**Short running title:**

**Adaptive actin networks ensure robust endocytosis**

(50 characters, including spaces)

**Full running title:**

# Adaptive actin organization counteracts elevated membrane tension to ensure robust endocytosis

(100 characters, including spaces)

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Main Figures 8
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Supp movies 5

## Highlights:

- Clathrin coat surface area and actin ultra-structure adapt to elevated membrane tension.
- The actin network is nucleated at the base of the clathrin-coated pit and grows upward.
- Actin ultra-structural organization is not tightly coupled to CME progression.
- Actin force generation is required earlier in CME progression under elevated membrane tension.


## Summary (~40-word):

Kaplan et al. revealed that actin assembly compensates for changes in plasma membrane tension by an adaptive force generating mechanism to ensure robust endocytosis. Under elevated membrane tension the network grows deeper, even in early endocytic stages, from the base upward.

## Abstract (160 words), 153 words

Clathrin-mediated endocytosis (CME) remains robust despite variations in plasma membrane tension. Actin assembly-mediated force generation becomes essential for CME under high membrane tension, but the underlying mechanisms are not understood. We investigated actin network ultrastructure at each stage of CME by super-resolution imaging. Actin and N-WASP spatial organization indicate that polymerization initiates at the base of clathrin-coated pits and that the actin network then grows away from the plasma membrane. Actin network organization is not tightly coupled to endocytic clathrin coat growth and deformation. Membrane tension-dependent changes in actin organization explain this uncoupling. Under elevated membrane tension, CME dynamics slow down and the actin network grows higher, resulting in greater coverage of the clathrin coat. This adaptive mechanism is especially crucial during the initial membrane curvature-generating stages of CME. Our findings reveal that adaptive force generation by the actin network ensures robust CME progression despite changes in plasma membrane tension.

78 ## Introduction

79 From yeast to humans, transient actin assembly is associated with the formation of
80 clathrin-coated endocytic vesicles. In yeast cells, actin assembly is always required to generate
81 forces to invaginate the plasma membrane against a high intrinsic turgor pressure for CME
82 (Kaksonen, Sun, and Drubin 2003; Aghamohammadzadeh and Ayscough 2009; Idrissi et al.
83 2012; Kukulski et al. 2012). In mammalian cells, actin assembly augments CME and becomes
84 increasingly critical as plasma membrane tension becomes increasingly elevated (Boulant et al.
85 2011; Kaur et al. 2014; Grassart et al. 2014). When actin assembly is perturbed in mammalian
86 cells, CME typically slows in a cell-type-dependent manner (Fujimoto et al. 2000; Merrifield,
87 Perrais, and Zenisek 2005; Grassart et al. 2014; Dambournet et al. 2018). A potential cause of
88 this reported variation between cell types might be differences in plasma membrane tension
89 (Pontes, Monzo, and Gauthier 2017). On an ultrastructural level, actin perturbation results in
90 accumulation of "U-shaped" membrane invaginations, reflecting difficulty in progressing to the
91 subsequent "omega-shaped" membrane stage (Fig. 1 A) (Fujimoto et al. 2000; Yarar, Waterman-
92 Storer, and Schmid 2005; Boulant et al. 2011; Almeida-Souza et al. 2018). These findings
93 suggest that actin assembly in general makes CME more efficient in mammalian cells,
94 potentially compensating for changes in plasma membrane tension.

95 Despite the fact that actin assembly appears to be associated with CME in all eukaryotes
96 and over a large range of membrane tensions, how actin assembly assists and whether it adapts to
97 changes in membrane tension are not known. Key to addressing these questions is investigating
98 the ultrastructure of actin networks at CME sites in vertebrate cells under different membrane
99 tensions. Platinum replica electron microscopy of cultured cells has provided some insights into
100 actin ultrastructure and force generation during mammalian CME (Collins et al. 2011). The

101 resulting electron micrographs led to the proposal that actin networks polymerize from a single
102 mother filament and therefore assemble around clathrin coats asymmetrically, ultimately forming
103 a collar-like arrangement around the vesicle neck (Fig. 1 A) (Collins et al. 2011). This actin
104 organization suggested that a pushing force is generated orthogonal to the direction of membrane
105 invagination, supporting neck constriction and fission. However, actin filaments do not only
106 make interactions with the vesicle neck, but also with the bud surface, because the clathrin coat
107 is embedded with actin-binding linker proteins like Hip1R and epsin, suggesting that actin
108 filaments might also help to pull the forming vesicle into the cell (Engqvist-Goldstein et al. 2001;
109 Messa et al. 2014; Sochacki et al. 2017; Clarke and Royle 2018).

110 Evidence suggests that such a pulling mechanism exists in budding yeast. Here, studies
111 support a model in which actin filaments are nucleated in a ring at the surface of the plasma
112 membrane surrounding the clathrin-coated pit (CCP), and in which the resulting filaments are
113 coupled to the clathrin coat surface (Kaksonen, Sun, and Drubin 2003; Carroll et al. 2012;
114 Skruzny et al. 2012, 2015; Picco et al. 2015; Mund et al. 2018). This assembly generates an
115 inward force parallel to the plasma membrane invagination as the actin network grows away
116 from the plasma membrane and pulls the coat with it against the high hydrostatic turgor pressure
117 (Picco et al. 2015). The feasibility of this model is supported by mathematical models (Liu et al.
118 2009; Berro, Sirotkin, and Pollard 2010; Carlsson and Bayly 2014; Dmitrieff and Nédélec 2015;
119 Wang and Carlsson 2017; Hassinger et al. 2017; Mund et al. 2018; Nickaeen et al. 2019;
120 Akamatsu et al. 2020). Because the endocytic machinery is highly conserved from yeast to
121 mammals, a similar mechanism for actin force generation seems likely to generate forces against
122 membrane tension in mammalian CME. However, ultrastructural evidence for such an
123 organization in mammalian CME, and for how this organization might respond to changing

membrane tension, are lacking. Importantly, several competing models for actin organization at CME sites in mammalian cells have been proposed, so it is now important to distinguish between these models (Engqvist-Goldstein et al. 2001; Messa et al. 2014; Sochacki et al. 2017; Clarke and Royle 2018; Boulant et al. 2011).

Here we used two-color, three-dimensional stochastic optical reconstruction microscopy (2c-3D STORM) to investigate actin organization at CME sites at different stages of internalization and established the relationship between actin organization and plasma membrane tension. We combined this method with live-cell imaging and experimental manipulations of membrane tension to investigate how elevated membrane tension affects actin network organization, and how actin assembly assists CME dynamics under a range of membrane tensions. Our data support an adaptive actin force-generating mechanism that ensures robust CME over a range of membrane tensions.

## Results

### *Actin organization suggests that force generation can be either parallel to, or orthogonal to, or both, the axis of clathrin-coated pit formation at different sites in the same cell*

Electron microscopy has been the method of choice to study actin ultra-structural organization at CME sites in mammalian cells (Collins et al. 2011; Akamatsu et al. 2020), but may not preserve all actin structures and is not amenable to achieving large sample sizes. Some electron microscopy (EM) procedures such as “unroofing” risk compromising structural integrity in order to obtain high resolution and unobstructed views. EM approaches can also lack the molecular specificity necessary to clearly identify specific proteins. Recent advances in super-resolution microscopy (SRM) provide new opportunities to examine cellular ultrastructures with low invasiveness and high molecular specificity (Sahl, Hell, and Jakobs 2017; Sigal, Zhou, and

148 Zhuang 2018). In particular, we have previously demonstrated the application of 3D-STORM to
149 study actin and related membrane cytoskeletal systems in mammalian cells (Xu, Babcock, and
150 Zhuang 2012; Xu, Zhong, and Zhuang 2013; Hauser et al. 2018) and CME in fission yeasts (Sun
151 et al. 2019).

152 Here we reveal actin ultra-structural organization at clathrin-coated structures (CCSs) and
153 clathrin-coated pits (CCPs) by 2c-3D STORM. Henceforth, we refer to CCSs as relatively flat
154 clathrin structures and CCPs as curved, invaginating clathrin structures. Our 2c-3D STORM
155 method preserves the sample by chemical fixation of intact cells, provides high molecular
156 specificity due to immunofluorescence labeling, and allows large numbers of sites to be imaged.

157 We conducted our experiments on a skin-melanoma cell line (SK-MEL-2) wherein 87%
158 of dynamin2-eGFP$^{EN}$ (DNM2-eGFP$^{EN}$) spots appear together with actin at the ventral plasma
159 membrane in live-cell fluorescence microscopy (Grassart et al. 2014). We previously used
160 genome editing to generate a clone of this cell line expressing DNM2-eGFP$^{EN}$ and clathrin light
161 chain A-tagRFP-T (CLTA-TagRFP-T$^{EN}$) at endogenous levels (Doyon et al. 2011). We used this
162 DNM2-eGFP$^{EN}$/ CLTA-TagRFP-T$^{EN}$ cell line in our 2c-3D STORM experiments because it
163 enabled us to complement our 2c-3D STORM data with live cell fluorescence microscopy using
164 the same cell line.

165 We resolved CCSs as round and elliptical shapes on the ventral surface in these cells
166 (Fig. 1 B). The majority of the CCSs appeared connected to filamentous actin visualized using
167 Alexa Fluor 647-tagged phalloidin (phalloidin-AF647) (Fig. 1 C and D). When we zoomed into
168 two representative clathrin coats, we could more clearly resolve the association with actin
169 networks (Fig. 1 E shows blowups of boxed regions in Fig. 1 D).

Knowing how actin networks are organized spatially in all dimensions at CME sites provides insights into actin's force generation mechanism(s). We rendered the high-resolution CCSs in three-dimensions by cropping an area of 310 nm x 310 nm around each clathrin and actin structure's center of mass to generate an x-z STORM image projection. To our surprise we observed two strikingly different actin filament spatial organizations in the x-z projections of the first two clathrin coats we examined, even though they were near each other in the same cell (Fig. 1 D). In the first example, a thin layer of actin filaments resided at the base of the clathrin coat (Fig. 1 F, inset 1), reminiscent of structures observed in the EM by Collins *et al.*, 2011. In contrast, actin filaments covered the second CCP completely in the x-z projection (Fig. 1 F, inset 2). This organization resembles actin interacting with the whole clathrin coat as indicated by thin section electron micrographs and SRM in budding yeast (Mulholland et al. 1994; Idrissi et al. 2008; Kukulski et al. 2012; Buser and Drubin 2013; Mund et al. 2018). These data indicate that distinct actin structures can coexist in the same cell, consistent with models for force generation parallel to and orthogonal to the invagination axis.

***CME site-associated actin networks grow from the CCP base to the tip of the coat and their organization is not coupled to CME progression***

We next aimed to analyze how actin is organized at CME sites and asked how actin organization relates to progress through CME. We first determined when actin filaments are first detectable at CCSs and CCPs over the course of endocytosis by manually selecting 992 high-resolution clathrin coats after applying quality control criteria explained in the Materials and Methods (Fig. S1 A). In our cell line we could detect actin associated with 74% of the clathrin coats. We developed a procedure to determine the endocytic stage of each structure and then

193 used these staged structures to reconstruct a timeline of actin organization over the course of
194 CME.

195 To classify the CME stages of the clathrin coats we used their x-z projections (side
196 profiles), similar to what had been done previously by electron microscopy (Avinoam et al.
197 2015). Clathrin coats were classified as early, intermediate, or late invaginations as follows:
198 using our STORM data, we measured clathrin coat width and height from x-z projections (Fig. 2
199 A, Fig. S1 B and C, see Materials and Methods). We then calculated the height to width ratios
200 and defined a shape index (SI). We manually defined clathrin coats as early when they were
201 shallowly curved and had a calculated SI index below 0.7 (Fig. 2 A and Fig. S1 D). Intermediate
202 (U-shaped) clathrin coats had an SI index higher than 0.7 and lower than 0.9 (Fig. 2 A and Fig.
203 S1 D). Late (omega-shaped) clathrin coats had a SI index higher than 0.9 (Fig. 2 A and Fig. S1
204 D). In representative x-z projections of clathrin coats we surprisingly could find a wide variety of
205 actin organizations in each of the three categories of CME stages (Fig. 2 A). We concluded that
206 irrespective of the endocytic stage at which the clathrin coats resided, we observed some that had
207 a thin actin network at the base of the pit, others with an intermediate level of coverage of the
208 clathrin, and others in which the actin completely covered the clathrin.

209 To extend our analysis to the entire data set, we quantified the overlap between clathrin
210 and actin in all our x-z projections (see Materials and Methods). We then plotted the shape index
211 versus the percentage of the clathrin coat covered by actin (Fig. 2 B). We could not detect a
212 significant correlation between the endocytic stage, as reported by clathrin coat shape, and actin
213 coverage of clathrin. This observation suggests that actin at individual CME sites in our cell type
214 can polymerize at all endocytic stages: early, intermediate, and late.

While actin distribution did not appear to be coupled to the CME stage, it was variable in our data set. We found actin covering more than half the coat at 70% - 75% of clathrin sites in all three endocytic stages (Fig. 2 B). Accordingly, a significant fraction (25-30%) of clathrin sites at all stages had only partial actin coverage. We observed that whenever actin is only partially covering clathrin, the network is always located at the base of the clathrin coat (Fig. 2 A). This observation suggests that actin polymerization is nucleated at the base of the pit and that the network then grows upward to and over the top of the clathrin coat. To test the validity of this conclusion for the entire data set, we calculated the distance between the centroid of clathrin and actin in the x-z projection images for each individual site and plotted the distance against the clathrin coverage by actin as a percentage (Fig. 2 C, see Materials and Methods). As the actin average position minus the clathrin average position is negative when the coverage is low, but is near zero when the coverage is high, we concluded that actin polymerization is initiated at the base of clathrin coats.

To reinforce the conclusion that actin polymerization is first nucleated at the clathrin coat's base in early coats, we next investigated the spatial distribution of the actin nucleation factor N-WASP at clathrin coats by 2c-3D STORM. Consistent with our conclusions about where actin assembly occurs at CCPs, N-WASP localized to the base of both early clathrin coats and late clathrin coats (Fig. 2 D, Fig. S1 E). More unexpectedly, at some CME sites N-WASP covered the entire clathrin coat in early and late stages (Fig. 2 E, Fig. S1 E).

In summary, we conclude that actin polymerization is nucleated at the base of clathrin-coated pits and grows to the coat's tip. However, unexpectedly, actin nucleation is not coupled to the stage of CME progression. A possible explanation for the variety of actin organizations we

observed associated with clathrin coats is that the organization responds to changes in membrane tension to adapt the magnitude and orientation of forces produced.

***Dynamics of clathrin-mediated endocytosis slow down under elevated membrane tension***

We next aimed to use 2c-3D STORM to identify the stages of clathrin coat deformation to which actin force generation contributes under elevated membrane tension. Previous EM studies identified a requirement at the "U" to "omega" transition, but our light microscopy-based analysis of a large number of sites at many CME stages provided increased sensitivity for detecting effects throughout the process. To do so, we first needed to establish conditions under which CME dynamics are affected by elevated membrane tension in our genome-edited SK-MEL-2 cells.

We analyzed CME dynamics in the SK-MEL-2 cells by imaging DNM2-eGFP$^{EN}$ and CLTA-TagRFP-T$^{EN}$ in real time by total internal reflection microscopy (TIRF) and concomitantly, elevated membrane tension by hypotonic media treatment (Fig. 3 A). (Doyon et al. 2011). CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$ fluorescence lifetimes were determined by single-particle tracking. In cells imaged in isotonic media kymographs of CLTA-TagRFP-T$^{EN}$ (magenta) and DNM2-eGFP$^{EN}$ (green) fluorescence tracks reveal many relatively short clathrin tracks with a burst of DNM2-eGFP$^{EN}$ signal at the end (Fig. 3 B, Supp. movie 1). For sites at which both CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$ were detected, the CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$ mean lifetimes were 47 ± 32.4 s (SD) and 38.8 ± 32.1 s (SD), respectively (Fig. 3 C and E). For CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$ tracks that were not associated with DNM2-eGFP$^{EN}$ or CLTA-TagRFP-T$^{EN}$, respectively, mean lifetimes were 16.8 ± 11.8 s (SD) and 18.9 ± 12.3 s (SD), respectively, characteristic of structures not associated with CME (Fig. 3 D and F) (Hong, Cortesio, and Drubin 2015). Neither isotonic media nor exchange to slightly

260 dilute 225 mOsm media for 2 min prior to imaging noticeably affected DNM2-eGFP$^{EN}$ and
261 CLTA-TagRFP-T$^{EN}$ lifetimes or CME initiation and completion rates for sites containing both
262 proteins (Fig. S2 A - D). Only 1 - 2% of these CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$
263 fluorescence tracks persisted over the entire 4.8 min movie in isotonic and 225 mOsm media
264 (Fig. S2 B and D).

265 An increase in CLTA-TagRFP-T$^{EN}$ lifetime was observed in response to 2 min incubation
266 in lower osmolarity 150 mOsm media, with a mean lifetime of 59.7 ± 52.7 s (SD) (compared to
267 49.1 ± 0.7 s for isotonic media exchange) (Fig. S2 E). In addition, mild effects on CME initiation
268 rate: 14.7 ± 5.0 s (SD) (compared to 24.5 ± 6.3 s for isotonic media), completion rate: 10.7 ± 3.9
269 s (SD) (compared to 18.8 ± 6.0 s for isotonic media), and percentage of persistent tracks: 3.23 ±
270 1.3 % (SD) (compared to 0.77 ± 0.9 % (SD) for isotonic media) were observed but only after
271 cells were cultured for 10 min in 150 mOsm media (Fig. S2 F).

272 We next tested the effect of exchange to 75 mOsm hypotonic media on CME dynamics.
273 After 2 minutes in 75 mOsm hypotonic media a more dramatic effect was observed. Example
274 kymographs of CLTA-TagRFP-T$^{EN}$ (magenta) and DNM2-eGFP$^{EN}$ (green) showed elongated
275 fluorescence tracks that very often lasted over the entire duration of a 4.8 min movie (Fig. 3 B,
276 Supp. movie 2). The mean lifetimes of CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$ tracks
277 containing both proteins were 128.4 ± 111.9 s (SD) (Fig. 3 C) and 125.1 ± 111.5 s (SD),
278 respectively (Fig. 3 E). We also observed a substantial decrease in CME initiation (2 min
279 hypotonic: 10.28 ± 1.5 $\mu m^{-2}$ $min^{-1}$ (SD) vs. isotonic: 22.18 ± 3.5 $\mu m^{-2}$ $min^{-1}$ (SD)) and completion
280 rates (2 min hypotonic: 5.32 ± 1.7 $\mu m^{-2}$ $min^{-1}$ (SD) vs. isotonic: 17.75 ± 3.4 $\mu m^{-2}$ $min^{-1}$ (SD)),
281 together with a strong increase in the percentage of persistent tracks (2 min hypotonic: 19.17 ±
282 9.2 % (S.) vs. isotonic: 0.65 ± 0.3 % (SD)) over the 4.8 min duration of a movie (Fi. 3 G – I, Fig.

S2 G). After 10 min of culturing, the CLTA-TagRFP-$T^{EN}$ and DNM2-$eGFP^{EN}$ lifetimes began to recover, most likely reflecting cellular adaptation to the hypotonic treatment (Fig. 3 C, E and G-I, Supp. movie 3). We did not detect effects of hypotonic media treatment on lifetimes of tracks containing only CLTA-TagRFP-$T^{EN}$ or only DNM2-$eGFP^{EN}$ (Fig. 3 D and F). We conclude that CME dynamics are markedly reduced in SK-MEL-2 cells transferred to 150 mOsm media, with an even stronger effect observed with 75 mOsm media.

To determine how membrane tension is affected by changes in media osmolarity, we performed membrane tether pulling experiments by atomic force microscopy (AFM) on our cells cultured under isotonic conditions and at 75 mOsm. Under isotonic media conditions, we measured an average tether force of 33.0 ± 7.4 pN (SD). This value increased to 48.0 ± 17.1 pN (SD) in 75 mOsm media (Fig. 3 J). We exchanged the isotonic media with 75 mOsm hypotonic media on the sample stage and pulled tethers from cells beginning 2 min after media exchange and up to 16 min after media exchange. These experiments allow us to relate a quantitative change in membrane tension to effects on CME dynamics, actin and clathrin ultra-structural organization.

***Actin force generation assists early clathrin coat deformation under elevated membrane tension***

Next we wanted to investigate when in the process of CME branched actin filament assembly becomes important under elevated membrane tension. Branched actin networks are generated by the Arp2/3 complex and activated by N-WASP (Pollard 2007). We inhibited Arp2/3-mediated actin polymerization using the small molecule CK666 (Hetrick et al. 2013). Since Arp2/3 inhibition can affect membrane tension (Diz-Muñoz et al. 2016), we first carefully established the experimental conditions so the effects of high membrane tension or CK666

306 would not mask one another. Once conditions were optimized, we performed STORM image
307 analysis to learn more about the CME stages at which branched actin network assembly is
308 required.

309 We first titrated CK666 and monitored the effect on CME dynamics in a 4.8-minute
310 movie starting after 2 min of CK666 treatment. We aimed to identify a minimal CK666
311 concentration that would show a rapid effect on CME dynamics. 100μM CK666 extended
312 lifetimes of CLTA-TagRFP-$T^{EN}$ associated with DNM2-$eGFP^{EN}$ to 78.9 ± 66.2 s (SD) after 2
313 mins of treatment compared to 56.2 ± 39.9 s for the DMSO control (Fig. S3 A)). 100μM CK666
314 did not affect CME completion frequency and therefore we also detected no increase in the
315 percentage of persistent CLTA-TagRFP-$T^{EN}$ tracks (Fig. S3 B and C), though we observed a
316 small decrease in the CME initiation rate (Fig. S3 D).

