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1 Membrane binding controls ordered self-assembly of animal septins

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3 Agata Szuba^{1#}, Fouzia Bano^{2§}, Gerard Castro Linares^{1,3}, François IV⁴, Manos Mavrakis^{4*}, Ralf P. Richter^{2*},
4 Aurelie Bertin^{5,6*}, Gijsje H. Koenderink^{1,3*}

5
6 ¹AMOLF, Department of Living Matter, Biological Soft Matter group, Science Park 104, 1098 XG Amsterdam, The
7 Netherlands

8 ²School of Biomedical Sciences, Faculty of Biological Sciences, School of Physics and Astronomy, Faculty of
9 Engineering and Physical Sciences, Astbury Centre for Structural Molecular Biology, and Bragg Centre for Materials
10 Research, University of Leeds, Leeds, LS2 9JT, United Kingdom

11 ³Department of Bionanoscience, Kavli Institute of Nanoscience Delft, Delft University of Technology, Van der
12 Maasweg 9, 2629 HZ Delft, The Netherlands

13 ⁴Institut Fresnel, CNRS, Aix-Marseille Univ, Centrale Marseille, 13013 Marseille, France

14 ⁵Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, CNRS UMR168, 75005, Paris, France

15 ⁶Sorbonne Université, 75005, Paris

16
17 *Corresponding authors: g.h.koenderink@tudelft.nl, r.richter@leeds.ac.uk, manos.mavrakis@univ-amu.fr,
18 aurelie.bertin@curie.fr

19 #Current address: Pollard Institute, School of Electronic & Electrical Engineering, University of Leeds, Leeds, LS2
20 9JT, United Kingdom

21 §Current address: Department of Clinical Microbiology, Målpunkt R, NUS, Umeå Universitet, Umeå, Sweden

22 23 Abstract

24 **Septins are conserved cytoskeletal proteins that regulate cell cortex mechanics. The mechanisms of**
25 **their interactions with the plasma membrane remain poorly understood. Here we show by cell-free**
26 **reconstitution that binding to flat lipid membranes requires electrostatic interactions of septins with**
27 **anionic lipids and promotes the ordered self-assembly of fly septins into filamentous meshworks.**
28 **Transmission electron microscopy reveals that both fly and mammalian septin hexamers form arrays**
29 **of single and paired filaments. Atomic force microscopy and quartz crystal microbalance demonstrate**
30 **that the fly filaments form mechanically rigid, 12 to 18 nm thick, double layers of septins. By contrast,**
31 **C-terminally truncated septin mutants form 4 nm thin monolayers, indicating that stacking requires**
32 **the C-terminal coiled coils on DSep2 and Pnut subunits. Our work shows that membrane binding is**
33 **required for fly septins to form ordered arrays of single and paired filaments and provides new**
34 **insights into the mechanisms by which septins may regulate cell surface mechanics.**

35 Introduction

36 Septins are a conserved family of cytoskeletal proteins [1] capable of forming filamentous scaffolds at
37 the cell cortex that participate in many processes such as cytokinesis, cell-cell adhesion, and
38 phagocytosis [2-5]. Most of what we currently know about the molecular mechanisms by which septins
39 function comes from studies of the budding yeast cell *S. cerevisiae*, where septins form hetero-octamers
40 [6-10] that form paired filaments [11-13]. During budding, septins form a collar encircling the bud neck
41 that acts as a scaffold to recruit proteins necessary for cell division [14-16], and that restricts lateral
42 diffusion of transmembrane proteins [17-19]. Compared to yeast septins, animal septins have a much
43 more variable cellular localization that changes with tissue type, developmental stage, and cell cycle
44 state [20, 21]. They are frequently found at curved regions of the plasma membrane such as the base of
45 dendritic spines and cilia, where they provide a diffusion barrier [22-24]. However, animal septins also
46 associate with flat regions of the cell cortex, where they influence the rigidity and contractility of the
47 actin-myosin cortex within single cells [25-27] and within multicellular tissues [28-30]. Cortical septins
48 also play a key role in cell division, where they are needed to stabilize the actomyosin ring [31] and
49 recruit proteins that mediate chromosome segregation and abscission [32-34].

50 Biochemical reconstitution studies have shown that mammalian septins can bind
51 phosphoinositides [4, 35-38], but animal septins have also been shown to bind actin filaments [39-41]
52 and various actin-binding proteins including anillin [42] and non-muscle myosin-2 [43]. Electron
53 microscopy of immuno-stained cells revealed localization of cortical septins with cortical actin in tissue
54 culture cells [44, 45], but the high density of the actin cortex in animal cells has made it impossible to
55 determine whether cortical septins directly interact with the plasma membrane. It is even unclear
56 whether cortical septins truly form filaments. Fluorescence microscopy has shown the presence of
57 cortical septin puncta, fibrous strands, or rings [25-27, 29], but has lacked the resolution to resolve the
58 precise nature of these structures. Septin-deficient cells exhibit a markedly reduced cortical rigidity [25-
59 27, 29], but it is unclear whether these effects imply a loss of membrane-bound septin filaments or a
60 loss of septin-mediated actin stabilization. A further complication is that microtubules have also been
61 found to interact with cortical septins [46].

62 Unlike in budding yeast, animal septins associate extensively with the actin and microtubule
63 cytoskeleton, and provide essential functions beyond cell division. Septin removal in nondividing cells
64 and tissues leads to dramatic phenotypes consistent with a loss of cortical integrity, notably the collapse
65 of early gastrulating embryos [47], loss of sperm integrity [48-50] and defects in neuron morphogenesis
66 [51, 52]. Despite the importance of animal septins in these processes, it remains unclear whether the
67 cortical septin pool in animal cells associates with the plasma membrane itself or with membrane-bound
68 actin filaments and microtubules [44, 53]. To resolve the functions of septins at the animal cell cortex, it
69 is necessary to understand the innate ability of septins to assemble alone and in association with the
70 membrane. Several reconstitution studies have been reported for native and recombinant septins from
71 various animal species, but nearly all of these studies considered septin assembly in bulk solution. Frog
72 (*Xenopus laevis*) septins were found to form paired filaments similar to yeast septins [54], while
73 recombinant nematode (*C. Elegans*) septins [55] and native and recombinant fly (*Drosophila*
74 *melanogaster*) septins [39, 56, 57] were observed to form bundles. Recombinant mammalian septin
75 hexamers have been shown to form rings and spirals that are reminiscent of structures formed by Shs1-
76 capped yeast septin octamers [58]. To the best of our knowledge, there are only two reports until now
77 of the organization of animal septins on lipid membranes, showing that septin-enriched fractions from

78 porcine brain extracts can tubulate giant liposomes and form filamentous meshworks encasing the
79 membrane tubes [36, 38].

80 Here we investigate the role of membrane-binding in animal septin assembly by reconstituting
81 recombinant animal septins on flat supported lipid bilayers. We focus on septin hexamers composed of
82 Pnut, DSep2, and DSep1 from the model organism *Drosophila*. We studied the septins on model cell
83 membranes composed of phosphatidylcholine (PC) lipids combined with either phosphatidylserine (PS)
84 or phosphatidylinositol(4,5)-bisphosphate (hereafter referred to as PI(4,5)P₂). PS is the most abundant
85 anionic lipid in the inner leaflet of the plasma membrane [59]. The cell membrane in the fly has been
86 reported to contain 3-10% PS, but the composition varies among tissues and developmental stages [60-
87 62]. PI(4,5)P₂ is an anionic signaling lipid previously shown to interact with yeast and mammalian septins
88 [35, 63, 64] and assumed to be important for septin-membrane interactions in the fly [65, 66]. We show
89 by fluorescence imaging that fly septin hexamers have a high affinity for membranes containing PS
90 and/or PI(4,5)P₂, and form dense membrane-associated meshworks. Nanometer-resolution imaging by
91 electron microscopy and by atomic force microscopy reveals that these meshworks comprise single and
92 paired filaments, which laterally associate into bundles that form tightly packed domains. We propose a
93 molecular model of the septin self-assembly mechanism that is consistent with these new data. Our
94 findings establish that membrane binding catalyzes animal septin polymerization and has a dramatic
95 impact on septin self-assembly, with C-terminal coiled-coils playing a key role in higher-order septin
96 filament organization.

97

98 Results

99

100 Septin hexamers form bundles in solution

101 To understand how membranes influence septin assembly, we begin by analyzing how septin oligomers
102 assemble in free solution. We focus on septin hexamers composed of DSep1, DSep2, and Pnut from the
103 model organism *Drosophila*, which have been previously characterized *in vivo* [39, 47, 67, 68] and *in*
104 *vitro* [39, 56, 57], and which are highly homologous to their human septin orthologs (DSep1, DSep2 and
105 Pnut are 75%, 68% and 65% identical to SEPT2, SEPT6 and SEPT7, respectively; see Methods section).
106 Based on phylogenetic analysis that classifies fly and human septins in distinct subgroups (DSep1/SEPT2
107 in the SEPT2 subgroup; DSep2/SEPT6 in the SEPT6 subgroup; Pnut/SEPT7 in the SEPT7 subgroup [69,
108 70]), and the recently demonstrated order of subunits in mammalian septin complexes [71-73], fly
109 septin hexamers should have a palindromic arrangement with the Pnut subunits in the center, the
110 DSep1 subunits at the termini, and the DSep2 subunits in between (**Figure 1A**). Each subunit carries a
111 flexible carboxy-terminal extension, a part of which is predicted to adopt a coiled-coil arrangement [74].
112 Using the coiled-coil prediction algorithm COILS (see Methods section), we predict the C-terminus of
113 DSep1 to form a coiled-coil that is 28 residues long, and the C-termini of Pnut and DSep2 to form coiled-
114 coils that are 86 residues long (**Figure 1B**). Our coiled-coil prediction analysis for human septins yields
115 the same predictions, *i.e.*, 28-residue-long coiled-coils for SEPT2 and 86-residue-long coiled-coils for
116 each SEPT6 and SEPT7 (**Figure 1C**). Considering a 1.5 Å rise per residue in an alpha-helix, we estimate the
117 coiled-coil of DSep1/SEPT2 to be 4 nm long, and the coiled-coils of DSep2/SEPT6 and Pnut/SEPT7 to be
118 13 nm long (**Figure 1A**). Hexamers form 24 nm-long rods, with the globular domains (G-domains) of the
119 septin subunits approximately 4 nm in diameter [39, 75]. The coiled-coil of DSep1 could thus extend as
120 much as the G-domain itself, while the coiled-coils of DSep2 and Pnut could even extend as much as 3-

121 fold the size of the G-domain. From the end of the α 6-helix to the start of the predicted coiled-coils,
122 there are stretches of 24, 15 and 15 residues for DSep1, DSep2 and Pnut, respectively (see **Figure 1B**),
123 that are predicted to be unstructured. Given a contour length per residue of ~ 0.4 nm, these lengths
124 translate to contour lengths of 6 to 10 nm. These regions thus likely act as a flexible hinge between the
125 G-domain and the coiled-coil, allowing the coiled-coils to pivot around their anchor points. This
126 prediction is consistent with observations that coiled coils did not show up in X-ray crystal structures or
127 particle-averaged electron microscopy (EM) analysis of yeast and mammalian septin oligomers [9, 75].
128 Moreover, EM images of yeast, *Drosophila*, and *C. elegans* septin oligomers showed direct evidence that
129 the coiled coils can sweep out a large volume [9, 39, 55].

130 To test the ability of the recombinant fly septin hexamers to polymerize in bulk solution, we
131 performed TIRF imaging of mEGFP-tagged septin hexamers (**Figure 1D**) after rapid dilution from a high
132 salt storage buffer containing 300 mM KCl to a low salt polymerization buffer containing 50 mM KCl. We
133 expect fly septin hexamers to form bundles under these conditions [39, 76]. To enable observation of
134 septins in the thin (100 nm) evanescent TIRF field, we pushed them down onto a coverslip passivated
135 with a neutral (PC) lipid bilayer with the crowding agent methylcellulose at a concentration of 0.1-wt%,
136 which is high enough to crowd the bundles to the surface yet low enough not to cause bundling. As
137 shown in **Figure 1D**, septin hexamers did not form any structures visible at the resolution of the light
138 microscope until the concentration reached 200 nM. When we increased the concentration further to
139 500 nM, the septin hexamers formed longer bundles. Bundle formation was very rapid, taking less than
140 2 minutes for completion. The bundles were rather straight and did not exhibit measurable thermal
141 bending undulations, indicating that they must be stiff.

142 To measure the width and mass of the septin bundles, we turned to scanning transmission
143 electron microscopy, which provides quantitative mass measurements based on the dark-field signal
144 from elastically scattered electrons emitted under a high angle [77]. The sample is raster-scanned with a
145 focused electron beam and the intensity in each image pixel is converted to the projected specimen
146 mass by including tobacco mosaic virus (TMV) rods with a well-defined mass-per-length (MPL) of 131
147 kDa/nm as an internal calibration. To test the accuracy of the mass mapping method, we first imaged
148 budding yeast septins, since these are already known to form paired filaments with a theoretical mass
149 per length of 23.2 kDa/nm [9]. As shown in **Figure 1E**, the yeast septins form thin semiflexible polymers
150 that are weaker in intensity than the TMV rods, consistent with their smaller mass per length (**Figure 1 –**
151 **Figure supplement 2A,B**). The average mass-per-length (based on 10 septin filaments from 3 images)
152 was ~ 20 kDa/nm, close to the mass-per-length expected for paired filaments. In rare cases the image
153 clearly showed a double-stranded structure with two filaments running in parallel with a small spacing in
154 the range of 17 nm – 27 nm, consistent with prior transmission EM findings [9] (see lower panel of
155 **Figure 1E**). By contrast, fly septins formed thick bundles that were stronger in intensity than the TMV
156 rods, indicating a larger MPL (**Figure 1F** and **Figure 1 – Figure supplement 2C**). Bundles formed at 200
157 nM had tapered ends and a thicker center. The example bundle in **Figure 1F** has a MPL of around 250
158 kDa/nm in the center (marked by an asterisk) and a corresponding center width of 31 nm. Given a
159 calculated MPL of 12.8 kDa/nm per fly septin hexamer, this number translates to around 20 hexamers
160 per cross-section. Considering that the inter-filament spacing has to be at least 4 nm, a width of 31 nm
161 and 20 hexamers per cross-section implies there are at least 3 monolayers. At 500 nM, the septin
162 bundles were thicker (**Figure 1G**) with maximal widths of up to 280 nm and MPL values up to ~ 5000
163 kDa/nm (**Figure 1 – Figure Supplement 2D**), corresponding to >400 hexamers per cross-section or a

164 thickness of at least 6 monolayers. This analysis suggests that septin bundles in solution do not grow as
165 flat sheets but as 3D bundles.

166 **Lipid membranes recruit septins and promote their assembly**

167 To investigate how membrane-binding affects septin hexamer polymerization, we deposited the purified
168 fly septin hexamers in polymerization buffer on glass-supported lipid bilayers (SLBs) composed of net-
169 neutral PC lipids doped with anionic lipids (**Figure 2A**). To test whether fly septin hexamers bind
170 PI(4,5)P₂, as reported for yeast septins [63, 64], we doped the PC membranes with different mole
171 percentages of PI(4,5)P₂ ranging from 1 to 8%. Already at 1% PI(4,5)P₂, septin hexamers formed
172 immobile bundles adhering to the membrane (**Figure 2B**). Importantly, membrane recruitment was
173 observed here in the *absence* of methylcellulose, showing that septin hexamers indeed bind to PI(4,5)P₂
174 lipids. As we raised the PI(4,5)P₂ content from 1% to 8%, we observed a striking transition from a sparse
175 arrangement of thick bundles to a dense protein layer. At intermediate PI(4,5)P₂ fractions of 2.5% and
176 5% we could still discern septin bundles, but at 8% PI(4,5)P₂ the protein density was too high to discern
177 any details of the ultrastructure.

178 To test whether fly septin hexamers selectively bind the PI(4,5)P₂ head group as reported for
179 yeast septins [63, 64] or simply bind through nonspecific electrostatic interactions, we next replaced
180 PI(4,5)P₂ by PS. As shown in **Figure 2C**, fly septin hexamers were also recruited to the PS-containing
181 bilayers and again showed a transition from a sparse distribution of thick bundles at low PS content to a
182 dense layer at high PS content. These experiments were again carried out in the absence of
183 methylcellulose (except for the reference image with 0% PS, where methylcellulose was required to
184 push septin bundles down into the TIRF field). The filamentous septin structures were already present as
185 soon as we could start imaging (~3 min after septin loading into the flow channel) and the structures did
186 not change in number or thickness over time (**Figure 2 – figure supplement 1A**). Apparently, septin
187 hexamers bind strongly and rapidly to the membrane. The total fluorescence intensity, which is a proxy
188 for the amount of membrane-bound septins, linearly increased as the PS content was raised from 5% to
189 20% (**Figure 2 – figure supplement 1B**). The observation that PS has a qualitatively similar effect
190 compared to PI(4,5)P₂ on septin recruitment and assembly on SLBs suggests that the membrane-binding
191 affinity of fly septin hexamers is governed by the net surface charge of the membrane rather than by
192 any specific affinity for PI(4,5)P₂. The transition from dilute septin bundles to dense septin films required
193 a larger PS mole fraction (10%) than PI(4,5)P₂ (2.5%), consistent with the larger net charge (-4) of the
194 head group of PI(4,5)P₂ due to the phosphate groups compared to the net charge (-1) on the head group
195 of PS [78-81]. Therefore, our findings suggest that the total negative surface charge of the membrane
196 governs septin hexamer adsorption, indicating that the septin-membrane interaction is primarily
197 electrostatic in origin.

198 To screen for the dependence of septin hexamer adsorption on bulk septin concentration, we
199 incubated bilayers containing 20 mole-% PS with solutions of septin hexamers over a wide range of
200 concentrations spanning from 10 to 300 nM (**Figure 2 – figure supplement 1C**). To obtain a bright
201 enough signal, we performed these experiments with 100% GFP-tagged hexamers. At low
202 concentrations (10-50 nM), we observed dim puncta and also brighter puncta, indicative of protein
203 aggregation (note that images are contrast-inverted). We could not distinguish clear evidence of
204 filaments. We suspect that the bright puncta are due to GFP-mediated aggregation because we
205 observed fewer bright puncta when we co-polymerized 10% GFP-tagged hexamers with 90% dark
206 hexamers. Remarkably, when we increased the septin concentration to values of 100 nM and higher, we

207 suddenly observed a fibrillar meshwork of septin filaments. At concentrations above 200 nM, we found
208 by off-TIRF-angle imaging that, in addition to the membrane-bound septin meshwork, also septin
209 bundles were present in the solution above the bilayer, consistent with the observed onset for bundling
210 in the absence of an adhesive membrane (**Figure 1D**). Fluorescence recovery after photobleaching
211 experiments showed that the membrane-bound septins exhibited negligible subunit exchange (**Figure 2**
212 – **figure supplement 2A**), indicating that they are stably anchored to the membrane. The membrane
213 underneath the septin film was nevertheless fluid, as indicated by FRAP measurements of rhodamine-
214 labeled PE tracer lipids (**Figure 2 – figure supplement 2B**).

215 To discern the ultrastructure of membrane-adsorbed septins, we turned to transmission
216 electron microscopy. We incubated septin hexamers with lipid monolayers, deposited these on EM
217 grids, and negatively stained the protein with 2% uranyl formate, following a protocol previously used
218 with yeast septins [63]. To test the role of membrane composition, we formed lipid monolayers by
219 mixing PC lipids with either 20 mol-% PS, 5 mol-% PI(4,5)P₂, or a combination of both that mimics the co-
220 existence of both lipids in the plasma membrane of cells [82, 83]. We chose a septin hexamer
221 concentration of 65 nM, below the bundling threshold (200 nM), to ensure that septin self-assembly was
222 initiated at the membrane. As shown in **Figure 3A-C**, septin hexamers formed densely packed arrays of
223 thin filaments in all three cases, which is qualitatively consistent with the dense meshworks observed by
224 TIRF microscopy. Close inspection of the EM micrographs revealed paired septin filaments (black arrows
225 point out examples) with an average center-to-center spacing of 5.7 ± 0.8 nm on monolayers containing
226 PS plus PIP(4,5)P₂ (see **Figure 3 – figure supplement 1A**). The width of the individual filaments was in the
227 range of 3.5 to 4 nm (3.6 ± 0.53 nm ($N = 60$) averaged over all lipid conditions; **Figure 3 – figure**
228 **supplement 1B**), consistent with the expected 4-nm width of fly septin hexamers [39].

229 On monolayers containing 20% PS plus 5% PI(4,5)P₂, we could observe clear examples of paired
230 filaments that were bridged at intervals of 24 nm by single or double hexamer rods (**Figure 3C**),
231 recognizable by their lengths clustering around either 24 nm or 48 nm (**Figure 3 – figure supplement**
232 **1C**), respectively. These bridges were either perpendicular to the filaments they bridged (blue
233 arrowheads) or under an angle (red arrowheads; see **Figure 3 – figure supplement 1C** for
234 quantification). We observed comparable arrays of tightly paired filaments connected by orthogonally or
235 diagonally oriented single filaments for mammalian septins on lipid monolayers of the same
236 composition (**Figure 3 – figure supplement 2**). Similar arrays of filaments were previously also observed
237 for budding yeast septins [58, 63], suggesting that this architectural feature is conserved within
238 eukaryotes. We do note a subtle difference for the mammalian septin hexamers as compared to the fly
239 septins. For the fly septin hexamers, the bridges between paired filaments are single or double isolated
240 hexamer rods. Instead, for the mammalian septin hexamers, the paired filaments (white arrows in
241 **Figure 3 – figure supplement 2**) are intersected with a distinct set of thinner continuous filaments (red
242 arrows), creating a network of interconnected and perpendicular filaments. It is unclear whether the
243 bridging filaments are single or paired; if paired, the filaments could be rotated or twisted to appear
244 thinner.

245 Since the sample preparation for the EM experiments requires drying and negative staining, we
246 corroborated the findings from the lipid monolayer assays by cryo-EM imaging on lipid bilayers using
247 large unilamellar vesicles (LUVs) comprised of PC and 5 mol-% PI(4,5)P₂ incubated with septin hexamers.
248 We chose a septin hexamer concentration of 160 nM, higher than the 65 nM concentration used in the
249 lipid monolayer experiments, because we expected reduced binding to the positively curved LUV

250 membranes [84]. As shown in **Figure 4**, the cryoEM images confirm the presence of paired septin
251 filaments on PI(4,5)P₂-containing lipid membranes (examples are traced out by double red lines in
252 **Figure 4A-B**). The average center-to-center distance between the filaments forming a pair was 5.7 ± 0.8
253 nm ($N = 40$, see **Figure 3 – Figure supplement 1** and **Figure 4 – figure supplement 1**), in excellent
254 agreement with the negative stain images of septin filaments on lipid monolayers. In addition, we
255 occasionally observed single filaments or hexamers (examples are traced out by single red lines in **Figure**
256 **4B**) including some that appeared to interconnect filaments at an orthogonal or oblique angle (blue lines
257 in **Figure 4B**). The density of filaments varied among vesicles (see **Figure 4** and **Figure 4 – figure**
258 **supplement 2**), which might be due to some variability in the proportion of charged lipids incorporated
259 within the vesicles. Besides, membrane binding could potentially be curvature-dependent, as was shown
260 for yeast septin octamers reconstituted on membrane-coated beads, rods, and wavy substrates [84, 85].
261 An indication for curvature-dependent binding in our data is that the diameter of control vesicles was
262 measured to be 358 ± 180 nm ($N = 40$) while the diameter of similar vesicles displaying septins was 542
263 ± 199 nm ($N = 24$), thus slightly higher, on average (**Figure 4 – figure supplement 3**). This may suggest
264 that septins preferentially interact with liposomes of lower curvatures. Vesicles decorated with septin
265 filaments typically appeared to have a faceted contour, whereas vesicles alone usually retained their
266 spherical shape in cryoEM. Note that the bilayer perpendicular to the electron beam is poorly defined
267 because of the missing wedge. The deformed membranes provide a first indication that septins were
268 bound to the membrane and able to flatten it. Some septin filaments clearly protrude a bit beyond the
269 vesicle they adhere to, suggesting they were pre-polymerized in solution and attached to one side of the
270 vesicle without fully wrapping around it. Cryo-electron tomography gave further clear evidence that
271 septin filaments are indeed membrane-bound (see **Figure 4C-D** and **Figure 4 - Movie 1**).

272

273 **Septins form thin and mechanically resilient membrane-bound arrays**

274 We further complemented EM imaging with AFM experiments, which allowed us to image septins on
275 flat lipid bilayers under hydrated conditions resembling the conditions in the TIRF experiments. We
276 performed these experiments on silica-supported lipid bilayers containing 20% PS, which have already
277 been extensively characterized in their quality by AFM and other biophysical techniques [86]. In
278 particular, the even inter-leaflet distribution of PS lipids in liposomes is approximately preserved upon
279 SLB formation on silica (and glass) supports, whereas this is not the case on mica [87], another
280 commonly used support for AFM. We first tested the dependence of septin hexamer assembly on septin
281 concentration by imaging septins at concentrations of 12, 24 and 60 nM (**Figure 5A-B**). We chose this
282 narrow concentration range based on the TIRF data, which showed that septin assembly is restricted to
283 the membrane surface as long as the septin hexamer concentration is below 200 nM. In most
284 experiments the samples were fixed with 1% glutaraldehyde (GTA) to prevent septin disruption by the
285 AFM tip.

286 At 12 nM, septin hexamers formed threads of typically several micrometers in length that were
287 sparsely and randomly distributed on the bilayer surface (**Figure 5A-B**, left column). These were mostly
288 isolated but could also be seen to meet, mostly at a shallow angle, and merge. Their apparent height
289 was mostly uniform at 5.1 ± 0.9 nm ($N = 29$), with a few notable exceptions around 12 nm ($N = 2$). The
290 apparent width showed two relatively broad but distinct populations: most isolated threads had a width
291 of 11.5 ± 1.6 nm ($N = 19$), whereas all merged threads and a few isolated ones were wider (16.6 ± 0.9
292 nm; $N = 12$) (**Figure 5C**, left column, and **Figure 5 – figure supplement 1**). These values are consistent

293 with a mixture of mostly single and paired filaments. This can be appreciated if one considers that the
294 minimal filament height is defined by the extension of the globular domain (4 nm) plus some additional
295 contribution by the coiled coils which, owing to the flexible hinge, may point in various directions and
296 additionally become flattened by the force exerted by the AFM tip. Moreover, the flexing of the coiled
297 coils and tip convolution effects (see Methods for details) can explain the relatively broad width
298 distributions, and why the apparent mean widths exceeded the widths of single and paired filaments as
299 seen by EM by about 8 nm on average. The sparse surface coverage observed at 12 nM is consistent
300 with the coverage expected for binding from a semi-infinite and still bulk solution to a planar surface.
301 Mass-transport limitations provide a robust upper limit for the amount of bound protein as $\Gamma \leq$
302 $2c\sqrt{Dt/\pi}$ [88]. With an incubation time $t = 15$ min, a septin concentration $c = 10$ nM, and diffusion
303 constant $D \approx 20 \mu\text{m}^2/\text{s}$ (a rough estimate for molecules with the size of septin hexamers in aqueous
304 solution), this gives $\Gamma \leq 0.10$ pmol/cm². Further assuming a footprint of 4 nm × 24 nm per septin
305 hexamer, the expected surface coverage is $\leq 6\%$, consistent with the sparse coverage observed by AFM.

306 At 24 nM, septin hexamers formed threads that were isolated in some places and concentrated
307 in areas of enhanced density in other places (**Figure 5A-B**, center column). Thread heights and widths
308 were comparable to the 12 nM conditions, although with a higher proportion of higher (up to 13 nm)
309 and wider threads (**Figure 5C**, center column), suggesting the increased presence of paired filaments
310 and the initiation of bundles made from more than two filaments.

311 At 60 nM, the septin hexamers formed threads that densely covered the bilayer surface (**Figure**
312 **5A-B**, right column) in closely apposed patches of aligned filaments, resembling nematic domains
313 observed for other 2D arrays of densely packed semiflexible biopolymers [89]. Salient features of the
314 patches were that the constituent threads varied in height and width, and that they formed a network
315 that is characterized by threads frequently joining and disjoining at a fairly shallow angle of
316 approximately 15 degrees. The smallest thread heights and widths observed were comparable to those
317 observed at 12 nM, indicating isolated single or paired filaments were still present. Other threads
318 appeared much wider (up to a few 100 nm; **Figure 5C**, right column). This suggests that many (up to
319 several tens of) single filaments may closely align on the lipid membrane, although the lateral resolution
320 was insufficient to reveal the individual filaments and their spacing. The wider threads also had an
321 elevated height, mostly between 8 and 13 nm and occasionally up to 18 nm (**Figure 5C**, right column),
322 suggesting that septins also stacked on top of each other.

323 Importantly, the morphologies of unfixed septins resembled those of GTA-fixed ones, though
324 imaging in this case was challenging because the filaments were more easily disrupted by the AFM tip
325 (**Figure 5 – figure supplement 2**). For one 60 nM unfixed sample, we came across bilayer areas where
326 the septin coverage was low enough to reveal septin filaments that were isolated or ran close together
327 and in parallel with others (**Figure 6**). Here we observed that the isolated filaments had heights of 4 nm,
328 corresponding to the height of a single septin hexamer and thus a single layer of septin filaments, while
329 the bundled areas had heights between 8 nm and 12 nm, suggesting that septin filaments can stack on
330 top of each other. We note that in the EM data there is no such clear evidence of layering. However, we
331 note several experimental differences between AFM and EM. In AFM we used solid-supported lipid
332 bilayers as a membrane substrate for septins, whereas in EM we used either lipid monolayers at the air-
333 water interface (for negative stain images) or vesicles (for cryoEM), where the curvature likely prevents
334 septins from reaching a high density of filaments.

335 Since AFM imaging involves mechanical scanning across the surface, it allowed us to
336 qualitatively test how firmly the septins are attached to the lipid bilayer by performing multiple
337 consecutive scans. In sparsely covered bilayer regions, wider bundles generally remained stable whereas
338 narrower bundles or isolated filaments (both GTA-fixed and unfixed) sometimes appeared ragged,
339 suggesting that the AFM tip moved them along the membrane (**Figure 5 – figure supplement 3A,B**).
340 Densely covered regions formed at 60 nM septins were completely unchanged after three consecutive
341 10-minute scans, again both for GTA-fixed and unfixed samples (**Figure 5 – figure supplement 3C,D**).
342 These observations suggest that lateral associations among septin filaments within bundles or dense
343 arrays may cause mechanical stabilization. Some image sequences clearly showed that entire bundles
344 can be laterally displaced along the membrane owing to lateral forces exerted by the AFM tip.
345 Interestingly, these events did not destroy the bundles but resulted in permanent local kinks, suggesting
346 the septin filaments are intrinsically stiff and ductile but the link to the membrane is fluid. We again
347 observed this behavior both for GTA-fixed and unfixed samples (**Figure 5 – figure supplement 4A,B**).

