

1 **Flat-to-curved transition during clathrin-mediated**
2 **endocytosis correlates with a change in clathrin-adaptor**
3 **ratio and is regulated by membrane tension**

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31 **Abstract**

32 Although essential for many cellular processes, the sequence of structural and
33 molecular events during clathrin-mediated endocytosis remains elusive. While it
34 was believed that clathrin-coated pits grow with a constant curvature, it was
35 recently suggested that clathrin first assembles to form a flat structure and then
36 bends while maintaining a constant surface area. Here, we combine correlative
37 electron and light microscopy and mathematical modelling to quantify the sequence
38 of ultrastructural rearrangements of the clathrin coat during endocytosis in
39 mammalian cells. We confirm that clathrin-coated structures can initially grow flat
40 and that lattice curvature does not show a direct correlation with clathrin coat
41 assembly. We demonstrate that curvature begins when 70% of the final clathrin
42 content is acquired. We find that this transition is marked by a change in the clathrin
43 to clathrin-adaptor protein AP2 ratio and that membrane tension suppresses this
44 transition. Our results support the model that mammalian cells dynamically
45 regulate the flat-to-curved transition in clathrin-mediated endocytosis by both
46 biochemical and mechanical factors.

47

48 Introduction

49 Clathrin-mediated endocytosis (CME) is an essential uptake pathway that
50 relocates membrane or extracellular cargo into the cell to regulate multiple cellular
51 functions and cell homeostasis¹. During CME, the clathrin coat is assembled to form
52 a clathrin-coated pit (CCP) that after dynamin-mediated scission from the plasma
53 membrane (PM) leads to the formation of a clathrin-coated vesicle (CCV)². This
54 process is coordinated by numerous adaptor and accessory proteins^{1,3}. Electron
55 microscopy (EM) of clathrin coated structures (CCS) has shown the architectural
56 complexity of the clathrin meshwork organized into hexagons and pentagons^{4,5}.
57 From this EM analysis, it was proposed that a CCV initiates as a flat clathrin lattice
58 that is then rearranged to form a curved CCP^{4,6,7}. However, for topological reasons
59 this requires a major ultrastructural rearrangement of the clathrin lattice which
60 appeared to be dynamically difficult and energetically costly⁸⁻¹². For these reasons,
61 this notion was replaced by a general belief that CCS grow with a constant curvature
62 (constant curvature model, Fig.1a)^{8,9,13} and that flat CCS are distinct from CCPs and
63 serve different purposes¹⁴⁻¹⁶. This model was supported by the finding that purified
64 clathrin triskelia self-assemble into curved clathrin baskets *in vitro*^{17,18}. Recently,
65 correlative light and electron microscopy (CLEM) analyses provided experimental
66 evidence that CCS first grow flat to their final size and then acquire curvature
67 (constant area model, Fig.1a)¹⁹. However, this study did not measure the dynamics
68 of CCP formation directly, and it did not identify the cellular factors that might
69 determine when the flat-to-curved transition occurs. Thus a comprehensive
70 understanding of the dynamic process of coat rearrangement, of the temporal
71 aspects of flat-to-curved transition and of what governs this ultrastructural
72 rearrangement during CME is still missing.

73 In this work, we combine mathematical modelling of individual endocytic
74 event dynamics and CLEM analysis to provide a comprehensive description of the
75 dynamic ultrastructural rearrangement of the clathrin coat during CME. We
76 demonstrate that CCPs indeed initially grow as flat arrays, but that their
77 reorganisation into curved structures occurs before reaching their full clathrin

78 content. We correlate this flat-to-curved transition with a change in the
79 AP2/clathrin ratio and show that it is governed by biophysical properties of the PM.
80 Our findings provide a unifying view of the dynamic process of coat rearrangement
81 during CME and our approach constitutes a methodological framework to further
82 study the fine-tuned spatio-temporal mechanism regulating coat assembly.

83

84 **Results**

85 **EM and CLEM analysis of CCS do not support existing growth models**

86

87 To address whether CCP formation follows the constant curvature model or the
88 constant area model (Fig.1a)¹³, we chose BSC-1 cells, a widely used cellular model to
89 study CME^{10,14,20}. BSC-1 cells present homogenous CME events in regard to both
90 lifetime as well as intensity profiles and lack the long-live flat clathrin-coated
91 plaques^{10,15} (Supplementary Fig.1 and Fig.1c-g). Both models predict different
92 growth profiles for the surface and projected area during CCP formation. The
93 constant curvature model implies that the projected area will quickly be smaller
94 than the surface area. In contrast, the constant area model implies that both
95 projected and surface areas initially show similar growth but then the projected
96 area should drop significantly as bending starts (Fig.1b).

97 To comprehensively characterise the ultrastructural organisation of CCS in BSC-1
98 cells, we performed TEM of metal replicas from unroofed PMs (Fig.1c). We
99 confirmed that CCS are not altered by the unroofing procedure using stimulated
100 emission depletion (STED) super-resolution microscopy of intact and unroofed cells.
101 The number and size distribution of CCS were indeed similar between intact and
102 unroofed cells (Supplementary Fig.2). CCS in TEM images of whole PM sheets were
103 counted, categorised as flat, dome or pit structures (Fig.1d-e) and their size was
104 measured as projected area (Fig.1a, f, and g). For the constant curvature model, we
105 would expect no flat structures at all and no dome structures that exceed the
106 projected area of pits (Fig.1a-b). In contrast our EM data reveals that around 50% of
107 the CCS in BSC-1 correspond to flat CCS (Fig.1e) and that a large fraction of the flat
108 and dome structures have a projected area larger than the projected area of the pits
109 (Fig.1g). Since BSC-1 cells do not have clathrin-coated plaques^{10,15}, these results
110 demonstrate that the constant curvature model cannot explain the CCS size
111 distribution, in agreement with the recent results by Avinoam et al¹⁹. Although the
112 existence of flat CCS seems to argue in favour for the constant area model, we would
113 expect that some flat structures have the same projected area as the surface area of

114 fully formed pits (Fig.1a-b). Since the surface area of a spherical pit ($4\pi r^2$, Fig.1b) is
115 four times larger than its projected area (πr^2 , Fig.1b), we would expect the mature
116 flat structures to have around four times the projected area of pits (Fig.1b). Instead,
117 we found no flat structures at all with a projected area four times larger than the
118 mean projected area of CCP (Fig.1g). Additionally, the constant area model would
119 imply that the projected area of dome structures (which resembles a hemisphere) is
120 reduced by a factor of two when converting to CCP. Instead we found only a slight
121 increase of the mean projected area of domes compared to pits (Fig.1g, distribution
122 and inset box/whiskers). Together, these observations argue against the constant
123 area model.

124 To further challenge the two growth models, we used a CLEM approach^{21,22} (Fig.2a).
125 BSC-1 cells were immunostained with a clathrin heavy chain antibody and the
126 fluorescence intensity of CCS (Fig.2b) was correlated to their size and
127 ultrastructural organisation measured using TEM of metal replicas (Fig.2c). For the
128 constant curvature model, we would expect the intensity to increase with increasing
129 contact angle (Fig.1a-b). In case of the constant area model, we would expect equal
130 intensity for the largest flat, domes, as well as all pit structures (Fig.1a-b). However,
131 our CLEM analysis clearly revealed that flat and dome structures have similar
132 fluorescence intensities while pits tend to display higher fluorescence intensities
133 (Fig.2b and d). In conclusion, our TEM and CLEM analyses argue that neither of the
134 proposed growth models fully explains the observed ultrastructural distribution
135 and corresponding fluorescence intensities of CCS in BSC-1 cells.

136

137 **Modelling of CCP assembly reveals bending of the coat before reaching** 138 **full surface area**

139

140 Although EM of metal replicas is a high-resolution microscopy technique revealing
141 detailed information about the size and ultrastructure of CCS, it only provides
142 snapshots of the dynamic process of CCP assembly²³. In contrast, live fluorescence
143 microscopy (FM) of CME mostly allows the characterisation of the dynamics of
144 different proteins during the formation of CCS but does not provide ultrastructural

145 information⁹. To obtain a more comprehensive dynamical picture, we used
146 mathematical modelling of clathrin growth behaviour to combine the ultrastructural
147 information from EM with the dynamic information obtained from total internal
148 reflection fluorescence (TIRF) microscopy of fluorescently tagged clathrin light
149 chain (CLC). As the formation of CCS is a complex process with numerous yet
150 unknown variables, here the modelling approach is used to combine and compare
151 the different data sets (FM, TEM and CLEM), with minimal assumptions on the
152 underlying mechanisms. Since we found a substantial number (around 50%) of flat
153 CCS with projected areas similar to pits (Fig.1e and g), we rule out the possibility
154 that the constant curvature model might be the dominant growth behaviour in BSC-
155 1 cells. We therefore modelled the growth of flat CCS first as circular planar discs
156 that grow to a finite size before bending (Fig.3a). Mathematically there is only one
157 type of growth equation that can explain why growth should stop at a finite patch
158 radius, namely association over the edge and dissociation over the area of the patch
159 (Supplementary Information). By fitting this growth equation and assigning the
160 three different morphologies to the individual intensity profiles obtained by TIRF
161 microscopy (Fig.3b and Supplementary Fig.1g-i), we could calculate the size and
162 morphology distribution of CCS as predicted by the constant area model (Fig.3c).
163 Comparison of the calculated distribution to the acquired EM data reveals that the
164 ratio between the flat, dome, and pit structures is biased toward flat structures
165 compared to the EM data (Fig.3g). Additionally, the means of the predicted size
166 distributions of flat and pit structures are clearly separated with a shift of the flat
167 projected area towards bigger sizes (Fig.3c and i box/whiskers and Fig.3h). In
168 agreement with our TEM and CLEM results, our mathematical modelling approach
169 thus demonstrates that the constant area model does not correctly describe the
170 assembly process of CCPs in BSC-1 cells.

171 Given the high proportion of flat structures (Fig.1e), we reasoned that CCS
172 start as flat structures and then acquire curvature before reaching the full clathrin
173 content (Fig.3d and Supplementary Information). In contrast to the patch growth
174 model (Fig.3a), now the system has an intrinsic mechanism to stop growth, namely
175 formation of a sphere. The patch growth is included as the initial regime. In contrast

176 to the constant curvature model, however, here we do not fix the radius of the
177 sphere. The resulting growth equation was again fitted to the individual intensity
178 profiles of CME events (Fig.3e) (Supplementary Information). As before, the ratio
179 between the flat, dome, and pit structures was biased towards flat structures
180 (Fig.3g) but now the calculated size and morphology distribution fit the EM data
181 better than the distribution according to the constant area model. The means of the
182 predicted projected area of both the flat and pit have similar sizes (Fig.3f and h,
183 box/whiskers). These findings strongly support a model where assembly of a CCP
184 initiates flat and then acquires curvature before it acquires its final clathrin content.

185 **Starting point of acquiring curvature is marked by a change in the** 186 **AP2/clathrin ratio**

187

188 The flat-to-curved transition of a CCS requires major ultrastructural reorganisation
189 of the coat¹¹. To acquire curvature, according to Euler's theorem the hexagonal
190 organisation of the clathrin triskelia needs to reorganise into a polyhedral assembly
191 including 12 pentagons¹¹. The clathrin lattice in flat structures is mostly composed
192 of hexagons^{4,5}. Although it has been shown using FRAP that the clathrin coat is
193 highly dynamic, which is a prerequisite for such rearrangement^{19,24,25}, it is still
194 puzzling what regulates the organisation of triskelia in the coat and what
195 coordinates the flat-to-curved transition. It was proposed before that the ratio of the
196 adaptor AP2 to clathrin changes within the growth of CCP^{22,26,27}. Therefore, we
197 correlated the relative amount of AP2 and the ultrastructural organisation of CCS.
198 We performed CLEM analysis using BSC-1 cells expressing AP2 fused to GFP (Fig.4a)
199 and correlated these results to clathrin immunostaining CLEM (Fig.4b). To find the
200 relationship between fluorescence intensity and the surface of CCS, the measured
201 projected area needs to be corrected for the curvature to obtain the surface area of
202 the CCS (Fig.1b). For flat structures, projected area and surface area are identical,
203 thus we used the linear regression of flat coats as a reference to correct the
204 projected area of both domes and pits. Assuming the geometry of a hemisphere for
205 domes and an almost complete sphere for pits we expect a correction factor of ≤ 2

206 for domes and $2 < x \leq 4$ for pit structures if the relationship between fluorescence
207 intensity and surface area is independent of curvature (Fig.1b). The correction
208 factors inferred for domes and pits were 1.4 and 2.8, respectively, which are within
209 the expected values (Fig.4d-e and Fig.1b). Strikingly, the correction factors for AP2
210 CLEM were smaller than expected, especially for the pit structures (domes: 1.2; pits:
211 1.7) (Fig.4c and e). This reveals that the AP2/clathrin ratio in a CCS differs
212 depending on its curvature and that this ratio is reduced within the coat as
213 curvature increases.

