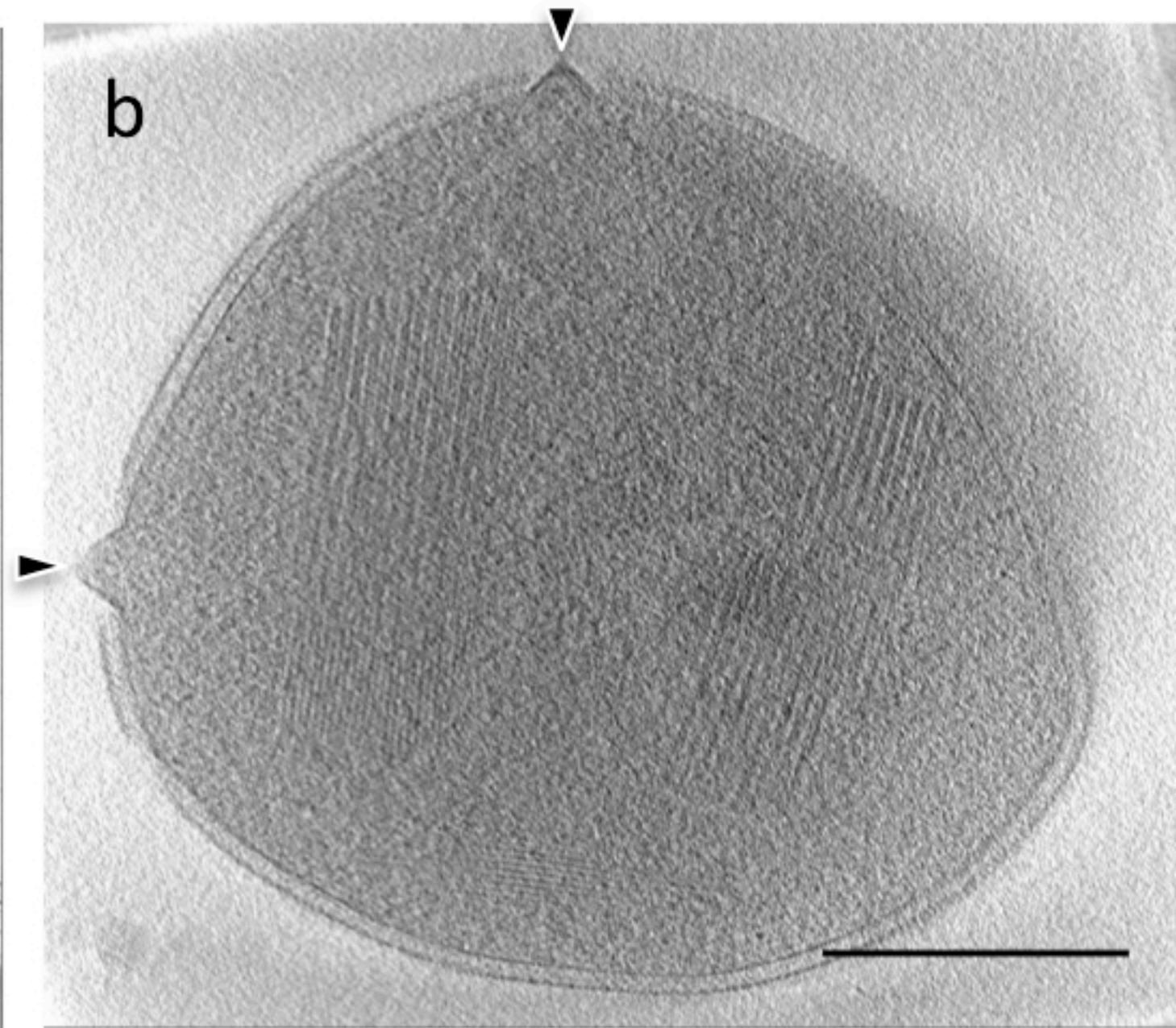
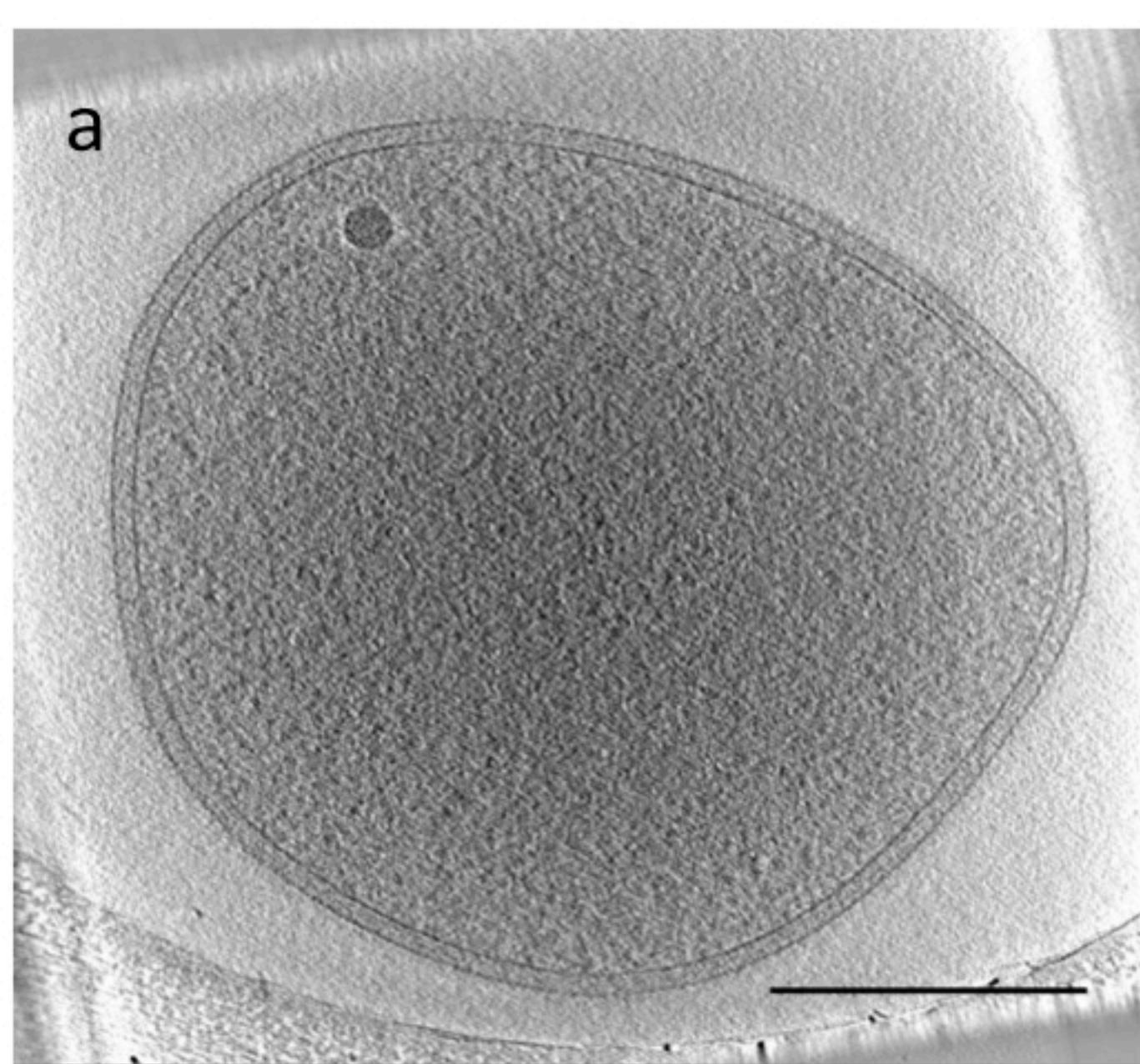
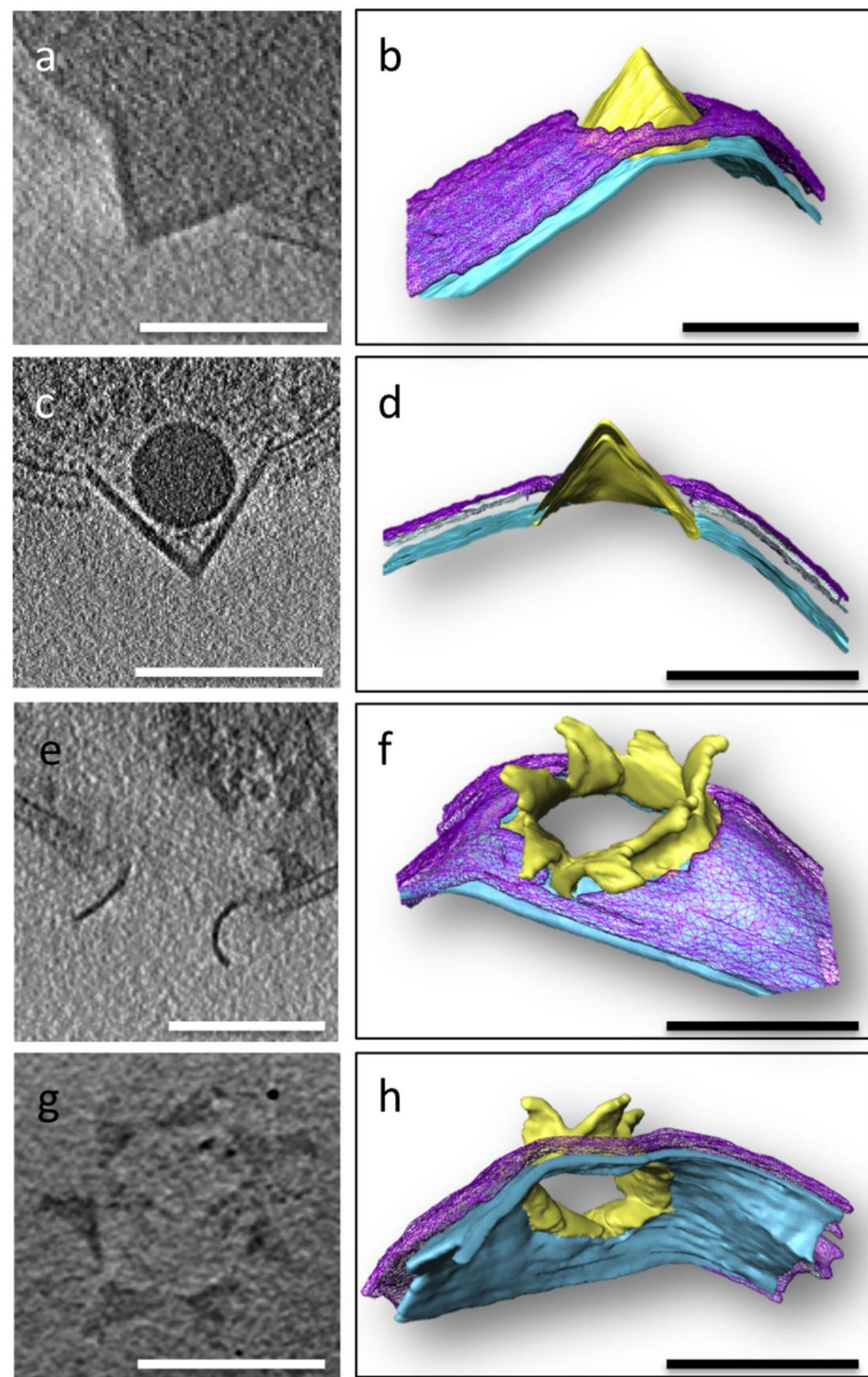