348

349 **QCM-D measurements show self-limiting septin assembly away from the membrane plane**

350 The AFM data suggest that fly septin hexamers form organized layers of stiff filaments on PS-containing
351 bilayers, with a limited thickness under conditions where assembly is initiated at the membrane (*i.e.*, at
352 septin concentrations below 200 nM). The maximum thickness of the septin films at 60 nM was
353 approximately 18 nm, according to the height profiles. However, we note that this value may
354 underestimate the actual geometrical height because the tip can potentially indent or otherwise disturb
355 the septin layer. To independently measure the film thickness, and to gain insight into the kinetics of
356 septin film formation, we therefore turned to quartz crystal microbalance with dissipation monitoring
357 (QCM-D), an acoustic technique that measures the mass and variations in the mechanical properties of
358 interfacial films in real time [90]. We first formed an SLB on the silica-coated QCM-D sensor by perfusing
359 the channel with a suspension of sonicated unilamellar vesicles (SUVs) and next perfused with a solution
360 of septin hexamers. To investigate the kinetics of SLB formation and septin binding, we monitored the
361 resulting shifts in the resonance frequency Δf , which is proportional to the areal mass density of
362 adsorbed biomolecules plus hydrodynamically coupled solvent, and in the dissipation ΔD , an indicator of
363 the mechanical properties of the adsorbed layer.

364 Typical QCM-D data are presented in **Figure 7**. The SUVs are composed of 80% PC and 20% PS,
365 as in the AFM experiments. SUV perfusion on a plain silica surface (**Figure 7A**) caused immediate
366 changes in Δf and ΔD in a two-stage process that is characteristic for the initial adsorption of intact SUVs
367 to the sensor surface followed by SUV rupture, spreading and coalescence [91]. The final shifts of $\Delta f = -$
368 25 ± 1 Hz and $\Delta D < 0.2 \times 10^{-6}$ ($N = 6$) are typical for confluent SLBs composed of PC and PS of high
369 quality, *i.e.*, with a negligible quantity of residual intact vesicles [86]. Subsequent rinsing with vesicle
370 buffer did not result in appreciable changes in Δf and ΔD , confirming the SLB was stable. Septin hexamer
371 perfusion at 60 nM on such a SLB (**Figure 7B**) produced an immediate decrease of Δf and a concurrent
372 increase of ΔD , indicating that septins adsorbed. Binding reached a plateau after about 50 minutes, and
373 subsequent perfusion with buffer caused no appreciable change in Δf , suggesting that septin hexamers
374 stably adsorb to the membrane. Moreover, the saturation of the signal despite continued perfusion with
375 septin hexamers indicates the presence of a mechanism that limits septin binding and film growth. We
376 observed similar binding kinetics and self-limiting levels of binding on SLBs containing 5% PI(4,5)P₂, or a
377 combination of 5% PI(4,5)P₂ and 20% PS (**Figure 7 – figure supplement 1A,B**). In contrast, we did not

378 observe any adhesion of septin hexamers to pure PC membranes (**Figure 7B**; dotted blue line with
379 diamonds). We conclude that the presence of negatively charged lipids is required for septin-membrane
380 binding, consistent with the TIRF observations, and that film growth is self-limiting.

381 The total frequency shift for septin binding was $\Delta f = 93 \pm 4$ Hz ($N = 4$) on SLBs containing 20%PS,
382 $\Delta f = 77 \pm 2$ Hz ($N = 2$) on SLBs containing 5% PI(4,5)P₂, and $\Delta f = 113 \pm 7$ Hz ($N = 2$) on SLBs containing both
383 5% PI(4,5)P₂ and 20% PS (**Figure 7 – figure supplement 1C**). Using the Sauerbrey equation (see Eq. [1]),
384 we estimate septin film thickness values of 15 nm on SLBs containing 20% PS, 13 nm on SLBs containing
385 5% PI(4,5)P₂, and 19 nm on SLBs containing both 5% PI(4,5)P₂ and 20% PS, from the respective frequency
386 shifts. This is consistent with the upper end of bundle heights measured by AFM (**Figure 5C**, right
387 column), which we had attributed to stacking into more than one layer.

388 The dissipation shifts are consistent with the septin film having soft molecular linkers within the
389 structure. To a first approximation, the $\Delta D/\Delta f$ ratio is a measure of elastic compliance (*i.e.*, softness)
390 [90], and for globular proteins the major source of compliance typically are flexible hinges linking the
391 proteins to the surface or inter-connecting protein domains [90, 92]. Membrane-bound septins attained
392 dissipation over frequency shift ratios $\Delta D/\Delta f$ between $(0.20 \pm 0.01) \times 10^{-6}/\text{Hz}$ at low coverage ($\Delta f = -15 \pm$
393 5 Hz) and $(0.09 \pm 0.01) \times 10^{-6}/\text{Hz}$ at high coverage ($\Delta f < -90$ Hz; $N = 4$; **Figure 7 – figure supplement 2**).
394 For comparison, monolayers of streptavidin attain $\Delta D/\Delta f$ ratios of $<0.01 \times 10^{-6}/\text{Hz}$ when physisorbed on
395 gold, and between $0.08 \times 10^{-6}/\text{Hz}$ (at low coverage) and $0.015 \times 10^{-6}/\text{Hz}$ (at high coverage) when linked
396 *via* biotins on a short, flexible linker to a supported lipid bilayer [92]. The higher $\Delta D/\Delta f$ ratios for septins
397 are consistent with a high degree of flexibility in the linkage between the coiled coils and the globular
398 domains of septin hexamers. Indeed, $\Delta D/\Delta f$ ratios close to $0.1 \times 10^{-6}/\text{Hz}$ have been reported for
399 monolayers of neutravidin with short (*i.e.*, several ten base pairs long) double-stranded DNA strands
400 grafted to it [93], and for monolayers of streptavidin with linear oligosaccharide chains grafted to it [94].
401 Possibly the compliance of the coiled coils themselves, and/or the linkage between septins and the
402 membrane, also contribute to the elevated $\Delta D/\Delta f$ ratio relative to fully globular proteins.

403 **Septin's coiled coil domains are essential for forming multilayers**

404 The data described above collectively demonstrate that fly septin hexamers form filaments that interact
405 with each other both in the plane of the membrane, and out-of-plane. Which septin domains mediate
406 these interactions? One candidate are the C-terminal coiled coils. In prior work on yeast septin
407 octamers, coiled coils have already been proposed to be involved in septin filament pairing and, on
408 membranes, in the formation of perpendicular octamer cross-bridges between two paired filaments [9,
409 63]. Consistent with this view, a recent structural study of human septin coiled-coils employing X-ray
410 crystallography, NMR, and modelling indicated that the coiled coils can form both parallel dimers that
411 stabilize septin filaments and antiparallel dimers that form cross-bridges [95]. A second candidate are
412 the G-domains, which have been proposed to be involved in lateral interactions among yeast septin
413 filaments on membranes [63].

414 In order to test the arrangement of septin hexamers in the presence of G-domain interactions
415 alone, we generated coiled-coil truncated fly septin hexamers (ΔCC mutant). Transmission EM
416 confirmed that fly septins with their C-termini truncated from all subunits form stable hexamers (**Figure**
417 **1 – figure supplement 1B**), similarly to C-terminally truncated human septin hexamers [75]. EM imaging
418 showed that in low salt polymerization buffer, the ΔCC mutant only formed short rods (**Figure 8A-B**)
419 with lengths varying continuously between ~ 20 and ~ 80 nm (**Figure 8 – figure supplement 1**). However,
420 on lipid monolayers the C-terminally truncated fly septin hexamers formed dense arrays of aligned

421 filaments (**Figure 8C-F**). Thus, membrane-binding promotes filament formation, mirroring earlier
422 observations for yeast septin octamers with C-terminally truncated Cdc11 subunits [63]. The width of
423 the Δ CC septin filaments was on average slightly smaller than that of full length septin filaments, around
424 3.5 nm (**Figure 3 – Figure supplement 1B**), likely due to the absence of coiled coils. In places we can see
425 three filaments side by side instead of two (indicated by triple blue lines in **Figure 8D**). The mutant
426 filaments were even more closely spaced than wild type septin filaments, with a center-to-center
427 distance for filaments within pairs of around 4.5 nm (**Figure 8G and Figure 3 – Figure supplement 1A**),
428 consistent with direct contact between the globular domains deprived of the CC domain of adjacent
429 filaments due to crowding and perhaps specific G-domain interactions.

430 The septin density on the lipid monolayers observed in EM images for the Δ CC mutant was
431 notably higher than for the full-length septins, even though the solution concentrations were the same.
432 This observation suggests that the Δ CC mutant perhaps has a reduced tendency to form multilayers. To
433 test this hypothesis, we performed QCM-D measurements for the Δ CC mutant on bilayers containing
434 20% PS. As shown in **Figure 7C**, the Δ CC mutant binds to the bilayers but the frequency shift is much
435 smaller than for the full-length septins, indicating a thinner layer. In this case, the $-\Delta f$ values remained
436 less than 10 Hz, consistent with a single and sparse septin monolayer. Thus, the EM and QCM-D data
437 demonstrate that the coiled coils are not needed for membrane binding, but they are needed for
438 filament pairing, the formation of cross-bridges, and the stacking of septins on top of each other.

439
440

441 Discussion

442

443 Species-dependence of septin-membrane interactions

444 We investigated the influence of membrane-binding on septin hexamer assembly by reconstituting
445 recombinant animal septins on supported lipid bilayers and imaging septin assembly with several
446 complementary techniques. Fluorescence imaging revealed that fly septin hexamers have a high affinity
447 for negatively charged lipid bilayers, which competes with the septin–septin lateral interactions that
448 prevail in bulk solution, and that they form dense membrane-associated meshworks. Electron
449 microscopy (EM) imaging revealed that these meshworks are predominantly comprised of paired
450 filaments and meshworks thereof. A similar organization was observed for mammalian septin hexamers,
451 consistent with earlier findings for septin-containing porcine brain extracts [36, 38]. Finally, atomic force
452 microscopy (AFM) of septins on lipid bilayers showed that septin filaments laterally associate into
453 bundles but also stack on top of each other. AFM imaging, fluorescence recovery after photobleaching
454 and QCM-D experiments indicate that the septins are immobile and firmly attached to the membrane.
455 By contrast, C-terminally truncated septin hexamers form monolayers and the filaments do not form
456 pairs and meshworks, showing that the coiled coils of septins are crucial for mediating septin-septin
457 interactions.

458 Prior *in vitro* studies of septin-membrane binding focused mainly on mitotic, Cdc11-capped
459 budding yeast septin octamers [63, 96]. We find several striking similarities between the membrane
460 interactions of animal (fly and mammalian) septin hexamers and Cdc11-capped yeast septin octamers.
461 First, membrane binding in both cases promotes septin polymerization. For fly septin hexamers, the
462 threshold for polymerization is lowered more than 10-fold, from 200 nM in solution to less than 12 nM
463 on negatively charged membranes (**Figure 9A**). For yeast septins, a similar enhancing effect was

464 observed on membranes containing PI or PI(4,5)P₂ [63, 96]. The polymerization-enhancing effect of
465 membrane binding is likely due to the increase of the effective septin concentration caused by 2D
466 confinement. In addition, membrane binding may orient septins in a manner that promotes their
467 polymerization, as seen for other proteins such as annexin A5 [97]. A second similarity between the
468 behavior of animal and yeast septins on membranes is that they both form paired filaments visible by
469 transmission electron microscopy, with a narrow spacing of ~2 nm, much narrower than the ~10 nm
470 spacing observed for yeast septin filaments formed in solution [9]. A third similarity is that paired
471 filaments of both animal and yeast septins are interconnected by bridges visible by electron microscopy,
472 which appear to be formed by short and thin (single) septin filaments having a length and axial spacing
473 that correspond to the length of a single protomer [63]. Similar arrays have been observed by electron
474 microscopy in yeast cells [98], but not yet in animal cells. Note that yeast septins were studied only by
475 transmission electron microscopy, which provides a 2D-projection. It is unknown whether yeast septins
476 form multilayers on membranes, as we see for fly septins by AFM and QCM-D.

477 Our work also reveals two striking differences between animal and yeast septins. The first
478 difference is their polymerization in solution. Fly septins form thick and rigid bundles with tapered ends
479 in solution, suggesting that the septins have strong lateral interactions that promote bundling. On
480 membranes, however, we observed by TIRF microscopy a gradual transition from dilute arrays of thick
481 bundles to dense arrays of paired filaments with increasing net surface charge. This observation
482 suggests a competition of septin-membrane interactions with lateral septin-septin interactions, which
483 suppresses the formation of thick bundles on membranes. By contrast, yeast septin octamers tend to
484 form paired filaments in solution [9, 63] (as confirmed by quantitative mass-mapping STEM in our study,
485 see **Fig. 1E**), although they do form bundles under certain conditions [99]. Thus, in solution fly septins
486 have a stronger propensity for bundling than yeast septins, but on membranes the two septins behave
487 similarly. The second striking difference between yeast and animal septins revealed here is their
488 membrane binding selectivity. We find that fly septins form similar structures on membranes containing
489 PS or PI(4,5)P₂, and that the main determinant of fly septin binding and filament organization in both
490 cases is the net surface charge of the membrane. By contrast, Cdc11-capped yeast septin octamers were
491 shown to be highly selective for PI, PI(4)P, PI(5)P and PI(4,5)P₂, while they did not interact with PS [10,
492 37, 63, 64, 85, 96, 100, 101]. The origin of the differences in lipid specificity remains unknown. It is
493 thought that septins interact with negatively charged phospholipids *via* a polybasic region close to the
494 N-terminus that is composed of a sequence of 1 to 7 basic amino acids [35, 100]. This stretch is very
495 similar to polybasic sequences found in gelsolin, profilin, G-protein-coupled receptor kinases and ion
496 channels [35], which are all reported to interact with PI(4,5)P₂. In the future perhaps molecular
497 dynamics simulations can identify the determinants of lipid selectivity [102].

498 It is noteworthy that the intricate, highly-organized septin filament meshworks observed at the
499 membrane of dividing budding yeast (Bertin et al 2012, Ong et al 2014) have not been documented to
500 date in animal cells. Although we cannot exclude that such assemblies exist in animal cells, this
501 difference could be due to inherent differences between yeast and animal septins at the molecular level.
502 Despite the homology between yeast and animal septins (on the order of 25-44% sequence identity),
503 the end subunits of the respective septin protomers, SEPT2/DSep1 and Cdc11/Shs1 in human/fly and
504 yeast, respectively, differ considerably in their C-terminal extensions. Cdc11/Shs1 have C-termini that
505 are 2-3 times longer than their animal counterparts (118 and 211 residues for Cdc11 and Shs1 vs 55 and
506 57 residues for SEPT2 and DSep1, respectively). Cdc11 and Shs1 termini are predicted to form coiled-

507 coils twice as long as the ones of SEPT2/DSep1, and with flexible hinges between the α 6 helix and the
508 coiled-coils 2-3 times longer than their animal counterparts (**Fig. 1B,C** and ref. [103]). Given the above
509 differences between yeast and animal septins, the very high conservation between fly and mammalian
510 septins (on the order of 65-75% identity), and the ubiquitous presence and essential functions of animal
511 septins, studying animal septin assembly promises to advance our understanding of how differences
512 between yeast and animal septins lead to differences in their organization and function.
513
514

515 **Towards a model of septin assembly on membranes**

516 What do the imaging experiments collectively teach us about the assembly mechanism of fly septin
517 hexamers on lipid membranes? A firm conclusion is difficult because there are still a number of
518 important unknowns. It is still under debate where the membrane-binding region is located [85, 104,
519 105]. We observe that fly septin hexamers are recruited to the membrane by electrostatic interactions
520 with anionic lipids (PS and/or PI(4,5)P₂). It is generally believed that the N-terminal polybasic domains of
521 septins interact with the membrane, which would leave the coiled coils free to rotate in the half-space
522 above the membrane, although a recent study on yeast septin octamers suggested that membrane
523 binding is aided by an amphipathic helix motif identified on the C-terminal side of the coiled-coil of
524 Cdc12 [85]. In our case, however, we observe that C-terminally truncated septins still bind membranes
525 and our data on full-length septins are consistent with the coiled coils being available for interactions
526 with coiled coils on neighboring septins. Since the coiled coils are connected to the body of the septin
527 complex *via* unstructured regions that can act as hinges, we assume that the coiled coils can rotate in
528 the entire half-plane above the membrane, so they are available for both in-plane and out-of-plane
529 interactions with coiled coils on neighboring septin subunits/filaments. The nature of these interactions
530 is still unknown (*i.e.*, assembly of two vs. four coiled coils, and parallel vs. antiparallel assembly). A
531 recent structural study of human septin coiled coils indicated that these can form both parallel dimers
532 that stabilize septin filaments and antiparallel dimers that form cross-bridges [95]. Nevertheless, our
533 imaging and QCM-D experiments do allow us to present a speculative model of membrane-templated
534 septin self-assembly, which is illustrated in **Figure 9**.

535 On the left in **Figure 9B**, we display the proposed organization of septin filaments in the
536 membrane-proximal layer. The hexamers form paired filaments by a combination of end-to-end
537 association mediated by homodimerization of the terminal DSep1 subunits of adjacent hexamers, and
538 lateral associations. Paired filaments in turn form in-plane bundles that occasionally branch and merge.
539 In addition, paired septin filaments are bridged by hexamers or dimers thereof. Similar bridges were
540 observed for yeast septin octamers on lipid monolayers, where the bridge length and spacing were also
541 multiples of the octamer length [63]. In the context of yeast septins, it was proposed that coiled coils
542 mediate septin filament pairing and the formation of perpendicular octamer cross-bridges between
543 paired filaments [9, 63]. The interpretation in that case was that at least some C-terminal extensions on
544 the terminal Cdc11 subunits of paired filaments were free to engage in interactions with C-terminal
545 extensions on the bridging filaments. We here propose that fly septins may similarly form bridges *via*
546 interactions of the C-terminal coiled coils of the terminal subunits (DSep1), facilitated by their
547 orientational flexibility conferred by the hinge regions. Indeed for the C-terminally truncated septin
548 hexamers, both pairing and cross-bridging are abrogated (**Figure 9C**).

549 To explain why septins form multi-layers, we hypothesize that not all coiled coils are engaged in
550 interactions within the first membrane-proximal septin layer, thus leaving some coiled coils free to
551 engage off plane. Pairing of the septin filaments in the membrane plane is very tight, with a spacing of
552 only 2 nm, much smaller than the 13-nm extended length of the long coiled coils on Pnut and DSep2
553 subunits. Similarly tight pairing was previously observed for yeast septins on lipid monolayers [63]. To
554 explain this, we consider that the coiled-coils may be oriented either upwards and engage transversely
555 with coiled coils on a second layer of septins (**Figure 9B** right, scenario 1), or parallel to the membrane
556 sandwiched between two layers of septins (**Figure 9B** right, scenario 2). According to scenario 1, we
557 would expect a layer height of 21 nm, which is larger than the maximum height of ~18 nm seen by AFM

558 for dense yet sub-saturated membranes, and the average heights in the range of 13 to 19 nm seen by
559 QCM-D (depending on membrane composition) for close-to-saturated membranes. According to
560 scenario 2, we would expect a layer height of ~12 nm, in better agreement with the typical heights seen
561 in AFM. However, the AFM images show that the bundle heights are variable. We therefore anticipate a
562 combination of both scenarios, or even alternate scenarios, given the length and orientational freedom
563 of the coiled coils and the known ability of coiled coils to form both parallel and antiparallel multimers
564 [95, 106, 107]. Importantly, layering is restricted to just two layers. Our interpretation is that coiled coils
565 are mostly engaged in in-plane associations while fewer are engaged in the recruitment of a new layer
566 of septins, so that with increasing layer height, there is a smaller and smaller probability of recruiting
567 additional material on top. This ultimately generates septin arrays that are very extensive in-plane, but
568 limited in thickness to a ~12 nm thick array with a layer of coiled coils sandwiched between two layers of
569 globular domains. We note that this model is valid under conditions where septin assembly is initiated
570 at the membrane (*i.e.*, at septin concentrations below 200 nM, consistent with physiological levels of
571 cytoplasmic septins in the range of 100-200 nM measured in fungi [108]). Thicker layers appear to be
572 rare, and may arise either from a different organization of the second septin layer or from an additional
573 septin layer. To elucidate the exact roles of the coiled coils and distinguish between contributions from
574 the short versus the long coiled coils, it could be interesting in the future to study mixtures of wild-type
575 and Δ CC septin hexamers or to study additional septin mutants with selective removal of either the
576 short or long coiled coils. Since AFM lacks chemical specificity, it would probably be necessary to use
577 superresolution fluorescence microscopy to resolve the potential exclusion of Δ CC septins within a
578 mixed septin assembly and to localize the coiled coils. In addition, computational modelling will be
579 essential to model the contributions of the short and long coiled coils and the G-domains of septins to
580 septin-septin and septin-membrane interactions.

581

582 **Biological implications**

583 Our observations of septin filament networks on model biomembranes suggest the possibility that
584 septins in cells may directly enhance the rigidity of the cell cortex by forming filaments on the inner
585 leaflet of the plasma membrane. Our AFM data suggest that membrane-bound septin filaments are
586 intrinsically stiff and ductile while the link to the membrane is fluid. It will be interesting to probe the
587 mechanical stability of the membrane-septin composite envelope in the future by reconstituting septins
588 on free-standing membranes such as giant unilamellar vesicles [64, 84]. With their ability to form rigid
589 filaments and extended meshworks thereof, septins could furthermore have an important role in
590 mechanical integration of the cell cortex, since the actin-myosin cortex in mesenchymal and epithelial
591 cells is known to turn over rapidly [109-112]. Although septins in cells are more dynamic than *in vitro*,
592 with typical *in vivo* half-times for fluorescence recovery on the order of 100 s (depending on septin
593 subunit), the turnover rate of septins is still slower than for actin (where the half-time for fluorescence
594 recovery is on the order of tens of seconds) [44]. In the context of amoeboid T-cell migration, it has for
595 instance been suggested that stable septin structures are important to ensure directional extension of
596 leading-edge protrusions [25]. Reconstitution of animal septins in the combined presence of membranes
597 and the actin/microtubule cytoskeleton promises to help explore the contribution of septins to cell
598 cortex mechanics. New 3D-superresolution techniques [113] and EM tomography [13] of the cell cortex
599 may be able to resolve the proximity of cortical septins to the cell membrane to answer the question
600 whether septins indeed interact directly with the membrane at the cell cortex. Cortical septins are

601 associated with both flat and curved membrane regions in the cell. Here we focused on flat membranes.
 602 It will be interesting to study whether animal septin binding and assembly depends on the degree of
 603 membrane curvature as in the case of yeast septin octamers, which preferentially bind membranes with
 604 a curvature in the micrometre range [37, 84, 85].

605

606 **Materials and Methods**

607

608 **Key resources table**

609

| Key Resources Table | | | | |
|---|---|----------------------|------------------------------|--|
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
| Strain, strain background (<i>Escherichia coli</i>) | BL21(DE3) | Agilent Technologies | Agilent Technologies: 200131 | |
| recombinant DNA reagent | pnEA-vH encoding His ₆ -D Sep1 (plasmid) | PMID: 24633326 | | |
| recombinant DNA reagent | pnCS encoding D Sep2 and Pnut-StrepII (plasmid) | PMID: 24633326 | | |
| recombinant DNA reagent | pnCS encoding mEGFP-D Sep2 and Pnut-StrepII (plasmid) | PMID: 27473911 | | |
| recombinant DNA reagent | pnEA-vH encoding His ₆ -D Sep1ΔC56 (plasmid) | this paper | | See Materials and Methods section, <i>Septin</i> |

| | | | | |
|------------------------------|--|----------------|--|---|
| | | | | <i>purification</i> |
| recombinant DNA reagent | pnCS encoding DSep2 Δ C111 and Pnut Δ C123-StrepII (plasmid) | this paper | | See Materials and Methods section, <i>Septin purification</i> |
| recombinant DNA reagent | pnEA-vH encoding mouse SEPT2 (plasmid) | PMID: 24633326 | | |
| recombinant DNA reagent | pnCS encoding human SEPT6 and human SEPT7-StrepII (plasmid) | PMID: 24633326 | | |
| Peptide, recombinant protein | <i>Drosophila melanogaster</i> septin hexamers composed of His ₆ -DSep1-, DSep2-, Pnut-StrepII | PMID: 24633326 | | |
| Peptide, recombinant protein | Coiled-coil truncation mutant of <i>Drosophila melanogaster</i> septin hexamers composed of His ₆ -DSep1 Δ C56, DSep2 Δ C111 and Pnut Δ C123-StrepII | this paper | | See Materials and Methods, Section <i>Septin purification</i> |
| Peptide, recombinant protein | <i>Drosophila melanogaster</i> septin hexamers composed of His ₆ -DSep1, mEGFP- | PMID: 27473911 | | |

Membrane binding controls ordered self-assembly of animal septins

| | | | | |
|--|---|----------------------|------------|---|
| | DSept2, and Pnut-StrepII | | | |
| peptide, recombinant protein | Mammalian septin hexamers composed of mouse His ₆ -SEPT2, human SEPT6, and human SEPT7-StrepII | PMID: 24633326 | | |
| peptide, recombinant protein | Budding yeast septin octamers composed of Cdc3, Cdc10, Cdc11 and His ₆ -Cdc12 | PMID: 18550837 | | |
| Commercial assay, kit | HisTrap FF crude column | GE Healthcare | 17-5286-01 | |
| Commercial assay, kit | StrepTrap [®] HP | GE Healthcare | 28-9075-46 | |
| Commercial assay, kit | 4-15% gradient Mini-PROTEAN [®] TGX [®] Precast Protein Gels | Bio-Rad | #4561084 | |
| Commercial assay, kit | Precision Plus Protein [®] Kaleidoscope [®] protein standard | Bio-Rad | 1610375 | |
| Biological Sample (<i>genus Tobamovirus</i>) | tobacco mosaic virus (TMV) rods | [114] PMID: 28017791 | | Gift from Dr. J.L. Pellequer (Institut de Biologie Structurale, Grenoble, France) |

610

611 **Septin purification**

612 Chemicals were bought from Sigma unless indicated otherwise. Recombinant *Drosophila* septin
613 hexamers composed of DSep1 with an N-terminal His₆ tag, DSep2, and Pnut with a C-terminal Strep tag
614 II (WSHPQFEK, 1058 Da) were purified from *Escherichia coli* BL21(DE3) cells (Agilent Technologies) using
615 a two-tag affinity purification scheme to aid the isolation of full-length complexes as explained in ref.
616 [76]. Using the same cloning strategy as for wild type complexes [62], a coiled-coil truncation mutant of
617 fly septin hexamers referred to as the ΔCC mutant was made with C-terminal truncations right after the
618 end of the α6-helix (DSep1ΔC56, DSep2ΔC111, PnutΔC123). Cell cultures for wild type and ΔCC septins
619 were grown at 37°C and expression was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside
620 (IPTG) when the optical density at 600 nm (OD600) reached a value between 2 and 3. Fluorescently
621 tagged septin complexes were obtained by tagging DSep2 with monomeric enhanced green fluorescent
622 protein (mEGFP) on its N-terminus [62]. To ensure GFP-folding, cell cultures for GFP-labeled septins
623 were grown at 37°C until the OD600 reached 0.6-0.8 and expression was induced overnight at 17°C.

624 Cell pellets collected after centrifugation (10 min, 2800 × *g*, 4°C) were lysed on ice with a tip
625 sonicator in *lysis buffer* (50 mM Tris-HCl pH 8, 500 mM KCl, 10 mM imidazole, 5 mM MgCl₂, 20 mM
626 MgSO₄, 0.1 mM guanosine diphosphate (GDP), 5% glycerol) supplemented with 0.25 mg/mL lysozyme
627 (5934-D, Euromedex), 1 mM phenylmethylsulfonyl fluoride (Sigma), Complete® protease inhibitor
628 cocktail (Roche), and 10 mg/L DNase I (Sigma). The clarified lysate obtained after centrifugation (30 min
629 at 20000 × *g*, 4°C) was first run on a nickel affinity column (HisTrap FF crude column, GE Healthcare)
630 equilibrated with *septin 1 buffer* (50 mM Tris-HCl pH 8, 500 mM KCl, 10 mM imidazole, 5 mM MgCl₂, 5%
631 glycerol) by eluting with 250 mM imidazole. The peak fractions were pooled and run on a streptavidin
632 affinity column (StrepTrap® HP, GE Healthcare) equilibrated with *septin 2 buffer* (50 mM Tris-HCl pH 8,
633 300 mM KCl, 5 mM MgCl₂, 5% glycerol) by eluting with 2.5 mM d-Desthiobiotin. Pooled fractions from
634 the elution peak were dialyzed against *storage buffer* (50 mM Tris-HCl pH 8, 300 mM KCl, 5 mM MgCl₂,
635 5% glycerol, 5 mM dithiothreitol (DTT)) at 4°C overnight and concentrated to ~3-5 mg/mL using Vivaspinn
636 6 concentrators (Sartorius, 6 ml, 100 kDa cut-off). Initial purifications used an inverted order of columns
637 and a gel filtration step (Superdex 200 HiLoad 16/60, GE Healthcare) instead of a dialysis step [39, 115].
638 Mammalian septin hexamers composed of mouse SEPT2 (98.6% identical to human SEPT2, differing in 5
639 out of 361 residues, based on sequence alignment with the Clustal Omega from
640 <https://www.ebi.ac.uk/Tools/msa/clustalo/>) with an N-terminal His₆ tag, human SEPT6, and human
641 SEPT7 with a C-terminal Strep tag II were purified in the same manner [39]. The SEPT7 isoform used was
642 as annotated in Ref. [116]. Budding yeast septin octamers (composed of Cdc3, Cdc10, Cdc11 and His₆-
643 Cdc12) were purified as described [9] and stored in 50 mM Tris-HCl buffer (pH 8) supplemented with 300
644 mM KCl. Purified septin complexes were flash-frozen in liquid nitrogen and stored at -80°C.

645

646 **Septin characterization**

647 The concentration of septin complexes was determined by measuring the optical absorbance of the
648 solutions at 280 nm with a UV-VIS spectrophotometer (Thermo Scientific, Nanodrop 2000) and
649 subtracting the contribution of scattering (typically ~2-3%) from absorbance measurements between
650 320-340 nm [117]. We used extinction coefficients of 0.545 L × g⁻¹ × cm⁻¹ for full-length *Drosophila*
651 septins, 0.584 L × g⁻¹ × cm⁻¹ for mEGFP-tagged septins, 0.645 L × g⁻¹ × cm⁻¹ for the ΔCC mutant, and 0.565
652 L × g⁻¹ × cm⁻¹ for the mammalian septins, with the values all calculated from the sequence [118]. Molar
653 concentrations were converted into weight concentrations using the calculated molecular weights of
654 306.9 kDa for full-length untagged *Drosophila* septins, 361.6 kDa for mEGFP-tagged septins, 237.8 kDa

655 for the Δ CC mutant, and 285.7 kDa for the mammalian septins. The purity and integrity of the proteins
656 was evaluated for each batch by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
657 using 4-15% gradient gels (Mini-PROTEAN® TGX® Precast Protein Gels, Bio-Rad) with a Precision Plus
658 Protein® Kaleidoscope® (Bio-Rad) protein standard (see **Figure 1 – figure supplement 1A-B** for full-
659 length and Δ CC septins, respectively). The length of the purified complexes (as a readout of the stability
660 of the hexameric arrangement) was evaluated by transmission electron microscopy (TEM). Solutions of
661 septins diluted to 65 nM with a buffer containing 300 mM KCl, to prevent polymerization, were
662 deposited on glow-discharged carbon coated copper grids (CF300-Cu, Electron Microscopy Sciences) and
663 incubated for at least 30 min. The samples were negatively stained with 2% uranyl acetate (Nanoprobes,
664 Yaphank, USA), air-dried, and examined with a FEI Tecnai Spirit (120 kV) electron microscope
665 (ThermoFisher, Waltham, USA). We observed rod-shaped oligomers, mostly having the expected 24 nm
666 length of a hexamer (**Figure 1 – figure supplement 1A-B**). We performed a total of 10 preparations of
667 the full-length fly septins and verified that the protein quality by SDS-PAGE and by TEM analysis was
668 constant. We used 2 preparations of C-terminally truncated fly septins, 1 preparation of yeast septins,
669 and 1 preparation of mammalian septins.