214 As the AP2/clathrin ratio depends on the curvature of CCS (Fig.4) and as AP2
215 partitions at different nanoscale zone in relation to the edge of the clathrin lattice
216 during the different stage of CCS formation²², we hypothesised that the change in
217 the AP2/clathrin ratio correlates with the stage at which a flat CCS bends to form a
218 CCP. Using cells expressing both AP2 fused to GFP and CLC fused to the fluorescent
219 protein tdTomato, we analysed the intensity profiles of AP2 and CLC during CME.
220 While AP2 profiles often show a distinct plateau phase, the intensity of CLC
221 continues to increase until the end of an endocytic event (Fig.5a and Supplementary
222 Fig.1). By normalising the fluorescence intensities of AP2 as well as CLC to the time
223 point when the AP2 signal plateaus, we can calculate the time offset between the
224 time AP2 signal reaches its plateau and the time point CLC reaches its maximal
225 intensity (Fig.5a-b). Similarly, we defined the intensity offset of clathrin over AP2
226 (Fig.5a and c). We found that the time offset was around 10s (Fig.5b) and that the
227 intensity offset of clathrin over AP2 was around 15% (Fig.5c). We hypothesised that
228 the time point when AP2 reaches its plateau and therefore the AP2/clathrin ratio
229 changes marks the starting point of bending. We performed another round of CCP
230 assembly modelling, this time using both AP2 and CLC intensity profiles and
231 defining the time point of flat-to-curved transition when AP2 reaches its plateau
232 phase (Fig.5e and Supplementary Information). At this precise time, the mean
233 clathrin content reached around 70% of its maximal value (Fig.5d). Using these new
234 parameters, the predicted ratio of flat, dome, and pit structures perfectly matched
235 the EM data (Fig.5f) and the means of the predicted projected area of both the flat

236 and pit CCS have similar sizes (Fig.5g-h, box/whiskers and 5i). This AP2/clathrin
237 ratio model better resembles the parameters measured in EM compared to both the
238 constant curvature and constant area models (Fig.3h and 5i). These findings
239 strongly support a model where flat-to-curved transition initiates at around 70% of
240 its maximal clathrin content and correlates with the concomitant change in the
241 AP2/clathrin ratio.

242 **High membrane tension inhibits the flat-to-curved transition of CCS**

243

244 By inducing curvature to the PM, the CCS needs to act against the plasma membrane
245 tension (PMT). Higher PMT has been shown to increase the lifetime of clathrin
246 events at the PM^{28,29} and modelling of the energetic cost of membrane bending
247 suggests that it affects the morphology of the CCS^{30,31}. But the effects of increasing
248 PMT on the ultrastructural organisation of CCS have not been investigated in living
249 cells. We monitored the dynamics of clathrin and AP2 during osmotic shock in
250 which the PMT was increased by applying hypotonic medium inducing osmotic
251 swelling of the cells²⁸ (Fig.6a). Following a short latency period, we observed that
252 CCS stalled at the PM. This effect was transient and cells quickly reverted to normal
253 clathrin dynamics (Fig.6b and d). By performing similar modelling of CCP assembly
254 using AP2 and CLC intensity profiles under osmotic shock, we showed that the CCS
255 display a longer AP2 plateau phase (Fig.6c and e) and that the time offset was
256 increased compared to mock treated cells (Fig.6f and 5b). According to our findings
257 that the change in AP2/clathrin ratio coordinates the flat-to-curved transition of
258 CCS, the delayed offset in the AP2/clathrin ratio under higher PMT suggests that the
259 flat-to-curved transition is suppressed and that the coats are flat under this
260 condition.

261 By looking at the AP2/clathrin ratio during osmotic shock we predicted that
262 during the stalling phase, 70% of the CCS would be flat (Fig.7a). To test this notion,
263 we performed EM of metal replica of CCS under osmotic shock (Fig.7b). We found an
264 accumulation of flat CCS under osmotic shock compared to normal conditions and
265 the frequency was comparable to our predictions from the AP2 and CLC profiles

266 (Fig7c). These flat structures, as well as the dome and pit structures, have the same
267 size distribution as under normal conditions (Fig7d box/whiskers and 7e). The EM
268 data confirms that under higher PMT the flat-to-curved transition of CCS is
269 inhibited. Using mathematical modelling and individual clathrin and AP2 intensity
270 profiles acquired under osmotic shock, we could predict the morphology of the
271 stalled CCS. This validates our model describing that the change in AP2/clathrin
272 ratio represents the precise moment at which the flat-to-curved transition occurs.
273

274 Discussion

275 The complex coordination of CCS formation during CME has been
276 investigated for decades^{32,33} and the field has been driven by the competition
277 between the constant curvature versus the constant area models¹³. Recent
278 technological advances, in particular in CLEM, favoured the constant area model¹⁹.
279 In this work, we implemented a multidisciplinary approach to combine information
280 from EM, fluorescence intensity profiles of individual endocytic events and
281 mathematical modelling of CCS growth to infer the ultrastructural rearrangement of
282 CCS during CME.

283 By modelling the growth behaviour according to the two proposed growth
284 models, we could calculate the expected size and morphology distribution of CCS
285 (Fig.1 and 3). We could clearly demonstrate that neither of the proposed models
286 explains the ultrastructural organisation and size distribution of CCS present in BSC-
287 1 cells. Instead, our data supports a model in which CCS first grow flat and then the
288 flat-to-curved transition occurs at around 70% clathrin content (Fig.8). Importantly,
289 we demonstrated that this transition is directly linked to PMT and correlates with a
290 change of the AP2/clathrin ratio within the coat. Increasing PMT results in
291 inhibition of the change in AP2/clathrin ratio and the subsequent stalling of the
292 ultrastructural rearrangement. Our model where a change in AP2/clathrin ratio
293 drives the flat-to-curved transition is consistent with our recent observation that
294 AP2 (and other adaptor/accessory proteins) partitions in different nanoscale area of
295 the clathrin coat and that the concentration of AP2 varies within these zones at
296 various stage of CCS assembly²².

297 Our growth behaviour of CCP assembly, determined in this work in BSC-1
298 cells, may also apply for other cell types. To test whether our growth model could
299 explain the ultrastructural distribution of CCS described in previous studies, we
300 extended our mathematical model and calculated the predicted contact angle
301 between the clathrin cage and the PM, the coated surface area and the radius of tip
302 curvature of the CCS (Supplementary Fig.3). By comparing the model to the
303 Avinoam et al. data¹⁹ set describing the distribution and ultrastructural organization

304 of CCS in SK-MEL-2 cells, we could demonstrate that our growth model could also
305 explain their observed distribution.

306 It was proposed earlier that the physical properties of the PM are influencing
307 the morphology of CCS because PMT energetically acts against curvature acquisition
308 of the clathrin array^{28,30,31}. We demonstrated the effect of increased PMT on CCS
309 assembly in cells. Under high PMT, the accumulation of stalled flat CCS at a stage
310 prior of the change in AP2/clathrin ratio reveals an important step of CCP formation
311 to overcome the PMT to acquire curvature. It is tempting to speculate that this
312 change of AP2/clathrin ratio is a key event mandatory for curvature acquisition. The
313 demonstrated interplay between AP2 and PMT shows that both biochemical and
314 physical factors regulate CME. Surprisingly, the increase in PMT only changes the
315 ratio between the different morphologies of CCS in favour of flat structures but does
316 not affect their size. One could assume that a higher energy barrier of the PM might
317 be counteracted by the clathrin system by the formation of larger flat CCS, which
318 could accumulate more energy for the bending process by molecular crowding of
319 clathrin itself as well as BAR proteins incorporated in the coat^{30,34-36}. Since under
320 high PMT the flat CCS still have the same size as under normal PMT we suggest that
321 there is an internal limitation of the coat size that might be regulated by certain
322 components of the coat. Proteins that regulate the size of CCV have been
323 reported^{37,38} and it might be possible that the size of a flat clathrin lattice is
324 controlled in a similar way. The fact that other cell types show much larger flat CCS
325 under normal conditions, commonly referred to as clathrin-coated plaques^{10,15},
326 illustrates that the clathrin machinery is capable of forming large flat structures
327 under certain conditions. But the factors necessary for clathrin-coated plaque
328 formation have not been described so far. These clathrin-coated plaques might
329 contribute to CME. Live-cell FM^{39,40} and EM⁴¹ of such structures support budding of
330 CCV from such plaques most probably from the edge, again illustrating the ability of
331 a flat CCS to rearrange into a CCP further supporting that our observed flat-to-
332 curved transition is indeed possible.

333 We could show that during coat assembly the AP2/clathrin ratio changes.
334 This finding is in agreement with other FM^{26,27,29} and CLEM²² studies. Mathematical

335 modelling demonstrated the correlation between the change in the AP2/clathrin
336 ratio and the time of coat bending. Several other adaptor and accessory proteins
337 have been proposed to influence the ultrastructure of the CCS. Depletion of FCHO 1
338 and 2 has been reported to alter the ordered hexagonal organisation of the flat
339 clathrin lattices⁴². Other proteins like CALM and NECAP have been proposed to
340 regulate the final size off a CCV^{37,38}.

341 Our findings provide a unifying view on the process of CCP assembly where
342 we demonstrate that CCPs initially grow as flat lattices and that change in
343 clathrin/adaptor ratio correlates with the onset of coat curvature acquisition prior
344 to the completion of coat polymerization. We propose that the proportion of
345 different coat proteins could ultimately define the morphology of a clathrin
346 structure and temporal changes of this proportion might initiate bending of the coat,
347 allowing for dynamical regulation by the cell.

348

349 **Author contributions**

350 DB and KAS performed CLEM experiments. DB performed FM experiments. FF
351 performed modelling. DB and FF designed experiments and modelling, analysed
352 data and wrote manuscript. KAS performed EM experiments. SK and H-GK
353 performed STED. J-PB, WJG and KR tracked FM data. JWT designed experiments
354 and oversaw EM and CLEM imaging. USS designed modelling, interpreted data and
355 wrote manuscript. SB designed experiments, interpreted data and wrote
356 manuscript.

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372 Core facilities for use of equipment.

373

374 **Methods**

375 **Cell lines and cell culture**

376 BSC-1 cells were obtained from ATCC. BSC-1 cells stably expressing AP2-eGFP were
377 created by transfecting BSC-1 cells with a plasmid expressing the sigma2 subunit of
378 AP2 fused to eGFP²⁰. Following selection with G418 (750 μ g.mL⁻¹) (Gibco), AP2-
379 eGFP expressing BSC-1 cells were grown in DMEM (Gibco) supplemented with 10%
380 fetal bovine serum, penicillin and streptomycin (Gibco) at 37 °C and 5% CO₂. For
381 passaging, cells were rinsed with PBS and incubated for 3-5 minutes with 0.05%
382 Trypsin/EDTA (Gibco) at 37 °C and 5% CO₂. After detaching, the cells were
383 resuspended in complete medium. Passaging was done every 2-3 days in a ratio of
384 1:5-1:10.

385

386 **Transfection**

387 Transfection of cells was done using Lipofectamine 2000 (Invitrogen). Cells were
388 plated in 6-well plates one day before transfection. The next day, cells were
389 transfected at 70-80% confluence. 2 μ g DNA and 8 μ l Lipofectamine 2000 were
390 separately mixed with 100 μ l OptiMEM (Gibco). The two solutions were mixed
391 together. After incubation for 20 minutes at room temperature, the transfection mix
392 was added drop-wise onto the cells. For generation of stable cell lines, the growth
393 medium was exchange for fresh growth medium after 8 hours. The cells were put
394 under selection two days after transfection.

395 For live-cell imaging of BSC-1 AP2-eGFP transiently expressing CLCa-tdtomato were
396 seeded 8 hours after transfection.