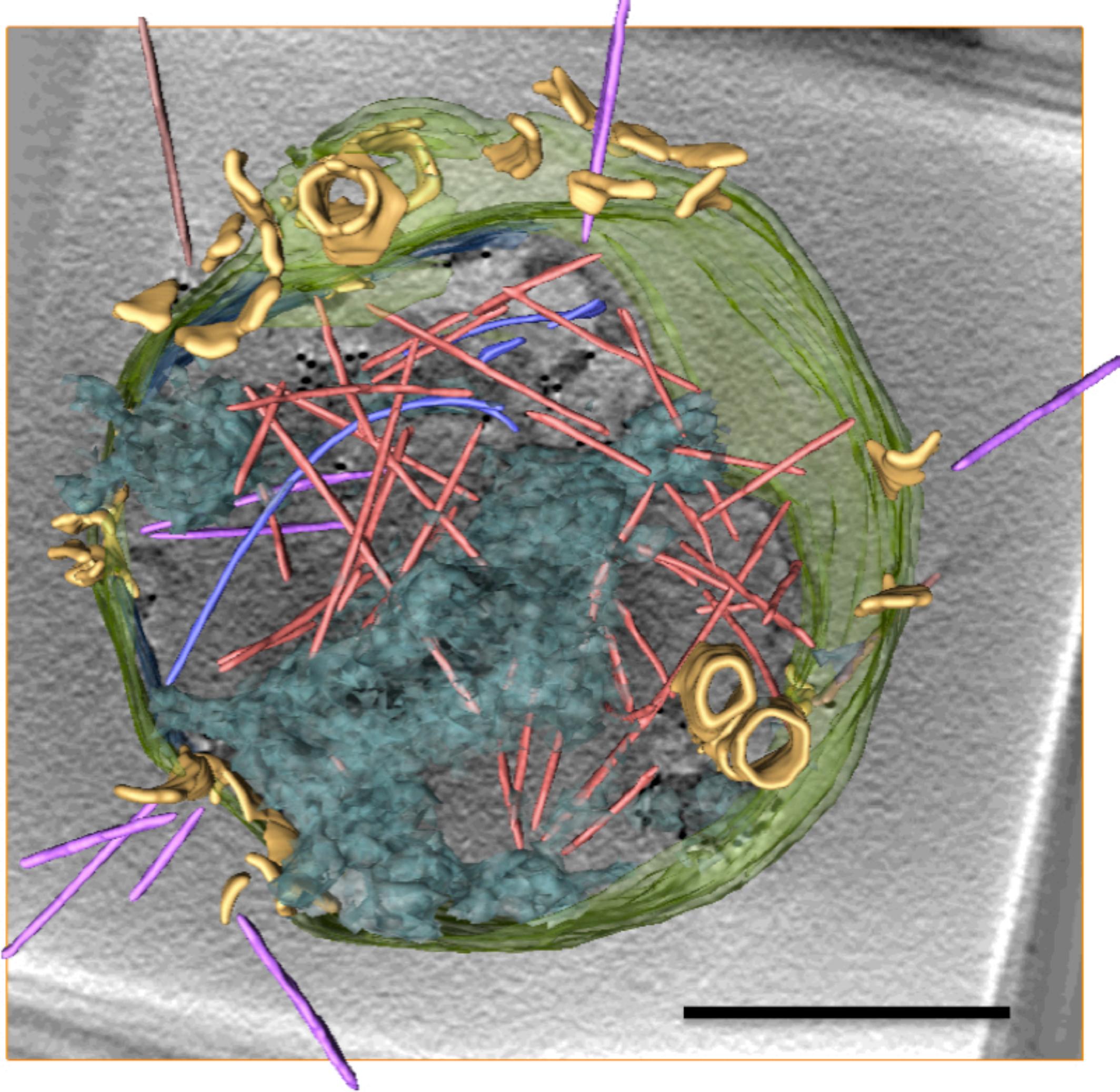
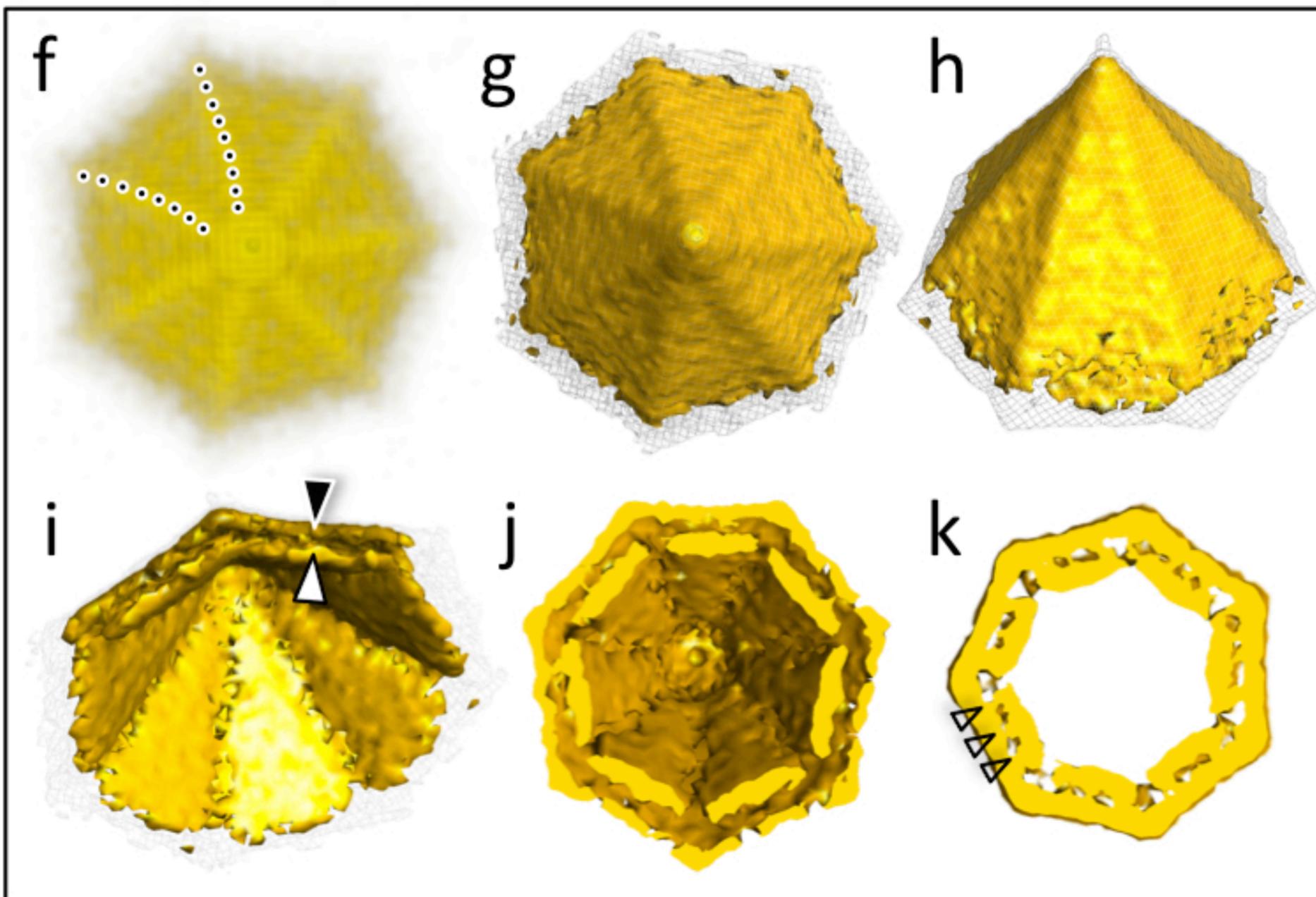
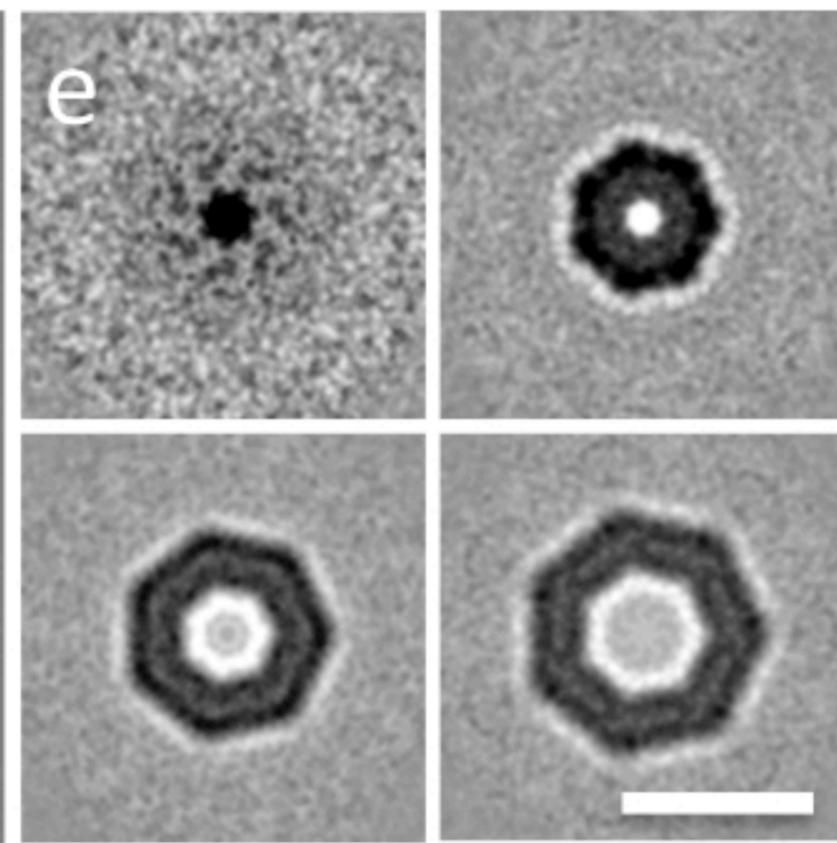