670

671 **Sequence alignment of fly and human septins**

672 Sequence alignment of fly and human septins was performed using the Clustal Omega multiple
673 sequence alignment program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) using the full-length
674 sequences of these septins. The % identity scores mentioned in the text are extracted from the percent
675 identity matrix output of the multiple sequence alignment. A score of 100% is given to two identical
676 amino acids and a score of zero otherwise.

677

678 **Septin coiled-coil size prediction**

679 To identify the regions of the C-termini of fly and human septins that can adopt a coiled-coil
680 conformation, we employed the coiled-coil prediction algorithm COILS ([https://embnet.vital-
681 it.ch/software/COILS_form.html](https://embnet.vital-it.ch/software/COILS_form.html)) using the full-length sequences of these septins. The output was
682 identical when we used the scoring matrix MTIDK (derived from myosins, paramyosins, tropomyosins,
683 intermediate filaments, desmosomal proteins and kinesins) or MTK (derived from the sequences of
684 myosins, tropomyosins and keratins [119]), and for both weighted and unweighted scans, indicating
685 little bias due to the high incidence of charged residues. **Figure 1A** displays the predicted structures for
686 fly septins starting right after the α 6-helix (ending in ...DRLAK for DSep1; ...RLEQ for DSep2; ...KLSE for
687 Pnut). For DSep1, the algorithm predicted a 28-residue-long coiled-coil (LGEKDR...AQMQR) with 99.8%
688 probability. An 86-residue-long C-terminal coiled-coil (QQTFEA..QQLATA) was predicted for DSep2 (47%
689 probability for the first 21 residues and 86-99.9% probability for the following 65 ones). An 86-residue-
690 long stretch in the C-terminus of Pnut (LTQMEE...HVTLEE) was further predicted to adopt a coiled-coil
691 conformation with >97.9% probability. **Figure 1B** shows the corresponding predictions for human
692 septins starting right after the α 6-helix (ending in ... RLKR for SEPT2; ...KLEE for SEPT6; ...KLAA for
693 SEPT7). The C-terminus of human SEPT2 was predicted to form a 28-residue-long coiled-coil
694 (LLEKEA...QMQRG) with 90.2% probability. The coiled-coil prediction for mouse SEPT2 is identical. An
695 86-residue-long stretch in the C-terminus of SEPT6 (QETYE...TAAELL) was predicted to adopt a coiled-
696 coil conformation with 74-100% probability. An 86-residue-long C-terminal coiled-coil

697 (LAQMEE..RILEQQ) was further predicted for SEPT7 with 99.5-100% probability. The coiled-coil
698 predictions were fully in line with the α -helix prediction output of the secondary structure prediction
699 programs PHYRE2 and PSIPREDv4.0 [120, 121]. From the end of the α 6-helix to the start of the predicted
700 coiled-coils, there are stretches of 24, 15 and 15 residues for DSep1, DSep2 and Pnut, respectively, and
701 stretches of 15, 15 and 19 residues for SEPT2, SEPT6 and SEPT7, respectively, that are predicted to be
702 unstructured. To depict septin coiled-coils, the crystal structures of which have not yet been published,
703 in **Figure 1C**, **Figure 1 - figure supplement 1A**, **Figure 7B** and **Figure 9**, we have used the available crystal
704 structures of coiled-coil dimers and tetramers from vimentin (PDB 3UF1) (coiled-coil side-views in the
705 figures) and the early endosomal SNARE complex (PDB 2NPS) (coiled-coil top-views in the figures). The
706 molecular structure visualization program PyMOL was used for isolating helical segments, and rotating
707 and coloring the dimeric and tetrameric coiled-coil segments shown in the images.

708

709 **Preparation of small unilamellar lipid vesicles**

710 Lipids were purchased from Avanti Polar Lipids (Birmingham, AL). The following lipids were bought and
711 stored at a concentration between 10 and 25 mg/mL in chloroform: 1,2-dioleoyl-*sn*-glycero-3-
712 phosphocholine (PC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (PS), and 1,2-dioleoyl-*sn*-glycero-3-
713 phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-PE). Furthermore, 1,2-dioleoyl-
714 *sn*-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate) (ammonium salt) (PI(4,5)P₂) was bought in
715 powder form and stored as a 0.5 mg/mL solution in a chloroform:methanol:water mixture (20:9:1
716 volume ratio). Small unilamellar vesicles (SUVs) were prepared by drying mixtures of PC/PS/rhodamine-
717 DOPE (79.7:20.0:0.3 molar ratio), PC/PI(4,5)P₂/rhodamine-PE (94.7:5.0:0.3), or
718 PC/PS/PI(4,5)P₂/rhodamine-DOPE (74.7:20.0:5.0:0.3) in glass vials using an air stream. PC/PS lipid
719 mixtures were resuspended in a filtered and degassed imidazole buffer of pH 7.4 (20 mM imidazole-HCl,
720 50 mM KCl, and 2 mM MgCl₂). The total lipid concentration of the stock solutions was 0.5 mM for TIRF
721 imaging and 2.5 mM for atomic force microscopy (AFM) and quartz crystal microbalance with dissipation
722 monitoring (QCM-D) experiments. PI(4,5)P₂-containing lipid mixtures were resuspended at a total lipid
723 concentration of 0.25-0.5 mM in a 50 mM citrate buffer of pH 4.8 containing equimolar amounts of
724 trisodium citrate and citric acid, 50 mM KCl, and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The
725 acidic pH promotes the formation of homogeneous and fluid bilayers by reducing the net charge on the
726 head group of PI(4,5)P₂ from -4 to -3 [122]. SUVs for TIRF experiments were obtained by sonication with
727 a tapered microtip (Branson, USA; 3 mm diameter) for 30 min in pulsed mode (30 s on/30 s off at 10%
728 amplitude). SUVs for AFM and QCM-D experiments were obtained by exposing the lipid suspensions to 5
729 freeze/thaw cycles followed by sonication with a microtip (FisherBrand; 2 mm diameter) for 30 min in
730 pulsed mode (1 s on/1 s off at 30% amplitude) with ice cooling. The SUVs were centrifuged (15 min at
731 15000 × *g*) to remove any titanium particles that might come off the sonicator tip, along with residual
732 lipid aggregates, although these were minimal as no white pellet could be observed after sonication. All
733 vesicles were stored in the fridge. PI(4,5)P₂-containing SUVs were used within 5 days, and all other
734 vesicles within 30 days. We confirmed by QCM-D measurements that SLB formation and septin binding
735 were unchanged over the course of these storage periods.

736

737 **Fluorescence microscopy**

738 Samples were prepared in home-made flow channels assembled from cleaned coverslips and
739 microscope slides that were rendered hydrophilic in base piranha (5% hydrogen peroxide, 5%

740 ammonium hydroxide in water) at 70°C. Flow channels were prepared by assembling a dried coverslip
741 and microscope slide with two parafilm spacers spaced apart by ~2 mm and fixed in place by melting on
742 a hotplate at 120°C. We used either 100 mol-% mEGFP-tagged hexamers or mixtures of 90 mol-%
743 untagged hexamers with 10 mol-% mEGFP-tagged hexamers (see captions). Septin assembly on
744 supported lipid bilayers (SLB) was performed on the microscope. SLBs were first formed by flushing the
745 SUV stock solution into the flow channels and allowing for SUV rupture and spreading (10 to 15 min).
746 Correct SLB formation was ascertained by checking that the fluorescence signal was spatially uniform
747 and, using fluorescence recovery after photobleaching measurements, that the bilayer was fluid.
748 Residual SUVs were removed by washing the SLBs with 5 channel volumes of septin *polymerization*
749 *buffer* (20 mM imidazole pH 7.4, 1 mM DTT, 0.1 mM MgATP, 50 mM KCl and 2 mM MgCl₂), containing 2
750 mM trolox to suppress blinking [123] and a 2 mM protocatechuic acid/0.1 μM protocatechuate 3,4-
751 dioxygenase mixture to suppress photobleaching [124]. Septin hexamers diluted to a concentration
752 between 10 nM and 1 μM in *polymerization buffer* were then flushed onto the SLBs and assembly was
753 allowed to proceed for 30 minutes at 20°C unless mentioned otherwise. In case of net-neutral (pure PC)
754 bilayers, we had to include 0.1 wt-% methylcellulose in the buffer to crowd the septins close enough to
755 the surface to allow visualization by TIRF microscopy. This methylcellulose concentration is low enough
756 such that it does not cause filament bundling [125]. In case of anionic bilayers, we did not use
757 methylcellulose to ensure that any surface recruitment was really due to membrane binding.

758 Most TIRF images were obtained with a Nikon Eclipse Ti-E inverted microscope equipped with a
759 TI-TIRF-E motorized TIRF Illuminator (Roper Scientific), a Nikon Apo TIRF 100×/1.49 N.A. oil immersion
760 objective, a 491 nm/50 mW Calypso laser (Cobolt, Solna, Sweden), and a QuantEM 512SC EMCCD
761 camera (Photometrics, Roper Scientific, Tucson, AZ, USA) using an exposure time of 50 ms. This
762 microscope was controlled with MetaMorph 7.5 software (Molecular Devices, Sunnyvale, CA, USA). TIRF
763 images in **Fig. 2 – Fig. supplement 1C** were obtained using a Nikon Ti2-E microscope equipped with a
764 Gataca iLAS2 azimuthal TIRF illumination system, 488-nm laser (Gataca laser combiner iLAS2), and Andor
765 iXon Ultra 897 EM-CCD camera using an exposure time of 75 ms. Images were processed (contrast
766 enhancement and look-up table inversion) and analyzed (integrated intensity calculations) with Fiji
767 software [126]. Fluorescence recovery after photobleaching was performed using a NikonA1 confocal
768 microscope using a 100× oil immersion objective and an argon laser (Coherent, CA, USA) at excitation
769 wavelengths of 488 nm for septins and 561 nm for rhodamine-PE lipids. The septins or the bilayer were
770 first bleached by briefly (1 s) illuminating a circular region with a radius of 5 μm using a high (70%)
771 output laser power. The fluorescence recovery was then measured for 5-7 minutes by illuminating the
772 sample using a low (0.1-2%) output laser power and 30 ms exposure time. For the first 30 seconds after
773 the bleach, images were acquired at 1 frame per second. For the remaining acquisition time, images
774 were acquired at a rate of 1 frame per 10 seconds.

775

776 **Scanning transmission electron microscopy of septin bundles**

777 To determine the morphology and mass per length (MPL) of septin bundles formed in solution, we
778 performed scanning transmission electron microscopy (STEM) using tobacco mosaic virus (TMV) rods
779 with a well-defined length (300 nm), width (18 nm) and MPL (131 kDa/nm) as an internal mass standard
780 [127, 128]. Carbon or formvar+carbon coated copper grids (Ted Pella, Redding, CA, USA) were first
781 incubated for 30 s with 3 μL TMV (0.01-0.02 mg/ml dispersion in phosphate-buffered saline [114]), a
782 kind gift from Dr. Jean-Luc Pellequer (Institut de Biologie Structurale, Grenoble, France), washed with

783 ultrapure water, and blotted with filter paper. Next, 5 μ L of a solution of septins preassembled for 1 h at
784 20°C at a concentration between 50 and 500 nM was deposited and left for 1 min. Finally, the grids were
785 washed with ultrapure water to remove excess salts and left to dry in air at 37°C. Images of 3072 \times 2207
786 pixels (16 bits) were acquired at different magnifications (from 15000 to 50000 \times) and at an acceleration
787 voltage of 10 to 20 kV, a current of 100 pA, and a pixel dwell time of 3 to 5 μ s on a FEI Verios 460 STEM
788 microscope. The MPL was determined by analysis in Fiji [126] following a published procedure [128].
789 Briefly, we selected images that contained both TMV rods and septin bundles. For intensity calibration,
790 boxes with a width of 25 to 45 nm (depending on the image resolution) and a length of 100 nm were
791 drawn along the TMV rods, to obtain the protein signal, and on both sides of each rod, to obtain the
792 background signal. The conversion factor from intensity (in counts) to mass (in kDa) per pixel was
793 determined from the integrated intensities of at least three TMV rods per image. A similar procedure
794 was then used to obtain the MPL of septin bundles, drawing 100 nm long boxes over the bundles that
795 encompassed their width (20 to 50 nm), together with equal-sized boxes on either side of the bundle for
796 background subtraction. The width of the septin bundles was measured by drawing a line in Fiji
797 perpendicularly across septin bundles and estimating the distance between the edges by eye (see
798 examples in **Figure 1 – Figure supplement 2B,C**).

799

800 **Transmission electron microscopy of septins on lipid monolayers**

801 Samples for transmission electron microscopy (TEM) were prepared by incubating septin hexamers with
802 lipid monolayers formed at an air-buffer interface. Teflon wells were filled with 20 μ L of a solution of 65
803 nM (fly) or 70 nM (mammalian) septin hexamers in a 50 mM Tris-HCl (pH 8) buffer with 50 mM KCl. A
804 drop (\sim 0.5 μ L) of a 0.5 g/L lipid solution in chloroform was deposited at the air-water interface in each
805 well to form a lipid monolayer. The Teflon block was kept overnight at 4°C in a humid chamber, while
806 stirring the solution in each well with a magnetic stirrer bar. Lipid monolayers with adsorbed septins
807 were collected by briefly placing hydrophobic grids (Electron Microscopy Sciences, Hatfield, PA, USA) on
808 the surface of the solutions with the carbon-coated side facing the solution. Grids with the collected
809 monolayers facing up were stained at once with 2% uranyl formate (Electron Microscopy Sciences) in
810 water by depositing 4 μ L on the grid and simultaneously blotting the excess solution. Samples were
811 imaged using a Tecnai Spirit electron microscope (Thermo Fischer) operated at an acceleration voltage
812 of 120 V. We repeated the experiments twice having each time 2 replicates. Filament width and distance
813 measurements were performed manually using Image J, based on line profiles.

814

815 **Cryo-electron microscopy of septins on large unilamellar lipid vesicles**

816 Samples for cryoEM were prepared by incubating septin hexamers with large unilamellar vesicles (LUVs).
817 Lipid mixtures at a total lipid content of 50 μ g per sample composed of 85 mol-% PC, 5 mol-% PI(4,5)P₂
818 and 10 mol-% PE (i.e., phosphatidylethanolamine) were dried in glass vials with argon and left in vacuum
819 for 2 h to remove chloroform. We included PE because earlier studies for yeast septin octamers showed
820 that PE favors septin-membrane binding [64]. We did not observe any qualitative changes in the
821 appearance of the fly septin filaments on LUVs made with or without PE present (data not shown). LUVs
822 were formed by hydrating the lipids in high salt buffer at a concentration of 0.5 mg/mL (20 mM
823 imidazole pH 7.4, 1 mM DTT, 300 mM KCl and 2 mM MgCl₂). The vesicles were next incubated for 30 min
824 with septins at a concentration of 160 nM (0.05 g/L) in high salt septin storage buffer, corresponding to
825 a lipid-to-protein weight ratio of 10:1. Meanwhile, lacey carbon coated grids (Electron Microscopy

826 Science, EMS, France) were plasma treated for 30 s to hydrophilize the surface and remove any
827 impurities. Vesicles diluted in septin *polymerization buffer* to a final concentration of 0.3 μM (0.1 g/L)
828 were applied [84]d to the grid straight after plasma treatment. Prior to the vitrification of the sample, 10
829 nm gold beads (EMS) were added in solution to be subsequently used as fiducial markers. Excess liquid
830 was blotted and the samples were vitrified in liquid ethane using an automatic plunge freezer (EM PG,
831 Leica, Wetzlar, Germany). Samples were imaged using a FEI Tecnai G2 microscope equipped with a LaB6
832 filament and a 4KX 4K CMOS camera F416 (TVIPS, Gauting, Germany) operated at an acceleration
833 voltage of 200 kV and a magnification of 50000 \times . Data collection was carried out using the EMTool
834 (TVIPS) software suite. Tilt series were acquired from -60 to 60 degrees using a saxton angular data
835 collection scheme. Individual images were collected with 0.8 electrons per \AA^2 for a total dose of less
836 than 70 electrons per \AA^2 . IMOD software [129] was primarily used for data processing and alignment for
837 individual images. The reconstructions were performed using either IMOD (Weighted back projection)
838 or Tomo3D (SIRT) [130]. The segmentation of the volumes was performed manually using IMOD. We
839 repeated the experiments three times. Filament width and distance measurements were performed
840 manually using Image J, based on line profiles.

841

842 **Atomic force microscopy of septins on supported lipid bilayers**

843 AFM samples were prepared on silicon substrates in order to approximate the conditions used for TIRF
844 imaging and QCM-D measurements. Silicon wafers (Prime Silicon, Les Ulis, France) were cut into squares
845 of $9 \times 9 \text{ mm}^2$, rinsed with absolute ethanol, blow-dried with N_2 gas, and treated with UV/ozone (Bioforce
846 Nanoscience, Ames, IA) for 30 min to remove organic contaminants and render the surface hydrophilic.
847 Each substrate was glued (Picodent Twinsil, Wipperfurth, Germany) on a 15-mm diameter metal disc
848 (Agar Scientific) covered with a hydrophobic protector film (Bytac surface protection laminate; Sigma).
849 An SLB was formed on the silicon wafer piece by incubating for 30 min with 100 μL of a 100 $\mu\text{g}/\text{mL}$ SUV
850 solution diluted in septin *polymerization buffer* with an enhanced KCl concentration to promote SUV
851 rupture (20 mM imidazole pH 7.4, pH 7.4, 1 mM DTT, 150 mM KCl and 2 mM MgCl_2). Residual SUVs were
852 washed off with 1 mL of septin *polymerization buffer*. The SLB was kept hydrated by leaving 100 μL
853 buffer on the wafer surface and 50 μL of septin solution was added to reach a final concentration of
854 either 12, 24 or 60 nM. After 15 min incubation at room temperature (19 $^\circ\text{C}$), unbound protein was
855 washed off with 1 mL of *polymerization buffer*. In most experiments, the samples were fixed for 1
856 minute with 1 wt-% glutaraldehyde (GTA) in *polymerization buffer* and washed. Unfixed septins had a
857 similar morphology as fixed septins but were difficult to image at high resolution because they were
858 easily disrupted by the scanning AFM tip. Reproducibility across biological replicates was checked by
859 performing experiments on 5 protein preps.

860 AFM images were acquired at room temperature (23 $^\circ\text{C}$) with a Nanoscope Multimode 8 system
861 (Bruker, CA, USA) on samples immersed in *polymerization buffer*. Images of various lateral dimensions (5
862 to 20 μm) at a resolution of 512×512 pixels and a scan rate of 0.8 Hz were recorded with silicon
863 cantilevers (ScanAsyst-Fluid+; Bruker) with a nominal spring constant of 0.7 N/m and a sharp pyramidal
864 tip with a nominal radius of 2 nm and a maximal radius of 12 nm. Images were recorded in Peak Force
865 Tapping mode with a typical driving frequency of 2 kHz and tapping amplitude of 50 nm. Images were
866 second-order flattened using open source software Gwyddion [131] to correct for sample tilt and
867 curvature of the xy piezo scanner. Analysis of septin thread heights and widths was performed by
868 constructing height profiles (perpendicular to the thread axis, and averaging 5 pixels along the axis) in

869 Gwyddion software. Heights were defined as the peak value above the flat membrane surface. Widths
870 were measured at a height of 1 nm below peak height. Sections across narrow threads generally showed
871 a single peak, but wider threads (> 30 nm in width) often showed multiple peaks. In the latter case,
872 widths were measured at 1 nm below the height of the two outermost peaks. We recall that tip
873 convolution effects lead to an enlarged effective width. Considering the expected tip shape, and the
874 height at which the widths were measured, we expect tip convolution to entail a width overestimation
875 by between 2 and 10 nm.

876

877 **QCM-D measurements of septin-membrane binding**

878 To analyse the kinetics of septin binding and self-organisation on lipid membranes, we employed a
879 surface analytical technique known as quartz crystal microbalance with dissipation monitoring (QCM-D).
880 This is an acoustic technique that measures the adsorption of biomolecules onto the surface of a
881 piezoelectric quartz crystal that is cyclically sheared at its acoustic resonance frequency by applying an
882 alternating current [132]. The adsorption of molecules to the sensor surface results in a frequency shift
883 Δf that, to a first approximation, is proportional to the acoustic mass (including hydrodynamically
884 coupled solvent) of the adsorbed film [133] and a dissipation shift ΔD that provides information on the
885 mechanical properties of the film [92, 134].

886 We used silica-coated QCM-D sensors (QSX303, Biolin Scientific, Sweden) that were cleaned in
887 an aqueous solution of 2% sodium dodecyl sulfate (30 min), rinsed with ultrapure water, blow-dried
888 with N_2 gas, and exposed to UV/ozone (BioForce Nanosciences, Ames, IA) for 30 min. The sensors were
889 then immediately mounted in the Q-Sense Flow Modules of the Q-Sense E4 system (Biolin Scientific) and
890 experiments were started within 15 min. Experiments were performed at a working temperature of
891 23°C. The instrument was operated in flow mode, meaning that sample solution was continuously
892 perfused through the measurement chamber at a rate of 20 $\mu\text{L}/\text{min}$ with a syringe pump (KD Scientific).
893 We sequentially incubated the sensors with SUVs to form a bilayer and then septins, interspersed by
894 rinsing steps. The surface was first equilibrated with *vesicle buffer* before flowing in the SUV suspension.
895 The adsorption of the SUVs onto the sensor surface and the subsequent formation of a supported lipid
896 bilayer (SLB) were monitored by measuring Δf and ΔD . The frequencies are measured in odd multiples
897 (overtones) of the fundamental resonance frequency of the piezoelectric sensor crystal. In our
898 experiments, the fundamental resonance frequency was 5 MHz and data were recorded at overtones $i =$
899 3 to 13. For further analysis, we selected data obtained at the 7th overtone, in view of its stable response
900 across all measurements. Other overtones provided qualitatively similar information and are thus not
901 shown. The frequency shifts were normalized according to $\Delta f = \Delta f_i/7$. Once Δf and ΔD reached stable
902 values, indicating that the SLB was ready, the surface was rinsed with *vesicle buffer* to remove residual
903 SUVs and then with septin *polymerization buffer* to equilibrate the ionic conditions. Next, the sensor was
904 perfused with 60 nM septins in *polymerization buffer* for at least 60 min. Finally, we tested the
905 reversibility of septin-membrane binding by rinsing the sensor with polymerization buffer. The
906 experiments were performed at least in duplicate. Reproducibility across biological replicates was
907 checked by performing experiments on 5 protein preps.

908 QCM-D measurements can provide information on the thickness of the adsorbed septin films.
909 Provided that the septin layer is sufficiently uniform, dense and rigid, the Sauerbrey equation [133]
910 relates the frequency shift to the areal mass density of the film, $m_{\text{QCM-D}}$, which equals the layer density
911 $\rho_{\text{QCM-D}}$ times the layer thickness $d_{\text{QCM-D}}$:

912
$$\Delta f = -C^{-1} \times m_{\text{QCM-D}} = -C^{-1} \times \rho_{\text{QCM-D}} \times d_{\text{QCM-D}} \quad (1)$$

913 The mass sensitivity constant C is $18 \text{ ng/cm}^2/\text{Hz}$ for QCM-D sensors with a fundamental resonance
914 frequency of 5 MHz and the mass density for protein films is 1.1 g/cm^3 to a good approximation [90]. For
915 sufficiently dense and rigid ($\Delta D/\Delta f \ll 0.4 \times 10^{-6}/\text{Hz}$) films, the film thickness $d_{\text{QCM-D}}$ is therefore
916 proportional to Δf with a proportionality constant of 6.1 Hz/nm .

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933

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1218

1219 **Main text figures – figure captions**

1220

1221 **Figure 1: Fly septin hexamers form bundles in solution.** (A) Schematic of the fly septin hexamer,
1222 showing its expected palindromic arrangement with long (13 nm) coiled-coil extensions of the Pnut and
1223 DSep2 (2) subunits, and shorter (4 nm) extensions on the DSep1 (1) subunits. The predicted septin
1224 coiled-coils are depicted to scale using available crystal-structures. (B) Structure predictions for the C-
1225 terminal regions of fly septins, starting from the end of the α 6-helix regions (aas – amino acids). (C)
1226 Corresponding predictions for human septins. (D) TIRF images of mEGFP-tagged fly septin bundles
1227 formed in solution and crowded onto net-neutral SLBs composed of PC by methylcellulose, with septin
1228 concentrations as indicated above each image. (E) STEM images of yeast septin filaments. Upper panel:
1229 septin filament and a TMV rod (see arrows). Lower panel: paired septin filament. (F) STEM image of fly
1230 septin bundle formed at a concentration of 200 nM surrounded by several TMV rods (one example is
1231 pointed out). The bundle center width and MPL (asterisk) are 31 nm and 250 kDa/nm, respectively. (G)
1232 STEM image of a fly septin bundle formed at a concentration of 500 nM, together with three TMV rods
1233 (one is indicated by an arrow). Note that TIRF and STEM images are all contrast-inverted, so septins
1234 appear dark against a light background.

1235 The following figure supplements are available for figure 1:

1236 **Figure 1 – Figure supplement 1.** Biochemical and morphological characterization of septins.

1237 **Figure 1 – Figure supplement 2.** Quantification of septin filament MPL from STEM images.

1238

1239 **Figure 2: Glass-supported lipid bilayers containing anionic lipids recruit septins and promote the**
1240 **assembly of dense septin meshworks.** (A) Purified fly septin hexamers are deposited on a glass-
1241 supported bilayer composed of net-neutral (PC) and anionic (PS or PI(4,5)P₂) lipids (sketch not to scale).
1242 (B) TIRF images recorded ~3 min after the deposition of septin hexamers (1 μ M; 10 mol-% mEGFP-
1243 tagged hexamers) on bilayers doped with PI(4,5)P₂ at mole fractions between 1 and 8% (see legend). (C)
1244 TIRF images obtained ~3 min after the deposition of septins (1 μ M) on bilayers doped with PS at mole
1245 fractions between 0 and 20% (see legend). Note that methylcellulose was used to crowd septin bundles
1246 to the bilayer for the neutral SLB (0% PS) but was left out in all other cases. All images are contrast-
1247 inverted, so septins are dark and membrane areas devoid of septins are light. Scale bars: 10 μ m.

1248

1249 The following figure supplements are available for figure 2:

1250 **Figure 2 – Figure supplement 1.** Dependence of septin adsorption on septin concentration and on the
1251 PS content of the SLBs.

1252 **Figure 2 – Figure supplement 2.** FRAP data testing the mobility of septins and lipids.

1253

1254 **Figure 3. Septin hexamers form dense arrays of tightly paired filaments on anionic lipid monolayers.**
1255 Electron micrographs of negatively stained fly septin hexamers (65 nM) after overnight incubation with
1256 lipid monolayers composed of PC combined with anionic lipids: (A) 20% PS, (B) 5% PI(4,5)P₂, and (C) 20%
1257 PS plus 5% PI(4,5)P₂. Images on the right show zoom-ins of the regions in the white-dashed boxes on the
1258 left. Black arrows point out examples of paired filaments recognizable by two linear filaments running in
1259 parallel. Blue arrows indicate examples of orthogonal hexamers, recognizable by their ~24 nm length,
1260 between adjacent paired filaments. The two red arrows point to the two ends of a longer (43 nm) cross-
1261 bridging filament that bridges two adjacent paired filaments under an oblique angle. Scale bars: 250 nm
1262 (left) and 50 nm (right).

1263

1264 The following figure supplements are available for figure 3:

1265 **Figure 3 – Figure supplement 1.** Quantification of filament dimensions from electron microscopy
1266 images.

1267 **Figure 3 – Figure supplement 2.** EM images of mammalian septin hexamers on lipid monolayers.

1268

1269

1270 **Figure 4. Septin hexamers form single and paired filaments on large unilamellar lipid vesicles.** CryoEM
1271 images of fly septin hexamers (160 nM) after a 30 minute incubation with LUVs containing 95% PC and
1272 5% PI(4,5)P₂. (A) Example image (left) with paired septin filaments traced out in red, connecting
1273 orthogonal filaments in blue and the vesicle membrane highlighted in yellow (right). (B) Another
1274 example image (left), with mostly paired and occasionally single filaments traced out in red, connecting
1275 orthogonal filaments in blue and membranes in yellow (right). Black dots are gold nanoparticles that
1276 were included as fiducial markers for tomography. The black curved lines in panels A, B come from the
1277 carbon lacey substrate. (C) Slice from 3D reconstructed cryo-tomogram (left) with segmented data
1278 (right). (D) Another example tomogram (left) with segmented data (right). Reconstructions show
1279 membrane in yellow and septin filaments in blue. Note that the bilayer perpendicular to the electron
1280 beam is poorly defined because of the missing wedge. Scale bars are 50 nm in A,B and 850 nm in C, D.

1281

1282 The following video and figure supplements are available for figure 4:

1283 **Figure 4 – Figure supplement 1.** Interfilament spacing for paired fly septin filaments.

1284 **Figure 4 – Figure supplement 2.** Additional cryoEM data.

1285 **Figure 4 – Figure supplement 3.** Quantification of vesicle size distributions.

1286 **Figure 4 – Video 1.** 3D tomographic reconstruction of septins bound to lipid vesicles.

1287

1288

1289

1290 **Figure 5. Septins form filaments and ordered arrays on lipid bilayers composed of 80% PC and 20% PS.**
1291 (A-B) AFM topographic images of membrane-bound fly septin structures formed at solution
1292 concentrations of 12 nM (left), 24 nM (middle) and 60 nM (right) and observed at a scan size of (A) $20 \times$
1293 $20 \mu\text{m}^2$. (B) Same samples, imaged at a scan size of $5 \times 5 \mu\text{m}^2$ (left and right) and of $6.7 \times 6.7 \mu\text{m}^2$
1294 (middle). Color bars on the right show the height scale. The samples were fixed with glutaraldehyde
1295 (GTA). (C) Scatter plots with marginal histograms of thread widths and heights determined from the
1296 corresponding images in (B). $N = 31$ (left), 26 (middle) and 27 (right) measurements were taken,
1297 respectively, across representative sets of well resolved threads.

1298

1299 The following figure supplements are available for figure 5:

1300 **Figure 5 – Figure supplement 1.** Examples of AFM height profiles across filaments and bundles.

1301 **Figure 5 – Figure supplement 2.** Images of non-fixed septin samples on lipid bilayers.