397

398 **Antibodies and Plasmids**

399 Anti-clathrin heavy chain antibody (X22, ab2731, 1:500 for immunofluorescence)
400 was purchased from Abcam. Anti-clathrin light chain antibody (Con.1, C1985, 1:500
401 for immunofluorescence) was purchased from Sigma-Aldrich. Secondary Alexa

402 Fluor 647 goat anti-mouse (1:1,000 for immunofluorescence) and goat anti-mouse
403 Atto594 (1:200 for immunofluorescence) were purchased from molecular probes.
404 Wheat Germ Agglutinin AlexaFluor 647 conjugate (W32466, 1:200 for
405 immunofluorescence) was purchased from molecular probes.
406 Mammalian expression vectors containing rat CLCa N-terminally fused to
407 tdtomato⁴³ and the rat AP2 subunit sigma2 C-terminally fused to eGFP²⁰ were used
408 for stable and transient expression of fluorescently tagged proteins.

409

410 **Live-cell microscopy**

411 Glass coverslips (TH. Geyer, 25mm diameter, No. 1.5H) were coated with poly-D-
412 lysine solution (Sigma-Aldrich, #P6407) at concentration 0.1mg ml⁻¹ for 5 minutes
413 at room temperature and washed three times with PBS. Cells were seeded on poly-
414 D-lysine-coated coverslips and live-cell microscopy was performed 12-16 hours
415 after seeding. Live-cell imaging of AP2-eGFP was performed with an inverted
416 spinning-disk confocal microscope (PerkinElmer), with a 60x (1.42 numerical
417 aperture, Apo TIRF, Nikon) or 100x (1.4 numerical aperture, Plan Apo VC, Nikon) oil
418 immersion objective and a CMOS camera (Hamamatsu Ocras Flash 4). An
419 environment control chamber was attached to the microscope to keep 37 °C and
420 5%CO₂. 10 minutes-long movies of representative cells were taken with one frame
421 every 3 seconds. Live-cell imaging of AP2-eGFP together with CLCa-tdtomato was
422 performed with an inverted Ti microscope (Nikon) with objective TIRF illumination,
423 with a 60x (1.49 numerical aperture, Apo TIRF, Nikon) oil immersion objective and
424 EMCCD camera (Andor iXon Ultra DU-897U). An on-stage incubation chamber was
425 used to keep 37 °C and 5%CO₂. 10 minutes-long movies of representative cells
426 were taken with one frame every 3 seconds.

427

428 **Osmotic shock experiments**

429 For live-cell microscopy of cells under osmotic shock, one cell was imaged for 5
430 minutes under normal conditions. Afterwards the medium was change to hypotonic

431 medium (1:1 ratio of medium to water with 10% FBS) and the same cell was imaged
432 for an additional 30 minutes. For TEM of cells under osmotic shock, cells were put
433 into hypotonic medium and unroofed after 10 minutes of osmotic shock. The
434 samples were then prepared for TEM.

435

436 **Unroofing**

437 Cells were seeded on poly-D-lysine-coated coverslips (25mm). Unroofing was
438 performed 16 hours after seeding. Cells were washed three times with stabilisation
439 buffer (30mM HEPES buffer, brought to pH 7.4 with KOH, 70mM KCl, 5mM MgCl₂).
440 Unroofing was performed in 2% paraformaldehyde in stabilisation buffer using two
441 short sonication pulses. Sample was immediately put into fresh 2%
442 paraformaldehyde solution and fixed for 20 minutes at room temperature.

443

444 **Immunofluorescence**

445 For immunofluorescence, intact cells growing on poly-D-lysine coated 12mm
446 coverslips (#1.5, Thermo Scientific) or unroofed PMs were fixed with 2-4%
447 paraformaldehyde for 20 minutes at room temperature. Intact cell were
448 permeabilised with 0.5% Triton X in PBS for 15 minutes. After blocking with PBS
449 1% BSA for 1 hour at room temperature, samples were incubated with primary
450 antibody diluted in PBS with 1% BSA for 1 hour at room temperature. After five
451 washes with PBS, samples were incubated with the secondary antibody. Wheat
452 germ agglutinin diluted in 1% BSA in PBS was incubated on cells for 30 minutes at
453 room temperature. After five washes with PBS, samples for STED were mounted
454 using Mowiol and samples for correlative light and electron microscopy were fixed
455 by incubation with 2% paraformaldehyde for 20 minutes at room temperature and
456 washed three times with PBS.

457

458 **Imaging of unroofed PMs for CLEM**

459 Widefield fluorescent images of unroofed PMs were taken with a Nikon N-STORM
460 microscope with a 100x oil immersion objective and an EMCCD camera (Andor Ixon
461 Ultra DU-897). To cover an area of 1mm² a montage of 15x15 images with an
462 overlap of 15% for stitching was taken. The imaged area was marked with a circle
463 (4mm in diameter) around the centre of the imaged area using an objective
464 diamond scribe. The immersion oil was carefully removed from the bottom of the
465 glass coverslip and the sample was prepared for EM.

466

467 **TEM of metal replica**

468 Coverslips with unroofed membranes were fixed with 2% glutaraldehyde in PBS
469 overnight. Samples were incubated with 0.1% tannic acid for 20 minutes at room
470 temperature. After four washes with water, the samples were incubated with 0.1%
471 uranyl acetate for 20 minutes at room temperature. After two washes with water,
472 samples were dehydrated with a series of ethanol solutions (15%-100%). Samples
473 were placed in each ethanol solution for 5 minutes. After replacing the 100%
474 ethanol solution, the samples were dried in a critical point dryer. The samples
475 were then put under vacuum until they were coated. The samples were coated with
476 JFDV JOEL Freeze Fracture Equipment with a first layer of platinum with an angle of
477 17° while rotating and with a second layer of carbon with an angle of 90° while
478 rotating. For better orientation, the marked area of the coated samples was imaged
479 with a phase contrast microscope. The samples were then cut to fit on the EM grids
480 (TED PELLA, 75 Mesh Copper, Support Films Formvar/Carbon). 5% hydrofluoric
481 acid was used to remove glass from the metal replica. The floating metal replica was
482 extensively washed with water and the carefully placed on a glow discharged EM
483 grid. Samples were dried on filter paper and again imaged with a phase contrast
484 microscope. TEM imaging was performed using a JEOL 1400 equipped with
485 SerialEM freeware for montaging. Montages of large membrane sheets at 12,000
486 magnification (1.82nm per pixel) with 10% overlap were imaged.

487

488 **Transformation of images for CLEM**

489 The fluorescence microscopy image and the electron microscopy montage of the
490 same membrane sheet were first manually and roughly overlaid using Photoshop.
491 MATLAB was used to transform the fluorescence image according to the electron
492 microscopy montage using three manually identified CCS. For the transformation
493 the centre of the clathrin structure in the electron microscope montage and the
494 centre of the fluorescence signal determined by a Gaussian fit were used as
495 landmarks.

496

497 **Tracking**

498 For tracking CME events, we used ilastik (<http://ilastik.org>). First the images were
499 segmented using the pixel classification and object classification workflow. For
500 tracking, the automatic tracking workflow was used. The maximal distance was put
501 to 5 to avoid merging of close tracks. Automatic tracking of CCS using AP2-eGFP
502 expressing BSC-1 cells was performed using a probabilistic particle tracking
503 method⁴⁴. The method combines Kalman filters with particle filters and probabilistic
504 data association with elliptical sampling (PDAE). For particle detection, a Laplacian-
505 of-Gaussian filter and connected-component labelling was used. Based on the
506 computed trajectories, the signal intensity of each tracked object (normalized to the
507 background intensity) was determined and intensity statistics over all trajectories
508 were computed. Also, the object size was determined for each time. In addition, the
509 lifetime of CCS was quantified and classified into different ranges.

510

511 **STED**

512 STED nanoscopy was carried out using the Two-color-STED system (Abberior
513 Instruments GmbH, Göttingen). Image acquisition was performed using a 100x
514 Olympus UPlanSApo (NA 1.4) oil immersion objective and 70 % nominal STED laser
515 power ($\lambda = 775$ nm, max. power = 1.2 W). Line accumulation was set to 4 and a pixel
516 size of 15 nm was used. Deconvolution of acquired images was done using

517 impector software (Abberior Instruments GmbH, Göttingen). Richardson-Lucy
518 deconvolution with a regularisation parameter of 0.001 was used and stopped after
519 30 iterations.
520

521 **Supplementary Information**

522 **Constant area model**

523 As the fluorescence intensity of labelled clathrin triskelia is proportional to the
524 number of incorporated clathrin triskelia or equivalently to the size of clathrin
525 covered membrane area, we model the assembly of CCV as surface growth.

526 Our mathematical description of the constant area model considers finite growth of
527 a flat circular patch with radius r . The flat patch grows at its edge (L) with rate k_{on}
528 but decays over the bulk with rate k_{off} , which can be expressed by $\frac{dA}{dt} = k_{\text{on}}L -$
529 $k_{\text{off}}A$. In (Fig. 3a) a sketch of the growth schematics is shown. The growth equation
530 can be simplified by plugging in the surface of a circular patch $A = \pi r^2$, it follows
531 $\frac{dr}{dt} = k_{\text{on}} - k_{\text{off}}\frac{r}{2}$ with steady state radius $r_{\text{ss}} = 2k_{\text{on}}/k_{\text{off}}$. By integrating this
532 equation, we find the patch area as a function of time

533

$$534 \quad A(t) = \pi \left(r_{\text{ss}} \left(1 - e^{-(k_{\text{off}}/2)t} \right) \right)^2.$$

535

536 In the observed fluorescence intensity tracks the intensity decreases after some
537 time until the intensity vanishes completely. Biologically, this indicates that the
538 clathrin coated vesicle pinches off the cell membrane and therefore, moves out of
539 the focus of the microscope. We model this by assuming an exponential decay of the
540 area with time constant τ , starting at time t_{decrease}

$$541 \quad A(t) = \begin{cases} \pi \left(r_{\text{ss}} \left(1 - e^{-(k_{\text{off}}/2)t} \right) \right)^2 & t \leq t_{\text{decrease}}, \\ \pi \left(r_{\text{ss}} \left(1 - e^{-(k_{\text{off}}/2)t_{\text{decrease}}} \right) \right)^2 e^{-t/\tau} & t > t_{\text{decrease}}. \end{cases} \quad (\text{Eq. S1})$$

542 As the steady state is only reached approximately we define an area plateau at 95%
543 of the steady state area and the corresponding time t_{plateau} . The constant area
544 model assumes that a flat patch transforms into a spherical pit as soon as the patch
545 reaches the area plateau. Here, we neglect the exact details of the transformation
546 process but classify the CCS as flat, dome (less than a half sphere) or pit according to

547 time. Before reaching the plateau, the area is considered flat. After reaching the
548 plateau we classify CCS in the first 40% of the remaining time until t_{decrease} to be
549 domes and in the last 60% of time to be pits. The exact choice is motivated by the
550 occurrences of domes and pits in EM which is roughly 2:3.

551 **Data fits**

552 To test whether the constant area model correctly describes the shape and size of
553 clathrin coated vesicles we fitted Eq. 1 to 4927 FM tracks of 4 different cells (Fig. 3b)
554 and calculated from the fitted area surface growth curves histograms which we
555 could compare to EM histograms. Therefore, we related the intensity of an FM track
556 to its corresponding area. Furthermore, the FM dataset was filtered before the
557 fitting. The exact details of our procedure are described in the following.

558 **Relate fluorescence intensity and surface area by means of** 559 **CLEM**

560 To relate the fluorescence intensity of a clathrin FM track to the corresponding
561 clathrin covered membrane area we use our clathrin CLEM data, relating the
562 projected surface size of CCS to their fluorescence intensity. We analyse flat CCS for
563 which the projected area directly corresponds to their surface. By fitting a line
564 through the origin to the clathrin CLEM data we get the slope β providing us with a
565 linear relation between size and intensity. As the local intensity background is
566 removed from the CLEM data we find

$$567 \quad I(A) = \beta A, \text{ (Eq. S2)}$$

568 where I is the intensity of the FM data, A is the area of the clathrin structure and β is
569 the proportionality constant. Fig. 4b shows the intensity of flat structures as a
570 function of their projected area (blue). We find $\beta = 4085 \text{ nm}^2$.