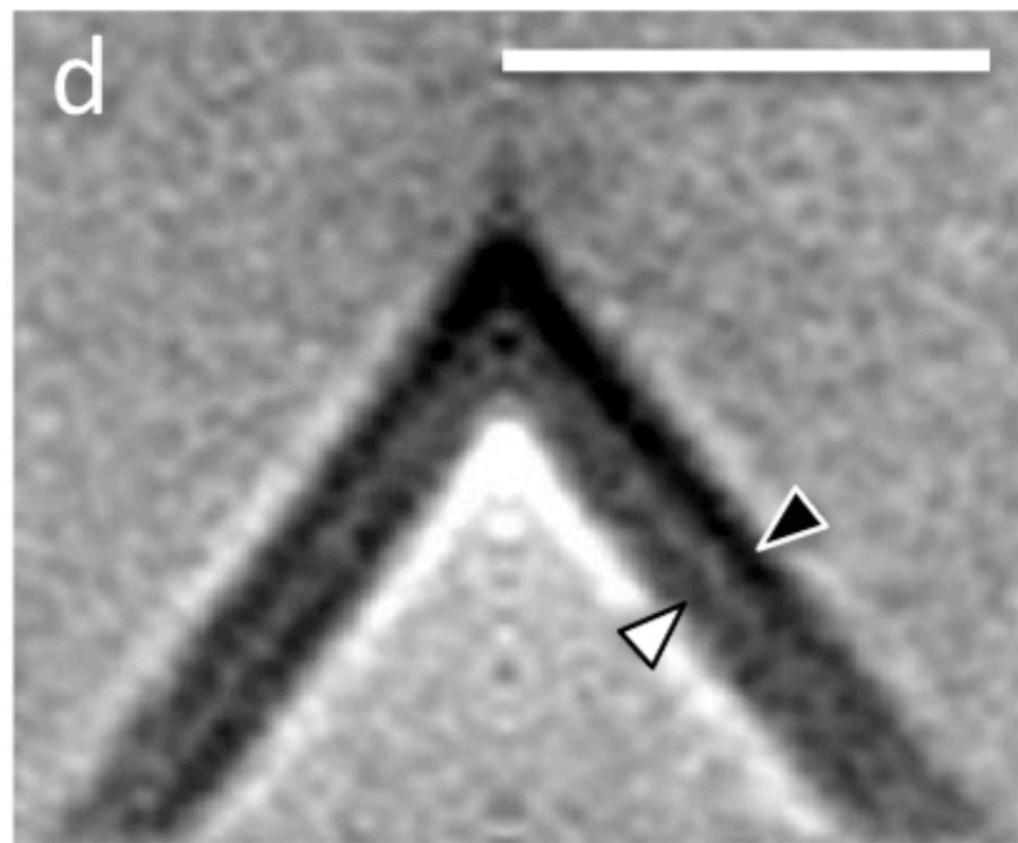
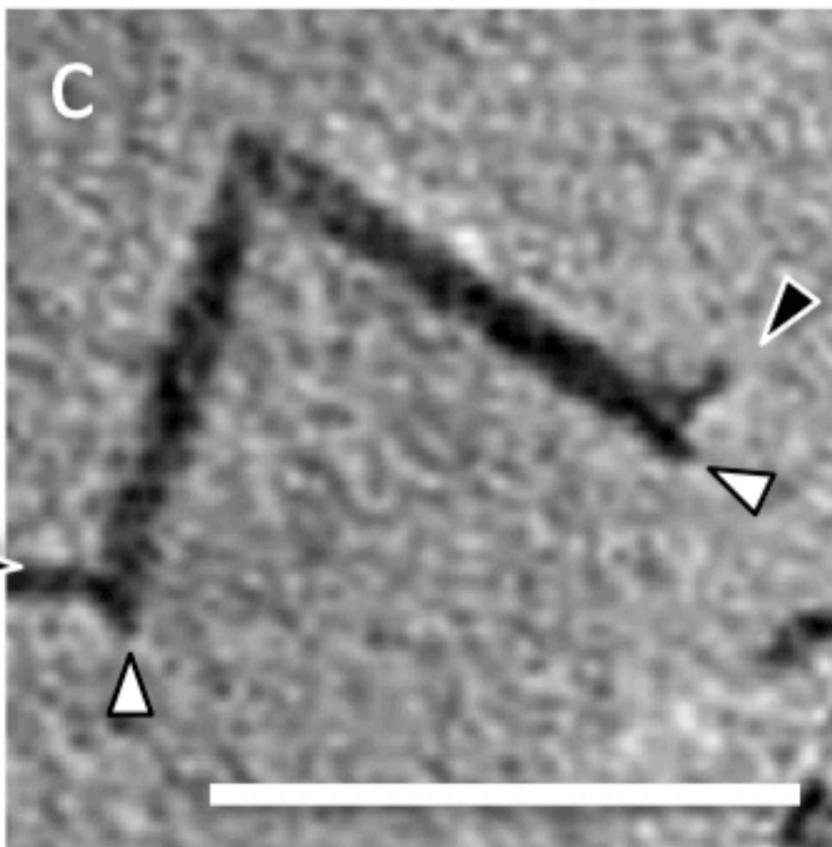
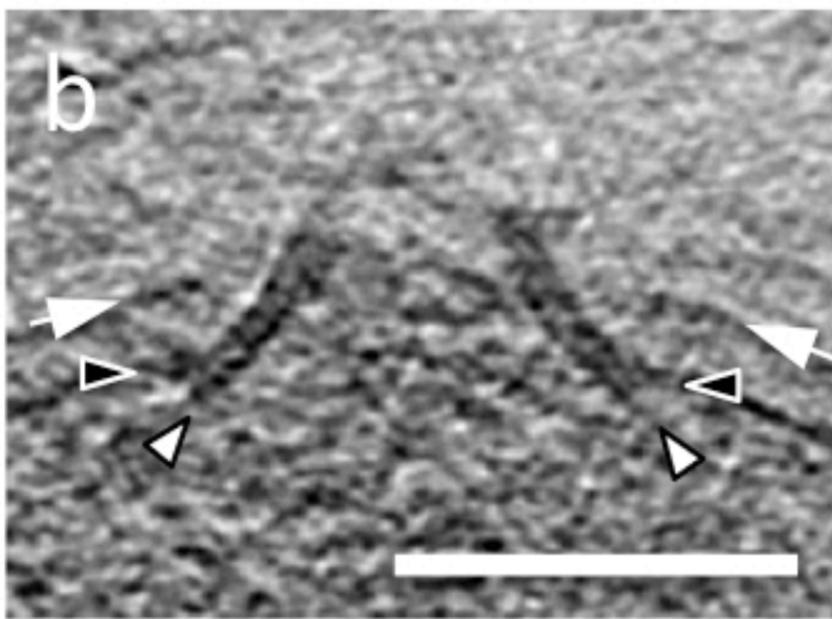
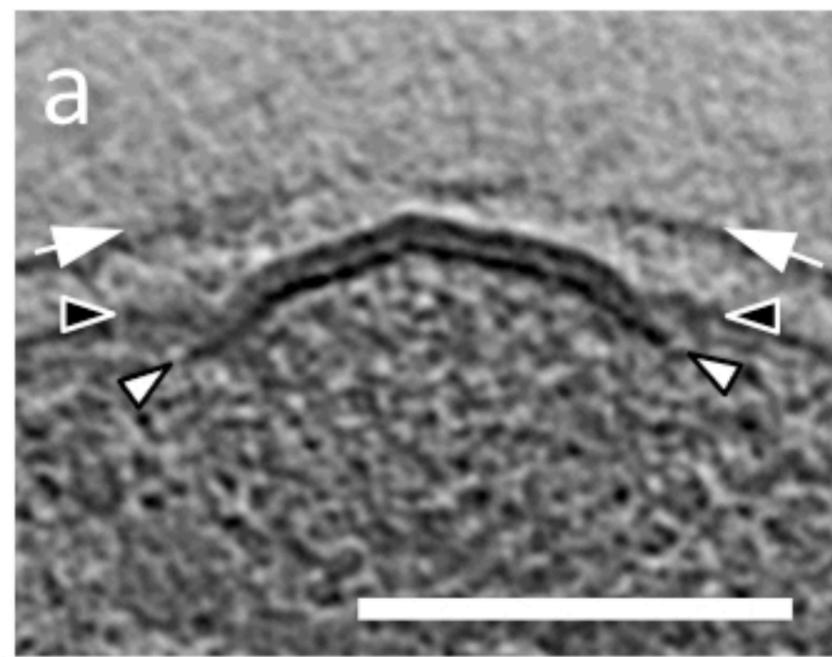