317 Kymographs showing the DNM2-$eGFP^{EN}$ and CLTA-TagRFP-$T^{EN}$ fluorescence signals
318 revealed elongated tracks upon 100μM CK666 treatment, with further elongation observed upon
319 simultaneous osmotic elevation of membrane tension (Fig. 4 A). As quantitatively analyzed in
320 Fig. 4B-E, compared to controls (Fig. 4 B and D) the combination of 100μM CK666 and
321 elevated membrane tension markedly lengthened the lifetimes of clathrin 96.3 ± 85.5 s (SD)
322 (compared to DMSO control 59.0 ± 51.9 s (SD)) and dynamin2 83.5 ± 85.3 s (SD) (compared to
323 DMSO control 46.9 ± 48.8 s (SD)) associated with each other at CME sites (Fig. 4 C and E).
324 Consistent with the results of Boulant et al. (2011), who examined the effects of actin inhibitors
325 on CME when membrane tension is elevated, our results reveal that Arp2/3 complex-mediated
326 actin assembly is required for normal CME dynamics under elevated membrane tension.

327 We next sought to determine the precise CME stages during which Arp2/3-branched
328 actin network force contributes to normal progression when membrane tension is elevated. We

329 used STORM to look for increased representation of CME stages upon drug and osmolarity
330 treatment. Cells were treated under the optimized CK666 and osmolarity conditions described
331 above, chemically fixed, and then immunolabeled for clathrin. CME sites in the STORM images
332 were staged using criteria described in Fig. 2 above, and their height and width in x-z projections
333 were quantified (Fig. 4 F). As in the above 2d-3D STORM experiments, the full progression
334 from a flat clathrin coat to a rounded vesicle could be clearly resolved in the x-z projections (Fig.
335 4 F, lower image panel). Here, we used clathrin coat height as a proxy to precisely quantify
336 when clathrin coat progression is affected. Control cells treated with DMSO showed an average
337 clathrin coat height of 98 ± 21 nm (SD) (Fig. 4 G, Fig. S3 E). The average height increased to
338 106 ± 27 nm (SD) when cells were treated with 100μM CK666, consistent with previous studies
339 reporting a high accumulation of U-shaped clathrin coats in electron micrographs of epithelial
340 cells treated with Latrunculin A and Jasplakinolide (Boulant et al. 2011).

341 Interestingly, when Arp2/3-mediated actin polymerization was inhibited in cells with
342 elevated membrane tension, the average clathrin coat height decreased to 96 nm ± 24 nm (SD)
343 (Fig. 4 G, Fig. S3 E). This height decrease was also reflected in the accumulation of smaller
344 shape indices compared to 100μM CK666 only treatment, together with a very mild effect on
345 clathrin coat width (Fig. S3 E – G). This result indicates that the transition from a shallow
346 invaginated coat to a U-shaped coat is slowed when Arp2/3-mediated actin filament assembly is
347 inhibited in cells with elevated membrane tension.

348 The above data support the conclusion that Arp2/3-mediated actin force generation
349 facilitates the U-shaped to omega-shaped clathrin coat transition under isotonic conditions, but
350 becomes critical for the early transition from a shallow invaginated to U-shaped coat when
351 membrane tension increases.

***Contribution of clathrin coat surface area to membrane remodeling***

Clathrin coat assembly can provide energy to bend the plasma membrane (Kaksonen and Roux 2018; Sochacki and Taraska 2019). Since we are able to extract clathrin height and width from our STORM data, we asked whether we detected an effect on clathrin coat surface area when membrane tension is elevated. Mathematical simulations suggest that when the membrane tension is low, increasing the clathrin coat area can provide sufficient energy for membrane vesicle formation (Hassinger et al. 2017). In addition, clathrin can polymerize into a fully formed pit on giant unilamellar vesicles under hypertonic media conditions (Saleem et al. 2015). Indeed, we find that 26% of our super-resolved clathrin coats in all stages of CME lacked detectable actin (Fig. 5 A and B, Fig. S 4 A and B). Of the clathrin coats lacking detectable actin, 5% show an associated diffraction-limited DNM2-eGFP$^{EN}$ signal (Fig. 5 B and Fig. S4 B). The DNM2-eGFP$^{EN}$ fluorescence serves as a read-out for membrane vesicle neck formation (Avinoam et al. 2015; Sochacki et al. 2017). We assume that fully formed clathrin coats without actin or DNM2-eGFP$^{EN}$ were either going to become associated with DNM2-eGFP$^{EN}$ or were already associated with DNM2-eGFP$^{EN}$ and recently underwent vesicle scission. Therefore, this is a population represented among the CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$ associated tracks that we observe in living cells, and we included them when performing our analysis of clathrin coat surface area. By classifying clathrin coats as being or not being associated with DNM2-eGFP$^{EN}$, and using the shape index information, we were able to organize the coats lacking actin into early, intermediate, membrane fission or later (after membrane fission) (Fig. 5 C).

For the clathrin coats showing detectable actin, 50% contained a DNM2-eGFP$^{EN}$ fluorescence signal (Fig. 5 D and E, Fig. S 4 C and D). We used the same classification to place each structure analyzed into a timeline as we did for the coats without actin (Fig. 5 F). As described below, this

375 classification enabled the detection of differences in clathrin coat surface area, not only
376 depending on membrane tension but also on actin.

377 Upon sorting the individual CCSs and CCPs into positions along the above established
378 timeline, we first found that under isotonic media conditions the coat surface area on average
379 increased in the transition from early (Without actin: 62055 ± 16951 $nm^2$ (SD), and with actin
380 54811 ± 17328 $nm^2$ (SD)) to intermediate endocytic stage (Without actin: 71048 ± 18854 $nm^2$
381 (SD), and with actin 61145 ± 17360 $nm^2$ (SD)) (Fig. 6 A and C and Table 1). This increase in
382 surface area was independent of actin. In subsequent endocytic stages, we did not detect any
383 changes in average coat surface area. We conclude that clathrin polymerization to enlarge the
384 coat surface area is likely to contribute energy to membrane bending in the transition from early
385 to intermediate endocytic stages, but then the coat surface area remains constant until final CCV
386 formation.

387 Under 75 mOsm hypotonic media conditions, the difference in coat surface area between
388 the early and intermediate stages vanished. However, we detected on average a substantially
389 larger surface coat area between the early-intermediate (Without actin: 69606 ± 17353 $nm^2$ (SD),
390 and with actin 68347 ± 21643 $nm^2$ (SD)) and the membrane fission stage (Without actin: 80071
391 ± 26077 $nm^2$ (SD), and with actin 73414 ± 25636 $nm^2$ (SD)) (Fig. 6 B and D and Table 1). After
392 fission, the coat surface area decreased again (Without actin: 67607 ± 22633 $nm^2$ (SD), and with
393 actin 59022 ± 22969 $nm^2$ (SD)) (Table 1). We speculate that in the early stages the clathrin coat
394 surface area must increase to a certain threshold to drive membrane bending under higher
395 membrane tension. Subsequent coat growth contributes energy for the U-shape to omega shape
396 transition of the clathrin coats. We observed similar surface area changes under hypotonic media
397 conditions irrespective of whether actin was present or lacking. However, the magnitude of the

398 coat surface area change was larger when actin was present (Table 1). We speculate that these
399 clathrin sites might have experienced locally higher membrane tension, reflected in the induction
400 of actin assembly.

401 Interestingly, we also observed that on average, the clathrin coat surface area was smaller
402 when actin was present compared to when actin was absent (Fig. 6A-D and Table 1). Under
403 isotonic conditions this difference was observed for each of the stages of endocytosis. Whereas
404 under hypotonic media conditions the coat surface area was smaller when actin was present, this
405 difference was not statistically significant. We conclude that when actin is absent, the coat
406 surface areas grow larger, possibly to contribute the energy required for membrane bending.
407 When actin was present we speculate that it either contributes enough force to bend the
408 membrane so further growth of the clathrin coat surface area is not necessary. Therefore, the coat
409 surface area is on average smaller for each stage of endocytosis. On the other hand, when actin is
410 present at the cortex this might restrict the clathrin coats from expanding their surface area.

411 Thirdly, we compared the average clathrin coat surface area between the isotonic media
412 condition (low membrane tension) and the 75 mOsm hypotonic media condition (high membrane
413 tension) over the course of endocytosis (Fig. S4 E). In the absence of actin, the average coat
414 surface area under higher membrane tension was larger even in the early stage (Hypotonic:
415 69456 ± 20014 $nm^2$ (SD), isotonic: 62055 ± 16951 $nm^2$ (SD)) (Fig. 6 A and B, Fig. S4 E, Table
416 1). Over the remaining stages of endocytosis, the coat surface area still showed the trend to be on
417 average either at the same size or of slightly larger size under higher membrane tension.

418 We detected a much larger difference between the coat surface area at low and high
419 membrane tension when actin was present. From the early stage to the membrane fission stage,
420 the average coat surface area was considerably larger when membrane tension was higher (Fig.

S4 E). These results are consistent with the possibility that clathrin polymerizes to cover a larger surface area under higher membrane tension to contribute energy to membrane remodeling.

Taken together, clathrin coat surface area differs depending on whether actin is present or absent, and on membrane tension. Our results suggest that the clathrin coat surface area might contribute to an adaptive force generating mechanism that responds to changes in membrane tension.

***Actin organization adapts to elevated membrane tension by increasing coverage of clathrin coats***

We found evidence that actin force generation contributes to the progression of early stages of CME under elevated tension. Since 75mOsm hypotonic media caused a pronounced slowdown of CME dynamics after only 2 min in our live cell experiments and strongly increased membrane tension of our SK-MEL-2 cells, we applied this same treatment to the cells and then chemically fixed them for 2c-3D STORM. When we super-resolved clathrin and actin by 2c-3D STORM in cells treated with 75 mOsm hypotonic media, the actin cytoskeleton remained intact and associated with CCSs (Fig. S5 A - D).

We observed that the average actin height dramatically increased for all endocytic stages after membrane tension was elevated by switching to 75 mOsm hypotonic media (Fig. 7 A and B, Table 2). In early clathrin coats, the average actin height increased from 123 nm ± 29 nm (SD) under isotonic media conditions to 158 nm ± 48 nm (SD). This growth of the actin network correlated well with an increase of average clathrin coverage by actin at early coats from 66% to 76% under 75 mOsm hypotonic media (Fig. 7 C, Table 2). Likewise, actin height and coat coverage of intermediate coats also increased significantly (Fig. 7 B, C and Table 2).

443 The average actin network height for clathrin coats at membrane fission reaches 161 nm
444 ± 39 nm (SD) under hypotonic media treatment, which differs dramatically from the height 135
445 nm ± 30 nm (SD) under isotonic conditions (Fig. 7B, Table 2). Actin covering clathrin coats at
446 membrane fission on average showed a moderate increase from 64% to 69% under hypotonic
447 conditions (Fig. 7C). The difference in average clathrin coverage of actin was even greater for
448 late coats (65% to 73%). These observations of higher average actin height and clathrin coverage
449 at late clathrin coats suggest that the force contribution of actin in late CME around the time of
450 vesicle scission is increased when membrane tension is elevated.

451 In response to hypotonic media treatment, actin was observed to grow over the clathrin
452 coats of all stages and, consistently, the actin network height increased as well (Fig. 7D and E,
453 Fig. S5 E and F). When we then plotted actin height against clathrin coverage, we found that on
454 average, actin height is also greater even when coverage of clathrin is in the lower percentage
455 range (Fig. 7 F and G). Our observations suggest that the actin network grows higher at clathrin
456 sites under elevated membrane tension by growing over the clathrin-coated pit and also towards
457 the plasma membrane.

458 Based on electron micrographs, it has been proposed that actin filaments envelope
459 clathrin coats asymmetrically in mammalian cells (Collins et al. 2011). We wanted to evaluate
460 whether higher symmetry around the clathrin coat might also contribute to higher force
461 generation. Therefore, we determined the position of the maximum actin signal for normalized x-
462 histogram intensity from x-z and y-z STORM image projections and calculated the distance of
463 the signal's maximum from the clathrin coat's center (Fig. 7 H, Fig. S5 G, see Materials and
464 Methods). Early stage coats under isotonic media conditions showed a wide distribution of actin
465 signal deviation from the center with a mean of 99.2 ± 51 nm (SD). Actin symmetry increased

466 for intermediate clathrin coats and coats undergoing membrane fission (intermediate clathrin
467 coat mean 84.1 nm ± 49 nm (SD); membrane fission clathrin coat mean 73.6 nm ± 49 nm (SD)).
468 Interestingly, late clathrin coats after fission showed a more asymmetric actin organization
469 (Mean 84.7 ± 52 nm (SD)), again. These data indicate that actin polymerization at the clathrin
470 coat's base starts off asymmetrical and reaches the highest symmetry at membrane fission. That
471 the symmetric actin organization was decreased in the late clathrin coats after membrane fission
472 might indicate a rearrangement of actin organization after vesicle scission, perhaps to propel the
473 released vesicle into and through the cytoplasm.

474 We observed that under hypotonic media treatment, compared to isotonic conditions,
475 actin networks grew more symmetrically even around the early coats (72.4 nm ± 44 nm (SD).
476 This parameter did not change appreciably during CME progression (Fig. 7 I and Fig. S5 H).

477 Overall, these observations lead us to conclude that actin grows higher in the z-dimension
478 at clathrin coats under elevated plasma membrane tension, the actin networks are more
479 symmetric and they achieve higher clathrin coat coverage. Such an adaptive mechanism for actin
480 organization presumably generates the required forces to ensure efficient progression of
481 mammalian CME under varying membrane tensions.

482

483 **Discussion**

484 By combining two-color, three-dimensional STORM imaging, quantitative live-cell TIRF
485 microscopy, and membrane tension measurements by AFM membrane tether pulling
486 experiments, we showed that actin organization and clathrin coat area adapt to membrane tension
487 changes at individual CME sites. This mechanism likely generates forces necessary for robust
488 endocytic progression over a range of membrane tension regimes. While STORM that we

applied to resolve individual CCSs cannot attain the resolution of EM, our approach had several advantages that allowed us to gain these new mechanistic insights; (1) it allowed us to sample much larger numbers of CME sites than is possible by EM, (2) we imaged the CME sites in intact cells that had not been subjected to unroofing or extraction protocols, and (3) we were able to use antibodies and fluorescent phalloidin to unambiguously identify specific proteins at CME sites.

**Actin assembly and organization adapt to elevated membrane tension**

We and others showed that elevating membrane tension can have a dramatic impact on CME dynamic progression in mammalian cells (Raucher and Sheetz 1999; Boulant et al. 2011; J. P. Ferguson et al. 2016, 2017; Willy et al. 2017; Bucher et al. 2018). Our results provide mechanistic insights into how the CME machinery adapts to elevated membrane tension to maintain robust CME progression. We showed that actin began to polymerize and that the amount of assembly and network organization adapted to membrane tension for three membrane tension regimes as defined by Hassinger et al. (2017) and now refined by our data obtained from measuring membrane tension of SK-MEL-2 cells by AFM membrane tether pulling (Fig. 8). It is important to note that these tension regimes do not necessarily apply to distinct cells. Since we detect differences in actin organization at individual CME sites within a single cell by 2c-3D STORM, these different membrane tension regimes might reflect local differences in tension within a single cell.

***Low membrane tension regime***

Clathrin coat assembly provides sufficient energy to bend the underlying plasma membrane into a full spherical shape when membrane tension is low (Fig. 8) (Saleem et al. 2015). We indeed found in our STORM data that 26% of clathrin coats are without actin in early,

intermediate and late CME stages. We further found that the clathrin coat area measured by STORM is on average larger when there is no associated actin. This observation is consistent with mathematical modeling, which indicates that an increased coat area can provide sufficient energy to bend the plasma membrane when membrane tension is low (0.002 pN/nm, (Hassinger et al. 2017)). Since 26% of the super-resolved clathrin coat structures were not associated with actin, we defined our low membrane tension regime as being below the tether force value of 27.5 pN This tether force value marks the upper border of 25% of the tether force values that are equal to and smaller than 27.5 pN in the plot in Figure 3 J for cells cultured in isotonic media. Assuming a 100 pN*nm bending rigidity of the plasma membrane, the calculated membrane tension for 27.5 pN is 0.096 pN/nm (Diz-Muñoz, Fletcher, and Weiner 2013). A small population of the super-resolved clathrin coats lacking associated actin are associated with dynamin2, which indicated that they undergo membrane neck formation and, presumably, scission. Interestingly, the CME sites that did not have detectable actin generally showed a very low actin cortex density in the x-y STORM image projections (Fig. S4 A and B). The lack of an actin cortex in those regions might reflect a lower membrane tension and therefore lack of a need for actin assembly in this membrane tension regime.

***Intermediate tension regime***

When membrane tension was elevated to an intermediate level, CME lifetimes slowed. 74% of super-resolved clathrin coats were associated with actin. When we observed clathrin coats in the late endocytic stage, 25% showed an actin coverage below 50% (Fig. 2 B). Therefore, we define the intermediate tension regime as being between the low membrane tension regime 0.096 pN/nm defined above and the measured average membrane tether force of 33 pN under isotonic media condition, resulting in a calculated membrane tension of 0.14 pN/nm

(when using a bending rigidity of 100 pN*nm). Clathrin coat assembly and membrane curvature inducing proteins still appear to provide sufficient energy to drive the shallow to U-shaped clathrin coat transition (Fig. 8, intermediate membrane tension). In addition, when we inhibited Arp2/3-mediated actin polymerization using CK666, clathrin coat progression stalled at the U-shaped stage, consistent with the effects of actin assembly inhibition in other cell types (Yarar, Waterman-Storer, and Schmid 2005; Boulant et al. 2011; Almeida-Souza et al. 2018). Thus, at intermediate membrane tension it appears that actin force generation is primarily required for the U-shaped to omega-shaped clathrin coat transition. The actin network organization at the base of the pit is well positioned to drive plasma membrane neck constriction and scission by generating forces orthogonal to the direction of membrane invagination.

***High membrane tension regime***

Our STORM observations indicate that as membrane tension is elevated further, actin force generation increasingly becomes required to drive coat deformation and membrane invagination during the early stages of CME (Fig. 8). We found that for 70% of early endocytic sites under isotonic conditions, actin covered over 50% of the coat surface area (Fig. 2 B). This higher membrane tension regime presumably starts above our measured mean calculated membrane tension of 0.14 pN/nm. When we inhibited Arp2/3-mediated actin polymerization using CK666 under high membrane tension, we found that coat deformation slows in the shallow coat regime. Actin assembly from the base of the CCP continues until the network covers the clathrin coat completely, allowing it to interact with proteins linking the actin network to the clathrin coat (Engqvist-Goldstein et al. 2001; Sochacki et al. 2017; Messa et al. 2014). Actin-binding linker proteins such as Hip1R and epsin1 cover the clathrin coat completely where they

557 are positioned to provide high membrane internalization efficiency by harnessing actin assembly
558 forces perpendicular to the plasma membrane (Akamatsu et al. 2020).

559 When the actin network fully covers the clathrin coat it resembled the radial organization
560 described by mathematical modeling for mammalian CME and the actin network organization
561 described for budding yeast (S. M. Ferguson et al. 2009; Hassinger et al. 2017; Mund et al. 2018;
562 Akamatsu et al. 2020). In yeast this actin organization drives endocytic membrane invagination
563 against the high resistance resulting from turgor pressure. Mathematical modeling showed that
564 this organization provides high forces perpendicular to the plasma membrane (Hassinger et al.
565 2017). Actin-generated forces parallel and orthogonal to the membrane invagination at high
566 tension may coexist to drive membrane invagination first and then scission.

567 Under high hypotonic media conditions such as 75 mOsm, we measured a mean tether
568 force of 48 pN that results in a mean membrane tension of 0.29 pN/nm (using 100 pN*nm as
569 bending rigidity for the calculation). CME dynamics dramatically slowed down when cells were
570 in this high membrane tension regime resulting in only ~40% of endocytic lifetimes that were
571 shorter than 50 sec and ~19% were longer than the 4.8 min movies we captured (Fig. 3 C). We
572 suggest here that we observed the limits of this adaptive mechanism that could have potentially
573 severe impact on dysregulation of the endocytic process.

574 **Clathrin coat surface area also responds to membrane tension and might contribute**
575 **energetically to membrane deformation**

576 Clathrin coat assembly and growth in area contribute energetically to membrane bending
577 (Saleem et al. 2015; Hassinger et al. 2017). In agreement with theoretical modeling and in vitro
578 findings, our STORM data revealed that the average clathrin coat surface area increased with

579 increasing membrane tension. Hence the clathrin coat itself might also be part of the adaptive
580 mechanism that responds to changes in membrane tension.

581 Mathematical modeling shows that under low membrane tension (0.002 pN/nm) an
582 average coat area increase of 9000 $nm^2$ can drive step-wise membrane remodeling from flat to
583 U-shaped and U-shaped to membrane neck closure (Hassinger et al. 2017). However, under the
584 model's defined high membrane tension (0.2 pN/nm), a surface of 28000 $nm^2$ alone does not
585 provide sufficient energy to achieve a flat to curved membrane transition. We and others
586 observed that the average clathrin coat surface area increased under high membrane tension
587 caused by hypotonic media conditions and thus appears to contribute energy for membrane
588 bending (Willy et al. 2019). In addition, we and others also found coat surface areas were larger
589 than the 28 000 $nm^2$ used in mathematical modeling studies, even under isotonic media
590 conditions when our SK-MEL-2 cells are at their equilibrated membrane tension of 0.14 pN/nm
591 (Avinoam et al. 2015; Bucher et al. 2018). Therefore, we speculate that when larger clathrin coat
592 surface areas are observed, the cells being observed might be experiencing either global or local
593 regions of higher membrane tension (> 0.14 pN/nm).

594 Interestingly, we measured the same average growth in clathrin coat surface area in cells
595 as was predicted by mathematical modeling to be necessary to drive the development of
596 membrane curvature. Under isotonic media conditions we detected an average growth of coat
597 surface area of 8993 $nm^2$ without actin and 6334 $nm^2$ when actin was present from the early into
598 the intermediate stage (Hassinger et al. 2017; Bucher et al. 2018). This observation can be
599 explained if actin compensates for membrane bending energy loss when there is less coat
600 growth. As has previously been reported for the same SK-MEL-2 cells that we use in our study,

601 the surface coat area stays constant throughout the remaining endocytic stages under isotonic
602 media conditions (Avinoam et al. 2015).