571 **Relate different FM datasets**

572 To calculate a size histogram from fluorescence intensity tracks we analyse live cell
573 FM data, that have a different intensity level than the fluorescence intensity of the
574 CLEM data. Therefore, we need to relate these two different data sets. As the CLEM
575 intensity I and the live cell FM intensity I' are both proportional to the number of
576 labelled clathrin triskelia, both intensities, which are background corrected, can be
577 related only by some factor α

$$578 \quad I' = \alpha I \rightarrow \alpha = \langle I' \rangle / \langle I \rangle, \text{ (Eq. S3)}$$

579 which can be calculated by dividing the means (indicated by $\langle \rangle$) of both data sets.

580 **Relate different FM datasets**

581 We next restricted the live cell FM data set to ensure that the calculated size
582 histogram is comparable to the EM histogram. In EM we only detect structures that
583 reach a threshold size. However, in the live cell FM data set we register only
584 detectable intensities, which exceed the local background signal. Therefore, we
585 relate the minimal detectable size in CLEM (A_T) to a threshold intensity (I_T), which
586 we relate to a threshold intensity (I'_T) that we use on the live cell FM tracks. As $I' =$
587 αI we find

$$588 \quad \frac{\langle I' \rangle - \langle I'_T \rangle}{\langle I' \rangle} = \frac{\langle I \rangle - \langle I_T \rangle}{\langle I \rangle}$$

589 As $\langle I' \rangle$ is a function of $\langle I'_T \rangle$ we can find the root of

$$590 \quad 0 = I_T \frac{\langle I' \rangle}{\langle I \rangle} - I'_T$$

591 by iteratively increasing $\langle I'_T \rangle$.

592 In EM we find that the minimal sized clathrin structure has an area of $A_T =$
593 5644 nm^2 , corresponding to a calculated intensity of $I_T = 2.305 \cdot 10^7 \text{ arb. unit}$. We
594 find the mean intensity in the clathrin CLEM data of all structures $\langle I \rangle = 1.766 \cdot$
595 10^8 arb. unit . To measure the mean intensity in the live cell FM data set we sample
596 from each FM track that lasts a least 24 seconds a number of intensities
597 proportional to its lifetime. From these sampled intensities, which we restrict to be

598 larger than the threshold value I'_T , we then compute the mean intensity. We obtain
599 $I'_T = 5.450 \cdot 10^3$ arb. unit, $\langle I' \rangle = 4.148 \cdot 10^4$ arb. unit. By combining the Eq. S2 and Eq.
600 S3 we finally arrive at

$$601 \quad A'(I') = \frac{I'}{\alpha\beta}$$

602 according to which we compute the surface of a clathrin structure from its intensity
603 in the live cell FM. Furthermore, we filter all FM tracks showing a mean intensity
604 which is smaller than I'_T , as those tracks would not be observable in EM.

605 **Data filtering**

606 To ensure that all structures correspond to real objects we assume for a productive
607 object a minimal lifetime of 24 seconds. We filter our data set for FM tracks with
608 multiple structures (defined as a FM track that shows at least two clear intensity
609 maxima) to allow for direct fitting of single tracks. Furthermore, we filter tracks that
610 start already with a medium intensity level $I'(t = 0) > 0.5\langle I' \rangle$.

611 **Parameter choice and data fitting**

612 The parameters for the fit are restricted by assuming that growth curves should at
613 least reach 90% and at most 120% of the maximal area value. Additionally, we
614 assume that vesicle pinch off (corresponding to a decrease in the intensity to 10%)
615 takes between 10 seconds to 20 seconds. Additionally, we require the fit to reach
616 99% of the steady state area before the area decrease happens and at least 10% of
617 that time until the 99% area level are reached. We implement the Python module
618 'Imfit' for fitting the area tracks where we use the method 'nelder' of the minimiser
619 function. In this way we obtain for each track four parameters (r_{ss} , k_{off} , τ , $t_{decrease}$)
620 that characterise the growth curve.

621 Calculation of the size histogram

622 From the growth curves, we calculated a histogram (Fig. 3c) to compare the
623 constant area model to the measured projected size EM histogram (Fig. 3h). We
624 proceeded as follows:

625 • From each of the fitted growth curves we uniformly drew a number of time
626 points, proportional to the time until the structure pinches off the cell membrane,
627 that is given by t_{decrease} (Fig. 3a and b). We used 4927 FM tracks of 4 different
628 cells giving us 4 million time points, which we used to calculate the size
629 histogram.

630 • We classified the chosen times and corresponding areas into three categories. If
631 $t < t_{\text{plateau}}$ the structure is flat (blue region in Fig. 3b). We assume that the
632 transformation process from flat to dome takes 40% of the plateau time (which
633 corresponds roughly to the ratio between domes and pits in EM) whereas from
634 dome to pit it takes 60%. This is supported by the notion that the clathrin array
635 reorganisation becomes increasingly complicated. Therefore, if $t_{\text{plateau}} < t <$
636 $t_{\text{plateau}} + \frac{2}{5}(t_{\text{decrease}} - t_{\text{plateau}})$ the structure is a dome (red region in Fig. 3B) and
637 otherwise it is classified as a pit (green region in Fig. 3b).

638 • We computed the projected area by assuming that the transformation within the
639 dome and pit phase is a linear function of time. Therefore, we divided the area by

640 $1 + \frac{5}{2} \frac{t - t_{\text{plateau}}}{t_{\text{decrease}} - t_{\text{plateau}}}$ for domes and $2 + \frac{5}{3} \frac{t - t_{\text{plateau}} - \frac{2}{5}(t_{\text{decrease}} - t_{\text{plateau}})}{t_{\text{decrease}} - t_{\text{plateau}}}$ for pits. This

641 factor equals 1 for a flat patch, 2 for a completed dome (half sphere) and 4 for a
642 completed pit (full sphere).

643 • We excluded times for which the corresponding area is below the area threshold
644 or where the area already decreases (white region in Fig. 3b).

645 • From the calculated projected areas, we then determined the size histogram (Fig.
646 3c), which we compare to the EM data (Fig. 3h).

647 **Curvature acquisition during growth: updated model**

648 In the updated growth model we assume that CCS first grow flat, start to invaginate
649 as they reach 70 % of their final size (which we determine by taking the inverse of
650 the intensity ratio of pit and flat structures in clathrin CLEM, which is 1.44) and
651 finally grow as a spherical cap until a full pit has formed. As before we model the
652 assembly of clathrin coated vesicles as surface growth.

653 Our mathematical description of the “curvature acquisition during growth
654 model “ considers a spherical cap with radius R that grows at its edge with rate k_{on}
655 which can be expressed by $\frac{dA}{dt} = k_{on}L$. In (Fig. 3d) a sketch of the growth schematics
656 is shown. The growth equation can be simplified by plugging in the surface of a
657 spherical cap $A = 2\pi R^2(1 - \cos(\varphi))$, such that the change in the angle $\frac{d\varphi}{dt} = \frac{k_{on}}{R}$ is
658 constant. In the limit of a small growth angle $\varphi = k_{on}t/R < 1$ we recover the growth
659 equation of a flat patch $A(t) = \pi(k_{on}t)^2$. For an almost complete pit the growth
660 equation also holds perfectly. However, the mathematical description of the growth
661 model approximates the flat patch by a spherical cap for intermediate flat patch
662 sizes. In this case, the error is negligible as the model interpolates between the
663 correctly addressed limiting cases and is not used to assign the shape of the CCS.

664 To define the ultrastructural organization of the clathrin lattice, we neglect,
665 as before, the exact details of the transformation process but classify the CCS as flat,
666 dome (less than a half sphere) or pit according to time relative to the time when
667 reaching the maximal area. Before reaching 70% of the maximal area the coat is flat.
668 After reaching 70% of the maximal area we classify CCS in the first 40% of the
669 remaining time to be domes and pits otherwise. The exact choice is motivated by the
670 occurrences of domes and pits in EM which is roughly 2:3.

671 As before we model that vesicles pinch off the membrane by an exponential
672 decay of the area with time constant τ , starting at time $t_{decrease} = \pi R/k_{on}$. The full
673 growth equation for the area as a function of time $A(t)$ reads

674

$$675 \quad A(t) = \begin{cases} 2\pi R^2 \left(1 - \cos\left(\frac{k_{\text{on}} t}{R}\right)\right) & t < \frac{\pi R}{k_{\text{on}}}, \\ 4\pi R^2 e^{-t/\tau} & t > \frac{\pi R}{k_{\text{on}}}. \end{cases} \quad (\text{Eq. S4})$$

676 **Data fits, parameter choice and data fitting**

677 To test whether the “curvature acquisition during growth” model correctly
678 describes the shape and size of clathrin coated vesicles we fitted Eq. S4 to 4927 FM
679 tracks of 4 different cells (Fig. 3e) and calculated from the fitted area surface growth
680 curves histograms which we could compare to EM histograms. Therefore, we
681 related the intensity of an FM track to its corresponding area. Furthermore, the FM
682 dataset was filtered before the fitting. The exact details of our procedure are the
683 same as before.

684 The parameters for the fit are restricted by assuming that growth curve
685 should at least reach 90% and maximal 120% of the maximal area value and that
686 the vesicle pinching off (corresponding to a decrease in the intensity to 10%) takes
687 between 10 seconds to 20 seconds. We implement the Python module ‘lmfit’ for
688 fitting the area tracks where we use the method ‘nelder’ of the minimiser function.
689 In this way we obtain for each track 4 parameters ($R, k_{\text{off}}, \tau,$) that characterise the
690 growth curve.

691 **Calculation of the size histogram**

692 From the growth curves we calculated a histogram (Fig. 3f) to compare “the
693 curvature acquisition during growth model” to the measured projected size EM
694 histogram (Fig. 3h). We proceeded in principle as before and only mention changes:

695

- 696 • We classified the chosen times (around 4 million time points) and corresponding
697 areas into three categories. If $A(t) < 0.7A_{\text{max}}$ the structure is flat (blue region in
698 Fig. 3e) and we call this time $t_{\text{transformation}}$. We assume that the transformation
699 process from flat to dome takes the first 40% of the remaining time until the

700 maximal area is reached (which corresponds roughly to the ratio between domes
701 and pits in EM) whereas from dome to pit it takes the rest of the time.

702 • We computed the projected area by assuming that the transformation within the
703 dome and pit phase is a linear function of time. Therefore, we divided the area by

704 $1 + \frac{5}{2} \frac{t - t_{\text{transformation}}}{t_{\text{decrease}} - t_{\text{transformation}}}$ for domes and $2 +$

705 $\frac{5}{3} \frac{t - t_{\text{transformation}} - \frac{2}{5}(t_{\text{decrease}} - t_{\text{transformation}})}{t_{\text{transformation}} - t_{\text{plateau}}}$ for pits. This factor equals 1 for a flat

706 patch, 2 for a completed dome (half sphere) and 4 for a completed pit (full
707 sphere).

708 **Curvature acquisition during growth: Flat-to-curved** 709 **transition corroborates with the change of clathrin/AP2** 710 **ratio**

711 Here, the only thing that changes compared to the updated model is that the
712 transition time $t_{\text{transformation}}$ is now given by the time when the AP2 intensity
713 plateaus, which we calculated by fitting eq. 1 to the AP2 FM intensity tracks.

714

715 **Live cell FM analysis resampling**

716 To determine the ratio of clathrin and AP2 during the process of CME, we
717 performed TIRF microscopy of BSC-1 AP2-eGFP cells transiently expressing CLCa-
718 tdtomato. We analysed the excess of clathrin in comparison with AP2 during the
719 formation of CCVs. Therefore, we calculated the ratio of the maximum clathrin
720 intensity divided by the intensity of clathrin at the time when AP2 shows an
721 intensity plateau (95% intensity level) and subtract this ratio from the ratio, which
722 we get for AP2.

723 In detail: we set a threshold for the AP2 intensity underneath fluorescence
724 intensity tracks are excluded (as before). We calculate the plateau time, defined as
725 the time when 95% of the AP2 plateau intensity is reached by fitting the constant

726 area model to the AP2 FM data. Additionally, we normalise all intensity values to the
727 intensity value when 95% of the AP2 intensity (clathrin intensity) is reached. For
728 each track (in total 754 tracks of one single cell) we calculate the difference of the
729 time when AP2 plateaus and clathrin reach the maximum intensity and determine
730 the corresponding histogram (cf. Fig 5b). Furthermore, we calculate the intensity
731 offset given by the normalised maximum clathrin intensity divided by the
732 normalised maximum AP2 intensity (cf. Fig. 5c).