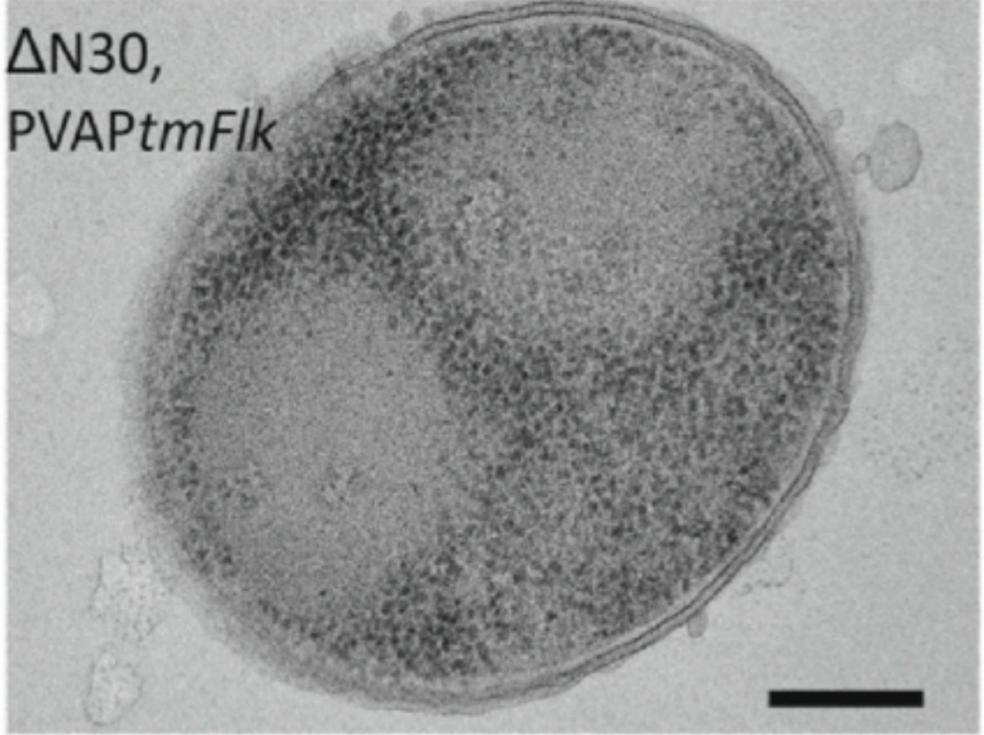
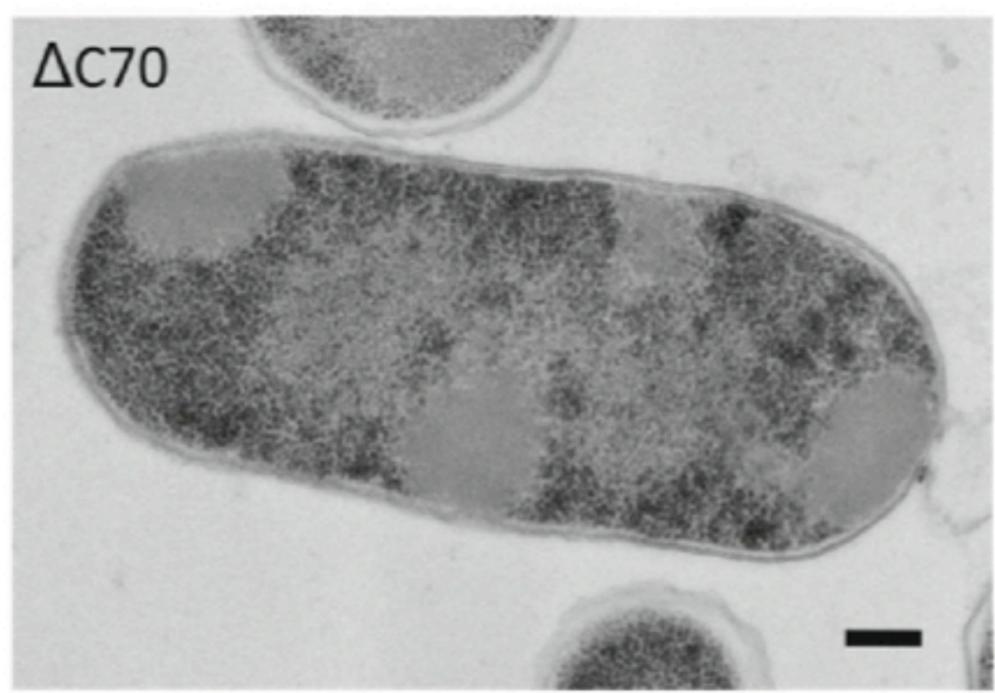
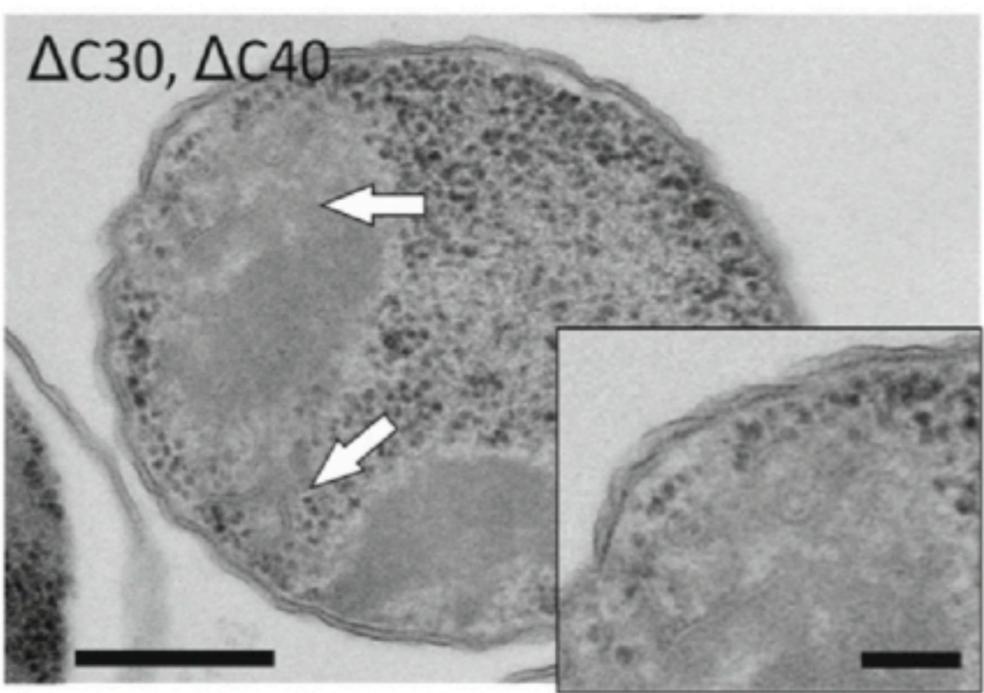