603 When we elevated membrane tension to 0.29 pN/nm by hypotonic media treatment, we
604 observed on average a larger surface area by approx. 7600 $nm^2$ in the early endocytic stage. At
605 the beginning of membrane neck formation this difference in surface area under elevated
606 membrane tension increased on average to approx. 10700 $nm^2$. These coat surface area
607 differences under elevated membrane tension appeared independent of actin. This observation
608 suggests that the increase in coat area contributes membrane bending energy to compensate for
609 increases in plasma membrane tension, as supported by the results reported here and elsewhere
610 (Willy et al. 2019).

611 **N-WASP spatial organization suggests an actin force generation control mechanism**

612 N-WASP spatial organization at CCSs and CCPs provides valuable mechanistic insight
613 into how actin network assembly contributes to force generation during CME. We found that N-
614 WASP localized at the base of early and late clathrin-coated pits, where it likely interacts with
615 SH3 domain-containing proteins present at the endocytic membrane neck (Schöneberg et al.
616 2017; Sochacki et al. 2017; Almeida-Souza et al. 2018). This organization is similar to that of the
617 homologous nucleation promoting factor Las17 in budding yeast (Mund et al. 2018). Filaments
618 nucleated at the base of CCPs would be able to interact with coat proteins such as Hip1R and
619 Epsin1/2/3 to generate forces to invaginate the plasma membrane (Hassinger et al. 2017; Mund
620 et al. 2018; Akamatsu et al. 2020). N-WASP at the base of CCPs is expected to activate the
621 Arp2/3 complex to nucleate actin networks to generate forces both orthogonal and parallel to the
622 plasma membrane to drive invagination and membrane neck closure, respectively.

623 Intriguingly, we also sometimes observed a strikingly different N-WASP spatial
624 organization in which it was distributed over the full clathrin coat. The type II nucleation factors
625 Abp1 and cortactin bind to actin filaments and to the Arp2/3 complex and could serve as binding
626 partners for N-WASP when actin surrounds the clathrin-coated pit (Le Clainche et al. 2007;
627 Pinyol et al. 2007; Helgeson and Nolen 2013; Guo et al. 2018). Such an organization might
628 reflect a distinct mechanism of actin filament assembly from the coat that is potentially important
629 to generate higher forces when actin already surrounds the clathrin-coated pit.

630 The results presented here provide a new understanding of how actin functions in
631 mammalian clathrin-mediated endocytosis by adapting its ultrastructural organization to changes
632 in plasma membrane tension, thereby ensuring the progression of a flat clathrin-coat to a round
633 clathrin-coated vesicle. Clathrin-coat surface area also responds to these changes in plasma
634 membrane tension. Future studies will be required to determine how clathrin machinery
635 components contribute to this adaptive mechanism.

636

637 **Materials and Methods:**

638

639 **Cell culture**: SK-MEL-2 cells from clone Ti13 (hCLTA$^{EN-1}$ /hDNM2$^{EN-1}$) were cultured in
640 DMEM/F12 with GlutaMax™ supplement (10565-018, Thermo Fisher Scientific) media
641 containing 10% fetal bovine serum (FBS) and 1,000 U/mL penicillin-streptomycin mix
642 (15140122, Thermo Fisher Scientific) and kept in a 37°C humidified incubator with 5% CO2
643 (cell source information (Doyon et al. 2011)). After each cell vial was thawed, cells were
644 checked after 2 passages for mycoplasma contamination. Cell line authentication was performed
645 by short tandem repeat validation.

646

647 **Antibodies and reagents**: The primary antibodies used were mouse anti-clathrin light chain (AB
648 CON.1, MA5-11860, Thermo Fisher Scientific), mouse anti-clathrin heavy chain (AB X-22,

649 MA1-065, Thermo Fisher Scientific) and rabbit anti-N-WASP (ab126626, Abcam). The
650 secondary antibodies used were Alexa Fluorophore 647 chicken anti-rabbit (A21443, Thermo
651 Fischer Scientific), goat anti-mouse (115-005-205, Jackson ImmunoResearch) conjugated to
652 CF680-NHS ester (Biotium 92139). Reagents and small molecule inhibitors used were DMSO
653 (D2650, Sigma Aldrich), CK666 (SML0006, batch # 0000012761, Sigma Aldrich) and
654 Phalloidin -AF647 (A22287, Fisher Scientific).
655
656 **Preparation of CF680-labeled secondary goat anti-mouse antibody**: CF680 NHS ester was
657 dissolved at a concentration of 3 mM in anhydrous DMSO. 1 µL of dye solution, 80 µL of a 1.25
658 mg/mL suspension of unlabeled goat anti-mouse IgG1 secondary antibody (115-005-205,
659 Jackson ImmunoResearch Laboratories, Inc.), and 10 µL of 1M sodium bicarbonate solution
660 were mixed and allowed to react for 15 min at room temperature. The reaction mixture was
661 added to an equilibrated NAP-5 column (Sigma GE17-0853-01) and flushed with PBS. The dye
662 conjugated antibody was collected from the first colored eluent fraction and a concentration of
663 0.12mg/mL was determined with a NanoDrop spectrophotometer.
664
665 **Sample preparation for two-color clathrin and actin imaging**; 18 mm round coverslips were
666 cleaned 20 min in 70% ethanol (Electron Microscopy Science, Cat # 72222-01). Cells were
667 detached with 500uL 0.05% trypsin (25300-054, Gibco), washed once in DMEM/F12 and
668 collected by centrifugation. Cells were counted using a hemocytometer and 20,000 cells/mL
669 were seeded onto 18 mm round coverslips in 12-well plates. Cells were incubated for 16 – 24
670 hours in culture media prior to preparation for imaging.
671 Cells were fixed first for 1-2 min in 0.3% (v/v) glutaraldehyde (GA) solution containing
672 0.25% (v/v) Triton in cytoskeleton buffer (CB: 10mM MES, 150mM NaCl, 5mM EGTA, 5mM
673 Glucose, 5mM $MgCl_2$, 0.005% $NaN_3$, pH6.1) and then immediately fixed for 10 min in 2% (v/v)
674 GA solution in CB. Both solutions were prepared fresh from a 70% GA stock (Electron
675 Microscopy Science, cat #16365) (protocol follows reference (Xu, Babcock, & Zhuang, 2012)).
676 After fixation, samples were washed once in CB and then incubated for 7 min in freshly prepared
677 CB containing 0.1% (w/v) $NaBH_4$. Subsequently, samples were washed 3 times for 10 min in CB
678 with gentle agitation on a shaker. Samples were then blocked for 30 min in 5% (w/v) BSA in CB
679 (Sigma Aldrich, A3733). For dense clathrin labeling, light (diluted 1:200) and heavy chain

680 (diluted 1:200) antibodies were used together in a 1% (w/v) BSA CB solution. Primary antibody
681 immunostaining was performed overnight at 4°C. On the next day, samples were washed twice in
682 1% (w/v) BSA CB for 5 min. The mouse secondary antibody-CF680 was used at a final
683 concentration of 0.40 µg/mL – 0.60 µg/mL in a 1% BSA - 1x CB solution. Samples were stained
684 for 30 min at room temperature in the dark and washed twice for 5 min in 1% (w/v) BSA CB
685 solution, and then for 10 min in CB solution. Samples were then placed into a solution of CB
686 containing 0.5µM Phalloidin-AF647 and kept at room temperature in the dark for a minimum of
687 2 hours. Samples were washed once with PBS before STORM imaging.
688
689 **Sample preparation for single-color clathrin and dual-color N-WASP imaging**: Cells were
690 prepared as for the two-color sample preparation on coverslips, and then fixed for 20 minutes in
691 3% (v/v) paraformaldehyde (PFA, 15710 Electron Microscopy Sciences) in CB (protocol follows
692 (Li et al. 2018)). Samples were washed quickly in CB and subsequently were incubated for 7 min
693 in freshly prepared 0.1% (w/v) $NaBH_4$ in CB solution. Subsequently, samples were washed 3
694 times for 10 min in CB with gentle agitation on a shaker and permeabilized afterwards in a 0.1%
695 Triton-PBS solution for 1-2 min. For single antibody clathrin staining, subsequent washing,
696 blocking and antibody incubation steps were similar to the two-color clathrin and actin sample
697 preparation protocol.
698 Dual-color immunolabeling was performed with primary antibody against N-WASP
699 (diluted 1:200), clathrin heavy and clathrin light chain (diluted 1:600 -1:1000) in 1% (w/v) BSA
700 in PBS over night at 4°C. Samples were washed the next day twice for 5 min in 1% (w/v) BSA in
701 PBS. Secondary antibody staining was first performed with Alexa Fluorophore 647 anti-rabbit
702 antibody (diluted 1:200) in 1% BSA (w/v) in PBS for 30 min at room temperature and kept in the
703 dark. After two 10 min long washes in PBS containing 1% (w/v) BSA, secondary antibody
704 staining was performed with CF680 anti-mouse antibody (diluted 1:600). The samples were
705 given three final washes in PBS for 10 min each.
706
707 **SRM imaging**: Dye-labeled cell samples were mounted on glass slides in a standard
708 STORM imaging buffer consisting of 5% (w/v) glucose, 100 mM cysteamine, 0.8 mg/mL
709 glucose oxidase, and 40 µg/mL catalase in 1M Tris- HCL (pH 7.5) (Huang et al, 2008; Rust et al,
710 2006). Coverslips were sealed using Cytoseal 60. STORM imaging was performed on a

711 homebuilt setup (Wojcik et al. 2015) based on a modified Nikon Eclipse Ti-U inverted
712 fluorescence microscope using a Nikon CFI Plan Apo λ 100x oil immersion objective (NA 1.45).
713 Dye molecules were photoswitched to the dark state and imaged using a 647-nm laser (MPB
714 Communications); this laser was passed through an acousto-optic tunable filter and introduced
715 through an optical fiber into the back focal plane of the microscope and onto the sample at an
716 intensity of ~2 kW $cm^2$. A translation stage was used to shift the laser beam toward the edge of
717 the objective so the light reached the sample at incident angles slightly smaller than the critical
718 angle of the glass-water interface. A 405-nm laser was used concurrently with the 647-nm laser
719 to reactivate fluorophores into the emitting state. The power of the 405-nm laser (typical range 0-
720 1 W $cm^{-2}$) was adjusted during image acquisition so that at any given instant, only a small,
721 optically resolvable fraction of the fluorophores in the sample was in the emitting state. For 3D
722 STORM imaging, a cylindrical lens was inserted into the imaging path so that images of single
723 molecules were elongated in opposite directions for molecules on the proximal and distal sides of
724 the focal plane (Huang et al. 2008). The raw STORM data were analyzed according to
725 previously described methods (Rust, Bates, and Zhuang 2006; Huang et al. 2008). Data were
726 collected at a frame rate of 110 Hz for a total of ~80,000 frames per image. Single and two-color
727 imaging was performed on cells labeled with Alexa Fluor 647 only or Alexa Fluor 647 and
728 CF680 with 647-nm excitation based on a ratiometric detection scheme (Bossi et al. 2008; Testa
729 et al. 2010; Gorur et al. 2017). In the two-color imaging scheme, light emitted from the AF647
730 and CF680 fluorophores was collected concurrently and split into two light paths using a long
731 pass dichroic mirror (T685lpxr; Chroma). Each light path was projected onto one half of an
732 Andor iXon Ultra 897 EM-CCD camera. Dye assignment was performed by localizing and
733 recording the intensity of each single molecule in each channel. Conventional imaging of 560-
734 and 488-nm dyes was performed immediately prior to STORM imaging using the appropriate
735 laser and filter set. Emission data were collected through the short wavelength reflected path of
736 the aforementioned optical setup and overlaid directly onto the final STORM image.
737
738 **Selection of clathrin-coated super-resolved structures for image analysis**: Clathrin-
739 containing structures were extracted from processed STORM images using a custom MATLAB
740 routine. Briefly, a kernel convolution with a disk of ~80 nm radius was performed on the super-
741 resolved clathrin image channel to accentuate possible clathrin-coated pits. The resulting image

742 was median filtered, and peaks were detected by sequentially identifying and cropping out
743 regions corresponding to the local image maxima. 310 nm x 310 nm wide square regions
744 centered at these peaks were cropped from all color channels and aligned. Subsequently, we
745 selected super-resolved clathrin coats by visual inspection for further quantitative image analysis
746 based on the following criteria: We excluded clathrin labeled structures for image analysis that
747 looked deformed, that covered almost the entire 310 nm x 310 nm wide square, or that contained
748 small punctuated clathrin structures which were not distinguishable from background noise or
749 small clathrin seeds (Fig. S1 A). Clathrin-coated structures selected for analysis were first
750 inspected to determine whether they appear round-shaped, elliptical-shaped or triangle-shaped in
751 the x-y projected super-resolved image. These images were 3D rendered to determine whether
752 the x-z projection resulted in the typical wider-crescent shape, U-shape or round-shape
753 appearance of the clathrin coat. If we could identify the stereotypical clathrin-coat shapes in both
754 projections, we included the clathrin-coat in the pool for further image analysis. We then
755 classified clathrin coats by manual inspection of DNM2-eGFP$^{EN}$ fluorescence signal intensity
756 into DNM2 negative or positive: When the 3x3 pixel kernel aligned with the super-resolved
757 clathrin-coat was at the same or lower intensity relative to the surrounding pixels, the coat was
758 classified as DNM2 negative (Fig. S4 A and C). When the 3x3 pixel kernel showed a very bright
759 middle and/or a bright pixel in the kernel aligned with the clathrin-coat, the coat was classified as
760 DNM2 positive (Fig. S4 B and D). Second, we assigned the clathrin-coats as being actin negative
761 when the number of localizations in the actin channel in the 310 nm x 310 nm ROI was below
762 50, since we could not distinguish whether these localizations came from background or
763 emanated from actin (Fig. S4 A and B). Clathrin-coated structures were classified as actin
764 positive when the number of localizations in the actin channel in the 310 nm x 310 nm ROI were
765 above 50 and actin signal overlapped with the clathrin signal.
766
767 **STORM image data display in figures**: Reconstructed super-resolution images were visualized
768 using the "insight" software package and saved in a 'png' file format (Huang et al. 2008). These
769 images were loaded into ImageJ, converted from an RGB image into an 8 bit image,
770 pseudocolored for clathrin = red hot, actin = cyan, and then converted back into an RGB image.
771

**Image analysis of reconstructed clathrin and actin side views**: Selected reconstructed super-resolved images of clathrin and actin were treated as digital images for further analysis. A custom-written Jupyter notebook script was used to project reconstructed side views of clathrin and actin onto their x- and z-axis to obtain histograms of the clathrin and actin pixel intensity signals normalized to their respective maximum intensity value (Fig. S1 B and C). From these normalized z-axis and x-axis pixel intensity histograms we read out the height and width of clathrin and actin at the $30^{th}$ percentile. This process resulted in more robust results than the read out at the full width at half maximum (Fig. S1 B and C).

*Actin and clathrin coat height:* The z-axis intensity histograms were used to report the actin and clathrin coat heights in the x-z projections. Before extracting the values, a mean filter (50) was run over the histograms to reduce signal fluctuations that interfere with the size measurement (Fig. S1 B and C).

*Actin-clathrin overlap calculation:* We calculated the total overlap between clathrin and actin using the indices obtained at the upper position of clathrin and lower position of actin at the $30^{th}$ percentile of the respective z - axis projected intensity histograms (Fig. S1 B). We then reported the overlap relative to the clathrin height in units of percentage.

*Clathrin-coat width:* We used the $30^{th}$ percentile of x-axis intensity histogram to report the clathrin coat width in the x-z projection. Before extracting the values, a median filter (100) was run over the histogram to smooth out signal fluctuations that interfere with the correct size determination (Fig. S1 B and C).

*Shape index calculation:* Shape indices of individual clathrin structures are defined as the ratio between clathrin coat height and clathrin coat width. This value allowed us to sort flat (low shape index) and rounded (high shape index) clathrin coats from each other.

*Asymmetry of actin signal around clathrin coat:* To evaluate the asymmetry of the spatial actin organization around the clathrin coat, we determined the difference in the positions of the peak actin and clathrin signals on both x-z and y-z projections of our images (Fig. S5 G). We obtained the center of the clathrin coat in nm by rendering the clathrin super-resolved image into a diffraction limited image, as explained in paragraph 'Selection of clathrin-coated super-resolved structures for image analysis'. We first identified the position of the actin maximum intensity in the x-z projection profile and y-z projection profile in nm. Then we measured the distance of these obtained position to the middle position of the clathrin intensity profile. This distance

measurement is proportional to the asymmetry of the actin position with respect to clathrin; namely a low distance corresponds to high symmetry and a high distance corresponds to high asymmetry in the position of the actin signal.

*Clathrin coat surface area:* We determined the width of clathrin coats in x and y and height in z from the x-z and y-z projections of the reconstructed, super-resolved images. x, y and z values were then used to calculate the coat surface area with the formula of a half ellipsoid by Knud Thompson's approximation:

$$A = \frac{4 \times \pi \times \left(\frac{\left((z \times x)^{1,6075} + (z \times y)^{1,6075} + (x \times y)^{1,6075}\right)}{3}\right)^{\frac{1}{1,6075}}}{2}.$$

**Total internal reflection fluorescence (TIRF) microscopy**:TIRF imaging was carried out on a Nikon Eclipse Ti2 inverted microscope with a CFI60 60x Apo TIRF objective and a Hamamatsu Orca-Flash 4.0 V2 sCMOS camera. eGFP and Tag.RFP-T fluorescence were excited using 488 nm and 561 nm lasers and detected using a Chroma HC TIRF Quad Dichroic (C-FL TIRF Ultra Hi S/N 405/488/561/638) and Chroma HC Quad emission filters BP 525/50 and BP600/50, respectively (Bellows Falls, VT). Unless mentioned specifically, channels were acquired sequentially at a 1.2 sec interval and 400ms exposure time over 4.8 minutes to 6 minutes. Real-time acquisition was achieved by a National Instruments (PXI 1033, Austin, TX) controller. The system was controlled with NIS-Elements software and maintained at 37°C by an OkoLab environmental chamber (Burlingame, CA).

**Hypo-osmotic media treatment**: SK-MEL-2 cells were plated on glass coverslips one day prior to osmotic treatment and imaging: 20,000 cells/ mL were seeded 16h – 24h prior to the experiment on 25 mm round #1.5 glass coverslips that had been cleaned with 70% ethanol (Warner Instruments, 64-0715). Isotonic imaging media contained Dublbecco's Modified Essential Medium and Ham's F-12 medium (DMEM/F12) without phenol red (11039, Thermo Fisher Scientific) with 5% v/v FBS. The media was diluted with an inorganic salt solution containing 10mM $CaCl_2$, 0.3mM $MgCl_2$ and 0.1mM $MgSO_4$ (CMM) to maintain concentrations of critical ions, while obtaining hypo-osmotic conditions by diluting the media containing components such as D-Glucose. 225 mOsm hypotonic imaging media contained 1:4 v/v CMM solution in DMEM/F12, 150 mOsm hypotonic imaging media contained 1:1 v/v CMM solution

832 in DMEM/F12, and 75 mOsm hypotonic imaging media contained 4:1 v/v CMM solution in
833 DMEM/F12. 5% v/v FBS was present in all hypotonic solutions.
834 Images of CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$ fluorescence in SK-MEL-2 cells were
835 acquired first in isotonic media over a course of 4.8 minutes. Subsequently, media was
836 exchanged on the stage to hypotonic media (either 225 mOsm, 150 mOsm or 75 mOsm) and
837 movies were acquired for 4.8 minutes, starting 2 minutes and 10 minutes after media exchange.
838 Media exchange on the stage did not affect CME initiation rates or fluorescence lifetimes beyond
839 the existing experimental intrinsic variability (Fig. S3 A).
840
841 **CK666 concentration titration**: 20,000 SK-MEL-2 cells/ mL were seeded in 8 well chambers
842 16h – 24h prior to the experiment (80826, ibidi, Fitchburg, WC). A CK666 (SML0006, batch #
843 0000012761, Sigma Aldrich) stock solution was prepared at 50mM in DMSO and kept at -20 $^{o}$C.
844 25μM, 50μM and 100 μM CK666 and equivalent 0.5% v/v DMSO, 1% v/v DMSO and 2%
845 DMSO v/v solutions for controls were prepared fresh in DMEM/F12 containing 5% FBS and
846 kept at 37°C until used. Cells were first imaged in DMEM/F12 containing 5% FBS solution as a
847 baseline control for 4.8 minutes. Subsequently, imaging solution was exchanged on the
848 microscopy stage to CK666 or DMSO containing imaging solution and another 4.8-minute
849 movie was acquired after 2 minutes of treatment. Each treatment was repeated twice and an area
850 of 1024 pixel x 1024 pixel was used to record 3-6 cells per experiment.
851
852 **CK666 in combination with hypo-osmotic media**: Cells were prepared as for the CK666
853 concentration titration experiment described above. Solutions of 2% v/v DMSO in DMEM/F12,
854 100 μM CK666 in DMEM/F12, 2% v/v DMSO in 1:1 v/v CMM solution in DMEM/F12 (150
855 mOsm hypotonic media) and 100 μM CK666 1:1 v/v CMM solution in DMEM/F12 (150 mOsm
856 hypotonic media) were prepared fresh and kept at 37°C until used. All solutions contained 5%
857 FBS. Cells were first imaged in DMEM/F12-5% FBS solution as a baseline control for 6
858 minutes. Subsequently, the imaging solution was exchanged on the microscopy stage to the
859 desired experimental solutions and a 6 minute movie was recoded after 4 minutes of incubation.
860
861 **Image analysis of TIRF live-cell microscopy data:** Fluorescent diffraction-limited spots of
862 DNM2-eGFP$^{EN}$ and CLTA-TagRFP-T$^{EN}$ in SK-MEL-2 cells were tracked in each channel over

863 the course of the movie using the detection and tracking software feature of the cmeAnalysis
864 software package in Matlab (Aguet et al. 2013). Subsequently, we cleaned up the background
865 signal and, based on the x and y positions of associated CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$
866 fluorescent tracks, separated them from CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$ that were not
867 associated with each other, using a custom-written Matlab script (Hong, Cortesio, and Drubin
868 2015; Ferguson et al. 2016; Dambournet et al. 2018). We obtained fluorescence lifetimes for
869 DNM2-eGFP$^{EN}$ and CLTA-TagRFP-T$^{EN}$ tracks that were associated and not associated with each
870 other and that appeared and disappeared within the duration of a movie. We classified the tracks
871 as "persistent" when they were already present in the first movie frame and lasted longer than the
872 movie.
873
874 **CME initiation rate and completion rate measurement:** We classified tracks as "complete"
875 when they appeared and disappeared over the course of the movie. When we classified tracks as
876 "initiated," they appeared within the course of the movies, excluding in the first frame, and lasted
877 longer than the movie. Initiated tracks include the number of complete tracks, as well. To
878 calculate rates, we used the binary cell mask image that was generated by the CME analysis
879 program in Matlab that highlights the cell area in which particle tracking was performed. ImageJ
880 was then used to calculate the area of the cell from the binary mask image.
881
882 **Tether pulling experiments using Atomic Force Microscopy:** Custom-cut 35-mm glass-
883 bottom dishes (Greiner Bio-One, #627860) were coated with fibronectin (50 ug/mL, Corning
884 #356008) for 30 minutes and washed with DPBS shortly before use. SK-MEL-2 cells were
885 seeded at a density of 0.15-0.20x$10^5$ cells/ml in DMEM/F12 GlutaMax™ supplement media
886 with 1% FBS and penicillin-streptomycin mix (Gibco™, #15140-122) in a 37°C humid
887 incubator with 5% $CO^2$ for 2-4 hours, and used directly for membrane tether pulling
888 experiments. OBL-10 cantilevers (Bruker) were mounted on a CellHesion 200 AFM (Bruker)
889 integrated into an Eclipse Ti inverted light microscope (Nikon), calibrated using thermal noise
890 method and coated with 2.5 mg/ml Concanavalin A (C5275, Sigma) for 1 hour at 30°C. After
891 rinsing the cantilever with DPBS, it was positioned at any location over the cell for tether pulling
892 using brightfield imaging. Approach velocity was set to 1 μm/s, contact force to 100–300 pN,
893 contact time to 300 ms–10 s, and retraction speed to 10 μm/s. After a 10 μm tether was pulled,

the cantilever position was held constant until the moment of tether breakage and at least 2 seconds afterwards. Sampling rate was set to 2000 Hz. After measurements of tether forces in control conditions, an inorganic salt solution containing 10mM $CaCl_2$, 0.3mM $MgCl_2$ and 0.1mM $MgSO_4$ was added to the medium (4:1 v/v) to achieve 75 mOsm hypotonic treatment. Tether forces were measured after media dilution for 2-16 minutes. Tether forces per cell are the average of at least 3 tethers. Cells were not used longer that 1 h for data acquisition. Force-time curves analysis was performed using the JPKSPM Data Processing Software.