1302 **Figure 5 – Figure supplement 3.** AFM experiment testing the mechanical stability of the septin
1303 ultrastructures.

1304 **Figure 5 – Figure supplement 4.** AFM experiment showing septin bundles can be displaced along the
1305 membrane.

1306

1307

1308 **Figure 6. Native ultrastructure of septin assemblies on lipid bilayers composed of 80% PC and 20% PS.**
1309 (A) AFM image of non-fixed septins at 60 nM, showing a bilayer region that happens to be sparsely
1310 covered with septin filaments. Scale bar for the left panel: $5 \mu\text{m}$; right panel shows zoom of the dashed
1311 rectangle in the left panel; color bar on the right shows the height scale. (B) Height profiles were
1312 determined along the numbered white lines in the images in A.

1313

1314 **Figure 7. QCM-D measurements of fly septin hexamer adsorption on supported lipid bilayers (SLBs)**
1315 **containing anionic lipids show that septins form thin, rigid films.** Shown are frequency shifts (Δf – blue
1316 lines with symbols) and dissipation shifts (ΔD - red solid lines). Arrows on top of the graphs indicate start
1317 and duration of sample perfusion; during remaining times, plain buffer (A – vesicle buffer; B and C –
1318 septin polymerization buffer) was flown over the sensor surface. (A) Small unilamellar vesicles (SUVs at
1319 $50 \mu\text{g}/\text{mL}$; 20% DOPS, 80% DOPC) were exposed to a plain silica surface to form a SLB. (B-C) Hexamers of
1320 wild type septin (B) and the ΔCC septin truncation mutant (C) (60 nM each), were exposed to 80% PC/
1321 20% PS SLBs (solid line with circle symbols). (B) also shows control frequency data for exposure of wild
1322 type septin (100 nM) to pure DOPC SLBs (dotted line with diamond symbols).

1323

1324 The following figure supplements are available for figure 7:

1325 **Figure 7 – Figure supplement 1.** Lipid composition dependence of septin adsorption.

1326 **Figure 7 – Figure supplement 2.** Coverage-dependent $\Delta D/\Delta f$ ratios for fly septins.

1327

1328 **Figure 8. Negative stain EM images of C-terminally truncated fly ΔCC -septins.** (A) ΔCC -septin hexamers
1329 in septin polymerization buffer. (B) Zoom-in of the boxed region in (A) shows that ΔCC -septin hexamers
1330 (84 nM) predominantly form monomers and short oligomers of hexamers with a length of $\sim 24\text{-}60 \text{ nm}$.
1331 (C) ΔCC -septin hexamers (210 nM) on a negatively charged lipid monolayer composed of 80% DOPC,

1332 10% PS and 10% PIP(4,5)P₂. (D) Zoom-in of the boxed region in (C) shows sheets of closely spaced, long
1333 filaments (red lines) and occasionally three filaments lined up side by side (trimer highlighted in blue).
1334 (E) Another example of the Δ CC-septin hexamers (210 nM) on a lipid monolayer composed of 80% PC,
1335 10% PS and 10% PIP(4,5)P₂. (F) Zoom-in of the boxed region in (E) again shows sheets of closely spaced,
1336 long, unpaired filaments. (G) Line scan (from area highlighted in panel F by an orange line) reveals a
1337 center-to-center (asterisks) spacing of \sim 4.5 nm. Scale bars are 200 nm (A,C,E) and 50 nm (B,D,F).
1338

1339 The following figure supplements are available for figure 8:

1340 **Figure 8 – Figure supplement 1.** Lengths of Δ CC-septin hexamer filaments formed in low-salt septin
1341 polymerization buffer.
1342

1343
1344 **Figure 9. Model of membrane-templated septin self-assembly.** (A) Diagram summarizing the regimes of
1345 fly septin self-assembly observed in the solution phase (top) and on anionic lipid membranes (bottom).
1346 (B-C) Left column: top views of the membrane proximal septin layer. Right column: side views of all
1347 septin layers. The coiled-coils are depicted to scale using available crystal-structures of coiled-coils (see
1348 Methods) in light-gray for coiled coils emanating from the bottom septin layer and dark-gray for coiled-
1349 coils from the top layer. (B) Full-length septin hexamers form meshworks of paired filaments on the
1350 membrane, which are bridged by hexamers and dimers thereof and form bundles with filaments joining
1351 or emerging at a shallow angle. A second layer of septin filaments is recruited by coiled-coil interactions
1352 oriented vertically (scenario 1) or in-plane (scenario 2). For simplicity, scenario 2 is depicted with coiled
1353 coils oriented perpendicular to the filament axis though they may in reality adopt a range of
1354 orientations. (C) Δ CC-septin hexamers lacking coiled coils form a monolayer of filaments interacting by
1355 excluded volume interactions, perhaps augmented by G-domain interactions. Dimensions indicated in
1356 the schematic are based on the known size of septin subunits from EM and calculated lengths of the
1357 coiled-coil regions, and are consistent with the measured filament widths and spacings in-plane (from
1358 EM images) and with the measured height of the septin films (from AFM and QCM-D).

1359 **Table supplement**

1360 **Table S1.** Overview of the analysis technique and assay conditions for each figure, i.e. the septin concentration, anionic lipid
 1361 content of the membrane, and type of membrane used (lipid monolayers in case of negative stain EM imaging, supported lipid
 1362 bilayers (SLB) in case of TIRF, AFM, and QCM-D, and large unilamellar vesicles (LUV) in case of cryoEM imaging).

| Figure | Assay | Technique | Septin concentration [nM] | Anionic lipid content |
|---------------|--|---------------|--|--|
| Fig. 1 | WT-fly septin in solution | TIRF and STEM | 100, 200, 500 | No membrane present |
| Suppl 1 | Fly septin in solution (WT and Δ CC mutant*) | TEM | 65 nM (wild type) 84 nM (Δ CC mutant) | No membrane present |
| Suppl 2 | WT-fly septin in solution | STEM | 500 | No membrane present |
| Fig. 2 | WT-fly septin on SLB | TIRF | 1000 | 1) 1-8 mole% PI(4,5)P ₂ 2) 5, 10, 20% mole% PS |
| Suppl 1 | WT-fly septin on SLB | TIRF | 10, 100, 150, 200, 300, 500 | 5, 10, 20% mole% PS |
| Suppl 2 | WT-fly septin on SLB | FRAP | 500 | 20% PS |
| Fig. 3 | WT-fly septin on monolayer | TEM | 65 | 1) 20% PS 2) 5% PI(4,5)P ₂ , 3) 20% PS & 5% PI(4,5)P ₂ |
| Suppl 1 | WT-fly septin on monolayer | TEM | 65 | Same as Fig. 3 |
| Suppl 2 | Mammalian septin on monolayer | TEM | 70 | 20% PS & 5% PI(4,5)P ₂ |
| Fig. 4 | WT-fly septin on LUVs | cryoEM | 160 | 5% PI(4,5)P ₂ |
| Suppl 1 | WR-fly septin on LUVs | cryoEM | 160 | 5% PI(4,5)P ₂ |
| Suppl 2 | WT-fly septin on LUVs | cryoEM | 160 | 5% PI(4,5)P ₂ |
| Video 1 | WT-fly septin on LUVs | cryoEM | 160 | 5% PI(4,5)P ₂ |
| Fig. 5 | WT-fly septin on SLB | AFM | 12, 24, 60 | 20% PS |
| Suppl 1 | WT-fly septin on SLB | AFM | 12, 24, 60 | 20% PS |
| Suppl 2 | WT-fly septin on SLB | AFM | 60 | 20% PS |
| Suppl 3 | WT-fly septin on SLB | AFM | 24,60 | 20% PS |
| Suppl 4 | WT-fly septin on SLB | AFM | 60 | 20% PS |
| Fig. 6 | WT-fly septin on SLB | AFM | 60 | 20% PS |
| Fig. 7 | Fly septin on SLB (WT and Δ CC) | QCM-D | 60 | 20% PS |
| Suppl 1 | WT-fly septin on SLB | QCM-D | 60 | 1) 20% PS 2) 5% PI(4,5)P ₂ , 3) 20% PS & 5% PI(4,5)P ₂ |
| Suppl 2 | WT-fly septin on SLB | QCM-D | 60 | 20% PS |
| Fig. 8 | Δ CC fly septin in solution and on monolayer* | TEM | 84, 210 | 10% PS + 10% PIP(4,5)P ₂ |
| Suppl 1 | Δ CC fly septin in solution | TEM | 84 | No membrane present |

1363 *We matched the weight concentrations for Δ CC-septin hexamers and full-length septin hexamers in EM experiments in high
1364 salt solutions (0.02 mg/mL). Due to the different molecular weights of these hexamers, this corresponds to slightly different
1365 molar concentrations (i.e., 84 nM for Δ CC-septins and 65 nM for full-length septins).

1366 Figure supplements: captions

1367
1368 **Figure 1 – Figure supplement 1. Biochemical and morphological characterization of septin hexamers.**
1369 (A) Left: SDS-PAGE gel of full-length fly septin hexamers with lanes showing: 1) nickel-column eluate, 2)
1370 StrepTrap-column eluate, 3) gel filtration eluate. Molecular weight markers in kDa are indicated on the
1371 left. The three bands of similar intensity represent the three subunits: Pnut-Strep (61.2 kDa), DSep2
1372 (48.5 kDa) and His₆-DSep1 (43.8 kDa). The fainter bands between the DSep2 and Pnut bands represent
1373 partially degraded Pnut-Strep, while the faint band located around 70 kDa is DnaK [39]. Right: negative-
1374 stain TEM image showing that fly septin hexamers (0.02 mg/ml, i.e., 65 nM) in a high-salt (300 mM KCl)
1375 storage buffer are present predominantly as hexamers or dimers thereof. (B) Left: SDS-PAGE gel of the
1376 Δ CC mutant with C-terminal truncations (DSep1 Δ C56, DSep2 Δ C111, Pnut Δ C123), showing 3 bands at
1377 the positions expected for the three septin subunits for the nickel-column eluate (lane 2) and
1378 subsequent StrepTrap-column eluate (lane 3). Right: negative-stain TEM image shows that the Δ CC-
1379 septin hexamers (0.02 mg/ml, i.e., 84 nM) in a high-salt (300 mM KCl) storage buffer are present mainly
1380 as hexamers.

1381
1382 **Figure 1 – Figure supplement 2. (A-C) Examples of MPL determination from STEM images for an image**
1383 **of a TMV virus rod (A), an image showing both a TMV virus rod and a paired yeast septin filament (B),**
1384 **and an image showing both a TMV virus rod and a fly septin bundle (C). The graphs show line profiles**
1385 **taken along the orange dotted lines (for TMV rods) and blue dotted lines (for septin filaments) in the**
1386 **corresponding images. The intensity can be converted to MPL as explained in the materials and methods**
1387 **section. (D) Quantification of the bundle size for fly septin hexamer bundles formed in bulk solution at**
1388 **500 nM. Scatter plot of the mass-per-length (MPL; in units of kDa/nm) versus the bundle width (in nm).**
1389 **The data were collected from 2 independent STEM-imaging sessions, 5 images, 16 bundles, and in total**
1390 **130 data points.**

1391
1392 **Figure 2 – Figure supplement 1: TIRF imaging shows that fly septin hexamers rapidly adsorb to anionic**
1393 **bilayers and rapidly form filaments. (A) TIRF images obtained at time points of ~3 min (top row) and 4 h**
1394 **(bottom row) after the deposition of fly septin hexamers (500 nM, 10 mol-% mEGFP-tagged) on PC**
1395 **bilayers doped with 5, 10 or 20 mol-% PS. The larger the DOPS content, the less bundled and the more**
1396 **dense and grainy the septin layer appears. Note that the 3 min and 4 h images do not show the same**
1397 **regions because experiments required keeping the glass slides in a humidified petri dish, so finding the**
1398 **same region afterwards was not possible. (B) Corresponding integrated septin intensities (each data**
1399 **point is based on one experiment with 3 different regions of interest in different sample locations),**
1400 **showing a linear increase with PS content (lines are fits) with insignificant changes from 3 min to 4 h of**
1401 **septin incubation. Note that the TIRF signal includes contributions from both membrane-bound septins**
1402 **and solution-phase septins within the TIRF excitation field. (C) TIRF images taken 30 min after depositing**
1403 **fly septin hexamers (100 mol-% EGFP-tagged) at different concentrations, as indicated above each**
1404 **image, on PC bilayers doped with 20 mol-% PS. TIRF reveals the presence of membrane-bound septin**
1405 **already at a concentration of 10 nM, but lacks the resolution necessary to reveal the organization. Dark**

Membrane binding controls ordered self-assembly of animal septins

- 1406 (i.e., brightly fluorescent) puncta are indicative of some protein aggregation, likely due to the GFP tag.
1407 All images are contrast-inverted, so septins are dark and membrane areas devoid of septins are light.
1408 Scale bars: 5 μm .

1409 **Figure 2 – Figure supplement 2. Fluorescence recovery after photobleaching experiments show that**
1410 **membrane-adhered fly septin layers are immobile and stable over time while the bilayer is fluid.** A
1411 solution of 500 nM septin hexamers containing 10 mol-% mGFP-labeled hexamers was incubated with
1412 glass-supported lipid bilayers containing PC, 20% PS, and 0.3% mol-% of fluorescently labeled lipids
1413 (rhodamine-PE). (A) The fluorescence of the septin meshwork does not recover after photobleaching a
1414 circular region with a radius $r = 5 \mu\text{m}$ (indicated by the white circle) at time $t = 0$ for 1 s on an
1415 observation time scale of nearly 7 min, indicating negligible mobility and turnover. (B) The fluorescent
1416 signal of the rhodamine-PE lipids does recover fully upon photobleaching. The time-dependent
1417 fluorescence recovery reveals an average lipid diffusivity $D \approx 1.2 \mu\text{m}^2/\text{s}$, both with and without adsorbed
1418 septins, as determined by extracting the recovery half time $\tau_{1/2} = 4r^2\gamma_D/D$ (where $\gamma_D = 0.88$) from a fit to
1419 the Soumpasis equation [135]. This diffusivity is characteristic of fluid lipid bilayers on solid supports
1420 [136]. Scale bar: 10 μm .

1421
1422 **Figure 3 – Figure supplement 1. Quantification of negative stain EM and cryoEM images of membrane-**
1423 **bound septin hexamers.** Data for wild type (WT) septin hexamers are shown together with data for C-
1424 terminally truncated ΔCC mutant septin hexamers. (A) Interfilament distance (center-to-center)
1425 between septin filaments within paired filaments. The figure legend denotes EM data for negatively
1426 stained WT septins on lipid monolayers (“WT negstain”) and for ΔCC mutant septins on lipid monolayers
1427 (“ ΔCC PS-PIP2”), and cryoEM data for WT septins on LUVs (“WT cryo”). The monolayers contained 20%
1428 PS + 5% PI(4,5)P₂, while the LUVs contained 5% PI(4,5)P₂. (B) Widths of individual septin filaments from
1429 EM images of negatively stained septins on lipid monolayers, for different lipid compositions. The figure
1430 legend denotes the septin variant (WT or ΔCC) and the lipid composition: 20% PS (“WT PS”), 5%
1431 PIP(4,5)P₂ (“WT PIP2”) or 20% PS plus 5% PIP(4,5)P₂ (“WT PS-PIP2” and “ ΔCC PS-PIP2”). (C) Lengths and
1432 angles of connecting bridges between paired septin filaments on PS-PI(4,5)P₂ monolayers. The bridge
1433 lengths are distributed within 2 clusters with lengths near 24 nm (corresponding to hexamers; left
1434 vertical line) or 48 nm (corresponding to double hexamers; right vertical line). Black horizontal lines in
1435 panels A and B are averages of the distributions. Number of measurements: in panel A: $N = 57, 40$ and
1436 36 for “WT negstain”, “WT cryo” and “ ΔCC mutant”, respectively. In panel B: $N = 28, 17, 15$ and 35 for
1437 “WT PS”, “WT PIP2”, “WT PS-PIP2” and “ ΔCC mutant”, respectively. In panel C, $N = 27$.

1438
1439 **Figure 3 – Figure supplement 2. Mammalian septin hexamers form paired filaments on lipid**
1440 **monolayers comprised of PC, 20 mol-% PS, and 5 mol-% PI(4,5)P₂.** Mammalian septin hexamers
1441 (comprised of mouse SEPT2, human SEPT6 and human SEPT7) incubated at 70 nM with a lipid
1442 monolayer containing 20% PS and 5% PIP2 form paired filaments connected by many cross-bridging
1443 filaments. The white arrows indicate examples of paired filaments (in areas where there are two obvious
1444 densities side by side and the filament as a whole looks thicker). Red arrows point at bridging filaments
1445 which seem to be thinner and run continuously across the paired filaments. It is unclear whether the
1446 bridging filaments are single filaments or (twisted, and therefore thinner) paired filaments. Scale bars:
1447 100 nm.

1448
1449 **Figure 4 – Figure supplement 1. Paired fly septin filaments (160 nM) on large unilamellar lipid vesicles**
1450 **composed of 85 mol-% PC, 10 mol-% PE and 5 mol-% PI(4,5)P₂ exhibit an average center-to-center**
1451 **spacing of ~5.7 nm.** (A, B, C) Three examples of cryoEM images, with (D, E, F) corresponding line profiles

- 1452 drawn along the blue lines. Stars denote approximate center positions of filaments. Scale bars: 50 nm.
1453 Further quantification is shown in **Figure 3 – Figure supplement 1**.

1454 **Figure 4 – Figure supplement 2. Fly septin hexamers form paired and occasionally single filaments on**
1455 **large unilamellar lipid vesicles.** Gallery of cryoEM images of septins (160 nM) after a 30 minute
1456 incubation with large unilamellar vesicles composed of 85 mol-% PC, 10 mol-% PE and 5 mol-% PI(4,5)P₂.
1457 Images are from different regions/samples, except for panel D, which shows a zoom-in of the boxed
1458 region in C. The images show septin filaments (linear structures) and vesicles of different sizes and
1459 shapes (circular objects). Most vesicles are covered with parallel sheets of tightly paired filament with
1460 narrow spacing (e.g. panel A), but some display rather spaced parallel filaments (e.g. panel E). Black dots
1461 are 10 nm diameter gold beads used as fiducials for cryo-tomography. Scale bars: 50 nm.

1462
1463 **Figure 4 – Figure supplement 3. Size distributions of large unilamellar lipid vesicles based on**
1464 **quantification of cryoEM data.** Diameters of LUVs (PC 80%, PI(4,5)P₂ 10%, PS 10%) measured from
1465 cryoEM images, without septins (left; *N* = 40) and with septins (160 nM) (right; *N* = 24). The horizontal
1466 lines represent the averages of the distributions.

1467
1468 **Figure 4 – Video supplement 1 [here: still image].** 3D tomographic reconstruction of fly septin hexamers
1469 (160 nM) bound to lipid vesicles composed of 85 mol-% PC, 10 mol-% PE and 5 mol-% PI(4,5)P₂. The
1470 membrane is in yellow while septin filaments are segmented in blue. Scale bar: 850 nm. The membrane
1471 (though only partly visible because a part is perpendicular to the electron beam and therefore invisible
1472 because of the missing wedge in tomography) is distorted because of the bound septin filaments.

1473
1474 **Figure 5 – Figure supplement 1. Examples of AFM height profiles across filaments and bundles.** (Left)
1475 AFM topographic images of GTA-fixed fly septins at concentrations of 12 nM (A), 24 nM (B), and 60 nM
1476 (C). The images were cropped from the images presented in Figure 5B, and color bars on the right show
1477 the height scale. (Right) Height profiles were determined along the numbered white lines in each
1478 corresponding image on the left. In A and B, they run perpendicularly to the filament/bundle axes, as
1479 done for height and width analysis in Figure 5C. In C, a horizontal line across many filaments/bundles
1480 was chosen to appreciate their rather dense packing: note that the AFM tip, owing to its finite size, is
1481 unable to reach 0 nm height (*i.e.* the SLB surface) between some tightly spaced filament/bundles.

1482
1483 **Figure 5 – Figure supplement 2. AFM images show that glutaraldehyde (GTA) fixation does not change**
1484 **the morphology of fly septins adsorbed to supported lipid bilayers.** Septin hexamers were incubated at
1485 60 nM on supported lipid bilayers made from 80 mol-% PC and 20 mol-% PS. The bundles and their
1486 liquid-crystalline organisation are preserved. (A) Image of unfixed septin filaments. (B) Image of septin
1487 filaments fixed by GTA treatment. Scale bar: 5 μm; color bar between images shows the height scale.
1488 The graphs below the images present height profiles along the solid white line depicted on each image.
1489 Note that the two samples were prepared independently, and that differences in surface density are
1490 likely due to slight variations in the sample preparation conditions. Occasional lines appearing empty (in
1491 A) correspond to phases when the AFM tip lost contact with the surface, as imaging without GTA
1492 required minimal forces to avoid perturbation of the septin organisation.

1493 **Figure 5 – Figure supplement 3. AFM imaging shows that membrane-bound fly septin bundles and**
1494 **dense septin arrays are more mechanically stable than isolated septin filaments (SLB made from 80**
1495 **mol-% PC and 20 mol-% PS), both in fixed (+GTA) and unfixed (-GTA) conditions.** (A) Three
1496 representative images taken at different surface areas in unfixed conditions (-GTA). (B) Three
1497 representative images taken at different surface areas after fixation with 1% GTA (+GTA). Septin
1498 concentration was 60 nM in A, and 24 nM in B. White arrows in A and B show examples of single septin
1499 filaments that appear perturbed and mechanically unstable, as indicated by a 'zigzag' shape, most likely
1500 due to the AFM tip moving the filament back and forth across the membrane. Pink arrows show
1501 (typically thicker) stable filaments or bundles. (C-D) Dense arrays of septins (60 nM; C – unfixed
1502 condition, D – after fixation with GTA) are mostly stable over time, as demonstrated by three
1503 consecutive scans of the same region (each scan taking 12 min in C, and 10 min in D; $t = 0$ min indicates
1504 the initiation of the first scan; white vertical arrows show the direction of the slow scan axes). Images in
1505 C show artefactual variations in contrast, including some scan lines without any contrast: unfixed
1506 samples required careful minimisation of the imaging force to minimise filament perturbation by the
1507 AFM probe, and fluctuations in the imaging force lead to transient loss of surface tracking. Fixed
1508 filaments in D withstood higher imaging forces and thus could be imaged more clearly. Scale bars: 5 μm ;
1509 colour bars on the right show the height scales.

1510

1511 **Figure 5 – Figure supplement 4. Fly septin filament bundles can be displaced along the membrane.**
1512 (Left) Three consecutive atomic force micrographs (1, 2 and 3) of the same surface area are shown of
1513 GTA-fixed septin filaments (60 nM) on supported lipid bilayers made from 80 mol-% PC and 20 mol-%
1514 PS. The alternating direction of the slow-scan axis is indicated (white arrows). Most of the septin
1515 filaments remain in place, but some fibrils are occasionally displaced along the horizontal fast-scan axis
1516 owing to lateral forces exerted by the AFM tip. Arrow sequence B-B'-B'' shows such a displacement
1517 event leading to a permanent local kink and filament relocation (B''). The disruption of the filament (B')
1518 in micrograph 2 is most likely apparent, owing to the line-by-line scanning nature of AFM image
1519 acquisition. The spacing between the two blue vertical lines, corresponding to the displacement of the
1520 filament bundle in B', is 170 nm. Arrow sequences A-A'-A'' and C-C'-C'' highlight additional examples of
1521 displaced fibrils. Images 2 and 3 were stretched/compressed along the vertical (slow scan) axis and
1522 skewed along the horizontal (fast scan) axis to correct for drifts during imaging and align these images
1523 with image 1. (Right) Equivalent data for unfixed septin filaments (60 nM) on supported lipid bilayers
1524 made from 80 mol-% PC and 20 mol-% PS. Note that this region represents a zoom on a small region in
1525 Figure 5 – Figure supplement 3C. Arrow sequences D-D'-D'' and E-E'-E'' highlight examples of displaced
1526 fibrils, whereas most other filaments remain in place. Larger variations in filament appearance across
1527 the images (*e.g.*, filament widening) are due to fluctuations in the surface tracking because the AFM
1528 probe was operated at a reduced peak force to minimise filament perturbations.

1529

1530 **Figure 7 – Figure supplement 1. Adsorption kinetics for fly septin hexamers (60 nM) on PC bilayers**
1531 **containing different anionic lipids (see legend).** (A-B) Representative QCM-D data for the binding of
1532 wild type septin hexamers (60 nM) to SLBs containing 5% PI(4,5)P₂ (A) or 20% DOPS and 5% PI(4,5)P₂ (B).
1533 Shown are frequency shifts (Δf – blue lines with symbols) and dissipation shifts (ΔD - red solid lines).
1534 Arrows on top of the graphs indicate start and duration of sample perfusion; during remaining times,
1535 plain septin polymerization buffer was flown over the sensor surface. (C) The level of adsorption as a

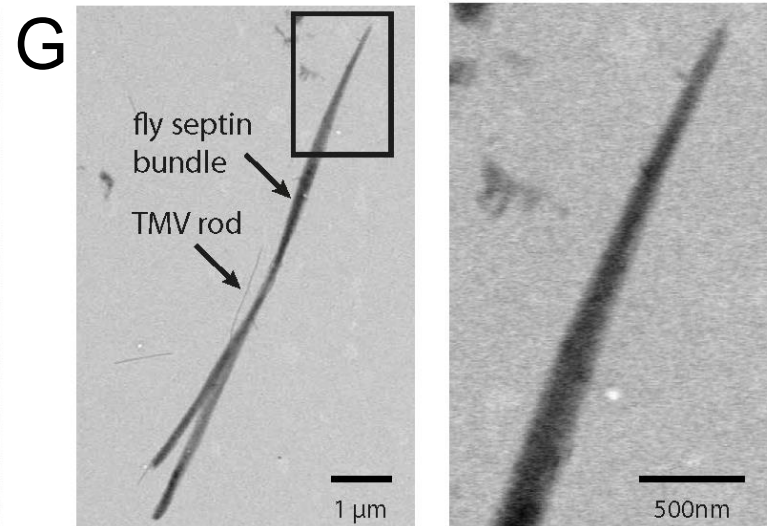
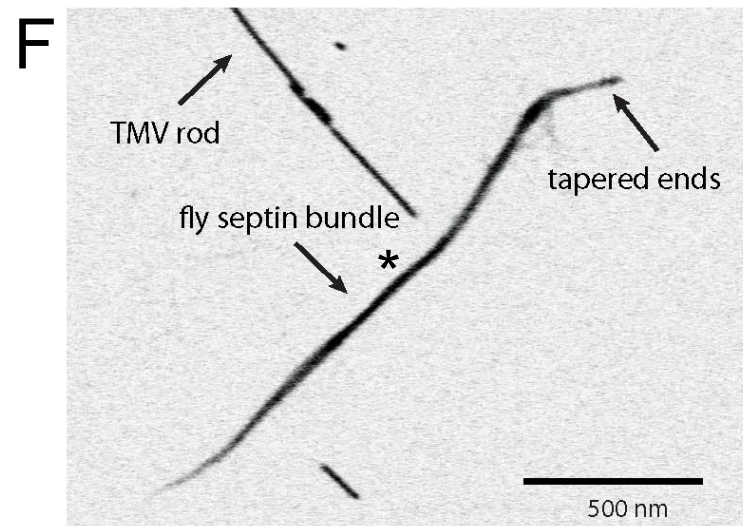
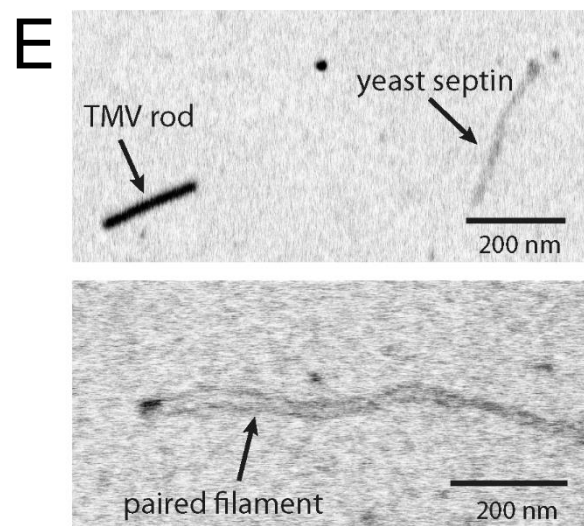
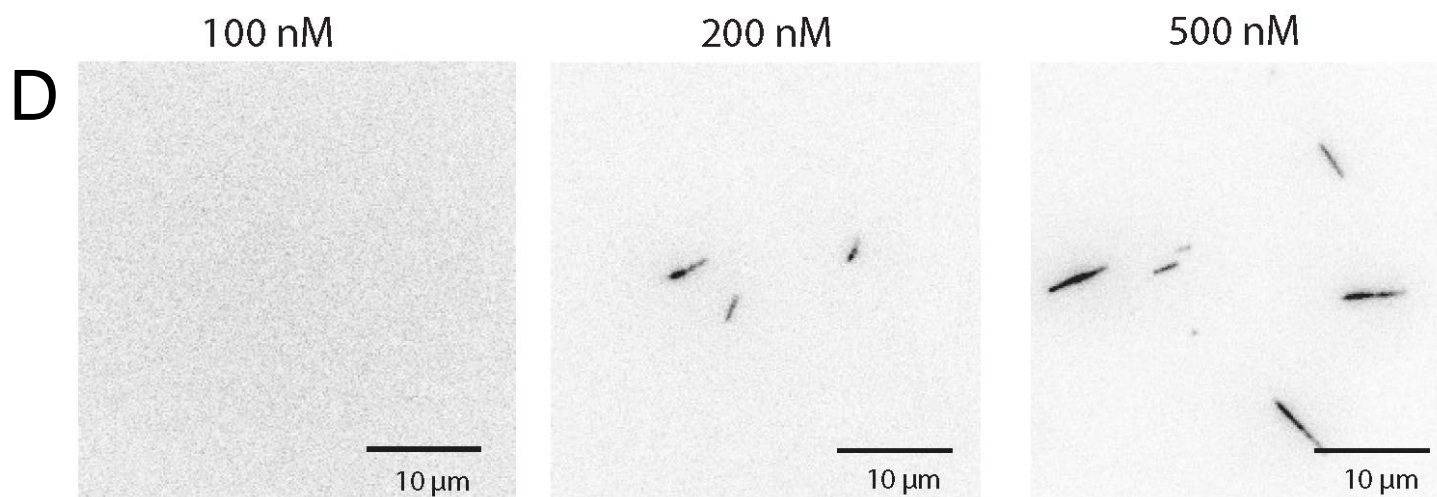
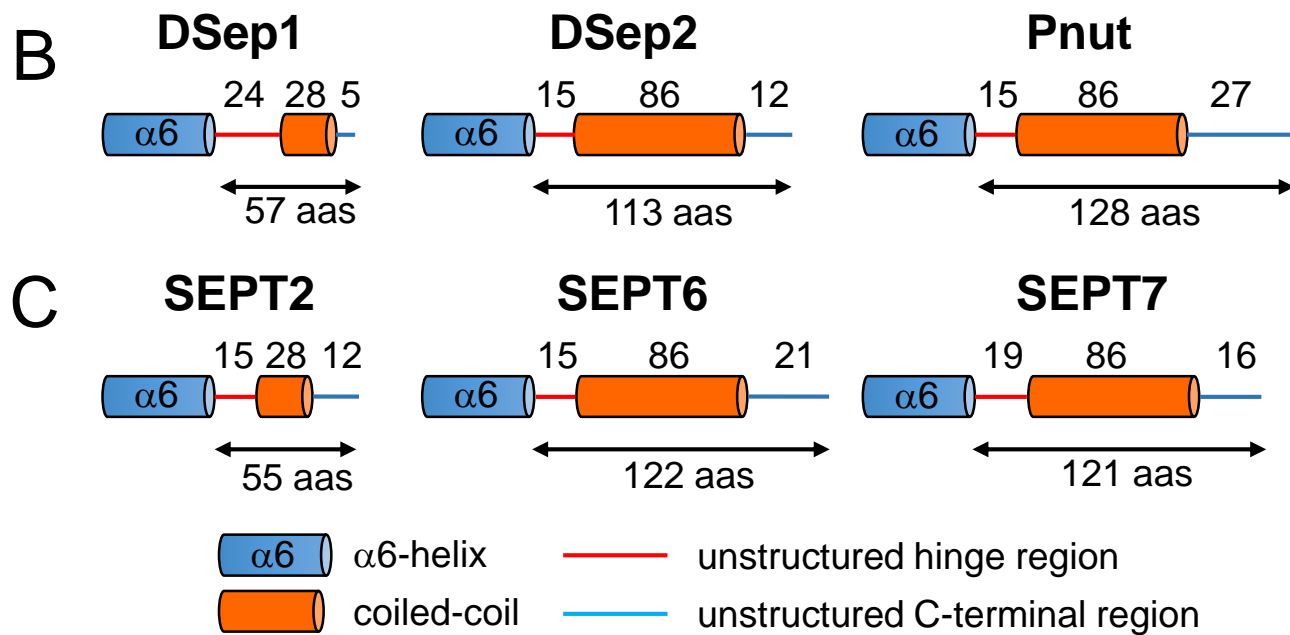
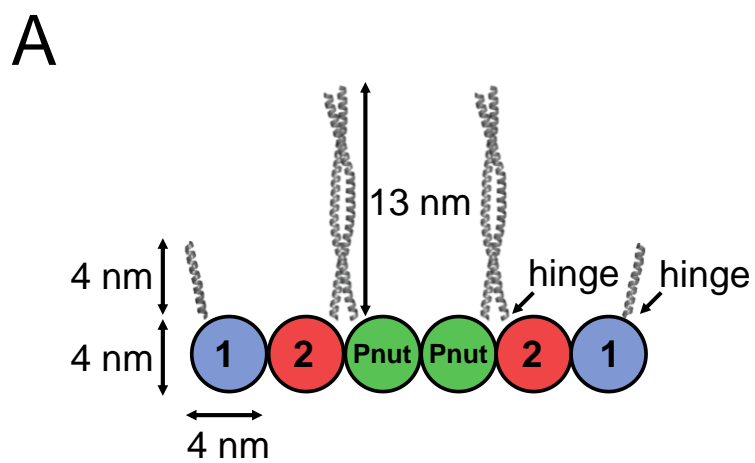
1536 function of SLB composition is expressed in terms of $-\Delta f$ (measured after an incubation time of 1 h),
1537 which is proportional to the areal mass density of septins hexamers including hydrodynamically coupled
1538 solvent. Error bars represent standard deviations based on N experiments per data point (see legend).