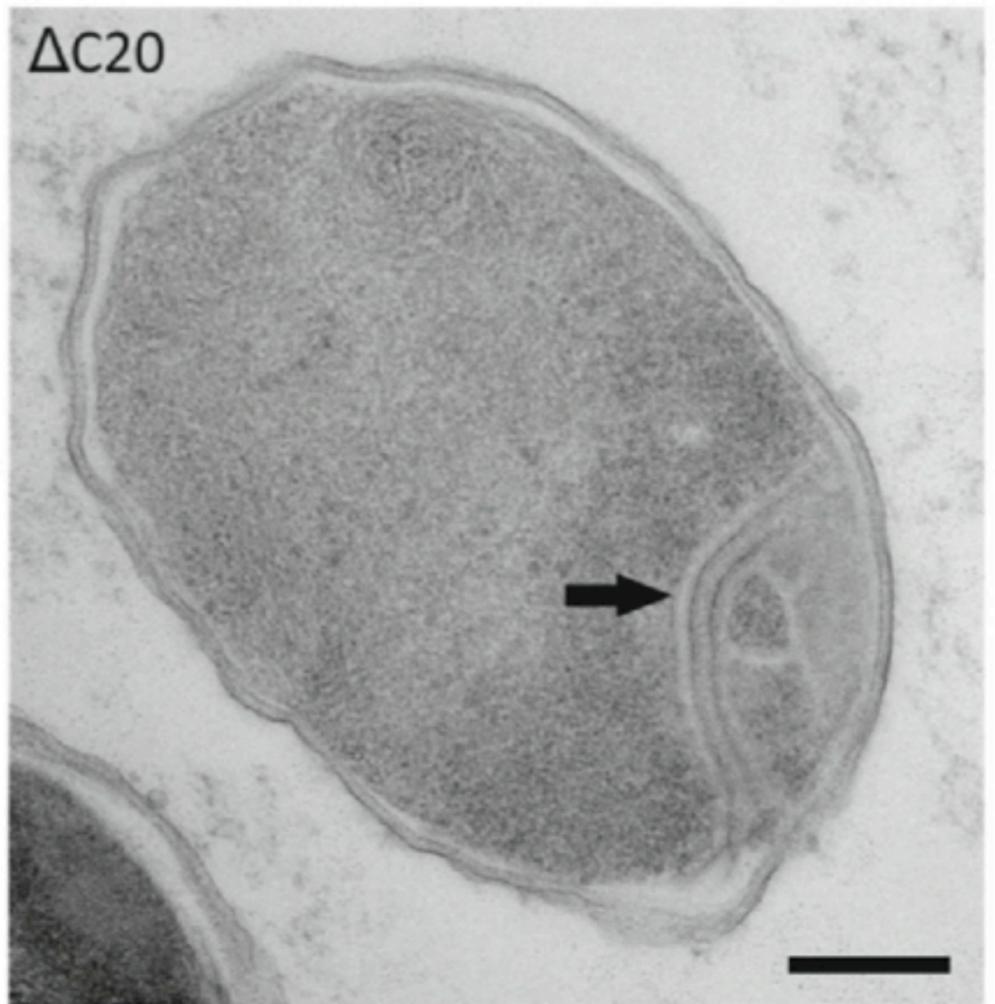
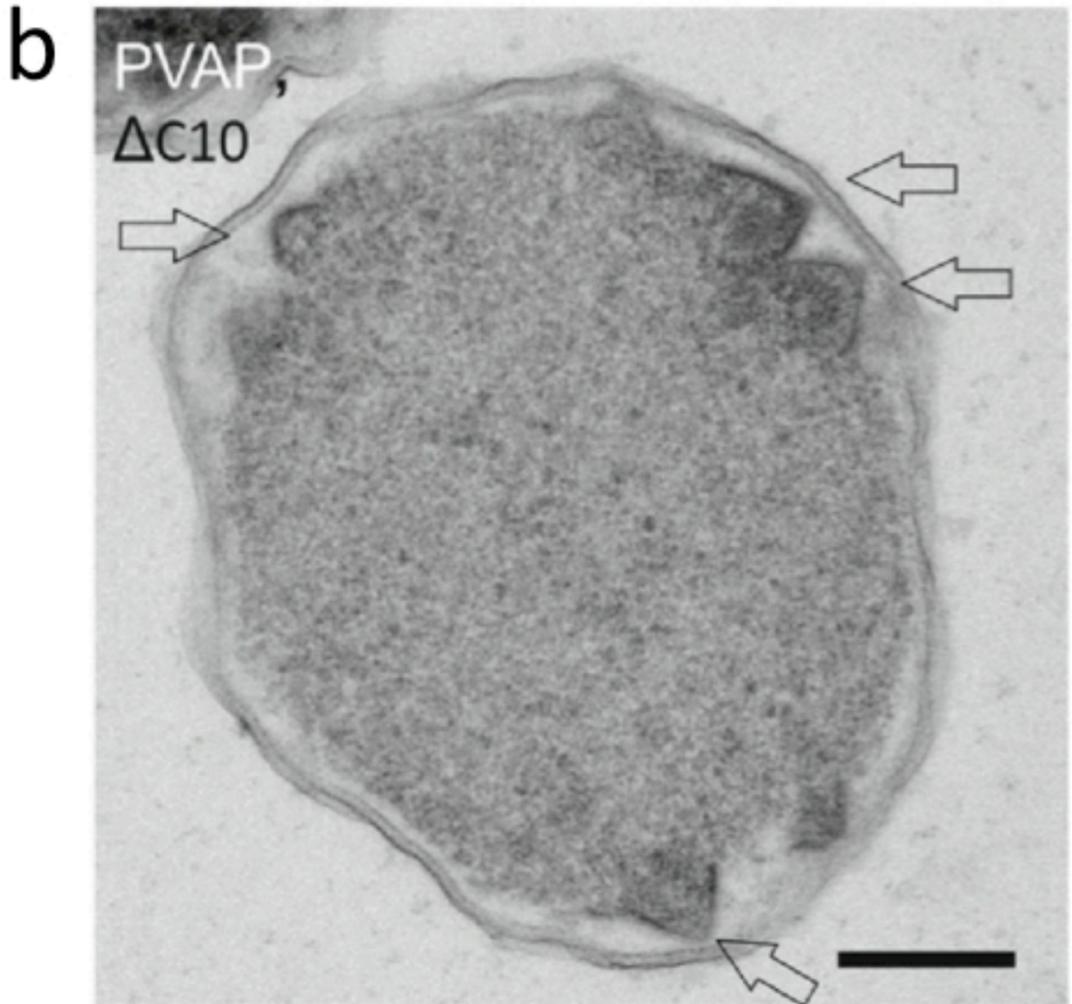
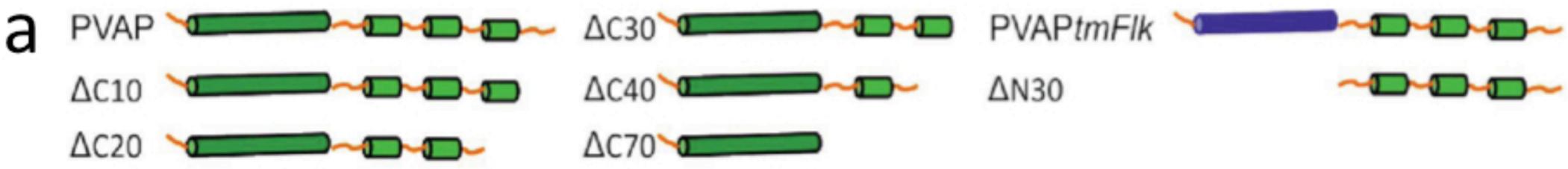