**Data analysis, statistical analysis and data plotting:** For statistical analysis and data plotting, Prism version 7.0e and Jupyter notebook 5.5.0 were used.

**Online supplemental material**: Fig. S1 gives an overview of clathrin structure selection and parameter extraction of super-resolved clathrin and actin. Representative x-y STORM image projections are shown of super-resolved clathrin, actin and NWASP, corresponding to the images in Fig. 2. In Fig. S2 we present additional data from live cell imaging of SK-MEL-2 cells endogenously expressing CLTA-TagRFP-$T^{EN}$ and DNM2-$eGFP^{EN}$ in hypotonic and isotonic control media. Fig. S3 establishes the CK666 concentration affecting CME in SK-MEL-2 cells and provides additional data on CK666 and concomitant hypotonic media treatment. Fig. S4 shows x-y STORM projections of actin and clathrin with or without a DNM2-$eGFP^{EN}$ diffraction limited fluorescence signal corresponding to x-z projections in Fig. 5. The table shows the statistical difference between clathrin coat surface area when cells are in isotonic vs. 75 mOsm hypotonic media. Fig. S5 provides additional data on the effect of hypotonic media treatment on actin organization and how actin asymmetry was quantified.

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**Author contributions**

C. Kaplan and D.G. Drubin conceived the study and experiments. C. Kaplan performed live cell data acquisition, SRM data analysis and live cell data analysis. Sam J. Kenny, Shirley Chen and Ke Xu performed SRM, super-resolution data reconstruction and supervised SRM imaging. Johannes Schöneberg supported the SRM data analysis. Ewa M. Sitarska and Alba Diz-Muñoz performed membrane tether pulling experiments by atomic force microscopy, data analysis and supervised AFM tether pulling experiments. C. Kaplan and D.G. Drubin wrote the manuscript with feedbacks from other authors.

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Abbreviations:

CME Clathrin-mediated endocytosis
CCV Clathrin coated vesicles
CCS clathrin coated structure
CCP Clathrin coated pits
SI shape index

## Figure legends

**Figure 1: Simultaneous two-color, three-dimensional stochastic optical reconstruction microscopy (2c-3D STORM) resolves clathrin structures highly connected to the actin cortex. (A)** Current model of actin (blue) organization at clathrin structures (red) based on platinum-replica EM data. The schematic shows clathrin coat progression from a shallow membrane invagination to a fully formed membrane vesicle and organization of the associated actin network. Perspectives representing the x-y projection (top profile) and the x-z projection (side profile) are shown. **(B)** STORM image of clathrin coats on the ventral surface of an SK-MEL-2 cell immunolabeled with the CF-680 antibody. **(C)** STORM field corresponding to (B) showing the actin cytoskeleton labeled with phalloidin-AF647. **(D)** Merged clathrin and actin STORM images from (B) and (C). Orange squares are areas shown in Figure 1E. **(B) – (D)** Scale bars: 5 μm. **(E)** Magnification of highlighted areas 1 and 2 in Figure 1D. STORM images of clathrin coats (left) and clathrin-coats merged with actin (right). Orange squares identify clathrin coats of interest. Scale bars are 250 nm. **(F)** STORM images of clathrin coats and actin showing the x-z projection generated by three-dimensional rendering of insets 1 and 2 from (E). The white dashed line shows the orientation to the plasma membrane. Color code scale bar shows the height in the z-dimension for actin. Scale bars: 100 nm.

**Figure 2: The actin network is nucleated from the base of endocytic pits and its assembly is not coupled to clathrin coat progression. (A**) Left schematic shows range of shape indexes (SI) calculated from height to width ratios that classify the endocytic stages of clathrin coats (red) into early (SI < 0.7), intermediate (0.7 < SI > 0.9) and late (0.9 < SI). STORM images show x-z projections of representative clathrin-coats (red) with different actin (cyan) coverages of clathrin.

The SI for each clathrin coat is given in the lower right corner of each panel. (**B)** Shape index of clathrin coats from all three endocytic stages in (A) is plotted against the percentage of clathrin covered by actin (actin coverage >0.05%). The percentage of clathrin coats with below 50% actin coverage and above 50% of actin coverage is shown in the upper left and right corner. **(C)** The schematic shows the clathrin coat in the side profile and actin either at the tip, covering the clathrin coat completely, or at the base of the clathrin coat. The dashed black lines indicate the average position of actin and clathrin in the z-dimension. "$D_z$" represents the resulting distance when subtracting the actin average from clathrin average position. Difference of average actin position to average clathrin position plotted against the percentage of actin covering clathrin (clathrin coverage >0.05%, n = 719). The inset predicts how the data plot would look when actin grows from the clathrin coat tip (red line) or when actin grows from the clathrin coat base. **(D**) - **(E)** Schematic of clathrin coat side profiles shows N-WASP localization. Merged STORM images show spatial localization of N-WASP-AF647 (rainbow) at clathrin coats (red) in x-z projections. **(D)** N-WASP is localized at the base of clathrin coats. **(E)** N-WASP is localized all over clathrin coats. All scale bars in (A), (D) and (E) are 100 nm.

**Figure 3: Clathrin-mediated endocytosis mechanosensitivity. (A)** Schematic shows actin organization in CME when cells are in isotonic media (left). Hypotonic media (right) causes water influx and stretches the cell membrane. However, how actin assembly at CME sites is affected was not known. **(B)** Kymographs show lifetimes of CLTA-TagRFP-$T^{EN}$ (magenta) and DNM2-$eGFP^{EN}$ (green) as viewed by total-internal reflection microscopy. Imaging was performed for 4.8 min. SK-MEL-2 cells were imaged in isotonic media, or 2 min or 10 min after hypotonic media exchange. **(C)** and **(D)** Normalized cumulative distribution fluorescence track

lifetime data are shown for CLTA-TagRFP-T$^{EN}$ associated with (C) or not associated with DNM2-eGFP$^{EN}$ (D). Control cumulative distribution curve is red, 2 min cumulative distribution curve is violet and 10 min cumulative distribution curve in orange. **(E)** and **(F)** Normalized cumulative distribution fluorescence track lifetime data for DNM2-eGFP$^{EN}$ associated with (E) or not associated with CLTA-TagRFP-T$^{EN}$ (F). Control cumulative distribution curve is light green, 2 min cumulative distribution curve is dark green and 10 min cumulative distribution curve in blue. **(C) – (F)** Experiment was repeated 4 times. Lifetimes were recorded in isotonic media (n = 8 cells) as well as 2 min (n = 8 cells) and 10 min (n = 9 cells) after 75 mOsm hypotonic media exchange. **(G)** Table shows the CLTA-TagRFP-T$^{EN}$ lifetimes when associated with or not associated with DNM2-eGFP$^{EN}$ and the corresponding statistics. **(H)** Table shows the DNM2-eGFP$^{EN}$ lifetimes when associated with or not associated with CLTA-TagRFP-T$^{EN}$ and the corresponding statistics. **(G)** and **(H)** The Kolmogorov-Smirnov statistical test was used. **(I)** Plots represent CME initiation rate for the lifetimes in (C), CME completion rate for the lifetimes in (C) and percentage of persistent tracks over the course of the movie for the lifetimes in (C). **(J)** Mean membrane tether values are plotted for measurements from cells in isotonic media (n = 18) or in 75 mOsm hypotonic media (n = 17). Mean values were obtained by pulling at least 3 tethers from each cell. Round symbols in the plotted 75 mOsm data represent mean tether values from 2 min to 10 min after hypotonic media exchange. Rectangular symbols represent mean tether values obtained between 10 min and 16 min after hypotonic media exchange. **(I)** and **(J)** Bars in plots show mean with SD. The Mann-Whitney statistical test was used.

**Figure 4: Arp2/3-mediated actin polymerization importance during CME increases under elevated membrane tension. (A)** Kymographs show dynamics of CLTA-TagRFP-T$^{EN}$

(magenta) and DNM2-eGFP$^{EN}$ (green) imaged by total-internal reflection microscopy for 6 min. SK-MEL-2 cells were imaged first in isotonic media. The media was then exchanged to either 150 mOsm hypotonic media, 100μM CK666 in isotonic media or 100μM CK666 in 150 mOsm hypotonic media, respectively. Images were collected 4 min after changing media. **(B)** Normalized cumulative fluorescence lifetime distribution data for CLTA-TagRFP-T$^{EN}$ associated with DNM2-eGFP$^{EN}$. Shown are the lifetime curves and statistics for cells cultured in isotonic media before exchange to the indicated experimental media conditions (T = 0). Color code shows the pretreatment control conditions for the treatment in (C). **(C)** Normalized fluorescence track lifetime cumulative distribution data for CLTA-TagRFP-T$^{EN}$ associated with DNM2-eGFP$^{EN}$ after exchange to media containing 2% v/v DMSO in isotonic media (yellow), 2% v/v DMSO in 150 mOsm hypotonic media (green), 150 mOsm hypotonic media (magenta), 100μM CK666 in both isotonic media (light blue) and 150 mOsm hypotonic media (dark blue) (T = 4 min after media exchange). (**D)** Normalized cumulative fluorescence lifetime distribution for pretreatment control data as in (B) except for DNM2-eGFP$^{EN}$ associated with CLTA-TagRFP-T$^{EN}$. **(E)** Normalized fluorescence lifetime cumulative distribution data as in (C) except for DNM2-eGFP$^{EN}$ associated with CLTA-TagRFP-T$^{EN}$ under the different treatment conditions. **(B)** – **(E)** Right tables show statistics for the normalized distribution functions. Kolmogorov-Smirnov statistical test was used. **(F)** Representative STORM images of immunolabeled clathrin-coated structures. Upper panel shows the x-y projections and lower panel the corresponding x-z projections. Scale bars: 100 nm. **(G)** Clathrin coat heights when cells where treated with only 2% v/v DMSO, 100μM CK666 in isotonic media and 100μM CK666 in 150 mOsm hypotonic media. Clathrin coat images for quantitative analysis were collected from at least 3 cells for each condition. Statistics are given in Figure S3 E. Mann-Whitney statistical test was used.

**Figure 5: Super-resolved clathrin coats organized into an endocytic timeline via their shape index, the DNM2-eGFP$^{EN}$ fluorescent signal and actin association. (A), (B), (D) and (E)** Schematics show side profiles of clathrin coats (red) and their association with DNM2 (green puncta) and actin (blue bar). Clathrin coats were quantified via their height to width ratio into early (SI < 0.9) and late (SI > 0.9) endocytic stages. Nine hundred and ninety-two clathrin coats were selected from 6 cells acquired in three independent dSTROM experiments to quantify their association with DNM2-eGFP$^{EN}$ and super-resolved actin. **(A)** The image panel shows examples of x-z STORM projection images of early (n = 85) and late (n = 118) clathrin coats without significant presence of DNM2-eGFP$^{EN}$ or actin. The white dashed line locates the plasma membrane. The image row below shows actin (cyan) in the AF647 channel. The third row of images shows the merged clathrin and actin STORM images. **(B)** Same organization of super-resolved clathrin, actin and merged image as in (A). These early (n = 31) and late (n = 22) clathrin coats are associated with DNM2-eGFP$^{EN}$, but not with actin. **(C)** Schematic of clathrin coats combines the information about coats without and with DNM2-eGFP$^{EN}$ from (A) and (B) and organizes a timeline of early coats without DNM2-eGFP$^{EN}$, intermediate coats with DNM2-eGFP$^{EN}$, coats undergoing membrane fission with DNM2-eGFP$^{EN}$ and late coats after fission without DNM2-eGFP$^{EN}$. **(D)** Same organization of super-resolved clathrin, actin and merged image as in (A). These early (n = 167) and late (n = 202) clathrin coats are not associated with DNM2-eGFP$^{EN}$, but are associated with actin. **(E)** Same organization of super-resolved clathrin, actin and merged image as in (A). These early (n = 221) and late (n = 156) clathrin coats are associated with DNM2-eGFP$^{EN}$ and actin. **(F)** Schematic of clathrin coats combines the information about coats with actin in combination without and with a DNM2-eGFP$^{EN}$ signal

from (D) and (E). Clathrin coats are organized into a timeline of being early in endocytosis without DNM2-eGFP$^{EN}$, intermediate in endocytosis with DNM2-eGFP$^{EN}$, in the membrane fission stage with DNM2-eGFP$^{EN}$ and being late after fission without DNM2-eGFP$^{EN}$.

**Figure 6: Effects of elevated membrane tension on clathrin coat surface area. (A) – (D)** Clathrin coat surface area is plotted in the early, intermediate, membrane fission and late endocytic stages for coats lacking actin under **(A)** isotonic media conditions, **(B)** lacking actin under 75 mOsm hypotonic media conditions, **(C)** with actin under isotonic media conditions, **(D)** and with actin under 75 mOsm hypotonic media conditions. **(A) – (D)** Bars show mean with standard deviation. Mann-Whitney statistical test was used.

**Figure 7: Actin organization at CME sites adapts to elevated membrane tension. (A)** Schematic shows actin organization at CME sites when cells reside in isotonic media (left cell) and after media is exchanged to hypotonic media (right cell). Hypotonic media causes water influx and stretches the cell membrane. As a consequence, the actin network grows higher, resulting in larger coverage of clathrin. **(B)** Plotted are actin heights in nm at clathrin coats measured from STORM x-z projections. Average actin height, SD, number of data points and p-values are listed in Table 1. **(C)** Percentage of clathrin covered by actin in STORM x-z projection images from the same dataset in (B) (actin coverage > 1% plotted). Average percentage, SD and number of data points are listed in Table 1. **(B) – (C)** Clathrin coats are classified as early (SI < 0.9) or late coats (SI > 0.9) based on their height to width ratio (SI) and as DNM2-eGFP$^{EN}$ positive or negative, and classified into early, intermediate, membrane fission and late stages. Bars show mean with standard deviation. Mann-Whitney statistical test was used

in (B). **(D) – (E)** Schematic shows adaptation of actin organization at early and late stage clathrin coats to hypotonic media treatment. MT = membrane tension. Corresponding representative STORM images show clathrin (red) and actin (cyan) in x-z projections. Event number in data set is given in the lower right corner. All scale bars are 100 nm. **(F) – (G)** Actin height in nm from (B) is plotted against clathrin coverage by actin from (C) in percentage from early and late clathrin coats. Grey dots and the grey regression line represent the data set from cells treated with isotonic media and black dots and black regression line represent the data set from cells treated with 75 mOsm media. **(H)** Schematic shows that the distance of the actin signal maximum (cyan) to the center of the clathrin coat (red) increases with asymmetric actin organization. Plot shows distances of the maximum actin signal from the clathrin coat's center in respective x-z and y-z projections. Early clathrin coats: N = 301, intermediate clathrin coats N = 353, clathrin coats at membrane fission N = 256, and late clathrin coats N = 402. **(I)** Schematic shows distance of the actin signal maximum to the center of the clathrin coat to represent asymmetric actin organization. Plot shows distances of the maximum actin signal from the clathrin coat's center in respective x-z projection and y-z projection under 75 mOsm hypotonic media. Mann-Whitney statistical test was used in **(B)**, **(H)** and **(I)**. **(B), (C), (F) – (H)** Actin heights at clathrin coats in isotonic media were analyzed from the same dataset as in Figure 2. Three independent dSTROM experiments with cells in isotonic (N = 6) and 75 mOsm hyptonic media (N = 7) were performed. **(B)**, **(C)**, **(H)** and **(I)** Bars in plot show mean and SD.

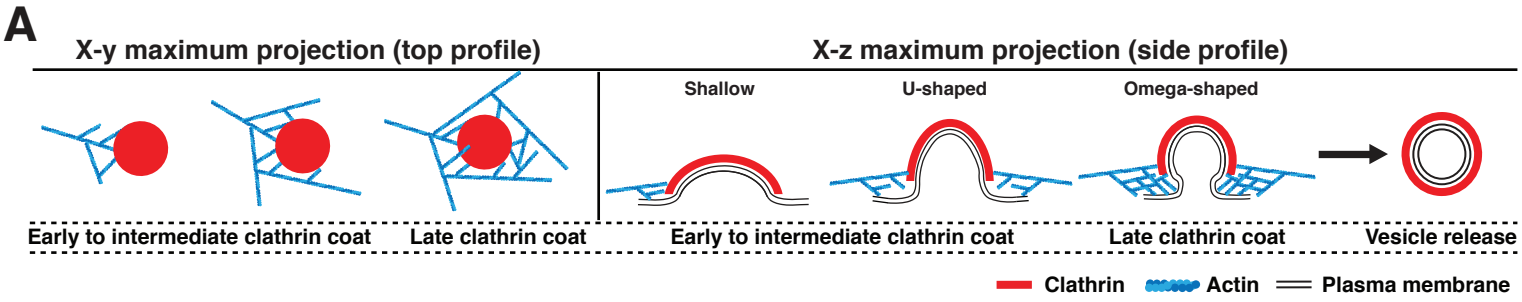
**Figure 8: Adaptive actin force generating mechanism counteracts elevated membrane tension to ensure robust CME progression.** The model proposes three scenarios in which membrane tension is low, intermediate and high, and how CME is accomplished efficiently by

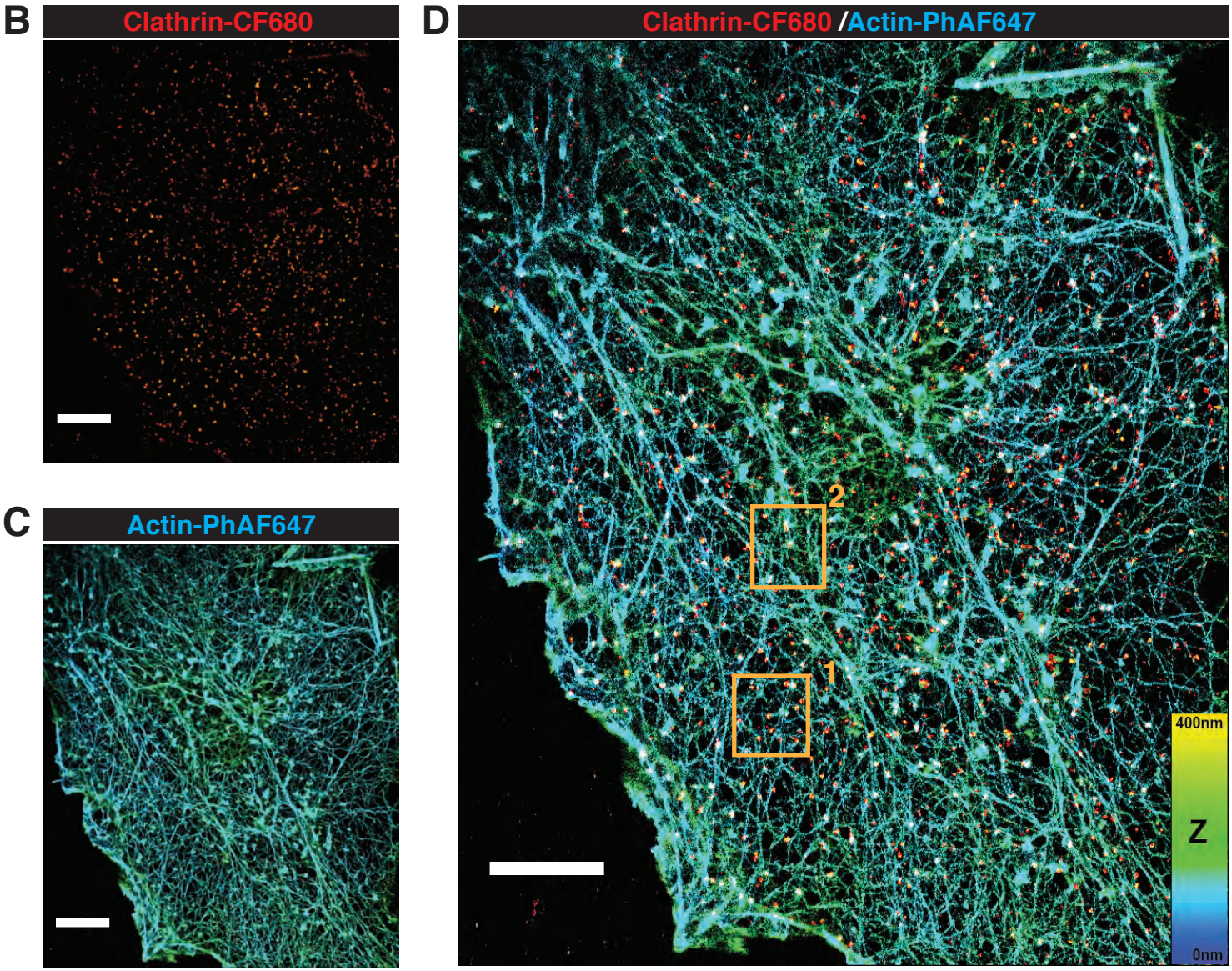
an adaptive actin network organization. Black arrows indicate actin forces exerted orthogonal and parallel to the membrane invagination.