1539

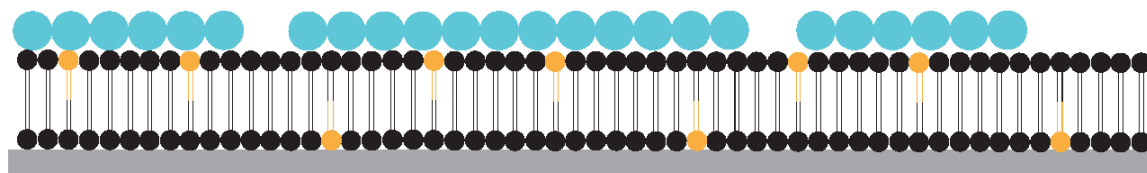
1540 **Figure 7 – Figure supplement 2. Fly septin filament and bundles are sensed as soft relative to**
1541 **membrane-bound globular proteins by QCM-D.** The plot shows $\Delta D/-\Delta f$ ratios (a measure of elastic
1542 compliance, or softness) as a function of $-\Delta f$ (a measure of coverage) for septin hexamers incubated at
1543 60 nM on an SLB made from 80% DOPC and 20% DOPS (data taken from Figure 7B). $\Delta D/-\Delta f$ ratios are
1544 relatively high, consistent with flexible hinges linking the protein to the membrane and/or inter-
1545 connecting protein domains.

1546

1547 **Figure 8 – Figure supplement 1.** Length of filaments formed by C-terminally truncated (ΔCC) fly septin
1548 hexamers (84 nM) formed in septin polymerization buffer, i.e., in the absence of a lipid mono-/bilayer.
1549 The length varies continuously from 20 to 80 nm, suggesting that subunits can be added individually to
1550 the ends of rods.



A



anionic lipid



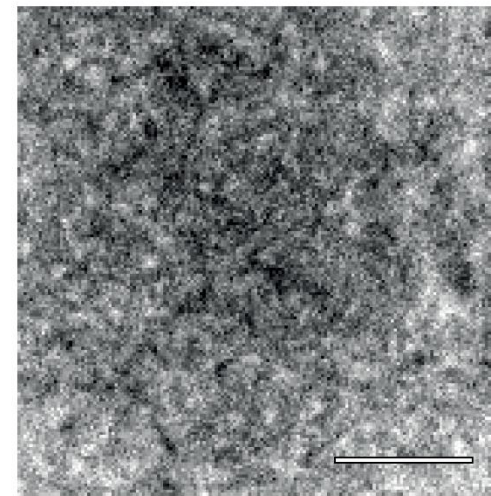
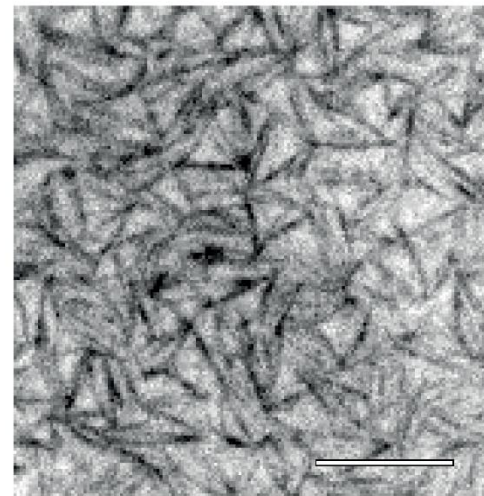
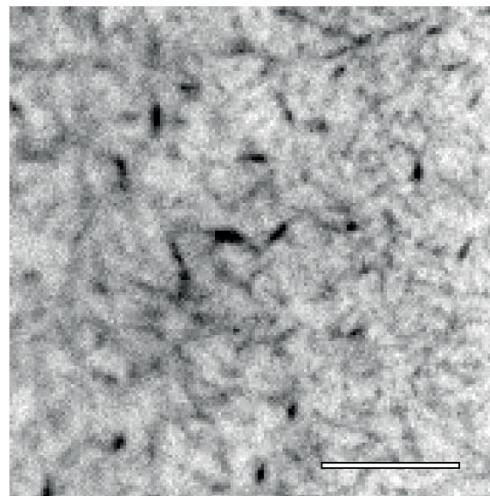
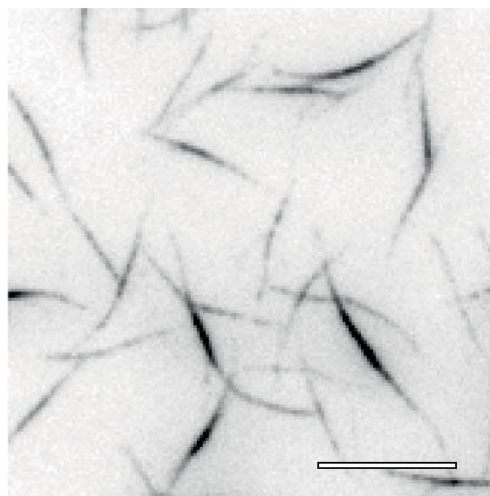
neutral lipid



septin hexamer

PI(4,5)P₂ 1%PI(4,5)P₂ 2.5%PI(4,5)P₂ 5%PI(4,5)P₂ 8%

B



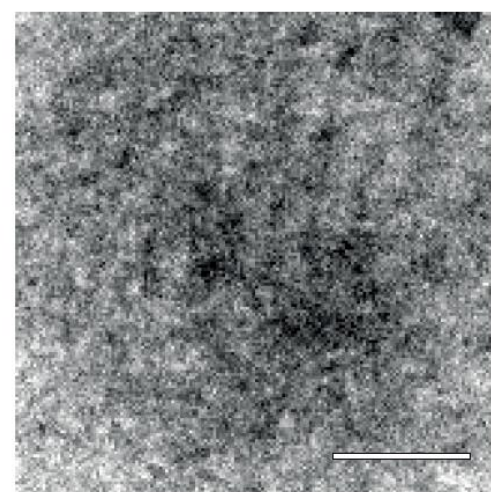
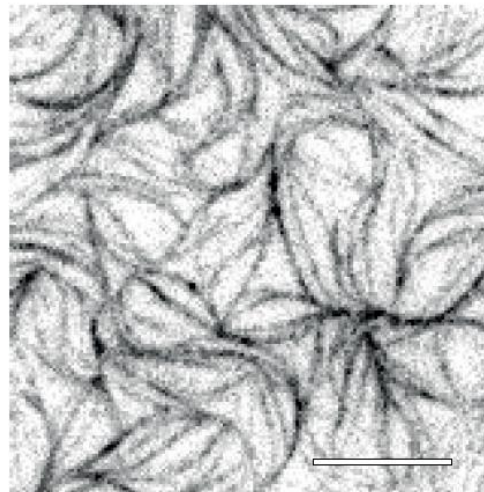
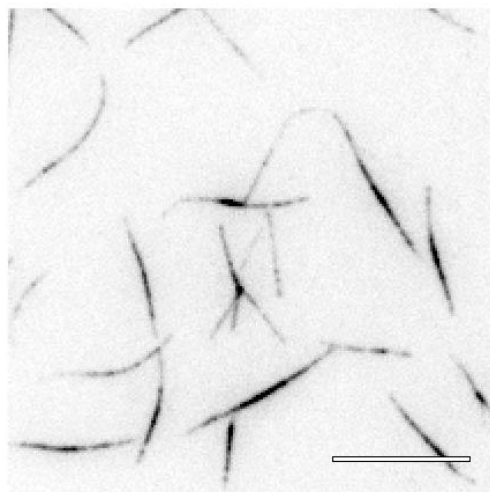
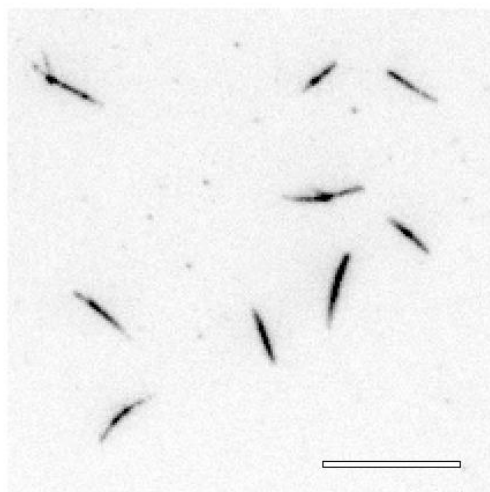
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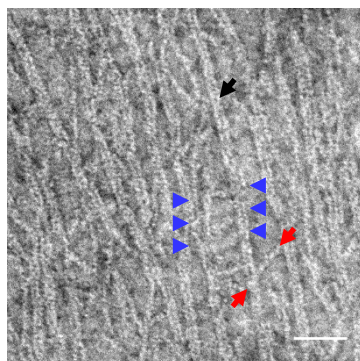
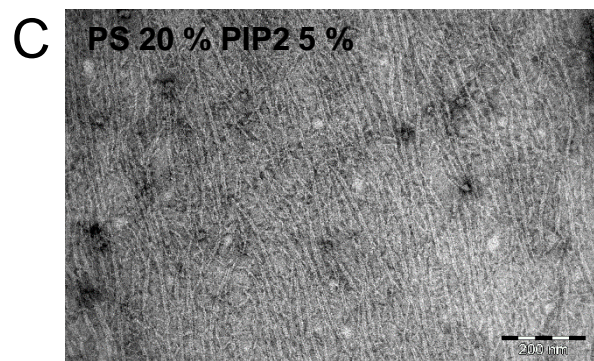
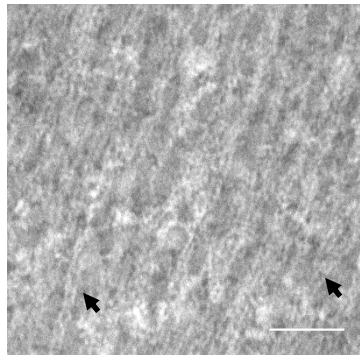
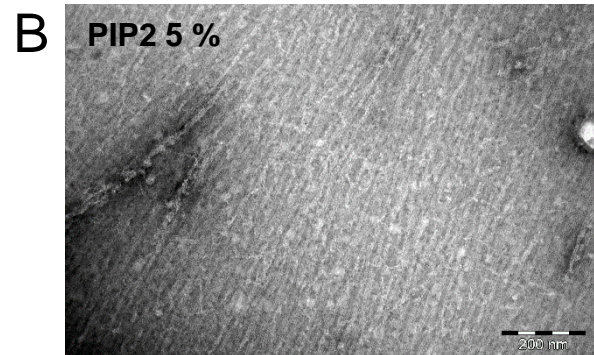
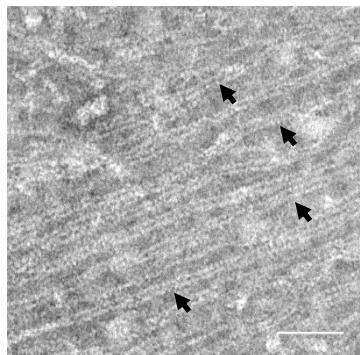
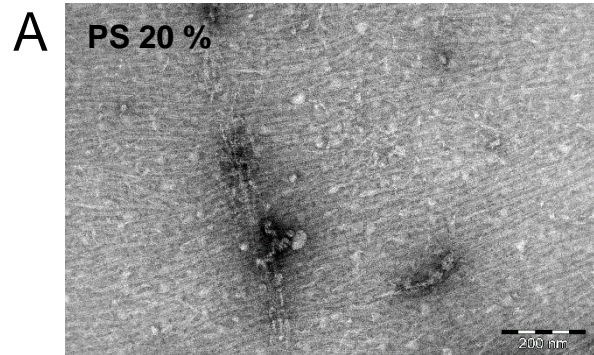
PS 5%

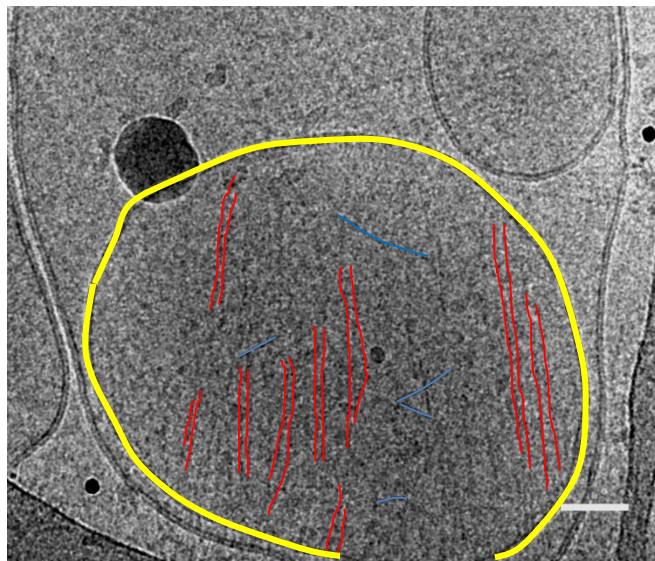
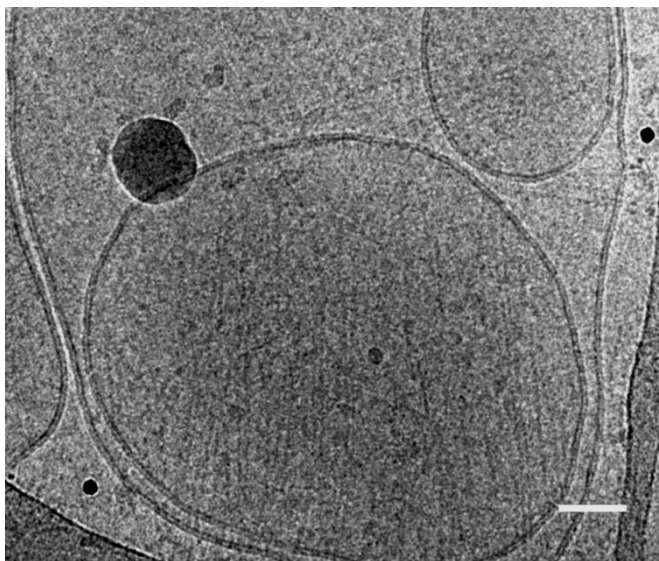
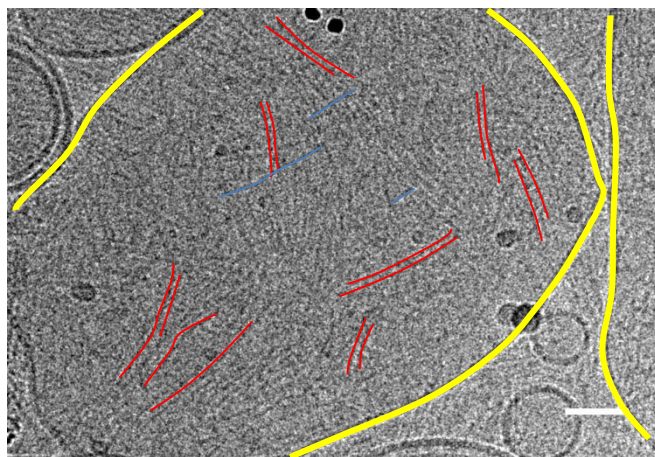
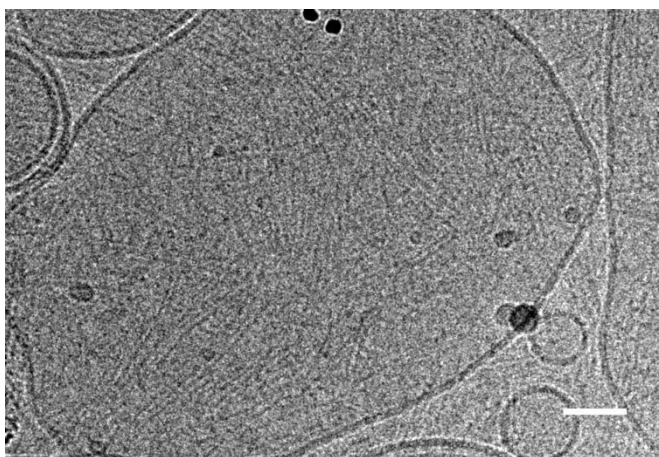
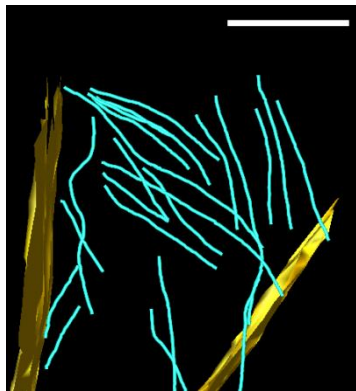
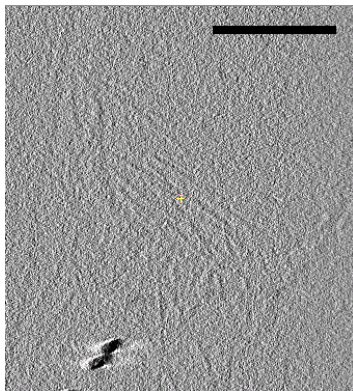
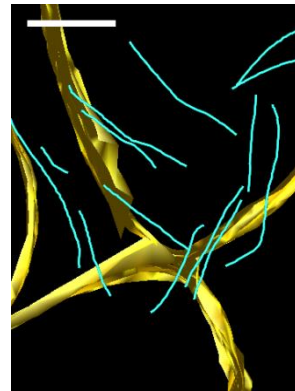
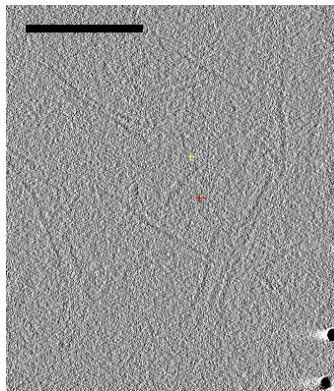
PS 10%

PS 20%

C





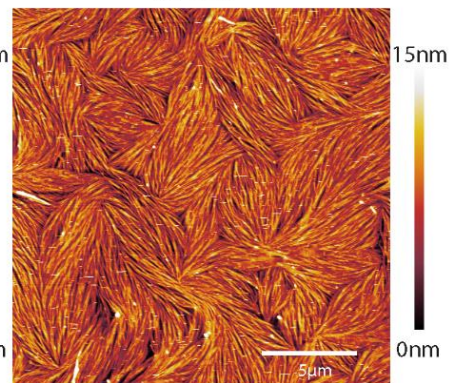
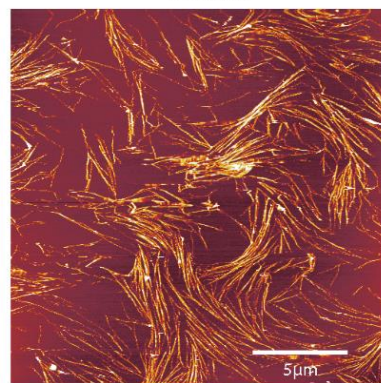
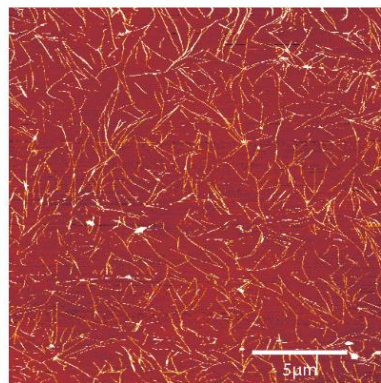
A**B****C****D**

12 nM

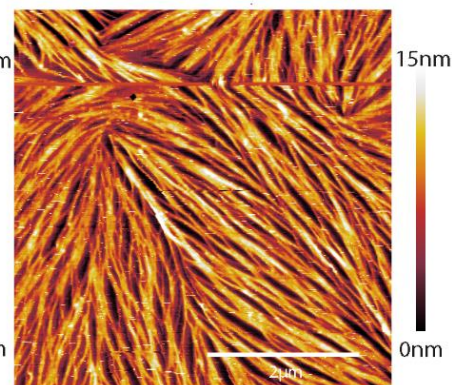
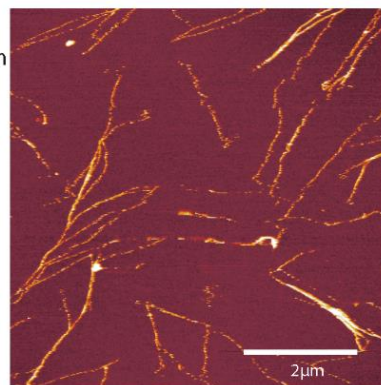
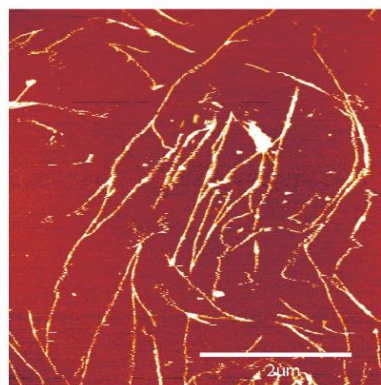
24 nM

60 nM

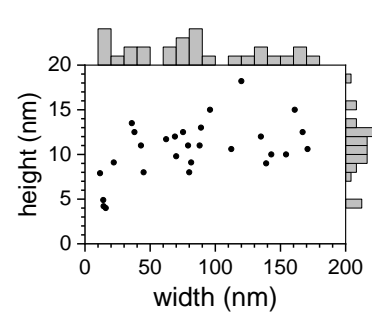
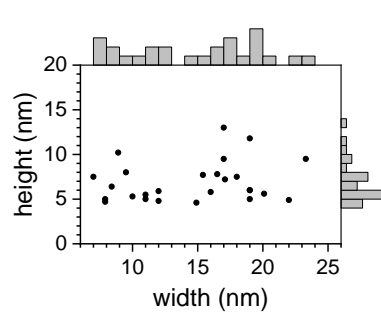
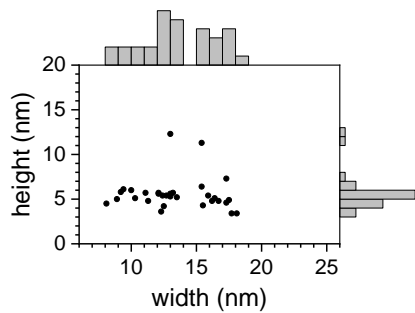
A

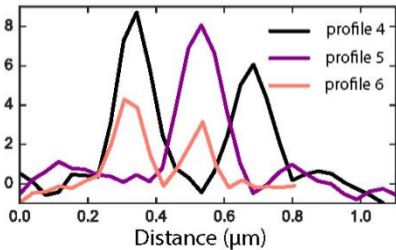
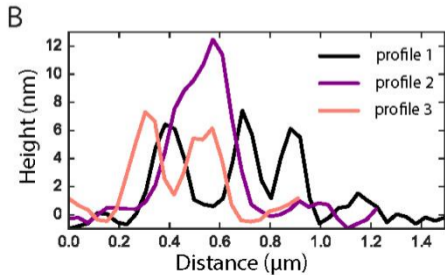
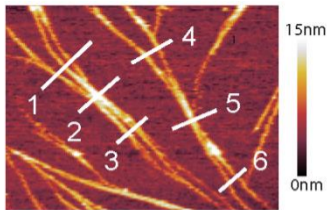
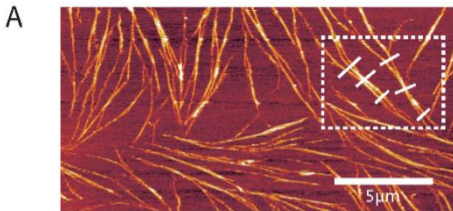


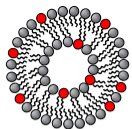
B



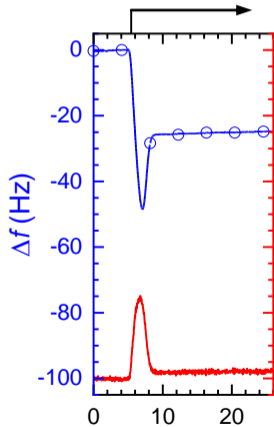
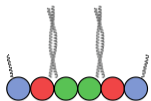
C



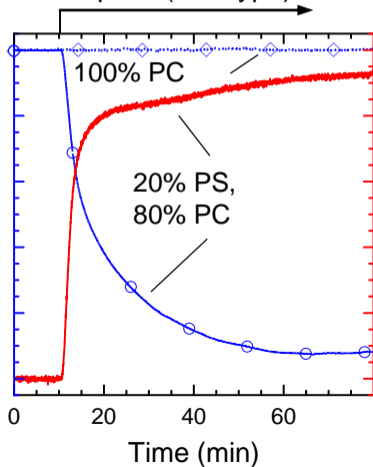
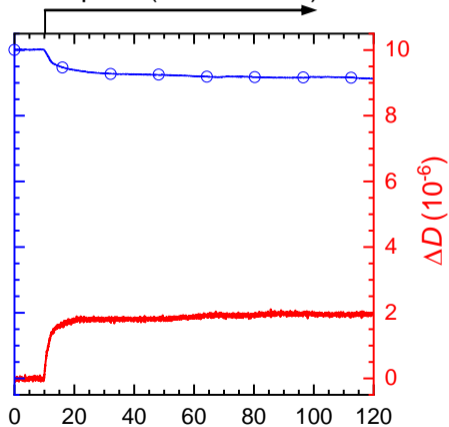