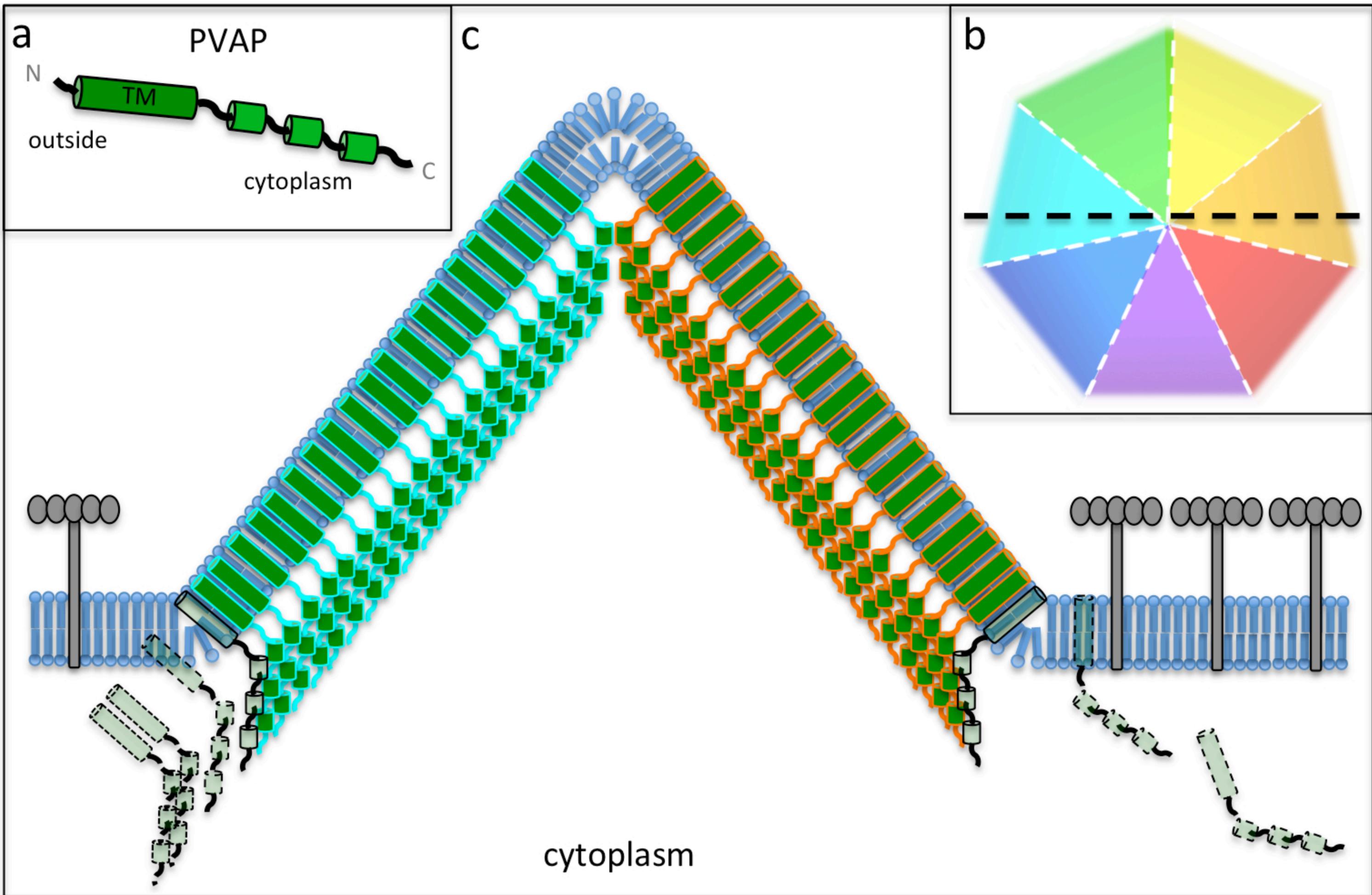
Figure 2











Supplementary Figure Legends

Supplementary Figure 1. Early stage of VAP assembly. Consecutive tomographic slices through nascent VAP at 3 h.p.i. in *S. islandicus* host cell. Black arrows indicate VAP. PM, plasma membrane. Scale bar: 100 nm

Supplementary Figure 2. Secondary structure prediction of PVAP. Sequence of wild type PVAP (black lettering) with predicted secondary structure as indicated. Blue, predicted α -helices (a); yellow, coils (c); red, strands (e). TM segment, predicted trans-membrane segment.

Supplementary Figure 3. VAPs in *E. coli* and *S. acidocaldarius*. Tomographic slices through *E. coli* (upper panel) and *S. acidocaldarius* (lower panel) cells expressing PVAP. VAPs in *E. coli* remain closed for longer than 2 weeks. VAPs in *S. acidocaldarius* open ~72 hours after PVAP induction; bars, 500 nm.

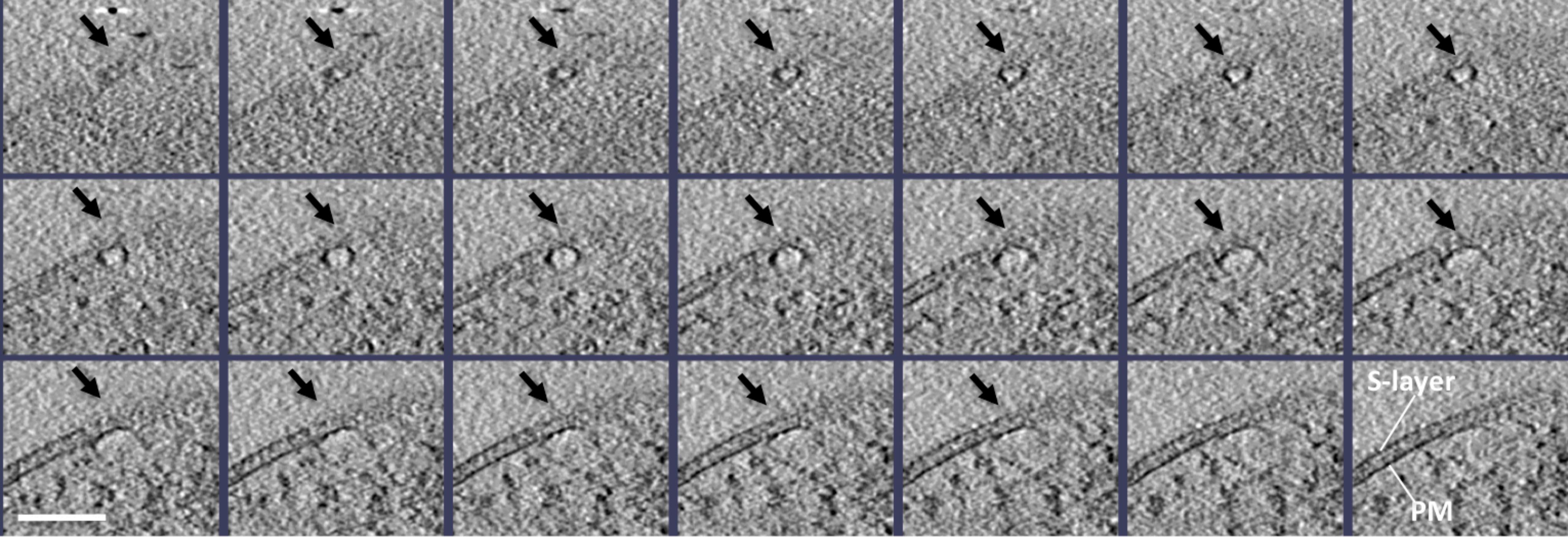
Supplementary Figure 4. Unsymmetrised subtomogram average of VAPs. Average of 57 VAPs obtained from *E. coli* cells overexpressing PVAP. Slices run perpendicular (a) or parallel (b) to the base of the pyramid. The unsymmetrized average shows clear 7-fold symmetry around an axis perpendicular to the base. Scale bars: 50 nm.