**Table 1: Statistics for clathrin coat surface area.** Table provides average clathrin coat surface area in $nm^2$ over the course of CME under normal and 75 mOsm hypotonic media conditions, without and with actin present. Mann-Whitney statistical test compares the clathrin coat surface area between the different clathrin coat stages when actin is lacking and when actin is present.

**Table 2: Statistics for actin height and coverage of clathrin coats.** Table provides average actin height in nm and average percentage of clathrin covered by actin over the course of CME under normal and 75 mOsm hypotonic media conditions. Statistical test: Mann-Whitney.

# Figure 1


A
X-y maximum projection (top profile)
X-z maximum projection (side profile)
Shallow
U-shaped
Omega-shaped
Early to intermediate clathrin coat
Late clathrin coat
Early to intermediate clathrin coat
Late clathrin coat
Vesicle release
Clathrin
Actin
Plasma membrane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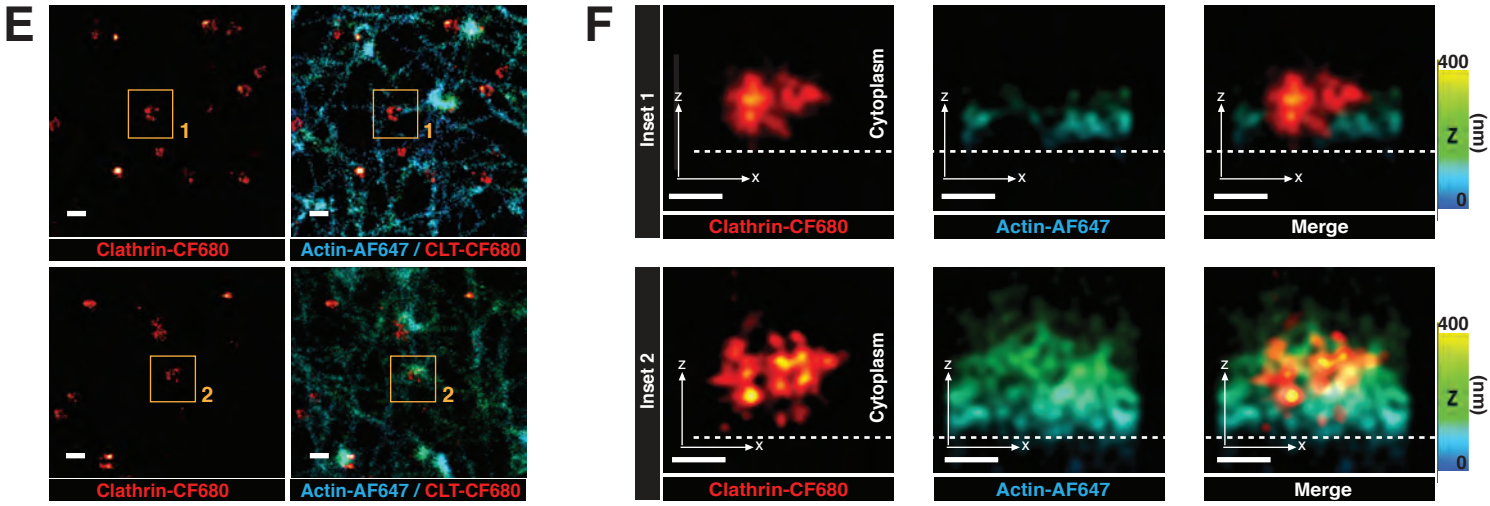
C
Actin-PhAF647
D
Clathrin-CF680 /Actin-PhAF647
2
1
400nm
Z
0nm


E
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1
Clathrin-CF680
Actin-AF647 / CLT-CF680
2
2
Clathrin-CF680
Actin-AF647 / CLT-CF680
F
Inset 1
Cytoplasm
Clathrin-CF680
Actin-AF647
Merge
400
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(nm)
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Cytoplasm
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Z
(nm)
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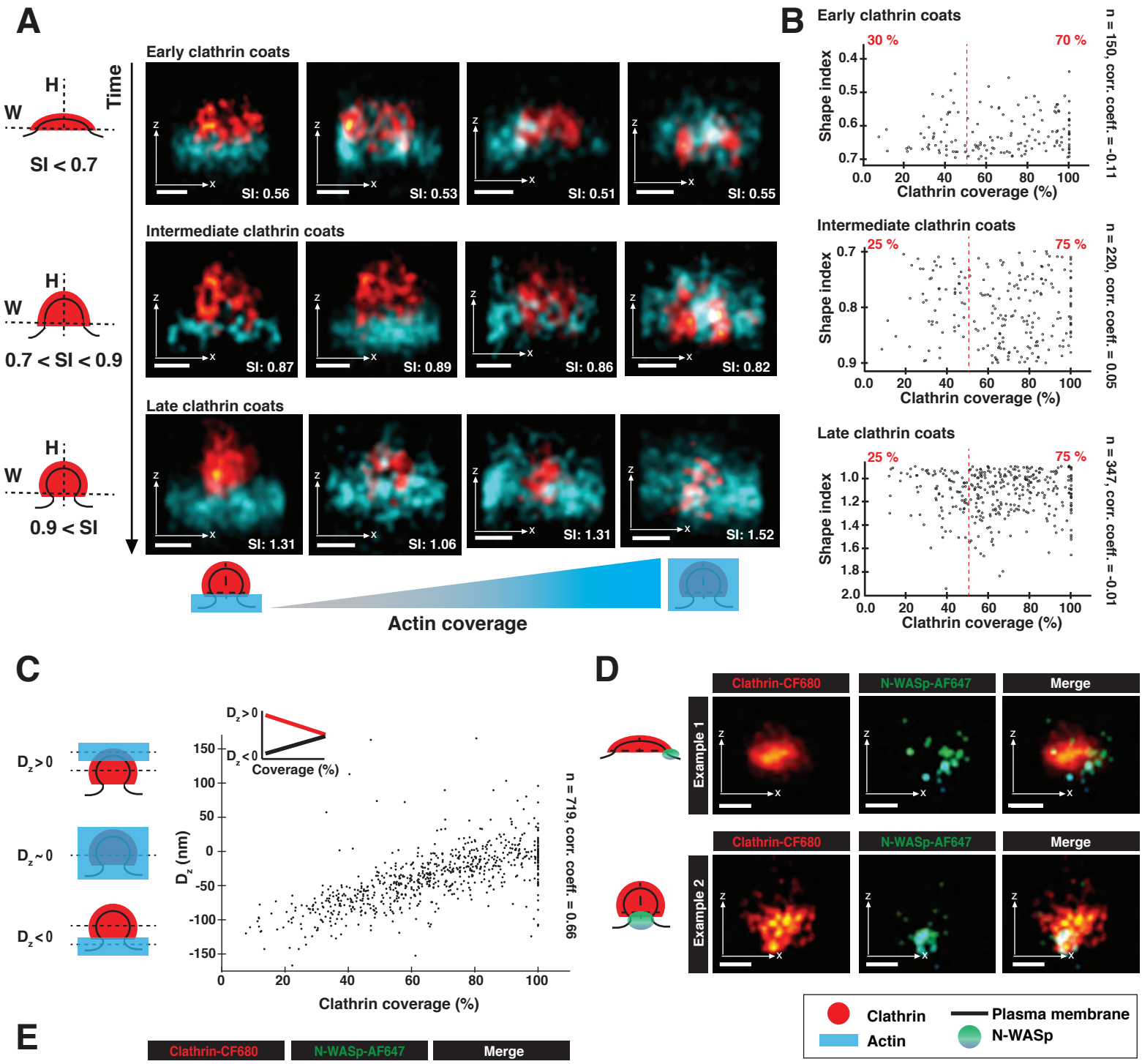
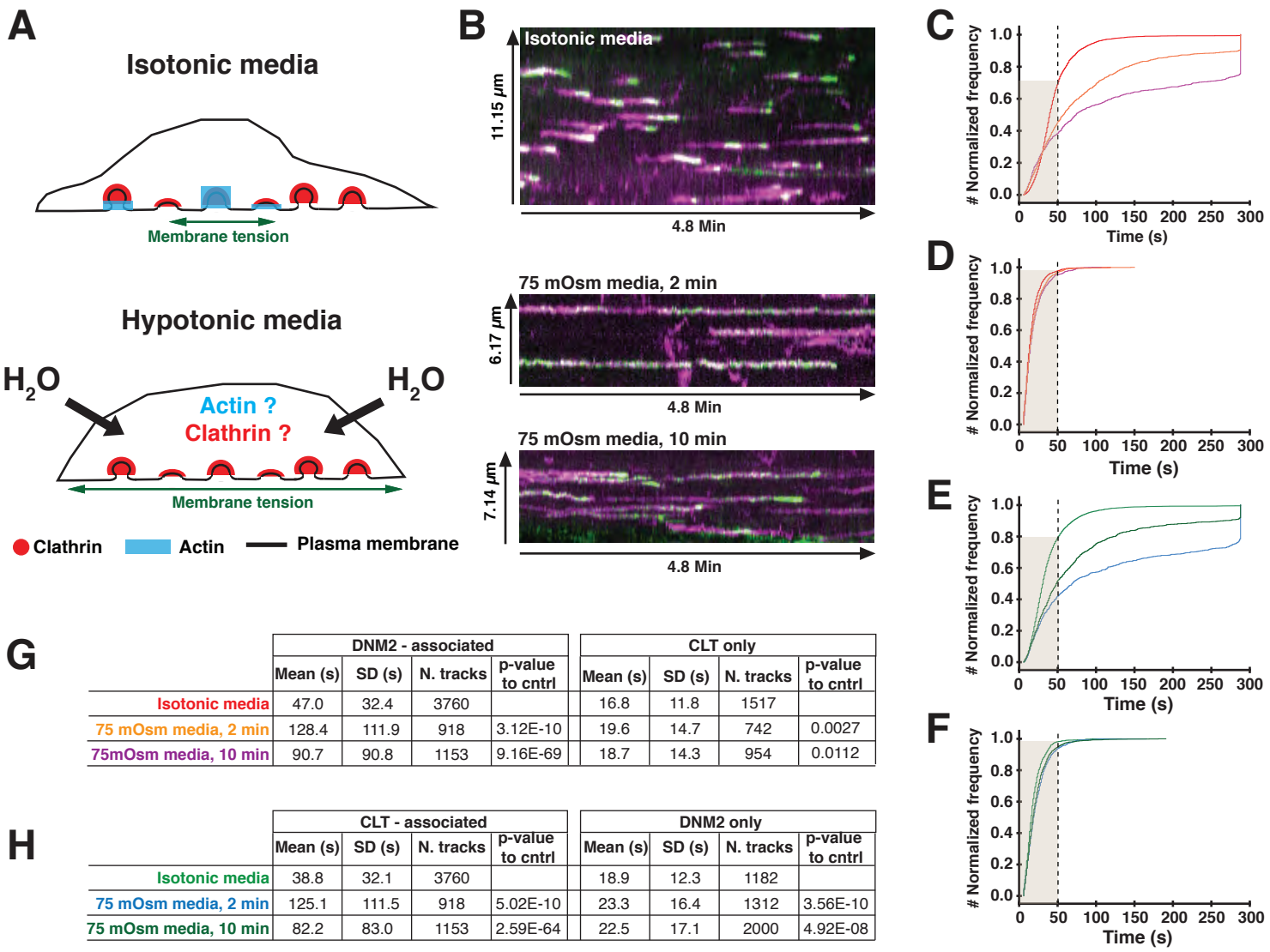
# Figure 2


A
Time
H
W
SI < 0.7
0.7 < SI < 0.9
0.9 < SI
Early clathrin coats
SI: 0.56
SI: 0.53
SI: 0.51
SI: 0.55
Intermediate clathrin coats
SI: 0.87
SI: 0.89
SI: 0.86
SI: 0.82
Late clathrin coats
SI: 1.31
SI: 1.06
SI: 1.31
SI: 1.52
Actin coverage
B
Early clathrin coats
30 %
70 %
Shape index
Clathrin coverage (%)
n = 150, corr. coeff. = -0.11
Intermediate clathrin coats
25 %
75 %
n = 220, corr. coeff. = 0.05
Late clathrin coats
n = 347, corr. coeff. = -0.01
C
$D_z > 0$
$D_z \sim 0$
$D_z < 0$
Coverage (%)
$D_z$ (nm)
Clathrin coverage (%)
n = 719, corr. coeff. = 0.66
D
Clathrin-CF680
N-WASp-AF647
Merge
Example 1
Example 2
Clathrin
Actin
Plasma membrane
N-WASp
E
Example 3
Example 4

# Figure 3

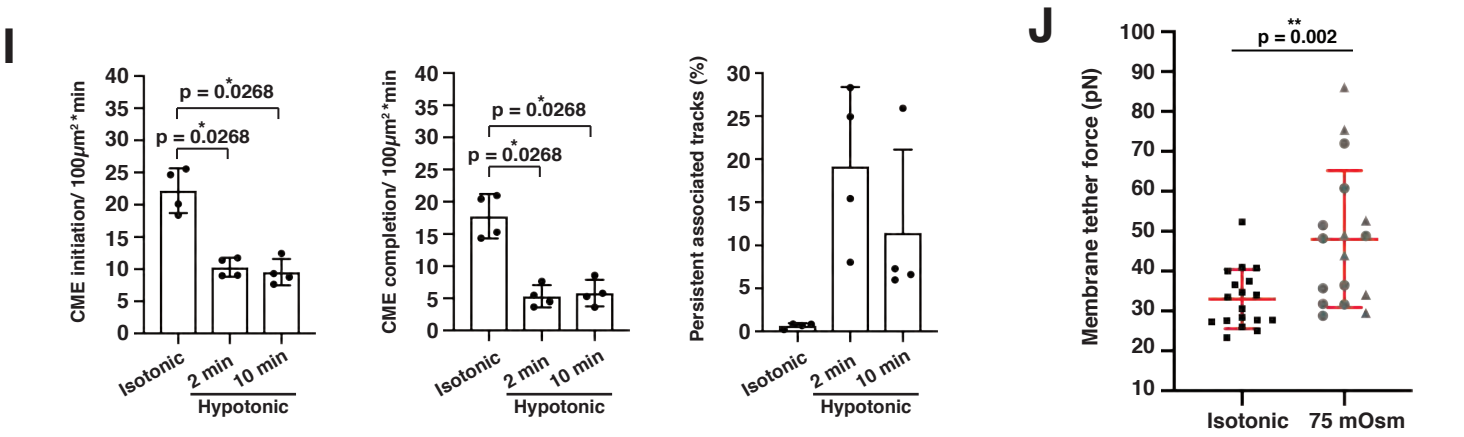



G

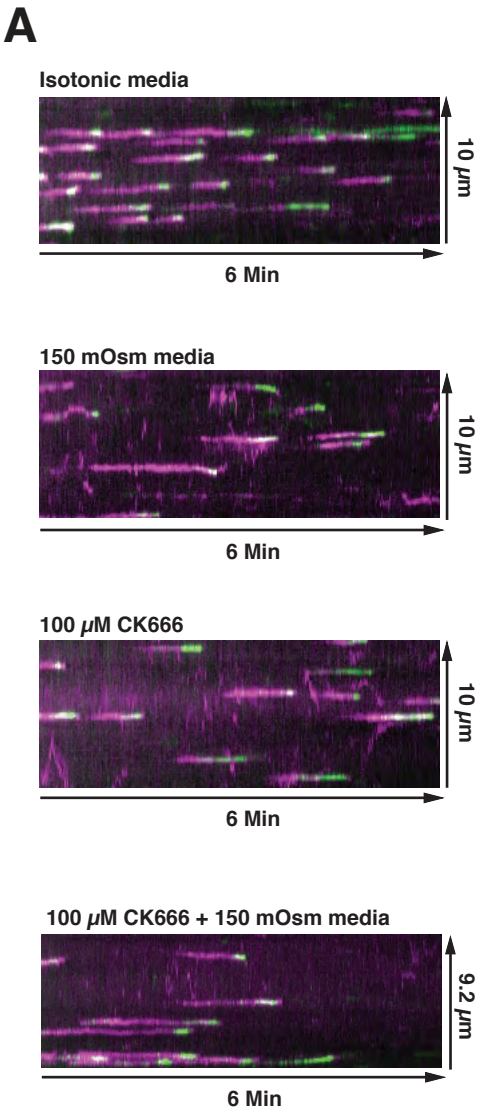
| | DNM2 - associated | | | | CLT only | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean (s) | SD (s) | N. tracks | p-value to cntrl | Mean (s) | SD (s) | N. tracks | p-value to cntrl |
| Isotonic media | 47.0 | 32.4 | 3760 | | 16.8 | 11.8 | 1517 | |
| 75 mOsm media, 2 min | 128.4 | 111.9 | 918 | 3.12E-10 | 19.6 | 14.7 | 742 | 0.0027 |
| 75mOsm media, 10 min | 90.7 | 90.8 | 1153 | 9.16E-69 | 18.7 | 14.3 | 954 | 0.0112 |

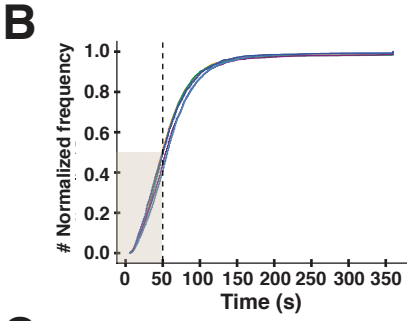
H

| | CLT - associated | | | | DNM2 only | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean (s) | SD (s) | N. tracks | p-value to cntrl | Mean (s) | SD (s) | N. tracks | p-value to cntrl |
| Isotonic media | 38.8 | 32.1 | 3760 | | 18.9 | 12.3 | 1182 | |
| 75 mOsm media, 2 min | 125.1 | 111.5 | 918 | 5.02E-10 | 23.3 | 16.4 | 1312 | 3.56E-10 |
| 75 mOsm media, 10 min | 82.2 | 83.0 | 1153 | 2.59E-64 | 22.5 | 17.1 | 2000 | 4.92E-08 |



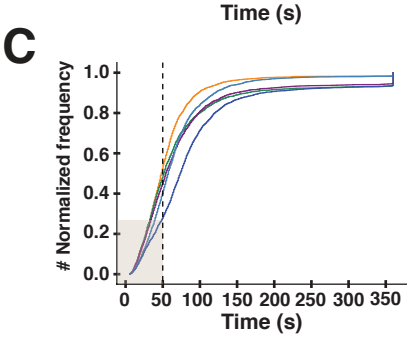
# Figure 4



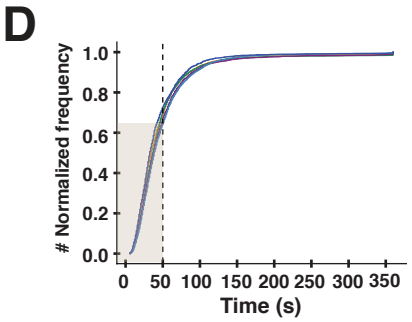

CLTA-tagRFP.t, associated

| Pre-treatment to | Mean (s) | SD (s) | N. tracks | N. cells | p-value to DMSO |
|---|---|---|---|---|---|
| DMSO | 59.0 | 47.2 | 2378 | 10 | |
| DMSO + 150 mOsmo | 61.5 | 52.8 | 4345 | 12 | 0.093 |
| 150 mOsm | 65.4 | 53.9 | 2173 | 9 | 3.88E-06 |
| 100 μM CK666 | 64.8 | 46.0 | 4219 | 10 | 1.18E-14 |
| 100 μM CK666 + 150 mOsm | 59.1 | 45.4 | 1405 | 9 | 0.902 |



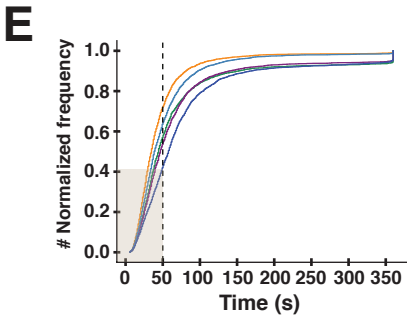

CLTA-tagRFP.t, associated

| Treatment | Mean (s) | SD (s) | N. tracks | N. cells | p-value to DMSO |
|---|---|---|---|---|---|
| DMSO | 59.0 | 51.9 | 3028 | 10 | |
| DMSO + 150 mOsm | 80.2 | 87.4 | 2286 | 10 | 2.13E-13 |
| 150 mOsm | 79.1 | 82.0 | 2762 | 8 | 7.15E-20 |
| 100 μM CK666 | 69.5 | 56.4 | 3124 | 10 | 7.46E25 |
| 100 μM CK666 + 150 mOsm | 96.3 | 85.5 | 2783 | 10 | 1.12E-119 |