733 **Calculation of the ratio histogram during the osmotic** 734 **shock**

735 To determine the ratio histogram of flat, dome and pit CCS during the
736 osmotic shock (Fig. 7a and 7c) we first defined a transition time $t_{\text{transformation}}$, when
737 flat CCS start to invaginate, where the normalised clathrin intensity exceeds the
738 normalised AP2 intensity by 5% (blue region in Fig. 6c). We assumed that the
739 transformation process from flat to dome takes the first 40% of the remaining time
740 until the vesicle pinches of the PM, given by t_{decrease} (red region in Fig. 6c) whereas
741 from dome to pit it takes the remaining 60% of time (green region in Fig. 6c). Next,
742 we started 50s after the osmotic shock and determined the morphology of all tracks
743 present at that time depending on the description above. We repeated this
744 procedure in steps of 5 seconds and average the number of structures over time
745 intervals of 100 seconds. In total we used 1356 FM tracks of one cell. The found
746 ratios of flat, dome and pit CCS were then plotted as a function of the time after the
747 osmotic shock (Fig. 7a).

748 To test the consistency of this approach we used it to calculate the ratios for
749 flat, dome and pit structures on the data without osmotic shock consisting of 4927
750 FM tracks of 4 different cells. Averaging over all tracks and considering only tracks
751 with lifetimes shorter than 90s and with AP2/clathrin discrepancy we obtained
752 47.8% flat CCS, 18.0% dome CCS and 34.2% pit CCS which is close to the determined
753 ratios in (Fig. 5g).

754 **Quantification of agreement between measured and** 755 **predicted size histograms**

756 To determine the level of agreement between measured (Fig. 3h) and
757 predicted size histograms (Fig. 3c, Fig. 3f and Fig. 5e) we calculated chi-squared

758 $\chi_j^2 = \sum_{i=1}^k \frac{(N_{ij} - np_{ij})^2}{np_{ij}}$ where we sum over all bins k the occurrences N_i of measured CCS

759 and compared it with the number of expected occurrences np_i , where p_i is the
760 predicted normalised frequency per bin, which we deduced from our models

761 (CAM=constant are model, UM=updated model and AP2=transition flat/dome
762 determined by the time when the AP2 intensity plateaus) and $n = \sum N_i$. We repeated

763 this for all CCS $j = \{flat, dome, pit\}$ such that we found three values for χ_j^2 for each
764 model and cell. We averaged these values for all CCS and four cells and found:

765 $\chi_{CAM}^2 = 1018$, $\chi_{UM}^2 = 649$ and $\chi_{AP2}^2 = 632$ which shows that the model plotted in
766 Fig. 5e describes the data best.

767 Additionally, we performed a Welch's t-test to calculate p-values for the null
768 hypothesis that the measured and predicted size distributions of a CCS have
769 identical mean values. We averaged over all CCS and four cells and found $p_{CAM} =$
770 0.60 , $p_{UM} = 0.75$ and $p_{CAM} = 0.78$.

771

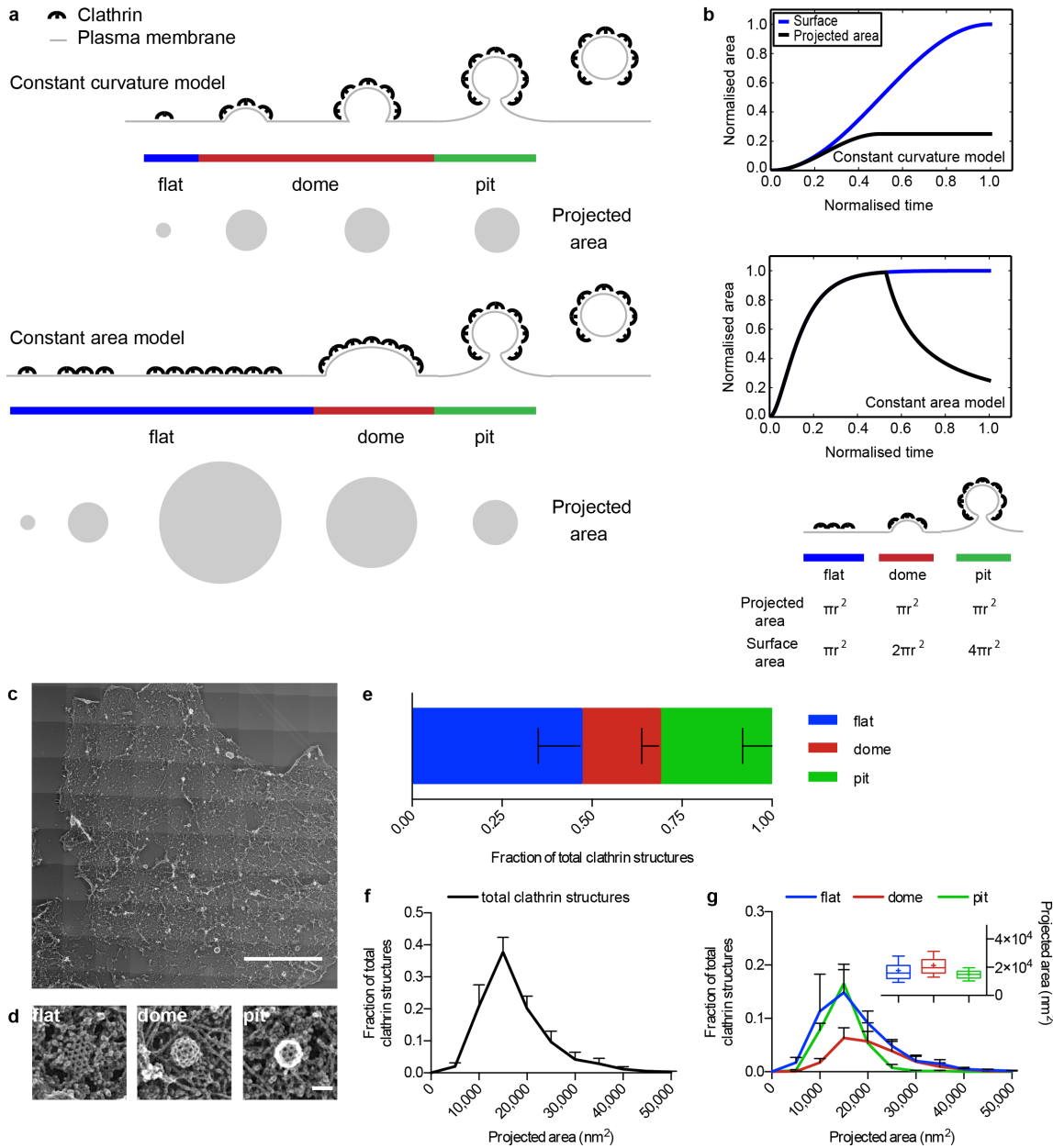
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886 **Figure 1: Comprehensive ultrastructural characterisation of CCS in BSC-1 cells**

887 **by TEM.** (a) Schematic of the constant curvature and constant area models. The

888 stages of different curvature (flat (blue), dome (red), pit (green)) and the variation

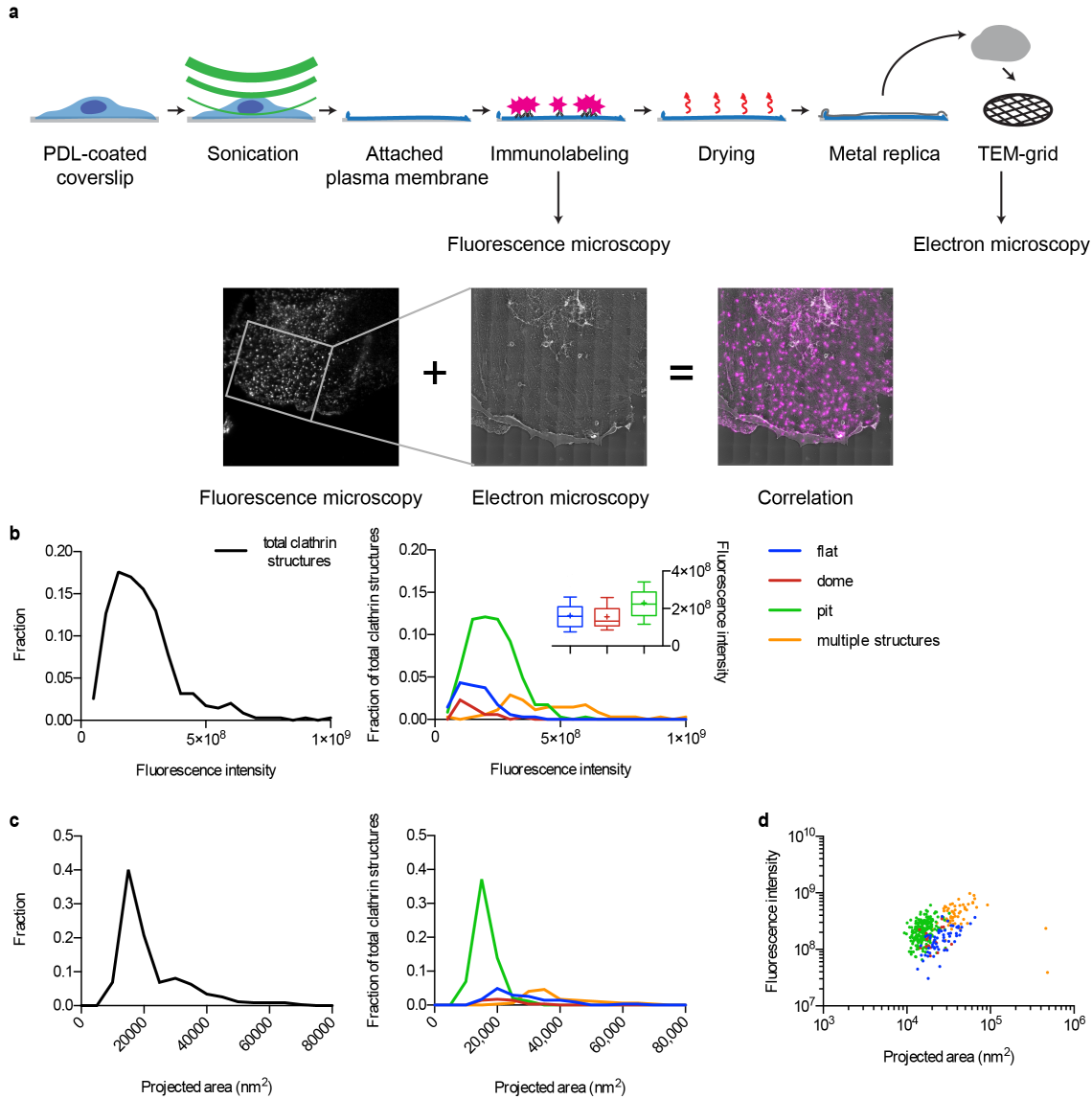
889 of projected area, which can be assessed during TEM imaging, is depicted for both

890 growth models. (b) Difference between projected area (black) and surface area

891 (blue) during the course of CCP formation according to the two models. The

892 schematic illustrates the relationship between projected area and surface area for