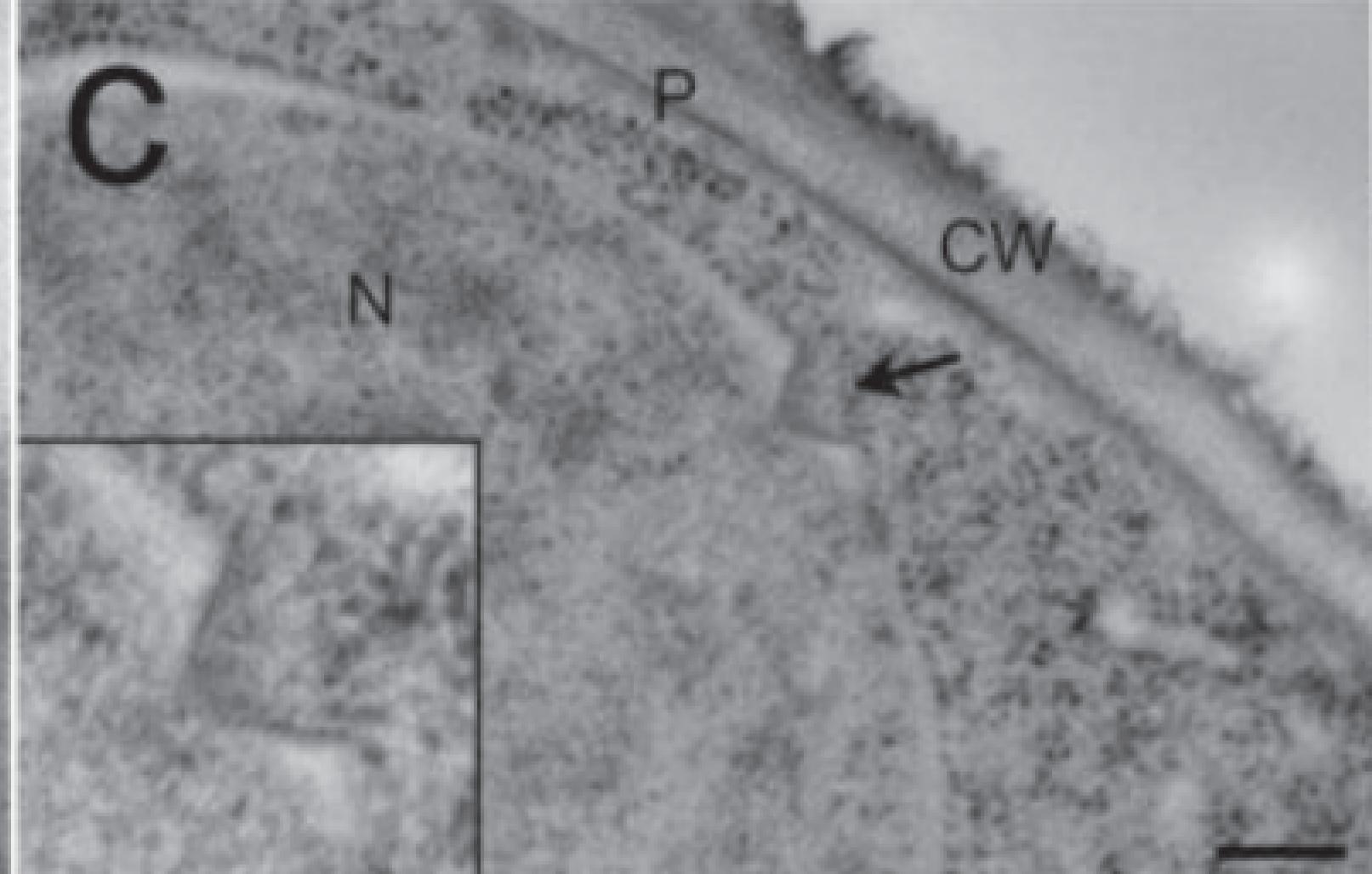
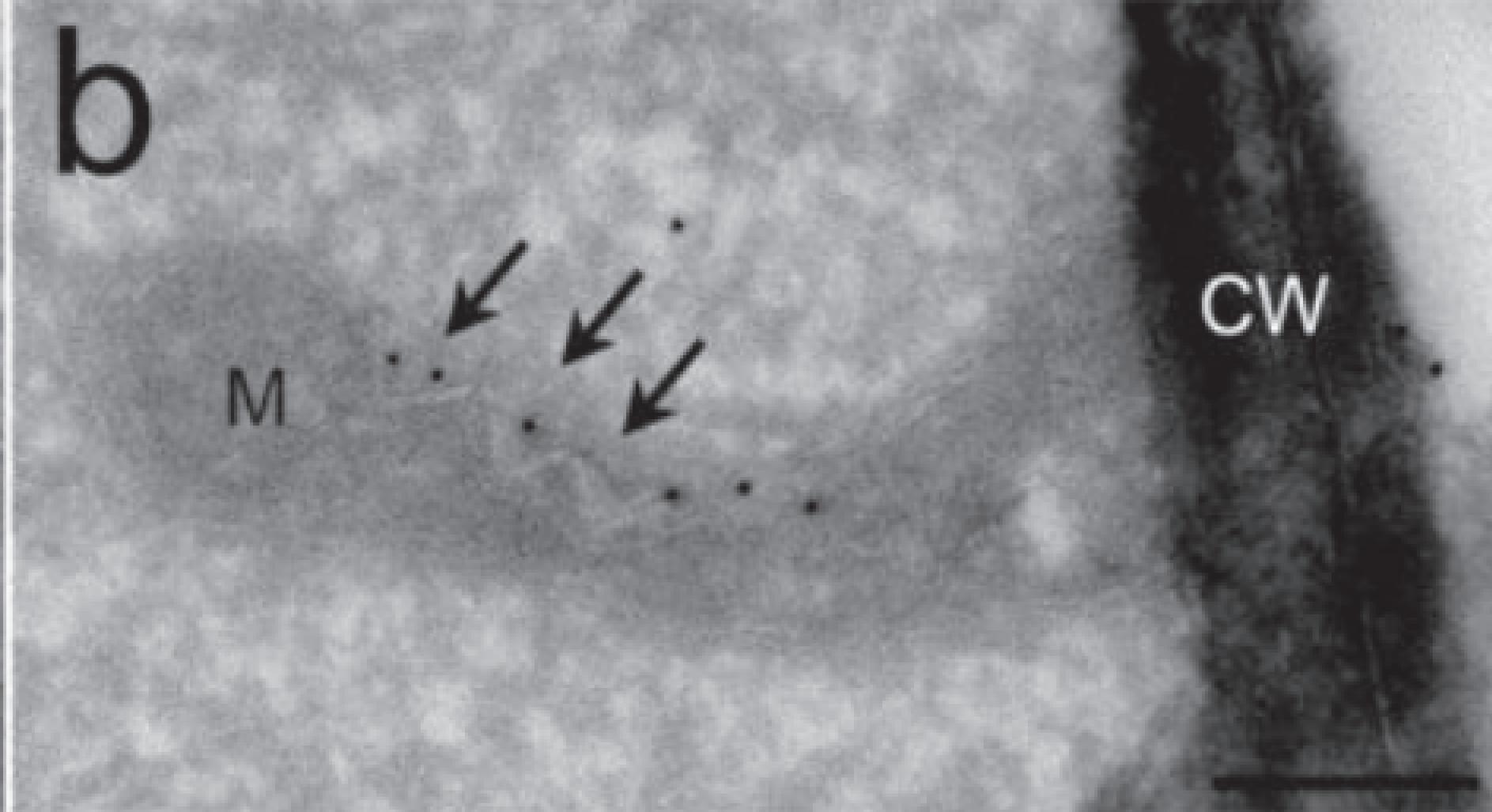
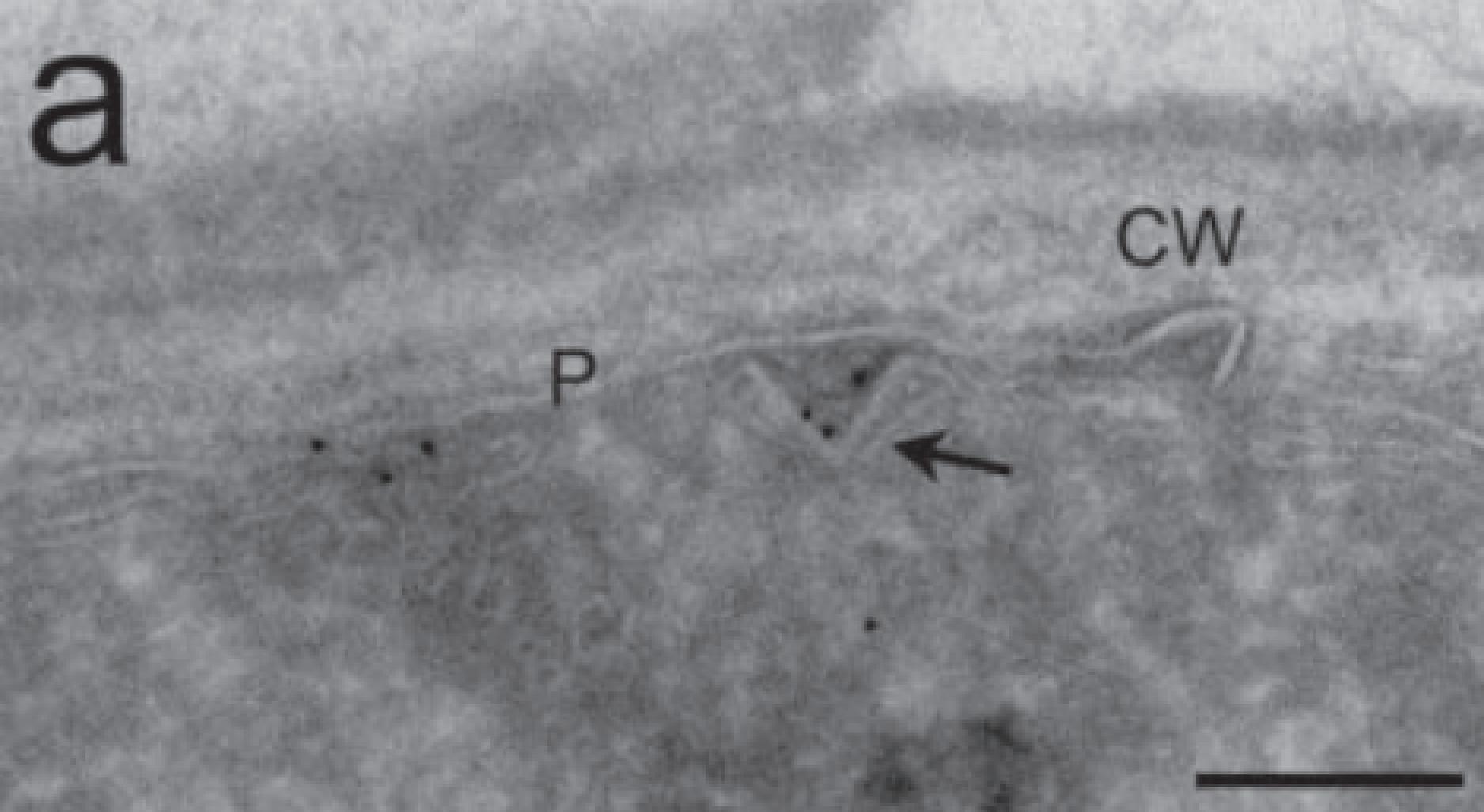
Supplementary Figure 5. Resolution estimate of VAP subtomogram average. (a) Local resolution for successive slices through the 3D map is shown in different colours. (b) Histogram showing the number of voxels for which a certain resolution was estimated. The estimated local resolution of the average is between 36 and 58 Å. Mean and median resolution are 43 and 42 Å, respectively.

Supplementary Figure 6. Flk-PVAP fusion protein localizes to *E. coli* membranes. SDS/PAGE of: 1, marker; 2, SIRV2-infected *S. islandicus*, 10 hpi; 3-4, membrane fraction of *E. coli* expressing Flk-PVAP fusion (PVAP_{tm}Flk); 5-6 cytosolic fraction of *E. coli* expressing PVAP_{tm}Flk. The *E. coli* samples were loaded undiluted or diluted 1:10. The positions of proteins with known molecular mass (in kDa) are indicated by bars based on the marker loaded in the first lane. (a) Coomassie Blue-stained gel. (b) Western blot of a duplicate gel with antibodies against SIRV2-PVAP. Mem, membrane fraction; Cyt, cytosolic fraction

Supplementary Figure 7. Analytical size exclusion chromatography of PVAP. Elution profile of PVAP on the Superdex75 column shows a symmetrical peak eluting at 70 kDa. SDS-PAGE of the peak fraction and Western-Blot analysis with antibodies against PVAP indicate protein bands at the level expected for the PVAP monomer, dimer, trimer and heptamer.

Supplementary Movie 1. 3D map of the VAP. Subtomogram average of 57 VAPs in closed state.