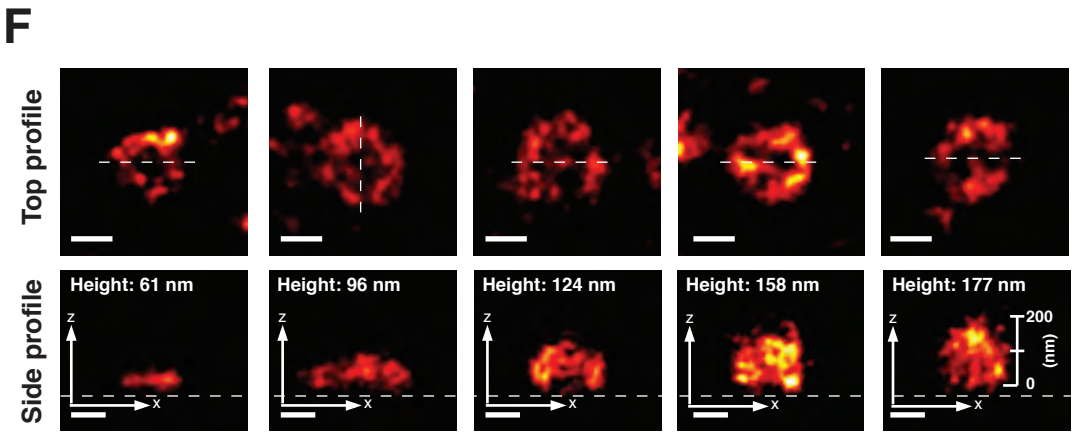

DNM2-eGFP, associated

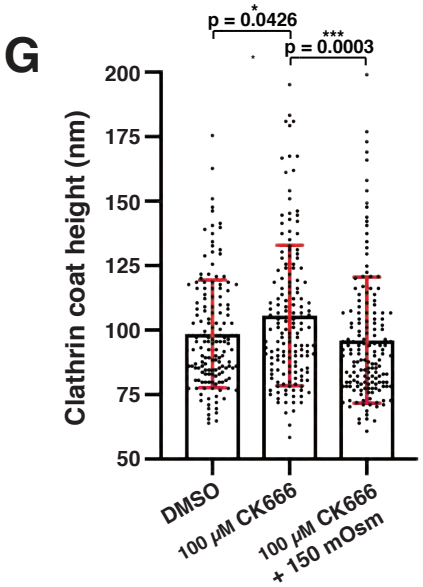
| Pre-treatment to | Mean (s) | SD (s) | N. tracks | N. cells | p-value to DMSO |
|---|---|---|---|---|---|
| DMSO | 46.7 | 45.9 | 2378 | 10 | |
| DMSO + 150 mOsm | 49.5 | 51.7 | 4345 | 12 | 0.004 |
| 150 mOsm | 51.1 | 51.5 | 2173 | 9 | 0.001 |
| 100 μM CK666 | 50.3 | 43.3 | 4219 | 10 | 2.57E-09 |
| 100 μM CK666 + 150 mOsm | 44.0 | 41.4 | 1405 | 9 | 0.094 |



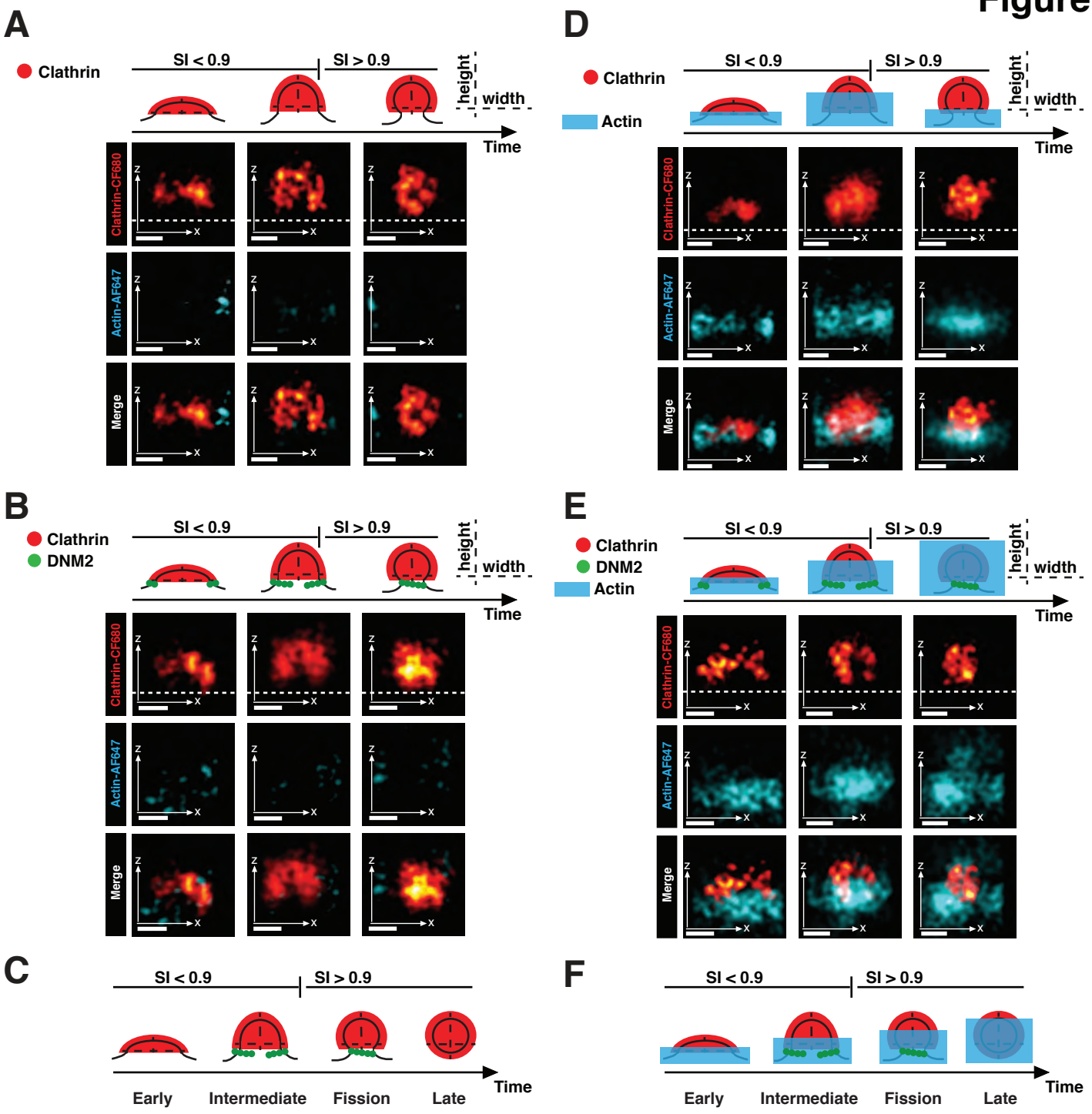

DNM2-eGFP, associated

| Treatment | Mean (s) | SD (s) | N. tracks | N. cells | p-value to DMSO |
|---|---|---|---|---|---|
| DMSO | 46.9 | 48.8 | 3028 | 10 | |
| DMSO + 150 mOsmo | 73.5 | 86.4 | 2286 | 10 | 4.27E-26 |
| 150 mOsm | 72.5 | 81.2 | 2762 | 8 | 3.51E-44 |
| 100 μM CK666 | 54.2 | 54.4 | 3124 | 10 | 8.39E-11 |
| 100 μM CK666 + 150 mOsmo | 83.5 | 85.3 | 2783 | 10 | 1.94E-115 |

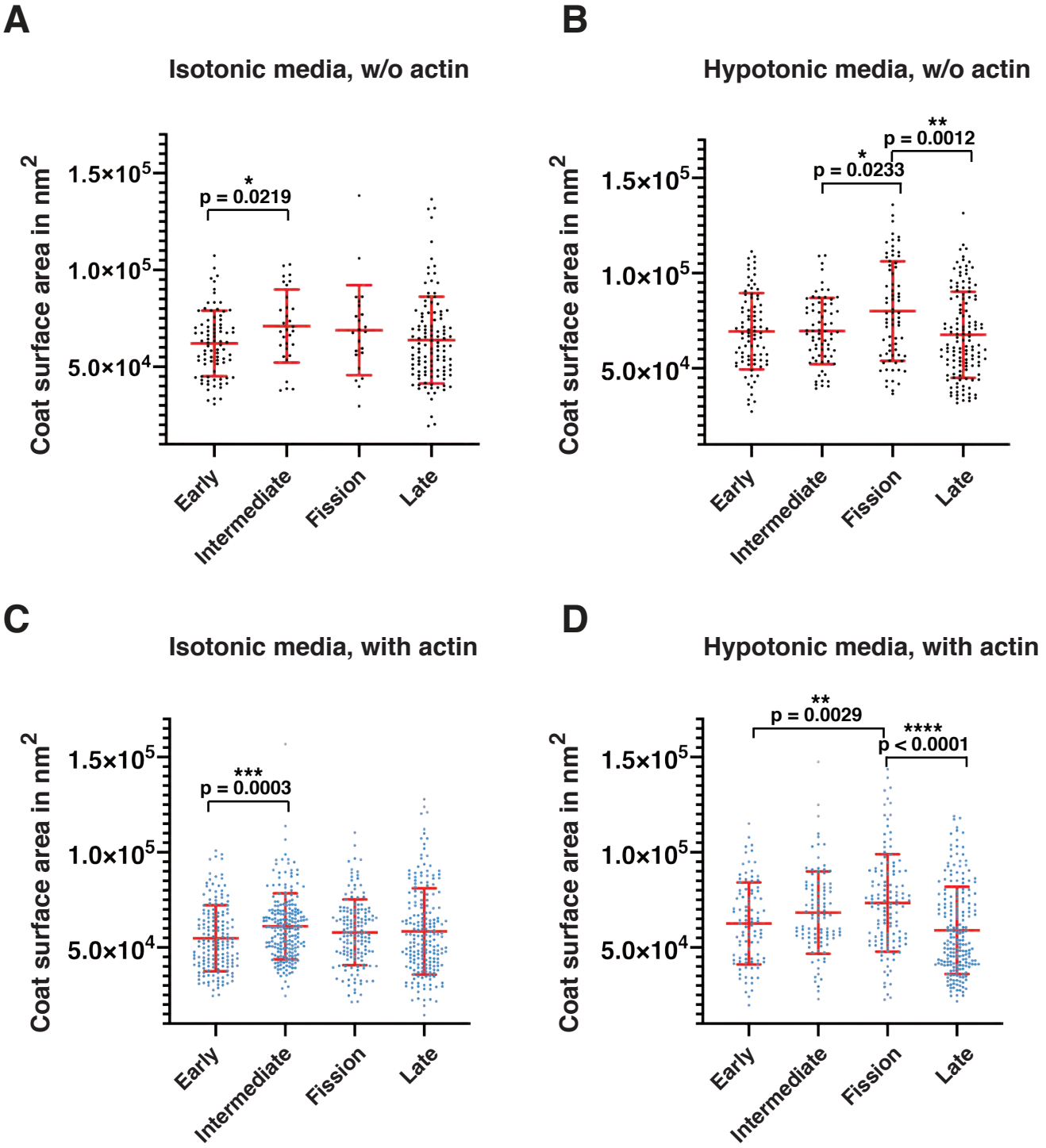


# Figure 5


A
Clathrin
SI < 0.9
SI > 0.9
height
width
Time
Clathrin-CF680
Actin-AF647
Merge
B
Clathrin
DNM2
C
Early
Intermediate
Fission
Late
D
Actin
E
F

# Figure 6


A
Isotonic media, w/o actin
Coat surface area in nm2
1.5×10^5
1.0×10^5
5.0×10^4
*
p = 0.0219
Early
Intermediate
Fission
Late
B
Hypotonic media, w/o actin
Coat surface area in nm2
1.5×10^5
1.0×10^5
5.0×10^4
*
p = 0.0233
**
p = 0.0012
Early
Intermediate
Fission
Late
C
Isotonic media, with actin
Coat surface area in nm2
1.5×10^5
1.0×10^5
5.0×10^4
***
p = 0.0003
Early
Intermediate
Fission
Late
D
Hypotonic media, with actin
Coat surface area in nm2
1.5×10^5
1.0×10^5
5.0×10^4
**
p = 0.0029
****
p < 0.0001
Early
Intermediate
Fission
Late

# Figure 7

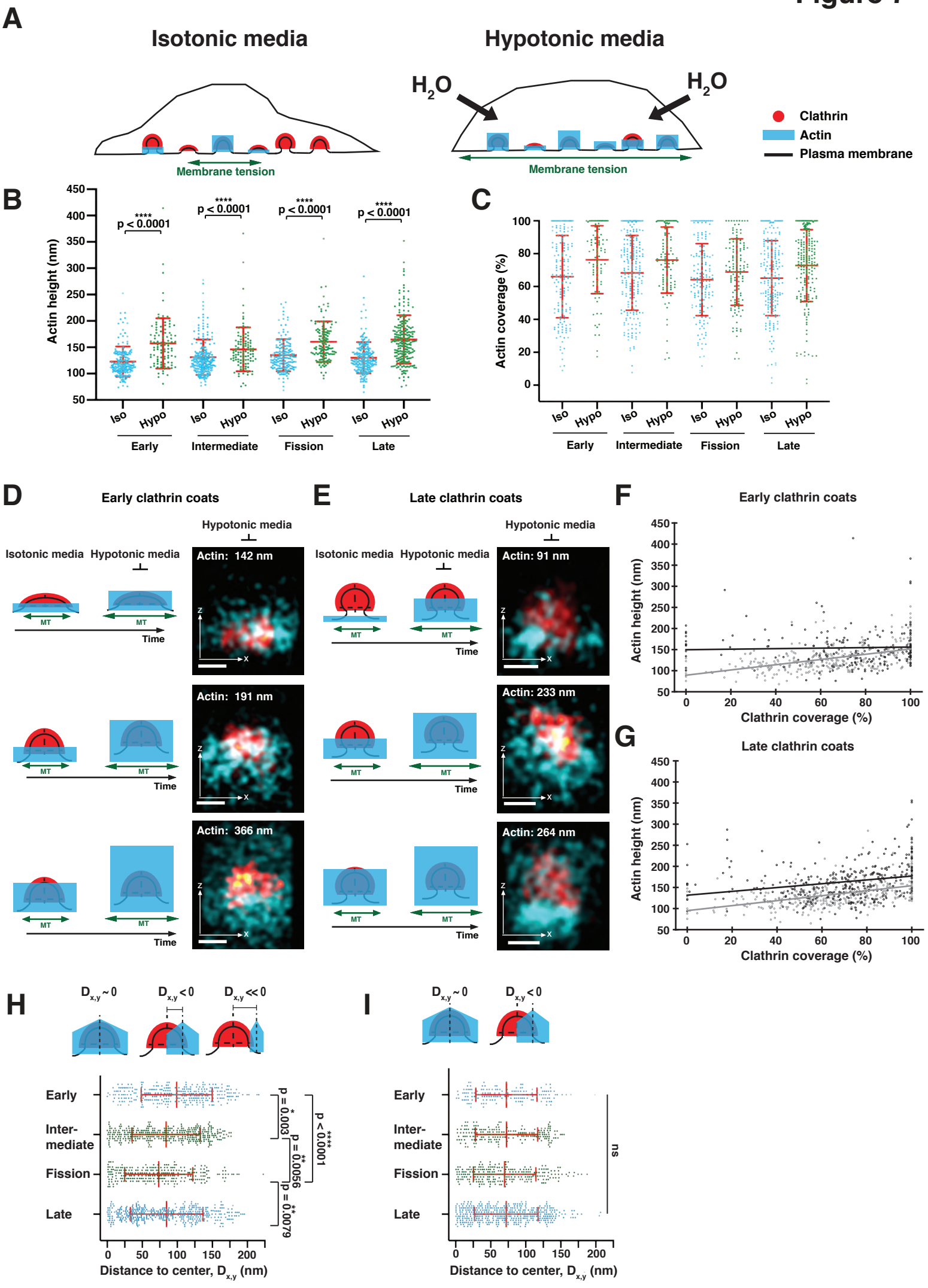

A
Isotonic media
Hypotonic media
$H_2O$
$H_2O$
Membrane tension
Membrane tension
Clathrin
Actin
Plasma membrane
B
Actin height (nm)
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
Iso
Hypo
Early
Intermediate
Fission
Late
C
Actin coverage (%)
D
Early clathrin coats
Isotonic media
Hypotonic media
MT
Time
Actin: 142 nm
Actin: 191 nm
Actin: 366 nm
E
Late clathrin coats
Actin: 91 nm
Actin: 233 nm
Actin: 264 nm
F
Early clathrin coats
Actin height (nm)
Clathrin coverage (%)
G
Late clathrin coats
H
$D_{x,y} \sim 0$
$D_{x,y} < 0$
$D_{x,y} \ll 0$
Early
Inter-mediate
Fission
Late
p = 0.003
p = 0.0056
p = 0.0079
p < 0.0001
Distance to center, $D_{x,y}$ (nm)
I
ns

Figure 8

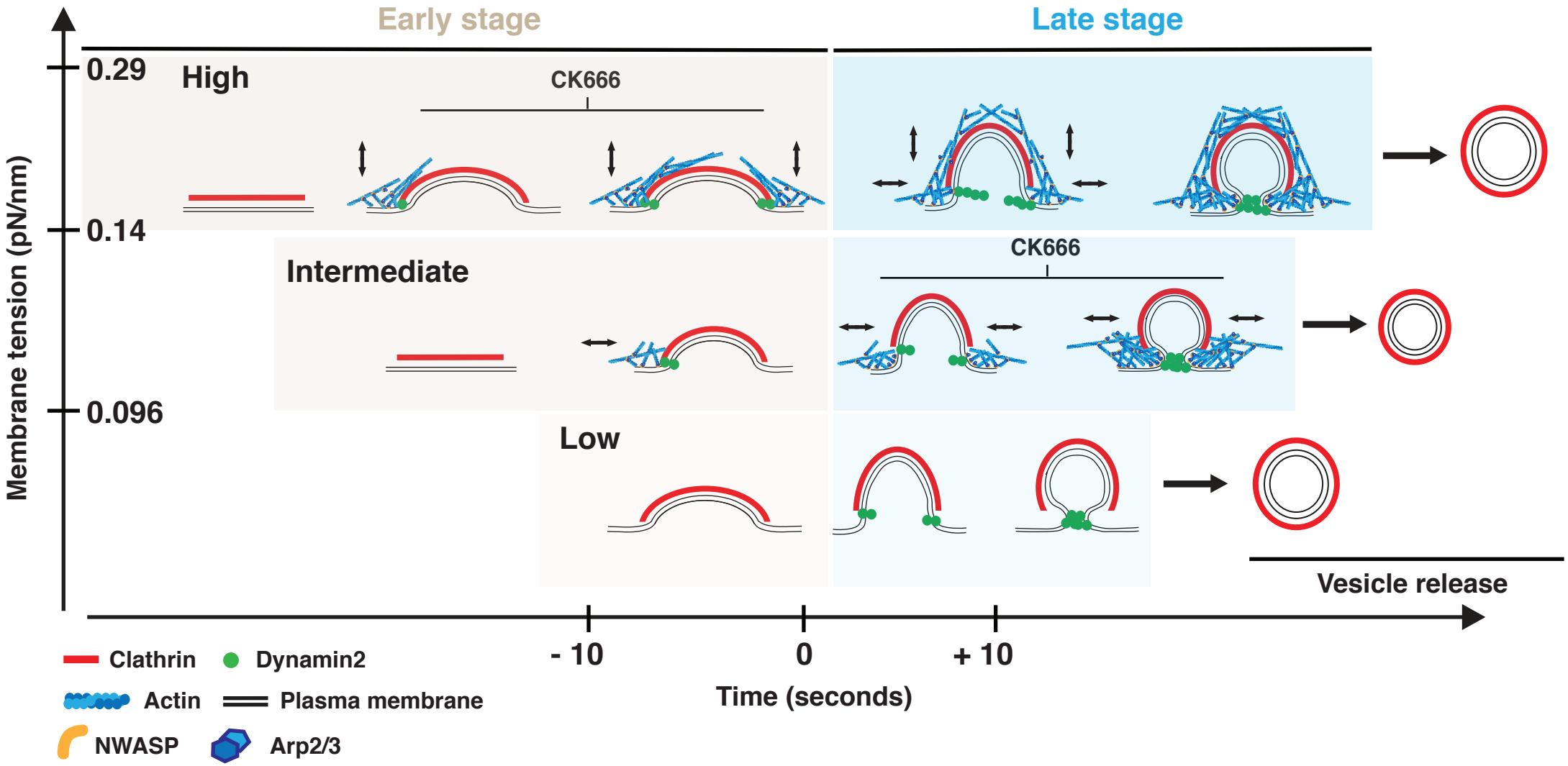

Early stage
Late stage
Membrane tension (pN/nm)
0.29
0.14
0.096
High
CK666
Intermediate
CK666
Low
Vesicle release
- 10
0
+ 10
Time (seconds)
Clathrin
Dynamin2
Actin
Plasma membrane
NWASP
Arp2/3

**Figure S1: Representative examples of ambiguous clathrin data, overview of 3D measurement methods for super-resolved clathrin and actin, and representative x-y projections.**

**(A)** Representative examples of immunolabeled clathrin structures that cannot be identified as individual clathrin coated events. dSTORM images show x-y projection of clathrin structures. First two columns show structures that do not show a clear round or elliptical shape. Third image column shows clathrin structures that extend over the entire region of interest. Fourth image column shows very small punctate structures. **(B)** Illustration of method to obtain actin and clathrin height and extent of overlap from super-resolved CCP images. x-z projection of a merged dSTORM image of clathrin and actin with its corresponding normalized pixel intensity histogram projected onto the z-axis. The black line marks the $30^{th}$ percentile of the z height histogram, which we used to calculate the height of clathrin and actin. We found that this metric, the "full z width at $30^{th}$ percentile max," was more reliable than the full width at half max. These raw intensity profiles are noisy based on the image quality, which depends on the fluorescent labeling quality of the target structure. Without filtering of the profile, the measured height of clathrin and actin are underestimated. Numbers correspond to positions used to calculate height (in z) of clathrin (1. and 2.) and actin (4. and 5.) and their average positions (3. and 6.). Lower panel shows the same dSTORM image after a mean filter was applied to reduce noise in the histogram of z-position to improve the reliability of the clathrin and actin height readout. We measured the overlap of clathrin and actin profiles by subtracting the upper position of actin from the lower position of clathrin. **(C)** x-z projected dSTORM image of a clathrin coat with its corresponding normalized pixel intensity histogram projected onto the x-axis unfiltered. Due to the image noise the measurement of clathrin coat width misses most of the image. The right

image shows the improved width measurement of the same clathrin coat after application of a median filter to smooth the histogram. **(D)** x-y projection dSTORM images corresponding to the x-z projections in Figure 2 A. The upper image corresponds to the first image in the row in Figure 2 A. Left images show clathrin (red), middle images actin (cyan) and the right image clathrin and actin merged, respectively. **(E)** x-y projection dSTORM images of clathrin (red) and N-WASP (rainbow) corresponding to the x-z projection in Figure 2 D and in Figure 2 E. **(A) – (E)** All scale bars are 100 nm.