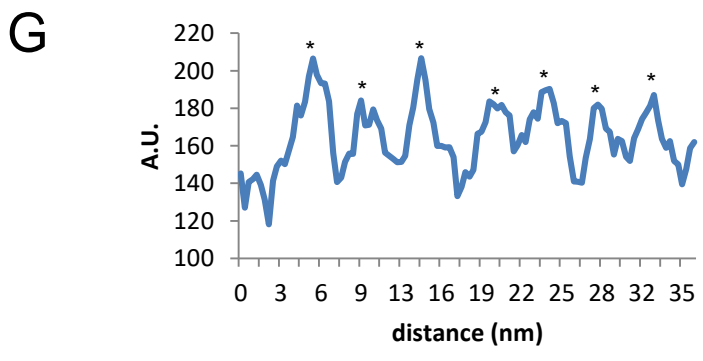
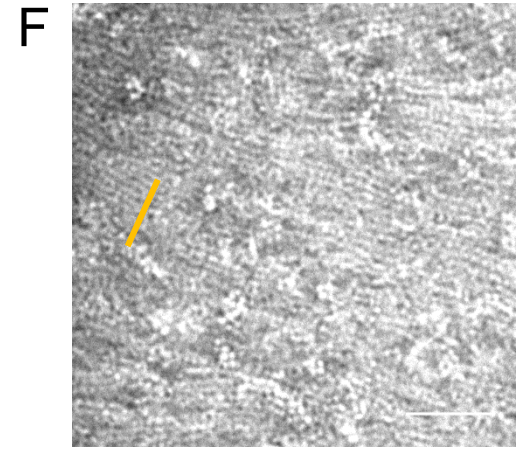
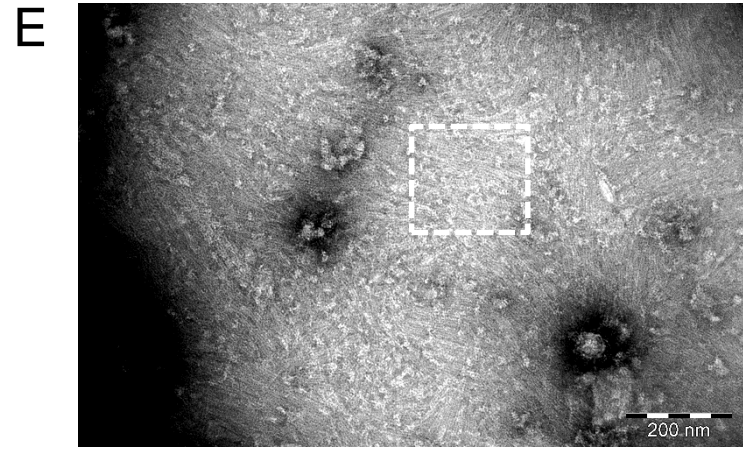
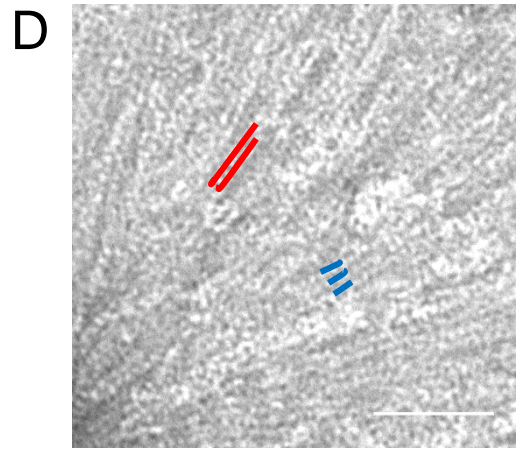
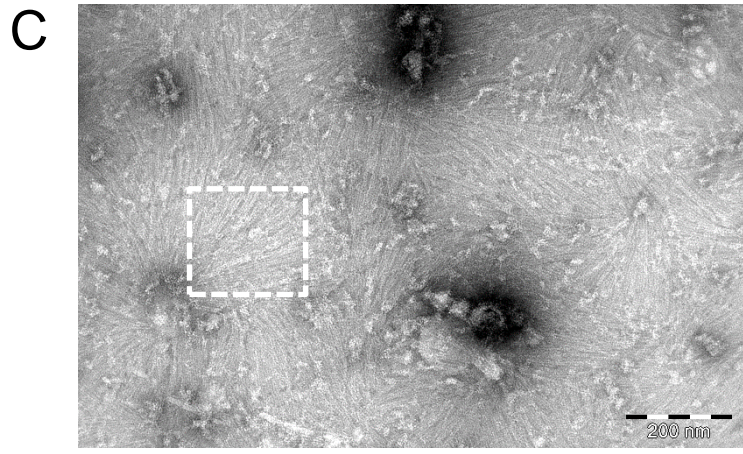
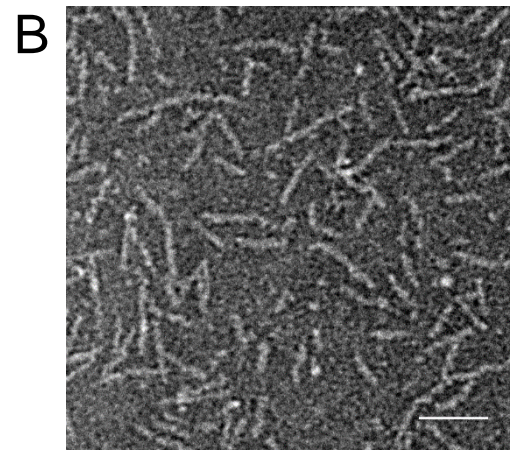
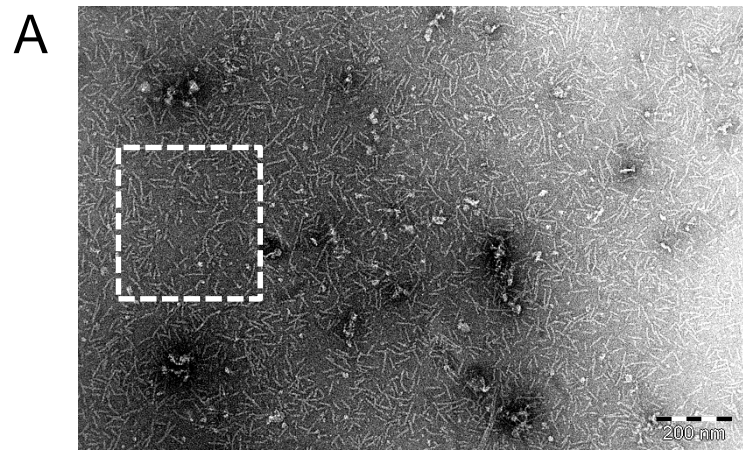
A

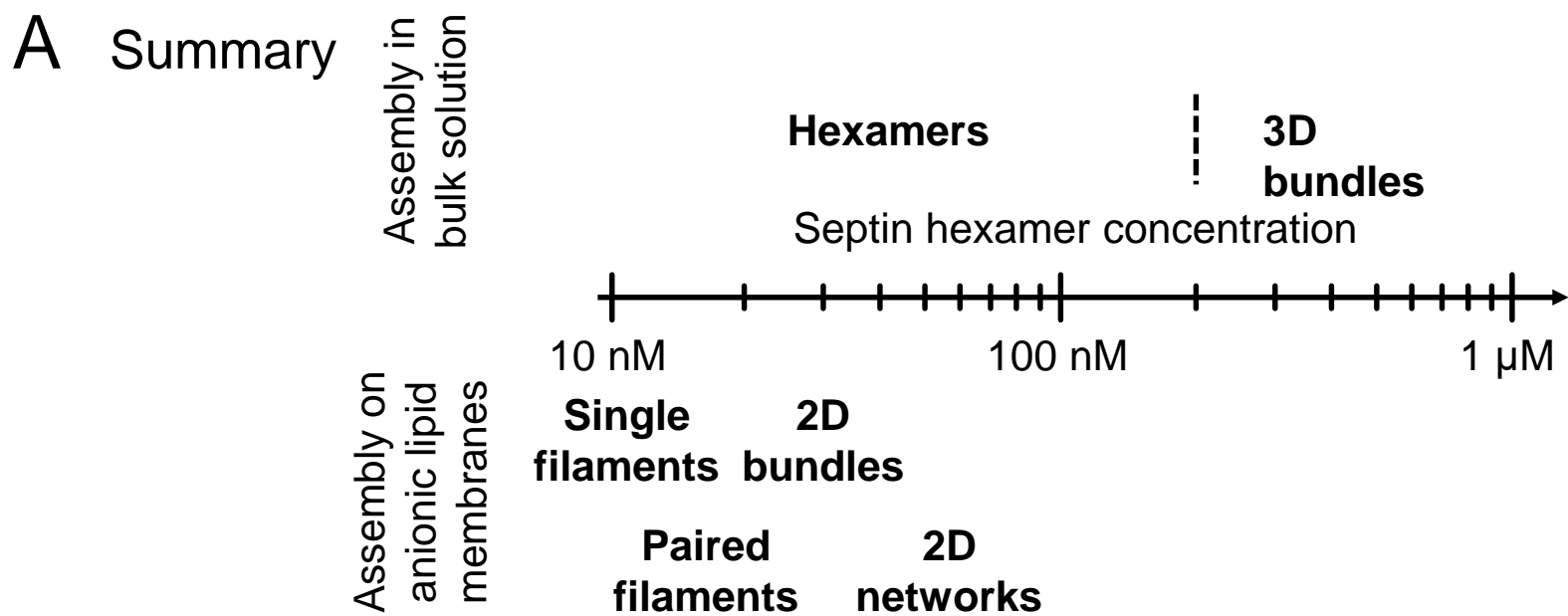
SUVs

**B**

Septins (wild type)

**C**Septins (ΔCC mutant)



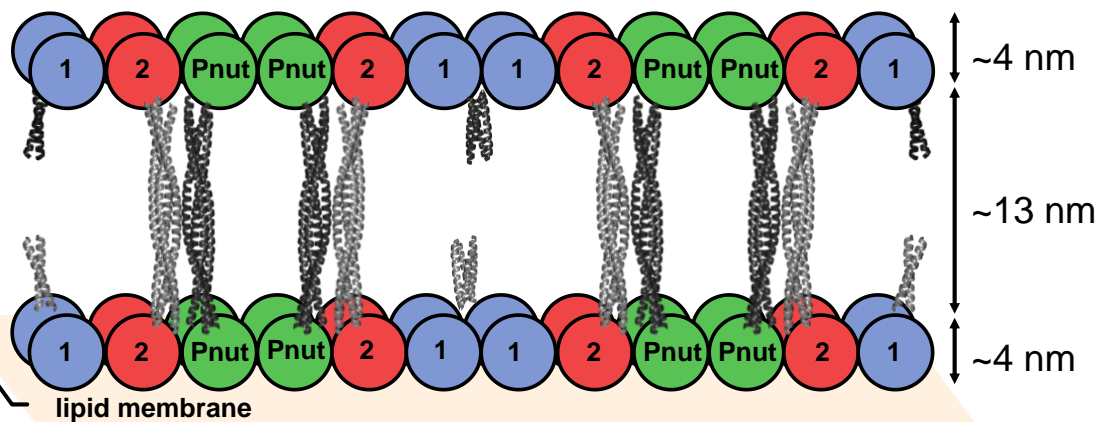
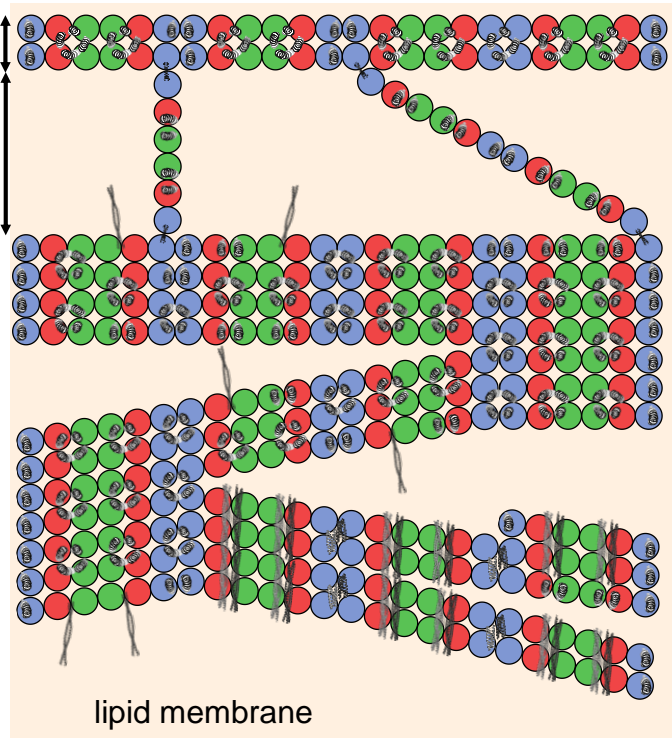


B Self-assembly of full-length septin hexamers on lipid membranes

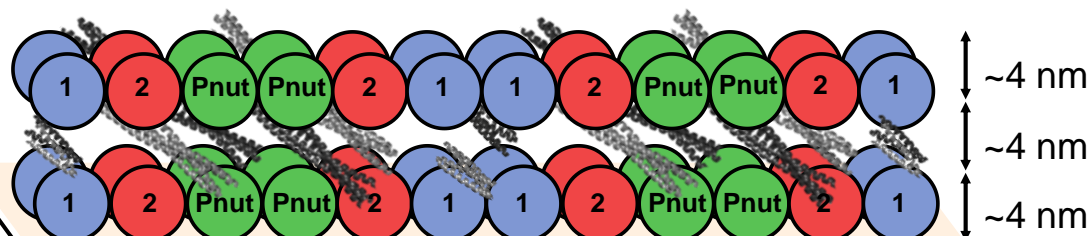
TOP VIEW
EM, AFM

SIDE VIEW
AFM, QCM-D

~8 nm
~24 nm

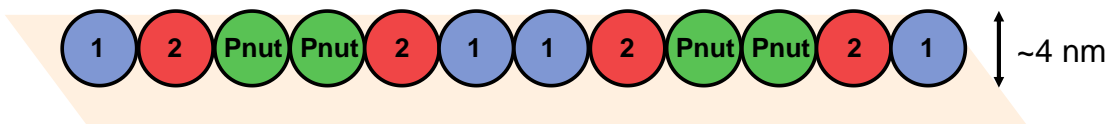
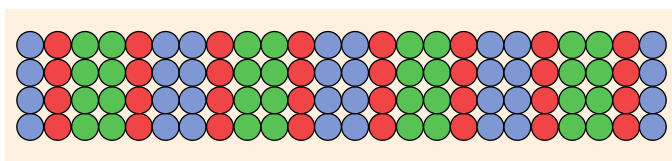


Scenario 1: coiled-coils perpendicular to the membrane

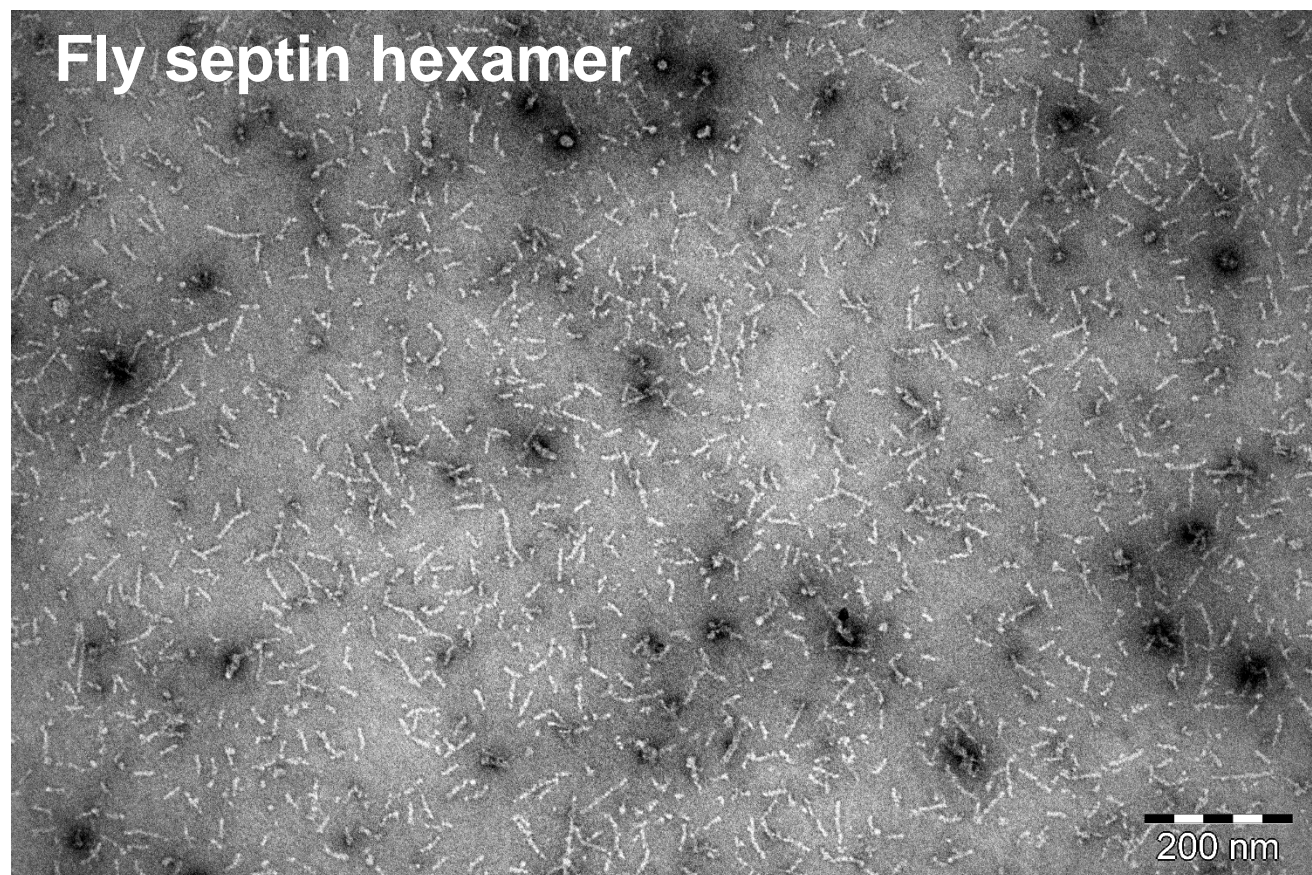


Scenario 2: coiled-coils parallel to the membrane

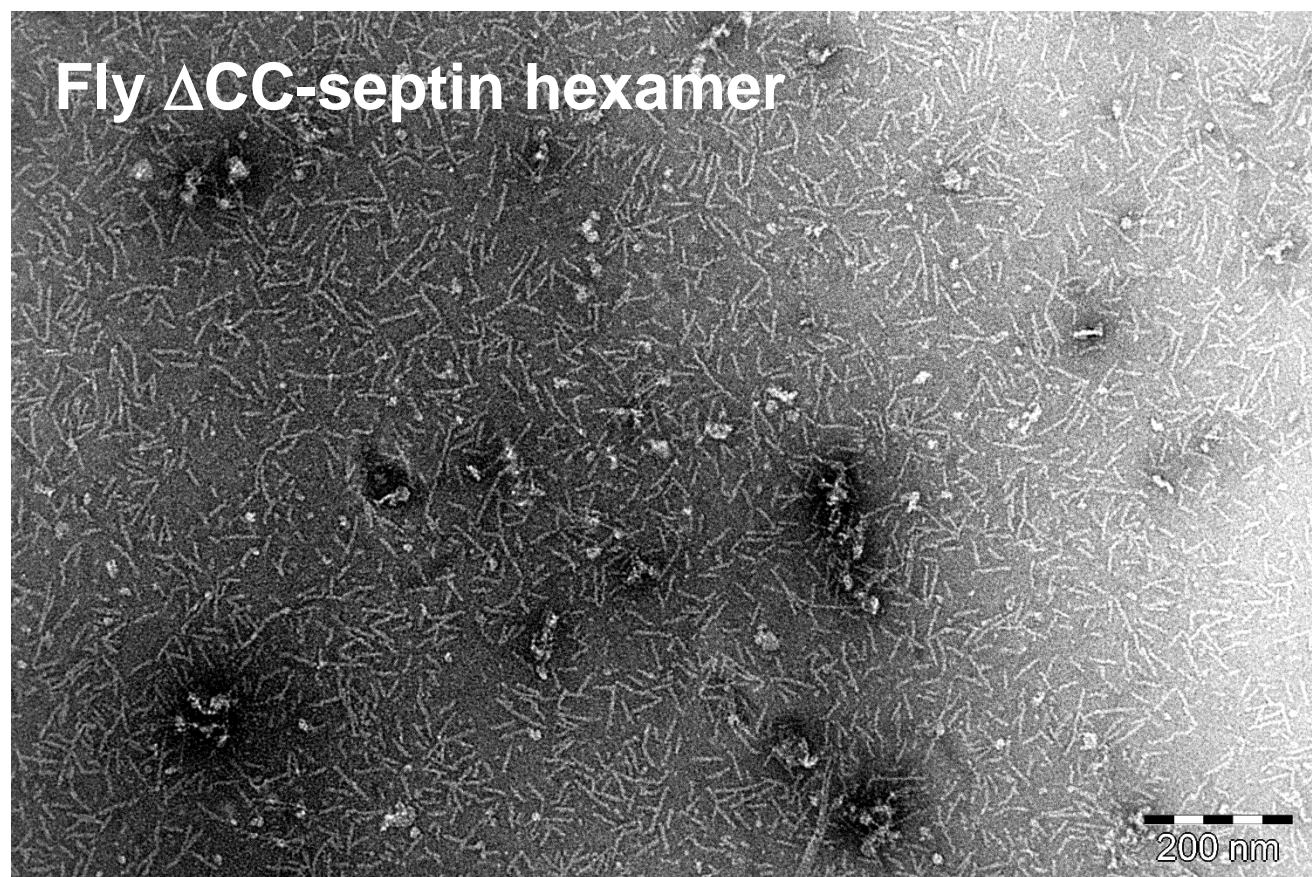
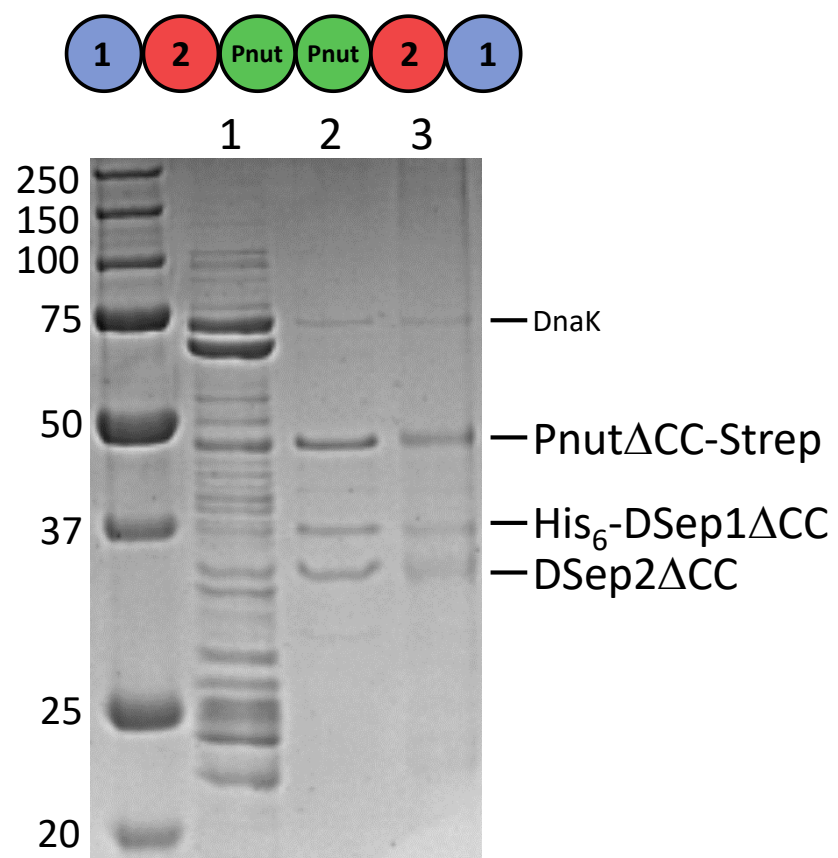
C Self-assembly of Δ CC septin hexamers on lipid membranes

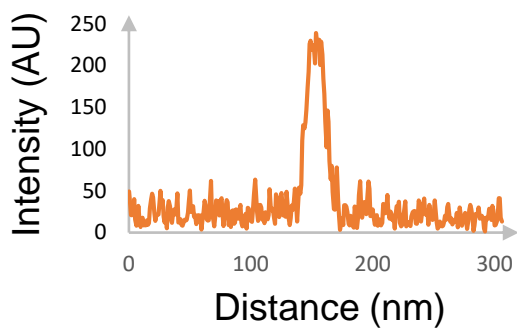
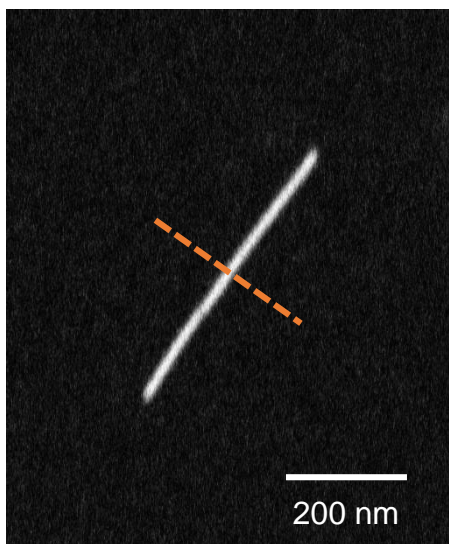
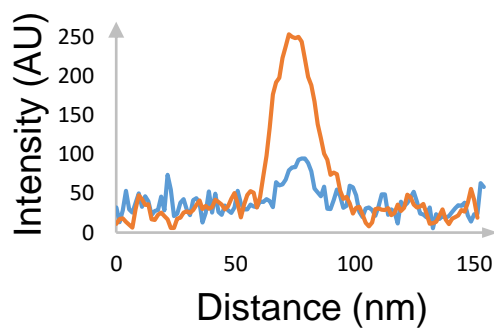
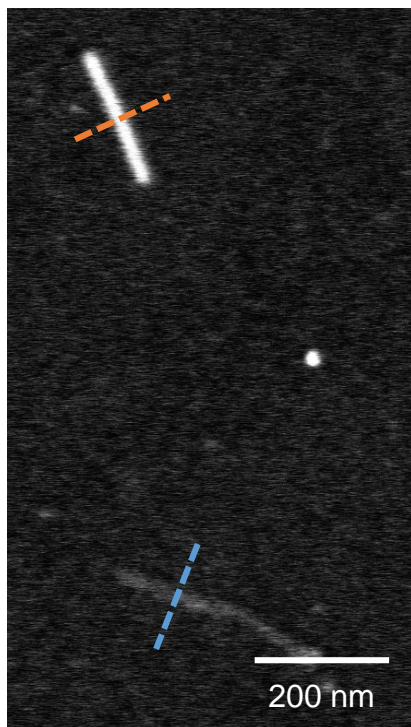
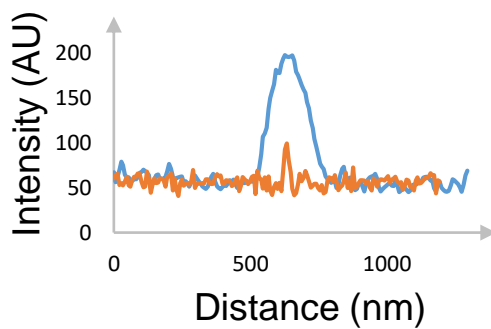
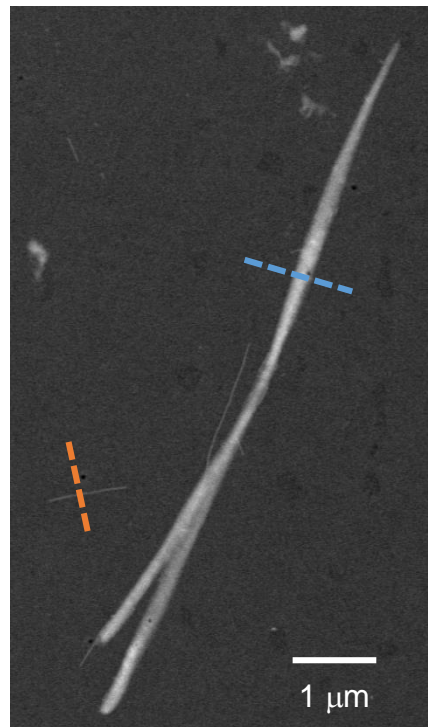
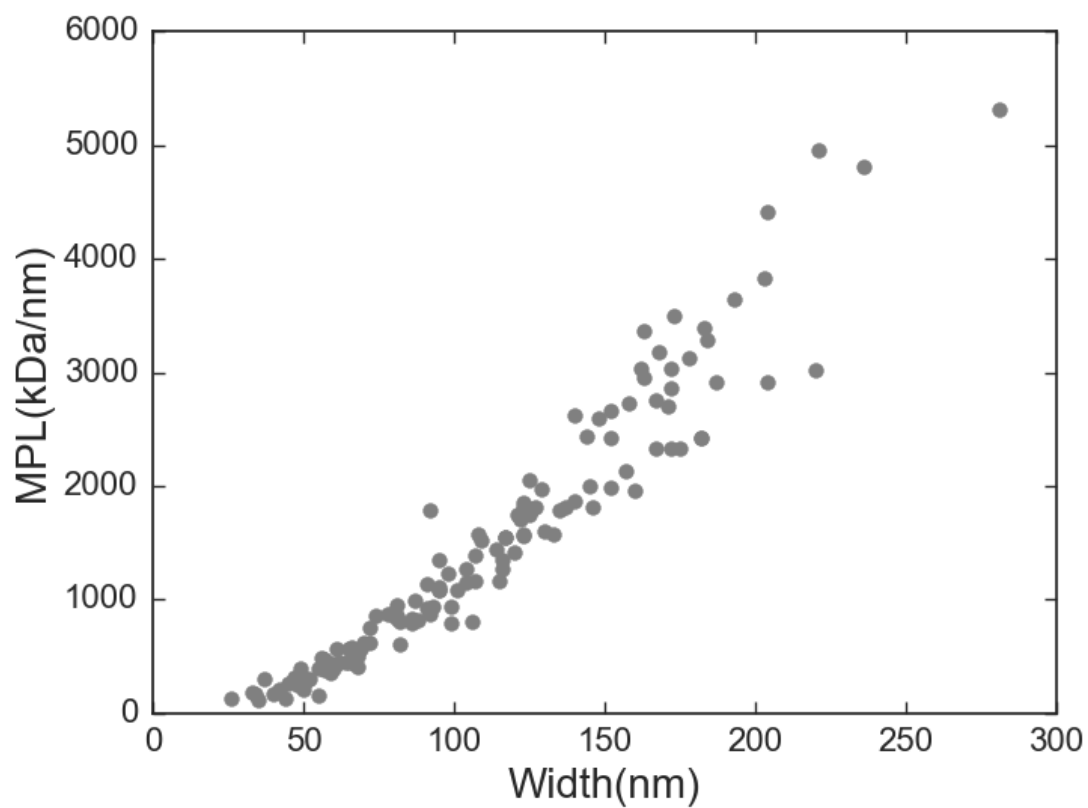


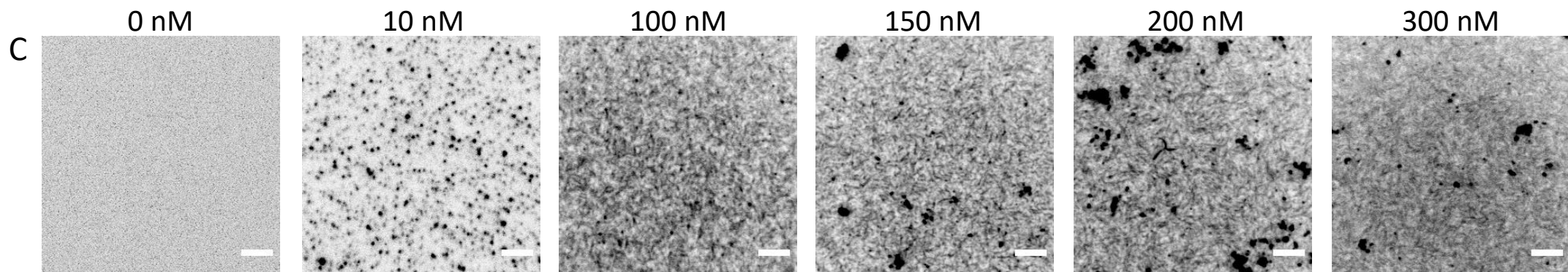
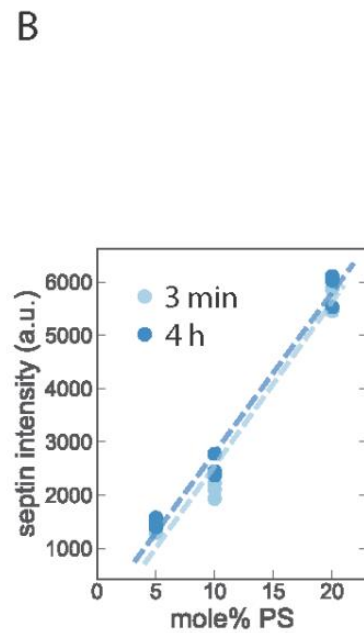
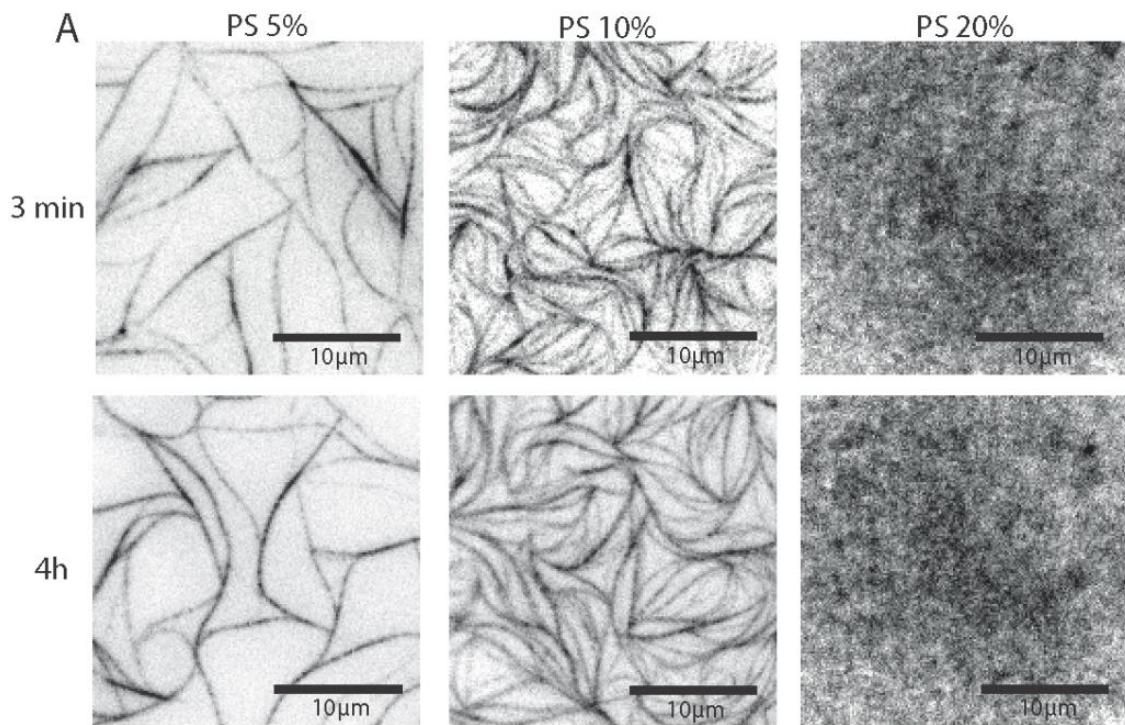
A