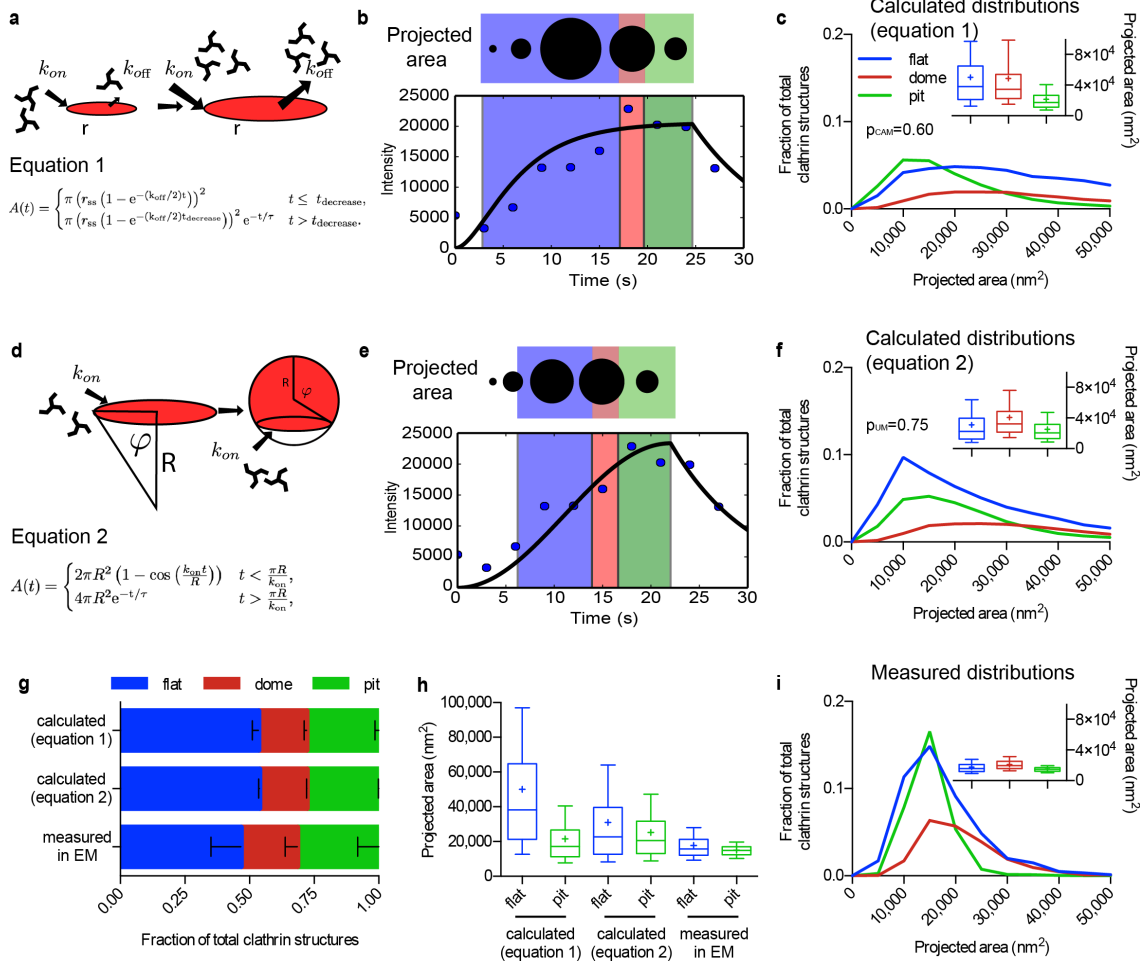
893 flat, dome (approximately a hemisphere), and pit (approximately a complete
894 sphere) CCS. (c) TEM of metal replica from unroofed PM, overview of whole
895 membrane, scale bar: 10 μ m. (d) Examples of flat, dome, and pit structures, scale bar:
896 100nm. (e) Fraction of flat (blue), dome (red) and pit (green) CCS in whole PM of
897 BSC-1 cell. (f) Projected area distribution of all CCS (black) measured by TEM. (g)
898 Projected area distribution of the different clathrin morphologies (flat, dome, pit). A
899 box/whiskers plot of the projected area is shown in the inset. Mid-line represents
900 median, cross represents the mean and the whiskers represent the 10 and 90
901 percentiles. Results are calculated from three different membranes (number of CCS
902 per membrane: 746, 869, and 739); means with SD are shown.
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906 **Figure 2: CLEM of CCS.** (a) Schematic representation of the CLEM approach (Upper
907 part). Cells growing on poly-D-lysine (PDL)-coated coverslips were unroofed by
908 sonication. Attached PMs were immunostained and imaged using FM. Samples were
909 then critical-point dried and the metal replica was created and lifted from the
910 sample onto a TEM grid for imaging in TEM. (Lower part) The FM and EM pictures
911 were then correlated to combine their information (See methods). Unroofed PMs
912 were immunostained using an anti-clathrin heavy chains antibody. The white inset
913 box represents the area observed by TEM. (b) Fluorescence intensity distribution
914 (clathrin heavy chain antibody, X22) of all CCS (black line, left panel) and of flat
915 (blue), dome (red), pit (green) CCS, and multiple structures which cannot be

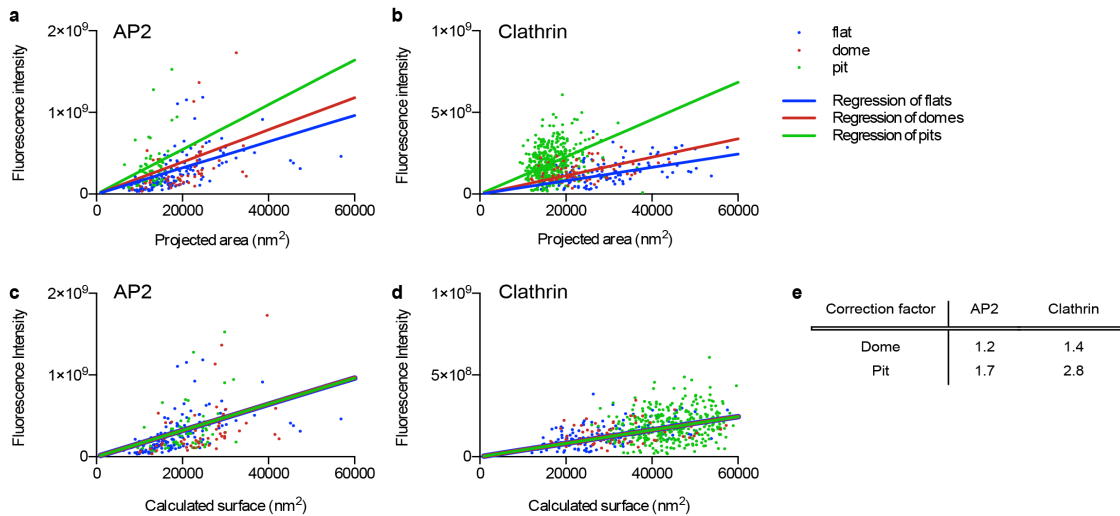
916 distinguished by fluorescence microscopy (orange) (right panel). A box/whiskers
917 plot of the fluorescence intensity is shown in the inset. Mid-line represents median,
918 cross represents the mean and the whiskers represent the 10 and 90 percentiles. (c)
919 Projected area distribution of all CCS (black line, left panel) and of the different
920 clathrin morphologies (right panel). (d) Correlation of size and fluorescence
921 intensity of all CCS sorted by their different morphologies. Graphs show one
922 representative CLEM result with a total of 347 CCS from one PM.



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924 **Figure 3: Mathematical modelling of CCS growth from intensity profiles of**
 925 **individual CME events.** (a) Mathematical representation of the constant area
 926 model, flat-to-curved transition happens at the time when clathrin reaches its final
 927 content. (b) Example of a clathrin intensity track fitted by the constant area model.
 928 Blue dots represent measured intensity of a single CME event; black line represents
 929 the fit with equation 1. The schematic illustrates the calculated size and curvature,
 930 flat (blue), dome (red), and pit (green). (c) Calculated projected area and curvature
 931 distributions of the CCS according to the constant area model for 4927 FM tracks of
 932 4 different cells. P-value of Welch's t-test to compare the predicted to the measured
 933 distribution in panel h. A box/whiskers plot of the projected area is shown in the
 934 inset. Mid-line represents median, cross represents the mean and the whiskers
 935 represent the 10 and 90 percentiles. (d) Mathematical representation of the updated
 936 growth model where a flat clathrin patch grows and the flat-to-curved transition

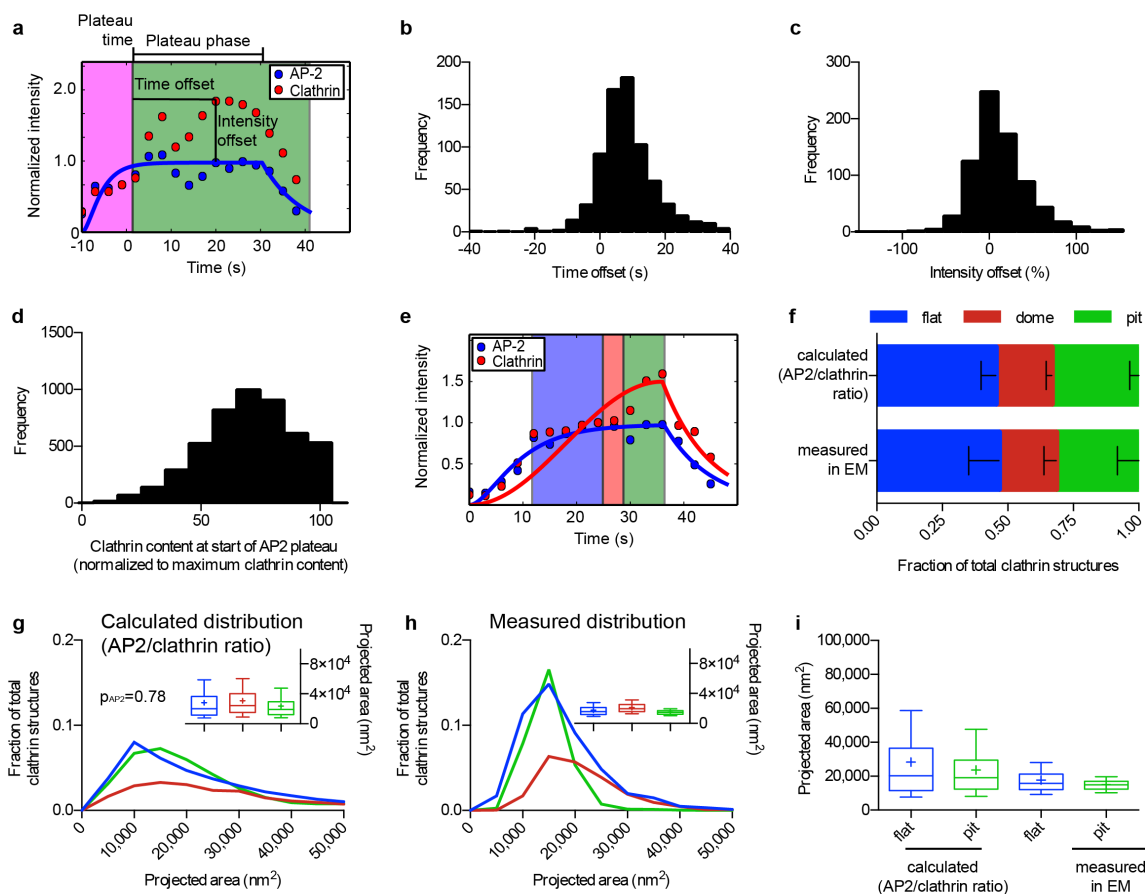
937 happens before reaching the final clathrin content. (e and f) same as (b and c) but
938 using equation 2. (g) Comparison of the predicted ratio of flat, dome, and pit
939 structures from both growth model (equation 1 (a) and equation 2 (d)) and the
940 distribution obtained from TEM imaging. Results are calculated for 4927 FM tracks
941 of 4 different cells; means with SD are shown. (h) Direct comparison of the projected
942 area distribution of flat and pit structures calculated by equation 1 and 2 as well as
943 measured in EM, box/whiskers plot. (i) Measured projected area and curvature
944 distributions of the CCS from TEM data as shown in Fig. 1.
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948 **Figure 4: The relative amount of AP2 and clathrin molecules per surface unit**
 949 **of a CCS is curvature dependent.** CLEM analysis of CCS labelled with AP2-eGFP (a)
 950 or clathrin heavy chain antibody (b). Flat (blue), dome (red), and pit (green). Lines
 951 in the corresponding colour show linear regression of the projected area and of the
 952 fluorescence intensity. CLEM analysis of CCS corrected according to the regression
 953 of flat structures labelled with AP2-eGFP (c) or clathrin heavy chain antibody (d).
 954 Projected areas of dome and pit structures of the CLEM analysis were multiplied by
 955 a correction factor to fit the linear regression of flat CCS. Lines in the corresponding
 956 colour show linear regression of the calculated surface and the fluorescence
 957 intensity. (e) Table shows correction factors for dome and pit structures for AP2-
 958 eGFP or clathrin heavy chain labelling used in (c) and (d).