**Figure S2: Hypotonic media treatment of SK-MEL-2 cells endogenously expressing CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$. (A), (C)** and **(E)** Normalized cumulative distribution data for CLTA-TagRFP-T$^{EN}$ and DNM2-eGFP$^{EN}$ fluorescence lifetimes when associated with or not associated with each other. Lifetimes were recorded under isotonic media conditions and then recorded after 2 min or 10 min of media exchange. Plot legend provides respectively: mean lifetime ± SD, number of tracks, p-value determined in Kolmogorov-Smirnov test with significance compared to the control. **(B), (D)** and **(F)** show CME initiation rate, completion rate and percentage of persistent tracks plotted for sites in which DNM2-eGFP$^{EN}$ and CLTA-TagRFP-T$^{EN}$ were associated with each other. Tables below show the corresponding statistics. Mann-Whitney statistical test was used to compare CME initiation rates and completion rates. **(A)** and **(B)** Control conditions in which only isotonic media exchange was performed. n = 3-4 (cells) for each condition. Experiments were repeated 3 times. **(C)** and **(D)** Media were exchanged from isotonic to 225 mOsm hypotonic. For each of the following conditions: control, 2 min, or 10 min after media exchange, n = 6 (cells). **(E)** and **(F)** Media were exchanged from isotonic to 150 mOsm hypotonic. For each condition of the following conditions: control, 2 min

and 10 min after media exchange, n = 6 (cells). **(C) – (F)** Experiments were repeated 5 – 6 times. **(G)** Statistics for CME initiation rates, completion rates and percentage of persistent tracks for CLTA-TagRFP-$T^{EN}$ associated with DNM2-$eGFP^{EN}$ corresponding to Figure 3 G – I.

**Figure S3: Effects of hypotonic media and concomitant CK666 treatment on CME progression. (A)** Normalized fluorescence lifetime cumulative distribution data for CLTA-TagRFP-$T^{EN}$ and associated DNM2-$eGFP^{EN}$ for CK666 concentrations of 25 μM, 50 μM and 100 μM. Respective controls were 0.5% v/v, 1% v/v and 2% v/v DMSO. Fluorescence lifetimes were acquired after 2 minutes of treatment. Experiments were repeated 2-3 times. Kolmogorov-Smirnov statistical test was used. **(B)** Corresponding CME completion rates for CLTA-TagRFP-$T^{EN}$ associated with DNM2-$eGFP^{EN}$ in Figure 4 B and C. **(C)** Percentage of persistent tracks for CLTA-TagRFP-$T^{EN}$ associated with DNM2-$eGFP^{EN}$ for corresponding imaging data in Figure 4 B and C. **(D)** Corresponding CME initiation rates for CLTA-TagRFP-$T^{EN}$ associated with DNM2-$eGFP^{EN}$ in Figure 4 B and C. **(E)** Statistics for clathrin coat height and width for data in Figure 4 G when cells were treated with 2% v/v DMSO, 100 μM CK666 and 100 μM CK666 in 150 mOsm hypotonic media. **(F)** Plotted clathrin coat width in nm for data in Figure 4 G. **(G)** Ratio of clathrin coat height to width for data in Figure 4 G and Figure S3 F. **(B), (D)** and **(F)** Mann-Whitney statistical test was used. **(B) - (D)** and **(F)** bars show mean with SD.

**Figure S4: Representative x-y projections of super-resolved clathrin coat associated with or without actin and/or a DNM2-$eGFP^{EN}$ fluorescence signal. (A) – (D)** x-y projection dSTORM images corresponding to the x-z projections in Figure 5 A, B, D and E. Left images show TIRF images of DNM2-$eGFP^{EN}$, middle images show super-resolved clathrin (red) in dSTROM and

right images show super-resolved actin (cyan), respectively. **(A)** Clathrin not associated with actin or DNM2-eGFP$^{EN}$. **(B)** Clathrin associated with DNM2-eGFP$^{EN}$, but lacking actin. **(C)** Clathrin associated with actin, but lacking DNM2-eGFP$^{EN}$. **(D)** Clathrin associated with actin and DNM2-eGFP$^{EN}$. **(A) – (D)** All scale bars are 100 nm. **(E)** Statistical significance of the difference in clathrin coat surface area between isotonic media and 75 mOsm hypotonic media. Mann-Whitney statistical test was used.

**Figure S5: Effect of hypotonic media treatment on actin organization. (A)** dSTORM image of CF-680 immunolabeled clathrin-coated structures (red) on the ventral cell surface when 75 mOsm hypotonic media was applied for 5 min. **(B)** Same area of the cell as in (A) showing the dSTORM image of phalloidin-AF647 labeled actin cytoskeleton (rainbow). **(C)** Same area of the cell as in (A) showing the corresponding DNM2-eGFP$^{EN}$ conventional microscopy image. **(D)** Merged clathrin and actin dSTORM images from (A) and (B). Color bar shows actin position in the z-dimension. **(A) – (D)** All scale bars are 5 µm. **(E)** and **(F)** dSTORM x-y projection images corresponding to images in Figure 7 D and E. All scale bars are 100 nm. (**G)** Upper panel shows an example of asymmetrically organized actin around a clathrin-coated pit. Normalized pixel intensity histograms of clathrin (red) and actin (blue) from x-z and y-z projections. Measurement is the distance of the peak actin signal from the center of the clathrin coat. Actin is asymmetrically organized since the distance from the x-axis to the center is large compared to the distance to the y-axis. The lower panel shows an example of symmetrically organized actin around a clathrin-coated pit. Respective distances of the actin maximum to the center of the clathrin coat are much smaller. **(H)** Table with statistics for the data in Figure 7 I. Mann-Whitney statistical test compares asymmetry of actin under isotonic and hypotonic media conditions.

**Supplementary movie 1:** Movie of CLTA-TagRFP-T$^{EN}$ (magenta) and DNM2-eGFP$^{EN}$ (green) in SK-MEL-2 cells in isotonic media.

**Supplementary movie 2:** Movie of CLTA-TagRFP-T$^{EN}$ (magenta) and DNM2-eGFP$^{EN}$ (green) in SK-MEL-2 cells after 2 minutes of media exchange to 75 mOsm media.

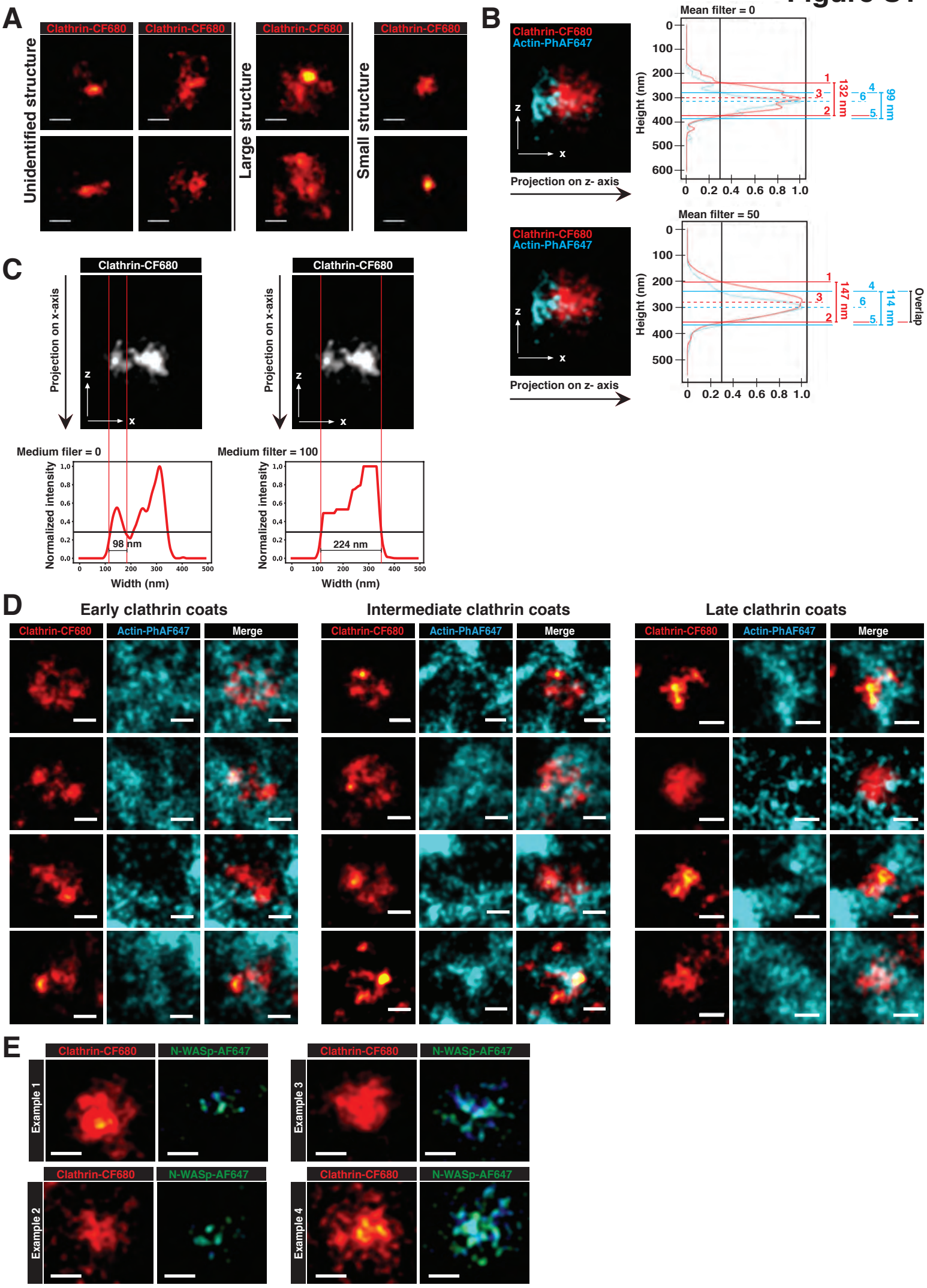
**Supplementary movie 3:** Movie of CLTA-TagRFP-T$^{EN}$ (magenta) and DNM2-eGFP$^{EN}$ (green) in SK-MEL-2 cells after 10 minutes of media exchange to 75 mOsm media.

**Supplementary movie 4:** Movie of CLTA-TagRFP-T$^{EN}$ (magenta) and DNM2-eGFP$^{EN}$ (green) in SK-MEL-2 cells in isotonic media before treatment with CK666 and 150 mOsm hypotonic media.

**Supplementary movie 5:** Movie of CLTA-TagRFP-T$^{EN}$ (magenta) and DNM2-eGFP$^{EN}$ (green) in SK-MEL-2 cells in 150 mOsm hypotonic media with 100 μM CK666. Movie acquisition started 2 minutes after media exchange.

.

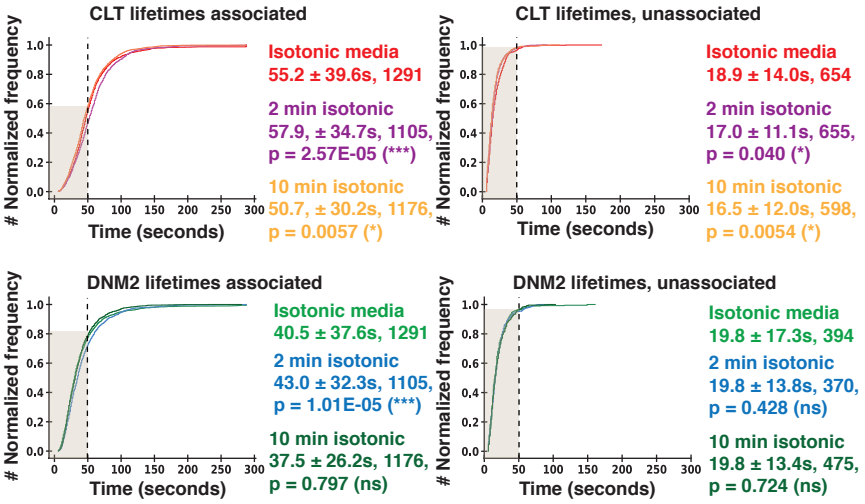
# Figure S1


A
Clathrin-CF680
Unidentified structure
Large structure
Small structure
B
Clathrin-CF680
Actin-PhAF647
Projection on z- axis
Mean filter = 0
Mean filter = 50
Height (nm)
132 nm
99 nm
147 nm
114 nm
Overlap
C
Clathrin-CF680
Projection on x-axis
Medium filer = 0
Medium filter = 100
Normalized intensity
98 nm
224 nm
Width (nm)
D
Early clathrin coats
Intermediate clathrin coats
Late clathrin coats
Clathrin-CF680
Actin-PhAF647
Merge
E
Clathrin-CF680
N-WASp-AF647
Example 1
Example 2
Example 3
Example 4

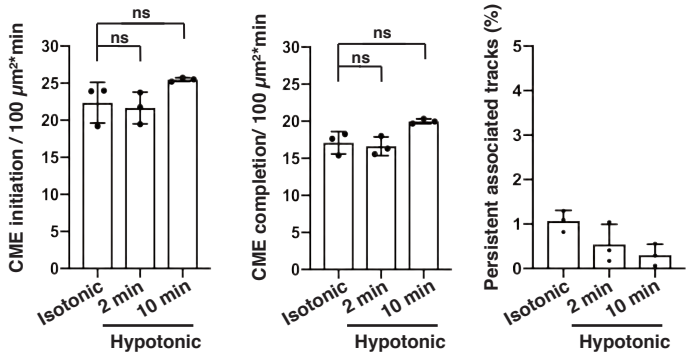
# Figure S2

## A

### Isotonic media, 300 mOsm



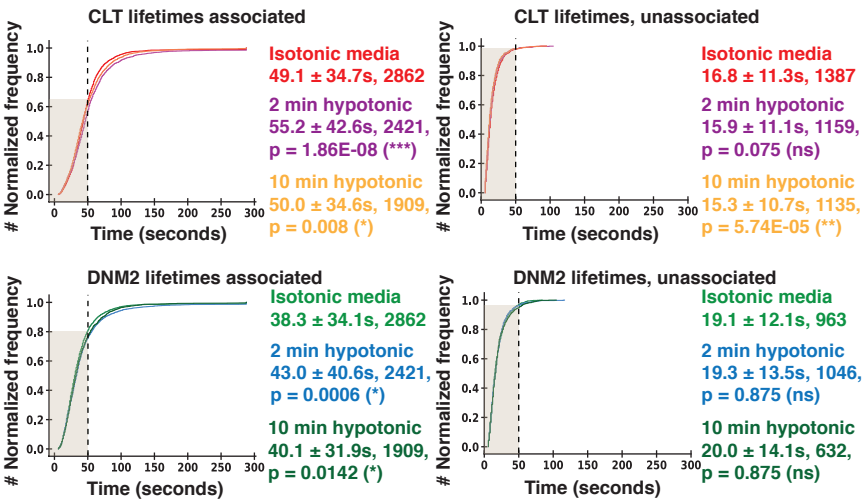

## B



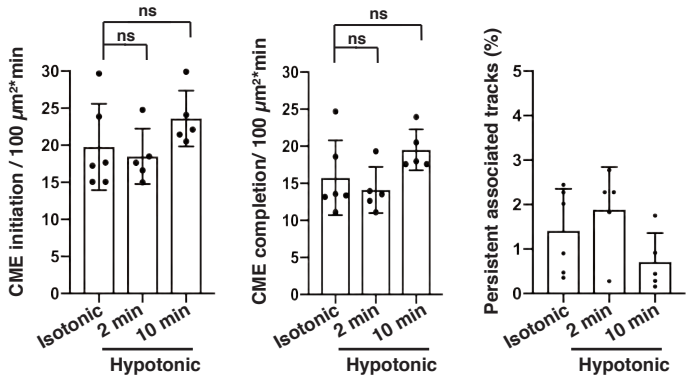

| | Mean initiation rate ± SD (100 μm²*min) | Mean completion rate ± SD (100 μm²*min) | Mean persistent tracks (%) ± SD |
|---|---|---|---|
| Isotonic | 19.19 ± 2.75 | 17.1 ± 1.51 | 1.1 ± 0.23 |
| Isotonic, 2 min | 19.46 ± 2.14 | 16.64 ± 1.26 | 0.54 ± 0.45 |
| Isotonic, 10 min | 25.22 ± 0.25 | 20 ± 0.33 | 0.30 ± 0.24 |

## C

### Hypotonic media, 225 mOsm



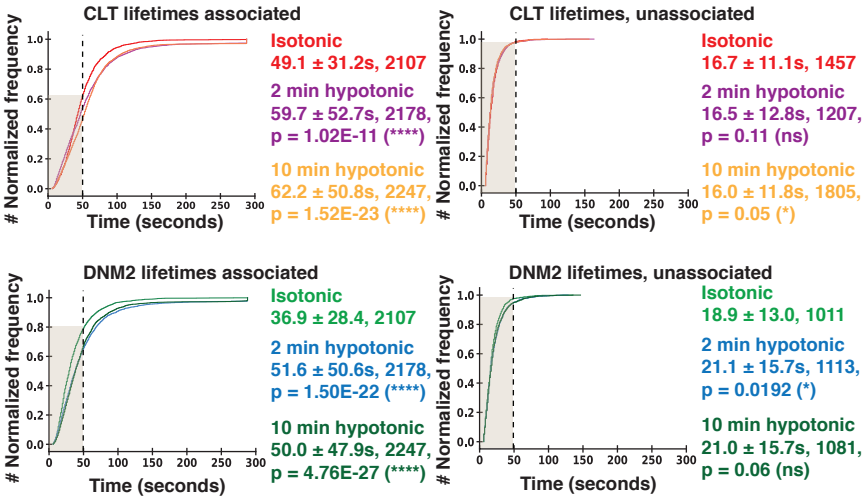

## D



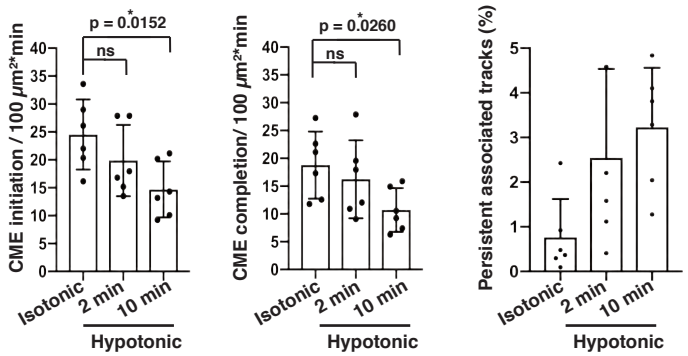

| | Mean initiation rate ± SD (100 μm²*min) | Mean completion rate ± SD (100 μm²*min) | Mean persistent tracks (%) ± SD |
|---|---|---|---|
| Isotonic | 19.76 ± 5.82 | 15.75 ± 5.03 | 1.41 ± 0.94 |
| 225 mOsm, 2 min | 18.50 ± 3.73 | 14.10 ± 3.12 | 1.89 ± 0.96 |
| 225 mOsm, 10 min | 23.61 ± 3.76 | 19.52 ± 2.76 | 0.71 ± 0.65 |

## E

### Hypotonic media, 150 mOsm




## F




| | Mean initiation rate ± SD (100 μm²*min) | Mean completion rate ± SD (100 μm²*min) | Mean persistent tracks (%) ± SD |
|---|---|---|---|
| Isotonic | 24.53 ± 6.29 | 18.78 ± 6.03 | 0.77 ± 0.86 |
| 150 mOsm, 2 min | 19.87 ± 6.39 | 16.24 ± 7.02 | 2.55 ± 1.99 |
| 150 mOsm, 10 min | 14.70 ± 5.00 | 10.72 ± 3.92 | 3.23 ± 1.34 |

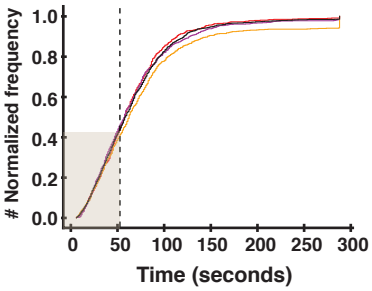
## G

| | Mean initiation rate ± SD (100 μm²*min) | Mean completion rate ± SD (100 μm²*min) | Mean persistent tracks (%) ± SD |
|---|---|---|---|
| Isotonic | 22.18 ± 3.46 | 17.75 ± 3.43 | 0.65 ± 0.30 |
| 75 mOsm, 2 min | 10.28 ± 1.48 | 5.32 ± 1.72 | 19.17 ± 9.21 |
| 75 mOsm, 10 min | 9.54 ± 2.05 | 5.82 ± 2.07 | 11.45 ± 9.66 |