B



A**B****C****D**



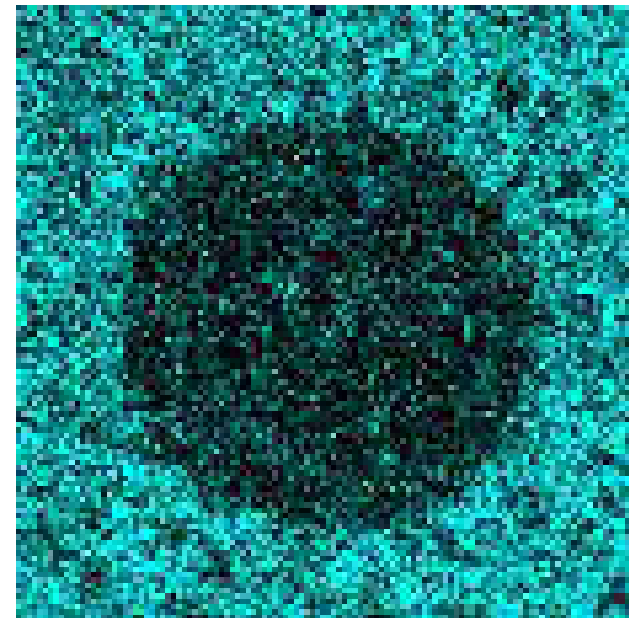
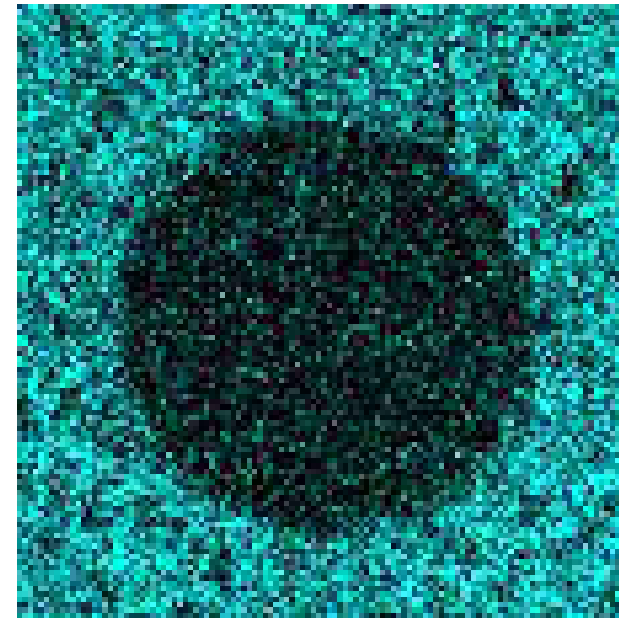
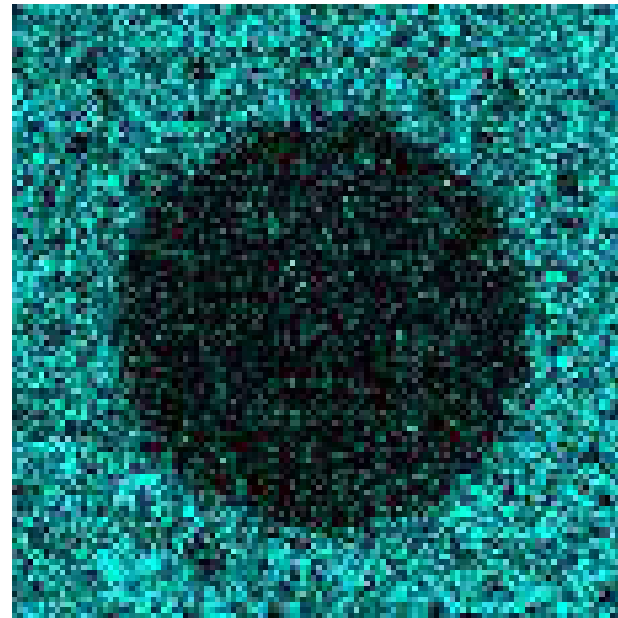
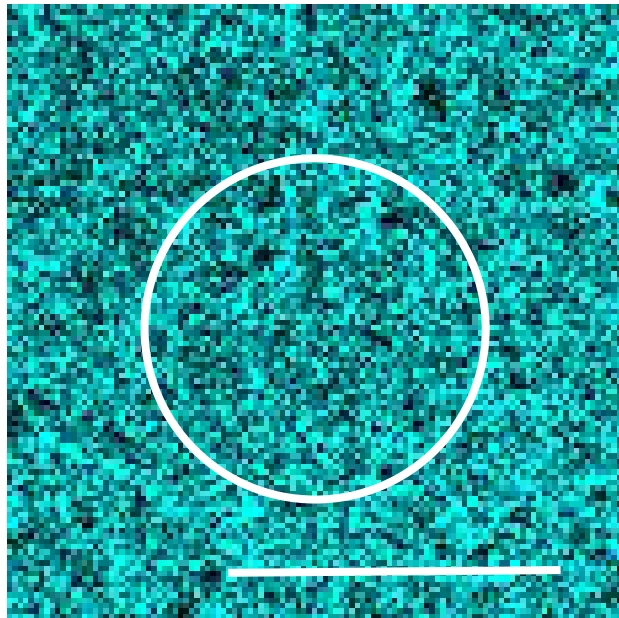
before bleaching
 $t=-12s$

moment of bleaching
 $t=0s$

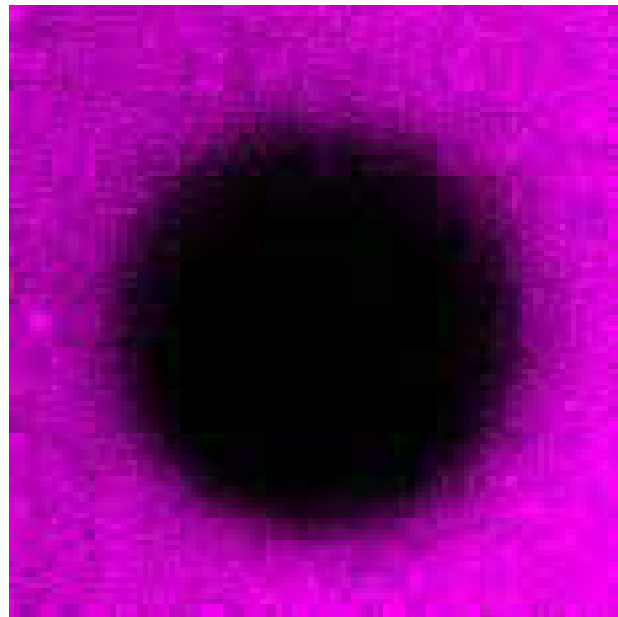
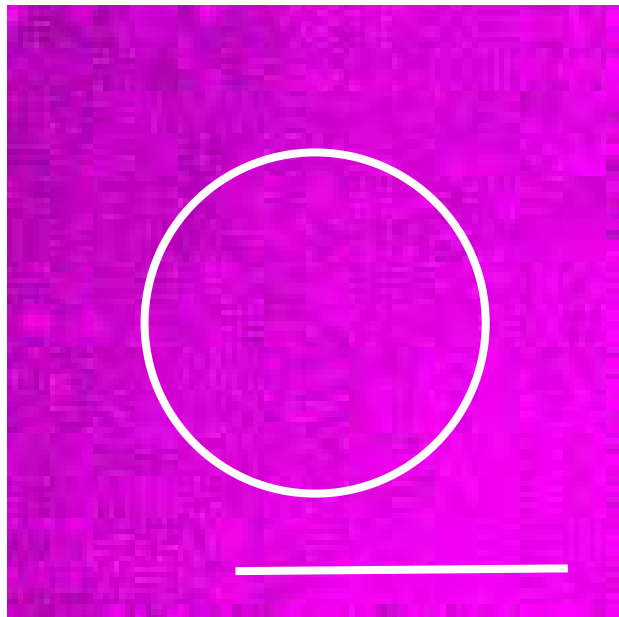
after bleaching
 $t=60s$

after bleaching
 $t=440s$

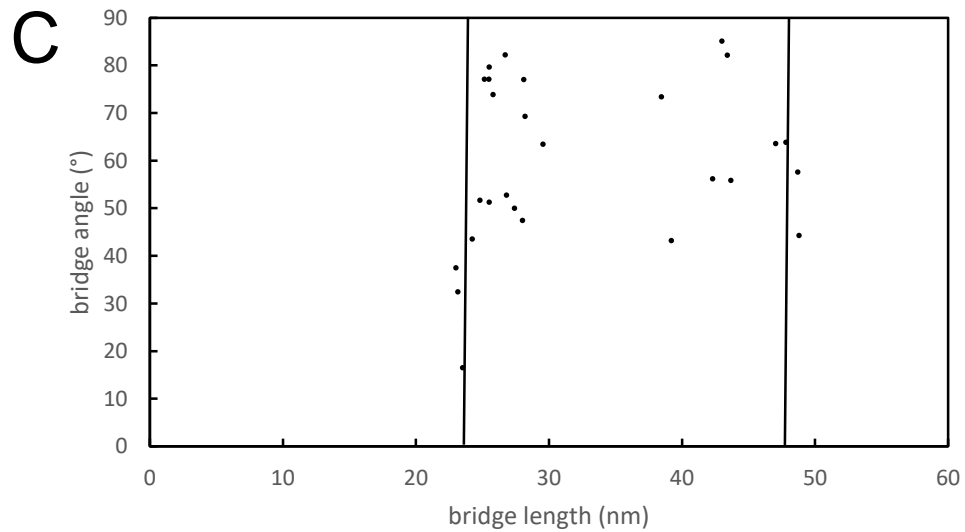
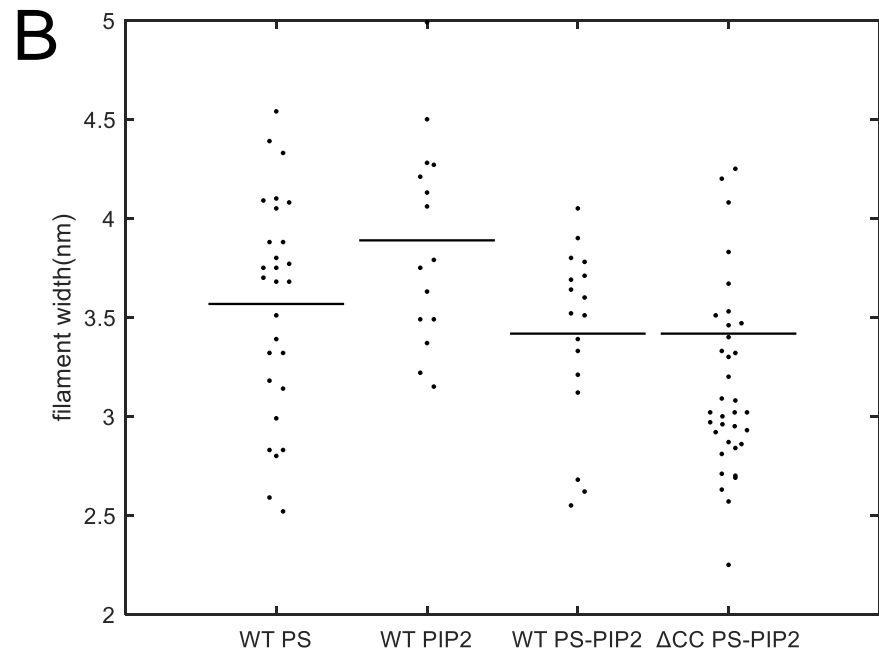
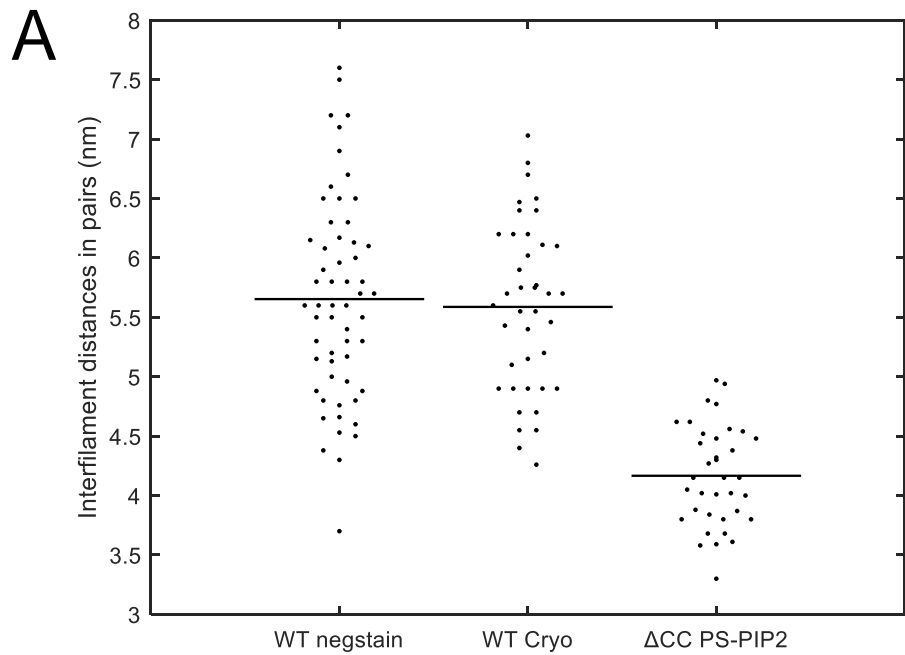
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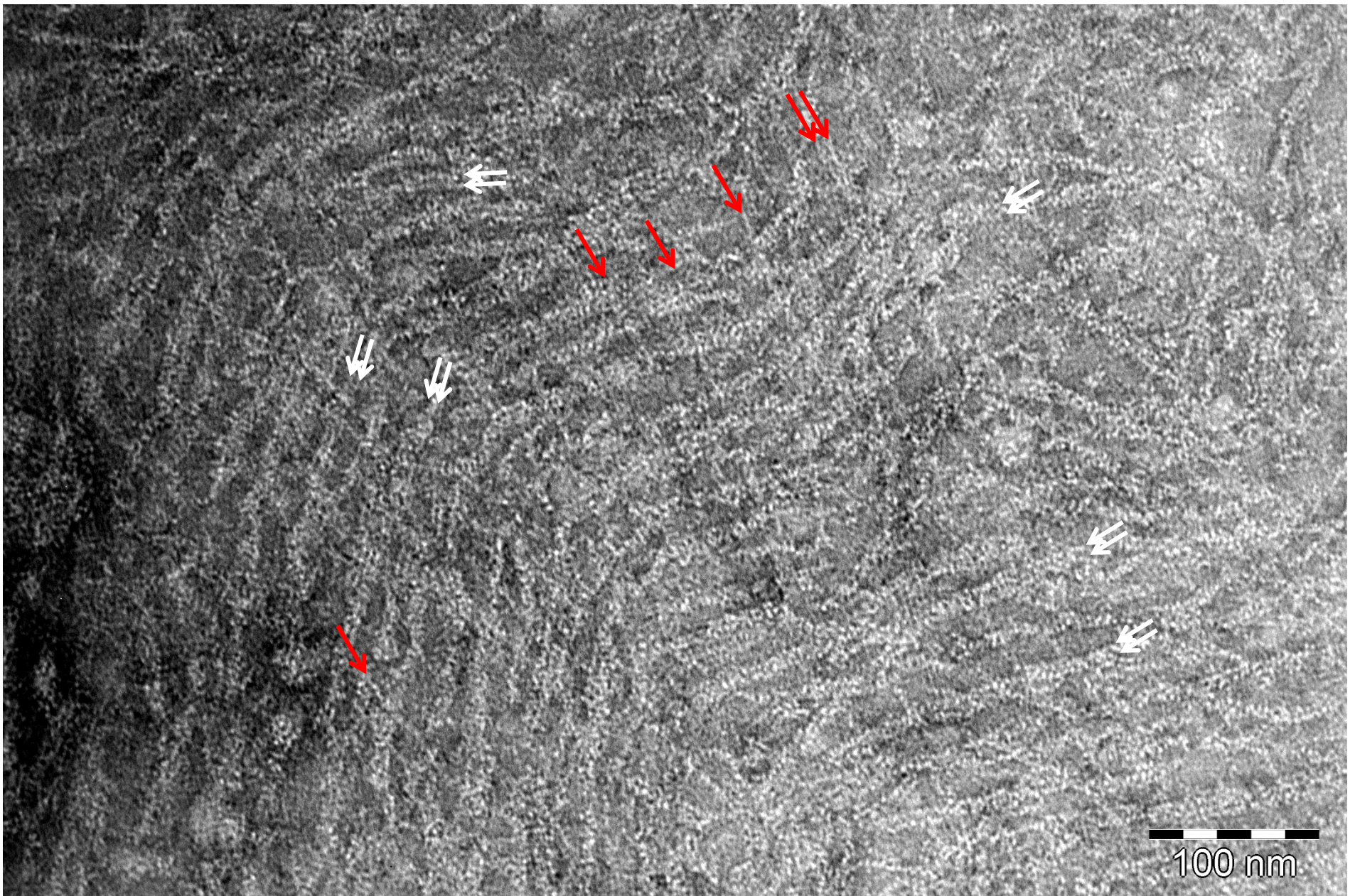
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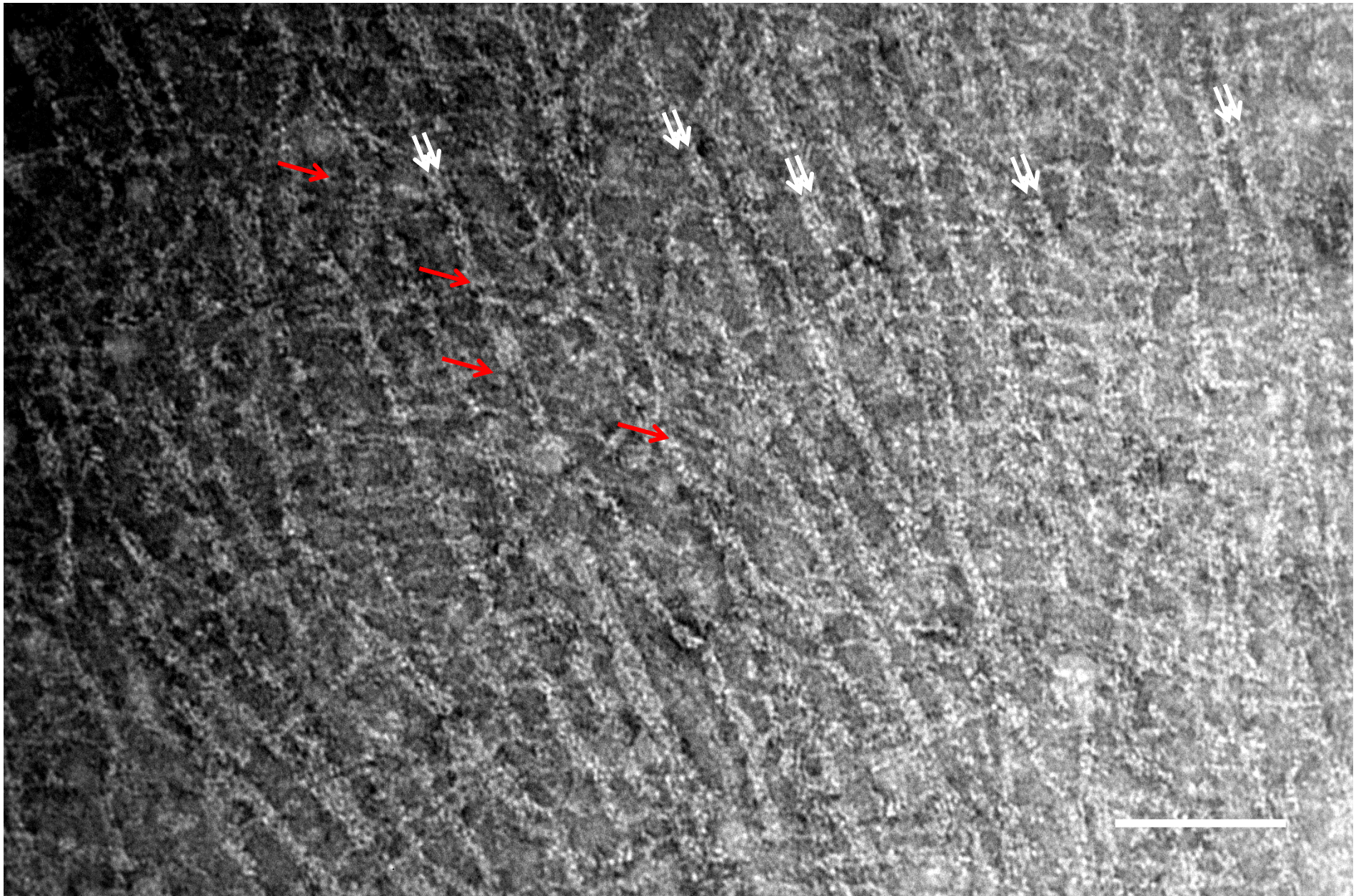


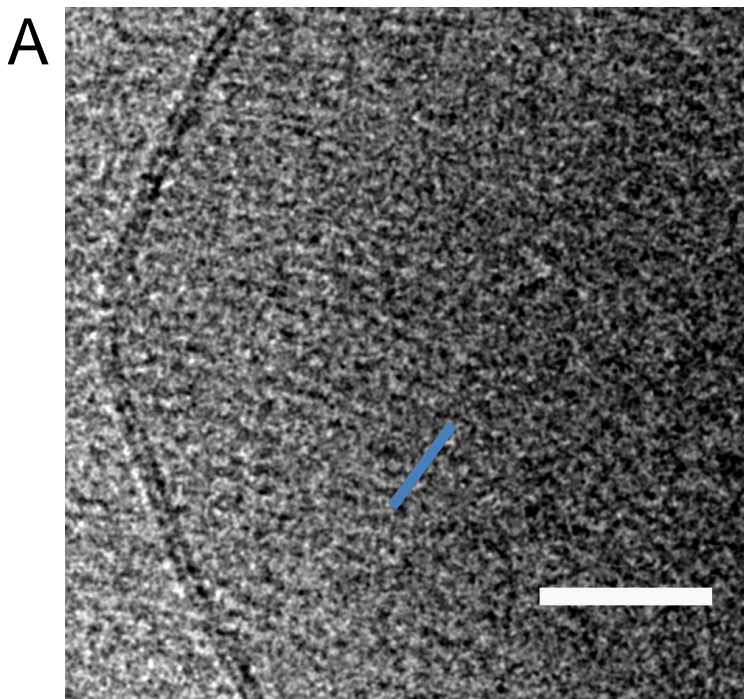


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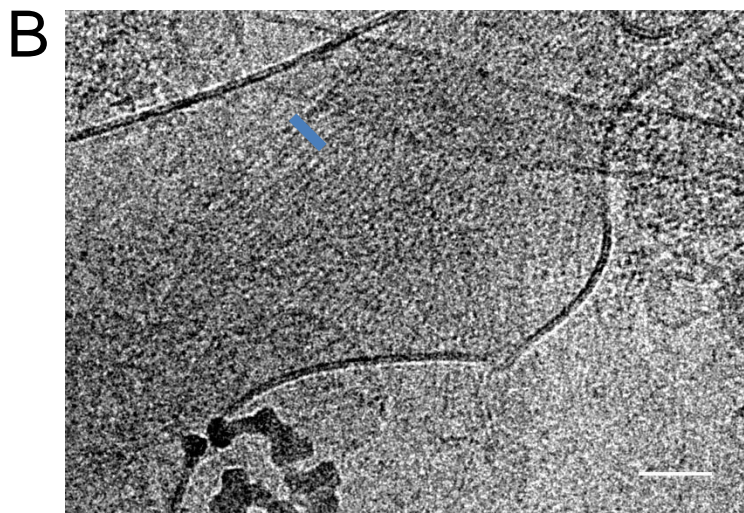
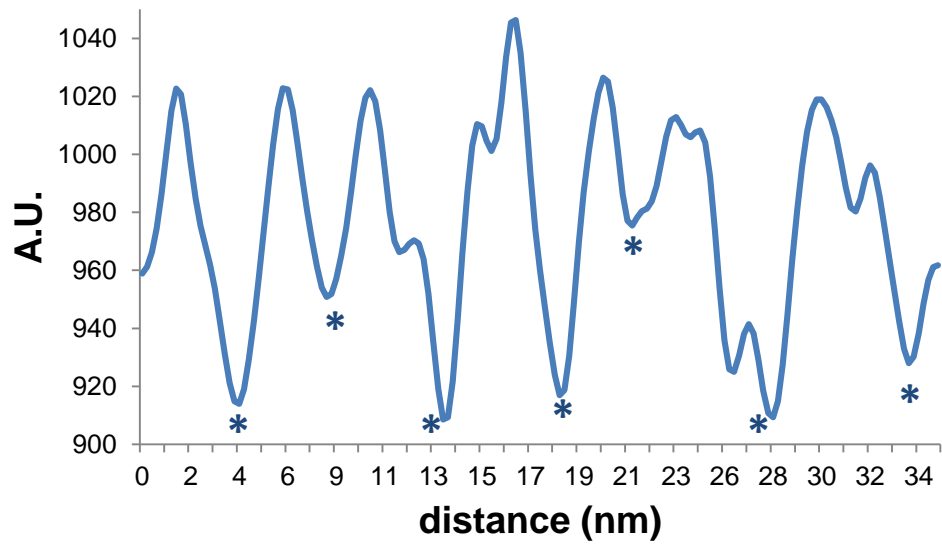


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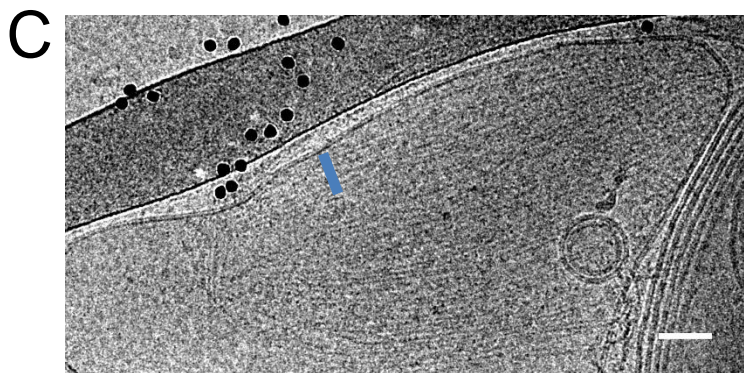
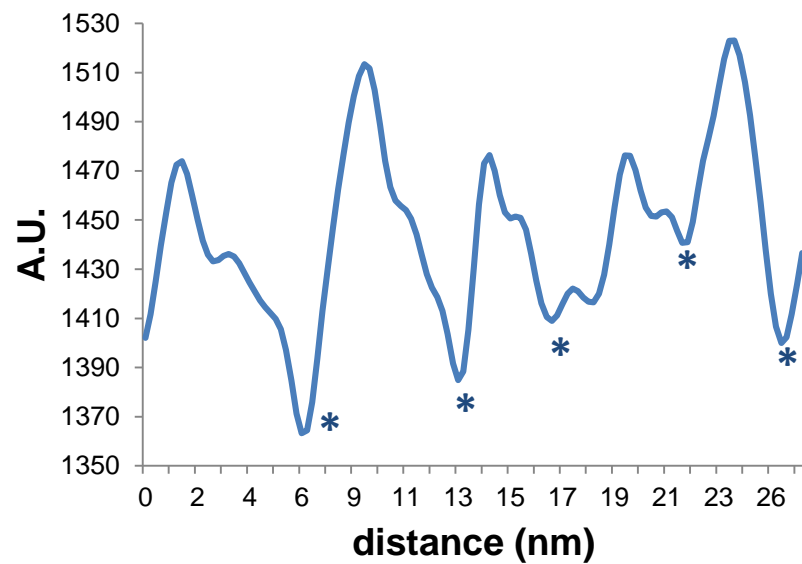