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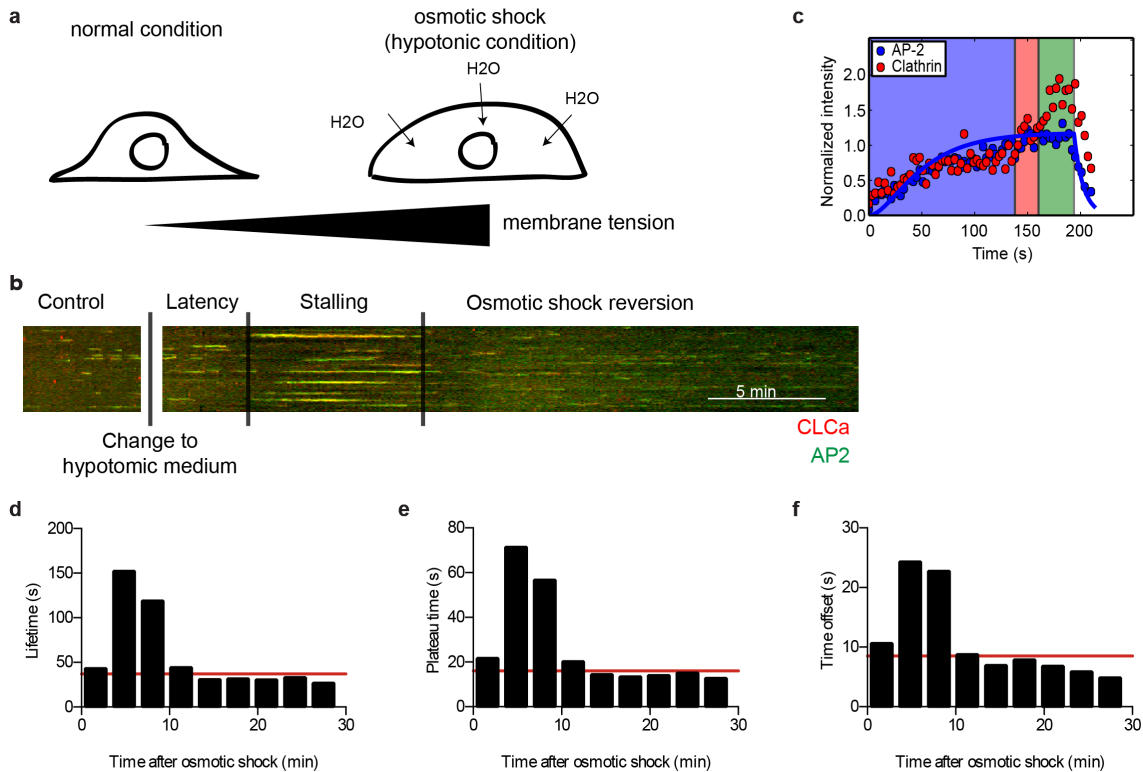


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962 **Figure 5: Change in the AP2/clathrin ratio is associated with flat-to-curved**
 963 **transition.** (a) Example of an AP2 (blue) and clathrin (red) intensity profile from an
 964 individual CME event. The AP2 profile was fitted to equation 1 to find the time when
 965 AP2 signal plateaus. For more information see Supplementary Information. The
 966 fluorescence intensity of AP2 and clathrin were normalised to the fluorescence
 967 intensity of the time when the fitted AP2 profile reaches its plateau. Time offset
 968 (difference between the time AP2 plateaus and clathrin reaches its maximum
 969 intensity) and intensity offset (excess of maximal clathrin signal over AP2 maximum
 970 intensity) are indicated in the profiles. Quantification of the time offset (b) and the
 971 intensity offset (c) for 754 tracks of one single cell. (d) Quantification of the clathrin
 972 content at the time when AP2 reaches its plateau from 4927 FM tracks. The clathrin
 973 signal was normalised to the maximal clathrin signal in each track. (e) Example of an
 974 AP2 (blue) and clathrin (red) profile fitted to equation 1 and 2, respectively. These
 975 fits were used to calculate the size and curvature distributions of the CCS in (f and

976 g). For more information see Supplementary Information. (f) Comparison of the
977 calculated ratio of flat, dome, and pit structures to the measured ratio in TEM.
978 Results calculated from 4927 FM tracks from 4 different cells; means with SD are
979 shown. (g) Calculated projected area of the CCS using a growth model where the
980 flat-to-curved transition corroborates with the change of clathrin/AP2 ratio (when
981 AP2 signal reaches its plateau phase) for 4927 FM tracks of 4 different cells. P-value
982 of Welch's t-test to compare the predicted to the measured distribution in panel f. A
983 box/whiskers plot of the projected area is shown in the inset. (h) Measured
984 projected area and curvature distributions of the CCS from TEM data as shown in
985 Fig.1. A box/whiskers plot of the projected area is shown in the inset. (i) Direct
986 comparison of the projected area distribution of flat and pit structures calculated
987 according to the AP2/clathrin ratio as well as measured in EM, box/whiskers plot.
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991 **Figure 6: Osmotic shock induces stalling of CCS.** (a) Illustration of the effect of

992 osmotic shock on BSC-1 cells. Hypotonic medium was applied to BSC-1 cells,

993 inducing osmotic swelling that results in an increase in PMT. The same BSC-1

994 expressing fluorescently tagged clathrin light chain and AP2 proteins was followed

995 from 5 minutes prior (internal control) until 30 minutes post hypotonic medium

996 application using spinning disc confocal microscopy. (b) Kymograph of AP2-eGFP

997 (green) and clathrin light chain a-tdtomato (red) expressing BSC-1 cells. The

998 dynamics of CCP was recorded during 5 min prior to osmotic shock until 30 minutes

999 post-osmotic shock. The time after applying the hypotonic medium can be divided

1000 into latency, stalling, and osmotic shock reversion time depending on the effect on

1001 CME dynamics. Scale bar: 5 minutes. (c) Representative AP2 (blue) and clathrin

1002 (red) intensity profile from an individual CME event during the time of stalling fitted

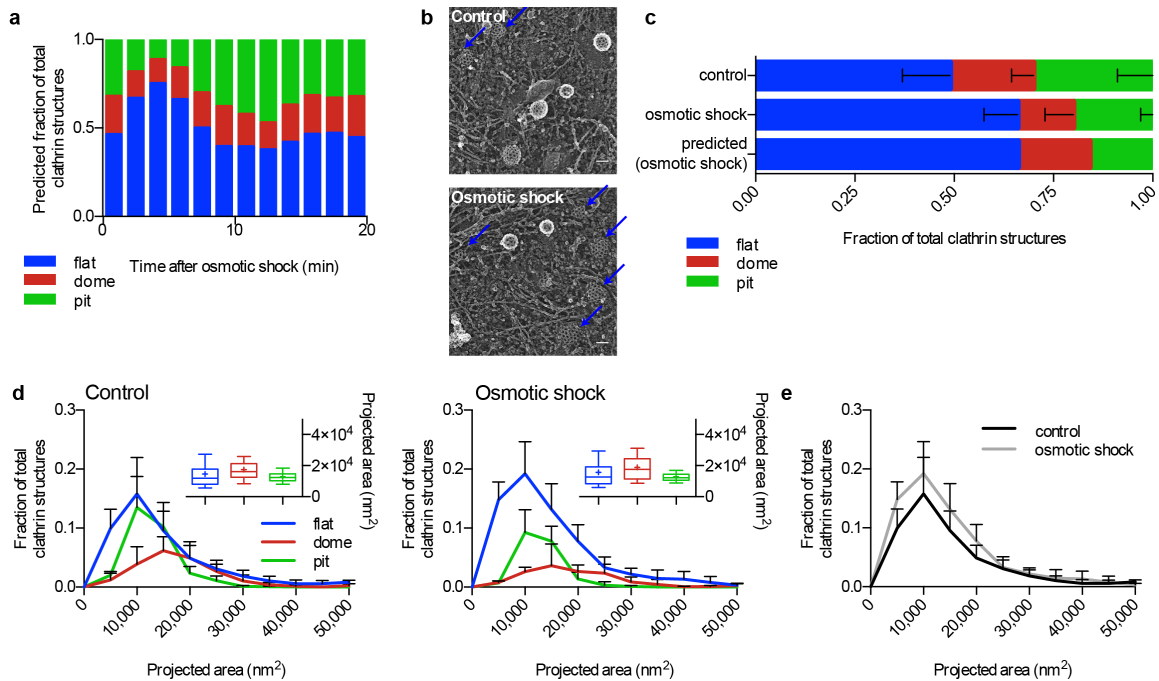
1003 to equation 1 to quantify the plateau time. (d) Quantification of the lifetime of CME

1004 events during osmotic shock experiments for 1607 tracks of one single cell. CME

1005 events were binned in 3 min intervals in respect to the onset of osmotic shock. Red

1006 line indicates lifetime of CME prior to osmotic shock. (e) Quantification of the

1007 plateau time of AP2 of individual CME events during osmotic shock experiments (as
1008 defined in Fig.5a) for 1607 tracks of one single cell. CME events were binned in 3
1009 min intervals in respect to the onset of osmotic shock. Red line indicates plateau
1010 time of CME prior to osmotic shock. (f) Quantification of the time offset between
1011 AP2 plateau and clathrin maximum of individual CME events during osmotic shock
1012 experiments (as defined in Fig.5a) for 1607 tracks of one single cell. CME events
1013 were binned in 3 min intervals in respect to the onset of osmotic shock. Red line
1014 indicates time offset of CME prior to osmotic shock.
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Figure 7: Osmotic shock blocks flat-to-curved transition of CCS. (a) Predicted

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ratio of flat (blue), dome (red), and pit (green) structures calculated from the

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binned AP2 and clathrin profiles of CME events (Fig.6c) during osmotic shock for

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1357 tracks. (b) Examples of CCS under normal and osmotic shock conditions.

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Blue arrows point to flat structures. (c) Comparison of measured and predicted

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frequency of flat, dome, and pit structures under normal and osmotic shock

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conditions. (d) Projected area distribution of the different clathrin morphologies

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under normal and osmotic shock conditions. A box /whiskers plot of the

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projected area is shown in the inset. Mid-line represents median, cross represents

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the mean and the whiskers represent the 10 and 90 percentiles. (e) Comparison

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of projected area distributions of flat CCS under normal and osmotic shock

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conditions. Results are calculated from four different membranes (number of CCS

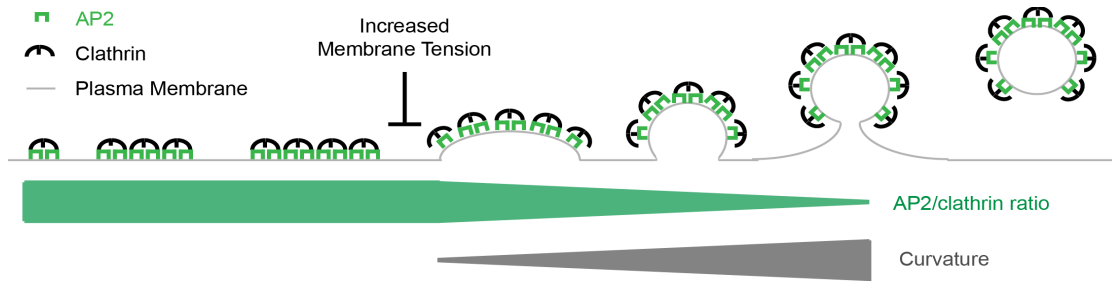
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per membrane: normal conditions 267, 308, 229, and 323; osmotic shock: 395,

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99, 351, and 201); means with SD are shown.