# Figure S3

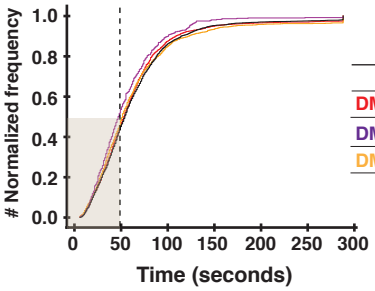
## A

### CK666 treatment; CLCA-TagRFP.T associated



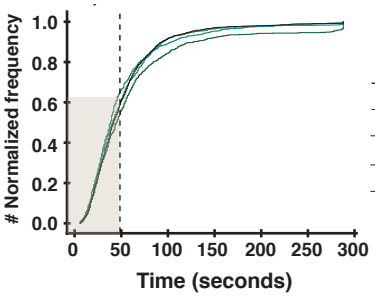

| | Mean(s) | SD(s) | N. tracks | N. cells | p-value to pre CK666 |
|---|---|---|---|---|---|
| pre CK666 | 68.0 | 48.8 | 1441 | 6 | |
| CK666 25μM | 66.2 | 45.4 | 744 | 6 | 0.7111 |
| CK666 50μM | 68.8 | 52.0 | 833 | 5 | 0.7925 |
| CK666 100μM | 78.9 | 66.2 | 835 | 8 | 0.0485 |

### DMSO treatment; CLCA-TagRFP.T associated



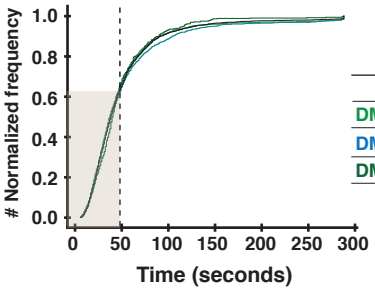

| | Mean(s) | SD(s) | N. tracks | N. cells | p-value to pre DMSO |
|---|---|---|---|---|---|
| pre DMSO | 65.2 | 50.0 | 2852 | 14 | |
| DMSO 0.5% (v/v) | 64.3 | 51.5 | 1469 | 5 | 0.2012 |
| DMSO 1.0% (v/v) | 66.3 | 56.7 | 1177 | 5 | 0.1405 |
| DMSO 2.0% (v/v) | 56.2 | 39.9 | 526 | 5 | 0.0011 |

### CK666 treatment; DNM2-eGFP associated



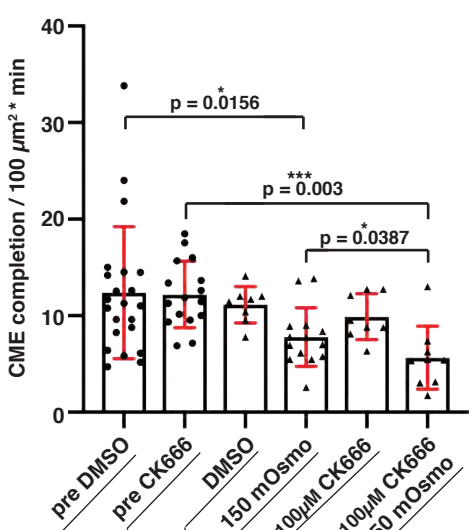

| | Mean(s) | SD(s) | N. tracks | N. cells | p-value to pre CK666 |
|---|---|---|---|---|---|
| pre CK666 | 52.6 | 42.3 | 1441 | 6 | |
| CK666 25μM | 51.1 | 44.6 | 744 | 6 | 0.0064 |
| CK666 50μM | 54.6 | 48.3 | 833 | 5 | 0.5346 |
| CK666 100μM | 64.7 | 64.1 | 835 | 8 | 0.0034 |

### DMSO treatment; DNM2-eGFP associated



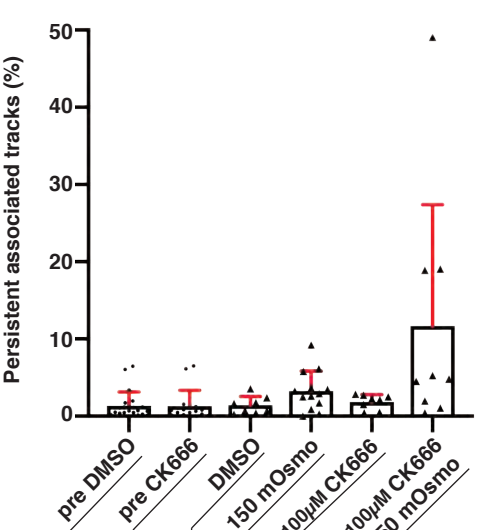

| | Mean(s) | SD(s) | N. tracks | N. cells | p-value to pre DMSO |
|---|---|---|---|---|---|
| pre DMSO | 51.0 | 45.9 | 2852 | 14 | |
| DMSO 0.5% (v/v) | 51.2 | 49.4 | 1469 | 5 | 0.5880 |
| DMSO 1.0% (v/v) | 54.4 | 52.6 | 1177 | 5 | 0.2858 |
| DMSO 2.0% (v/v) | 49.0 | 37.8 | 526 | 5 | 0.0583 |

## B



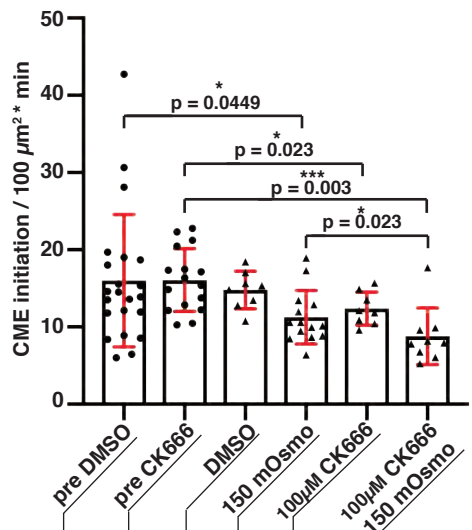

| | pre DMSO | pre CK666 | DMSO | 150 mOsmo | 100μM CK666 | 100μM CK666 + 150 mOsmo |
|---|---|---|---|---|---|---|
| Mean | 12.4 | 12.2 | 11.1 | 7.8 | 9.9 | 5.7 |
| SD | 6.8 | 3.4 | 1.9 | 3.0 | 2.4 | 3.3 |
| N. | 22 | 16 | 8 | 14 | 8 | 9 |

## C




| | pre DMSO | pre CK666 | DMSO | 150 mOsmo | 100μM CK666 | 100μM CK666 + 150 mOsmo |
|---|---|---|---|---|---|---|
| Mean | 1.4 | 1.3 | 1.4 | 3.3 | 1.9 | 11.7 |
| SD | 1.8 | 2.0 | 1.2 | 2.6 | 0.9 | 15.7 |
| N. | 22 | 16 | 8 | 13 | 8 | 9 |

## D

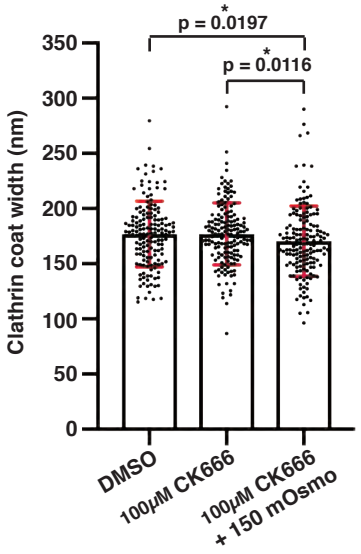



| | pre DMSO | pre CK666 | DMSO | 150 mOsmo | 100μM CK666 | 100μM CK666 + 150 mOsmo |
|---|---|---|---|---|---|---|
| Mean | 16.0 | 16.0 | 14.8 | 11.3 | 12.4 | 8.8 |
| SD | 8.5 | 4.1 | 2.4 | 3.5 | 2.2 | 3.7 |
| N. | 22 | 16 | 8 | 14 | 8 | 9 |

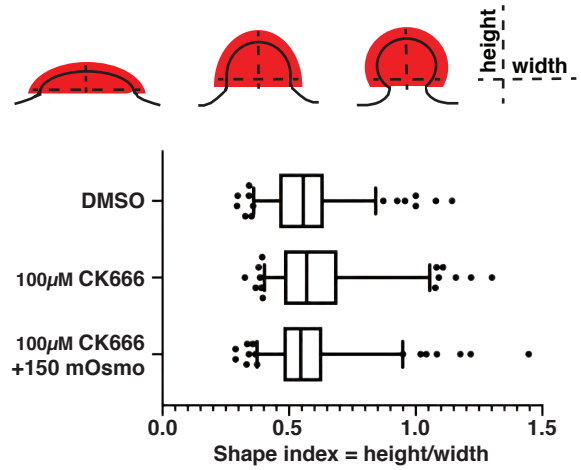
## E

| | Mean height (nm) | SD (nm) | N. of values | Mean width (nm) | SD (nm) | N. of values |
|---|---|---|---|---|---|---|
| DMSO | 98 | 21 | 154 | 177 | 30 | 154 |
| 100μM CK666 | 106 | 27 | 158 | 177 | 28 | 158 |
| 100μM CK666 + 150mOsmo | 96 | 24 | 159 | 171 | 32 | 159 |

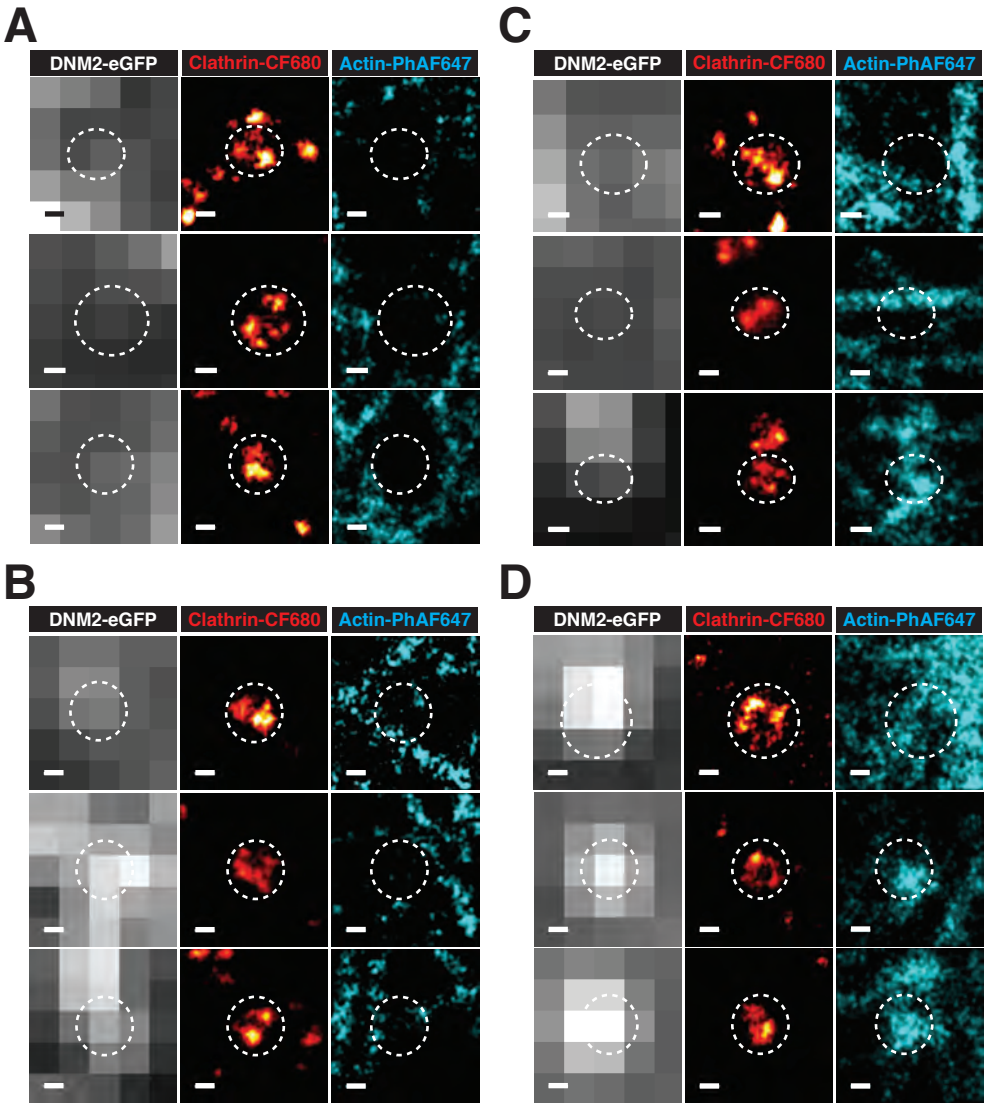
## F




## G



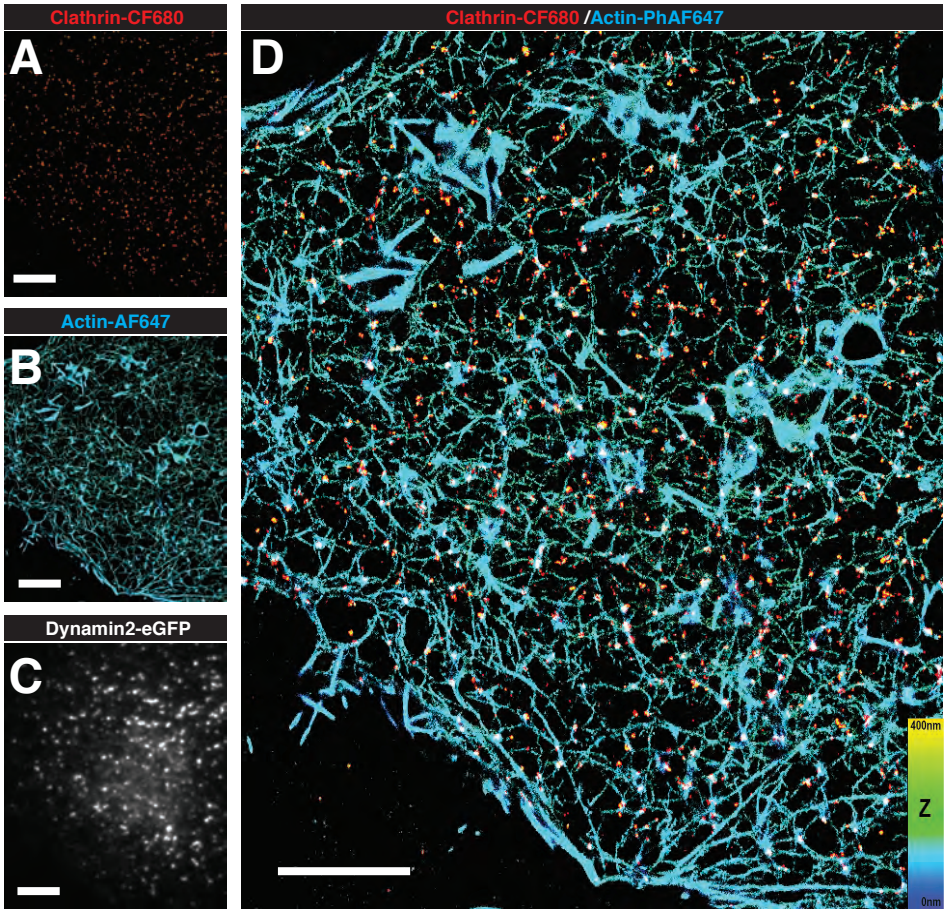
# Figure S4

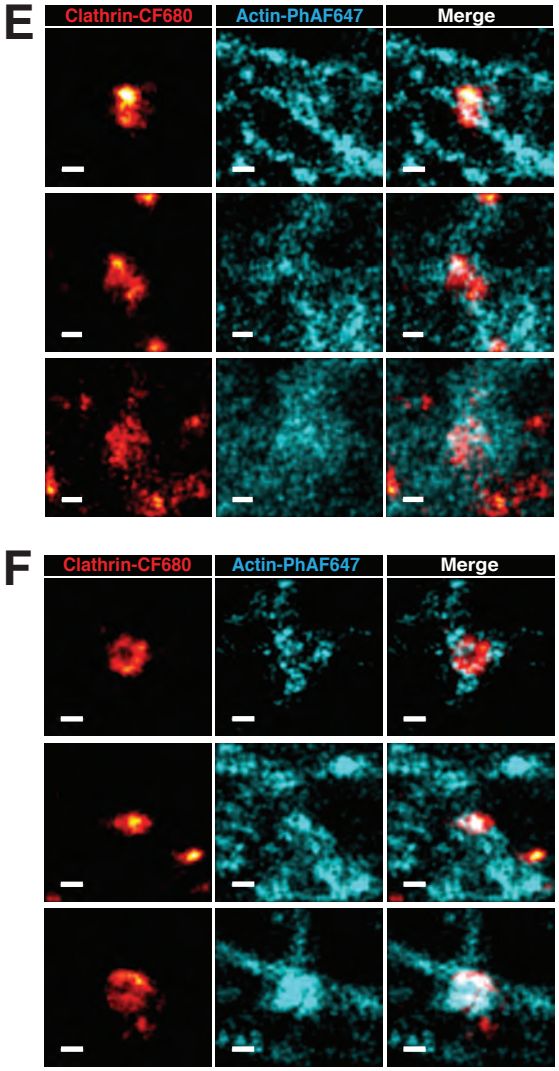


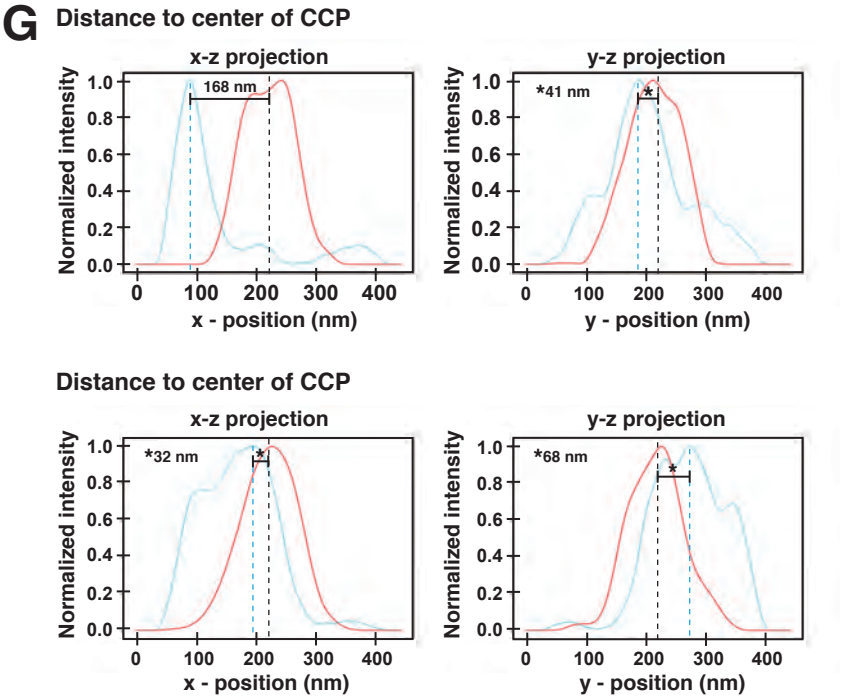

**E**

| Surface area | Early | Intermediate | Fission | Late |
|---|---|---|---|---|
| **W/o actin** | 0.0148 * | ns | ns | ns |
| **With actin** | 0.0035 ** | 0.0027 ** | < 0.0001 **** | ns |

# Figure S5




H

| | 75 mOsm | | | |
|---|---|---|---|---|
| | Mean deviation (nm) | SD (nm) | N. of values | p value |
| Early | 72 | 44 | 155 | < 0.0001 |
| Intermediate | 73 | 44 | 190 | 0.0087 |
| Fission | 70 | 45 | 236 | ns |
| Late | 72 | 45 | 401 | 0.0010 |

# Table 1

| | | Isotonic media | | | | Hypotonic media | | | |
|---|---|---|---|---|---|---|---|---|---|
| W/o Actin | Surface area | Early | IM | Fission | Late | Early | IM | Fission | Late |
| | Mean ($nm^2$) | 62055 | 71048 | 68911 | 63762 | 69455 | 69606 | 80071 | 67607 |
| | S.D. ($nm^2$) | 16951 | 18854 | 23305 | 22419 | 20014 | 17353 | 26077 | 22633 |
| | N. | 85 | 31 | 23 | 117 | 85 | 70 | 72 | 132 |
| With Actin | Surface area | Early | IM | Fission | Late | Early | IM | Fission | Late |
| | Mean ($nm^2$) | 54811 | 61145 | 57936 | 58450 | 62666 | 68347 | 73414 | 59022 |
| | S.D. ($nm^2$) | 17328 | 17360 | 17268 | 22654 | 21515 | 21643 | 25636 | 22969 |
| | N. | 168 | 211 | 156 | 201 | 96 | 101 | 126 | 204 |
| | p-value | ** 0.0013 | ** 0.0028 | * 0.0234 | * 0.0263 | * 0.0353 | ns | ns | *** 0.0005 |

# Table 2

| | Isotonic | | | Hypotonic | | | Isotonic | | | Hypotonic | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Height (nm) | SD (nm) | N. | Height (nm) | SD (nm) | N. | Coverage (%) | SD (%) | N. | Coverage (%) | SD (%) | N. |
| **Early** | 123 | 29 | 167 | 158 | 48 | 96 | 66 | 25 | 163 | 76 | 21 | 88 |
| **Intermediate** | 131 | 34 | 211 | 146 | 42 | 101 | 68 | 23 | 209 | 76 | 20 | 100 |
| **Fission** | 135 | 30 | 156 | 161 | 39 | 127 | 64 | 22 | 153 | 69 | 20 | 126 |
| **Late** | 130 | 30 | 202 | 165 | 46 | 204 | 65 | 23 | 196 | 73 | 22 | 198 |