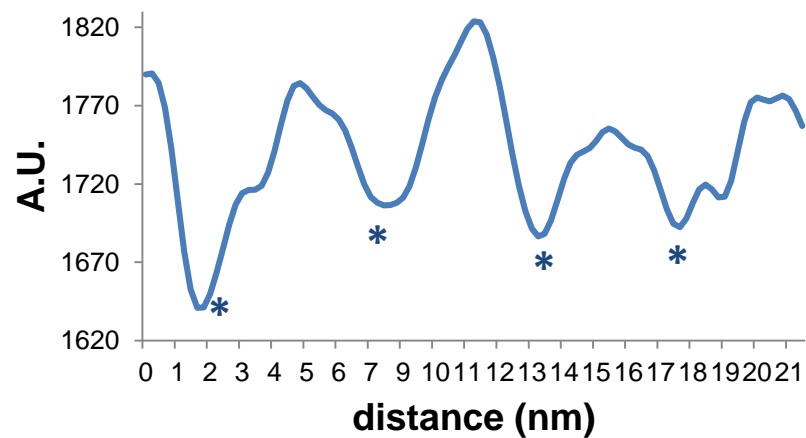
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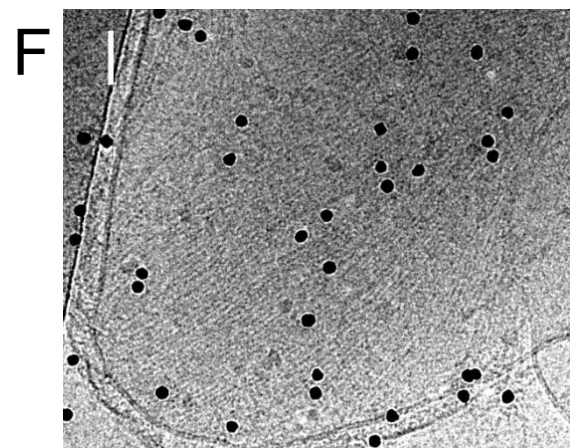
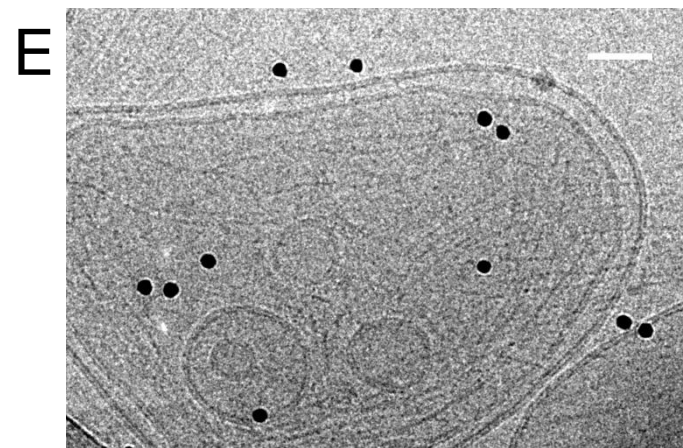
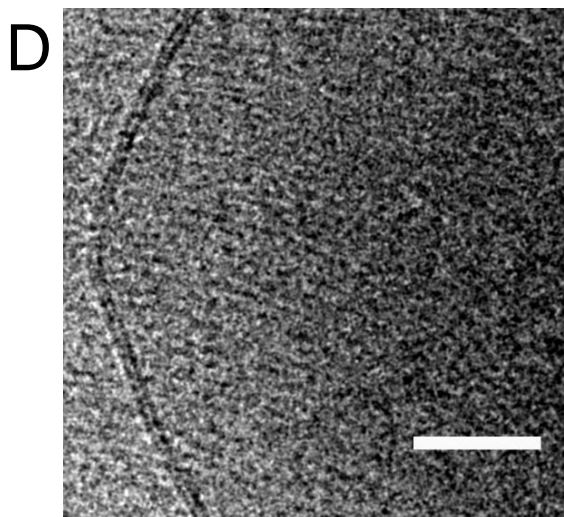
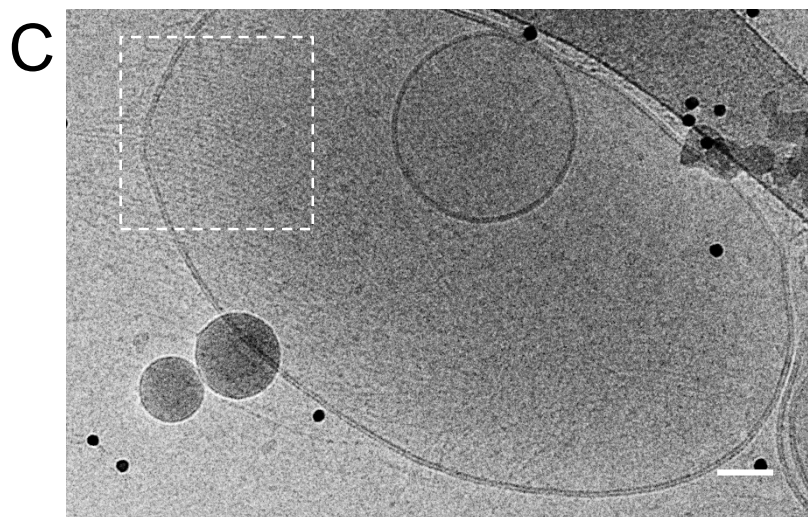
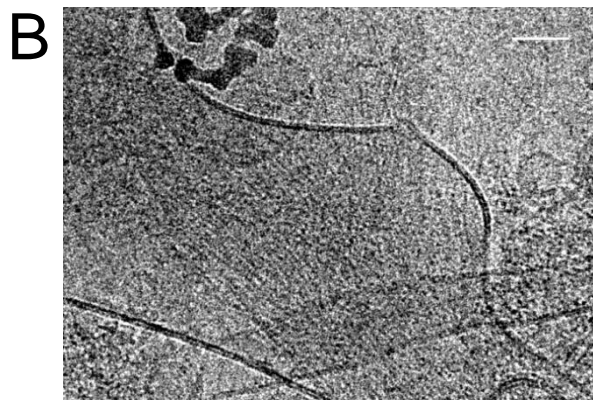
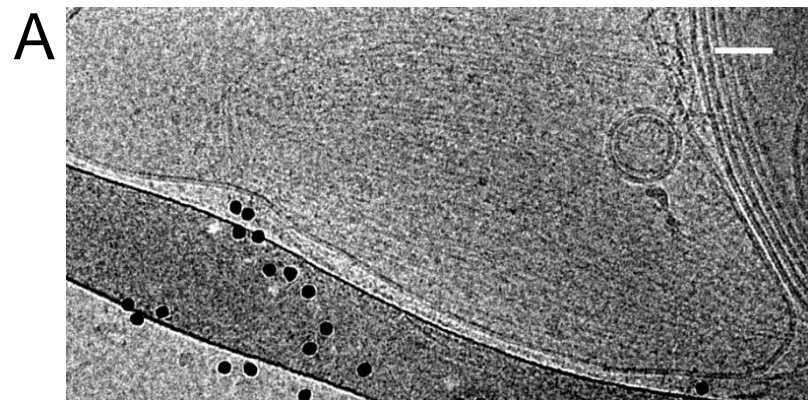


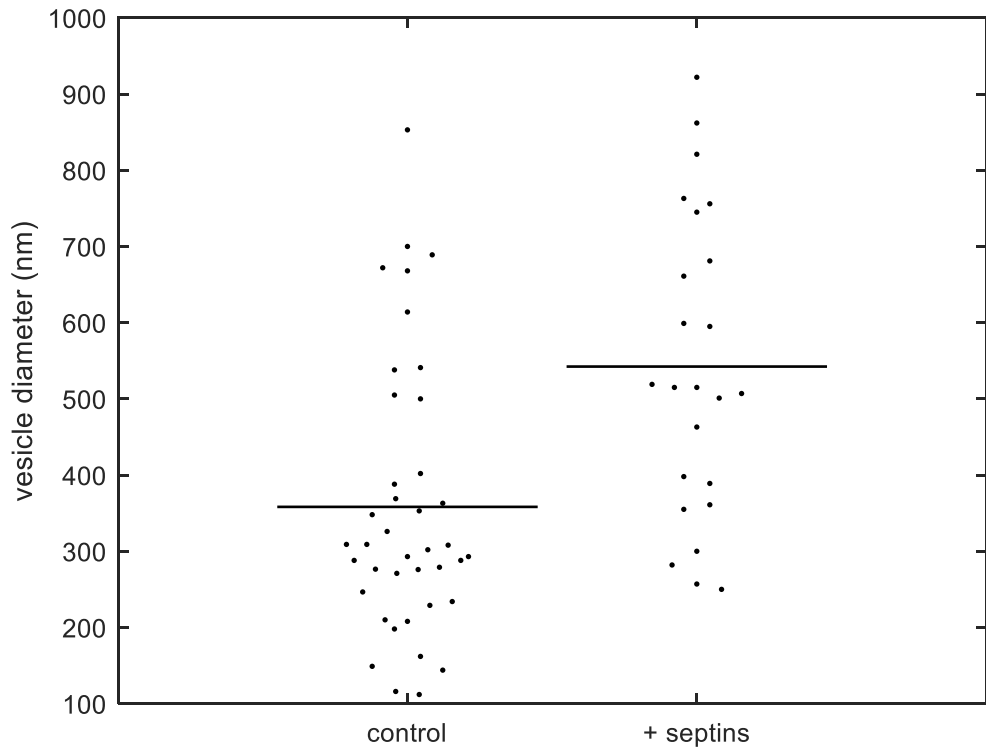
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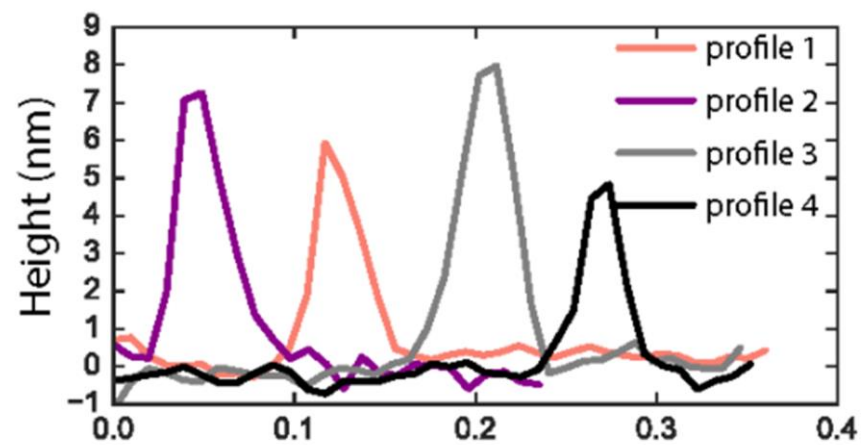
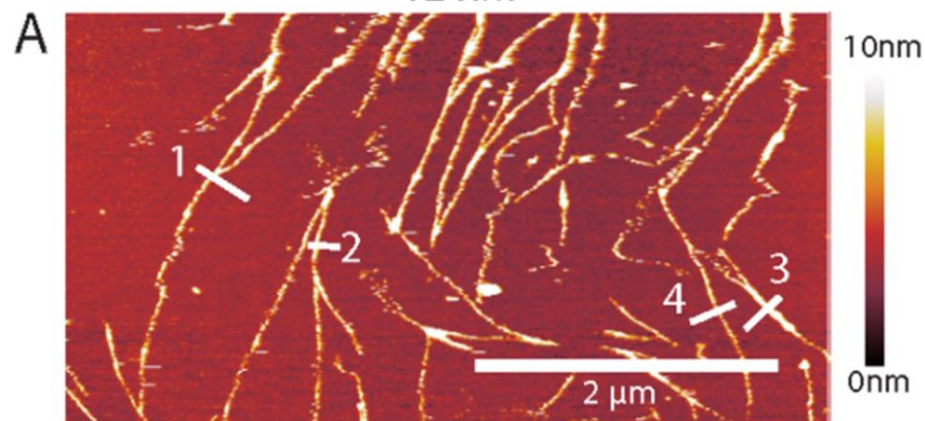
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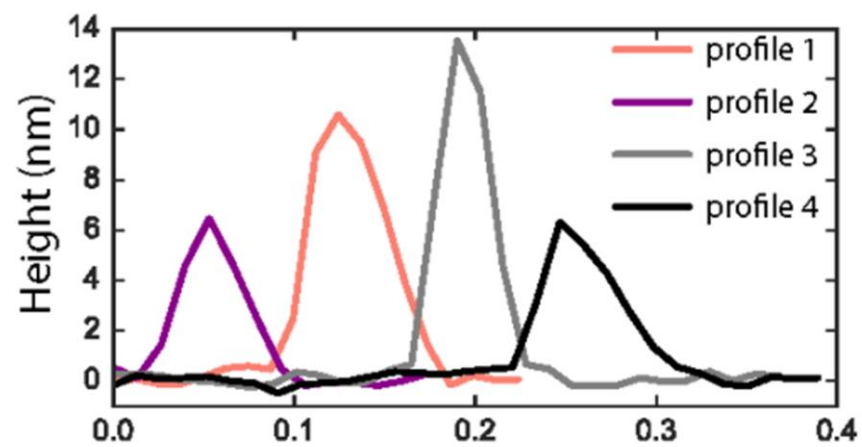
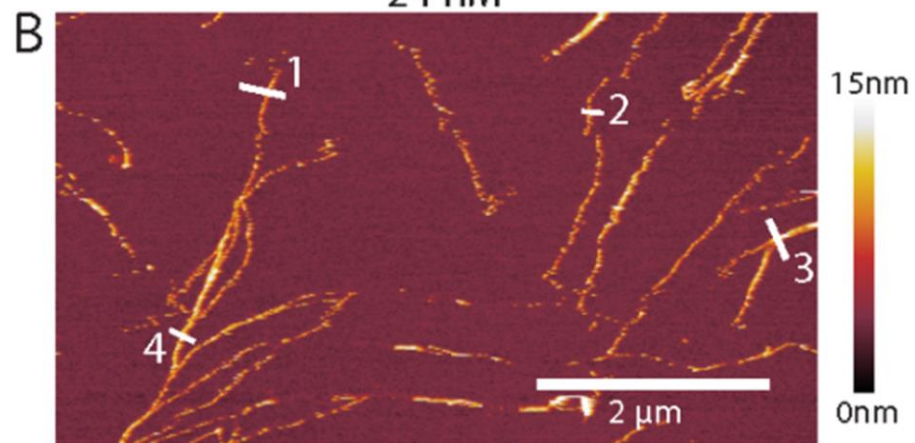




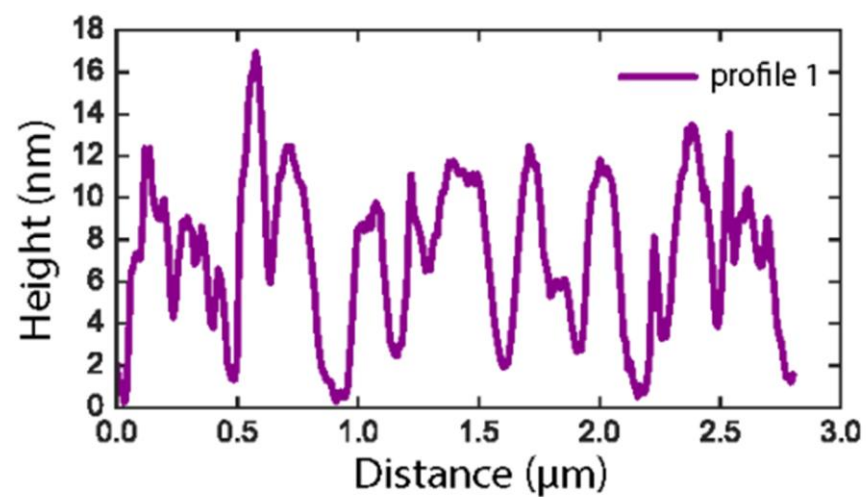
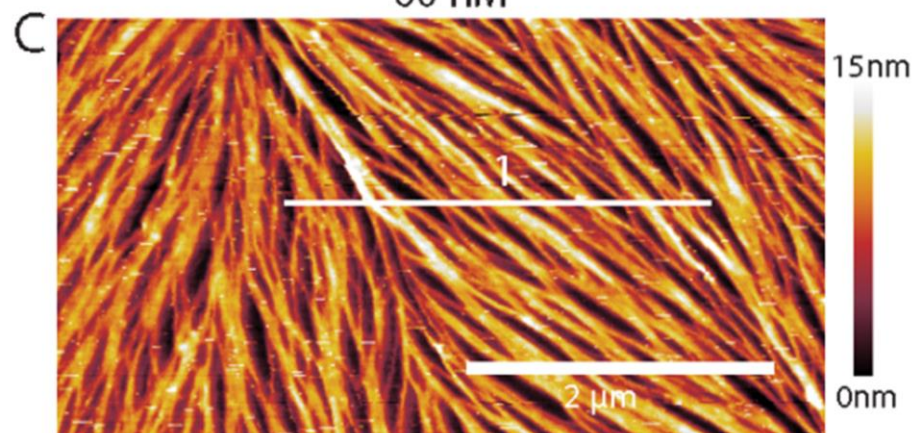
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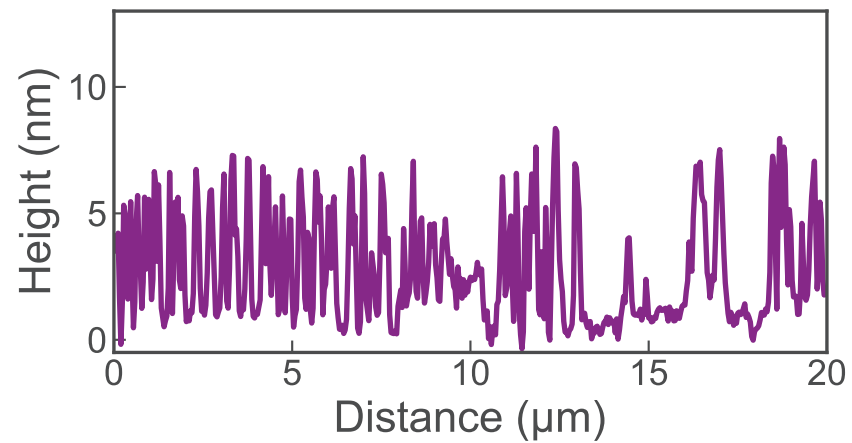
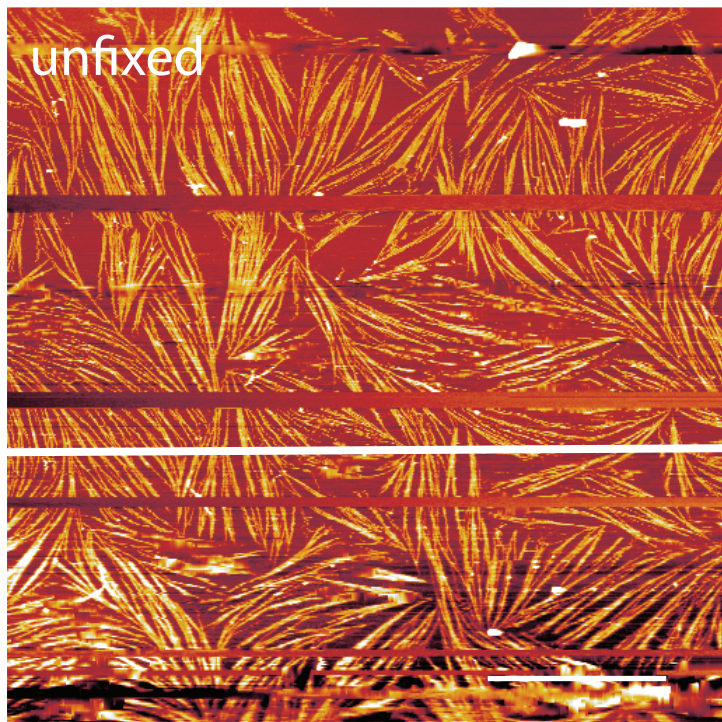
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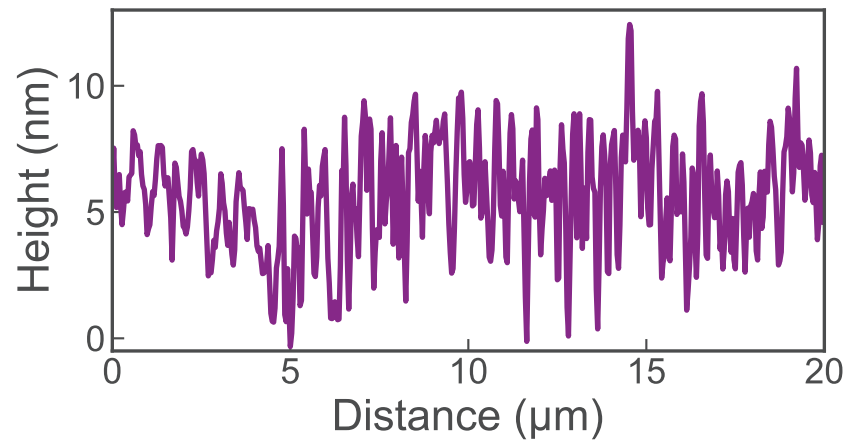
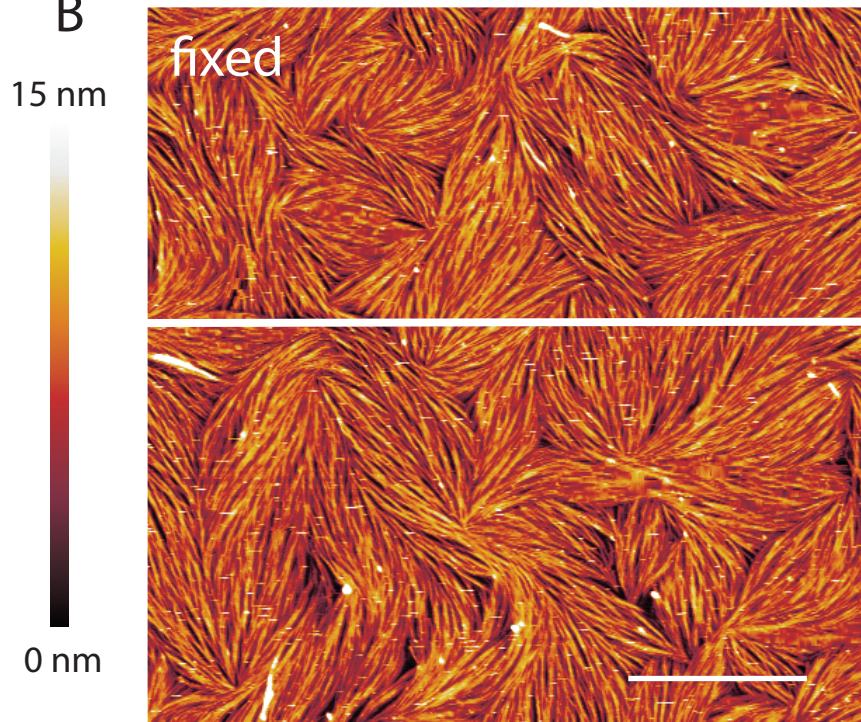
60 nM

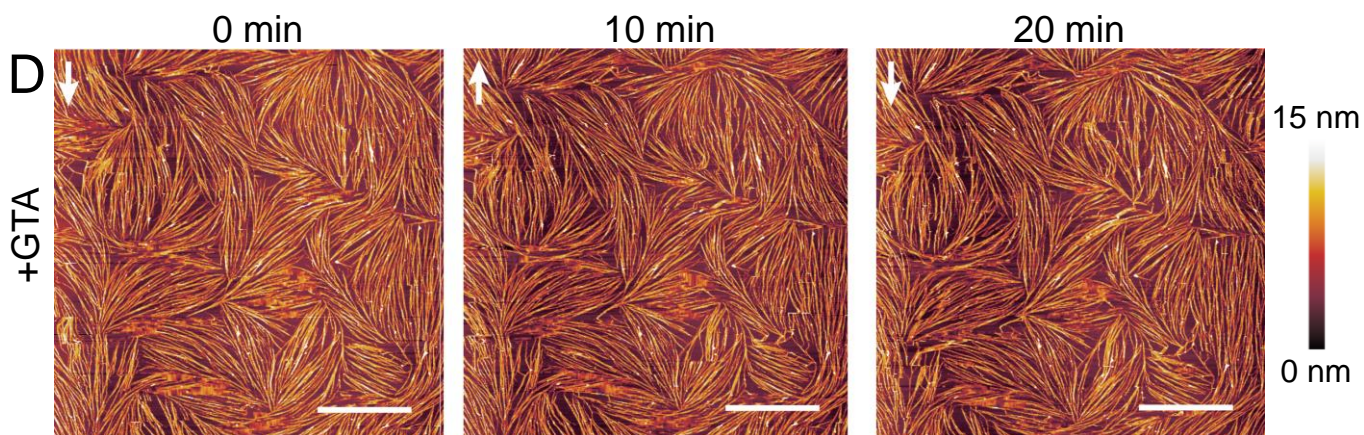
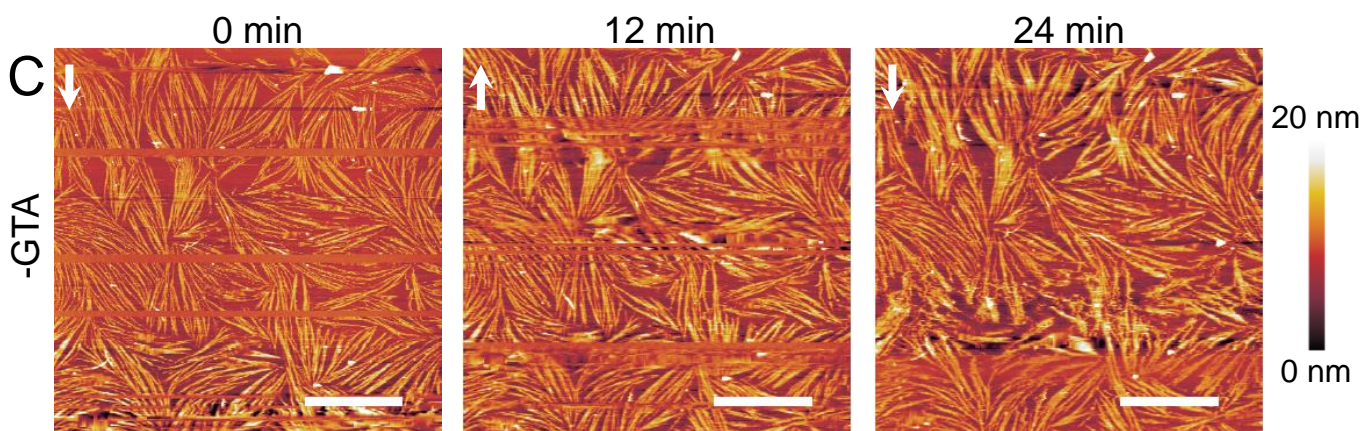
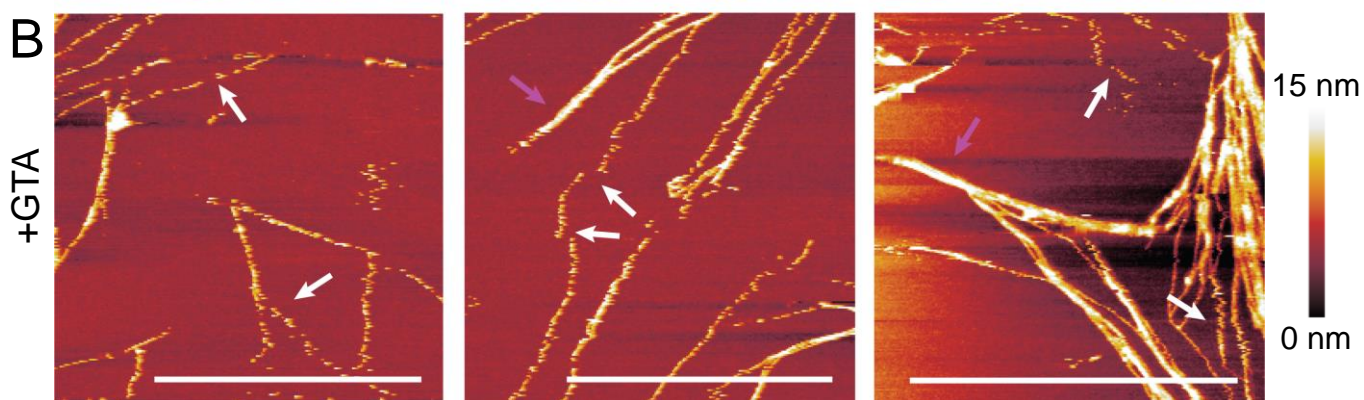
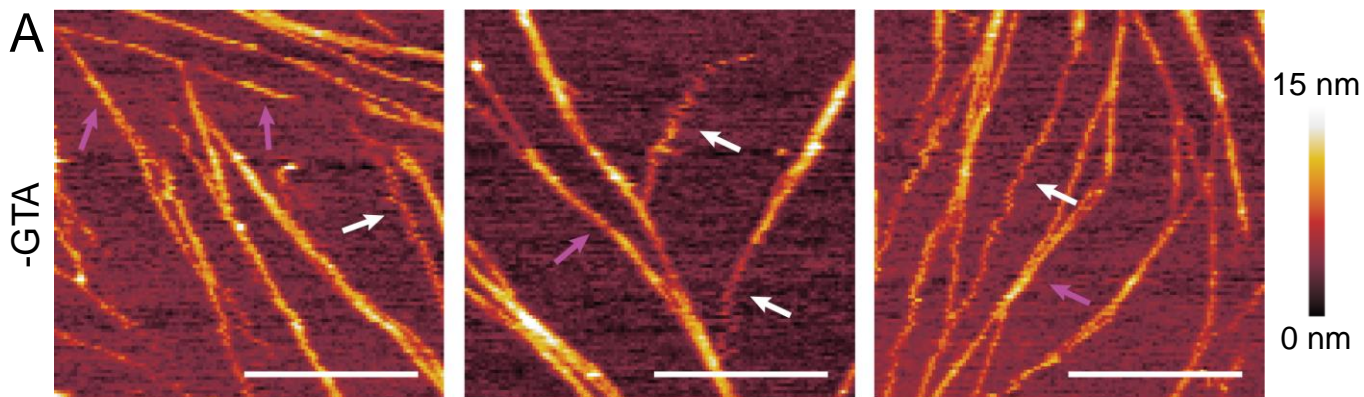


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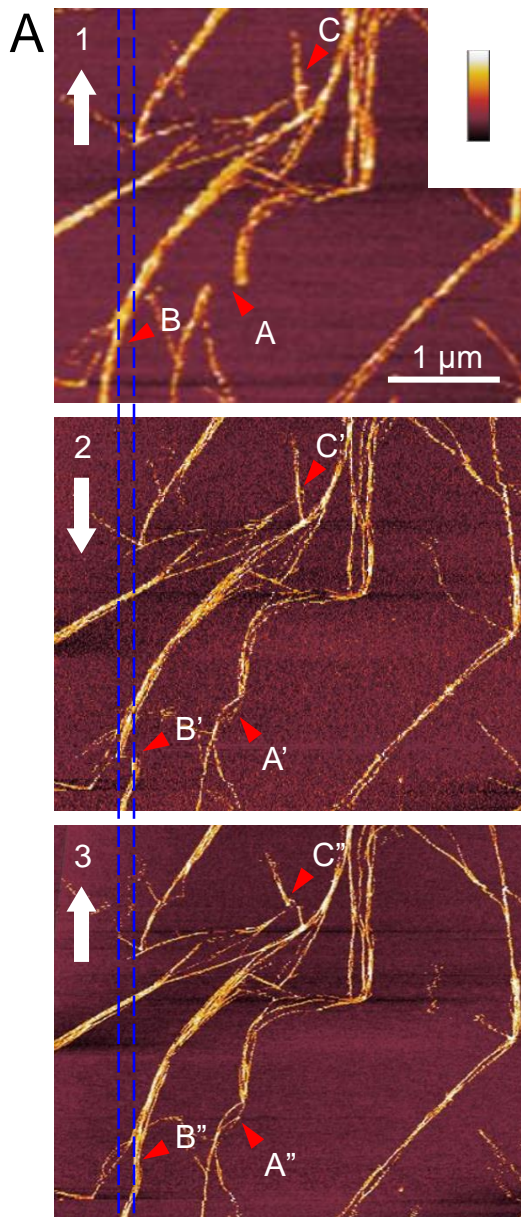


B





+ GTA



- GTA