Supplementary Methods

Virus and host strains The SIRV2 virus stock was prepared and the *S. islandicus* LAL 14/1 strain grown as described previously (1). For analysis of morphological changes upon viral infection, cells were synchronized by dilution of precultures in fresh medium. The cultures were grown for ~12 hours until an OD of 0.1-0.2 was reached and SIRV2 was added directly to the cultures as described (1).

Plasmid constructs and transformation of *S. acidocaldarius* For the expression of *PVAP* in *S. acidocaldarius* M31, SIRV2_ORF98 (NCBI RefSeq ID: NP_666583) was amplified from SIRV2 genomic DNA and cloned into the lacS gene locus in the pMZ1 plasmid (2) using the NcoI and BamHI sites. SIRV2_ORF98 (*PVAP*) and the araS promoter were transferred from pMZ1 to the pSVA1450 plasmid (3) using the NcoI and EagI sites, which yielded pTQ26. pTQ26 was transformed to *S. acidocaldarius* M31. The preparation of competent cells, methylation of the plasmid, and electroporation were carried out as described (4) using a Bio-Rad Gene Pulser Xcell electroplater with 1-mm cuvettes at 1,500 V, 600 Ω , and 25 μ F. Selection of *PVAP* expressing colonies and induction of expression were performed as before (5).

Plasmid constructs and transformation of *E. coli* For the overexpression of *SIRV2_ORF98* (*PVAP*) C-terminal truncation mutants in *E. coli* Rosetta(DE3)pLys (Novagen Merck), SIRV2_ORF98 was amplified from SIRV2 genomic DNA with different reverse primers resulting in PCR products of 297 (full length), 267 (-10 AA), 237 (-20 AA), 207 (-30 AA), 177 (-40 AA), 87 (-70 AA) bp. PCR amplification of a 216 bp product starting 81 bp downstream of the ATG resulted in a *PVAP* mutant lacking the N-terminal transmembrane domain. The same sequence was used for a fusion with the 75 bp transmembrane segment of the *E. coli* *Flk* gene, preceded by the 27 N-terminal bp of *PVAP*. This oligonucleotide sequence was synthesized by GeneArt® (Invitrogen). All *PVAP* gene mutants were cloned into the T7 promoter-driven expression vector pSA4 using the NcoI and BamHI sites (6). The pSA4 vector contains an isopropyl β -d-1-thiogalactopyranoside-inducible promoter that was used for the expression of a C-terminally His-tagged protein. Analysis of *PVAP* expression cultures by high pressure freezing and freeze substitution was performed as described (5). The location of the *Flk*-*PVAP* fusion was analyzed by isolation of membranes as described in ref (11), followed by SDS PAGE, western blotting and immunolabelling with antibodies raised against *PVAP* as described (5).

Plasmid constructs and transformation of *S. cerevisiae* For the overexpression of *SIRV2_PVAP* (*PVAP*) in *Saccharomyces cerevisiae*, *SIRV2_PVAP* was amplified from SIRV2 genomic

DNA and cloned in the expression vector pCM190 (7) using PstI and XbaI sites. *S. cerevisiae* was transformed with the plasmid according to Gari et al. (7). After selection on plates without uracil a single colony was picked and grown at 30 °C overnight in a pre-culture in selective uracil-free medium, and with 10 microgram/ml doxycyclin. After one day, cells were diluted 1/1000 in medium without doxycyclin.

Immuno-electron microscopy Yeast cells were fixed with 4% paraformaldehyde in 0.1M Hepes, pH 5.4 for 2 h at RT. The cells were then washed with 50 mM NH₄Cl in PBS to quench free aldehyde groups and pelleted in 12% gelatin in PBS. The gelatine pellet was solidified on ice and cut into small blocks, which were infiltrated over night at 4°C with 2.3M sucrose for cryoprotection, mounted on aluminium pins and frozen in liquid nitrogen. Thin sections were cut with a UC6/FC6 (Leica microsystems, Vienna, Austria) and picked up in a 1:1 mixture of 2.3M sucrose and 2% methylcellulose (8). Labelling for PVAP was done as described previously (5).

High-pressure freezing and freeze-substitution *E.coli* cells were taken up in cellulose capillary tubes (Leica Microsystems GmbH, Vienna, Austria) as described in (9). *S. cerevisiae* cultures were concentrated by filtration. Tubes or cell concentrates were placed into brass planchettes filled with 1-hexadecen (Agar Scientific, Stansted, United Kingdom). Samples were frozen in a HPM 010 high pressure freezer (Baltec, now Abra Fluid AG, Widnau, Switzerland). Freeze-substitution was performed in anhydrous acetone containing 2% osmium tetroxide (Merck, Germany). Small cracks were introduced under liquid nitrogen in solid 1-hexadecen by pre-cooled fine point forceps (No 5, Dumont, Switzerland) to allow perfusion of the substitution mix. Freeze-substitution was carried out at -90°C for 24h, and at -60°C and -30°C for 8h each in a freeze substitution device (Leica Microsystems GmbH, Vienna, Austria). Afterwards the temperature was raised to 0°C and the samples were washed with dry acetone and embedded stepwise in EPON. After heat polymerization thin sections were cut with an Ultracut UCT microtome (Leica Microsystems GmbH, Vienna, Austria). Sections were collected on 200 mesh Formvar-coated copper grids and post-stained with 4% uranylacetate and Reynold's lead citrate. Images were recorded with a JEOL 1010 electron microscope at 80 kV equipped with an Olympus Keen View camera (Olympus Soft imaging systems, Münster, Germany).

Whole cell cryo-tomography *S. islandicus* cells were harvested shortly after infection, concentrated by low-speed centrifugation (3000 rpm for 10 min) and plunge-frozen directly in the growth medium. For this, cell pellets were resuspended in an equal volume of fresh medium. *E. coli* cells overexpressing PVAP were harvested at the same conditions, washed once in 50mM Tris, 300mM NaCl, pH7 and plunge-frozen in the same buffer. Before freezing, suspensions were

mixed with an equal volume of 10 nm colloidal protein-A gold suspension (Aurion, Wageningen, The Netherlands). 3 μ l of this mixture were added to a 300 mesh R2/2 glow-discharged Quantifoil grid, blotted and rapidly injected into liquid ethane using a homemade plunge freezer. Tomograms were recorded with a Polara G2 Tecnai field emission transmission electron microscope (FEI, Hillsboro, USA) operated at 300 kV, equipped with a Gatan Tridiem energy filter and 2x2 k CCD camera (Gatan Inc., Pleasanton, USA). Zero-loss filtered images were collected using the Digital Micrograph software (Gatan Inc., Pleasanton, USA). Tomographic tilt series were recorded with the FEI tomography software (FEI Company, Hillsboro, USA). Tilt series were generally collected in a range of -60° to $+60^\circ$ in steps of 1.5° or 2° , at 6-9 μ m defocus and magnifications of 41.000x, 34.000x or 27.500x, corresponding to a pixel size of 0.5766nm, 0.709nm or 1.073nm, respectively. Tomograms were reconstructed using the IMOD software package (10) and de-noised by non-linear anisotropic diffusion (NAD) (11).

Subtomogram averaging For subtomogram averaging of VAPs, 57 pyramid volumes were cut out from a single tomogram of a PVAP overexpressing *E. coli* cell, aligned and averaged using the PEET software (12,13) as described before (14). The average volumes were averaged further by applying 7-fold rotational symmetry. 3D maps obtained by subtomogram averaging were displayed and analyzed in 3Dmod (IMOD,(34)) or UCSF chimera (15). The resolution of the map was estimated using the ResMap software (16).

PVAP purification The codon-optimized gene coding for PVAP was synthesized (Genscript) and inserted in to the expression plasmid pET26b (Novagene) using the restriction sites NdeI and XhoI. *E. coli* BL21DE3/Rosetta/pLysS cells were transformed with the resulting plasmid and transformants were selected using kanamycin (Kan, 50 μ g/ml) and chloramphenicol (Cam, 34 μ g/ml). An overnight pre-culture of a single colony was transferred to 12L "terrific broth" (TB) medium containing Kan and Cam and incubated in the shaker at 160 rpm until the optical density at 600nm (OD_{600}) reached 0.8 - 1.0. Protein expression was induced with 1mM IPTG (isopropyl- β -D-1-thio-galactopyranoside) at 37°C. After 2 hours, cells were pelleted, resuspended in lysis buffer (50 mM Tris pH 7.0, 300 mM NaCl, 0.5 mM PMSF) and disrupted with a Microfluidizer (M-110L, Microfluidics Corp., Newton, MA). Unbroken cells were removed by 30 min. centrifugation at 14,000g. The membrane fraction was pelleted by centrifugation of the supernatant at 100.000g for 90 min. at 4°C, resuspended in 50 mM Tris pH 7.0, 300 mM NaCl and diluted to a protein concentration of 5 mg/ml. Membranes were solubilized by adding of N-laurylsarcosine to a final concentration of 1.5%. Non-solubilized protein was removed by centrifugation for 60 minutes at 100.000g. The supernatant containing the His-tagged protein was loaded onto a Ni-NTA column and unspecifically bound proteins were removed in several

washing steps. The protein was eluted in buffer containing 500 mM imidazole and concentrated using Amicon spin columns (Millipore) with a 30 kDa cutoff prior to loading onto a gel filtration column (Superdex75). Purified protein was eluted using 50 mM Tris pH 7.0, 300 mM NaCl and 0.05 % N-laurylsarcosine as running buffer.

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