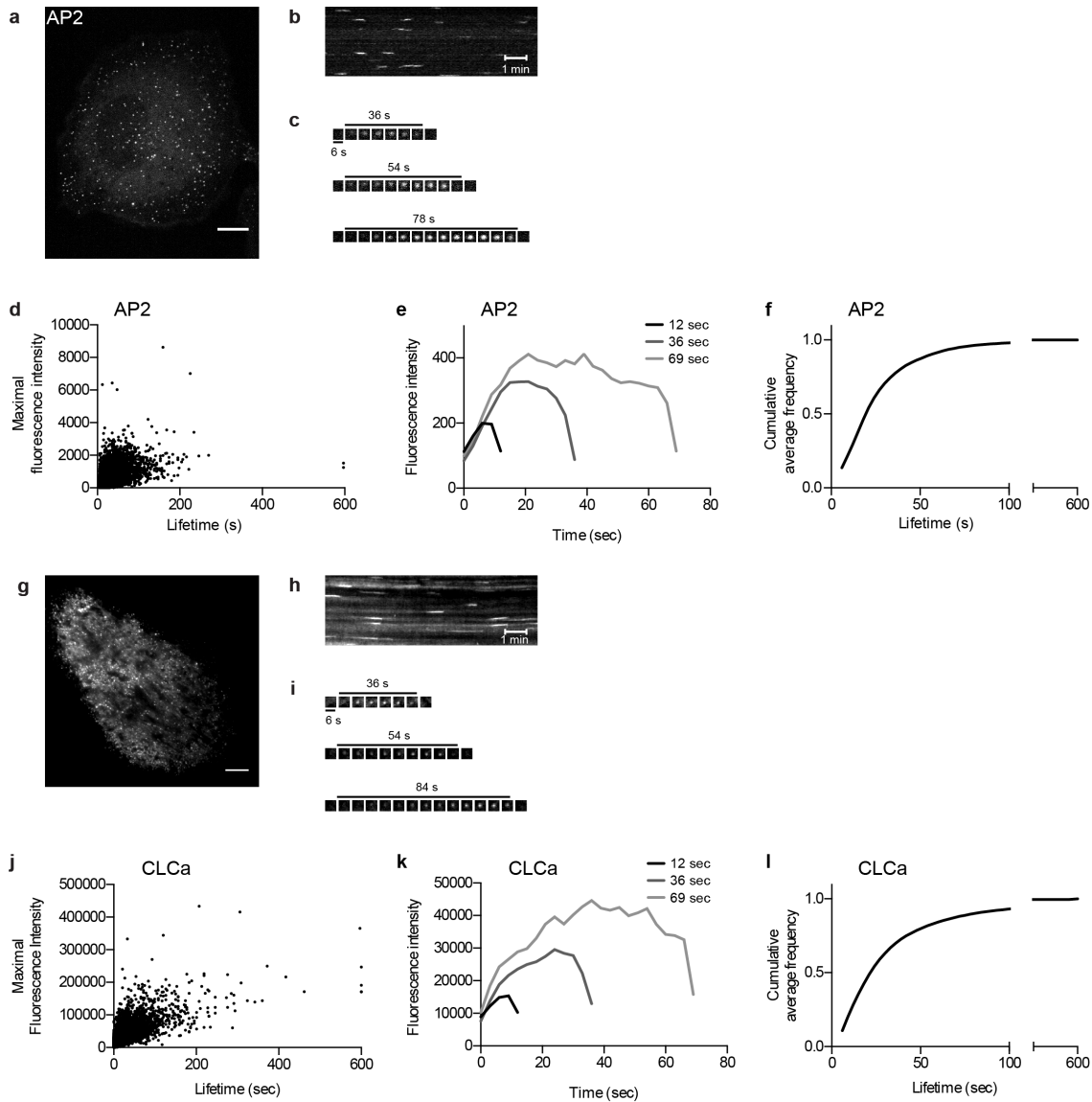
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1034 **Figure 8: Model of CCP assembly.** Schematic representation of the growth model
1035 of CCS. CCS initiate as flat clathrin array. They first grow in size in a flat morphology
1036 with a constant AP2/clathrin ratio. When they reach around 70% of their full
1037 clathrin content, the AP2/clathrin ratio starts to decrease and the CCS start
1038 acquiring their curvature. CCPs keep growing by adding additional clathrin
1039 molecules until formation and release of CCV into the cytoplasm. The flat-to-curved
1040 transition of CCS can be inhibited by increasing PMT resulting in an accumulation of
1041 flat structures. We propose that flat-to-curved transition is concomitant with
1042 bypassing the energy barrier necessary to curve the PM and that this critical step in
1043 CME is coordinated by the uncoupling of clathrin and AP2 characterised by their
1044 abrupt ratio decrease.

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Supplementary Figure 1: Characterisation of clathrin dynamics in BSC-1 cells.

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Spinning disc live-cell microscopy of BSC-1 AP2-eGFP cells. (a) Representative

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image of a BSC-1 AP2-eGFP cell. Scale bar: 10 μ m. (b) Kymograph of a 10 minute long

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movie following AP2-eGFP signal. Scale bar: 1min (c) Representative individual CME

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events. (d) Dot plot showing the lifetime and the maximal fluorescence intensity of

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AP2-eGFP of all CME events from one cell in a 10 minute long movie. Total number

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of tracked CME events: 14,694. (e) Average fluorescence intensity profiles (AP2-

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eGFP) of CME events with lifetime of 12, 36, and 69 seconds. Data are acquired from

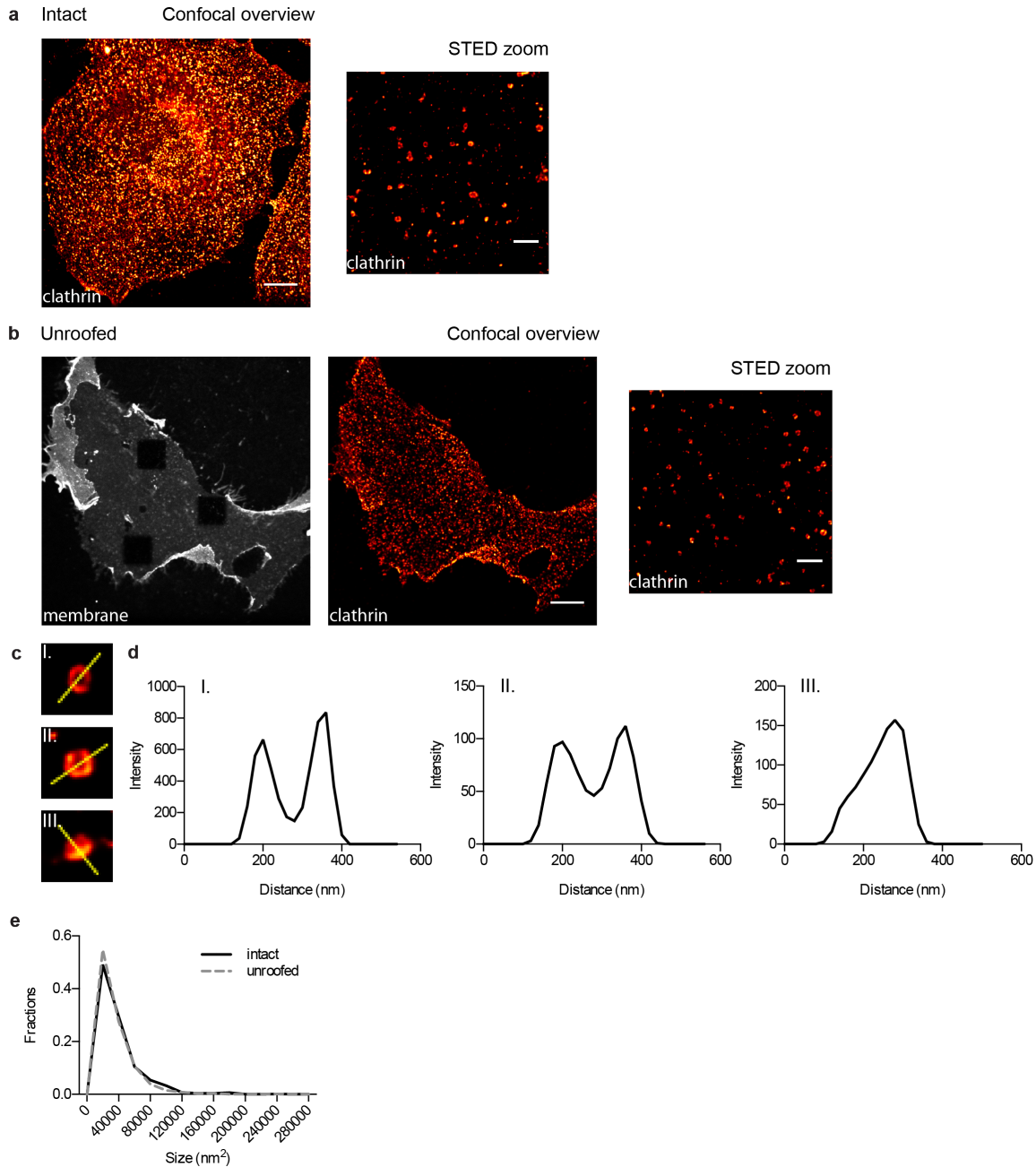
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one cell during a 10 minute long movie. Number of tracks used for calculating the

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average intensity profiles: 1,525 for 12sec-lifetime; 604 for 36sec-lifetime; 230 for

1058 69sec-lifetime. (f) Cumulative average frequency of CME lifetime. The average was
1059 calculated from five different cells. Number of total tracks per cell: 14,037; 16,386;
1060 14,694; 16,797; 19,631. (g) TIRF live-cell microscopy of BSC-1 cells expressing
1061 clathrin light chain a (CLCa)-tdtomato. Representative image of a BSC-1 cell
1062 expressing CLCa-tdtomato. Scale bar: 10 μ m. (h) Kymograph of a 10 minute long
1063 movie following CLCa-tdtomato. Scale bar: 1min (i) Representative individual CME
1064 events. (j) Dot plot showing the lifetime and the maximal fluorescence intensity of
1065 CLCa-tdtomato of all CME events from one cell in a 10 minute long movie. Total
1066 number of tracked CME events: 4,763. (k) Average fluorescence intensity profiles
1067 (CLCa-tdtomato) of CME events with lifetime of 12, 36, and 69 seconds. Data are
1068 acquired from one cell during a 10 minute long movie. Number of tracks used for
1069 calculating the average intensity profiles: 175 for 12sec-lifetime; 75 for 36sec-
1070 lifetime; 21 for 69sec-lifetime. (l) Cumulative average frequency of CME lifetime.
1071 The average was calculated from four different cells. Number of total tracks per cell:
1072 2,429; 4,763; 5,443; 16,797; 8,393.



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Supplementary Figure 2: Comparative STED nanoscopy analysis of CCS in

1076 **intact vs. unroofed cells.** (a) STED nanoscopy of intact BSC-1 cells. CCS were

1077 immunostained with an anti-clathrin light chain (CLC) antibody. Left: confocal

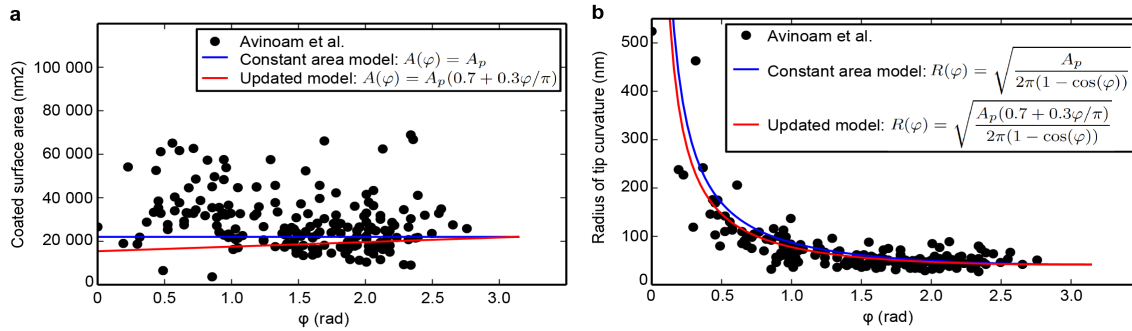
1078 overview, scale bar: 5 μ m. Right: STED view, scale bar 1 μ m. (b) STED nanoscopy of

1079 unroofed BSC-1 cells. BSC-1 cells were unroofed by sonication; the remaining

1080 attached PM was stained with wheat germ agglutinin (WGA). CCS were

1081 immunostained with a an anti-clathrin antibody. Left: confocal overview, scale bar:

1082 5 μ m. Right: STED view, scale bar 1 μ m. (c) Example of different CCS and their
1083 intensity line profile. Yellow line marks the axes measured for the intensity line
1084 profiles. (d) Analysis of the size distribution of CCS in intact and unroofed cells. CCS
1085 from five cells per conditions with three STED pictures per cell. Number of analysed
1086 CCS for intact cells: 820 and unroofed cells: 1169.



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1089 **Supplementary Figure 3: Updated growth model explains the CLEM data from**

1090 **Avinoam et al.** (a) Clathrin coated surface area as a function of the growth angle ϕ .

1091 The constant area model where we assume $A=22000\text{nm}^2$, corresponding to the

1092 most probable surface area¹² is plotted (blue). Our updated model where we assume

1093 that the surface starts to bend when 70% of the final clathrin content is reached and

1094 grows the last 30% linear with ϕ plotted (red). The data (black dots) were extracted

1095 from Avinoam et al.¹⁹. (b) Radius of tip curvature as a function of the growth angle

1096 ϕ . Again, the constant area model (blue) and our updated model (red) are plotted.

1097 The radius of the tip curvature is given assuming that the clathrin structure exhibits

1098 the shape of a spherical cap. Then the tip curvature reads $R =$

1099 $\sqrt{A(\theta)/(2\pi(1 - \cos(\theta)))}$. The data (black dots) were extracted from Avinoam et al.

1100 ¹⁹.